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Hypoxia-inducible Factor-1 (HIF-1) in Pancreatic Cancer Cell Aggressiveness and Therapeutic Resistance and the Potential Role for Pancreatic Endocrine Cells in Islet Transplantation

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Stockholm 2014

Published and printed by Karolinska Institutet, Printed by Universitetesservice-AB.

ISBN [978-91-7549-415-9]

To my lovely family

ABSTRACT

Tissue hypoxia results from an inadequate supply of oxygen (O_2) that compromises biologic functions in various tissues, including both normal and malignant. It is known that a heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1) mainly mediates this critical adaptation. It regulates the expression of more than 100 genes encoding key factors in cell proliferation and survival, glucose metabolism, invasion, angiogenesis and erythropoiesis. In this thesis I have investigated the roles of HIF-1α in endocrine β-cells of pancreatic islets (paper I) and in exocrine ductal epithelial cells (paper II and III) as well as their surrounding stromal cells (paper IV) of pancreatic ductal adenocarcinoma (PDAC).

Pancreatic islet transplantation is a biological replacement strategy for diabetes mellitus, however the benefits of islet transplantation are only short-term due to the lost grafts over time. Several strategies have been explored to improve the efficacy of islet transplantation. We previously reported a combination of islet preculture and recipient treatment with exendin-4 improved the metabolic outcome of a suboptimal number of rat islets transplanted to diabetic athymic mice. In paper I, we aimed to investigate mechanisms of effects of exendin-4 on islet function and viability in the rodent islet transplantation model with special focus on HIF-1α expression. Our data revealed that short-term preculture with exendin-4 followed by recipient treatment improved the outcome of both free and macroencapsulated islet grafts due to a larger surviving endocrine cell volume. Furthermore, this study has indicated for the first time that the protective effects of the glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4 may be mediated via the HIF-1 pathway.

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignant disease with fatal prognosis. It is characterized by a rapid progression, early metastasis, diagnosis at an advanced stage, and a limited response to chemotherapy and radiotherapy. Since tumour hypoxia is strongly associated with tumour propagation, malignant progression, and resistance to therapy, the study of HIF-1α has emerged a central issue in tumour physiology and cancer therapy. In paper II, we aimed to investigate the relationship of excess glucose, glucose reprogramming and cell migration in human PDAC cells with respect to HIF-1α expression. We found that excess glucose induced HIF-1α expression, increased ATP contents and stimulated migration in MiaPaCa2 pancreatic cancer cells. In addition, non-hypoxic factors contributed to this action in MiaPaCa2 cells as well.

The drug-resistant nature of PDAC cells results in a lack of effective chemotherapies, which contributes to the high mortality in patients with pancreatic ductal adenocarcinoma. The microenvironment (such as interactions between cell surface integrins, extracellular matrix components and intra-tumoural hypoxia) is responsible for innate drug resistance. In order to screen new drugs for PDAC treatment, an *in vitro* model as a more predictive platform is strongly required in this field. In paper III we aimed to develop a 3D model of human PDAC cells, and to further explore mechanisms underlying the transition from 2D to 3D cultures that might be responsible for chemoresistance, including HIF-1 pathway. We successfully established a new high-throughput 3D cell culture drug screening system for pancreatic cancer, which displays

increased chemoresistance resulting from enhancement of ECM production, glycolysis and expression of miRNA, hypoxia-inducible genes as well as chemoresistance genes. Our finding is supporting the concept of cell adhesion mediated drug resistance in PDAC. To increase utility and predictive value of our 3D tumour cell model for preclinical drug discovery, in paper IV we generated a hetero-spheroid model with pancreatic stellate cells (PSCs) surrounding a core of cancer epithelial cells, as can be observed in sections from patients with PDAC. Furthermore, gene expression was up-regulated in hetero-spheroids of PDAC cells, including E-cadherin, β-catenin, fibronectin, collagen I, lumican, COX2 and PPP1R1B, compared to mono-spheroids. In addition, we found that HIF-1α expression in hetero-spheroids was associated with the enhanced expression of ECM proteins, cancer stem cell marker (CD24), gene PPP1R1B (DARP-32) and hypoxia-inducible genes, which might alter sensitivity of cancer to chemotherapy.

In conclusion, the present work demonstrates that HIF-1α is an important transcription factor for displaying protective effects of exendin-4 on islet grafts after islet transplantation, understanding the mechanism of Warburg effect in pancreatic cancer cells and describes the development of an organotypic *in vitro* culture system for PDAC, facilitating the examination of tumor-stroma interactions and improving predictability of drug screening system in pancreatic ductal adenocarcinoma.

LIST OF PUBLICATIONS

This thesis includes the following papers, which will be referred to by their Roman numerals in the text:

I. **Xiaohui Jia,** Amit Sharma, Makiko Kumagai-Braesch, Annika M. Wernerson, Anne K. Sörenby, Shinji Yamamoto, Feng Wang, and Annika B. Tibell.
Exendin-4 Increases the Expression of Hypoxia-Inducible Factor-1α in Rat Islets and Preserves the Endocrine Cell Volume of Both Free and Macroencapsulated Islet Grafts.
Cell Transplantation 2012, **21 (6)**: 1269-83.

II. Zhiwen Liu, **Xiaohui Jia**, Yijie Duan, Huijie Xiao, Karl-Gösta Sundqvist, Johan Permert and Feng Wang.
Excess glucose induces hypoxia-inducible factor-1α in pancreatic cancer cells and stimulates glucose metabolism and cell migration.
Cancer Biology & Therapy 2013, **14 (5)**: 1-8.

III. Paola Longati, **Xiaohui Jia**, Johannes Eimer, Annika Wagman, Michael-Robin Witt, Stefan Rehnmark, Caroline Verbeke, Rune Toftgård, Matthias Löhr† and Rainer L Heuchel†.
3D pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing.
BMC Cancer. 2013, **13**:95.

IV. **Xiaohui Jia**, Paola Longati, K.-Jessica Norberg, Salvatore Nania, Johannes Eimer, J.-Matthias Löhr†, Rainer Heuchel†
Correctly oriented and activated pancreatic stellate cells engulfing pancreatic ductal adenocarcinoma cells to form an avascular, stroma-containing mini-tumour:
Establishment of the model and first description. (Manuscript)

† Equal contributors

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LIST OF ABBREVIATIONS

APH	acid phosphatase
ARD1	arrest-defective-1
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
CBP	CREB-binding protein
COX-2	cyclooxygenase-2
CSC	cancer stem cells
C-TAD	C-terminal transactivation domain
DR	dense desmoplastic reaction
ECM	extracellular matrix
EPF	E2-endemic pemphigus foliaceus
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblast growth factor
FIH	factor-inhibiting HIF
GLP1	glucagon like-peptide 1
GLUT	glucose transporter
H&E	Hematoxylin and eosin
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HK	hexokinase
HSP90	heat-shock protein 90
IBMIR	instant blood-mediated inflammatory reaction
IFP	interstitial fluid pressure
iPAS	inhibitory PAS
IGF	insulin-like growth factor
IL-1β	interleukin-1β
INS	insulin
IPGTT	intraperitoneal glucose tolerance test
KH	Krebs-Henseleit
LDH	lactate dehydrogenase
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase
MDR1	multidrug resistance protein 1
MMP	matrix metalloproteinase
N-TAD	N-terminal transactivation domain
O_2	oxygen
ODDD	oxygen-dependent degradation domain
OXPHOS	oxidative phosphorylation
PanIN	pancreatic intraepithelial neoplasia

PAS family	PER, AHR, ARNT and SIM family
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet-derived growth factor
PDK-1	pyruvate dehydrogenase kinase-1
PHD	prolyl hydroxylase domain proteins
PPP1R1B	protein phosphatase1, regulatory subunit1B
PSC	pancreatic stellate cell
PSC-c	conditioned PSCs
RBX1	ring box protein-1
ROS	reactive oxygen species
SNED1	sushi, nidogen and EGF-like domains 1
SSAT	spermidine/spermine-N^1-acetyltransferase
TGF	transforming growth factor
TNF	tumour necrosis factor
UCP	ubiquitin carrier protein
VHL	von Hippel-Lindau tumour suppressor
α-SMA	α-smooth muscle actin
2D	two-dimensional
3D	three-dimensional

1 INTRODUCTION

1.1 Hypoxia and HIF signalling pathway

1.1.1 Definition of hypoxia

Oxygen (O_2) has a central role in biology because it is used during respiration. The normal O_2 levels inside the human body vary from ~21% [corresponding to a partial pressure (PO_2) of ~150 mmHg at sea level] in the upper airway to ~1% at the corticomedullary junction of the kidney. *In vitro*, cultured cells are usually maintained in 20% O_2 (95% air and 5% CO_2) and this concentration is referred to 'normoxia' in spite of the fact that most cells in the human body are exposed to much lower O_2 levels. Whatever the specific set point, complex homeostatic mechanisms serve to maintain the cellular O_2 concentration within a narrow range *in vivo* [1].

Hypoxia is defined as a reduction of O_2 supply to the tissues of the body below physiological levels [2]. However, this is a relative term. Hypoxia can occur continuously, as when individuals ascend and remain at high altitude, or intermittently, as in individuals with sleep apnea. Hypoxia can be divided into an acute phase, in which rapid but transient responses are mediated through posttranslational modification of existing proteins, and a chronic phase, in which delayed but durable changes are mediated through altered gene transcription and protein synthesis. Finally, hypoxia can be systemic, as in the case of ascent to high altitude, or local, as in the case of myocardial ischemia associated with coronary artery disease [1].

Hypoxia plays essential roles in the pathophysiology of anemia, kidney, coronary artery, pulmonary and hematological diseases, wound healing, inflammation and cancer [1, 3 and 4]. However, hypoxia has also an important and beneficial role in regulating angiogenesis and controlling stem cell differentiation and cell fate during mammalian embryogenesis [5–7].

1.1.2 The hypoxic response

Oxygen is critical for the survival of multicellular organisms because it is used in aerobic metabolism, which turns carbohydrates into the energy needed to power essential cellular processes such as protein synthesis. Consequently, cells have to sense and respond to hypoxic conditions. The earliest recognized pathway is that hypoxic cells switch their primary pathway of energy production from oxidative phosphorylation (OXPHOS) to glycolysis. Hypoxia also induces erythropoietin production in renal cells (to increase haemoglobin production) and tyrosine hydroxylase synthesis in neural cells (involved in catecholamine production). One of the

most well studied hypoxic responses is production of growth factors that induce angiogenesis. Other hypoxia inducible genes are involved in pH homeostasis, oxygen sensing, cell death, genomic stability, proliferation as well as cell migration and they all aim at adapting cells to the hypoxic stress [8, 9]. Moreover, hypoxic tumour cells evolve to take advantage of some adaptive responses and to avoid others and an aggressive tumour phenotype is probably achieved through a stepwise selection process, in part driven by hypoxia. A number of studies have verified that hypoxia-inducible factors (HIFs) mediate this critical adaptation [8]. In addition, hypoxic cells also activate non-HIF-mediated mechanism for adaptation to hypoxic microenvironment [10–12].

1.1.3 HIF signalling pathway

1.1.3.1 Hypoxia-inducible factors (HIFs)

As mentioned above, HIFs is the master regulator of O_2 homeostasis and it facilitates both O_2 delivery and adaptation to O_2 deprivation by regulating a large number of genes involved in diverse processes like metabolism, apoptosis and angiogenesis [3]. HIFs are transcription factor consisting of an α-subunit and a β-subunit also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). There are three isoforms of the HIF-α subunit (HIF-1α, HIF-2α and HIF-3α) and three isoforms of HIF-1β (ARNT, ARNT2 and ARNT3), which all belong to the basic helix-loop-helix proteins (bHLH) of the PAS family (PER, AHR, ARNT and SIM family) [13–17]. These subunits contain a N-terminal bHLH and two PAS domains that mediate DNA binding and heterodimerization, shown at figure 1 [18, 19]. HIF-1α and HIF-2α also contain an oxygen-dependent degradation domain (ODDD) and two transactivation domains referred to as the N-TAD and C-TAD (N- and C-terminal transactivation domain, respectively). The ODDD domain is responsible for the negative regulation of HIF-α in normoxia [20] and both TADs have been shown to interact with co-activators such as CBP/p300 to activate gene transcription [21, 22]. Therefore the α-subunit is the regulatory subunit, while the β-subunit is constitutively expressed. Either HIF-1α or HIF-2α is able to dimerize with HIF-1β in the nucleus and binds to the consensus sequence 5'-(A/G) CGTG-3' present in the hypoxia response element (HRE) of O_2-controlled target genes.

HIF-1α was the original HIF isoform identified by affinity purification using oligonucleotides from the erythropoietin locus, while HIF-2α and HIF-3α were identified by homology searches or screens for interaction partners with HIF-1β [13-15]. HIF-1α is expressed in most cell types, whereas HIF-2α shows tissue-specific expression in endothelial cells, glial cells, type II pneumocytes, cardiomyocytes, fibroblasts of the kidney, interstitial cells of the pancreas and duodenum, and hepatocytes [23]. Although HIF-1α and HIF-2α are structurally similar in their

DNA binding and dimerization domains and undergo similar proteolytic regulation, they have distinct transcriptional targets due to their different transactivation domains. It has been proposed that they regulate both common and unique target genes [24–26], and are differentially regulated depending on the duration and severity of hypoxia exposure [27]. For instance, HIF1α (and not HIF2α) appears to be particularly important for activating anaerobically glycolytic gene expression [24]. HIF-3α is also expressed in specific tissues such as adult thymus, lung, brain, heart, and kidney. And it is the more distantly related isoform and encodes at least one splice variant that is dominant-inhibitory to HIF called inhibitory PAS (iPAS) [28, 29]. HIF-1β is identical to a previously described constitutive nuclear protein, ARNT, which has roles in other transcription pathways [30]. ARNT2 and ARNT3 mainly participate in O_2-independent pathways such as development of the hypothalamus and regulation of circadian clocks, respectively.

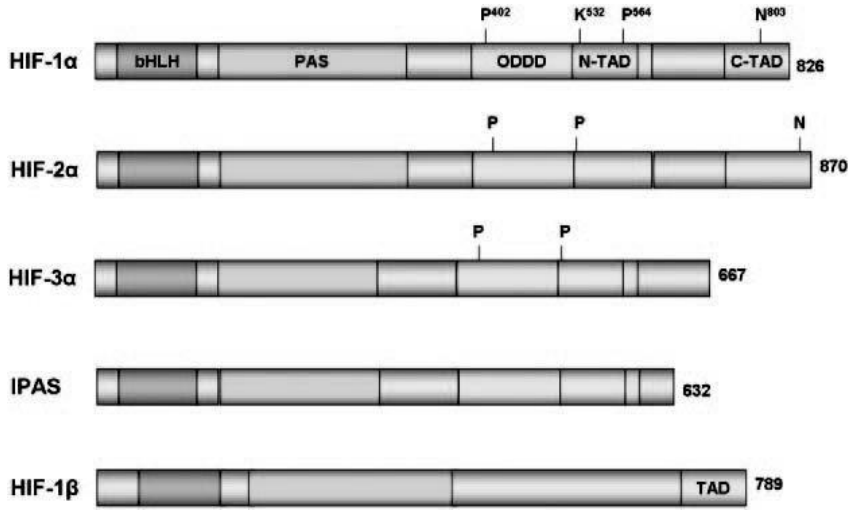

Figure 1. Domain structures of human HIF transcription factors. The three HIF-α paralogues (HIF-1α, HIF-2α and HIF-3α, including the HIF-3α splice variant iPAS) and HIF-1β all belong to the bHLH and PAS protein family. HIF-α contains an ODDD that mediates O_2-regulated stability through the hydroxylation of two proline (P) residues and the acetylation of a lysine (K). The proline residues are conserved in HIF-2α and HIF-3α. HIF-1α and HIF-2α also contain two transaction domains (C-TAD and N-TAD), whereas HIF-1β has only one TAD. The total number of amino acids of each subunit is marked at the end of the domain structure. Figure 1 is adapted from Ke Q, Costa M: **Hypoxia-Inducible Factor-1 (HIF-1)**. *Mol Pharmacol* 2006, **70**:1469–80.

1.1.3.2 Regulation of HIF-1α

HIF-1α is constitutively transcribed and translated in mammalian cells, but it has a very short half-life of less than 5min and is highly regulated by O_2 [20, 31]. Tight regulation of the stability and subsequent beneficial effects of HIF-1α is mainly controlled by its post-translational modifications (see Figure 2), such as hydroxylation, ubiquitination and phosphorylation [32]. And modification of HIF-1α occurs within several domains, such as the C-terminal ODDD.

