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Review

Prevention of carcinogenesis and cancer metastasis by bovine lactoferrin

By Hiroyuki TSUDA,*),†) Katsumi FUKAMACHI,*) Jiegou XU,*) Kazunori SEKINE,*) Shigetoshi OHKUBO,*),**) Nobuo TAKASUKA,**) and Masaaki IIGO**)

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Abstract: Increasing attention is being paid to chemopreventive agents for individuals at high risk of cancer. We have concentrated on bovine lactoferrin (bLF), an 80 kDa iron-binding glycoprotein known to have anti-microbial and immunoprotective effects. Lactoferrin is particularly abundant in colostrum, and is also present in tears, saliva and seminal and uterine secretions. However, only little is known regarding its influence on carcinogenesis. We have shown preventive effects of bLF and its fragment peptide, lactoferricin (bLFcin), consisting of a 25 amino acid sequence without iron binding capacity, on chemically-induced colon carcinogenesis in the rat and transplanted carcinoma cell metastasis in the mouse. The mechanisms are wide-spectrum, including elevation of caspase-1 and IL-18 in the small intestine, enhancement of the cell killing activity of cytotoxic T and natural killer (NK) cells, and anti-inflammatory and anti-angiogenic effects. It also inhibits the induction of liver CYP1A2, a carcinogen activating enzyme, and induces apoptosis in the colon epithelium of carcinogen treated rats. Thus, bLF possesses multi-functional potential to suppress carcinogenesis and is a good candidate for practical application in humans.

Key words: Cancer chemoprevention; lactoferrin; lactoferricin; multi-function; IL-18.

Introduction. The purpose of cancer prevention is to cause a delay in onset of carcinogenesis and to suppress promotion from precancerous lesions to cancer. Cancer can be prevented either by avoiding risk factors such as smoking, a high caloric diet, physical inactivity and exposure to environmental carcinogens,[1] or by increasing intake of chemopreventive agents from foods.[2] The latter method may be particularly practical because the agents can be taken simply by changing the current dietary style or as supplements.[3] As target subjects for chemoprevention, two groups should be considered, one with precancerous lesions and the other with lifestyles related to elevated cancer risk. Whichever of the two, it is necessary that exposure be chronic, possibly for life. In this regard, there are advantages to using natural compounds because adverse effects are generally known and therefore the required procedures for forwarding to clinical trials can be reduced as compared to newly developed compounds. Since carcinogenic processes are multistage and complex,[4] agents possessing multiple mechanisms of action offer the most promising candidates. In this review, basic studies on cancer chemoprevention by bovine lactoferrin (bLF) and its peptide fragment lactoferricin (bLFcin) are discussed, focusing on multi-functional mechanistic aspects.

Stages in neoplastic development and inhibition of carcinogenesis. In the prevailing paradigm, three stages of neoplasia can be recognized, "initiation", "promotion" and "progression", as illustrated in Fig. 1. In the initiation stage, carcinogens such as heterocyclic amines, polycyclic aro-

*) Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan.

**) Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo, Japan.

†) Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan (htsuda@med.nagoya-cu.ac.jp).

Abbreviations: bLF, bovine lactoferrin; bLFcin, lactoferricin; NK, natural killer; NSAID, non-steroidal anti-inflammatory drugs; RO/NOSS, reactive oxygen and/or nitrogen oxide species-induced stress; ACF, aberrant crypt foci; AOM, azoxymethane.

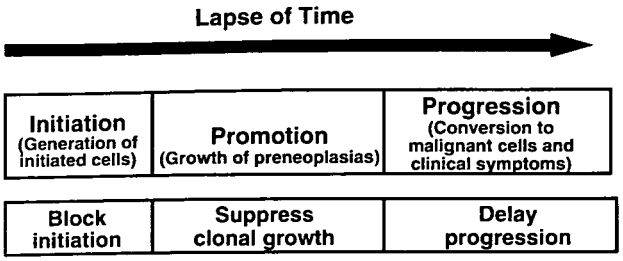

Fig. 1. Stages of carcinogenesis and corresponding methods for cancer chemoprevention.

matic hydrocarbons and nitrosamines are metabolized to proximate and ultimate forms by drug-metabolizing enzymes such as phase I xenobiotic-metabolizing enzyme species, including CYP 450s[5] and also some phase II enzymes, such as sulfotransferases. Generally, however, phase II enzymes such as glutathione transferases protect cells from activated carcinogens by converting them to more readily excreted forms. They may also act as oxygen radical scavengers. Accordingly, initiation is a process which is dependent on a balance of enzyme activity between activation and detoxification.[6] Initiated cells eventually undergo clonal growth to become preneoplastic lesions from which malignant lesions are thought most likely to arise.

Chemopreventive agents which interfere with the initiation process by blocking metabolic activation of carcinogens are defined as "blocking agents". Compounds acting to reduce cancer development in the post-initiation stage are defined as "suppressing agents".[7] For the analysis of mechanisms, chemopreventive agents can act during initiation, post-initiation or both.

Since human beings are continuously exposed to various environmental carcinogens,[1),8] "initiated cells" can always be expected to exist, awaiting stimuli to commit to neoplastic development (promotion) and further malignant conversion (progression). Practical application of chemopreventive agents should therefore be focused on both initiation and promotion stages.

Mechanisms of inhibition of carcinogenesis. The following actions are proposed as possible mechanisms for cancer chemoprevention.

1) Anti-oxidant action

Reactive oxygen species and/or nitrogen oxide species-induced stress (ROS/NOSS) and downstream events are clearly important for carcinogenesis. ROS/NOSS can induce DNA adducts and their excision, which may cause DNA damage leading to impaired gene regulation. Anti-oxidants, such as polyphenols, carotenoids and curcumin are therefore expected to inhibit carcinogenesis.[9)-11)]

2) Anti-inflammatory action

Chronic inflammation and associated elevated levels of cell proliferation appear to predispose to cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) act to strongly inhibit cycloxygenase activity resulting in reduction of prostaglandin formation and effects on neoplasia.[12),13)] For example, COX2 inhibitors, nimesulide and celecoxib, have been found to markedly inhibit colon carcinogenesis in the rat.[14),15)]

3) Anti-hormone action

Enhanced cell proliferation through hormone-receptor signaling is a risk factor of cancer development. For example, estrogen is a growth factor in the absence of progesterone and is the major causative agent for breast and ovarian cancer. Thus the estrogen antagonist tamoxifen citrate has been used for subjects at high risk of breast cancer.[16),17)]

4) Modulation of immune activity

Since the immune system can influence inflammatory cell reactions through the function of various cytokines, it is important in host defense during the early stages of carcinogenesis. Compounds which influence immune status and suppress carcinogenesis include alpha-tocopherol, unsaturated fatty acids and proanthocyanidins.[18)-21)]

5) Modulation of xenobiotic-metabolizing enzyme activity

The balance between the phase I activating and phase II detoxifying enzymes plays important roles in determining initiation of carcinogenesis. Thus, intervention in carcinogen metabolism, for example 8-methoxypsoralen inhibition of tobacco-specific 4-(methylnitrosamino)-l-(3-pyridyl)-l-butanone (NNK) activation in the lung, causes inhibition of carcinogenesis.[5),22),23)]

6) Inhibition of angiogenesis

The question of whether preneoplasias or early stage tumors can continue to grow is dependent to a large extent on the availability of nutrients; consequently, anti-angiogenic agents are clearly of importance.[24),25)] Anti-angiogenic effects have been shown for both NSAIDs and retinoids.[26)]

7) Regulation of signal transduction

Interference with signaling pathways downstream of receptors responsible for cell growth, dif-

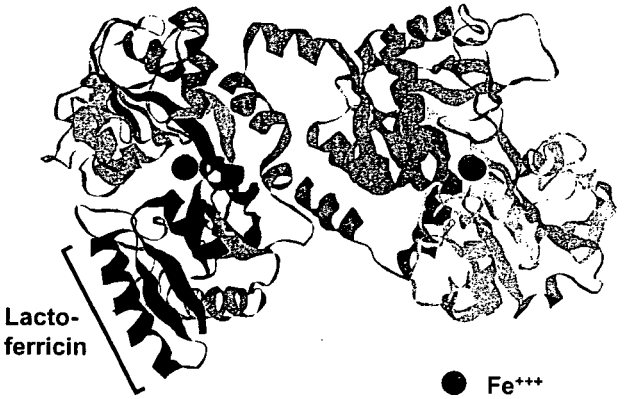

Fig. 2. Structure of bovine lactoferrin (courtesy of Professor Keiichi Shimazaki of Graduate School of Agriculture, Hokkaido University).

Table I. Distribution of lactoferrin in different body fluids

Organ		Concentration (/ml)
Human	Colostrum	5–10 mg
	Ordinary milk	2 mg
	Tears	0.4–1.2 mg
	Nasal secretion	0.1 mg
	Uterine secretion	0.5–1.0 mg
	Salivary juice	5–10 μg
	Urine	1 μg
Bovine	Colostrum	0.5–1.0 mg
	Ordinary milk	0.4 mg

ferentiation and apoptosis is clearly important for cancer prevention. For example, *d*-limonene may act by inhibiting Ha-ras oncogene activation through reduction of H-ras p21 isoprenylation.[27]

Milk components and lactoferrin. The whey fraction of milk is actually composed of a large number of ingredients such as alpha-lactoalbumin, beta-lactoalbumin, immunoglobulin, bovine serum albumin and lactoferrin. Very little is known about their individual effects on carcinogenesis, although alpha-lactoalbumin has been shown to be a potent Ca^{2+} elevating and apoptosis-inducing agent.[28),29]

We have concentrated attention on lactoferrin, an 80-kD member of the transferrin family with two iron-binding sites per molecule, which is well known to have antimicrobial properties against bacteria, fungi and viruses.[30)–32] The complete amino acid sequence of bLF has been determined and the protein comprises 689 amino acids, folding into N- and C- globular lobes (Fig. 2), each capable of binding up to 1.4 mg iron per 1 g of protein. In its native state in milk, iron saturation is 20~30%. Lactoferricin (bLFcin), a peptide fragment composed of 15 amino acids produced by pepsin hydrolysis of bLF has the sequence NH_2-Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe-COOH, with Cys-Cys cyclic formation. bLFcin does not have any iron binding site.[33),34]

In humans, lactoferrin also exists in various secretions, for example, tears, saliva and seminal fluid. Levels of lactoferrin in colostrum are particularly high (5–10 mg/ml) and newborn infants receiving mother's milk ingest lactoferrin at 1–2 g/day. Concentrations of lactoferrin in different body fluids are listed in Table I. Lactoferrin is well known to act against bacteria and stimulate immune responses, activating NK cells and neutrophils, enhancing lymphokine-activated killer (LAK) activity and augmenting macrophage cytotoxicity.[35),36]

bLF and prevention of chemical carcinogenesis. We have generated data on the preventive effects of bLF on carcinogenesis in the colon and other organs (see below). Another focus of interest is its influence on metastasis. Therefore, we have studied effects on prevention of cancer and metastases by oral administration of bLF and bLFcin.

