Regulation of energy metabolism in human skeletal muscle cells and adipocytes:

Effects of SENP2 knockdown and conditioned medium from human pancreatic carcinoma cells

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Oslo, August 2022

Solveig A. Krapf

List of Publications

Paper I

Jenny Lund, **Solveig A. Krapf**, Medina Sisteka, Hege G. Bakke, Stefano Barthesaghi, Xiao-Rong Peng, Arild C. Rustan, G. Hege Thoresen, Eili T. Kase

SENP2 is vital for optimal insulin signaling and insulin-stimulated glycogen synthesis in human skeletal muscle cells.

Current Research in Pharmacology and Drug Discovery, 2021;(2):100061

Paper II

Solveig A. Krapf, Jenny Lund, Hege G. Bakke, Tuula A. Nyman, Stefano Barthesaghi, Xiao-Rong Peng, Arild C. Rustan, G. Hege Thoresen, Eili T. Kase

SENP2 is an important regulator of energy metabolism in adipocytes and its knockdown increases selected markers of browning.

Submitted to **Adipocytes**

Paper III

Solveig A. Krapf, Jenny Lund, Malin Lundkvist, Marianne G. Dale, Hege G. Bakke, Tuula A. Nyman, Arild C. Rustan, G. Hege Thoresen, Eili T. Kase

Pancreatic cancer cells show lower oleic acid oxidation and their conditioned medium inhibits oleic acid oxidation in human myotubes.

Pancreatology, 2020;20(4):676-682

Paper IV

Solveig A. Krapf, Jenny Lund, Hege G. Bakke, Tuula A. Nyman, Awais Ur Rehman Saqib, Arild C. Rustan, G. Hege Thoresen, Eili T. Kase

Pancreatic cancer cell conditioned primary myotubes display increased protein/leucine turnover and lipid accumulation and reduced glucose uptake.

Submitted to **Pancreatology**

Abbreviations

ACACA/ACCα	Acetyl-CoA carboxylase 1
ACBP	Acyl-CoA-binding protein
ACLY	Acetyl-CoA by ATP-citrate lysase
ACS	Acyl-CoA synthetase
ASM	Acid soluble metabolites
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
CA	Cell associated
CoQ	Coenzyme Q
CO2	Carbon di oxide
CtyC	Cytochrome complex
CPT	Carnitine palmitoyltransferase
DAG	Diacylglycerol
ETC	Electron transport chain
FA	Fatty Acid
FAD	Flavine adenine dinucleotide
FABP	Fatty acid binding protein
FAO	Fatty acid oxidation
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FATP	Fatty acid transport protein
FAS	Fatty acid synthase
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FFA	Free fatty acid
GLUT	Glucose transporter
GS	Glycogen synthase
G-6-P	Glucose 6 phosphate
HFD	High Fat diet
HK	Hexokinase
hPEC	Human pancreatic epithelial cells

IGF	Insulin-like growth factor
IMTAG	Intramuscular triacylglycerol
IR	Insulin receptor
IRS-1	Insulin receptor substarte-1
JNK	c-Jun N-terminal kinases
KD	Knock down
KO	Knock out
LD	Lipid droplets
LPL	Lipo protein lipase
LXR	Liver X receptor
MAG	Monoacylglycero
MAPK	mitogen -activated protein kinase
MCT	Monocarboxylate transporter
MHC/MYH	Myosin heavy chain
mTOR	mammalian target of rapamycin
NADH	Nicotineamide adenine dinucleotide
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OA	Oleic Acid
PANC-1	Pancreatic cancer cell line 1?
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1- alpha
PI3	Phosphoinositide 3-kinase
PKB/akt	protein kinase B
PL	Phospholipids
PPAR	Peroxisome proliferator-activated receptor
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
SCR	Scramble
SENP	SUMO/Sentrin specific protease

SENP2-KD	SUMO/Sentrin specific protease knockdown
shRNA	Short hairpin Ribonucleic acid
SPA	Scintialtion proximity assay
SUMO	Small ubiquitin-related modifier
TBC1D4/AS160	TBC1 domain family member 4/Akt substrate of 160kDA
TCA	Tricarboxylic acid
TAG	Triacylglycerol
TBC1D1	TBC1 domain family member 1
TNF	Tumor necrosis factor
TLC	Thin layer chromatography
T2DM	Type 2 diabetes mellitus
UCP	Uncoupling protein
WAT	White adipose tissue
WHO	World health organization
WC	Waist circumference
WHR	Waist hip ratio

Abstract

Energy metabolism is a fundamental and vital process in all the body's cells, and disturbed energy metabolism is be involved in diseases such as obesity and cancer. Lifestyle choices including diet with high fat and sugar content and lack of exercise are predisposing factors for these conditions. Chronic obesity is often associated with many comorbidities, including type 2 diabetes mellitus (T2DM) and cardiovascular disease. Also, T2DM have been linked to an increased risk of several forms of cancer. Skeletal muscle and fat cells exerts important roles in the regards of metabolism and whole body homeostasis. While adipose tissue is an excellent storage tissue and takes care of access fat, skeletal muscle serves as the main site of insulin-stimulated glucose disposal, processes that often are disturbed in obesity and T2DM. Altered energy metabolism is also one of the hallmarks of cancer, and several of the cancers' success factors can be related back to a change in energy metabolism. In addition, cancers can impact energy metabolism in other tissues, such as skeletal muscle.

The peroxisome proliferator-activated receptors (PPARs) are important metabolic regulators. PPARs are also targets for the post-translational protein modificator prosess SUMOylation (SUMO = small ubiquitin related modifier) with by revers binding to target proteins alters their function, location and/or half-life. Recently, the SUMO/sentrine-specific protease (SENP)2, which deSUMOylate SUMOs, has been identified as a regulator of cellular pathways and in skeletal muscle cells and adipose tissue. To our knowledge, previous studies on SENP2 have only been performed in rodents and we wanted to explore the impact of knocking down this protein in primary human myotubes and adipocytes.

Myotubes with knock-down of SENP2 (SENP2-KD) showed blunted insulin-stimulated Akt phosphorylation and glycogen synthesis, while basal glucose uptake and cell-associated glucose were increased and fractional glucose oxidation was reduced. In contrast, the SENP2-KD adipocytes had both reduced glucose uptake and oxidation. More similar was the fatty acid (FA) handling in the two cell types. Both myotubes and adipocytes showed increased FA oxidation after SENP2-KD, as well as decreased lipid accumulation and lipid incorporation into triacylglycerol (TAG). Although the underlying molecular mechanisms are not fully elucidated, SENP2 seem to play a role in energy metabolism in both myotubes and adipocytes also in humans.

To study energy metabolism in a pancreatic cancer cell line (PANC-1) and to explore crosstalk between cancer cells and primary human myotubes, we first compared energy metabolism in a human PANC-1 cells to non-malignant human pancreatic cells (hPEC). PANC-1 cells tended to be more glycolytic compared to control hPEC. Glucose oxidation and oleic acid uptake was higher, while oleic acid oxidation and reserve capacity was lower. PANC-1 also took up more glucose from the media and released more lactic acid compared to hPEC. The differences in energy metabolism were reflected in gene expressions of transporters and nuclear receptors as well as pathway analysis of the secretome. Thereafter we explored whether conditioned media from the two cell types had impact on the energy metabolism of primary human myotubes. PANC-1-conditioned myotubes had an imbalance in protein turnover resulting in a total decrease of protein content compared to myotubes exposed to conditioned medium from hPEC-1 cells. Also, lipid uptake in PANC-1-conditioned myotubes was increased whereas fatty acid oxidation was unchanged, leading to accumulation of intracellular lipids in the muscle cells conditioned with PANC-1 media.

In summary, the work presented in this thesis provide insight into cellular processes and regulatory molecules that are affected by SENP2-KD and cancer cell metabolism as well as impact of conditioning myotubes with cancer cell media.

1. Introduction

1.1 Energy metabolism and metabolic diseases

Metabolism is the chemical processes occurring within all living organism which transforms metabolites to fulfill biological functions. The catabolic pathways breaks down molecules to produce energy and maintain life [1]. A cell's basic metabolic state is the maintenance of energy demand, i.e. to maintain the ion balance between the cell and extracellular medium. The demand increases beyond basal when the cell grows, moves or performs other actions [1]. As any homeostatic process, the metabolic process can be disrupted or put out of balance. An imbalance between intake and output leads to disruption of this homeostatic process, (**Figure 1**).

Figure 1: Metabolism in balance. When energy intake matches energy usage the metabolic process will be in balance. Image created with Biorender.com.

Type 2 diabetes mellitus (T2DM) is a worldwide public health threat and a significant contributor to morbidity and mortality. T2DM develops due to insulin resistance and is characterized by impaired insulin-dependent glucose metabolism in metabolic important tissues, including skeletal muscles, liver and adipose tissue (reviewed in [2]). Insulin is the hormone secreted by beta (β) cells of the islets of Langerhans which by stimulating uptake of molecules such as sugar into fat, skeletal muscle and liver, controls metabolism [3], (**Figure 7)**. Excess lipid storage and expansion of WAT is the primary contributor to obesity, a strong risk factor for development of insulin resistance, which further can lead to T2DM [4]. Over the past decades a drastic increase in the prevalence of overweight and obesity has been seen worldwide [5]. In 2016, as many as 39% of adults were

overweight and 13% obese [6]. The definition of overweight and obesity is in practice assessed by body mass index (BMI) [7]. As classified by the World Health Organization (WHO) BMI is considered normal if it lies in the 18.50 - 24.99 kg/m^2 range and overweight or obese if the BMI ≥ 25 kg/m^2 or ≥ 30 kg/m^2, respectively [5]. However, BMI classification can be problematic due to ethnic variations in body composition [8], age and chronic diseases [9], which limits the usefulness of BMI to assess health risk. Therefore, it has been suggested that measurement of waist circumference or waist-to-hip ratio, which reflect visceral (abdominal) fat, may be more suitable for classification of overweight and obesity [10]. For Caucasian European females/males, the threshold values for waist circumference (WC) and waist hip ratio (WHR) are considered above normal if they are higher than 88/102 cm and 0.85/0.09, respectively [11] **(Figure 2**).

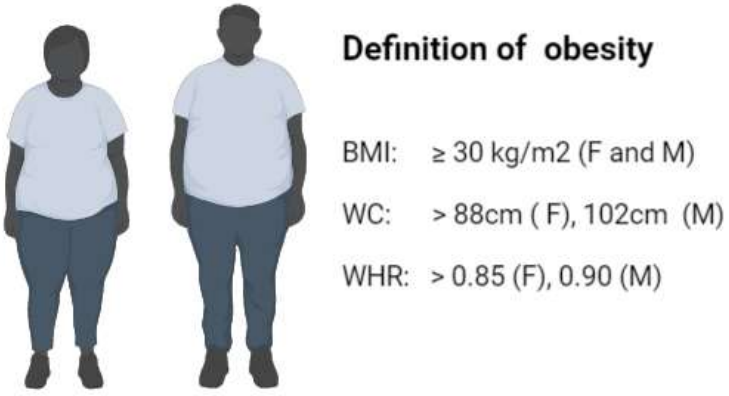

Figure 2. Cut of values for obesity definitions by BMI, WC and WHR. Obesity is defined by body mass index (BMI) ≥ 30 kg/m^2 in female and male, waist circumference (WC) > 88 cm in female and 102 cm in male and waist to hip ration (WHR) >0.85 in female and 0.90 in male [11]. Image created with Biorender.com.

1.2 Myotubes

1.2.1 Myotube energy metabolism

In non-obese adult individuals, skeletal muscle contributes to approximately 40% of the body weight. It is a major organ for metabolism of lipids and glucose, and the largest insulin-sensitive organ, accounting for more than 80% of insulin-stimulated glucose disposal [12,13]. Carbohydrates and fatty acids (FAs) are the main fuel sources for skeletal muscle [14]. As lipid storage is large and potentially inexhaustible, FA oxidation is the main metabolic activity in skeletal muscle during fasting [15-17]. Carbohydrate storage on the other hand is limited to glycogen storages in muscle,

liver and glucose in circulation with around 400-500 g, 60-100 g, and 4-5 g respectively, during rest [15]. Glucose is taken up in skeletal muscle and follows one of three major pathways:

1. Stored as glycogen, during rest/inactivity and available for rapid utilization at a later time
2. Oxidized for energy production, by making adenosine triphosphate (ATP)
3. Acting as a precursor for lipid synthesis

The uptake of glucose across the plasma membrane is considered the rate-limiting step for glucose utilization during rest, and facilitated by a family of glucose transporters (GLUTs) [18]. In humans GLUT1 and GLUT4 are responsible for the majority of the glucose uptake into muscle with slightly different roles [19-22]. While GLUT1 is the main transporter during basal conditions, GLUT4 is translocated from intracellular vesicles during insulin stimulation or contraction. Glucose is then taken up through a different pathway [23-26]. Several factors are proposed involved in the regulation of GLUT4 included the Rab8A, Rab13 [27], as well as the Rab GTPase-activating protein, TBC1 domain family member 1 (TBC1D1) [28], and TBC domain family member 4 (TBC1D4, also known as Akt substrate of 160kDa (AS160)) [29]. When insulin binds its receptor phosphorylation of insulin receptor substarte-1 (IRS-1), Pi 3-kinase(PI3K) and protein kinase B (PKB/Akt) leads to receptor activation [30], and GLUT4 is translocated from intracellular vesicles to cell surface by the phosphorylation of PKB/Akt [30,31]. Glucose then gets taken up into the cells and phosphorylated to glucose 6 phosphate (G-6-P) by hexokinase (HK) followed by glycolysis to generate pyruvate, ATP and nicotineamide adenine dinucleotide (NADH). Under aerobic conditions pyruvate is transferred into mitochondria and converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDC).

Alternatively, glucose can be converted into glycogen by glycogen synthase (GS) and stored [32]. As the muscle cells have limited ability to store glycogen, leftover glucose goes through lipogenesis and is converted into lipids [33]. This conversion take place through so called *de novo* lipogenesis where pyruvate from both stored or plasma glucose can be decarboxylated in the mitochondria to acetyl coenzyme A (acetyl-CoA) by PDC [34]. However as glucose oxidation rate is mainly determined by the activity of PDC [35], phosphorylation of PDC by pyruvate dehydrogenase kinase (PDK) 4 (which inhibits the activity of PDC) reduces glucose oxidation by inhibiting pyruvate to enter Tricarboxylic acid (TCA) cycle [36,37]. Thus increased PDK4, reduces PDC activity and by extension glucose oxidation [38]. Acetyl-CoA is then converted to citrate and

directed back to plasma from mitochondria for fatty acid (FA) and cholesterol synthesis [39] (Figure 3).

FAs are delivered to skeletal muscle bound to albumin, or derived from triacylglycerol (TAG) in chylomicrons or very low-density lipoproteins liberated by lipoproteinlipase (LPL) [40]. The FA enters by passive diffusion or protein mediated transport. The main proteins regulating this uptake is fatty acid translocase (FAT/CD36) and FA transport protein 4 (FATP4) [41]. Inside the skeletal muscle cell the FAs binds to cytoplasmic FA-binding protein (FABP) [42]. Dependent on the cells metabolic status, the FAs can either be:

1. Oxidized in mitochondria for energy production as ATP
2. Esterified to monoacylglycerol and diacylglycerol (MAG and DAG, respectively), to be stored as triacylglycerol (TAG) in lipid droplets (LD)s or incorporated into phospholipids (PLs) for use in cellular membranes or as lipid second messengers [43].

In order to be oxidized, intracellular FAs firstly get activated to FA-CoA (as acyl-CoA) mediated by acyl-CoA synthetase (ACS) [44], before shuttled to the outer mitochondrial membrane by acyl-CoA-binding protein (ACBP) [42,43]. Carnitine palmitoyltransferase (CPT) 1 is situated on the mitochondria outer membrane [43]. CPT conjugates and transport FAs over the outer membrane, as the mitochondrial membrane is impermeable to acyl-CoA [45]. Once inside the mitochondria, regeneration of acyl-CoA takes place by the help of CPT2 which is located in the inner mitochondrial membrane [46]. Furthermore, FA acyl-CoA can be degraded via β-oxidation producing acetyl-CoA which enters TCA cycle and produces NADH and FADH2 that gets delivered to the electron transport chain (ETC) for ATP production [47] (**Figure 3**).

