Expression of an Intestine-Specific Transcription Factor (CDX1) in Intestinal Metaplasia and in Subsequently Developed Intestinal Type of Cholangiocarcinoma in Rat Liver

Ping Ren,* Debra G. Silberg,[†] and
Alphonse E. Sirica*

From the Department of Pathology,* Division of Cellular and Molecular Pathogenesis, Medical College of Virginia, Campus of Virginia Commonwealth University, Richmond, Virginia; and the Department of Medicine,[†] Division of Gastroenterology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

CDX1 is a caudal-type homeobox intestine-specific transcription factor that has been shown to be selectively expressed in epithelial cells in intestinal metaplasia of the human stomach and esophagus and variably expressed in human gastric and esophageal adenocarcinomas (Silberg DG, Furth EE, Taylor JK, Schuck T, Chiou T, Traber PG: Gastroenterology 1997, 113: 478–486). Through the use of immunohistochemistry and Western blotting, we investigated whether CDX1 is also uniquely associated with the intestinal metaplasia associated with putative precancerous cholangiofibrosis induced in rat liver during furan cholangiocarcinogenesis, as well as expressed in neoplastic glands in a subsequently developed intestinal type of cholangiocarcinoma. In normal, control adult rat small intestine, specific nuclear immunoreactivity for CDX1 was most prominent in enterocytes lining the crypts. In comparison, epithelium from intestinal metaplastic glands within furan-induced hepatic cholangiofibrosis and neoplastic epithelium from later developed primary intestinal-type cholangiocarcinoma each demonstrated strong nuclear immunoreactivity for CDX1. CDX1-positive cells were detected in hepatic cholangiofibrotic tissue as early as 3 weeks after the start of chronic furan treatment. We further determined that the percentages of CDX1-positive neoplastic glands and glandular nuclei are significantly higher in primary tumors than in a derived, transplantable cholangiocarcinoma serially-propagated *in vivo*. Western blotting confirmed our immunohistochemical results, and no CDX1 immunoreactivity was detected in normal adult rat liver or in hyperplastic biliary epithelial cells. These findings indicate that CDX1 is specifically associated with early intestinal metaplasia and a later developed intestinal-type of cholangiocarcinoma induced in the liver of furan-treated rats. (*Am J Pathol* 2000, 156:621–627)

Intestinal metaplasia in the intra- and extrahepatic biliary tract has been reported to be associated with cholangiocarcinoma and gallbladder cancer in humans,^{1–5} and with biliary-tract adenocarcinoma induced in experimental rat^{6–8} and hamster⁹ models of cholangiocarcinogenesis. Intestinal metaplasia has also been detected in the livers of patients with hepatolithiasis¹⁰ or with choledochal cysts,¹¹ two conditions that are of increased risk for human cholangiocarcinoma development. It also has been found to be a common option of hepatic oval stem-like cells in relation to cholangiofibrosis induced in the liver of rats exposed to the carcinogen 2-acetylaminofluorene.¹² In addition, Hughes et al¹³ presented preliminary data demonstrating an intestinal antigen termed 17NM that is expressed in 44% of analyzed cases of cholangiocarcinoma in the livers of Thai patients from a region where infection with the liver fluke *Opisthorchis viverrini*, a causative agent for human cholangiocarcinoma, is endemic. In human gallbladder cancer, intestinal metaplasia has also been reported to be associated with a more aggressive phenotype.^{14,15} However, until now there have been no studies aimed at identifying possible genetic factors that may be regulating aberrant differentiation along the intestinal lineage in liver in association with human or experimental animal cholangiocarcinogenesis.