1) Inhibition of colon and liver carcinogenesis by concurrent administration with carcinogen (blocking effects)

The inhibitory influence of bLF on colon carcinogenesis by a food carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), was investigated. Rats were treated with PhIP alone or concurrently with 0.2% bLF (approximately 100 mg/kg/day) for 10 weeks. Effects were evaluated by counting the numbers of colon aberrant crypt foci (ACF), a postulated preneoplastic lesion for colon carcinomas. bLF caused a significant decrease in ACF. Similar effects were observed for preneoplastic lesion development in the liver induced by 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). One possible mechanism is down-regulation of CYP1A2 expression, associated with a decrease in metabolic activation of the carcinogen and DNA adduct formation, resulting in reduction of preneoplastic development.[37] bLF also inhibited ACF development induced by another colon carcinogen, 1,2-dimethylhydrazine. This inhibition

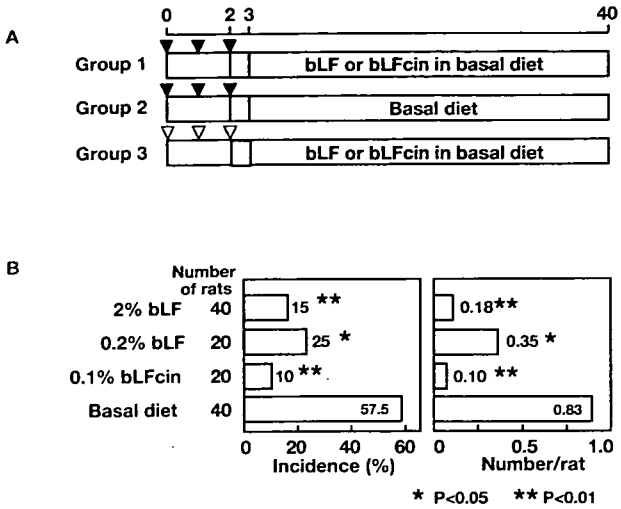

Fig. 3. A, Experimental protocol for the assay of suppressive the effects of bLF on AOM-induced colon carcinogenesis in the rat. AOM (▼) was subcutaneously injected into male F344 rats at 15 mg/kg, 3 times in two weeks, followed by feeding of the basal diet containing bLF at 2.0 or 0.2% (approximately 1000 or 100 mg/kg/day, respectively) or 0.1% (50 mg/kg/day) bLFcin for weeks 3 to 40 (Group 1). The control group was given AOM alone (Group 2). For the test of toxicological effects, rats were initially treated with saline (▽) in place of AOM, then fed bLF or bLFcin as in Group 1 (Group 3). B, Incidence and number of adenocarcinomas in the colon. Figures indicate observed values. Statistics was performed by Fischer's exact probability for the incidence and Dunnet's t test for the number values. *, $P < 0.05$, **, $P < 0.01$.

was associated with enhancement of NK cell activity.[38]

2) Inhibition of colon carcinogenesis in the post-initiation stage (suppressing effects)

To evaluate preventive activity of bLF and bLFcin in the post-initiation stage of colon carcinogenesis, male rats were initially administered a colon carcinogen, azoxymethane (AOM), and then fed a diet containing bLF or bLFcin from weeks 3 to 40. Control rats received the basal diet alone after AOM treatment (Fig. 3A). Both the incidences and multiplicity (number of tumors/rat) of adenocarcinomas in animals receiving bLF and bLFcin were clearly reduced as compared to the control group (Fig. 3B). No obvious toxicity was noted in major organs. The results provided clear evidence of an inhibitory effect of bLF against colon tumor development when given in the post-initiation stage.[39),40] Furthermore, carcinogenesis in rats treated with different carcinogens inducing tumors in the tongue, liver, esophagus, lung, bladder and thyroid was inhibited by administration of bLF during the post-initiation period.[41]

Inhibition of hereditary carcinogenesis. For assessment of preventive effects on hereditary intestinal polyposis, the Apc^{Min} mouse, an animal model of familial polyposis coli, was used. Female C57BL/6J$^{Min/+}$ (Apc^{Min}) mice were fed a basal diet containing bLF for 8 weeks. Significant reduction of the polyp number in the jejunum was observed with 2% bLF ($P < 0.05$, Dunnet's t test ,68% of the control).[42] Since COX2 expression plays a major role in tumor development in Apc^{Min} mice, bLF effects may be linked to its anti-inflammatory actions.[43),44]

Prevention of lung metastasis of transplanted carcinomas in mice. Intraperitoneal injection of human lactoferrin has been shown to inhibit lung metastasis of mouse B16-F10 melanoma cells from subcutaneously inoculated tumor cells.[36] Since the intraperitoneal route is not suitable for practical use because lactoferrin may cause allergic reactions, we studied the effects of orally administered bLF and bLFcin at doses of 30, 300 and 1000 mg/kg on tumor metastasis. Lung metastatic colony counts were clearly decreased at the higher doses (Fig. 4A). Thus, anti-metastatic effects were demonstrated by oral application to tumor-bearing mice.[45]

Mechanisms of inhibitory effects on carcinogenesis and metastasis. Levels of interleukin 18 (IL-18) in the mucosa propria of the small intestine were measured in C57BL/6 mice given bLF and bLFcin intragastrically at 30, 300 and 1000 mg/kg/day for 7 days. Metastatic foci counts were clearly decreased at the higher doses. Significant increases in IL-18 protein levels were also observed in mice treated with 30 and 300 mg/kg bLFcin and a tendency for increase was noted with bLF (Fig.4B). In transplanted tumor-bearing mice, a marked increase in the number of cytotoxic T and NK cells in the mucosal layer of the small intestine and peripheral blood cell population was found after treatment with bLF but not bovine transferrin. The production of IL-18 and caspase-1 in intestinal epithelial cells was also enhanced and the number of interferon (IFN)-γ positive cells increased (Fig. 5).[46] Accordingly, we conclude that IL-18 was induced by bLF in mice with transplanted tumors as well as in mice without transplanted tumors.

In mice, orally and intraperitoneally administered bLF was also found to significantly inhibit angiogenesis stimulated by tumor cells *in vivo* in a dorsal air sac assay (Fig. 6).[47] Our findings in mice

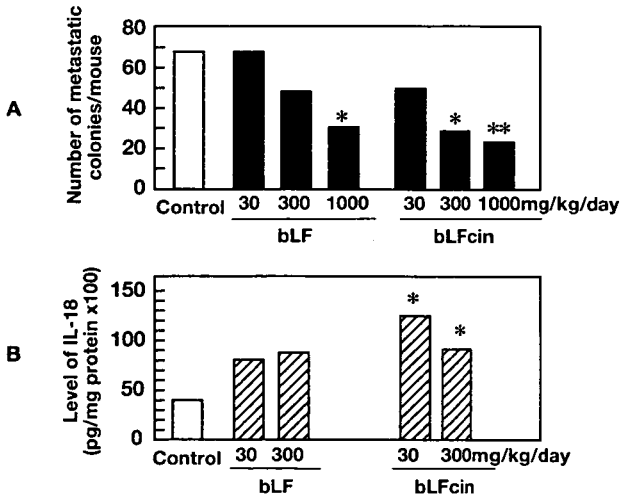

Fig. 4. Prevention of metastasis of transplanted cancer cells to the lung (**A**) and assay of IL-18 in the small intestine in mice (**B**). 3LL (mouse Lewis Lung carcinoma) cells were subcutaneously inoculated into C57BL/6 mice; then bLF and bLFcin were administered for days 3-7 and 10-14 at 30, 300 and 1000 mg/kg/day by gavage. Lung metastatic colony counts were clearly decreased in both bLF and bLFcin treated groups (Mann-Whitney *U* test). For the IL-18 protein level assay, mice were given bLF or bLFcin, both at 30 and 300 mg/kg/day, by gavage for 7 days and killed. Levels of IL-18 in the mucosal epithelium and mucosa propria tissue were assayed. bLFcin treatment induced a clear increase in IL-18 protein levels (Dunnet's *t* test). bLF treated animals showed tendency of increased IL-18 protein levels. *, $P < 0.05$, **, $P < 0.01$.

clearly indicated that IL-18 induction played a pivotal role in bLF-mediated inhibition of angiogenesis, and this inhibition resulted in reduction of cancer development.[19),21),26),48)]

Discussion. The objective of cancer chemoprevention is to cause a delay in the onset of clinical cancers so that they do not cause suffering but rather occur at the natural termination of life, as conceived in the "Natural-end cancer" concept.[49)] The great interest in developing chemicals suitable for chemopreventive agents has generated a massive literature. However, in spite of the large number of compounds for which efficacy has been proven in experimental models, few clinical trials have been performed and none has, so far, proven a net benefit conferred by natural compounds.

Compounds such as anti-estrogens and NSAIDs actually have been used for high risk patients, but they require careful observation for adverse effects because they are used for far longer periods than originally envisaged as medicines.[50)] Thus, usage should be based on thorough examinations regarding beneficial (preventive) and adverse (promotion of carcinogenesis or toxic) effects.[21),51)] Unfortunately for most micronutrients only limited data are available on toxicity.[52)] In this regard, use of bLF, a protein with high homology to human lactoferrin, is advantageous because human lactoferrin is ingested by neonates with mother's milk and is already permitted for use as a food additive.[30)] Furthermore, ingestion of bLF for up to 75 weeks did not show any adverse effects in any of the experiments so far conducted (manuscript in preparation).

The results of our series of investigations provide clear evidence of the inhibitory potential of bLF against chemically-induced carcinogenesis, especially in the colon, with actions as both a blocking and a suppressing agent. In addition, bLF reduces metastasis of transplanted tumors. Thus bLF is a promising chemopreventive agent for human colon carcinogenesis. It is likeky to also be active against metastasis of colon and lung tumor cells, as well as inhibiting development of neoplastic lesions in the tongue, esophagus, lung and bladder. Furthermore, its mechanisms of action have been partly clarified and include induction of IL-18 and increases in the numbers of interferon gamma-positive cells and cytotoxic T-cells and augmentation of intestinal immunity.