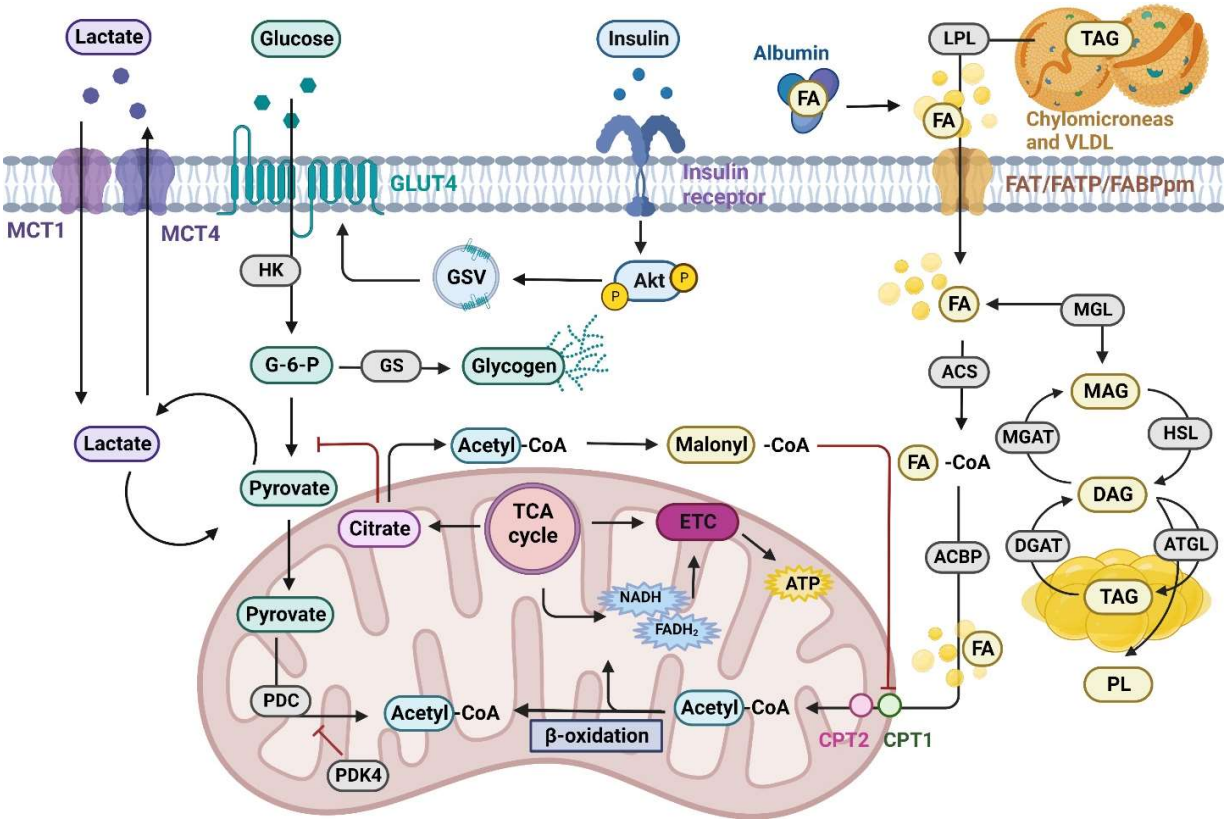

Figure 3. Energy metabolism in skeletal muscle cells. Glucose and fatty acids (FA)s are substrates for adenosine triphosphate (ATP) production. Binding of insulin to its receptor activates a phosphorylation cascaded, including protein kinase B (PKB/Akt), leading to translocation of the glucose transporter (GLUT)4 to the cellular membrane, which facilitates glucose uptake into the cell. Inside the cell glucose is either converted to glycogen for storage by glycogen synthesis (GS) or taken up into the mitochondria after conversion to pyruvate by the enzyme hexokinase (HK). Inside the mitochondria pyruvate is decarboxilated into acetyl-CoA by pyruvate dehydrogenase complex (PDC) before directed towards the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) for ATP production. FA, does either come bound to albumin or as triacylglycerol (TAG) in chylomicrons and very low density lipoproteins (VLDL). Before take up to the cell lipo protein lipase (LPL) liberated the TAG and FAs are take up into the cell via fatty acid transporters (FAT/CD36, FATP, FABPpm). Inside the cell FA is converted to FA-CoA by acyl-CoA synthase (ACS) then either incorporated into monoacylglycerol (MAG), followed by canalization into di acyl glycerol (DAG) by monoacylglycerol acyltransferase (MGAT) and lastly to TGA by diacylglycerol acyl transferase (DGAT). When energy demands goes up, TAG, DAG and MAG are hydrolyzed respectively by adipose triglyceride lipase (AGTL), hormone-sensitive lipase (HSL), and mono acyl glycerol lipase (MGL). FA-CoA, either from TAG or plasma can then be shuttled to mitochondria by acyl-Coa protein (ACBP) and further transported into the matrix by carnitine palmitoyltransferase (CPT)1 and 2. Inside the mitochondria FA-CoA undergo β-oxidation to acyl-CoA which enters TCA cycle. The TCA cycle generates nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) which is further oxidized by the complexes of the ETC creating ATP. Under conditions of excess energy,

either from glucose or FA is converted to citrate and re-directed to cytosol from mitochondria. Citrate exert a negative feedback on glycolysis by inhibiting formation of pyruvate. Citrate is also synthesized to malonyl-CoA by ATP citrate lyase (ACL) and acetyl-CoA carboxylase (ACC), and act as a inhibitor of CPT1 reduce FA oxidation. When FA levels are elevated pyruvate dehydrogenase kinase 4 (PDK4) can be induced to suppress PDC and reduce glucose oxidation redirecting to FA storage. Image created with Biorender.com.

The ability of skeletal muscle to take up glucose in response to insulin is reduced with increased insulin resistance [48,49]. The mechanisms underlying insulin resistance is not fully elucidated, though, an increased level of plasma free fatty acids (FFA)s and TAGs, as well as hyperglycemia are proposed as causal factors [50-55]. Moreover, abnormal lipid metabolism with accumulation of lipotoxic intermediates and TAG and mitochondrial dysfunction in skeletal muscle have been linked to insulin resistance and diabetes [56]. However, the phenomenon of accumulation of intramyocellular triacylglycerol (IMTG) also occurs in insulin-sensitive endurance-trained athletes, termed the "athletes paradox" [57,58]. In these subjects increased IMTG positively correlates with insulin sensitivity and FA oxidation [57,59]. The lipid turnover, i.e., balance between storage and utilization, of IMTG in endurance trained athletes may help elucidate dysregulation of glucose and lipid metabolism in skeletal muscle [57,59,60].

1.3 Adipocytes

1.3.1 Adipocyte energy metabolism

Humans are considered to have two main types of metabolic fat tissue [61]. Besides white adipose tissue (WAT), which mainly is involved in storage, there is also thermogenic fat, namely brown adipose tissue (BAT) and beige adipose tissue (often referred to as brite, which stands for brown in white). These thermogenic fats are essential in maintenance of body temperature and energy metabolism [62]. WAT in lean, healthy individuals are confined to dedicated deposits. However, during conditions such as obesity WAT can increase ectopically, mostly within the visceral cavity [63]. The classical BAT, on the other hand, is located in dedicated BAT deposits while beige adipocytes are sporadically resided within WAT [62].

White adipose tissue

Human WAT consist of visceral adipose tissue (which is adipose tissue lining internal organs) and subcutaneous adipose tissue (which is the adipose tissue beneath the skin) [64]. The latter again compromises of a deep and a superficial subcutaneous adipose tissue compartment [64]. While visceral adipose tissue only accounts for 6-20 %, subcutaneous adipose tissue contributes with approximately 80-90 % of total adipose tissue, depending on sex, ethnicity and age specific differences [64].

Adipose tissue plays a role in lipid metabolism as well as circulation of glucose and FA. Most of the body's energy reserves are stored as TAG in WAT, which either comes from *de novo* lipogenesis or fatty acid uptake from plasma [65]. *De novo* lipogenesis is when fatty acid molecules arise from non-lipid substrates such as carbohydrates [66]. This process in humans mostly occurs in the liver, adipose tissue and mammary glands [66]. While most TAG originates from dietary sources, *de novo* lipogenesis is only considered a minor contributor [67], however, this may be more significant in individuals on high carbohydrate diet [67]. The pathway of *de novo* lipogenesis from glucose to fatty acids includes the uptake of glucose by glucose transporters followed by glycolysis to generate pyruvate. Pyruvate is converted into acetyl-CoA and via the TCA into citrate within the mitochondria [65]. Citrate exits the mitochondria and is converted back into the acetyl-CoA by ATP-citrate lysase (ACLY) [65]. Followed by carboxylation into malonyl-CoA by acetyl-CoA carboxylase (ACACA). Further malonyl-CoA is converted to palmitate by the rate limiting enzyme fatty acid synthase (FAS(N)). Palmitate is then in the end converted to different complex fatty acids, MAG, DAG and TAG [65]. However, in WAT the TAG deposits are mainly the uptake of pre-existing circulating TAGs in chylomicrons or very low density lipoproteins. These are then metabolized into FAs by lipoprotein lipase (LPL) outside of the endothelial lining as the particles are too large to penetrate the capillary lining. The FFAs are then taken up into the adipose tissue, converted and stored as TAGs. Hydrophobic fatty acids can diffuse across the cell membrane due to an intracellular drop in pH in cells exposed to fatty acids [68]. Long chain fatty acids are either passively diffused or transported by membrane proteins or lipid rafts [68]. Four proteins have been functionally linked to fatty acid transport in adipocytes:

1. Plasma membrane fatty acid binding protein (FABPpm)
2. Fatty acid translocase (FAT/CD36)
3. Caveolin-1
4. Fatty acyl CoA synthetases (FATP and ACSL) [69].

WAT is not only important for storage but also for FA availability by lipolysis generating substrate for β-oxidation and energy production in mitochondria [70]. The process of lipolysis is similar as in muscle cells and includes ATGL, HSL and MGL, hydrolyzing TAG, DAG and MAG respectively [69]. And β-oxidation is followed by TCA cycle and ATP production through the ETC complexes [71] (**Figure 4**).

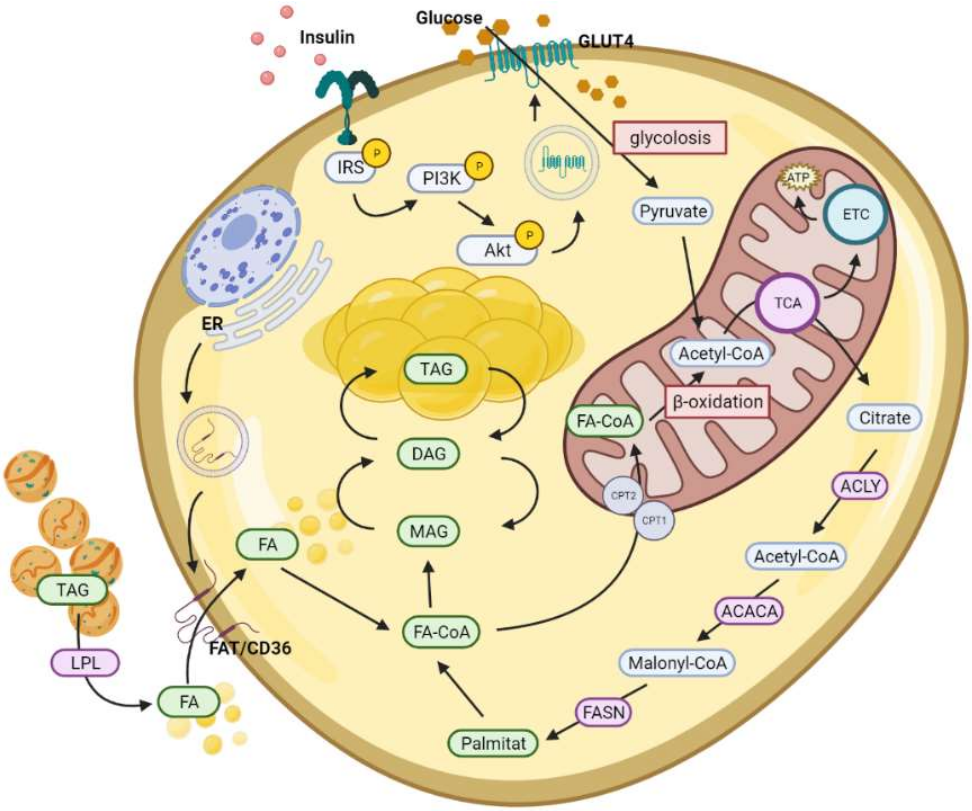

Figure 4. Energy metabolism in white adipose tissue. Insulin secreted from pancreatic β-cells induces glucose uptake into adipocytes via the insulin-dependent glucose transporter (GLUT) 4. Inside the adipocyte glucose is converted to pyruvate through glycolysis and further to acetyl-CoA. During the tricarboxylic acid (TCA) cycle in mitochondria, Acetyl-CoA turns in to citrate, which exits the mitochondria and turns back into Acetyl-CoA by ATP-citrate lysase (ACLY) and then to malonyl-CoA by acetyl-CoA carboxylase (ACACA). Via the fatty acid synthase enzyme malonyl-CoA is converted into palmitate. Palmitate is the converted to FA-CoA. FA-CoA also comes from FA acids taken up into the cells. Circulating TAG stored in chylomicron or very low-density lipoproteins (VLDL) is metabolized into FAs

by lipoprotein lipase (LPL) followed by uptake into the cell via the fatty acid transporter. Here it is converted to FA-CoA just as glucose through *de novo* lipogenesis. FA-CoA then either is converted into mono-, di- or tri- acylglycerol's for storages or sent to and taken up in mitochondria through carnityl transfecrace (TCA) 1 and 2, converted to acetyl-CoA through β-oxidation. Acetyl-CoA can also be oxidized to create energy through electron transport chain (ETC) after TCA. Image created with Biorender.com.

Brown (and brite) adipose tissue

Brown adipose tissue (BAT) was first reported in the 1950s and only thought to exist in infants [72], however, in 2009 several independent groups identified thermogenic adipose tissue also in adult humans [73,74]. Brown adipocytes are typically smaller than white with several small lipid droplets instead of one large [75]. Resent years has recognized adipose tissue as a highly metabolically active organ [76]. With the major function of BAT being non-shivering thermogenesis as a response to cold environments[77]. However, a high fat diet (HFD) might also activate a diet-induced thermogenesis in BAT [78]. BAT contain a larger number of mitochondria compared to WAT, brite adipose tissue is somewhere in between and is the reason for their extensive ability to produce heat. This occurs by uncoupled respiration, a process where unlike most cells, mitochondrial proton gradient can be released without ATP production in BAT [79]. Thermogenesis is mediated by uncoupler protein (UCP)1 [80]. As much as 2-5% of resting metabolic rate in humans can be contributed to active BAT [77]. And activation of BAT is as mentioned, triggered by cold or HFD. Inhibition of UCP1 when thermogenesis is not required, occurs by nucleotide binding to UCP1[79]. UCP1 has only been found in BAT and is therefore an ideal tissue-specific marker [77] (**Figure 5**).

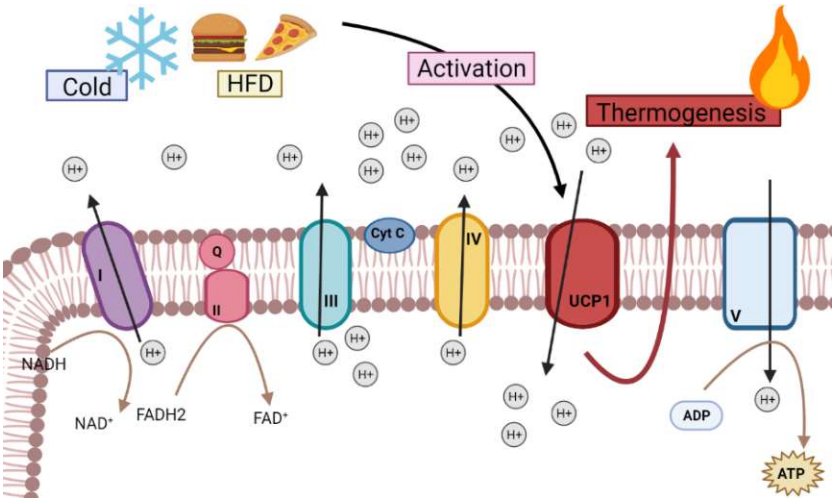

Figure 5 Thermogenesis in BAT. The electron transport chain (ETC) compromise of 4 enzyme complexes (I-IV), plus coenzyme Q (CoQ) and cytochrome C (CytC) which transfer electrons to create a proton gradient and produce adenosine triphosphate (ATP). Uncoupling of electron transport chain (ETC) by uncoupling protein (UCP)1 leads to free passage of electrons across the inner mitochondrial membrane which increases energy expenditure and lead to heat production [79]. Image created with Biorender.com.

1.3.2 Adipocytes and metabolic disease

Excess lipid storage and expansion of WAT is the primary contributor to obesity. During the development of obesity, adipose tissue expands by recruitment of new adipocytes (hyperplasia) or by increasing the size of adipocytes (hypertrophy) [63]. However the capacity to expand is not unlimited. Overexpansion of adipocytes may lead to inadequately handling of excess lipids and is associated with increased FA release that raises plasma FFA and results in lipotoxicity [81]. This can further result in visceral deposits of lipids as well as in ectopic sites, such as liver, skeletal muscle, and pancreatic β-cell [63,82]. Chronically elevated FFA can also cause insulin resistance in other tissues such as skeletal muscle and liver [83,84]. Excess adipose mass, i.e. obesity, is associated with low grade inflammation [85], and linked to T2DM and atherosclerosis [86]. It is now recognized that adipocytes function as an endocrine organ producing a variety of cytokines and bioactive peptides referred to as adipokines [87,88]. These are important for the dynamics of cross-talk between metabolic organs [85]. Adipokines released by adipocytes are several and diverse including: interleukin-6, adiponectin, resistin, angiotensinogen, plasminogen activator inhibitor-1, tumor necrosis factor and leptin [12]. Imbalance of these adipokines is connected to disorders of chronic inflammation, insulin resistance and type 2 diabetes.

BAT, however, is inversely correlated to BMI and increased energy expenditure [89]. Animal models on HFD show that BAT transplantation increases glucose tolerance and insulin sensitivity as well as reducing adipocyte tissue inflammation [90]. The inflammatory profile of macrophages is enhanced by WAT and impaired by BAT, suggesting that BAT is less prone to tissue inflammation associated with obesity [91]. Therefore, in obese individuals recruitment and reactivation of BAT could be of significant importance to reduce inflammation and increase energy expenditure [77].

1.4 SENP2 and SUMOylation of PPAR

Peroxisome proliferated receptors (PPARs) are members of a nuclear receptor family consisting of three isoforms: PPAR-alpha (α), -delta/beta (δ/β) and -gamma (γ) [92]. The PPARs show different tissue distribution yet all play an important role in lipid metabolism and glucose homeostasis [93]. PPARα is predominantly expressed in the liver and has been demonstrated to play a central role in lipid metabolism, decreasing dyslipidemia associated with metabolic syndrome [94,95]. During fasting, PPARα is activated by adipose-derived FAs enhancing FA oxidation in the liver [96]. PPARδ enhances fatty acid oxidation and energy consumption in skeletal muscles and contributes to amendment in muscle fiber type to a more oxidative fiber type [97]. PPARγ which is mainly expressed in adipose tissue, though also seen in muscle and liver tissue, has an important role in increasing insulin sensitivity in these tissues [98-101]. PPARs work as a heterodimer together with the retinoid X receptor (RXR), binding to the specific DNA response element peroxisome proliferators response elements (PPREs) located in the promotor of target genes [102].