CDX1 is a caudal-related homeodomain intestine-specific transcription factor, whose gene was first identified in normal embryonic and adult mouse intestine.¹⁶ More recently, this 37-kd protein transcription factor was selectively localized by immunohistochemistry to mucosal epithelial nuclei of both normal adult mouse and human small intestine and colon.¹⁷ CDX1 has been hypothesized to play a regulatory role in intestinal differentiation during development, as well as in possibly contributing to maintaining the differentiated phenotype of the intestinal

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Address reprint requests to Dr. Alphonse E. Sirica, Department of Pathology, Medical College of Virginia Campus of Virginia Commonwealth University, P.O. Box 980297, Richmond, Virginia 23298-0297. E-mail: asirica@hsc.vcu.edu.

epithelial cell.^{17,18} That CDX1 may also be functioning as a candidate transcription factor in the development of intestinal metaplasia in the human stomach and esophagus is further suggested by the immunohistochemical findings of Silberg et al,¹⁷ who detected strong nuclear immunoreactivity for CDX1 in areas of Barrett's esophagus with intestinal metaplasia, as well as in epithelial cells in intestinal metaplasia of human gastric mucosa. These investigators further noted variability in expression of CDX1 in human gastric and esophageal adenocarcinomas, leading them to postulate more than one cellular pathway in the development of these carcinomas.

Based on these findings in human stomach and esophagus, we used immunohistochemistry, Western blotting, and the same anti-CDX1 antibody described by Silberg et al,¹⁷ to investigate whether CDX1 may also be selectively expressed in intestinal metaplasia associated with putative precancerous cholangiofibrotic tissue preferentially induced in the right/caudate liver lobes of rats during the early stages of furan cholangiocarcinogenesis.^{19,20} We further determined whether CDX1 expression may also be significantly associated with a primary intestinal type of cholangiocarcinoma also formed in the right/caudate liver lobes of rats after long-term furan treatment.^{7,8} In addition, we assessed the stability of CDX1 expression in the neoplastic glandular epithelium of a transplantable cholangiocarcinoma originated from a furan-induced tumor,^{7,21,22} as a function of increasing serial passages *in vivo*.

Materials and Methods

Reagents

Furan (99+%) was purchased from Aldrich Chemical Co., Milwaukee, WI. Phenylmethylsulfonyl fluoride, sodium fluoride, sodium orthovanadate, dithiothreitol, aprotinin, leupeptin, polyoxyethylene sorbitan monolaurate (Tween 20) and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co., St. Louis, MO. Hybond ECL nitrocellulose protein blotting membranes and ECL Western blotting detection reagents were obtained from Amersham Life Sciences, Arlington Heights, IL. The Vectastain Elite avidin-biotin-peroxidase (ABC) immunostaining kit and avidin-biotin blocking kit were purchased from Vector Laboratories, Burlingame, CA.

Animal Experimentation and Tissue Specimens

All animal experimentation was conducted according to National Institute of Health Guidelines for the Care and Use of Laboratory Animals and only after approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee (Animal Protocol 9703-2367). Young adult male Fischer 344 rats, 116–190 g, were purchased from Harlan Sprague-Dawley, Indianapolis, IN, and were used to generate the various tissue samples analyzed in this study. These included: 1) normal adult rat liver and small intestine; 2) massive bile ductular hyper-

plasia induced in livers of bile duct-ligated/furan-treated rats;^{23,24} 3) intestinal metaplasia associated with hepatic cholangiofibrosis, preferentially induced in the right liver lobe of rats administered furan in corn oil by gavage at a dose of 45 mg/kg, 5 times/week, over a 3- to 15-week period, as previously described;^{8,19,23} 4) discrete tumor mass of furan-induced primary intestinal-type of cholangiocarcinoma harvested at 16 months after initiation of the furan treatment,⁷ and a transplantable cholangiocarcinoma, designated E5A, which originated from a furan-induced primary tumor and which was serially passaged for up to 18 *in vivo* passages in the right inguinal fat pad of Fischer 344 recipient rats, as previously described.^{21,22} Three to four rats composed each experimental group, and, after removal, all tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until use.

