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Morphological and molecular characterization of human ductal pancreatic adenocarcinoma cells undergoing duct-like differentiation

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1 Introduction

1.1 Epithelial Cells

In vertebrates two of the major tissue types are epithelial- and connective tissue. They present two extremes in which the structural roles played by the matrix and by cell-cell contacts are different. In connective tissues extracellular matrix is plentiful and cells are sparsely distributed within it. The matrix is rich in fibrous polymers, especially collagen (Loehr, 1996), and bears most of the mechanical stress to which the tissue is subjected (Leonova and Raphael, 1997; Mogensen et al., 1998). The cells are attached to components of the matrix but direct attachments between these cells are relatively seldom. In epithelial tissues, by contrast, cells are tightly bound together into sheets. Extracellular matrix is scanty and consists mainly of a thin mat called the "basal lamina", which underlies the cellular sheet. Here the cells themselves, rather than the matrix, bear most of the mechanical stresses by means of strong intracellular protein filaments which transmit mechanical stress from one cell to the next. These filaments are directly or indirectly attached to transmembrane proteins in the plasma membrane (Sato and Kamiya, 2000), where specialized junctions are formed between the surface of adjacent cells and with the underlying basal lamina (see Fig.1).

Epithelial cell sheets line all the cavities and inner surfaces of the body, and the specialized junctions between the cells enable these sheets to form barriers to the movement of water, solutes, and cells from one body component to another (Forslind, 1994; Leonova and Raphael, 1997). Epithelial sheets almost always rest on a supporting bed of connective tissue which may attach them to other tissue (such as muscle) that do not themselves have either strictly epithelial or strictly connective-tissue organization. Both compartments influence each other not only during organogenesis but also in carcinogenesis (Condon and Bosland, 1999). It is indisputable that carcinogenesis depends on mesenchymal factors (Mori and Miyazaki, 2000). Epithelial structures are found in almost all organs which have developed from the ectoderm, mesoderm, entoderm and are characterized by at least two different membrane areas exhibiting different functions. Cells with functionally different membrane sides are called "polar". In contrast to plant cells which are surrounded by a cell wall, animal cells have to maintain

tissue stability by developing close and durable connections with their neighboring cells, these are the cell junctions (compare Fig.1).

Figure 1

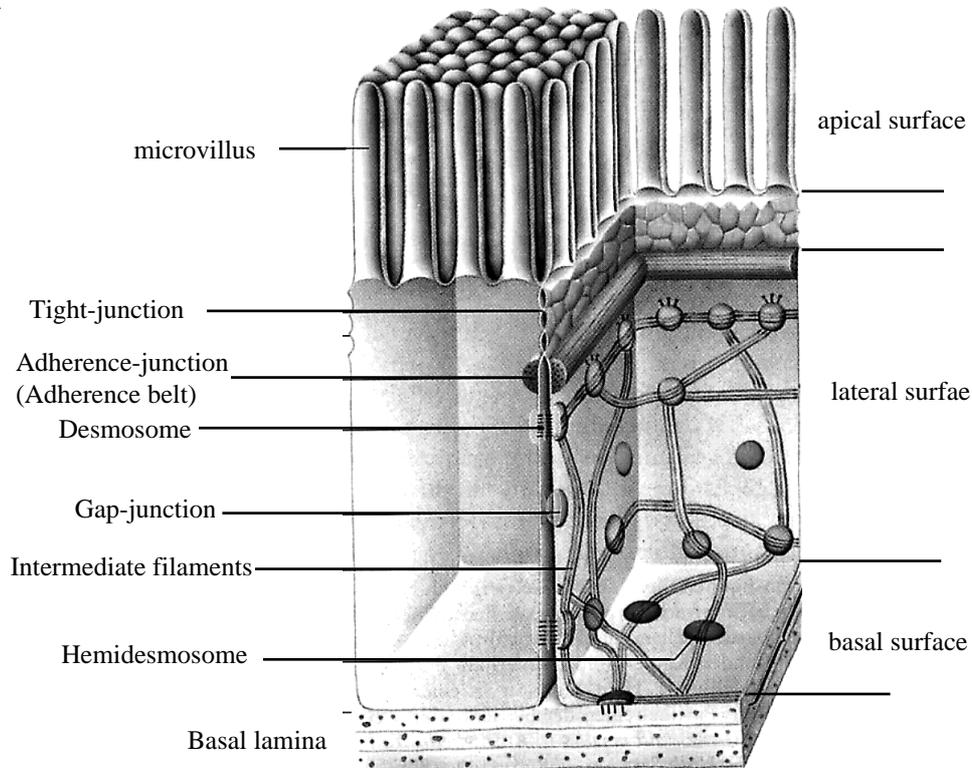


Fig.1: Distribution of various cell-contacts between epithelial cells of the small intestine. After: *Molekulare Zellbiologie*, de Gruyter 1996, by Harvey Lodish, Second Edition, p 653

Cell junctions

Despite the many structural and biochemical differences among various types of epithelia, all have at least one important function in common: they serve as selective permeability barriers, separating fluids on each side having different chemical compositions.

Tight junctions

Tight junctions play two distinct roles in this selective barrier function (Alberts et al., 1994). The epithelial cells lining the small intestine keep most of the gut contents in the lumen. At the same time, however, the cells must transport selected nutrients across the cell sheet from the lumen to the extracellular fluid permeating the connective tissue on the other side, from

where the nutrients diffuse into small blood vessels. This transcellular transport requires two sets of membrane-bound carrier proteins: one is confined to the apical surface (facing towards the lumen; covered with microvilli to enlarge the surface) of the epithelial cell and transports molecules from the lumen of the gut into the cell; the other, which is confined to the basolateral (basal and lateral) surfaces allows the same molecules to leave the cell by facilitated diffusion into the extracellular fluid on the other side. As this directional transport is to be maintained, the carriers must not be allowed to migrate between the apical and basolateral membranes. Furthermore, the spaces between epithelial cells must be sealed to prevent already transported molecules from diffusing back into the lumen of the gut. Tight junctions between the epithelial cell block these kinds of diffusion.

The molecular structure of tight junctions is still uncertain although major structural molecules like ZO-1, occludin or symplekin have been identified (Chen et al., 2000).

Anchoring junctions

Anchoring junctions enable epithelial cells to function as robust structural units by connecting the cytoskeletal elements of a cell either to those of another cell or to the extracellular matrix (compare Fig. 1). They occur in three structurally and functionally different forms: *adherence junctions*, *desmosomes* and *hemidesmosomes*.

E-cadherin and β -catenin are the most important molecules in adherence junctions which are the first junctions observed when dissociated cells were allowed to re-associate (Lincz et al., 1997). Cadherins are responsible for Ca^{2+} -dependent cell-cell adhesion. Virtually all vertebrate cells express one or more cadherins, each encoded by a separate gene, the particular set expressed being characteristic of the cell type. *In vitro*, the removal of extracellular calcium or treatment with an cadherin-blocking antibody disrupts the tissue, and if cadherin-mediated cell-adhesion is left intact, antibodies against other adhesion molecules are without effect. Under *in vivo* conditions, mutations which inactivate the function of cadherins cause embryos to fall apart early in development or prevent organ development. Cadherins mediate cell-cell adhesion by a homophilic mechanism (compare Fig. 2). In tumors the loss of E-cadherin mediated cell-cell adhesion is a critical step towards metastasis (Perl et al., 1998). The

cytosolic partners of E-cadherin in epithelial cells connecting the transmembrane-domain of E-cadherin with the actin-filaments of neighboring cells are the catenins, in particular α -, β - γ -catenin as well the protein pp120 (compare Fig. 2). β -catenin is of particular interest with respect to its additional function as transcription factor. Cytosolic β -catenin normally is degraded in a complex including the APC-gene product and the glycogen-synthase-kinase-3- β (GSK3 β). In the case of a mutated APC-gene being often found associated with colorectal cancer (Gryfe et al., 1997), degradation is not taking place facilitating the transport of β -catenin into the nucleus of cells and the initiation of transcription of oncogenes like c-myc (Eberhart et al., 2000; Sparks et al., 1998).

Figure 2: Roles of cadherins and catenins in cell-cell contacts

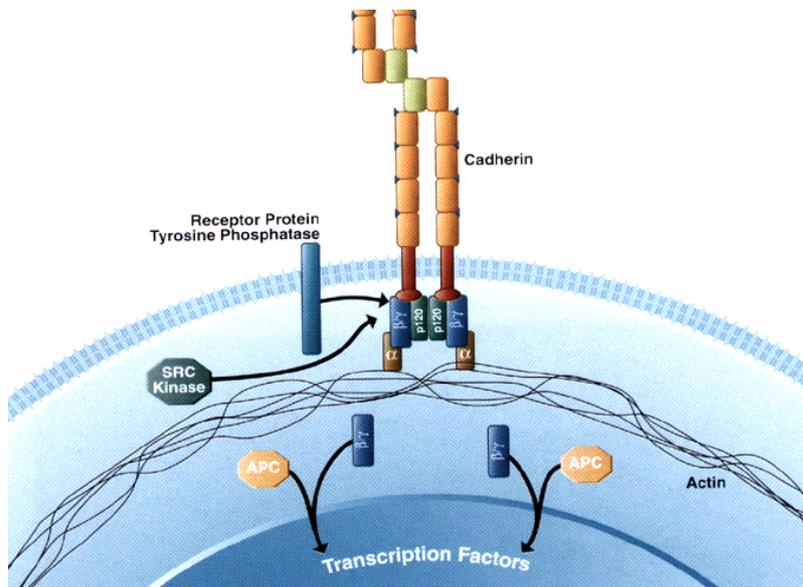


Fig.2: The roles of cadherins and catenins in cell-cell contact and transcription. From Clontechiques 03/99

In contrast to adherence junctions in cell-cell contact, adhesions between the cell and the matrix exists in so called "focal adhesions" comprising of integrins as cell-surface matrix receptors and a cytosolic complex consisting of attachment proteins like talin, α -actinin and vinculin (Burrige et al., 1988) but also paxillin and the focal adhesion kinase (FAK) (Tremblay et al., 1996).

Desmosomes and hemidesmosomes act as rivets to distribute tensile or shearing forces through an epithelium. Desmosomes are button-like proteins of intercellular contact. On the cytosolic

surface of each interacting plasma membrane is a dense plaque composed of proteins like plakoglobin or desmoplakin. These plaques are connected to the actin-filaments of their cells and by transmembrane linker proteins (members of the cadherin-family) to the cytoplasmic plaque of the neighboring cell (Buxton and Magee, 1992).

In hemidesmosomes the transmembrane linker proteins belong to the integrin-family used in focal adhesions rather than to the cadherin family used in desmosomes (Schwarz et al., 1990).

Gap junctions

Gap junctions are the most wide spread junctions shared by practically all animal species. Gap junctions allow direct communication and exchange of small molecules like ions, sugars, amino acids, nucleotides between cells while macromolecules are held back. Gap junctions couple cells chemically and electrically. They consist of proteins called connexins which form transmembranous channels. These channels are closed at a low pH or a high Ca^{2+} -concentration and open at a high pH or low Ca^{2+} -concentration (Bennett and Verselis, 1992).

Cell adhesion in human cancer is a critical point. Upon malignant transformation epithelial cells dramatically render their biological properties. To metastasize a cell of a typical solid tumor must be able to loose the adhesion to its original neighbors, escape from the tissue of origin, burrow through other tissues until they reach a blood- or lymphatic vessel, cross the basal lamina and endothelial lining of the vessel so as to enter the circulation, exit from the circulation elsewhere in the body, survive and proliferate in a new environment (Fidler, 1991). The loss of E-cadherin has been described to be associated with invasion and metastasis (Perl et al., 1998; Takeichi, 1993).

1.2 Morphology and physiology of the human pancreas

The pancreas is the largest secreting gland in the human body and consists of the endocrine and the exocrine system. Morphologically it is divided into five areas: uncinata process, head, neck, body and tail (compare Fig. 3). The endocrine system secretes its products directly into the blood. The responsible cell types are organized in the islets of Langerhans. These islets of Langerhans are ovoid, 76 x 175 μm collections of cells scattered throughout the pancreas (compare Fig. 4 and 5). They make up about 2% of the volume of pancreas. In human, there are 1-2 million islets and the cells in the islets can be divided into different types on the basis of their staining and morphology. There are at least 4 distinct types of cells (alpha, beta, delta and gamma) in human pancreatic islets. Alpha cells: about 20% of total islet cells, secrete glucagon. Beta cells: most abundant (60-75%), locate in the center of each islet, secrete insulin. Delta cells: secrete somatostatin. Less common, gamma cells: secrete pancreatic polypeptide.

Figure 3

B= body
H= head
N= neck
T= tail
Un= uncinata

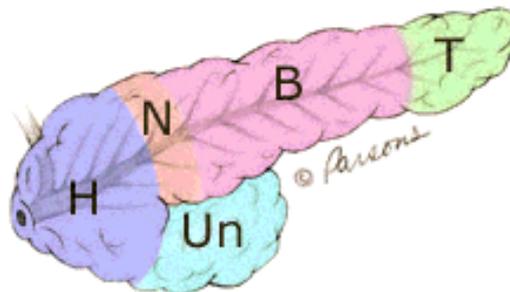


Fig. 3: Structure of the human pancreas (schematic) uncinata process: The part of the gland that bends backwards and underneath the body of the pancreas. Two very important blood vessels, the superior mesenteric artery and vein cross in front of the uncinata process. Head: The widest part of the gland. It is found in the right part of abdomen, nestled in the curve of the duodenum which forms an impression in the side of the gland. Neck: The thin section between the head and the body of the gland. Body: the middle part of gland between the neck and the tail. The superior mesenteric blood vessels run behind this part of the gland. Tail: the thin tip of gland in the left part of abdomen in close proximity with the spleen. This figure was taken from: Chapter 65 "Functions of the Pancreas" in R. Greger and U. Windhorst (eds).

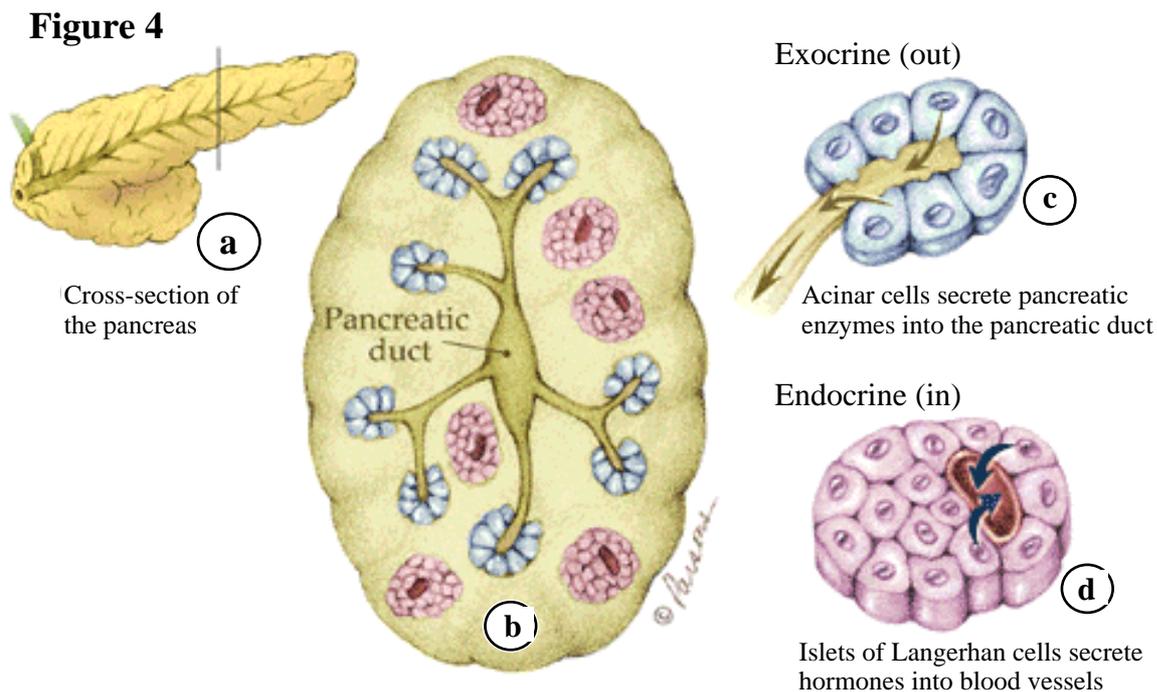


Fig. 4: Structure of the human pancreas: Schematic cross-section through the body as indicated in panel a. The exocrine compartment consisting of globular acini (enlarged in panel c) is connected to the main pancreatic duct by "Schaltstücke", intra- and interlobular ducts and secretes pancreatic enzymes or pre-forms related to the digestive process like: α -Amylase, Lipase, Trypsinogen 1, 2, 3, Chymotrypsin and others into the main pancreatic duct. The endocrine compartment organized in the islets of Langerhans (enlarged in panel d) consists of four specialized cell types: alpha, beta, delta and gamma cells which secrete insulin, glucagon, somatostatin and pancreas polypeptide into blood vessels. This figure was taken from: Chapter 65 "Functions of the Pancreas" in R. Greger and U. Windhorst (eds)

The exocrine pancreas is a secreting gland and characterized by the combination of ductal and acinar cells. The acinar unit of the pancreas is comprised of a group of acinar cells representing a three-dimensional goblet surrounding the acinar lumen (compare Fig. 2, panel c). Ultrastructurally these cells contain extensive Golgi apparatuses and numerous secretory granules containing zymogen components near their apical membranes. Acinar cells secrete enzymes which are related to digestive processes. The most important ones are: Amylase, Lipase, Trypsin and Chymotrypsin. Except Amylase and Lipase these enzymes are secreted as pre-forms being either activated by enterokinases or acids of the bile duct. The pancreatic ductal system connects secretory cells of the exocrine pancreas to the duodenum. Ducts arise at the acinar unit of the pancreas and function by adding secretions finalizing the composition of the pancreatic juice (mainly bicarbonate-rich fluid) and facilitate the transport of secretions

of the acinar cells to the duodenum where they are needed to digest carbohydrates, proteins and fat. Approximately 1.5 l of pancreatic juice is produced per day. These intralobular duct cells also express glycoproteins of the mucin-family like MUC-1 (Monges et al., 1999). Near the junction of the acinus the centroacinar cell is located, whose nature remains unclear. It is claimed to represent a distinct cell type while others argue it is either a ductal or an acinar cell.

The morphology of the ductal cells change in moving away from the acinar unit towards the duodenum. The cells generally become taller when approaching the stage of interlobular ducts. The whole ductal compartment containing all cells from the smallest intra-, interlobular, and intercalating ducts to the ductus pancreaticus present proximately 5% of the total mass of the pancreas (Lohr and Friess, 1996). The vast majority of this tissue, nearly 80% (Githens, 1988) consists of acinar cells (compare Fig. 5).

Figure 5

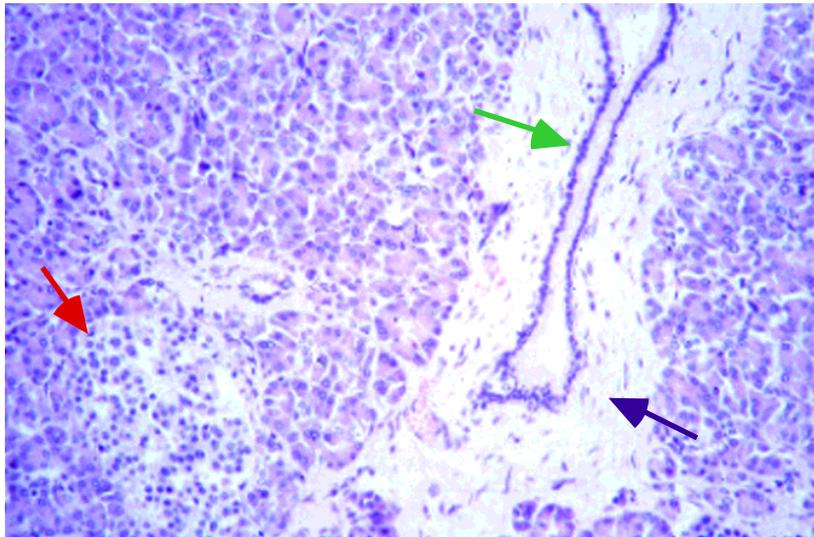


Fig. 5: Structure of the human pancreas: Cross-section through the body of a human pancreas. All functional components of the pancreas are visible in this figure. The islets of Langerhans containing the endocrine cells of the pancreas are indicated with the red arrow. Acini display the major component of pancreatic tissue filling 80% of this image being connected by a loose fibrous structure containing the thin vessels nutritifying the acini. The right half of the image shows a pancreatic duct (green arrow) cut crossways surrounded by a periductal fibre zone (blue arrow).

1.3 Pancreatic carcinoma

Pancreatic carcinoma belongs to the frequent causes of death caused by malignant disease in western Europe and is placed on the fifth position of U.S cancer statistics.

Most patients suffering from a pancreatic carcinoma are diagnosed in advanced stages of this disease when metastasis are frequently observed. A high number of these patients are classified as inresectable and have a very poor prognosis. Radiochemotherapy and other therapies failed, the five-year survival rate varies between 0.4% and 2% worldwide (Murr et al., 1994; Takeda et al., 1998). In contrast to colorectal carcinoma where a pre-screening is possible with the "hemocult-test", no such systems are available for an early diagnosis of pancreatic carcinoma. With increasing age the incidence of the pancreatic carcinoma rises continuously. Pre-dispositions are factors like smoking, fat-enriched nutrition and alcohol-induced chronic pancreatitis (Chari et al., 1996; Gates, 2000; Hart, 1999; Mangray and King, 1998; Tominaga and Kuroishi, 1998). The chronic pancreatitis as a pre-disposing event is critically discussed although a publication from Lowenfels and co-workers give some evidence (Lowenfels et al., 1993). Model systems to address pre-malignant lesions in pancreatic carcinoma or early steps in the carcinogenesis are highly desired. Attempts to establish cell lines from primary duct cells are difficult and have not led to the expected success so far (Jesnowski et al., 1998). Further uncertainty has been caused by reports about transdifferentiation of former acinar cells into cells exhibiting ductal characteristics (Schmid et al., 1999). These observations cause difficulties to construct a suitable model system for the investigation of early pancreatic cancer. The scenario becomes even more complicated if one considers the role of the mesenchymal compartment during carcinogenesis which is also of major importance for the proper development of the pancreas during embryogenesis and reveals its importance in carcinogenesis by the fibrosis observed in pancreatic carcinoma (Lohr et al., 1999; Sparmann et al., 1997).

Suspecting the metabolically hyperactive acinar cell to be the germinal cell in many cancers, acinar cell carcinomas represent an extremely small percentage of pancreatic cancers as well as neuroendocrine cells from the islets of Langerhans (Mangray and King, 1998). The vastmajority (90%) is classified as ductal adenocarcinomas (Cubilla and Fitzgerald, 1975;

Kloppel et al., 1980; Kozuka et al., 1979). A characteristic feature of ductal adenocarcinomas is their localization in the head of the pancreas accompanied by a massive proliferation of stroma cells (desmoplasia) with an infiltration of well differentiated duct-like structures. Desmoplastic tissue contains fibroblasts as the major cellular compartment connected with proteins of the extracellular matrix (ECM) as the non-cellular compartment (Loehr, 1996). This noticeable desmoplasia of pancreatic adenocarcinoma is shared with carcinomas of the prostate gland and mammary gland also arising from epithelial cells of the corresponding organ (Loehr, 1996). The influence of the mesenchymal compartment is critical for proliferation, secretion and differentiation of pancreatic epithelial cells (Hahn et al., 1987; Stallmach et al., 1991). Subsequently the components - epithelial cell and stroma (including ECM) are classified as a functional unit influencing each other during development, inflammation or carcinogenesis. Carcinogenesis as a "multi-step-event" by means of a step-wise accumulation of genetic alterations leading to an instable malignant genotype has been shown for colorectal carcinoma (Fearon and Vogelstein, 1990).

Lacking model systems for the detection of early lesions during pancreatic carcinoma and the difficulty to obtain and isolate primary pancreatic duct cells or highly differentiated pancreatic tumor cells from early stages of pancreatic carcinoma make it difficult to define such a multi-step-carcinogenesis for the pancreatic carcinoma.

1.3.1 Pancreatic carcinoma: Molecular basis

Due to the ductal origin of the vast majority of pancreatic adenocarcinomas, pancreatic cancer cells express several ductal marker antigens like the carbonic anhydrase type II (CAII) (Nishimori et al., 1999), cytokeratines CK7, CK8, CK18, CK19 (Bouwens et al., 1994; Schussler et al., 1992), the Carcinoembryonic antigen CEA (Kim et al., 1990) and the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Hollande et al., 1998). Ductal cells are negative for Amylase and Lipase and can be discriminated from acinar cells which are positive, by immunocytochemical staining for these two antigens.

The development of neoplasia is coupled with a dysregulation of important cellular processes like the programmed cell death (apoptosis), proliferation, differentiation of cells and cell adhesion (Goss and Groden, 2000; Nollau et al., 1997). The genetic background of these dysregulations has been shown in experiments where human tumor DNA was transfected into several cell types and induced a neoplastic transformation (Andersen et al., 1981; Jhappan et al., 1990; Krontiris and Cooper, 1981; Perucho et al., 1981). Experimental results from other tumors like the colorectal carcinomas led to the discovery of several oncogenes or tumorsuppressor genes involved in the development of sporadic pancreatic carcinoma. Recent developments in molecular biology helped to extent the knowledge about new oncogenes and tumorsuppressor genes during the past few years.

The most frequent and best characterized genetic alterations in human pancreatic carcinoma affect the Ki-ras protooncogene (74-100%), p16^{ink4} (up to 98%), p53 (43-76%) and DPC4 (53%). See table 1 for more summarized details and references.

Table 1: Most frequent genetic alterations in pancreatic carcinoma

Gene	Chromosomal localization	Alteration	Frequency	Reference
Ki-ras	12p13	point mutations codon 12,13,61	74-100%	Dergham et al., 1997 Rozenblum et al.,1997
p53	17p13	point mutation exons 5, 6, 7, 8; LOH	43-76%	Dergham et al., 1997 Rozenblum et al., 1997 Redstone et al.,1994
p16	9p21	homozygous deletion, point mutation, hypermethylation	27-98%	Rozenblum et al., 1997 Huang et al., 1996 Caldas et al., 1994
DPC4	18q21	homozygous deletion, point mutation, LOH	50%	Hahn et al., 1996 Rozenblum et al., 1997
DCC	18q21	LOH	n.d	Simone et al., 1994 Hohne et al., 1992 Barton et al., 1995
BRCA2	13q12.3	LOH	27%	Goggins et al., 1997
Rb1	13q14	LOH, point mutation	0-10%	Schutte et al., 1997 Barton et al., 1995 Huang et al., 1996

After: Pancreatic Cancer; Molecular and Clinical Advances by J.P. Neoptolemos and N.R. Lemoine; Blackwell Science 1996, pages 119-121. (n.d = not determined)

1.3.2 Oncogenes

Ki-ras

Ras oncogenes were first recognized as the transforming genes of Harvey and Kirsten murine sarcoma viruses (Ha-MSV, Ki-MSV). These highly oncogenic retroviruses were generated during passage of murine leukemia viruses through laboratory strains of rats (Harvey, 1964; Kirsten and Mayer, 1967). Subsequent molecular analyses showed that Ha-MSV and Ki-MSV contained sequences in their genomes that were not present in the genomes of the parental viruses; these newly acquired sequences appeared to be the result of viral transduction of cellular genes and were thus likely to be responsible for oncogenic transformation. This was confirmed when it was shown that subgenomic fragments of Ha-MSV containing only cell-derived sequences were able to transform cells. The ras oncogene family consists of three human genes: Ki-ras, Ha-ras and N-ras. They represent the most frequently mutated dominant

oncogenes in human neoplasia (Bos, 1989). The gene products are small GTP-binding proteins localized at the inner cell membrane, having a molecular weight of 21kDa (p21). Ras proteins play a role in the signal transduction of receptor tyrosin-kinases like the EGF-receptor as well as they are further involved in processes like cellular differentiation, cell cycle progression and secretion (Bollag and McCormick, 1991). Ras-proteins are activated by the binding of GTP. The intrinsic GTPase hydrolyzes GTP into GDP and monophosphate. After that, ras-proteins return into the inactive form facilitated by GTPase-activating proteins (GAPs). Point mutations in the Ki-ras gene lead to a constitutive activation of the ras-signaling cascade by inhibiting the hydrolysis of GTP. Further the ras oncogene product p21ras requires farnesylation for its transforming activity. This key post-translational modification is catalyzed by p21ras farnesyltransferase, which transfers farnesyl from farnesylpyrophosphate to the cysteine of the CA1A2X carboxyl-terminal tetrapeptide of p21ras. Inhibitors of farnesyltransferases were therefore thought to display potential therapeutic agents. (Prendergast, 2000; Reuter et al., 2000)

1.3.3 Tumor suppressor genes

Tumor suppressor genes mostly function to inhibit the cell growth and cell cycle; they act to prevent the development of neoplasia. Mutations in tumor suppressor genes cause the cell to ignore one or more of the components of the network of inhibitory signals, removing the brakes from the cell cycle and resulting in a higher rate of uncontrolled growth -- cancer.

p53

The p53 protein blocks cells from passing beyond G1 in the cell cycle by binding to a transcription factor called E2F (Vaishnav and Pant, 1999). This prevents E2F from binding to the promoters of proto-oncogenes such as c-myc and c-fos. Transcription of c-myc and c-fos is needed for mitosis so blocking the transcription factor needed to turn on these genes prevents cell division. Normally, damage to cellular DNA initiates increased expression of p53 which leads to arrest of the cell cycle. This interruption permits DNA-repair to occur before the cell resumes the cell cycle and normal proliferation. If repair of the DNA is not

successful, the cell undergoes apoptosis, programmed cell death. When p53 mutates, damaged cells are not arrested in G1 and DNA-repair does not take place. This failure to arrest DNA-damaged cells will be repeated in subsequent cell cycles permitting other mutations to accumulate culminating in neoplastic transformation, tumor formation and cancer (Geske et al., 2000; Wang and Harris, 1997). Point mutations and deletion of the chromosomal locus of p53 on chromosome 17p are the most abundant alterations in human malignancies (Hollstein et al., 1991; Kalthoff et al., 1993; Levine, 1992).

