HEPATOCYTIC DIFFERENTIATION OF NORMAL BUT NOT NEOPLASTIC CULTURED RAT PANCREATIC DUCT CELLS

JIM-RAY CHEN

Department of Pathology
McGill University, Montréal

July, 1995

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ISBN 0-612-08084-6

RÉSUMÉ

Cette thèse est consacrée à l'étude de certains aspects du potentiel de différentiation des cellules normales et néoplastiques (spontanément et par transformation chimique) du canal pancréatique du rat, mises en culture et soumises à l'influence de deux micro-environnements.

Cette étude se fait par implantation *in vivo* de cellules dans des sites intrapéritonéal et sous-cutané, méthode qui permet non seulement de révéler le potentiel intrinsèque des cellules implantées mais reflète également les effets du micro-environnement sur leur expression phénotypique.

Les résultats de l'étude indiquent que lorsque les cellules de culture normales et capables de se propager, dérivées de l'épithélium du canal pancréatique du rat adulte, sont implantées *in vivo*, elles développent les caractéristiques phénotypiques des hépatocytes et que l'ampleur de cette expression phénotypique dépend du micro-environnement dans lequel ces cellules sont implantées. Lorsqu'elles sont implantées dans un site sous-cutané, les cellules développent partiellement les caractéristiques des hépatocytes et conservent une partie de leur phénotype pancréatique. Par contre, lorsque les mêmes cellules sont implantées dans un site intrapéritonéal, elles expriment l'intégralité des propriétés phénotypiques des hépatocytes adultes. Les lignées cellulaires du canal pancréatique transformées spontanément et chimiquement n'affichent aucune des caractéristiques phénotypiques de la lignée hépatocytaire après implantation *in vivo*. Nous en concluons donc 1) que les cellules du canal pancréatique pourraient fort bien être les cellules souches des hépatocytes pancréatiques transdifférentiés; 2) que la transformation néoplastique de ces lignées cellulaires entraîne la disparition d'une partie ou de la totalité des voies biochimiques qui modulent la réponse cellulaire à la différentiation hépatocytaire dans ces cellules.

ABSTRACT

This thesis represents an effort to examine certain aspects of the differentiation potential of normal and neoplastic (spontaneously- and chemically-transformed) cultured rat pancreatic ductal cells under the influence of two microenvironments.

The technique of *in vivo* implantation of cells to subcutaneous and intraperitoneal sites is used as it not only reveals the intrinsic potential of the implanted cells but also reflects the effects of the microenvironment on phenotypic expression.

The results of these studies indicate that when implanted *in vivo* normal propagable cultured cells derived from the duct epithelium of adult rat pancreas develop phenotypic features of a hepatocyte, and that the extent of this phenotypic expression is influenced by the microenvironment in which these cells are implanted. When localized subcutaneously, the cells displayed partial differentiation toward hepatocytes but retained some of their ductal phenotype. In contrast, when the same cells were implanted intraperitoneally, they expressed the full phenotypic properties of mature hepatocytes. Both spontaneously- and chemically-transformed pancreatic ductal cell lines did not display phenotypic differentiation along the hepatocytic lineage after *in vivo* implantation. It is concluded that (1) pancreatic ductal cells can be the progenitor cell for pancreatic hepatocytes; (2) Neoplastic transformation of these cell lines results in partial or total loss of hepatoctyic differentiation.

Table of Contents

PREFACE

During embryogenesis, the pancreas arises from a protrusion of the foregut endoderm into the mesoderm to form a ramifying system of tubules. These primitive tubular epithelial cells then give rise to acinar or endocrine cells. The cells of the branching epithelium that do not differentiate into acinar or endocrine cells remain as the duct cells. *In vivo* studies have indicated that some duct cells retain the ability to differentiate into islet cells or hepatocytes under experimental manipulation. It is therefore postulated that a duct cell may represent a facultative stem cell in this organ. Direct evidence supporting this hypothesis has not been forthcoming, since temporal sequences of events and cause-and-effect relationships are difficult to determine in *in vivo* models. Direct proof requires the isolation and culture of duct cells from adult pancreas for these studies.

This thesis presents an effort to examine certain aspects of the differentiation potential of ductal cells derived from the adult rat pancreas. It relies on the ability (1) to isolate ductal fragments from adult Fischer-344 rat pancreas, (2) to propagate pure duct epithelial cells in monolayer culture, and (3) to generate a large number of clonal cell populations of these duct epithelia for biochemical and molecular characterization. Using these cells, the following hypotheses were tested:

(1) Normal pancreatic duct epithelial cells can form a propagable cell line with pancreatic-hepatobiliary "stem cell" properties.

(2) When implanted *in vivo*, these cultured normal epithelial cells will demonstrate further differentiation capacity, and the level

of differentiation will be modulated by the microenvironment in which they are placed.

(3) Following neoplastic transformation, these duct epithelial cells lose their differentiating capacity and retain their primitive phenotype.

This thesis is composed of 6 chapters:

Chapter 1 consists of a literature review in 5 sections. The first two sections present a review of pancreatic embryology and development, histology and anatomy Section 3 reviews the isolation techniques used, cultures and characteristics of pancreatic duct epithelium of different species. Section 4 emphasizes the differentiation capacity of duct cells, particularly the induction of pancreatic hepatocytes in *in vivo* models. Sections 5 reviews the general features of pancreatic cancer, animal models of pancreatic carcinogenesis, and histological classification of acinar neoplasms in the rat models. This chapter will provide the basic knowledge to understand the work reported in this thesis.

Chapter 2 illustrates a refined method of establishing a propagable cell line (RP-2) directly from an isolated duct fragment. The growth and phenotypic properties of RP-2 are characterized by various techniques.

Chapter 3 describes the fate of the RP-2 cells after implantation at two anatomic sites- subcutaneous tissue and intraperitoneal cavity, and the phenotypes of these cells *in vivo*.

Chapter 4 describes the growth and phenotypic characteristics of spontaneously- and chemically- transformed epithelial cells of ductal origin following subcutaneous and intraperitoneal implantation.

Chapter 5 consists of a general discussion and the conclusions of this thesis.

Chapter 6 gives perspectives for future work which may provide further information relevant to the studies presented in this thesis.

ACKNOWLEDGMENT

I wish to express my sincere appreciation to my supervisors, Drs. W.P. Duguid and M.-S Tsao, for initiating the project and providing continued support and guidance throughout the project and thesis preparation.

I wish to thank Dr. J. Shepherd (former graduate student of Department of Pathology) for providing tumor cell lines used in Chapter 5, Dr. S. Githens (University of New Orleans) for the protocols of histochemical staining and biochemical assay of carbonic anhydrase, Dr. N. Marceau (Laval University) for providing the anti-HES6, -BDS7, and -4G7 antibodies, Dr. L.A. Culp (Case Western Reserve University) for pRSV*lacZ* plasmid and the protocol for β-galactosidase histochemical staining, Dr. G. Yeoh (University of Western Australia) for anti-TAT antibody, Dr. J.K. Reddy (Northwestern University) for anti-amylase antibody, Dr. T. Nakamura (Kyushu University) for anti-HGF antibodies, and Dr. D.C. Lee (University of North Carolina at Chapel Hill) for the TGF-α cDNA probe. I also thank Dr. K.G. Nelson (NIEHS, Research Triangle Park) and Dr. A. Giaid for advice on *in situ* hybridization. I thank Ms C. Luty, A. Torrisi, and the staff members of Histology and Electronmicroscope Lab for their technical assistance. I thank Mrs S. Shepherd and Ms D. O'Connor for secretarial assistance.

I am also grateful to Dr. E. Zorychta (Director of Graduate Studies, Department of Pathology) for her encouragement during my Ph.D. program.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AEC	Aminoethyl Carbazole
AFP	Alpha-Fetoprotein
ALP	Alkaline Phosphatase
AZA	Azaserine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
BOP	*N*-nitrosobis(2-oxopropyl)amine
BHP	*N*-nitrosobis(2-hydroxypropyl)amine
CA	Carbonic Anhydrase
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleic Acid
D-PBS	Dulbeco's Phosphate Buffered Saline
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
GGT	Gamma-Glutamyl Transpeptidase
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid
HBSS	Hanks' Buffered Salt Solution
HGF	Hepatocyte Growth Factor
IGF	Insulin-like Growth Factor
ITPG	Isopropyl-β-D-Thiogalactopyranoside
LacZ	β-galactosidase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
M-MLV	Murine- Moloney Leukemia Virus
MOPS	3-[N-Morphoino-Propanesulfonic Acid

mRNA	messenger Ribonucleic Acid
NBT	Nitroblue Tetrazolium
RLEC	Rat Liver Epithelial Cells
RNA	Ribonucleic Acid
RT	Reverse Transcription
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
TAT	Tyrosine Aminotransferase
TGF-α	Transforming Growth Factor-aplha
TGF-β	Transforming Growth Factor-beta
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

CHAPTER 1 LITERATURE REVIEW

1.1 DEVELOPMENT AND DIFFERENTIATION OF THE PANCREAS

The purpose of this section is to review the normal sequence of pancreatic development and differentiation. This will provide the fundamental background, an understanding of which, is necessary to investigate alteration in pancreatic growth and development. Knowledge of pancreatic development has mainly been obtained from studies in rats, mice, and chickens, especially rats. This section, unless otherwise mentioned, deals primarily with the rat.

1.1-1 MORPHOLOGICAL DEVELOPMENT

Rat pancreatic development is initiated by dorsal evagination of foregut splanchnopleure at the level of the liver diverticulum on day 11 of gestation (20 somites). The ventral evagination appearing 12 hours later (28-30 somites) is a caudal bud of the hepatic diverticulum (Spooner et al., 1970; Pictet et al., 1972; Githens, 1986). Despite the 12-hour difference between the times of their appearance, they both exhibit indistinguishable patterns of morphological and biochemical differentiation. Nevertheless the dorsal and ventral rudiments differ in one respect. The former contains more glucagon cells and demonstrates a fivefold higher glucagon specific activity, but has fewer pancreatic polypeptide cells than the latter (Spooner et al., 1970; Orci, 1982). The characteristic pattern of pancreatic lobules separated by mesenchyme and drained by ducts appears on day 12 and is well developed at about day 16

and 17 when the two outpouchings fuse together to become a single pancreatic mass (Picket et al., 1972).

The epithelial cells of these evaginations proliferate as branching tubules in the surrounding mesenchyme of splanchnic mesoderm origin. They are called protodifferentiated cells (Githens, 1986). These protodifferentiated cells are ultrastructurally indistinguishable from other cells of the embryonic gut through day 14, with the exception of glucagon cells, which can be identified immunocytochemically on day 11 (Yoshinari and Daikoku, 1982) and exhibit their mature appearance on day 12 (Rall et al., 1973). Cytodifferentiation of acinar cells begins on day 15 (Parsa et al, 1969; Uchiyama and Watanabe, 1984) and zymogen granules are first seen on day 16 (Pictet et al., 1972). Endocrine (Githens, 1986) and acinar (Oates and Morgan, 1989) cells continue to differentiate from protodifferentiated cells in the ductal epithelium during the early postnatal period in rats, although cell division of the already differentiated cell types contributes substantially to the increase in the number of these cells. The cells of the branching epithelium that do not differentiate into endocrine or acinar cells become committed duct cells (Richardson and Spooner, 1977; Githens 1989). The proportion of duct cells in the exocrine cell population decrease sharply from 57% on postnatal day 2 to 13% on day 33. This is due to a more rapid rate of proliferation of acinar cells (Githens, 1989).

1.1-2 BIOCHEMICAL DIFFERENTIATION DURING DEVELOPMENT

Studies of prenatal and postnatal enzyme profiles show that

the accumulation of exocrine pancreatic enzymes and hormones is a two-phase process (Figure 1-1). The primary transition occurs at the onset of the formation of the pancreatic outpouching, during which the exocrine enzymes and insulin accumulate to detectable levels. The levels of products are maintained for a period of 2-3 days. At this time there is no evidence of cytodifferentiation of either exocrine or insulin cells; thus it is proposed that the specific products are distributed in all or at least in a large number of cells, each containing a small number of molecules of the exocrine enzyme and/or insulin. This initial period is followed by the protodifferentiated state lasting for several days. During this time, a rapid exponential growth of protodifferentiated cells accompanied by lobulation occurs without significant changes in the levels of exocrine enzymes and insulin. In the second transition period, the synthesis of exocrine enzymes and insulin increases dramatically by several orders of magnitude, coincident with the appearance of differentiated insulin and acinar cells. A postnatal tertiary transition involves a further modulation, usually a decrease in enzyme activity. The adult levels of secretory enzymes are acquired shortly after weaning (Rutter et al., 1968; Robberecht et al., 1971; Pictet and Rutter, 1972; Sanders and Rutter, 1974).

The prenatal and postnatal modulation of pancreatic exocrine enzymes are regulated by various factors. Glucocorticoids and thyroxine are two hormones that play pivotal roles in regulating the development of the pancreatic enzymes (Rall et al., 1977; Kumegawa, 1980). Gastrointestinal hormones including secretin, cholecystokinin, gastrin, and bombesin have been shown to regulate pancreatic

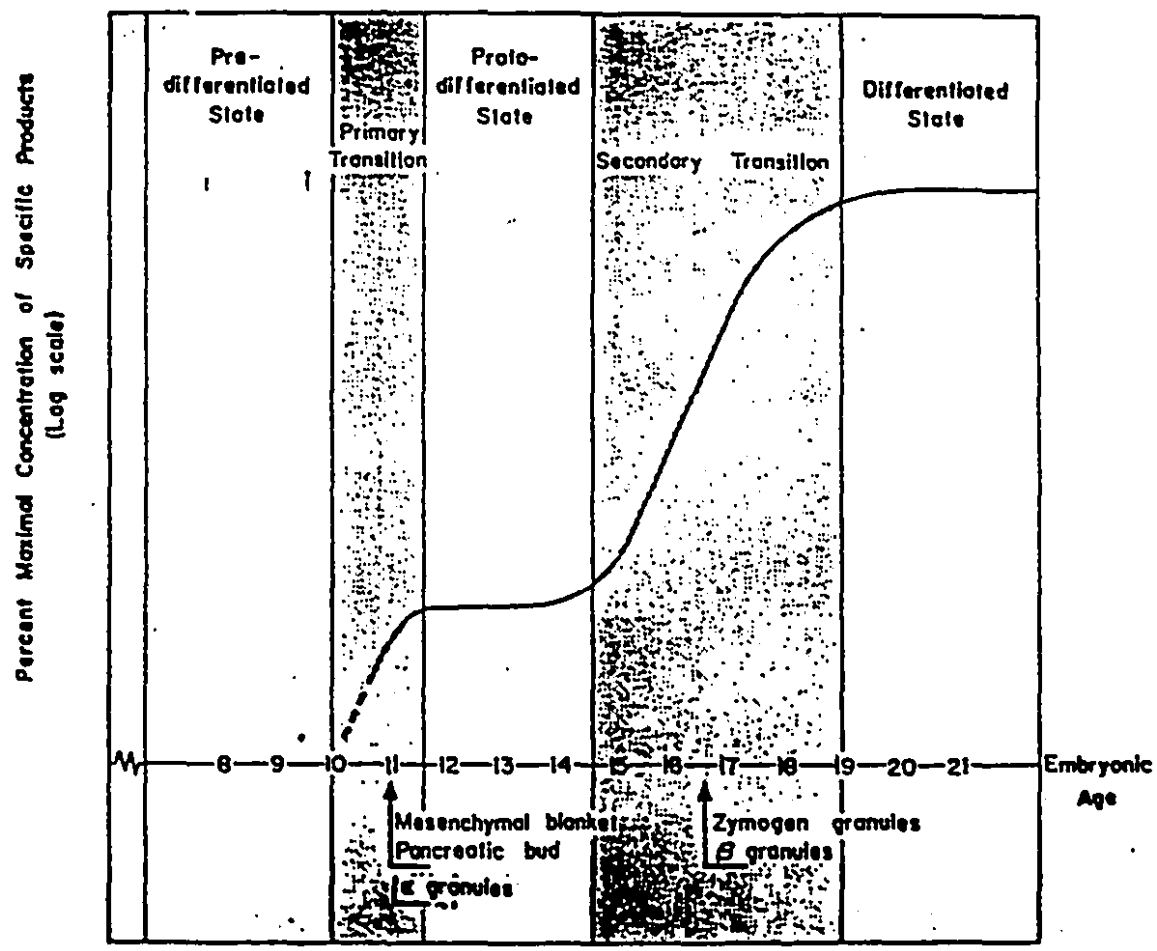

Figure 1-1 Model for the development of the exocrine pancreas and insulin cells in the rat (schematic). (From Pictet and Rutter, 1972, with permission).

secretion, cell replication as well as development (Mangino et al., 1992). Insulin is another important hormone that regulates pancreatic exocrine functions. In humans, insulin deficiency, as found in insulin-dependent diabetes, has been shown to be accompanied by functional abnormalities in the exocrine pancreas (Lee, 1939). Insulin also appears to be indispensable for the maintenance of rat pancreatic acinar cells (Logsdon, 1986) or fetal pancreata in culture (Parsa and Marsh, 1976). Nutrition is the most important exogenous factor, particularly when malnutrition affects development adversely. Changes in the levels of the exocrine enzymes at the time of weaning represent a physiologic response to the changes in dietary composition, switching from a diet high in fat to a diet high in carbohydrate (Lee et al., 1982).

1.1-3 ENDOCRINE DEVELOPMENT

Pancreatic endocrine tissue appears early during fetal development. By day 11, glucagon-containing cells are detectable in the early embryonic pancreas by electron microscopy (Pictet et al., 1972) and by immunocytochemistry (Yoshinari and Daikoku,1982). Insulin cells are first detected in buddings of the epithelium on day 12.5 by immunocytochemistry (Yoshinari and Daikoku,1982) and on day 15 by electron microscopy (Rall et al., 1973). Immunoreactive somatostatin cells appear first at the periphery of primitive islets on day 15.5 while PP cells first appear at birth (Sumnaer et al., 1977). Embryonic islet-like structures appear in the pancreatic diverticulum around day 15 in the rat and they obtain their definitive arrangements on day 18.5. Their size and number then rapidly

increase during the subsequent days, particularly during the first day of birth (Freie et al., 1975).

Very few markers of islet precursor cells have been reported and those which have been identified include tyrosine hydroxylase (TH), acid β-galactosidase (β-gal), and alkaline phosphatase (ALP). The finding that TH, the first enzyme of the catecholamine biosynthetic pathway, is transiently expressed in the differentiating islet cells led some investigators to suggest that pancreatic endocrine cells, like neural cells, are derived from neural crest (Pearse and Polak, 1971, Teitelman and Lee, 1987). On the basis of transgenic mice studies, Alpert and colleagues (1988) reported the controversial findings that not only do differentiating pancreatic endocrine cells synthesize TH but that developing neurons in the neural tube transcribe the insulin gene. This should imply a related molecular biological profile rather than a common histogenetic origin, since cultured endoderm and mesoderm of early rat embryos with the neural crest removed are still able to develop a pancreas with a normal complement of endocrine cells (Pictet et al., 1976). *In vitro* studies show that populations of fetal pancreatic cells committed to differentiation into mature endocrine cells evanescently co-express TH and high acid β-gal activity (Beattie et al., 1994). The expression of acid β-gal peaks at 18-24 weeks of gestation in man and declines to low levels in adult islets. Some cells of the primitive epithelium contain ALP and this enzyme activity also appears to be associated with the process of early endocrine cell development. As the primitive endocrine cells leave the duct epithelium system to form the islets of Langerhans, ALP activity disappears in the majority of

cells but is preserved in peripherally located cells of the adult islets (Githens, 1983). Whether the ALP positive cells are A or D cells remains unknown.

1.1-4 ROLE OF MESENCHYME IN DEVELOPMENT

It has already been noted that the embryonic pancreas is composed of an epithelial component of endodermal origin and a mesenchymal component of mesodermal origin. The mesenchymal component of the rat pancreas comprises as much as 55% of the organ volume on day 18 and declines to the adult proportion of less than 12% on postnatal day 30 (Githens, 1986). It has been long appreciated that an epithelial-mesenchymal interaction is necessary for pancreatic cytodifferentiation (Golosow and Grobstein, 1964) and this interaction can occur in the absence of cell contact (Grobstein, 1964). Furthermore fetal mesenchyme is important in regulating the proportion of cell types during development. 12-day embryonic epithelium cultured without mesenchyme can only give rise to endocrine cells, primarily glucagon cells. If mesenchyme is supplied, relatively normal development occurs, with acinar cells dominating (Pictet and Rutter, 1972). It also appears that specificity of the epithelial-mesenchymal interaction exists in different organs. Salivary gland mesenchyme is essential for successful development of embryonic salivary gland epithelium, whereas normal differentiation of primitive pancreatic epithelium can be induced by mesenchyme of various embryonic sources and extracts of entire chicken embryo (Ingber, 1993). A mesenchymal factor (MF) found in particulate fractions of homogenates of midgestational young rat

(Rutter et al., 1968) or chicken (Ronzio and Rutter, 1973) embryos stimulates DNA synthesis and amylase synthesis in embryonic rat pancreata stripped of their mesenchymal component. Older rat embryos or adult organ extracts are inactive. MF may be glycoprotein in nature, based on its sensitivity to periodate, but efforts to purify MF to homogeneity have been unsuccessful.

1.2 ANATOMY AND HISTOLOGY OF THE PANCREAS

1.2-1 ANATOMY OF THE PANCREAS

Animal models have an essential role in elucidating the etiology and pathogenesis of human diseases. The rodent models including rats, hamsters, and mice are particularly useful in studying the pathogenesis of various pancreatic diseases, the success of which is largely based on the extensive knowledge of the anatomy and histology of this species. In the following sections emphasis will be on the rodent species, especially rats, but comparison with the human will be mentioned.

In contrast to human pancreas which is located retroperitoneally, the pancreas of the rat is an intraperitoneal organ, which makes it easily accessible to experimental manipulation.

The rodent pancreas, a light pinkish organ, is composed of three distinct lobes. The gastric and splenic lobes are located anterior and posterior to the stomach respectively, and the duodenal lobe is situated lateral to the descending limb of the duodenum. These three segments are joined posteromedially near the proximal end of the duodenum to form the head of the gland.

Each lobe has one main duct. The duct of the duodenal lobe enters the common bile duct directly, while these of the gastric and splenic lobes meet in the head to form the pancreatic common duct. This structure in turn opens into the common bile duct. This duct enters the duodenum posterolaterally, passing initially through the head of the gland.

The exocrine pancreas is composed of compound tubuloalveolar glands (Pour, 1978) separated into lobules by fine connective tissue. Each microscopic pancreatic lobule is composed of a large population of acinar cells that synthesize a variety of digestive enzymes and a minor portion of duct cells that comprise the conduit system. The islets of Langerhans occupy part of the lobules. Lobular arterioles supply a larger proportion of blood to the islets and a smaller proportion directly to the exocrine tissue. All of the blood draining from the islets, however, passes through capillaries supplying exocrine tissue before exiting from the lobules via lobular venules. Thus the exocrine tissue immediately surrounding an islet normally is subjected to a very high concentration of islet hormones (Bendayan and Ito, 1979; Bockman, 1986; Williams and Goldfine, 1986)

1.2-2 ACINAR TISSUE

Acinar cells are recognized histologically by the presence of zymogen granules. The zymogen-containing acinar cells are pyramidal in shape and comprise most of the pancreatic parenchyma. They are traditionally visualized as being arranged in small spheroidal groups resembling bunches of grapes, each bunch

bordering a stem representing a termination of the duct system. However, the concept of rounded acini tagged to the duct system like grapes to the stem appears oversimplified. By using three-dimensional reconstruction and retrograde injection, it is concluded that the pancreatic architecture actually has a tubular arrangement. The branching tubules of various diameters curve acutely, anastomose, and end blindly (Bockman, 1976, 1980). The findings led some investigators to hypothesize that the so-called tubular complexes seen in human and experimental pancreatic cancers, cystic fibrosis, and pancreatitis are derived from acinar cells through the process of de-differentiation (Bockman, 1981; Flaks, 1984; Scarpelli, 1984).

Each acinus is surrounded by distinct basal laminae. Using specific monoclonal antibodies, it was shown that only laminin and collagen type IV are present in the basal laminae, while fibronectin and collagen type I are predominantly located in the interstitial connective tissue surrounding ducts and blood vessels (Kern, 1986).

Based on morphologic, biochemical, and physiologic differences, the pancreatic acinar tissue has been divided into periinsular and teleinsular regions. The periinsular acinar cells are larger and contain a higher concentration of zymogen granules. The periinsular tissue also has higher uptake of amio acids than the teleinsular tissue. Furthermore, the tissue in the two regions differ in their response to various secretagogues (Hellman et al., 1962; Malaisse-Lagae et al., 1975; Bendayan and Ito, 1979). Such differences may be due to the fact the periinsular exocrine tissue is subjected to higher concentrations of islet hormones. Both insulin (Palla et al.,

1968; Korc et al., 1981) and glucagon (Alder, 1977) have been shown to play a role in the regulation of the biosynthesis and secretion of acinar enzymes. The structural and functional partitions of the acinar tissue in relation to the islets of Langerhans seem to indicate that acinar/endocrine interaction occurs in the pancreas in normal and pathological conditions. This concept is further supported by the findings that both transdifferentiated hepatocytes (Reddy et al., 1984; Hoover and Poirier, 1986) and pancreatic tumors in transgenic mice overexpressing c-*myc* (Sandgren, 1991) were usually located adjacent to the islets.

1.2-3 DUCT SYSTEM

The pancreatic duct system produces both a bicarbonate-rich fluid and mucin, as well as providing a pathway for these substances and the digestive enzymes secreted by the acinar cells to reach the duodenum (Githens, 1988). It consists of a branching network beginning with the main pancreatic duct, which subdivides into progressively smaller ducts. Four levels of ducts are classified according to the number of epithelial cells seen in cross section (Githens et al., 1980): intercalated (containing two to three centroacinar cells), intralobular (three to five cells), interlobular duct (15-60 cells), and main duct (75-100 cells). As the circumference of the ducts decreases, so does the height of the epithelium (ranging from columnar to flattened cuboid) and the amount of surrounding connective tissue (ranging from considerable to virtually none). In guinea pigs (Bensley, 1911) and hamsters (Pour, 1978; Takahashi and Pour,1978; Pour and Wilson, 1980), a meshwork of small

ductules are noted around some islets (periinsular ductules). These further give rise to minute ductules which penetrate into the islets (intrainsular ductules). The lumina of these intrainsular ductules are practically invisible under light microscopy and can best be demonstrated by injection of India ink into the pancreatic duct system.

Previous studies in the rat pancreas indicated that the duct epithelium was composed of homologous cell types with marked ultrastructural similarities (Githens et al., 1980). Later it was shown that the rat pancreatic ducts, according to the differences in their ultrastructural features, chiefly consist of principal cells and 5 other types of specialized cells (Madden and Sarras, 1989). While principle cells make up the majority of duct cells observed, differential distribution of specialized cells is observed among the various classes of pancreatic ducts. The types and frequency of rat pancreatic duct epithelial cells are summarized in Table 1-1. The principal cells share many ultrastructural similarities throughout the ductal system but differ in size and shape. They are characterized by apical membrane projections and cytoplasmic vesicles, both of which increase in size in accord with the size of the ducts. Specialized cells can be morphologically divided into five categories: light cells, basal cells, goblet cells, endocrine cells, and brush cells. Light cells differ from the principal cells merely by their clear, lightly staining cytoplasm. Basal cells have a low nuclear-to-cytoplasmic ratio and are distinguished from lymphocytes by the presence of multivesicular bodies and numerous mitochondria. Some investigators have postulated that basal cells may act as progenitor

Table 1-1 Summary of the types and frequency of epithelial cells located in each class of pancreatic ducts of mature rats based on ultrastructural and cytochemical analysis (after Madden and Sarras, 1988)

	Ductal type frequency, % of total cells analyzed[a]			
Cell type	Intercalated	Intralobular	Interlobular	Main
Principal	95	90	69	55
Basal	0	4	14	11
Goblet	0	0	2	2
Light	5	6	11	31
Brush	0	0	3	0
Pancreatic polypeptide	0	0	1	1

a: In this study, the following total of ductal cells were counted for each duct class from ultramicrographs: main, 200; interlobular, 200; intralobular, 50; and intercalated, 20.

cells in human pancreatic ductal epithelium (Kern, 1986). Goblet cells are easily recognized by the large number of cytoplasmic mucin vacuoles. Brush cells, only found in interlobular ducts, are characterized by the presence of long microvilli along their apical plasma membrane. Endocrine cells are present within the rat pancreatic ductal epithelium. They include PP (pancreatic polypeptide), A (glucagon), and B (insulin) cells (Bendayan, 1987; Madden and Sarras, 1989). In mature rats, PP cells are the predominant cell type located in the ducts. The main and interlobular ducts of human and rat pancreata are innervated by both adrenergic and chlorinergic nerves (Kodama, 1983, Madden and Sarras, 1989). The biochemical and phenotypic characteristics of duct cells will be elaborated in the following section. Scanning electron microscopic studies have shown that most, if not all, of the duct cells in a variety of species (guinea pig, human, hamster, rat and sheep) possess a single cilium (Althoff et al., 1976; Githens, 1988, Hootman and Logsdon, 1988; Madden and Sarras, 1989). These primary cilia are characterized by the presence of nine peripheral doublets without the central microtubules. The function of the cilium is unknown, but it has been speculated to perform some sort of sensory function and is not believed to be motile (Bockman, 1986).

1.2-4 ISLETS OF LANGERHANS

The endocrine tissue of the rat pancreas is composed of the islets of Langerhans. There are about 5,000 islets present in the rat pancreas (approximately one million for a human pancreas) comprising 1 to 2% of the pancreatic mass (Williams and Goldfine,

1986). The islets are randomly distributed throughout the pancreas, but in humans, islets appear to be disproportionately concentrated in the tail. Occasionally islets can be found in the duodenal wall of rats (Bendayan and Park, 1991).

A typical islet is composed of about 5,000 endocrine cells. Islets contain four major types of endocrine cells, which synthesize and secret insulin (B Cell), glucagon (A cell), somatostatin (D cell), and pancreatic polypeptide (PP cell) (Pour, 1978, Orci, 1982). In some species there are other minor cell types producing vasoactive intestinal polypeptide (D1 cell), 5-hydroxytryptamine (EC cell), and bombesin (P cell) (Lack et al., 1990). Immunocytochemical studies of the islets in different species have shown that endocrine cells are not randomly distributed within an islet. In the rat, rabbit and hamster (but not in all species), B cells occupy the central mass of each islet with other three cell types scattered at the periphery (Orci, 1982). PP cells are found primarily in the most caudal portion of the rat pancreas, which extends along the small intestine, whereas A cells are more prominent in the islets of the rest of the organ (Orci et al., 1976).

The B cells make up the largest proportion of cells and are often arranged in cords along vascular channels. Ultrastructurally the insulin granules have rectangular profiles and a crystalline matrix and are surrounded by a halo. The granules of A cells are round, with closely apposed membranes and an electron dense center. D cells have large, pale granules with closely applied membrane. PP cell granules, approximately 170 mm in diameter, are membrane bound with a characteristic dense core surrounded by a

halo (Orci, 1982; Madden and Sarras, 1989).

The islet is highly vascularized, and is fed directly by one to three arterioles, depending on the islet size. Each arteriole penetrates the islet through the non-B cell mantle and enters directly into the B cell core, where it branches into a number of capillaries. The endothelial cells of islets capillaries are fenestrated, whereas those in the surrounding exocrine tissue have few or no fenestrations (Williams and Goldfine, 1986; Bonner-Weir, 1991). The efferent vessels vary in pattern depending on the islet size. Large islets (those greater than 250 μm diameter) are selectively located near the larger ducts and blood vessels. Their efferent vessels coalesce into collecting venules within the islet capsule and so they probably have no effect on surrounding exocrine tissue. In small islets the efferent vessels extend into the exocrine tissue for 50 to 100 μm before coalescing into collecting venules. The vascular pattern of the small islets and their abundance leads to an effective islet-acinar portal system. There are two parallel portal systems present within the pancreas: (1) an intraislet portal system that carries hormones from core to mantle in the proposed B cells > A cells >D cells sequence, and (2) an islet-acinar portal system that conveys hormones from small islets directly to the adjacent acinar tissue. It has been postulated that a portion of the islet hormones in the acinar interstitial fluid is removed via the lymph, while the remaining portion may reenter the pancreatic vascular system to supplement the hormone contribution of the large islets to the hepatic venous system (Samols et al., 1988; Stagner and Samols, 1990; Bonner-Weir, 1991; Samols and Stagner, 1991).

1.2-5 INTERMEDIATE CELLS OF THE PANCREAS

Intermediate cells in the pancreas are characterized by the presence of features of both exocrine cells and different types of islet cells. Intermediate cells have been identified in the pancreas of different species and in all combinations of mixed cell types (Melmed et al., 1972; Melmed, 1979). The origin and biological significance of intermediate cells is controversial. Some investigators suggest that intermediate cells are derived from an abnormal differentiation of the endocrine cells (Becker et al., 1978) whereas others have hypothesized that these cells result from cell fusion during fetal development (Kabayashi, 1966). Some believe that they arise by the "transformation" of differentiated pancreatic cells in response to a metabolic disturbance (Patent and Alfert, 1967; Pictet et al., 1967).

Intermediate cells are usually situated near the periphery of islets and their frequency is generally inversely proportional to the degree of segregation of the exocrine and endocrine pancreas. They are mostly found in the frog in which exocrine and endocrine elements are poorly segregated (Melmed et al., 1972). They are less frequently encountered in the pancreas with a definite separation of these two elements but usually become more prominent in the face of metabolic disturbances, such as in rats fed with raw soybean flour (Melmed et al., 1973), or in mice (Patent and Alfert, 1967) and guinea pigs (Johnson, 1958) treated with alloxan. Therefore intermediate cells found in the normal pancreas are likely to represent the pre-existence of a population of cells capable of more than one specialized function, rather than the product of a "transformation" process. The frequent occurrence of these cells in

different disease states is most likely "a reactive response to an unbalanced homeostasis" (Melmed, 1972).

1.3 CULTURE AND CHARACTERIZATION OF PANCREATIC DUCT EPITHELIUM

1.3-1 ISOLATION AND CULTURE OF PANCREATIC DUCT EPITHELIUM

The duct cells (exclusive of duct cells) comprise only approximately 4% of the volume of the pancreas, which makes it difficult to study their biochemical and physiologic characteristics. While considerable insight into the properties of duct cells has been gained by studies of the intact pancreas (Case and Argent, 1986), further understanding of the role of duct cells and their phenotypes in normal and abnormal pancreatic functional states depends on the development of techniques for the isolation and culture of these cells.

Several approaches have been employed to isolate ducts from different species. The first uses extract of fetal tissue, thereby requiring only minimal or no enzyme treatment and/or mechanical agitation due to the low content of connective tissue. After maintenance in culture for 9 days under optimal conditions, explants of fetal rat pancreas form monolayers of epithelial cells having the morphological characteristics of pancreatic duct epithelium (Wallace and Hegre, 1979). Explants of fetal mouse pancreas cultured on the dermal surface of irradiated pigskin have also been successfully maintained for at least 12 weeks. This approach results in a culture

containing a mixture of all pancreatic cell types (Hirata et al., 1982). The culture of human mid-trimester fetal pancreas gives rise to duct-like cells probably of interlobular origin that express carbonic anhydrase and mucin, and that may be passaged up to 20 weeks (Harris and Coleman, 1987). The proportion of true duct cells in these fetal or neonatal preparations is not known, as some or many of the cells may be acinar cells that have not fully developed.

One approach has used the main duct of human (Jones, 1980) and bovine (Stoner et al., 1978; Sato et al., 1983) pancreas as the starting material. The easily recognized main duct is isolated by gross dissection and the epithelial cells are obtained either by scraping or by perfusing the duct lumen with neutral protease and collagenase solutions that dissociate cells from the underlying stroma. This method provides relatively pure, viable duct cells, which readily attach to culture vessels and can be successfully maintained or passaged in culture for 30 weeks. This straightforward procedure is nevertheless not feasible for the isolation of cells from smaller ducts, as scraping and perfusion are not possible. Additionally these cells are of little use in the studies of fluid-electrolyte secretion, because interlobular ducts rather than main ducts are believed to be the major sites of this process (Case and Argent, 1986; Hootman and Williams, 1987).

One technique utilized explant cultures of human and bovine pancreatic ducts (Jones et al., 1977) and human (Parsa et al., 1981b) or hamster (Resau et al., 1983) pancreas, which could be maintained for 60 to 85 days. Throughout the culture period, ductal cells maintained their morphological appearance but showed limited

proliferation. In contrast, the acinar cells exhibited degradation and loss of zymogen granules accompanied by an accumulation of cystic duct-like structures that contained mucins (Resau et al., 1983). These explants have been used chiefly in the study of *in vitro* chemical carcinogenesis.

To date there are no reports of successful isolation and culture of human interlobular and/or intralobular pancreatic duct cells. The apparent importance of the smaller duct classes in various disease processes makes the development of such a technique a high priority. Substantial progress in the isolation and culture of interlobular ducts has come from the work of Githens's group with the rodent species (Githens, 1988). Their approach is briefly described as follows. Pancreas of adult Sprague-Dawley rats is minced, digested with proteolytic enzymes (collagenase, chymotrypsin, and papain), and sieved to separate duct fragments from acinar and islet tissue. The collected duct tissues are then embedded in rat-tail collagen to create a natural environment. Within 1-2 day in culture, the epithelial ends coalesce and the cut ends of the ducts seal, and the duct lumina start to enlarge because of continued fluid and electrolyte secretion, making them easily distinguishable from other tissues. A second digestion is mandatory to remove the periductal connective tissue and other contaminating cells and this results in highly purified cysts consisting essentially only of epithelial cells. Based on size, these cysts have been suggested as being of interlobular duct origin. The yield of the cysts is greatly improved by including epidermal growth factor, cholera toxin, and bovine pituitary extract in the culture medium (Githens et

al., 1989). Fragments of duct epithelium derived from enzyme-dissociated cysts can be cultured on a porous membrane (HATF filters) coated with extracellular matrix, which significantly enhances the growth of the cells with the retention of their differentiated features (Heimann and Githens, 1991). Similar strategies have been successfully employed in hamsters (Githens et al., 1987; Hubchak et al., 1990), guinea pigs (Hootman and Logsdon, 1988), and Rhesus monkeys (Githens et al., 1994).

The above approaches of Githens and other investigators contrast with that of Tsao and Duguid, who dissociated the whole rat pancreas and established propagable cell lines from tiny clusters of cells judged to be duct cells by morphological criteria (Tsao and Duguid, 1987). This procedure excluded the need for the cumbersome culture and maintenance of cysts, but it failed to provide information on what parts of the ductal system these duct fragments were isolated. Nevertheless the propagable nature of the cell line provides an infinite supply of cells for all kinds of studies, an advantage not available from other approaches.

Interlobular ducts can also be isolated from the pancreas of copper-deficient rats (Arkle et al., 1986). A copper-deficient diet causes a non-inflammatory atrophy of the acinar tissue while the duct cells remain structurally and functionally intact. This provides two advantages. First, the proportion of duct cells in the gland is increased and second, the content of the harmful digestive enzymes is greatly reduced.

1.3-2 PHENOTYPIC AND BIOCHEMICAL MARKERS OF DUCT CELLS

Although adult pancreatic duct cells superficially resemble primitive pancreatic duct-like cells, studies show that the adult epithelium has both gained and lost features as compared with the protodifferentiated epithelium (Githens, 1988). The timing of the changes taking place has been studied only twice, and the findings suggest that it occurs around 18 weeks in the human (Parsa et al., 1984) and day 18-19 in the rat (Githens, 1989). Most studies have been performed on human specimens using various antibodies against cell surface antigens or mucin (Githens, 1988). Many of these antisera are prepared against human pancreatic cancer tissue or cell lines, suggesting that duct cells bear a closer relationship to pancreatic cancer cells than do acinar or islet cells.

Several biochemical markers are localized preferentially if not exclusively in the duct epithelium, when compared to the acinar and islet cells. Since rats are used throughout this study, Table 1-2 summarizes only the biochemical markers of the rats. A few points need special attention. Carbonic anhydrase (CA) is a monomeric zinc metalloenzyme that catalyses the reversible hydration of carbon dioxide and is encoded by a multigene family that yields at least seven isoenzymes (Stolle et al., 1991). CA II is expressed in the duct cells of human (Kumpulainen, 1984) and mouse (Githens, 1992) pancreas, but not in acinar or islet cells. Histochemical studies of the rat pancreas show that CA is mainly present in duct cells and capillaries, but it is also expressed in acinar cells to a lesser extent.