Mucin Histochemistry and CDX1 Immunohistochemistry

Mucin-producing glands were identified in 6- μ m-thick cryostat tissue sections of furan-induced cholangiofibrotic tissue and of cholangiocarcinoma, using the periodic acid-Schiff staining reaction, and were verified in 5- μ m sections of corresponding paraffin-embedded tissues by the mucicarmine staining reaction, as previously described.^{19,20} Immunohistochemical detection of CDX1 protein was performed on 6- μ m-thick, 4.0% paraformaldehyde-fixed cryostat tissue sections according to our adaptation of the standard ABC method of Hsu et al,²⁵ as previously described,^{7,22} and using the affinity-purified primary antibody termed Cdx1-CPSP.¹⁷ This antibody was generated in rabbit against a synthetically produced peptide encoding the carboxy terminus of mouse CDX1 (PSPVPVKEEFLP) corresponding to amino acids 257–268, as described by Silberg et al.¹⁷ Optimum immunostaining was obtained by incubating tissue sections overnight at 4°C with a 1:200 dilution of Cdx1-CPSP.

CDX1 Western Blot Analysis

Western blotting was carried out as previously described^{21,22} on whole-tissue protein extracts prepared from 1) normal adult rat liver; 2) normal adult rat small intestine; 3) a highly purified cell fraction of hyperplastic bile ductular epithelial cells isolated from the liver of a bile duct-ligated/furan-treated rat;²⁶ 4) furan-induced primary cholangiocarcinoma; and 5) E5A transplantable rat cholangiocarcinoma at *in vivo* passages 1, 5, 11, and 18, respectively. Briefly, lysates were prepared by homogenizing tissue or cell samples at 4°C in RIPA buffer containing 1.0 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium fluoride, 0.1 mmol/L sodium orthovanadate, 0.5 mmol/L dithiothreitol, 5 μ g/ μ L aprotinin, and 5 μ g/ μ L leupeptin. Samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C in a Sorvall RC-5B Refrigerated Super-speed Centrifuge, and supernatant was collected. For each sample, 50 μ g of extracted protein along with a cocktail of molecular weight standards were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electro-

phoresis and then transferred to the nitrocellulose membrane. Individual membranes were blocked for 2 hours at room temperature with blocking buffer containing 5% nonfat dry milk in 10 mmol/L Tris, pH 8.0, 165 mmol/L NaCl, 0.05% Tween 20. Membranes were incubated with a 1:200 dilution of Cdx1-CPSP affinity-purified antibody in the solution of 10 mmol/L Tris, pH 8.0, 165 mmol/L NaCl, 0.05% Tween 20, for 2 hours at room temperature. After rigorous washing, the membranes were then incubated for 1 hour at room temperature with a 1:10,000 dilution of secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA). Enhanced chemiluminescence was used to develop the blot, according to instructions provided with the Amersham ECL kit.

Quantification of CDX1-Positive Glands and Nuclei and Statistical Analysis

Percentages of CDX1-positive glands were determined from microscopic counts of glands immunoreactive for CDX1 over total glands observed in random areas of immunohistochemically stained preparations of furan-induced hepatic cholangiofibrotic tissue, primary cholangiocarcinomas, and transplantable cholangiocarcinomas at different stages of serial passage *in vivo*. Similarly, the percentages of CDX1-positive nuclei were calculated from microscopic counts of nuclei showing positive CDX1 immunostaining over the total nuclei counted in random tissue section areas of normal rat small intestine crypts and villi, in metaplastic glands of furan-induced cholangiofibrotic tissue, and in neoplastic glands of primary and transplantable rat cholangiocarcinoma. Each data point was determined from tissue sections prepared from three rats per group, with total gland counts made ranging from 934 to 1878 and total nuclear counts ranging from ~1600 to ~3500. In addition, measurements of mucin-positive and CDX1-positive glands in hepatic cholangiofibrotic tissue at different time points of furan treatment were performed on mirror cryostat tissue sections prepared from the same tissue sample. Tissue area measurements in square millimeters were facilitated by use of a High Resolution Video Imaging System (Olympus Corp., Melville, NY), using Image-Pro Plus software from Media Cybernetics, Silver Spring, MD. Student's *t* test was used to determine statistical significance.