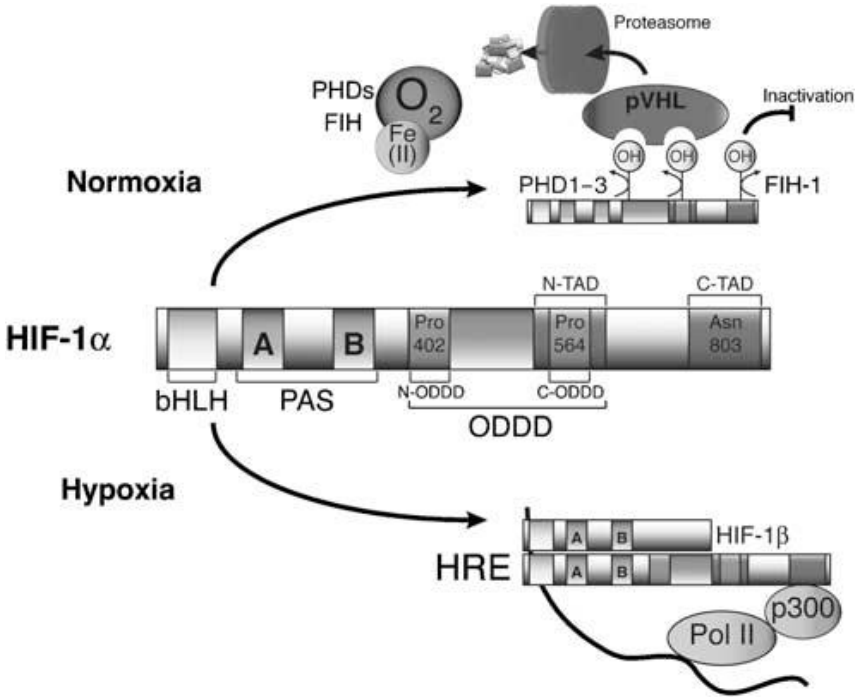

Figure 2. Regulation of HIF-1α protein by O_2-dependent prolyl hydroxylation and proteasomal degradation. There are three hydroxylation sites in the HIF-1α subunit: two prolyl residues in the oxygen-dependent degradation domain (ODDD) and one asparaginyl residue in the C-terminal transactivation domain (C-TAD). Under normoxic conditions, prolyl hydroxylation is catalyzed by the Fe^{2+}-, O_2- and 2-oxoglutarate-dependent PHDs. The hydroxylated prolyl residues allow capture of HIF-1α by the von Hippel–Lindau protein (pVHL), leading to ubiquitination and subsequent proteasomal degradation. Asparaginyl hydroxylation is catalyzed by an enzyme termed as factor-inhibiting HIF (FIH) at a single site in the C-TAD. This hydroxylation prevents cofactor recruitment. In the absence of hydroxylation due to hypoxia or PHD inhibition, HIF-1a translocates to the nucleus, heterodimerizes with HIF-1β and binds to hypoxia-response elements (HREs) in the regulatory regions of target genes. Figure 2 is adapted from Weidemann A, Johnson RS: **Biology of HIF-1alpha**. *Cell Death Differ* 2008, **15**:621-7.

Prolyl hydroxylation by PHDs-signaling for polyubiquitination

Under normoxic conditions, synthesized HIF-1α is rapidly hydroxylated by specific prolyl hydroxylase domain proteins (PHD 1–3) on proline residues (Pro402 or Pro564) located within ODDD [33, 34]. The PHDs use O_2 and 2-oxoglutarate (α-ketoglutarate) as substrates to create prolyl-hydroxylated HIF-1α and succinate. Ascorbate helps to maintain Fe in the ferrous iron; therefore it is required as a cofactor in the above reaction. Inactivation of the PHDs by 2-oxoglutarate analogs such as dimethyloxalylglycine can increase the half-life of HIF-1α. In addition, effect of hypoxia can be mimicked by iron chelators and metal irons (such as Co^{2+}) because PHDs contain Fe^{2+} in their catalytic centers. Cobalt appears to not only bind HIF-1α but also is thought to compete with or displace Fe^{2+} from the Fe^{2+} binding site in the PHD [35]. In addition to the enzymatic inhibition of the PHDs, hypoxia induces a paradoxical burst of reactive oxygen species (ROS), which converts Fe^{2+} to Fe^{3+}. As a consequence, this alteration inhibits PHD activity and promotes HIF-1α stabilization [36–38]. This implies that hypoxia has both direct and indirect roles on PHD function.

Due to the O_2-dependent activity of PHDs, they are considered as oxygen sensors, monitoring the progressive increase in HIF-1α abundance. Moreover, PHD2 was particularly demonstrated to be the most important isoform in oxygen sensing, since only knockdown of PHD2 is sufficient to stabilize HIF-1α protein levels under normoxic conditions [31, 39, 40]. It had been demonstrated

PHD2 localized primarily in the cytoplasm, whereas PHD1 was in the nucleus and PHD3 was in both compartments [41]. Despite its primary localization in the cytoplasm, PHD2 is able to shuttle between the cytoplasm and the nucleus, thereby contributing to HIF-1α degradation in both compartments. Interestingly, expression of PHD2 is reciprocally up-regulated by HIF-1α proteins under hypoxia and this may represent a way by which HIF-1α self-regulates its expression [40, 42].

Polyubiquitination by pVHL-signaling for O_2-dependent degradation

Once HIF-1α is hydroxylated by PHDs, the von Hippel-Lindau tumour suppressor protein (pVHL) then captures HIF-1α. The pVHL is the recognition component of an E3 ubiquitin ligase complex that includes Elongin C, Elongin B, cullin-2, and RBX1 (ring box protein-1) [43]. Binding of HIF-1α to this multiprotein E3 complex causes polyubiquitination of HIF-1α, eventually leading to its degradation by 26S proteasome [44, 45]. In hypoxia however, enzymatic activity of PHDs is inhibited, HIF-1α protein then escapes the VHL capture and proteasomal degradation. Furthermore, mutations in VHL gene that are associated with renal cell carcinoma and sporadic tumours prevent this ubiquitin, resulting in an accumulation of HIF-1α and continuous over-expression of hypoxia-inducible genes [46].

The pVHL E3 ligase complex predominantly localized in the cytoplasm and can shuttle back and forth between the nucleus and cytoplasm, leading to HIF-1α degradation in both compartments [47, 48]. Importantly, pVHL mutants that interfere with its nuclear-cytoplasmic trafficking can no longer regulate hypoxia-inducible genes [48]. In addition, pVHL itself is also tightly regulated by the ubiquitin-proteasome pathway, which is mediated by the E2-endemic pemphigus foliaceus (EPF) ubiquitin carrier protein (UCP). EPF-UCP is an E2 ubiquitin-conjugating enzyme [49]. A co-expression of UCP and HIF-1α correlating with decreased pVHL was observed not only in different human normoxic cell lines in a cell line–dependent manner, but also in non-hypoxic area of human primary and metastatic tumours, such as liver, colon and breast tumours [50]. This may provide an explanation for the elevated levels of HIF-1α that are observed in non-hypoxic regions of such tumours.

In addition, spermidine/spermine-N^1-acetyltransferase (SSAT)2 was identified as one of HIF-1α binding partners by a yeast two-hybrid screen [51]. SSAT2 binds to the PAS A-subdomain of HIF-1α (residues 81–200) and promotes its O_2-dependent ubiquitylation/degration by stabilizing the interaction of pVHL and elongin C (see Figure 3). Although mutation in SSAT2 conservative residues (Ser82 and Thr83) interferes with its acetyltransferase activity on physiological substrate

of SSAT2, it doesn't eliminate the ability of SSAT2 to promote HIF-1α degradation [51]. Thereby, the inhibitory activity of SSAT2 on HIF-1α is believed to result from the combined effect of its acetyltransferase activity and physical interactions with HIF-1α, pVHL and elongin C.

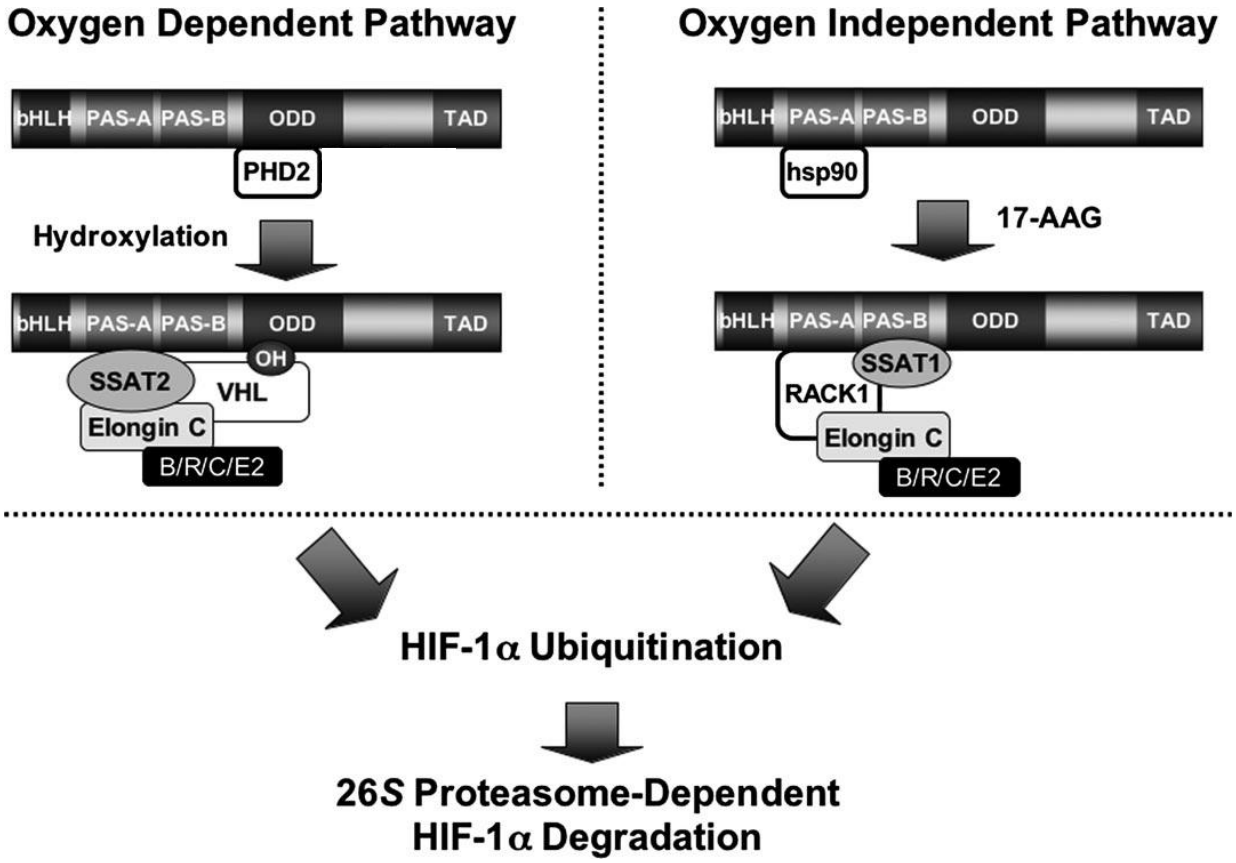

Figure 3. The O_2-dependent and O_2-independent pathways for HIF-1α ubiquitination and degradation. *Left*, SSAT2 is required for O_2- and PHD2/VHL-dependent ubiquitination of HIF-1α. *Right*, SSAT1 is required for O_2-independent and RACK1-dependent ubiquitination of HIF-1α in response to treatment with the HSP90 inhibitor 17-AAG. In both cases, ubiquitination is mediated by recruitment of the Elongin C ubiquitin-ligase complex, which also includes Elongin B, RBX1, cullin-2, and an E2 ubiquitin carrier protein (*B/R/C/E2*). Figure 3 is adapted from Baek JH, Liu YV, McDonald KR, Wesley JB, Hubbi ME, Byun H, Semenza GL: **Spermidine/spermine-N1-acetyltransferase 2 is an essential component of the ubiquitin ligase complex that regulates hypoxia-inducible factor 1alpha.** *J Biol Chem* 2007, **282**:23572–80.

Polyubiquitination mediated by RACK1 pathway for O_2-independent degradation

In addition to the pVHL mediated HIF-1α degradation, the O_2-independent degradation pathway also has an important role in controlling HIF-1α levels. It involves the heat-shock protein 90 (HSP90) and receptor of activated protein C kinase (RACK1), which compete for binding to HIF-1α [52]. RACK1 homodimerizes and recruits Elongin C and other components of the E3 ligase complex to HIF-1α in an O_2-independent manner, leading to HIF-1α ubiquitylation and 26S proteasome-dependent degradation. HSP90 competes with RACK1 for binding to the PAS A-subdomain of HIF-1α and inhibition of Hsp90 causes O_2- and pVHL-independent HIF-1α degradation [53]. RACK1-HIF-1α binding is dependent upon the presence of SSAT1, which

stabilizes the interaction between these two proteins, and SSAT1 acetyltransferase activity is required in this process (see Figure 3). Although homologous proteins SSAT1 and SSAT2 (described earlier) both bind to and promote HIF-1α ubiquitylation, they do so by completely different and complementary molecular mechanisms: SSAT2 binds to the PAS A-subdomain of HIF-1α and promotes O_2- and pVHL-dependent regulation, whereas SSAT1 binds to the PAS B-subdomain (residues 201-329) and promotes O_2-independent, RACK1-dependent HIF-1α degradation [53].

The RACK1 pathway is also regulated by calcium through the activity of calcineurin, a calcium/calmodulin-dependent and serine/threonine-specific protein phosphatase. Calcineurin dephosphorylates RACK1 in a calcium-dependent manner, thus promotes HIF-1α expression by blocking RACK1 dimerization and inhibiting RACK1-mediated HIF-1α degradation [54]. Thus, it indicates that intracellular calcium levels can regulate HIF-1α expression by modulating calcineurin activity and RACK1 dimerization.

Asparagine hydroxylation by FIH-1-preventing CBP/p300 binding

Under different post-translational modifications described above, HIF-1α protein is stabilized but it is not sufficient for full transcriptional activation of HIF-1. A second level of HIF-1α regulation occurs in the nucleus through the hydroxylation of an asparagine residue at the C-terminal end of HIF-1α [55]. HIF-1α contains two transactivation domains, N-TAD and C-TAD, employing recruitment of coactivators such as p300 and its paralogue CREB-binding protein (CBP), steroid receptor coactivator-1, and the transcription intermediary factor 2 [56–59]. The CBP/p300 is essential for linking transcription factors with coactivator complexes and the basal transcriptional machinery, and is thus indispensable for robust transcriptional activation. Moreover, CBP/p300 proteins have histone acetyltransferase activity that is necessary for chromatin modification prior to transcription.

FIH-1 (factor inhibiting HIF-1) is another α-ketoglutarate-dependent dioxygenase that hydroxylates Asn803 and thereby prevents interaction of HIF-1α C-TAD with the coactivators CBP/p300 [60–62]. In contrast to the prolyl hydroxylation that enables protein–protein interaction, the asparaginyl hydroxylation prevents protein recruitment. Because FIH-1 also requires O_2 for catalytic activity, C-TAD of HIF-1α remains unmodified under hypoxia and can efficiently interact with CBP/p300 to activate transcription of HIF-1 target genes [61]. Replacement of Asn803 with alanine permits coactivator binding with HIF-1α in normoxia [55]. Utilization of O_2 as a substrate allows FIH-1 to serve as a second O_2 sensor. The estimated Km

values of FIH-1 and the PHDs for O_2 were first reported to be approximately 90 and 250 mM, respectively [63, 64]. Although these values were determined *in vitro* using relatively short peptides as substrates, they do provide a guide as to the O_2 sensitivity of these enzymes within cells. Thus as the severity of hypoxia increases, the PHDs would be inactivated first, maximal activation of the HIF-1 pathway requires more severe hypoxia to inactivate FIH-1 [65]. Indeed, overexpressed FIH-1 can still play a catalytic effect at 0.2% O_2 concentration, whereas the PHDs were inactive under the same conditions [66].