Table 1-2 Biochemical Markers of Pancreatic Cells (Modified from Githens, 1988)

Property	Acinar	Islet	Duct		
			intralobular	interlobualr	main
Carbonic anhydrase[a]	±	-	+	+	
Alkaline phosphatase	-	±[b]	-	-	-
Glutathione-S-transferase B & C	±		+	+	
transferase E	±		+	+	
Na^+-K^+ ATPase	-		++	+++	++
Sulfated mucin	-		+	+	
Sialomucin	-		++	+	
GABA[c] binding	-	±[d]			
Epoxide hydrolase	±		+	+	
GGT[e]	+++	-		+	+
CKs 8/18[f]	+	-	+	+	+
CKs 7/19[f]	-	-	+	+	+

a: Also present in capillaries; *b*: This enzyme is located in the peripheral cells of the islets, but it is not known whether the alkaline phosphatase positive cells are glucagon or somatostatin cells; *c*: gamma-amino butyric acid; *d*: Somatostatin (D) cells only; *e*: gamma-glutamyl transpeptidase; *f*: cytokeratin. See text for details.

To date it is not known what types of isoenzymes are expressed in duct and acinar cells (Heimann and Githens, 1991). Both CA and Na^+-K^+ ATPase are important in fluid and electrolyte secretion (Case and Argent, 1986). Glutathione-S-transferase and epoxide hydrolase are important in detoxification reactions (Githens, 1988). Intralobular duct epithelium and endocrine cells of the fetal and neonatal rat pancreas contain binding sites for γ-amino butyric acid, which have disappeared in the mature pancreas except in somatostatin cells (Gilon et al., 1987). This shift in binding localization may also reflect the presence of islet cells or their precursors in the developing intralobular duct epithelium. There are some 19 different cytokeratin polypeptides in human (and perhaps in rat) tissues, present in different combinations in distinct epithelial cell types (Moll et al., 1982). Though the role of cytokeratins is not well defined, it has been suggested that their specific composition is important for the performance of differentiated functions in epithelial cells. The cytokeratins are frequently expressed in distinct pairs consisting of one acidic (type I) and one neutral/basic (type II) cytokeratin (Moll et al., 1982). In the rat pancreas two cytokeratins equivalent to the human cytokeratins 8 (type II) and 18 (type I) are present in both mature acinar and ductal cells, while two cytokeratins 7 (type II) and 19 (type I) are expressed specifically in ductal cells (Marceau, 1990). The cytokeratin expression in the adult rat liver parallels that of the adult pancreas. While hepatocytes express cytokeratins 8 and 18, biliary duct and oval cells express 7, 8, 18, and 19 (Marceau, 1990).

The data in Table 1-2 suggest that interlobular and intralobular ducts of the rat pancreas are usually not distinguishable from one another with respect to the listed markers except for a few differences. The main duct has not been included in enough studies for any generalization to be made. Further investigation will reveal clear-cut differences between different size classes of ducts.

1.4 DIFFERENTIATION CAPACITY OF PANCREATIC DUCT EPITHELIUM

1.4-1 ACQUISITION AND MAINTENANCE OF DIFFERENTIATION

The zygote of a developing organism gives rise to a complex multicellular organism through three processes: mitosis, to increase cell number; morphogenesis, the organization of cells into precise spatial patterns; and differentiation, the process by which progenitor cells produce a spectrum of morphologically and functionally different progeny. Overt differentiation is usually preceded by an event known as commitment or determination: committed cells do not show the specific phenotypic features characteristic of the given cell type, but are destined ("have made a developmental choice") to a specialized course of development and differentiation (Okada, 1986a; Alberts et al., 1989a; Watt, 1991). Once a cell has become differentiated it may cease cell division, in the case of terminally differentiated cells, or it may divide to produce progeny that normally resemble it in the type of differentiation.

The differentiated state in the adult organism is normally stable and is passed on to the progeny as parental cells divide. The expression of a differentiated state does not necessarily imply irreversible changes, instead it may require continuous regulation by a combination of intrinsic and extrinsic cellular controls (Blau and Baltimore, 1991). The mechanisms controlling intrinsic programs of an autonomous pathway of cellular differentiation remain largely unknown. It is generally accepted that cell differentiation occurs against the background of a constant genome without any structural changes. There are few exceptions to this generalization (Bird et al., 1981; Okada, 1986a). However it remains controversial whether the process of differentiation is accomplished through differential recruitment of genes (DiBerardino et al., 1984) or by irreversible gene repression (Caplan and Ordahl, 1978). Additionally the intrinsic program is modulated by extrinsic factors including hormones, vitamins, growth factors, cell adhesion molecules, and components of the extracellular matrix, which either independently or most likely in coordination, participate and govern this phenomenon (Cross and Dexter, 1991; Sporn and Roberts, 1991; Ingber, 1993).

1.4-2 TRANSDIFFERENTIATION

It has now been convincingly shown that fully differentiated cells, after an appropriate stimulus, can change their commitment and convert into an entirely different phenotype under a permissible environment. Classically, the term "metaplasia" has been frequently utilized at the histologic level to designate a qualitative change in a cell population in which one adult cell type is replaced by another

cell type, such as the squamous metaplasia of the columnar epithelium of mucus glands, or of the respiratory tract in response to protracted physical or chemical irritation (Diberardino et al., 1984). This term does not define the change at the cellular level regarding the histogenetic origin of the new cell types. It is now proposed that "transdifferentiation" is a more appropriate term for defining the process of cell type conversion as described below. This phenomenon is usually the result of experimental manipulation. Such alterations are associated with distinct morphologic changes and are accompanied by the elaboration of new gene products, indicating activation of genes that are normally repressed in that cell type (Bird et al., 1981; Okada, 1986b). Examples of transdifferentiation include the following categories:

1. Transdifferentiation between cell types belonging to the same class

   A well-documented example in this category are the changes occurring between three distinct cell types (iridophore, melanophore and xanthophore) all belonging to the pigment cell class in amphibians (Ide, 1986).

2. Transdifferentiation between cell types belonging to the same cell lineage

   This type of transdifferentiation occurs between cell types that are derived from a common embryonic origin and which are segregated in later stages of development. The examples include transdifferentiation of chromaffin cells to sympathetic neuron (ectoderm), and conversion of cell types within the family of connective-tissue cells (mesoderm), both of which have been

demonstrated *in vivo* and *in vitro* (Unsicker et al., 1978; Aloe and Levi-Montalcini, 1979; Nathanson, 1986). Conversion of pancreatic duct cells into hepatocytes in rats (Rao et al., 1990b) and hamsters (Makino et al., 1990) (endoderm) which has been observed *in vivo* also falls into this category. Another example (differentiation/ transdifferentiation) occurring in the pancreas is nesidioblastosis, which represents the conversion of duct cells to endocrine cells (Boquist and Edström, 1970; Cantenys et al., 1981; Rosenberg et al., 1983; Weaver et al., 1985; Sarventinick and Gu, 1995).

3. Transdifferentiation between cell types belonging to a different cell lineage

This type of transdifferentiation is best represented by the conversion of pigmented epithelial cells of chicken embryonic retina (neuroepithelium) into lens (epidermis) (Eguchi and Okada, 1983).

In almost all examples known, the transdifferentiated process results in the production of a single cell type, not multiple. Production of multiple cell types, though a rare event, has been convincingly shown in *in vitro* conditions. Isolated, mononucleated striated muscle cells of the medusa can be activated by collagenase treatment to transdifferentiate to various cell types, including nematocytes, intestinal, secretory, and presumably nerve cells (Schmid and Alder, 1984).

1.4-3 DEVELOPMENT OF PANCREATIC HEPATOCYTES IN *IN VIVO* MODELS

The induction of hepatocytes in the pancreas of adult rats and hamsters has been achieved by using the following protocols.

A. RAT MODELS

1. Copper Depletion-Repletion Model

In this model, adult male Fischer-344 rats were maintained on a copper-deficient diet supplemented with a copper-chelating agent-0.6% trien (triethylenetetramine tetrahydrochloride) or 0.6% penicillamine for 7-9 weeks and then returned to a normal diet (Rao et al., 1986b; Rao et al., 1989; Rao et al., 1990). The latter was abandoned due to high mortality rates resulting from its high toxicity to multiple enzyme systems in several organs. Another protocol employed a 10-week copper-deficient diet coupled with a single intravenous injection of 4-hydroxyaminoquinoline 1-oxide (4-HAQO), which also resulted in a high mortality (Rao et al., 1986c).

At the end of the copper-depletion regimen, most of the acinar tissue has been ablated except that around the main pancreatic duct in the duodenal segment. The duct system and the islets of Langerhans appeared morphologically unaltered, but there was marked proliferation of spindle (oval) cells that either lined duct-like spaces or were present in the interstitium around the ductules (periductular). At this time individual hepatocyte-like cells could be found within the ductules and in the interstitium. After returning to a normal diet, foci of hepatocyte-like cells continuously increased in number and size in the following weeks and they became grossly visible after several months.

These cells were morphologically indistinguishable from the parenchymal cells of the liver but were not arranged in the normal trabecular pattern seen in adult rat liver. They contained abundant glycogen and liver-specific proteins and enzymes including albumin,

urate oxidase, carbamoylphosphate synthetase I (CPS I), and glutamine synthetase (GS). The co-expression of CPS I and GS in pancreatic hepatocytes is unusual as in the liver, these two enzymes are distinctly distributed in two areas. CPS-I is present in a homogeneous manner in all liver cells except for its absence in a single layer of hepatocytes surrounding the central vein. In contrast, GS is localized exclusively to a narrow zone of pericentral hepatocytes surrounding the central vein (Usuda et al., 1988; Yeldandi, 1990). Functionally these pancreatic hepatocytes demonstrated similar responses to carcinogen treatment (Rao et al., 1991) and androgen regulation (Dwivedi et al., 1990) as the liver parenchymal cells. These protocols were highly reproducible for the induction of pancreatic hepatocytes, being 80% for penicillamine and almost 100% for trien.

Employing sequential examination of different stages of the pancreatic lesions, it was confirmed that both ductular and periductular cells served as progenitor cells of the transdifferentiated hepatocytes (Rao et al., 1990).

2. Ciprofibrate Model

Transdifferentiated hepatocytes can be induced in the pancreas of adult male Fischer-344 rats fed with a hypolipidemic hepatic peroxisome proliferator- ciprofibrate(2-[4(2,2-Dichlorocyclopropyl) phenoxy]2-methyl propionic acid) (Reddy et al., 1984). When ciprofibrate was added to the diet at a dosage of 10 mg/Kg body weight for 60-72 week, approximately 25% of rats developed one or several foci of hepatocytes. Unlike in the copper depletion-repletion protocol, the pancreas showed no evidence of acinar atrophy or fatty

replacement. These hepatocytes were usually observed in the vicinity of the islets of Langerhans with extensions into the surrounding acinar tissue. Transmission electron microscopic examination revealed cell junctions between the hepatocytes and islet cells. Some of the cells around the islets showed transitional forms displaying features of both hepatocyte and acinar/endocrine cells. The identifying marker for hepatocytes is the presence of numerous peroxisomes with uricase-containing crystalloid nucleoids, which is the *sine qua non* for the identity of hepatocytes (Hruban and Swift, 1964).

3. Methyl-Deficient Diet Model

In this model, adult male Fischer-344 rats were fed with different combinations of methyl-deficient diets with or without an initial injection of diethylnitrosoamine (DEN) (Hoover and Poirier, 1986). At the end of the dietary regimen (52 or 77 weeks), foci of cells with the phenotypic features of hepatocytes were found immediately adjacent to the islets of Langerhans. The incidence of this transdifferentiated process was proportional to the severity of the methyl group deficiency, ranging from 1.4% to 25%.

4. Cadmium Chloride Model

Cadmium chloride was accidentally found to be an efficacious agent for the induction of pancreatic hepatocytes in experiments designed to determine the carcinogenicity of repeated exposures to cadmium. After receiving multiple subcutaneous injections of cadmium chloride for 18 consecutive weeks, foci of hepatocytic cells were found within the pancreas of both Wistar and Fischer-344 rats (Konish et al., 1990). Wistar rats showed more tolerance to cadmium

and were also more susceptible to its inducing effects. The incidence was 93% for Wistar rats and 50% for Fischer rats. The number and incidence of hepatocytic foci were proportional to the increases in dose. The pancreas frequently showed acinar atrophy, fatty replacement, and interstitial fibrosis similar to that seen in the copper depletion-repletion model but without spindle cell proliferation.

B. HAMSTER MODELS

In the pancreas of aged hamsters, eosinophilic cells morphologically resembling hepatocytes have been observed and are interpreted as a metaplastic alteration (Pour et al., 1976; Takahashi and Pour, 1978). The change occurs spontaneously, is uncommon and is always associated with pancreatic exocrine atrophy. The incidence varies considerably from one hamster colony to another. For instance, in one study only 4 (0.67%) of 877 animals showed this change (Pour et al., 1976) while in a second, 26 (6%) of 426 animals did (Takahashi and Pour, 1978). As neither morphologic, cytochemical, nor immunohistochemical characterization of these cells were published, it is difficult to compare these with the findings reported by other investigators (Scarpelli and Rao, 1981; Rao et al., 1982a; Makino et al., 1990). Their presence exclusively in aged animals and their infrequency suggest that genetic factors (Pour et al., 1976; Takahashi and Pour, 1978) or environmental mutagens (Scarpelli and Rao, 1981) may play a role in their pathogenesis. Also large eosinophilic hepatocyte-like cells were observed in 90% of hamster pancreatic organ explants exposed to carcinogens-methylnitrosourea (MNU) and *N*-methyl-*N*-nitro-N'-nitro guanidine

(MNNG) and in 45% of the controls (Resau et al., 1985). No phenotypic characterization of these cells was reported.

1. Methyl-Deficient Diet Model

To date this is the only available well-characterized *experimental* hamster model. In this protocol, male Syrian golden hamsters were injected with a pancreatotoxic dose of ethionine (the metabolic antagonist of methionine and consequently an inhibitor of transmethlylation reactions) for 8 consecutive days while being fed a methyl-deficient diet to induce acinar cell injury, followed on the 9th day by a large dose of L-methionine to initiate pancreatic regeneration. Sixty hours after initiation of regeneration (approximately during the peak of DNA synthesis), a single subcutaneous injection of the pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) was administered. Foci of large eosinophilic cells were observed 2-10 months later in the pancreas which also showed acinar atrophy and fatty replacement. These cells demonstrated mature hepatocytic phenotypes and also responded to peroxisome proliferator (methyl clofenapate) as do normal liver parenchymal cells (Scarpelli and Rao, 1981; Rao et al., 1982a; Makino et al., 1990).

The presence of atrophy and fatty replacement of acinar tissue is also found associated with most rat models except the rat peroxisome proliferator model. Chronic 2,6-dichloro-*p*-phenylydiamine administration also induced pancreatic hepatocytes in both male and female Fischer-344 rats (McDonald and Boorman, 1989). However, only the female rats had significant acinar atrophy and fatty replacement. These changes were not observed in hamster

pancreatic explant organ cultures treated with or without carcinogens (Resau et al., 1985). Although such structural changes may be important in this transdifferentiated process, other mechanisms may also play crucial roles in initiating such phenotypic alterations.

1.4-4 NESIDIOBLASTOSIS (ISLET NEOGENESIS) IN *IN VIVO* MODELS

Nesidioblastosis, a term first used by Laidlaw (1938), represents the formation of new islet cells singly or in groups connecting and branching out directly from ducts or ductules. These new islet cells appear to arise from progenitor cells in the duct epithelium, called variously as "nesidioblast" (Laidlaw,1938), "Trbübe Zelle" (Neubert, 1942), "immature β cells" (Bencosme, 1955) or "islet precursor cells" (Pour et al., 1978; Pour and Wilson, 1980). Nesidioblastosis can be observed postnatally *in vivo* in man (Bani et al., 1985), hamsters (Pour, 1978) and rats (Githens, 1988). Apparent islet neogenesis has been experimentally induced in rats and hamsters using different protocols. The experimental procedures used in the rat model included ligation of the adult pancreas duct (Boquist and Edström, 1970), injection of streptozotocin in neonatal rats (Cantenys et al., 1981), and feeding of soy bean trypsin inhibitor (Weaver et al., 1985). Those employed in hamsters include feeding of ethionine or excess methionine (Boquist, 1970), injection of a nitrosoamine carcinogen (Pour, 1978) and chronic duct obstruction (Rosenberg et al., 1983). Islet neogenesis also occurs when fragments of the late fetal or early postnatal pancreas are cultured

(Dudek et al., 1980; Amory et al., 1988) or are implanted into the anterior chamber of the eye (Coupland, 1960). Apparent islet neogenesis is also observed in interferon-gamma transgenic mice (Sarventnick and Gu, 1992). Additionally, adult interlobular and main ducts in many species normally contain scattered endocrine cells (Kumegawa et al., 1980; Alumets et al., 1983, Bendayan, 1987; Madden and Sarras, 1989).

1.4-5 HISTOGENESIS OF TRANSDIFFERENTIATION

The cells in a multicellular organism are divided into three types: "labile", "stable", and "permanent", depending on their regenerative capacity. The pancreas contains stable cells that undergo cell division only after experimental manipulation (Lehv and Fritzgerald, 1968; Githens, 1988). According to some investigators it may even lack stem cells (Leblond, 1964). In this organ transdifferentiated cells may arise as a result of conversion of one of the differentiated cell types. Early studies indicated that pancreatic hepatocytes in both rats (Reddy et al., 1984) and hamsters (Scarpe''' and Rao, 1981) were derived from the acinar cells, however subsequent studies suggested that they more likely originated from the duct and/or periductal cells (Makino et al., 1990; Rao et al., 1990). The role of acinar cells in this process remains intriguing, because transitional cells displaying features of both hepatocytes and acinar/endocrine cells have been noted in the rat pancreas (Reddy et al., 1984). In contrast, such intermediate cells were never found in the hamster model (Scarpelli et al., 1984). These examples reflect the difficulties in elucidating histogenesis in

an *in vivo* experimental system because of the multiplicity of epithelial cell types present in organs, species difference and the generally dynamic nature of the process. A solution to this dilemma is to establish an *in vitro* experimental system which offers two main advantages: simplicity (individual cell types can be studied in isolation and it is easy to manipulate the culture environment) and it provides easy accessibility of cells for analyses at cellular and molecular levels. This approach has been employed by Eguchi and Okada (1987) to confirm the histogenetic origin of Wolffian regeneration of the lens from the dorsal iris originally described by Coluci in 1891.

1.4-6 MECHANISMS OF TRANSDIFFERENTIATION

There are two different pathways of transdifferentiation into new cell types; single (direct) and multiple (indirect) step (Okada, 1986b). Single step differentiation involves the direct conversion between two distinct cell types without the intervention of progeny generation. This type of transdifferentiation usually occurs without DNA synthesis and cell division and features the appearance of "chimeric" cells containing the phenotypic features of more than one cell type (Schmid and Adler, 1984; Ide, 1986).

In other cases, transdifferentiation requires a multi-step process for its completion. Early in the process, intermediate cells not having the specific phenotype of either the original or the new cell types are formed. These cells are usually capable of differentiating into either the original or a new cell type, and therefore are at least bipotential (Okada, 1983; Itoh and Eguchi,

1986). This process is accompanied by active DNA replication and cell proliferation. As to the development of pancreatic hepatocytes, it is still not clear whether it involves a single or a multi-step process (Rao, 1990b).

The process of *in vitro* transdifferentiation can be initiated and modulated by growth conditions and/or the culture media used. For instance, chick embryo extract and fetal calf serum can induce the conversion of chicken retinal pigment cell into lens cells, whereas adult serum failed (de Pomerai and Gali, 1981). This transdifferentiated process is also greatly enhanced by the addition of ascorbic acid or phenylthiourea with hyaluronidase in the culture medium (Itoh, 1976; Eguchi et al., 1981). The conversion of 3T3 fibroblasts into adipocytes is stimulated by a number of agents, including insulin, glucocorticoids and fetal calf serum, and is inhibited by phorbol esters and tumor necrosis factor-α (TNF-α) (Sul, 1991).

The mechanisms that initiate transdifferentiation *in vivo* are complex as judged by its occurrence under various conditions, such as those involved in the induction of pancreatic hepatocytes by most protocols (Reddy et al., 1984; Hoover and Poiries, 1986; Konishi et al., 1990; Rao et al., 1990). In some cases it may be attributed to removal of growth-suppressive factors caused by the loss of the organized state of the tissue, loss of interaction between adjacent cells and changes in the environment of the cells as is seen during the regeneration of amputated urodelen amphibian limb (Dunis and Namenwrith, 1977). Transdifferentiation can also occur without the loss or structural alteration of the tissue through the unknown

actions of chemical carcinogens (Tsonis and Eguchi, 1981) and peroxisome proliferator (Reddy et al., 1984).

Studies on the molecular events of transdifferentiation to date mostly concern changes in molecular markers that characterize the original and terminal cell types. It is noteworthy that the original cells usually express a low level of the genes that are the markers of the new cell type. Liver epithelial cells capable of differentiation into hepatocytes after *in vivo* transfer (Coleman et al., 1994) express low levels of albumin and α-fetoprotein *in vitro* (Tsao et al., 1984). In lens transdifferentiation, a low level of transcript of δ-crystallin gene is found *in situ* in these non-lenticular ocular tissues which have the capacity of lentoid differentiation *in vitro* (Agata et al., 1983). The biological significance of this phenomenon is still not clear. It seems likely that "transdifferentiation is accomplished not by the activation of completely dormant genes, but by enhancement of expression of genes that are already active" (Okada, 1986).

Studies on the regulatory genes controlling the process of transdifferentiation are just beginning. Molecular events maintaining the stability of a differentiated cell include chromatin structure, DNA methylation, DNA protein interactions, and DNA rearrangements (DiBernardino et al., 1984). Modification of any of these may initiate transdifferentiation. For instance, Swiss 3T3 fibroblasts treated with hypomethylating agents (5-azacytidine or 5-aza-2'-deoxycytidine) can differentiate into chondrocytes, adipocytes, and striated muscle cells (Taylor and Jones, 1979). Transfection of MyoD converted fibroblasts into myoblasts (Davis et al., 1987) and activated muscle-specific genes in a variety of non-

muscle cells (Weintraub et al., 1989). MyoD and other members of the gene family encode DNA-binding proteins that positively regulate muscle differentiation (Blau and Hughes, 1990). To date very little is known about the molecular events taking place during the induction of pancreatic hepatocytes.

1.5 PANCREATIC CANCER

1.5-1 GENERAL FEATURES OF PANCREATIC CANCER

Carcinoma of the exocrine pancreas is the fourth or fifth common cause of cancer death in North America, in both males and females, and it remains one of the most mortal of human malignancies (Boring et al., 1992; Reddy, 1995). Adenocarcinoma of duct cell origin is by far the most frequent histologic type, accounting for about 80 percent of malignant tumors (Cubilla and Fitzgerald, 1975; Pour et al., 1979; Klöppel and Fitzgerald, 1986). These highly fatal cancers have deceptively silent growth, such that by the time they are diagnosed they are rarely resectable. When first seen, 90% of patients have regional lymph node metastasis and 80% have liver metastasis (Gordis and Gold, 1986). Conventional methods of treatment including surgery, radiotherapy, and chemotherapy offer little hope of cure, and the median survival time, regardless of therapy, is 2 to 3 months after diagnosis. The 1-year relative survival rate is about 8% and the 5-year survival rate is about 2% (Baylor and Berg, 1973; Cancer of the Pancreas Task Force Group 1981; Gudjonsson, 1987; Warshaw and Swanson,1988). Its incidence varies from country to country, being high in the United States and Denmark and low in Japan and Italy. Incidence rates of pancreatic

cancer increase steadily with age. Approximately 80% of cases of pancreatic cancer occur between ages 60 and 80. The ratio of the number of male to female cases is approximately 1.1:1 (Gordis and Gold, 1986). Tumors may arise anywhere in the pancreas, but most studies show a fairly standard distribution: head of pancreas, 60%; body of pancreas, 15 to 20%; tail of pancreas, 5%.

So far there is no evidence that the prognosis is significantly better in patients with early diagnosis. Consequently, it seems clear that the main hopes for improving the outcome will relate to therapy and to the development of effective measures for prevention. Prevention of pancreatic cancer will require the identification of specific etiologic factors associated with high risk of tumors, and a reduction or elimination of exposure to these factors. The epidemiologic association of diabetes and pancreatic cancer has been reported by many investigators, and the incidence of pancreatic cancer in diabetics is two to five times higher than that of the non-diabetic populations (Cuzick and Babiker, 1989; Lack et al., 1990; Jain et al., 1991). To date, cigarette smoking is the only environmental factor that has been found to be consistently associated with pancreatic cancer (Howe et al., 1990; Warshaw and Castillo, 1992). There appears to be no association between risk of pancreatic cancer and consumption of coffee or alcohol (Gold et al, 1985; Norrel et al., 1986; Clavel et al., 1989; Jain et al., 1991; Ghadirian and Howe, 1994); likewise, critical analysis of the available data does not support a casual relationship with chronic primary pancreatitis (Warshaw and Castillo, 1992). A strong association between tropical pancreatitis and pancreatic cancer may reflect an

environmental etiology, as these cases are invariably associated with multiple intrapancreatic ductal canaliculi in a background of malnutrition (Thomas et al., 1990). Familial occurrence of pancreatic cancer, though extremely rare, has been reported (MacDermott and Kramer, 1973; Ehrenthal et al., 1987; Ghadirian et al., 1991). Nevertheless there is no evidence to date to suggest that such a predisposition results in pancreatic cancer occurring at earlier ages. It has now been established that familial relapsing pancreatitis (hereditary chronic pancreatitis) is a risk factor for pancreatic cancer (Conrath, 1986; Pour, 1990). Some autosomal dominant disorders such as Li-Fraumeni's syndrome (Malkin et al., 1990) and neurofibromatosis (Keller and Logan, 1977) have also been reported as sharing some associations with pancreatic cancer.

1.5-2 ANIMAL MODELS OF PANCREATIC CARCINOGENESIS

Exogenous carcinogens include chemical carcinogens, ionizing radiation and oncogenic viruses. Chemical carcinogens are extremely diverse in structure and include both natural and synthetic compounds. They can be generally classified into one of two categories: (1) direct-acting compounds, which do not require chemical transformation for their carcinogenicity; or (2) indirect acting or procarcinogens that require metabolic activation *in vivo* to become ultimate carcinogens (Hopkins, 1987). Neoplastic transformation of cells *in vitro* and the induction of cancer *in vivo* by chemical carcinogens is a dynamic process involving at least three distinct and well-characterized stages: (1) **Initiation** results from

exposure of cells or tissues to an ultimate carcinogen, which causes heritable and irreversible genetic damage in the initiated cells. Initiation alone is not sufficient for tumor formation, and it only confers on the cells a genetic capacity to become tumorigenic. (2) **Promotion** encompasses the induction of clonal proliferation of initiated cells to form distinct tumor cell populations. Promoters by themselves are nonmutagenic and nontumorigenic but they alter the differentiation programs of the cells. (3) **Progression** denotes further phenotypic and genotypic changes resulting in an increasing capacity for invasiveness and metastasis. (Farber and Cameron, 1980; Land et al., 1983; Klein and Klein, 1985; Knudson , 1985; Farber, 1987; Nicolson, 1987; Weinstein, 1988; Alberts et al., 1989b; Weinberg, 1989).

The spontaneous incidence of neoplasms in the exocrine pancreas in rats, hamsters, and other rodents used experimentally is quite low (Rowlatt and Roe, 1967; Takahashi and Pour, 1979). Therefore, to gain a precise understanding of the events that characterize pancreatic carcinogenesis and of the factors that initiate and modulate those events, a well-defined model is essential. Interest in pancreatic chemical carcinogenesis increased after Druckrey and co-workers' report of inducing pancreatic carcinoma in guinea pigs with *N*-Methyl-*N*-nitrosourea (MNU) (Druckrey et al., 1968). Since then a diverse group of more than 15 chemicals has been reported to be carcinogenic for the pancreas (Longnecker, 1986) (Table 1-3). Most of these pancreatic carcinogens are procarcinogens except MNU and *N*-(*N*-Methyl-*N*-nitroso-carbamoyl)-L-ornithine (MNCO). Most chemicals are species-specific in the

Table 1-3 Known Pancreatic Chemical Carcinogens (after Longnecker, 1986)

Chemical	Abbreviation	Species
Aflatoxin B1	-	Monkey
Azaserine	-	Rat
Clofibrate, ethylchloro-phenoxyisobutyrate	-	Rat
7,12-dimethylbenz(a)-anthracene	DMBA	Rat
4-Hydroxyaminoqui-noline-1-oxide	4-HAQO	Rat
N-Methyl-*N*-nitrosourea	MNU	Guinea pig
N-(*N*-Methyl-*N*-nitroso -carbamoyl)-L-ornithine	MNCO	Rat, hamster
Nafenopin, [2-methyl-2-(*p*-1,2,3,4-tetrahydro-1 -naphthyl)-pheoxy]-propionic acid	-	Rat
Nitrofen, 2,4-dichloro-1(4-nitrophenoxy)benzene	-	Rat
N-nitroso(2-hydroxypropyl) -(2oxopryplyl)amine	HPOP	Rat

induction of neoplasia, but some agents have proven to be equally effective in several species (Table 1-3).

By far the greatest portion of human pancreatic exocrine tumors are classified as adenocarcinomas of ductal/ductular origin (Cubilla and Fitzgerald, 1975; Pour et al., 1979). However, considerable controversy exists regarding the precise cell of origin because of the finding that under certain conditions acinar cells can also form a tubular arrangement (Bockman , 1981; Reddy et al., 1986; Pettengill et al., 1993). Ideally, an animal model of the human disease should closely parallel the latter with respect to its histogenesis, biological behavior and morphology. Unfortunately current studies focusing on the histogenesis of pancreatic tumors in animal models have not provided a definite answer as to the cell origin. In rats and mice tumors consist predominantly of acinar cell carcinomas whereas in hamsters and guinea pigs the great majority of the lesions are adenocarcinomas (Longnecker, 1984; Rao, 1987; Pour, 1994). While there is little doubt about the histogenesis of acinar cell carcinogenesis in the rat model (Scarpelli et al., 1984; Longnecker, 1984, 1986), considerable debate exists as to not only the cell of origin but the mechanisms of histogenesis in hamsters. Some investigators believe these ductal adenocarcinomas are derived from acinar cells that undergo "de-differentiation/metaplastic transformation" during carcinogenesis eventually resulting in the histologic appearance of "pseudoductular transformation" (Bockman, 1981; Flaks, 1984; Scarpelli, 1984; Reddy, 1995). This mechanism is also used to explain the occurrence of ductal adenocarcinomas in the rat model. In contrast, Pour strongly defends the concept that ductal

carcinomas in the hamster model originate from ductular/ centroacinar cells (Pour, 1984, 1988). After duct obstruction and administration of carcinogens it has been convincingly shown that ductal carcinomas can arise from the main duct in hamsters (Rosenberg et al., 1984). Longnecker (1984) prefers to believe that both alternatives are possible. Though investigators from all sides of the controversy have accumulated volumes of data to support their hypotheses, absolute proof for either side is not forthcoming. The reason is that sequential observations of the pancreatic lesions using histologic, ultrastructural, and immunocytochemical studies can only provide static pictures of the process instead of the dynamic interactions that are taking place, i.e., that the relative proportion of tumors arising as a result of transformation of acinar cells and ductal cells is not known with certainty. Thus, the availability of propagable and pure cultures of acinar or ductal cells of pancreas would greatly help us resolve the controversy and understand the biochemical facets of chemical carcinogenesis (Frazier and Longnecker, 1993).

To date the most fruitful approach to *in vitro* pancreatic carcinogenesis has been the model system developed by Parsa and co-workers who exposed the organ cultures of embryonic rat and human pancreas and adult human pancreas to dimethyl-nitrosamine (DMN), MNU, and *N*-Nitrosobis(2-hydroxy-propyl)amine (BHP) (Parsa et al., 1980, 1981, 1984). Following 2-10 weeks of treatment, the pancreatic explants showed malignant changes, including duct cell hyperplasia, ductal carcinomas (predominant), and acinar cell carcinomas. Tumors were produced when these carcinogen-treated

pancreatic explants were introduced into nude mice. These studies demonstrated that the pancreas can activate indirect acting carcinogens such as DMN and BHP, but they fail to delineate the histogenesis due to the multiplicity of cell types. Acinar cells have not been maintained in long-term cultures, limiting the usefulness of isolated primary cultures of rat acinar cells to studies of DNA damage and repair (Longnecker et al., 1984).

1.5-3 HISTOLOGIC CLASSIFICATION OF PANCREATIC TUMORS IN AZASERINE-TREATED RATS

The induction of exocrine pancreatic carcinomas by azaserine (O-diazoacetyl-L-serine) has provided the most extensively studied experimental model of pancreatic carcinogenesis in rats, although similar carcinomas are induced by other agents, including HPOP, 4-HAQO, and DMBA (Table 1-3) (Longnecker et al., 1981; Longnecker, 1986). Azaserine, a natural product of a *Streptomyces*, was initially investigated as a potential antibiotic. Azaserine has subsequently been identified as a pancreatic carcinogen and has been demonstrated to induce a spectrum of focal proliferative abnormalities in acinar cells that eventually culminate in the development of carcinomas. The sequential proliferative changes include atypical acinar cell foci (microscopic in size), atypical acinar cell nodules (AACN) (1 to 10 mm in diameter), acinar cell adenomas, carcinomas *in situ* (CIS), and adenocarcinomas (Scarpelli, 1984; Longnecker, 1986). AACN are the most frequently reported abnormality in the rat pancreas following systemic exposure to a pancreatic carcinogen. These clearly seem to be the precursor lesions

to acinar cell adenomas and carcinomas. Roebuck et al (1984) described two phenotypic subtypes of azaserine-induced AACN: acidophilic and basophilic foci (AF and BF). A similar classification was also reported in rats treated with 4-HAQO (Rao et al., 1982b). In both models, AF have been shown to stain intensely for ATPase activity, as do carcinomas (Bax et al., 1986). AF also appear to have greater proliferative potential than BF which have a low mitotic activity and generally remain small.

No hyperplastic or atypical changes have been identified within the epithelium of the intralobular or the main pancreatic ducts. Lesions such as cystic ductal complex (cystic adenoma) and tubular ductal complexes (pseudoductular hyperplasia) are rarely found in azaserine-treated rats. They are found more characteristically in carcinogen-treated hamsters (Pour, 1984) or in rats treated with MNCO and DMBA (Longnecker, 1986). The tubular ductal complex consists of ductlike structures, usually a scant fibrous stroma and a narrow lumen lined by cuboidal or columnar epithelium. Some of these epithelial cells contained sparse zymogen granules, suggesting that the tubules were derived from acinar cells. Intraductal hyperplasia, and ductular and intraductal CIS, frequently encountered in carcinogen-treated hamsters, are rare or nonexistent in rats .

The carcinomas include a wide range of histologic variants. Study of a series of 332 carcinomas revealed 7 types which were highly divergent in differentiation (Table 1-4) (Longnecker et al., 1984; Longnecker, 1986). The well-differentiated carcinoma clearly produced zymogen and retained acinar or tubular configuration in

Table 1-4 A Histologic Classification of Azaserine-Induced Pancreatic Carcinoma (after Longnecker et al, 1984)

Histologic type	%
Acinar cell carcinoma, pure	
Well-differentiated	30
Poorly differentiated	43
Acinar cell carcinomas, mixed (with)	
Ductlike carcinoma	18
Undifferentiated carcinoma	5
Cystic carcinoma	1
microadenocarcinoma	1
Undifferentiated carcinoma	5

many areas. Ultrastructurally the tumor cells retain abundant rough endoplasmic reticulum and varying numbers of atypical-shaped zymogen granules. The great majority of the tumors are designated as poorly differentiated acinar cell carcinoma. Here, the tumor cells remain as a tubular pattern but they contain little or no zymogen granules. Some of the carcinomas show a variable histologic appearance from area to area. Acinar cell differentiation dominates in such mixed tumors, which also contain areas of undifferentiated carcinoma or one of the patterns described below. Several of the carcinomas showed areas with duct-like structures and varying degrees of desmoplasia. This is the most frequent histologic appearance among those lesion recognized as localized carcinomas. A few of the tumors were cystic in areas. The cells lining some of these cystic spaces were cuboidal or columnar and lacked evidence of zymogen granules in the cytoplasm. In other areas, cysts have been lined by multilayered glandular cells, some of which contained zymogen granules. This suggests that all such patterns can arise from acinar cell precursor lesions. Only a small percentage of carcinomas were entirely undifferentiated. These tumors contain pleomorphic cells without a glandular/tubular pattern or evidence of zymogen production.

Several acinar cell carcinomas of the rat pancreas were established as transplantable tumor lines. Reddy and Rao (1977) were the first to report a transplantable pancreatic acinar cell carcinoma in the F344 rat strain. Longnecker and co-workers subsequently also reported the establishment of azaserine-induced pancreatic carcinomas as transplantable tumors in the Wistar rats

(Longnecker et al., 1979). They were maintained by serial transplantation in the peritoneal cavity or subcutaneous tissue of either syngeneic hosts or athymic nude mice. Irrespective of the transplantation location, the neoplasm appeared as an encapsulated, soft, gray-white mass with extensive vascularization. Histologically the tumors consisted of solid masses or cords of small cuboidal or columnar cells with prominent nuclei. Ultrastructural examination of the tumors revealed a continuum of cells from undifferentiated forms virtually lacking zymogen granules to well-differentiated forms with abundant, mature secretory granules.

In contrast to the rat model, the predominant lesions in the hamsters are duct adenocarcinomas (Longnecker, 1984; Rao, 1987; Pour, 1994). *N*-nitrosobis(2-oxopropyl)amine (BOP) treatment induces a wide range of proliferative abnormalities and eventually carcinomas. A detailed classification of these lesions has been reported by Pour and Wilson (1980).

CHAPTER 2

ESTABLISHMENT AND CHARACTERIZATION OF PROPAGABLE CULTURED RAT PANCREATIC DUCTAL EPITHELIAL CELLS

2.1 SUMMARY

A propagable epithelial cell line (termed RP-2) was established directly from an isolated pancreatic duct fragment of an adult male Fischer-344 rat. These cells grew as a monolayer with a doubling time of approximately 24 hr, and have been propagated for more than 16 passages. Ultrastructurally they were polygonal cells with complex basolateral membrane processes, short surface microvilli and intercellular desmosomes. Differentiation features of either acinar or endocrine cells were not noted. They retained certain enzymatic activities which are expressed by pancreatic duct cells *in vivo*, including carbonic anhydrase, alkaline phosphatase, and γ-glutamyl transpeptidase. They neither produced mucin nor accumulated glycogen particles. They expressed epidermal growth factor receptor, insulin-like growth factor II and transforming growth factor (TGF)-β1, but not TGF-α. Immunocytochemically they were positive for cytokeratin 8 but negative for cytokeratin 19, albumin, α-fetoprotein, and surface antigens of rat liver biliary epithelial cell (BDS7) and hepatocyte (HES6). These cells expressed mRNA of CA II, and also low levels of albumin and α-fetoprotein mRNA as detected by the reverse transcription-polymerase chain reaction. The results show that pancreatic duct epithelial cells of adult rats can be continuously propagated *in vitro* and they retain some of their *in vivo* differentiated phenotypes.

2.2 INTRODUCTION

Though pancreatic duct epithelium comprises only a small portion of the pancreatic cellular volume, the duct system is essential for pancreatic exocrine function, including the elaboration of mucin (Forstner and Forstner, 1986) and a large volume of bicarbonate rich fluid to facilitate the flow of secreted products of the acinar cells (Kuijpers and de Pont, 1987; Case and Argent, 1986). The duct system also provides a conduit for these products and digestive enzymes to reach the duodenum. The understanding of the regulation of acinar secretion is well documented, since acinar tissue comprises 90 % of the pancreatic parenchyma and is easy to isolate and study *in vitro* (De Lisle and Williams, 1986; Logsdon and Williams, 1986). In contrast, the duct system *in vivo* are less accessible and comprises only a small portion of the pancreatic mass. Thus, the regulatory processes in the ductal system is more difficult to study, hence less understood. A complete understanding of the mechanisms which regulate the differentiation, growth and secretion of pancreatic ductal epithelium is critical, since the pathogenesis of a majority of human pancreatic diseases, including cystic fibrosis, acute and chronic pancreatitis, and carcinoma seems to be the result of aberrations in these control mechanisms (Pour et al., 1982; Park and Grand, 1981; Sarles, 1986).

Most of our modern understanding of the biochemical and physiological properties of the pancreatic ducts has been gained from studies of ducts *in situ*, of isolated duct fragments, or of duct-epithelium enriched tissue (Arkle et al., 1986; Githens, 1988). While such studies are important, they are usually complicated by a

contamination of the duct preparation by varying amounts of other cell types, and by the inability to propagate these cells in long-term culture. The isolation and culture of pure populations of pancreatic duct cells may provide an improved system to define further the properties of these cells. Our laboratory was the first to successfully establish a propagable cultured pancreatic duct epithelial cell line from normal adult Fischer-344 rats (Tsao and Duguid, 1987). This approach was advantageous over other methods of culturing the pancreatic duct cells by its ability to provide a continuous large number of cells for various studies, however, it was disadvantaged by an uncertainty as to the *in vivo* anatomic origin of these cultured cells. I have refined further the methodology and have established a duct epithelial cell line directly from an isolated duct fragment. I demonstrate here that these cultured epithelial cells partially maintained the phenotype of adult rat pancreatic duct cells.