Mutations in the p53 gene result in abnormal proteins exhibiting an extended half-life and therefore being more stable than wild-type p53. Observations indicate that a normal p53 gene is dispensable for embryonic development, but that its absence predisposes animals to neoplastic disease (Donehower et al., 1992).

p16

The p16 tumor suppressor gene is thought to play an important role in cell cycle regulation by encoding for protein products being able to inhibit the progression from G1 to S phase in the cell cycle. Recently, the p16 gene has been found to be mutated or deleted in a variety of different types of primary human malignant tumors and human-derived malignant tumor cell lines. p16 is localized on chromosome 9p21 and was identified as the first member of the INK4-family of cyclin-dependent-kinases (cdk). During the cell cycle cells have to pass a restriction point which decides if the cell "is allowed" to proceed into the S-phase of the cell cycle when the genetic material is doubled. For the progression of the G1-phase activation of cyclin D1 by cdk4 is required. The complex of cyclin D1/cdk4 phosphorylates the retinoblastoma gene product Rb. This phosphorylation prevents the binding of Rb to the transcription factor E2F. Unbound E2F now acts as transcription factor and leads to progression into the S-phase of the cell cycle. p16 inhibits G1/S-progression by inactivating the cdk4/cyclinD1-complex. This prevents phosphorylation of Rb and therefore G1/S-progression. Loss of p16 activity turns off the inhibitory effect at the cdk4/cyclinD1 level and leads to an increased cell cycle progression. Up to 98% of pancreatic carcinoma show inactivation of p16 by either homozygous deletion in both alleles or a mutation in one and deletion of the other allele (Caldas et al.,

1994; Huang et al., 1996; Naumann et al., 1996). Patients in which pancreatic carcinoma was associated with inherited melanoma, beared p16-mutations in the germ line (Hussussian et al., 1994; Kamb et al., 1994).

DPC4 (Smad4)

The DPC4-gene is localized on chromosome 18q21.1. High sequence similarities of the human exons 1, 2 and 11 to the Mad-protein (Mother against decapentaplegic) of *Drosophila melanogaster* and the sma-2, sma-3 and sma-4 proteins of *Caenorhabdites elegans* are reported (Liu et al., 1996). These proteins display a part of the signal-transduction pathway of TGF- β . Therefore a role of the DPC4 gene product, relying on the sequence similarities, in the TGF- β pathway is hypothesized. TGF- β signaling is mediated by phosphorylation of Smad2 and Smad3 followed by complex-formation with Smad4. Smad-proteins mediate or resemble the TGF- β signaling pathway which is initiated by ligands of the TGF- β -superfamily including TGF- β , Activin and BMP's (bone morphogenic proteins). While TGF- β is known as a negative regulator of the cell cycle (Adnane et al., 1998; Alexandrow and Moses, 1997), inactivation of such regulation pathways by loss of DPC4 is likely. 90% of the analyzed pancreatic carcinoma showed an allelic loss on chromosome 18q whereas 30% exhibited a homozygous deletion of the locus 18q21.1 (Hahn et al., 1996; Hahn et al., 1996). In other tumors like colorectal carcinoma and breast cancer, the frequency of DPC4 deletion was approximately 10% (Moskaluk et al., 1997; Schutte et al., 1996; Takagi et al., 1996). Interestingly a correlation between inactivation of DPC4 and p16 was reported (Rozenblum et al., 1997). In all investigated cases inactivation of DPC4 coincided with p16-inactivation but not vice versa supporting the idea that loss of p16-activity provides a selective advantage for the development of DPC4 mutations (Rozenblum et al., 1997). Next to the described genetic alterations there are some less frequent mutations known.

DCC

The DCC-gene consists of 29 exons overspanning 1.4 mega basepairs in direct vicinity to the DPC4-gene localized on chromosome 18q. Recent works suggested genetic alterations in the DCC-gene in pancreatic carcinoma (Hohne et al., 1992; Simon et al., 1994). Later data showed that the complete suppression of the DCC expression is a rare event as well as a deletion of the DCC locus (Barton et al., 1995). Due to the enormous size of this gene no sequence analysis was performed in pancreatic carcinoma so far.

BRCA2

It is estimated that about 5-10% of breast cancer cases may be due to inherited predisposition. Until now, two main susceptibility genes have been identified: BRCA1 and BRCA2 (Osorio et al., 2000). A significant number of these family members developed pancreatic carcinoma (Teng et al., 1996). In patients apparently developing sporadic pancreatic carcinomas, germ line mutations in the BRCA2-gene were identified (Goggins et al., 1996). 27% of all analyzed cases showed a LOH (loss of heterozygosity) in tumors. In 10% of these cases abnormalities in the second allele were detected mainly identified as germ line mutations.

Rb1

Genetic alterations in the retinoblastoma gene 1 localized on chromosome 13q14 were detected in less than 10% in the investigated pancreatic carcinomas (Barton et al., 1995; Huang et al., 1996b). Tumors with point mutations in the p16-gene generally show no coexisting Rb1-mutation which would moderate the effects of a loss of p16 activity. Since p16-mutations are frequent events in pancreatic carcinoma, Rb1-mutations are rather infrequent (Schutte et al., 1997).

1.3.4 Chromosomal abnormalities in pancreatic carcinoma

Beside the described mutations in single genes chromosomal abnormalities, changing the whole structure of the genome are observed in pancreatic carcinoma (Bardi et al., 1993; Griffin et al., 1994; Griffin et al., 1995). Investigations on metaphase chromosomes discovered

additive alterations on chromosome 7 and 20 as well as loss of the chromosome arms 1p and 6q to be decisive for pathogenesis of pancreatic carcinoma. Extensive studies on 17 pancreatic carcinomas showed frequent allelic losses for the chromosome arms 1p (50%), 6p (50%), 6q (50%), 8p (56%), 9q (96%), 10p (50%), 10q (50%), 12p (50%), 17p (95%), 18q (88%), 21q (61%) and 22q (61%) (Lumadue et al., 1995). These data correlate with the chromosomal loci of several tumor suppressor gene like p53 (17p), DPC4 (18q) and p16 (9p).

1.3.5 Growth factors and cytokines

The genetic profile of pancreatic carcinoma consists not only of the described scopes like inactivation of tumor suppressor gene, activation of oncogenes and protooncogenes or chromosomal aberrations. It is further characterized by altered signal transduction pathways of cytokines and growth factors and their corresponding receptors (Friess et al., 1999; Ketterer et al., 1998). Overexpression of growth factors provide an advantage for tumor cells mostly resulting in an increased proliferation rate. These growth factors like transforming growth factor alpha (TGF- α), epidermal growth factor (EGF) and its receptor, acidic and basic fibroblast growth factors (aFGF, bFGF) have influence on the autocrine growth regulation of tumor cells (Korc et al., 1992; Yamanaka et al., 1993). Beside the autocrine growth stimulation of tumor cells, growth factors like TGF- β which is expressed in many pancreatic carcinoma cells, support the proliferation of mesenchymal cells like fibroblasts in a paracrine manner leading to the massive increase in the mesenchymal cells associated with pancreatic carcinoma called desmoplasia (Ellenrieder et al., 2000; Kalthoff et al., 1991). Since it is known that overexpression of growth factors and their receptors is not restricted to pancreatic carcinoma being also found in chronic pancreatitis, it is likely that only the combination of overexpression and the described genetic defects leads to carcinogenesis.

Increasing knowledge of the molecular mechanisms in pancreatic carcinoma made molecular biological techniques interesting for diagnosis and therapy. Identification of new genes associated with early events in pancreatic carcinoma and the development of suitable model systems resembling precursor-lesions should be the aims of on-going research.

Although tumors of different patients differ genetically, most of them share mutations in

Ki-ras, p53 and p16 which are of pathological importance in most carcinomas. Especially the frequent activations of a ras-oncogene (Kalthoff et al., 1993b) in combination with mutations of a tumor suppressor gene, in particular p16 (Naka et al., 1998), are likely to contribute to the malignant phenotype of pancreatic adenocarcinomas. Model systems are required not only to detect new genetic alterations but for helping to unravel the process of progression from a pre-lesion towards a pancreatic carcinoma.

1.4 Therapeutic options

At the time of diagnosis nearly 90% of the patients have infiltrated lymph nodes and 80% show metastasis in the liver. Over the last ten years, resection rates have increased from less than 3% to over 10% in many published series and during the same period, operative mortality has fallen from 25% to less than 10% (Cameron et al., 1993; Russell, 1990; Trede et al., 1990). Despite these improvements in surgical technique, the five-year survival rates in these same series ranged from 11 to 24% and few, if any, patients were actually cured of their disease (Gudjonsson, 1987). Epidemiological studies paint an even bleaker picture. Bramhall et al. (1995) reported on a series of 13,560 patients with pancreatic cancer in whom only 2.6% underwent resection with a five-year survival of 5.5% and an overall five-year survival of 0.2% (Bramhall et al., 1995).

The belief that surgery alone has had a major impact on long-term survival in pancreatic cancer is overoptimistic. Although it may have had a marginal impact on improving five-year survival, the most important determinants remain disease stage (Baumel et al., 1994; Cameron et al., 1991; Funovics et al., 1989; Trede et al., 1990). Next to surgical treatment of pancreatic cancer, other strategies have been applied like radiotherapy, chemotherapy or combinations either used as single methods or in adjuvant therapy. However, the results are still disappointing (Andre and Balosso, 1999; Huguier and Mason, 1999; Kornek et al., 2000). A combination of radical surgery and combined modality adjuvant therapy may improve the five year survival to 20% or more (Douglass, 1987). Other forms of putative therapies are discussed and strategies for genetic interventions are formulated (Takeda et al., 1998).

There are five broad approaches to genetic intervention for cancer therapy and each has its own advantage and drawbacks for clinical application.

1) Replacement or augmentation of tumor-suppressor gene function.

Possible candidate-genes are the p53- (Baker et al., 1990) or p16^{INK}-gene (Serrano et al., 1995).

2) Blockade of dominant gene expression.

Most experimental settings concerning this idea of therapy have made use of antisense oligonucleotides or ribozymes to interrupt male-function of target-genes like p53, p16, DPC4 (Georges et al., 1993; Mukhopadhyay and Roth, 1993; Mukhopadhyay et al., 1991).

3) Genetically directed enzyme producing therapy (GDEPT).

The principle of GDEPT is to take advantage of transcriptional differences between normal and tumor cells, or between one tissue and the rest of the individual. Expression of a gene encoding a prodrug-activating enzyme is directed by a tumor- or tissue-specific gene promotor so that only these cells should become susceptible to the toxic effects of the prodrug when it is subsequently administered systemically. Most studies have been done with the deaminase gene and the herpes simplex virus thymidine kinase (HSV-tk) gene (Howard et al., 2000; Howard et al., 1999).

4) Genetic inmodulation.

Downregulation of HLA-system components is a widely observed phenomenon in various common tumor types (Cabrera et al., 1992). Local expression or delivery by e.g. virus-based systems of exogenous cytokines at the side of a tumor can elicit impressive anti-tumor responses (Provinciali et al., 1994; Lopez et al., 1992).

5) Polynucleotide- and Peptide-vaccination

Inoculation of experimental animals with plasmids encoding a variety of proteins has been shown to stimulate both antibody and T-cell immune responses which are specific and sometimes long-lived. The tumor-associated antigen, CEA, for example has been utilized for active adoptive immunotherapy. Early reports from Curiel et al. showed that intramuscular injection of a plasmid expressing the human CEA complementary DNA could stimulate a humoral

response against CEA in mice (Conry et al., 1994). The same group has since demonstrated this approach can produce immunoprotection against tumor challenge with CEA-expressing colon carcinoma cells (Conry et al., 1995). For peptide-vaccination studies with ras-proteins have been set up. However, gene-therapeutic approaches are only useful in stages of disease where only one gene-defect rather than a multi gene-defect is present or if the time course of the major events in malignant transformation is known. So far surgery seems to be the only therapy with curative chances in pancreatic cancer but hope is present based on new insights in the molecular mechanisms underlying this disease. Earlier diagnosis together with the knowledge of early genetic alterations and their appearance in the time course of pancreatic cancer are a prerequisite for successful treatment approaches either based on surgery or gene-therapy. Therefore defined model systems are required enable to:

- 1) Discover the early genetic changes in pancreatic carcinoma.
- 2) Resemble normal duct epithelial cells helping us to gain an impression of what "normal" means and to set up a "multi-step-carcinogenesis" model by step-wise introducing putative transforming candidate genes into these cells and monitoring their transforming effects on "normal" cells.
- 3) Integrate the mesenchymal compartment into the scenario of pancreatic cancer as a long-time underestimated component in the development of this disease.

1.5 *Experimental models in pancreatic cancer*

1.5.1 *In vivo models*

In vivo models are primarily based on animal experiments and although not part of this work, it should be mentioned they are important for the understanding of the complex situation found in patients. However, more and more options arise to transfer *in vivo*-experiments to the *in vitro*-level.

Chemically induced cancers

Pancreatic cancer has been experimentally induced in rodents with at least 20 different chemical carcinogens. Most of these chemicals are known mutagens which seem to act as initiators of carcinogenesis by damaging DNA. Nitrosamines used in hamsters induce pancreatic carcinoma with a ductal phenotype, remarkably similar to the human clinical situation in morphological and molecular features (Birt et al., 1981). Recent studies on the effects of castration, testosterone replacement and estrogen treatment of tumor foci in male rat, indicate that testosterone promotes and estrogen inhibit, the growth of preneoplastic lesions in the pancreas. This is of relevance to human pancreatic cancer since the male to female ratio of 2:1 in pancreatic cancer incidence might be related to the long term effects of sex steroid hormones (Longnecker and Sumi, 1990). Although these data suggest a role for hormonal treatment of pancreatic cancer (Bourhis et al., 1987; Greenway, 1987) most studies using hormones had no positive effect so far.

Xenotransplantation models

Various different models have been described either using established cell lines for inoculation in mice, hamster or rat or pieces of an original tumor. Primarily these transplantations were carried out by subcutaneous inoculation. Recent publications showed that orthotopic transplants behave different and must be considered to reflect the patients situation better than subcutaneous transplants (Cui et al., 1998). Future perspectives in this field are "humanized" mouse models combining human tumor cells with human mesenchymal or human endothelial cells in a mouse which e.g. expresses human MHC. These model systems will be even closer to the patients situation.

Transgenic models

The introduction of genes into the germ line of mammals is a technology which offers exciting possibilities for generating precise animal models of human diseases and for understanding carcinogenesis. Since the development of embryonic stem cell technology in the early 1980s and the generation of the first knockout mice about a decade ago, an explosive

growth in the number of targeted mouse mutants published every year has been seen as reviewed in (Brandon et al., 1995; Brandon et al., 1995). It has become feasible to produce mice with designed genetic alterations ranging from simple gene disruptions and point mutations to large genomic deletions encompassing several centimorgans, or even to generate specifically engineered chromosomal translocations. Such mice have since become an valuable new tool to dissect the functions of individual components of complex biological systems, namely to produce mouse models of human inherited diseases. Homologous recombination in embryonic stem cells (ES cells) is a routine technique being used to modify the mouse genome at any chosen locus (Doetschman et al., 1987; Thomas and Capecchi, 1987). The introduction of the foreign DNA is either performed by micro-injection into the pronucleus or by retroviral transduction of embryonic cells (Kilby et al., 1993). This type of manipulation may not only be used to inactivate (knock-out) a gene but also to generate "knock-in" mice in which, the coding region of a different gene or a regulatory element is introduced into the locus. In contrast to knock-out mice where a "loss of function" is the aim, a "gain of function" is developed in knock-in mice. Furthermore, genes may be replaced by their homologues from different species, to produce, for example, humanized antibodies or to exchange murine genes for disease-associated alleles from humans (Moore and Melton, 1995; Zou et al., 1994). The p53-gene, relevant in pancreatic cancer has been knocked out in mice already and lead to suppression of p53-dependent apoptosis in these animals (Nikiforov et al., 1996).

1.5.2 *In vitro* models

The most common *in vitro* models are based on cell lines. Human pancreatic cell lines are of particular interest and a wide spectrum is available (e.g. Capan 1 and 2, Panc 1, Colo 357, MiaPaCa-2, Aspc-1, BxPC-3) [Kalthoff, 1993 #101]. These cell lines have been used to study many aspects of pancreatic cancer, such as cellular de-differentiation, the promoting or inhibiting effect of gastrointestinal hormones and other growth factors. Hall and Lemoine showed the human acinar phenotype was not stable and could transdifferentiate into a ductal phenotype *in vitro* (Hall and Lemoine, 1992). Although many studies have been performed and are still performed using cell lines, the two-dimensional growth of cells alters their behavior in many

ways (Pawlik et al., 2000; Mansbridge et al., 1994; Roberts and Soames, 1993).

Three-dimensional cell culture systems have been established decades ago (Moscona, 1952; Holtfreter, 1947) trying to overcome some disadvantages of two-dimensional cell culture systems. It has been shown that multicellular aggregates called "spheroids" generated from tumor cells resemble the *in vivo* situation of a solid tumor better than conventional monolayer cultures of cancer cells. Carlsson and co-workers showed the outer cell layers of spheroids exhibited a higher proliferation rate than cells inside the spheroid due to the accumulation of toxic products or the lack of oxygen (Carlsson and Acker, 1988). Spheroids have been used as models for the analysis of tumor cell invasion (Bräuer and Hülser, 1990; Bräuer and Hülser, 1990), tumor-host interaction (Wilson and Lord, 1987), and cell-cell contacts (Bridges et al., 1991; Knuechel et al., 1990). A special characteristic of spheroids is the induction of morphological and functional cell-differentiation. This was already recognized by Mc Allister and Co-workers (1967) when they observed a striking cytological similarity between primary hamster tumors, corresponding soft-agar colonies and tumors induced by cultivated tumor cells. These observations were later supported by findings in other tumor systems (Knuechel et al., 1990). In contrast to spheroids obtained from tumor cells, spheroids obtained from normal cells e.g. freshly isolated tissue from chicken or mouse embryos, differentiated as observed in the *in vivo* tissue and showed typical morphological structures (Mattieu et al., 1981). Normal cells isolated from adult, differentiated tissue was shown to remain in an active and functional state for several weeks if cultivated as spheroids (Boxberger et al., 1997; Boxberger et al., 1993).

For the exocrine pancreas only one experimental system was described using explanted primary duct epithelial cells so far. In this study Oda and co-workers were able to grow primary pancreatic duct cells in monolayer cultures if these cells were supplied with an underlying feederlayer which consisted of collagen type I and fibroblasts (Oda et al., 1998). Although this experiment showed the cultivation of primary duct cells of the exocrine pancreas is possible, cell culture systems are required that can be held in culture for at least some weeks. Further these cells should maintain their properties even after being frozen and thawed and must assure reproducibility if experiments are repeated. Due to the low numbers of

passages in culture investigated so far and the different origin of donor tissue (different patients with different pre-lesions or treatments) it will be difficult to establish such a model system with primary pancreatic duct epithelial cells.

1.6 Aim of this study

This work is based on the following observation: Earlier observations with a subclone of the cell line A818, called A818-1, developed three-dimensional “ball-like” structures consisting of a single layer of pancreatic duct epithelial cells surrounding an inner lumen upon the prevention of adherence to substratum. Later on, this subclone lost the ability to form these structures. Consequently, other subclones of A818 were screened for their ability to form luminal structures and the subclone A818-6 was found.

The following hypothesis was formulated:

Although being carcinoma cells, all A818-6 cells which are part of this three-dimensional structure exhibit features of “normal” pancreatic duct epithelial cells while their corresponding monolayer cells do not. Therefore A818-6 monolayer cells were propagated as the “malignant” phenotype and A818-6 hollow sphere cells as “benign” phenotype of one cell line.

Following this hypothesis the purpose of this work was to show how “normal” A818-6 hollow sphere cells are in comparison to their corresponding monolayer cells and to find out whether this model is suitable to study important questions associated with development of pancreatic cancer.

The work was subdivided into three parts:

1) Establishment of the A818-6 cell culture system including the following steps:

- a) Determination of the best seeding density of cells.
- b) Determination of the best coating material for cell culture plates.
- c) Determination of the best cell culture medium and supplements.
- d) Establishment of a cell-collecting method using a micromanipulator to divide three-dimensional structures from undesired cells.

2) Characterization of the A818-6 cell culture system:

- a) Morphologically
 - Cell adhesion
 - Polarity
 - Differentiation marker antigens
- b) Physiologically
 - Proliferation
 - Secretion
 - Differentiation marker antigens

3) Analyses on morphoregulatory factors using the A818-6 system as a model:

- a) Growth factors and cytokines
 - stimulatory
 - inhibitory
- b) Mesenchymal influence
 - Co-cultures
 - Conditioned medium
- c) Epimorphin

2 Materials and Methods

2.1 Materials

2.1.1 Cell culture media, components and material

Basal Medium	Producer/Company
RPMI 1640	Life Technologies, Karlsruhe, Germany
Supplements	
FCS 10 %	Hyclone Labs, Logan, UT, USA
Glutamine 2mM	Life Technologies, Karlsruhe, Germany
Sodium pyruvate 1mM	Life Technologies, Karlsruhe, Germany
Penicillin 100 units/ml	Life Technologies, Karlsruhe, Germany
Streptomycin 100 µg/ml	Life Technologies, Karlsruhe, Germany

Basal Medium	Producer/Company
Fibroblast Growth Medium	PromoCell, Heidelberg/Germany
Supplements	
FCS 10 %	Hyclone Labs, Logan, UT, USA
Insulin 5µg/ml	PromoCell, Heidelberg/Germany
bFGF 1ng/ml	PromoCell, Heidelberg/Germany
Gentamicin 50ng/ml	PromoCell, Heidelberg/Germany
Amphotericin B 50ng/ml	PromoCell, Heidelberg/Germany

Basal Medium	Producer/Company
DMEM	Life Technologies, Karlsruhe, Germany
Supplements	
FCS 10 %	Hyclone Labs, Logan, UT, USA
Glutamine 2mM	Life Technologies, Karlsruhe, Germany
Sodium pyruvate 1mM	Life Technologies, Karlsruhe, Germany
1x NEAA	Life Technologies, Karlsruhe, Germany

Reagents	Producer/Company
PBS	Life Technologies, Karlsruhe, Germany
HBSS	Life Technologies, Karlsruhe, Germany
Trypsin/EDTA	Life Technologies, Karlsruhe, Germany
Trypan Blue solution (0.5%)	Life Technologies, Karlsruhe, Germany
Collagen R	Serva, Heidelberg, Germany
Matrigel	Serva, Heidelberg, Germany

All standard laboratory plastic ware, tissue culture flask and dishes of tissue culture grade were obtained from Nunc (Roskilde, Denmark). Culture dishes of microbacteriological grade and plastic pipettes were bought from Greiner (Solingen, Germany).

Incubators	Producer/Company
B6	Heraeus, Hanau, Germany
3164 Water Jacketed	Forma Science, Marietta, Ohio, USA

Centrifuges	Producer/Company
Centrifuge 5402	Eppendorf-Netheler-Hinz, Hamburg, Germany
Biofuge pico	Heraeus, Hanau, Germany
Rotina 48R	Hettich, Tuttlingen, Germany

Photometers	Producer/Company
BioPhotometer	Eppendorf-Netheler-Hinz, Hamburg, Germany
HT-2	Anthos Labtech, Salzburg, Austria

Microscopes	Producer/Company
Axiovert +	Zeiss, Jena, Germany
Mircomanipulator 5179	Eppendorf-Netheler-Hinz, Hamburg, Germany
Axioskop 2	Zeiss, Jena, Germany
BH-2	Olympus, Wuppertal, Germany
LSM 510	Zeiss, Jena, Germany
EM 10	Zeiss, Oberkochen, Germany

2.1.2 Bacteriological reagents

Reagents	Producer/Company
Agarose	Life Technologies, Karlsruhe, Germany
Ampicillin	Sigma Chemical Co, St. Louise, MO, USA
Bacto-Agar	Difco Labs, Detroit, MI, USA
Bacto-tryptone	Difco Labs, Detroit, MI, USA
Yeast Extract	Difco Labs, Detroit, MI, USA
LB-Medium	Composition
Ampicillin (50µg/ml)	For liquid medium mix tryptone, yeast extract, NaCl and adjust to 1l with distilled water.
Bacto-tryptone (10.0 g)	For solid LB-plates add agar.
Yeast Extract (5.0 g)	Autoclave 20 min. at 120°C
NaCl (5.0 g)	Let cool to 50°C and add Ampicillin (50µg/ml)
Bacto-Agar (15.0 g)	

2.1.3 Bacteria

Bacteria	Genotype	Producer/Company
E.coli TOP10F' K12-derived strain	F' {lacIq, Tn10(TetR)} mcrA Δ (mrr-hsdRMS-mcrBC) φ 80lacZ Δ M15 Δ lacX74 deoR recA1 araD139 Δ (ara-leu)7697 gal/U gal/K rpsL (StrR) endA1 nupG	Invitrogen, Leek, Netherlands

2.1.4 Plasmids

Plasmid	Producer/Company
pBluescript SK (+/-)	Stratagene, La Jolla, CA, USA
pcDNA3.1	Invitrogen, Leek, Netherlands
Tjmother	Viagene, San Diego, CA, USA
pEGFP/neo	Clontech, Palo Alto, CA, USA
	Dr. Dull at Cell Genesis Corp
	Dr. Dull at Cell Genesis Corp.
	Dr. Robins, Pittsburgh, USA

2.1.5 Molecular biological reagents

Enzymes	Producer/Company
DNATaq-Polymerase	Life Technologies, Karlsruhe, Germany
SuperscriptII Reverse Transcriptase	Life Technologies, Karlsruhe, Germany
RNase Inhibitor	Perkin Elmer, Hamburg, Germany
T4-Ligase	Boehringer, Mannheim, Germany

Restriction enzymes	Producer/Company
ClaI	Boehringer, Mannheim, Germany
EcoRI	Boehringer, Mannheim, Germany
BamHI	Boehringer, Mannheim, Germany
Sal I	Boehringer, Mannheim, Germany

Kit-systems	Producer/Company
BCA-Assay	Pierce, Rockfors, IL, USA
Endofree Plasmid Kit	Quiagen, Hilden, Netherlands
PolyTract 1000 mRNA	Promega, Madison, WI, USA
RNA-Clean	AGS, Heidelberg, Germany
ECLWestern blot detection	Amersham-Buchler, Braunschweig, Germany
Gel-Extraction-Kit	Quiagen, Hilden, Netherlands

Miscellaneous	Producer/Company
Oligo-dT	Perkin Elmer, Hamburg, Germany
dNTP's	Pharmacia, Uppsala, Sweden

2.1.6 Staining reagents

Reagents	Producer/Company
Mayers Hemalaun Sol.	Merck, Darmstadt, Germany
DAPI	Boehringer, Mannheim, Germany
Annexin V/Propidium iodine	Molecular Bioprobes, Eugene, OR, USA
Trypan Blue solution (0.5%)	Life Technologies, Karlsruhe, Germany
Vectastain/Elite	Vector Labs, Burlingame, CA, USA
Vectashild	Vector Labs, Burlingame, CA, USA
DAB	Vector Labs, Burlingame, CA, USA
Ethidium Bromide	Merck, Darmstadt, Germany
Coomassi-Blue	Merck, Darmstadt, Germany

2.1.7 Antibodies and recombinant proteins

Primary antibodies against cell-adhesion associated molecules	Producer/Company
E-Cadherin	Transduction Laboratories, Lexington, KY, USA
α -catenin	Transduction Laboratories, Lexington, KY, USA
β -catenin	Transduction Laboratories, Lexington, KY, USA
γ -catenin	Transduction Laboratories, Lexington, KY, USA
Integrin 1- β	Transduction Laboratories, Lexington, KY, USA
Integrin 6- β	Alexis, Grünberg, Germany
Integrin V- α	Alexis, Grünberg, Germany
Desmoglein	Transduction Laboratories, Lexington, KY, USA
Symplekin	Progen, Heidelberg, Germany
p120	Transduction Laboratories, Lexington, KY, USA
L1	Transduction Laboratories, Lexington, KY, USA
β -actin	Sigma, Saint Louise, MI, USA
Paxillin	Transduction Laboratories, Lexington, KY, USA
FAK	Transduction Laboratories, Lexington, KY, USA
Primary antibodies against cell-cycle associated molecules	
Cyclin B	Transduction Laboratories, Lexington, KY, USA
Cyclin D1	Transduction Laboratories, Lexington, KY, USA
Cyclin D3	Transduction Laboratories, Lexington, KY, USA
cdc2	Transduction Laboratories, Lexington, KY, USA
cdk2	Transduction Laboratories, Lexington, KY, USA
cdk4	Transduction Laboratories, Lexington, KY, USA
p21/Cip	Transduction Laboratories, Lexington, KY, USA
p27/Kip	Transduction Laboratories, Lexington, KY, USA
p16/INK	Transduction Laboratories, Lexington, KY, USA
Ki67	
Primary antibodies against differentiation marker antigens	
carbonic anhydrase II (CAII)	Dr. Nishimori, Kochi, Japan
mucin-1	Prof. Olijia Finn, Pittsburgh, USA
CEA	Ch. Wagener, UKE, Hamburg, Germany
BGP	M. Neumeier, UKE, Hamburg, Germany
NCA 95	Ch. Wagener, UKE, Hamburg, Germany

Primary antibodies against miscellaneous antigens	Producer/Company
HGF/SF c-met IL-13 c-Raf p53 VEGF GSK3- β TGF- β (pan-specific) G- γ 7 Epimorphin	R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany Pharmingen, San Diego, CA, USA Transduction Laboratories, Lexington, KY, USA Sigma, Saint Louise, MI, USA R&D Systems, Wiesbaden, Germany Transduction Laboratories, Lexington, KY, USA R&D Systems, Wiesbaden, Germany Santa Cruz Inc., Santa Cruz, CA, USA Y. Hirai, Sumitomo Industries, Yokohama, Japan
Rekombinant Proteins (cytokines, growth factors)	
TGF- α TGF- β HGF/SF TNF- α Epimorphin Activin A Inhibin Fas-Ligand TRAIL EGF bFGF IFN- γ	R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany Knoll/BASF, Ludwigshafen, Germany Y. Hirai, Sumitomo Industries, Yokohama, Japan R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany R&D Systems, Wiesbaden, Germany PromoCell, Heidelberg/Germany Knoll/BASF, Ludwigshafen, Germany

2.1.8 Cell lines

The following cell lines were used in this study and either bought from the American Type Culture Collection (ATCC) or established in our laboratory (see origin). The characterization in columns 2-3 was taken from the original publication or is based on personal communication with Prof. Dr. Klöppel (Institute of Pathology, CAU, Kiel, Germany).