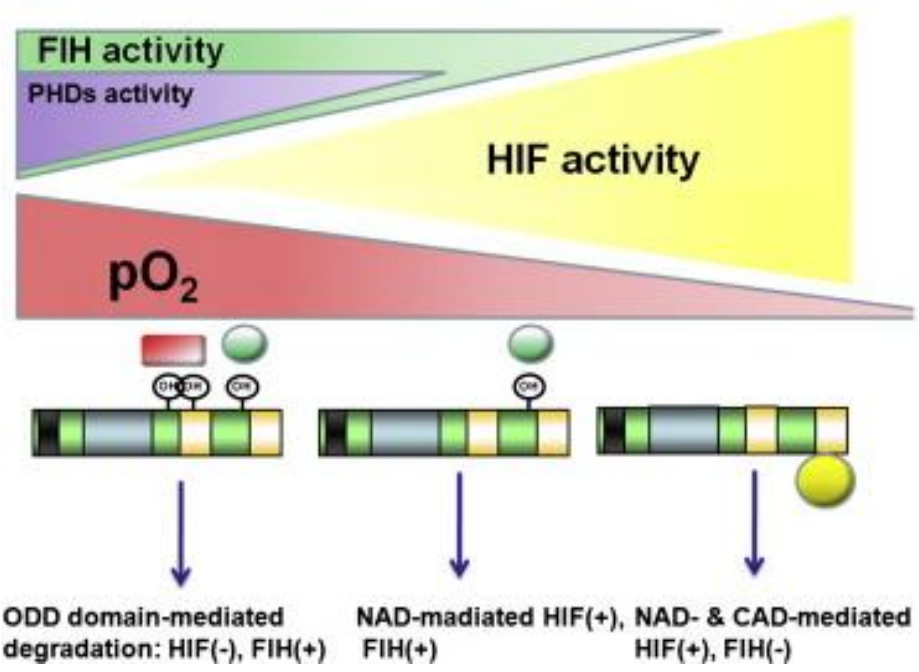

Figure 4. Hypothesized sequential activation of HIF-1α in gradients of hypoxia: based on the *in vitro* derived Km values of PHDs and FIH for O_2, as O_2 concentrations drop, the PHDs lose catalytic activity first due to their weaker affinity for O_2, increasing HIF-1α stability, while C-TAD activity is still repressed due to the hydroxylation of Asp803 by FIH-1. At lower O_2 tensions, catalytic activity of FIH-1 is reduced leading to the full transcriptional activation of HIF-1. Figure 4 is adapted from Kizaka-Kondoh S, Tanaka S, Harada H, Hiraoka M: **The HIF-1-active microenvironment: an environmental target for cancer therapy.** *Adv Drug Deliv Rev* 2009, **61**:623–32.

The transcription of FIH-1 itself is not affected by O_2 concentration and FIH-1 does not influence HIF-1α stability. FIH-1 is mainly located in the cytoplasm even under hypoxia, but a small portion resides in the nucleus as well [41]. Like PDHs, FIH-1 also requires 2-oxoglutarate, Fe^{2+}, ascorbate and O_2 for catalysis of hydroxylation. However pyruvate and tricarboxylic citric acid cycle intermediates, such as isocitrate, oxaloacetate, succinate, and fumarate, inhibit PHD2, but apparently do not inhibit FIH-1 [67–70].

Phosphorylation by MAPK-enhancing transactivation

Despite hydroxylases play a central role in sensing O_2 tension and regulating HIF-1α, mitogen-activated protein kinase (MAPK) signaling is required for the transactivation activity of HIF-1α [71, 72]. It has been shown that MEK-1/p42/p44 MAPK pathway activates the transactivational ability of HIF-1α, but does not regulate its stabilization and DNA binding activity [71]. MAPK

has been correlated with C-TAD activity as enhanced activation of MAPK stimulates both HIF-1α and p300 and conversely inhibition of MAPK restricts transcription by disruption of HIF-C-TAD/p300 interaction [72]. Moreover, phosphorylation of two distinct serine residues (Ser-641/643) inside the inhibitory domain of HIF-1α by p42 MAPK promotes the nuclear accumulation and activity of HIF-1α by blocking its chromosome maintenance region 1-mediated nuclear export [73].

In addition to the post-translational modification of HIF-1α described above, the small ubiquitin-like modifier (SUMO) modification of HIF-1α might contribute to repressing transactivation [32], though a varied outcome of hypoxia-induced SUMOylation in HIF-1α has been reported [74–77]. For example, SUMOylation of HIF-1α has been suggested to both increase HIF-1α stability and transcriptional activity [74, 75], however in other studies, it has been suggested to decrease activity and enhance pVHL-mediated ubiquitination [76, 77]. Moreover, *S*-nitrosation on cysteine 800 of HIF-1α has been shown to increase its transactivation via enhancement of its interaction with CBP/p300 [78]. However, another quantitative interaction study with purified proteins and peptides demonstrated a significant decrease in p300 binding upon Cys800 S-nitrosylation [79].

Additionally, translation level of HIF-1α is modulated by many growth factors and cytokines, such as insulin (INS), insulin-like growth factor-2 (IGF-2), tumour necrosis factor-α (TNF-α), epidermal growth factor (EGF) and interleukin-1β (IL-1β), resulting in the HIF-1α accumulation under either hypoxia or non-hypoxia conditions [80–82]. Although complex and cell-type dependent, it has been suggested that activation of phosphatidylinositol 3-kinase (PI3K) or MAPK pathways plays a role in the phosphorylation of HIF-1α and subsequent protein stabilization [81–83].

1.1.3.3 HIF-1 mediated hypoxic response

As described above, PHDs and FIH-1 are repressed in the absence of O_2, thereby HIF-1α protein is able to accumulate and translocate into the nucleus, where it dimerizes with HIF-1β and activates the transcription of a number of target genes displaying an HRE motif [83]. It has been found that HIF-1 is expressed in all nucleated cells of metazoan species and functions as a master regulator of oxygen homeostasis [1]. It regulates the expression of more than 100 genes encoding key factors in cell proliferation and survival, glucose metabolism, invasion, angiogenesis and erythropoiesis [81–84] (see Figure 5). Therefore, hypoxia and HIF-1 have been correlated with

the embryonic development and both protective responses and pathogenesis in varies diseases such as organ transplantation and cancer, respectively [81].

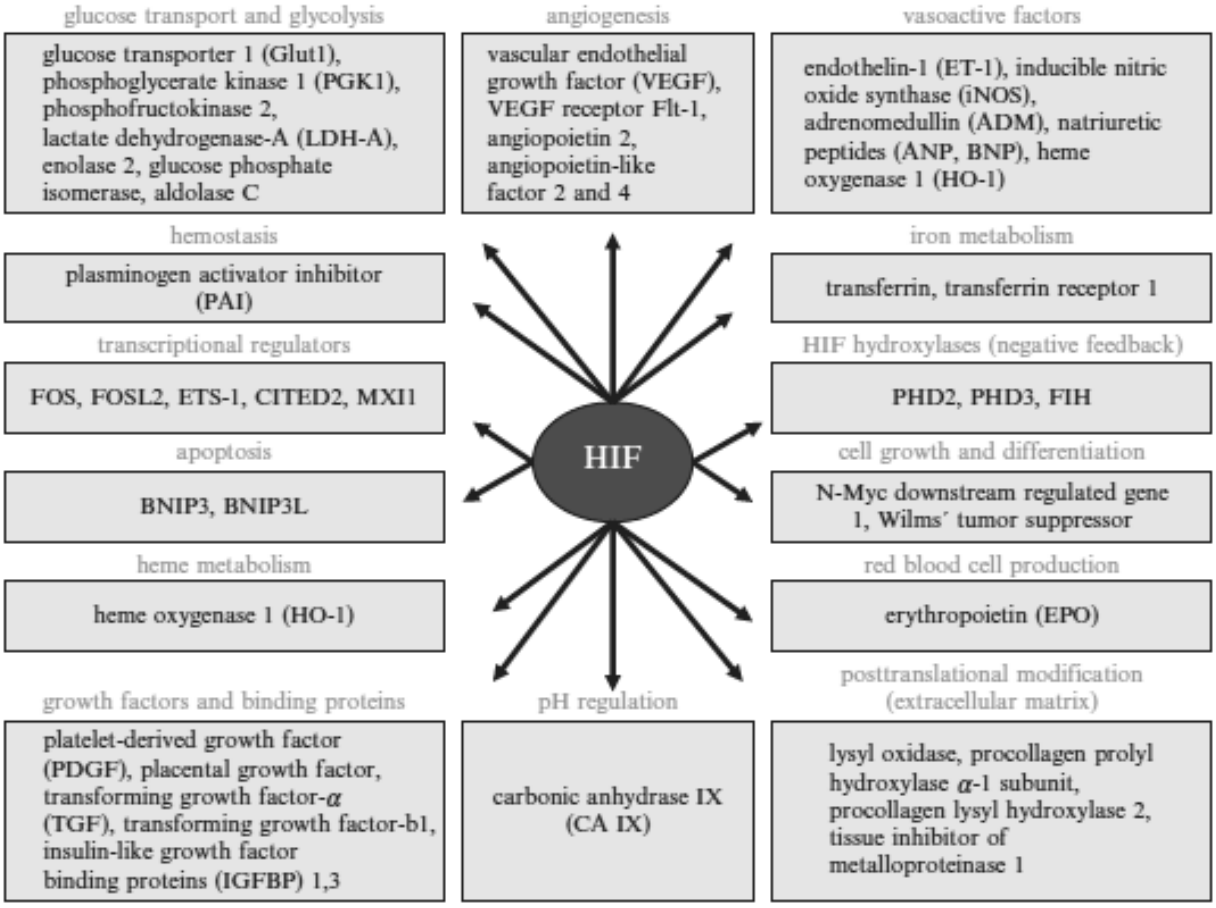

Figure 5. Transcriptional targets of HIF-1. A representation of the increasing number of genes is modulated by HIF-1, which are involved in various adaptive processes in response to hypoxia. Figure 5 is adapted from Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu M, Simons JW, Semenza GL: **Modulation of hypoxia-inducible factor 1α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells : implications for tumor angiogenesis and therapeutics**. *Cancer Res* 2000, **60**: 1541-45.

1.2 Pancreatic diseases and therapy

1.2.1 Cell components of the pancreas

Pancreas is the second largest gland in the human body and it is functionally divided into an endocrine portion (about 1-2% of the total weight of the pancreas) and an exocrine portion (about 80%) [85, 86]. The lobules are connected by connective tissue septa that contain the blood vessels, nerves, lymphatics, and excretory ducts (constituting about 18% of this organ).

The part of pancreas with endocrine function is made up of about 1.5 million cell clusters entitled the islet of Langerhans (see Figure 6) that are scattered throughout the exocrine tissue [86, 87]. It consists of glucagon-producing α cells, insulin-producing β-cell, somatostatin-producing δ-cell, ghrelin producing ε-cell and pancreatic polypeptide-producing PP-cell, which work together to

regulate the glucose metabolism. The size of the islets varies between approximately 20-250 μm in diameter with the majority of islets being <100 μm [86, 87].

As the most abundant and important cell type, β-cells comprise 65-80% of the islets of Langerhans, sensing the glucose level in the blood and secreting insulin to counteract hyperglycemia [88]. Besides insulin, islet amyloid polypeptide (also known as amylin) is also produced by β-cells, which plays a role in glucose homeostasis [85, 89]. The second most common cell type in the islet is α-cell (15–20% of islet mass) that produces glucagon as a negative regulator of insulin, which mainly stimulates glycogenolysis and gluconeogenesis in the liver. Besides endocrine cells, the islet of Langerhans also contains endothelial cells, nerves, fibroblasts and dendritic cells [85]. Particularly, islets are highly vascularized mini organs that receive 5-10 times higher blood flow than the exocrine part of the pancreas [90]. β-cells are able to consume large amounts of O_2 serving for the high demand of mitochondrial respiration during insulin secretion [91], thereby they are sensitive to hypoxic conditions that may occur during islet isolation and after transplantation.

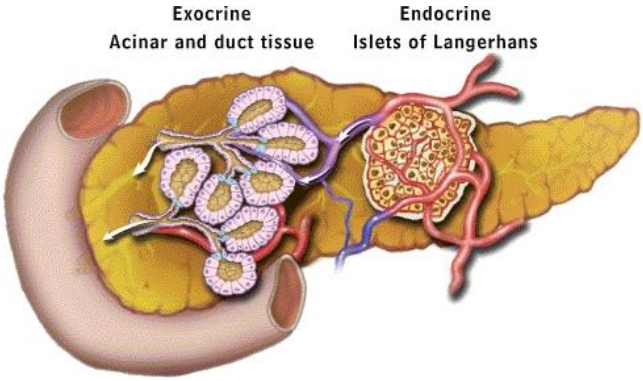

Figure 6. The figure represents exocrine and endocrine tissues in pancreas. It is from Pandol SJ: **The Exocrine Pancreas.** *San Rafael (CA): Morgan & Claypool Life Sciences* 2010.

The exocrine portion of pancreas has ducts that are arranged in clusters of acinar cells (known as acini) (see Figure 6). Acinar cells are tall, pyramidal or columnar epithelial cells, which are specialized to synthesize, store, and secrete digestive enzymes. With their broad bases on a basal lamina, apices of acinar cells converge on a central lumen that is the origin of the secretory duct. In response to distension and/or food in stomach and duodenum, digestive enzymes, H_2O and $NaHCO_3$ are secreted into the duodenum through the pancreatic ductal system. Tight junctions between acinar cells form a band around the apical aspects of the cells and act as a barrier to prevent passage of large molecules, such as the digestive enzymes. The junctional complexes also provide for the paracellular passage of H_2O and ions [92].

The duct cell epithelium consists of cells that are cuboidal to pyramidal and contain the abundant mitochondria necessary for ATP-dependent ion transport. The lumen of the acinus also contains the centroacinar cell, which is unique to pancreas. This cell has ductal cell characteristics but is

also likely a progenitor for different cell types for the pancreas. The duct cells as well as the centroacinar cells produce carbonic anhydrase, which is important for their ability to secrete bicarbonate [92].

Another cell type of exocrine pancreas that is becoming important because of its role in pathologic states is the pancreatic stellate cell (PSC) [93, 94]. In normal pancreas, this star-shaped cell represents approximately 4% of the resident cells and is located in the periacinar and interlobular space. And quiescent PSCs can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm [95]. The role of PSCs in normal tissue is probably to lay down the basement membrane to direct proper formation of the epithelial structures. Their roles in pathologic states, such as chronic pancreatitis and pancreatic cancer, have been of considerable interest. PSCs participate in disease pathogenesis after transforming from a quiescent state into an "activated" state (also known as a "myofibroblastic" state). Characteristic features of this transition include an enhancement in the production of extracellular matrix (ECM), including type I and III collagens, laminin, fibronectins, as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases. Other important features of activation include loss of vitamin A lipid droplets, proliferation, increased expression of α-smooth muscle actin (α-SMA) and up-regulation of various cytokines and growth factors, such as TGF-β1, PDGF and vascular endothelial growth factor (VEGF), etc [93, 94]. Sustained activation of PSCs has an increasingly appreciated role in the fibrosis that is associated with chronic pancreatitis and with pancreatic cancer [96]. Particularly, the activated PSCs are emerging as a key participant in both the rate of growth of the cancer and the development of resistance to chemotherapy [93].

1.2.2 Diabetes mellitus and pancreatic islet transplantation

1.2.2.1 Type 1 diabetes mellitus

The different forms of diabetes mellitus are characterized by deficient insulin release in relation to the insulin demand. According to World Health Organization, there are approximately 347 million adults with diabetes worldwide. Type 2 diabetes mellitus is the most common type of this disease, comprising 90% of diabetic patients, and is largely the result of excess body weight and physical inactivity. It is characterized by a combination of impaired insulin production from the pancreatic β-cells and insulin resistance in peripheral tissues [97]. Although the incidence of type 2 diabetes is higher than type 1, the latter remains the most common chronic condition in children. Type 1 diabetes is primarily due to autoimmune-mediated destruction of β-cells, resulting in absolute insulin deficiency. Patient with type 1 diabetes require life-long intake of

exogenous insulin to control blood glucose levels and to prevent ketoacidosis [97, 98]. And many of them develop complications related with cardiovascular disease, nephropathy, neuropathy and retinopathy, which account for most of the increased morbidity and mortality associated with the disease [98]. Moreover, some of patients could develop resistance to insulin therapy, who may experience repeated hypoglycaemia or diabetic ketoacidosis. Transplantation of pancreas or pancreatic islets could provide cure of diabetes or improvement of blood glucose control in such recipients.

1.2.2.2 Pancreatic islet transplantation

Pancreatic islet transplantation is a biological replacement strategy for treating selected patients with frequent hypoglycemic events or severe glycemic lability. It is considered less invasive with a minor operation and thus the patient will not have severe complications as compared to the major surgery in whole pancreas transplantation. Although significant progress has been made in the islet transplantation field, many obstacles still remain to the application of islet transplantation in the clinic. Two of the most important limitations are severe shortage of donor and the currently inadequate treatments for preventing islet rejection [99, 100].