2.3 MATERIALS AND METHODS

2.3.1) Establishment of Cell Lines:

A male Fischer-344 rat (Charles River Breeding Laboratories, Wilmington, MA) weighing 120-150 g was used in this study. The animals was anesthetized with ether and killed by cervical dislocation. The gastric and splenic lobes of the pancreas were isolated and placed in an ice-cold equal mixture of Richter's improved medium with zinc option (IMEMZO) (Irvine Scientific, Santa Ana, CA) and calcium-magnesium-free Hanks' Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island, NY) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamycin. In a

100-mm Petri dish, the peri-pancreatic adipose tissue was carefully removed and the pancreas was minced into 1-2 mm diameter fragments using a scalpel blade.

The minced tissue suspension was washed repeatedly with an ice-cold equal volume mixture of IMEMZO and HBSS solution, and then incubated in a 10-15 ml solution of serum-free IMEMZO containing 0.1% collagenase XI, 0.1% hyaluronidase, 0.1% soybean tyrpsin inhibitor (Sigma Chemical, St. Louis, MO), and 10 μg/ml deoxyribonuclease I (DNase I) (Boehringer Mannheim Canada, Laval, Que) in a 50-ml sterile conical centrifuge tube (Becton Dickinson, Mississauga, Ont). After incubation for 30-45 min at 37°C and with intermittent agitation for every 5-10 min, the digest was centrifuged at 1,000 r.p.m. for 5 min at 4°C. The pellet was resuspended in IMEMZO containing 4 μg/ml insulin (Gibco, BRL), 40 μg/ml gentamycin, 2.6 mM sodium bicarbonate, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), and 10% fetal bovine serum (FBS) (ICN Biomedical, St. Laurent, Que), and was placed in a 100-mm tissue culture plate (Becton Dickinson). The resulting suspension consisted of small clumps of epithelial cells, acinar tissue, islets, duct fragments and blood vessels. When viewed with a phase contrast inverted microscope, the duct fragments were identified by their characteristic cobblestone-appearing epithelial lining, while the smaller arterioles and venules were lined by elongated or flat appearing endothelial lining, or by the presence of red blood cells in their lumens. Nerve fragments were identified by their frayed-rope-like appearance. The duct fragments were aspirated using a siliconized Pasteur pipette and suspended in 0.5 to

1 ml medium in 60-mm tissue culture dishes. Within 24 hr, the duct fragment attached to the plate and epithelial cells started to explant and form a cell monolayer. Cultures were monitored continuously and photographed. Cells at different passage levels were frozen and stored in liquid nitrogen. When a large area of monolayer cultured cells had formed, subculture was performed by dissociation with a Ca^{++}-Mg^{++}-free HBSS solution containing 0.05% trypsin and 0.53 mM EDTA, and then replated at a density of 1 X 10^5 cells in 100-mm tissue culture plates. Cells were counted using a ZM Coulter cell counter (Coulter Electronic, Hialeah, FL).

2.3.2) Cell Proliferation Assay:

To estimate the population doubling time of cultured cells, 5 X 10^4 RP-2 cells at passages 6, 10, and 15 were seeded in six-well tissue culture plates (Becton Dickinson). Triplicate wells of cells were trypsinized at various times afterwards (typically every 24 hr) and counted with the ZM Coulter cell counter. The doubling time was calculated from growth curves generated by 3 separate experiments.

2.3.3) Ultrastructural Studies:

Ultrastructural studies were performed on trypsin-dissociated cultured cells and on monolayer cultured cells fixed *in situ*. The cells were fixed in a phosphate buffer containing 4 % paraformaldehyde and 1 % glutaraldehyde, and then post-fixed in osmium tetroxide. The samples were processed and embedded in Epon 812. Transmission electron microscopic (TEM) studies were carried out using a Philips electron microscope Model 201.

2.3.4) Histochemistry:

Cells at passages 5 and 10 were grown to confluence in Lab-Tek tissue culture slide chambers (Nunc, Naperville, IL). Periodic acid-Schiff (PAS) stain with or without prior digestion by diastase and alcian blue (pH 2.5) stain were used respectively to detect the presence of glycogen and mucin. Histochemistry for γ-glutamyl transpeptidase (GGT) (Rutenberg et al., 1969) was performed on cells and frozen sections of adult rat pancreas fixed in acetone. After a thorough washing in 0.05 M Tris-HCl buffer, pH 7.4, cells/sections were incubated for one hour in a solution containing 0.1 M Tris-HCl, pH 7.4, 1.3 mM tetrazotized *o*-dianisidine, 0.43 mM γ-L-glutamine-4-methoxy-2-naphthylamide (Sigma Chemical, St. Louis, MO), 0.1 M NaCl and 3.8 mM glycylglycine. After a rinse in normal saline for 3 min, they were incubated in 0.1M $CuSO_4$ for 2 min. The GGT activity was revealed by the formation of a reddish-brown deposit.

The histochemistry for alkaline phosphatase (ALP) was performed on cells and frozen sections of adult rat pancreas fixed in PBS containing 2 % (v/v) formaldehyde/0.2 % (v/v) glutaraldehyde. The reaction solution consisted of a Tris-HCl buffer, pH 9.5, containing 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 1 mg/ml nitroblue tetrazolium (Lin et al., 1992).

2.3.5) Quantitative Biochemical Assays:

RP-2 cultured cells at passage 10 which have reached confluence were used for biochemical assays. Confluent cultures were used in all instances to reduce a variation in enzyme level related to the proliferative state of the cells (Tsao et al., 1985).

To measure the specific activity of GGT, cells were washed with D-PBS, lysed in 0.1% Triton-X100 solution and then collected by scraping into microcentrifuge tubes. After 10 min centrifugation at 10,000 r.p.m. the supernatant was assayed immediately or stored at -80°C prior to measurement. The quantitative colorimetric endpoint assay contained the γ-glutamyl-*p*-nitroanilide as a GGT substrate. A GGT kit of Sigma Diagnostics was used.

To measure the carbonic anhydrase (CA) activity, cells grown in 100 mm tissue culture plates were rinsed twice in cold D-PBS, scraped and sonicated for 1 min in 1-2 ml of cold 0.3 M sucrose solution, and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was stored at -80°C. Cell scrapings in sucrose solution were used since trypsin and detergent (sodium dodecyl sulfate and Triton X-100) treatment inhibits significantly the CA activity (Brion et al., 1988), which was assayed by a modified method of Brion et al. (1988) as described by Heimann and Githens (1992). This colorimetric assay employs an imidazole-Tris buffer with *p*-nitrophenol as the pH indicator. The basic principle of the test is that the hydration of CO_2 acidifies the solution, and this is noted by a change in the color of the solution. A 50 μl of cell extract was added to a 50 μl of assay buffer containing 40 mM imidazole, 20 mM Tris base, 1 mM *p*-nitrophenol, and 0.1% bovine serum albumin at a final pH of 9.6. The solution was continuously bubbled with CO_2 at 4 ml/min. The time required for the yellow color to vanish and the solution appear colorless was recorded. Control tests were performed by including in the solution 1 mM acetazolamide (Sigma Chemical), which is a strong CA inhibitor. Purified CA-II from bovine

erythrocytes (Sigma Chemical) was used to establish a standard curve of activity. The assay may detect as little as 2 ng of CA-II activity, and the specific activity was expressed as mg CA-II equivalents per mg protein. Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Lab., Richmond, CA).

2.3.6) Immunocytochemistry:

This was performed using the streptavidin-biotin-peroxidase system (Zymed Laboratories, San Francisco, CA). Cells at passages 5 and 10 cultured in Lab-Tek tissue culture slide chambers were fixed in situ with either cold methanol at -20°C or 10% phosphate-buffered formaldehyde at room temperature. After air drying, they were either immunstained immediately or after storage at -80°C. The endogenous peroxidase was quenched in PBS containing 0.28% periodic acid for 45 seconds, and this was followed by a 10 min incubation with 10% normal goat serum to suppress a non-specific binding of IgG. For paraffin sections, the periodic acid incubation was replaced by a 10 min incubation in methanol containing 3% hydrogen peroxide. Antigen-antibody complex formation was visualized with aminoethyl carbazole (AEC). Polyclonal antibodies against rat albumin (ALB) and α-fetoprotein (AFP) were obtained from Nordic Immunological Laboratories (Capistrano Beach, CA) and were used at 1:6000 and 1:2000 respectively. The mouse monoclonal antibodies against human cytokeratin 19 (Amersham Canada, Oakville, Ont) and rat cytokeratin 8 (Germain et al.,1988) were used at 1:50 dilution. The mouse monoclonal antibodies HES6 and BDS7 respectively recognize the surface antigens of hepatocytes

and biliary epithelial cells in adult rat liver (Germain et al.,1988). HES6 was used at 1:200 dilution while BDS7 was used undiluted. The rabbit polyclonal antibody against porcine α-amylase (Hansen et al., 1981) was used at 1:200 dilution. Polyclonal antibodies against insulin, glucagon, chromogranin and somatostatin were purchased from Dako (Carprinteria, CA) and used at 1:150 to 250 dilutions, as suggested by the manufacturer. Paraffin or frozen sections of adult rat pancreas and liver were used as positive controls, while the negative controls included the replacement of primary antibodies by non-immune sera, or by their omission.

2.3.7) Isolation of Total Cellular and Tissue RNA and Northern Blot Analysis:

Total cellular RNA was isolated from confluent cultured cells using a simplified protocol of Chomczynski and Sacchi (1987). Cells in 100 mm tissue culture dishes were washed once with ice-cold Dulbeco's phosphate-buffered saline (D-PBS) (Gibco) and lysed in 2 ml of 4 M guanidine isothiocyanate solution, pH 7.0, containing 25 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% sarcosyl (N-lauryl sarcosine). After one minute vortex, 200 μl of 2 M sodium acetate at pH 4.0, 2 ml diethylpyrocarbonate-treated(DEPC)-H_2O saturated phenol, and 400 μl chloroform: isoamyl alcohol (49:1) were sequentially added, with each step mixed by inversion. The mixture was incubated on ice for 15-30 min, and then centrifuged at 10,000 r.p.m. at 4°C for 20 min. The aqueous phase was collected and precipitated at -20°C with an equal volume of isopropanol for at least one hour. After 20 min centrifugation at 10,00 r.p.m., the RNA pellet

was dissolved in DEPC-treated H20. The amount of total RNA was estimated from the absorbence at 260 nm, using an Ultrospec II spectrophotometer (LKB Biochrom, Cambridge, England).

Total cellular RNA was isolated from tissue using a previously described method (Chirgwin et al., 1979). Briefly, approximately 1 gram of frozen tissue was cut into 2-3 mm cubic fragments and homogenized by a Brinkman's Polytron in 6 ml solution containing 4 M guanidine isothiocyanate (GIT) (ICN Biomedical), pH 7.0, 25 mM sodium citrate, 0.1 M β-mercaptoethanol, and 0.5% sarcosyl. The homogenate was layered on to a 3.3 ml cushion of 5.7 M cesium chloride (CsCl) (ICN biomedical) solution, pH 5.0, containing 25 mM sodium acetate in a 15 ml polyallomer tube, and centrifuged at 32,000 r.p.m. for 20 hr at room temperature. The clear gelatinous pellet of RNA was dissolved in DEPC-H_2O containing 0.3 M sodium acetate, pH 6.0. After phenol-chloroform extraction, the aqueous phase containing the RNA was precipitated with 3 volumes of absolute alcohol at -80° C. Following a 15 min centrifugation at 10,000 r.p.m., the pellet was redissolved in DEPC-H2O.

Thirty microgram of the total RNA sample was denatured at 65° C for 10 min in a solution containing 20 mM 3-[N-morphoino-propanesulfonic acid (MOPS), 50% deionized formamide, and 6% formaldehyde, and was electrophoretically separated in 1% agarose gel containing 0.66 M formaldehyde in an aqueous buffer containing 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA, pH 7.0. After the gel was washed for 1 hr in 20X SSC, with one wash change after 30 min, the RNA was transferred to Hybond-N membranes (Amersham) by capillary blotting for 16-24 hours in 20X SSC

solution. The air-dried membrane was exposed to the ultra-violet light of UV-transilluminator (Photodyne, New Berlin, WI) for 3 min to cross link the RNA to the membrane.

2.3.8) Nucleic Acid Hybridization with cDNA Probes:

The cDNA probes were, 2.3 kb *Eco*RI insert of prTGF0.2 for rat TGF-α (Lee et al., 1985); 2.4 kb *Cla*I insert of pE7 for the epidermal growth factor receptor (Merlino et al., 1984); a rat kidney γ-glutamyl transpeptidase (GGT) cDNA clone 37 (Laperche et al., 1986); 1.6 kb insert of pMurbeta-2 for TGF-β1 (Dernyk et al., 1986) and 780 bp insert for IGF-II (Brown et al., 1986). cDNA probe for β-actin was purchased from Clontech (Palo Alto, CA).

This was performed according to a slightly modified procedure of Church and Gilbert (1984). 10-100 ng of cDNA fragments was labelled with [^{32}P]-dCTP to a high specific activity (approximately 1 X 10^9 c.p.m/μg) using the Oligolabelling kit (Pharmacia, Piscataway, NJ). Membrane was prehybridized at 42°C for 1-2 hr in a solution containing 0.5 M Na_2HPO_4, pH 7.2, 5% bovine serum albumin (BSA) fraction V, 1 mM EDTA, 5% sodium dodecyl sulphate, 0.1% boiled sheared salmon sperm DNA and 50% deionized formamide. Hybridization was carried out in the same solution containing the ^{32}P-labeled probe at 42°C for 16-18 hours. The membrane was subsequently washed 4 times for 10 min at room temperature in 2 X SSC-0.1% SDS, and twice for 30 min at 55-60°C in 0.3 X SSC-0.1% SDS. After a further rinse in 1 X SSC, the membrane was blotted semi-dry and exposed with an intensifying screen to Kodak XAR-5 films at -80°C.

2.3.9) Phenotyping by Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

RT-PCR is a highly sensitive method for the detection of RNA transcripts (Rappolee et al., 1991). For most genes with introns, PCR primers are designed to include sequences separated by at least one intron, hence the PCR products of RNA can be easily distinguished from those of the contaminating genomic DNA fragment. However this may be a problem if the gene to be studied lacks introns. In such cases, an exhaustive DNase treatment of the RNA is essential to obtain meaningful RT-PCR results. Ten microgram of total cellular RNA isolated from confluent cultured RP-2 cells at passage 10 was subject to RNase-free DNase I (Promega, Madison, WI) treatment at 37°C for 30 min in a 30 µl reaction mixture, which contained 2-3 U DNase I /µg RNA in 1 X buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 µg/µl bovine serum albumin). The reaction mixture was then extracted once with phenol, once with phenol: chloroform: isoamyl alcohol (25:24:1), and precipitated with absolute ethanol at -20°C.

Two microgram of DNase-treated RNA was reverse transcribed (RT) with a standard first stand reaction condition (Kawasaki, 1991). The RT step contained 1 X RT buffer (10 mM Tris-HCl, pH 8.3, 15 mM KCl and 0.6 mM $MgCl_2$), 3.3 µM random hexamer primer (Pharmacia), 0.5 mM deoxyribonucleoside triphosphates (dNTPs), 1 U/µl recombinant RNase inhibitor (Pharmacia) and 13.3 U/µl Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in a total volume of 30 µl. The reaction mixture was incubated at 42°C for one hour, then heat-inactivated at 70-80°C for 10 min.

The quality of the RT reaction was checked by performing a PCR reaction (Mullis and Faloona, 1987) using paired primers for the β-actin gene. The RT reaction was considered satisfactory if an obviously large amount of 283-bp β-actin product was visible by ethidium bromide fluorescent staining. Using paired primers, 2 or 3 μl of the RT product was amplified for 30 PCR cycles. The sequences of these genes were obtained from the Genebank file contained in Entrez (National Center for Biotechnology Information, Bethesda, MD), and the PCR primers were selected using the Oligo 4.0 (Plymouth, MN) software. The sequences and positions of all primers and the PCR product size are summarized in Table 3-1. PCR was performed using a Perkin-Elmer DNA Thermal Cycler, with each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C. A 50 μl PCR reaction contained 1 X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1 % Triton X-100), 1.5 mM $MgCl_2$, 200 μM dNTP mix, 2 units of Taq DNA polymerase (Promega). The reaction products were separated electrophoretically in a 2 % agarose gel. The cDNA products were identified by their desired size and subsequent confirmation by restriction enzyme mapping. As positive controls, RNA from tissue expressing high levels of the desired gene (adult rat liver for albumin and aldolase B, new-born liver for α-fetoprotein and adult rat kidney for carbonic anhydrase II) were used. Negative controls included the omission of RT products in PCR reactions.

Table 2-1 The sequences of primers and their positions in each cDNA sequence and the predicted size of PCR product

cDNA	Primer sequences/Nucleotide position	Product size(bp)/ Restriction site[a]
Albumin	5'-CTCCTCCTCCTCTTCATCTCC-3'/19	619/*Hind* III
	5'-CTTTCTCTTTCACGGCATCAA-3'/617	
α-FP	5'-CCCTCTACCATTCTCTGCGAC-3'/147	261/*Hinf* I
	5'-TAAACCCCCAAAGCCTCACGA-3'/387	
aldolase B	5'-TGCCAATGGGAAGGGTATCT-3'/140	805/*Kpn* I
	5'-TAGAGGGCAACGGTAGATAG-3'/925	
CA II[b]	5'-GCACAACGGACCAGAGAACG-3'/35	584/*Pst* I
	5'-TCAGAGAGCCAGGGTATGTC-3'/599	
β-actin	5'-TCTAGGCACCAAGGTGTGATGGT-3'/34	283
	5'-GGTCTCAAACATGATCTGGGTCA-3'/294	

a: Restriction site present in each cDNA used for quick screening the identity of PCR products

b: The primers represents sequences of mouse carbonic anhydrase I I (CA II) gene. The sense and anti-sense primers show 85% (17/20) and 45% (9/20) homology to corresponding area in rat carbonic anhydrase II gene

2.4 RESULTS

Purification and growth characteristics of duct epithelial cells:

A duct fragment with small branches was aspirated and cultured in a 60 mm plate (Figure 2-1 A). Twenty four hours after initial plating, the fragment attached to the tissue culture dish and some of the epithelial cells started to migrate out, spread and proliferate (Figure 2-1 B). Forty eight hours later, the initial duct fragment had almost completely dissolved and formed a monolayer of predominantly epithelial cells (Figure 2-1 C). These cells assumed a polygonal shape and grew in a cobblestone arrangement (Figure 2-1 D). Both arterioles and venules did not develop into monolayer cells as did the duct fragments. The cells continued to proliferate and formed a colony which was devoid of fibroblast contamination. After the first subculture, a cell line (RP-2) was obtained. This was designated as passage 1.

At passage 6, these cells had a population doubling time of 30.8 $\pm$ 1.1 hr (n=3), however, with continuing subcultures, the doubling time decreased to 23.2 $\pm$ 1.4 hr (n=3) at passage 10, and 18.5 $\pm$ 1.3 hr (n=3) at passage 15. The line has subsequently been propagated for 16 passages (80-90 doublings) without any morphologic evidence of senescence (Figure 2-3 A).

Ultrastructure:

Transmission electron microscopic studies of cultured cells at passage 10 revealed polygonal cells which were often joined by tight junctions and desmosomes (Figure 2-2), supporting their epithelial

nature. Most cells demonstrated a relatively simple cytoplasmic organization including mitochondria, short strands of rough endoplasmic reticulum and a well-developed Golgi complex. Glycogen particles were not seen. The cell formed complex basolateral microvillus processes, however, cilia were not observed. When the cells at passage 15 were grown as a monolayer, polarity was maintained as evident from the presence of microvilli at the apical cell surfaces (Figure 2-3 B). There was no significant ultrastructural differences between cells at different passages. These cells were also indistinguishable at the light and electron microscopic levels from the previously established cell line RP-F344-1 (Tsao and Duguid, 1987). This cell line has been propagated for 22 passages (110-130 doublings) without any morphologic evidence of senescence.

Phenotypic and genotypic properties of RP-2 cells:

RP-2 cells retained few biochemical phenotypic markers of pancreatic duct epithelial cells *in vivo*. They showed low levels of γ-glutamyl-transpeptidase (GGT) activity (19.1 ± 12.7 mU/mg protein) which was undetectable histochemically. The specific activity of carbonic anhydrase (CA) in these cells (0.35 ± 0.11 mg CA/mg protein) was similar to that of normal cultured rat pancreatic duct epithelial cells reported by Heimann and Githens (1991). The cells stained negatively for glycogen and mucin, but demonstrated alkaline phosphatase (ALP) activity.

Using immunocytochemistry, the cells stained negatively for albumin and α-fetoprotein (AFP). The great majority of the cells

stained positively for cytokeratin 8 but negatively for cytokeratin 19. There was no immunoreactivity with the monoclonal antibodies HES6 and BDS7.

The RT-PCR technique demonstrated the presence of low levels of mRNA transcripts for CA II, albumin and AFP (Figure 2-4) in RP-2 cells, however, these transcripts were not detectable using the Northern hybridization technique. The aldolase B mRNA was not detected by either technique. Northern blot analysis (Figure 2-5) showed that these cells expressed mRNAs of EGF receptor, TGF-β1, and IGF-II, but mRNAs of TGF-α and GGT were not detectable. The phenotypes of RP-2 are summarized in Table 2-2.

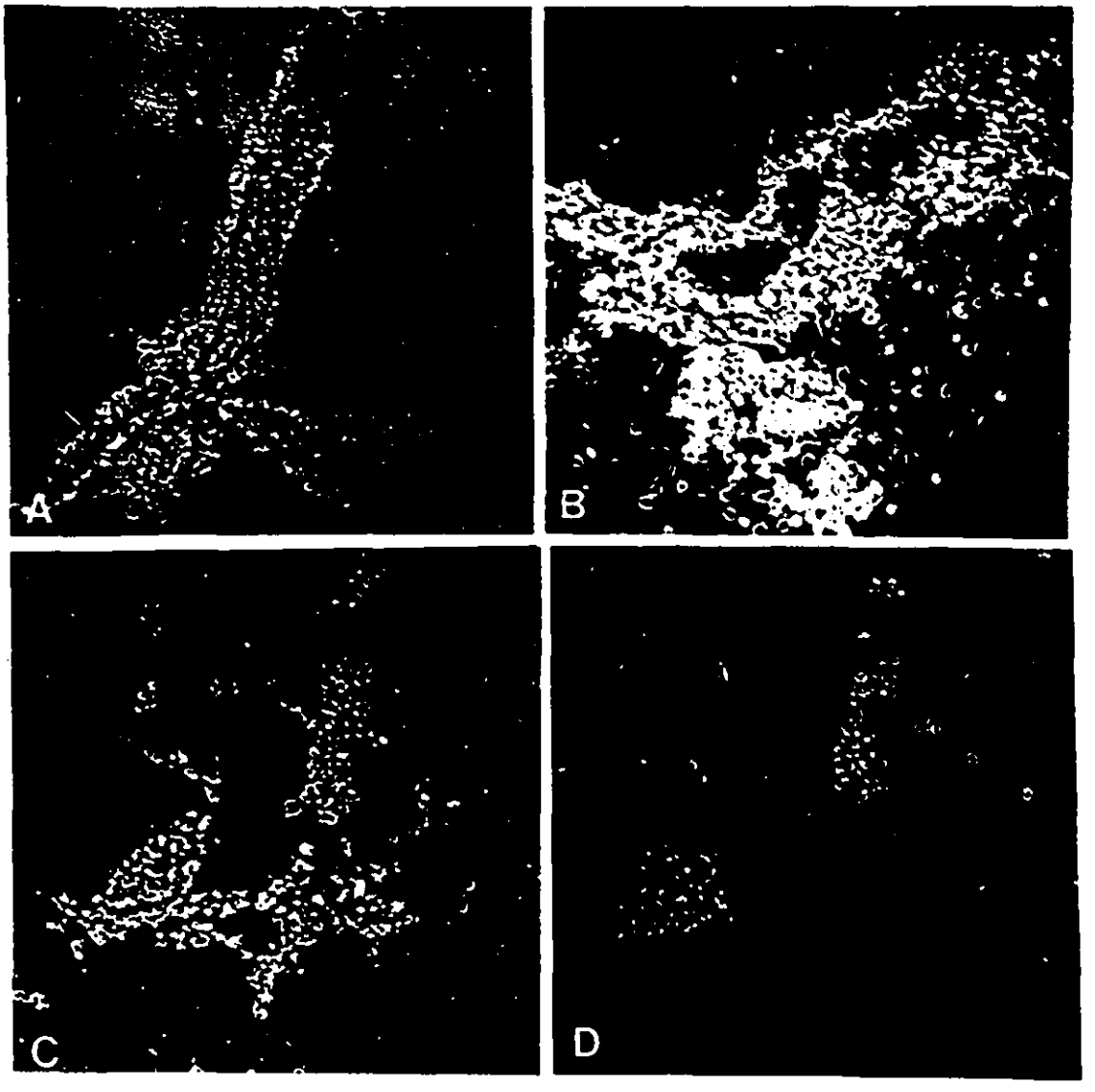

Figure 2-1 Phase-contrast microscopic illustrations show the establishment of a monolayer cell line from a rat pancreatic duct fragment. (A) A freshly isolated branching duct fragment was aspirated from the collagenase digest of a rat pancreas by using a Pasteur pipette, then were cultured in 60 mm plates. (B) One day later, the fragment attached and the epithelial cells started to migrate onto the culture plate. (C) At day two after plating, the cells showed further spreading. (D) At day 3, the duct fragment has completely dissolved and a monolayer culture of cobble-stone appearing cells was formed. (Magnification: X 400).

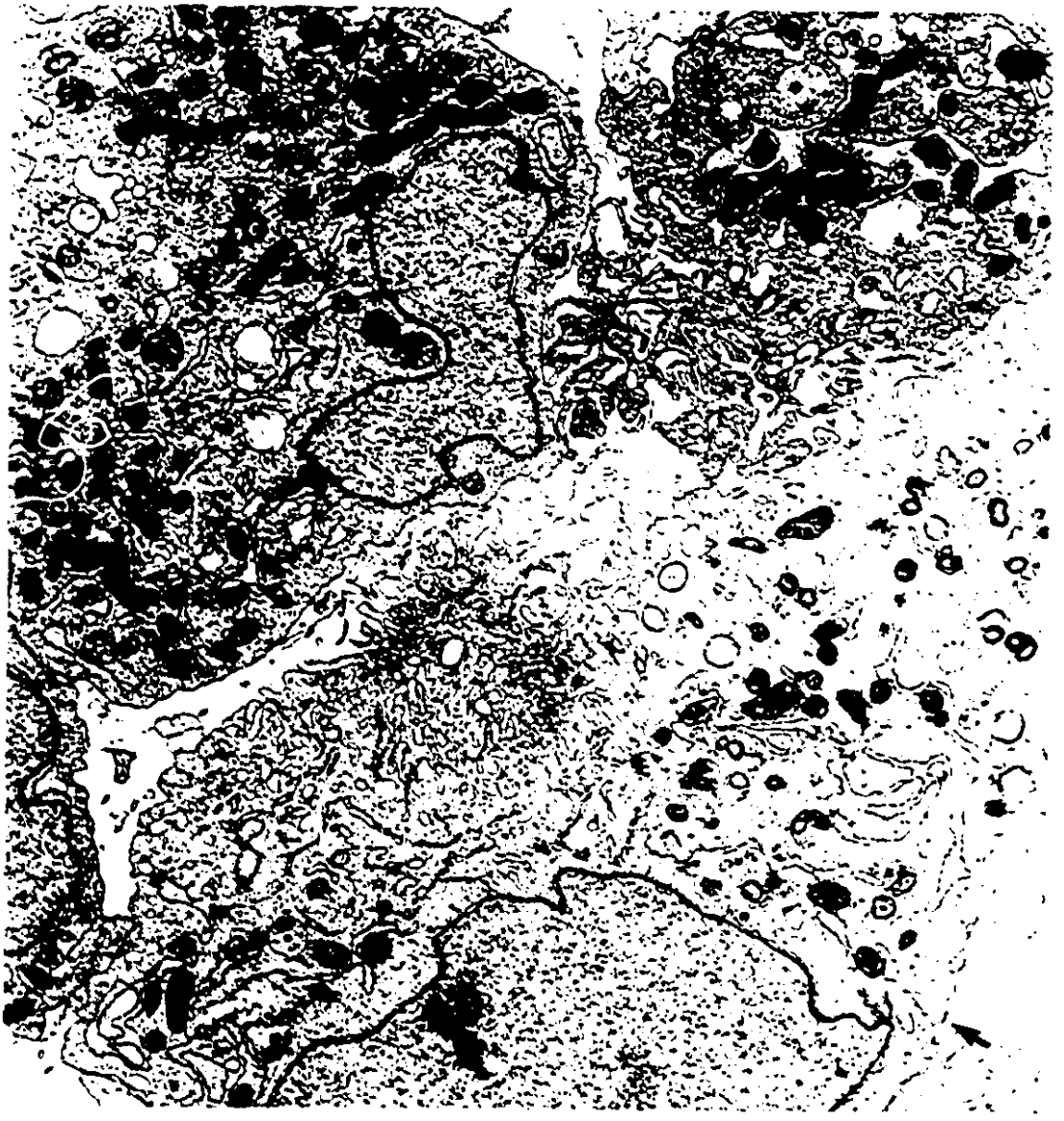

Figure 2-2 An electron microscopic picture of RP-2 cells at passage 10 showing the irregular nuclei, microvillus plasma membrane projections, apical and lateral cell processes and cell junctions (arrow head). (Magnification: X 5,730)

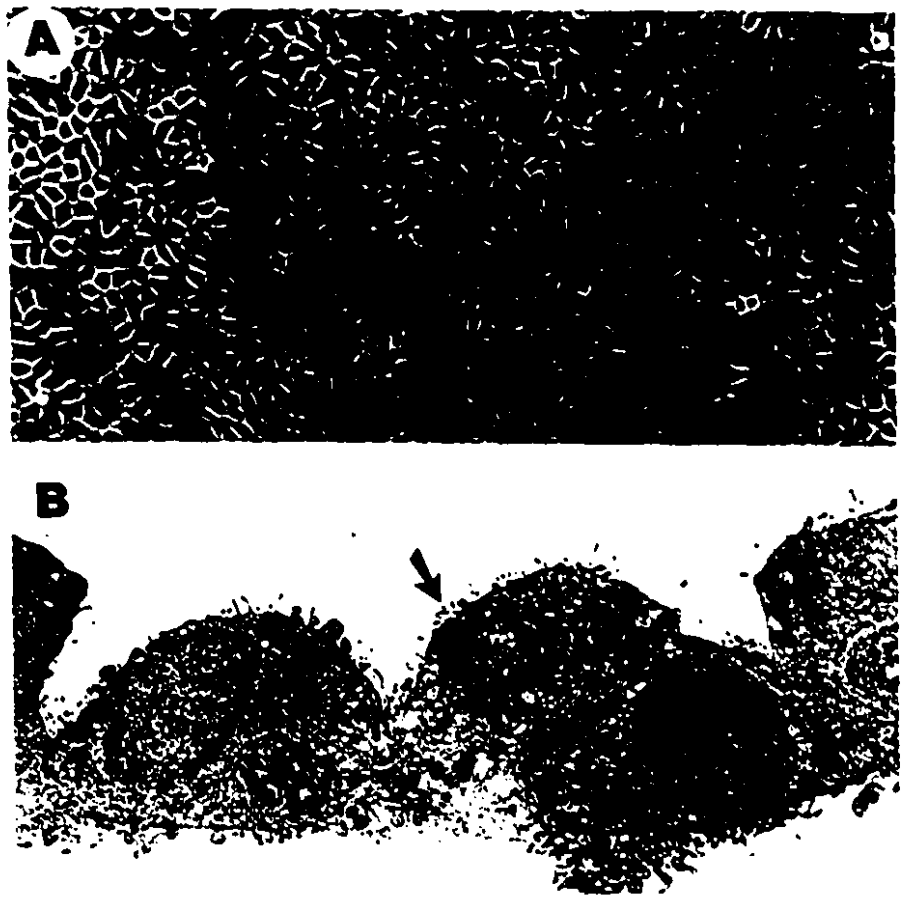

Figure 2-3 (A) Phase-contrast micrograph of RP-2 cells at passage 15. The cells are tightly packed, cuboidal in shape, and demonstrate large nuclei containing prominent nucleoli. (B) An electron micrograph of RP-2 cells at passage 15 growing on a tissue culture dish. The maintenance of cell polarity is evident by the presence of microvilli at their apical surfaces (arrow). (Magnification: A: X 250; B: X 3580)

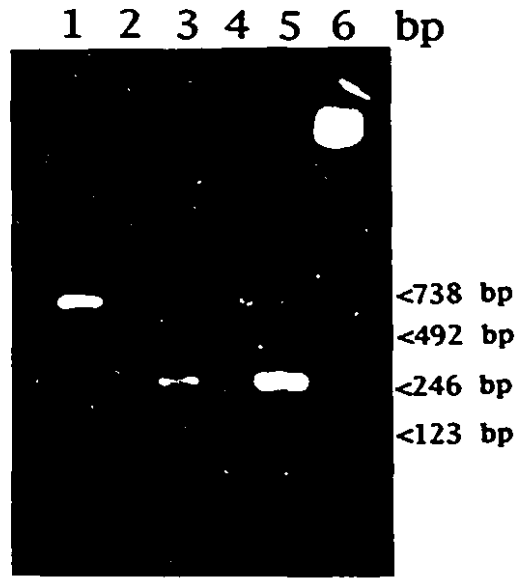

Figure 2-4 The RT-PCR analyses of mRNA expression in cultured rat pancreatic duct epithelial cells at passage 10. Lane 1: CA II (582 bp); lane 2: albumin (619 bp); lane 3: α-fetoprotein (261 bp); lane 4: aldolase B (805 bp); lane 5: β-actin (283 bp); and lane 6: 123 bp ladder molecular weight marker.

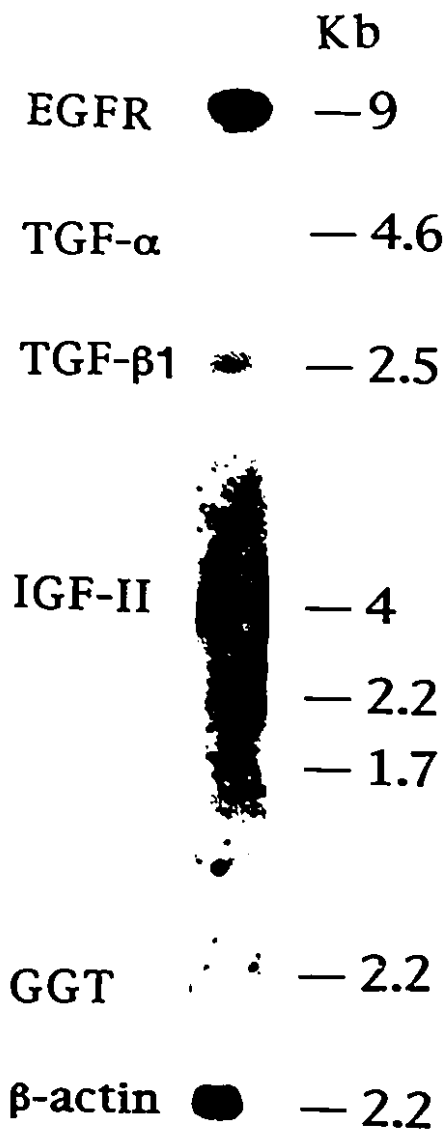

Figure 2-5 Northern blot analyses of RP-2 cells at passage 10 showing the mRNA expressions of EGFR, TGF-α, TGF-β1, and IGF-II, GGT, and β-actin.

Table 2-2 The Major Phenotypic Properties of RP-2 Rat Pancreatic Duct Epithelial Cells

Express epidermal growth factor receptor (ERGF-R)
Express insulin-like growth factor-II and transforming growth factor-β1
Express carbonic anhydrase (0.35 ± 0.11 mg CA/mg protein) and alkaline phosphatase activities
Express low levels of γ-glutamyl transpeptidase activity (19.1 ± 12.7 mU/mg protein)
Express low levels of albumin and α-fetoprotein mRNA
Express cytokeratin 8 but not cytokeratin 19
Do not express transforming growth factor alpha
Do not store glycogen
Do not produce mucins
Do not express rat liver biliary epithelial cell antigen recognized by monoclonal antibody BDS 7 and hepatocyte antigen for monoclonal antibody for HES 6

2.5 DISCUSSION

In this study, a propagable epithelial cell line was established directly from an isolated pancreatic duct fragment of an adult male rat. The ductal origin of the cells was demonstrated by direct and sequential observations of the development of epithelial monolayer cells from the single isolated duct fragment. These cells maintained certain duct epithelial phenotypes as judged by the detection of low levels of CA II, GGT, and ALP activities. They were indistinguishable from the original cell line (RP-F344-1) (Tsao and Duguid, 1987) at both the light and electron microscopic levels, thus confirming their previous conclusion that ductal cells of normal adult rat pancreas can be continuously propagated *in vitro* to form cell lines.

The phenotypic properties of RP-2 (Table 2-2) are very similar to those of normal rat liver epithelial cells (RLEC) which have been posited to originate from the intrahepatic bile ductular cells (Tsao et al., 1984; Grisham et al., 1990). The RLEC has also been shown to resemble phenotypically the hepatic "oval" cells. While our knowledge of the properties and biology of rat liver "oval" cells is quite significant, very few studies have implicated the involvement of a similar cell in the pathogenesis of pancreatic diseases. Periductular "oval" cells have been observed in the pancreata of rats fed with copper-deficient diet, and they were postulated to be one of the progenitor cells of pancreatic hepatocytes (Rao et al., 1990a). When established as a cell line (Rao and Reddy, 1991), these cells showed ultrastructural features of epithelial cells, were negative for GGT but stained positively for OV-6 antibodies. The relationship and

similarity between RP-2 cells and these oval cells remains to be investigated.

Bisgaard and Thorgeirsson (1990) reported that propagable normal epithelial cell lines established from the rat liver and pancreas have identical phenotypes, thus suggesting their common origin from the pancreatico-hepatobiliary stem cells. This hypothesis is supported by the similar properties between these cell lines. For instance, the anaplastic carcinoma produced by the spontaneously transformed rat liver (Tsao et al., 1990; Tsao and Zhang, 1992) and pancreatic (Shepherd et al., 1993) epithelial cell lines are indistinguishable at the light and electron microscopic levels. The existence of a common progenitor or stem cell in the pancreas and liver is also supported by the ability to induce hepatocytic differentiation in the pancreata of rats or hamsters following a copper-depleting diet (Rao et al., 1990) or after carcinogen treatment (Scarpelli and Rao, 1981), and by the induction of pancreatic acinar cell differentiation in the liver of rats treated by polychlorinated biphenyls (Kimbrough, 1975; Rao et al., 1986a). Recently, the status of the RLEC as the facultative stem cell in liver has been strengthened by their ability to differentiate into hepatocytes and integrate into the parenchymal hepatic cell plates following *in vivo* transplantation (Coleman et al., 1994). Though pancreatic ductal and periductular (oval) cells have been implicated as the progenitor cells of the transdifferentiated hepatocytes (Rao et al., 1990 a,b), direct supporting evidences has been lacking. With the availability of cultured pancreatic duct epithelial cell lines such as the RP-2, *in vivo* transplantation of these cells in different anatomic sites should

provide more information on the differentiation potential of rat pancreatic ductal cells.

When cells are grown *in vitro*, they are known to release various autocrine factors into the culture medium, a process known as "conditioning" which may influence their own growth (Kinard et al., 1990). Cultured rat fetal islets synthesize and release IGF-I and also a small amount of IGF-II (Romanus et al., 1985), while both RP-2 cells and RLEC express IGF-II and TGF-β1 (Grisham et al., 1990). Insulin, and IGF-I and -II, are three homologous peptides that bind with different affinities to three distinct receptor types (Czech, 1989). An autocrine stimulation of cell proliferation by IGF-I and -II has been suggested by their frequent expression in many types of tumors (Heldin and Westermark, 1989). IGF-I is mainly produced in the liver, whereas IGF-II is also produced in a variety of other tissues (Cross and Dexter, 1991). The rat pancreas expressed appreciable levels of IGF-I mRNA, but IGF-II mRNA has not been detected (Brown et al., 1986; Hynes et al., 1987). Because these studies were performed using the Northern blot hybridization technique with total RNA, the cellular localization of these IGFs could not be demonstrated. Furthermore Northern hybridization may not be sensitive enough to detect low levels of IGF-II expression, especially when IGF-II was expressed by the duct cells. Further *in situ* hybridization and immunocytochemical studies on rat pancreata of different ages may provide further insights into the developmental regulation of IGF expression at the cellular level

Five species of IGF-II RNA transcripts have been detected in rat cells, they are respectively 6, 4, 2.2, 1.7, and 1.2 kilobase pairs

(Graham et al., 1986); they are probably produced by alternative processing of a single primary RNA transcript. A 3.8 kb species has also been described by Brown and colleague (1986), but it was usually indistinguishable from the 4-kb due to their approximate sizes. Although the relative abundance of these RNAs transcripts varies in different tissues, the 4 kb RNA is the predominant species in all tissues (Brown et al., 1986). Our results revealed the expression of three mRNA species of approximately 4 (dominant), 2.2, and 1.7 kb length. The levels of the 6 and 1.2 kb RNAs were not detectable. It is worth noting that the cultured rat liver epithelial cells also elaborate large amount of IGF-II, whose initial isolation was in fact from the conditioned medium of the Buffalo rat liver epithelial cells (Dulak and Temin, 1973).