Neoplastic development of the human endocervix has been shown to be associated with down-regulation of lactoferrin mRNA expression in early carcinomas, accompanied by a pronounced elevation in cell proliferation.[53)] Furthermore, it is reported that neutrophilic lactoferrin transactivates the p53 tumor suppressor gene through activation of nuclear factor-kB (NF-kB) and consequently regulates p53-responsive oncogenes.[54)] In addition, treatment of human breast cancer cells with human lactoferrin resulted in growth arrest at the G1 to S transition of the cell cycle by a p53-independent mechanism.[55)] In animal studies, a protective influence of lactoferrin against the growth of solid tumors and development of experimental metastases in mice was also reported.[36)]

The mechanisms of action of cancer prevention by bLF is summarized in Fig. 7. bLF exhibits six out of ten classic chemopreventive functions. Furthermore, bLF possesses unique characteristics regarding the immune system, not always shared by compounds such as carotenoids and polyphenols. Increases in IL-18 and enhancement of cytotoxic T cell and NK cell activities may share common functions

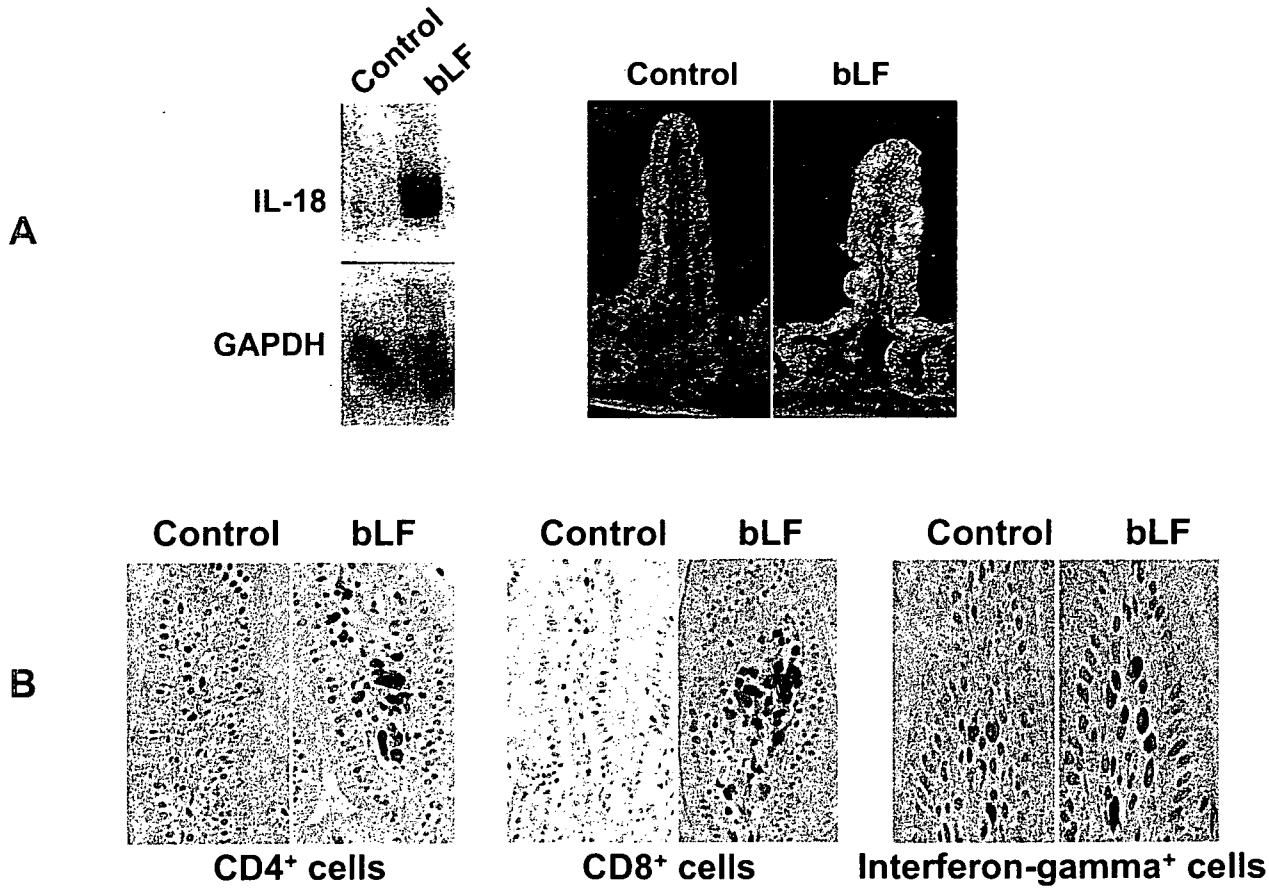

Fig. 5. Immunostaining of IL-18. Induction of IL-18, CD4, CD8 and interferon-gamma in the epithelial cells and mucosa propria of the small intestine of mice. The highly metastatic Co26Lu cells, derived from mouse colon carcinoma, were implanted subcutaneously into BALB/c mice. The mice were then treated with bLF and bovine transferrin at 300 mg/kg/day by gavage for 3 days and killed on day 4. Levels of IL-18 were clearly increased (**A**). The number of white blood cells positive for CD4, CD8 and interferon-gamma were also increased in the mucosa propria (**B**). These alteration were not observed after treatment with bovine transferrin (not shown).

for both cancer prevention and inhibition of metastasis. Further studies on the responsiveness of preneoplastic cells and carcinoma cells to such cytotoxic factors are obviously required.

In conclusion, bLF and bLFcin are promising chemopreventive agents of colon and less markedly the tongue, esophagus, lung, bladder and thyroid carcinogenesis (in rats) and carcinogenesis of the small intestine/jejunum (Apc^{Min} mice). They also prevent metastasis of transplanted carcinomas. Their mechanisms of action include: 1) inhibition of induction of CYP1A2, an activating enzyme for carcinogenic heterocyclic amines; 2) induction of cytotoxic $CD8^+$, $CD4^+$, $IFN\gamma^+$ and NK cells in the small intestine and blood; 3) induction of IL18 and caspase 1 in small intestine epithelium; 4) induction of apoptosis in carcinogen-initiated colon epithelium by activation of Fas, caspase-8 and caspase-3; and 5) inhibition of angiogenesis. Based on these observations, bLF has been forwarded for a clinical trial in patients with small colon polyps at the National Cancer Center Hospital, Japan.

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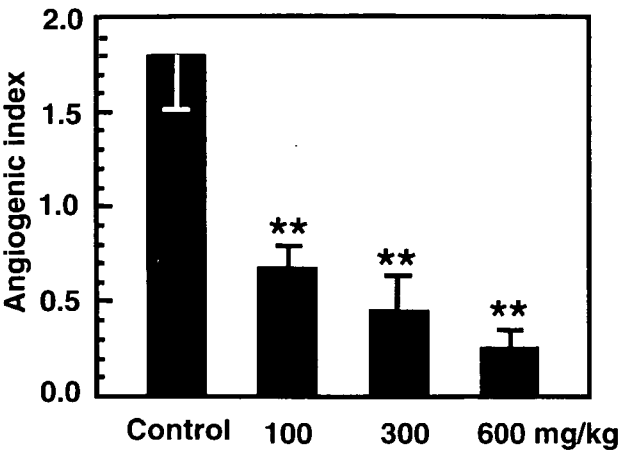

Fig. 6. Inhibition of angiogenesis by bLF. Millipore chambers filled with 3LL (Lewis lung carcinoma) cells (4×10^6 cells) were subcutaneously implanted into ICR mice. The mice were given bLF or bLFcin by gavage from day 2 to 6 and killed on day 7. The skin adjacent to the chamber was removed for observation of angiogenesis. The length of the blood vessels was scored as 0 for no induction and 3 for maximal induction (Mann-Whitney *U* test). bLF dose-dependently inhibited angiogenesis. **, $P < 0.01$.

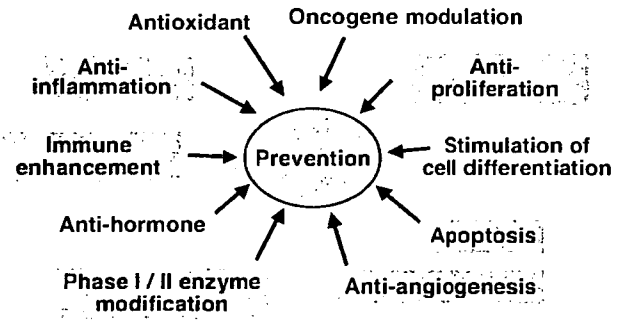

Fig. 7. Summary of the mechanism of action of cancer chemoprecention. bLF, by itself, possessses six out of ten well known chemopreventive functions.

preparation of the manuscript. Experiments by the authors were conducted according to the "Guidelines for Animal Experiments of the National Cancer Center" of the Committees for Ethics of Animal Experimentation of the respective institutes.

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Ductal origin of pancreatic adenocarcinomas induced by conditional activation of a human Ha-*ras* oncogene in rat pancreas

Shinobu Ueda[1,2,3], Katsumi Fukamachi[1], Yoichiro Matsuoka[2,5], Nobuo Takasuka[2,6], Fumitaka Takeshita[3], Akihiro Naito[2], Masaaki Iigo[2,6], David B.Alexander[1], Malcolm A.Moore[1], Izumu Saito[4], Takahiro Ochiya[3] and Hiroyuki Tsuda[1,2,*]

[1]Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan, [2]Experimental Pathology and Chemotherapy Division and [3]Section for Studies on Metastasis, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan and [4]Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108-8639, Japan

Present address: [5]Second Department of Pathology, Kansai Medical University, 10–15 Fumizono-cho, Moriguchi, Osaka, Japan and [6]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo, Japan

*To whom correspondence and requests for reprints should be addressed. Tel: +81 52 853 8991; Fax: +81 52 853 8996; Email: htsuda@med.nagoya-cu.ac.jp

Pancreatic ductal adenocarcinoma is one of the most debilitating malignancies in humans. Currently, radiation and chemotherapy are ineffective, with median survival times after treatment of <12 months. Animal models that reflect the human condition and can be used to explore screening and therapeutic approaches are clearly desirable. One feature of human pancreatic adenocarcinoma is an exceedingly high frequency of K-*ras* mutation. The present study was conducted to determine if targeted activation of a human oncogenic-*ras* transgene in rat pancreas would induce carcinomas correspondent to human pancreatic ductal adenocarcinomas. We established transgenic (Hras250) rats in which expression of a human Ha-*ras*^{G12V} oncogene is regulated by the *Cre/lox* system. Targeted pancreatic activation of the transgene was accomplished by injection of *Cre*-carrying adenovirus into the pancreatic ducts and acini through the common bile duct. Adenoviral infection of injected animals was exclusive to the pancreas; infected cells could be identified in duct, intercalated duct, centroacinar and, less frequently, acinar cells, but not in endocrine islet cells. Four weeks after injection, proliferative lesions in the duct epithelium, intercalated ducts and centroacinar cells, but not acinar cells, were widespread. Tumorigenesis in other tissues was not observed. Most lesions, including atypical duct proliferative lesions, PanIN-like lesions and carcinomas, were positive for cytokeratins 19 and 7, cyclooxygenase 2 and MMP-7 but negative for amylase and chymotrypsin. Many adenocarcinoma lesions were positive for EGF and EGFR. Duct epithelial and atypical duct proliferative lesions and carcinoma lesions were all positive for transduced Ha-*ras*^{G12V} oncogene expression. The cytogenesis of pancreatic ductal type carcinoma was depicted. This model exhibits important similarities to the human disease and promises to advance our understanding of the behavior of pancreas adenocarcinomas and expedite screening and therapy.

Abbreviations: AB, alcian blue; CK, cytokeratin; COX2, cyclooxygenase 2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; MMP, matrix metalloproteinase; PAS, periodic acid-Schiff's; PCNA, proliferating cell nuclear antigen; Rb, retinoblastoma; RT–PCR, reverse transcriptase polymerase chain reaction.

Introduction

Cancers of the pancreas are diagnosed in ~1 in 10 000 people/year in the United States and are the fifth leading cause of cancer death. Most patients die within 1 year of diagnosis (1), and the 5 year survival rate is <5% (2,3). Ductal adenocarcinomas are diagnosed in ≥95% of the patients with pancreatic cancer. Of these patients, only ~10–15% can undergo potentially curative resection. Even surgical resection, however, offers a very low cure rate; the 5 year survival rate is <10%. Radiation and chemotherapy have so far proven ineffective (1,4). Clearly, our current understanding of this disease is far from sufficient.