Clinical islet transplantation

Modern clinical experience with islet transplantation can be dated to 1977 when Najarian and his colleagues injected approximately 50,000 "impure" islets into a diabetic patient's intraperitoneal space and reported some decrease in the blood glucose [101]. Over the next decade, optimization of the technique of islet isolation and use of immunosuppressive drugs with less deleterious side effects became focal points in the field [102, 103]. Until 2000, interest in islet transplantation was reawakened after Shapiro et al. reported successful outcome in seven patients who all became insulin independent and still being fully functional during the one-year follow-up. Their novel approach utilized freshly isolated islets from multiple donors injected into the main portal vein in the liver on C-peptide negative type 1 diabetic patients with several episodes of hypoglycemic unawareness, using a steroid-free immunosuppressive protocol [104]. The success achieved in the Edmonton protocol transformed islet transplantation from an experimental procedure to a true alternative to conventional diabetes therapies in patients with glycemic lability. Clinical protocols to optimize islet survival and function in post-transplantation improved dramatically with the introduction of the Edmonton protocol [105]. Results of the Collaborative Islet Transplant Registry published in 2009 [106] reported, after achievement of insulin independence, 70% of islet-alone recipients retained it at 1 year after transplantation, as did 55% at 2 years, 45% at 3 years, and 36% at 4 years. Despite the success rate declined in long-term follow-up, islet

transplantation affords C-peptide positivity, nearly normal hemoglobin A1c levels, fewer episodes of hypoglycemia, reduction of diabetic complications [107, 108], and improved quality of life [109], thus showing an emerging role in contemporary type 1 diabetes management.

Obstacles of success in islet transplantation

Several reasons cause the loss of graft function with time: loss of islet mass during isolation and culture, loss of islet graft immediately after transplantation by instant blood mediated inflammatory response (IBMIR) [110] and during the first week after transplantation by the prolonged inflammatory process as well as the shortage of O_2 and other nutrients, and gradual loss of the surviving islets with time due to chronic rejection caused by reoccurrence of autoimmunity [111]. Moreover, the immunosuppressive drugs are not only beneficial to islet graft in preventing rejection, but also harmful to them due to toxic effects [112].

Generally about 50–70% of pancreatic islet cells undergo apoptosis during isolation, culture, and the peritransplant period [87]. During isolation, islets are separated from their pervious nourishing microenvironment and subjected to devascularization, denervation, and hypoxia. A culture period following isolation may provide the islets with a needed recovery prior to transplantation, whereas data from both human and mice islet transplantation suggest that fresh isolated islets are more effective than cultured islets at reserving hyperglycemia [113, 114]. Indeed, fresh islets have shown a higher O_2 and adenosine triphosphate (ATP) contents than cultured islets [113].

Clinically, islets were injected via portal vein but more than 50% of islet mass was destroyed within one hour [115] due to the damaging rapid clotting process termed IBMIR [110]. Once transplanted, islets require rapid revascularization for survival and proper glucose sensing, and at this time point, they also face allorejection, recurrent autoimmunity, inflammatory and metabolic stress, which all lead to the early islet graft loss [87]. Therefore, the periods immediately after islet isolation and transplantation are particularly crucial for long-term survival of islets.

Several immunosuppressive drugs are found to be associated with significant toxicity and thereby cause a number of adverse effects [116] in islet recipients, including painful mouth ulceration, peripheral oedema, proteinuria, hypercholesterolaemia and hypertension. In addition, they also increase the risk for infection and certain malignancies as designed to weaken the immune response in recipients. Therefore, several strategies to reduce immunosuppressive drugs have

been studies, such as induction of peripheral tolerance and development of immunopretective encapsulation [117–119].

Strategies to improve outcome of islet transplantation

There are many strategies to improve outcome of islet transplantation. In this thesis, I focused two aspects: enhancement of islet robustness by drugs and protection of islets from rejection by immunopretective encapsulation.

1) Usage of drugs to increase islet robustness: In order to improve the transplantation efficiency, a variety of approaches, such as anti-inflammatory, anti-apoptosis, metabolic and growth stimulation and immunomodulation have been investigated. Exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist, is currently used in the management of type 2 diabetes and has shown to improve the outcome of islet transplantation [120–123]. It has a number of effects including glucose-dependent stimulation of insulin secretion [124], the suppression of glucagon secretion [125], the slowing of gastric emptying, the inhibition of food intake, and the modulation of glucose trafficking in the peripheral tissues. With regard to islet transplantation, the special interest is its ability to inhibit β-cell apoptosis and increase β-cell proliferation [126–128]. When clinical protocols using exendin-4 in islet transplantation are being designed, it is important to discuss the optimal treatment period. The initial short period following islet transplantation has been considered as a critical time to preserve the quantity and quality of islet. In recent clinical trials, exenatide (recombinant GLP-1 analogue) treatment combined with tumour necrosis factor (TNF) receptor antagonist, etanercept, from the time of initial islet transplantation showed promising effects for engraftment and long-term graft survival [123, 129, 130]. However, nausea and vomiting were major adverse effects of exendin-4 therapy that limited patient compliance for long-term administration [129, 130]. Pretreatment of isolated islets prior to transplantation could be also an effective way to improve transplantation outcome. One advantage of islet preculture would reduce the dosage of drug given on the patient systemically and thus it is a safer option. In an earlier study, we demonstrated that a combination of islet preculture and recipient treatment with exendin-4 improves the metabolic outcome of a suboptimal number of rat islets transplanted to diabetic athymic mice [121]. However, that study was not designed to compare the individual impact of islet preculture or recipient treatment on graft outcome. In this thesis, I compared the effect of exendin-4 treatment on different aspects before and after islet transplantation (Paper I).

Current methods of islet preparation cause hypoxic stress that has been considered as one of the major contributors to β-cell death. Animal studies have demonstrated that about 80% of

intraportally injected islets are hypoxic at 1 day after transplantation [131]. Revascularization of islets takes at least 1 week, but the impaired vascular density does not seem to recover fully [132, 133]. Adaptive responses to hypoxia are triggered following the expression HIF-1α and induce a series of genes, for example in glucose metabolism adaptation, angiogenesis, apoptosis, and cell growth [81, 82, and 83]. It has been reported that HIF-1α is an important molecule to regulate β-cell function in pancreatic islets even in normoxic conditions [134]. A recent report also showed that decreased apoptosis is caused by HIF-1α, resulting in increased β-cell mass in post-transplantation [135]. These findings suggest that HIF-1α is a protective factor and is required for successful islet transplant outcomes. Therefore, anti-apoptotic effect of exendin-4 was studied in paper I with a special focus on HIF-1α expression in isolated islets.

2) Usage of immunoprotective devices for islet transplantation: Encapsulation of islets is attractive methods in order to avoid chronic usage of immunosuppressive drug. There are different strategies for cell encapsulation involving enclosure of cellular groups within an immunoprotective biomaterial, such as micro- and macro-encapsulation devices. Microencapsulation is the encapsulation of single or small groups of islets in a gel capsule [136], which offers the advantage of increased O_2 and nutrient transport due to the large surface area-to-volume ratio. Major limitations of micro-encapsulation are insufficient encapsulation and instability of the capsule, which can lead to graft rejection over time. This can be overcome by macro-encapsulation of the graft inside diffusion chambers such as the TheraCyte™ (TheraCyte Inc, Irvine, CA, USA) device used in this thesis. The advantages of this device are that it requires minor surgery and can be retrieved if needed.

This planar diffusion chambers is shaped like a teabag (see Figure 7) and composed of a double membrane of polytetrafluoroethylene. The inner membrane (pore size: 0.45μm) provides immunoisolation that enables the diffusion of nutrients and O_2, whereas the outer membrane (pore size: 5μm) facilitates angiogenesis. In an xenotransplantation study, neonatal porcine islets within TheraCyte™ device transplanted subcutaneously survive and reverse diabetes for up to 16 weeks in diabetic autoimmune non-obese diabetic mice [118]. More recently, a study using an allogeneic rat model has showed the TheraCyte™ device protects the allografts also in immunized recipients [119] that further highlights the potential for using macro-encapsulation to avoid immunosuppressive therapy in clinical islet transplantation.

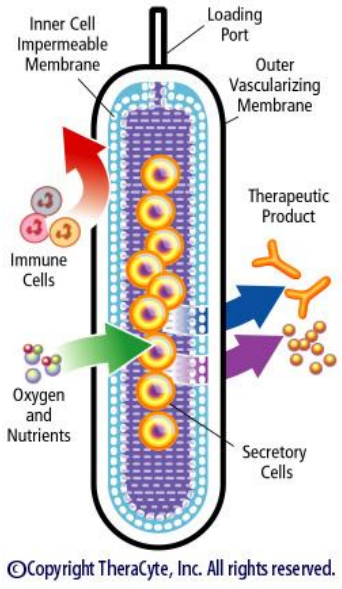

Figure 7. The TheraCyte™ device is a thin membrane bound polymeric chamber, which protects allogeneic cells from rejection by the recipient and, when implanted subcutaneously, induces the development of blood capillaries close to the membranes. This vascularization feature provides a rich blood supply to nourish the tissues within the membranes, aids in the communication of implanted cells with the host and assures rapid uptake of therapeutic molecules. (http://www.theracyte.com/TheTechnology.htm; TheraCyte, Inc.)

However, the major drawback in macro-encapsulation is the limitation of O_2 diffusion and nutrient transport. Until such an implantable device is revascularised, islet graft is exposed to hypoxia and other nutrient deprivation due to a poor blood supply and the superficial location of the transplant, which is associated with a risk of mechanical stress and damage to the graft. Proliferation of fibroblasts, occurring within the devices one month after implantation, is another limitation of macroencapsulation for a long-term islet graft survival. Our previous studies showed that it would need two-three months to get well vascularization of the empty membrane [137], and a preimplantation of the TheraCyte™ device three months before the islet transplantation could significantly reduce curative doses of macroencapsulated rat islets to the same dose as non-encapsulated islet transplantation [138]. It is still of great interest that any drug can improve graft outcome in the model of the macroencapsulated islet transplantation. In paper I, I investigated the effects of exendin-4 treatment on the macroencapsulated islet transplantation in rodent models.

1.2.3 The tumour microenvironment in pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is associated with a 5-year survival rate of less than 5% and a median survival of 6 months after diagnosis, thereby exhibiting the poorest prognosis of all solid tumours [139, 140]. This dismal prognosis is a result of the late diagnosis of the disease, the lack of biomarkers allowing early screening, the early metastatic dissemination and ultimately the resistance to systemic therapies. Known risk factors for the disease include cigarette smoking, chronic and hereditary pancreatitis, late onset diabetes mellitus and familial cancer syndromes [141]. Although target therapies have seen significant advances in the treatment for many tumour types, including melanoma, lung and colorectal cancer [142], similar success has not occurred in PDAC, which remains a lethal disease. Gemcitabine, the current standard-of-care chemotherapeutic was approved mainly on the basis of patient benefit and produced only a modest increase in survival [143, 144]. One explanation for the poor response of patients to systemic therapies was provided in an accurate PDAC mouse model by the demonstration that chemotherapies are poorly delivered to PDA tissues because the presence of the dense stromal

matrix correlated with a deficient vasculature [145]. A deeper knowledge of the underlying mechanisms is the key to better understand the tumour progression and metastasis as well as to identify novel therapeutic strategies capable of overcoming the chemoresistance in PDAC.

1.2.3.1 The stromal microenvironment and chemoresistance

One of the most prominent histological features of PDAC is the presence of an abundant tumour stroma [146], which displays an extensive stromal reaction accounting for up to 90% of the tumour volume. The stromal microenvironment is a complex structure composed of ECM proteins, activated fibroblasts and PSCs, inflammatory cells as well as blood and lymphatic vessels that distort the normal architecture of pancreatic tissue. It is not a static entity but is constantly changing in composition especially in the progression from pre-neoplastic pancreatic intraepithelial neoplasia (PanIN) to invasive PDAC. Early PanIN lesions may be associated with small amounts of normal stroma surrounding the normal pancreatic ducts from which the PanINs arise. By contrast, with PanIN III lesions there may be the beginning of enhanced stroma formation, and progression to invasive carcinoma is often associated with a readily evident increase in stroma formation that ultimately results in extensive stroma. Often, there is an associated inflammatory infiltrate [146].

Interactions between cancer cells and stromal cells and extracellular matrix have been proposed to stimulate the extensive fibrotic, desmoplastic reaction. Cancer cell derived growth factors [146–148], such as fibroblast growth factors (FGFs), TGF-β, IGF-1, hepatocyte growth factor (HGF), PDGF-BB, and EGF become sequestered within the stroma, which thus acts as a storage site for these factors. The invading cancer cells produce matrix metalloproteinases (MMPs) that release these growth factors. Upon activation by growth factors, cytokines or oxidant stress, PSCs transform into a myofibroblast-like phenotype and promote fibrogenesis and ultimately may create a highly desmoplastic, hypovascular and hypoxic tumour microenvironment [149, 150]. Moreover, there is an altered gene expression profile in the cancer-associated stroma, including altered integrin expression that may promote cancer cell motility, increased expression of cyclooxygenase-2 (COX-2), PDGF, VEGF-A, collagen I, WNT5a and HIF-1α that enhance stromal neo-vascularization, promote cancer cell growth and resistance to hypoxia [151–156]. Therefore, PSCs have emerged as key players in PDAC promotion and progression, as described above [93, 94].

In addition, the stromal component has been suggested to control drug sensitivity in cancer [157]. *In vitro*, PSCs induce PDAC cancer cell resistance to both gemcitabine and radiation [158]. This

effect may partly be mediated by PSC secretion of ECM protein collagen I and IV, laminin and fibronectin, which have been shown to have anti-apoptotic effect [159]. Moreover, the rigidity of the ECM compresses blood vessels in cancer tissue, which leads to reduce perfusion that ultimately impedes the delivery of drugs to cancer cells [145]. However, the lack of significant functional perfusion in PDA results not only from a sparse vasculature but also from a profound degree of vascular collapse, which are known to contribute to elevated interstitial fluid pressure (IFP) [160]. Extremely high IFP was found in autochthonus PDAC, ranging from 75 to as high as 130 mmHg, which forms a barrier to transcapillary transport [161, 162]. This barrier is an obstacle in tumour treatment, as it results in inefficient uptake of therapeutic agents. Lowering the tumour IFP by hyaluronidase with a combination use of gemcitabine increased apoptosis in both PSCs and PDAC tumour epithelial cell compartments in a KPC mice model [161].

Despite the preponderance of desmoplasia and stromal cells found in pancreatic cancer specimens and the fact that fibrosis increases the risk of PDAC, studies of the tumour–stroma interactions in this disease have lagged behind. One of the major reasons of poor understanding is the lack of suitable models to study these relationships. *In vitro* co-culturing of cancer cells and stromal cells has demonstrated profound effects on cancer cell invasion [163, 164], however, most of studies were investigated using two-dimensional cell culture systems, which are vastly different from patient situation. Many ECM proteins are also typically absent in such *in vitro* models, which remove an important regulation of PDAC cell behavior [165].

Creation of cell cultures in three dimension changes the polarity of cells and confers resistance to apoptosis [166]. Cells in three-dimensional (3D) culture exhibit similar responses to chemotherapy as cells *in vivo* and could be used as an important tool in pancreatic cancer biology [167, 168]. Different techniques can be used to create 3D cultures including suspending cells and ECM proteins in gels, attaching cells to 3D scaffolds composed of ECM proteins, growing cells on microbeads surrounded by ECM proteins and standard cell culture media, and so on [169]. Among current 3D culture techniques, cellular spheroids are a simple 3D model that can be generated from a wide range of cell types and form due to the tendency of adherent cells to aggregate. Scaffolds are not required for spheroid formation. Spheroids can be readily imaged by bright-light, fluorescence, and confocal microscopy. Consequently, 3D culture system using cell spheroids has been investigated in cancer researches, such as modelling solid tumour growth and metastasis and are also used in a multitude of therapeutic studies, e.g. for high throughput screening [170].

Optimal *in vitro* models to study pancreatic cancer cell interaction with the adjacent stroma

should be 3D and contain appropriate types of ECM proteins and stromal cells. Previously we developed a novel 3D PDAC cell culture system using PDAC Panc-1 cells, which displays a strongly increased chemoresistant property [168]. However a single cell type cannot accurately reflect the complex tumour microenvironment in PDAC. A bigger challenge for an ideal model of investigation on tumour–stromal interaction is to develop a 3D cell culture system containing the following elements: a pancreatic cancer cell with metastatic feature, activated PSCs, ECM containing pre-dominantly collagen and laminin, and the presence of endothelial cells, immune cells and neurons, whose presence is also important to the creation and regulation of tumour stroma [171]. Attempts to create this type of complex model are underway in several laboratories including my group. Therefore, I explored the development of a multicellular tumour spheroid model including ductal epithelial cells and PSCs in paper III and IV and investigated the sensitivity of our 3D cell culture system of PDAC on anti-cancer drugs.

1.2.3.2 The hypoxic microenvironment and metabolic reprogramming

A striking characteristic of the desmoplasia seen in pancreatic cancer is hypovascularity resulting in hypoxic conditions that contribute to tumour progression and metastatic potential [172-174]. Tumour hypoxia usually occurs at a distance of 100–200 micrometer from blood vessels and cells that fail to adapt to O_2 and nutrient deprivation undergo cell death by apoptosis and/or necrosis, see Figure 8 [175]. Paradoxically, hypoxia can also stimulate tumour development by local adaptation that provokes a more aggressive tumour phenotype often in combination with chemoresistance. A molecular explanation for adaptation results, for example, from increased aerobic glycolysis facilitated by increased expression of glucose transporters and glucose metabolizing enzymes, angiogenesis and/ or expression of drug export pumps, e.g. multidrug resistance protein 1 (MDR1) [83, 176 and 177].