The three mammalian transforming growth factor β (TGF-β) isoforms are multifunctional polypeptides which exercise major influences on cell growth, differentiation, adhesion, migration, angiogenesis, extracellular matrix formation and immune functions (Derynck et al., 1986). In human pancreas, immunoreactivities for TGF-β1, -β2, and -β3 have been demonstrated in the islet cells, acinar cells and ductal cells. In the ductal cells, all three isoforms show a similar intensity and pattern of immunostaining and are more frequently observed in the smaller distal ductules than in the larger pancreatic ducts (Yamanaka et al., 1993). In contrast, the distribution of these three TGF-β isoforms in rat pancreas is less clear. These TGF-β isoforms may exert their biologic effects through autocrine and paracrine mechanisms (Yamanaka et al., 1993). It appears that cultured RP-2 and RLEC retain their capacity to secrete

TGF-β1, but TGF-β1 may have no biological effect as it is usually secreted in a latent and inactive form by the normal cells (Liu et al., 1988).

CHAPTER 3

Hepatocytic Differentiation of Propagable Cultured Rat Pancreatic Duct Epithelial Cells Following *In Vivo* Implantation

3.1 SUMMARY

We have investigated the differentiation potential of propagable cultured rat pancreatic duct epithelial cells after *in vivo* implantation in isogeneic Fischer-344 rats. Cells genetically labelled with *Escherichia coli* β-galactosidase (*lacZ*) reporter gene were embedded in a mixture of collagen and Matrigel (basement membrane matrix), and implanted either subcutaneously or intraperitoneally. Tissues from the two locations were harvested 4-8 weeks later. The great majority of the *lacZ*-labelled epithelial cells colonizing both sites phenotypically resembled hepatocytes, however, they demonstrated different degrees of hepatocytic differentiation. Less than 5% of *lacZ*-labelled cells formed ductular structures. The hepatocyte-like cells from the subcutaneous implantation site expressed mixed phenotypes of both hepatocyte and ductal cell, including the expression of α-fetoprotein (AFP), tyrosine aminotransferase, γ-glutamyl transpeptidase, carbonic anhydrase II (CA II), and cytokeratin 19. In contrast, the hepatocyte-like cells colonizing the mesentery showed the phenotype of mature hepatocytes, including abundant glycogen storage and a lack of AFP and CA II expressions. Neither acinar cell nor endocrine differentiation was seen. These findings demonstrate that pancreatic ductal cells can be the progenitor cell for transdifferentiated hepatocytes.

3.2 INTRODUCTION

The induction of hepatocytes in the pancreas of rats and hamsters following treatment with chemical carcinogens (Scarpelli and Rao, 1981) or copper-depleting diets (Rao et al., 1986b), and the development of pancreatic acinar cells in rat liver following treatment with polychlorinated biphenyls (Kimbrough, 1973; Rao et al., 1986a) are examples of metaplasia or transdifferentiation. Initially it was postulated that the pancreatic hepatocytes were derived from the acinar cells (Scarpelli and Rao, 1981, Reddy et al., 1984), hence representing transdifferentiation (Okada, 1986). However, subsequent studies suggested that they originated from the duct epithelial and/or "periductal" cells (Rao et al., 1990; Makino et al., 1990; Reddy et al., 1991). An elucidation of the histogenetic origin of transdifferentiated hepatocytes in the pancreas is complicated by the multiplicity of epithelial cell types present in this organ, and the dynamic process of *in vivo* experiments. A more direct approach is to establish an *in vitro* model using cell lines derived from specific pancreatic epithelial cell types, and to investigate if they are subsequently capable of differentiating into other cell types. Although pancreatic acinar (Brannon et al., 1985) and islet (Takaki,1989) cells in primary cultures can be maintained functionally for an extended period of time, they have not been shown to be capable of continuous proliferation *in vitro* to form cell lines. In contrast, we and others have reported the establishment of propagable cell lines from normal pancreatic duct epithelial cells from adult rats (Tsao and Duguid, 1987) and hamsters (Hubchak et al., 1990), and of a periductal (oval) cell line from rats fed a copper-

depleting diet (Rao and Reddy, 1991, Ide et al., 1991). In this study, we have genetically labelled cultured normal rat pancreatic duct epithelial cells with a bacterial enzyme whose activity can be detected by histochemistry and have examined the differentiation capacity of these ductal cells in two *in vivo* microenvironments.

3.3 MATERIALS AND METHODS

3.3.1) Cell line and Culture:

RP-2 is a pancreatic duct epithelial cell line established from the pancreas of an adult male Fischer-344 rat, as described in Chapter 3. RP-2 cells at passage 10 were used in this study. Cells were cultured in Richter's improved minimal essential medium with zinc option (IMEMZO) (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum (ICN Biomedical, St. Laurent, Que), 4 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamycin at 37°C in a humidified 5% CO_2 atmosphere. Subcultures were routinely performed before the cultures reached confluence, at a plating density of 1X 10^5 cells per 100 mm tissue culture dish (Beckon Dickinson, Mississauga, Ont). Cell number was determined using a ZM Coulter cell counter (Coulter Electronics, Hialeah, FL).

3.3.2) Genetic Labelling:

RP-2 cells at passage 10 were genetically labelled by transfection with the *E. coli* β-galactosidase (*lacZ*) gene. The transfected pRSV*lacZ*expression vector (Figure 3-1) contained a 3.7-kb *Hind*III-*Xba*I fragment of the bacterial *lacZ* gene (Lin and Culp, 1991). Transfection was performed using the Lipofectin Reagent

(Gibco-BRL, Grand Island, NY), which is a 1:1 (w w) liposome formulation of the cationic lipid dioleoyl phosphotidylethanolamine (DOPE) and N-[1(-2,3-dioleyloxy)propyl]-N,N,N-trimethlyammonium chloride (DOTMA) in membrane filtered water. The action of liposome-mediated transfection is schematically depicted in Figure 3-2. Cells were plated in 35 mm tissue culture plates at the density of 1-2 x 10^5 cells per plate and cultured until they were 40-60% confluent. In a 12 x 75 mm polysterene tube, 5 μg DNA in 100 μl serum-free IMEMZO (SFIM) medium was gently mixed with equal volume of SFIM containing 25 μg LIPOFECTIN Reagent, and this mixture was incubated at room temperature for 10-15 minutes. After washing the cells twice with SFIM, 0.8 ml of SFIM was gently mixed with the LIPOFECTIN Reagent DNA-complexes and then added to the culture plate. After incubation for 24 hours at 37°C, the medium was replaced with IMEMZO containing 10% FBS and the cells were incubated for another 48-72 hours. Stably transfected cells were selected by culturing in a medium containing 800 μg/ml G-418 sulfate (Gibco-BRL). Untransfected cells usually started to die 72 hr later. After 14 days, the surviving transfected cells were pooled and then reseeded at a plating density of 500 cells per 100 mm tissue culture plate (Beckon Dickinson) and grown in a medium containing G-418 sulfate. Using cloning cylinders, several cell strains were isolated from individual colonies. Cells forming these colonies were designated as passage 1 and they were expanded by further subcultures in 100 mm tissue culture plates. Transfected RP-2 cells of strain 2 and 5 at passage 2 were randomly chosen and used for implantation.

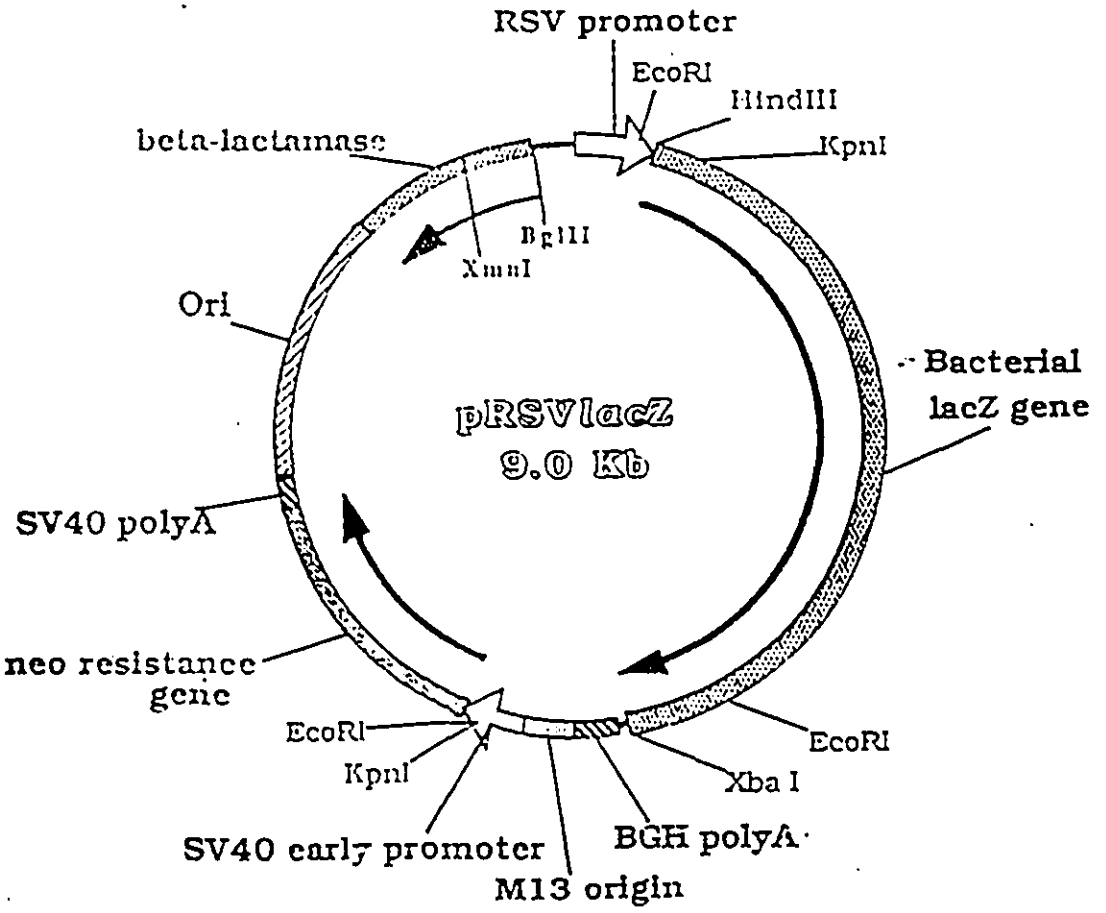

Figure 3-1 The expression vector pRSV *lacZ* carried a 3.7 kb *Hind*III-*Xba*I fragment bacterial *lacZ* gene in a 5.2 kb pRc/RSV plasmid (Invitrogen) (Lin and Culp, 1991). The pRc/RSV plasmid contains the neomycin resistance marker gene for easy selection of stable transfectants and the enhancer/ promoter sequences of Rous sarcoma virus long terminal repeat (RSV LTR) for high levels transcription of the recombinant gene.

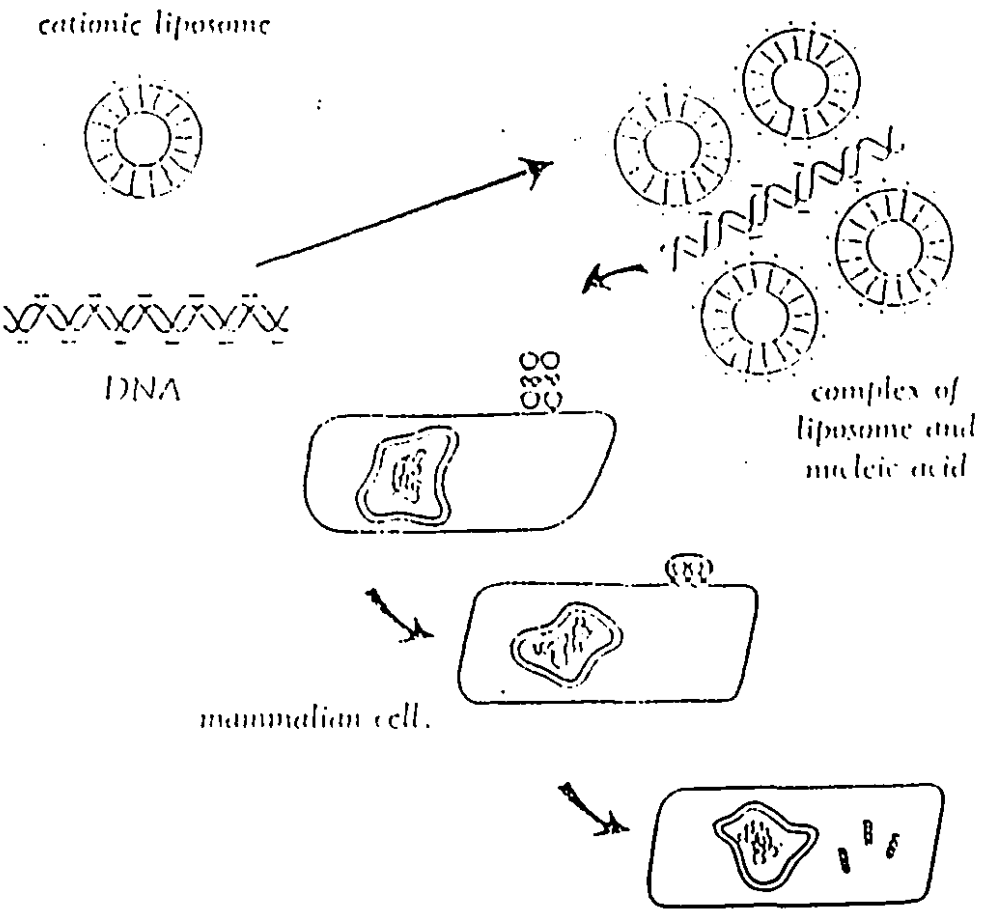

Figure 3-2 Schematic illustration of the action of liposome-mediated transfection. Lipofectin Reagent® (Gibco, BRL) is a liposome formula of a cationic acid and a neutral lipid that interacts with DNA to form a lipid-DNA complex. The fusion of the lipid-DNA complex with cells results in efficient uptake and expression of the DNA. The efficiency of this process is influenced by several parameters, including the culture medium, the concentration of Lipofectin Reagent, the concentration of DNA, and the incubation time.

3.3.3) Preparation of Rat Tail Collagen Solution:

Type I rat collagen was prepared using a published method (Montesano and Orci, 1985). Tails from adult rats were collected and stored at -20° C in plastic bags to prevent dehydration. Prior to processing, tails were thawed and sterilized for about 20 min in 70% ethanol. Using sterile equipment, each tail was fractured starting from the tip. The tendon attached to the distal piece was drawn out from the tail and was then cut free and collected again in 70% ethanol.

After air drying overnight, the tendons were weighed and transferred to a flask containing suitable amount of 0.017 M acetic acid (approximately 4 g of tendon dissolved in each liter). This solution was kept stirring for 48 hr at 4°C to promote dissolution of collagen. The solution is first passed through double layers of sterile gauze to remove undissolved fibers and then centrifuged at 15,000 r.p.m. for 30 min. The supernatant was carefully layered on top of one tenth volume of chloroform and allowed to sit at 4°C for overnight. The collagen solution was subsequently removed aseptically and used without further purification.

3.3.4) *In Vivo* Implantation:

Male Fischer-344 rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 120-150 g were used in all experiments. All animal were fed with normal chow diet.

Male rats were used for subcutaneous implantation because of their lack of mammary glands. Under ether anesthesia, a skin incision was made along the midline of the upper abdomen. The skin

and underlying fascia were bluntly dissected from the abdominal muscle, and cells suspended in an extracellular matrix gel were placed in the space between the skin and the rectus muscle. The extracellular matrix gel was reconstituted at 4°C from 6.4 parts of collagen solution (approximately 3.33 mg/ml), 1.8 parts of 5X concentrated IMEMZO, and 1 part of an aqueous solution containing 200 mM HEPES, 200 mM $NaHCO_3$, and 50 mM NaOH (Altmann and Quaroni, 1990), and 1 part of Matrigel™ solution (Collaborative Research, Bedford, MA). 2 X 10^6 RP-2 cells at passage 12 or *lac*Z-transfected cells were used in each implantation. Cell-free gel was implanted in control animals. The skin was closed with 2-0 nylon suture and the animal allowed to recover.

Intraperitoneal implantation was carried out in a similar fashion except that the gels were placed between the mesenteric fat and omentum in the peritoneal cavity.

Four to eight weeks after implantation, the animals were sacrificed by cervical dislocation and tissue from the implantation site was harvested. Part of the tissue was fixed immediately in 10% buffered formaldehyde for routine paraffin embedding, and a small portion was fixed in 1% glutaraldehyde-4% paraformaldehyde solution for electron microscopic studies. For *in situ* hybridization, tissue was fixed in freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS) (Gibco-BRL), pH 7.4 for 4-6 hr and then stored in 25% sucrose/PBS at 4°C. When available, tissue was also snap-frozen in liquid nitrogen and stored at -80°C.

Transfected cells and fresh implantation tissue were also subjected to an enzymatic reaction for bacterial *LacZ*. Rat liver,

pancreas, kidney and skeletal muscle were also subjected to this reaction as controls for mammalian β-galactosidase activity. For cultured cells, they were rinsed with PBS three times and then fixed at 4°C in 2% (v/v) formaldehyde/0.2%(v/v) glutaraldehyde in PBS for 5 min. After rinsing with PBS, they were incubated at 37°C in a solution containing 20 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), 2 mM $MgCl_2$, 20 mM potassium ferricyanide, and 20 mM potassium ferrocyanide in PBS. For staining of intact organs, the 2-3 mm tissue fragments were rinsed thoroughly with PBS and then fixed at 4°C in 2%(v/v) formaldehyde/0.2%(v/v) glutaraldehyde in PBS for 60 min. After rinsing with PBS three times, they were incubated at 4°C overnight in the X-gal staining solution with the addition of Nonidet-P40 and sodium deoxycholate to the final concentration of 0.02% (vol/vol) and 0.01% (vol/vol), respectively. A positive reaction was indicated by the formation of bluish precipitation. After the tissue was rinsed sequentially in PBS containing 3% dimethyl sulfoxide (DMSO) and PBS alone, it was processed for paraffin embedding (Lin et al., 1992).

3.3.5) Histochemistry, Ultrastructural and Enzyme Histochemical Studies:

Paraffin sections were routinely stained with hematoxylin-eosin, periodic acid-Schiff (PAS) with or without prior digestion with diastase, and reticulin stains. Frozen sections of unfixed tissue were stained with Sudan IV and were used in various enzymatic histochemistry.

Histochemistry on tissue sections for bacterial β-galactosidase

was performed using the same reaction as described above. Alkaline phosphatase (ALP) and γ-Glutamyl transpeptidase (GGT) (Tsao et al., 1985) were performed as described in Chapter 2, page 57. For carbonic anhydrase (CA) histochemistry (Githens et al., 1987), unfixed frozen sections were dipped in a solution containing 8.75 mM $CoSO_4$, 53 mM H_2SO_4, 5.85 mM KH_2PO_4, 0.5% Triton X-100, and 157 mM $NaHCO_3$ for 2 seconds, removed from the solution, and then drained for 28 seconds in front of a fan to promote CO_2 removal. This procedure was repeated up to 15 times. After the last dip and drain, the slides were placed in a rinsing solution (0.67 mM KH_2PO_4 and 0.15 M NaCl, pH 5.9) for 2 min and dipped in $(NH_4)_2S$ solution for 15 seconds. After washing in two changes of distilled water, the sections were counter stained with nuclear fast red (Sigma Chemical), dehydrated, and mounted in Permount. A positive reaction is indicated by the deposits of black brownish cobalt sulfide precipitate. The specificity of the reaction is confirmed by including acetazolamide in the solution, which strongly inhibits the reaction.

Transmission electron microscopic studies of 0.1-0.2 μm sections of epon-embedded tissue were carried out using a Philips electron microscope Model 201.

3.3.6) Immunocytochemistry:

This was performed using the streptavidin-biotin-peroxidase system (Zymed Laboratories, San Francisco, CA). Formalin-fixed paraffin-embedded sections are used in most cases. It is clear that some antigenic determinants are masked by formalin fixation and paraffin embedding, a phenomenon postulated to result either from

intermolecular cross-links formed between formalin and proteins or from the binding of other molecular structures to the epitope (Shi et al., 1991). Traditionally protease digestion of formalin-fixed sections has been used to resolve this dilemma. It has been shown that microwave heating of tissue sections is even more efficient in retrieving the masked epitope. Antigen retrieval by microwave heating offers several advantages, including (a) enzymatic predigestion can be omitted; (b) the incubation time of the primary antibodies can be significantly reduced, or dilution of primary antibodies can be increased; and (c) certain antibodies which are typically unreactive with formalin-fixed tissues give excellent staining (Shi et al., 1991).

A Sharp microwave model Carousel II operating at a frequency of 2,450 MHz with six power level settings was used (highest power setting- 600 w). Following deparaffinization and rehydration, the sections were placed in a Coplin jar containing boiling 0.1 M citric acid buffer, pH 6.0, and then twice microwaved for 5 min at the lowest power setting. The sections were allowed to cool in the same buffer for 20-30 min at room temperature. Endogenous peroxidase was quenched in hydrogen peroxide (3%) methanol solution for 10 min, followed by a 10 min incubation with 10% normal goat serum to suppress non-specific binding of IgG. The sections were then incubated with primary antibodies in a moist chamber at room temperature overnight. Antigen-antibody complex formation was visualized with either aminoethyl carbazole (AEC) or 3,3'-diaminobenzidine (DAB), which yielded red and brownish colored deposits respectively. Polyclonal antibodies against rat albumin

(ALB), α-fetoprotein (AFP) and transferrin were obtained from Nordic Immunological Laboratories (Capistrano Beach, CA) and were used at 1:6000, 1:2000 and 1:6000 dilution, respectively. The rabbit polyclonal antibody against rat tyrosine aminotransferase (TAT) was used at 1:600 dilution (Chou and Yeoh, 1987). A mouse monoclonal antibody against *E. coli* β-galactosidase was obtained from Oncogene Science (Uniondale, NY) and used at 1:10 dilution. The mouse monoclonal antibodies against human cytokeratin 19 (Amersham Canada, Oakville, Ont) and rat cytokeratin 8 (Germain et al., 1988) were used at 1:50 dilution. The mouse monoclonal antibodies HES6 and BDS7 respectively recognize the surface antigens of hepatocytes and biliary epithelial cells in adult rat liver (Germain et al., 1988). HES6 was used at 1:200 dilution while BDS7 was used undiluted. The rabbit polyclonal antibody against porcine α-amylase (Hansen et al., 1981) was used at 1:200 dilution. Polyclonal antibodies against insulin, glucagon, chromogranin and somatostatin were purchased from Dako (Carprinteria, CA) and used at 1:150 to 250 dilution, as suggested by the manufacturer. Tissues for the positive controls included rat liver and pancreas. Negative controls included replacement of the primary antibodies with non-immune serum or PBS.

Most antibodies were used on formalin-fixed paraffin-embedded sections. We find that HES6 antibody which was previously reported to be reactive only with frozen tissue sections (Germain et al., 1988), worked well on paraffin sections treated by antigen retrieval procedure. In contrast, immunostaining with BDS7 or antibodies to cytokeratin 8 and 19 could only be performed on

cold acetone fixed frozen sections.

3.3.7) Isolation of Total Genomic DNA and Southern Blot Analysis:

Genomic DNA was isolated according to the standard method (Sambrook et al., 1989). Trypsinized cells are digested at 37°C overnight with 100 μg/ml proteinase K (Boehringer Mannheim Canada, Laval, Que) in an aqueous solution containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 25 mM EDTA pH 8.0 and 1% SDS. After extraction once with an equal volume of TE buffer-saturated phenol, once with an equal volume of phenol and chloroform, and once with chloroform alone, the aqueous phase containing DNA was precipitated with 1/10 volume of 5 M NaCl and 2 volumes of absolute alcohol. The DNA was spooled out with glass rod, washed with 70% ethanol, air-dried, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The concentration of the DNA was estimated using a spectrophotometer at a wavelength of 260 nm.

The stable integration of the *LacZ* gene was confirmed by Southern blot analyses of the *Eco*RI digested DNA of transfected cell lines/strains. Fifteen microgram of DNA was digested overnight with 5U/μg *Eco*RI (Pharmacia, Piscataway, NJ), and resolved by electrophoresis in a 0.8% agarose gel in 40 mM Tris-HCl buffer, pH 7.9, containing 50 mM sodium acetate and 1 mM EDTA. Following electrophoresis, the gel was treated sequentially in the following solutions- 0.25 N HCl for 20 min (depurination), 0.2 N NaOH/0.6 M NaCl for 40 min (denaturation), and finally 0.5M Tris-HCl, pH 7.6/ 1.5 M NaCl for 40 min (neutralization). The DNA is then transferred

on to a Hybond-N nylon membrane (Amersham) in 20X SSC solution (SSC: standard saline citrate; 1X SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0). The air-dried membrane was cross-linked by exposure to the ultraviolet light. It was then hybridized to a 3.7-kb *HindIII-XbaI* fragment of the *E. coli* β-galactosidase cDNA labelled with [^{32}P]-dCTP to a high specific activity using the Random Primer labelling kit (Amersham). Hybridization and washing were performed according to Church and Gilbert (1984) as described in Chapter 2, page 62 and membranes were exposed to Kodak XAR-5 film at -80° C using an intensifying screen.

3.3.8) Isolation of Total Cellular and Tissue RNA and Northern Blot Analysis:

Total cellular RNA was isolated from confluent cultured cells, kidney and liver of adult rat, liver of a one-day old newborn Fischer-344 rat and from the subcutaneous implanted tissue. This procedure and Northern blot analysis are described in Chapter 2, page 60.

3.3.9) Gene Cloning by Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

This procedure is schematically depicted in Figure 3-3.

a) RT-PCR

Large amount of cDNA fragments of rat ALB (n19-638), AFP (n147-408), CA II (n35-619), and aldolase B (n140-945) were generated by the RT-PCR using the published procedure (Rappolee et al., 1991) as described in Chapter 2, page 63.

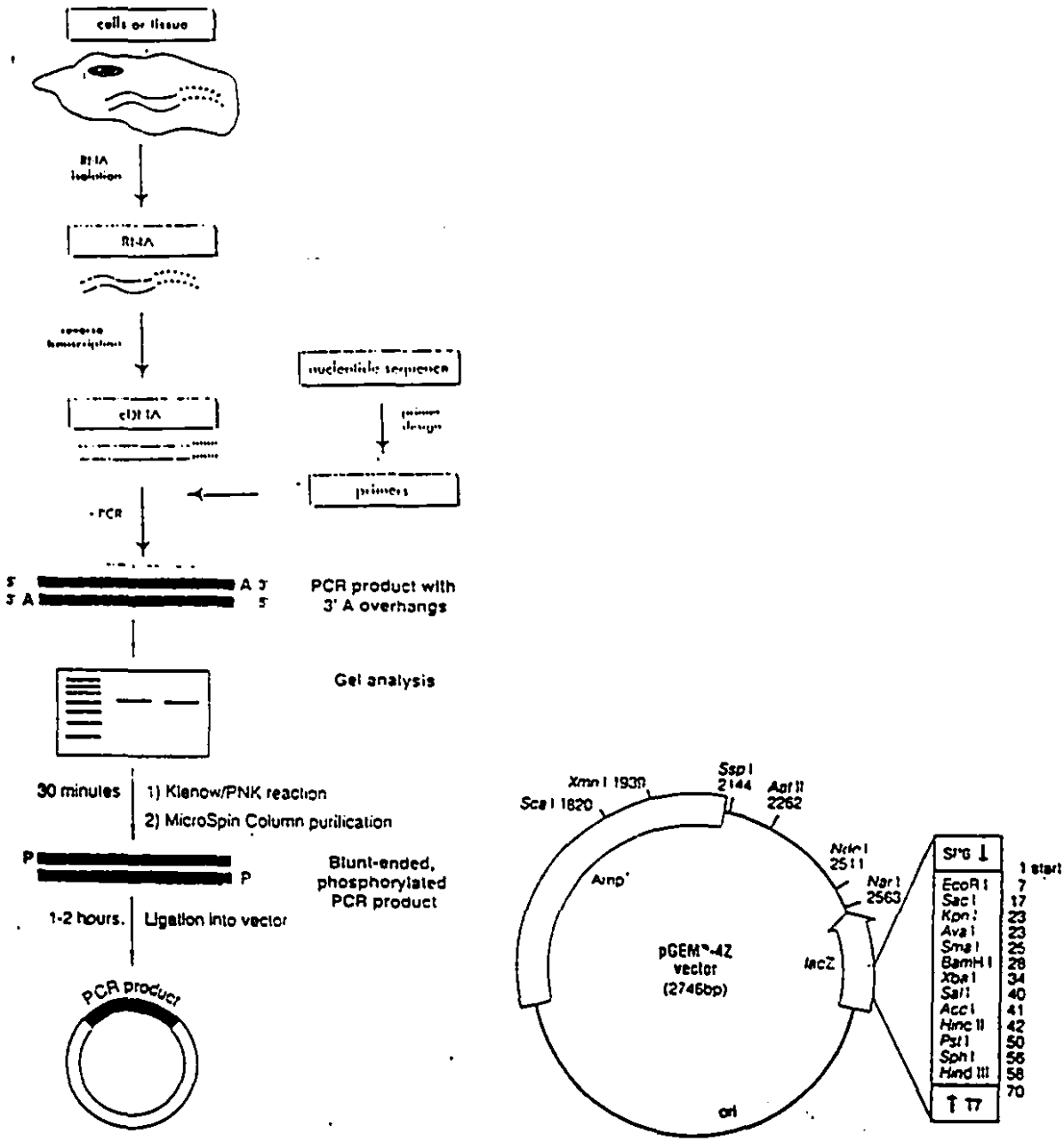

Figure 3-3 The flowchart of the gene cloning process by reverse transcription-polymerase chain reaction (RT-PCR) and the map of cloning vector- pGEM®-4Z (Promega).

b) Preparation of the PCR Product and the Vector

The PCR product was subjected to electrophoresis in a 1.5% agarose gel in 1X TAE buffer containing 10 μg/ml ethidium bromide. The band was excised from the gel under UV light visualization. The DNA was extracted from the gel using Qiaex kit (Qiagen). The eluted DNA was concentrated by ethanol precipitation and dissolved in TE buffer at the concentration of 25 ng/μl. Many thermostable polymerases possess a template-independent polymerization activity that adds a single dATP to the 3' end of the template (Clark et al., 1988), which leads to difficulty in subsequent cloning of fragments generated by PCR. To circumvent this problem, the cDNA fragment was then simultaneously blunt-ended and kinased using the Klenow fragment of *E coli* polymerase I and T4 polynucleotide kinase using Sureclone Ligation Kit (Pharmacia). In brief, a 20 μl mixture containing 2 μl 2X blunting/kinasing buffer, 6.5 units of Klenow, 7 units of T4 kinase, and optimal amount of purified cDNA fragments was incubated at 37°C for 30 min. The reaction mixture was extracted once with phenol-chloroform and purified with Microspin column containing Sephacryl S-200 resin.

The blunt-ended dephosphorylated vector was prepared according to the established protocol (Sambrook et al., 1989). In brief, 4 μg of pGEM-4Z was digested with 30 units of *Sma*I restriction enzyme in 20 μl volume. After digestion, the enzyme was then heat inactivated at 70°C for 10 min. 2 μl of calf intestinal alkaline phosphatase (CIAP), 4 μl of 10X CIAP buffer (0.5M Tris-HCl, pH 9.3, 10mM $MgCl_2$, 1 mM $ZnCl_2$, and 10 mM spermidine) and 14 μl of water were added and incubated for 15 min at 37°C. After the

addition of another aliquot of CIP, the reaction mixture was incubated at 55°C for 30 min. CIAP was then inactivated at 75°C for 10 min in the presence of 5 mM EDTA, pH 8.0. The blunt-ended dephosphorylated pGEM-4Z was gel-purified as described above, quantitated by ethidium bromide staining, and reconstituted at a concentration of 50 ng/μl in TE buffer.

c) Subcloning of the PCR product to the linearized vector

After DNA polymerase and kinase treatment, the PCR product was ligated into the blunt-ended and dephosphorylated pGEM-4Z vector. For optimal ligation efficiency, an equimolar ratio of insert ends to vector ends is needed. The number of ends are calculated using the following formula. The number of pmole ends= 2 X amount (in ng) X 10^3/ length of DNA (in bp) X 649 dalton. The ligation reaction was carried out using TaKaRA ligation kit (Takara Biochemical, Berkeley, CA). Typically, a solution of 20 μl ligation buffer, 1 μl pGEM-4Z (50 ng/μl), 4 μl insert, and 5 μl T4 DNA ligase was incubated at 16°C for a minimum of 30 min.

d) *E. coli* Competent Cell Transformation

Ligated PCR product and pGEM-4Z were transformed into subcloning-efficiency competent *E coli* DH5α cells (Gibco BRL) according to the protocol of the manufacturer. Briefly, 2 or 3 μl ligation mixture (1-10 ng) was gently mixed with 50 μl of competent cells and incubated at 4°C for 30 minutes. The cells were then heat shocked at 37°C for 20 seconds and chilled on ice for 2 minutes. 950 μl S.O.C medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$. 10 mM $MgSO_4$, 20 mM glucose) was added to

the cell suspension followed by incubation in a 37°C shaker at 225 r.p.m. for one hour to allow expression. The cell suspension at different dilutions was then spread on LB agar plates containing ampicillin (100 μg/ml), X-gal (40 μg/ml), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (ITPG). After 16-18 hr growth at 37°C, white colonies were screened for the presence of recombinant clones, using PCR primers specific for sequences flanking the cloning site of pGEM-4Z. The sequences of the paired primers (5'-3') are CGGCCAGTCAATTGGATTTAGGTandACGCCAAGCTCTAATACGACTCA.

e) Plasmid Isolation and Sequencing

Plasmids were isolated using a Plasmid Midi Kit (Qiagen, Chatworth, CA), which was based on a modified alkaline lysis procedure (Sambrook et al., 1989). A positive colony containing the recombinant plasmid was selected and cultured in 50 ml of LB medium containing 100 μg/ml ampicillin overnight at 37°C to a cell density of 1 X 10^9 cells per ml (approximately 1-1.5 A_{600} units/ml). Cells were harvested by centrifugation and resuspended in 50 mM Tris/HCl, 10 mM EDTA, pH 8.0, and 100 μg/ml RNase A. The cells were lysed using 0.2 M NaOH and 1% SDS. Protein and genomic DNA were precipitated with 3.0 M potassium acetate, pH 5.5. Following centrifugation, the supernatant was collected and subjected to liquid chromotography using Qiagen tip-100. The remaining contaminants were removed by washing, and the eluted plasmid DNA was precipitated with isopropanol and resuspended in TE buffer. The concentration was measured using a spectrophotometer at a wavelength of 260 nm.

cDNA inserts of the recombinant clones were sequenced by the

Sanger's dideoxy-mediated chain-termination method (Sambrook et al., 1989) using the Sequenase™ Version 2.0 T7 DNA sequencing kit (Amersham). In brief, 3-5 μg of template DNA was denatured with 0.1 volume of 2 M NaOH/2 mM EDTA at 37°C for 30 minutes. The mixture is neutralized by adding 0.1 volumes of 3M sodium acetate, pH 5.2 and the DNA was precipitated with 2-4 volumes of absolute ethanol. The pellet was washed with 70% ethanol, air dried and then re-dissolved in 7 μl H2O. 1 μl (0.5 pmole) of pUC/M13 forward primer (5'-GTTTTCCCAGTCACGA-3') and 2 μl of annealing buffer (40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl) were added, and the reaction was heated at 65°C for 2 minutes then cooled slowly to < 35°C over 15-30 minutes. Then a mixture of labelling mix (dGTP, dCTP and dTTP), P^{32}-dATP (3,000 Ci/mmol), and T7 DNA polymerase was added to the annealed template-primer and the reaction was incubated at room temperature for 5 minute to allow initiation of DNA synthesis from the primer. 3.5 μl of the above labeling reaction was then added to four tubes containing each termination mixtures (ddGTP, ddATP, ddCTP, and ddTTP) and incubated at 37°C for 5 minutes. The reaction was stopped by the addition of 95% formamide and 20 mM EDTA. After heat denaturation, 2 or 3 μl of the sample was loaded directly onto a 31 x 38.5 x 0.4 cm 6% acrylamide/0.15% bisacrylamide/8M urea/50 mM Tris borate/EDTA pH 8.3/0.1% ammonium persulfate/0.1 % *N,N,N',N'*-tetramethylethylenediamine gel that has been preheated to a 50°C surface temperature. Electrophoresis was performed at 1500 V for 2 and 4 hours respectively. Following electrophoresis, the gel was dried in a

Bio-Rad gel drier. Autoradiography was carried out at -80°C for 1-2 hours.

3.3.10) Northern Blot Analysis Using cRNA Probes:

The orientation of the cloned cDNA insert was determined by hybridization analyses on membranes containing RNA isolated from tissues expressing the desired gene. Thirty microgram of RNA was electrophoretically separated in 1 % formaldehyde-agarose gel in an aqueous buffer containing 5 mM sodium acetate, 1 mM EDTA, and 20 mM 3-[N-morphoino-propanesulfonic acid (MOPS), pH 7.0. Northern transfer was carried out in 10 X SSC solution, and the membrane was cross-linked as described. Template plasmids were linearized with a suitable restriction endonuclease to avoid the generation of 3' protruding ends (overhang), which was reported to produce extraneous transcripts (Schenborn and Mierendorf, 1985). ^{32}P-UTP labelled sense and anti-sense cRNAs were generated from the linearized cDNA templates using the SP6/T7 Riboprobe Gemini II Core System (Promega). The reaction mixture of 20 µl contained 1 X transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl), 10 mM dithiothreitol, 1 U/µl RNase inhibitor, 0.5 mM each ATP, CTP, and GTP, 10 µM UTP, 50 µCi [α-^{32}P]-UTP, 1 µg of linearized template DNA, and 1U/µl of SP6 or T7 RNA polymerase. The reaction was incubated at 37°C for 2 hr, and the template was then removed by RQ1 RNase-free DNase (Promega) at 37°C for 15 minutes. After extraction once with TE-saturated phenol:chloroform and once with chloroform, the unincorporated nucleotides were removed by ethanol precipitation, and the probes

were used for Northern or *in situ* hybridization without further purification. Duplicate membranes containing total RNA isolated from the kidney, brain, skeletal muscle, and adult and fetal liver of rats were used to determine the orientation of the cloned cDNA and the specificity of the probes. Membranes were prehybridized at 50-60° C for 2 hr in a solution containing 50 mM Na_2HPO_4, pH 6.5, 0.8 M NaCl, 1 % SDS, 1 mM EDTA, 50% deionized formamide, 100 μg/ml salmon sperm DNA and 0.5 ng/ml yeast tRNA. After adding the labelled cRNA probes, hybridization was carried out overnight at 60° C in the same solution minus the salmon sperm DNA and yeast tRNA. Washing was performed at 70-75° C in a solution containing 0.0125 M NaCl and 0.125% SDS for 3 to 5 times of 10 min each. Membranes were exposed to Kodak XAR-5 film for 5-10 days. The orientation of the cloned cDNA insert in pGEM4Z vector was listed in Table 3-1.

Table 3-1 Restriction endonuclease used to linearize the templates and the orientation of the cloned cDNA insert in pGEM4Z vectors determined by Northern hybridization

cDNA/Restriction enzyme[a]	RNA polymerase	cRNA[b]
Albumin/*Eco*R I	T7	anti-sense
Albumin/*Xba* I	SP6	sense
α-fetoprotein/*Eco*R I	T7	anti-sense
α-fetoprotein/*Hind* III	SP6	sense
aldolase B/*Eco*R I	T7	sense
aldolase B/*Hind* III	SP6	antisense
CA[c] II/*EcoR* I	T7	antisense
CA II/*Hind* III	SP6	sense

a: cDNA insert cloned the pGEM4Z vector were linearized at either end using suitable restriction endonuclease that is not present in the insert. The linearized template containing either T7 or SP6 RNA polymerase promoter sequences is then used in *in vitro* transcription to generate "run-off" transcripts.

b: Determined by Northern blot hybridization.

c: carbonic anhydrase

3.3.11) *In Situ* Hybridization:

To preserve mRNA in the tissue, all solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) (BDH Biochemical). Tissues were either fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 for 4-6 hr and then stored in 25% sucrose/PBS at 4° C or fixed overnight in 10% buffered formalin and processed for routine paraffin embedding. Generally the latter gives weaker signals but provides superior morphology when compared to the former (Gibson and Polak, 1984).