Animal models are used extensively in the study of human disease. In the Syrian hamster, pancreatic ductal neoplasms similar to those in man can be induced by propylnitrosamines (5). These tumors are characterized by a high rate of K-*ras* activation (6), a characteristic that is believed to be of particular importance in human pancreatic cancers: while activated Ras is associated with ~40% of all human cancers, the figure for pancreatic adenocarcinomas is >90% (7–9). The hamster model (10–12), however, suffers from a number of disadvantages. These include the necessity of using a potent non-specific carcinogen, the small size of the animal and the inability to raise hamsters in pathogen-free conditions.

Attention has now become concentrated on the use of activated oncogenes for the generation of pancreatic lesions in transgenic animals. In most mouse models reported to date, however, pancreatic lesions are derived from acinar cells. For example, transgenic mice carrying a K-*ras*^{G12D} gene with an elastase promoter (13) or an H-*ras*^{G12V} gene with an elastase promoter (14) both lead to the development of pancreatic ductal lesions, which are probably derived from acinar cells. In humans, the cytogenesis of human pancreatic ductal adenocarcinomas is unclear; whether they are derived directly from duct cells has not yet been unequivocally established. However, it is important to consider the possibility that rodent models in which pancreatic tumors are derived from elements that do not give rise to human pancreatic tumors may not appropriately model the human condition.

In another model system, using transgenic mice with a K-*ras*^{G12V} transgene under the control of the cytokeratin 19 (CK19) promoter, there was some hyperplasia of ductal

epithelial cells, but no discernable morphological changes in acinar or islet cells (15). Moreover, the hyperplastic lesions did not progress into tumors. Recently, human-like pancreatic premalignant lesions have successfully been generated in mice in which oncogenic K-ras^{G12D} protein is expressed at endogenous K-ras levels in pancreatic progenitor cells (16), and when this oncoprotein is expressed in *Ink4a/Arf* null progenitor cells the resulting tumors become highly invasive and metastatic (17). While these two animal models are strikingly similar to human pancreatic intraepithelial neoplasias and ductal carcinomas, they do not specify the cellular compartment from which the lesions arise and tumor induction is independent of exogenous control.

We have been focusing on the advantages of rat models (18) and initially established a transgenic rat carrying a human c-Ha-*ras* proto-oncogene. These animals are highly susceptible to carcinogens, developing mammary, skin, esophagus and bladder tumors depending on the carcinogen and route of exposure (18–21). Interestingly, in these tumors there is an extremely high incidence of mutation of the human c-Ha-*ras* transgene. Therefore, we proceeded to establish a transgenic rat carrying a human Ha-*ras*^{G12V} oncogene in which expression is regulated by the *Cre/lox* system (22). Targeted expression of Cre recombinase specifies the tissues or cell types in which the Ha-ras^{G12V} oncoprotein is expressed.

The present study was conducted to determine if transgenic rats carrying a conditionally expressed human Ha-*ras*^{G12V} oncogene would be susceptible to induction of ductal pancreatic adenocarcinomas when Ha-ras^{G12V} protein expression was induced in pancreatic tissue. Targeted activation of Ha-*ras*^{G12V} was accomplished by injection of a *Cre*-carrying adenovirus into the pancreatic ducts through the common bile duct. Preneoplastic lesions derived from ductal and centroacinar cells that developed into adenocarcinomas were indeed induced. This rat model is a promising experimental system to provide a better understanding of pancreatic ductal adenocarcinoma cell origin and behavior and expedite experimental screening and therapies of one of the most lethal human carcinomas.

Materials and methods

Recombinant DNA constructs and conditional transgenic rats

The CALNLHras (CAG promoter-*loxP* sequence–neo-resistant gene-*loxP* sequence–Ha-*ras*^{G12V}) switching unit was constructed following the original protocol of Kanegae (22). Briefly, total RNA was extracted from cultured T24 cells (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) bearing an activated c-Ha-*ras* gene (23,24) using ISOGEN (NIPPON GENE, Toyama, Japan). The RNA was treated with DNase I (amplification grade; Life Technologies, Gaithersburg, MD), and reverse transcription polymerase chain reaction (RT–PCR) was performed with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) using oligo(dT)$_{12-18}$ to prime first-strand DNA synthesis. The primers 5′-ATGACGGAATATAAGCTGGTGGTGGTG-3′ and 5′-TCAGGAGAGCACACACTTGCAGC-3′ were used to amplify Ha-*ras*^{G12V}. The resultant 570-bp amplicon was cloned into pCR2.1-TOPO using the TOPO TA Cloning system (Invitrogen), and the sequence was confirmed with a Thermo Sequenase Cy 5.5 Terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ). The complete Ha-*ras*^{G12V} sequence was subcloned into the *Swa*I site of pCALNLw (Riken Bioresources Center DNA Bank) to produce pCALNLHras. pCALNLHras was digested with SalI and HindIII, run out on an agarose gel, and the CALNLHras cassette was cut out and purified using a QIAquick Gel Extraction kit (QIAGEN). A total of 1 to 2 pl of the purified cassette at a concentration of 3.0 ng/μl 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 (25). Transgenic founder rats were mated with Sprague–Dawley rats, and offspring were screened for the presence of the transgene by PCR and Southern blot analysis of genomic DNA isolated from tail biopsies at the age of 3 weeks.

Preparation of adenovirus vectors

Recombinant adenovirus vectors carrying the *Cre* gene (AxCANCre) or the *GFP* gene (AxCAGFP) and a control empty adenovirus vector (AxCAwt) were prepared as described previously (22). The virus stock was concentrated and purified at 1.0×10^{10} pfu/ml as reported previously (26).

Tumor induction and pathological examination

Twenty-eight 7-week-old male and female Hras250 rats were divided into two groups. One group received AxCANCre (21 rats) and the other group received AxCAwt (7 rats). To determine the site of infection, an adenovirus carrying the *GFP* reporter gene (AxCAGFP) was injected. All injections were into the pancreatic duct through the common duct according to the method of Taniguchi *et al.* (27). Animals were injected with 150 μl adenovirus solution (4×10^9 pfu/ml in DMEM) with a 31-G needle. A vital tracking dye (Indigo carmine, 2 mg/ml) was added to the vector suspension for visualization (27). Animals were killed by exsanguination from the inferior vena cava under deep ethyl ether anesthesia. The pancreas was excised and fixed in phosphate-buffered 10% formalin or acetone and processed for paraffin embedding for histological observation. All experiments were conducted according to the 'Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences' and the 'Guidelines for Animal Experiments of the National Cancer Center' of the Committee for Ethics of Animal Experimentation of the respective institutes.

Immunostaining

Tissues were fixed in 4% formaldehyde fixative and embedded in paraffin. Primary antibodies against green fluorescent protein (GFP; CLONTECH Laboratories, CA) diluted 1 : 100; CK19 (Abcam, Cambridge, UK), diluted 1 : 100; cytokeratin 7 (CK7) (DAKO, Carpinteria, CA), undiluted; cyclooxygenase 2 (COX2) (IBL, Takasaki, Japan), diluted 1 : 20; epithelial growth factor (EGF; Biomedical, Stoughton, MA), diluted 1 : 100; EGF-receptor (EGFR; UBI, Lake Placid, NY), diluted 1 : 25; matrix metalloproteinase-7 (MMP-7; Santa Cruz, CA), diluted 1 : 50; proliferating cell nuclear antigen (PCNA; DAKO), diluted 1 : 50; anti-α-amylase (Sigma, St Louis, MO), diluted 1 : 200; and chymotrypsin (Biogenesis, Poole BH177DA England, UK), diluted 1 : 100 were incubated with slides for 2 h at room temperature to overnight at 4°C, and staining was performed using the biotin peroxidase complex (ABC) method (Vectastain ABC kits, Vector Laboratories, Burlingame, CA). For PCNA staining, section slides were autoclaved for 15 min in a 10 mM citrate buffer (pH 6.0) and then allowed to cool for 30 min before incubation with antibodies. For visualization of the transfer of GFP by adenovirus AxCAGFP *in vivo*, on Day 1 after injection of AxCAGFP the pancreas, liver, duodenum and spleen were examined. For mucin staining, periodic acid-Sciff's (PAS) and alcian blue (AB) staining were performed. Immunostaining of mucins using human antibodies such as MUC1 and MUC5a/c was negative throughout the normal pancreas tissue and neoplastic lesions including those of PanIN morphology, possibly owing to lack of cross-reactivity (rat antibodies are not currently available).

Detection of recombination and expression of the transgene in vivo

Pancreatic tissues were isolated 4 and 5 weeks after injection of AxCANCre or AxCAwt and genomic DNA and proteins were extracted using standard methods (28). Genomic DNA was used as the template for PCR reactions for detecting transgene recombination. The primers (Figure 1, arrows) were CAGp-f: 5′-CGTGCTGGTTGTTGTGCTGTCT-3′ (in the CAG promoter region) and Hras3-4r: 5′-CCTCCACTCCCTGCCGGGTC-3′ (in the Ha-*ras*^{G12V} coding region). PCR amplification was performed with TaKaRa Ex *Taq* TM (TAKARA SHUZO). Samples were denatured at 95°C for 5 min and then amplified for 33 cycles at 95°C for 40 s and 72°C for 2 min. A final extension at 72°C for 10 min was performed after the last cycle. Anti-pan Ras antibodies (Upstate, Charlottesville, VA) and goat anti-mouse IgG-HRP (Southern Biotechnology Associates, Birmingham, AL) were used to detect total Ras protein, and a Ras activation kit (Upstate) was used to detect activated Ras protein. All samples were subjected to SDS–PAGE in a 4–20% gradient gel (Daiichi Pure Chemicals, Tokyo, Japan) and electroblotted onto Immobilon-P membranes (Millipore, MA). Before incubation with antibodies, the blots were blocked with 5% skim milk in TPBS [0.1% Tween-20 in phosphate-buffered saline (PBS)]. Blots were incubated with antibodies according to the manufacturer's instructions. Visualization was carried out with the ECL+plus Western Blotting Detection System (Amersham Biosciences).