PPARs are also known targets for SUMOylation (SUMO = small ubiquitin related modifier) [103,104], which is a post-translational protein modification system with reversible activity [105.] SUMOs covalently bind to target proteins and alter their function, location and or half-life [106]. There are five known family members of SUMOs identified in vertebrates, SUMO-1-3, are well characterized and ubiquitously expressed in humans [107,108]. The two latter SUMO-4 and -5 are restricted to specific tissues and their proof of function still needs to be confirmed [109,110] . Though SUMO is a ubiquitin-related modifier, it has a much larger range of action than the tagging of proteins for protesomal degradation that ubiquitin does [108.]. SUMOs are involved in several disease processes including cancer, cardiomyopathies and neurodegenerative disorders such as Huntington's disease [108]. The different PPARs are prone to many different SUMO modifications,

occurring at specific sites and associated with inhibition of activity [111]. However, there is still a lot to be elucidated [111].

Once SUMOylated, PPARs are also a target for desumoylation by a SUMO/sentrine-specific protease (SENP), especially SENP2[112]. The SENPs are a family of cysteine proteases that desumoylate different target proteins [113] and the six members of the family include SENP1-3, and SENP5-7 [113]. The SENPs regulate different cellular pathways and play important roles in numerous processes in the body by maturing and desumoylating SUMOs [114-117]. SENP2 is of special interest as it increases the activity of PPARδ and PPARγ by desumoylation [118,119]. Do Koo *et al.* described how increased SENP2 expression in skeletal muscle cells leads to increased PPARδ activity and subsequently PPARδ target genes, such as the regulator of fatty acid oxidation, carnitine palmitoyl transferase 1B (CPT1B) and the insulin sensitivity and lipid content mediator Acyl-CoA synthease long chain family member 1 (ACSL1) [119]. Further they described how these PPARδ target genes lead to increased fatty acid oxidation in the mitochondria [119]. These findings identified SENP2 as an important regulator of fatty acid metabolism in skeletal muscle. Later Do Koo *et al*. went on to describe how the adipokine leptin (which regulates food intake and energy expenditure [120,121]) caused a gradual dose-dependent increase in SENP2 mRNA and protein levels in C2C12 myotubes [122]. He showed how leptin increased the PPARD and PPARG mobilization to the promotor leading to rapid and prolonged increase in fatty acid oxidation (FAO)-related genes. However, suppression of SENP2 through KD diminished the prolonged leptin-induced expression of FAO– related genes, though the rapid FAO response was unchanged [122]. Leptin is secreted from adipocytes and plays an important role in the utilization shift between carbohydrates and FA during starvation[123]. Leptin also acts directly by regulating gluconeogenesis in the liver and insulin sensitivity by increasing energy expenditure in skeletal muscle [124,125].

These findings made it clear that factors or substances from adipocytes can regulate the SENP2 expression in myotubes. Additionally it has become evident that the SENP2 also has metabolic impact in adipocytes [122].

Chung *et al*. established a critical role of SENP2 in adipogenesis by desumoylation and stabilization of C/EBPβ thereby promoting expression of PPARγ which is important for mature adipocyte differentiation [126]. Subsequently, two individual groups explored the impact of SENP2 on energy metabolism by creating an adipocyte-specific *Senp2* knock out mice (*Senp2*-aKO), with

somewhat conflicting results. Both groups found that their *Senp2*-aKO mice had reduced adipose lipid storage/accumulation compared to the control. However, while Lee *et al*. found that the SENP2-KO enhanced formation of beige adipocytes from WAT, in the absence of cold exposure or treatment with a browning inducer, and concluded that SENP2-Ko had a beneficial metabolic effect in the adipocytes [127]. Zheng et al. found that the SENP2-KO induced insulin resistance in mice fed on HFD, and that SENP2 protects against lipotoxicity during obesity [128]. The two studies used different knockout (KO) approaches, which may explain the somewhat contradicting results and conclusions. *Zheng et al*. deleted exon 13 and 14 in the C-terminal region, whereas *Lee et al*. deleted exon 3 as they believed the KO-methodology by Zheng et al. could have allowed production of truncated SENP2. Though these studies have all been conducted in mice, they still implicate a the importance of SENP2 for metabolism (**Figure 6**).

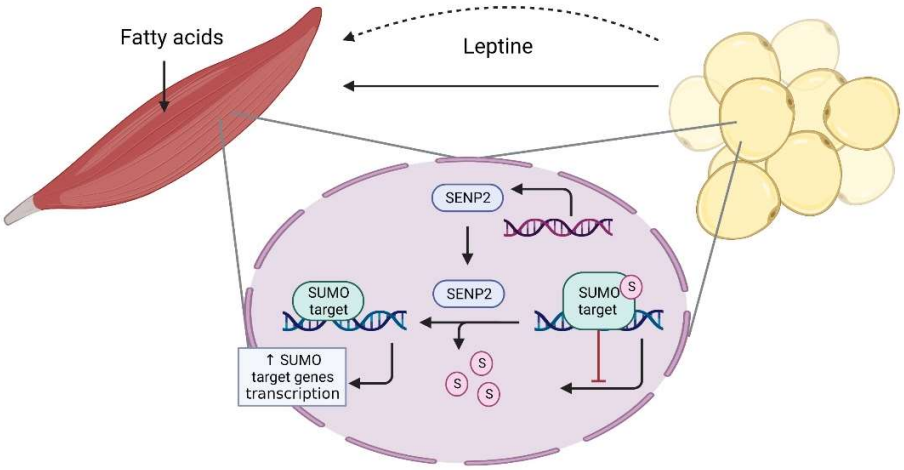

Figure 6: Desumoylation of SUMO target by SENP2. According to literature small ubiquitin-related modifier (SUMO)s inhibits the activity of a range of target genes, including PPARs. SUMO/sentrin specific protease (SENP)2 desumoylate SUMOs from SUMO targets and increases gene expression of SUMO targets. Several factors can impact the degree of SENP2 quantity in muscle, fatty acids and leptin exposure are proposed. Image created with Biorender.com.

1.5 Pancreatic cancer cells

1.5.1 Pancreas

The pancreas plays a key role in metabolism and energy homeostasis by releasing various digestive enzymes and hormones [129], an overview over endocrine and exocrine pancreas (**Figure 7**).

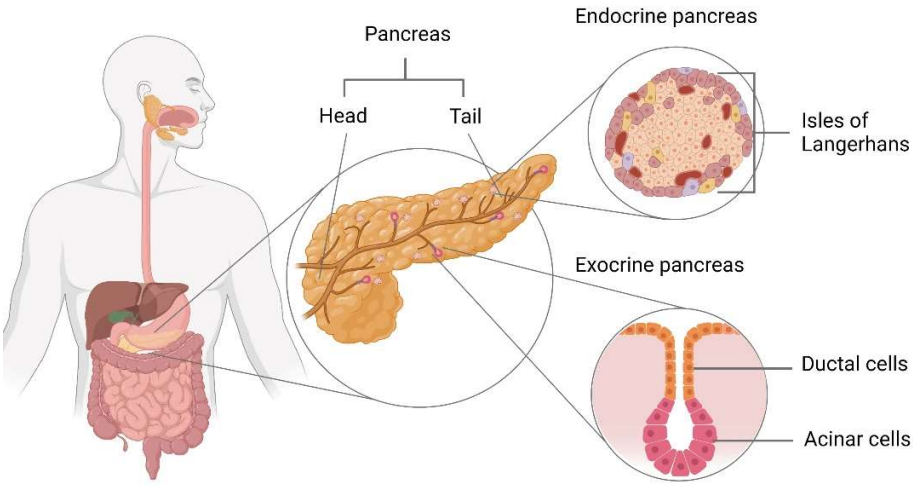

Figure 7: Anatomy of pancreas. Pancreas is located in the stomach adjacent to the duodenum. The pancreas consists of several types of cells such as the endocrine cells found in the isles of Langerhans, the exocrine acinar cells as well as non-secretory cells such as pancreatic ductal cells. Image created with Biorender.com.

The islets of Langerhans are clusters of cells in the pancreas consisting of several different cell types. These cells release different hormones, such as: glucagon-producing α-cells [130], C-peptide- and insulin-producing β-cells [130], pancreatic polypeptide-producing γ-cells [131] and somatostatin-producing δ-cells [130]. These hormones, especially glucagon and insulin allows the islets of Langerhans to maintain blood glucose levels between 4-6 mM [129]. In between meals the blood glucose levels goes down and glucagon is released from the α-cells to promote glycogenolysis in the liver which increases blood glucose levels [132]. Contrary, after a meal insulin is secreted from β-cells due to elevated exogenous glucose levels [133]. Insulin promotes glycogenesis [134], lipogenesis [135] and incorporation of amino acids into protein [136] (**Figure 8**). The insulin enhances glucose uptake in muscle and fat tissue by the insulin dependent GLUT4 [137], described in detail under muscle and fat cell energy metabolism.

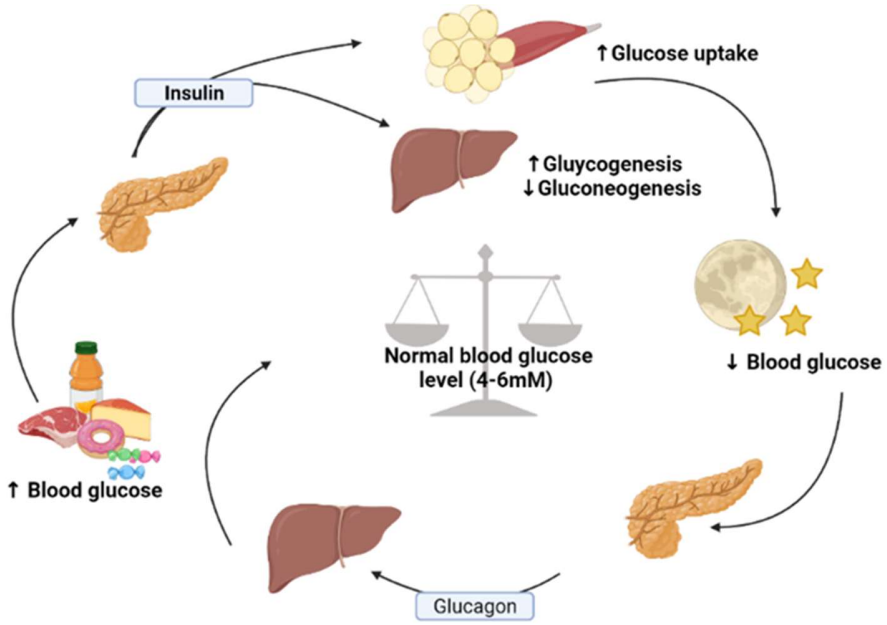

Figure 8. The glucagon/insulin blood glucose regulation. After a meal blood glucose levels goes up and insulin is secreted from the pancreatic β-cells which leads muscle and fat to take up more glucose and decreasing gluconeogenesis and increasing glycogenesis in the liver to maintain normal blood glucose levels. In between meals, during fasting, the blood glucose levels goes down triggering release of glucagon from the pancreatic α-cell which works on the liver to increase glycogenolysis and gluconeogenesis and again restore normal blood glucose levels. Image created with Biorender.com.

Secretion from the exocrine pancreas is important for digestion of a meals components making them available for processing and absorption by the intestinal epithelium [138]. Exocrine pancreas is the source of several enzymes that are part of the iso-osmotic, alkaline pancreatic juice [139]. While goblet cells secrete the mucus, the acinar cells secrete the digestive enzymes. These include the proteolytic enzymes such as trypsin, chymotrypsin, carboxypeptidase, and elastase which digest proteins. The pancreatic lipolytic enzymes are lipase, phospholipase, and esterase which digest fats. The glycolytic (carbohydrate digesting) enzymes are lactase and amylase, which breaks down starch into maltose, maltotriose, and dextrins. And the nucleolytic enzymes include ribonuclease and deoxyribonuclease, which break down nucleic acids into mono- and oligonucleotides [139]. The enzymes are finally secreted into the pancreatic duct which transports the digestive enzymes into the duodenum[129]. Just before the duodenum of the small intestine, the pancreatic duct meets up with bile duct coming from the gall bladder and subsequent release of all the contents to process the food [129]. These pancreatic duct cells are the primary site of cancer in the pancreas [140]. Pancreatic duct adenocarcinoma constitutes 90% of all pancreatic cancers [140].

1.5.2 (Pancreatic) cancer cells and metabolic disease

Cancer is a generic term for a large group of diseases that can affect any part of the body. According to the WHO about 30-50% of cancers can be prevented by avoiding risk factors such as tobacco and alcohol and implementation of a healthy diet and exercise [141]. A healthy cell goes through several steps in the process of malignancy: Initiation, promotion and progression, and factors that can affect these steps can be associated with the cancer [142]. Epidemiological studies have shown an increased risk of several forms of cancer in individuals with type 2 diabetes [143,144]. Diabetes may influence the malignancy process by hyperinsulinemia, hyperglycemia and chronic inflammation leading to secondary effects such as increased bioavailable sex hormones and inflammatory cytokines which may contribute to the cancer progression, however, there are still many unknowns [142]. Individuals with diabetes have a two-fold greater relative risk of pancreatic cancer compared to individuals without diabetes [144], the increased risk of pancreatic cancer in diabetic patients has been demonstrated by several studies [145]. The reason is thought to be due to hyperglycemia caused by decreased insulin activity, low peripheral glucose uptake and changes in insulin signaling in T2DM patients, which can cause cancer [146,147]. However, pancreatic cancer has also been linked to the onset of diabetes [148]. It has been suggested that pancreatic cancer cells products can alter the metabolism in muscle and induce peripheral insulin resistance [149]. As many as 80% of pancreatic cancer patients have impaired glucose tolerance or new onset of type 2 diabetes at the time of diagnosis [150]. As surgical removal of the tumor improves the insulin resistance in pancreatic cancer patients [151], insulin resistance is believed to be caused by the presence of the pancreatic tumor itself rather than being a risk factor for developing cancer [152,153]. Case control studies have revealed that 25-50% of patients diagnosed with pancreatic cancer had developed T2DM 1-3 years before being diagnosed with cancer [154], indicating that T2DM could potentially be seen as an early sign or marker for pancreatic cancer [155,156].

The majority of cancer cells express insulin-like growth factor (IGF), which is a growth promoting hormone that increases cell proliferation and glucose use [157,158]. The hyperglycemia and increased insulin binding IGFR leads to activation of Mitogen-activated protein (MAPK) and PI3K/Akt/(mammalian target of rapamycine (mTOR) pathways which promotes cell proliferation and decreases apoptosis in these patients [155]. This partly explains the link between hyperglycemia, T2DM and the increased cancer risk[155].

1.6 Crosstalk pancreatic cancer cells and myotubes

Communication between cells in a multicellular organism is highly important for coordination of their functions and development. The communication is often via soluble factors such as growth factors, chemokines, cytokines and neurotransmitters [159]. Both skeletal muscle and adipose tissue produce and secret cytokines and other proteins [85,160]. These factors when secreted from skeletal muscle are termed myokines [161-163], and when secreted by adipose tissue are referred to as adipokines [87,88].

Metabolic alterations in cancer cells are as previously described a well-known phenomenon. However, it has also been suggested that secreted peptides from pancreatic cancer cells can alter the energy metabolism in muscle [149,150]. Metabolic changes have been seen to occur in other tissues other than the tissue containing the cancer itself. As one of the main regulators of glucose and lipids in the human body and a reservoir of amino acid storage, skeletal muscle has a profound impact on whole-body homeostasis. As previously described, the pancreatic cancer risk is increased by T2DM as well as linked to the onset of T2DM, and in the latter thought to be induced by secreted factors from the cancer. This will not be further discussed here.

1.7 Cachexia and pancreatic cancer

Another typical feature of cancer is cachexia (muscle wasting), especially seen in striated muscle [164]. Cachexia is also caused by substances secreted from, or other types of interactions with, the tumor, which leads to increased basal metabolic rates and energy expenditure as well as loss of skeletal muscle mass due to an imbalance in protein synthesis and degradation [165,166]. Cachexia is found to occur in more than half of all patients with cancer and is responsible for more than 20% of cancer related deaths [166]. An even higher incidence of cachexia is seen with pancreatic cancer compared to other cancers [166]. As reviewed by Fearon *et al*. cancer cachexia is divided into three clinical stages. First stage is pre cachexia, described as a weight loss of no more than 5% over 6 months with anorexia and metabolic changes [167]. Second stage cachexia, is when the weight loss increases over 5% or BMI is under 20, often also seen is reduced food intake and systemic inflammation [167]. For the third and last stage there is refractory cachexia, the degree of cachexia can vary. The cancer disease at this stage is both pro catabolic and not responsive to treatment, and

the expected survival is less than three months [167]. The three stages of cachexia are illustrated in **Figure 9**.

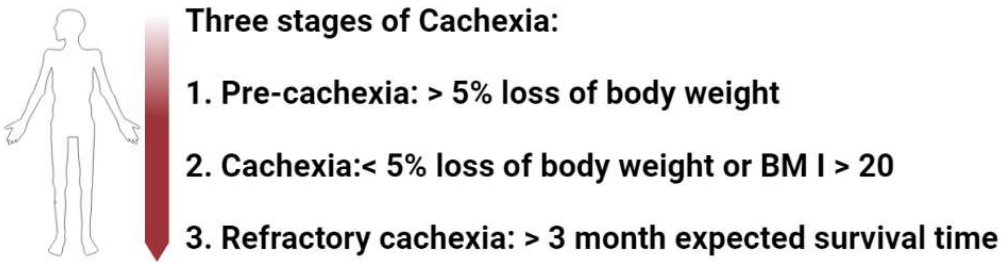

Figure 9. The tree stages of cachexia. Cancer cachexia proceeds through three stages: 1. pre-cachexia defined as a weight loss of no more than 5% of body weight over 6 months. 2. Then follows cachexia where weight loss has proceeded 5% or body mass index (BMI) is under 20. 3. The last stage is refractory cachexia where the patients no longer responds to treatment and the expected survival is less than three months. Image created with Biorender.com.