4 μm of formaldehyde-fixed tissues or 10 μm of frozen tissues were sectioned on 3-amino-propyltriethoxysilane- or polylysine-coated slides (Sigma Chemical, St. Louis, MO). Paraffin sections are first deparaffinized and rehydrated, and then immersed in 0.2 N HCl for 30 min at room temperature (this removes ribosomes from mRNA). Subsequently paraffin and frozen sections are treated in the same way. Permeabilization of tissue is necessary to enhance the accessibility of target mRNA to the probe. Sections were first treated with 20 μg/ml proteinase K (Boehringer Mannheim Canada) in 0.1 M Tris-HCl, pH 7.4 and 0.05 M EDTA at 37° C for 20 min, and the reaction was stopped by immersing in 4% paraformaldehyde solution for 10 min. After three washes in PBS, sections were immersed in a solution of 0.25% acetic anhydride in 0.1 M triethanolamine (Sigma Chemical), pH 8.0, to reduce non-specific noise possibly through blocking charged amino groups on the tissue (Gibson and Polak, 1984), dehydrated in ethanol, and then air-dried.

^{32}P- or ^{35}S-labelled anti-sense or sense cRNA probes were prepared as described in the previous section, and the length of the

probes was reduced to about 200 bp by partial alkali hydrolysis as followed (Cox et al., 1984). 180 μl of cRNA probe solution was mixed with 20 μl of 1 X alkali hydrolysis buffer (40 mM $NaHCO_3$ and 60 mM Na_2CO_3, pH 10.2) and incubated at 60°C for the required time according to the following formula: T = Lo - Lf/K Lo Lf, where T = incubation time in min, Lo = initial transcript length in kilobases, Lf = desired probe length in kilobases, and k = the rate constant for hydrolysis, which is approximately 0.11 kilobases per min. At the end of incubation, the solution was neutralized with 0.1 volume of 3M sodium acetate, pH 5.2 and precipitated with 2.5 volumes of absolute ethanol. The specificity of the labelled probe is determined using a Beckman L6000 scintillation counter.

1-5 X 10^6 cpm/section of probes were mixed in 20-40 μl of hybridization buffer (2X SSC, 100 μg/ml hering sperm DNA, 100 μg/ml yeast tRNA, 10% dextran sulfate, 50% deionized formamide, and 5 X Denhardt's solution; 100X Denhardt's solution is 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin), and added on to the sections and incubated for 16 hr at 50-55°C in a humidified chamber containing 50% formamide/2X SSC. In the case of ^{35}S-labelled probe, dithiothreitol (DTT) was added to a final concentration of 10 mM to reduce nonspecific "stickiness" of the probe to the tissue sections. Unhybridized probes were washed in 2X SSC solution followed by treatment with 20 μg/ml RNase A (ICN Biochemical) and 10U/ml RNase T1 in 10 mM Tris-HCl, pH 7.4 and 0.15 M NaCl at 37°C for 30 min. The sections were washed in 2X SSC/50% formamide at 50°C for 30 min. The final stringent wash was performed in 0.1X SSC for 15 min twice at 60°C. After

dehydration in increasing concentration of ethanol, sections were air-dried and then coated with autoradiographic emulsion (Amersham), and incubated at 4° C in a light-tight box for 5-14 days. Prior to development, the sections were equilibrated at room temperature for 30 min to avoid condensation on the emulsion. They were subsequently developed in Kodak D19 developer at 18° C for 3 min, washed in water for 1 min, and then fixed for 5 min in 24% sodium thiosulfate (ICN Biomedical). After washing in running tap water for 10 min, they were counterstained with hematoxylin and coverslipped. Step sections were stained with hematoxylin and eosin for histological evaluation. Sections hybridized with a sense probe or sections treated with RNase solution before hybridization served as negative controls. Photography was performed with a Zeiss microscope (Carl Zeiss, Thornwood, NY) under bright-field illumination to reveal histological details and under dark-field illumination for best visualization of silver grain distribution. Photographs were taken on Kodak TMAX 100 film.

3.4 RESULTS

Genetic labelling of normal pancreatic duct epithelial cells:

The liposome-mediated transfection method was very efficient in labelling these propagable cultured rat pancreatic duct epithelial cells with the *E. coli* β-galactosidase reporter gene. From more than 50 colonies present in a 100 mm tissue culture plate, five cell strains (RP2-*LacZ* C1-5) were isolated. The integration of the bacterial *lacZ* gene in these cell strains was confirmed by Southern analysis showing its random integration into the genomic DNA (Figure 3-4), by the expression of the *lacZ* mRNA (data not shown), and by a positive histochemical staining for bacterial β-galactosidase enzyme activity (Figure 3-5). The parental untransfected cells did not show any of these genotypes and phenotypes.

Phenotypic properties of labelled duct epithelial cells:

Prior to implantation, the *in vitro* phenotypic properties of these *lacZ* labelled cell strains were examined to check whether the integration of *lacZ* gene in the host genome and the production of *lacZ* resulted in any appreciable phenotypic changes. Table 4-2 summarizes the *in vitro* phenotypic properties of these *lacZ* labelled duct epithelial cell strains, as studied using immunocytochemistry, enzyme histochemistry and mRNA expression. All five duct epithelial cell strains showed identical phenotypes similar to those of the parental cell lines. These cells expressed carbonic anhydrase (CA) II, alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) activities, but were negative for glycogen storage and mucin.

RT-PCR technique also demonstrated the presence of low levels of mRNA transcripts for CA II, albumin (ALB) and α-fetoprotein (AFP), but they were not detectable by Northern hybridization (Figure 3-6). Aldolase B mRNA was not detected by either techniques (Figure 3-6). Immunohistochemistry did not detect the presence of ALB, AFP, transferrin, tyrosine aminotransferase (TAT), chromogranin, insulin, glucagon, somatostatin and α-amylase (data not shown) in these cells. The great majority of the cells stained positively for cytokeratin 8 but negatively for cytokeratin 19. Immunohistochemistry with the monoclonal antibodies HES6 and BDS7 were also negative.

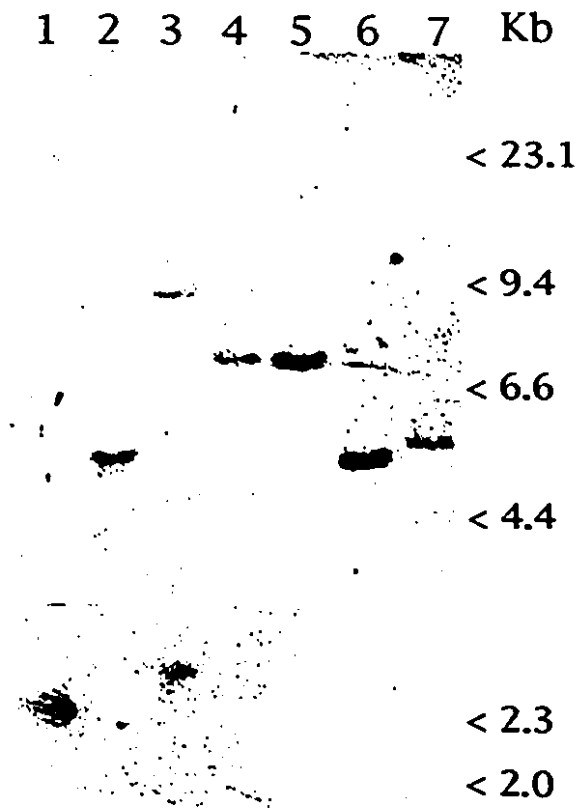

Figure 3-4 The Southern blot analyses of DNA from the parental cell line (Lane 1), *lacZ* gene-transfected stock cell line (Lane 2), and five clonal cell strains (Lane 3-7 correspond to strains 1-5) showing the random integration of *lacZ* gene in the genome of the transfected cells.

Figure 3-5 Transfected RP-2 cell strains (*LacZ* C5) histochemically stained with *E. coli* β-galactosidase showed heterogeneous staining of the cells. (Magnification: X 1,000)

Table 3-2 Phenotypic profiles of adult rat hepatocytes, cultured *lacZ*-labelled cells, and *lacZ*-positive hepatocyte-like cells located subcutaneously and intraperitoneally

Phenotypes	Adult parenchymal hepatocytes	Cultured *lacZ*-labeled RP-2 cells	hepatocyte-like cells located subcutaneously	hepatocyte-like cells located intraperitoneally
albumin[a]	+++	-	+	+++
α-fetoprotein	-	-	+	-
transferrin	+++	-	+	+++
TAT	+++	-	+	+++
aldolase B[b]	+	-	+	+
CA II	-	+	+	-
cytokeratin 8	+	+	+	+
cytokeratin 19	-	-	-	-
ALP	-	+	-[c]	-
GGT	-	+	+	-
HES6	+	-	-	+
BDS7	-	-	-	-
glycogen storage	+	-	-[d]	+

Abbreviation: CA II: carbonic anhydrase II; TAT: tyrosine aminotransferase; HES6: surface antigen of rat hepatocyte; BDS7: surface antigen of rat biliary duct epithelial cells; ALP: alkaline phosphatase; GGT: γ-glutamyl transpeptidase; +: positive; -: negative

a: The relative levels of albumin, α-fetoprotein, transferrin, and TAT were determined by the intensity of immunostaining. For other phenotypic markers, only - or + was marked.

b: Determined by mRNA expression.

c: A strong staining for ALP activity was localized to the periphery of the cell nests bordering the connective tissue

d: only a few cells (< 2%) contained a scant cytoplasmic glycogen deposit

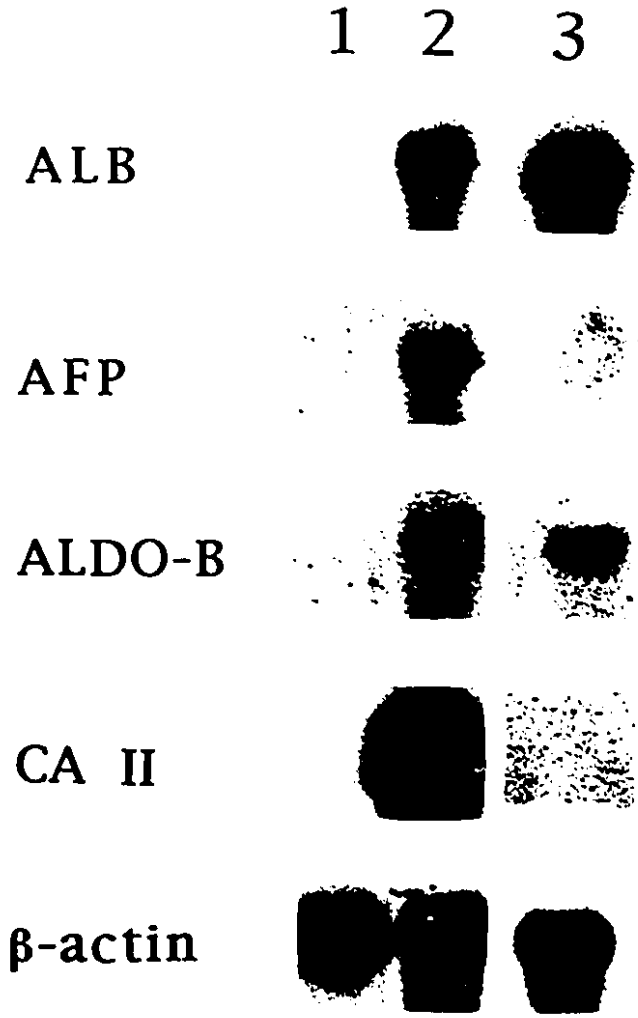

Figure 3-6 Northern blot analyses showing the relative expression of albumin (ALB), α-fetoprotein (AFP), aldolase B (ALDO-B), and carbonic anhydrase (CA II) in the transfected cell strain used in implantation (Lane 1), in the subcutaneous tissue containing the implanted cells (Lane 2), and in adult rat liver (Lane 3). RNA was hybridized with P^{32} labelled cRNA (ALB and AFP) or cDNA (CA II and ALDO-B) probes. The mRNA sizes are 2.3 kb for ALB, 2 kb for AFP, 1.6 kb for ALDO-B, and 1.5 kb for CA II.

Subcutaneous Implantation:

Two RP-2 cell strains (RP2-LacZC2 and RP2-LacZC5) were randomly chosen for *in vivo* implantation study. Prior to implantation, cell strains were grown in 35 mm tissue culture plates and stained histochemically for β-gal activity. We found consistently that more than 95% cells stained positively for β-gal in the G-418 containing medium. The abdominal subcutaneous tissue was selected due to its easy accessibility and the absence of host epithelial cells. Extracellular matrix as cell embedding medium was essential for the localization, improved colonizing efficiency and survival of these implanted cells.

4-8 weeks after implantation, a piece of pinkish-white fat-like tissue localized over the rectus abdominis muscle was found. No similar tissue was noted in the control animals implanted with only extracellular matrix gels. Following incubation in the reagent solution for bacterial β-galactosidase enzyme activity, numerous blue spots appeared within the tissue (Figure 3-7A). These blue foci represent aggregates of cells whose cytoplasm contained numerous blue granules (Figure 3-7B). The same enzymatic reaction performed on fresh tissues of rat kidney, liver, pancreas and muscle did not produce blue foci or cells containing cytoplasmic blue granules (data not shown).

Hematoxylin and eosin staining of paraffin sections of formalin-fixed tissue showed nests of epithelial cells with abundant eosinophilic but finely vacuolated cytoplasm scattered among adipocytes (Figure 3-8A). Focally, small ducts lined by low columnar epithelial cells were noted among the epithelial cell nests (Figure

3-8A). They comprised less than 5% of the *lacZ*-positive cells.

The large epithelial cells demonstrated positive immunostaining with antibodies to ALB (Figure 3-7B), AFP, transferrin, TAT, cytokeratin 8, cytokeratin 19 and bacterial β-galactosidase. They stained negatively for HES6 and BDS7 antibodies, and antibodies to several pancreatic enzymes/ hormones, including α-amylase, insulin, glucagon, chromogranin and somatostatin. Sudan IV stain demonstrated that the cytoplasmic vacuoles in these epithelial cells represented lipid globules (Figure 3-9A), and only a few of these cells (< 2%) contained a scant cytoplasmic glycogen deposit. Strong staining for ALP activity was localized to the periphery of the cell nests bordering the connective tissue (Figure 3-9B). In contrast to the nests of epithelial cells, the ductal epithelium showed strong cytoplasmic alkaline phosphatase activity. Enzyme histochemistry showed strong activity for CA and weak activity for GGT in the cytoplasm of these epithelial cells (data not shown).

Transmission electron microscopic examination revealed the presence of bile canalicular-like structures between adjoining epithelioid cells. Other organelles were sparse but lipid globules were evident (Figure 3-10). Occasionally some cells contained rectangular- to round-shaped granules surrounded by a halo zone (Figure 3-11). Using the electron-immunogold technique, the granules stained negatively with antibodies against insulin. Granules of endocrine (glucagon, proinsulin, c-peptide, etc.) and non-endocrine (melanin, lysosomes or peroxisomes) may have similar ultrastructure. Their exact nature remains to be investigated.

Northern blot analysis of RNA isolated from the subcutaneous tissue confirmed the presence of mRNA transcripts for albumin (2.3 kb), α-fetoprotein (2 kb), aldolase B (1.6 kb), and CA II (1.5 kb) (Figure 3-6). These mRNA transcripts were also detected using the RT-PCR technique (Figure 3-12). *In situ* hybridization studies confirmed that the CA II (Figure 3-13), albumin, aldolase B, and AFP mRNA were expressed in the large vacuolated hepatocyte-like cells.

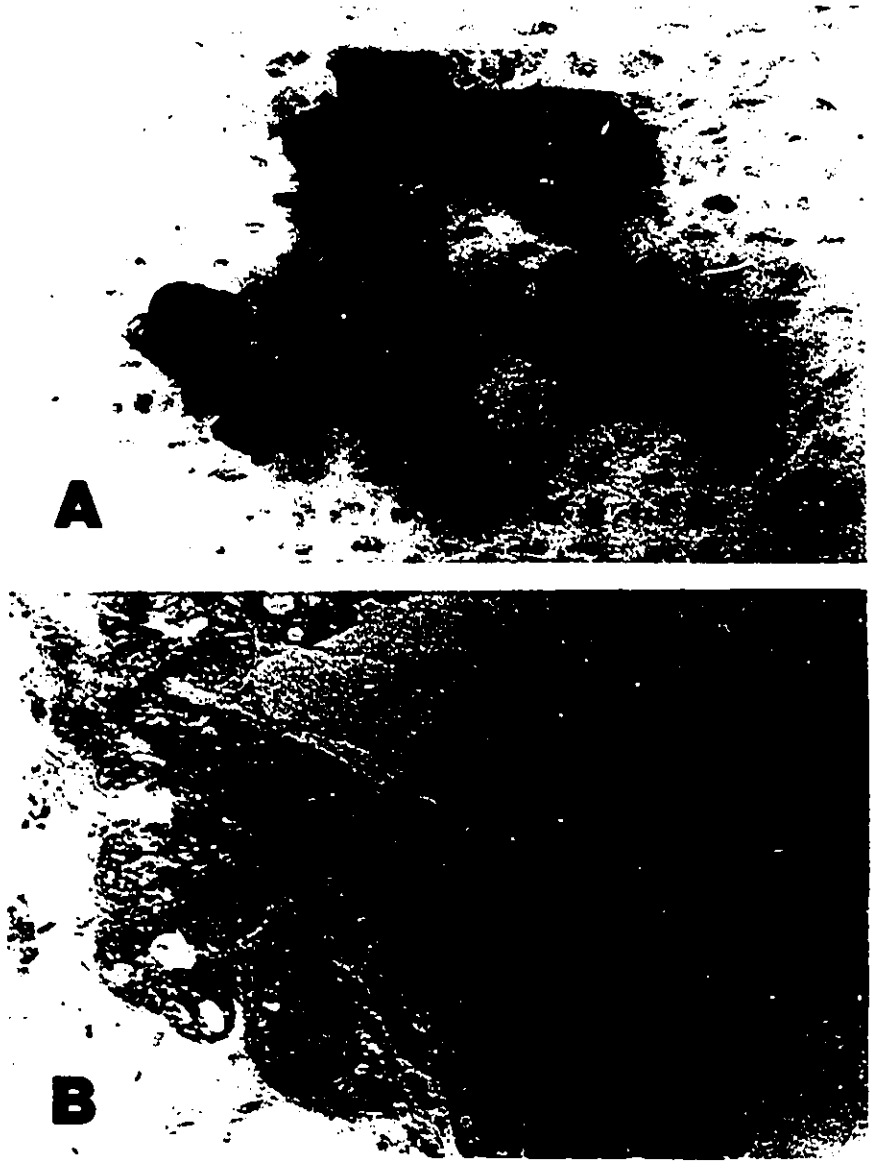

Figure 3-7 Morphologic and histologic examination of the subcutaneously implanted cells after 8 wk implantation. (A): Incubation of the subcutaneous tissue containing implanted cells with the enzymatic reaction solution for bacterial β-galactosidase reveals the presence of *lacZ*-transfected cells. (B): A histologic section of the *lacZ*-stained tissue shown in (A) reveals blue cytoplasmic granules in the these large hepatocyte-like cells, confirming the *in vitro* origin of these cells. (Magnification: A: X 50; B: X310).

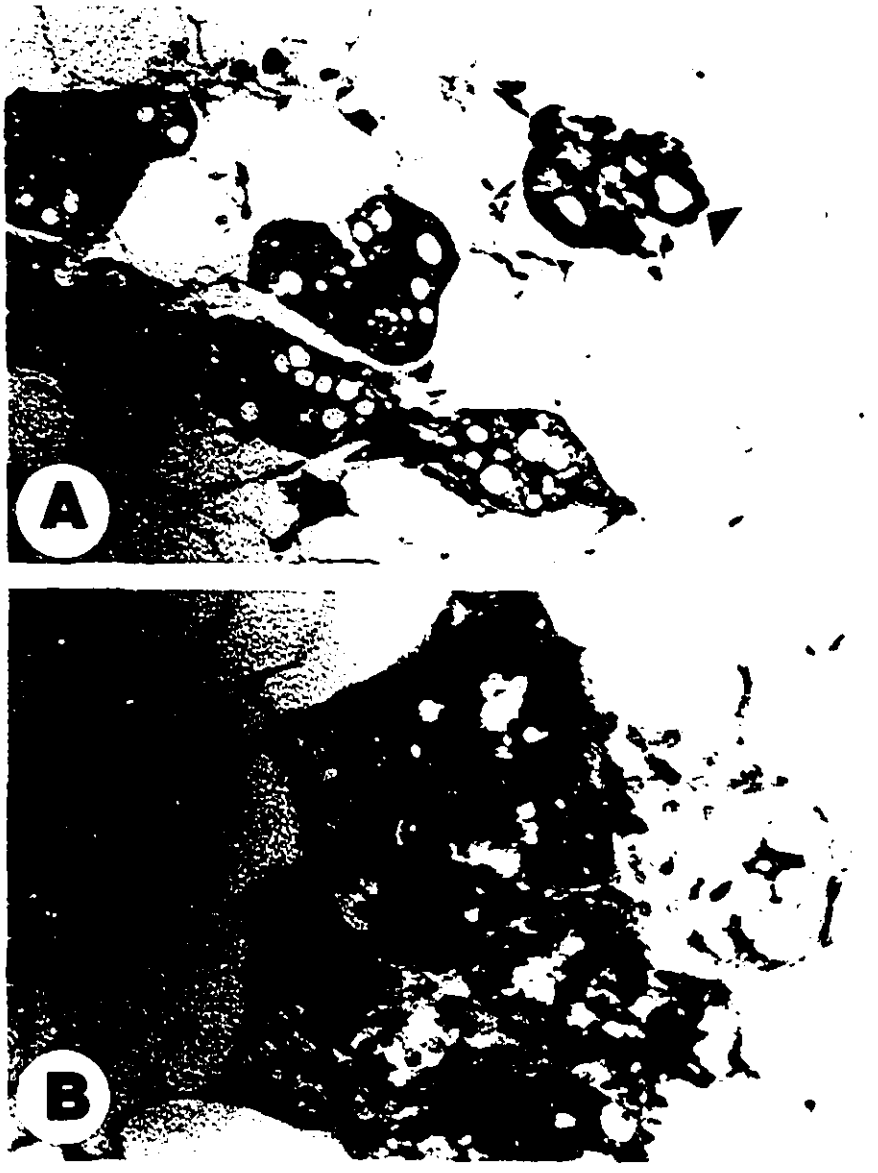

Figure 3-7 Morphologic and histologic examination of the subcutaneously implanted cells after 8 weeks of implantation. (A): Microscopically nests of large epithelial cells with finely granular and vacuolated cytoplasm are scattered among adipocytes. Occasional ductal structures lined by low cuboidal epithelial cells are noted adjacent to these nests of hepatocyte-like cells (arrow head). (B): Immunohistochemistry for rat albumin shows a strong positive staining in the hepatocyte-like cells. (Magnification: A and B: X 500).

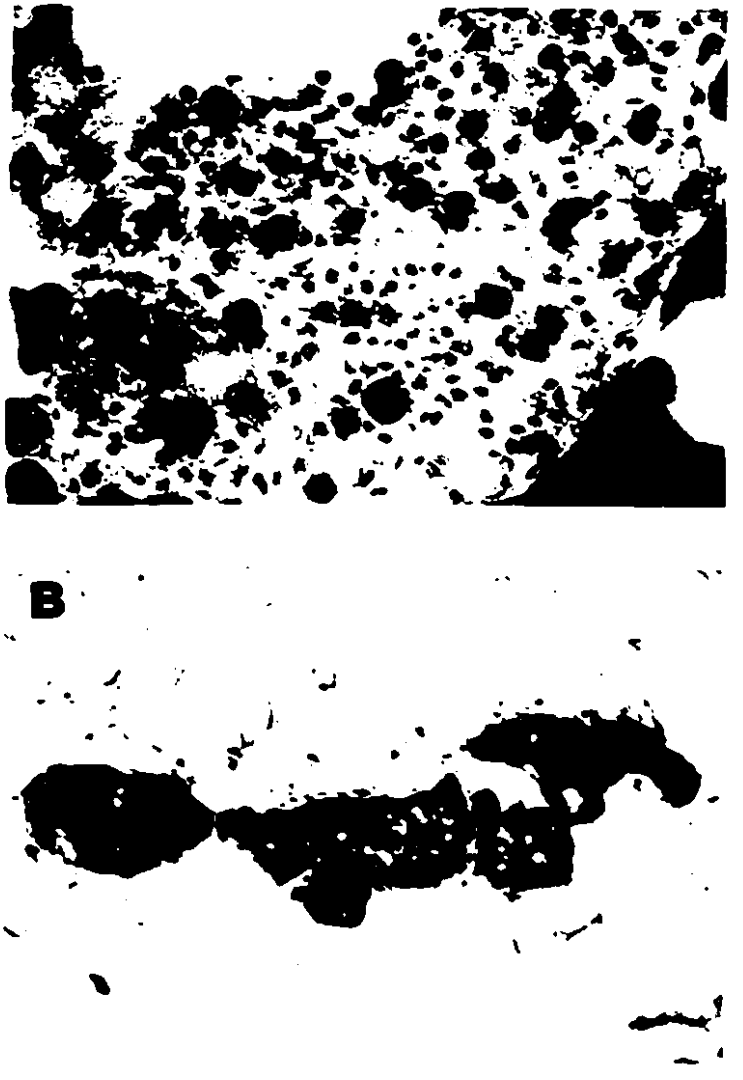

Figure 3-9 Histochemical studies of the subcutaneously implanted cells. (A) Sudan IV fat staining confirmed the nature of multiple vacuoles in these subcutaneous epithelial cells. (B) Alkaline phosphatase histochemistry showed the presence of strong activity in the rim of cells bordering the connective tissue, while the rest of the cells stained negatively. (Magnification: A: X 325; B: X 130).

Figure 3-10 Electron microscopic photography of the subcutaneously implanted cells showed the presence of bile canalicular-like structures between adjoining hepatocyte-like cells. (Magnification: X 9280).

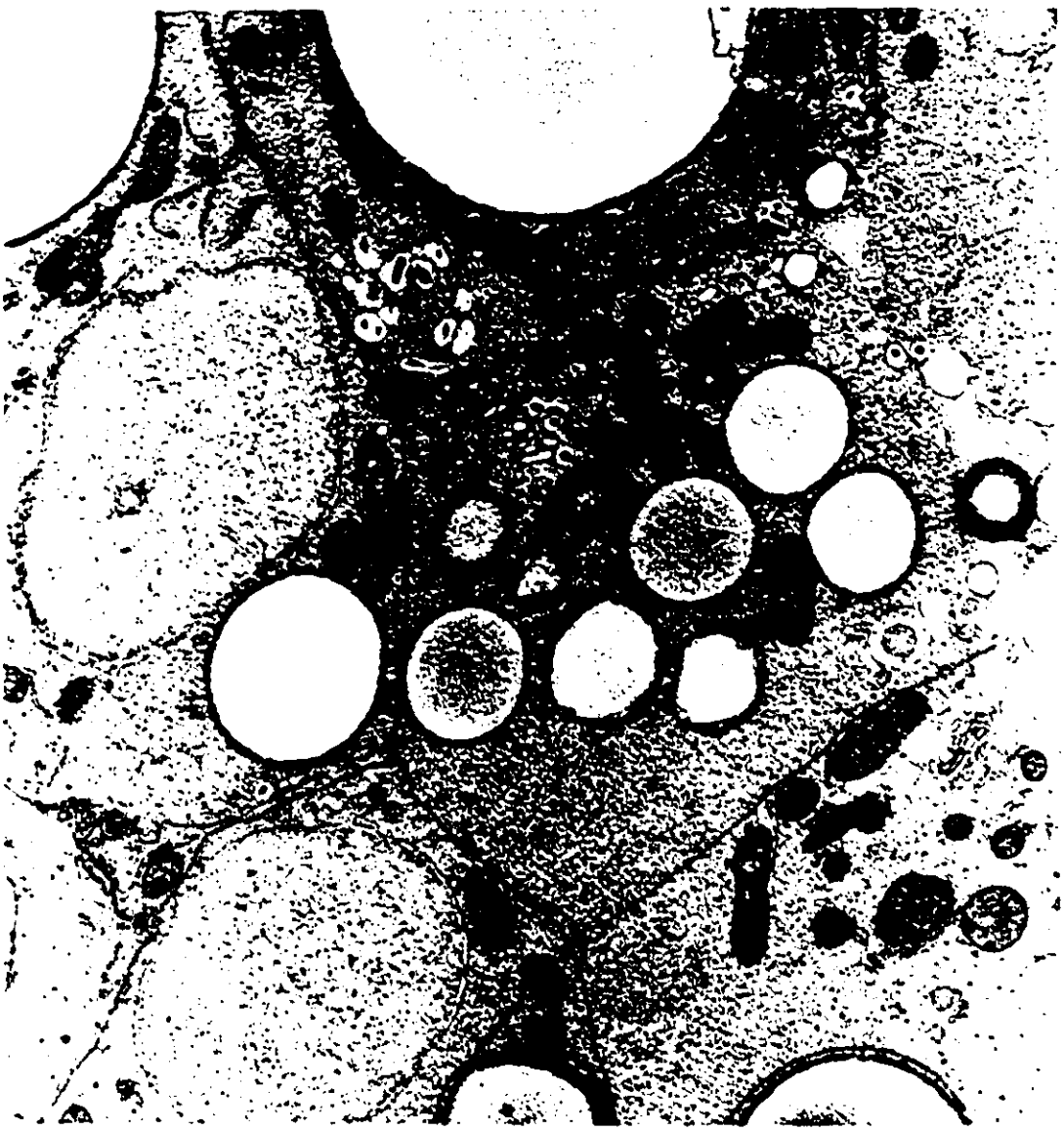

Figure 3-11 Electron microscopic photography of the subcutaneously implanted cells showed prominent cytoplasmic fat droplets, and cell junctions. Occasionally some cells contained rectangular- to round-shaped granules surrounded by a clear halo (arrowhead) (Magnification: X 5370).

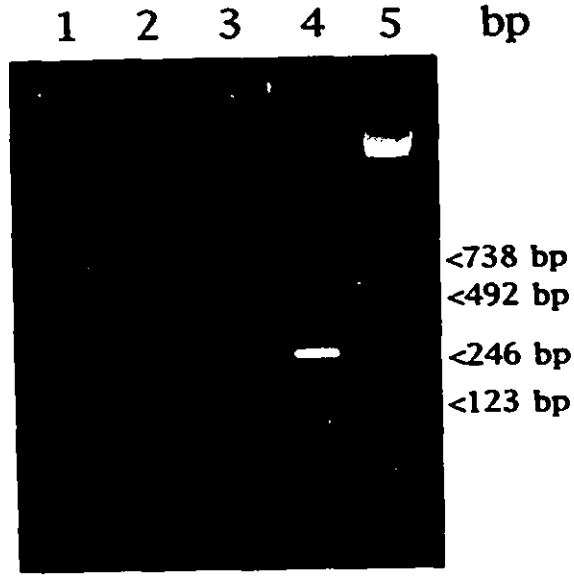

Figure 3-12 RT-PCR analysis of mRNA expression in the subcutaneous tissue containing the implanted cells. 2 μg total RNA was reverse transcribed using Moloney murine leukemia virus, and detection of various mRNAs by PCR was performed using specific sets of primers listed in Table 3-2. PCR products were analyzed in a 2% agarose gel containing 40 mM Tris-acetate/1 mm EDTA , pH 7.6, and stained with ethidium bromide. Lane 1, albumin (619 bp); lane 2, aldolase B (805 bp); lane 3, α-fetoprotein (261 bp); lane 4, β-actin (283 bp); and lane 5, molecular weight marker (123 bp ladder).

Figure 3-13 *In situ* hybridization with ^{32}P-labelled CA II cRNA of a tissue section from the subcutaneous tissue after 8 weeks of implantation. (A) The bright-field photomicrograph showed nests of hepatocyte-like cells. (B) The corresponding dark-field photomicrograph showed intense silver granules in the hepatocyte-like cells. (C) Only background silver granules were observed in the negative control using the sense cRNA probe. (Magnification: A, B and C: X 60).

Intraperitoneal Implantation:

Six weeks after implantation with cell-containing gel matrix, a whitish mass was found wrapped around the mesentery of a segment of small bowel. The hematoxylin and eosin stained section of this tissue showed trabeculae and nests of large polygonal epithelial cells scattered in a desmoplastic mesentery (Figure 3-14). The cells histologically resembled the mature hepatocyte of adult rat liver, demonstrating central nuclei and granular eosinophilic cytoplasm (Figure 3-14A) devoid of lipid globules. They were intensely PAS-positive and were negative after predigestion with diastase, confirming the presence of glycogen (Figure 3-15B). Immunohistochemitry staining using monoclonal antibody to the *E. coli* β-galactosidase yielded a strong positive reaction, confirming the *in vitro* origin of these cells (Figure 3-16A). As a negative control, hepatocytes of normal adult rats stained negatively with the same antibody (data not shown). Similar to the subcutaneous site, the hepatocyte-like cells were predominant, representing approximately 95% of the *lacZ*-positive cells, however, *lacZ* positive small ductules were also noted among these hepatocyte-like cells (Figure 3-16A). Compared to the primitive ductal structures seen in the subcutaneous site (Figure 3-7A), these ducts were usually well-organized and larger (Figure 3-15A, 3-16A). Using the same titre of primary antibodies, these hepatocyte-like cells showed stronger immunoreactivity for TAT (Figure 3-16 B), ALB, transferrin than the subcutaneous cells. They stained positively for HES6 but negatively for BDS7 antibodies. Staining with antibodies against various pancreatic enzymes/hormones also proved negative.

Transmission electron microscopic examination revealed polygonal cells with a round nucleus, numerous mitochondria, abundance of smooth endoplasmic reticulum, and bile canalicular structures between adjoining cells (Figure 3-17).

In situ hybridization studies using tissue sections confirmed the presence and localization of ALB (Figure 3-18 and 3-19) and aldolase B mRNA transcripts in the hepatocyte-like cells, whereas mRNA of AFP and CA II were not detected. Phenotypic comparisons between the adult rat parenchymal hepatocytes, cultured *lacZ*-labelled cells and subcutaneously- and intraperitoneally-implanted RP-2 cells are summarized in Table 4-2.

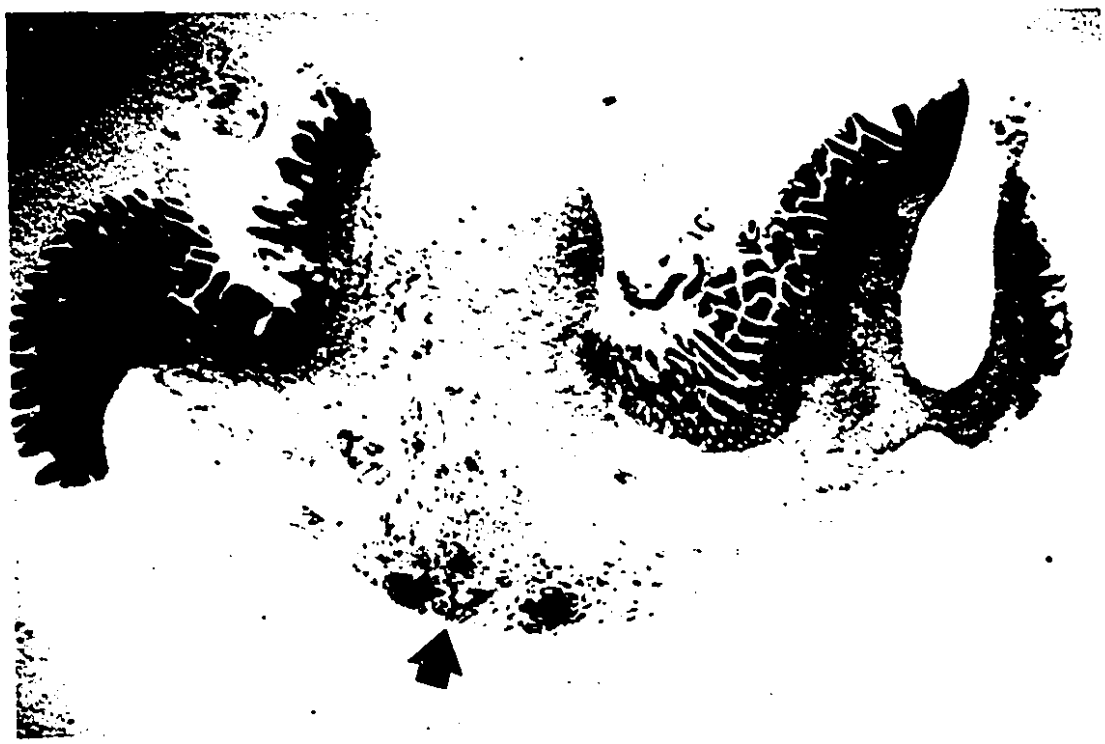

Figure 3-14 A histologic section of the intraperitoneal implanted tissue showed trabeculae and nests of large polygonal epithelial cells (arrow head) in a desmoplastic mesentery. (Magnification: X 60).

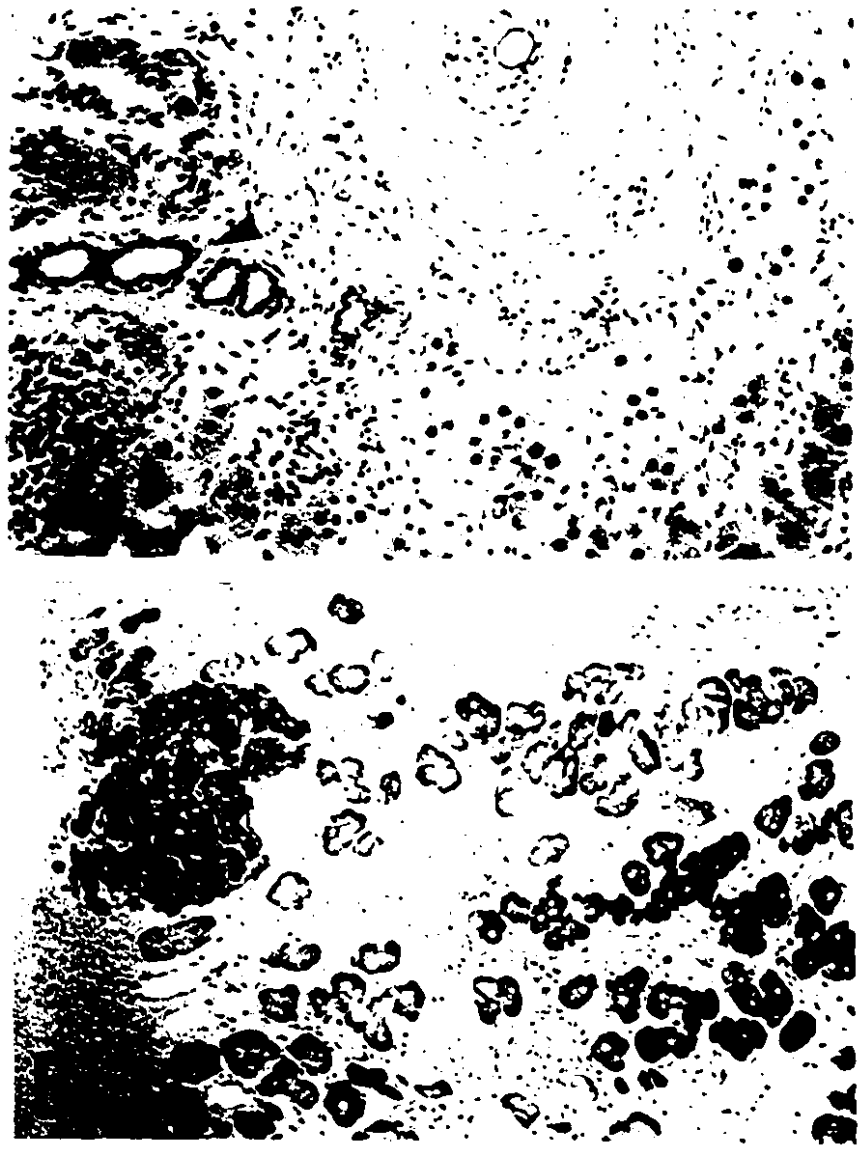

Figure 3-15 Microscopic analysis of the intraperitoneal tissue after 6 weeks of cell implantation. (A) An H&E section shows islands/cords of hepatocyte-like cells with eosinophilic granular cytoplasm within a desmoplastic stroma. Ductal structures were also formed focally (arrow head). (B) The hepatocyte-like cells contain abundant glycogen as shown by the presence of diastase sensitive periodic acid-Schiff (PAS) positive material. (Magnification: A and B: X 310).

Figure 3-16 Microscopic analysis of the intraperitoneal tissue after 6 weeks of cell implantation. (A) Both hepatocyte-like and ductal cells show strong immunostaining with antibody against the bacterial β-galactosidase. (B) The hepatocyte-like cells demonstrate strong immunostaining for tyrosine aminotransferase. (Magnification: A and B: X 500).