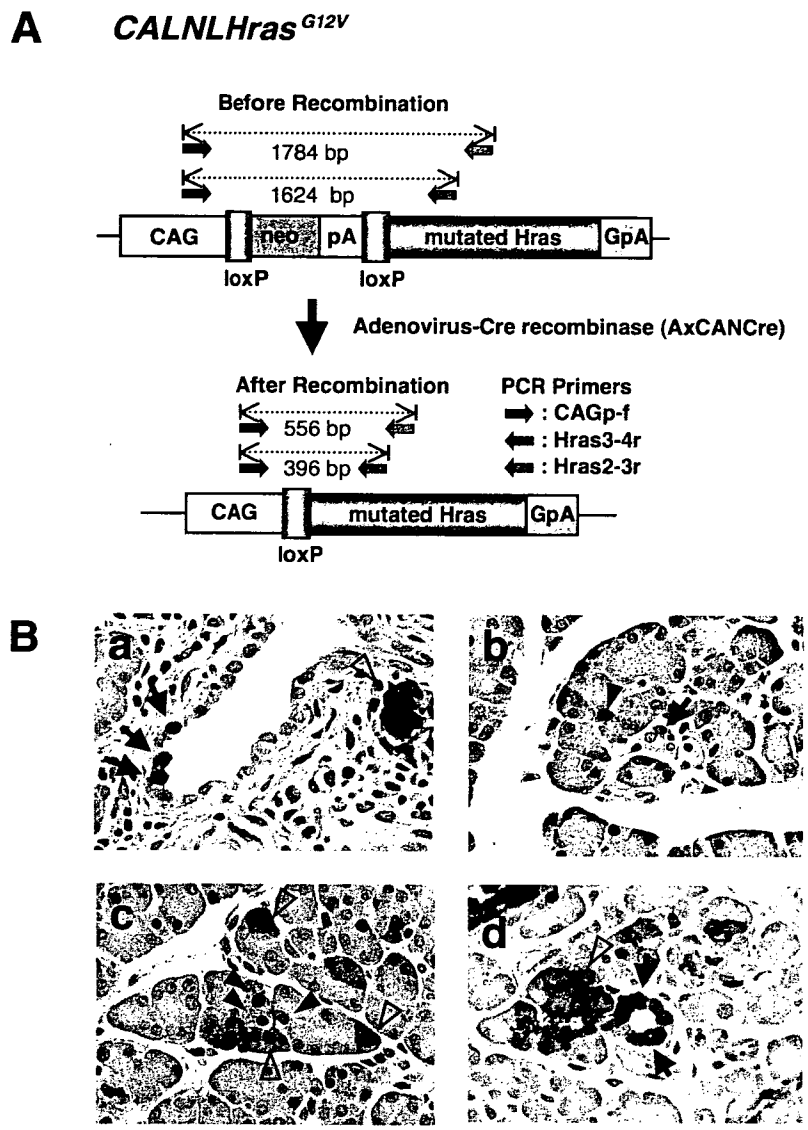

Fig. 1. (**A**) The *CALNLHras*^{G12V} transgene is comprised of a hybrid CMV enhancer/chicken beta-actin promoter (CAG), a cassette for the neomycin resistance gene flanked by loxP sites, and a sequence containing human Ha- *ras*^{G12V}. Infection with the *Cre recombinase*-carrying adenovirus AxCANCre 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-*ras*^{G12V} gene expression unit. pA, SV40 early poly(A) site; GpA, rabbit-β-globin poly(A) site. Arrows, primers for the detection of recombination of the transgene. (**B**) Localization of adenovirus infection in the pancreas. At Day 1 after injection of the *GFP*-carrying adenovirus AxCAGFP into the pancreata of transgenic Hras250 rats, GFP is clearly shown in epithelial cells of a duct (panel a, arrows), intercalated ducts (panels b and d, arrows), centroacinar cells (panels b and c, arrowheads) and acinar cells (panels a–d, open arrowheads) (×132).

Detection of recombination of the transgene by laser capture microdissection (LCM)

Microdissection was performed using a PixCell IIe Laser Capture Microdissection Instrument (Arcturus Engineering, Mountain View, CA). DNAs were isolated from the captured samples and were used as templates for PCR reactions for detecting transgene recombination. The primers (Figure 1, arrows) were CAGp-f: 5′-CGTGCTGGTTGTTGTGCTGTCT-3′ (in the CAG promoter region) and Hras2-3r: 5′-GATCTGCTCCCTGTAC-TGGTGG-3′ (in the Ha-*ras*^{G12V} coding region). PCR amplification was performed with TaKaRa Ex *Taq* TM (TAKARA SHUZO). Samples were denatured at 95°C for 5 min and then amplified for 40 cycles at 95°C for 40 s, 60°C for 30 s and 72°C for 1 min. A final extension at 72°C for 10 min was performed after the last cycle.

RT–PCR analysis

Total RNA from gross tumors isolated 4 and 5 weeks after adenovirus injection was prepared using the ISOGEN method (NipponGene) according to the manufacturer's protocol. Total RNA derived from AxCAwt-injected rat pancreas (control) was isolated as follows: pancreas samples were frozen using liquid nitrogen, the frozen sample was ground to a powder under liquid nitrogen, RNA was extracted using ISOGEN and the extracted RNA was spun (12 h at 41 000× *g* at 4°C) through a 5.7 M cesium chloride cushion containing 25 mM acetate (pH 6.0) and 1 mM EDTA and resuspended in RNA Secure (Ambion, Austin, TX). A total of 500 ng of total RNA (from control pancreas and tumor samples) was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Reverse-transcription reaction mixtures were diluted 1 : 5 and 1 μl was

used for PCR. Real-time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA) using TaKaRa Ex *Taq* HS (TAKARA SHUZO) with SYBR Green in the reaction mixture according to the manufacturer's instructions. The initial amplification of nested PCR (*Ink4a* and *Arf*) was also performed in a SmartCycler II using TaKaRa Ex *Taq* HS, but without SYBR Green in the reaction mixture. The second amplification of nested PCR was performed in an iCycler (Bio-Rad, Hercules, CA) using 0.5 μl of initial PCR product in a 20 μl reaction with TaKaRa Ex *Taq* HS according to the manufacturer's instructions. The following primers were used: initial amplification of *p16-Ink4a*, 5'-GCGGGCACTGCTGGAA-3' and 5'-CACCTGGGCGTGCTTGA-3'; nested amplification of *p16-Ink4a*, 5'-GGGGCTTCACCAAACGC-3' and 5'CGTTCCCCGTTCCCAGCGGAGGAGA-3'; initial amplification of *p19-Arf*, 5'-TCGTGGCCTTGGTGTTGA-3' and 5'- CACCTGGGCGTGCTTGA -3'; nested amplification of *p19-Arf*, 5'-AGGGCCGCAGCCACAT-3' and 5'- CCGCAAATACCGCACGAC-3'; *Madh4*, 5'-TCAGCACCACCCGCCTAT-3' and 5'-CTGCCGCAAATCAAAGACC-3'; *p53*, 5'-GATGCCCGTGCTGCCGAGGAGT-3' and 5'-TGGGCCAGGAACCAGTTTGCATAGA-3'; *Mdm2*, 5'-GTGGCCCAGATGCTCCTGTCA-3' and 5'-TGGCTCGATGGCGTTCAGAGA-3'; *Rb*, 5'-ATAGGCGGTATTTAAGGTAGCGTCAG-3' and 5'-AGCCCTTGACCTAAACCCAACTAAC-3'; *p21-Waf1/Cip1*, 5'-GTGGCCTTGTCGCTGTCTTG-3' and 5'-TGGGCACTTCAGGGCTTTCT-3'; *cyclin D1*, 5'-GGGGATTCAGGACGACTCTTA-3' and 5'-AGCGGCGGCAAGAATGT-3'; *cyclin D2*, 5'-CGATGCCCTGACGGAGCTG-3' and 5'-CTCTTGCCGCCCGAATGG-3'; *cyclin D3*, 5'-CTTCAGAAATCCCGATAGACGC-3' and 5'-GCCACCAGCCCAAACCTT-3'; *β-actin*, as an internal control, 5'-CCGTAAAGACCTCTATGCCAACA-3' and 5'-CGGACTCATCGTACTCCTGCTT-3'. Nested PCR parameters were as follows: initial amplification of *p16-Ink4a*, denature at 95°C for 3 min, amplify for 25 cycles at 95°C for 15 s and 60.5°C for 64 s; nested amplification of *p16-Ink4a*, denature at 95°C for 3 min, amplify for 20 cycles at 95°C for 20 s, 60°C for 20 s and 72°C for 30 s; initial amplification of *p19-Arf*, denature at 95°C for 3 min, amplify for 25 cycles at 95°C for 15 s and 61°C for 64 s; nested amplification of *p19-Arf*, denature at 95°C for 3 min, amplify for 20 cycles at 95°C for 20 s, 60°C for 20 s and 72°C for 27 s. For real-time PCR, samples were denatured for 3 min at 95°C and amplified for 35 cycles. Cycling parameters were as follows: *Madh4*, 95°C for 15 s and 61°C for 77 s; *p53*, 95°C for 15 s and 72°C for 30 s; *Mdm2*, 95°C for 15 s and 67.5°C for 27 s; *Rb*, 95°C for 15 s and 66°C for 30 s; *p21-Waf1/Cip1*, 95°C for 15 s and 64°C for 37 s; *cyclin D1*, 95°C for 15 s and 62°C for 60 s; *cyclin D2*, 95°C for 15 s and 70°C for 22 s; *cyclin D3*, 95°C for 15 s and 61°C for 80 s; *β-actin*, 95°C for 15 s and 63°C for 29 s. PCR amplicons that were run out on agarose gels were generated using the same conditions as for real-time PCR except that reactions were stopped when the amplifications were in the log linear phase: *Madh4*, 28 cycles; *p53*, 30 cycles; *Mdm2*, 29 cycles; *Rb*, 30 cycles *p21-Waf1/Cip*, 31 cycles; *cyclin D1*, 27 cycles; *cyclin D2*, 27 cycles; *cyclin D3*, 33 cycles; and *β-actin*, 24 cycles.

Results and discussion

Generation of rats with conditional regulation of activated c-Ha-ras expression

We obtained three founder rats carrying the *CALNLHras*^{G12V} transgene (Figure 1) transmittable to descendant generations (Hras218, 246 and 250). In these rats, Cre recombinase excises the stuffer DNA between the CAG promoter and the Ha-*ras*^{G12V} gene, thereby initiating gene expression (Figure 1A). Southern blotting analysis indicated that Hras218 and 250 had one copy, and Hras246 had three copies of the transgene. Hras250 rats were used for these studies.

Adenovirus-mediated AxCAGFP expression

For detection of adenovirus-mediated gene transfer into adult rat pancreatic cells, a *GFP*-carrying adenovirus (AxCAGFP) was injected into the pancreatic ducts through the common bile duct. Immunostaining for GFP after injection indicated a clear localization of gene transfer into pancreatic duct cells (Figure 1B, panel a, arrows), intercalated duct cells (Figure. 1B, panels b and d, arrows) and centroacinar cells (Figure 1B, panels b and c, closed arrowheads). A small number of acinar cells were also positive for GFP (Figure 1B panels a–d, open arrowheads). Islet cells were negative for GPF. Liver, spleen and intestines were also examined for infection; these organs were negative (not shown).

Detection of transgene activation

For transgene activation, Hras250 rats were injected with an adenovirus carrying the *Cre* gene (AxCANCre); injection of an adenovirus without an exogenous insert (AxCAwt) served as a negative control. Four weeks after injection, genomic DNA was isolated from the pancreata of AxCANCre and AxCAwt animals and subjected to PCR. A 1784-bp band corresponding to the unmodified transgene was detected in the pancreata of both animals (Figure 2A, left panel). In addition, a 556-bp band corresponding to the recombinant transgene was generated by PCR in the AxCANCre group (Figure 2A, left panel). Activation of the transgene was assessed by western blot analysis. An extremely high level of active Ras was detected in the pancreata of the AxCANCre animals compared with AxCAwt animals (Figure 2A, right panel), indicating AxCANCre-mediated activation of the transgene.