Abbreviations: Pa.: Pancreas; Ca.: Carcinoma; PT.: primary tumor; AS.:Acites;

LM.: Liver metastasis; n.d.: not determined

Cell line	Primary tumor	Tumor-grading	
A818-2	Pa. Adeno-Ca., AS	n.d.	PhD-work Heringlake et al. 1991
A818-4	Pa. Adeno-Ca., AS	highly differentiated; Grade I	Kalthoff et al., 1993 Oncogene. 8:289-298
A818-5	Pa. Adeno-Ca., AS	n.d.	PhD-work Heringlake et al. 1991
A818-6	Pa. Adeno-Ca., AS	highly differentiated; Grade I	Lehnert et al., 2000 Ph.D.-thesis
A818-7	Pa. Adeno-Ca., AS	highly differentiated; Grade I	PhD-work Heringlake et al. 1991
Capan-1	Pa. Adeno-Ca., LM	well differentiated; Grade II	ATCC, HTB 79
Capan-2	Pa. Adeno-Ca., PT	highly differentiated; Grade I	ATCC, HTB 80
Panc-1	Pa. Adeno-Ca., PT	low differentiated; Grade II-III	ATCC, CRL-1469
BxPC3	Pa. Adeno-Ca., PT	medium differentiated; Grade II	ATCC, CRL-1687
AsPC-1	Pa. Adeno-Ca., AS	medium differentiated; Grade II	ATCC, CRL-1682
Kif-5	skin-fibroblasts		own laboratory
PTF-994	Pa.-fibroblasts		own laboratory
293T	fetal-kidney-cell		ATCC, CRL 1575
L-428	Hodgkin´s disease		DSMZ, Braunschweig, Germany DSM ACC 197

2.1.9 Animals

Mice	Producer/Company
SCID-bg	Charles-River; Sulzfeld, Germany

2.1.10 Chemicals

All fine chemicals were obtained from the following Companies and their grade of purity was at least *per analysis*.

Merck, Darmstadt, Germany

Sigma-Aldrich, Saint Louise, MI, USA

Serva, Heidelberg, Germany

2.2 Methods

2.2.1 Standard cell culture

Cell lines were cultured in RPMI 1640 supplemented with 10 % FCS, 2mM glutamine, 1mM sodium pyruvate, 100 units penicillin/ml, 100 µg streptomycin/ml. 293T- cells were cultured in DMEM supplemented with 10 % FCS, 2mM glutamine, 1mM sodium pyruvate, 1x non essential amino acids (NEAA). Fibroblasts were grown in Fibroblast Growth Medium containing all supplements and 10% FCS. Prior to immunoprecipitation experiments tumor cells and fibroblasts were cultured under serum-free conditions for 24 h. All cell lines were routinely checked for mycoplasma contamination by the PCR-based mycoplasma detection kit from TaKaRa (by Bio Whittaker Europe, Taufkirchen/Germany).

Fresh medium was added every second or fourth day, depending on the cell line. Old medium was discarded, cells were washed with PBS (pre-warmed to 37°C) once to remove all cell debris and new medium (pre-warmed to 37°C) was added (15 ml/T75-flask). Cells were split after they reached a confluency of approximately 80%. The old medium was discarded, cells were washed with PBS and Trypsin/EDTA-solution (0.25% Trypsin/ 0.01% EDTA) was added. Cells were incubated at 37°C for 5-30 min., depending on the cell line. Trypsin-activity was stopped by adding an equal amount of medium and afterwards cells were centrifuged at 1,000 rpm for 5 min. The supernatant was discarded, cells were resuspended in fresh medium

and seeded into a new cell culture flasks. The average splitting-ratio was 1:3. Prior to some experiments cells were counted in a Neumeier-Chamber using Trypan Blue solution (0.5% in PBS) to discriminate living from dead cells.

For long-term monolayer culture, cells were grown in T75 cell culture flasks. For long-term storage cells were frozen in liquid nitrogen. The storage solution consisted of FCS/10% DMSO.

2.2.2 Three-dimensional cell culture

2.2.2.1 Suspension on solid agarose

For three-dimensional growth $1-5 \times 10^5$ cells were seeded into pre-coated 6-well plates. The coating solution contained agarose at 31mg/ml mixed with RPMI 1640 (without supplements, pre-warmed to 37°C) at a ratio of 1:3. 1ml was added to each well. The agarose-solution was autoclaved prior to use. This coating prevented cell adhesion to the tissue culture dishes. Cells were seeded in a total volume of 5 ml cell culture medium per well. As it was impossible to remove all medium without losing the cells in suspension, new cell culture medium was added by removing 2-3 ml of the old medium carefully and replacing it with fresh one.

2.2.2.2 Collagen type I gels

10 ml NaOH (0.34 M) were added to 20 ml of ten-fold concentrated RPMI 1640 medium and used as stock solution. In 0.4 ml of this solution 5×10^4 - 1×10^5 cells were resuspended, mixed with 1.7ml of collagen-solution and poured into one well of a 6-well plate. The gel became solid after 15 min. and was overlaid with 2 ml of cell culture medium.

2.2.2.3 Matrigel

Matrigel is a complex mixture components of mammalian extracellular matrix derived from Engelbreth-Holm-Swarm tumor cells. All pipettes and culture dishes were cooled to -20°C prior to use. The cell culture medium was placed on ice one hour before use. Matrigel was carefully thawed on ice. 5×10^4 - 1×10^5 cells or A818-6 hollow spheres were quickly resuspended in 2 ml of cold medium and mixed with 2 ml of Matrigel. 2 ml of this solution was poured into one well of a 6-well plate and incubated at 37°C for 30 minutes. After Matrigel became solid, 2-3 ml of cell culture medium were added.

2.2.3 Co-cultivation of tumor cells with fibroblasts

Fibroblast co-cultures were carried out with dermal-derived-fibroblasts (KIF-5) and pancreas-derived fibroblasts (PTF994). These fibroblasts were co-cultured with the tumor cell lines A818-6, Capan-1, Capan-2, BxPC3 and Panc-1. In a first experimental set-up, fibroblasts were mixed at a ratio of 1:1 with tumor cells after trypsinization. The mixture was seeded into agarose-coated culture-dishes. As a control, tumor cells were seeded without fibroblasts. In a second experimental set-up, fibroblasts were seeded under standard cell culture conditions and grown in monolayer culture. After reaching confluency, freshly trypsinated tumor cells were seeded on top of these fibroblast monolayer cultures and were incubated for 48 h. In a third set-up, A818-6 pre-mature hollow spheres (5-6 days old) were seeded on top of fibroblast monolayer cultures. Two days before co-culturing fibroblasts with tumor cells, fibroblast were adapted to RPMI 1640 medium by mixing both media at a ratio of 1:1. At the day of the experiment both, fibroblasts and A818-6 cells were incubated in RPMI 1640 medium. Fibroblast-free protein from fibroblast-stimulated A818-6 hollow spheres was obtained by transferring hollow spheres to non-coated tissue culture plates. Fibroblasts adhered to this plates but hollow spheres did not.

2.2.4 Immunostaining

2.2.4.1 Immunohisto-/cytochemistry

For immunohisto-/cytochemistry either human or murine pancreatic tissue or cell lines were used. After surgical treatment of patients or mice, one fraction of the resected pancreatic tissue was immediately frozen in liquid nitrogen. Another fraction was inspected by pathologists to determine tumor-type and grade. Prior analysis 5 mm blocks of tissue were cut of the primary tissue block and cut in 5µm-thin slices using a cryotom. Three to five of this slices were mounted on a microscopic slide, air-dried for at least 3 h and fixed in acetone for 5 min. Cells from cell lines were harvested by trypsinization or scraping and cytocentrifugation onto microscopic slides. Completely developed hollow spheres were centrifuged onto protein pre-coated microscopic slides from Marienfeld (Darmstadt, Germany). Cells on microscopic slides were fixed by incubation with acetone for 5 min. Fixed tissue and cells were stored at -20°C in plastic bags under vacuum conditions.

Prior analysis, the frozen samples were allowed to reach room temperature and were re-hydrated in PBS. Staining was then carried out by incubating for 1 h with a primary antibody followed by three washes with PBS. Primary antibodies were used in concentrations ranging from 5-20 µg/ml. The incubation time for the secondary, horseradish peroxidase-conjugated antibody was 30 min. The secondary antibodies were used at concentrations recommended by the supplier and were diluted in PBS containing 1% human serum. Primary antibodies were diluted in PBS/1% bovine serum albumin (BSA). Detection was carried out with diaminobenzidine (DAB) as a substrate for the horseradish peroxidase resulting in a brown precipitate in case of a reaction. All samples were counter-stained with hemalaun which stains nuclei of cells blue.

Evaluation of results with the antibodies KiS5 and KiS2 was carried out microscopically by counting positive (brown) cells and negative cells (blue). 200 to 300 cells were counted in total, repeated three times. The medians and standard deviations were calculated and results expressed as "% of positive/negative cells".

2.2.4.2 Immunofluorescence

The indirect immunofluorescence staining was performed in 5 ml plastic tubes from Nunc (Denmark). Monolayer cells were scraped off the plates, transferred to tubes and centrifuged. Hollow spheres were directly transferred into the tubes. The supernatants were discarded and a single PBS wash was performed. 2 ml of ice cold methanol (70%) was added to pre-fix and permeabilize cells followed by incubation on ice for 3 minutes. All cells were then washed with ice cold PBS containing 0.05% sodium azide three times and stained with the primary antibody for 1h. All primary antibodies were used in concentrations ranging from 5-20 µg/ml. After washing three times with ice cold PBS, incubation with the secondary antibody (Cy3-conjugated goat-anti-mouse IgG+IgM) was carried out for 30 min. A final fixation step was carried out by adding 4% Paraformaldehyde to the stained cells after washing. Primary antibodies were diluted in PBS/1% BSA. The secondary antibodies were used at concentrations recommended by the supplier and were diluted in PBS containing 1% human serum. Visualization was carried out with an Axioskop2-fluorescence-microscope for epifluorescence or with a laser-scanning microscope LSM 510 for confocal microscopy both from Carl Zeiss Jena, Germany.

2.2.5 Electron microscopy

Electron microscopy was performed by Prof. Markus M. Lerch at the University of Münster, Germany as part of a cooperation within the DFG-grant this work was founded by.

For transmission electron microscopy confluent cells were harvested by scraping and cells or hollow spheres in suspension were concentrated by 100 g centrifugation, fixed in ice cold 2% glutaraldehyde/2% formaldehyde solution at pH 7.4 with 0.1M cacodylate buffer. Fixed cells and spheres were post-fixed in 1% osmiumtetroxide and embedded in Epon. Silver thin sections were contrasted with uranyl and lead and viewed on a Zeiss EM 10 electron microscope.

2.2.6 Cytokine and growth factor treatment of A818-6 cells

Cytokines, growth factors and apoptosis-inducing factors were either added to freshly seeded A818-6 cells or completely developed hollow spheres (for concentrations compare Tab.1 in Results). In a second experimental setting a HGF-neutralizing antibody or a epimorphin-neutralizing antibody (MC-1) were used in a concentration of 50 µg/ml to neutralize exogenously added HGF/epimorphin or to block intrinsic HGF/epimorphin produced by the cells. All cytokines and corresponding antibodies were added on day 2, 5 and 7 after seeding and were replaced together with fresh cell culture medium. These experiments were performed in 6-well plates and the cultures were checked by microscope daily.

2.2.7 Transfection of established cell lines and generation of transient VSV-G pseudotyped retroviral supernatants

The established "producer cell line" for the production of retroviral supernatants 293T was used to generate retroviruses carrying the gene encoding for the "enhanced green fluorescent protein" (EGFP). These retroviruses were used to transduce fibroblasts and A818-6 cells enabling them to express the EGFP-gene and to become fluorescent under UV-light.

24 h prior to transfection, 1×10^6 293T cells were seeded into Primaria plates (Falcon # 3803). 10 mg of each retroviral plasmid vector (pEGFP/neo), 7 mg of the packaging plasmid pKAT 1.gag/pol.ATG and 7 mg of pCMV-VSV-G were then co-transfected into 293T cells using calcium phosphate co-precipitation (Wigler et al., 1979). The next day, fresh medium was added and viral supernatants were harvested 24 and 48 h later, filtered through 0.45 µm low protein binding Acrodisc™ filters (#4184, Gelman Sciences, Ann Arbor, MI) and stored at -80°C.

2.2.8 Transduction of A818-6 cells and fibroblasts

1 x 10⁵ A818-6 cells and Kif-5 fibroblasts were seeded into 6 cm² dishes containing complete medium and 8 mg/ml polybrene. 24 h later, pEGFP/neo retroviral supernatants generated from 293T producer cell line were used to infect these cell lines at a multiplicity of infection (MOI) of 10. For long term evaluation of gene expression and pro-vector copy analysis, pEGFP/neo-transduced A818-6 cells were selected in G418 for two weeks using 1,000 µg/ml. For fibroblasts 500 hollow spheres µg/ml G418 were used for selection. After successful transduction the retroviral mRNA is converted into DNA by the virus reverse transcriptase and integrated into the host-genome leading to a permanent expression of the EGFP-protein under a CMV-promotor which is part of the retroviral construct.

2.2.9 Proliferation assays with A818-6 cells

2.2.9.1 KiS5/KiS2-staining

KiS5 and KiS2 are antibodies which were developed by the group of Prof. Parwaresch at the Institute of Pathology, CAU, in Kiel. KiS5 detects the proliferation associated antigen Ki67 being expressed in all phases of the cell cycle except the G₀-phase. KiS2 binds to a 100-kd nuclear protein expressed in S, G₂, and M phases of the cell cycle but not G₁/G₀ (Heidebrecht, 1997 and Rudolph, 1998).

2.2.9.2 Telomerase assay (telomeric repeat amplification protocol; TRAP-assay)

The telomerase assay was performed under guidance of Dr. Klaus Heidorn from the Institute of Pathology, CAU in Kiel as previously described (Klapper et al., 1998; Krupp et al., 1997). Protein was extracted from A818-6 hollow spheres or from A818-6 monolayer cells using standard CHAPS buffer (Krupp et al., 1997). A 5'-fluorescein labeled reverse primer "CX-ext" was used to obtain fluorescence-labeled PCR products. The inclusion of an internal amplification standard (ITAS) permitted quantitative comparisons, and ITAS was prepared by PCR essentially as described (Wright et al., 1995). As template the gene of the RNA component of *Thermus aquaticus* RNase P was used (Hartmann and Erdmann, 1991; Klapper et al., 1998). The

values shown in Results were obtained by comparison with a calibration curve for extracts (0.04-0.2 µg) from the Hodgkin's disease human tumor cell line L-428. Aliquots of the TRAP-assay (2 µl) were analyzed by capillary electrophoresis (ABI prism 310). One example of results is shown below (Fig. 6). Integrated values were added up for all telomerase products (black peaks) representing five telomeric hexamer repeats and calibrated by dividing by the value for ITAS. Values and standard deviations calculated from all data are summarized in a graph in Results (Fig.11). The raw data of this experiment are listed in the addendum to Results.

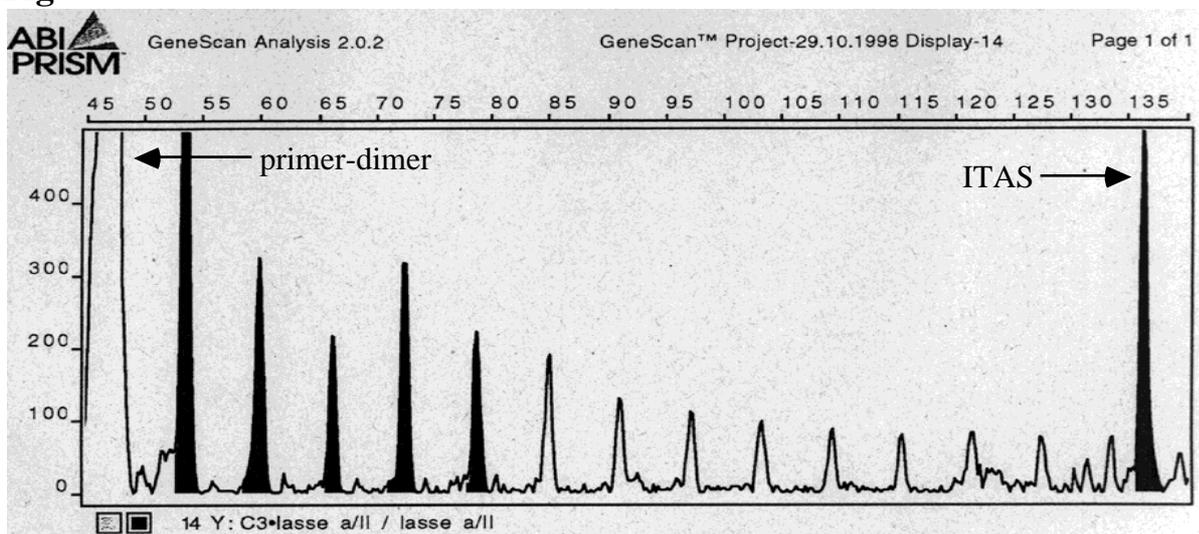
Fig.6

Fig. 6: Example for one measurement of telomerase activity in A818-6 monolayer cells.

2.2.9.3 Analyses of cell cycle associated proteins

Cell cycle associated proteins were analyzed by Western blot as explained under point 2.2.11. The amounts of different cell cycle associated proteins were compared between A818-6 monolayer cells and A818-6 hollow sphere cells using monoclonal antibodies to detect these proteins in protein-lysates.

2.2.10 SDS/Polyacrylamide Gel Electrophoresis

Sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) is a method used to fractionate molecules according to their size. SDS-PAGE was used to separate the different proteins in protein-lysates obtained after the lysis of cells from each other following the following protocol: (Laemmli)-gel preparation. Volumes given are sufficient for small (8 cm x 10 cm x 0.75 mm) gel format (10 ml). Volumes were scaled up as needed.

1. Pouring the Separating Gel

A gel-system from Biometra was used. First a separating gel was prepared by mixing the components given below. TEMED was added just prior pouring the gel. This separating gel was overlaid with n-butanol and allowed to polymerize. A stacking gel of 4% was poured on top of the separating gel. A comb with 10 slots was inserted before the gel polymerized.

15% Separating-gel	Composition
distilled water	2.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
20% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	5.0 ml
10% (w/v) ammonium persulfate	0.05 ml
TEMED	0.005 ml
Total Volume	10.005 ml

4% Stacking-gel	Composition
distilled water	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate	0.025 ml
TEMED	0.005 ml
Total Volume	5.05 ml
Sample-buffer (Laemmli) 4x	Composition
distilled water	4.0 ml
0.5 M Tris-HCl	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
beta-mercaptoetanol	0.4 ml
0.05% (w/v) bromophenolblue	0.2 ml
5x Running buffer, pH 8.3 (1 liter)	Composition
Tris Base	15g
Glycine	72g
SDS	5g
	distilled water to 1 liter

Sample-preparation and electrophoresis:

The Biometra electrophoresis chamber was assembled according to the manufacturers instruction and filled with 1x running buffer. The comb with 10 slots allowed to load up to 40µl of probe per slot. Protein-lysates were mixed with the concentrated sample-buffer at a ratio of 4:1, boiled at 95°C for 5 min. and then directly loaded on the gel. The run was started with applying 60W until all samples were collected at the border between stacking- and separation-gel. Separation was carried out at 120W for approximately 2 h. The blue color bromophenol blue was used as a marker for the migration-distance of probes. After the run the glass plates were carefully removed and the gel was prepared for blotting or staining e.g. with Coomassi-blue.

Coomassi-blue staining of PAGE-gels

Gels were transferred into Coomassi-blue solution (approximately 200 ml), shaken gently and incubated for up to one hour at RT. Destaining was performed by transferring the gel into destaining buffer. After replacing the buffer up to three times protein bands are visible without background.

Staining-solution	Composition
Coomassie Blue R-250 Ethanol Water Acetic acid	0.25g 100 ml 100 ml 25 ml Dissolve the dye completely and then add acetic acid. Fill up to 250 ml with water.
Destaining-solution	Composition
Ethanol Acetic acid	400 ml 100 ml Fill up to 1,000ml with water and store at RT

*2.2.11 Western blot analysis*Buffers:

Blotting-buffer (10x)	Composition
Tris-Base Glycin Methanol	121.14 g 142.6 g 20% (v/v) Dissolve Tris and Glycin in 800 ml of distilled water and fill up to 1 liter. Add methanol when preparing 1x buffer by mixing 1part of 10x buffer, 7 parts distilled water and 2 parts of methanol.
PBST	Composition
PBS Tween 20 Thimerosal	400 ml 100 ml 0.01% (w/v)

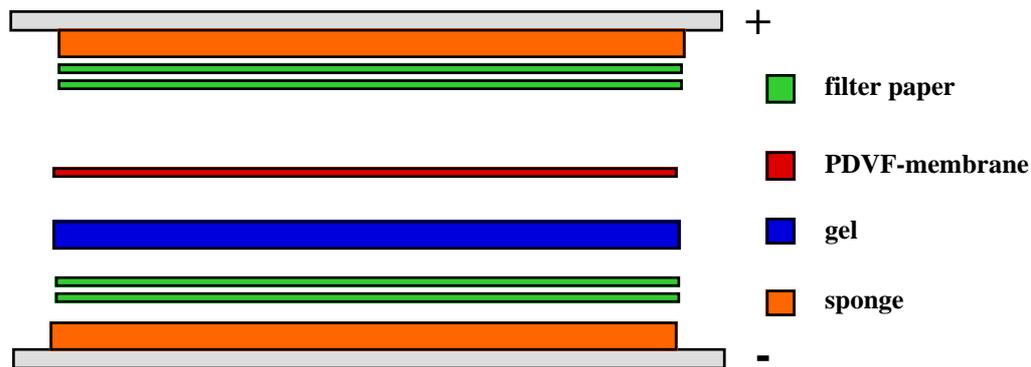
Blocking-buffer	Composition
PBST skim milk powder	100 ml 5g Dissolve milk powder in 100 ml
RIPA-buffer	Composition
PBS NP40 Sodium-deoxycholate SDS Protease Inhibitor (Complete Stock Boehringer)	1.0% 0.5% 0.1% 1 tablet added to 50 ml

Protein Extraction from adherent cells and A818-6 hollow spheres

Cells were washed with PBS once and 500 μ l RIPA-buffer was added per well of a 6-well plate. 3 ml were used for a T25-flask. All steps were carried out on ice. After lysis, cell debris was removed by centrifugation at 14,000 rpm for 15 min. The supernatant was either aliquoted and stored at -80°C or a BCA-assay was performed to measure the total amount of protein in a probe. The BCA-assay was obtained from Pierce, Rockfords, IL, USA and performed as recommended by the manufacturer. The results were obtained as colorimetric values measured by a photometer (anthos II). The concentrations were determined using a titration of bovine serum albumin from 1,000 $\mu\text{g}/\text{ml}$ to 15.625 $\mu\text{g}/\text{ml}$ total protein. A818-6 hollow spheres were centrifuged, the pellet was washed with PBS. After further centrifugation the supernatant was discarded and cells were lysed in RIPA-buffer added to the centrifugation tube.

SDS-PAGE

After the protein concentration was determined SDS-PAGE was performed as described above to separate the proteins by size. In general, 20 μg total protein was loaded per slot. A protein-size marker (RainbowTM, Amersham, Braunschweig) was used to check the correct sizes of the expected protein bands after Western blot analysis.

Western blot assembly and performance

Filter paper was obtained from Whatman and the PDVF-membrane (polyvinylidene difluoride membrane) was bought from Immobilon P, Millipore Corp. Bedford, USA.

Prior to blotting the PDVF-membrane was washed in methanol for 5 minutes followed by distilled water for another 5 min. and blotting buffer. Filter paper was pre-wet in blotting buffer. The blot was assembled as shown above and the transfer was performed at 400 mA for 1.5 h. After proteins were transferred to the PDVF-membrane the membrane was incubated in blocking buffer at 4°C over night to block all unspecific binding-sides. The transfer efficiency and later the correct protein size was checked by using a Rainbow™ protein marker.

Detection

After blocking the membranes with blocking buffer they were washed in PBST and then incubated with the primary antibody. Primary antibodies were diluted in 10 ml PBST/1% skim milk in concentrations of 5-10 µg/ml. Incubation with the primary antibody was carried out at RT for an hour and gentle agitation. Subsequently the membranes were excessively washed with PBST and the secondary antibody was added. Secondary antibodies were diluted in 10 ml PBST/1% skim milk as well and were conjugated with the enzyme horseradish-peroxidase (HRP). Incubation was carried out for 30 min. at RT and gentle agitation followed by excessive washes with PBST. The membranes were placed in plastic covers and ECL-solution was added as recommended by the manufacturer. ECL is a substrate for HRP and its metabolism results in light-emission which can be detected with a x-ray film (Kodak Biomax). All blots

were normalized with an antibody against β -actin (42kDa) to prove the loading of equal amounts of protein per lane.

2.2.12 Immunoprecipitation

Immunoprecipitation was performed with supernatants from A818-6 hollow spheres or monolayer cultures, fibroblasts and co-cultures from hollow spheres and fibroblasts. 1-3 μ g of primary antibodies were added to approximately 2 ml of cell free supernatant and rotated at 4°C overnight. Protein-G-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was equilibrated for the corresponding cell culture medium over night and then added to the primary-antibody solution followed by rotation for 30 min. at 4°C. Sepharose-beads were collected by centrifugation at 14,000 rpm for 2 min. and washed four times with TNE-buffer. The pellet was then resolved in 1x Laemmli-buffer, boiled for 5 minutes at 95°C and immediately loaded onto a 15% SDS-PAGE-gel for separation. Detection was carried out as described for Western blot analyses.

TNE-buffer	Composition
Tris pH8,	0.5M
NaCl,	0.15M
NP40,	0.1%
EDTA	0.125M

2.2.13 RNA- and mRNA-isolation from cultured cells

RNA-isolation

Total-RNA was isolated from adherent cells and A818-6 hollow spheres using the RNA-Clean™ system combining the classical "Guanidin-thiocyanate method" with a Phenol-chloroform extraction to a "single-step method".

Cells grown in 6-well plates were lysed with 1ml of RNA-Clean per well on ice, 6 ml were used for T75-flasks. The viscous solution (genomic DNA) was transferred into 2 ml Eppendorf-tubes and 100 μ l chloroform was added per ml of lysate. The mixture was vortexed for 15 seconds and incubated on ice for 10 min. followed by centrifugation at 14,000 rpm for 5 min.

The clear, upper phase was transferred into a new tube taking care of not pipetting material from the interphase which contains proteins and carbohydrates. An equal volume of iso-propanol was added and the tubes were placed on ice for 30 min. after a gentle agitation. The following centrifugation was performed at 14,000 rpm for additional 30 min. After this step a RNA-pellet is visible at the bottom of the tube. The supernatant was discarded and the pellet was washed with icecold ethanol (70%) to remove salts. This step was repeated two times followed by air-drying of the pellet. RNA-pellets were stored dry at -80°C. Prior use, pellets were re-hydrated in 30-50 µl distilled, RNase-free water or TE-buffer (100mM Tris-HCl, pH7.5, 10mM Na₂EDTA).

mRNA-isolation

The PolyTract 1000™ mRNA Kit from Promega, Madison, WI, USA was used for mRNA-isolation from total RNA. This system uses magnetic beads conjugated with oligo-dT strands. These oligo-dT strands anneal with the 3'-polyA-tails of mRNA. The beads with the “caught” mRNA are collected with a magnet washed with SCC-buffer and eluted with water. The isolation was carried out strictly following the manufacturers manual using between 0.1-1mg of total RNA prior isolated with RNA-Clean™.

Agarose gel electrophoresis for RNA

CAVE: Use only RNase free glassware and plastics! Work with gloves! Prepare all solutions using DEPC treated and sterilized miliQ water.

2-3 g agarose were dissolved in 170 ml DEPC treated water, 20 ml 10 x MOPS were added and the solution was cooled in a waterbath to 50 °C. Formaldehyde was filtered over Whatman 3MM and 10.2 ml were added to agarose. The gel was poured immediately. 25 µl sample buffer was added to the RNA samples (1-3µg poly(A) or 10-30µg total in 5 µl DEPC water). The samples were heated for 15 min. at 65 °C, 1 µl ethidiumbromide was added and loaded on gel. The run was stopped after bromophenol blue had migrated 10 cm. The gel was photographed on UV transilluminator without further staining.

10 x MOPS/EDTA	Composition
MOPS Na-acetate EDTA	0.2 M 50 mM 10 mM
Sample buffer	Composition
10xMOPS deionised formamide formaldehyde DEPC water glycerol bromophenol blue (10% solution)	150 μ l 750 μ l 240 μ l 100 μ l 100 μ l 80 μ l Keep in small portions frozen at -20 °C.

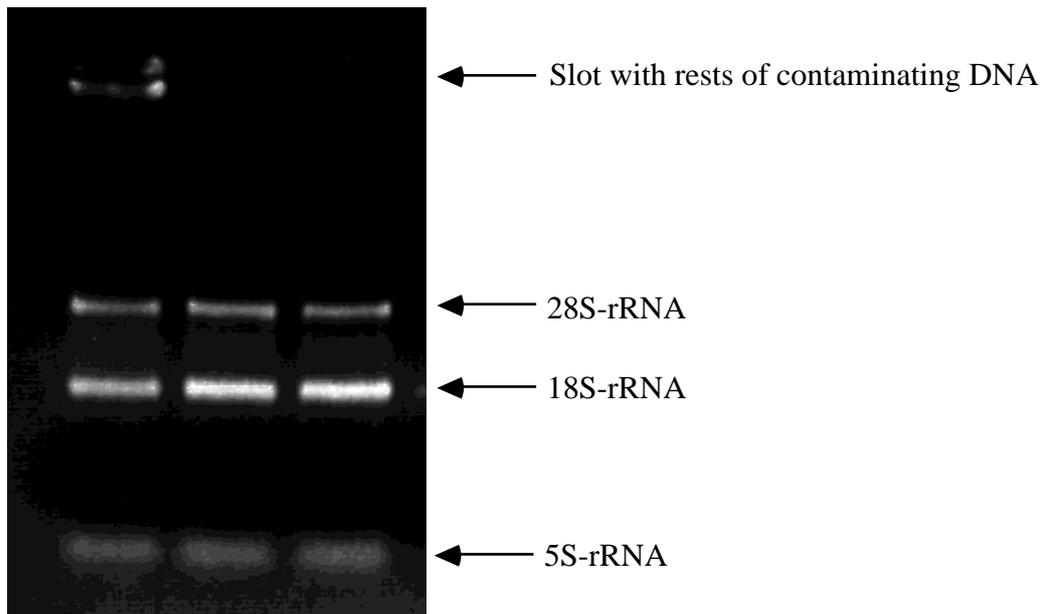
Figure 7

Fig.7. Representative picture of a 1%-RNA-gel with ribosomal RNA bands

2.2.14 Polymerase chain reaction (PCR)

PCR has been used as a standard method for the amplification of DNA since years and many different protocols are used. Therefore a standard PCR protocol is explained here and details different from this protocol are described in the corresponding result-section. PCR-reactions

were performed in 50 µl volumes. All PCR reactions were carried out as "hot-start" PCRs. This modification reduces unspecific annealing of primers in the first reaction-cycle. Subsequently, a 50 µl-reaction was set up in two reaction tubes. The first contained PCR-buffer, dNTPs, MgCl₂ and template-DNA, the second tube contained PCR-primers and Taq-polymerase. The mix in the first tube was covered with a drop of mineral oil, placed in the PCR-cycler and heated to 80°C. This temperature was kept for 5 min. and the content of tube number two was added.