Results

As was recently shown for human and mouse small intestine,¹⁷ positive immunohistochemical staining with Cdx1-CPSP was also detected in the nuclei of the enterocytes of normal adult rat small intestine, with a significantly greater percentage of CDX1-positive nuclei ($P \leq 0.007$) having been detected in the small intestinal crypts than at the tips of the villi or upper one third of the surface epithelium (Figure 1). In comparison, no CDX1 immunoreactivity was detected in the hepatocytes, bile duct epithelium, or other cell types of normal adult rat liver (Fig-

Figure 1. Distribution of CDX1-positive nuclei in enterocytes lining crypts and villi of normal adult rat small intestine. Percentages were determined from random counts made on cryostat tissue sections of small intestine from three separate rats. Total enterocyte nuclei analyzed, 1697. Each value represents the mean $\pm$ SD.

ure 2A), nor in hyperplastic bile ductular epithelium massively induced in the liver of bile duct-ligated/furan-treated rats (data not shown). Hyperplastic biliary epithelium and hepatocytes in livers of rats treated with furan alone without bile duct ligation also did not show detectable immunohistochemical staining for the CDX1 protein (Figure 2B). In sharp contrast, CDX1-positive cells were detected in hepatic cholangiofibrotic tissue as early as 3 weeks after the start of chronic furan treatment (Figures 2C and 3), and became a predominant feature of metaplastic glandular structures associated with cholangiofibrosis after longer-term furan treatments (Figures 2D and 3), as well as of neoplastic glands in subsequently developed primary cholangiocarcinoma (Figure 2E). Neoplastic glands of transplantable cholangiocarcinoma originating from a furan-induced tumor also exhibited a more variable CDX1 nuclear immunoreactivity during early *in vivo* passages than those of primary tumor, as exemplified by the photomicrograph (Figure 2F).

Histochemically detected mucin-producing intestinal metaplastic glands per square millimeter of cholangiofibrotic tissue, formed in the right liver lobe of rats at

Figure 2. Immunohistochemical demonstration of CDX1 protein in furan-induced rat putative precancerous hepatic cholangiofibrotic tissue and associated primary and transplantable rat cholangiocarcinoma compared with normal adult rat liver and hyperplastic intrahepatic biliary epithelium. **A:** Representative photomicrograph demonstrating absence of CDX1 immunoreactivity in hepatocytes (h) and cell types of the portal tract (p) of normal adult rat liver (original magnification, $\times 132$). **B:** Hyperplastic biliary ducts (arrows) in portal area of liver from a 15-week furan-treated rat, showing no CDX1 immunoreactivity in nuclei of ductal epithelium (original magnification, $\times 132$). **C:** CDX1-positive cells appearing in rat liver after 3 weeks of furan treatment (original magnification, $\times 132$). **D:** Strong nuclear immunoreactivity for CDX1 in metaplastic glands in cholangiofibrotic tissue induced in rat liver at 15 weeks of furan treatment (original magnification, $\times 66$). **E:** Representative neoplastic gland in furan-induced rat primary cholangiocarcinoma characterized by prominent nuclear immunoreactivity for CDX1 (original magnification, $\times 330$). **F:** Nuclear immunoreactivity for CDX1 detected in neoplastic glands of a transplantable cholangiocarcinoma at *in vivo* passage 5, which originated from a furan-induced primary rat liver tumor (original magnification, $\times 132$).

different time points after the start of chronic furan treatment, very closely paralleled immunohistochemically detected CDX1-positive glands in mirror cryostat sections of the same cholangiofibrotic tissue (Figure 3). Of further importance in assessing the identity of the CDX1-positive cells in furan-induced hepatic cholangiofibrosis is that no significant differences were noted between the numbers of mucin-positive glands and corresponding numbers of CDX1-positive glands counted per square millimeter of cholangiofibrosis tissue area at the different time points analyzed.