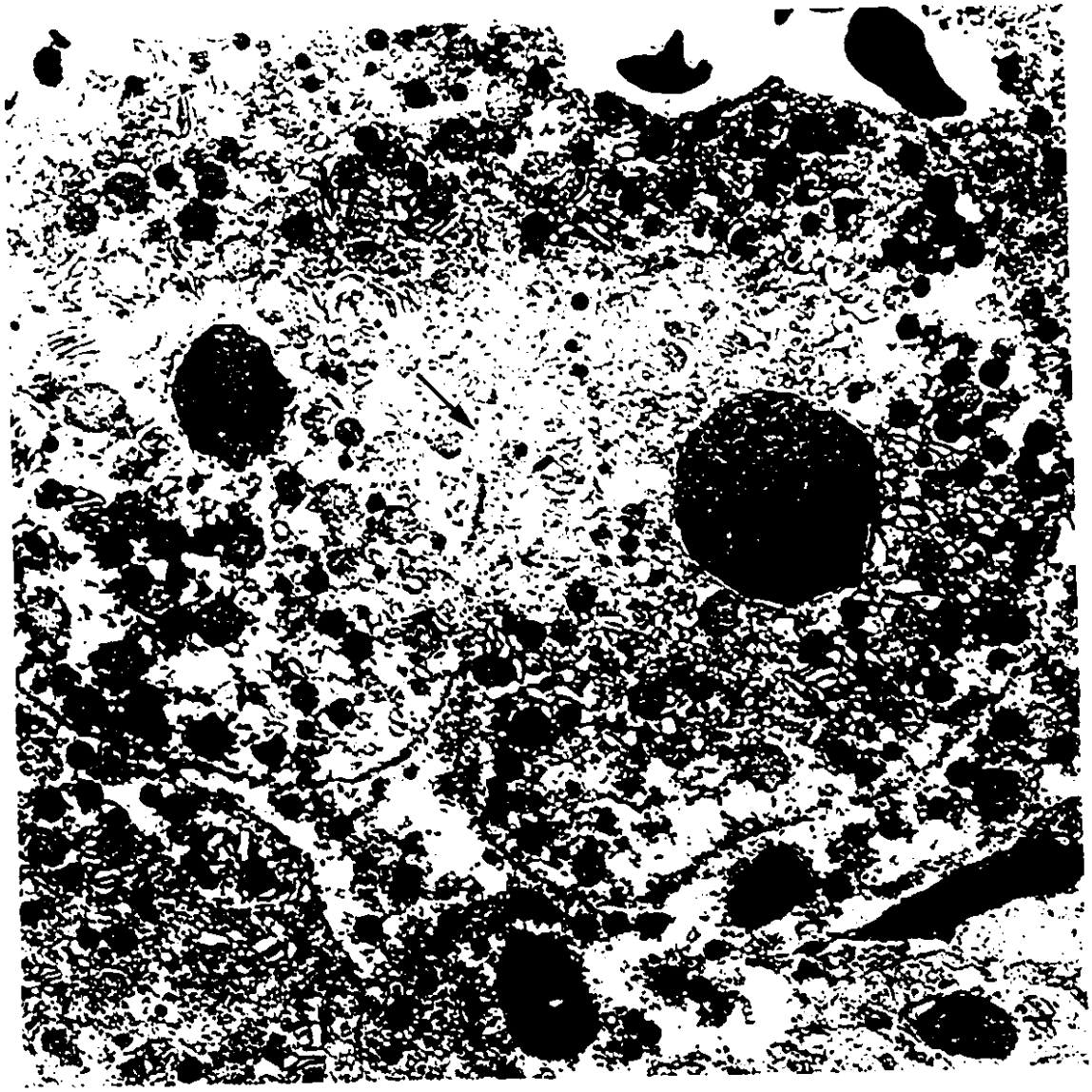

Figure 3-17 Electron microscopic photography of the intraperitoneally implanted cells showed polygonal cells with a round nucleus, numerous mitochondria, abundance of smooth endoplasmic reticulum, and bile canalicular structures between adjoining cells (arrow) (Magnification: X 9280).

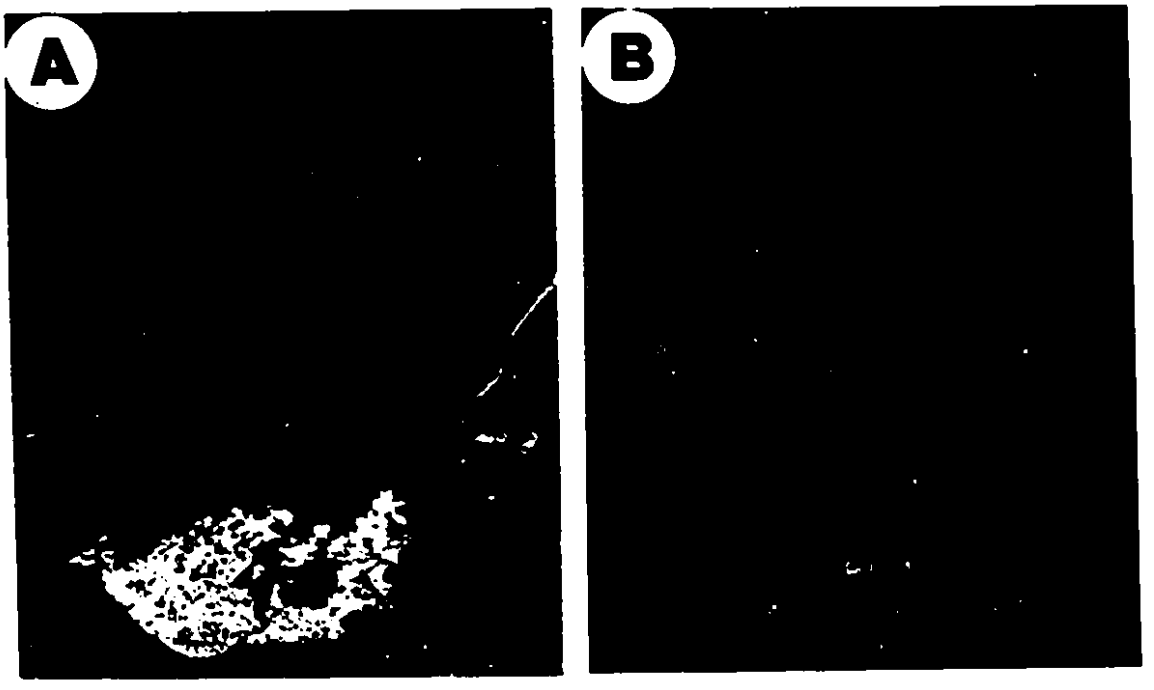

Figure 3-18 (A) *In situ* hybridization with ^{32}P-labelled albumin cRNA of a tissue section from the intraperitoneal tissue after 6 weeks of implantation. The dark-field photomicrograph showed trabeculae and nests of intensely labelled cells in the mesentery; (B) An absence of silver granules in the negative control using the sense cRNA probe. (Magnification: A and B: X 40).

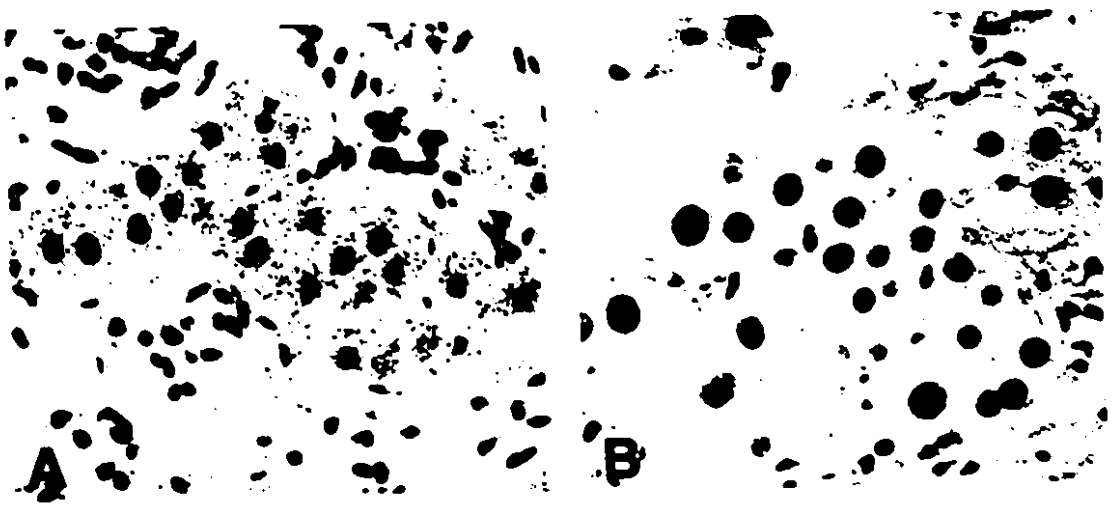

Figure 3-19 The high power bright-field photomicrograph of Figure 3-18. (A) An abundance of albumin mRNA was noted in the intraperitoneal hepatocyte-like cells; (B) and an absence of silver granules in the negative control using the sense cRNA probe. (Magnification: A and B: X 380).

3.5 DISCUSSION

We have demonstrated that after *in vivo* implantation, propagable cultured cells derived from the duct epithelium of adult rat pancreas developed the phenotypic features of a hepatocyte, and that the extent of this phenotypic expression is influenced by the microenvironment in which these cells are localized. The propagable normal pancreatic epithelial cell line used in this experiment was established from a duct fragment selectively picked using a Pasteur pipette under a phase contrast microscope This was performed to confirm specifically that the cells we used were of ductal origin. These cultured cells show low level expression of *in vivo* phenotypes of ductal cells such as CA II, ALP and GGT, as well as low level expression of ALB and AFP mRNAs. The expression of ALB and AFP has been reported in the "oval" cells of rat liver *in vivo* and *in vitro*, and in cultured normal rat liver epithelial cells (Tsao et al., 1984; Sirica et al., 1990). "Periductal" oval cells from copper-deficient rats also express albumin *in vivo* and *in vitro* (Rao et al., 1990; Ide et al., 1993). These results support the hypothesis that the cells of duct epithelia of adult rat pancreas and hepatobiliary tree share common phenotypic and histogenetic properties (Bisgaard and Thorgeirsson, 1990).

The acquisition and maintenance of cell differentiation results from synergistic interplay between intrinsic and environmental factors; the latter include growth factors, cell surface molecules, and extracellular matrix proteins (Watt, 1991). The environment can regulate not only the decision to differentiate but also the range of genes expressed during the differentiation process. However, the

responses to such environmental stimuli depend on the intrinsic programming mechanism of the cells (Watt, 1991; Smith et al., 1988). Thus, implantation of cells in different cellular milieu will reveal not only the nature of these cells, but also reflect the effects of the microenvironment on their phenotypic expression. The results of our study represent the most direct evidence yet to support the ductal cell origin of transdifferentiated pancreatic hepatocytes. When localized in the systemic circulatory compartment, the cells displayed partial differentiation toward hepatocytes but retained some of their ductal phenotype. In contrast, when the same cells were implanted intraperitoneally, they expressed the full phenotypic properties of mature hepatocytes, including their morphological appearance and the loss of AFP and CA II expression. Further studies are needed to determine if the modulating effect of the microenvironment on the extent of differentiation of these cells is related to an exposure to the portal circulation. Coleman et al (1993) reported that when cultured rat liver epithelial cells were transplanted into the liver parenchyma, they fully differentiated into mature hepatocytes and became integrated into the hepatic cell plates, thus becoming morphologically indistinguishable from native parenchymal cells. We have demonstrated further that direct contact with host hepatocytes is not a prerequisite for inducing hepatocytic differentiation, suggesting that factors other than cell surface molecules are important in the induction of this differentiation pathway. Wu et al (1994) have reported that in some hepatocyte cell lines derived from mice transgenic for transforming growth factor-α, the expression of liver specific proteins such as ALB, TAT and transferrin gradually

diminished when these cells were continuously cultured and passaged in serum containing medium. However, a short term culture in serum-free medium can restore the high levels of expression of these genes. These results confirm that the expression of hepatocyte specific genes is modulated by extracellular factors.

Though hepatocytes can be identified in the pancreas of aged rats (Chiu, 1987), the incidence is exceptionally low, indicating that the normal pancreatic microenvironment is not conducive for hepatocytic differentiation. Although acinar cells possess the ability to proliferate after partial resection (Lehv and Fitzgerald, 1968) or subtotal acinar cell necrosis (Fitzgerald et al., 1968), complete regeneration is not seen in global acinar cell necrosis (Scarpelli and Rao, 1981; Reddy et al., 1984; Hoover and Porier, 1986; Konishi et al., 1990; Rao et al., 1990). The extensive destruction/atrophy of the acinar cell population may alter the reticulin framework of the pancreatic acini. Instead, proliferation of ductular and periductular cells occurs, and these cells are committed to hepatocyte rather than acinar cell differentiation. Our findings that pancreatic duct cells can differentiate toward hepatocytes following *in vivo* implantation, in th· ·ence of stringent extracellular matrix requirements, suggests a pr· ·nsity for hepatocyte differentiation for the hepatobiliary-pancreatic duct epithelial "stem" cells of adult animals. In contrast, acinar or endocrine cell differentiation does not seem to occur. It is possible that the differentiation into these cells requires a more precisely defined microenvironment such as the composition and organization of the extracellular matrix and growth factors. These may be present in *in vivo* situations in which nesidioblastosis and

islets from duct cells can occur (Boquist and Edstrom, 1970; Pour, 1970; Cantenys et al., 1981; Rosenberg et al., 1984; Weaver et al., 1985; Rosenberg et al., 1988)

The definition of differentiation potential and lineage of duct epithelial cells in the adult pancreas may have important implications for the treatment of various pancreatic diseases. There is ample evidence that pancreatic ductal cells from various animal species can be propagated in culture to yield a large number of these multipotential "stem" cells. Hubchak et al (1990) have demonstrated that when propagable hamster pancreatic duct epithelial cells are grown on basement membrane matrix, they can recapitulate the morphology of differentiated ductal cells. Our results have demonstrated the possible use of these cells as "stem" cells for hepatocyte differentiation. We are currently investigating the capacity of these cells to differentiate along the acinar or endocrine cell lineages. An understanding of the precise conditions and mechanisms undertaken by these cells to pursue specific differentiation pathways would have a significant implication in pancreatic and liver bio-engineering technologies. Moreover, these stem cells could also be used to introduce new genes into the pancreas for gene therapy purposes.

CHAPTER 4

HEPATOCYTIC DIFFERENTIATION IN NEOPLASTIC RAT PANCREATIC CELL LINES

4.1 SUMMARY

The hepatocytic differentiation potential of neoplastically transformed pancreatic duct epithelial cell lines was investigated. Two cell lines derived respectively from tumors formed by spontaneously- and azaserine-transformed ductal cells were implanted into either the subcutaneous or intraperitoneal sites of isogeneic Fischer-344 rats. Both cell lines produced aggressively growing tumors subcutaneously and intraperitoneally 1-2 weeks following *in vivo* implantation. The tumors at both sites are histologically indistinguishable. Spontaneously-transformed cells (RPC5T) produced anaplastic carcinomas, while the azaserine-transformed cells (AsC4TC) produced poorly-differentiated adenocarcinomas. Neither differentiated along the hepatocytic lineage, nor did they demonstrate lineage and ultrastructural markers for acinar or islet cell differentiation. Both the cell lines and their derived tumors had some phenotypic similarities to the parental normal duct cells. The tumor cell lines and their derived tumors showed enhanced expression of α-fetoprotein, particularly that induced by azaserine. The results showed that in neoplastically transformed cells, differentiation is largely determined by the intrinsic nature of the cells rather than by the microenvironment in which they grow.

4.2 INTRODUCTION

Microenvironmental milieu can have a profound influence on the genotypic and phenotypic stabilities of tumors cells (Nicolson, 1984; Honsik et al., 1986; Nicolson, 1987). Differentiation inducers/modulators including nutrients, oxygen, ions, enzymes, extracellular matrix, hormones, growth factors and their influence may vary in different microenvironments (Watt, 1991). These constituents play interacting but complex roles in modulating gene expression and the phenotypic properties of tumor cells (Schirrmacher, 1980; Bissel et al., 1983; Klein and Klein, 1985; Nicolson, 1987). Evidence has shown that neoplastic cells also maintain a certain degree of differentiation plasticity and are partially or differentially responsive to environmental stimuli. Mouse embryonal carcinoma cells can develop into phenotypically normal cells and tissues after they are implanted into mouse blastocysts (Mintz and Illmensee, 1975; Pierce et al., 1979). Certain chemically induced malignant rat liver epithelial cell lines can morphologically differentiate into hepatocytes upon intrahepatic implantation but remain tumorigenic in the subcutaneous site (Coleman et al., 1993). Furthermore, Pettengill et al (1994) have shown that neoplastic acinar cells can lose their differentiated features and acquire ductal phenotypes when regrafted into an isogeneic host.

In the previous chapter, we have demonstrated the ability of normal cultured rat pancreatic duct cells to differentiate along the hepatocytic lineage when implanted subcutaneously or intraperitoneally. In this study, we have examined whether

neoplastic rat pancreatic cell lines demonstrate a similar differentiation capacity following *in vivo* implantation.

4.3 MATERIALS AND METHODS

4.3.1) Cell Lines and Culture:

The RP-F344-1 used in this study was derived from a propagable cultured rat pancreatic duct epithelial cell line of an adult Fischer-344 rat (Tsao and Duguid, 1987). Cells at passage 10 were either spontaneously transformed by 5 cycles of "selective" culture condition (Lee et al., 1989) or chemically transformed by 4 treatment with 0.3 mM azaserine (Shepherd et al., 1993). Tumors which formed in isogeneic animals after subcutaneous transplantation of transformed cells gave rise to tumor cell lines (Shepherd et al., 1993). A spontaneously-transformed tumor cell line was established from an anaplastic carcinoma, and an azaserine-transformed tumor cell line was established from a moderately/poorly differentiated adenocarcinoma. They were designated as RPC5T and AsC4TC, respectively. Figure 5-1 showed the morphologic features of the two cell lines. The cells were routinely grown in Richter's improved minimal essential medium with zinc option containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamycin (ICN Biomedical, St Laurent, Que) at 37°C in a humidified 5% CO_2 atmosphere. The media were changed twice a week. Subculture was performed by dissociating the colonizing cells with 0.05% trypsin and 0.53 mM EDTA (Gibco, Grand Island, NY).

4.3.2) *In Vivo* Implantation:

Male Fischer-344 rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 120-150 g were used in all experiments. All animal were fed with normal chow diet. 2 X 10^6 trypsin-harvested RPC5T or AsC4TC cells were embedded in a mixture of rat tail collagen and Matrigel (Collaborative Research, Bedford, MA) and implanted either subcutaneously or intraperitoneally as described in Chapter 3, page 87. Cell-free gel was implanted in control animals.

Animals were sacrificed when subcutaneous tumors reached 1-2 cm in diameter, or when ascites became apparent by abdominal enlargement. The animals were sacrificed by cervical dislocation and tumor tissue from the implantation site was harvested. Part of the tumor tissue was fixed immediately in 10% buffered formaldehyde for routine paraffin embedding, and small cubes (1-2 mm^3) of tumor tissue were fixed in 1% glutaraldehyde-4% paraformaldehyde solution for electron microscopic studies. Tissue was also snap-frozen in liquid nitrogen and stored at -80 C for enzyme histochemistry and RNA isolation.

4.3.3) Histochemical and Ultrastructural Studies:

Paraffin sections were routinely stained with hematoxylin-eosin, periodic acid-Schiff (PAS) with or without prior digestion with diastase and alcian blue (pH 2.5). Frozen sections of unfixed tissue were used for γ-glutamyl transpeptidase (Rutenberg et al., 1969), alkaline phosphatase (Lin et al., 1992), and carbonic anhydrase (Githens et al., 1987) histochemistry, as described in Chapter 2, page 57. Comparison of the relative levels of enzyme expression between

cultured cells and tumor tissues was achieved by scoring the intensity of the staining in a fixed period of time (15 min). Since the specific activity is expressed as mU/ mg protein, the above approach was preferred because of a large amount of proteins derived from non-tumor origins.

Transmission electron microscopic studies of 0.1-0.2 μm sections of epon-embedded tissue were carried out using a Philips electron microscope Model 201.

4.3.4) Immunocytochemistry:

This was performed using the streptavidin-biotin-peroxidase system (Zymed Laboratories, San Francisco, CA) coupled with microwave antigen retrieval treatment (Shi et al., 1991). The same panel of antibodies described in Chapter 3 were used. Antigen-antibody complex formation was visualized with aminoethyl carbazole (AEC). Tissues for the positive controls included adult rat liver and pancreas. Newborn rat liver was used as the positive control for α-fetoprotein. Negative controls included replacement of the primary antibodies with non-immune serum or PBS.

4.3.5) Isolation of Total Cellular and Tissue RNA and Northern Blot Analysis:

Isolation of total cellular RNA from confluent cultured cells and tumor tissues were performed by the established methods (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987) as described in Chapter 2, page 60. Thirty μg of denatured RNA sample were resolved in a

1% formaldehyde-agarose gel and transferred to Hybond-N nylon membrane (Amersham Canada, Oakville, Ont).

4.3.6) Nucleic Acid Hybridization With cDNA Probes:

The cDNA probes were labelled with [^{32}P]-dCTP to high specific activities, using the Oligolabelling kit of Pharmacia (Dorval, Que). The probes included purified cDNA fragments of rat ALB (n19-638), AFP (n147-408), CA II (n35-619), aldolase B (n140-945), α-amylase (n 55-609), a rat kidney GGT clone 37 (Laperche et al., 1986), and β-actin (Clontech, Palo Alto, CA). Hybridization and washing were performed according to a slightly modified procedure of Church and Gilbert (1984) as described in Chapter 2, page 62 and membranes were exposed to Kodak XAR-5 film at -80°C using an intensifying screen.

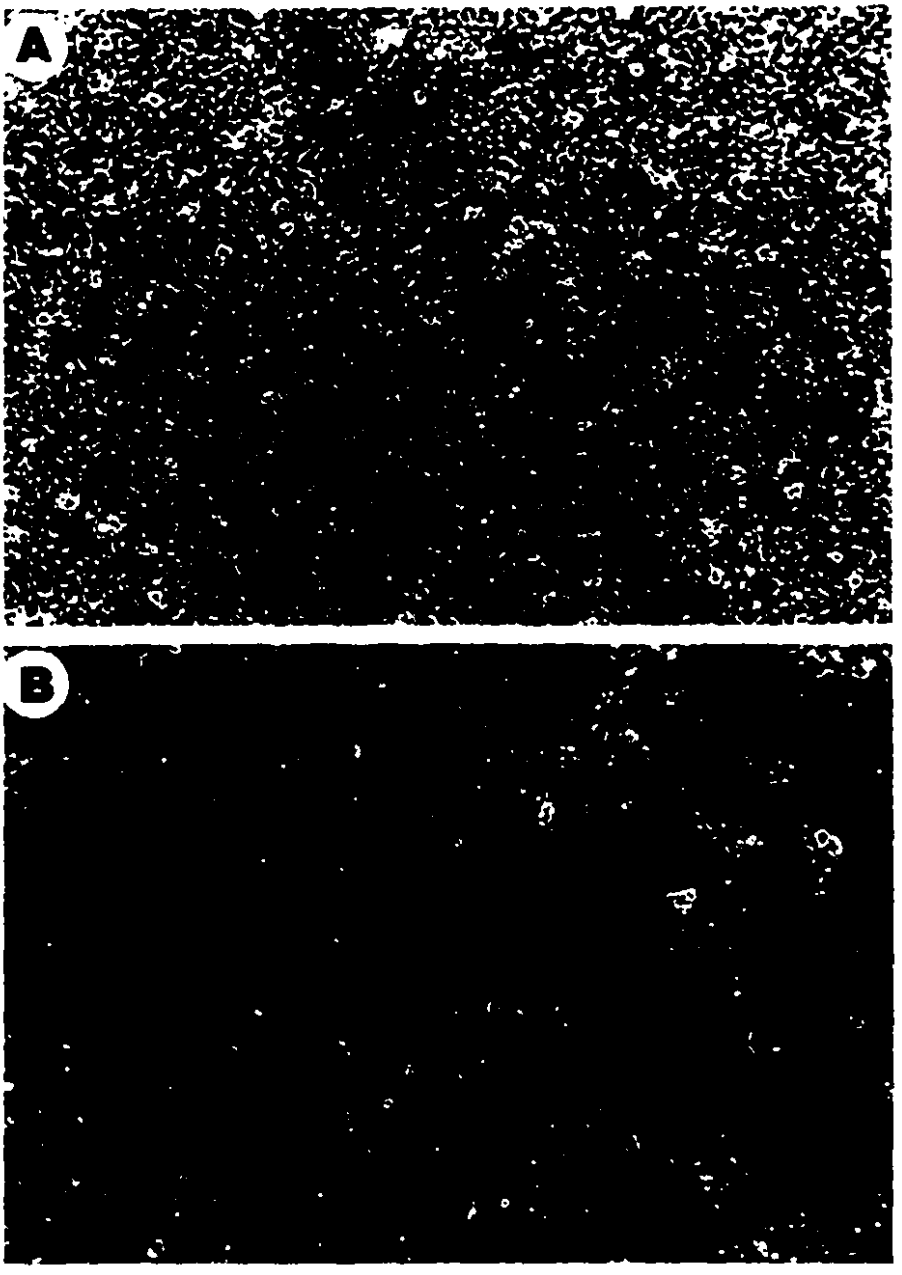

Figure 4-1 The microphotographs of the tumor cell lines. (A) RPC5T, a spontaneously-transformed tumor cell line derived from an anaplastic carcinoma; (B) AsC4TC, an azaserine-transformed tumor cell line derived from a moderately/poorly differentiated adenocarcinoma. (Magnification: X 180).

4.4 RESULTS

Phenotypic properties of labelled duct epithelial cells:

Table 4-1 summarizes the *in vitro* phenotypic properties of both RPC5T and AsC4TC cell lines, as studied using immunocytochemistry, enzyme histochemistry and Northern blotting. Immunohistochemistry did not detect the presence of ALB, transferrin, tyrosine aminotransferase (TAT), chromogranin, insulin, glucagon, somatostatin nor α-amylase in either cell line. Immunostaining for AFP was weakly positive in AsC4TC cells and negative in RPC5T cells. Immunostaining with the monoclonal antibodies HES6 and BDS7 were also negative. 50-60% of RPC5T and AsC4TC cells stained positively for cytokeratin 8 but negatively for cytokeratin 19. Both cell lines were negative for glycogen storage and mucin. RPC5T cells expressed strong activity for ALP and weak activity for CA and GGT, while AsC4TC cells showed strong activity for GGT and CA and weak activity for ALP.

Northern blotting detected the presence of AFP (2-kb) and GGT (2.2-kb) mRNA transcripts in AsC4TC cells (Figure 4-2). AFP mRNA transcript was barely detected by Northern blotting in RPC5T cells, but it was easily detected by the more sensitve RT-PCR. mRNA transcripts of albumin, aldolase B, CA, and α-amylase were not detectable by Northern hybridization in both cell lines.

Table 4-1: Phenotypic profiles of spontaneously-transformed (RPC5T), azaserine-transformed (AsC4TC) cells, and their derived tumors located subcutaneously and intraperitoneally

	RPC5T			AsC4Tc		
Phenotypes	Cell Line	Sub Tumor	IP Tumor	Cell Line	Sub Tumor	IP Tumor
albumin[a]	-	-	-	-	-	-
α-fetoprotein	+	+	+	+	+	+
transferrin	-	-	-	-	-	-
TAT	-	-	-	-	-	-
aldolase B[b]	-	-	-	-	-	-
CA II	+	+	+	++	+++	+++
cytokeratin 8	+	+	+	+	+	+
cytokeratin 19	-	+	+	-	+	+
ALP	++	+++	+++	+	+++	+++
GGT	+	+	+	+++	++	++
HIS6	-	-	-	-	-	-
BDS7	-	-	-	-	-	-
mucin	-	-	-	-	-[c]	-
glycogen storage	-	-	-	-	-	-
α-amylase	-	-	-	-	-	-
islet hormone[d]	-	-	-	-	-	-

Abbreviation: Sub: subcutaneous; IP: intraperitoneal; CA II: carbonic anhydrase II; TAT: tyrosine aminotransferase; HIS6: surface antigen of rat hepatocyte; BDS7: surface antigen of rat biliary duct epithelial cells; ALP: alkaline phosphatase; GGT: γ-glutamyl transpeptidase; +: positive; -: negative

a: The relative levels of ALP, GGT, and CA were determined by the intensity of staining of enzyme histochemistry. For other phenotypic markers, only + or - was marked.

b: Determined by mRNA expression.

c: Mucin production was limited to the cells in the glandular structures.

d: Including insulin, glucagon, somatostatin, and chromogranin.

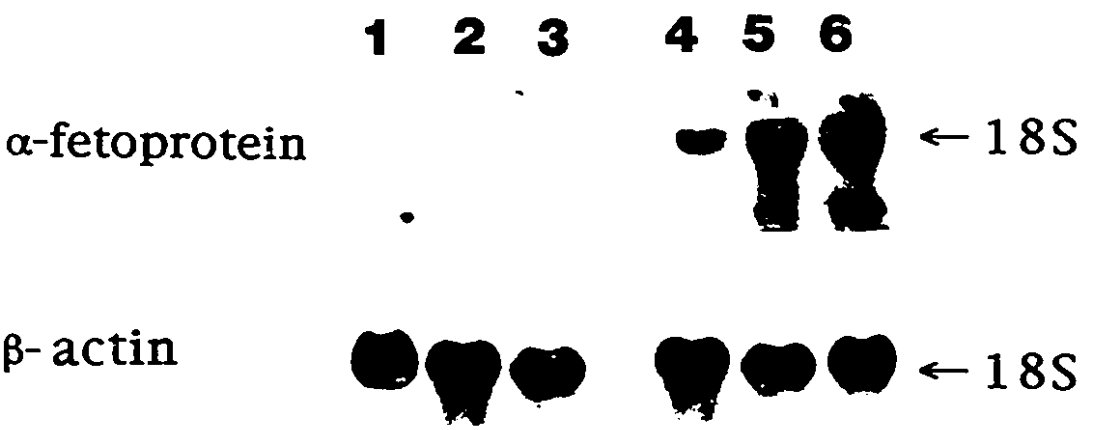

Figure 4-2: Northern blot analysis showed the expression of a 2-kb α-fetoprotein mRNA in RPC5T cell line (Lane 1), subcutaneous RPC5T tumor (Lane 2), intraperitoneal RPC5T tumor (Lane 3), AsC4TC cell line (Lane 4), subcutaneous AsC4TC tumor (Lane 5) and intraperitoneal AsC4TC tumors (Lane 6). β-actin (2.2-kb) was used as the indicator for the amount of RNA loaded in each lane.

Gross Morphology of Implanted Tumors:

Both RPC5T and AsC4TC cells were highly tumorigenic following *in vivo* implantation. Tumors implanted at both locations were palpable and became visible in 1-2 weeks. Subcutaneous tumors were solitary masses and measured up to 2 cm in diameter. RPC5T subcutaneous tumors had ill-defined borders with prominent invasion into the surrounding tissues, whereas those produced by AsC4TC were more circumscribed. When intraperitoneally transplanted, both cell lines produced numerous tumors of 0.5 to 1 cm in diameter in the mesentery, omentum, and visceral surfaces of various abdominal organs, and they were usually accompanied with ascites. In the case of RPC5T, metastatic nodules could be found in lungs and thoracic lymph nodes. No tumors were found in the control animals.

Phenotypic Characterization of R^{D}C5T Subcutaneously- and Intraperitoneally-Implanted Tumor:

The morphology of subcutaneous and intraperitoneal RPC5T tumors were histologically indistinguishable. All tumors consisted of anaplastic carcinomas showing sheets of pleomorphic epithelial cells with scant cytoplasm and irregular or round euchromatic nuclei (Figure 4-3 A). Stromal fibrosis (desmoplasia) was noted focally. Table 4-1 summarized the phenotypic profiles of the tumors at both locations. The results showed that they had similar phenotypic characteristics. The great majority of tumors cells demonstrated positive immunostaining with antibodies to cytokeratin 8 (Figure 4-3 B) and cytokeratin 19. Immunostaining with antibodies to AFP was

weakly positive. They stained negatively for liver specific proteins, HES6 and BDS7 antibodies, and antibodies to several pancreatic enzymes/ hormones, including α-amylase, insulin, glucagon, chromogranin and somatostatin. The tumors cells did not accumulate glycogen or produce mucin. Enzyme histochemistry showed strong staining for ALP activity but that for GGT and CA was weakly positvie.

Northern blotting analysis showed a 2-kb transcript of AFP mRNA in both tumors (Figure 4-2). mRNA transcripts of albumin, aldolase B, GGT, CA, and α-amylase were not detected by Northern blotting.

Ultrastructurally, the pleomorphic tumor cells in both locations contained moderately abundant dilated rough endoplasmic reticulum and mitochondria. They also formed numerous bile-canalicular-like intercellular lumina (Figure 4-4). No features of acinar or endocrine differentiation was noted.

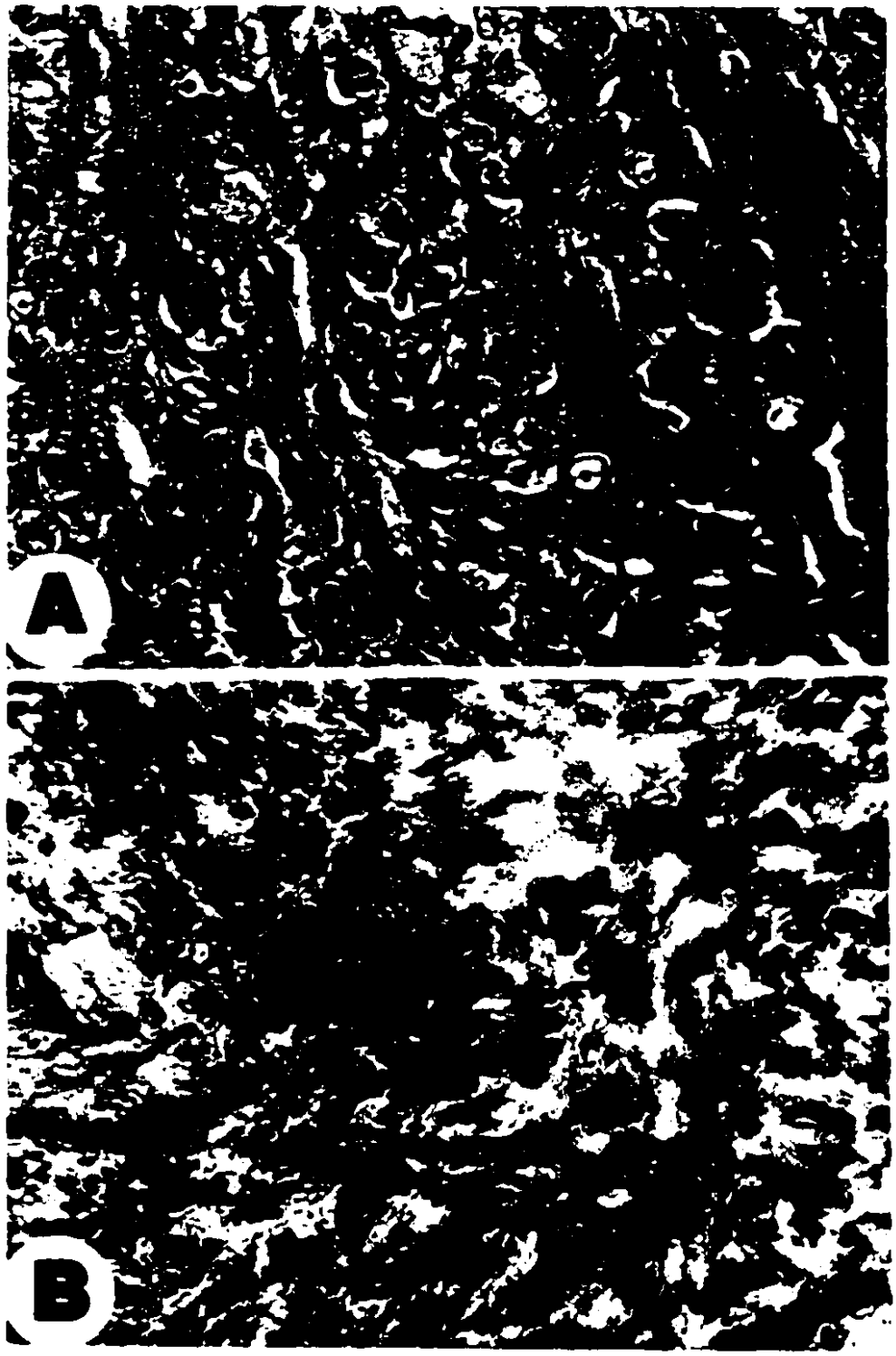

Figure 4-3: Microscopic analysis of the RPC5T tumors. (A) An H&E section shows an anaplastic carcinoma consisting of mainly pleomorphic epithelial cells. (B) The great majority of the tumor cells demonstrate strong immunostaining for cytokeratin 8. (Magnification: A and B: X 380).

Figure 4-4: Transmission electron microscopic appearance of the RPC5T tumors. The anaplastic carcinomas, as represented by the light microscopic appearance shown in Figure 4-3A, consist of epithelial cells containing moderately abundant dilated rough endoplasmic reticulum and mitochondria. The cells formed numerous tiny bile-canalicular-like intercellular lumens (arrow). (Magnification: X 5730)

Phenotypic Characterization of AsC4TC Subcutaneously- and Intraperitoneally-Implanted Tumor:

The morphology of subcutaneous and intraperitoneal AsC4TC tumors were also histologically indistinguishable. All tumors were poorly-differentiated carcinomas showing monotonous-appearing cuboidal or round cells which grew in trabecular and/or solid manner. The cells had basophilic cytoplasm and round euchromatic nuclei with one or two prominent nucleoli (Figure 4-5 A). Glandular structures having alcian blue-stained apical brush borders were noted focally. Tumors at both sites also demonstrated similar phenotypic features (Table 4-1).

The tumors cells demonstrated positive immunostaining with antibodies to cytokeratin 8, cytokeratin 19, and AFP (Figure 4-5 B). The AFP level was higher than that in RPC5T tumors, as judged by the intensity of immunostaining and Northern blotting (Figure 4-2). They stained negatively for liver-specific proteins, HES6 and BDS7 antibodies, and antibodies to several pancreatic hormones and enzymes. None of the cells contained cytoplasmic glycogen. Mucin production was limited to the cells in the glands Enzyme histochemistry showed strong cytoplasmic staining for ALP and CA and moderate activity for GGT. Northern blot analyses showed the mRNA transcripts of AFP (Figure 4-2), GGT, and CA II whereas those of albumin, aldolase B and α-amylase were not detected.

Ultrastructurally, cells in poorly differentiated areas contained moderately abundant dilated rough endoplasmic reticulum and mitochondria. They also formed small glandular spaces (Figure 4-6), confirming that it was a poorly-differentiated adenocarcinoma.

Figure 4-5: Microscopic analysis of the AsC4TC tumors. (A) An H&E section shows a poorly-differentiated carcinoma consisting of monotonous-appearing cuboidal or round cells arranged in trabecular and /or solid manner. The cells have a basophilic cytoplasm and round euchromatic nuclei with one or two prominent nucleoli. (B) Most of the tumors cells demonstrate positive immunostaining for α-fetoprotein. Fibroblasts are not stained (arrow) (Magnification: A and B: X 380).

Figure 4-6: Transmission electron microscopic examination of the AsC4TC tumors. The poorly-differentiated tumor, depicted in Figure 4-5A, contains moderately abundant dilated rough endoplasmic reticulum and mitochondria and formed small glandular spaces (arrow), confirming that it is a poorly-differentiated adenocarcinoma (Magnification: X 5730).

4.5 DISCUSSION

In this study we examined the differentiation potential of spontaneously (RPC5T)- and azaserine (AsC4TC)-transformed tumor cell lines when implanted in two different microenvironments. Both cell lines remained highly tumorigenic and produced aggressively growing tumors subcutaneously and intraperitoneally. Tumors at both sites were histologically indistinguishable and resembled their parental tumors. RPC5T cells produced anaplastic carcinomas, while AsC4TC cells produced poorly-differentiated adenocarcinomas. The extent of *in vivo* phenotypic expression of both tumor cell lines was somewhat modified after implantation, including the modulation of enzyme activities (ALP, CA, and GGT) and re-expression of cytokeratin 19. Unlike the normal pancreatic duct epithelial (RP-2) cells, they did not differentiate along the hepatic epithelial pathway. Neither did they demonstrate lineage and ultrastructural markers for acinar or islet cell differentiation.

It is conceivable that not all malignant cells can be modulated by their microenvironments. Sachs (1984) has proposed that as tumors develop, they shift from inducible pathways of gene expression to constitutive ones, thus explaining the nonresponsiveness of malignant cells. Our observations suggest that both tumor cell lines have lost part or all of their cellular targets (cell-surface receptors, cell adhesion proteins, etc.) that mediate the cellular response to microenvironmental influences. For example, both tumor cell lines displayed a marked down regulation or absence of epidermal growth factor receptor (EGFR) expression when compared to their parental normal pancreatic duct epithelial cells

(Shepherd et al., 1993). EFGR and its ligands- EGF and transforming growth factor-α (TGF-α) have been shown to play important roles in regulating the growth and differentiation of the pancreas and other tissues (Lee and Han, 1990, Githens, 1993).