Thermal cycling was carried out as follows: denaturation at 95°C for 1min., annealing at 69°C for 1 min., and extension at 72°C for 2 min. Every third cycle the annealing temperature was reduced by 2°C until the temperature of 59°C was reached at which 30 cycles were performed. A second variant of cycling was used which only differed by the annealing temperatures. In this variant the temperature was lowered from 65°C to 55°C. All PCR-products were analyzed by electrophoresis through 1% agarose gels. Some of the products were cut out of the gel, eluted from the agarose using a gel-extraction kit (Quiagen Hilden, Netherlands) and either subjected to restriction enzyme digestion or again used as template for PCR.

2.2.15 Reverse Transcription PCR (RT-PCR)

For analyses of RNA-transcripts an amplification of the material is often essential. Therefore the RNA is converted into cDNA using the reverse transcriptase. This enzyme is a RNA-depending DNA-polymerase synthesizing a complementary DNA from a RNA-template. Commonly MMLV-RTase from the *Moloney murine leukemia virus* is used which lacks RNase H-activity and has its optimal temperature at 42°C. In this study the SuperScript™II reverse transcriptase was used. Oligo (dT)₁₂₋₁₈ primer were used to prime all RT-reactions to select for the poly-A tails of mRNA.

RNA (1-5 µg) and 1µl of Oligo (dT)-primer were mixed in an Eppendorf tube, filled up to 12 µl with RNase-free water and incubated at 70°C for 10 min. to denature possible secondary structures. After this step the tube was immediately placed on ice. 4 µl 5xRT-buffer, 1 µl dNTPs and 2 µl DTT were added and the tube was incubated at 42°C for 2 min. followed by the addition of 1 µl SuperScriptII to start the reverse transcription. Incubation at 42°C was

carried out for 1 h followed by 15 min. inactivation at 70°C. Depending on the expected amount of transcribed cDNA, 1-5 µl of the reaction were used as a template in the PCR.

Desoxyribonucleotide-mix	Composition	End-concentration
dATP 100mM	10 µl	10 mM
dCTP 100mM	10 µl	10 mM
dGTP 100mM	10 µl	10 mM
dTTP 100mM	10 µl	10 mM
distilled water	60 µl	
Mastermix for a 50µl-reaction	Composition	End-concentration
10xPCR-buffer/-Mg ⁺⁺	5 µl	1x
10mM dNTPs	1 µl	0.2 mM
50mM MgCl ₂	1.5µl	1.5 mM
DNA Taq-Polymerase	5 µl	2.5 Units
Template DNA	2 µl	
Primer each 10µM	1µl each= 2µl	each 0.2µM
distilled water	38 µl	

2.2.16 Restriction enzyme digestion

Digestion of PCR-products or genomic DNA was performed with restriction enzymes from Boehringer, Mannheim, Germany and the corresponding buffers (composition of all restriction enzyme buffers are listed in the Boehringer Catalogue 2000). Reactions were set up in 20 µl-scales as follows: 2 µl 10x restriction enzyme buffer, 2-10 µg DNA in 10 µl TE buffer, 1 µl enzyme, 7 µl distilled water. This reaction mix was incubated at 37°C for 2 h. The products were analyzed by electrophoresis through 1% agarose gels.

Agarosegels for analyses of PCR-products or digestions were prepared as follows:

1g of agarose was dissolved in 100 ml 1xTBE-buffer by cooking in a microwave. After the solution had cooled to 60°C, 4 µl ethidium bromide (10 mg/ml) were added and the gel was poured immediately.

3 Results

3.1 A818-6 hollow sphere development analyzed by light microscopy

Under standard cell culture conditions, A818-6 cells grew as a monolayer culture (Fig. 8a) with a doubling time of 69 h. When adherence of these cells to culture substratum was prevented by coating tissue culture plates with solid agarose, hollow sphere development was observed within a time frame of 10-14 days after the initial seeding. The development of hollow spheres was subdivided into three phases: i) initiation ii) maturation and iii) senescence. The phase of initiation proceeded from the day of seeding to day 6-8 of the development. Signet-ring-like cells with an expanded vacuole (white arrows Fig. 8b) appeared. Six to eight days after seeding, "pre-mature" hollow spheres represented the predominant component of the culture (Fig. 8c). Other remaining structures were identified as compact spheroids (black arrow Fig. 8c) and signet-ring-like cells. The phase of maturation started after "pre-mature" hollow spheres were re-transferred into non-coated standard cell culture flasks. During the next 4-6 days, hollow spheres became more homogenous in size and shape (Fig. 8d). In comparison to earlier stages of the development, only few apoptotic cells were visible inside the hollow spheres. In contrast to other remaining cell clusters, hollow spheres did not attach to the surface of tissue culture grade flasks. Only after mechanical disruption or trypsinization hollow sphere cells re-attached to the surface of the dish and grew again as a monolayer culture (Fig. 8e). These "re-monolayer" cells were able to develop new hollow spheres after they were subjected to three-dimensional growth conditions again. The A818-6 system can therefore be described as reversible. After more than three weeks in culture some A818-6 hollow spheres entered the phase of senescence. A high heterogeneity was observed when hollow spheres became senescent. While some began to alter their phenotype after three weeks, others remained unchanged for more than eight weeks and longer (Fig. 8f). In light microscopy cells of hollow spheres which became senescent appeared more condensed (blue arrow Fig. 8f). This process was observed for 2-3 days ending in a status of maximal condensation (red arrow Fig. 8e).

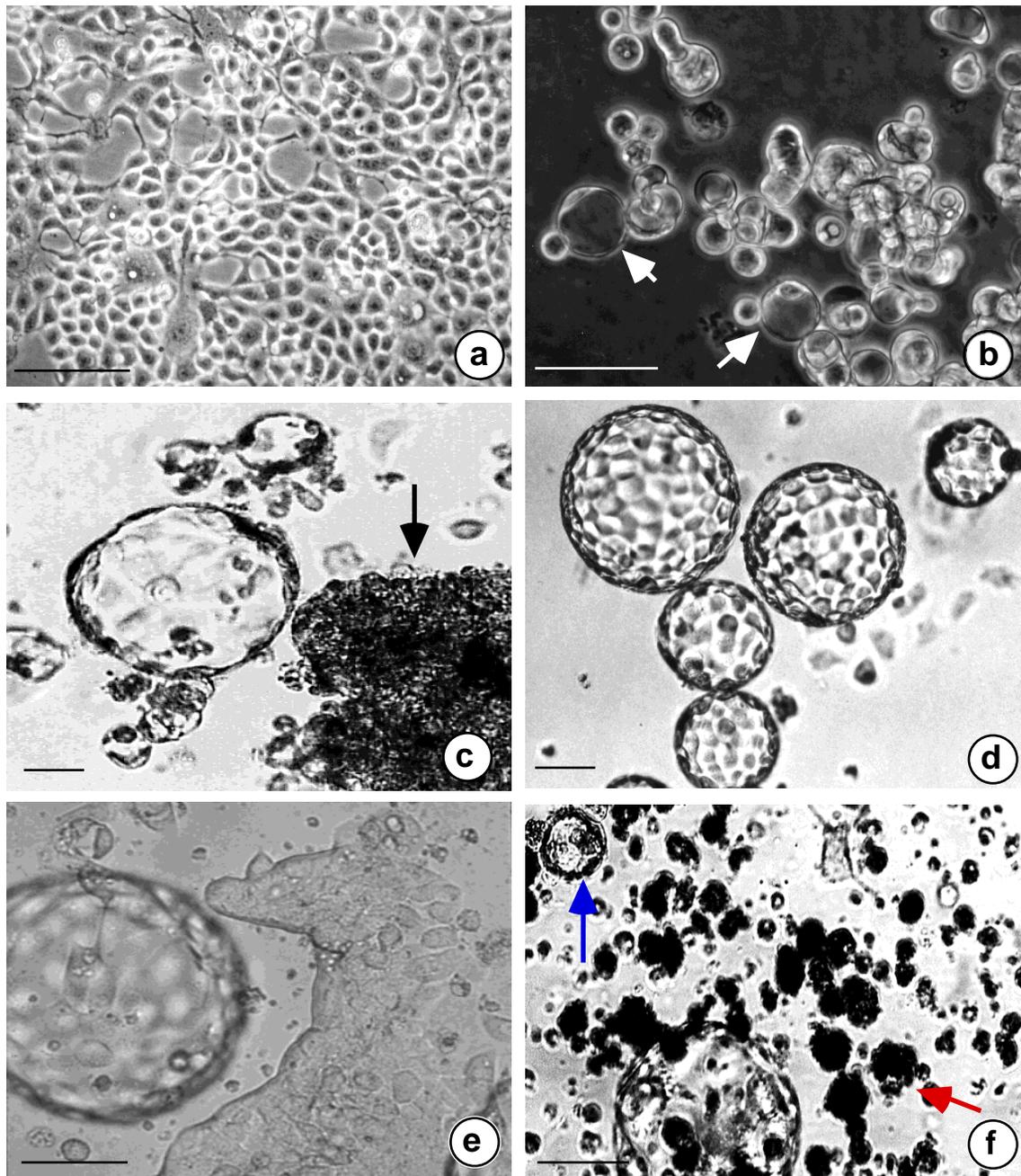
Figure 8

Fig.8: Analysis of A818-6 hollow sphere development by light microscopy. Panel a: A818-6 monolayer culture under standard cell culture conditions. Panel b: Signet-ring-like cells of A818-6 after 1-2 days on solid agarose. White arrows indicate typical cells. Panel c: A818-6 cells in three-dimensional culture after 5-6 days. Pre-mature hollow spheres next to remaining compact spheroid-structures (black arrow). Panel d: Mature A818-6 hollow spheres after 10-12 days in three-dimensional culture and re-transfer to uncoated cell culture plates. Panel e: A818-6 cells growing as re-monolayer after mechanical disruption of a hollow sphere. Panel f: A818-6 hollow spheres becoming senescent. The blue arrow indicates an early state, the red arrow the final state of the senescence-process. Bars indicate 50 μm .

Next to suspension cultures, A818-6 cells were grown in semi-solid support like matrigel and collagen I-gels. Embedded as monolayer cells they spread out and formed thin, tube-like structures as previously shown for the subclone A818-1 (Lehnert et al., 1999). Embedded as mature A818-6 hollow spheres they remained unchanged for more than two weeks (data not shown).

3.2 A818-6 hollow sphere development analyzed by electron microscopy

In electronmicroscopic analyses the initial step in hollow sphere development consisted of the formation of cell clusters with multiple cell-cell contacts between neighboring cells (Fig. 9a). These cell clusters no longer had any cell-substratum adhesions but were held together by multiple adherence junctions between A818-6 cells. Some of these cells contained large vacuoles and an either central or lateral space within the cluster formed and dilated progressively. In the next phase of development, mature hollow spheres were found abundantly now consisting of a single cell layer (Fig. 9b) which was sealed towards the outside of the sphere by clearly defined tight junctions between neighboring cells. No tight junctions sealing the internal surface of hollow-sphere-forming cells were detected but lateral cell-cell contacts corresponded ultrastructurally to adherence junctions. At the same time interval the plasmalemma facing the outside of the sphere had developed an often dense and highly developed surface structure of microvilli (Fig. 9c). During later phases of hollow sphere development occasional cell death by apoptosis was found. Cells undergoing apoptosis were initially found in situ within the single cell layer of hollow spheres still in contact with unaffected neighboring cells and were then apparently sequestered to the lumen of the sphere as indicated by apoptotic or partially digested cells in the sphere's lumen (Fig. 9d).

Overall, the whole process of A818-6 hollow sphere development was accompanied by a high amount of apoptosis. Approximately 50% of the initially seeded cells underwent apoptosis during the first two days of hollow sphere development.

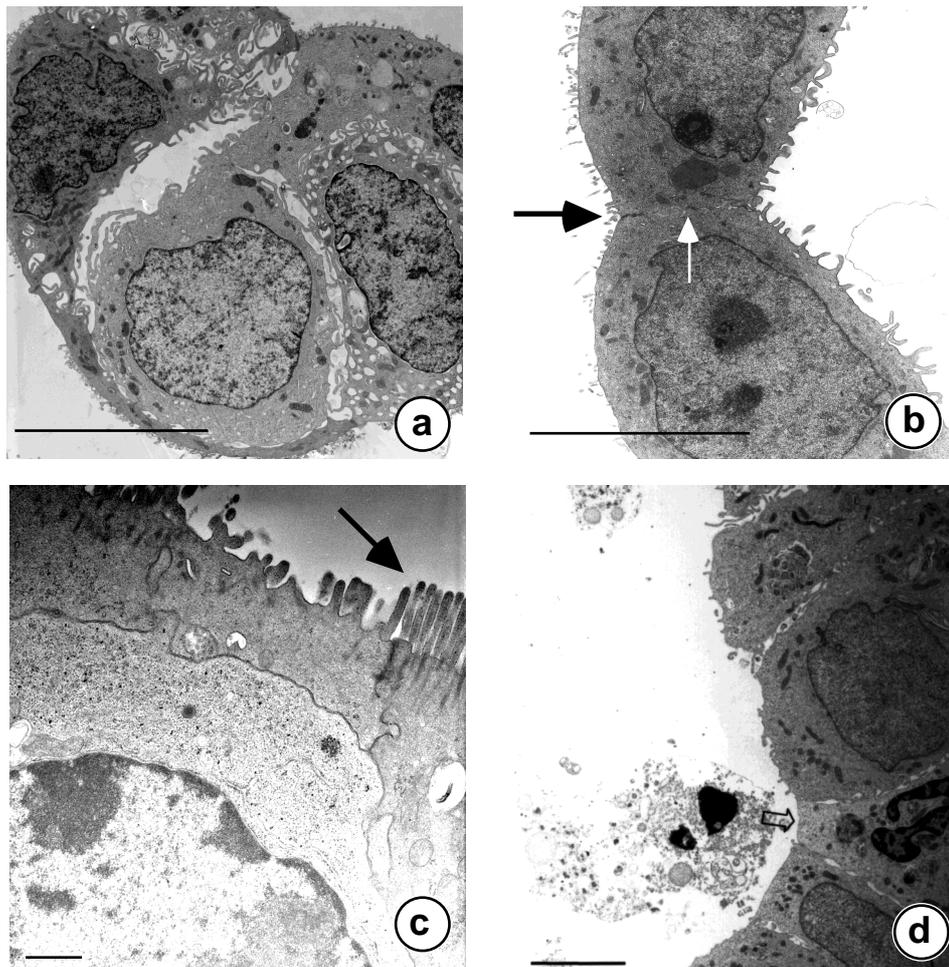
Figure 9

Fig. 9: Ultrastructural analyses at different stages of A818-6 hollow sphere development. After two days in culture, clusters of A818-6 cells formed with multiple cell-cell contacts between neighboring cells (panel a, bar =10 μ m). Mature hollow spheres (10-12 days old) developed tight junctions (black arrow, panel b) and adherence junctions constituting the polarized epithelial phenotype (white arrow, panel b). Bar =10 μ m. Highly developed microvilli were found on the apical surface of A818-6 hollow sphere cells being older than ten days (black arrow, panel c). Bar = 2 μ m. Cells with condensed chromatin but intact junctions to neighboring cells undergoing apoptosis were observed throughout the entire hollow sphere development. These cells were sequestered to the lumen of A818-6 hollow spheres (panel d) Bar =10 μ m.

3.3 Proliferation

3.3.1 KiS5/KiS2-staining

Proliferation of hollow sphere- and monolayer cells was monitored by immunoperoxidase staining for the proliferation-associated antigens Ki67 and p100 with the antibodies KiS5 and KiS2. The Ki67 antigen is detectable in all phases of the cell cycle except in the G₀-phase. Therefore, cells being negative for Ki67 are arrested in the G₀-phase of the cell cycle. The p100 antigen is expressed in all phases of the cell cycle except G₁/G₀. KiS2-staining was used to separate those KiS5-positive cells which are in the G₁-phase of the cell cycle from those being in S/M or G₂. Based upon these data a distribution of A818-6 monolayer and hollow sphere cell in the different phases of the cell cycle was evaluated and summarized in table 2. During hollow sphere formation a dramatic decrease in proliferation was observed. While 95% of A818-6 monolayer cells were positive for Ki67, only 31.5% of A818-6 hollow sphere cells were positive (Fig.10 a, b and table 2). This means approximately 70% of A818-6 hollow sphere cells are arrested in the G₀-phase of the cell cycle. Staining against the p100 antigen with the monoclonal antibody KiS2 revealed that 23.3% of the Ki67-positive cells in monolayer cells were in the G₂/S-or M-phase while only 2.2% of hollow sphere cells were found in these proliferative phases (Fig.10 c, d and table 2).

Figure 10 a KiS5-staining of A818-6 monolayer cells

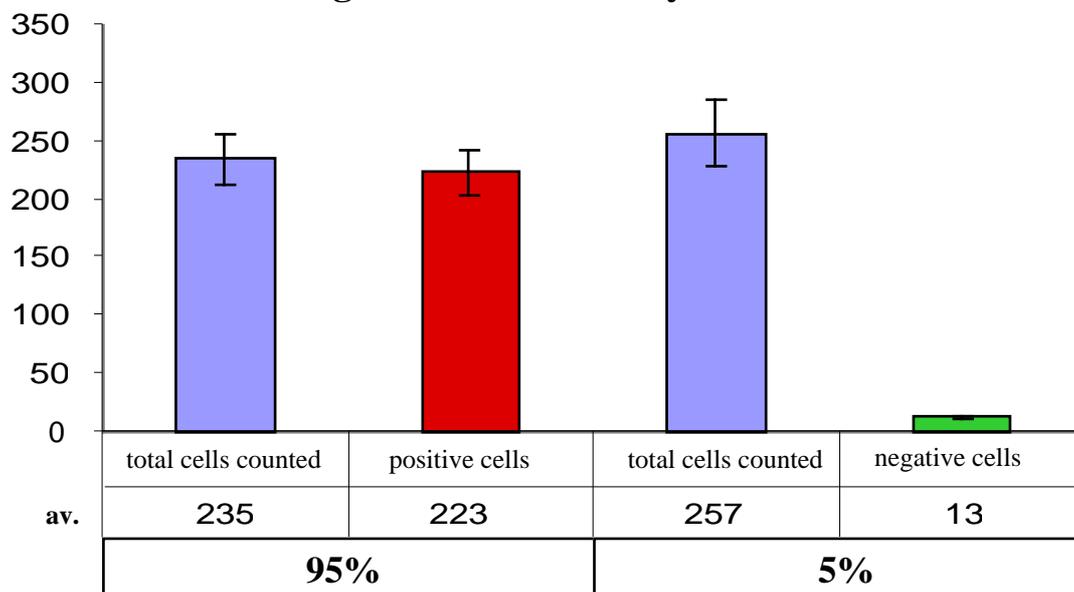


Figure 10 b KiS5-staining of A818-6 hollow sphere cells

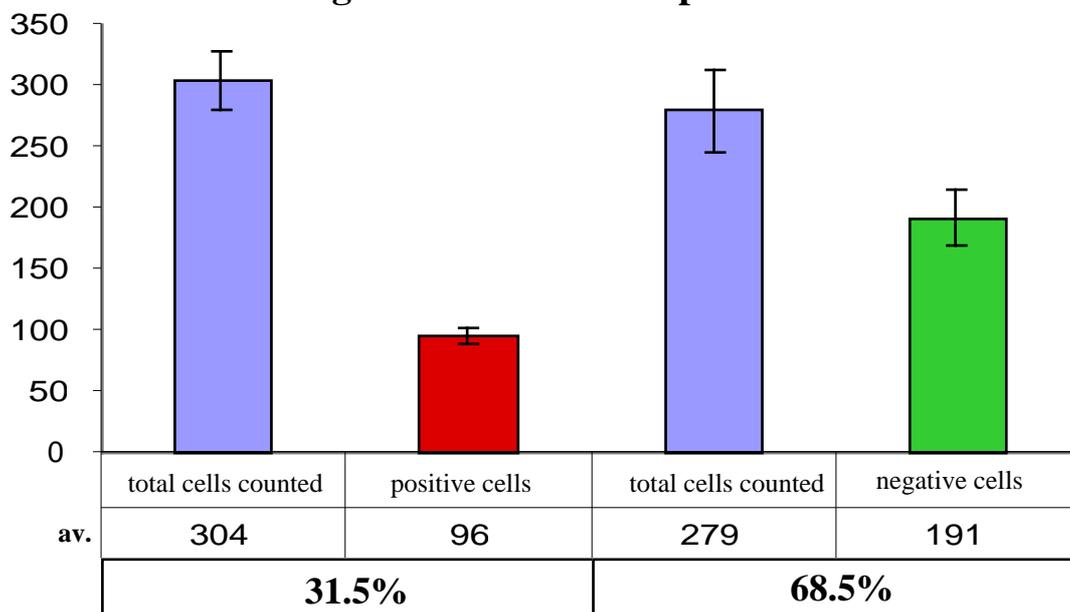


Fig.10 a and b: Results obtained by immunocytochemistry with the antibody KiS5 against Ki67 in A818-6 monolayer- and hollow sphere cells. Blue columns represent the amount of cell that were counted for the evaluation of Ki67 positive cells (red columns) and negative cells (green columns). The averages of all evaluations are indicated by (av.). The relation of averages is expressed in %.

Figure 10 c KiS2-staining of A818-6 monolayer cells

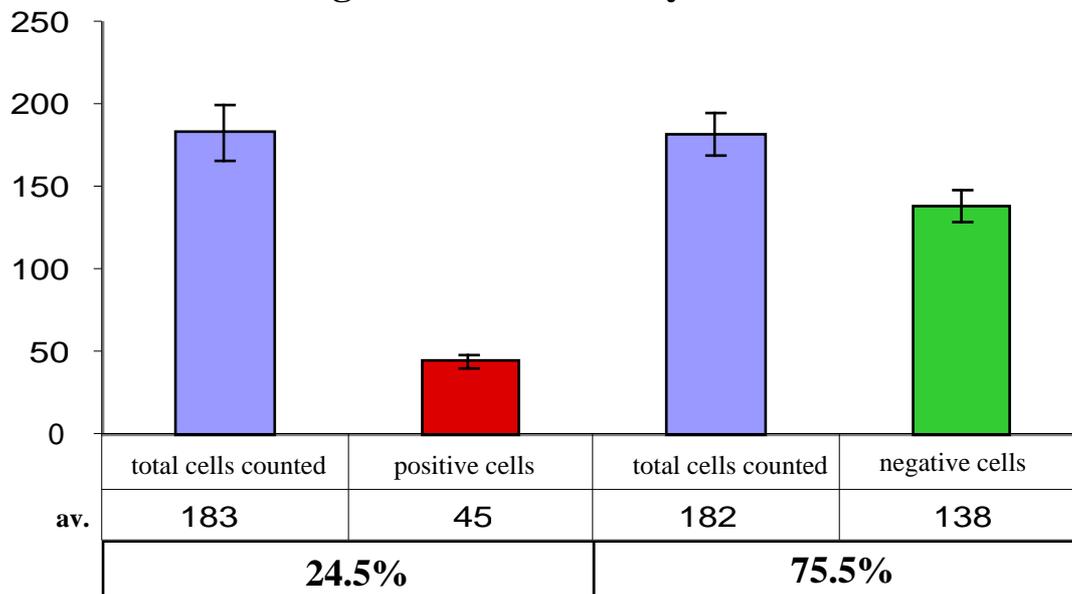


Figure 10 d KiS2-staining of A818-6 hollow sphere cells

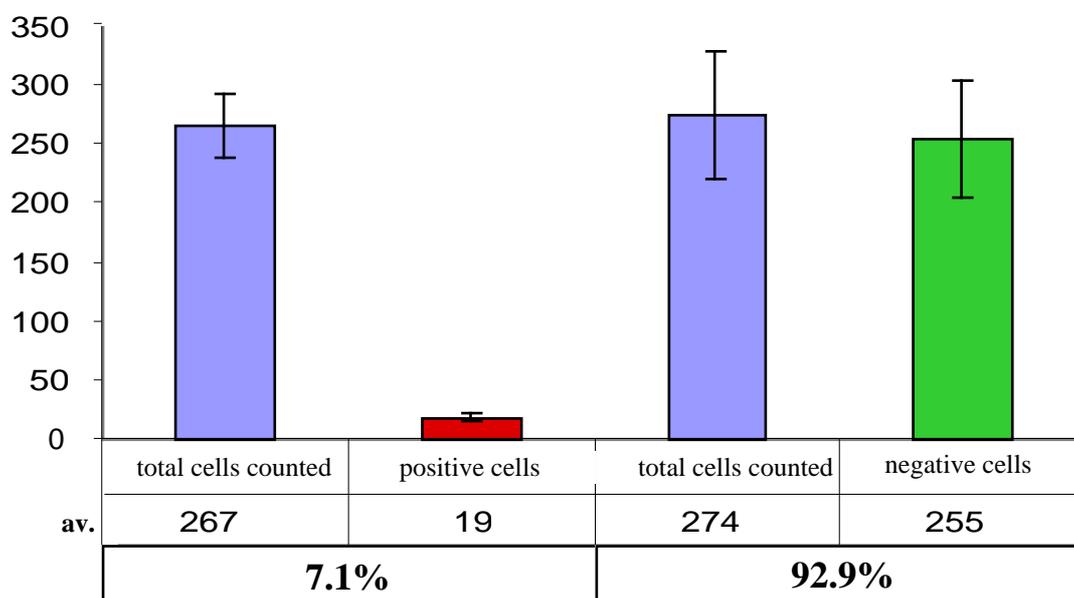


Fig.10 c and d:Results obtained by immunocytochemistry with the antibody KiS2 against p100 in A818-6 monolayer- and hollow sphere cells. Blue columns represent the amount of cell that were counted for the evaluation of p100 positive cells (red columns) and negative cells (green columns). The averages of all evaluations are indicated by (av.). The relation of averages is expressed by %.

Table 2 Distribution of A818-6 monolayer- and hollow sphere cells in phases of the cell cycle

	A818-6 monolayer cells	A818-6 hollow sphere cells
G0	5%	68,50%
G1	71,70%	29,30%
G2/S/M	23,30%	2,20%

Tab. 2: Distribution of A818-6 monolayer- and hollow sphere cells in the different phases of the cell cycle determined by immunocytochemistry with the antibodies KiS5 and KiS2 against the proliferation associated antigens Ki67 and p100.

3.3.2 Telomerase assay

A telomerase-assay was carried out as previously described (Klapper et al., 1998) using a modified TRAP-assay. Recent publications showed a correlation between the status of differentiation of cells and the telomerase activity (Krupp et al., 1997). Therefore measurement of telomerase activity can be used as a further indicator for differentiation in A818-6 hollow sphere cells. In comparison to the corresponding monolayer, the relative telomerase activity was found to be approximately six-fold reduced in hollow spheres (Fig.11).

Figure 11 Telomerase activity in A818-6 cells

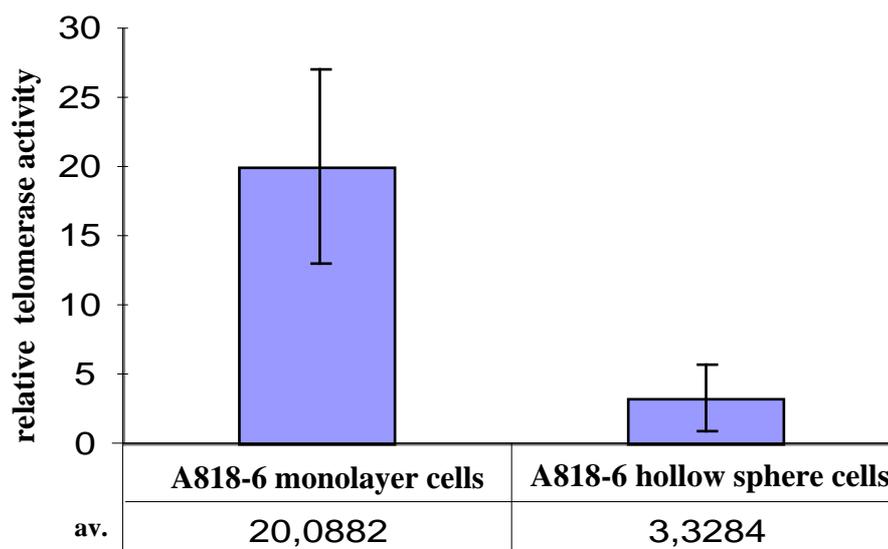


Fig. 11: Comparison of the relative telomerase activity in A818-6 monolayer- and hollow sphere cells. Averages (av.) and standard deviations are indicated.

3.3.3 Analyses of cell cycle associated proteins

Further analyses on cell cycle associated proteins by Western blot analysis supported the findings observed by immunocytochemistry and telomerase-assay. The cell cycle is regulated by interaction of promoting and inhibitory proteins. Growth arrest is often accompanied by up-regulation of cell cycle inhibitors like p27Kip and p21/Cip and down-regulation of cell cycle promoters like cyclin B. Consequently expression levels of these factors were analyzed. The cell cycle inhibitor p27/Kip was expressed in larger amounts in hollow spheres than in monolayer cells. The inverse result was obtained when the expression of the cell cycle promotor cyclin B was compared between monolayer cells and hollow sphere cells (Fig.12 a). cyclin D1 (Fig. 12 c) was also detected in higher amounts in monolayer cells whereas other cell cycle associated proteins like p21Cip (Fig.12 b) were found in equal amounts in hollow sphere and monolayer cells or were not detected (table 3).