CDX1 nuclear immunostaining was observed to be most prominent in the metaplastic glands in advanced cholangiofibrotic tissue and in neoplastic glands of pri-

mary cholangiocarcinoma, with the percentages of both CDX1-positive glands and glandular nuclei becoming significantly diminished in transplantable cholangiocarcinoma by *in vivo* passage 5, compared with primary tumor, and not detected in neoplastic glands of transplantable cholangiocarcinomas at *in vivo* passages 11 and 18 (Figures 4, A–C, and 5). It is interesting that the loss of CDX1 immunoreactivity appeared to correlate with a marked decrease in histochemical reactivity for mucin as a function of increased *in vivo* serial passaging of the transplantable cholangiocarcinoma relative to the primary tumor. Specifically, neoplastic glands in primary cholangiocarcinomas were observed to exhibit strong histochemical reactivity for mucin, exemplified by the photomicrographs in Figure 4 (D and F), whereas a majority of the neoplastic glands in transplantable cholangiocarcinoma at *in vivo* passage 18 showed either weak or no immunoreactivity for mucin (Figure 4F).

The Western blots (Figure 6) validate our immunohistochemical results and further demonstrate that CDX1 protein is not expressed in normal adult rat liver or in isolated rat hyperplastic bile ductular epithelial cells. However, compared with normal adult rat small intestine as a positive control, CDX1 protein is specifically detected by Western blotting in lysates from furan-induced putative precancerous hepatic cholangiofibrotic tissue, characterized by prominent intestinal metaplasia, as well as in lysates prepared from the primary intestinal type of cholangiocarcinoma and derived transplantable tumors at least up to *in vivo* passage 5, but not detected at *in vivo* passages 11 and 18, respectively.

Figure 3. Time course correlating the appearance of mucin-positive glands (**A**) with CDX1-positive glands (**B**) formed in rat liver at different times of furan exposure. Each value represents the mean $\pm$ SD determined from measurements made on serial cryostat tissue sections of right liver lobe that were individually obtained from three rats per time point and separately reacted for mucin and for CDX1 protein, respectively.

Figure 4. CDX1 nuclear immunoreactivity compared with histochemically positive mucin in neoplastic glands of furan-induced primary and derived transplantable rat cholangiocarcinoma. Representative photomicrographs demonstrating CDX-1 nuclear immunoreactivity in a neoplastic gland of a primary cholangiocarcinoma (A), in a neoplastic gland of transplantable rat cholangiocarcinoma at *in vivo* passage 5 (B), and in neoplastic glands of transplantable rat cholangiocarcinoma at *in vivo* passage 18 (C). Representative photomicrographs demonstrating histochemically detected mucin (m) in neoplastic glands of primary cholangiocarcinoma (D and E) and of transplantable rat cholangiocarcinoma at *in vivo* passage 18 (F). Original magnifications: A and B, $\times 330$; C, E, and F, $\times 132$; D, $\times 66$.

Discussion

Our findings demonstrate for the first time that, in the furan rat model of hepatic cholangiocarcinogenesis, the intestinal-specific transcription factor CDX1 is distinctively expressed in the epithelial cells of both early appearing intestinal metaplasia associated with cholangiofibrosis in right/caudate liver lobes and in the later

developed intestinal type of cholangiocarcinoma that originated in the same liver lobes. Previously, we have shown that the cellular composition of intestinal metaplastic glands that formed by 3–4 weeks in right/caudate liver lobes of furan-treated rats closely resembles that of the crypts of Lieberkühn of normal adult rat small intestine.²⁰ This similarity in cellular composition is strongly reinforced by the fact that nuclear immunoreactivity for CDX1 was observed by us to be most prominent in both intestinal metaplastic glands in liver from furan-treated