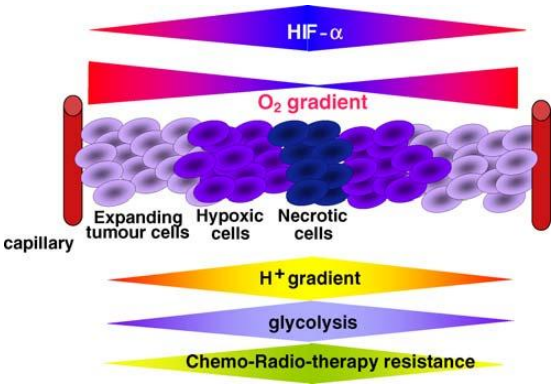

Figure 8. The characteristics of a hypoxic tumour mass. Blood capillaries carry O_2 to tissues, but since O_2 has a diffusion limit, its concentration decreases as the distance from capillaries increases. Macroscopic examination of solid tumours reveals the presence of expanding tumour cells in proximity to capillaries and a central region of necrotic cells. This gradient of cell viability parallels that of a decreasing gradient of O2, which is accompanied by an increase in HIF-1α levels, a decrease in the extracellular pH and an increase in the resistance to chemo- and radio-therapy. Figure 8 is adapted from Brahimi-Horn MC, Chiche J, Pouysségur J: **Hypoxia and cancer.** *J Mol Med (Berl)* 2007, **85**:1301-07.

As the key regulator in the adaptations to the hypoxic conditions, HIF-1α expression is observed in both pancreatic cancer cells and the surrounding stromal cells [179]. HIF-1 plays a pivotal role in hepatic metastasis through its association with the expression of angiogenic factors in PDAC patients [180]. Recently, Lu and Kang [181] concluded that each step of the metastasis process 'from the initial epithelial-mesenchymal transition to the ultimate organotropic colonization' can potentially be regulated by hypoxia, suggesting a master regulator role of hypoxia and HIFs in metastasis. This hypoxia-induced metastatic phenotype may also explain the recent provocative findings that antiangiogenic therapy increases metastasis in preclinical models [181-184]. It is also likely that the already fibrotic and hypovascular microenvironment of pancreatic cancer is one of the reasons for the failure of antiangiogenic therapies in PDAC in the clinical setting [185]. Additionally, hypoxia provides a niche for slow-cycling, highly drug-resistant cells, which may be identical to the proposed cancer stem cells (CSC) [185, 186]. Thus standard chemotherapy agents fail because they are unsuccessful at targeting the cell within the hypoxic microenvironment, which might be those that most need to be eliminated [187].

It has been known for a long time that hypoxic cancer cells switch their primary pathway of energy production from OXPHOS in mitochondria to glycolysis in the cytoplasm. Otto Warburg first observed an anomalous characteristic of cancer cell energy metabolism in 1920's [188, 189]: even in the presence of oxygen, cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed aerobic glycolysis or Warburg effect. Such reprogramming of energy metabolism is seemingly counterintuitive, in that cancer cells must compensate for the ~18-fold lower efficiency of ATP production afforded by glycolysis relative to mitochondrial oxidative phosphorylation. However the high glycolytic rate allows these cells to balance their energy demands and supply the anabolic precursors for de novo nucleotide and lipid synthesis [190]. This strategy is considered to provide a growth advantage for the tumour cells, though it renders cells highly dependent on substrate availability for survival [191]. Another important feature of this hypoxic microenvironment in tumour is acidosis, which is a consequence of increased glycolysis leading to production of lactate and of defective vascular evacuation of metabolic lactic acid and CO_2 [192]. It has been reported the acidic environment has a strong impact on the activity of the p-glycoprotein drug transporter responsible for multidrug resistance [193].

Several mechanisms for the enhanced glycolysis in tumour cells have been investigated and suggested as increased expression of the glycolytic enzymes and glucose transporters or decreased expression of mitochondrial oxidative enzymes and transporters [194]. This switch has

now been revealed to implicate, at least in part, [195] HIF-1 induced glucose capture, glycolytic flux and inhibition of OXPHOS. HIF-1α enhancement promotes the expression of glucose transporter (GLUT), hexokinase (HK), phosphofructokinase type-1 and -2, aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase, enolase, pyruvate kinase and lactate dehydrogenase (LDH) [196, 197], which leads to a stimulation of the glycolytic flux. HIF-1α also favors the glycolytic flux by repressing OXPHOS through induction the expression of pyruvate dehydrogenase kinase-1 (PDK-1), an inhibitor of pyruvate dehydrogenase, the enzyme that drives pyruvate into the TCA cycle for mitochondrial respiration [198, 199]. As described above, oncogenic events have been linked to stabilization of HIF-1α in the presence of adequate O_2, such as RAS-MAPK and PI-3K/AKT signaling [200]. Moreover, mutations of the TCA cycle tumour suppressors, succinate dehydrogenase and fumarate hydratase can also lead to stabilization of HIF-1α. In particular, prolyl hydroxylation of HIF-1α requires α-ketoglutarate as a substrate, which is converted to succinate, thereby inhibiting the degradation of HIF-1α [200]. Furthermore, intracellular ROS levels paradoxically increase under hypoxia where mitochondria appear to be their main source of production [201]. Studies have shown that these mitochondria-derived ROS are both necessary and sufficient to stabilize and activate HIF-1 [202, 203] most probably via modulation of PHD activity [204]. This signaling effect of ROS in activation of HIF-1 pathways thereby proposes a possible feedback loop, which would attenuate the toxic burst of hypoxic ROS [205].

Apart from microenvironment factors described above, other potential HIF regulators have been identified, such as extracellular glucose. Glucose is also delivered by the blood circulation and diffuses into tissues for cell capture, like oxygen. A decrease in the amount of glucose provided to hypoxic cells was shown to result in a decrease in the level of HIF-1α [205] or to have no effect [206]. The explanation for this difference may lie in either the different cell lines or the level of hypoxia (0.1 or 1.0% O_2). But HIF-1α expression is increased in malignant and non-malignant cells with high extracellular glucose [206, 207]. However, it is still uncertain whether excess glucose stimulates HIF-1 expression in pancreatic cancer and if glucose-induced HIF-1α is correlated with tumour aggressiveness. Therefore, I investigated the effect of high glucose on HIF-1 expression in PDAC cells in paper II with regard to metastasis.

In addition to hypoxia induced HIF-1 expression, oncogenic activation or loss of tumour suppressors is widely involved in metabolic alterations of human cancers, which independently, or cooperatively with hypoxic adaptive responses contributes to tumourigenesis. For example, K-Ras oncogenes can enhance glucose transport and glycolysis in transformed cells [10]. Most

glycolytic enzyme genes and glucose transporter genes are directly or indirectly induced by c-Myc transcription factor [11]. Akt, independent of HIF-1, can also activate glycolysis through induction of glucose transporters and HK-II [11]. The loss of p53 leads to derepression of the transcription of the GLUT1 and GLUT4 and loss of glycolysis repression [12]. Perhaps one function of oncogenic pathways is to drive cell-autonomous nutrient uptake and program proliferative metabolism, whereas one function of tumour suppressor pathways is to prevent nutrient utilization for anabolic processes.

2 AIMS

The overall objective of this thesis was to investigate the roles of HIF-1α in endocrine β-cells of pancreatic islets and in exocrine ductal epithelial cells as well as their surrounding stromal cells of pancreatic ductal adenocarcinoma.

Specific aims were:

- To investigate mechanisms of effects of exendin-4 on islet function and viability in a rodent islet transplantation model with special focus on HIF-1α expression. (Paper I)

- To investigate relationship of the role of excess glucose on glucose reprogramming and cell migration in human PDAC cells with respect to HIF-1α expression. (Paper II)

- To develop a high-throughput 3D model of human PDAC cells, and to further explore mechanisms underlying the transition from 2D to 3D that might be responsible for chemoresistance, including hypoxia-inducible factor-1 pathway. (Paper III)

- To improve the development of a 3D model of human PDAC cells and PSC cells, and to evaluate its potential properties similar to a stromal and hypoxic PDAC *in vivo*. (Paper IV)

3 MATERIALS AND METHODS

3.1 MATERIALS AND MODELS

3.1.1 Animals (Paper I)

The animal experiments were approved by the local Animal Ethics Committee (S19-07, S177-06 and S9-05). All animals were maintained in accordance with the requirements of the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals.

Male Sprague Dawley rats (weighting 300g) were used as islet donors, whereas male inbred athymic mice (nu/nu Black 6, weighing 25 g) served as recipients. These immunoincompetent nude mice cannot reject cellular grafts, thus the survival of the transplanted islets is not influenced by any rejection reaction. Diabetes was induced at mice by the injection of streptozotocin (250 mg/kg body weight) into the penile vein under inhalation anesthesia using Enflurane. Mice were considered diabetic if its non-fasting blood glucose level exceeded 20 mmol/l (>360 mg/dl) for 2 consecutive days and used as transplant recipients. During the experiments, the animals had free access to tap water and pelleted food. Prior to the glucose tolerance tests, the animals were fasted overnight.

3.1.2 Cell lines and incubation (Paper I-IV)

INS-1E cells were a kind gift from Dr. P. Maechler [208]. Cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol under standard conditions (37°C, 95% air and 5% CO_2). Due to the variable hypoxia within islets, the rat insulin producing cells were used to evaluate the effects of exendin-4 on energy production. During incubation with various exendin-4 treatments, cells were first cultured in serum-free RPMI-1640 medium under standard conditions for 20 hours. Then they were changed into new serum-free medium with various exendin-4 concentrations and incubated in normoxia (95% air and 5% CO_2) or hypoxia (1% O_2, 5% CO_2 and 94% N_2) for 2 hours. The concentrations of O_2 and CO_2 were monitored using a gas meter. Two hours incubation with hypoxia was determined according to our preliminary test (data were not shown). (Paper I)

Wild-type MiaPaCa2 human PDAC cells (wt-MiaPaCa2) and a MiaPaCa2 sub-line (namely si-MiaPaCa2) were prepared as described in previous study of our group, and the latter lacked HIF-1α [209]. Human PDAC cell lines BxPC-3 and Panc-1 [210] were bought from the European

Collection of Cell Cultures. All cells were cultured under standard conditions in Dulbecco's modified Eagle's media (DMEM) with 5.6 mM glucose containing 10% FBS. In order to evaluate the effect of excess glucose in PDAC cells, cells were maintained with a physiological blood glucose level. When cells reached to 80% confluent, experimental incubation was set up, using DMEM (Invitrogen, 11966, originally glucose-free) or KH buffers supplemented with known amounts of glucose. Cells were incubated in normoxia (95% air and 5% CO_2) or hypoxia (1% O_2, 5% CO_2 and 94% N_2). The incubation time of hypoxic treatment was determined according to pervious publication of members of our group [209]. During hypoxic incubation, a gas meter (Dansensor) was used to monitor the O_2 and CO_2 concentrations (Paper II)

Human PDAC cell lines AsPC-1, BxPC-3, Capan-1, Paca44 and Panc-1 [210, 211] as well as a normal human pancreatic ductal epithelial cell line HPDE were obtained from the American Type Tissue Collection. The PSC cell line derived from patients with chronic pancreatitis and subsequently transformed [212]. KPC cells were established from a mouse PDAC model, carrying pancreas-specific Kras and p53 mutations (Kras$^{LSL\text{-}G12D/+}$;Trp53$^{LSL\text{-}R172H/+}$;p48-Cre; hence KPC) [213]. Cells were cultured under standard culture conditions in DMEM/F12 or phenol red-free DMEM/F12 medium containing 10% fetal calf serum. In order to train PSC to grow as 3D, they were cultured on the 1% agarose gel (Invitrogen, life technology) for at least three months as conditioned PSCs (PSC-c), and then they were seeded back to cell culture flask at one day before preparing spheroids. (Paper III and IV)

3.1.3 Rat islet preparation and culture (Paper I)

Rat pancreatic islets were isolated using collagenase digestion and discontinuous density gradient purification. In inhalation-anesthetized rats, pre-cold collagenase P solution (0.7mg/ml and 20ml/rat, *Roche Diagnostics GmBh*) was injected into the pancreas via the common bile duct. Rats were euthanized by heart exsanguinations after removal of the pancreas. Enzymatic digestion of pancreas was carried out at 37°C. Then the density gradient centrifugation was performed at 4°C, where pelleted digested tissue was resuspended in Histopaque-1119 and culture medium RPMI 1640 was carefully added to form a sharp interface. After centrifugation islets were collected from the interface. Islet purity was about 90%, as estimated semiquantitatively under microscope.

Islets were floating cultured in culture medium RPMI 1640 with exendin-4 (0 or 0.1 nM) under standard conditions for 20 hours, supplemented with 10% FBS, 2 mmol/l L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. At the following day, islets were handpicked and used

for transplantation.

3.1.4 The TheraCyte™ device and macroencapsulation (Paper I)

The TheraCyte™ device with a volume of 4.5 µl (see Figure 9) was used for islet macroencapsulation in this thesis. In order to improve the procedure of macroencapsulation in islet transplantation and investigate the effects of exendin-4 on macroencapsulated islets, a suboptimal number of islets (500) were cultured for 20 hours in either the presence or absence of exendin-4 (0.1 nM). Then islets were handpicked and loaded into the device using a neonatal venflon cannula attached to a Hamilton syringe. After loading, the port was sealed with glue to avoid islet leakage and growth of host tissue into the device lumen.

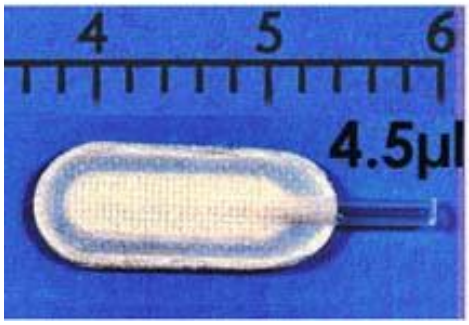

Figure 9. The TheraCyte™ device with a lumen volume of 4.5 µl was used in Paper I.

3.1.5 Islet transplantation model and recipient management (Paper I)

Renal sub-capsular islet transplantation

Thirty rat islets cultured for 20 hours were handpicked and washed once with HBSS. Then free islets were transplanted beneath the kidney capsule of mice with inhalation anaesthesia (Enflurane) as previously described [121]. The kidney capsule is already considered as a successful islet transplantation site in experimental islet transplantation. In our model, the diabetic athymic mice were repeatedly cured by 50 rat islets but not by 30 islets, as shown in a previous study [121]. To mimic the clinical situation, we used a marginal mass of 30 free islets in this study.

Encapsulated islet transplantation

Macroencapsulated islets were implanted subcutaneously on the back of inhalation-anaesthetised mice. As fresh implantation, islets were inserted *in vitro* into the device's lumen just before implantation.

After transplantation, non-fasting blood glucose levels and weights of the animals were measured daily during the first week. Measurements were always done prior to injecting the study drug or insulin. Thereafter, mice with blood glucose levels below 10 mM for 2 consecutive days were assessed twice a week. The mice with hyperglycemia (≥ 11 mM) were checked daily. The mice with blood glucose levels above 20 mM received a mixture of short- and long-acting human recombinant insulin subcutaneously as previously described [121].

Animals that could not maintain their general condition on insulin treatment due to severe diabetes were sacrificed earlier, in accord with regulations pertaining to animal welfare.

An intraperitoneal glucose tolerance test (IPGTT) was performed in all cured mice after 4 weeks. Before the IPGTT, mice were fasted for at least 6 hours. Glucose (20%) was injected i.p. (10 l/g) and the blood glucose level determined before injection and then at 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes after the injection. Healthy mice were used as normal controls and tested simultaneously. After IPGTT, graftectomy was done to rule out a recurrence of native pancreatic function in all cured and partially cured animals. The mice were then sacrificed by cervical dislocation.

3.1.6 Three-dimensional (3D) cell culture model (Paper III and IV)

In order to create an easy technique for high-throughput screening application, we used a crowding agent methylcellulose, a cellulose-derived inert compound, to help cells to aggregate and form spheroids. Moreover, exogenous ECM components were not required in our method avoiding its possible effects on cell signaling [169]. Cells (2500 cells/well for mono-spheroids; 1750 cells/well PDAC cells mixed with 750 cells/well PSC-c for hetero-spheroids) were seeded onto round bottom non-tissue culture treated 96 well-plates in 100 µl DMEM-F12 medium, containing 10% FCS and supplemented with 0.24% methylcellulose. Spheroids were grown under standard culture conditions and harvested at different time points for RNA, protein isolation and drug testing. In Paper III, growth kinetics of mono-spheroid was performed by cell counting after trypsinization. In Paper IV, the diameter of different spheroids referred as the growth curve was evaluated using a light microscope by the same investigator (P. L.). A general image analysis of cell spheroids was performed a fluorescent microscope (Axiophot, Zeiss) equipped with a CCD camera (AxioCam MRc, Zeiss) and imported into image analyzer software (AxioVision Rel. 4.6, Zeiss).