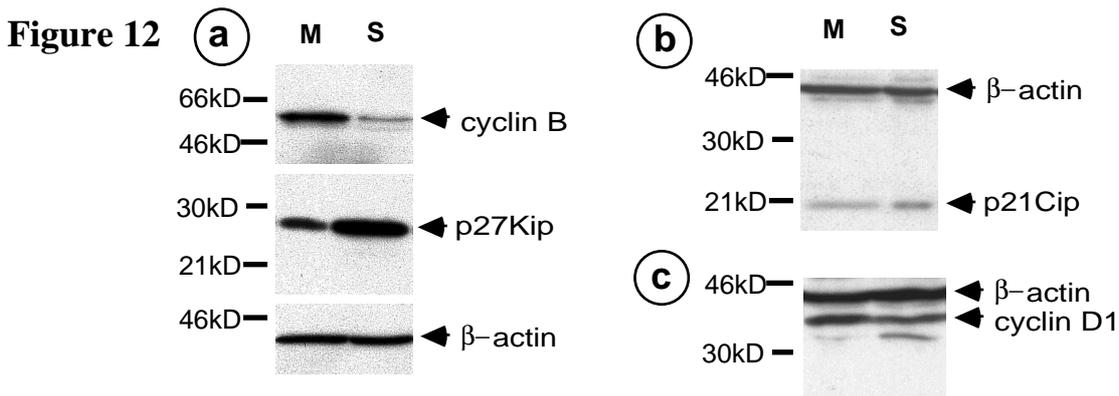


Fig.12: Western blot analysis of the expression of p27/Kip, cyclin B, cyclin D1 and p21Cip. Comparison between A818-6 monolayer- (M) and hollow sphere (S) cells. Panel a: Unequal expression levels were observed for cyclin B and p27 whereas cyclin D1 and p21Cip were expressed in equal amounts in A818-6 monolayer- and hollow sphere cells (panel b). Panel c: cyclin D1 slightly overexpressed in A818-6 monolayer cells compared to the expression in hollow sphere cells. Proteins are indicated by arrows.

Table 3 Expression levels of other cell cycle associated proteins in A818-6 monolayer and hollow sphere cells

protein	expression
cyclin D3	eq.
cdc2	eq.
cdk2	eq.
cdk4	eq.
Rb	eq.

Tab..3: Expression of cell cycle associated proteins in A818-6 cells. Equal expression in monolayer- and hollow sphere cells is indicated by (eq.).

3.4 Expression of differentiation marker antigens

3.4.1 Expression of mucin-1

In normal ductal epithelial cells of the pancreas, highly glycosylated proteins of the mucin family are facing towards the lumen of the duct. Subsequently the expression of mucin-1 was analyzed in A818-6 cells. A818-6 hollow sphere cells expressed mucin-1 (MUC-1) antigen at the outer surface being therefore identified as the apical membrane, which resembles the lumen orientated membrane in pancreatic ducts *in vitro* (Fig. 13 a). A818-6 monolayer cell exhibited a heterogeneous staining for MUC-1 with only single cells showing an apical staining (Fig. 13 b).

Figure 13

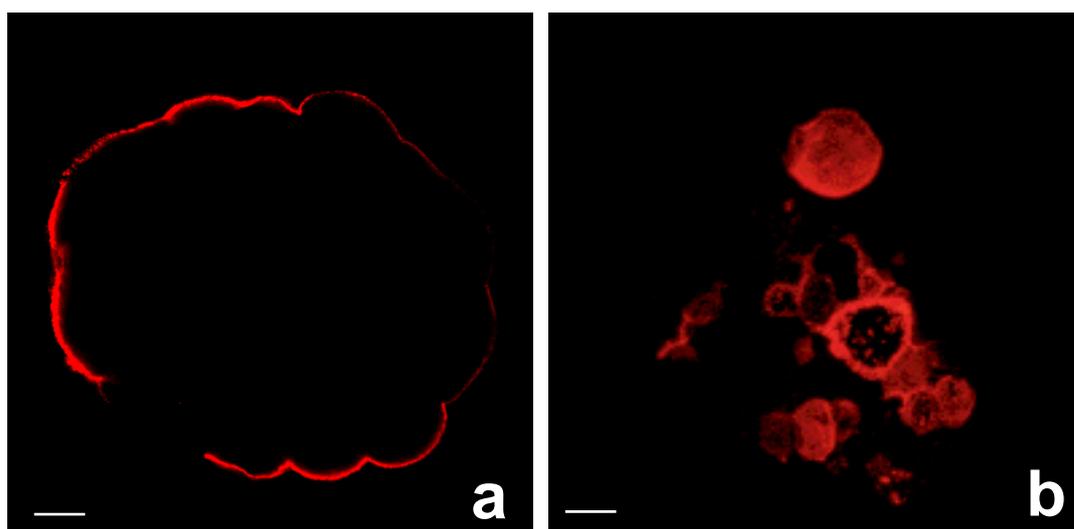


Fig.13: Immunofluorescence staining of hollow sphere cells for duct cell differentiation marker antigens. Mucin-1 staining depicts the apical membrane side of A818-6 hollow spheres (panel a). Heterogeneous staining of mucin-1 in A818-6 monolayer cells (panel b). Analyses were performed using confocal laser scanning microscopy. Bars indicate 10 μ m.

3.4.2 Expression of CD43

Leukosialin (CD43) is normally known to be expressed on cells of the hematopoietic system, especially on immunocompetent cells. It is speculated, that one main function of CD43 is to prevent cell contact between these cells (Manjunath et al., 1993). Recent works on CD43 showed expression on several tumor tissues as well (Santamaria et al., 1996). It may be hypothesized tumors require the ability to express CD43 as a protection against cytotoxic T-lymphocytes.

Testing this hypothesis immunofluorescence analyses were carried out with an antibody against CD43, known to react positively with tumor tissues. Scraped A818-6 monolayer cells were mixed with A818-6 hollow spheres and stained for CD43. In Fig. 14 a, one hollow sphere is surrounded by several monolayer cells as observed by light field microscopy. Fig. 14 b shows exactly the same observation field in epifluorescence. Only monolayer cells were stained whereas A818-6 hollow sphere cells were negative (compare black arrows from panel a with white arrows in panel b).

Figure 14

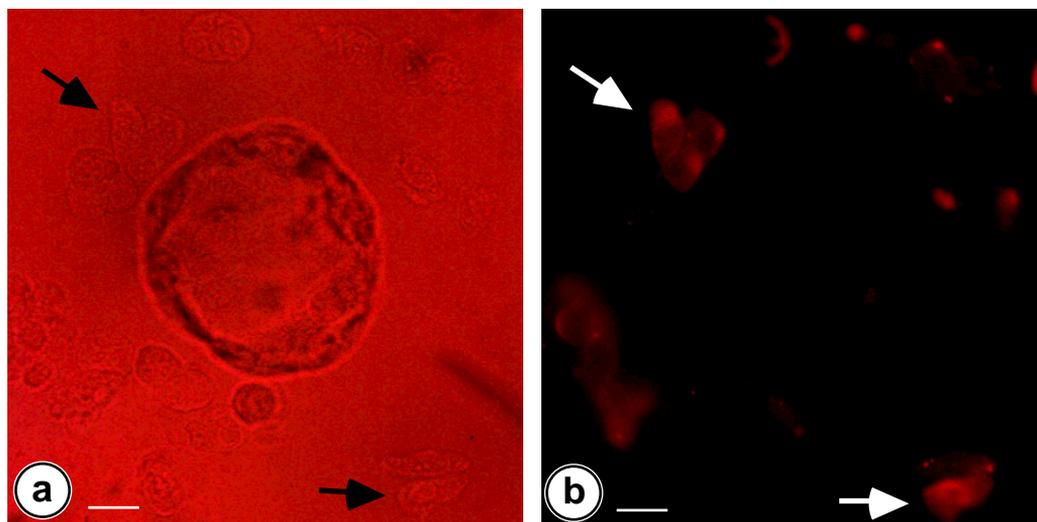


Fig.14: Comparison of CD43-expression between A818-6 monolayer- and hollow sphere cells. Both panels represent the same observation field. In panel b the light was turned off and epifluorescence remained. Black and white arrows indicate the identical cells in both panels. This experiment was analyzed by epifluorescence. Bars indicate 10 μ m.

3.4.3 Expression of β -catenin and E-cadherin

Between differentiated ductal epithelial cells, cell-adhesions are formed at the basolateral cell surface. E-cadherin and β -catenin are the most important molecules in these adherence junctions. In this study β -catenin exhibited a strictly lateral staining in A818-6 hollow sphere cells as detected by confocal laser scanning microscopy (Fig. 15 a). This localization of β -catenin correlated with the ultrastructural observations (compare Fig. 9, panel b). E-cadherin was detected laterally in A818-6 hollow spheres as well but did not exhibit such a clear staining pattern than β -catenin (Fig.16 a). Furthermore α - and γ -catenin were found showing a weaker but also laterally localized staining (data not shown). A818-6 monolayer cells revealed a heterogeneous staining for both E-cadherin and β -catenin (Fig.15 b, 16 b).

Figure 15

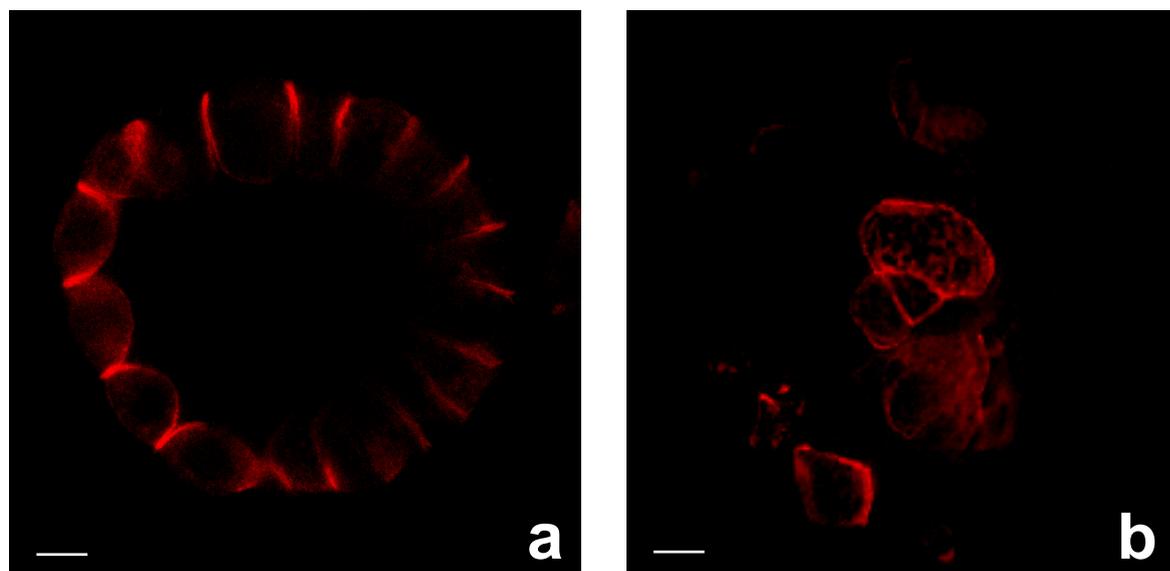


Fig. 15: Detection of β -catenin in A818-6 hollow sphere cells (panel a) and monolayer cells (panel b) by immunofluorescence. Analyses were performed using confocal laser scanning microscopy. Bars indicate 10 μ m.

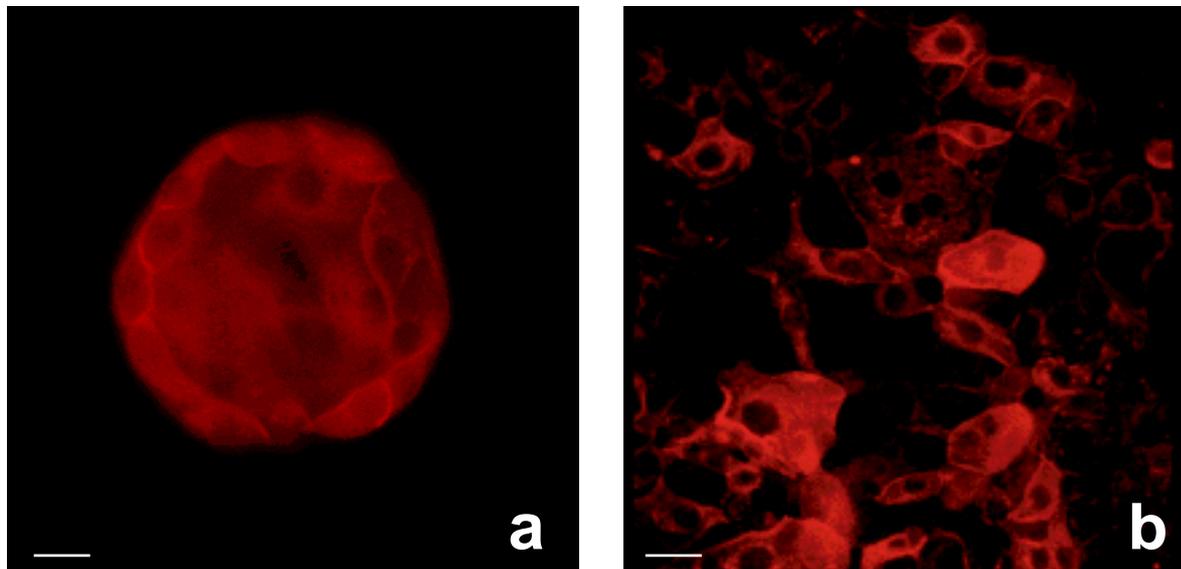
Figure 16

Fig. 16: Detection of E-cadherin in A818-6 hollow sphere cells (panel a) and monolayer cells (panel b) by immunofluorescence. Analyses were performed using epifluorescence microscopy. Bars indicate 10 μ m.

3.4.4 Expression of other cell adhesion molecules

Beside cadherins and catenins, other molecules involved in cell adhesion were analyzed and next to immunofluorescence-, Western blot experiments were carried out as a more quantitative method for some antigens.

As shown by electron microscopy in Fig. 9 b, tight junctions were developed from A818-6 hollow sphere cells. Symplekin is a major molecule involved in the development of tight junctions and stored in the nucleus of cells until it is needed. Subsequently symplekin was analyzed in A818-6 cells and was found to be recruited to the membranes of A818-6 hollow sphere cells during hollow sphere development while monolayer cells exclusively showed a nuclear staining (Fig. 17 a, b).

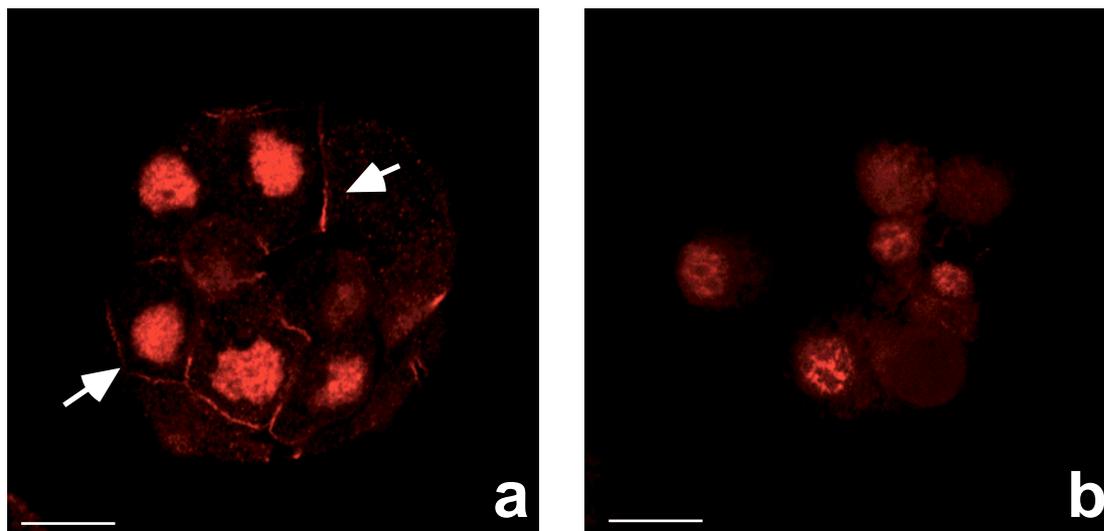
Figure 17

Fig.17: Detection of symplekin in A818-6 hollow sphere cells (panel a) and monolayer cells (panel b) by immunofluorescence. White arrows indicate the membrane recruited symplekin in A818-6 hollow sphere cells. Analyses were performed using confocal laser scanning microscopy. Bars indicate 10 μ m.

In Western blot analyses performed for other cell adhesion molecules only paxillin showed a quantitative difference between A818-6 monolayer- and hollow sphere cells (Fig.17) while all others exhibited equal amounts in both phenotypes (table 4).

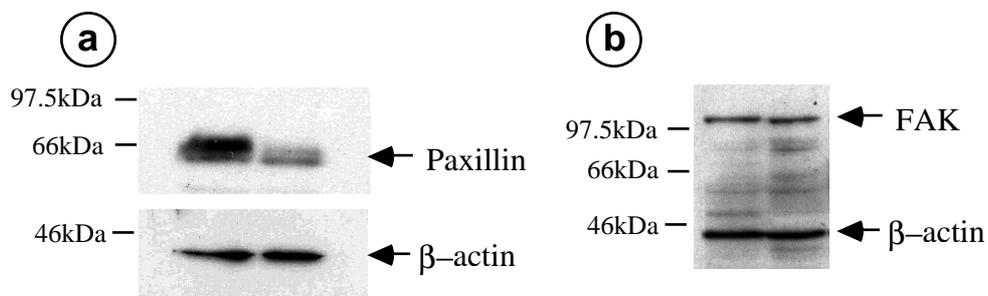
Figure 18

Fig.18: Western blot analysis for paxillin and focal adhesion kinase (FAK) in A818-6 monolayer (M)- and hollow sphere (S) cells. Panel a: Upregulation for paxillin was found in A818-6 monolayer cells compared with hollow spheres while an equal expression was observed for the focal adhesion kinase (panel b).

Table 4 Expression levels of other cell adhesion associated proteins in A818-6 monolayer and hollow sphere cells

protein	expression
α - Catenin	eq.
β - Catenin	eq.
γ - Catenin	eq.
Desmoglein	eq.
Integrin β -1	eq.
pp120	eq.

Tab. 4: Expression of cell adhesion associated proteins in A818-6 cells. Equal expression in monolayer- and hollow sphere cells is indicated by (eq.).

3.4.5 Expression of carbonic anhydrase II

As bicarbonate secretion of ductal epithelial cells is an important physiological feature, carbonic anhydrase II (CAII) is a reliable marker for this type of cells. Reliable marker antigens for acinar cells display the enzymes lipase and amylase. Consequently CAII was detected in hollow sphere cells by indirect immunofluorescence staining (Fig.19) as well as in the corresponding monolayer cells (data not shown). Immunocytochemistry with antibodies against lipase and amylase were performed as controls and no reaction was observed.

Figure 19

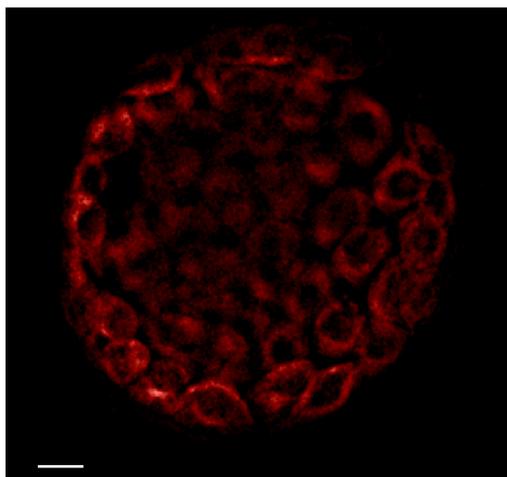


Fig.19: Cytosolic staining for carbonic anhydrase II in A818-8 hollow sphere cells as assessed by immunofluorescence and confocal laser scanning microscopy. Bar indicated 10 μ m.

3.4.6 Expression of members of the carcinogenic embryonic antigen family

The carcinoembryonic antigen (CEA) is a tumor marker in serum of patients used in clinical praxis to detect recurrence of a malignant disease. Tumor cells secrete CEA-180 as the soluble form in an unpolar fashion. Immunohistochemically, A818-6 hollow spheres were negative for CEA-180. In some cases single cells exhibited an apical staining being too weak to be documented. Other members of the CEA-family like BGP and NCA 95 showed an apical staining in hollow spheres and like CEA heterogeneous staining in monolayer cells. Subsequently the secretion of CEA was measured in A818-6 cells with an automated ELISA protocol for patient sera and A818-6 monolayer cells were found to secrete approximately double amounts of CEA-180 than hollow sphere cells (table 5). For this experiment 100 and 200 hollow spheres containing approximately 200 cells were collected with a micromanipulator (Eppendorf-Netheler-Hinz, Hamburg, Germany) and transferred into one well of a 24-well plate. Cell culture medium was changed after 24 h followed by 48 h of incubation. Then the supernatant was taken, centrifuged at 14,000 rpm to remove all cells and measured. Corresponding monolayer cells reach confluency at the day of analyses and the number of cells was counted after the supernatant was taken.

Table 5 Secretion of CEA-180 in A818-6 monolyer and hollow sphere cells

sample	CEA-180	number of cells
100 hollow spheres	0.1 ng/ml	20,000
200 hollow spheres	0.6 ng/ml	40,000
A818-6 monolayer	8.2 ng/ml	270,000
RPMI1640	0.0 ng/ml	○

Calculation: $27,0000 \div 40,000 = 6.75$ (relation between number of cells)

$$8.2 \text{ ng/ml} \div 6.75 = \underline{1.21 \text{ ng/ml}}$$

Equilibrated to the number of cells :

40,000 hollow sphere cells secrete **0.6 ng/ml** CEA-180

40,000 monolayer cells secrete **1.21 ng/ml** CEA-180

3.5 Influence of cytokines and growth factors on A818-6 hollow sphere development

Hollow sphere development was found to be influenced by exogenously added cytokines. Hollow sphere development was inhibited by Interferon- γ in a concentration dependent manner. At a concentration of 20 U/ml, hollow spheres were able to develop but only in reduced numbers. A concentration of 200 U/ml prevented hollow-sphere development, was cytotoxic for mature hollow spheres and the corresponding monolayer (Tab. 6, 7). TNF- α lead to cell death of monolayer- and hollow sphere cells at a concentration of 1000 U/ml. TGF- β was found to prevent hollow sphere formation at a concentration of 10 ng/ml but had neither influence on the integrity of mature hollow spheres nor on monolayer cells. All other cytokines and growth factors tested, showed no visible influence on hollow sphere formation over a period of 8 days. With exogenously added HGF/SF or Activin A, no visible changes in hollow sphere development occurred. Neutralizing antibodies against HGF/SF and TGF- β added to freshly seeded cells at a concentration of 1 μ g/ml had no influence on hollow sphere formation. From all cytokines and growth factors tested, only basic fibroblast growth factor (bFGF) was able to stimulate growth and development of A818-6 hollow spheres. The development was accelerated in speed and mature hollow spheres returned into a proliferative state without loosing the highly differentiated phenotype.

Table 6 Influence of cytokines and growth factors on A818-6 hollow sphere development

Cytokine	Concentration		Development of hollow spheres	Integrity of hollow spheres
Contr.	no cytokine		8-12 days	standard
IFN- γ	0.2-20	U/ml	no influence	smaller
IFN- γ	200	U/ml	inhibition	no spheres
TGF- β	10	ng/ml	inhibition	no spheres
TGF- α	10	ng/ml	no influence	no influence
EGF	10	ng/ml	no influence	no influence
TNF- α	1,000	U/ml	cell death	no spheres
HGF/SF	100	ng/ml	no influence	no influence
Activin A	2-8	ng/ml	no influence	no influence
bFGF	5	ng/ml	acceleration	larger

Tab.6: Cytokine treatment of A818-6 during hollow sphere development. Concentrations are indicated. Cell culture medium containing cytokines and growth factors was replaced on day 2, 4 and 7. Standard hollow spheres had a 100 μ m diameter. Size assessments like larger/smaller refer to this standard size.

Table 7 Influence of cytokines on mature hollow spheres

Cytokine	Concentration		Integrity of hollow spheres
control	no cytokine		standard
IFN- γ	0.2-20	U/ml	smaller
IFN- γ	200	U/ml	no spheres
TGF- β	10	ng/ml	no influence
TGF- α	10	ng/ml	no influence
EGF	10	ng/ml	no influence
TNF- α	1,000	U/ml	no spheres
HGF/SF	100	ng/ml	no influence
Activin A	2-8	ng/ml	no influence
bFGF	5	ng/ml	larger

Tab.7: Cytokine treatment of mature A818-6 hollow spheres. Concentrations are indicated. Cell culture medium containing cytokines and growth factors was replaced on day 2, 4 and 7. Standard hollow spheres had a 100 μ m diameter. Size assessments like larger/smaller refer to this standard size.

Next to TNF- α , an agonistic antibody to Fas-Ligand (ApoI) and the TRAIL-Factor were tested for induction of apoptosis. A818-6 hollow spheres were found to be not sensitive for both, ApoI at a concentration of up to 1 μ g/ml or TRAIL at a concentration of 200 ng/ml or 500 ng/ml. Treated hollow spheres survived for more than 2 weeks without any sign of apoptosis. The monolayer responded with few apoptotic cells after 72 h.

3.6 Mesenchymal co-culture

3.6.1 A818-6 monolayer cells seeded on top of a confluent fibroblast monolayer

So far only bFGF was found to stimulate A818-6 hollow sphere development. Subsequently the question was raised whether direct contact of A818-6 cells to mesenchymal cells may stimulate hollow sphere development since it is known that mesenchymal support is a prerequisite for proper development of luminal structures in the pancreas.

Fibroblast co-cultures were carried out with A818-6 cells, foreskin-derived KIF-5-fibroblasts and pancreas-derived PTF-994-fibroblasts. When A818-6 cells were seeded onto a confluent fibroblast monolayer culture, invasive growth and the development of plaques of A818-6 tumor cells was detected (Fig. 20 a). When pre-mature A818-6 hollow spheres, shown in Fig. 19 b were seeded onto a confluent fibroblast monolayer culture, these hollow spheres increased in size by increasing the number of cells per hollow sphere and developed a homogenous shape within 48 h on both types of fibroblasts (Fig. 20 c, d).

Figure 20

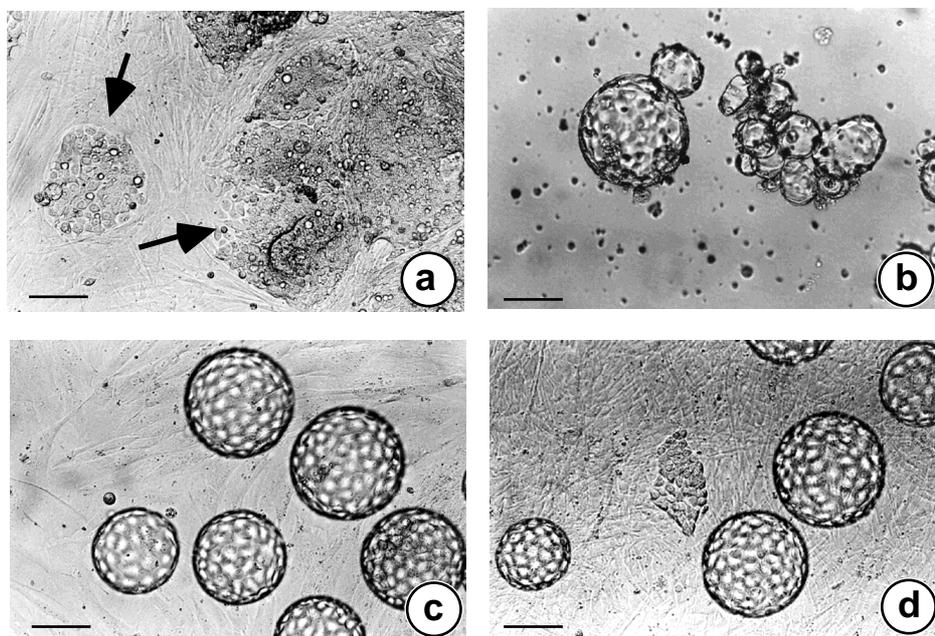


Fig. 20: Co-culture with A818-6 cells, Kif5- and PTF994-fibroblasts. Arrows in panel a indicate invasive plaques of A818-6 monolayer cells. Panel c: Pre-mature hollow spheres after 48 h of culture on top of Kif5-fibroblasts. Panel d: Pre-mature hollow spheres after 48 h of culture PTF994-fibroblasts. Bars indicate 100 μm .

3.6.2 A818-6 cells in suspension co-culture with fibroblasts

In a second experimental set up, A818-6 monolayer cells were mixed with fibroblasts and seeded on agarose-coated dishes. This procedure was also found to enhance A818-6 hollow sphere development. Two days after initial co-culture of A818-6 cells with KIF-5-fibroblasts development of hollow spheres was seen, which was normally observed after 6-8 days in cultures without fibroblasts (Fig. 21 a) Some of these hollow spheres already contained more than 150 cells. Later "giant-spheres" with an diameter of up to 500 μm developed. Remarkably a different result was obtained with pancreas-derived PTF994-fibroblasts. The size of the developing hollow spheres was standard but the total amount of hollow spheres was increased in co-culture with PTF994 (Fig. 21 b). In accordance with Kif5-results the speed of hollow sphere development was increased as well.

Figure21

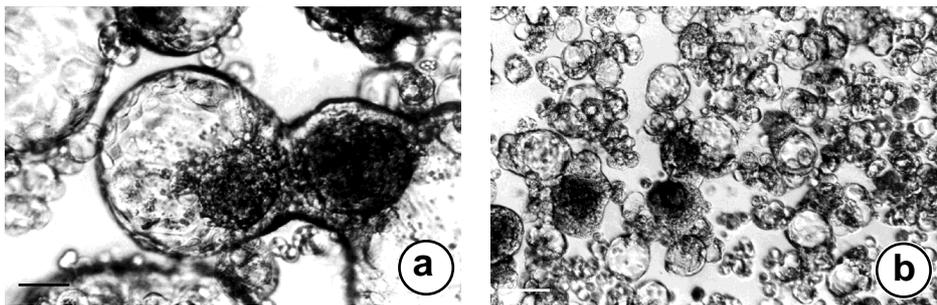


Fig.21: Suspension co-culture with A818-6 cells, Kif5 and after of co-seeding on solid agarose. Panel a: A818-6 cells with development of "giant-spheres". Panel b: A818-6 cells seeded with development of an increased number of hollow spheres. Bars indicate 100 μm .

The same experiment was repeated with EGFP-transduced fibroblasts exhibiting a green fluorescence when exposed to UV-light and revealed a localization of fibroblasts in the lumen of hollow spheres closely associated to the basal side of A818-6 cells (Fig 22 a-d). Fibroblast-conditioned medium was not able to replace the direct presence of fibroblasts in the same manner (data not shown).