Gross and histological observation of pancreatic duct lesions

Animals were killed 4, 5 and 9 weeks after injection of AxCANCre or AxCAwt. At Week 4, many grossly visible whitish nodules of ~1–3 mm in diameter were observed throughout the pancreas in the AxCANCre group (Figure 2B, left photograph), but not in the AxCAwt group (Figure 2B, right photograph). Histological examination determined that these nodules were adenocarcinomas (Figure 2C). Incidence of tumors in the AxCANCre group was 100% (21 out of 21 animals). One animal died at Week 4, presumably as a result of tumor-associated pancreatic malfunction. By Week 9, a total of seven of the AxCANCre animals had died, all with severely damaged pancreas. No neoplastic lesions were evident in organs other than the pancreas (data not shown). None of the AxCAwt group displayed any pancreatic lesions (or lesions in other tissues) even after 9 weeks (not shown).

Histological characterization of the lesions

Early proliferative lesions that developed in AxCANCre-treated animals were largely divided into three types: intraductal epithelial lesions, centroacinar cell lesions and ductular (intercalated duct) lesions. The epithelial cells in the intraductal lesions tended to be tall columnar and the lesions were quite similar to those of human intraductal PanIN-1 (Figure 3) or PanIN-2/3 (Figure 4) (29,30). The cells were positive for CK19 and CK7. These cells were also positive for COX2 and EGF. Compared with the PanIN-1 lesions, the more advanced PanIN-2/3 lesions exhibited a more dramatic increase in COX2. The lesions also began displaying increased EGFR in the cell membranes and MMP-7 in the cytoplasm near the apical border. PanIN-2/3 lesions with obvious intraluminal growth and papillary projection showed some irregular localization of β-catenin. The lesions were partly positive for mucopolysaccharides, as demonstrated by PAS and AB. Lesion cells were negative for amylase and chymotrypsin (Figure 4, amylase data not shown). The presence of COX2 in the cytoplasm indicates similarity to human pancreatic carcinomas. Co-expression of EGF and EGFR indicates that tumor growth may be controlled in an autocrine manner, and the presence of MMP-7, another feature of human pancreatic carcinomas (31–34), indicates progression toward malignancy.

Early centroacinar cell lesions (Figure 5A, arrowheads) and ductular proliferative lesions (Figure 5A, arrows) exhibited a transitional figuration to an incomplete duct-like

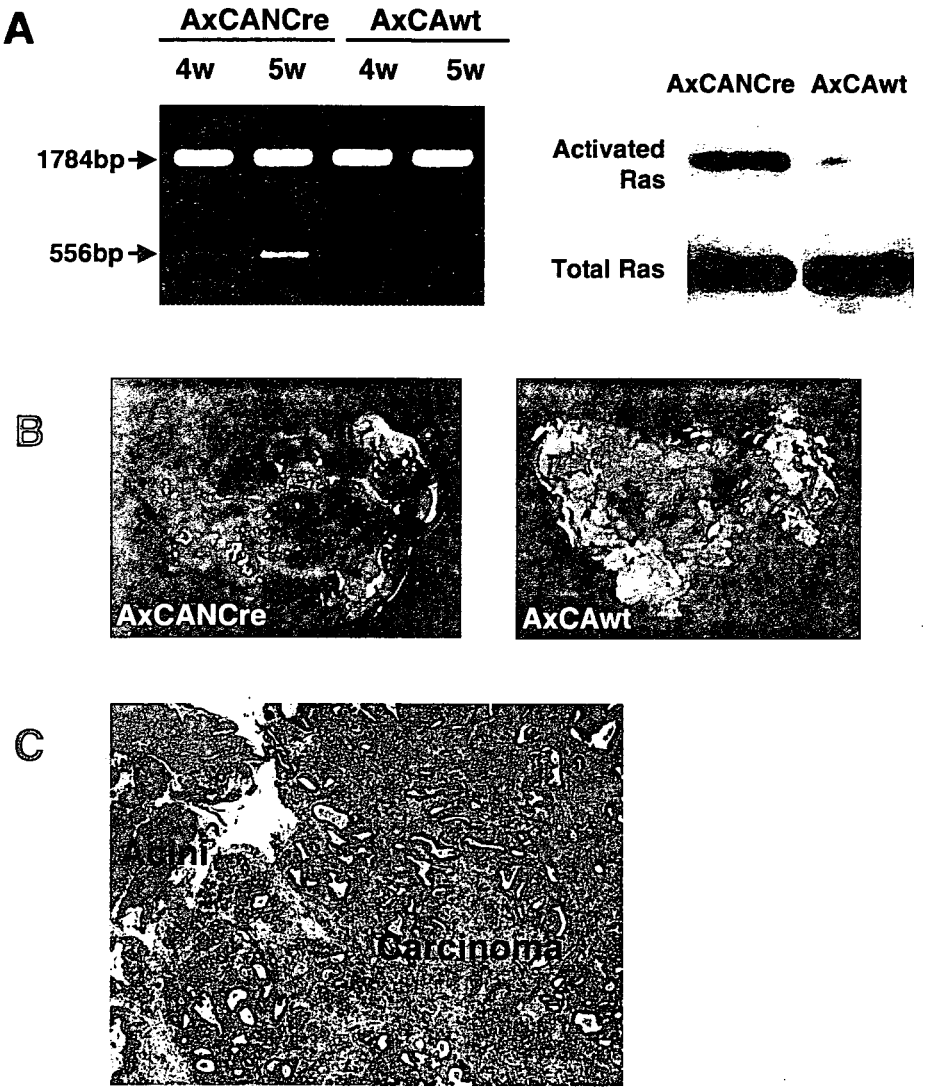

Fig. 2. (**A**) Induction of recombination of the *CALNLHras*^{G12V} transgene and activation of Ras expression four weeks after injection of AxCANCre into the pancreatic duct. A 556-bp band is apparent in pancreas tissue of Hras250 rats injected with AxCANCre, but not in rats injected with the empty control adenovirus AxCAwt, indicating recombination of the transgene (left panel). Activated Ras is clearly detected in the pancreas of AxCANCre-injected rats by western blot; very little activated Ras was detected in AxCAwt-injected animals (right panel). (**B**) Macroscopic appearance of pancreatic tumors induced by human activated c-Ha-*ras* at Week 4. Many nodules ~1–3 mm in diameter were observed in the pancreata of the AxCANCre group at Week 4 (left). No pancreatic lesions were induced in rats injected with AxCAwt (right). (**C**) (×33) Most of the nodular lesions were diagnosed as adenocarcinoma (carcinoma) with obvious invasion of the surrounding acinar structure (acini).

arrangement with nuclear atypia and increased labeling for PCNA (Figure 5A, right panel). No PCNA labeling was observed in acinar cells. These ductular lesions also showed expansive growth into the surrounding acinar structure (Figure 5B). Most of the cells comprising the lesions were positive for CK19, CK7, COX2 (not shown), EGF, EGFR (not shown) and MMP-7; were weakly positive for AB in the cytoplasm and at the apical boarder; and were negative for chymotrypsin and amylase (Figure 5B and C).

Advanced lesions, including adenocarconomas of moderately differentiated morphology, exhibited expansive growth into the surrounding acinar structure (Figures 6 and 7). These lesions shared several common characteristics with early proliferative epithelial and ductular lesions: many of the component cells were positive for CK19, CK7, EGF, EGFR, COX2 and MMP-7; weakly positive for mucopolysaccharides; and negative for chymotrypsin and amylase. Carcinoma tissue occasionally showed invasion of liver capsular tissue with accompanying fibrous reaction (not shown). Proliferative lesions in the acinar component were not observed. Comparison of cellular characteristics comprising the different lesions are summarized in Table I.

Thus, the histopathological appearance of these adenocarcinomas closely resembles that described in humans (29,30). Most human pancreatic neoplasms have a ductal morphology (29). The exact cellular origin of these lesions, however, is not certain. For example, rats treated with the non-specific carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) develop

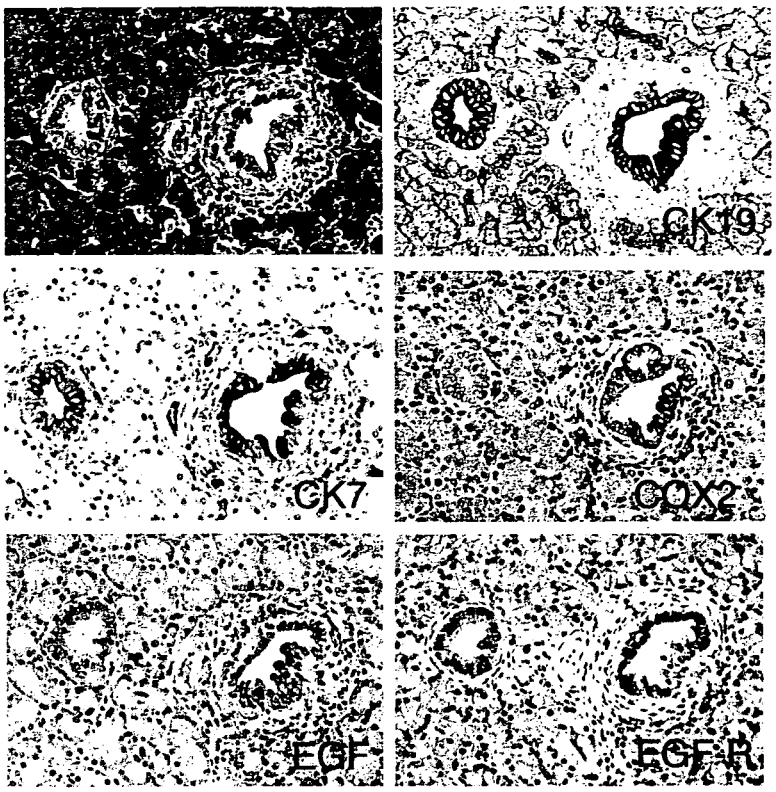

Fig. 3. (×66) Semi-serial sections through an early intraductal proliferative lesion in a pancreatic duct. Lesion cells are slightly tall columnar with some cellular and nuclear dysplasia resembling a human PanIN-1 lesion. The lesion is positive for CK19 and CK7 with increases in COX2 and EGF.

pancreatic tumors that exhibit ductal marker expression in the early stages of tumorigenesis (35), but the possibility that these tumors could arise through dedifferentiation of acinar cells has attracted a great deal of attention (36). *In vivo* experiments have provided evidence that suggests that injured or carcinogen-treated acinar cells may assume a ductal phenotype in the rat (36) and in the hamster (37). Also, transdifferentiation of neoplastic acinar cells to give rise to duct-like structures in the rat has been described previously (38). Moreover, several models for the induction of duct-like tumors in transgenic mice appear to involve acinar cell precursors (13,14). *In vitro* studies of acinar cell preparations have also shown transition of acinar cells to duct-like cells (39–41).

Our results clearly indicate that the pancreatic lesions induced in the Hras250 rat express duct characteristics but do not express an acinar phenotype. Thus, our findings suggest that centroacinar and intercalated duct epithelial cells were the precursors of adenocarcinomas, and that de-differentiation of acinar cells was not a contributing factor. Our results agree with the observation made in the Pten knockout mouse that centroacinar cell proliferation may be a cause of pancreatic ductal carcinomas (42). A schematic diagram of Hras250 rat pancreatic carcinogenesis is summarized in Figure 8. Precursor lesions are hyperplastic and dysplastic proliferation of centroacinar cells, intercalated ducts and duct epithelium exhibiting PanIN-like lesions, but not of acinar cells. Irrespective of cell origin the eventual morphology is adenocarcinoma with a ductular phenotype.