3.2 METHODS

3.2.1 Quantitative real-time PCR (qPCR) (Paper I-IV)

Total RNA from rat islets or cultured cells as well as spheroids was extracted using the RNeasy or the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RNA integrity and concentration were analyzed using agarose gel electrophoresis and Nanodrop® Spectrophotometer ND-100 (Saveen& Werner AB, Linhamn, Sweden).

In paper I, real-time PCR was performed by the ABI Prism 7000 instrument using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and predesigned primer sets

(QuantiTect® primers, Qiagen) for rat Hif-1α, pancreatic and duodenal homeobox-1 (Pdx-1), B-cell lymphoma 2 (Bcl2), and 18S ribosomal RNA. Data were analyzed by ABI Prism 7000 SDS software. In paper II, HIF-1α mRNA was determined in a TagMan duplex real-time PCR, using 18S rRNA as an endogenous control. In Paper III and IV, we also used SYBR green (Fermentas, Thermo Scientific Life Science Research) binding to amplified cDNA for target genes and ribosomal protein L13 were used as the endogenous control genes in human PDAC cells. Then qPCR was performed in the C1000™ Thermal Cycler equipped with CFX96™ Real-time PCR System (BIO-RAD). Further detailed information regarding oligonucleotide primers is described in original papers. The ΔΔCT method was used for result calculation in all included papers.

3.2.2 Western blotting (Paper I-IV)

For protein extraction, the nuclear and cytosolic proteins were sequentially extracted from either isolated islets or cultured cells according to the protocol of Schreiber et al [214], in paper I and II. In paper III, IV and some experiments of paper II, commercial cell lysis buffers such as RIPA and M-PER® were also used to prepare whole-cell proteins. After separated by SDS-PAGE gel electrophoresis, the proteins were then transferred to Immobilon-P membrane (Millipore) where they were exposed to a primary antibody specific to the target protein. Thereafter a HRP (horse radish peroxidase)-conjugated secondary antibody was applied. The HPR cleaves a chemiluminescent substance, and the reaction product produces bioluminescence that can be detected by photographic film or CCD camera. The image was analyzed by densitometry using ImageJ software. The antibodies used in this thesis are described in original papers.

3.2.3 Insulin measurement (Paper I)

The total amount of insulin in renal subcapsular islet grafts was detected in the whole graft-bearing kidneys. Both kidneys were removed immediately after the mice were sacrificed by cervical dislocation and were quickly frozen in liquid nitrogen and kept at −80°C until use. Insulin was extracted from kidneys by means of acid ethanol. The insulin concentrations in homogenates were then determined using ELISA (Mercodia). The Merocodia Insulin ELISA is a two-site enzyme immunoassay utilizing the direct sandwich technique with two monoclonal antibodies directed against separate antigenic determinants of the insulin molecule.

3.2.4 Intracellular ATP assay (Paper I and II)

For the measurement of ATP, serum-free medium or Krebs-Henseleit (KH) buffer with study objects were used for cell incubation. In paper I, ATP assays were performed on cells after the hypoxic incubation using an ATP bioluminescent somatic cell assay kit. Since the bioluminescent

kits require specialized luminescence instrumentation and utilize luciferase that can be difficult to maintain in active form. In paper II, ATP content in cells was determined using a newly developed ATP colorimetric assay kit from BioVison. This kit is designed to be a robust, simple method that utilizes the phosphorylation of glycerol to generate a product that is easily quantified by colorimetric method. Cellular proteins were determined using a BCA assay kit. Original ATP values were normalized by protein contents.

3.2.5 Measurement of glucose consumption and lactate content (Paper II and III)

In paper II, glucose consumption and lactate production were determined in removed buffers after cell incubation using a biochemical analyzer. Before incubation, cell culture medium was changed to KH buffers with different glucose concentrations. Cellular proteins were determined using a BCA assay kit. Glucose consumption was calculated by subtracting measured glucose from original glucose concentrations. KH buffer does not contain lactate, so lactate contents in used buffers indicated lactate-production rates in cells (hence glycolysis levels in the cells). The original data of glucose consumption and lactate production were normalized with protein contents.

Since culture media were not changed during experiments, lactate contents in removed media were determined using a biochemical analyzer at the end-point of the experiments, considered as lactate accumulation. Concentration of lactate was calculated by subtracting measured lactate from original glucose concentrations in the cell-free medium. And it was normalized with protein contents as described above.

3.2.6 ROS measurement (Paper II)

Intracellular ROS levels were detected *in vitro* using a fluorescent probe CM-H_2DCFDA according to the manufacturer's recommendations. CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA, useful as an indicator for ROS in cells. The probe becomes fluorescent after oxidation by H_2O_2, $OH^{\bullet}$, $ROO^{\bullet}$ and $ONOO^{-}$. At the end of the incubation period with different glucose concentrations and O_2 tensions, wt-MiaPaCa2 cells were loaded with 5 μM ROS probe in pre-warmed phosphate buffer, incubated for 15 min at 37°C. After washing the cells twice with PBS, fluorescence was detected at 480 nm (excitation) and 525 nm (emission) using a flow cytometer.

3.2.7 Histological Studies (Paper I, III and IV)

Hematoxylin and eosin (H&E) staining

For visualization of cells in spheroids, formalin-fixed spheroids were embedded in paraffin, and sectioned into 7 μm thick sections. They were then stained with H&E and observed in a light microscope. (Paper III and IV)

In paper I, H&E staining was performed on sections (5 μm thick) of macroencapsulated islet grafts with a distance of 74 μm, prior to analyses of volume densities and vascular profiles. Volume densities (i.e., fractional volumes and absolute volumes) and vascular profiles were evaluated by morphometry. Procedures in detail are described in original papers. All examinations were performed blindly on coded specimens by the same investigator (M.K.). The micrographs were counted twice and the intraobservation variability was found to be less than 10%.

Electron microscopy analysis

In order to better investigate the cellular morphology in detail, especially the cell-cell contact in the spheroids, the electron microscopy analysis was performed in studies of paper III. Spheroids were fixed in phosphate buffer (pH7.4) containing 4% glutaraldehyde and 1% paraformaldehyde, and subsequently embedded and processed. Imaging was performed on a Tecnai 12 Spirit Bio TWIN transmission electron microscope at the Central Electron Microscopy Unit of Karolinska Institutet.

Immunofluorescent staining

In studies of paper I, we used direct and indirect immunofluorescent staining methods to identify insulin and glucagon on the same macroencapsulated islet grafts, respectively. Nuclei were stained with DAPI. The slides were examined using an Olympus fluorescent microscope and the localization and number of specific endocrine cells were evaluated using the Cell^F Imaging Software. For paper III and IV, indirect immunofluorescent staining method was performed to stain ECM proteins and other specific proteins expressed either in epithelial cells or PSCs. The antibodies used in this thesis are described in original papers. DAPI was used for nuclei staining. The slides were examined using a Zeiss fluorescent microscope.

3.2.8 Cell migration assay (Paper II)

In paper II, we used a Boyden chamber with a porous membrane (pore diameter=12 μm) coated

with fibronectin to analyze the effect of high glucose on cell migration in hypoxic MiaPaCa2 cells. IGF-I (50ng/ml) was used in the lower compartment of chamber as an additional chemotactic agent. After a 16-h normoxic or hypoxic incubation, the membrane was removed. Cells that migrated to the opposite side of the membrane were counted under a light microscope.

3.2.9 Cell viability assay for drug test (Paper III)

Most routinely used cell viability assays (i.e. trypan blue dye exclusion assay and MTT assay) are designed for monolayer cultured cells or a single cell suspension, they could not be easily adapted for application in 3D cultures. In order to estimate the efficacy in spheroid versus monolayer cells of different compounds, a slightly modified acid phosphatase (APH) assay [215] was performed in different cells, including 3 human cancer cell line, one human non-tumour cell line and one rat cell line, at the end of the exposure period in studies of paper III. APH assay, which is based on quantification of cytosolic acid phosphatase activity, was reported as most suitable tool to determine cell viability in complex 3D cultured cells for the anti-cancer drug screening [215]. To validate the APH assay on our 3D culture system, a regrowth assay and the secondary APH assay were performed after drug testing.

4 RESULTS AND DISCUSSION

4.1 Paper I:

Exendin-4 increases the expression of hypoxia-inducible factor-1α in rat islets and preserves the endocrine cell volume of both free and macroencapsulated islet grafts.

In a previous study, we demonstrated that a combination of islet preculture and recipient treatment with exendin-4 improves the metabolic outcome of a suboptimal number of rat islets transplanted to diabetic athymic mice [121]. However, that study did not compare the individual impact of islet preculture or recipient treatment on graft outcome. In addition, the effects of exendin-4 on macroencapsulated islets, which are another useful islet transplantation model established in our group [138], have not yet been studied.

In paper I we therefore compared the individual effect of islet preculture and recipient treatment on free islet graft outcome, using three different treatment protocols of exendin-4 (see Table 1). We found that islet preculture with exendin-4 followed by a short-term recipient treatment improved the metabolic outcome when a marginal mass of islet graft was transplanted under the kidney capsule. Similar effects of exendin-4 treatment were observed when islets were transplanted in an immunoprotective device subcutaneously.

Table 1. Ex-4 treatment protocols for mice transplanted with 30 free islets under the kidney capsule.

Groups	Ex-4 in islet culture medium	Mouse treatment with Ex-4[a]
1	-	-
2	+	-
3	-	+
4	+	+

[a] From day 0 to 7, recipient mice were treated (i.p.) with 100 ng exendin-4 (+) or with vehicle only(-).

Our finding from *in vivo* experiments was further confirmed by the total insulin content data of free islet transplantation and histological analysis of the macroencapsulated islet graft. Significantly greater insulin content was found in the group given combined exendin-4 treatment. As the only insulin storage cells in pancreas, the higher introcellular insulin content indicates the better-maintained β-cell mass in the presence of exendin-4 and suggests that this effect is related to improved β-cell survival. Moreover, the exendin-4-treated group had significantly larger endocrine volume, less graft necrosis, and more blood vessels around the capsule in macroencapsulated grafts. The majority of endocrine cells were positive for insulin, while only a few cells were glucagon positive.

We further investigated the protective effects of HIF-1 on islet graft with exendin-4 treatment. It has been known that transplanted islets can be damaged by severe inflammation and hypoxia before the vascularization in the grafts is established. However, a number of genes are also up-regulated for better cell survival followed by overexpression of HIF-1 [3, 81 and 84]. Our western blotting confirmed the existence of stabilized HIF-1α protein in the isolated rat islets. Markedly, expression of HIF-1α protein is increased by extendin-4 in a dose-depended manner after an overnight culture. On the contrary, expression of the pro-apoptotic enzyme caspase-3 was attenuated in the islets (see Figure 10). Furthermore, the anti-apoptotic gene Bcl2 and a β-cell differentiation factor, Pdx-1, as well as HIF-1α were up-regulated on the mRNA expression level in the exendin-4-treated islets (see Figure 11). These findings indicate that HIF-1 up-regulation by exendin-4, in concentrations used here, confers a protective effect in islets. In addition, exendin-4-treated INS-1E cells produced more ATP than control cells under hypoxic conditions, indicating that exendin-4 increases glycolysis in islet graft and thereby improves its energy production.

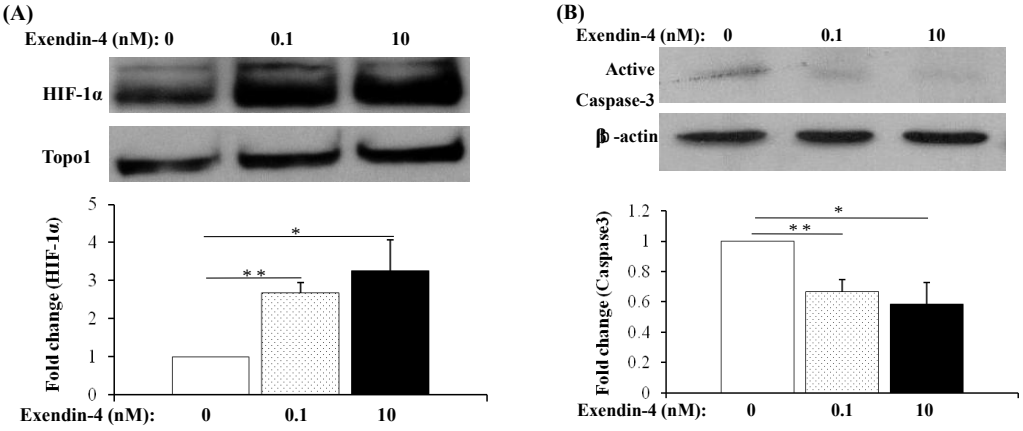

Figure 10. Western blot analyses assessing hypoxia inducible factor (HIF)-1α (A) and active caspase-3 (B) expression levels with/without exendin-4 treatment. The upper part of each panel shows specific bands of indicated proteins, whereas the lower part shows the fold change of target protein level/loading control protein level compared to the respective control samples (n = 6 in each group). $^{**}p < 0.01$, $^{*}p < 0.05$.

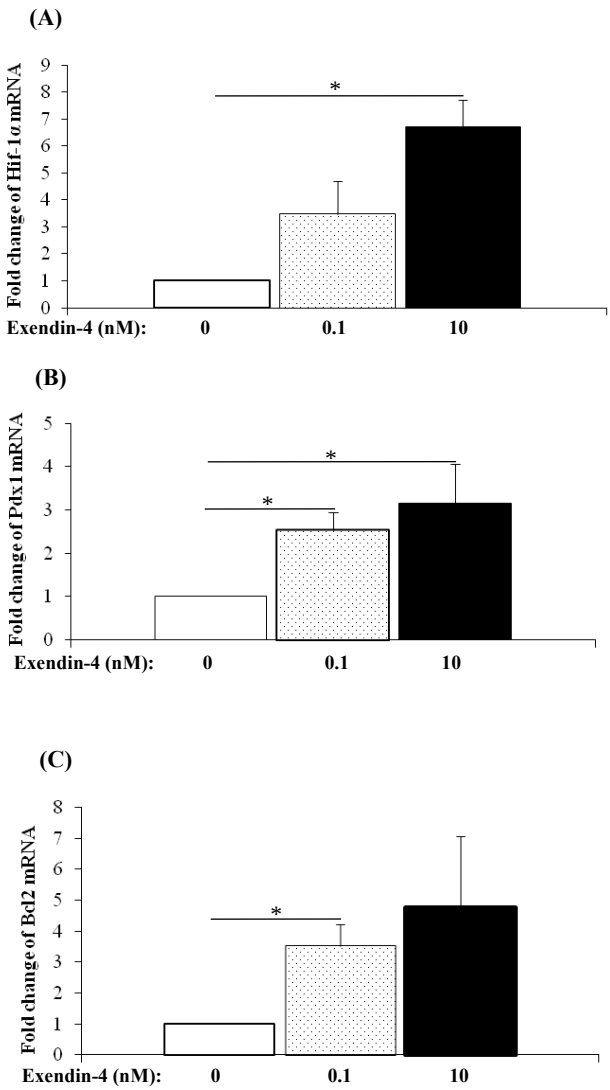

Figure 11. Real-time PCR analysis of Hif-1α (A), pancreatic and duodenal homeobox 1 (Pdx-1) (B), and B cell lymphoma 2 (Bcl2) (C) gene transcripts in isolated islets with/without exendin-4 treatment. Fold changes of target gene mRNA level/the endogenous control mRNA level are com- pared to the respective control samples (n = 3–6). *p < 0.05.

Although our *in vitro* data showed that a 20-h incubation of islets with exendin-4 improved islet viability, we observed that only the preculture of islets with exendin-4 (without recipient treatment) did not improve metabolic control after islet transplantation in the *in vivo* study. Combined with our previous finding that a short-term recipient treatment with exendin-4 improved metabolic control after islet transplantation when evaluated a few days after the discontinuation of the treatment [121], we propose that the anti-apoptotic effects of exendin-4 initiated *in vitro* may be complemented by a direct effect of exendin-4 on transplanted grafts as well as indirect effects of exendin-4 on the recipient mice such as glucose-dependent stimulation of insulin secretion [124] and the suppression of glucagon secretion [125].

Our data from the rodent models indicate that a shorter initial exendin-4 treatment may be of value to preserve islet graft mass. This study has indicated for the first time that the

protective effects of the GLP-1 receptor agonist exendin-4 may be mediated via the HIF-1 pathway.