2. Aims

The overall aim of this thesis was to study regulation of energy metabolism in skeletal muscle, adipocytes and pancreatic cancer cells, as well as the interplay between and within these cells. Specifically, by comparison of impact of conditioning of skeletal muscle cells with media from pancreatic cancerous or primary cells and knocking down genes of specific metabolic interest.

More specific the objective of the different studies were:

1. Exploring the SUMO/sentrin specific protease 2 (SENP2)'s metabolic regulation in human skeletal muscle cells, by using a lentiviral knockdown technique **(paper I)**.

2. Exploring the SUMO/sentrin specific protease 2 (SENP2)'s metabolic regulation in human fat cells, by using a lentiviral knockdown technique **(paper II)**.

3. Study metabolic regulation and energy metabolism in a pancreatic cancer cell line PANC-1 compared to a primary pancreatic cell hPEC and investigate the impact of conditioning human skeletal muscle cells with media from a pancreatic cancer cell line PANC-1 compared to primary pancreatic cells hPEC **(paper III)**.

4. Investigate the impact of conditioning human skeletal muscle cells with media from a pancreatic cancer cell line PANC-1 compared to media from primary pancreatic cells hPEC **(paper IV)**.

3. Summary of papers

PAPER I: *SENP2 is vital for optimal insulin signaling and insulin-stimulated glycogen synthesis in human skeletal muscle cells.*

In this paper the aim was to study the metabolic implications of knocking down the SENP2 gene in primary human skeletal muscle cells. The impact of SENP2 on fatty acid and glucose metabolism as well as insulin sensitivity in human skeletal muscle was investigated, using cultured primary human myotubes. Acute (4 h) oleic acid oxidation was reduced in SENP2-knockdown (SENP2-KD) cells compared to control cells, with no difference in oleic acid uptake. After pre-labeling (24 h) with oleic acid, total lipid content and incorporation of oleic acid into TAG was decreased, while incorporation into other lipids, as well as complete oxidation and β-oxidation of oleic acid was increased in SENP2-KD cells. Basal glucose uptake (i.e., not under insulin-stimulated conditions) was higher in SENP2-KD cells, whereas oxidation of glucose was similar to control myotubes. Further, basal glycogen synthesis was not significantly different in SENP2-KD myotubes, but both insulin-stimulated glycogen synthesis and AktSer473 phosphorylation was completely blunted in SENP2-KD cells.

In conclusion, SENP2 seems to play an important role in fatty acid and glucose metabolism in human myotubes. Interestingly, SENP2 also appears to have a pivotal role in regulating myotube insulin sensitivity, main findings are summarized in figure 10.

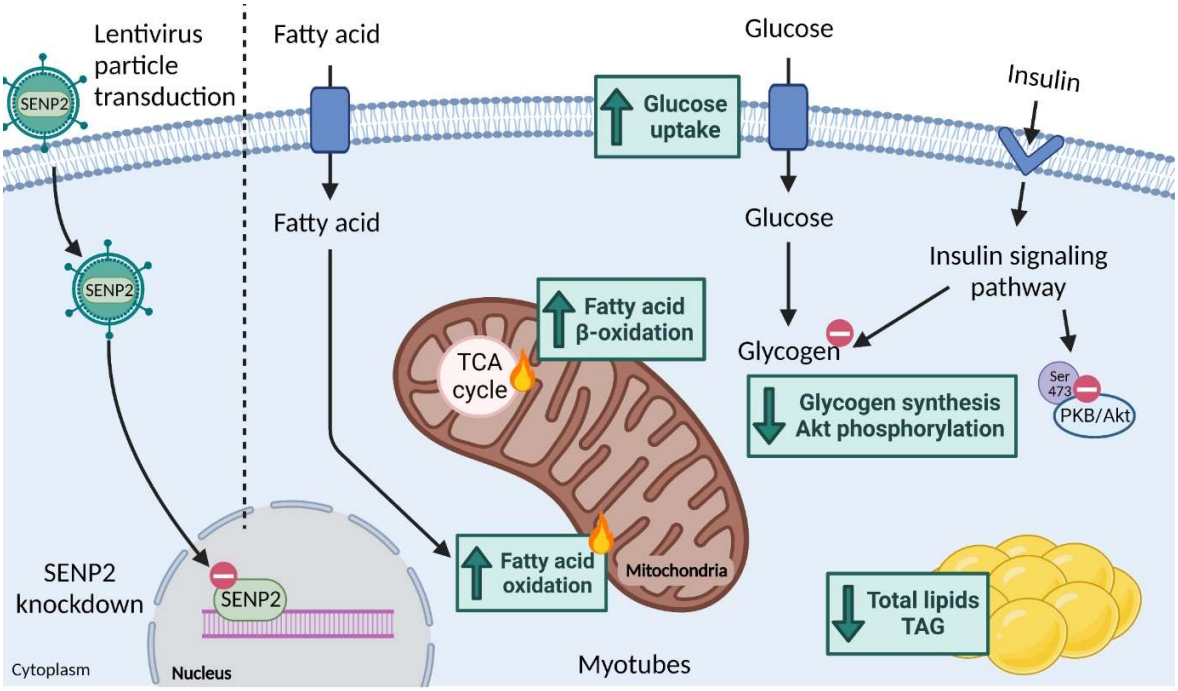

Figure 10. Summary of metabolic differences and gene expression in SENP2-KD myotubes compared to control myotubes. Knockdown of SENP2 mRNA led to increased fatty acid (FA) oxidation and β- oxidation, while total lipid and triacylglycerol (TAG) was decreased. Glucose uptake was increased after knockdown of SUMO/sentrin specific protease (SENP)2 though insulin-induced glycogen synthesis and Akt phosphorylation was blunted.

PAPER II: *SENP2 is an important regulator of energy metabolism in adipocytes and knockdown increases markers of browning.*

In this study we investigated the impact of SENP2 on fatty acid and glucose metabolism in primary adipocytes. We used a lentiviral, sh-RNA approach to knock down the SENP2 gene in the adipocytes. Approximately 60% KD was confirmed at gene level though differentiation markers where sustained. KD of SENP2 led to decreased glucose uptake and oxidation. The accumulation and distribution of oleic acid into complex lipids was also decreased in SENP2-KD cells, while oleic acid oxidation was increased compared to control cells. Furthermore, *de novo* lipogenesis and incorporation into TAG was reduced in SENP2-knockdown cells. Expression of metabolically important regulators was increased, and proteomics data revealed many positively regulated proteins related to mitochondrial function and several markers of browning.
In conclusion, SENP2 seems to be an important regulator of energy metabolism in primary human adipocytes and the results also indicate a potential role of SENP2 in adipocyte browning, main findings are summarized in figure 11.

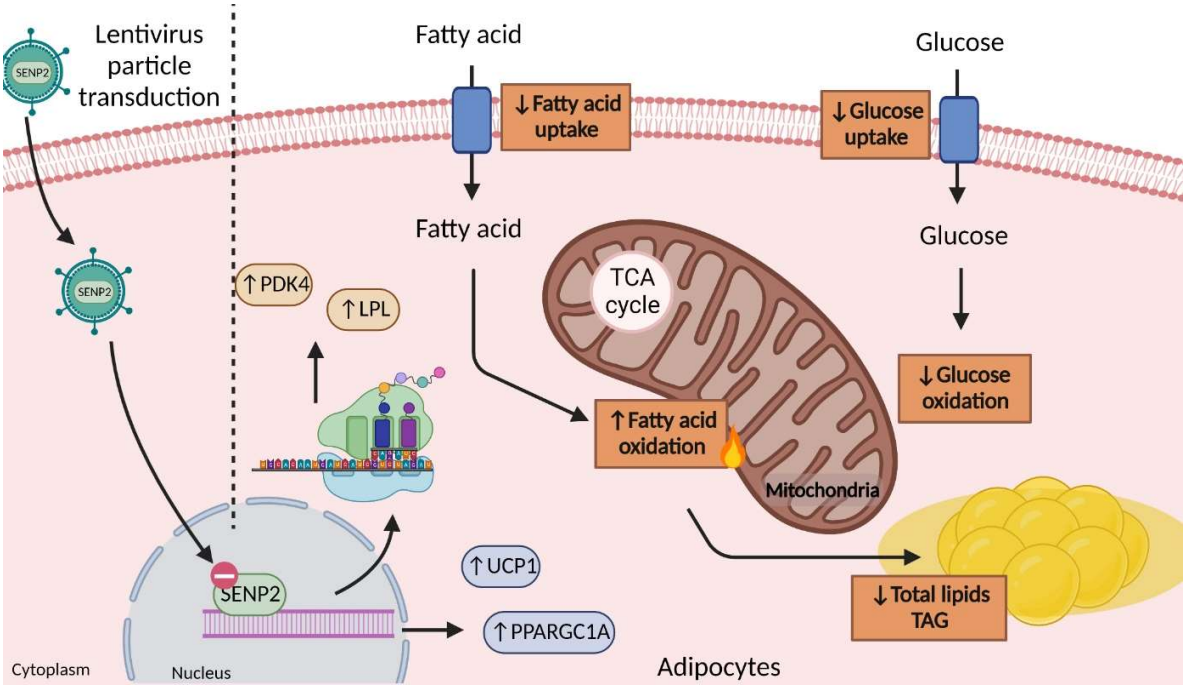

Figure 11. Summary of metabolic differences and gene expression in SENP2-KD adipocytes compared to control adipocytes. Knockdown (KD) of SUMO/sentrin specific protease (SENP2) mRNA led to decreased fatty acid uptake and total lipid and triacylglycerol (TAG) incorporation, while fatty acid oxidation was increased. Glucose uptake and oxidation was decreased by SENP2 KD while *UCP1* and *PARGAC1A* mRNA expression and PDK4 and LPL protein expression was increased.

PAPER III: *Pancreatic cancer cells show lower oleic acid oxidation, and their conditioned medium inhibits oleic acid oxidation in myotubes.*

Herein we explored the metabolic differences between a pancreatic cancer cell line (PANC-1) and primary pancreatic cells (hPEC). Metabolic alterations are well known to occur in cancer cells and are important mediators of the cancer course. Using radioactive labeled substrates, qPCR and proteomics, we examined differences between the two cell types in regard to uptake and consumption of different substrates (fatty acid, glucose and lactic acid), gene expression and release of substances. Glucose oxidation and oleic acid uptake was higher, while oleic acid oxidation and reserve capacity was lower in PANC-1 compared to hPEC. Measurements of glucose and lactic acid concentrations in harvested media from the two cell types revealed that PANC-1 took up more glucose and released more lactic acid compared to hPEC. The differences in energy metabolism were reflected in gene expressions of transporters and nuclear receptors as well as pathway analysis of the secretome.

In conclusion, metabolic characterization of PANC-1 cells revealed a more glycolytic phenotype compared to hPEC, with lower oleic acid oxidation and higher secretion of proteins into the media that also reduced oleic acid oxidation in myotubes, main findings are summarized in figure 12.

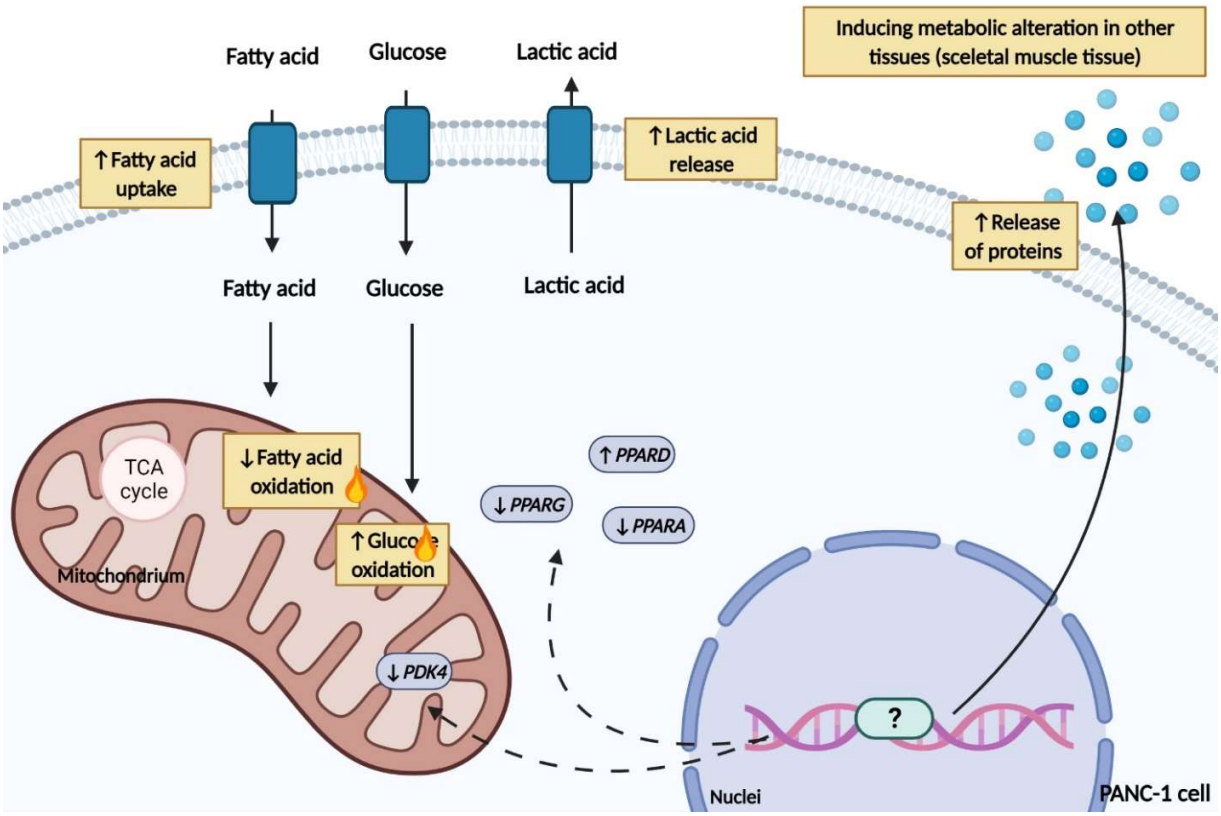

Figure 12. Energy metabolism in PANC-1 compared to hPEC cells. Pancreatic cancer cell line (PANC- 1) cells had higher fatty acid (FA) uptake and higher FA and glucose oxidation as well as higher lactic acid release compared to human pancreatic epithelial cell (hPEC) cells. PANC-1 cells also had higher transcription of several metabolically relevant genes including *PDK4*, *PPARA*, *PPARD* and *PPARG* and a higher release of protein inducing metabolism in other tissues.

PAPER IV: *Human myotubes exhibit insulin resistant and cachexic traits after conditioning with media from pancreatic cancer cells.*

Myotubes conditioned with media from a pancreatic cancer cell line, PANC-1, and a primary human pancreatic cell, hPEC, was used to explore the tissue crosstalk and impact of pancreatic cancer cells on muscle cell metabolism of protein, glucose, and lipids. We found that the PANC-1-conditioned myotubes had an imbalance in protein turnover resulting in a total decrease of protein content compared to myotubes exposed to conditioned medium from hPEC-1 cells. Decreased glucose uptake and blunted insulin-stimulated glycogen synthesis was also observed in PANC-1 conditioned cells. Furthermore, lipid uptake was increased whereas fatty acid oxidation was similar, leading to accumulation of intracellular lipids in the muscle cells conditioned with PANC-1 media. These findings were supported by gene expression and secretome analyses.

In conclusion, pancreatic cancer cells produce and release peptides that act on skeletal muscle tissue and affect their metabolism and protein turnover in a way resembling cachexia, main findings are summarized in figure 13.

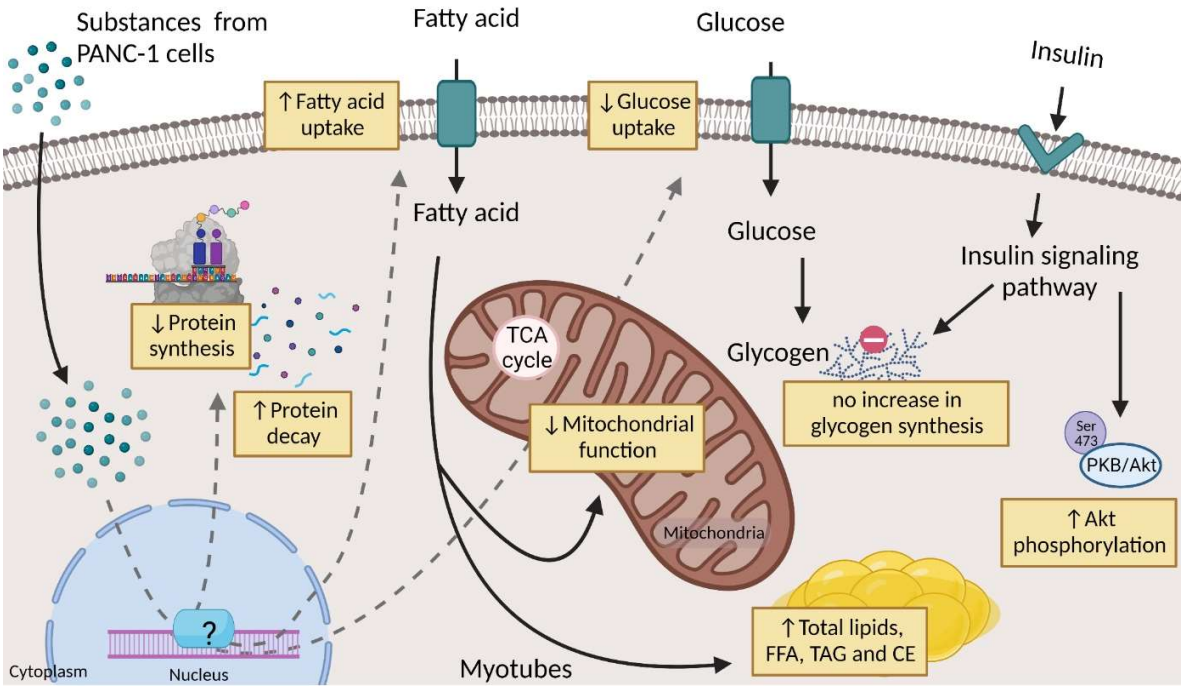

Figure 13. Energy metabolism in myotubes conditioned with media from PANC-1 and hPEC cells. Conditioning myotubes with media from pancreatic cancer cell line (PANC-1) cells induced several metabolic differences including decreased protein synthesis and increased protein decay compared to human pancreatic epithelial cell (hPEC) conditioned myotubes. Also increased fatty acid (FA) uptake and total lipid storage and decreased mitochondrial function was found in PANC-1-conditioned myotubes. Glucose uptake was also decreased in PANC-1 conditioned-cells, and there was no effect of insulin treatment on glycogen synthesis, although Akt phosphorylation was increased.