Detection of recombination of the transgene

To identify in which cellular compartment the transgene underwent recombination, samples were removed using LCM (Figure 9A), the DNA was extracted and PCR was performed. A 1624-bp band corresponding to the unmodified transgene was detected in all captured samples (Figure 9B). A 396-bp band corresponding to transgenes that had undergone recombination was generated by PCR in tumor tissue (Figure 9A, panel a and 9B, lanes 3 and 4), mixed populations of proliferating centroacinar cells and intercalated ducts (Figure 9A, panel b and 9B, lanes 5 and 6), a PanIN-2/3-like lesion (Figure 9A, panel c and 9B, lanes 7 and 8) and in normal-looking acinar cells surrounding mixed populations of proliferating centroacinar cells and intercalated ducts (Figure 9A, panel d and 9B, lanes 9–11). A recombinant transgene was not detected in acinar (Figure 9B, lane 1) or ductal cells (Figure 9B, lane 2) obtained from the pancreata of AxCAwt-treated animals. Pancreatic cancers exhibit multiple genetic and molecular alterations, several of which have been identified in the last few years (8,43–46). It is generally well accepted that K-*ras* gene mutation is a highly frequent and early event in pancreatic carcinogenesis (6–9,43–46) Similarly, in the Hras250 rat, the initiating signal in pancreatic ductal adenocarcinogenesis is expression of the Ha-ras^{G12V} oncogene.

Molecular analysis of pancreatic adenocarcinomas

We conducted a molecular analysis of the tumors to determine the status of pathways that are commonly altered

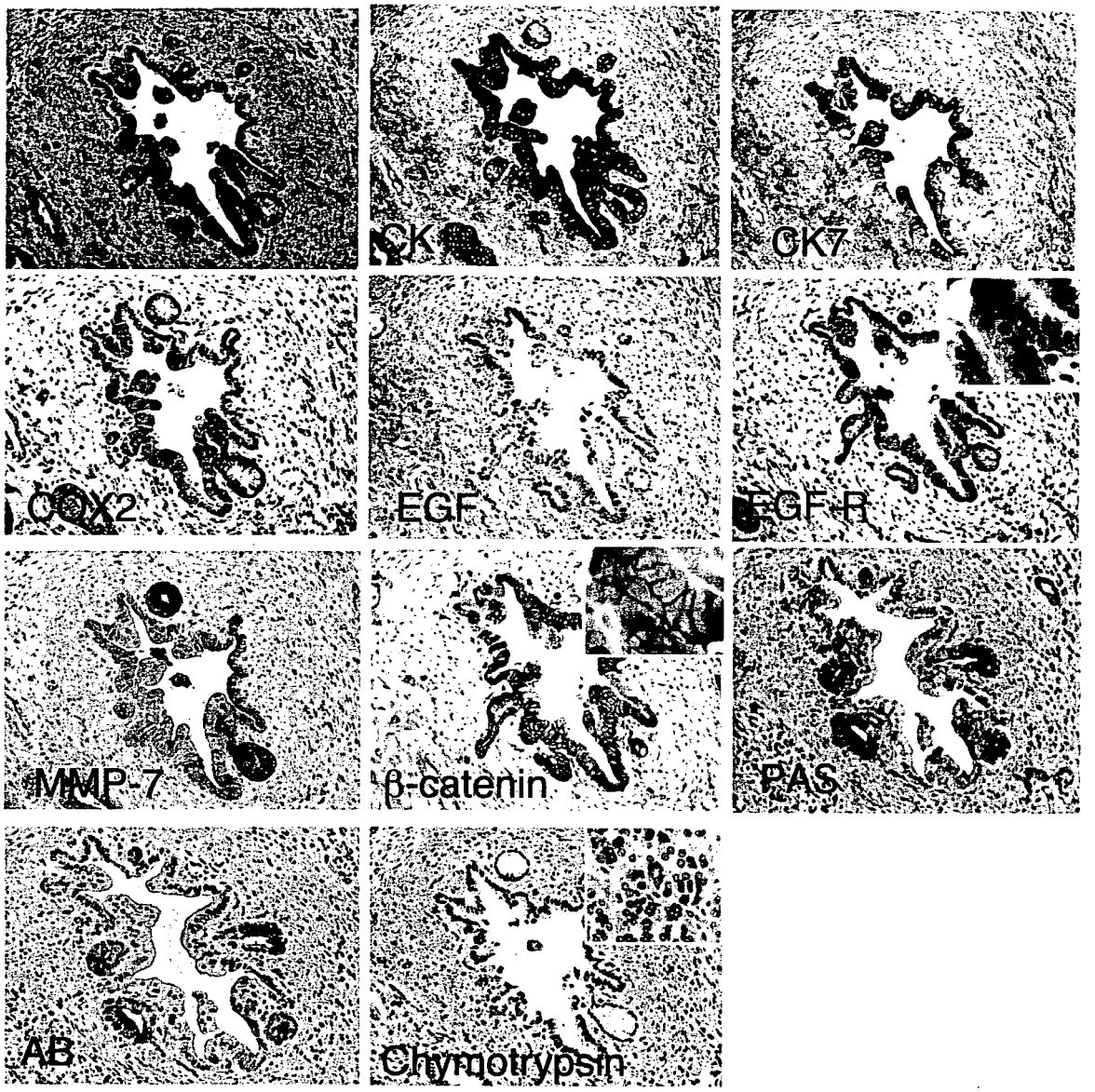

Fig. 4. (×66) Semi-serial sections through an intraductal proliferative lesion in a pancreatic duct. The lesion is positive for CK19 and CK7 and shows increases in EGF, EGFR, COX2 and MMP-7 (insets are ×5 higher magnification). The cellular arrangement is irregular, as can be seen with β-catenin staining, partly exhibiting papillary formation resembling PanIN-3 lesions. Some epithelial cells have mucinous material positive for PAS and AB staining. Chymotrypsin is negative (inset, acini on the same slide).

in human pancreatic adenocarcinomas (8,43–46). We assessed the expression profile of *Madh4, p21-Waf1/Cip1, p53, Mdm2, p19-Arf, Rb, p16-Ink4a, cyclin D1, cyclin D2* and *cyclin D3* in pancreatic adenocarcinomas from AxCANCre-injected animals and pancreatic tissue from AxCAwt-injected animals (control pancreas) using PCR (Figure 10).

Madh4 (MAD homolog 4), also known as Smad4 and Dpc4, is an important regulator of the transforming growth factor-β (TGF-β) signaling pathway (47). In tumor tissue, expression of *Madh4* was depressed. Decreased expression of *Madh4* is expected to depress differentiation and anti-proliferative signaling by TGFβ, resulting in enhanced growth.

p21-Cip1/Waf1 (cyclin-dependent kinase inhibitor 1A, Cdkn1a), an inhibitor of cyclin-dependent kinases (48,49), is another protein important in antiproliferative pathways (50,51). Like *Madh4*, expression of *p21-Cip1/Waf1* was depressed in tumor tissue, and, as with *Madh4*, the expected consequence of decreased *p21-Cip1/Waf1* expression is enhanced proliferation.

p53 signaling is believed to be disrupted in virtually all metastatic cancers (52–55). In contrast to *Madh4* and *p21-Cip1/Waf1*, we could not detect any obvious downregulation

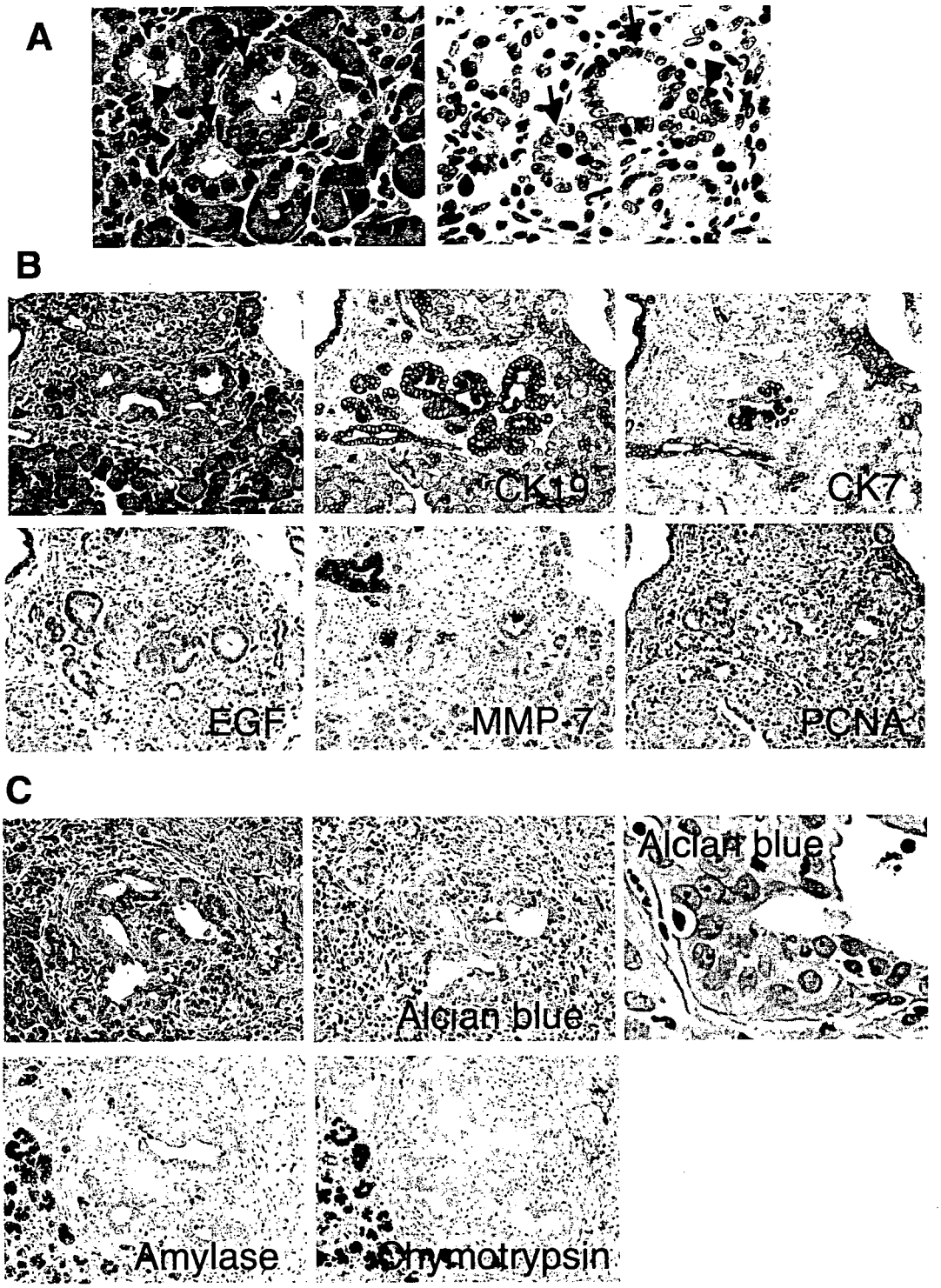

Fig. 5. (**A**) (×132) Proliferation of intercalated duct (arrows) and centroacinar cell lesions (arrowheads) (left). Cells exhibit incomplete duct-like arrangements with slight nuclear atypia. An obvious increase in PCNA labeling was observed (right). (**B**) (×66) These atypical ductular proliferative lesions exhibit compression of the surrounding acinar structure. The cells in the lesions were variably positive for CK19, CK7, EGF, MMP-7 and PCNA. (**C**) (×66) Another view of an atypical ductular proliferative lesion. The higher magnification (×264) panel clearly shows that AB is weakly positive in the cytoplasm in some cells. Amylase and chymotrypsin are negative.