4.2 Paper II:

Excess glucose induces hypoxia-inducible factor-1α in pancreatic cancer cells and stimulates glucose metabolism and cell migration.

Although aerobic glycolysis has now been generally accepted as a metabolic hallmark of cancer, the underlying mechanisms are still unclear. One compelling idea to explain the Warburg effect is that the altered metabolism of cancer cells confers a selective advantage for survival and proliferation in the unique tumour microenvironment. As the early tumour expands, it outgrows the diffusion limits of its local blood supply, leading to hypoxia and stabilization of HIF-1α [175, 178]. In addition, HIF-1α can be affected by other environmental characteristics, such as nutrients, pH and ECM [216]. Increased extracellular glucose has been reported to regulate HIF-1α expression in benign cells [207, 217]. Since pancreatic cancer is frequently associated with diabetes [218], it offers cancer cells a microenvironment with high glucose concentration. We hypothesized that hyperglycemia in pancreatic cancer patients may facilitate cancer cells to reprogram glucose metabolism via HIF-1α and thereby increase cancer cell aggressiveness. In order to investigate whether the excess glucose-induced HIF-1α stimulates aerobic glycolysis in PDAC cells, a MiaPaCa2 subline (namely si-MiapaCa2) was used in paper II, which stably expresses HIF-1α-specific small interfering RNA.

We found that the expression of hypoxia-induced HIF-1α was increased by extracellular glucose stimulation (5.6-22.2 mM) in wt-MiaPaCa2 human pancreatic cancer cells (see Figure 12). Furthermore, high glucose not only stabilized HIF-1α proteins, but also up-regulated HIF-1α transcription resulting in enhanced HIF-1α mRNA level in both normoxia and hypoxia. Whereas HIF-1α-production rates might be still slower than HIF-1α-degradation rate at protein level in the normoxic cells, HIF-1α protein was expressed in MiaPaCa2 cells under hypoxia rather than normoxia. Furthermore, the excess glucose-induced HIF-1α-production requires PI-3K activity in MiaPaCa2 cells. We found that excess glucose stimulated the expression of PI-3K (p85 subunit) in a dose-dependent manner in hypoxia and glucose induced HIF-1α was attenuated by PI-3K inhibitor in hypoxic wt-MiaPaCa2 cells.

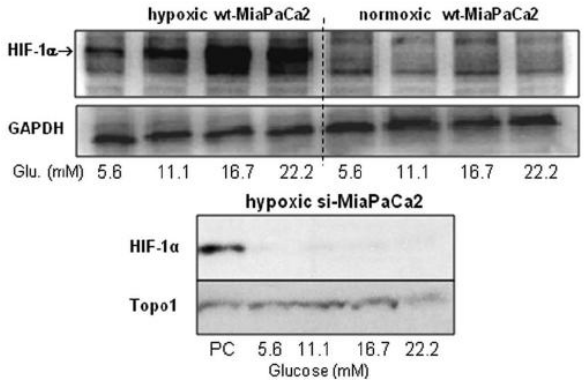

Figure 12. Wt-MiaPaCa2 (upper) and si-MiaPaCa2 (below) pancreatic cancer cells were incubated with different amount of glucose in hypoxia or normoxia for six hours. HIF-1α was determined by western blotting, using GAPDH and Topo1 as loading control.

Clearly, si-MiapaCa2 cells were devoid of HIF-1α in both normoxia and hypoxia. And in hypoxic si-MiapaCa2 cells, HIF-1α expression was not up-regulated upon excess glucose stimulation (see Figure 12). We first investigated the expression of HK-II in wt- and si-MiaPaCa2 cells exposed to different amounts of glucose in hypoxia or normoxia, which is one of the key glycolytic enzymes in glycolysis. Although the induction of HK-II expression was up-regulated by increased glucose levels in all examined cell types, excess extracellular glucose induced greater HK-II expression in wt-MiaPaCa2 cells than in si-MiaPaCa2 cells In hypoxia. Therefore glucose might stimulate HK-II expression through not only HIF-1α but also other non-hypoxic factors. To further assess glucose metabolisms in MiaPaCa2 cells with excess extracellular glucose, we determined glucose consumption, lactate production and intracellular ATP in wt- and si-MiaPaCa2 cells in normoxia and hypoxia after a 6-hour-incubation with different amounts of glucose. KH buffers with different amounts of glucose were used as culturing media during experiment to avoid interference from other components in culture media. For example, it has been recently shown that hypoxic cancer cells also use glutamine as a carbon fuel source for survival [219]. When glucose becomes the sole carbon fuel source in studied cells, glucose consumption represents cellular glucose uptake in glycolysis and lactate production in culture media is another index of glycolysis levels. A practical ratio between these two parameters compared to an ideal value, which happened in glycolysis, was further investigated to show the levels of glycolysis relative to other glucose metabolisms. However we found hypoxia-increased glycolysis in MiaPaCa2 cells is independent of extracellular glucose, though HIF-1α expression improved ATP production. As described above, other intracellular regulators of glucose metabolisms, such as Akt, c-Myc and TP53, may regulate the hypoxia-induced glycolysis [10, 11]. As various mechanisms of aerobic glycolysis modulation were reported in different cancer cell types, we further investigated the mitochondrial activities in MiaPaCa2 cells with increased glucose stimulation in hypoxia and normoxia. The glucose-induced HIF-1α stimulated both PDK-1

expression and ROS reduction in MiaPaCa2 cells, which indicate a less mitochondrial activities associated with glucose-induced HIF-1α. In addition, cell migration in MiaPaCa2 cells was stimulated by glucose via both HIF-1α dependent and independent mechanism.

We demonstrated excess glucose stimulates HIF-1α expression, increases ATP contents and stimulates migration in MiaPaCa2 PDAC cells, however, extracellular glucose and hypoxia may not regulate glucose metabolisms and cell migration depended on HIF-1α alone. It is possible that PDAC cells appear to use glycolytic metabolism before exposure to hypoxic conditions. For example, leukemic cells are highly glycolytic [220], yet these cells reside within the bloodstream at higher oxygen tensions than cells in most normal tissues. More recently, a study using a Pdx1-Cre;Ink4a/Arf$^{fl/fl}$;LSL-Kras^{G12D} mouse model of pancreatic cancer showed hypoxia increases the "glycolytic" switch of pancreatic cancer cells from OXPHOS to lactate production and increased lactate efflux from hypoxic cancer cells favors the growth of neighboring normoxic cancer cells. Their study partly confirmed our hypothesis whereas HIF-1α was not investigated in their study. Therefore, more studies are required to clarify the effects of glucose and hypoxia on pancreatic cancer cells and a better pancreatic cancer experimental model should be considered to study glucose metabolism of pancreatic cancer.

4.3 Paper III:

3D pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing.

Although improvements in cancer treatment modalities have been achieved, current cure rates are not satisfactory for many forms of advanced cancer diseases [221], such as PDAC. There is a large medical need for novel, efficient anti-cancer agents. Consequently, an *in vitro* model as a more predictive platform is strongly required for drug screening.

Most of published 3D culture studies in PDAC used biomaterial scaffolds (hydrogels or 3D porous scaffolds) to replicate the natural extracellular matrix required for native cell function. To avoid the existence of exogenous ECM components in our 3D culture system, we designed a model as spheroids of PDAC cells by adding the crowding agent methylcellulose in suspension culture. A single cell spheroid per well can be easily and reproducibly formed using our technique and thus be used for high-throughput drug screening. We used 2500 cells/well in U-bottom 96-well plates, which allow the diameter of spheroid below 500 μm during the 7-day growth period. Different PDAC cell lines were

tested, including Panc-1, MiaPaCa2, BXPC3 and ASPC-1, and showed different ability to form spheroids. Panc-1, carrying both KRAS and p53 mutation, was then selected for further testing in paper III. Cellular morphological analysis revealed that cells were homogeneously distributed in spheroid without big central necrosis and their spherical structures contained a hollow perceptible lumen indicating a spatial organization in 3D similar to that *in vivo* tumour. Moreover, tight junctions were identified between adjacent cells in Panc-1 cell spheroids. This is in agreement with a higher expression of E-cadherin was observed in 3D cultured Panc-1 cells rather than 2D cultured cells. Panc-1 cells formed relatively compact and round spheroids (see Figure 13), but cell proliferation was not increased as much as in 2D cultured cells. However cell viability was similar in both 2D and 3D cultured cells during 7-day culture, approximately 90%.

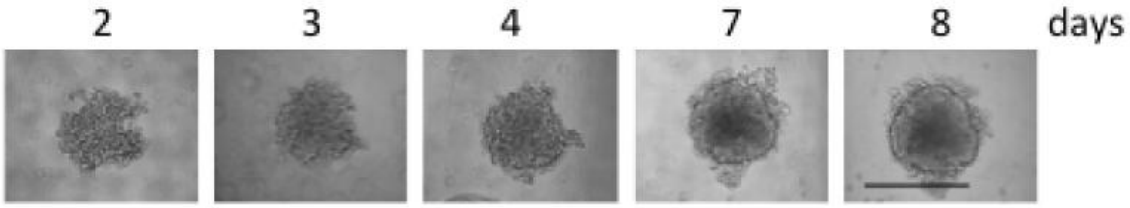

Figure 13. Development of a single representative Panc-1 spheroid, photographed from day 2 to day 8 by counting with the Boyden chamber the cell number of trypsinized spheroids and taking pictures of spheroids at fixed time points. Scale bar represents 500 μm.

Based on our current knowledge regarding chemoresistance in PDAC [222-225], we investigated glucose metabolism and expression of genes related to stroma, hypoxia, as well as chemoresistance in 2D and 3D cultured PDAC cells and compared cellular response to anti-cancer drugs using the same cell setting. Firstly, we found that endogenous ECM components were stimulated when cultured cells underwent 2D-to-3D transition. For example, the mRNA expression of FN, COL6A1 and COL1A was higher in 3D during the sphere formation (contact making) as well as compaction phase and the protein expression of collagen I and fibronectin I was observed from one day-7-spheroid section by immunohistochemistry. In addition, the proteoglycan lumican and a new stromal marker SNED1 (sushi, nidogen and EGF-like domains 1) were also up-regulated in our 3D cultured Panc-1 cells, which display associated chemoresistant property. Furthermore, miRNA-146a was strongly up-regulated when Panc-1 cells were grown in 3D, indicating the forced immobilization of cancer cells in the spheroid. Secondly, glycolysis was stimulated in association with an increased hypoxia-inducible gene expression. As the end product of glycolysis, we compared lactate accumulation in 2D and 3D cultured Panc-1 after a 10-day-culture. Lactate accumulation increased significantly more in 3D than 2D cell culture

medium after 5 days, indicating a metabolic switch to increased glycolysis in 3D. Moreover, HIF-1α was stabilized and expressed in both 2D and 3D cultured Panc-1 cells, whereas 3D cultured cells exhibited a highest content particularity at day 4. Our result suggests both hypoxic and non-hypoxic factors modulate the expression of HIF-1α in Panc-1 cells. Following HIF-1α, the expression of genes of glucose transporters, glycolytic enzymes, growth factors and proliferation was all up-regulated in 3D cultured cells, including GLUT1 and 12, HK-II, LDHA, VEGFA, PDGFB and PTGS2. Thirdly, chemoresistance-related genes were up-regulated in 3D cultured Panc-1 cells, including miRNAs miR-21 and miR-335, PPP1R1B (protein phosphatase1, regulatory subunit1B) and SNED1. Although MDR genes and transporter proteins were reported to contribute to chemoresistance in PDAC [226, 227], we did not observe this type of changes in our 3D model system, on the contrary, we found a decreased expression at least for MRP1/ABCC1. Finally and as expected, significantly higher chemoresistance was detected in 3D cultured cells upon testing of various drugs, including gemcitabine and new designed substances. Interestingly, both 2D and 3D cultured Panc-1 cells were sensitive to the anti-metabolites MT100 as well as allicin and the flavonoid AXP (genistein isoform), indicating those new drugs may have potential effects on pancreatic cancer treatment (see Figure 14). The above findings were then validated and confirmed on a mouse cell line, freshly established from the current state-of-the-art pancreatic cancer mouse model with Kras and p53 mutations in the pancreas.

In summary, this study presented a 3D culture model in PDAC that acquires a more stroma-rich phenotype and thereby displays a strongly increased chemoresistance. Our data even support two of the three mechanisms that are proposed to underlie chemoresistance according to the novel hypothesis of cell adhesion mediated drug-resistance (CAM-DR) [228]: spheroid formation and matrix/fibronectin production (the third mechanism being related to the stroma). Taken together, the switch from 2D to 3D growth affects several “hallmarks of cancer” and leads to a more aggressive cancer phenotype [229] (see Figure 15).

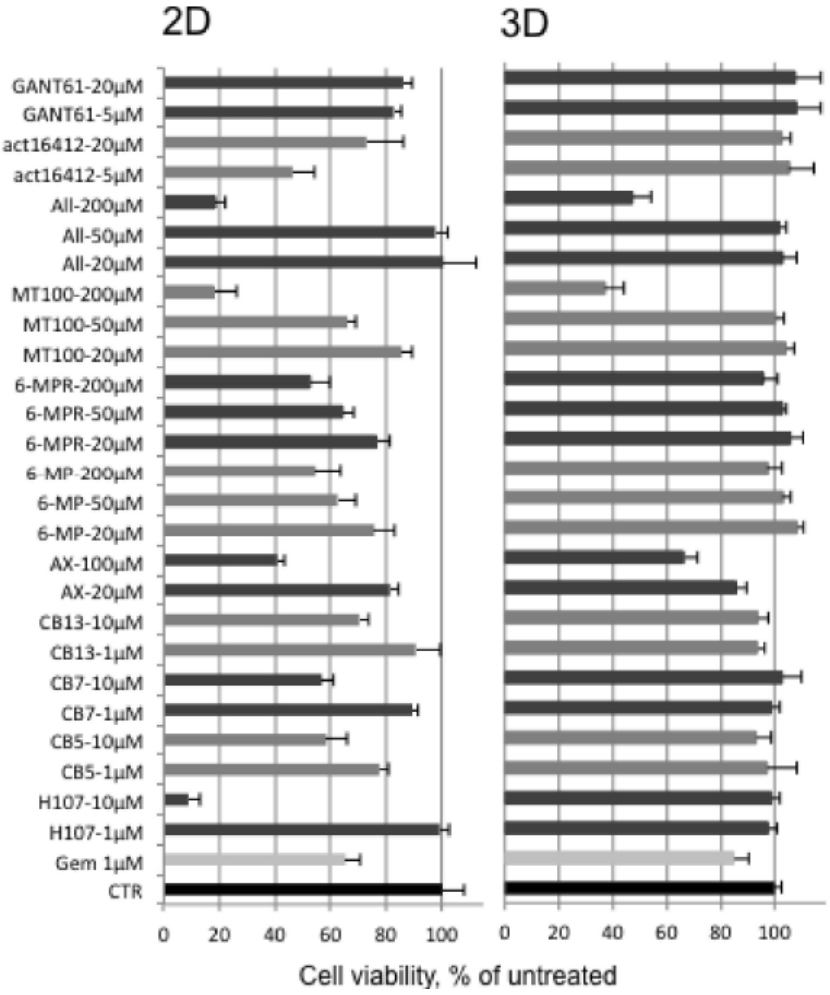

Figure 14. Comparison of chemoresistance between 2D and 3D culture using multiple cytotoxic compounds. Histogram summarizing the results from viability assays performed on 2D and 3D Panc-1 cell cultures. Different drugs were used at the indicated concentrations. Data are plotted as percentage of the respective untreated control (CTR) and each drug was tested three times in octuplets.
Gem: gemcitabine.
All: allicin.
AX: AXP-107-11.

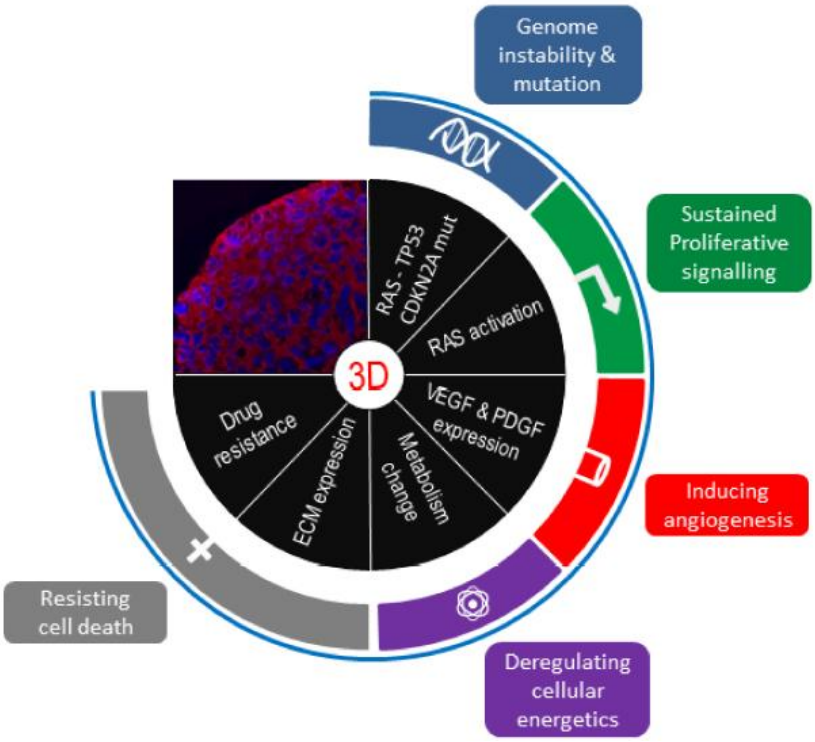

Figure 15. Representation of the characteristics of the 3D spheroid model in relation to the holistic hallmarks of cancer according to Hanahan and Weinberg [229] observed in the reductionist pancreatic cancer mono-spheroid model. Figure 15 is adapted from Longati P, Jia X, Eimer J, Wagman A, Witt MR, Rehnmark S, Verbeke C, Toftgård R, Löhr M, Heuchel RL: **3D pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing.** *BMC Cancer* 2013, **13**:95.