Implantation of cells to different *in vivo* sites will not only provide a stringent test on the cell commitment, but also reveal the role of potential microenvironmental regulators (Blau and Hughes, 1990). Such experiments can usually delineate the mechanisms on how a defined microenvironment regulates cell growth and differentiation and lay the foundation to probe such a process at the molecular level. After Mintz and Illmensee (1975) and Pierce et al (1979) showed that mouse embryonal carcinoma cells demonstrated the ability to develop into phenotypically normal cells and tissues upon implantation into mouse blastocysts, it was subsequently determined that malignant cell contact with the trophoectoderm and a factor in blastocele fluid were both required (Pierce et al., 1984) . Similarly, Gerschenson et al (1986) has been able to decrease significantly the formation of melanoma tumors in the mouse embryo, but only if they were injected into the embryonic skin at precisely the time when premelanocytes migrate into the skin. Whether the embryonic environment can regulate the differentiation of both normal and neoplastic pancreatic duct epithelial cell remains to be investigated. This possibility is likely since Dudek et al (1991) have shown that fetal mesenchyme can induce islet cytodifferentiation in adult rat pancreatic ductal epithelium.

AFP belongs to a group of oncofetal proteins that are present at high levels in embryonic and neoplastic tissues. AFP levels, which

are low in the serum and liver of adult animals and humans, are very high in the endodermal cells of the yolk sac and embryonic liver, as well as in the majority of germ cell tumors and hepatomas (Wepsic, 1983; Tamaoki and Fausto, 1984; Lemire and Fausto, 1991). AFP is transiently expressed in the pancreas during ontogenesis (Ruodlahti and Seppala, 1979). In human pancreatic neoplasms, AFP is frequently detectable in pancreatoblastomas (Iseki et al., 1986; Morohoshi et al., 1990). AFP may also be expressed in duct (McIntire et al., 1975; Wood and Moossa,1977) and acinar cell carcinomas (Kawamoto et al., 1992; Kimura et al., 1992; Nojima et al., 1992; Hoorens et al., 1993). The frequency is generally quite low except for the results of McIntire et al (1975) who reported elevated AFP serum levels in 24% of patients with pancreatic cancer. It appears that AFP is not a specific or consistent tumor marker for pancreatic cancers.

The expression of AFP is not reported in acinar tumors in azaserine-treated rats. The 2.0-kb (18S) AFP mRNA expressed by both RPC5T and AsC4TC cell lines and their derived tumors is slightly smaller than the previously reported 2.1-2.3 kb of mature rat AFP mRNA (Petropoulos et al., 1985; Lemire and Fausto, 1991). Cultured normal rat liver epithelial cell line (WB-F344) and its chemical carcinogen-treated clonal derivatives also express a 2.0-kb AFP mRNA (Tsao, 1993). Tumor cell lines derived from N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-transformed WB-F344 cells consistently expressed higher levels of AFP mRNA than tumor cell lines derived from spontaneously transformed WB-F344 cells (Tsao, 1993). Our results showed that tumor cells originating from

azaserine-transformed pancreatic duct cells expressed higher levels of AFP mRNA than the normal and spontaneously-transformed ones. It has been shown that the enhanced expression of AFP in MNNG-treated cultured rat liver epithelial cells is related to the DNA-hypomethylating effect of MNNG (Tsao, 1993). DNA hypomethylation has been correlated with enhanced gene expression and activation of specific genes during differentiation (Razin and Riggs, 1980; Jaenisch and Jahner, 1984). Interestingly, azaserine exerts its effect by forming methlyguanine adducts that alkylate and damage DNA (Zurlo et al., 1982). However, evidence shows that the methylation pattern of the AFP gene is not sufficient to explain the activation of AFP gene expression (Kunnath and Locker, 1983; Locker et al., 1987).

Just as the microenvironment is important in delineating gene expression and the phenotypic properties for normal cells, it is also equally important for neoplastic cells. Detailed studies of the growth conditions of tumors must be made, if we are to understand how malignant cells are regulated. The development of appropriate cell culture systems, particularly the use of serum-free chemically defined medium, has made it possible to identify growth or differentiation factors in various cell types. This approach has been particularly fruitful in identifying growth and differentiation in normal blood cells and leukemic myeloid cells (Fibach et al., 1972; Weisinger and Sachs, 1983; Sachs, 1984). Currently there are many human and rodent tumor cell line established from duct and acinar cell carcinomas (Fraizer, 1993). It would therefore be useful to apply similar approaches to identify the factors involved in regulating the growth and differentiation of duct and acinar cells. This may

eventually unravel the underlying mechanisms that regulate the balance between multiplication and differentiation. The biological treatment of cancer in the future may involve the directing of differentiation of malignant to benign cells by gene transfer as an alternative to immune modulation and/or cytotoxic therapy.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSION

Although the rat liver and pancreas arise from a common endodermal anlage in the floor of the foregut, they do not have common morphological nor functional properties. It is proposed that once the precursor (stem) cells are committed, they differentiate into pancreas or liver cell lineages and lose the ability for dual differentiation (Rutter et al., 1973). In liver, the hepatoblasts give rise to both differentiated hepatocytes and bile duct epithelium (Germain et al., 1988; Fausto, 1990; Shiojiri et al., 1991). In pancreas, the protodifferentiated epithelial cells give rise to acinar or endocrine cells. The cells of the branching epithelium that do not differentiate into acinar or endocrine cells remain as the duct cells (Githens, 1986, 1988). In adult rat liver and pancreas, uncommitted stem cells has not been identified, however, there were reports that hepatocytes can appear in the adult rat and hamster pancreas (Rao et al., 1990; Makino et al., 1990; Reddy et al., 1991) and acinar cells in the adult rat liver (Kimbrough, 1973; Rao et al., 1986a) when these organs are injured experimentally. Pancreatic acinar and ductular epithelia were found associated with large intrahepatic and with extrahepatic ducts in 4% of human livers (Terada, 1990). Wolf et al (1990) also reported the presence of numerous microscopic foci of exocrine pancreas distributed throughout the liver of one patient with severe posthepatic cirrhosis. Exocrine pancreatic tissue has also been observed in hepatic tumors of trout following exposure to various hepatocarcinogens (Hendricks et al., 1981) and diethylnitrosamine (Lee et al., 1989). This transdifferentiation of

pancreas to liver or vice versa raises the possibility that there is a significant but latent developmental capability in adult cells that requires only the correct circumstances to be unleashed.

Given the greater resemblance of the duct cells to the protodifferentiated epithelium, this cell compartment appears to be the most likely location for the putative stem cells (Githens, 1986, 1989). Evidence supporting this hypothesis includes the capacity of pancreatic duct cells to differentiate into islet cells (Boquist and Edström, 1970; Pour, 1970; Rosenberg et al., 1983; Dudek et al., 1991) and hepatocytes (Rao et al., 1990; Makino et al., 1990; Reddy et al., 1991; Sar). As a first step in the quest to identify stem cells in the adult pancreas, duct cells from adult rats (Tsao and Duguid, 1987; Heimann and Githens, 1991) and hamsters (Hubchak et al., 1990; Takahashi et al., 1995) have been isolated and cultured. In this study a cell line (termed RP-2) was established from a duct fragment selectively picked using a Pasteur pipette by a refined methodology of Tsao and Duguid (1987). The ductal origin of these cells was confirmed by the sequential observation of the duct fragment developing into a monolayer of epithelial cells as well as by their ultrastructural features and biochemical properties. RP-2 cells have subsequently been propagated for 16 passages (80-90 doublings) without any morphologic evidence of senescence. The extensive subcultures possible with these cells fulfills one of the criteria for a stem cell population: the potential for extensive if not unlimited proliferation. In contrast, pancreatic acinar (Brannon et al., 1985) and islet cells (Takaki,1989) have not been shown to be capable of continuous proliferation *in vitro* to form cell lines. Furthermore RP-2

phenotypically resemble normal cultured rat liver epithelial cells (termed WB) that are posited to originate from intrahepatic bile ductular cells (Tsao et al., 1984; Grisham et al., 1990). Bisgaard and Thorgeirsson (1990) have also reported the establishment of propagable epithelial cell lines with almost identical phenotypes from both adult organs. These *in vitro* data support the hypothesis that propagable normal epithelial cell lines from the rat liver and pancreas may represent progenies from a common pancreatico-hepatobiliary stem cell.

By utilizing *in vivo* implantation, the results of this study represent the most direct evidence to support the ductal cell origin of transdifferentiated pancreatic hepatocytes. When localized in the systemic circulatory compartment (the subcutaneous site), RP-2 cells display differentiation toward hepatocytes but retain some of their ductal features. In contrast, when RP-2 cells are located in the mesentery, they show the full phenotypic properties of mature hepatocytes, including their morphological appearance and the loss of α-fetoprotein and CA II expression (whereas cells in the subcutaneous site show α-fetoprotein and CA II expression). Further studies are required to determine whether the microenvironmental influence on the degree of differentiation of these cells is related to the portal circulation. Portal blood "hepatotropic factors" have been shown to be necessary for liver allograft survival (Lee et al., 1975) and for implanted islets of Langerhans (Mauer et al., 1974). Less islet tissue is required to ameliorate diabetes when the portal vein route is used in comparison to the number of islets necessary when they are implanted to other sites. Failure to lower bilirubin levels

when hepatocytes or slices of liver are implanted subcutaneously has been posited to be caused by the lack of hepatotropic factors in the blood perfusing the site (Matas et al., 1976), indicating the importance of hepatotropic factors in maintaining the differentiated state of implanted tissues.

It is interesting to note that the subcutaneously located hepatocyte-like cells accumulated cytoplasmic fat, whereas the intraperitoneal ones did not. None of the hepatocyte-like cells induced in rat and hamster models had cytoplasmic fat, but hepatocyte-like cells in the pancreas of aged hamsters accumulated fat. The latter had also been shown to transform to fat cells in a background of acinar atrophy and fat replacement resulting from duct obstruction or infection (Takahashi and Pour, 1978; Pour et al., 1979; Pour and Wilson, 1983). Walters (1966) and Geever et al (1977) believed that pancreatic fatty changes may be similar to that occurring in the liver as a consequence of dietary deficiency. In this study, the fatty changes in the subcutaneous hepatocyte-like cells may have been the result of (1) obstruction caused by lack of conductive pathways; and/or (2) differing nutrients and factors in systemic and portal circulation.

Coleman et al. (1993) have shown that WB cells can integrate into the hepatic plates and morphologically differentiate into hepatocytes upon intrahepatic transplantation. No cells were found to differentiate into biliary epithelial cells in their studies. The authors speculated that this may be due to lack of contact with the portal tract mesenchyme or to the fact that WB cells did not possess a bipotential capacity. The latter is probably unlikely because well-

formed ducts are noted in all the tissues examined. Nevertheless it is difficult to determine whether these ductal cells developed along the biliary or pancreatic duct cell lineages because of their low occurrence in the implanted cell population.

To study factors influencing the growth and functions of ductal epithelial cells, the need for a serum-free chemically defined medium is evident. While serum-free media have been developed to maintain the functional capacity for acinar (Brannon et al., 1985) and endocrine (Kinard et al., 1990) cells over an extended period of time, the growth of most pancreatic duct epithelial cell lines (Sato et al., 1983; Tsao and Duguid, 1987; Hubchak et al., 1990; Heimann and Githens, 1991) largely remains dependent on the presence of fetal bovine serum. Heimann and Githens (1991) reported that the addition of ethanolamine (EA), transferrin (T), and bovine serum albumin (BSA) substantially restored the poor growth of rat duct epithelium in the absence of serum. However, the growth rate in this serum-free medium was only half that of the cells grown in the serum-supplemented medium. The RP-2 cells grown in the same serum-free medium soon developed senescence as judged by their morphologic appearances. Optimization of the concentrations of EA, T, and BSA and/or the addition of other additives may be required to restore the growth of the duct epithelium to that found in the presence of serum. The potential additives may include gastrointestinal hormones such as secretin, bombesin, and gastrin which greatly stimulated DNA synthesis in hamster pancreatic duct cells (Mangino et al., 1992).

Carcinoma of the exocrine pancreas remains one of the most mortal of human malignancies (Boring et al., 1992) and conventional methods of treatment offer little hope of cure (Gudjonsson, 1987; Warshaw and Swanson,1988). The analysis of microenvironmental factors which might either promote or inhibit tumor development, and their mechanisms of action may be of paramount importance in future cancer treatment. Such studies may help to offer explanations for tumor progression and metastasis, and also to understand the failures of conventional therapy. This new knowledge may be the stimulus for the design of future strategic therapies.

CONCLUSIONS

(1) Propagable normal rat pancreatic duct epithelial cell lines can be consistently established from isolated duct fragments of collagenase-dissociated pancreatic tissue. These cells can be continuously propagated *in vitro* and they retain some of their *in vivo* differentiated phenotypes.

(2) When implanted *in vivo*, cultured rat pancreatic duct cells demonstrate a capacity for differentiation toward the hepatocyte phenotype and this process is influenced by the microenvironment. The subcutaneously-implanted cells show some differentiation toward immature hepatocytes but retain ductal features whereas the intraperitoneally-implanted cells express the phenotypic properties of mature hepatocytes;

(3) Both spontaneously- and azaserine-transformed pancreatic ductal cell lines did not display phenotypic differentiation along the hepatocytic lineage after *in vivo* implantation. It is suggested that both tumor cell lines have lost part or all of their biochemical pathways that mediate the cellular responses to hepatoctyic differentiation.

CHAPTER 6
FUTURE PROSPECTIVE

A. Molecular characterization of the model of *in vivo* transplantation of rat pancreatic duct cells

The model described in Chapter 3 provides an opportunity to study the molecular mechanisms that regulate gene expression during differentiation in liver and pancreatic epithelial cells. This involves transcription factors that regulate cell-specific gene expression, hence determining the specific cellular phenotypes. To identify such factors, various assays are required.

(1) The role of known liver-specific gene regulatory elements

At least five nuclear DNA-binding proteins that activate the expression of liver-specific genes have been identified and cloned (Johnson, 1990). For example, hepatocyte nuclear factor 1 (HNF-1) can up-regulate the expression of α- and β-fibrinogen, albumin, α-fetoprotein, and aldolase B. Studies using immunocytochemistry and Northern and/or *in situ* hybridization will unravel their roles and their relative importance in this metaplastic process.

(2) Transfection of known liver-specific gene regulatory elements into pancreatic ductal cells

The influence of these regulatory genes can also be assessed in an *in vitro* system by DNA transfection. The success of this assay depends on the choice of the recipient cells (Blau, 1988). Some cells may repress the transfected gene, whereas others may lack components for the expression of the novel phenotype. In this regard, this study has shown that pancreatic duct cells are suitable recipient cells because of their propensity for differentiation into

hepatocytes. This will determine whether single or combinatorial forced ectopic expression of these genes can convert pancreatic duct cells into those with hepatocytic phenotype, similar to the capacity of MyoD protein to promote differentiation of fibroblasts into myoblasts (Davis et al., 1987).

(3) Cloning of unknown regulatory factors by the mRNA differential display technique

The above two approaches take full advantage of the availability of known transcription factors, but a network of other unknown regulatory factors may participate in such a complicated process as occurs in most situations. Differential and subtractive hybridization is the traditional method used to identify and isolate the complementary DNA (cDNA) of differentially expressed genes. However this approach gives incomplete recovery and selects for only either under- or overexpressed genes. The screening is also time-consuming. The same results can now be achieved using a simpler and faster technique called mRNA differential display which involves the reverse transcription of mRNAs followed by the polymerase chain reaction (Liang and Pardee, 1992). This approach can also be used to clone mRNAs differentially existing between normal and malignant epithelial cell lines used in this study.

B. Pancreatic ductular morphogenesis in three-dimensional extracellular matrix gel

When isolated pancreatic duct fragments were embedded in rat tail collagen or agarose medium, the cut ends would seal and the fragments would develop into cystic structures, ensheathed by a

layer of connective tissue. These cysts continued to increase in size and larger cysts would form short tubular processes. Our preliminary studies show that this phenomenon can be modulated by alterations in the components of embedding matrices and the addition of exogenous factors. These isolated cysts are grown in a mixture of collagen and Matrigel (basement membrane matrices extracted from Englebreth-Holm-Swarm mouse tumor) instead of pure collagen. When a neutralizing antibody against hepatocyte growth factor (HGF) is added to the culture medium, the cysts started to develop new protruding tubules, which developed into interconnecting secondary and tertiary branches with lumen formation (Figure 6-1). Such a phenomenon is not observed in ductal cysts exposed to control medium or media containing either HGF or EGF or TGF-β. In contrast, small and medium size cysts treated with HGF and EGF, but not anti-HGF, developed some branching tubules (Figure 6-2). These preliminary studies suggest that tubular morphogenesis may occur in association with the appropriate extracellular matrix. The action of factors which regulate such processes may vary according to duct size. Since these cysts are surrounded by proliferating fibroblasts, a rich source of HGF (Bhargava et al., 1992), it is hypothesized that HGF inhibits ductular morphogenesis in the large ducts but promote it in medium and smaller ducts. To define precisely the factors and conditions which promote morphogenesis in this system, future work includes:

(1) Isolation and characterization of pancreatic ductal cysts of different sizes to establish the identity and nature of the ductal fragments.

(2) Quantitative measurements of neo-ductular formation in the presence of HGF and EGF in small cysts and anti-HGF antibody in the large cysts.

(3) Determination of the specificity of HGF and anti-HGF for neo-ductular formation and whether the effect can be mimicked by other growth factors.

(4) Assessment of the role of periductal mesenchymal cells in neo-ductular formation.

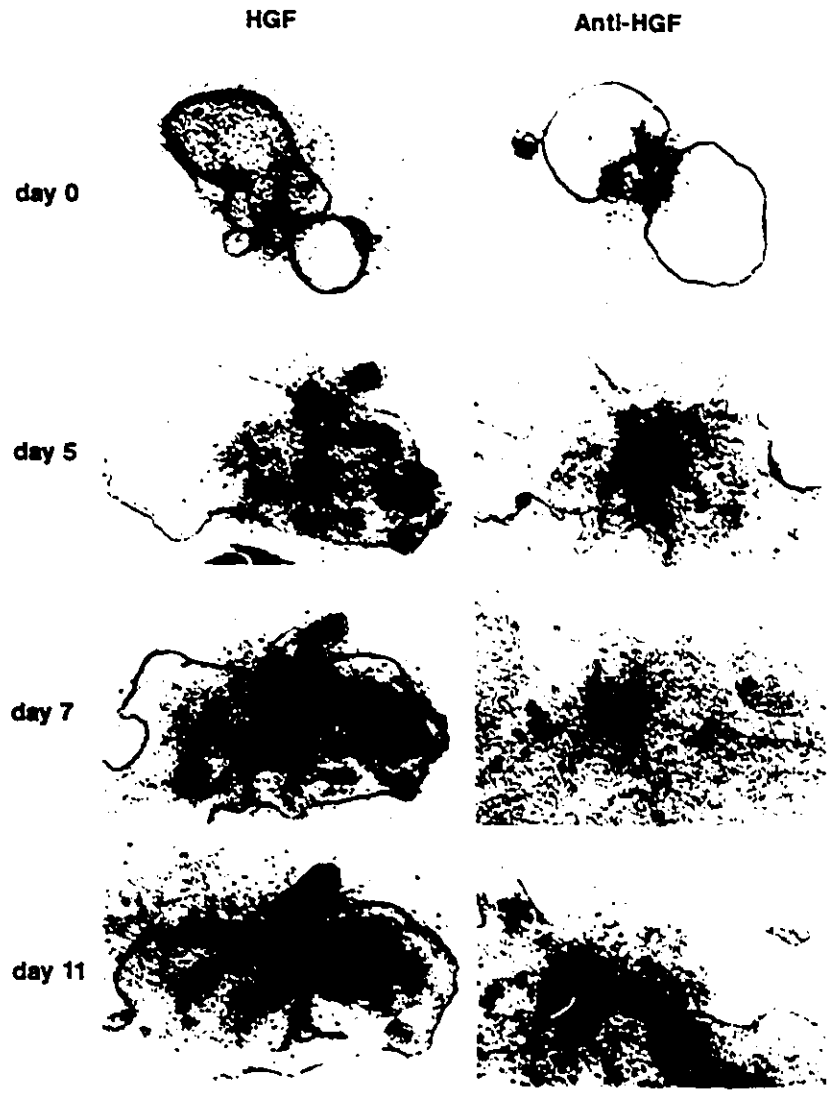

Figure 6-1: Phase-contrast microphotographs demonstrating the temporal development of ductular morphogenesis in large ductal cysts embedded in a diluted Matrigel™ solution (Collaborative Research, Bedford, MA), after treatment with neutralizing anti-HGF antibodies (1:3,000, obtained from T. Nakamura, Kyushu University). No such phenomenon was observed after treatment with 1 ng/ml of HGF or 10 ng/ml EGF (day 0: X80; HGF-day 5, 7,11: X120; Anti-HGF-day 5,7, 11: X200).

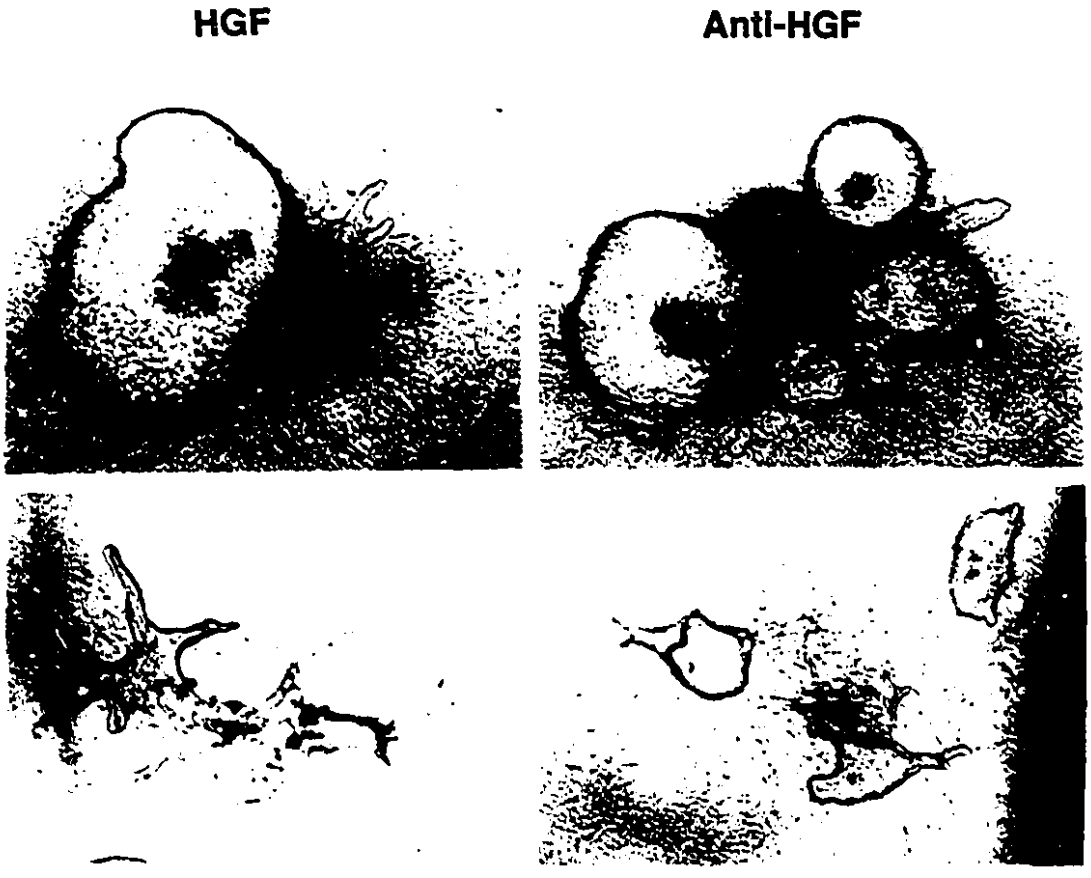

Figure 6-2: Phase-contrast microphotographs demonstrating the development of ductular branches from smaller ducts after 12 days incubation with 1 ng/ml HGF, but not in the presence of anti-HGF antibody (1:3,000). (Magnification: X 200).

References

Agata K, Yasuda K, and Okada TS (1983). Gene coding for a lens-specific protein, δ-crystallin, is transcribed in non-lens tissues of chicken embryo. Develop Biol, 100:222-226

Alberts B, Bray D, Lewis J, Raff M, Roberts K, and Watson JD (1989a). Differentiated cells and the maintenance of tissue. In: Molecular Biology of the Cell. pp 952-995. second edition. Garland Publishing; New York and London

Alberts B, Bray D, Lewis J, Raff M, Roberts K, and Watson JD (1989b). The molecular genetics of cancer. In: Molecular biology of the cell. pp 1203-1216. second edition, Garland Publishing Inc; New York and London

Alder G (1977). Effect of glucagon on the secretory process in the rat exocrine pancreas. Cell Tissue Res, 182:193-204

Aloe L and Levi-Montalcini R (1979). Nerve growth factor-induced transformation of immature chromaffin cells in vivo into sympathetic neuron: effect of antiserum to nerve growth factor. Proc Natl Acad Sic USA, 76:1246-1250

Alpert S, Hanahan D, and Teitelman G (1988), Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neuron. Cell, 53:295-308

Althoff J, Pour P, Malick L, and Wilson RB (1976). Pancreatic neoplasms induced in Syrian golden hamsters. Am J Pathol, 83:517-530

Altmann GG, and Quaroni A (1990). Behavior of fetal intestinal organ culture explanted onto a collagen substratum. Development, 110:1-18

Alumets J, Hakansom R, and Sundler F (1983). Ontogeny of endocrine cells in porcine gut and pancreas. An immunocytochemical study. Gastroenterology, 85:1359-1372

Ames BN, Durston WE, Yamasaki E, and Lee DF (1973). Carcinogens are mutagens: a simple test system combining liver homogenates for

activation and bacteria for detection. Proc Nat Acad Sci, 70:2281-2285

Amory B, Mourmeaux J-L, and Remacle C (1988). In vitro cytodifferentiation of perinatal rat islet cells within a tridimensional matrix of collagen. In Vitro Cell Dev Biol, 24:91-98

Arkle S, Lee CM, Cullen MJ, and Argent BE (1986). Isolation of ducts from the pancreas of copper-deficient rats. Quart J Exp Physio, 71:249-265

Bani D, Bani Sacchi T, and Biliotti G (1985). Nesidioblastosis and intermediate cells in the pancreas of patients with hyperinsulinemic hypoglycemia. Virchows Arch [Cell Pathol], 48:19-32

Bax J, Feringa AW, van Garderen-Hoetmer A, Woutersen RA, and Scherer E (1973). Adenosine triphosphatase, a new marker for the differentiation of putative precancerous foci induced in rat pancreas by azaserine. Carcinogenesis, 7:457-462

Baylor SM and Berg JW (1973). Cross-classification and survival characteristics of 5000 cases of cancer of the pancreas. J Surg Oncol, 5:335-558

Beattie GM, Levine F, Mally MI, Otonkoski T, O'brien JS, Salomon DR, and Hayek A (1994). Acid β-galactosidase: a developmentally regulated marker of endocrine cell precursors in the human fetal pancreas. J Clin Endocrinol Metab, 78:1232-1240

Becker K, Wendel U, Przyrembel H, Tsotsalas M, Muntefering H, and Bremer H (1978). Beta cell nesidioblastosis. Eur J Pediatr, 127:75-89

Bencosme SA (1955). The histogenesis and cytology of the pancreatic islets in the rabbit. Am J Anat, 96:103-151

Bendayan M (1987). Presence of endocrine cells in pancreatic ducts. Pancreas, 2:293-297

Bendayan M and Park IS (1987). Presence of extrapancreatic islets of Langerhans in the duodenal wall of the rat. Diabetologia, 34:604-606

Bensley RR (1911). Studies on the pancreas of the guinea pig. Am J

Anat, 12:297-388

Bhargava M, Joseph A, Kensel J, Halaban R, Li Y, Goldberg I, Setter E, Donovan MA, Zarnegar R, Michalopoulos GA, Nakamura T, Faletto D, and Rosen EM (1992). Scatter factor and hepatocyte growth factor: activities, properties and mechanism. Cell Growth and Diff, 3:11-20

Bissel MJ, Hall HG, and Parry G (1983). How does the extracellular matrix direct gene expressions? J Theor Biol, 99:31-68

Bird AP, Truman DES, and Clayton RM (1981). The molecular basis of differentiation and competence. In: Stability and switching in cellular differentiation. pp 61-64. Edited by Clayton RM and Truman DES. Plenum Press, New York

Bisgaard HC and Thorgeirsson SS (1990). Evidence for a common cell of origin for primitive epithelial cells isolated from rat liver and pancreas. J Cell Physio, 147:333-343

Blau HM (1988). Hierarchies of regulatory genes may specify mammalian development. Cell, 53:673-674

Blau HM and Hughes SM (1990). Cell lineage in vertebrate development. Curr Opin Cell Biol, 2:981-985

Blau HM and Baltimore D (1991). Differentiation requires continuous regulation. J Cell Biol 112:781-783

Bockman DE (1976). Anastomosing tubular arrangement of the exocrine pancreas. Am J Anat, 147:113-118

Bockman DE (1980). Architecture of normal pancreas as revealed by retrograde injection. Cell Tissues Res, 205:445-451

Bockman DE (1981). Cell of origin of pancreatic cancer: experimental animals tumors related to human pancreas. Cancer, 47:1528-1534

Bockman DE (1986). Anatomy of the pancreas.. In: The exocrine pancreas: biology, pathobiology, and disease. pp 1-7. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Bonner-Weir S (1991). Anatomy of the islet of Langerhans. In: The endocrine pancreas. pp 15-27. Edited by Samols E, Raven Press, New York

Boquist L (1970). Regeneration of exo- and endocrine pancreas: morphological studies of lesions induced by ethionine or excess methionine. Acta Pathol Microbiol Scand [A], 78:323-334

Boquist L and Edström C (1970). Ultrastructure of pancreatic acinar and islet parenchyma in rats at various intervals after duct ligation. Virchows Arch [Pathol Anat], 349:69-79

Boring CS, Squires TS, and Tang T (1992). Cancer statistics, 1992. Cancer J for Clinicians, 42:19-38.

Bos JL (1989). *ras* oncogenes in human cancer: a review. Cancer Res, 49:4682-4289

Brannon PM, Orrison BM, and Kretchmer N (1985). Primary culture of rat pancreatic acinar cells in serum-free medium. *In Vitro* Cell Dev Biol, 21:6-14

Brion LP, Scheartz JH, Zavillowitz BJ and Schwartz GJ (1988). Micro-method for the measurement of carbonic anhydrase activity in cellular homogenates. Anal Biochem, 175:289-297

Brown AL, Graham DE, Nissley SP, Hill DJ, Strain AJ, and Rechler MM (1986). Development regulation of insulin-like growth factor II mRNA in different rat tissues. J Biol Chem, 261:13144-13150

Cancer of the Pancreas Task Force Group (1981). Staging of cancer of the pancreas. Cancer, 47:1631-1639

Cantenys D, Portha B, Dutrillaux MC, Hollande E, Roze C, and Picon L (1981). Histogenesis of the endocrine pancreas in newborn rat after destruction by streptozotocin: an immunocytochemical study. Virchows Arch [Cell Biol], 35:109-122

Caplan AL and Ordahl CP (1978). Irreversible gene repression model for control of development. Science, 201:120-130

Case RM and Argent BE (1986). Bicarbonate secretion by pancreatic duct cells: mechanism and control. In: The exocrine pancreas:

biology, pathobiology, and disease. pp 213-244. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Chiu T (1987). Focal eosinophilic hypertrophic cells in the rat pancreas. Toxicol Pathol, 15:331-333

Chirgwin JM, Przybyla AF, MacDonald RJ, and Rutter WJ (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18:5294-5299

Chomczynski P and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156-159

Chou JY, and Yeoh GCT (1987). Tyrosine aminotransferase gene expression in a temperature-sensitive adult rat liver cell line. Cancer Res, 47:5415-5420

Clark JM (1988). Novel non-template nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerase. Nuc Acids Res, 16:9677-9686

Clavel F, Benhamou E, Auquier A, Tarayre M, and Flamant R (1989). Coffee, alcohol, smoking, and cancer of the pancreas: a case-control study. Int J Cancer, 43:17-21

Coleman WB, Wennerberg, Smith GJ, and Grisham JW (1993). Regulation of the differentiation of diploid and some aneuploid rat liver epithelial (stemlike) cells by the microenvironment. Am J Pathol, 142:1373-1382

Conrath SM (1986). The use of epidemiology, scientific data, and regulatory authority to determine risk factors in cancers of some organs of the digestive system. 6. Pancreatic cancer. Regul Toxicol Pharmacol, 6:193-210

Coupland RE (1960). The survival and growth of pancreatic tissue in the anterior chamber of the eye of the albino rat. J Endocrinol, 20:69-77

Cox KH, Delon DV, Angerer LM, and Angerer RC (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Develop Biol, 101:485-502

Church GM and Gilbert W (1984). Genomic sequencing. Proc Natl Acad Sci USA, 81:1991-1995

Cross M and Dexter TM (1991). Growth factors in development, transformation, and tumorigenesis. Cell, 64:271-280

Cubilla AL and Fitzgerald PJ (1975). Morphological patterns of primary non-endocrine human pancreatic carcinoma. Cancer Res, 35:2234-2248

Cuzick J and Babiker AG (1989). Pancreatic cancer, alcohol, diabetes mellitus and gall-bladder disease. Int J Cancer, 43:415-421

Czech MP (1989). Signal transmission by the insulin-like growth factors. Cell, 59:235-238

Davis RL, Weintraub H, and Lassar AB (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell, 51:987-1000

De Lisle RC and Williams JA (1986). Regulation of membrane fusion in secretory exocytosis. Ann Rev Physio, 48:225-238

Derynck R, Jarrett JA, Chen EY, And Goeddel DV (1986). The transforming growth factor-β precursor. J Biol Chem, 261:4377-4379

DiBerardino MA, Hoffner NJ, and Etkin LD (1984). Activation of dominant genes in specialized cells. Science, 224:046-952

Druckrey H, Ivankovic S, Bucheler J, Preussmann R, and Thomas C (1968). Erzeugung bon Magen- und Pancreas-Krebs beim Meerschweinchen durch Methlynitroso-Harnstoff und-Urethan. Z Krebsforsch, 71:167-182

Dudek RW, Freinkel N, Lewis NJ, Hellerstrom C, and Johnson RC (1980). Morphologic study of cultured pancreatic fetal islets during maturation of the insulin stimulus-secretion mechanisms. Diabetes, 29:15-21

Dudek RW, Lawrence IE, Hill RS, and Johnson RC (1991). Induction of islet cytodifferentiation by fetal mesenchyme in adult pancreatic ductal epithelium. Diabetes, 40:1041-1048

Dulak NC, and Temin HM (1973). A partially purified polypeptide fraction from rat liver cell conditioned with multiplication-stimulating activity for embryo fibroblasts. J Cell Physiol, 81:153-160

Dunis DA and Namenwirth N (1977). The role of grafted skin in the regeneration of X-irradiated axolotl limbs. Dev Biol, 56:97-109

Dwivedi RS, Yeldandi AV, Subbarao V, Feigelson P, Roy AK, Reddy JK, and Rao MS (1990). Androgen regulated expression of the α_{2u}-globulin gene in pancreatic hepatocytes of rat. J Cell Biol, 110:263-267

Ehrenthal D, Haeger L, Griffin T and Compton (1987). Familial pancreatic adenocarcinoma in three generation. A case report and a review of the literature. Cancer, 59:1661-1664

Eguchi G, Masuda A, Karasawa Y, Kodama R, and Itoh Y (1981). Microenvironments controlling the transdifferentiation of vertebrate pigmented epithelial cells in *in vitro* culture. In: Stability and switching in cellular differentiation. Edited by Clayton RM and Truman DES. pp. 209-221 Plenum press, New York

Eguchi G and Okada TS (1973). Differentiation of lens tissue from the progeny of chick retinal pigment cells cultured in vitro: A demonstration of a switch of cell types in clonal cell culture. m Proc Natl Acad Sci USA, 70:-1495-1499

Farber E and Cameron R (1980). The sequential analysis of cancer development. Adv Cancer Res, 31:125-226

Farber E (1987). Possible etiologic mechanisms in chemical carcinogenesis. Environ Health Perspect 75:65-70

Fausto N (1990). Hepatocyte differentiation and liver progenitor cells. Curr Opin Cell Biol, 2:1036-1042

Fibach E, Landau T, and Sachs L (1972). Normal differentiation of myeloid leukemic cells induced by a differentiation-inducing protein. Nature New Biology, 237:276-278

Flaks B (1984). Histogenesis of pancreatic carcinogenesis in the hamster: ultrastructural evidence. Environ Health Perspect, 56:187-203

Forstner G and Forstner J (1986). Mucus: biosynthesis and secretion. In: The exocrine pancreas: biology, pathobiology, and disease. pp 283-286. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Freie HMP, Pasma A, and Bouman PR (1975). Quantitative analysis of pancreatic islet development and insulin storage in the foetal and newborn rat. Acta Endocrinologia, 80:656-666

Frazier ML and Longnecker DS (1993). Cell lines of the human and rodent exocrine pancreas. In: The exocrine pancreas: biology, pathobiology, and disease. 2nd edition, pp 565-574. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press; New York

Geever EF, Seifter E, and Levenson SM (1970). Pancreatic pathology, chemically defined liquid diets and bacterial flora in the rat. Br J Exp Pathol, 51:341-347

Germain L, Noel M, Nourdeau H, and Marceau N (1988). Promotion of growth and differentiation of rat ductular oval cells in primary culture. Cancer Res, 48:368-378

Gerschenson M, Graves K, Carson SD, Wells RS, and Pierce GB (1988). Regulation of melanoma by embryonic skin. Proc Natl Acad Sci USA, 83:730707310

Ghadirian P, Boyle P, Simard A, Baillargeon J, Maisonneuve P, and Perret C (1991). Reported family aggregation of pancreatic cancer with a population-based case control in the Francophone community in Montréal, Canada. Int J Pancreatol, 10:183-196

Gibson SJ and Polak JM (1990). Principle and application of complementary RNA probes. In situ hybridization-principles and

practice. pp 81-94. Edited by Polak JM and McGee JO'D, Oxford University Press

Gilon P, Remacle C, Janssens de Varebeke PH, Pauwels G, and Hoet JJ (1987). GABA content and localization of high affinity GABA uptake during the development of the rat pancreas. Cell Mol Biol, 33:573-585

Githens S, Holmquist DRG, Whelan JF, and Ruby JR (1980). Characterization of ducts isolated from the pancreas of the rat. J Cell Biol, 85:122-135

Githens S (1983). Localization of alkaline phosphatase and adenosine triphosphatase in the mammalian pancreas. J Histochem Cytochem, 31:697-705

Githens S (1986). Differentiation and development of the exocrine pancreas in animals In: The exocrine pancreas: biology, pathobiology, and disease. pp 21-32. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press; New York

Githens S, Finley JJ, Patke CL, Schexneyder JA, Fallon KB, and Ruby JR (1987). Biochemical and histochemical characterization of cultured rat and hamster pancreatic ducts. Pancreas, 2: 427-438

Githens S (1988). The pancreatic duct cells: proliferative capabilities, specific characteristics, metaplasia, isolation, and culture. J Pediatr Gastroen Nutri, 7:486-506

Githens S (1989). Development of Duct cells In: Human Gastrointestinal development. pp 669-683. Edited by Lebenthal ED, Raven Press, New York

Githens S, Schexnayder JA, Desai K, and Patke C (1989). Rat pancreatic interlobular duct epithelium: isolation and culture in collagen gel. In Vitro, 25:679-688

Githens S, Schexnayder JA, and Frazier ML (1992). Carbonic anhydrase II gene expression in mouse pancreatic duct cells. Pancreas, 7:556-561

Githens S, Patke CL, and Schexnayder JA (1994). Isolation and culture of Rhesus monkey pancreatic ductules and ductule-like epithelium. Pancreas, 9:20-31

Ghadirian P and Howe GR (1994). Nutrition, alcohol, coffee, and pancreatic cancer. Int J Pancreatol, 16:204-207

Gold EB, Gordis L, Diener MD, Selter R, Boitnott JK, Bynum TE, and Hutcheon DF (1985). Diet and other risk factors for cancer of the pancreas. Cancer Res, 55:460-467

Golosow N and Grobstein C (1962). Epitheliomesenchymal interaction in pancreatic morphogenesis. Devl Biol, 4:242-255

Gordis L and Gold EB (1986). Epidemiology and etiology of pancreatic cancer. In: The exocrine pancreas: biology, pathobiology, and disease. pp 621-626. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press; New York

Grisham JW, Tsao M-S, Lee DC, and Earp HS (1990). Sequential changes in epidermal growth factor/receptor ligand function in cultured rat liver epithelial cells transformed chemically *in vitro*. Pathobiology, 58:3-14

Grobstein C (1964). Cytodifferentiation and its control. Science, 143:643-650

Gudjonsson B (1987). Cancer of the pancreas: 50 year of surgery. Cancer, 60: 2283-2303