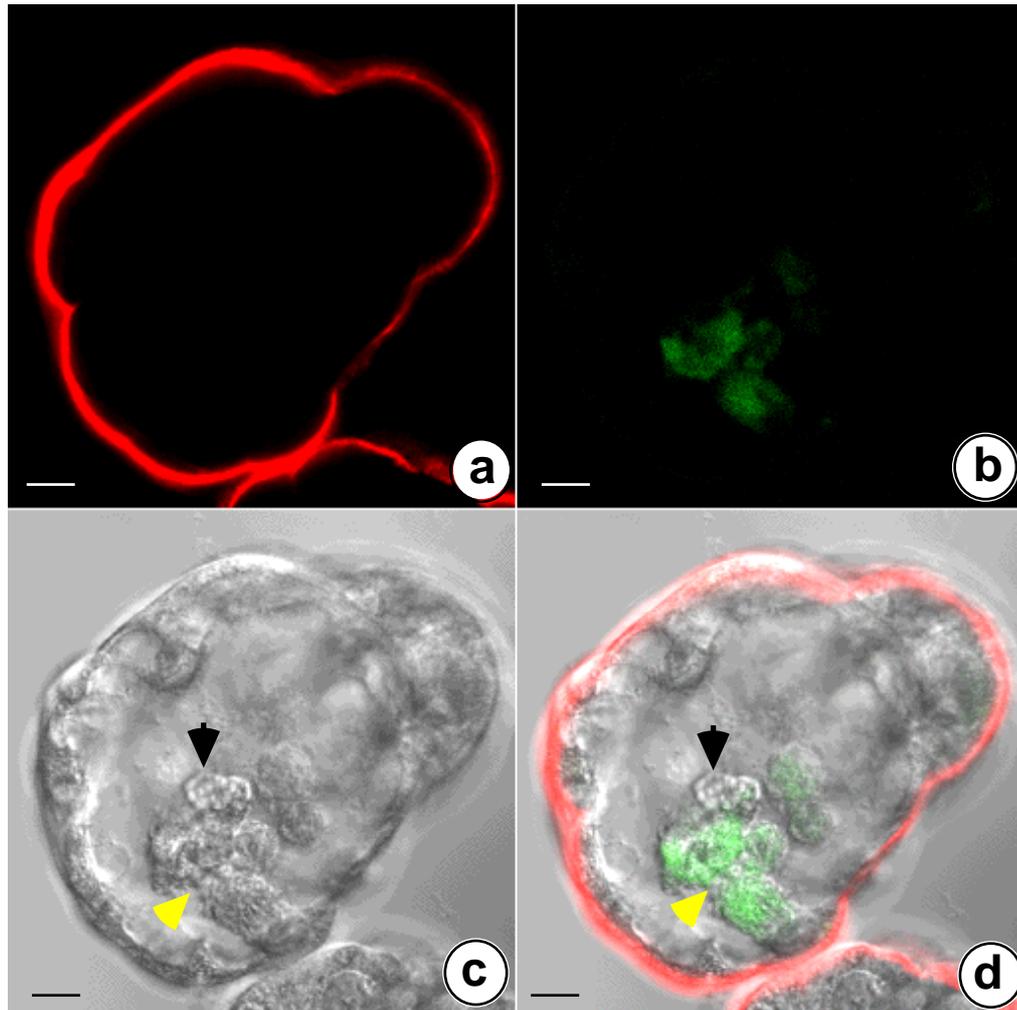
Figure 22

Fig. 22: Confocal laser scanning analysis of co-culture experiments of A818-6 cells with Kif-5 fibroblasts transduced with the gene coding for the enhanced green fluorescent protein (EGFP). After six days of hollow sphere development, A818-6 cells were stained for the mucin-1 antigen. Mucin-1 displayed a staining of apical membranes of A818-6 hollow sphere cells (panel a). Fibroblasts are depicted by EGFP-derived fluorescence (panel b). Panel c displays a differential interference contrast image of the analyzed hollow sphere with fibroblasts closely associated with the basal side of hollow sphere cells (yellow arrow and panel c and d) accompanied by a putative sequestered, non-fluorescing single A818-6 cell (black arrow panel c and d). Bars indicate 10 μ m.

3.6.3 Influence on cell cycle associated proteins

Analysis of the cell cycle associated proteins cyclin B and p27/Kip after co-culture revealed a downregulation of p27/Kip and a weak upregulation of cyclin B in A818-6 hollow spheres which were seeded on top of fibroblast monolayers (Fig. 23), underlining the observations made at the microscopic level (compare Fig. 20 c, d).

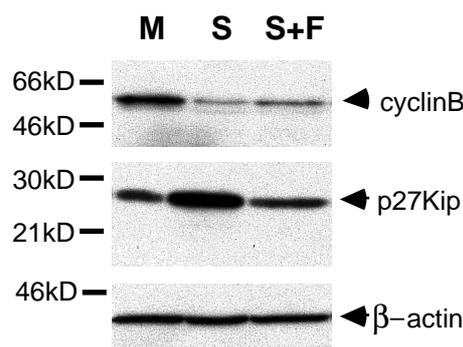
Figure 23

Fig.23: Regulation of p27Kip and cyclin B in A818-6 cells under mesenchymal influence analyzed by Western blot analysis. A818-6 hollow spheres without Kif-5 co-culture (S) exhibited a lower expression of cyclin B and an increased expression of p27/Kip compared to the corresponding monolayer (M). In hollow spheres seeded onto fibroblasts (S+F), the expression of p27 was downregulated and the expression of cyclin B was slightly upregulated compared to hollow spheres without co-cultured fibroblasts. Lanes M and S are equivalent with Fig.12, panel a.

3.6.4 Growth of A818-6 cells on pre-formed fibroblast-spheroids

A third co-culture model was set up to optimize contact between tumor cells and fibroblasts after it was observed that fibroblasts attached to the basal side of A818-6 hollow spheres. Kif 5-EGFP-fibroblasts were seeded onto solid agarose and formed compact spheroids (Fig. 24 a). The following day, A818-6 cells were seeded into the well containing fibroblast-spheroids. During the following three days A818-6 cells attached to the fibroblast-spheroids and grew around them as a single layer epithelium (Fig. 24 b, c). These complex structures were called "fibro spheres".

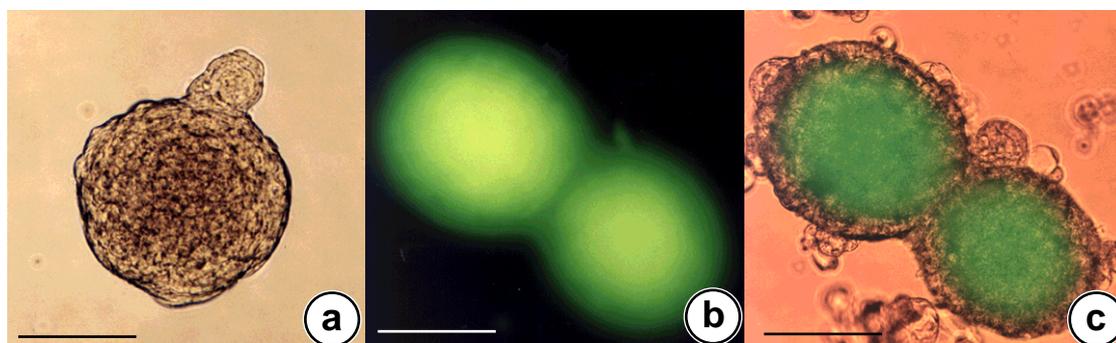
Figure 24

Fig.24: Culture of A818-6 cells on pre-formed fibroblast spheroids. Panel a: Kif-5 spheroid 24 h after seeding onto solid agarose. Panel b: Epifluorescence of EGFP-transduced fibroblast spheroids. Panel c: Phase-contrast/ Epifluorescence overlay showing fibroblast spheroids from panel b surrounded by A818-6 cells. Bars indicate 100 μ m.

Approximately 50% of all fibroblast-cores were surrounded by a single layer of A818-6 cell while others showed multilayers of A818-6 cells. In these structures the epithelial cells lost their direct contact to the fibroblast core (data not shown).

To assure the single layer character of A818-6 cells surrounding the fibroblast-cores immunocytochemical staining for β -catenin was carried out. A818-6 cells were found to be organized in a polar fashion with their basal side attaching to the fibroblast-core as assessed by confocal laser scanning microscopy (Fig. 25 a-d).

Figure 25

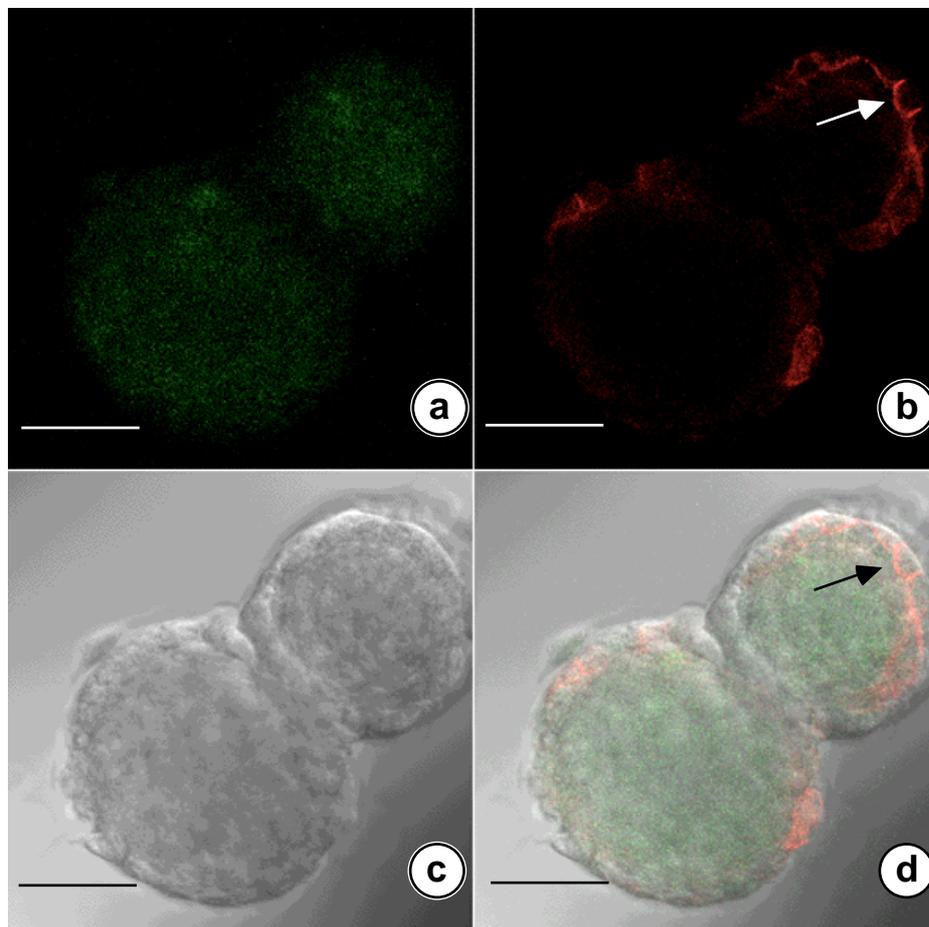


Fig. 25: Confocal laser scanning analysis of co-culture experiments of A818-6 cells grown on Kif-5 fibroblasts transduced with the gene coding for the enhanced green fluorescent protein (EGFP). After six days of fibro sphere development, A818-6 cells were stained for the β -catenin antigen. Beta-catenin displayed a staining of basolateral membranes of A818-6 cells (panel b, c). Fibroblasts are depicted by EGFP-derived fluorescence (panel a, d). Panel c displays a differential interference contrast image of the analyzed fibro sphere. Black and white arrows in panel b and d indicate a typically polarized A818-6 cell as part of a hollow sphere which surrounds a fibroblast-core. Bars indicate 100 μ m.

Investigations on the development of an extracellular matrix as a product of both tumor- and mesenchymal cells revealed that fibroblasts produced fibronectin (Fig. 26 a-d). A818-6 hollow sphere cells were found to produce and secrete laminin at their basement membranes. While only a low expression of laminin was detected in hollow spheres developed without mesenchymal core (Fig. 27), laminin was induced as a direct reaction to the co-culture with fibroblast spheroids (Fig. 28 a-d).

Figure 26

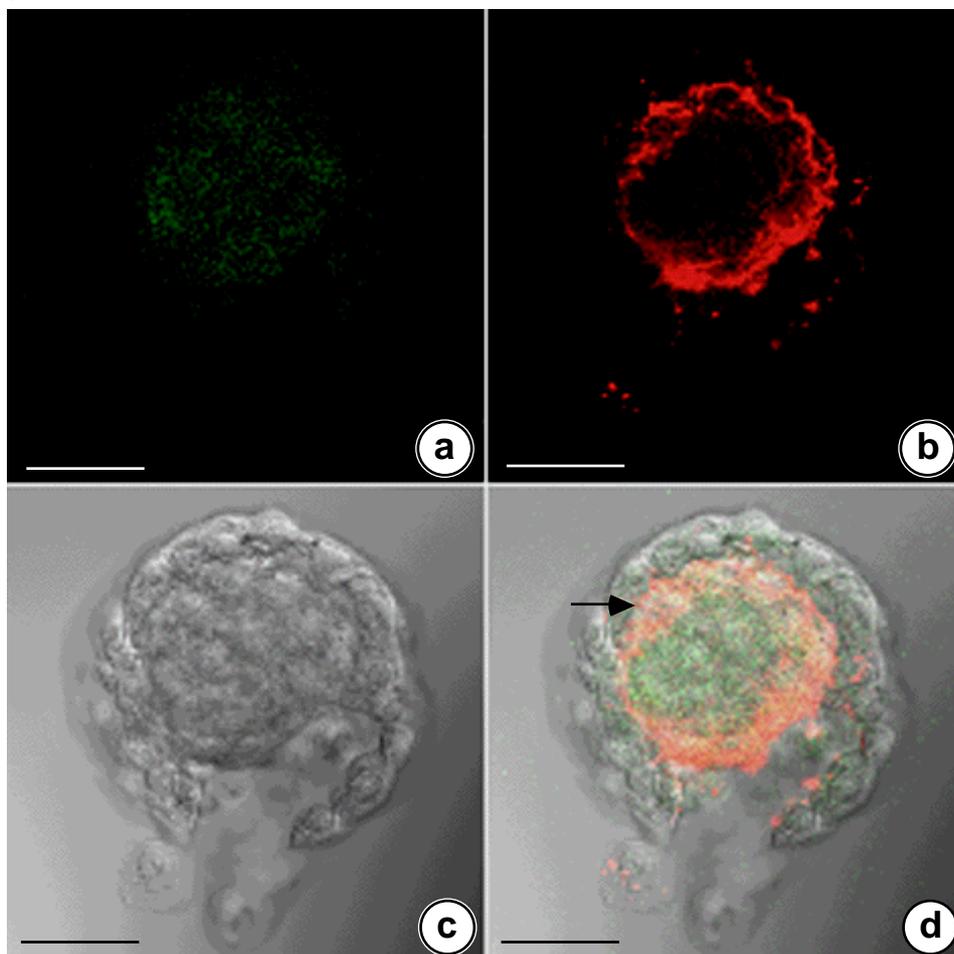


Fig. 26: Confocal laser scanning analysis of co-culture experiments of A818-6 cells grown on Kif-5 fibroblasts transduced with the gene coding for the enhanced green fluorescent protein (EGFP). After six days of fibro sphere development, fibroblasts were stained for the fibronectin antigen. Fibronectin displayed a mainly cytosolic staining in fibroblasts (panel b, d). Fibroblasts are depicted by EGFP-derived fluorescence (panel a, d). Panel c displays a differential interference contrast image of the analyzed fibro sphere. The black arrow in panel d indicates the boarder between polarized A818-6 cells and the fibroblast core. Bars indicate 100 μm .

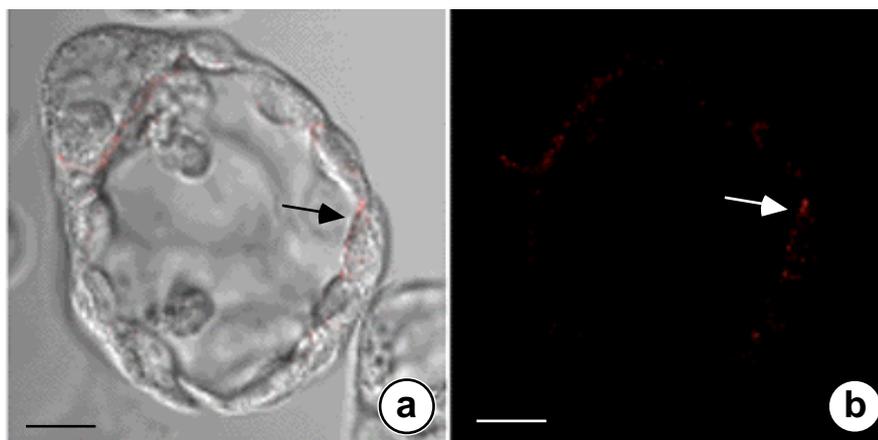
Figure 27

Fig. 27: Confocal laser scanning analysis of A818-6 hollow spheres stained against laminin. Laminin displays a weak but basal staining in A818-6 hollow sphere cells (black and white arrows). Bars indicates 10 μm .

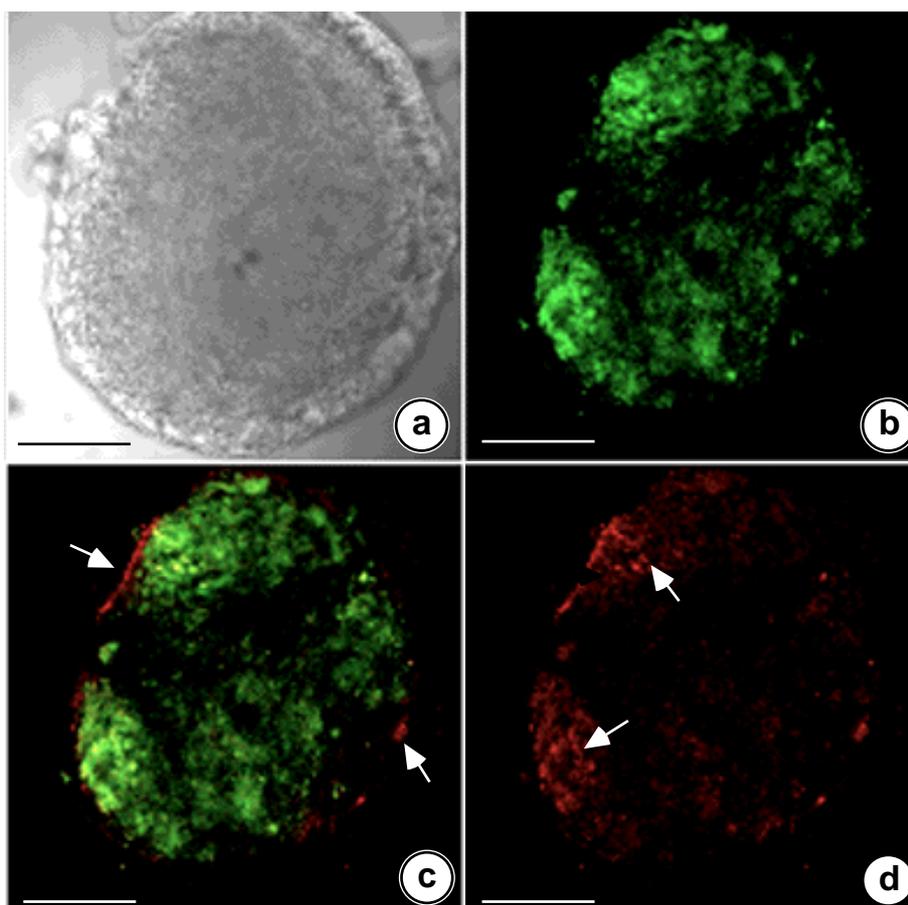
Figure 28

Fig. 28: Confocal laser scanning analysis of co-culture experiments of A818-6 cells grown on Kif-5 fibroblasts transduced with the gene coding for the enhanced green fluorescent protein (EGFP). After six days of fibro sphere development, fibroblasts were stained with an antibody against laminin. Laminin is mainly expressed from A818-6 cells at the boarder between epithelial and mesenchymal cells (white arrows panel c) but also from fibroblasts (white arrows panel d). Fibroblasts are depicted by EGFP-derived fluorescence (panel b, c). Panel a displays a differential interference contrast image of the analyzed fibro sphere. Bars indicate 100 μm .

3.6.5 *The role of epimorphin*

Epimorphin was recently described as a mesenchymal factor modulating morphogenesis of murine mammary ducts, skin, liver and lung *in vitro*. Hirai and co-workers (1998) showed that a monoclonal antibody to epimorphin was able to prevent the development of luminal structures in murine mammary epithelial cell. This finding suggested to analyze the role of epimorphin in the A818-6 system. A rat-monoclonal antibody MC-1 was previously described to react with native epimorphin (Hirai, 1993; Hirai et al., 1993) and was able to inhibit branching morphogenesis in mammary duct cells (Hirai et al., 1998).

3.6.5.1 *Inhibition of A818-6 hollow sphere development by a monoclonal antibody*

(MC-1) against epimorphin

Incubation of freshly seeded A818-6 cells with 50 µg/ml of MC-1 under three-dimensional culture conditions resulted in the inhibition of A818-6 hollow sphere development (Fig. 29 a-b). A rat IgG-isotype control for human IL-13 as control (50 µg/ml) showed no effect on A818-6 hollow sphere development (Fig. 29 c). So far, it has been generally assumed that fibroblasts are essential for the mediation of an epimorphin effect by a 150kDa form, associated to the membrane of fibroblasts. However, in this experiment an epimorphin effect independent of the presence of fibroblasts was found in A818-6 cultures. Therefore, epimorphin must act as a soluble factor in an autocrine manner in the A818-6 system. Neither a neutralizing antibody against HGF/SF nor recombinant HGF/SF added to freshly seeded cells had influence on hollow sphere formation even though HGF/SF and its receptor c-met were detected in A818-6 monolayer and hollow sphere cells by immunocytochemistry with intense staining for the receptor and only faint staining for the ligand (data not shown). Exogenously added recombinant epimorphin (31kDa-isoform) was not able to induce luminal structures in A818-4 cells lacking epimorphin expression so far.

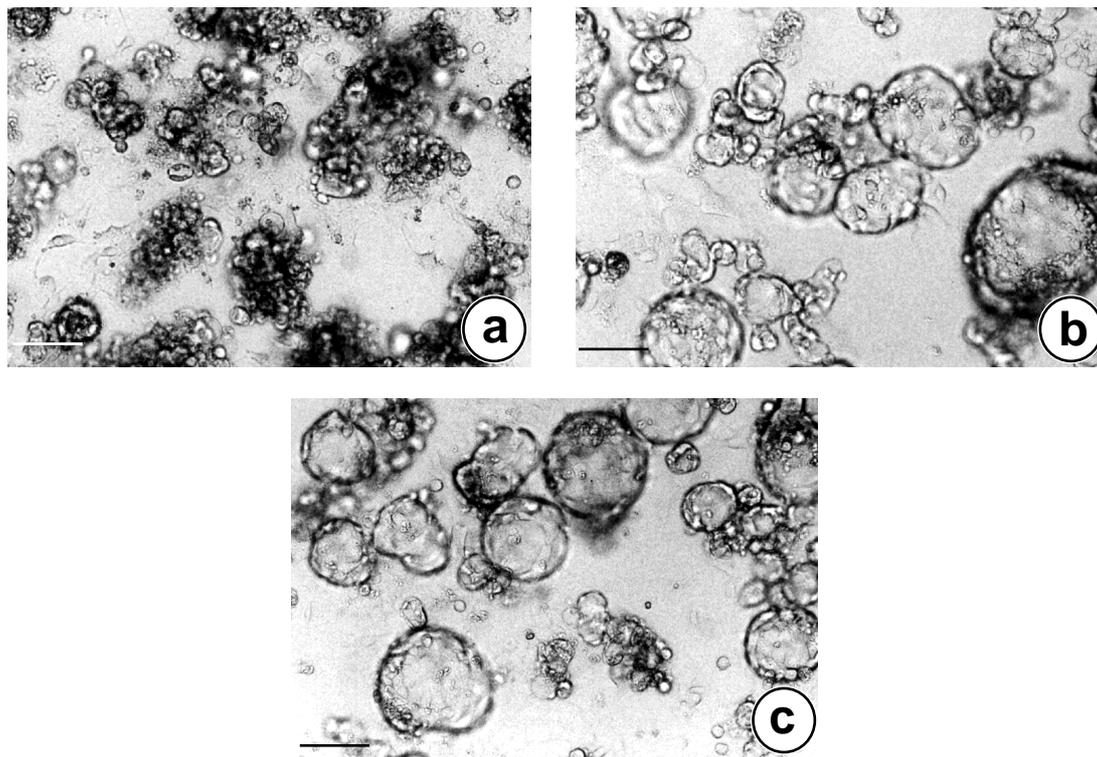
Figure 29

Fig. 29: Inhibitory influence of a monoclonal antibody against epimorphin, MC-1 on A818-6 hollow sphere development. A818-6 cells were cultured on solid agarose for eight days in the presence of MC-1 (50 µg/ml) (panel a). A818-6 cells cultured on solid agarose for eight days without MC-1 (panel b). A818-6 cells cultured on solid agarose for eight days in the presence of an antibody against human IL-13 used as an isotype control (panel c). The antibodies were added on day 2, 5 and 7 after seeding. Bars indicate 50 µm.

3.6.5.2 Expression pattern in cell lysates of A818-6 monolayer cells, A818-6 hollow sphere cells and fibroblasts

All forms of epimorphin previously described by others were detected with an affinity-purified antibody against epimorphin as assessed by Western blot analyses (Fig. 30 a, b). The 150kDa-tetramer of epimorphin was only detected in the lysates of fibroblasts (Fig. 30 b) whereas it was not detectable by Western blot analysis in lysates of A818-6 monolayer- and hollow sphere cells. No difference in the expression pattern of 70kDa-dimers was observed when fibroblasts, A818-6 monolayer cells and A818-6 hollow sphere cells were compared. Differences were observed in the expression pattern of monomeric 34kDa- and 31kDa-isoforms of epimorphin. Both isoforms were expressed in A818-6 monolayer cells while three-dimensional hollow spheres exclusively expressed the larger 34kDa-isoforms. Fibroblasts expressed larger amounts of the smaller, 31kDa-isoform, than of the 34kDa-isoforms (Fig. 30

a). Subsequently, cell lines not developing luminal structures were compared with cell lines, growing with a luminal phenotype under our cell culture conditions. Here it was observed that all cell lines capable of lumen-formation expressed the larger 34kDa-isoforms of epimorphin occasionally together with the 31kDa-isoform. Cell lines not capable of lumen-formation expressed only the smaller 31kDa-isoform or none (Fig. 30 c). The cell lines Capan-2 and BxPC-3 that expressed the 34kDa-isoforms responded upon fibroblast co-culture with stimulation of the development of luminal structures. Cell lines only expressing the 31kDa-isoform or none (Capan-1, A818-4, Panc 1) showed no induction of lumen-formation in fibroblast co-culture (data not shown).

Figure 30

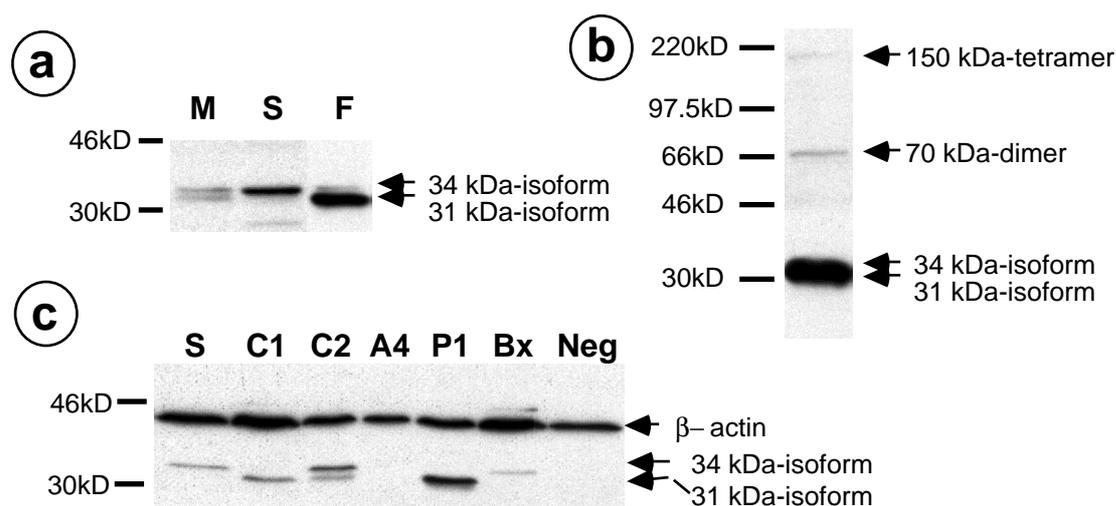


Fig. 30: Detection of epimorphin in protein lysates of pancreatic carcinoma cell lines and fibroblasts. Panel a: Western blot analyses of epimorphin in protein lysates of A818-6 monolayer cells (M), hollow sphere cells (S) and Kif-5 fibroblasts (F). Monolayer cells expressed the 34kDa- and 31kDa-isoforms while hollow sphere cells lacked the 31kDa-isoforms. Fibroblasts expressed larger amounts of the 31kDa-isoforms of epimorphin than of the 34 kDa-isoform (a, lane F). Panel b: The 150kDa tetrameric complex, 70kDa dimer and monomers of epimorphin detected in the lysate of Kif-5 fibroblasts. To make the tetrameric form visible a long exposure time was used which prevents visualization of the monomeric isoforms as two bands. Panel c: Western blot analysis of epimorphin in protein lysates of the pancreatic carcinoma cell lines Capan-1 (C1) Capan-2 (C2), A818-4 (A4), Panc-1 (P1), BxPC-3 (Bx) and A818-6 hollow sphere cells (S) as control also taken as negative control by omitting the primary antibody (Neg). All cell lines being capable of lumen-formation (A818-6, Capan-2, BxPC-3) expressed the 34kD-isoform of epimorphin occasionally together with the 31kD-isoform. Cell lines not developing luminal structures (Capan-1, A818-4, Panc1) expressed no epimorphin or only the 31kDa-isoforms. Epimorphin isoforms are indicated by arrows.

3.6.5.3 Detection of soluble epimorphin in supernatants of A818-6 hollow spheres, fibroblasts and co-cultures of both

Supernatants of A818-6 cells and fibroblasts were analyzed for putative soluble forms of epimorphin. Immunoprecipitations followed by Western blot analyses were carried out with the monoclonal MC-1 antibody and an affinity purified polyclonal antibody against epimorphin. First it was essential to prove that MC-1 detects the same antigens in the immunoprecipitate of the affinity purified antibody and vice versa. Supernatants from fibroblasts were used as source and both antibodies were found to detect the same antigens (Fig. 31a). Subsequently it was found that both antibodies were able to detect the monomeric 34kDa- and 31kDa-isoforms of epimorphin and the dimeric form of approximately 70kDa in the supernatants of A818-6 hollow spheres, Kif-5 fibroblasts and co-cultures from fibroblasts and hollow spheres. No tetrameric complex of 150kDa was detected in any of the supernatants. Epimorphin was neither found in the pure cell culture medium nor detected in the precipitate of an anti-human IL-13 (rat-IgG) antibody used as control (Fig. 31b).