4. Methodological considerations

4.1 Cultured cells as an in vitro model

Human cell cultures are important models for *in vitro* studies of cellular functions. They allow for controlled environments that are free of systemic variation, and can be used for the study of basic cell biology, interaction of drugs and other chemicals with the cell and to perform genetic manipulations otherwise not possible or allowed in *in vivo* human studies [168].

4.1.1. Cultured primary human myotubes

Satellite cells are progenitors of skeletal muscle fibers allowing for regeneration after an injury [169,170]. They can be differentiated into primary human myotubes to create a model system for intact human skeletal muscle. The human muscle cells used in this thesis were obtained from *m. vastus lateralis* (**paper I and IV**). The isolation of satellite cells from all biopsies was performed at the same location and by the same trained personnel. Multinucleated myotubes were established by activation of satellite cells, proliferation of myoblasts and differentiation into myotubes based on the method from Henry *et al.* [171], modified according to Gaster *et al*. and Bourlier *et al*. [172,173]. Myotubes are preferred over myoblast for experimental use as key proteins for glucose [174], and lipid [175], metabolism is increased, and resembles adult skeletal muscle [176]. Human myotubes also have the most relevant genetic background, as opposed to rodent cell cultures [31].

Generally, cultured primary cells are characterized by low mitochondrial oxidative capacity with a preference for carbohydrates over lipids as fuel source [82]. In cultured human myotubes the ratio of GLUT1:GLUT4 tends to be higher compared to adult skeletal muscle [82,177], which results in lower insulin responsiveness on glucose transport [174,178]. Typically, insulin increases glucose uptake by 40-50% in myotubes [179,180]. However, despite the reduced insulin-responsiveness, the mechanisms involved in glucose uptake *in vivo* are conserved *in vitro*. [174]. Several characteristics of the *in vivo* phenotype are conserved in culture, for example, the diabetic phenotype is conserved in cultured myotubes [181,182]. Biopsies from T2DM exhibit altered glucose [171,181], and lipid [183-185] metabolism in line with characteristics of obesity and T2D. Also preserved in cultured myotubes is ability of the skeletal muscle to switch between energy sources such as glucose and FA, called metabolic switching [186]. The oxidative capacity of skeletal muscle *in vivo* depends on fiber type composition, where the slow type I fibers are more oxidative and the fast type II fibers more glycolytic. However, in regards to muscle fiber type *in vitro*, it has been demonstrated that cultured

myotubes co-express both MHC isoforms regardless whether the source of the biopsy was fast or slow twitch single muscle fibers [187]. The precise mechanism by which skeletal muscle cells are able to retain the *in vivo* characteristic are not known. However, a combination of genetic and epigenetic mechanism are likely to be involved, as reviewed in [188]. Though several traits seems to be intrinsic and conserved *in vitro* it has been mentioned that the ability of the myoblast to fuse and differentiate into myotubes as well as metabolic processes such as fuel uptake, glycogen synthesis, glucose and FA oxidation gradually can be impaired with increased passaging of the muscle cells [189]. Therefore, experiments performed in this thesis where from passages that have normal responses (here passage 3 and 4). Altogether, though some limitations, the cell model used in this thesis appear to be valuable for studying skeletal muscle metabolism, both after knockdown (KD) of genes or conditioning with medium from other cells. It has the most relevant genetic background and represent the best available alternative system to intact skeletal muscle that can be used to study human disease [171,188,190].

4.1.2. Cultured primary human adipocytes

Primary pre-adipocytes has been successfully cultured from several species including humans [191]. Human pre-adipocytes (human adipose-derived stromal/stem cells (hASC)) allows for differentiation into adipocytes using a differentiation cocktail and is used as an *in vitro* model system for *in vivo* adipose tissue [192]. The pre-adipocytes used in this theses was obtained from the abdominal region of female patients during elective surgery at the Shalgrenska hospital in Gøteborg. All biopsies was performed at the same location and by the same trained personnel. We used a standard differentiation cocktail mix of dexamethasone, insulin and 3-isobutyl-1-methylxanthine (IBMX), commonly abbreviated MDI [193], and triiodothyronine (T3) and rosiglitazone which are PPAR regulators and improves the degree of differentiation [192,194].

Differentiated mature adipocytes (MA) is another option for studying adipose tissue in culture. MAs share many features with hASC, however, they are terminally differentiated and cannot be further expanded after isolation [195], they are also more difficult to handle and unable to adhere to culture surfaces [196]. The use of mature adipocytes is also associated with rapid phenotypic loss and dedifferentiation into fibroblast like cells [197]. This taken together excluded the use of MAs for our experiments.

When comparing primary cells isolated from adipose tissue to established cell lines, different developmental patterns has been observed [198]. Several studies have shown that endocrine and metabolic stress during preadipocyte development *in vivo* can increase disease risk later in life, reviewed in [199]. To mimic the normal exposure occurring *in vivo,* the *in vitro* model system of differentiation of hASC can be modified by media cocktails and extracellular matrices [200,201]. When the proteome from hASC cell lysate was compared to proteome from adipocytes, it was found that 72% aligned with proteins identified in human or rodent adipocytes, or adipose tissue [202]. However, 20-25% of the hASC secretome was uniquely identified proteins [202]. Therefore, some discrepancies are definitely to be expected. Also, primary pre-adipocytes cultured *in vitro* reflect immature WAT where lipid accumulation occur in small multilocular lipid droplets (LD), not reflecting the mature WAT *in vivo*, which contain a large unilocular LD [203]. Hence, LD dynamics may be limited in 2D cultures [203], however, in our experiments LD dynamic has only been explored in a before and after manner, where the differences seen reflects the impact of the intervention. Despite limitations, cultured primary human adipocytes as an *in vitro* models for adipose tissue can give valuable information about lipid and glucose metabolism, as well as gene and protein expression patterns related to browning [203]. Phenotypic and transcriptional evaluation have demonstrated that the main basic properties are kept at least for 6 passages [204], and the experiments performed in this thesis where from passages 1 and 2.

Donor characteristics in primary human myotubes and adipocytes

Several factors can affect energy metabolism *in vivo* including age, BMI, gender and exercise. For example, increased age *in vivo* has been associated with reduced skeletal muscle mass [205], impaired insulin sensitivity [206,207] and ectopic lipid accumulation [207,208], in addition to decreased mitochondrial content and function [209]. Metabolic processes in cultured myotubes have also been found to be affected by BMI [205,210]. Likewise, defects in fat cell metabolism have been seen under obesity [211], including decreased mitochondrial function [212]. These obesity-related disturbances on cellular metabolism are also found to be preserved in differentiated adipocytes [213]. Another major source in body composition, hormonal regulation and physiological function is gender [179,214], however, gender differences does not appear to be present in cultured myotubes, unless cultured in the presence of sex hormones [215,216]. For cultured adipocytes, on the other hand, metabolic gender differences seems to be kept in preadipocytes, though abolished in *in vitro* differentiated adipocytes [213]. Cultured myotubes and adipocytes used in this thesis were established from

relatively homogenous cohorts. For myotubes, only biopsies from male donors were used, the age varied between 44-49 years and BMI between 21-27.9 kg/m^2 (Table 1), while for adipocytes only biopsies from female donors where used, the age varied from 39-66 years and BMI 24.1-34.2 kg/m^2 (Table 2). Though possible variation due to age, gender and BMI, these where not features that where compared in this thesis.

Table 1. Summary of donor characteristics for myotubes used in paper I, III and IV. Range of values for selected features of the donors. BMI, body mass index.

Subject	Gender	Age, years	BMI, kg/m2
1	Male	40	23.7
2	Male	42	25.0
3	Male	49	27.9
4	Male	47	26.9
5	Male	47	26.6
6	Male	48	25.0
7	Male	55	21.0
8	Male	48	25.0
9	Male	47	22.2
10	Male	44	25.8
11	Male	49	27.0
12	Male	47	26.6

Table 2. Summary of donor characteristics for adipocytes used in paper II. Range of values for selected features of the donors. BMI, body mass index.

Subject	Gender	Age, years	BMI, kg/m^2
1	Female	57	25.8
2	Female	49	26.4
3	Female	54	24.1
4	Female	66	26.1
5	Female	39	34.2

4.1.3 Cultured pancreatic cells

The human pancreatic cells used in this thesis include a human pancreatic cancer cell line PANC-1 and primary, human pancreatic cells (hPEC) (**paper III-IV)**.

PANC-1 is a cancer cell line originated from a pancreatic-duodenectomy specimen removed from a 56-year-old Caucasian male [217]. The cell line was establishment in 1975, and believed to be the first continues tumor-cell line of pancreatic origin [217]. The PANC-1 cancer cell line has mutations

sites in all four of the of the most common mutation sites including: KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), TP53 (encoding the p53 protein), CDKN2A (also known as p16 or p16INK4a), and SMAD4 (SMAD family member 4, also known as DPC4; deleted in pancreatic carcinoma locus 4) as reviewed in [218]. Migration pattern seem to predominantly occur as single cell, and be greater than for many of the other pancreatic cancer cell lines, however regarding invasiveness the results seem to differ between different research groups, though PANC-1 seem less invasive than several of the other cell lines such as CAPAM-1 and BxPC-3 [218]. Cancer cell lines are *in vitro* model systems that are very valuable and widely used in cancer and drug discovery [219]. The main reason for their use is their ability to provide an unlimited source of biological material that is able to grow indefinably. It has been confirmed that the genomic diversity of their respective cancers is conserved in most of the cell lines and, thereof , can be used as *in vitro* model systems of the diseases from which they were derived [219,220]. However, there are several pancreatic cancer cell lines with phenotypic and genotypic differences [221]. These cell lines originate from different donors and there can be differences in which genes that are mutated, as well as, site of mutation within that gene [221]. Further, there is found differences in, among others, adhesion ability, invisibility and pro-angiogenetic factors in these cells [221]. It is also important to consider that the transcriptomic profile of cell lines greatly differs from that of the *in vivo* tumor [219,220]. Therefore the comparisons made can solemnly be used to imply differences between this cancer cell line and healthy pancreatic cells.

Human primary pancreatic epithelial cells (hPEC) are isolated from normal human pancreatic tissue. They are primary cells and should therefore only be used for a few passages, and not undergo repetitive thawing and freezing [222]. However, they give an opportunity to study the process of carcinogenesis and also used as comparison to a cancer cell line [222]. Many of the morphological, immunohistological, and secretory characteristics of normal pancreatic duct epithelial cells was found to be retained in the cells after several passages [222]. Lately also immortal human pancreatic duct epithelial cell lines (HPDE) has been established and compared to pancreatic cancer cell lines [223,224]. This is both valuable as *in vitro* models for mechanistic studies of human pancreatic ductal carcinogenesis or to serve as appropriate control comparing to pancreatic ductal cancer cell lines [224]. Though such immortal human pancreatic duct epithelial cell lines exist we have chosen to use a human primary pancreatic epithelial cell as they retain the

morphological and functional characteristics of their tissue of origin cell lines display differences in their expression [225].

4.2 Methods to study gene function of specific genes in primary human cells

Transfection is a powerful tool to study gene function in mammalian cells. Various types of nucleic acids including deoxyribonucleic acids (DNAs), ribonucleic acids (RNAs) as well as RNA interference (RNAi) which are small, non-coding RNAs such as siRNA, shRNA, and miRNA can be used for transfection [226-228]. Transfection can be classified into stable or transient transfection, the primary create a sustained long-term effect by integration of nucleic acid into the host cell genome, while the latter do not require integration of nucleic acid into the host cell genome [229,230]. The choice will depend on application and type of cells, as not all methods are employable under all circumstances [231]. The choice will also depend on various strategies for introduction of the nucleic acid into the cell. However, the most common way is by viral delivery [231]. In **paper I and II** we too knocked down a gene transcript using the transduction approach of RNAi, specifically shRNA using a lentivirus delivery system. RNAi is a widely used system, utalizing double stranded RNA (dsRNA) to induces sequence-specific gene silencing by targeting mRNA for degradation [232]. The shRNAs are capable of DNA integration and consist of two complementary 19–22 bp RNA sequences linked by a short loop of 4–11 nucleotides similar to the hairpin found in naturally occurring miRNA [233]. Creating a cell line using shRNA is a time-consuming task which may take months [233]. Also, primary and non-adherent cells, such as immune cells and non-dividing cells are difficult to transfect [233]. Lentivirus and adenovirus have made it possible for cells that are refractory to transfection, such as primary cells, to become permissive to shRNA [233].

To be able to study the role of SENP-2 in skeletal muscle myoblast, cultured adipocytes were transduced with lentivirus containing shRNA for SENP-2 to create a knockdown of the gene **(Paper I and II**).

For muscle cells, satellite cells were established from biopsies of musculus vastus lateralis and a cell bank was established [234]. A mixture of cells from different donors was cultured followed by transduction with shRNA viral particles targeting SENP2 or control and frozen down as transduced myoblasts. These myoblast were differentiated into myotubes before each experiment. For adipocytes, SENP2-KD cells three donors or a mix of three donors of pre-adipocytes were seeded into desired format for experiments followed by transduction with shRNA viral particles targeting SENP2 or control.

4.3 Methods for evaluating metabolism in cultured cells

Functional studies using radioactive labeled substrates to study fuel uptake and oxidation (substrate oxidation assay) and fatty acid uptake and distribution (lipid filtration, thin layer chromatography (TLC)) and acid soluble metabolites (ASM) as well as glucose uptake and incorporation in to glycogen (glycogen synthesis) and scintillation proximity assay (SPA) to study leucine turnover was applied in this thesis to describe metabolic processes.

The substrate oxidation assay [235] was used in **paper I, II, III and IV** to study uptake, complete oxidation (CO_2 production) and accumulation (cell associated radioactivity (CA)) of the radioactive labeled substrates fatty acid, glucose and lactic acid. Substrate oxidation method can also be used to measure **t**he maximal mitochondrial oxidative capacity, with the use of the mitochondrial uncoupler, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). The impact of electron transport chain inhibitors, such as antimycin A, oligomycine and rotenon, on oxidation can also be measured.

ASM (**paper I, II and IV**) was measured reflecting incomplete fatty acid oxidation (β-oxidation) [236] modified by Bakke et al. [237]. It is radioactivity released into the media which provides the possibility to estimate the mitochondria's rate of FA β-oxidation.

TLC was used to study the incorporation of radiolabeled oleic acid (OA) and acetate into lipid classes as intracellular lipids (**paper I-IV**) [238]. When assessing effect on de novo lipogenesis we looked at incorporation of acetic acid into intra cellular lipids as it demonstrates the cells ability to synthesis lipids from other sources that exogenous FA.

Glycogen synthesis assay was used to study incorporation of radiolabeled glucose into glycogen (**I and IV)** as well as measuring insulin response [239].

Scintillation proximity assay (SPA) is useful to study real time accumulation and decay of for example protein (leucine) (**paper IV**). The uptake was monitored over 24 hours (measurements at 0, 2, 4, 6, 8 and 24 hours). At 24 hours the experimental medium was changed to a medium without radioactive leucine, followed by measure of the decline in radioactivity for 6 hours (at time points 0, 2, 4 and 6) as a measurement of protein breakdown. It is a method allowing for continuous monitoring and valuable in use on primary cells which can be sensitive under culturing [235]. The

method however is limited by the need of the cells to be adherent [235]. However, the latter not a problem for our selected cell models of choice.

4.4 Identification of protein and gene expression

Also, gene expression analysis (qPCR, microarray) and protein analysis (immunoblotting and proteomics) were used in this thesis to describe and potentially explain results of the functional studies and various metabolic processes.

Real-time qPCR is a useful method for investigation of defined number of genes that is anticipated to be regulated and involved in the process (**paper I-IV**). This method provides a sensitive and accurate quantification of the gene of interest, and is a well-established technique that gives specific and reproducible results [240]. qPCR records the amplification of a PCR product by a combination of detection of target template and increase in fluorescent signal associated with production formation [241]. During the exponential phase of the PCR amplification, the amplicons detected are directly proportional to the initial number of target sequences [241]. This is unlike traditional end-point PCR where the numerical dominant amplicons not necessarily reflect abundance of sequence in the environment as the amplification of targets often comes from plateau face of the PCR reaction where there may be depletion of critical reagents and therefore slows down or stops the DNA amplification [241]. Also qPCR allows real-time visualization of reaction without the need for agarose gen needed in traditional end-point PCR [242]. The sample preparation is the most crucial step in qPCR, as contaminated or degraded material will not give accurate results, we used commercial kits as they are simple, quick and user friendly ways to improve the sample preparation [242]. Choice of detection method is also important for the experiment and while SYBR® Green fluorescent dye which binds non-specifically to double-stranded DNA as it is generated [242]. The other method is the use of the TaqMan® probes. Probes binds downstream of the primer and only emits fluorescence signal when the 5′ end of the probe, labelled with a fluorescent reporter, is cleaved from the 3′ end. At the 3′ a quencher molecule which prevents fluorescent emission when in close proximity to the reporter is situated. The cleaving occur as the DNA polymerase extends the primer [242]. Though the latter is a more specific method, we used SYBR® Green in our experiments as it is more cost beneficial and easy to use while TaqMan® probes method is more expensive [243]. However, the value of the data set should also be considered in relationship to primer and probe specificity [241].