Hansen LJ, Mark MM, Reddy JK (1981). Immunohistochemical localization of pancreatic exocrine enzymes in normal and neoplastic acinar epithelium of rat. J Histochem Cytochem, 29:309-313

Harris A and Coleman L (1987). Establishment of a tissue culture system for epithelial cells derived from human pancreas: a model for the study of cystic fibrosis. J Cell Sci, 87:695-703

Heimann TG and Githens S (1991). Rat pancreatic duct epithelium cultured on a porous support coated with extracellular matrix. Pancreas, 6:514-521

Helden C-H and Westermark B (1989). Growth factors as transforming proteins. Eur J Biochem, 184:487-496

Hellman B, Wallgren A, and Peterson B (1962). Cytologic characteristics of the exocrine pancreatic cells with their regard to their positions in relation to the islet of Langerhans. A study in normal and obese-hyperglycemic mice. Acta Endocrinol, 39:465-473

Hendricks JD, Meyers TR, and Shelton DW (1981). Histologic progression of hepatic neoplasia in rainbow trout (Salmon gairdneri). Natl cancer Inst Monogr, 65:321-335

Hirata K, Oku T, and Freeman AE (1982). Duct, exocrine and endocrine components of cultured fetal mouse pancreas. In Vitro, 18:789-799

Honsik CJ, Diamant M, and Olson L (1986). Generation of stable cellular phenotypes in a human malignant cell line conditioned by alterations in the cellular microenvironment. Cancer Res, 46:940-949

Hootman SR and Williams JA (1987). Stimulus-secretion coupling in the pancreatic acinus. In: Physiology of the gastrointestinal tract, 2nd ed. pp 1129-1146. Edited by Johnson LR. Raven Press, New York

Hootman SR and Logdson CD (1987). Isolation and monolayer culture of guinea pig pancreatic duct epithelial cells. In Vitro Cell Dev Biol, 24:566-574

Hoover KL and Poirier LA (1986). Hepatocyte-like cells within the pancreas of rats fed methly-deficient diets. J Nutr, 116:1569-1575

Hopkins NH (1987). The genetic basis of cancer. In: Molecular biology of the gene, 4th ed. pp 1006-1057. Edited by Watson JD, Hopkins NH, Roberts JW, Steitz JA, and Weiner AM. Benjamin/ Cumming Press

Howe GR, Jain M, Burch JD, and Miller AB (1990). Cigarette smoking and cancer of the pancreas: evidence from a population-based case-control study in Toronto, Canada. Int J Cancer, 47:323-328

Hruban Z and Swift H (1964). Uricase: localization in the hepatic microbodies. Science, 146:1316-1318

Hubchak S, Mangino MM, Reddy MK, and Scarpelli DG (1990). Characterization of differentiated Syrian golden hamster pancreatic duct cells maintained in extended monolayer culture. In Vitro Cell Dev Biol, 26:889-897

Hynes MA, van Wyk JJ, Brooks PJ, D'Ercole AJ, Jansen M, and Lund PK (1987). Growth hormone dependence on somatomedin-C/insulin -like growth factor-I and insulin-like growth factor-II messenger ribonucleic acids. Mol Endocrinol, 1:233-242

Ide H (1986). Transdifferentiation of amphibian chromatophores. Curr Top Develop Biol, 20:79-88

Ide H, Subbarao V, Reddy JK, and Rao S (1993). Formation of ductular structures in vitro by rat pancreatic epithelial oval cells. Exp Cell Res, 209:38-44

Iseki M, Suzuki T, Koizumi Y, Hirose M, Laskin WB, Nakazawa S, and Ohaki Y (1986). Alpha-fetoprotein-producing pancreatoblastoma: a case report. Cancer, 57:1833-1835

Itoh Y (1976). Enhancement of differentiation of lens and pigment cells by ascorbic acid in cultures of neural retinal cells of chick embryo. Develop Biol, 54:157-162

Itoh Y and Eguchi G (1986). In vitro analysis of cellular metaplasia from pigmented epithelial cells to lens phenotypes: a unique model system for studying cellular and mechanisms of "transdifferentiation". Develop Biol, 115:353-362

Ingber DE (1993). Extracellular matrix as a regulator of epithelial polarity, cell growth, and tissue pattern. 2nd edition, pp 369-380. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press; New York

Jaenisch R and Jahner D (1984). Methylation, expression and chromosomal positions of genes in mammals. Biochim Biophys Acta, 782:1-9

Jain M, Howe GR, Louis PSt and Miller AB (1991). Coffee and alcohol as determinants of risk of pancreas cancer: a case-control study from Toronto. Int J Caner, 47:384-389

Johnson DD (1958). Alloxan administration in the guinea pig. A study of the regenerative phase in the island of Langerhans. Endocrinology, 62:189-200

Johnson PF (1990). Transcriptional activators in hepatocytes. Cell Growth Diff, 1:47-51

Jones RT, Barret LA, Van Haafen C, Harris CC, and Trump BF (1977). Carcinogenesis in the pancreas. I. Long-term explant culture of human and bovine pancreatic ducts. J Natl Cancer Inst, 58:557-565

Jones RT, Trump BF, and Stoner GD (1980). Culture of human pancreatic ducts. Methods Cell Biol, 21B:429-439

Kabayashi K (1966). Electron microscope studies of the Langerhans islets in the total pancreas. Arch Histochem Jpn 26:439-482

Kawamoto S, Hiraoka T, Kanemitsu K, Kimura M, Miyauchi Y, and Takeya M (1991). Alpha-fetoprotein-producing pancreatic cancer. a case report and review of 28 cases. Hepato-Gastroenterol, 39:282-286

Kawasaki ES (1991). Amplification of RNA. pp 21-27. In: PCR Protocols, A Guide to Methods and Applications. Edited by Innis MA et al. San Diego, Academic Press

Keller RT and Logan GM (1977). Adenocarcinoma of the pancreas associated with neurofibromatosis. Cancer, 39:12640-1266

Kern HF (1986). Fine structure of the human exocrine pancreas. In: The exocrine pancreas: biology, pathobiology, and diseases. pp 9-19. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Kimbrough RD (1973). Pancreatic type tissue in livers of rats fed polychlorinated biphenyls. J Natl Cancer Inst, 51:679-682

Kimura N, Yonekura H, Okamoto H, and Nagura H (1992). Expression of human regenerating gene mRNA and its product in normal and neoplastic human pancreas. Cancer, 70:1857-1863

Kinard F, de Clercq L, Billen B, Amory B, Hoet J-J, and Remacle C (1990). Culture of endocrine pancreatic cells in protein-free, chemically defined medium. In Vitro Cell Dev Biol, 29:1004-1010

Klein G and Klein E (1985). Evolution of tumors and the impact of molecular oncology. Nature, 315:190-195

Klöppel G and Fitzgerald (1986). Pathology of nonendocrine pancreatic tumors. In: The exocrine pancreas: biology, pathobiology, and diseases. pp 649-674. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Knudson AG (1985). Hereditary cancer, oncogenes, and anti-oncogene. Cancer Res, 45:1437-1443

Kodama T (1983). A light and electron microscopic study on the pancreatic ductal system. Acta Pathol Jpn, 33(2);297-321

Konishi N, Ward JM, and Waalkes M (1990). Pancreatic hepatocytes in Fischer and Wistar rats induced by repeated injections of cadmium chloride. Toxicol Appl Pharmacol, 104:149-156

Korc M, Owerbach D, Quinto C, and Rutter WJ (1981). Pancreatic islet-acinar cell interaction: amylase messenger RNA levels are determined by insulin. Science, 213:351-353

Kuijpers GAJ and de Pont JJHHM (1987). Role of proton and bicarbonate transport in pancreatic cell function. Annu Rev Physio, 49: 87-103

Kumegawa M, Maeda N, Yajima T, Takuma T, Ikeda E, and Hanai H (1980). Permissive role of L-thyroxine in induction of pancreatic amylase by cortisol in neonatal rats. J Endocrinol, 86:497-500

Kumpulainen T (1984). Immunohistochemical localization of human carbonic anhydrase isozymes. Ann NY Acad Sci, 429:359-368

Kunnath L and Locker J (1983). Developmental changes in the methylation of rat albumin and α-fetoprotein. EMBO, 2: 317-324

Lack EE, Khettry U, and Legg MA (1990). The pancreas and extrahepatic biliary system. In: Principles and practice of surgical

pathology. pp 1347-1395. Edited by Silerberg SG, Churchill Livingstone, New York

Laidlaw GF (1938). Nesidioblastoma, the islet tumor of the pancreas. Am J Pathol, 14:125-134

Land H, Parada LF, and Weinberg RA (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature, 304:713-718

Laperche Y, Bulle F, Aissini T, Chobert M.-N., Aggerbeck M, Hanoune J, and Guellaen G (1986). Molecular cloning and nucleotide sequence of rat kidney γ-glutamyl transpeptidase cDNA. Proc Natl Acad Sci USA, 83:8937-8942

Leblond CP (1964). Classification of cell population on the basis of their proliferative behaviour. Natl Cancer Inst Monogr, 14:119-150

Lee BC, Hendricks JD, and Bailey GS (1989). Metaplastic pancreatic cells in liver tumors induced by diethylnitrosamine. Exp Mol Pathol, 50:104-113

Lee DC, Rose TM, Webb NR, and Todaro GJ (1985). Cloning and sequencing analysis of a cDNA for rat transforming factor-α. Nature, 313:489-491

Lee DC and Han KM (1990). Expression of growth factor and their receptors in development. In: Peptide growth factors and their receptors. pp 611-654. Edited by Sporn MB and Roberts AB. Springer-Verlag, New York

Lee LW, Tsao M-S, Grisham JW, and Smith JD (1989). Emergence of neoplastic transformants spontaneously or after exposure *N*-methyl-*N'*-nitro-N-nitrosoguanidine in population of rat liver epithelial cells cultured under selective and non-selective conditions. Am J Pathol, 135:63-71

Lee PC, Kim OK, and Lebenthal E (1982). Effect of early weaning and prolonged nursing on development of the rat pancreas. Pediatr Res, 16:470-473

Lee PC (1989). Functional development of the exocrine pancreas. In: Human Gastrointestinal development. pp 651-668. Edited by Lebenthal ED, Raven Press, New York

Lee S, Broelsch CE, Flamant YM, Chandler JG, Charters AC III, and Orloff (1975). Liver regeneration after portacaval transportation in rats. Surgery, 77:144-149

Lehv M and Fitzgerald PJ (1968). Pancreatic acinar cell regeneration IV. Regeneration after surgical resection. Am J Pathol, 53:513-535

Lemire JM and Fausto N (1991). Multiple α-fetoprotein RNA in the adult rat liver: cell type-specific expression and differential regulation. Cancer Res, 51:4656-4664

Liang PL and Pardee A (1992). Differential display of eukaryotic messenger RNA by means ofg the polymerase chain reaction. Science, 257:967-971

Lin WC and Culp LA (1991). Selectable plasmid vectors with alternative and ultrasensitive histochemical marker genes. BioTechniques, 11:344-351

Lin WC, Pretlow TP, Pretlow II TG, and Culp LA (1992). High resolution analyses of two different classes of tumor cells in situ tagged with alternative histochemical marker genes. Am J Pathol, 141:1331-1342

Liu C, Tsao MS, and Grisham JW (1988). Transforming growth factors produced by normal and neoplastically transformed rat liver epithelial cells in culture. Cancer Res, 48:850-855

Locker J, Hutt S, and Lombardi B (1987). α-fetoprotein gene methylation and hepatocarcinogenesis in rats fed a choline-devoid diet. Carcinogenesis. 8:142-246

Logsdon CD (1986). Stimulation of pancreatic acinar cell growth by CCK, EGF and insulin *in vitro*. Am J Physiol, 251:G487-494

Longnecker DS, Lilja HS, French J, Kuhlman E, and Noll W (1979). Transplantation of azaserine-induced carcinomas of pancreas in rats. Cancer Lett, 7:197-206

Longnecker DS, Roebuck BD, Yager JD, Lilja HS, and Seigmund B (1981). Pancreatic carcinoma in azaserine-treated rats: Induction, classification and dietary modulation of incidence. Cancer, 47:1562-1572

Longnecker DS, Wiebkin P, Schaeffer BK, and Roebuck BD (1984). Experimental carcinogenesis in the pancreas. Int Rev Exp Pathol, 26:177-229

Longnecker DS (1984) . Lesions induced in rodent pancreas by azaserine and other pancreatic carcinogens. Environ Health Perspect, 56:245-251

Longnecker DS (1986). Experimental models of exocrine pancreatic tumors. In : The exocrine pancreas: biology, pathobiology, and disease. pp 443-458. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Longnecker DS, Curphey TJ, Kuhlmann ET and Roebuck BD (1988). Inhibition of pancreatic carcinogenesis by retinoids in azaserine-treated rats. Cancer Res, 42:19-24

MacDermott RP and Kramer P (1973). Adenocarcinoma of the pancreas in four siblings. Gastroenterology, 65:137-139

Mack TM, Yu MC, Hanisch R, and Henderson BE (1986). Pancreas cancer and smoking, beverage consumption, and past medical history. J Natl Cancer Inst, 76:49-60

Maden ME and Sarras MP Jr (1989). The pancreatic ductal system of the rat: cell diversity, ultrastructure, and innervation. Pancreas, 4:472-485

Malaisse-Lagae F, Ravazzola M, Robberecht P, Vandermeers A, Malaisse WJ, and Orci L (1975). Exocrine pancreas: evidence for topographic partition of secretory function. Science, 190:795-797

Makino T, Usuda N, Rao S, Reddy JK, Scarpelli DG (1990). Transdifferentiation of ductular cells into hepatocytes in regenerating hamster pancreas. Lab Invest, 62:552-561

Malkin D, Li FP, Strong LC, Fraumeni JF, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, and Friend SH (1990). Germ-line p53 mutations in familial syndrome of breast cancer, sarcoma, and other neoplasms. Science, 250:1233-1238

Mangino MM, Hubchak S, and Scarpelli DG (1992). Stimulation of DNA synthesis in pancreatic duct cells by gastrointestinal hormones: interaction with other growth factors. Pancreas, 7:271-279

Marceau N (1990). Cell lineage and differentiation programs in epidermal, urothelial and hepatic tissue and their neoplasms. Lab Invest, 63:4-20

Matas AJ, Sutherland DER, Steffes MW, Mauer SM, Lowe A, Simmons RL, and Najarian JS (1976). Hepatocellular transplantation for metabolic deficiencies: decrease of plasma bilirubin in Gunn rats. Science, 192:892-894

Mauer SM, Sutherland DER, Steffes MW, Leonard RJ, Najarian JS, Michael AF, and Brown DM (1974). Pancreatic islet cell transplantation. Effects on the glomerular lesions of experimental diabetes in the rat. Diabetes, 23:748-753

McIntire KR, Waldmann TA, Moertel CG, and GO VWL (1975). Serum α-fetoprotein in patients with neoplasms of the gastrointestinal tract. Cancer Res, 35:991-996

Melmed RN, Benitez CJ, and Holt SJ (1972). Intermediate cells of the pancreas. J Cell Sci, II:449-475

Melmed RN, Turner RC, and Holt SJ (1973). Intermediate cells of the pancreas. II. The effect of dietary soybean trypsin inhibitor on acinar-beta cell structure and function in the rat. J Cell Sci, 13:279-295

Melmed RN (1979). Intermediate cells of the pancreas. Gastroenterology. 76:196-201

Merlino GT, Xu Y.-H., Ishii S, Clark AJL, Semba K, Toyoshima K, Yamamoto T, and Pastan I (1984). Amplification and enhanced expression of the epidermal growth factor gene in A431. Science, 224:417-419

Miller EC and Miller JA (1966). Mechanisms of chemical carcinogenesis: nature of proximate carcinogens and interactions with macromolecules. Pharmacol Rev, 18:805-838

Miller EC and Miller JA (1981). Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. Cancer, 47:2327-2745

Mintz B and Illmensee (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Pro Natl Acad Sci USA, 72:3585-3589

Moll R, Franke W, Schiller D, Geiger B, and Krepler (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell, 31:11-24

Montesano R and Orci L (1985). Tumor-promoting phorbol ester induces angiogenesis *in vitro*. Cell, 42: 469-477

Morisset J and Jolicoeur L (1980). Effect of hydrocortisone on pancreatic growth in rat. Am J Physiol, 241:G37-G42

Morohoshi T, Sagawa F, and Mitsuya T (1990). Pancreatoblastoma with marked elevation of serum alpha-fetoprotein: an autopsy case report with immunocytochemical study. Virchow Arch [A], 416:265-270

Mullis KB and Faloona FA (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol, 155:335-350

Nathanson MA, Kilfer SR, and Searls RL (1978). Formation of cartilage by non-chondrogenic cell types. Develop Biol, 64:99-117

Neubert K (1927). Bau und entwicklung des menschichlichen pankreas. Arch Entwicklngsmechn organ, 111:29-118

Nicolson GL (1984). Generation of phenotypic diversity and progression in metastatic tumors. Cancer Metastasis Rev, 3:25-42

Nicolson GL (1987). Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. Cancer Res, 47:1473-1478

Nojima T, Kojima T, Kato H, Sato T, Koito K, Nagashima K (1992). Alpha-fetoprotein-producing acinar cell carcinoma of the pancreas. Human Pathol, 23:828-830

Norrel SE, Ahlbom A, Erwald R, Jacobean G, Lindberg-Navier I, Olin R, Tornberg B, and Wiechel KL (1986). Diet and pancreatic cancer: a case-control study. Am J Epidemiol, 124:894-902

Oates P and Morgan RGH (1989). Cell proliferation in the exocrine pancreas during development. J Anat, 167:235-242

Okada TS (1986a). Introduction: Can specialized cells change their phenotype? Curr Top Dev Biol, 20: xxv-xxx

Okada TS (1986b). Transdifferentiation in animal cells: fact or artifact. Develop Growth and Differ, 28:213-221

Orci L, Baetens D, Ravazzola M, Stefan Y, and Malaisse-Lagae (1982). Pancreatic polypeptide and glucagon: non-random distribution in pancreatic islets. Life Sci, 19:1811-1816

Orci L (1982). Macro- and micro-domains in the endocrine pancreas. Diabetes, 32:538-565

Palla JC, Ben Abdeljlil A and Desnuelle P (1968). Effect of insulin on the rate of biosynthesis of some pancreatic enzymes. Gut, 9:254

Park RW and Grand RJ (1981). Gastrointestinal manifestation of cystic fibrosis: a review. Gastroenterology, 81:1143-1161

Parsa I, Marsh WH, and Fitzgerald PJ (1976). Pancreas acinar cell differentiation. I. Morphologic and enzymatic comparison of embryonic rat pancreas and the pancreatic anlage grown in organ culture. Am J Pathol, 57:457-487

Parsa I and Marsh WH (1976). Long-term organ culture of embryonic rat pancreas in a chemically defined medium. Am J Pathol, 82:119-125

Parsa I, Marsh WH and Sutton AL (1980). An *in vitro* model of human pancreas carcinogenesis: Two-stage MNU-TPA effect. Am J Pathol, 98:649-662

Parsa I, Marsh WH and Sutton AL (1981a). An *in vitro* model of human pancreas carcinogenesis: Effects of nitroso compound. Cancer, 47:1543-1551

Parsa I, Marsh WH, Sutton AL, and Butt KMH (1981b). Effects of dimethylnitrosamine on organ-cultured adult human pancreas. Am J Pathol, 102:403-411

Parsa I, Bloomfield RD, Foye CA and Sutton AL (1984). Methylnitrosourea-induced carcinoma in organ-cultured fetal human pancreas. Cancer Res, 44:3530-3538

Patent GJ and Alfert M (1967). Histologic changes in the pancreatic islets of alloxan-treated mice, with comments on β-cell regeneration. Acta Anat, 66:504-519

Pearse AGE and Polak JM (1971). Neural crest origin of the endocrine polypeptide (APUD) cells of the gastrointestinal tract and pancreas. Gut, 12:783-788

Pettengill OS, Faris RA, Bell RHJr, Kuhlmann ET, and Longnecker DS (1993). Derivation of ductlike cell lines from a transplantable acinar cell carcinoma of the rat pancreas. Am J Pathol, 143:292-303

Petropoulos CJ, Yawsen P, Panzica M, Fausto N (1985). Cell lineages in liver carcinogenesis: possible clues from studies of the distribution of α-fetoprotein RNA sequences in cell populations isolated from normal, regenerating, and preneoplastic rat livers. Cancer Res, 45:5762-5768

Pictet RL, Orci L, Gonet AE, Rouiller C, and Renold AE (1967). Ultrastructural studies of the hyperplastic islets of Langerhans of spiny mice (Acomys cohirinus) before and during development of hyperglycemia. Diabetologia, 3:188-211

Pictet RL, Clark WR, William RH, and Rutter WJ (1972). An ultrastructural analysis of the developing embryonic pancreas. Dev Biol, 29:436-467

Pictet RL and Rutter WJ (1972). Development of the embryonic endocrine pancreas. In: Handbook of Physiology, Section 7: Endocrinology, Vol I. Edited by Steiner DF and Freinkel. pp. 25-66. American Physiological Society, Washington, DC

Pictet RL, Rall L, Phelps P, and Rutter WJ (1976). The neural crest and the origin of the insulin-producing and other gastro-intestinal hormone producing cells. Science, 191:191-193

Pierce GB, Lewis SH, Miller GJ, Motitz E, and Miller P (1979). Tumorigenicity of embryonal carcinoma as an assay to study control of malignancy by the blastocyst. Proc Natl Acad Sci USA, 76:6649-6655

Pierce GB, Aguilar D, Hood C, and Wells RS (1984). Trophectoderm in control of murine embryonal carcinoma. Cancer Res, 44:3987-3996

de Pomerai DI and Gali MAH (1981). Serum factors affecting transdifferentiation in chick embryo neuro-retinal cultures. In: Stability and switching in cellular differentiation. Edited by Clayton RM and Truman DES. pp. 199-207 Plenum press, New York

Pour PM, Mohr U, Althoff J, Cardesa A, and Kmoch N (1976). Spontaneous tumors and common disease in two colonies of Syrian hamsters. III. Urogenital system and endocrine glands. JNCI, 56:949-961

Pour PM (1978). Islet cells as a component of pancreatic ductal neoplasm. I. Experimental study: ductular cells, including islet cell precursors, as primary progenitor cells. Am J Pathol, 90:295-316

Pour PM, Althoff J, Salmasi SZ, and Katherine S (1979). Spontaneous tumors and common diseases in three types of hamsters. JNCI, 63:797-811

Pour PM, Sayed S, and Sayed G (1982). Hyperplastic, preoneoplastic and neoplastic lesions found in 83 human pancreas. Am J Clin Pathol, 77:137-152

Pour PM and Wilson RB (1980). Experimental tumors of the pancreas. In Tumors of the pancreas. pp 37-158. Williams and Wilkins, Baltimore, MD

Pour PM (1984). Histogenesis of exocrine pancreatic cancer in the hamster model. Environ Health Perspect, 56:229-243

Pour PM (1988). Mechanism of pseudoductular(tubular) formation during pancreatic carcinogenesis in the hamster model. Am J Pathol, 130:335-344

Pour PM (1990). In: Chronic pancreatitis, H. Beger and M. Büchler (eds.), pp 106-112. Springer Verlag, Heidelberg

Pour PM (1994). Hamster model for carcinogenesis of the pancreas. Int J Pancreatol, 16:288-289

Razin A and Riggs AD (1980). DNA methylation and gene function. Science, 210:604-609

Rall L, Pictet R, Githens S, and Rutter WJ (1973). Early differentiation of glucagon-producing cells in embryonic pancreas: a possible role of glucagon. Proc Natl Acad Sci USA, 70:3478-3482

Rall L, Pictet R, Githens S, and Rutter WJ (1977). Glucocorticoids modulate the *in vitro* development of the embryonic rat pancreas. J Cell Biol, 75:398-409

Rao MS, Reddy MK, Reddy JK, and Scarpelli DG (1982). Response of chemically induced hepatocyte-like cells in hamster pancreas to methyl clofenate, a peroxisome proliferator. J Cell Biol, 95:50-56

Rao MS, Bendayan M, Kimbrough RD, and Reddy JK (1986a). Characterization of pancreatic-type specific tissue in the liver of rat induced by polychlorinated biphenyls. J Histochem Cytochem, 34:197-201

Rao MS, Subbarao V, and Reddy JK (1986b). Induction of hepatocytes in the pancreas of copper-depleted rats following copper repletion. Cell Differ 18:109-117

Rao MS, Scarpelli DG, and Reddy JK (1986c). Transdifferentiated hepatocytes in the rat pancreas. Curr Top Dev Biol, 20:63-78

Rao MS (1987). Animal models of exocrine pancreatic carcinogenesis. Cancer Metast Rev, 6:665-676

Rao MS, Dwivedi RS, Yeldandi AV, Subbarao V, Tan X, Uamdn MI, Thanhada S, Nemali MR, Kumar S, Scarpelli DG, and Reddy JK (1990). Role of periductal and ductular epithelial cells of the adult rat pancreas in the in the pancreatic hepatocyte lineage. Am J Pathol, 134:1069-1086

Rao MS, Subbarao V, Sato K, and Reddy JK (1991). Alterations of pancreatic hepatocytes in rats exposed to carcinogens. Am J Pathol, 139:1111-1117

Rao MS and Reddy JK (1991). Replicative culture in vitro of pancreatic epithelial oval cells derived from rats after copper-deficiency induced acinar cell depletion. J Tissue Cult Methods, 159:121-124

Rappolee DA, Mark D, Banda MJ, and Werb Z (1990). Wound macrophages express TGF-α and other growth factors in vivo: analysis by mRNA phenotyping. Science, 241:708-712

Reddy JK and Rao MS (1977). Transplantable pancreatic carcinoma of the rat. Science, 198: 78-80

Reddy JK, Rao MS, Qureshi SA, Reddy MK, Scarpelli DG, and Lalwani ND (1984). Induction and origin of hepatocytes in rat pancreas. J Cell Biol, 98:2082-2090

Reddy JK, Kanwar YS, Rao MS, Watanabe TK, Reddy MK, Parsa I, Longnecker DS, and Tafuri S (1986). Duct-like morphogenesis of Longnecker pancreatic acinar carcinoma cells maintained *in vitro* on seminiferous tubular basement membrane. Cancer Res, 46:347-354

Reddy JK (1995). Progress in pancreatic cancer: Implications of phenotypic and molecular plasticity. Lab Invest, 72:382-386

Richardson KEY and Spooner BS (1977). Mammalian pancreas develpment: regeneration and differentiation *in vitro*. Dev Biol, 58:402-420

Robberecht T, Deschodt-Lanckman M, Camus J, Bruylands J, and Christophe J (1971). Rat pancreatic hydrolase from birth to weaning and dietary adoption after weaning. Am J Physiol, 221:376-378

Romanus AJ, Rabinovitch A, and Rochler MM (1985). Neonatal rat islet cell cultured synthesize insulin-like growth factor I. Diabetes, 34:696-702

Rosenberg L, Brown RA, and Duguid WP (1983). A new approach to induction of duct epithelial hyperplasia and nesidioblastosis by cellophane wrapping of the hamster pancreas. J Surg Res, 35:63-72

Rosenberg L, Brown RA, and Duguid WP (1984): Development of experimental cancer in the head of the pancreas by surgical induction of injury. Am J Surg, 147:136-151

Rosenberg L, Duguid WP, Brown RA, and Vinik AI (1988): Induction of nesidioblastosis will reverse diabetes in Syrian golden hamster. Diabetes, 37:334-341

Rowlatt U and Roe FJC (1967). Epithelial tumors of the rat pancreas. J Natl Cancer Inst, 39:18-32

Ronzio RA and Rutter WJ (1973). Effects of a partially purified factor from chick embryos on macromolecular synthesis of embryonic pancreatic epithelial. Dev Biol, 30:307-320

Rutenberg AM, Kim H, Fishbein JW, Hanker JS, Wasserkrug HL, and Selegman AM (1969). Histochemical and ultrastructural demonstration of γ-glutamyl transpeptidase activity. J Histochem Cytochem, 17:517-526

Ruoslahti E and Seppälä (1979). Aplha-fetoprotein in cancer and fetal development. Adv Cancer Res, 29:275-246

Rutter WJ, Kemp JD, Bradshaw WS, Clark WR, Ronzio RA, and Sanders TG (1968). Regulation of specific protein synthesis in cytodifferentiation. J Cell Physio, 72(Suppl 1):1-18

Rutter WJ, Pictet RL, Morris PW (1973). Toward molecular mechanisms of developmental process. Annu. Rev Biochem, 42:601-646

Sachs L (1984). Normal regulators, oncogenes and the reversibility of malignancy. Cancer Surv, 3:219-228

Samols E, Stagner J, Ewart RBL, and Marks V (1988). The order of islet cellular perfusion is B-A-D in the perfused rat pancreas. J Clin Invest, 82:350-354

Samols E and Stagner J (1991). Intraislet and Islet-acinar portal systems and their significance. In: The endocrine pancreas. pp 93-124. Edited by Samols E, Raven Press, New York

Sambrook J, Fritsch EF, and Maniatis T (1989). Molecular cloning, A laboratory Manual, Cold Spring Laboratory, Cold Spring Harbour, N.Y.

Sanders TG and Rutter WJ (1974). The developmental regulation of amylolytic and proteolytic enzymes in the embryonic rat pancreas J Biol Chem, 249:3500-3509

Sarles H (1986). Chronic pancreatitis: etiology and pathophysiology. In: The exocrine pancreas: biology, pathobiology, and disease. pp 527-540. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press, New York

Sarventnick NE and Gu D (1992). Regeneration of pancreatic endocrine cells in interferon-gamma transgenic mice. Adv Exp Med Biol, 321: 85-89

Sato T, Sato M, Hudson EA, and Jone RT (1983). Characterization of bovine pancreatic duct cells isolated by a perfusion-digestion technique. In Vitro, 19:651-660

Scarpa A, Capelli P, Mukai K, Zamboni G, Oda T, Iacono C, and Hirohashi S (1993). Pancreatic adeoncarcinomas frequently show p53 gene mutations. Am J Pathol, 142:1534-1543

Scarpelli DG and Rao MS (1981). Differentiation of regenerating pancreatic cells into the hepatocyte-like cells. Proc Natl Acad Sci USA, 78:2577-2581

Scarpelli DG, Rao MS, and Reddy JK (1984). Studies of pancreatic carcinogenesis in different animal models. Environ Health Perspec, 56:219-227

Schecter AL, Stern DF, Vaisyanathan L, Decker SJ, Drebin MI, and Weinberg RA (1984). The neu oncogene: an erbB related gene encoding a 18500 Mr tumor antigen. Nature, 312:513-516

Schenborn ET and Mierendorf RC (1985). A novel transcription property of SP6 and T7 polymerases: dependence on template structure. Nucl Acids Res, 13:6223-6236

Schmid V and Adler H (1984). Isolated, mononucleated, striated muscle can undergo pluripotent transdifferentiation and form a complex regenerate. Cell, 38:801-809

Schirrmacher V (1980). Shifts in tumor cell phenotypes induced by signals from the microenvironment. Relevance for the immunobiology of cancer metastasis. Immunobiology, 157:89-98

Sell S, Skelly H, Leffert HL, Muller-Eberhard U, and Kida S (1975). Relationship of the biosynthesis of α-fetoprotein, albumin, hemopexin, and haptoglobin to the growth state of fetal rat hepatocyte culture. Ann NY Acad Sci 259:45-58

Sheh-Ong GLC, Keath EJ, Piccoli SP, and Cole MD (1982). Novel *myc* oncogene RNA from abortive immunoglobulin gene recombination in mouse plasmacytoma. Cell, 31: 443-452

Shepherd JG, Tsao M.-S., and Duguid WP (1990). Genotoxicity of pancreatic chemical carcinogens to propagable cultured normal pancreatic epithelial cells. Exp Mol Pathol, 14:1027-1033

Shi SR, Key ME, and Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunocytochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem, 39:741-748

Shiojiri N, Lemire JM, and Fausto N (1991) Cell lineages and oval cell progenitors in rat liver development. Cancer Res, 51:2611-2620

Sirica AE, Mathis GA, Nobuya S, and Elmore LW (1990). Isolation, culture, and transplantation of intrahepatic biliary epithelial cells and oval cells. Pathobiology, 58:44-64

Smit VTHBMK, Boot AJM, Smits AMM, Fleuren GJ, Cornelisse CJ, and Bos JL (1988). K-ras mutation codon 12 mutations occur very

frequently in pancreatic adenocarcinomas. Nucleic Acid Res. 167:7773-7782

Smith AJ, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahi M, and Rogers D (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature, 336:688-690

Spooner BS, Walther BT, and Rutter WJ (1970). The development of the dorsal and ventral mammalian pancreas in vivo and in vitro. J Cell Biol, 47:235-246

Sporn MB and Roberts AB (1985). Introduction: autocrine, paracrine, and endocrine mechanisms of growth control. Cancer Survey, 4:627-632

Sporn MB and Roberts AB (1991). Interaction of retinoid and transforming growth factor-β in regulation of cell differentiation and proliferation. Mol Endocrinol, 1:3-7

Stagner JI and Samols E (1990). Evidence for a rapidly dynamic islet-acinar portal system. Diabetes, 1990; 39 (suppl 1)

Stolle CA, McGowan MH, Heim RA, Varia M, and Neubauer JA (1991). Nucleotide sequence of a cDNA encoding rat brain carbonic anhydrase II and its deduced amino acid sequence. Gene, 109:265-267

Stoner GD, Harris CC, Bostwick DG, Jones RT, Trump BF, Kingsbury EW, Fineman E, and Newkirk C (1978). Isolation and characterization of epithelial cells from bovine pancreatic duct. In Vitro, 14L581-590

Sul HS (1989). Adipocyte differentiation and gene expression. Curr Opin Cell Biol, 1:1116-1121

Sumnaer S, Hakanson R, and Larsson LI (1977). Ontogeny of the rat pancreatic polypeptide (PP) cells. Exp Cell Res 178, 303-306

Tahira Y, Hayashi K, Ochiai M, Tsuchida N, Nagao M and Sugimura T (1986). Structure of the c-Ki-*ras* gene in a rat fibrosarcoma induced by 1,8-dinitropyrene. Mol Cell Biol, 6:1349-1351

Taiki T and Fausto N (1984). Expression of the α-fetoprotein gene during development, regeneration, and carcinogenesis. In: Recombinant DNA and cell proliferation, pp 145-148. Edited by Stein GS and Stein JL. Academic Press, Orlando, FL.

Takahashi T, Moyer MP, Cano M, Wang QJ, Mountjoy CP, Sanger W, Adrian TE, Sugiura H, Katoh H and Pour PM (1995). Differences in molecular biological, biological and growth characteristics between the immortal and malignant hamsters pancreatic cells. Carcinogenesis, 16:931-939

Takaki R, (1989). Culture of pancreatic islet cells and islet hormone producing cell lines "morphological and functional integrity in culture". In Vitro Cell Dev Biol, 25:763-768

Taylor SM and Jones PA (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell, 17:771-779

Teitelman G and Lee JK (1987). Cell lineage analysis of pancreatic islet cell development: glucagon and insulin cells arise from catecholaminergic precursors present in the pancreatic duct. Dev Biol., 121:454-466

Terada T, Nakanuma Y, and Kakita A (1990). Pathologic observations of intrahepatic peribiliary glands in 1000 consecutive autopsy liver. Heterotropic pancreas in the liver. Gastroenterology, 98:1333-1337

Thomas PG, Augustin P, Ramesh H, and Rangabashyan N (1990). Observation and surgical management of tropical pancreatitis in Kerala and South India. World J Surg, 14:32-42

Tsao M.-S., Smith JD, Nelson KG, and Grisham JW (1984). A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. Exp Cell Res, 154:38-52

Tsao M.-S., Grisham JW, Chou BB, Smith JD (1985). Clonal isolation of populations of γ-glutamyl transpeptidase-positive and -negative cells from rat liver epithelial cells chemically transformed *in vitro*. Cancer Res 44:5134-5138

Tsao, M.-S., and Duguid, W.P (1987). Establishment of propagable epithelial cell lines from normal adult rat pancreas. Exp Cell Res. 168: 365-375

Tsao M.-S., Duong M. and Batist G (1989). Glutathione and glutathione-S-transferase in clones of cultured rat liver epithelial cell that express varying activity of γ-glutamyl transpeptidase. Mol Carcinogenesis 2:144-149

Tsao M.-S. (1991). Enhanced expression of alpha-fetoprotein gene in cultured rat liver epithelial cells treated with N-methyl-N'-nitro-N-nitrosoguanidine. Pathobiology, 61:25-30

Tsonis DA and Eguchi G (1981). Carcinogens on regeneration. Effects of *N*-methyl-*N'*-nitro-N-nitrosoguanidine and 4-nitroquinoline -1-oxide on limb regeneration in adult newts. Differentiation, 20:52-60

Unsicker K, Kirsch B, Otten J, and Thoenen H (1978). Nerve-growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. Proc Natl Acad Sic USA, 75:3498-3502

Usuda N, Reddy JK, Hashimoto T, and Rao MS (1988). Immunocytochemical localization of liver-specific proteins in pancreatic hepatocytes of rat. Eur J Cell Biol, 46:29z9-306

Uchiyama Y and Watanabe M (1984). A morphometric study of developing pancreatic acinar cells of rat during prenatal life. Cell Tissue Res, 237:117-122

Verlaan-de Varies M, Bogaard ME, van den Elst H, van der EB AJ, and Bos JL (1986). A dot-blot screening procedure for mutated ras oncogenes using synthetic oligonucleotides. Gene, 50:311-320

Wallace DH and Hegre OD (1979). Development *in vitro* of epithelial-cell monolayers derived from fetal rat pancreas. In Vitro, 15:270-277

Walters MN (1966). Adipose atrophy of the exocrine pancreas. J Pathol Bactriol, 92:547-557

Warshaw AL and Swanson RS (1988). Pancreatic cancer in 1988. Possibilities and probabilities. Ann Surg 208: 541-553

Watt FM (1991). Cell culture model of differentiation. FASEB, 5:287-294

Weaver CV, Sorenson RL, and Kaung HC (1985). Immunocytochemical localization of insulin-immunoreactive cells on the pancreatic ducts of rats treated with soybean trypsin inhibitor. Diabetologia, 28:781-785

Weinberg RA (1989). Oncogenes, anti-oncogenes, and the molecular basis of multi-step carcinogenesis. Cancer Res, 49:3713-3717

Weinstein IB (1988). The origins of human cancer: molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment-Twenty-seventh GHA Clowes Memorial Award Lecture. Cancer Res, 48:4135-4143

Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, and Miller AD (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci USA, 86:5434-5438

Weinsinger G and Sachs L (1983). DNA-binding protein that induces cell differentiation. EMBO Journal, 2:2103-2107

Wepsic HT (1983). Overview of oncofetal antigens in cancer. Ann Clin Lab Sci, 13261-266

Williams JA and Goldfine ID (1986). The insulin-acinar relationship. In: The exocrine pancreas: biology, pathobiology, and disease. pp 347-360. Edited by Go VLW, Gardner JD, Brooks FP, Lebenthal E, DiMagno EP, and Scheele GA. Raven Press; New York

Willis RW, Defeo D, Shih TY, Gonda MA, Young HA, Tsuchida N, Lowy DR, Scolnick EM (1981). The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebra gene. Nature, 292:506-511

Wood RAB and Moossa AR (1977). The prospective evaluation of tumor-associated antigens for the early diagnosis of pancreatic cancer. Br J Surg, 64:718-723

Wolf HK, Burchette JL, Garcia JA, and Michalopoulos G (1990). Exocrine pancreatic tissue in liver: a metaplastic process?. Am J Surg Pathol, 14:500-595

Wu JC, Merlino G, Fausto N (1994). Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor α. Proc Natl Acad Sci USA, 91:674-678

Wynford-Thomas D (1991). Oncogenes and anti-oncogenes; the molecular basis of tumor behaviour. J Pathol, 165:187-201

Yeldandi AV, Tan X, Dwivedi RS, Subbarao V, Smith DDJr, Scarpelli DG, Rao MS, and Reddy JK (1990). Coexpression of glutamine synthetase and carbamoylphosphate synthetase I genes in pancreatic hepatocytes of rat. Proc Natl Acad Sci USA, 87:881-885

Yamanaka Y, Friess H, Buchler M, Beger HG, Gold LI, and Korc M (1993). Synthesis and expression of transforming growth factor β-1, β-2, and β-3 in the endocrine and exocrine pancreas. Diabetes, 42:746-756

Yeger H, Cullinane C, Flenniken A, Chilton-MacNeil S, Campell C, Huang A, Bonetta L, Coppes MJ, Thorner P, and Williams BR (1992). Coordinate expression of Wilms' tumor genes correlates with Wilms' tumor phenotypes. Cell Growth and Differ, 3:855-864

Yoshinari M and Daikoku S (1982). Ontogenetic appearance of immunoreactive endocrine cell in rat pancreatic islets. Anat Embryol, 165:63-70

Zurlo J, Curphey TJ, Hiley R, and Longnecker DS (1982). Identification of 7-carboxymethylguanine in DNA from pancreatic acinar cells exposed to azaserine. Cancer Res, 42:1286-1288