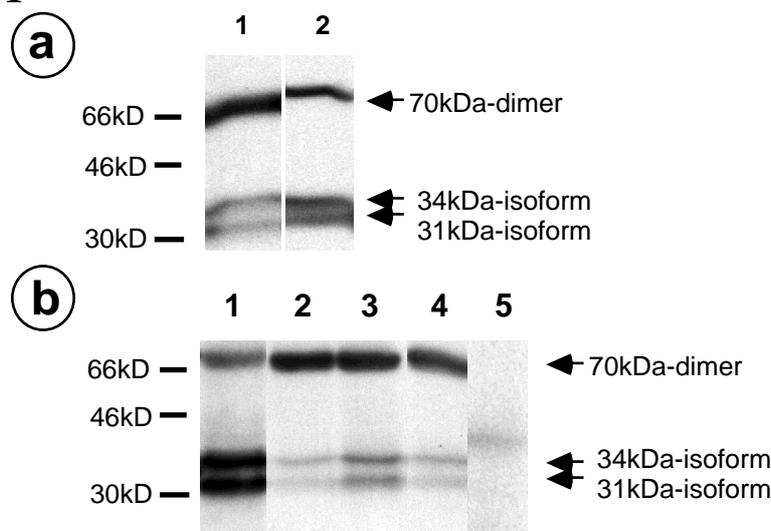
Figure 31

Fig. 31: Detection of epimorphin in the supernatants of A818-6 hollow spheres and Kif-5 fibroblasts by immunoprecipitation with the monoclonal antibody MC-1 and an affinity purified polyclonal antibody against epimorphin.

Panel a: Western blot analysis following immunoprecipitation from fibroblast (Kif-5) supernatants to confirm the detection of the same antigen (human epimorphin) by both antibodies used under these conditions. In lane 1 the precipitate obtained with the monoclonal antibody MC-1 was probed with the polyclonal rabbit serum against epimorphin, affinity purified against recombinant epimorphin. In lane 2, the precipitate obtained with the affinity-purified antibody was probed with the monoclonal rat-IgG- antibody against epimorphin MC-1. Epimorphin isoforms and dimers are indicated by arrows. Panel b: Western blot analysis following immunoprecipitation from A818-6 hollow sphere supernatants, Kif-5-fibroblast supernatants and supernatants obtained from co-cultures (pre-mature hollow spheres and fibroblasts). Precipitates obtained with both antibodies (MC-1 and the affinity purified polyclonal antibody against epimorphin) were resolved by SDS-PAGE (15%) and transferred to a nitrocellulose membrane. In lane 1, epimorphin was precipitated from hollow sphere supernatant by MC-1 and detected by MC-1. In lane 2 epimorphin was precipitated from hollow sphere supernatant and detected by the affinity purified polyclonal antibody. Epimorphin was also precipitated and detected by the affinity purified polyclonal antibody from co-culture supernatants (lane 3) and fibroblast supernatants (lane 4). In lane 5 an anti-human IL-13 (rat-IgG) antibody was used for precipitation from hollow sphere supernatants. This precipitate was probed with MC-1 on the succeeding Western blot (negative control). Epimorphin isoforms and dimers of the monomers are indicated by arrows.

3.6.5.4 Detection of epimorphin mRNA

On the mRNA level epimorphin was detected by RT-PCR in A818-6 monolayer- and hollow sphere cells (Fig. 32). Due to primer design the expected fragment size for epimorphin was 446bp. The most prominent band in Fig. 32 was found to exhibit the expected size. The upper, prominent band was cut out to prove the amplification of correct epimorphin-fragment. The DNA was eluted from the gel and digested with the restriction enzyme *Cla*I which should produce two fragments of 301bp and 145bp in case of the correct amplicon. As shown in Fig. 32 these fragments were obtained after the digestion with *Cla*I.

Figure 32

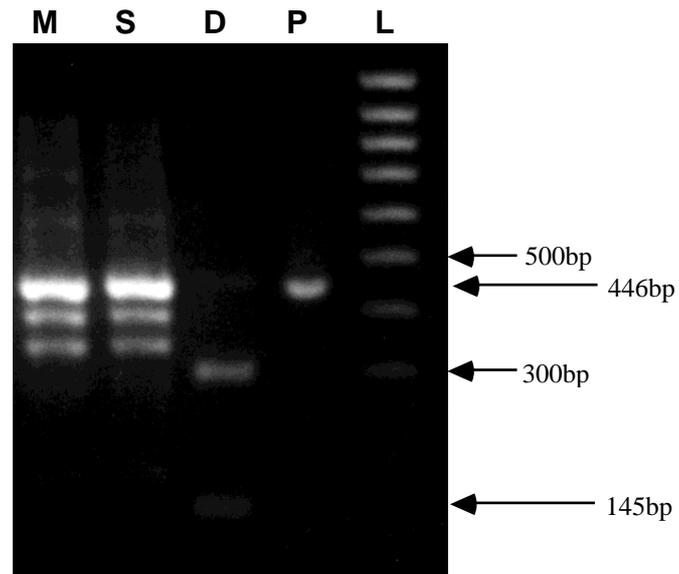


Fig. 32: RT-PCR for human epimorphin from RNA samples of A818-6 monolayer cells (M) and hollow sphere cells (S). The upper band of 446 bp was excised from the gel and used as probe (P) in a digest with the restriction enzyme *Cla*I (D). Fragment sizes are indicated as determined with a 100bp-DNA ladder (L) as standard.

4 Discussion

4.1 Discussion of methods

4.1.1 Generation of A818-6 hollow spheres

Epithelial cells have been cultured under various conditions and in most of the cases the two-dimensional monolayer-cell culture was the method of choice. Advantages of monolayer cultures with tumor cells are a quick access to mass production of cells and a high reproducibility of experiments due to the standardized culture conditions. On the other hand one must keep in mind that monolayer cultures are the most artificial cell culture type because under physiological conditions all cells are embedded in three-dimensional environment and this environment is essential for the regulation of growth and differentiation (Hollingsworth, 1999). Therefore experiments carried out in monolayer cultures gain performance by selling expressiveness. To overcome this discrepancy, researches thought about three-dimensional cell culture types coming closer to the *in vivo* situation with a high reproducibility and a good performance with Holtfreter and co-workers as pioneers (Holtfreter, 1944).

Basic work on morphology with three-dimensionally grown, multicellular aggregates was done during the 50ies and 60ies (Halpern et al., 1966; Moscona, 1952) until Inch and co-workers began to utilize aggregates to study the response of cell aggregates to radiotherapy in the early 70ies (Inch et al., 1970). They coined the term "multicellular spheroids". Since cell survival curves were similar in radiation experiments either with aggregates or with solid tumors, multicellular spheroids were henceforth considered *in vitro* tumor models for mimicking basic biological properties of cancer cells *in vivo*. Through their fundamental investigations Inch and associates inaugurated a continuously increasing number of studies on cell aggregates in various fields of experimental cancer research (Chuang and Liao, 2000; Graff et al., 2000; Quarrie et al., 1999).

Beside the support of cells with microcarriers or sponges one basic principle of three-dimensional suspension cell culture is to prevent the adherence of cells to substratum. This may either be achieved by stirring cells around in liquid media using so called "spinner-flasks" (Inch et al., 1970) or by coating cell culture flasks with non-adhesive material like agarose. Instead of coating flasks of cell culture grade, culture flasks of microbiological grade were used which

also reduced adherence. However, it has to be considered that depending on the way three-dimensional structures are generated, experimental outcomes may be altered. In cultures of A818-6 cells on solid agarose for example approximately 50% of the cells underwent apoptosis during hollow sphere development. If culture flasks of microbiological grade were used, some cells attached to the surface and grew out as monolayers, not becoming apoptotic. In these cultures the ratio between apoptotic cells and hollow sphere-developing cells was altered already by changing the way adhesion was prevented. If the coating-method was used to generate A818-6 hollow spheres, it was further important how much agarose was poured into each well. The more agarose was poured, the more concave the coating layer became due to the adhesion of agarose to the walls of the well.

This concave coating layers forced cells to role to the middle of the plate. A higher density of cells in the middle of the plate resulted in a higher amount of compact spheroids instead of hollow spheres.

Subsequently, the cell density was another important factor which has been standardized during many experiments. Less than 1×10^5 cells seeded per well of a 6-well plate were insufficient to produce enough hollow spheres for the experiments while more than 5×10^5 cells aggregated to large spheroid-like complexes leaving only few cells which developed into hollow spheres during 8-10 days. However, even under high density conditions hollow spheres developed from spheroid-like structures building "hollow buds" which were released. In contrast to normal hollow sphere development this procedure needed additional two weeks. Closely associated with the problem of aggregation to complex spheroids was the time of trypsinization of A818-6 cell prior to subjecting them to suspension culture. Grown as monolayer cultures these cells exhibited a strong affinity to substratum and needed significantly longer to be removed from the plates by trypsin/EDTA than other pancreatic carcinoma cell lines like Capan-2 or Panc-1. An average time of 30 min. was found to exhibit the best results with respect to the hollow sphere outcome. Shorter times lead to stronger aggregation of cells to spheroid-like structures while longer times lead to smaller hollow spheres. These observations suggest a critical role for integrins and cadherins as important molecules in either cell-cell adhesion or cell-matrix adhesion (Takeichi, 1993; Stallmach et al., 1991). The shorter the

trypsin-incubation time, the more adhesion molecules remain on the cell surfaces which may allow cells to quickly accumulate to spheroid-structures of under three-dimensional growth conditions whereas longer trypsin-incubation may lead to loss of most adhesion molecules and a therefore less efficient hollow sphere formation.

A818-6 cells subjected to a rotation culture (InVitro, Mannheim, Germany) also developed hollow spheres but smaller ones. However, hollow sphere development was observed under all tested conditions and conditions described in Material and Methods are optimized for high amounts of hollow spheres.

A high purity of hollow sphere cultures was essential to investigate differences between A818-6 hollow sphere- and monolayer cells. As mentioned before, approximately 50% of the initially seeded cells underwent apoptosis during hollow sphere development. It was impossible to remove all cell debris by standard centrifugation methods. Additionally proteins from dead cells contaminated the culture medium leading to false results in Western blot analyses for example.

Therefore a glass capillary adjusted to a micromanipulator was used to collect hollow spheres needed for an experiment.

4.1.2 Co-culture methods

Two basic co-culture methods were used in this study. The first was to mix two cell populations in equal amounts and to seed them together into pre-coated wells. The second was to pre-culture fibroblasts as confluent monolayers and to seed either trypsinated A818-6 cells or pre-mature hollow spheres on top of them.

In all co-culture experiments with two cell populations having different medium-requirements it is a prerequisite to define cell culture conditions both populations are satisfied with. In the case of fibroblast medium it was important to consider the influence of basic fibroblast growth factor onto hollow sphere development (compare 3.6.1, Results) and even not tested in detail, insulin as part of fibroblast medium might have an influence on A818-6 cells as well. Therefore RPMI1640 was used in all co-culture experiments. Experiments using the first variant of co-culture methods were only performed for the investigation of events that

could have been analyzed by microscopy including changes in shape or size of hollow spheres or differences in the speed of hollow sphere development. Once mixed, it was not possible to separate mesenchymal from epithelial cells to obtain protein-lysates or RNA-samples from only one cell type. Therefore Western blot analyses e.g. with fibroblast-stimulated A818-6 cells were only performed with hollow spheres being seeded on top of fibroblasts from where they could be easily removed without fibroblasts.

Co-culture experiments became much easier after EGFP-transduced fibroblasts were used. Unfortunately, pancreas-derived fibroblasts showed only unsatisfying fluorescence after EGFP-transduction. So far, no success was achieved in establishing a pancreas derived fibroblast cell line with satisfying EGFP-fluorescence. Skin-derived fibroblasts showed a satisfying fluorescence and were therefore used for most of the experiments. However it has been described that fibroblasts from different organs have a different influence on tumor cells (Okumura et al., 1992). Therefore it is essential to proceed the work of A818-6 co-cultures with organ-derived fibroblasts like PTF994 in the future.

4.1.3 Immunocytochemistry

First immunocytological experiments with A818-6 cells were performed by collecting monolayer- and hollow sphere cells by spinning them onto microscopic slides followed by staining as described in 2.2.4.1. This procedure worked well with monolayer cells but hollow spheres were lost during the multiple washing steps. From 100 hollow spheres collected on a microscopic slide 5 to 10 remained after the entire staining process. Even different coating agents like poly-L-lysine or chicken-albumin did not improve this method. Subsequently the only experiments performed with cytopins were stainings for the proliferation associated antigens Ki67 and p100 by using approximately 1,000 hollow spheres per slide from which 80-100 remained after the entire staining process. All other immunocytochemical experiments were carried out with cells stained in suspension (see 2.2.4.2, Methods) because staining on slides caused a waste of material. This staining procedure was especially adapted to A818-6 hollow spheres and permitted to maintain the three-dimensional shape of A818-6 hollow spheres throughout the whole process. For microscopical investigation coverslips were placed

on small wax-clumps allowing hollow spheres to remain three-dimensional even under microscopic conditions. This staining method worked well for membrane associated and nuclear antigens whereas cytosolic antigens sometimes lead to an eclipse of fluorescence caused by at least two cell layers above each other making it difficult to determine exact staining patterns. The disadvantage of the method was that monolayer cells were kept in suspension, floating around. Another critical point was the "trapping" of the antibodies in the lumen of hollow spheres after they were permeabilized for staining of cytosolic or nuclear antigens. In most cases excessive washing with PBS overnight solved that problem. In some cases it was possible to determine the specificity of an antibody-reaction by analyzing the experiments with a laser scanning microscope. The unspecific binding (trapping) of the secondary antibody in the negative control was subtracted from the sample by closing the pinhole of the laser. Only fluorescence brighter than the background remained and was attributed to a specific reaction of the primary antibody.

Taken together the cultivation and staining of A818-6 hollow spheres was standardized to permit reproducibility and comparability of all experiments.

4.2 Discussion of results

4.2.1 A818-6 hollow sphere development

As mentioned before, the three-dimensional culture of A818-6 hollow spheres must be seen in the tradition of earlier spheroid-cultures which resembled *in vitro* tumors. In contrast to these tumor models, the A818-6 systems displays a differentiation model using a pancreatic adenocarcinoma cell line which is able to revert the malignant into a partially benign phenotype. So far only one other comparable model system was described for the human pancreas also taking advantage of a highly differentiated adenocarcinoma cell line, Capan-1. Hollande and co-workers described a similar phenomenon for this cell line as it was described here for A818-6. However, Capan-1 cells were tested under culture conditions of A818-6 cells and did not display a luminal phenotype. Additionally Capan-1 "hollow spheroids" mostly consisted of more than a single layer of epithelial cell building the described phenotype (Hollande et al., 1998; Hollande et al., 1990). For other cell types and cells from other

species lumen-formation is well described. Experiments with Mardin-Darby canine kidney (MDCK) cells and a colon carcinoma cell line (LIM 1863) suggest a mechanism for the development of luminal structures from epithelial cells. MDCK cells were described to form tubular structures when grown in three-dimensional collagen gels (McAteer et al., 1986) and share the phenomenon of "clutching" with LIM1863 cells (Lincz et al., 1997). On the ultrastructural level clutching is characterized by the encapsulation of one epithelial cell by another as a very early step in the process of lumen-formation. A818-6 cells exhibited comparable stages relatively early in hollow sphere development (compare Results Fig. 9 a) but did not always show a complete encapsulation of cells. This finding is in accordance with MDCK cells which also do not display a complete encapsulation but are able to form luminal structures (Lincz et al., 1997). Clutching allows direct communication between cells to facilitate proper orientation and can also be regarded as a sign of differentiation; as the transitional state between a group of unpolarized cells and the formation of a highly organized three-dimensional structure.

A different hypothesis about how A818-6 hollow spheres start their development is to postulate a single cell as progenitor. Especially "signet-ring-like" cells as a characteristic phenotype in an early stage of hollow sphere development may bear this function. However, the detailed mechanism of A818-6 hollow sphere development will only be unraveled by an extended ultrastructural analysis combined with an on-line documentation as performed for LIM 1863 cells (Lincz et al., 1997). Unless these studies have not been performed the model described for LIM 1863 and MDCK cells is taken as the most likely one for A818-6 hollow sphere development as well. During A818-6 hollow sphere development approximately 50% of the initially seeded cells developed a luminal phenotype while most other cells underwent apoptosis or aggregated in spheroid-like structures. Therefore one might consider A818-6 hollow sphere cells as a subpopulation of the A818-6 cell line. On the other hand not all cells from a re-monolayer (monolayer which grew out after mechanical disruption of one hollow sphere) were able to generate new hollow spheres suggesting a regulative process turned on under special circumstances like contact inhibition (Soriano et al., 2000; Rashid and Basson, 1996). The aspect of contact inhibition is of particular interest with regard to the upregulation of the

cell cycle inhibitor p27/Kip found during hollow sphere development. Involvement of p27/Kip into epithel-cell-differentiation has been described in general (Lloyd et al., 1999) and in association with E-cadherin (St. Croix et al., 1998). However, the question arised whether A818-6 hollow sphere cells represent the "most benign" phenotype of the A818-6 system. When normal epithelial cells are either detached from their neighboring cell or their substrate, they undergo "anoikis" (apoptosis after detachment) (Frisch, 1999). Approximately 50% of A818-6 also not "survive" the detachment from their substrate. Since it has been described that β -catenin dysregulation leads to resistance against anoikes (Orford et al., 1999), it will be interesting to further analyze the regulation of β -catenin in the A818-6 system.

4.2.2 Development of junctional complexes

Loss of E-cadherin (Perl et al., 1998; Bracke et al., 1997) and changes in the expression of catenins (Sparks et al., 1998; Hague et al., 1997) displaying major components of adherence junctions, generally lead to a loss of contact inhibition, can change the tissue integrity and were described to be crucial for the development of a variety of malignant progressions. Especially the staining pattern for β -catenin in hollow spheres confirmed the identification of lateral adherence junctions at the electronmicroscopical level and underlines the strictly polar orientation of A818-6 cells in hollow spheres. A818-6 monolayer cells exhibited a heterogeneous staining pattern for cadherins and catenins. These findings are in accordance with the role of p27/Kip as an important indicator for differentiation in this system. Adherence junctions were the first cell-cell contacts recognized in A818-6 hollow sphere development as assessed by electron microscopy and according to the phenomenon of "clutching" (Lincz et al., 1997) they were found sporadically distributed. The anchorage of actin skeletons of cells are a prerequisite for the development of a proper cell polarity and the important role of cadherins and catenins in this context has been studied in *Drosophila melanogaster* previously (Grawe et al., 1996).

Mature A818-6 hollow spheres were ultrastructurally characterized by well-developed microvilli covering their apical membranes which were sealed by tight junctions further denoting the polarized epithelial development. Microvilli and tight junctions are characteristic

features of normal duct epithelial cells of the pancreas (Gumbiner, 1987; Stevenson and Keon, 1998). Additionally, the process of tight junction formation was found accompanied by translocation of symplekin from the nucleus to the membrane of A818-6 hollow sphere cells. This recruitment was not observed in monolayer cells. Recruitment seems to be a general mechanism for tight junction proteins which are stored somewhere else in the cell and then transported to the place they are needed (Yap et al., 1995). As the development of tight junctions reflects a high degree of differentiation the recruitment of a major component of the "zonula occludens", symplekin, underlines the process of increasing differentiation of A818-6 cells during hollow sphere formation (Keon et al., 1996).

4.2.3 Polarity of A818-6 hollow sphere cells

It still remains unclear why A818-6 hollow spheres exhibit a polarity with the apical membranes facing towards the surrounding cell culture medium as shown by various results including those from immunostaining with antibodies against mucin-1 and β -catenin. A similar orientation of cells was described for the pancreatic adenocarcinoma cell line Capan-1 also forming so called "hollow spheroids" (Fanjul and Hollande, 1993). These cells exhibited an inverted polarity after a Capan-1 tumor was heterotransplanted into nude mice (Murat et al., 1989) which was explained by the presence of extracellular matrix produced by fibroblasts of the connective tissue of the mouse. A818-6 hollow sphere cells were not tumorigenic in nude mice (data not shown) and therefore a comparative *in vivo* example is lacking. However, A818-6 cells supplied with a "mesenchymal cushion" consisting of fibroblasts attached to this fibroblast-core with their basement membranes and developed "fibro spheres" in which tumor cells showed the same orientation as found in hollow spheres (compare Fig. 24-26, Results). These results suggest a more physical reason. Under suspension cell culture conditions a ball-structure as a highly ordered structure exhibits the smallest entropy and may therefore be preferred. Even a cell culture model derived from normal cells of the human nasopharynx has been described to exhibit this "inside-out" polarity (Boxberger et al., 1993). On the other hand it is the apical membrane of a pancreatic epithelial cells that is in contact with a liquid like the pancreatic juice and not the basolateral membrane. If the surrounding cell culture

medium is interpreted as the former pancreatic juice, A818-6 hollow sphere cells behave normal when exposing their apical membranes towards the medium. Similar to Capan-1 (Levrat et al., 1988), A818-6 cell formed domes when grown to confluency as monolayer cultures and hollow spheres may be seen as the three-dimensional equivalent to these domes. Domes are a reflection of the presence of two media of different ionic concentrations separated by a planar barrier of cells connected by tight junctions (Fanjul and Hollande, 1993). In hollow spheres, differences in the concentration may be caused by the metabolic waste which must be secreted into the lumen of the sphere due to their polarity and might further display a reason for the limited life span of hollow spheres (Boxberger et al., 1993). Additionally, if tight junctions between A818-6 hollow sphere cells are not leaky (Blackburn et al., 1988; Rizzolo, 1997), the single layer of epithelial cells displays a functional barrier forcing the cells to obtain nutrients completely from the apical side (Cerejido et al., 1989; Handler, 1989) which required the encapsulated cell culture medium as storage-pool for nutrition. Next to β -catenin found associated to the basolateral membranes of A818-6 hollow sphere cells, integrin- β -1 and integrin- β -5 were detected by immunocytochemistry at the basal membranes of hollow sphere cells denoting the polarized phenotype (data not shown). Further laminin being described as an important factor in the establishment of functional and structural polarity (Ecay and Valentich, 1992; Moller et al., 1991) was observed at the basement membranes of A818-6 hollow spheres (compare Fig. 26, Results) pointing to an essential role of laminin in the development of A818-6 hollow spheres as well.

4.2.4 Proliferation of A818-6 cells

As an increase in proliferation is one of the most characteristic features associated with malignant transformation (Koli and Keski-Oja, 1996; Tennenbaum et al., 1996; Muir, 1995), it was demanding to analyze the differences in proliferation rates between A818-6 monolayer- and hollow sphere cells. Although no DNA-profile was generated to investigate the distribution of A818-6 cells in different phases of the cell cycle, a partial analysis of cell cycle distribution was possible with the antibodies KiS5 and KiS2 (Frahm et al., 1999; Rudolph et al., 1998; Heidebrecht et al., 1997; Rudolph et al., 1997; Heidebrecht et al., 1996). A massive decrease

in proliferation monitored by p27/Kip, cyclin B, Ki67 expression and telomerase assay during hollow sphere development characterized A818-6 hollow sphere cells as normal duct epithelial cells of the pancreas with the majority of cells being arrested in the G₀-phase of the cell cycle. From 31.5% of Ki67 positive cells in A818-6 hollow spheres only 2.2% were found in the G₂/S or M-phase of the cell cycle whereas approximately 24% were observed for monolayer cells (compare Fig. 10, Results). This nearly 10-fold decrease is likely to be ascribed to the re-stored ability of contact-inhibition in A818-6 hollow sphere cells underlined by findings with p27/Kip in Western blot analysis (Chen et al., 1999).

Of particular interest in the context of proliferation in A818-6 cells is the downregulation of the telomerase activity found in A818-6 hollow sphere cells. Human somatic cells have essentially no detectable telomerase activity. Telomerase is linked to tumor genesis and is a valuable marker for malignant growth (Morales et al., 1999; Krupp et al., 1997). It has been shown that the ectopic expression of the telomerase catalytic subunit (hTERT) in combination with two oncogenes (the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras) results in direct tumorigenic conversion of normal human epithelial and fibroblast cells (Hahn et al., 1999). Therefore, the regulation of the telomerase activity during hollow sphere development may display an important functional aspect concerning the reversibility of the A818-6 system (compare Results 3.1 Fig. 8 e and 3.3.2) and should be analyzed in ongoing studies.

4.2.5 Expression of differentiation marker antigens

As bicarbonate secretion of ductal epithelial cells is an important physiological feature, carbonic anhydrase II (CAII) is a marker for this type of cells (Nishimori et al., 1999). Marker antigens for acinar cells are the enzymes lipase and amylase (Krishnamurthy and Zamboni et al., 1996; Dayal, 1995). Consequently, CAII was detected in hollow sphere cells by indirect immunofluorescence staining determining the ductal origin of A818-6 cells underlined by negative staining results for lipase and amylase. Although widely accepted carbonic anhydrase, lipase and amylase as marker antigens should be interpreted with caution considering reports about transdifferentiation of former acinar cells into cells exhibiting

characteristics of ductal cells (Schmid et al., 1999). However, in this study the focus was set on clarifying the differences in the status of differentiation between two phenotypes of one cell line and therefore markers revealing differences within the system were required. Factors like p27/Kip, mucin-1, β -catenin, carbonic anhydrase, symplekin e.g. have been discussed above.

A further group of marker antigens suitable to prove the different status in differentiation between A818-6 monolayer and hollow spheres is displayed by members of the carcinoembryonic antigen family. In particular CEA-180 is a known and frequently used tumor marker detected in the serum of patients (Allum, 1990; Konomi et al., 1982). Former studies showed CEA was absent in normal tissue but detected in tumor tissue as assessed by immunohistochemistry (Kim et al., 1990). In A818-6 hollow sphere cells CEA was only detected in single cells by immunocytochemistry while most of the monolayer cells exhibited a positive reaction with an antibody against CEA. Furthermore A818-6 hollow sphere cells being positive for CEA exhibited an apical staining while monolayer cells were also stained cytosolically. These findings are in accordance with a theory saying that normal cells rapidly release CEA into the lumen of the digestive tract due to their polarity and not into the circulation like tumor cells (Kuroki et al., 1988). This theory is based on the observation that CEA concentrations in normal sera are very low. Secretion analysis of supernatants from A818-6 cells showed hollow sphere cells secreted only half of the amount of monolayer cells which also denotes CEA as a marker for differentiation in this system (compare 3.4.6, Results).

Next to mucin-1 another member of the mucin-family became interesting for the A818-6 system by means of different expression in monolayer- and hollow sphere cells. Leukosialin (CD43) was originally detected in the hematopoietic system and described as a "janus-headed" molecule either showing a cell-adhesion function (Manjunath et al., 1993) or the direct opposite by providing a repulsive barrier around cells (Zhang et al., 1997; Sanchez-Mateos et al., 1995). It has been speculated that tumor cells even not being of hematopoietic origin acquire the ability to express CD43 to passively repulse cells from the immune system (Santamaria et al., 1996). To test this hypothesis immunofluorescence analyses were carried

out with an antibody against CD43, known to react positively with tumor tissue (Santamaria et al., 1996). Only A818-6 monolayer cells were found to be positive for CD43 whereas hollow spheres resembling a normal duct-like structure, were negative in this study (compare 3.4.2, Results). These results support the idea of CD43 as a passive defense of tumor cells against attacks from the immune system and further suggest CD43 as an indirect differentiation marker in the A818-6 system.

4.2.6 *Influence of cytokines and growth factors*

Investigations on the effects of cytokines and growth factors were undertaken to obtain hints for essential signal transduction pathways responsible for A818-6 hollow sphere development. Investigations of the pathways regulating normal growth of epithelial cells have revealed the existence of two major growth-factor signaling cascades required for proliferation. One pathway is activated by IGF-1 or high insulin concentration. The other is triggered by EGF, TGF- α , or members of the FGF family (Aaronson et al., 1990). The insulin-like growth factor pathway was not addressed in this study. Structure and biological activity of EGF and TGF- α are quite similar and both growth factors are ligands of the same receptor (epidermal growth factor receptor, EGF-R) (Luetteke and Lee, 1990) being detected in A818-6 immunocytochemically (data not shown). However, no visible stimulation on A818-6 hollow sphere development was observed with both ligands. Many growth factors have been reported to be overexpressed in pancreatic and other carcinomas including EGF and TGF- α (Aaronson et al., 1990; Friess et al., 1996) suggesting that exogenously added higher amounts of already overexpressed growth factors exhibit no further stimulation due to the high present concentrations (Karnes et al., 1992). Growth stimulation of A818-6 cells was only observed with exogenously added bFGF a factor which also belongs to the pathway triggered by EGF and TGF- α in normal epithelial cells (Aaronson et al., 1990). Pancreatic cancers overexpress basic FGF (bFGF) and the type I FGF receptor (FGFR-1), and overexpression of bFGF has been correlated with decreased patient survival (Wagner et al., 1998). A818-6 hollow sphere cells were growth stimulated without disrupting or altering the highly differentiated hollow sphere structure pointing to a bFGF function in differentiation as observed during human embryogenesis

(Tienari et al., 1995) or fibre cell differentiation of the human lens (Leenders et al., 1997). On the other hand, Hirai and co-workers were able to show for HGF/SF that it was not the growth factor itself being responsible for luminal differentiation in mammary epithelial cells. In fact HGF/SF and other growth factors provided the proliferation stimulus necessary for differentiation, while epimorphin exhibited the morphoregulatory function (Hirai et al., 1998). Interferon- γ has been described to cause cell death in the human epidermoid carcinoma cell line A431 either grown in conventional monolayer culture or in three-dimensional spheroids (Ridge et al., 1991; Chang et al., 1987) as a cause of acceleration of terminal differentiation. These observations are similar to those obtained with exogenously added IFN- γ in cultures with A818-6 cells. Further IFN- γ has been described to exhibit apoptosis inducing capabilities like tumor necrosis factor alpha (TNF- α) (Munker and Andreeff, 1996). Like IFN- γ , TNF- α also induced cell death in A818-6 cultures. TNF- α is a multipotent molecule exhibiting many functions in either health or disease. Studies on the biological effects of TNF- α carried out in tissue culture, organ perfusion systems, animals, human volunteers and cancer patients suggest a general consensus for TNF- α as a key mediator of inflammation and the mammalian host response to injury or invasion by microbes, parasites or neoplasia (Tracey, 1994). Yet, the status of TNF- α receptors p55 and p75 (Kalthoff et al., 1993a) has not been specified in A818-6 cells and therefore it is difficult to interpret TNF- α effects. Eventually an effect is mediated through the induction of IFN- γ or TGF- β by TNF- α (Tracey, 1994), whereas further results from experiments with TGF- β suggest a different mechanism than for IFN- γ . TGF- β was found to inhibit A818-6 hollow sphere development but had no effect on mature hollow spheres or monolayer cells (compare 3.5, Results). New experiments revealed an upregulation of the cell cycle inhibitor p21/Cip after addition of recombinant TGF- β . The same result was observed after transduction of A818-6 cells with a constitutive active TGF- β I receptor. In these cells A818-6 hollow sphere development was massively slowed down. These findings are in accordance with the general ability of TGF- β to induce cell cycle inhibitory proteins, in particular p21 and the role of TGF- β 1 in contact inhibition (Lloyd et al., 1999; Miyazaki et al., 1998).