Further, for assessing the levels of selected relevant proteins to address the functional changes downstream of the gene expression, immunoblotting was used (**paper I, III and IV**). Immunoblotting is a time-consuming process and the reproducibility is impacted by variation in any of the multiple steps the procedure possess [244]. Another method to identify and quantify large number of proteins, either secreted (**paper III**) or cellular (**paper II**) is the use of high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics [245]. Proteomics is the large-scale study of proteoms, it allows for identification and quantification of overall proteins present content of a cell, tissue, organism or in media harvested from cultured cells (e.i the proteins these cells release into the media) (**paper III and IV**) [246]. Unlike immunoblotting which is a semi quantitative method and highly dependent on the quality of the antibodies being used [244], proteomics analysis confirms the presence of proteins and provides a direct measure of quantity [247]. Proteomics not only allows for characterizing protein mixtures in systems, however, also determine relationships between proteins, resolve their function, and identify protein–protein interactions of interest [248]. Though highly sensitive and resolute, LC-MS/MS methods tend to be less robust in the detection of low abundance proteins compared to the high abundance proteins [249]. However, both methods can give valuable information regarding differences proteins after interventions, either for a few selected or as a more complete picture. In **paper II**, proteomics was used to analyze the changes in protein expression following SENP2-KD in adipocytes, while in **paper III** (and **IV**) this method was used to look at differences in protein secretion in PANC-1 versus hPEC cells.

4.5. Methods for examining impact of cross talk

The secretome is proteins and other substances secreted from particular cells, possible working via paracrine and autocrine mechanisms to regulate physiological processes such as growth, differentiation, proteolysis, apoptosis, signaling, cell adhesion, and development of the extracellular matrix [250]. To investigate the impact of pancreatic cells, both a cancerous cell line and primary cells, on muscle cells, media was harvested from hPEC and PANC-1 cells after incubation of the cells for 48 h followed by conditioning of muscle cells with these media in **paper III and IV**. The same media was used to measured differences in glucose and lactate concentration of consume and release of the two cells, in **paper III**, and for proteomics studies in **paper III and IV.**

4.6. Data analyses and statistics

Unpaired t-test was used between groups and paired t-test within groups, using GraphPad version 8 and 9 For time-dependent experiments linear mixed model, using SPSS version 28 was used. This allowed for comparison of differences between groups and in-between groups at the same time taken to account that not all observations are independent.

5. General discussion

The studies presented in this thesis explore energy metabolism in cultured primary human myotubes, adipocytes and hPEC as well as in a cultured PANC-1. In **paper I and II** changes in energy metabolism was studied after knockdown of the SENP2 protein, looking at glucose and fatty acid uptake and distribution. **Paper I** focused on changes occurring in myotubes after knockdown while **paper II** focused on the changes occurring in adipocytes. In **paper III** and **IV** the study focus was on energy metabolism in cancer cells and implications of cancer on the metabolism in peripheral tissues focusing on primary human myotubes. While **paper III** explored the energy metabolism in a PANC-1 compared to a healthy primary cell of the same origin, hPEC, **Paper IV** focuses on the metabolism in primary human myotubes after conditioning with media from PANC-1 and hPEC cells.

5.1 SENP2-KD in primary human myotubes and adipocytes

Both skeletal muscle and fat cells perform important roles regarding metabolism and whole body homeostasis [251-255]. While adipose tissue is an excellent storage-tissue and takes care of excess fat [214], skeletal muscle serves as the main site of insulin-stimulated glucose disposal [256,257]. As described under section

1.4 SENP2 and SUMOylation of PPAR , i*n vivo* and *in vitro* mice studies have previously described how the SENP2 protein is important for fuel metabolism in myotubes and adipose tissue [119,122,127,128,258]. Though rodents can be good models for research on basic human conditions [259-261], there are still many differences between the species [262-264]. Compared to larger animals, such as humans, rodents have a large surface area to body volume, which is linked to a higher exchange of heat to the environment and thereof follows a metabolic rate which is about 3–5 fold greater compared to humans [265]. As discussed under the section **4. Methodological considerations**, **4.1.1. Cultured primary human myotubes** and **4.1.2. Cultured primary human adipocytes**, though with some limitations, primary human skeletal muscle cells and primary human adipocytes has the most relevant genetic background and represent the best available alternative system to intact skeletal muscle and adipose tissue that can be used to study human disease [171,188,190,203,204]. We therefore wanted to use our primary human cell model to further elucidate SENP2's metabolic impact on skeletal muscle and adipose tissue in humans.

Compared to control cells, in SENP2-KD myotubes glucose uptake and cell associated glucose were increased, and the fractional oxidation was reduced (**paper I**), while in SENP2-KD

adipocytes both glucose uptake and oxidation was reduced (**paper II**). At basal, fasting state, skeletal muscle are involved in clarence of about 25% of plasma glucose [266], increasing to 70-85% after a meal [13], while adipose tissue accounts for only a small proportion (about 10-15%) of overall glucose disposal [267]. However, alterations in adiposity have profound implications for glucose homeostasis [13,266]. While there was no significant difference in the PDK4 expression in SENP2-KD or SCR myotubes, the PDK4 expression was increased in the SENP2-KD adipocytes. PDK4 expression is usually associated with reduced glucose oxidation [268,269]. The difference in PDK4 expression between the two tissues may explain the reason for difference in glucose oxidation in myotubes and adipocytes after SENP2-KD. However, as stated, glucose energy metabolism in fat and muscle tissue differs [270], therefore SENP2-KDs metabolic implications in the tissues are not surprisingly different. No significant difference was found in the GLUT1 expression between SENP2-KD myotubes and SCR to explain the increased glucose uptake seen in these cells. In SENP2-KD adipocytes we looked at both GLUT1 and the insulin-dependent glucose transporter GLUT4[12] where non was significantly regulated in these cells.

At basal conditions, there was no difference in glycogen synthesis between SENP2-KD and SCR myotubes **(Paper I)**. Insulin promotes uptake of glucose into muscle subsequently to glycogen storage, an important step in lowering the blood glucose content [271]. Akt phosphorylation also play a crucial role in the glucose homeostasis via the PI3K-Akt axis leading to GLUT4 translocation and increased glucose uptake [30,31,272]. To study the insulin response in SENP2-KD myotubes, we measured insulin-induced glycogen synthesis and Akt phosphorylation**.** Though we had an increased basal glucose uptake in SENP2-KD compared to SCR myotubes, both the insulin-stimulated glycogen synthesis and Akt phosphorylation was blunted in SENP2-KD myotubes. Glycogen synthesis in SENP2-KD adipocytes was not studied in this thesis; though it has been shown in murine models that glycogen is storage in adipocytes, though in very low quantities [273]. Although not presented in **paper II,** we performed some experiments where we studued insulin-stimulated glucose uptake in the adipocytes. In spite of the increased GLUT4 expression in SENP2-KD adipocytes, we found no significant differences between the insulin-induced glucose uptake in SENP2-KD and SCR adipocytes (data not shown). This is in accordance with the study of Lee *et al*. which found no difference in glucose tolerance between control and *Senp2*-aKO mice when fed with normal chow-fed diet [13]. However, in the same study, *Senp2*-aKO mice showed improved glucose tolerance when fed with HFD [127].

This was interesting finding that made us wonder the impact of SENP2 on insulin signaling in SENP2-KD myotubes. One question is where did the excess glucose in SENP2- KD myotubes go? This is not known, as we did not explore other potential pathways to elucidate the glucose-faith in the SENP2-KD myotubes. However, one could purpose that the excess glucose potentially could have been converted to lipids via *de novo* lipogenesis and incorporated in intracellular as intramuscular triacylglycerol's. The contribution to the intramyocellular fatty acid pool is, however, usually small [274]. *De novo* lipogenesis will be discussed in more detail later. The excess glucose could also have turn into non-oxidized glucose metabolites like lactate and pyruvate. Lee *et al*. whom looked in to glucose handling in their Senp2-aKO mice. Had also found that though there was no significant difference in glucose tolerance between control and *Senp2*-aKO mice, the *Senp2*-aKO had similar insulin tolerance though the *Senp2*-aKO had lower glucose levels than control after insulin injection [275]. We were unfortunately not able to explore the Akt phosphorylation in the SENP2-KD adipocytes. However these results imply a role of SENP2 in insulin handling in both myotubes and adipocytes.

We also explored FA metabolism in SENP2-KD myotubes (**Paper I**) and adipocytes (**Paper II**). SENP2-KD lead to increased fatty acid oxidation both in cultured human myotubes (**paper I**) and adipocytes (**paper II**), however in myotubes this only occurred after prolonged (24 h) incubation with oleic acid while in adipocytes acute (4 h) oleic acid incubation was sufficient to see an increase. Further, looking into the lipid accumulation and distribution into complex lipids in SENP2-KD myotubes (**paper I**), we found that the lipid accumulation and distribution was lower than in the control cells for all lipid-groups. However, when adjusting for the lower total uptake there was increased distribution into all groups except TAG, which was still decreased. A somewhat similar tendency was seen in the SENP2-KD adipocytes (**Paper II**). In the SENP2-KD adipocytes total lipid accumulation and incorporation into PL and TAG was significantly lower, however, when adjusting for the total lipid uptake DAG and FFA was significantly increased while TAG was unchanged (Figure 14). Our findings align with the previous findings of reduced lipid storage in adipocyte specific knockout of SENP2 [127,128]. In C2C12 myotubes, on the other hand, increased SENP2 activity was seen after palmitate exposure leading to increased fatty acid oxidation [119], which does not correspond to our findings in SENP2-KD myotubes. However,

when looking at acutely available oleic acid after SENP2-KD in human myotubes we did observe both a reduced fatty acid oxidation and reduced oxidative reserve capacity **(Paper I)**. Also, recent research from mice studies with liver-specific knockout of SENP2 found that SENP2 deficiency upregulated genes involved in fatty acid oxidation and downregulated genes involved in lipogenesis [276].

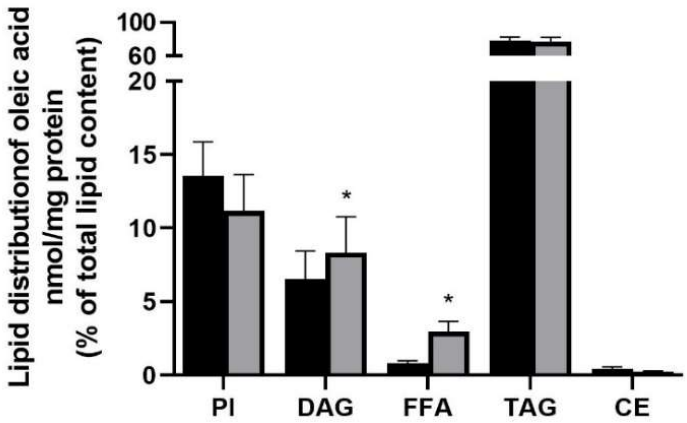

Figure 14. Lipid distribution of oleic acid in SENP2-KD adipocytes as percent of total lipid content. Adipocytes established from pre.adipocytes was incubated with [1-^{14}C]oleic acid (18.5 kBq/ml, 100 µmol/l) for 4 h following followed by separation of lipids by thin layer chromatography and quantified by liquid scintillation. Data are presented as ±SEM (n = 4).

Abnormal lipid metabolism with accumulation of lipotoxic intermediates and TAG in skeletal muscle have been linked to insulin resistance and diabetes [50-56]. Oxidation of FA have been reported to be impaired in skeletal muscle and adipose tissue of individuals with obesity and T2DM [177,277-280]. T2DM and obesity are also characterized by disturbances in WAT functions that can impact metabolic health [281]. As adipose tissue is not only a storage organ but also important endocrine organ, secreting adipokines which effects the whole body [282]. It is well established that excess adipose tissue predisposes toward development of insulin resistance [83,84,86]. Improved and increased fatty acid oxidation could ameliorated these conditions. The increased fatty acid oxidation, as seen in our SENP2-KD myotubes and adipocytes, could be the reason for the observed reduction in accumulation of intra muscular TAG.

A less discussed, but equally important topic, is the association between several forms of insulin resistance and loss or absence of adipose tissue [283,284]. Adipose tissue influences many processes,

including energy metabolism, inflammation, and pathophysiological changes such as cancer and infectious disease [285]. Therefore, loss of this important tissue, for example as can occur under cachexia [286], is not desirable.

5.2 SENP2 in relation to cancer

SENP2 has also been highlighted in regards to cancer, with tumor suppressor or anti-tumor effects and as a potential target for cancer therapy [287-289]. Tumor suppressor effects of SENP2 has been described in hepatocellular carcinoma [290,291], potentially as a negative regulator of proliferation, migration and invasion as seen in osteosarcoma cells [287]. Though not included in the scope of **paper III**, SENP2 plays an important role also in pancreatic tissue and cancer [288-290,292,293]. To combine the research conducted in this thesis, SENP2 expression was explored in PANC-1and hPEC cells. The SENP2 expression in PANC-1 was significantly lower than in hPEC cells (approximately 80%) Figure 15.

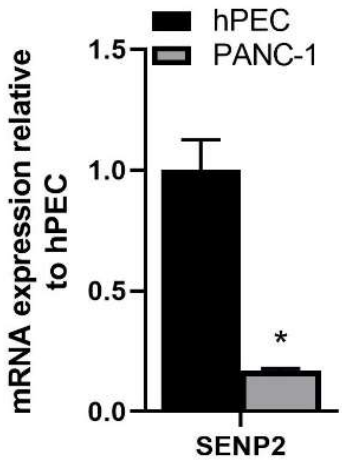

Figure 15. SENP2 expression in hPEC and PANC-1 cells. Cells established from a pancreatic cancer cell line (PANC-1) and primary human pancreatic epithelial cell (hPEC) where cultured. mRNA was isolated and gene expression of SUMO/sentrin specific protease 2 (SENP2) was assessed by qPCR. Data are presented as ±SEM (n = 4).

5.3 Cancer-muscle crosstalk

Some cancers, such as pancreatic cancer, present symptoms very late. About 80-85 % of PDAC patients have locally advanced or distant metastasis at time of diagnosis [294]. Due to the low incidence of PDAC, screening programs in the general population is thought to be more harmful then helpful; resulting in large amount of false positive results [295]. Knowing more about this

devastating form of cancer could help us define individuals with elevated risk, and early detection could prevent mortality. Therefore, in **paper III** we explored metabolic differences in hPEC and PANC-1. We found PANC-1 cells to be more glycolytic compared to hPEC. Thus, the PANC-1 cells took up and oxidized more glucose and secreted more lactic acid compared to hPEC cells. Most cancer cells lean towards a glycolytic phenotype, studies on PDAC cell lines have shown differences in glycolytic preference [296,297]. Also, PDK4 expression was lower in PANC-1 cells. PDK4 inhibits the PDC complex in converting pyruvate to acetyl-CoA in the mitochondria, and in that way increased PDK4 makes the cell favor FA oxidation over glucose [38]. The decreased PDK4 expression here can therefore potentially explain the increased glucose oxidation seen in the PANC-1 cells. In cancer cells, glucose transporters are typically expressed at a higher concentration to compensate for decreased ATP production [298]. In our study we found higher GLUT4 expression but lower GLUT1 expression in the PANC-1 cells compared to hPEC cells.

Metabolic changes in cancer cell have a big impact on the proliferation, metastases and survival of the cancer cell [299]. It is now obvious that the cancer also induce changes in other tissues [149,150], potentially to increase its own success rate. In **paper IV** we explored total protein content in myotubes conditioned with media from PANC-1 (cPANC-1)-exposed and conditioned media from hPEC (chPEC)-exposed. We found that the total protein content was significantly lower in the cPANC-1- exposed compared to chPEC-exposed myotubes. To further explore the difference seen in protein content we traced cellular accumulation and decay of leucine in the conditioned myotubes as a measure of protein turn-over. We found that over a 24 h period less leucine was accumulated in cPANC-1-exposed myotubes and over a 6 h period more leucine was decayed from cPANC-1-exposed compared to chPEC-exposed myotubes, suggesting an over-all decrease in intracellular protein content in cPANC-1-exposed myotubes. These results were accompanied by decreased myosin heavy chain (*MYH)2* mRNA expression in cPANC-1-exposed compared to chPEC-exposed myotubes.

Both rat and mice studies have shown decrease in mitochondrial content during cancer cachexia [300-302]. Also changes in morphology has been observed in human patients [303]. A decrease of metabolic flux through the TCA cycle [304], and activity of all complexes have been consistently described [305-308]. Our findings, from **paper IV,** complement these as we observed a mitochondrial dysfunction in the PANC-1-exposed cells. The mitochondrial dysfunction was manifested by a

decreased response to mitochondrial inhibitors and un-couplers, as well as a reduction in the mRNA expression of the ETC complex III-related gene *UQCRB*.

In **paper IV**, when treating muscle cells with conditioned media from PANC-1 and hPEC cells we found a significant decrease in uptake and oxidation of glucose in cPANC-exposed myotubes. Decreased insulin-stimulated glucose transport has previously been demonstrated and confirmed in skeletal muscle cells from PDAC patients [309-311]. The same decrease in insulin-stimulated glucose transport was, however, not seen in L6 myotubes after conditioning with media from human PDAC patients [310]. These researchers' conclusion was that skeletal muscle insulin resistance in PDAC patients can not directly be caused by circulating factors from the tumor, however, our result tend to disagree. There are some discrepancies between the methods, such as difference in exposure time to the conditioned media (24 h vs 4 days) and choice of species used for myotubes (rat vs human) which could explain the difference in outcome.

In skeletal muscle of obese subjects decreased insulin receptor (IR) binding capacity and impaired IR phosphorylation is demonstrated [312], however Liu *et al*. found that the insulin resistance associated with pancreatic cancer seemed to be a post-IR defect [151]. According to Liu and coworkers, skeletal muscle IR density, affinity and mRNA level were normal in pancreatic cancer patients, also IR kinase activity was normal in pancreatic cancer patients with and without diabetes [151]. Several studies have found that the insulin resistance seen in pancreatic cancer patients is associated with a decrease in skeletal muscle glycogen [151,310]. In **paper IV**, no difference in basal glycogen synthesis was observed between cPANC-1-exposed and chPEC-exposed myotubes, however, only chPEC-exposed myotubes had a significant increase in glycogen after insulin treatment.