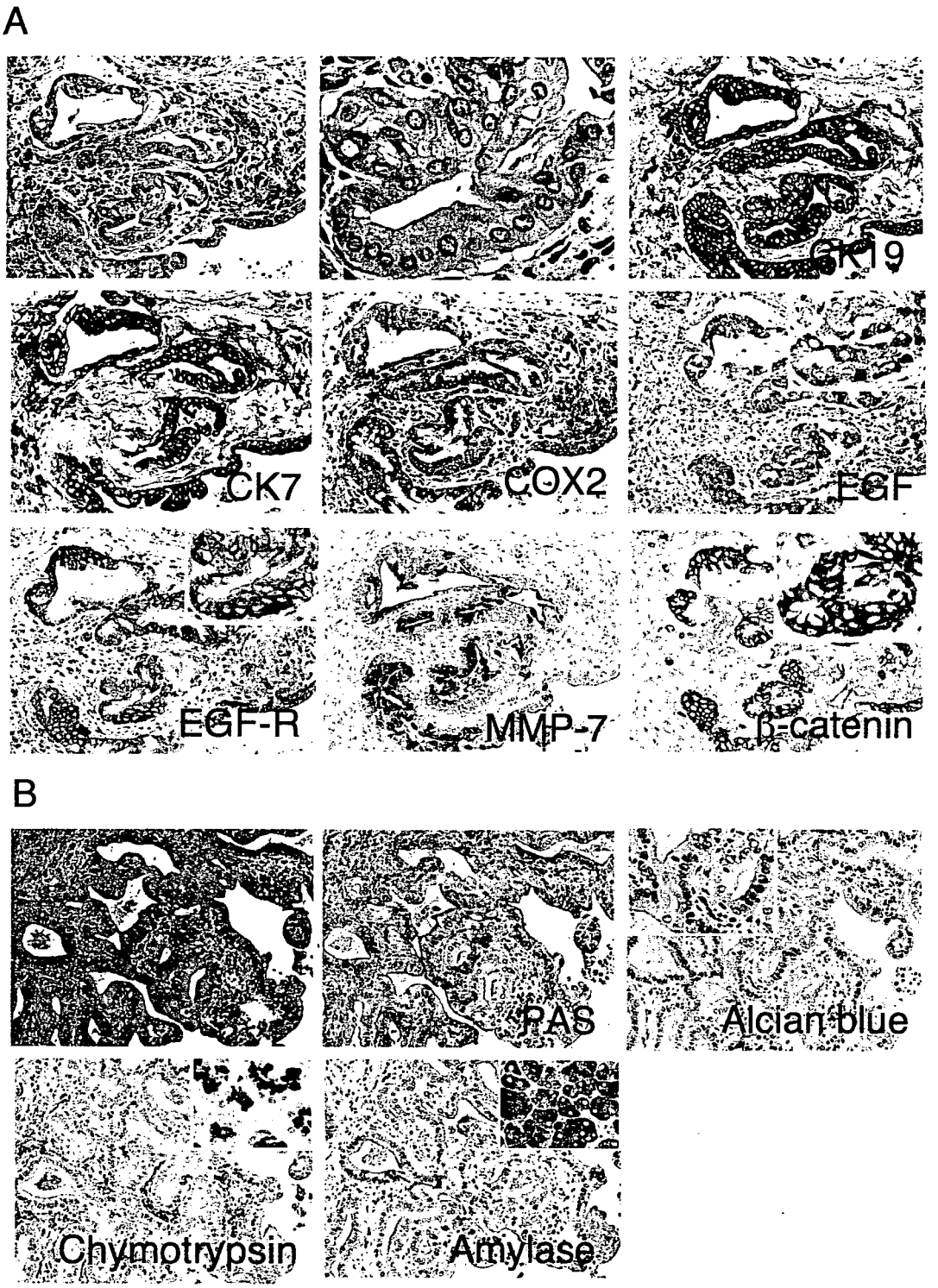

Fig. 6. In larger lesions, cells with moderate nuclear atypia exhibit a fusiform arrangement. (A) (×33) Many of the component cells were positive for CK19, CK7, EGF, EGFR, COX2 and MMP-7. Middle top (×123) is a higher magnification view of left top. In contrast to acinar cells, although the cytoplasm is slightly eosinophilic, no granular material was observed. β-catenin is localized primarily in the cell membrane, but is also seen in the cytoplasm. Insets (×66) are from the same lesion as the top middle panel. (B) (×33) Similar lesion as in A. The apical borders of the tumor cells are slightly positive for PAS and AB staining (inset ×66), but entirely negative for chymotrypsin and amylase [insets are intact acini from the respective slides (×66)].

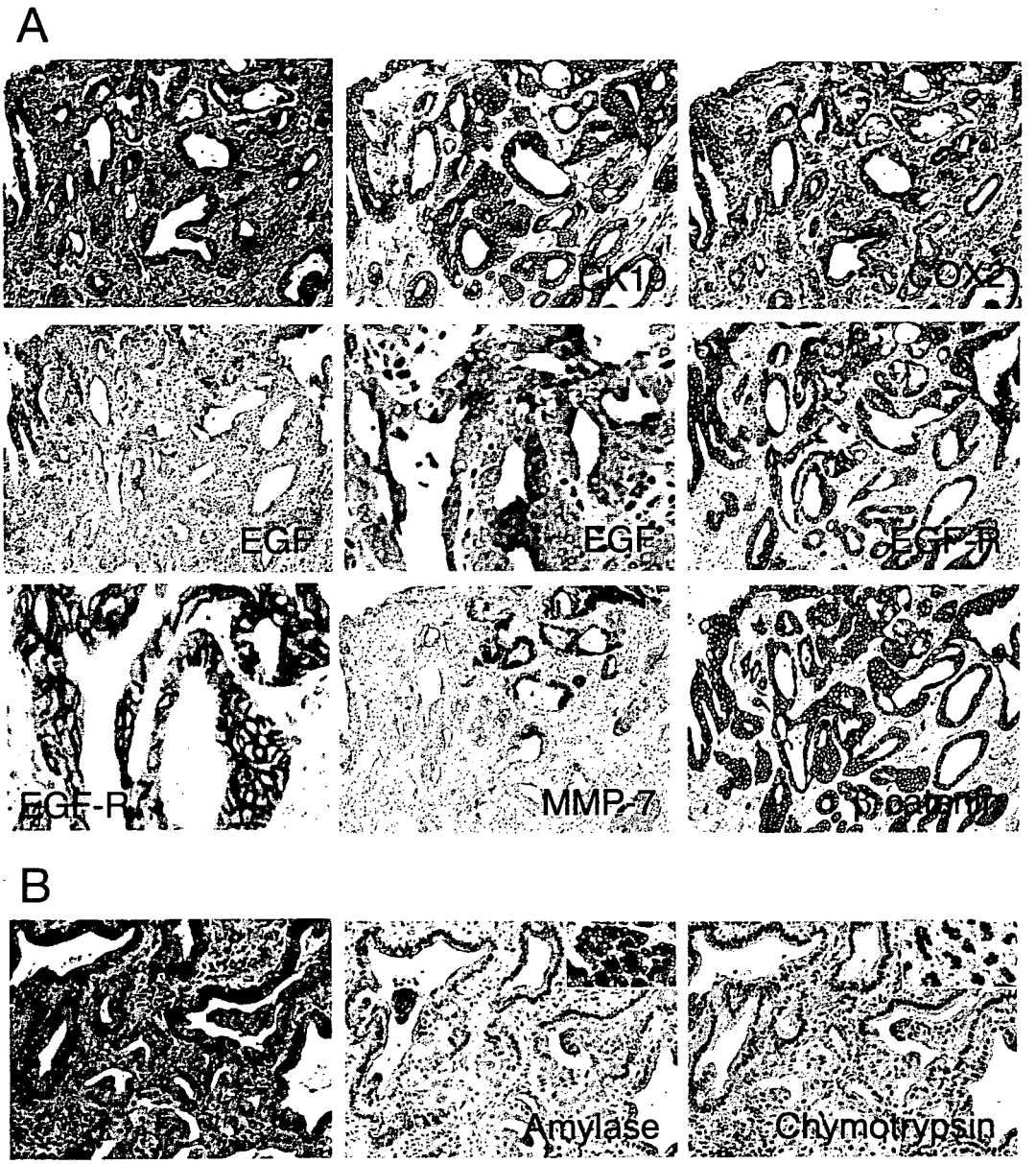

Fig. 7. (A) (×33) Moderately differentiated adenocarcinoma, exhibiting characteristics similar to those in Figure 6. Fusiform glands are irregularly arranged and surrounded by fibrous tissue resembling a desmoplastic morphology. Many of the component cells were positive for CK19, COX2, EGF (×33 and ×99), EGFR (×33 and ×99) and MMP-7. β-catenin is localized primarily in the cell membrane. (B) (×66) Similar lesion as in A. Carcinoma cells are negative for both chymotrypsin and amylase. Inset (×66) are acini from the respective slides.

of *p53* in tumor tissue. Two key regulators of p53 are Mdm2 (56,57) and p19-Arf (57). Mdm2 promotes p53 protein degradation and p19-Arf stabilizes the p53 protein. *Mdm2* and *p19-Arf* expression levels were unchanged in the tumor tissue. Therefore, in the pancreatic adenocarcinomas that developed in the Hras250 animals, the p53 signaling pathway is likely to be intact.

Inactivation of the retinoblastoma (Rb) signaling pathway is also thought to be a central theme in tumorigenesis (52–55). As with *p53*, there was no consistent change in *Rb* expression in tumor tissue compared with control pancreas. Important components of the Rb signaling pathway are the D-type cyclins and their associated kinase subunits, CDK4 and CDK6, and p16-Ink4a (cyclin-dependent kinase inhibitor 2A; Cdkn2a). Cyclin D-CDK4/6 complexes phosphorylate Rb and promote movement through the cell cycle (58), while p16-Ink4a binds to CDK4 and CDK6, inhibiting their activation by D-type cyclins, and represses movement through the cell cycle (59). Defects in *Ink4a/Arf* signaling have been shown to be important in pancreatic carcinogenesis: mutations in the *Ink4a/Arf* locus resulting in a null phenotype are frequently observed in human pancreatic cancers and are connected with a worse prognosis (43–46,60), and expression of K-ras^{G12D} in *Ink4a/Arf* null pancreatic progenitor cells results in induction of