Figure 5. Percent CDX1-positive glands and associated glandular nuclei determined for furan-induced primary rat cholangiocarcinoma (pChC) harvested at 16 months after initiation of furan treatment, and derived transplantable cholangiocarcinoma at different *in vivo* passages (E5Ap5, E5Ap11, and E5Ap18) compared with cell types of normal adult rat liver (NL), hyperplastic bile ductular epithelial cells (HBDE) induced in the liver of a bile duct-ligated/furan-treated rat, and intestinal metaplastic glands (IM) in 15-week furan-induced precancerous hepatic cholangiofibrotic tissue. Each value represents the mean $\pm$ SD determined from random counts made on immunohistochemically stained cryostat tissue sections as detailed in the Materials and Methods. Nuclear counts were made in random fields on a total of 30 glands per cholangiofibrotic tissue or cholangiocarcinoma samples analyzed. IM nuclei versus pChC nuclei, $P \leq 0.001$; pChC nuclei versus E5Ap5 nuclei, $P \leq 0.001$.

Figure 6. Western blot analysis of CDX1 protein expression in rat primary cholangiocarcinoma (pChC) and in serially transplanted cholangiocarcinoma (E5A p1, p5, p11, and p18) compared with normal adult rat liver (NL) and small intestine (SI), hyperplastic bile ductular epithelial cells (HBDE) isolated from the liver of a bile duct-ligated/furan-treated rat, and furan-induced hepatic precancerous cholangiofibrotic tissue containing intestinal metaplastic glands (IM). RIPA, no tissue buffer control. A and B represent two separate Western blots.

rats and in crypts of normal adult rat small intestine, compared with epithelial cells lining the middle and upper regions of small intestinal villi.

Strong histochemical reactivity for mucin is an important and characteristic phenotypic feature of intestinal metaplastic glands in hepatic cholangiofibrotic tissue and of neoplastic glands of the intestinal-type of cholangiocarcinoma induced in rat liver by furan.^{8,19,20} As demonstrated by our time course data (Figure 3), the appearance of CDX1-positive glands in liver at different weeks of exposure of rats to furan clearly mirrored that of mucin-positive glands formed in the same analyzed tissue, strongly suggesting that CDX1 expression is essentially localized to the mucin-producing glands in the furan-induced hepatic cholangiofibrotic tissue.

The protein product of the chicken homolog caudal-type homeobox gene termed *Chox-cad* has been reported to be detected by immunohistochemistry in bile ductular and endothelial cells, as well as in zone 1 hepatocytes and putative hepatic progenitor cells in regenerating chicken liver at 6 and 18 hours after partial hepatectomy.²⁷ In contrast, it is noteworthy, concerning cellular specificity, that we did not detect CDX1 nuclear immunoreactivity in the parenchymal and nonparenchymal cell types of normal adult rat liver, nor in hyperplastic biliary cells/oval cells or hepatocytes in rat liver after furan treatment alone, nor in hyperplastic bile ductules massively induced in the liver of bile duct-ligated/furan-treated rats.

We previously postulated that, in the furan rat model of cholangiocarcinogenesis, the intestinal metaplasia and associated cholangiofibrosis formed in the liver do not simply reflect reactive changes, but are directly related to the subsequent development of a primary hepatic intestinal-type of cholangiocarcinoma.^{7,21} This is supported by the following cumulative findings: 1) like the earlier appearing intestinal metaplastic glands in putative precancerous hepatic cholangiofibrotic tissue, the neoplastic glands of the primary intestinal-type tumors induced by furan in rat liver also exhibit evidence of goblet cell, Paneth cell, and serotonin-positive neuroendocrine cell differentiation^{7,8}; 2) the specific immunochemical data generated in this study clearly demonstrate that CDX1 expression is also a prominent feature of the mucin-producing neoplastic glands of furan-induced primary hepatic tumors; 3) both intestinal metaplastic glands in hepatic cholangiofibrotic tissue and neoplastic glands in subsequently developed primary hepatic intestinal-type cholangiocarcinomas exhibit proliferating cell nuclear antigen-labeling indices that are three- to fourfold higher than those determined for hyperplastic biliary ducts/ductules induced by furan in rat liver²²; and 4) both earlier appearing intestinal metaplastic glands and later developed neoplastic glands of resulting cholangiocarcinomas were recently shown by us to be characterized by coordinate overexpression of c-Met and c-Neu receptor tyrosine kinases when compared with normal and hyperplastic rat intrahepatic biliary epithelia.²²