4.4 Paper IV:

Correctly oriented and activated pancreatic stellate cells engulfing pancreatic ductal adenocarcinoma cells to form an avascular, stroma-containing mini-tumour: *Establishment of the model and first description.*

By understanding the pivotal role of PSCs in pancreatic cancer progression, metastasis and chemoresistance, we aimed to improve the current 3D model of PDAC as the multicellular tumour spheroids including ductal epithelial cells and PSCs, which represent the tumour-stromal interaction characteristic of *in vivo* solid tumours.

However the development of 3D multicellular tumour spheroids is more complex than cell spheroids of single cell type. How to organize heterogeneous cells in spheroids, particularly reflecting an *in vivo* situation of a pancreatic carcinoma was a big challenge of this study. We adapted PSCs to non-adhesive growth by a three-month culture on the agarose gel. Interestingly such conditioned PSCs (PSC-c) were able to form the hetero-spheroids with human pancreatic cancer cells in a novel, unexpected way. The Panc-1/PSC-c hetero-spheroids grew as firm spheroids with the PSCs in a cortical layer, similar to the patient situation, where nests of tumour cells are surrounded by a web of stromal cells (see Figure 16). Moreover, PSC-c became activated when growing in hetero-spheroids with cancer cells. We found that α-SMA and α-vimentin protein, activated PSCs markers, were strongly expressed in the outer layer of hetero-spheroids suggestive of the location of PSCs within the hetero-spheroids. As expected, higher ACTA2 mRNA levels in hetero-spheroids were observed when compared to the same number of PSCs from mono-spheroids. In addition, various pancreatic epithelial cells were tested to form hetero-spheroids with PSC-c, including human pancreatic tumour cell lines Paca44, the normal human pancreatic ductal epithelial cell line HPDE and KPC cells derived from a mouse PDAC model.

To investigate the feature of multicellular PDAC spheroids in the presence of PSCs, we compared the expression of genes associated with adhesion, proliferation, ECM and chemoresistance between mono- and hetero-spheroids. The mRNA expression of E-cadherin, an important protein component of tight junctions, was up-regulated in the hetero-spheroids compared to mono-spheroids. As the dedicated binding partner, E-cadherin and β-catenin were strongly expressed over the interphase between cancer cells and PSCs in the hetero-spheroids. The expression of fibronectin and collagen I was up-regulated in hetero-spheroids. Particularly, the immunofluorescence staining in hetero-spheroids showed a network-like fibronectin expression compared to the more diffuse and homogeneous

appearance of fibronectin in mono-spheroids, suggesting that enhanced ECM production in hetero-spheroids is similar to the patient situation. In addition, mRNA expression of lumican, COX2 and PPP1R1B was up-regulated in hetero-spheroids compared to mono-spheroids, which play important roles in collagen fibrillogenesis, cell proliferation and migration, metastasis as well as chemoresistance [230-233].

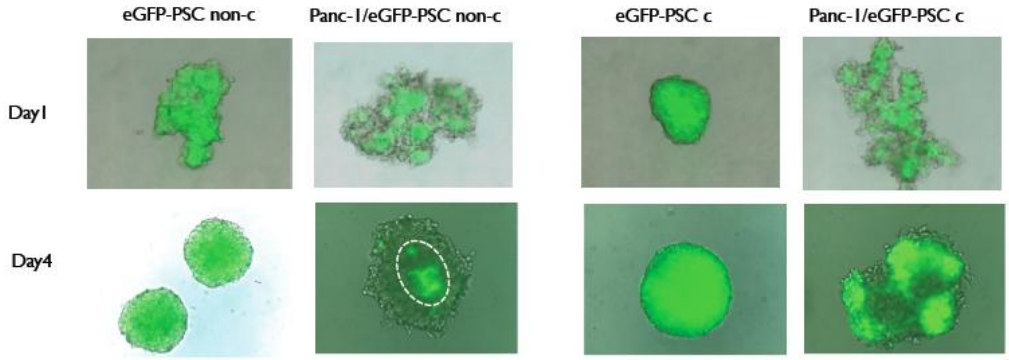

Figure 16. Development of mono- and hetero-spheroids using non-conditioned eGFP-PSC (PSC non-c), conditioned eGFP-PSC (PSC-c) and Panc-1 human PDAC cells. Pictures were taken on day1 and day 4. The PSC non-c cells are found inside the white, stippled circle, i.e. in the center of the spheroid.

Tumour hypoxia has been known for decades to negatively affect therapy outcome of cancer for decades. We applied the hypoxia incubations on mono- and hetero-spheroids and investigated the role of hypoxia in our 3D cell culture model. Interestingly, hetero-spheroids shrank in the absence of O_2 after two-day- or five-day-culture. Moreover, we found ECM component fibronectin, stromal marker α-SMA and growth factor PDGF-B were all up-regulated on the mRNA expression level in hypoxia. In addition, we applied a 6-hour-incubation of hypoxia on 4-day-spheroids, when spheroids were normally used for drug test in our previous study. We found the mRNA expression of hypoxia-inducible genes was enhanced, such as PDGF-B, VEGF, GLUT-1, ZEB-1 and LOX (lysyl oxidase), following a slightly enhanced HIF-1α mRNA expression. Furthermore, a strong enhancement of gene PPP1R1B and cancer stem cell marker CD24, both related to chemoresistance, was found in the hetero-spheroids after hypoxia incubations (see Figure 17). Interestingly, we found the protein expression of HIF-1α varied in different type of nomoxic spheroids and there was even a slightly expressed HIF-1α protein in 2D cultured cells. The 3D cultured Panc-1 cells did express HIF-1α protein, but HIF-1α protein was not found in PSC-c mono-spheroids. Thus Panc-1/PSC-c spheroids had a lower expression of HIF-1 α protein compared to pure Panc-1 mono-spheroids. As a low differentiation PDAC cell line, Panc-1 cells carry both KRAS and TP53 mutation. On one hand, HIF-1α protein may be stimulated by oncogenes in these cells and thereby expressed HIF-1α in normoxia; on the other hand the specific 3D

structure in our PDAC cell spheroids may induce the mild ‘intra-tumour hypoxia’ and enhance HIF-1α protein expression in spheroids. Taken together, our data suggest the expression of HIF-1α may result in alter the sensitivity of tumour to chemotherapy due to increased malignancy and invasiveness of human PDAC.

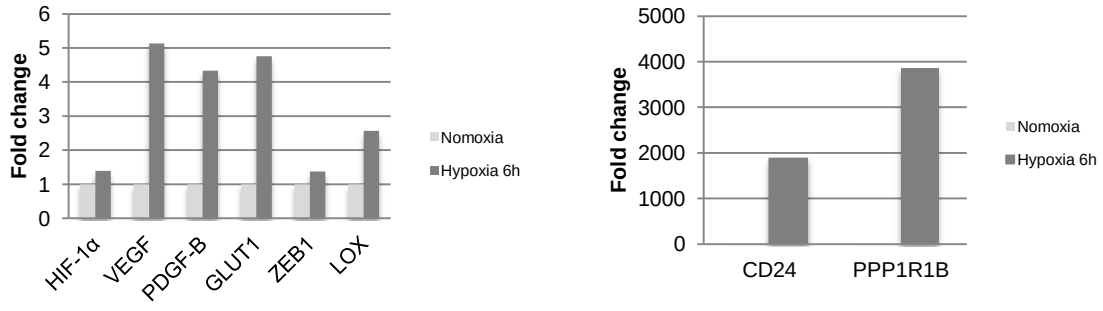

Figure 17. Gene expression analysis in Panc-1/PSC-c spheroids with normoxic and hypoxic incubation using qRT-PCR in left and right.

In summary, the 3D hetero-compound spheroid model of pancreatic cancer cells engulfed by PSCs represents a valuable model, allowing for the first time to study the interaction and mutual dependency of these two principal cell types found in human PDAC in a way more close to the patient. Further, it allows high-throughput drug screening on both the stromal cells and the tumour cells. And more studies are required to identify the features of our novel hetero-compound spheroid model in association with chemoresistance.

5 GENERAL CONCLUSIONS

- Evaluation of the effects of exendin-4 on free and encapsulated islet grafts in a rodent model revealed that preculture with exendin-4 followed by recipient treatment improved the outcome of both free and macroencapsulated islet grafts due to a larger surviving endocrine cell volume.

- Exendin-4 may improve islet graft resistance to hypoxia during the peritransplant period by increasing the expression of HIF-1α.

- In PDAC MiaPaCa2 cells, excess glucose increases HIF-1α and ATP in hypoxic wt-MiapaCa2 cells; however extracellular glucose and hypoxia regulate glucose metabolisms independent of HIF-1α. And cell migration is stimulated by glucose via both HIF-1α dependent and independent mechanism.

- Development of a high-throughput 3D cell culture drug screening system for pancreatic cancer, which displays a strongly increased chemoresistance.

- HIF-1α and its downstream hypoxia-inducible genes are up-regulated in this cell spheroid model of pancreatic cancer epithelial cells, which stimulates glycolytic switch when cells undergo the transition from 2D to 3D cultures.

- Development of a novel 3D hetero-spheroid model with PSCs in the correct, i.e. outer orientation combined with cancer epithelial cells in the center. The expression of genes associated with adhesion, proliferation, ECM and chemoresistance was up-regulated.

- HIF-1α expression in the hetero-spheroids of human PDAC is associated with the increased ECM production and enhanced expression of gene PPP1R1B (DARP-32) as well as cancer stem cell marker (CD24), which might enhance tumour chemoresistance.

6 ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone that has helped and supported me during my PhD studies, for knowledge and enthusiasm. I have learned many things, not just about science but also about life. In particular, I would like to thank:

Professor Matthias Löhr, my supervisor. Your enthusiasm, encouragement and love for research have created a fantastic working environment. It is grateful that I have being a part of your lab. I benefit a lot from your guidance and encouragement and I appreciate the support and freedom that I have been given to explore my own ideas.

Makiko Kumagai-Braesch, my co-supervisor, for opening the door of the research field of islet transplantation to me, for excellent guidance and for always taking the time to help me! Although I then switched to research pancreatic cancer, your hardworking ethic has influenced me a lot and will be beneficial to all my life.

Paola Longati, my co-supervisor, for excellent guidance, invaluable personal support and friendship. Thank you for believing in me and encouraging me when I need it! I really enjoyed the time when we worked together wherever in the lab or in the kitchen ☺

Rainer L. Heuchel, my co-supervisor, for sharing your knowledge on pancreatic cancer and for thorough review of my applications, manuscript and this thesis and all helpful comments and corrections. You showed me what a good German work ethic is like!

Feng Wang, my former co-supervisor, for introducing me to this exciting pancreatic cancer research field and for sharing your vast knowledge and experience on the scientific writing.

Professor Jörgen Larsson, my former supervisor, for valuable guidance, encouragement and financial support.

Hélène Jansson for your quick and helpful answers to my questions all the time! Although your work is out of my projects, you helped a lot for a foreign Ph.D. student to reach her goal in our division!

Annika B. Tibell, for giving me the opportunity to work in the division of Transplantation Surgery with all its excellent working facilities. Thank you, I had a great time at islet transplantation group.

Every past and present member of the PacLab: *Annika Wagman* for being a nice person and my reference book in Sweden, always taking the time to help me with everything from lab work to my life. *Irene Engström* and *Beth Hagman* for their excellent technical assistance. *Elisabeth Dungner* for being a nice person and all your help in the lab. *Johannes Eimer* for his contribution on the development of our 3D pancreatic cancer cell model. *Ying, Jessica, Xuan* and *Salvatore* and all the students from Merch for creating a nice and relaxed atmosphere in the lab.

My former colleagues and co-authors in islet transplantation group: *Amit Sharma* for showing me the excellent technique of islet transplantation and for being a great support when I first started. *Annika M. Wernerson* for sharing your vast knowledge of pathology. *Anne K. Sörenby* for showing me the technique of macroencapsulation of islet transplantation. *Shinji Yamamoto* for being a company during islet transplantation.

My former colleagues in department of clinical immunology: *Jining Liu* for your help with molecular biology and for as my first Chinese friend in KI. No matter where you are in Stockholm or Göteborg, I miss the time when you talked about science and life with me! *Bo Xu* for your help of taking care of my cells and for being a nice person. All the best for your life with Jining in Göteborg! *Zhiwen Liu* and *Eva Bergdahl* for your warm help on my experiments and for lunch companies during that time. Enjoy your life! *Mehmet Uzunel* for showing me how to isolate total RNA and perform real-time PCR. *Jan Holgersson* for your group meeting and helpful suggestions on my project. *Randa Diab, Stefan Gaunitz, Reeja Maria Cherian* and *Mats Alheim*, for good company in the lab.

My co-authors of pancreatic cancer studies: *Michael-Robin Witt, Stefan Rehnmark, Caroline Verbeke, Rune Toftgård, Zhiwen Liu, Yijie Duan, Huijie Xiao, Karl-Gösta Sundqvist* and *Johan Permert* for excellent collaboration.

TheraCyte™ Inc. and especially *Tom Loudovaris*, for kindly providing the immunoprotective devices used in this thesis.

Stig Linder, Dept. of Onco-Pathology, Karolinska Institutet, for providing us with the microtubulin-inhibitors. *Yehuda Miron* and *Talia Miron* from the Weizman Institute, Rehova, Israel, for providing us with the allicin and MT drugs used in this thesis.

The staff in the Animal Department, for good advice about handling of animals!

Jun Su and *Xiuqin Dong*, my extra mentors, for personal support, encouragement and

friendship.

Sylvie Le Guyader for teaching me how to operate the confocal microscopy and sharing your experience on research, though my data cannot be included in this thesis.

My friends in NOVUM: *Karolina Kublickiene* for practical tips before my half-time control and organizing the wonderful course. *Sebastian Sjöqvist* for bebing a former lab roommate. My Chinese friends for the scientific discussion, shared information and lunch company: *Jianping Liu, Xu He, Chunyan Zhao, Liang Yu, Sun Jia, Ting Zhaung, Ying Qu, Jiaxue He, Jian Yan, Meng Li, Ting Jia, Yan Li, Jiaqi Huang, Yimeng Yin, Zhilun Li, Lijuan Hu* and *Xiang Hua*.

All the Chinese friends that have had the pleasure to know from the Ph.D. courses and meetings of KI: *Bin Zhao, Xiao Wang, Xiaonan Zhang, Na Wang, Yao Shi, Xu Zhang, Ming Lu, Ying Sun, Lili Li, Jia Liu, Fang Zong, Miao Zhao, Yahong Han, Xiaofeng Zheng.*

All my great friends in Stockholm for cheerful dinners, shopping and waiting time during our daughter's course!

My family: *My parents* for your unconditional love and support and for always believing in me. Without your help for taking care of my children, I would not concentrate my Ph.D. study and accomplish my dream. 爸爸妈妈，谢谢你们对我无尽的爱及对我学业和家庭的支持！And my *parent-in-law* for your understanding, endless support and love. *Emily* and *Ella*, my sweet girls, for your love, kisses and understanding. Mom will have time to play with you when I get the Ph.D. degree ☺. *Hailong*, my husband, for your love and support during these years. I love you, too!

The work included in this thesis had been financially supported through the regional agreement on medical training and clinical research between Stockholm County Council and Karolinska Institutet (project number 20060710, 20080443), Swedish Research Council (52120083389 and 2007–4034), research grants from Karolinska Institute, the Foundation for International Surgical Cooperation, the Foundation for Oncological Cooperation and Barndiabetesfonden (2007, 2008, and 2009), Cancerfonden (CAN 2008/982, CF 120771 and 080312) and an EU FP7 program, "EPC-TM-Net", Vetenskapsrådet (VR, K2013-67X-22322).

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