Further, when we explored the Akt phosphorylation in cPANC-1-exposed and chPEC-exposed myotubes, the phosphorylation level in cPANC-1-exposed myotubes was higher at basal and rose significantly after insulin treatment, indication no cPANC-1-induced insulin resistance at the level of Akt. Akt is rapidly activated by phosphorylation after insulin binds IR, further leading to translocation of GLUT 4 to cell membrane from intracellular vesicles [30,31]. Therefore, an activation of Akt upon insulin treatment support the findings of Liu *et al* [84] that the insulin resistance is caused by a post-IR defect. However, in contrast to this, insulin-stimulated PI3-kinase/akt activity has been found to be decreased in skeletal muscle from patients with pancreatic cancer compared with

controls [311]. However, in that study glycogen synthesis was not investigated and the authors concluded that both PI3K activity or glycogen synthase activity could be reasons for the reduction of insulin-stimulated glucose transport associated with pancreatic cancer patients [311].

6. Future perspective

To further explore the SENP2 function the obvious next step would be to overexpress SENP2 in muscle and fat cells. Though differences before and after KD was pretty clear in our cells, KD only decreases RNA and protein content, unlike knock out (KO) where the gene is abolished. As previously described, KO is a very difficult procedure in primary cells, and complete abolishment of a gene may also lead to other issues dependent on the importance of the gene. An overexpression experiment, however, would allow to explore the implication of SENP2 on metabolism even more, and it is easier to increase expression several 100 folds while a KD is limited to the amount of the natural occurring protein content.

More work is needed to conclude on SENP2 as a potential future drug target for treatment of obesity and T2D, though our experiments show an important role of SENP2 in energy metabolism in both human myotubes and adipocytes. SENP2 had a lower total lipid accumulation and increased fatty acid oxidation in both adipose and myotubes as well as decreased TAG storage. In skeletal muscle increasing fatty acid oxidation and/or decreasing lipid storage in skeletal muscles can reverse insulin resistance [313-315], that increased fatty acid oxidation reduced TAG content in and inflammatory levels in adipocytes [279]. These findings implies a potential benefit SENP2-KD in the combat of obesity in both adipose and skeletal muscle tissue. However, the handling of glucose and impact of insulin varied between the two cell types, which implies a somewhat different role of SENP2 in the two tissues. This needs to be further elucidated.

For my own satisfaction, it would have been interesting to look further into the effects of SENP2 in the hPEC and PANC-1 cells. Though we found that SENP2 expression was lower in PANC- 1 compared to hPEC, it would have been interesting to see whether KD of SENP2 in hPEC cells would have made these cells more metabolically equal to PANC-1 cells. Also, I would have liked to study the SENP2 expression in PANC-1 conditioned and hPEC conditioned myotubes. Conditioning myotubes with media from these two cell types had a large effect on the metabolism in myotubes and potentially SENP2 may have had a role.

It would have been interesting to explore the lipid distribution also in the PANC-1 cells compared to hPEC cells. We know that PANC-1 cells took up more OA and oxidized more, and it is assumed the rest was used for proliferation, however, TLC could possibly give us more answers in that regard.

I would also recommend to explore the effect of PANC-1 conditioned media on adipocytes. As mentioned, adipose tissue is an important metabolic tissue and only small changes in glucose and fat metabolism can change the whole body homeostasis. There is also evident in that cancer-cachexia not only can lead to loss of skeletal muscle mass, but potentially also loss of fat mass [167]. Cancer cachexia is involved in adipocyte lipolysis inducing lipid loss [316], and increasing circulating FA which may benefit the tumor [317]. Also, upregulation of the browning process, which promotes energy expenditure during cancer cachexia, has been reported [318]. To our knowledge, similar work on primary human adipocytes has not been previously exerted, and it would give us a broader insight into the effects of pancreatic cancer on whole body metabolism. By having isolated tissues/cells under *in vitro* conditions, instead of *in vivo* conditions, we can be sure that the changes occurring is caused by the pancreatic cancer cell (cell line) directly and not indirectly through other cells such as immune cells.

7. Conclusion

In conclusion our work shows that:

- Knock down of SENP2 in primary human myotubes and adipocytes increased fatty acid oxidation and decreased total lipid and TAG accumulation in both cell types. Glucose uptake was increased in skeletal muscle cells and decreased in adipocytes followed by a decrease in glucose oxidation in adipocytes. SENP2 seems to have similar effect on fatty acids in skeletal muscle and fat cells, while the glucose and insulin handling differ in the two cells after SENP2-KD. Our results demonstrate a potential role of SENP2 in the combat against the ever rising pandemic of obesity and T2D (main findings summarized in **Figure 14**).

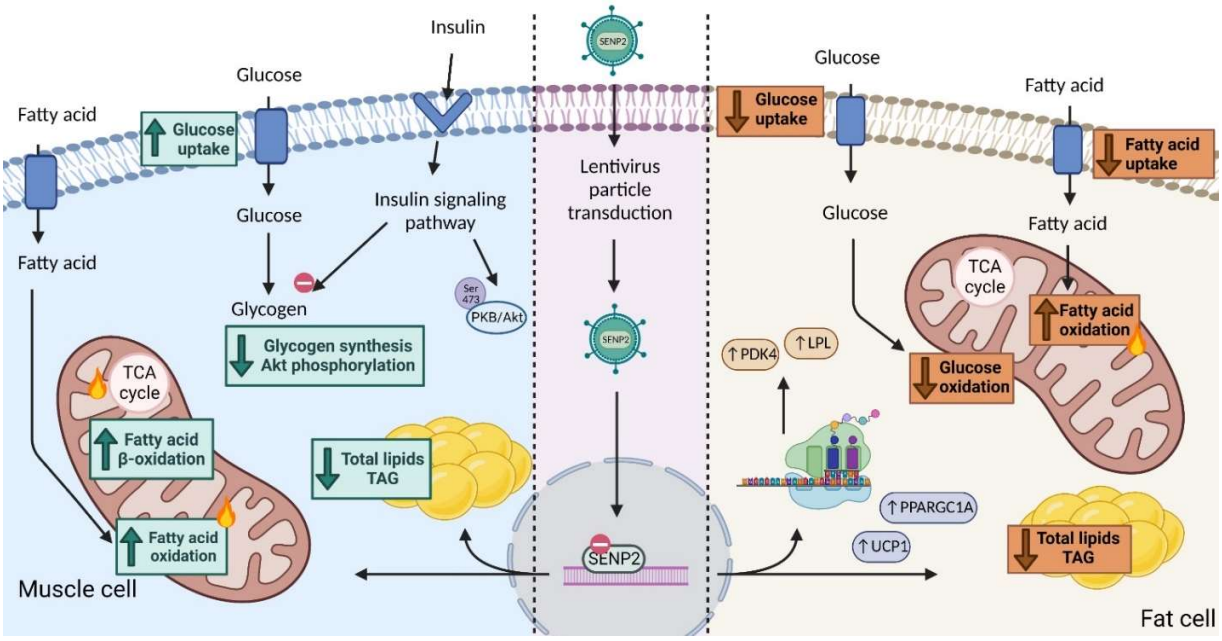

Figure 14. Summary of main findings after SENP2-KD in human primary myotubes and adipocytes. SUMO/Sentrin specific protease 2 knock down (SENP2-KD) increases fatty acid oxidation and reduces total lipid and triacylglycerol (TAG) accumulation in primary human myotubes and adipocytes. In myotubes glucose uptake increases after SENP2-KD while glycogen synthesis and Akt phosphorylation are blunted after insulin stimulation. In adipocytes glucose uptake and oxidation is reduced after SENP2-KD

- We found PANC-1cells to be metabolically very different from hPEC cells. They were more glycolytic, with more active glucose oxidation, and a lower oleic acid oxidation. They also secreted a greater amount of substances into the conditioned media compared to hPEC. Results from myotubes conditioned with media from the two cells types also showed how the energy metabolism could be affected in other tissues than the tissue containing the cancer. PANC-1 conditioned myotubes showed changes in leucine, glucose and oleic acid metabolism and that deciphering these effects could help in diagnosing and treatment of pancreatic cancer (main findings summarized in **Figure 15**).

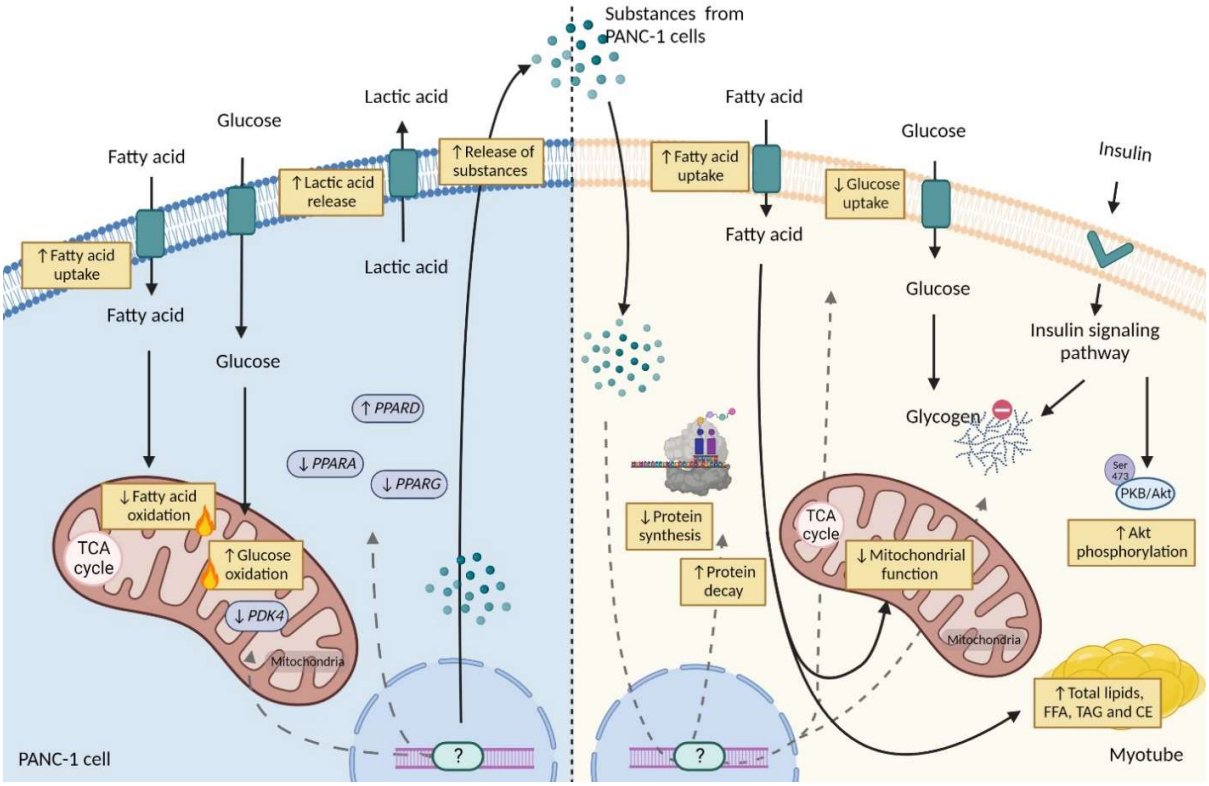

Figure 15. Summary of main findings from metabolic studies in PANC-1 compared to hPEC and in myotubes after konditioning with media from the two cell types. Pancreatic cancer cell line (PANC-1) had higher fatty acid (FA) uptake, though lower FA oxidation than human pancreatic epithelial cell (hPEC). glucose oxidation and lactic acid release was also increased in the PANC-1 cells. PANC-1 cells also had a higher secreation of substances potentially acting on other tissues. Myotubes conditioned with media from PANC-1 cells had increased FA uptake and total lipid accumulation compared to myotubes conditioned with hPEC media. Protein synthesis was decreased, while protein decay was increased. The function and glucose uptake was decreased though akt phosphorylation was increased.

8. References

1 Fernandez-de-Cossio-Diaz, J. & Vazquez, A. A physical model of cell metabolism. *Scientific Reports* **8**, 8349, doi:10.1038/s41598-018-26724-7 (2018).

2 Czech, M. P. Insulin action and resistance in obesity and type 2 diabetes. *Nature medicine* **23**, 804 (2017).

3 Wilcox, G. Insulin and insulin resistance. *Clin Biochem Rev* **26**, 19-39 (2005).

4 Sun, K., Kusminski, C. M. & Scherer, P. E. Adipose tissue remodeling and obesity. *The Journal of clinical investigation* **121**, 2094-2101, doi:10.1172/JCI45887 (2011).

5 Ng, M. *et al.* Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **384**, 766-781, doi:10.1016/s0140-6736(14)60460-8 (2014).

6 World Health Organization (WHO). *Obesity and overweight*, <https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight> (2022).

7 Aronne, L. J. Classification of obesity and assessment of obesity-related health risks. *Obes Res* **10 Suppl 2**, 105s-115s, doi:10.1038/oby.2002.203 (2002).

8 Seidell, J. C. & Halberstadt, J. The global burden of obesity and the challenges of prevention. *Ann Nutr Metab* **66 Suppl 2**, 7-12, doi:10.1159/000375143 (2015).

9 Müller, M. J. & Geisler, C. Defining obesity as a disease. *European Journal of Clinical Nutrition* **71**, 1256-1258, doi:10.1038/ejcn.2017.155 (2017).

10 Janssen, I., Katzmarzyk, P. T. & Ross, R. Waist circumference and not body mass index explains obesity-related health risk. *Am J Clin Nutr* **79**, 379-384, doi:10.1093/ajcn/79.3.379 (2004).

11 World Health Organization (WHO). *Waist Circumference and Waist-Hip Ratio*, <http://apps.who.int/iris/bitstream/handle/10665/44583/9789241501491_eng.pdf;jsessionid=0D4C0143AD59B9722D76694D10234426?sequence=1> (2011).

12 Defronzo, R. A. Dysfunctional fat cells, lipotoxicity and type 2 diabetes. *International Journal of Clinical Practice* **58**, 9-21, doi:https://doi.org/10.1111/j.1368-504X.2004.00389.x (2004).

13 DeFronzo, R. A. *et al.* The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**, 1000-1007, doi:10.2337/diab.30.12.1000 (1981).

14 van Loon, L. J., Greenhaff, P. L., Constantin-Teodosiu, D., Saris, W. H. & Wagenmakers, A. J. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *The Journal of physiology* **536**, 295-304, doi:10.1111/j.1469-7793.2001.00295.x (2001).

15 Wasserman, D. H. Four grams of glucose. *Am J Physiol Endocrinol Metab* **296**, E11-21, doi:10.1152/ajpendo.90563.2008 (2009).

16 Houmard, J. A. Intramuscular lipid oxidation and obesity. *Am J Physiol Regul Integr Comp Physiol* **294**, R1111-1116, doi:10.1152/ajpregu.00396.2007 (2008).

17 Dagenais, G. R., Tancredi, R. G. & Zierler, K. L. Free fatty acid oxidation by forearm muscle at rest, and evidence for an intramuscular lipid pool in the human forearm. *J Clin Invest* **58**, 421-431, doi:10.1172/jci108486 (1976).

18 Ren, J. M. *et al.* Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* **268**, 16113-16115 (1993).

19 Devaskar, S. U. & Mueckler, M. M. The mammalian glucose transporters. *Pediatr Res* **31**, 1-13, doi:10.1203/00006450-199201000-00001 (1992).
20 Mueckler, M. Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. *Diabetes* **39**, 6-11, doi:10.2337/diacare.39.1.6 (1990).
21 Mueckler, M. M. The molecular biology of mammalian glucose transporters. *Current opinion in nephrology and hypertension* **1 1**, 12-20 (1992).
22 Zierath, J. R. & Kawano, Y. The effect of hyperglycaemia on glucose disposal and insulin signal transduction in skeletal muscle. *Best Pract Res Clin Endocrinol Metab* **17**, 385-398, doi:10.1016/s1521-690x(03)00040-x (2003).
23 Gumà, A., Zierath, J. R., Wallberg-Henriksson, H. & Klip, A. Insulin induces translocation of GLUT-4 glucose transporters in human skeletal muscle. *Am J Physiol* **268**, E613-622, doi:10.1152/ajpendo.1995.268.4.E613 (1995).
24 Lauritzen, H. P. Insulin- and contraction-induced glucose transporter 4 traffic in muscle: insights from a novel imaging approach. *Exerc Sport Sci Rev* **41**, 77-86, doi:10.1097/JES.0b013e318275574c (2013).
25 Ploug, T., van Deurs, B., Ai, H., Cushman, S. W. & Ralston, E. Analysis of GLUT4 Distribution in Whole Skeletal Muscle Fibers: Identification of Distinct Storage Compartments That Are Recruited by Insulin and Muscle Contractions. *Journal of Cell Biology* **142**, 1429-1446, doi:10.1083/jcb.142.6.1429 (1998).
26 James, D. E., Strube, M. & Mueckler, M. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* **338**, 83-87, doi:10.1038/338083a0 (1989).
27 Sun, Y., Bilan, P. J., Liu, Z. & Klip, A. Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. *Proceedings of the National Academy of Sciences* **107**, 19909-19914, doi:doi:10.1073/pnas.1009523107 (2010).
28 Pehmøller, C. *et al.* Genetic disruption of AMPK signaling abolishes both contraction- and insulin-stimulated TBC1D1 phosphorylation and 14-3-3 binding in mouse skeletal muscle. *American journal of physiology. Endocrinology and metabolism* **297**, E665-E675, doi:10.1152/ajpendo.00115.2009 (2009).
29 Sakamoto, K. & Holman, G. D. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *Am J Physiol Endocrinol Metab* **295**, E29-37, doi:10.1152/ajpendo.90331.2008 (2008).
30 Kido, Y., Nakae, J. & Accili, D. Clinical review 125: The insulin receptor and its cellular targets. *J Clin Endocrinol Metab* **86**, 972-979, doi:10.1210/jcem.86.3.7306 (2001).
31 Shepherd, P. R., Withers, D. J. & Siddle, K. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem J* **333 (Pt 3)**, 471-490, doi:10.1042/bj3330471 (1998).
32 Jensen, T. E. & Richter, E. A. Regulation of glucose and glycogen metabolism during and after exercise. *The Journal of physiology* **590**, 1069-1076, doi:10.1113/jphysiol.2011.224972 (2012).
33 Dashty, M. A quick look at biochemistry: Carbohydrate metabolism. *Clinical Biochemistry* **46**, 1339-1352, doi:https://doi.org/10.1016/j.clinbiochem.2013.04.027 (2013).
34 Peters, S. J. Regulation of PDH activity and isoform expression: diet and exercise. *Biochem Soc Trans* **31**, 1274-1280, doi:10.1042/bst0311274 (2003).
35 Lee, I.-K. The role of pyruvate dehydrogenase kinase in diabetes and obesity. *Diabetes Metab J* **38**, 181-186, doi:10.4093/dmj.2014.38.3.181 (2014).