Possible relationships between CDX1 expression and some of the growth-related properties described above now need to be investigated. It is possible that CDX1 may

be directly or indirectly affecting intestinal metaplastic cell proliferation during furan cholangiocarcinogenesis, as suggested by the fact that CDX1 nuclear immunoreactivity is primarily localized to crypt cells forming the proliferative compartment of intestine. This would be consistent with our observation that crypt-like intestinal metaplastic glands induced in rat liver by furan exhibit significantly higher proliferating cell nuclear labeling indices than those of hyperplastic bile ductules in liver of bile duct-ligated or furan-treated rats.²² Moreover, it has been shown that a decrease of CDX1 expression by antisense mRNA, together with overexpression of another intestinal homeobox gene, CDX2, results in an inhibition of cell proliferation in modified cultured Caco2 human colonic adenocarcinoma cells,²⁸ suggesting that the ratio of CDX2 to CDX1 transcription factors may be important for determining the degree of proliferation versus differentiation shown by enterocytic cells. Conditional expression of CDX2 in IEC-6 cells, an undifferentiated intestinal cell line, was also demonstrated to inhibit cell proliferation, followed by a period of growth resulting in multicellular structures exhibiting morphological features of both enterocyte-like and goblet-like cells.²⁹

Our immunohistochemical data indicate that CDX1 is a useful marker for selectively delineating intestinal metaplasia and primary intestinal-type cholangiocarcinoma compared with hyperplastic biliary ducts/ductules. In addition, progressive loss of CDX1 nuclear immunoreactivity in neoplastic glandular epithelium of transplantable cholangiocarcinoma as a function of increasing serial passage *in vivo* appeared to be linked to a less differentiated phenotype, as supported by a marked decrease in mucin reactivity in the cancerous glands of late-passage tumor compared with those of primary cholangiocarcinoma. Similarly, nuclear expression of CDX1 protein was demonstrated to be progressively decreased in colonic epithelial cells in human adenomatous polyps and adenocarcinomas, compared with normal crypts in the same histological specimen.¹⁷ Likewise, CDX2 expression was reported to be markedly reduced in high-grade dysplasia and invasive carcinoma appearing in the later stages of both human and rat colorectal carcinogenesis,³⁰ and both CDX1 and CDX2 mRNA expression was shown to be down-regulated in human colorectal carcinogenesis.³¹

Recently, we described the establishment and partial characterization of a novel rat cholangiocarcinoma cell line from tumorigenic cells isolated from a transplantable furan-induced tumor at *in vivo* passage 12.³² At present, our preliminary data indicate that, like the parent tumor, this cultured rat cholangiocarcinoma cell line does not exhibit nuclear CDX1 immunoreactivity. We also now have preliminary immunohistochemical data demonstrating CDX2 nuclear immunoreactivity in neoplastic epithelium of the primary intestinal-type of cholangiocarcinoma induced by furan in rat liver. We are currently investigating CDX2 expression relative to that of CDX1 during furan cholangiocarcinogenesis and are planning to conduct transfection experiments using our cholangiocarcinoma cell line to directly assess the roles played by CDX1 compared with CDX2 in regulating neoplastic cell growth

versus intestinal differentiation in intestinal-type cholangiocarcinoma.

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