4.2.7 Mesenchymal co-culture

Although effects of factors like EGF, TGF- α and in particular HGF/SF on the morphogenesis of epithelial cells have been reported (Birchmeier et al., 1997; Brinkmann et al., 1995; Barton et al., 1991), no effects on either hollow sphere development or mature hollow spheres were observed. Therefore co-culture experiments have been undertaken to unravel the importance of the direct presence of mesenchymal cells to mediate effects either by direct cell-cell contact or by secreting soluble factors into the cell culture medium. Since it has been known for a long time the mesenchymal influence is a prerequisite for a proper development of the human pancreas (Polak et al., 2000; Ramalho-Santos et al., 2000; LeBras et al., 1998), the major importance of the mesenchyme in carcinogenesis is more and more recognized (Chung, 1995; Condon and Bosland, 1999; Hirai, 1993). A818-6 cells were stimulated by the direct presence of either organ-derived fibroblasts or skin-derived fibroblasts. Three experimental set ups were chosen to co-culture A818-6 cells with fibroblasts (compare 3.6, Results). Similar results for both fibroblast types were obtained after either trypsinated A818-6 cells or pre-mature hollow spheres were seeded on top of confluent fibroblast monolayers. Both fibroblast types supported the invasiveness of A818-6 monolayer cells as shown by the development of invasive tumor cell plaques within the confluent fibroblast monolayers or the growth of pre-mature hollow spheres into larger spheres of approximately equal size and shape (compare 3.6.1, Results). Growth stimulating effects of fibroblasts on epithelial cells have been contributed to mesenchymally derived growth factors like HGF/SF, KGF, EGF, VEGF e.g. secreted by the fibroblasts and acting via paracrine mechanisms (Zeigler et al., 1996; Hlatky et al., 1994; Nusrat et al., 1994). Subsequently conditioned medium of fibroblasts was tested for the ability to replace the direct presence of fibroblasts in the A818-6 co-culture experiments. Some stimulatory effect was observed when A818-6 hollow spheres were subjected to fibroblast-conditioned medium (without bFGF and insulin) but this stimulation was less effective than the effect with fibroblasts present in culture. This finding is surprising because pre-mature hollow spheres were stimulated when seeded onto confluent fibroblast monolayer cultures. Due to their "ball-like" structure direct contact to mesenchymal cells was limited to few A818-6 hollow sphere cells at a given time or by "rolling" over the fibroblasts.

Unless culture plates remained unmoved in the incubator for most of the time it is unlikely that hollow spheres have discovered a method of "self-movement" during the time they were not investigated. However, experiments with conditioned media have been found to be critical (Otonkoski et al., 1996). It further has been shown to be of crucial importance for the observed effects whether normal fibroblasts from the same organ, tumor-derived fibroblasts or fibroblasts from a different organ including their corresponding supernatants were taken for the experiments (Rossi et al., 2000; Brouty-Boye et al., 1994). These observations made by others support the results obtained after co-culturing trypsinated A818-6 cell with fibroblasts at a ratio of 1:1. Here differences between pancreas- and skin-derived fibroblasts were detected (compare 3.6.2, Results). Taken together, A818-6 cells were stimulated by fibroblast co-cultures. The "malignant" monolayer-phenotype showed an enhanced invasiveness by means of a higher proliferation rate of the cells growing as invasive plaques in confluent fibroblast monolayer cultures, while A818-6 hollow sphere cells were growth stimulated without disturbance of the highly differentiated hollow sphere phenotype. The fact that both cell types induce each other makes it difficult to speculate whether the secreted factors of the "benign" hollow sphere phenotype induce fibroblasts to secrete differentiation stimulating factors or whether the secreted mesenchymal factors were the same under conditions used in this study and the receptor status of A818-6 cells changed with respect to the differentiation status of monolayer- or hollow sphere cells. Therefore it is useful to determine the expression status of interesting factors independently in both compartments and then perform combining experiments. Such a procedure was performed for one mesenchymally derived factor called epimorphin.

4.2.8 *The role of epimorphin in the A818-6 system*

Recently epimorphin has emerged as an important morphoregulator derived from mesenchymal cells being also found in epithelial cells. Epimorphin has further been found to be responsible for the branching morphogenesis and lumen formation in breast epithelial cells of the mouse and is known to be a leading factor in the reconstitution of the liver (Hirai et al., 1998; Watanabe et al., 1998). This study shows that certain isoforms of epimorphin are important

factors for the development of luminal structures of pancreatic duct epithelial cells. As described for hair follicle growth and lung epithelial tubular formation (Hirai et al., 1992), the antibody MC-1 was found to be a potential inhibitor of A818-6 hollow sphere development. In accordance with previous work on mammary epithelial cell development (Hirai et al., 1998), no effects on hollow sphere development by using a neutralizing antibody against HGF were found. All reported isoforms of epimorphin (34kDa- and 31kDa-monomers) as well as the dimeric and tetrameric form were detected in fibroblasts whereas the 150kDa-tetramer of epimorphin was not detectable in lysates of A818-6 cells as assessed by Western blot analysis. Interestingly, a strict correlation between the ability of lumen-formation of pancreatic duct cells and the expression pattern of the 34kDa- and 31kDa-isoforms of epimorphin in cell lysates was observed. So far it is believed the extracellular and membrane-associated tetrameric complex (150kDa) represent the morphoregulatory form of epimorphin (Zhang et al., 1999; Hirai et al., 1998). In this study all monomeric isoforms and the dimeric form of epimorphin were detected in the supernatants of A818-6 cells and fibroblasts but no tetrameric form of 150kDa being restricted to fibroblast extracts. As immunoprecipitation data analyzing conditioned medium from A818-6 hollow spheres without co-cultivated fibroblasts revealed only the 34kDa- and 31kDa-isoforms which are able to either form homo- or heterodimeric complexes, morphogenic activity is attributed to these smaller forms. Interestingly, at least one 31kDa-isoform of epimorphin was reported to lack a putative membrane spanning domain further denoting a soluble character of at least one epimorphin-isoform (Hirai et al., 1998). Additionally, dimer-formation should only appear in case of undegraded monomers of epimorphin pointing to an ectodomain of epimorphin with functional interaction sites (Gilboa et al., 1998; Miller et al., 1995). The different affinities of the monoclonal antibody MC-1 and the affinity purified polyclonal antibody for either native or denatured forms of epimorphin are explained by the history of their production (Hirai et al., 1998; Hirai et al., 1992). This study was able to prove the presence of epimorphin and soluble epimorphin in particular in human pancreatic adenocarcinoma cells. Moreover, for the first time a functional effect of epimorphin on human epithelial cells exhibiting duct-like development was shown. Whether soluble epimorphin displays the active form of this competent morphoregulator also in other

tissues and whether quantitative differences in the ratio between monomeric and dimeric forms, as they are shown in Fig. 30 and 31, are of functional relevance need be addressed in further studies.

4.3 Conclusions

So far, most systems which are regarded as reflecting a situation comparable to normal tissues required an artificial three-dimensional environment. Some systems depended on exogenously added components like growth factors or other cytokines. The A818-6 hollow sphere system analyzed in this thesis developed spontaneously upon one single stimulus; the prevention from adherence. Although not being the first system which was described to exhibit the ability of luminal differentiation under three-dimensional conditions, the A818-6 system is the only system derived from cells of the human exocrine pancreas developing single layered epithelial, luminal structures. Furthermore, it recovers a functional contact inhibition as indicated by a dramatic decrease in proliferation during and after hollow-sphere formation. The polarized expression of members of the CEA-family, BGP and NCA as well as β -catenin or mucin-1 displays exactly the situation found in normal pancreatic tissue. Polarity is underlined by secretion of CEA 180 into the supernatant of hollow sphere cultures or the development of microvilli on apical membranes. When hollow spheres are disrupted or trypsinated and seeded into normal tissue culture flasks, cells grow as monolayer culture. When these cells are trypsinated again and seeded under three-dimensional growth conditions, hollow sphere formation takes place. This system is able to differentiate and de-differentiate in a reversible manner. Keeping in mind that A818-6 cells are derived from pancreatic adenocarcinoma and bear distinct genetic alterations these cells must "walk on a thin line" between malign and benign taken hollow sphere development as a hallmark for a benign phenotype. Yet unknown "major-switches" responsible for luminal differentiation are activated and deactivated in a reversible manner suggesting the A818-6 system as an interesting tool to unravel questions associated with the development of cell-cell contacts or the loss of the latter as observed during metastasis. In this thesis epimorphin has been found to present a potent candidate for such a "major switch" due to its dramatic influence on A818-6 hollow

sphere development and should be analyzed in more detail in ongoing studies. The reversibility combined with a quick access to high amounts of hollow spheres grown under standardized conditions display the practical advantage of this system enabling a researcher to confirm and extend the results without the limit of material resources as often associated to experiments carried out with normal tissue or primary cell cultures.

4.4 Future directions

If the general question about the future development of biological research is posed, the statements may be summarized as follows: Molecular biological techniques will be further improved allowing high throughput analyses for proteins like they are possible of nucleic acids today. Due to the increasing amounts of new sequences arising from the human genome project displaying potential genes of therapeutic relevance, a further focus will be put on bioinformatics in the future. Bioinformatics allow to navigate through the jungle of new sequences and to determine potential candidate genes by performing *in silico* experiments like virtual Northern blots following sequence alignments. However, beside these technical improvements a third field was lined out as important for future development. Biological read-out systems are essential to prove the biological function of sequences found by either virtual or classical methods for gene-hunting.

The unique three-dimensional shape of A818-6 hollow spheres displays a read-out criterion which is easy to document and can help to unravel gene functions being associated with cell-adhesion, cell cycle control, cell polarity, secretion of ductal epithelial cells, proliferation, contact inhibition and many more. Results may be either obtained by treating A818-6 cells with potential therapeutic agents or by transducing A818-6 monolayer cells with a gene of interest and subjecting these cells to three-dimensional growth conditions. Primary experiments have been set up to use hollow spheres as a model for functional epithelial barrier. Administration of infected gall or pancreatic juice from the bile duct may help to understand more about the mechanisms of acute pancreatitis since it has been described that bacterial invasion is associated with destruction of the tight junctions complexes (Arendt, 1991). As these experiments with infected juices were carried out in cats, the A818-6 system may be used to reduce animal

experiments in the future. Further experimental set up will use the barrier function to investigate the capability of phagocytosis in A818-6 cells. Preliminary results show different behavior of A818-6 hollow sphere cells in comparison to monolayer cells when they are fed with small fluorescing particles. The malignant monolayer took up particles quickly and in high amounts while hollow sphere cells phagocytosed only a few. Results from these experiments might help to develop methods for retrograde administration of agents coupled to particles which are phagocytosed by cancer cells of ductal origin without harming the normal cells.

5 Summary

Standard cell culture conditions must be considered as artificial especially with respect to the environment cancer cells are grown in. While tumors in patients exhibit a three-dimensional shape isolated cells are forced to grow as monolayer cultures two-dimensionally. Early experiments of Holtfreter and co-workers (Holtfreter, 1947; Holtfreter, 1944) showed the environment cells are grown in has a major impact on their behavior. Inch and co-workers (Inch et al., 1970) started experiments using compact aggregates of tumor cells (spheroids) for radiotherapy experiments opening a field of "in vitro mini-tumor" research using spheroids leading to relevant results in cancer research, in particular, analyses on tumor sizes (necrosis in tumors larger than 0.5- 0.9 mm in diameter) or angiogenesis.

This thesis characterizes a subclone of the pancreatic carcinoma cell line A818 called A818-6, developing hollow spheres under three-dimensional growth conditions. Hollow spheres consist of a single layer of 50-200 epithelial cells surrounding an inner lumen. In contrast, to A818-6, a subclone called A818-4 and many other cell lines tested formed compact spheroids as the only three-dimensional phenotype. A dramatically reduced proliferation rate compared to the corresponding monolayer was observed in hollow spheres as assessed by immunocytological staining against proliferation associated antigens like Ki67, p100 and p27/Kip or by TRAP-assay measuring telomerase activity. Hollow spheres developed in FCS-containing RPMI-1640-medium without additionally added cytokines. A818-6 hollow sphere formation and integrity was influenced by Interferon- γ , TGF- β , bFGF and TNF- α lead to cell death. Exogenously added HGF neither showed an effect on hollow sphere formation nor on the integrity of completely developed hollow spheres. Moreover, no changes were observed when cells were treated with a neutralizing antibody for HGF. Interestingly, hollow spheres showed intensive immunoreactivity for both, the HGF-receptor (c-met) and its ligand HGF. Immunostaining against BGP, NCA95, mucin-1 and β -catenin revealed a polar organization of hollow spheres further underlined by ultrastructural analyses showing tight junction formation at apical membranes of hollow sphere cells which were additionally covered with highly developed microvilli. Immunohistochemically, hollow spheres were negative for CEA-180. Interestingly, released CEA was found in the supernatant. Due to their polarity, hollow sphere cells must

secrete CEA apically which resembles the normal situation in gastrointestinal epithelial tissues. Mechanically disrupted hollow spheres not only attached but also grew as monolayer with the same doubling time as the founder cells. Co-culture of A818-6 cells with fibroblasts stimulated the development of hollow sphere structures in general and increased differentiation in 5-6 day old hollow spheres. Epimorphin was recently described as a mesenchymal factor modulating morphogenesis of murine mammary ducts, skin, liver and lung *in vitro*. Soluble 34kDa- and 31kDa-isoforms of epimorphin were detected in the culture supernatant of A818-6 cells. In lysates of A818-6 cells the 34kDa-, 31 kDa-isoforms and the dimers were detected. Lysates of fibroblasts additionally showed a 150kDa-tetramer of epimorphin. The tetrameric form was only detected in fibroblast lysates. A neutralizing monoclonal antibody against epimorphin (MC-1) blocked the development of hollow sphere structures from A818-6 cells efficiently. Cells treated with MC-1 were maintained in a developmental stage characteristic for days 2-3 of normal hollow sphere development. In this novel system for human duct-like differentiation of pancreatic epithelial cells strong evidence for an autocrine function of epimorphin as a major mediator for morphogenesis is provided. This is the first study showing a functional effect of epimorphin in cells of human origin.

Taken together, A818-6 hollow spheres resemble normally differentiated duct-like structures and will serve as an excellent model to study differentiation of pancreatic epithelial cells on morphological and molecular basis.

6 Zusammenfassung

Standard Zellkulturbedingungen müssen besonders im Hinblick auf die Umgebung, in der Krebszellen gezüchtet werden, als artifiziell angesehen werden. Tumore im Patienten sind dreidimensionale Gebilde, während man isolierte Krebszellen in Kulturflaschen zwingt, unter zweidimensionalen Bedingungen zu wachsen. Frühe Experimente von Holtfreter und Mitarbeitern (Holtfreter, 1947; Holtfreter, 1944) zeigten, dass die Form der Zellkultur maßgeblichen Einfluss auf das Verhalten von Zellen hat. Inch und Mitarbeiter (Inch et al., 1970) begannen, kompakte Tumorzellaggregate (Sphäroide) mit Radiotherapie zu behandeln, und eröffneten damit ein neues Feld der "in vitro-Minitumor"-Forschung, welches relevante Ergebnisse besonders in den Bereichen Tumorgöße (Nekrose in Tumoren, die größer als 0,5-0,9mm im Durchmesser werden) und Angiogenese hervorbrachte. Diese Dissertation charakterisiert einen Subklon der Pankreasadenokarzinomzelllinie A818, die A818-6 Zellen. Dieser entwickelt unter dreidimensionalen Kulturbedingungen "hollow spheres". "Hollow spheres" sind einschichtige Epithelhohlkugeln die aus 50 bis 200 Epithelzellen bestehen, welche ein inneres Lumen umschließen. Im Gegensatz zu A818-6 erzeugen ein anderer Subklon (A818-4) und viele andere getestete Zelllinien nur kompakte Sphäroide als einzige dreidimensionale Wachstumsform.

Ein dramatischer Rückgang der Proliferationsrate wurde während der Entwicklung von A818-6 hollow spheres beobachtet, gemessen an dem Vergleich der Expression von proliferations-assoziierten Antigenen wie Ki67, p100, p27/Kip oder der relativen Telomeraseaktivität in A818-6 "monolayer"- oder "hollow sphere Zellen". Die "hollow sphere" Entwicklung vollzog sich in RPMI1640-Medium ohne Zusatz von Zytokinen oder Wachstumsfaktoren, konnte aber durch exogene Zugabe bestimmter Faktoren wie Interferon- γ , TGF- β und bFGF beeinflusst werden. Die Zugabe von TNF- α führte zur Apoptose. Keine Veränderungen wurden mit rekombinantem HGF oder einem HGF-neutralisierenden Antikörper festgestellt. Interessanterweise exprimierten A818-6 Zellen sowohl HGF als auch seinen Rezeptor c-met. Immunzytologische Experimente mit Färbungen gegen Antigene wie BGP, NCA95, Muzin-1 oder β -catenin offenbarten die strikt polare Orientierung der A818-6 Zellen im "hollow sphere". Diese Polarität konnte desweiteren durch elektronenmikroskopische Analysen

untermauert werden. Diese zeigten "tight junctions" zwischen den apikalen Membranen von "hollow sphere" Zellen, welche zusätzlich noch hoch entwickelte Mikrovillistrukturen trugen. Immunzytochemisch waren "hollow spheres" negativ für CEA-180. Interessanterweise wurde sezerniertes CEA-180 aber im Zellkulturüberstand von "hollow spheres" gefunden. Entsprechend ihrer Polarität mit den apikalen Membranen Richtung Zellkulturmedium muss CEA apikal sezerniert werden. Dieses entspricht der Situation, welche im normalen gastrointestinalen Gewebe gefunden wird. Zellen von mechanisch zerstörten "hollow spheres" wuchsen in normalen Kulturflaschen an und bildeten neue Monolayer-Kulturen, die den Ausgangszellen entsprachen. Co-Kultur Experimente mit A818-6 Zellen und Fibroblasten stimulierten die Entwicklung der "hollow spheres" im allgemeinen und steigerte den Differenzierungsgrad von 5-6 Tage alten "hollow spheres". Epimorphin wurde kürzlich als mesenchymaler Faktor beschrieben, welcher massiven Einfluss auf die Morphogenese von murinen Brustdrüsengängen, der Haut, Leber und Lunge hat. Lösliche 34kDa- und 31kDa-Isoformen von Epimorphin wurden im Zellkulturüberstand von A818-6 "hollow spheres" gefunden. In Proteinlysaten von A818-6 Zellen fanden sich die 34kDa- und 31kDa-Isoform sowie eine dimere Form. Proteinlysate aus Fibroblasten zeigten zusätzlich noch eine tetramere Form von 150 kDa, welche aber nur in Fibroblasten gefunden wurde. Ein epimorphin-neutralisierender Antikörper (MC-1) war in der Lage, die Entwicklung der "hollow spheres" zu unterbinden, wobei die Zellen nicht getötet wurden, sondern auf Entwicklungsstufen der Tage 2-3 wie unter unbehandelten Bedingungen festgehalten wurden. Somit konnte ein autokriner Mechanismus für Epimorphin in der Differenzierung von Pankreasadenokarzinomzellen zu gangähnlichen Strukturen gezeigt werden.

Dieses ist die erste Studie, welche einen morphoregulatorischen Effekt von Epimorphin in menschlichen Zellen zeigt.

Zusammengefasst verhalten sich A818-6 "hollow sphere" Zellen in grundlegender Hinsicht wie normale Pankreasgangepithelzellen und eignen sich deshalb besonders als Modell zum weiteren Studium von Differenzierungsvorgängen in normalen Pankreasgangepithelzellen.

7 References

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Raw data proliferation**KiS5-staining****A818-6 monolayer**

counted cells	positive cells	counted cells	negative cells
255	242	224	11
238	226	281	14
212	202	267	14
705	670	772	39

235 223,333333 257,333333 13 average
 21,6564078 20,1328918 29,7040962 1,73205081 standard deviation

KiS5-staining**A818-6 hollow spheres**

counted cells	positive cells	counted cells	negative cells
278	88	254	174
312	98	268	184
323	102	317	217
913	288	839	575

304,333333 96 279,666667 191,666667 average
 23,4591844 7,21110255 33,0807094 22,5018518 standard deviation

KiS2-staining**A818-6 monolayer**

counted cells	positive cells	counted cells	negative cells
183	45	192	145
167	41	168	127
201	49	188	142
551	135	548	414

183,666667 45 182,666667 138 average
 17,0098011 4 12,858201 9,64365076 standard deviation

KiS2-staining**A818-6 hollow spheres**

counted cells	positive cells	counted cells	negative cells
298	19	212	197
248	15	308	286
256	23	304	283
802	57	824	766

267,333333 19 274,666667 255,333333 average
 26,8576494 4 54,3077649 50,5404129 standard deviation

Telomerase-assay raw data

Monolayer	Spheres	
32,354	4,815	
20,359	7,434	
19,429	0,845	
17,571	1,69	
10,728	1,858	
20,0882	3,3284	average
7,02	2,44	standard deviation

PCR-Primer to human epimorphin

EPIMOR-SENSE

5'-TACCAAGACCTTTGGTGAAGGG-3'

EPIMOR-ASENSE

5'-AAGCTATGGGAGCATAAGCAGC-3'

Primers used for the RNaseP template in telomerase-assay (TRAP-assay)F286: 5'-GCTTGCCAAATCCGTCGAGCAGAGTTCGGGATGGGCCGCTTGAGGC-3'R376: 5'-GTGCCCTTACCCTTACCCTTACCCTAACGCCTCCGGGACGAGGCGTAAG-3'

The primers include the sequences for TS and CX-ext respectively (underlined).

Abbreviations

APC	Gene product of adenomatous poliposis coli gene
APS	Ammonium Persulfate
BGP	Biliary Glycoprotien
CAII	Carbonic Anhydrase type II
Cdk's	Cyclin-dependend Kinases
cDNA	Complementary Desoxyribonucleic Acid
CMV	Cytomegalovirus
DMSO	Dimethyl Sulfoxid
DNA	Desoxribonucleic Acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked Immuno-Sorbent Assay
FCS	Fetal Calf Serum
G418	Aminoglycoside antibiotic geneticin
gag	Retroviral structural gene encoding the group antigen (capsid, nucleocapsid, matrix, protase proteins)
h	hour
HBSS	Hank's Balanced Salt Solution
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HRP	Horeseradish Peroxidase
IGF	Insulin-like Growth Factor
ITAS	Internal amplification standard
MLV	Murine Leukemia Virus, moloney strain
MOI	Multiplicity of infection
Muc-1	Human Mucin-1
NCA	Nonspecific Crossreacting Antigen

PBS	Phosphate Buffered Saline
pol	Retroviral structural gene encoding reverse transcriptase including polymerase RNase H and integrase
PDVF	Polyvinillidene difluoride
RNA	Ribonucleic Acid
RT	Room Temperature
SCID-bg	Severe Combined Immunodeficiency Defect-beige
SDS	Sodiumdodecyl Sulfate
TEMED	N,N,N,N-Tetramethylethylenediamine
TGF's	Transforming Growth Factors
TNF's	Tumor Necrosis Factors
TRAIL	TNF-Related Apoptosis Inducing Ligand
VEGF	Vascular Endothelial Growth Factor
VSV-G	Vesicular Stomatitis Virus Glycoprotein

All other abbreviations are explained directly in the text.

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Summary

Motivated young molecular biologist experienced in several techniques of molecular biology and cell biology. Coordinator of cooperations with other Universities and Companies focussing on the exchange of research results for a better output of biological research. Part of the organizing team of oncology congresses at the Clinic for General Surgery in Kiel. Experiences in oral presentations of research data. Training supervisor for medical students since 1998. Head of an own project which is funded by the "Deutsche Forschungs Gemeinschaft" (DFG) since 1997 and was elongated till 2001. Good knowledge of recent developments in the field of oncology, especially gastrointestinal diseases and pancreatic cancer in particular. Set up of an unique *in vitro* cell culture model which is part of cooperations with pharmaceutical companies.

Interested into various working fields that deal with complex problems, teamwork and organization preferentially embedded into a "natural science environment".

Education/Experience

Christian Albrechts University, Molecular Oncology Research Unit and Clinic for General Surgery :1995-Present

Ph.D.-Student: August 1997 till present

The main topic of this Ph.D.-thesis is the characterization of a pancreatic adenocarcinoma cell line which displays a model system for the analyses of three-dimensional differentiation of cancer cells and offers several opportunities for further investigations on molecular and pre-clinical levels. The work includes the preparation of publications, presentations of the data at international congresses (see attached publication list). Winner of the Award "Hans-Chiari-Preis" for good research in the field of pancreatic diseases November 1998. I plan to finish my thesis and to obtain the Ph.D.-degree in November 2000.

Research Assistant: February 1997 till August 1997.

Being educated in techniques of retroviral genetransfer.

Diplombiologe (M.S. biology)

12.02.1997 at Christian Albrechts University, Kiel. Grade: "sehr gut" (A-level)

Diploma-work (final exam work to achieve M.S. degree): January 1996 till January 1997.

Diploma-work dealt with the development of an Ras-specific ribozyme against codon 12 mutated Ki-ras-mRNA. The work was set up in cooperation with the Institute Of General Microbiology and the Institute Of Hematopathology of the Christian Albrechts University, Kiel. Following techniques were used: *in vitro* transcription, 5'-endlabeling of RNAs, ribozyme cleavage-assays, kinetic analyses of ribozymes, ELISA-techniques, Northern blotting, cell culture work with pancreatic carcinoma cell lines, transfection assays with different methods,

³H-thymidine-assays.

Study of biology at Christian Albrechts University, Kiel:

March 1992 till 12.02.1997. This period is including the diploma-work mentioned above as well as all taken courses and jobs as a student research assistant in different fields of biology. All assistantships were done beside the normal courses and exams.

Assistant student in the research group of molecular oncology:

May 1995 to February 1997. Gene expression analyses of cell membrane associated growth factor receptors by Northern blot and ELISA-techniques; cell culture work; extraction methods for nucleic acids.

Assistant student in the Institute of Microbiology:

March 1994 to April 1995. Cultivation of hyperthermophilic organisms. Different staining methods; DNA isolation out of several bacteria and hyperthermophilic organisms.

Vordiplom (comparable to B.A. biology but not an independent degree in Germany):

March 1993. Grade: "gut" (B-level). Main courses after the Vordiplom: 1) Microbiology, 2) Zoology, 3) Biochemistry

Assistant student in the center of applied ecology:

April 1992-December 1993. Chemical analyses of groundwater probes.

Study of biology at the Justus Liebig University, Giessen, Germany:

October 1990 to March 1992: General courses of mathematics, physics, botany, zoology, chemistry

Military Service: 1989-1990

High school: June 1980 till June 1989 at the "Auguste-Viktoria-Schule" in Itzehoe, Germany.

Graduation from College with the "Abitur" and the grade 2,0 (B-level)

Elementary school: 1976-1980

Parents: Ernst and Petra Lehnert (maiden-name: Kumbartzki)

Born: July 20th, 1969 in Itzehoe, Germany (Nationality , German)

Technical Skills

Experience with advanced methods of general microbiology and advanced experience in molecular biology. Knowledge of *in vitro* transcription methods, ribozyme analyses, ribozyme cleavage assays, kinetic characterization of an ribozyme against the multidrug resistance gene *mdr-1*. Development of an functional ribozyme against *k-ras-mRNA* in pancreatic cancer. Five years of cell culture experience. Knowledge of retroviral genetransfer methods. Good knowledge of protein detection assays and other general molecular biological techniques like PCR , Sequencing etc. Further training in bioinformatics. Since two month training in handling DNA-Chip technology.

Publications/Congresses (oral presentations)

Lasse Lehnert, Markus M. Lerch, Yohei Hirai, Marie-Luise Kruse, Wolff Schmiegel and Holger Kalthoff: Autocrine stimulation of human pancreatic duct-like development by soluble isoforms of epimorphin *in vitro*. Submitted to *The Journal of Cell Biology*, 24.07.2000

Deutsche Krebsgesellschaft, Krebskongress 2000, 20.03.-24.03.2000, Berlin

20. Jahrestagung des Deutschen Pankreasclub, 18.11.-20.11.1999, Leipzig

Gesellschaft für Gastroenterologie in Westfalen e.V., 9. Jahreskongress, 05.11.-06.11.1999, Münster

Lehnert, L., Trost, H., Schmiegel, W., Roeder, C. & Kalthoff, H. Hollow spheres: a new model for analyses of differentiation of pancreatic duct epithelial cells. *Ann N Y Acad Sci* **880**, 83-93 (1999).

XXXI Meeting of the European Pancreatic Club, 30.07.-31.07.1999, Lüneburg

Deutsche Gesellschaft für Chirurgie, Chirurgenkongress 1999, März 1999, München

Differenzierung einer Pankreas-Adenokarzinomlinie zu polarisierten Epithelhohlkugeln:

Ein *in vitro* Modell zum Studium von Vorläuferläsionen des Pankreas

Langenbeck's Archives of Surgery, Supplement I, Forumband 1999, 285-289

19. Jahrestagung des Deutschen Pankreasclub, 16.11.-28.11.1998, Münster, Verleihung des "Hans-Chiari-Preises" für Grundlagenforschung

K. K. Singh, P. Schluff, L. Lehnert and G. Krupp. (1996)

Design of hammerhead ribozymes to distinguish single base changes in substrate RNA.

Antisense Nucleic Acid Drug Dev 6:165-168

Presentation of the published data from above during the congress of "Therapeutic Antisense Oligonucleotides" in June 1996 in Rome/Italy.

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Further I appreciate the great support of all technical assistants and PhD-students in the Molecular Oncology research group. Some of them know me since I joined the group as a volunteer and even have not quitted the job. It would crack the frame to mention all the little and large things these people helped me with and for sure this help was indispensable. Thanks!

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To my parents: Ernst und Petra

Euch gebührt der größte Dank. Ihr habt immer an mich und meine Entscheidungen geglaubt und mich unterstützt, auch wenn der "allgemeine Trend" dagegen sprach. Dank für die hoffentlich nicht allzuvielen schlaflosen Nächte, die ich Euch bereitet habe.

Euer Sohn Lasse

Rückseite

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