36 Pilegaard, H. & Neufer, P. D. Transcriptional regulation of pyruvate dehydrogenase kinase 4 in skeletal muscle during and after exercise. *Proc Nutr Soc* **63**, 221-226, doi:10.1079/pns2004345 (2004).
37 Zhang, S., Hulver, M. W., McMillan, R. P., Cline, M. A. & Gilbert, E. R. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutr Metab (Lond)* **11**, 10-10, doi:10.1186/1743-7075-11-10 (2014).
38 Badin, P.-M. *et al.* Regulation of skeletal muscle lipolysis and oxidative metabolism by the co-lipase CGI-58. *Journal of Lipid Research* **53**, 839-848, doi:https://doi.org/10.1194/jlr.M019182 (2012).
39 Shi, L. & Tu, B. P. Acetyl-CoA and the regulation of metabolism: mechanisms and consequences. *Curr Opin Cell Biol* **33**, 125-131, doi:10.1016/j.ceb.2015.02.003 (2015).
40 Wang, H. & Eckel, R. H. Lipoprotein lipase: from gene to obesity. *American Journal of Physiology-Endocrinology and Metabolism* **297**, E271-E288, doi:10.1152/ajpendo.90920.2008 (2009).
41 Nickerson, J. G. *et al.* Greater Transport Efficiencies of the Membrane Fatty Acid Transporters FAT/CD36 and FATP4 Compared with FABPpm and FATP1 and Differential Effects on Fatty Acid Esterification and Oxidation in Rat Skeletal Muscle*. *Journal of Biological Chemistry* **284**, 16522-16530, doi:https://doi.org/10.1074/jbc.M109.004788 (2009).
42 Watt, M. J. & Hoy, A. J. Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *Am J Physiol Endocrinol Metab* **302**, E1315-1328, doi:10.1152/ajpendo.00561.2011 (2012).
43 Faergeman, N. J. & Knudsen, J. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem J* **323 (Pt 1)**, 1-12, doi:10.1042/bj3230001 (1997).
44 Doege, H. & Stahl, A. Protein-mediated fatty acid uptake: novel insights from in vivo models. *Physiology (Bethesda)* **21**, 259-268, doi:10.1152/physiol.00014.2006 (2006).
45 Campbell, S. E. *et al.* A Novel Function for Fatty Acid Translocase (FAT)/CD36: INVOLVEMENT IN LONG CHAIN FATTY ACID TRANSFER INTO THE MITOCHONDRIA*. *Journal of Biological Chemistry* **279**, 36235-36241, doi:https://doi.org/10.1074/jbc.M400566200 (2004).
46 Longo, N., Frigeni, M. & Pasquali, M. Carnitine transport and fatty acid oxidation. *Biochim Biophys Acta* **1863**, 2422-2435, doi:10.1016/j.bbamcr.2016.01.023 (2016).
47 Houten, S. M. & Wanders, R. J. A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation. *J Inherit Metab Dis* **33**, 469-477, doi:10.1007/s10545-010-9061-2 (2010).
48 Sugden, M. C. & Holness, M. J. Skeletal muscle lipid metabolism and the adipomuscular axis. (2006).
49 Storlien, L., Oakes, N. D. & Kelley, D. E. Metabolic flexibility. *Proceedings of the Nutrition Society* **63**, 363-368 (2004).
50 Tomás, E. *et al.* Hyperglycemia and insulin resistance: possible mechanisms. *Annals of the New York Academy of Sciences* **967**, 43-51 (2002).
51 Petersen, K. F. & Shulman, G. I. Etiology of insulin resistance. *The American journal of medicine* **119**, S10-S16 (2006).

52 Aas, V., Kase, E., Solberg, R., Jensen, J. & Rustan, A. Chronic hyperglycaemia promotes lipogenesis and triacylglycerol accumulation in human skeletal muscle cells. *Diabetologia* **47**, 1452-1461 (2004).
53 Aas, V. *et al.* Lipid metabolism in human skeletal muscle cells: effects of palmitate and chronic hyperglycaemia. *Acta Physiologica* **183**, 31-41 (2005).
54 Aas, V., Rokling-Andersen, M. H., Kase, E. T., Thoresen, G. H. & Rustan, A. C. Eicosapentaenoic acid (20: 5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *Journal of Lipid Research* **47**, 366-374 (2006).
55 Aas, V. *et al.* Chronic hyperglycemia reduces substrate oxidation and impairs metabolic switching of human myotubes. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1812**, 94-105 (2011).
56 Martins, A. R. *et al.* Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. *Lipids in health and disease* **11**, 30 (2012).
57 van Loon, L. J. & Goodpaster, B. H. Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state. *Pflugers Arch* **451**, 606-616, doi:10.1007/s00424-005-1509-0 (2006).
58 Russell, A. P. Lipotoxicity: the obese and endurance-trained paradox. *Int J Obes Relat Metab Disord* **28 Suppl 4**, S66-71, doi:10.1038/sj.ijo.0802859 (2004).
59 Turcotte, L. P. & Fisher, J. S. Skeletal muscle insulin resistance: roles of fatty acid metabolism and exercise. *Phys Ther* **88**, 1279-1296, doi:10.2522/ptj.20080018 (2008).
60 Walther, T. C. & Farese, R. V., Jr. Lipid droplets and cellular lipid metabolism. *Annu Rev Biochem* **81**, 687-714, doi:10.1146/annurev-biochem-061009-102430 (2012).
61 Gesta, S., Tseng, Y.-H. & Kahn, C. R. Developmental Origin of Fat: Tracking Obesity to Its Source. *Cell* **131**, 242-256, doi:10.1016/j.cell.2007.10.004 (2007).
62 Ikeda, K., Maretich, P. & Kajimura, S. The Common and Distinct Features of Brown and Beige Adipocytes. *Trends in Endocrinology & Metabolism* **29**, 191-200, doi:https://doi.org/10.1016/j.tem.2018.01.001 (2018).
63 Chait, A. & den Hartigh, L. J. Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. *Front Cardiovasc Med* **7**, 22-22, doi:10.3389/fcvm.2020.00022 (2020).
64 Karastergiou, K., Smith, S. R., Greenberg, A. S. & Fried, S. K. Sex differences in human adipose tissues - the biology of pear shape. *Biol Sex Differ* **3**, 13-13, doi:10.1186/2042-6410-3-13 (2012).
65 Ameer, F., Scandiuzzi, L., Hasnain, S., Kalbacher, H. & Zaidi, N. De novo lipogenesis in health and disease. *Metabolism* **63**, 895-902, doi:https://doi.org/10.1016/j.metabol.2014.04.003 (2014).
66 Song, Z., Xiaoli, A. M. & Yang, F. Regulation and Metabolic Significance of De Novo Lipogenesis in Adipose Tissues. *Nutrients* **10**, 1383, doi:10.3390/nu10101383 (2018).
67 Björntorp, P. & Sjöström, L. Carbohydrate storage in man: speculations and some quantitative considerations. *Metabolism* **27**, 1853-1865, doi:10.1016/s0026-0495(78)80004-3 (1978).
68 Ehehalt, R. *et al.* Uptake of long chain fatty acids is regulated by dynamic interaction of FAT/CD36 with cholesterol/sphingolipid enriched microdomains (lipid rafts). *BMC Cell Biol* **9**, 45-45, doi:10.1186/1471-2121-9-45 (2008).

69 Thompson, B. R., Lobo, S. & Bernlohr, D. A. Fatty acid flux in adipocytes: the in's and out's of fat cell lipid trafficking. *Molecular and cellular endocrinology* **318**, 24-33, doi:10.1016/j.mce.2009.08.015 (2010).
70 Rambold, A. S., Cohen, S. & Lippincott-Schwartz, J. Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics. *Dev Cell* **32**, 678-692, doi:10.1016/j.devcel.2015.01.029 (2015).
71 Console, L. *et al.* The Link Between the Mitochondrial Fatty Acid Oxidation Derangement and Kidney Injury. *Front Physiol* **11**, doi:10.3389/fphys.2020.00794 (2020).
72 Wegener, F. [Brown lipoma and brown fatty tissue in man]. *Beitr Pathol Anat* **3**, 252-266 (1951).
73 Nishio, M. & Saeki, K. The Remaining Mysteries about Brown Adipose Tissues. *Cells* **9**, 2449, doi:10.3390/cells9112449 (2020).
74 van Marken Lichtenbelt, W. D. *et al.* Cold-activated brown adipose tissue in healthy men. *N Engl J Med* **360**, 1500-1508, doi:10.1056/NEJMoa0808718 (2009).
75 Cinti, S. The role of brown adipose tissue in human obesity. *Nutr Metab Cardiovasc Dis* **16**, 569-574, doi:10.1016/j.numecd.2006.07.009 (2006).
76 Kershaw, E. E. & Flier, J. S. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* **89**, 2548-2556, doi:10.1210/jc.2004-0395 (2004).
77 Maliszewska, K. & Kretowski, A. Brown Adipose Tissue and Its Role in Insulin and Glucose Homeostasis. *Int J Mol Sci* **22**, 1530, doi:10.3390/ijms22041530 (2021).
78 Rothwell, N. J. & Stock, M. J. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* **281**, 31-35, doi:10.1038/281031a0 (1979).
79 Ricquier, D. Uncoupling protein 1 of brown adipocytes, the only uncoupler: a historical perspective. *Front Endocrinol (Lausanne)* **2**, 85-85, doi:10.3389/fendo.2011.00085 (2011).
80 Carpentier, A. C. *et al.* Brown Adipose Tissue Energy Metabolism in Humans. *Front Endocrinol (Lausanne)* **9**, 447-447, doi:10.3389/fendo.2018.00447 (2018).
81 Kahn, S. E., Hull, R. L. & Utzschneider, K. M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444**, 840-846, doi:10.1038/nature05482 (2006).
82 Hardy, O. T., Czech, M. P. & Corvera, S. What causes the insulin resistance underlying obesity? *Curr Opin Endocrinol Diabetes Obes* **19**, 81-87, doi:10.1097/MED.0b013e3283514e13 (2012).
83 DeFronzo, R. A. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* **37**, 667-687, doi:10.2337/diab.37.6.667 (1988).
84 DeFronzo, R. A. Pathogenesis of type 2 diabetes: Metabolic and molecular implications for identifying diabetes genes. *Diabetes Reviews* **5**, 177-269 (1997).
85 Ouchi, N., Parker, J. L., Lugus, J. J. & Walsh, K. Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology* **11**, 85-97, doi:10.1038/nri2921 (2011).
86 Pickup, J. C. & Crook, M. A. Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia* **41**, 1241-1248, doi:10.1007/s001250051058 (1998).
87 Ouchi, N., Kihara, S., Funahashi, T., Matsuzawa, Y. & Walsh, K. Obesity, adiponectin and vascular inflammatory disease. *Curr Opin Lipidol* **14**, 561-566, doi:10.1097/00041433-200312000-00003 (2003).
88 Berg, A. H. & Scherer, P. E. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res* **96**, 939-949, doi:10.1161/01.Res.0000163635.62927.34 (2005).

89 Saito, M. *et al.* High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* **58**, 1526-1531, doi:10.2337/db09-0530 (2009).
90 Shankar, K. *et al.* Role of brown adipose tissue in modulating adipose tissue inflammation and insulin resistance in high-fat diet fed mice. *Eur J Pharmacol* **854**, 354-364, doi:10.1016/j.ejphar.2019.02.044 (2019).
91 Dowal, L. *et al.* Intrinsic Properties of Brown and White Adipocytes Have Differential Effects on Macrophage Inflammatory Responses. *Mediators Inflamm* **2017**, 9067049, doi:10.1155/2017/9067049 (2017).
92 Tyagi, S., Gupta, P., Saini, A. S., Kaushal, C. & Sharma, S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *J Adv Pharm Technol Res* **2**, 236-240, doi:10.4103/2231-4040.90879 (2011).
93 Willson, T. M., Brown, P. J., Sternbach, D. D. & Henke, B. R. The PPARs: From Orphan Receptors to Drug Discovery. *Journal of Medicinal Chemistry* **43**, 527-550, doi:10.1021/jm990554g (2000).
94 Berger, J. P., Akiyama, T. E. & Meinke, P. T. PPARs: therapeutic targets for metabolic disease. *Trends Pharmacol Sci* **26**, 244-251, doi:10.1016/j.tips.2005.03.003 (2005).
95 Barter, P. J. & Rye, K.-A. Is There a Role for Fibrates in the Management of Dyslipidemia in the Metabolic Syndrome? *Arteriosclerosis, Thrombosis, and Vascular Biology* **28**, 39-46, doi:doi:10.1161/ATVBAHA.107.148817 (2008).
96 Bouwens, M., Afman, L. A. & Müller, M. Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid beta-oxidation: functional role of peroxisome proliferator activated receptor alpha in human peripheral blood mononuclear cells. *Am J Clin Nutr* **86**, 1515-1523, doi:10.1093/ajcn/86.5.1515 (2007).
97 Reilly, S. M. & Lee, C.-H. PPARδ as a therapeutic target in metabolic disease. *FEBS letters* **582**, 26-31 (2008).
98 Hevener, A. L. *et al.* Muscle-specific Pparg deletion causes insulin resistance. *Nature medicine* **9**, 1491 (2003).
99 Miles, P. D., Barak, Y., He, W., Evans, R. M. & Olefsky, J. M. Improved insulin-sensitivity in mice heterozygous for PPAR-γ deficiency. *Journal of Clinical Investigation* **105**, 287 (2000).
100 Fajas, L. *et al.* The organization, promoter analysis, and expression of the human PPARγ gene. *Journal of Biological Chemistry* **272**, 18779-18789 (1997).
101 Gavrilova, O. *et al.* Liver peroxisome proliferator-activated receptor γ contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *Journal of Biological Chemistry* **278**, 34268-34276 (2003).
102 Ricote, M. & Glass, C. K. PPARs and molecular mechanisms of transrepression. *Biochim Biophys Acta* **1771**, 926-935, doi:10.1016/j.bbalip.2007.02.013 (2007).
103 Yamashita, D. *et al.* The transactivating function of peroxisome proliferator-activated receptor γ is negatively regulated by SUMO conjugation in the amino-terminal domain. *Genes to Cells* **9**, 1017-1029, doi:10.1111/j.1365-2443.2004.00786.x (2004).
104 Rytinki, M. M. & Palvimo, J. J. SUMOylation attenuates the function of PGC-1α. *Journal of Biological Chemistry* **284**, 26184-26193 (2009).
105 Hay, R. T. SUMO: a history of modification. *Molecular cell* **18**, 1-12 (2005).

106 Kurepa, J. *et al.* The Small Ubiquitin-like Modifier (SUMO) Protein Modification System in Arabidopsis: ACCUMULATION OF SUMO1 AND -2 CONJUGATES IS INCREASED BY STRESS *. *Journal of Biological Chemistry* **278**, 6862-6872, doi:10.1074/jbc.M209694200 (2003).
107 Eaton, E. M. & Sealy, L. Modification of CCAAT/Enhancer-binding Protein-β by the Small Ubiquitin-like Modifier (SUMO) Family Members, SUMO-2 and SUMO-3 *. *Journal of Biological Chemistry* **278**, 33416-33421, doi:10.1074/jbc.M305680200 (2003).
108 Barry, J. & Lock, R. B. Small ubiquitin-related modifier-1: Wrestling with protein regulation. *The International Journal of Biochemistry & Cell Biology* **43**, 37-40, doi:https://doi.org/10.1016/j.biocel.2010.09.022 (2011).
109 Liang, Y.-C. *et al.* SUMO5, a Novel Poly-SUMO Isoform, Regulates PML Nuclear Bodies. *Scientific Reports* **6**, 26509, doi:10.1038/srep26509 (2016).
110 Bohren, K. M., Nadkarni, V., Song, J. H., Gabbay, K. H. & Owerbach, D. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem* **279**, 27233-27238, doi:10.1074/jbc.M402273200 (2004).
111 Wadosky, K. M. & Willis, M. S. The story so far: post-translational regulation of peroxisome proliferator-activated receptors by ubiquitination and SUMOylation. *American Journal of Physiology-Heart and Circulatory Physiology* **302**, H515-H526, doi:10.1152/ajpheart.00703.2011 (2012).
112 Huang, C. *et al.* SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. *The EMBO journal* **28**, 2748-2762 (2009).
113 Nayak, A. & Müller, S. SUMO-specific proteases/isopeptidases: SENPs and beyond. *Genome biology* **15**, 422 (2014).
114 Kang, X. *et al.* SUMO-specific protease 2 is essential for suppression of polycomb group protein-mediated gene silencing during embryonic development. *Molecular cell* **38**, 191-201 (2010).
115 Reverter, D. & Lima, C. D. Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates. *Nature structural & molecular biology* **13**, 1060 (2006).
116 Békés, M. *et al.* The dynamics and mechanism of SUMO chain deconjugation by SUMO-specific proteases. *Journal of Biological Chemistry* **286**, 10238-10247 (2011).
117 Mikolajczyk, J. *et al.* Small Ubiquitin-related Modifier (SUMO)-specific Proteases PROFILING THE SPECIFICITIES AND ACTIVITIES OF HUMAN SENPs. *Journal of Biological Chemistry* **282**, 26217-26224 (2007).
118 Chung, Sung S. *et al.* SUMO modification selectively regulates transcriptional activity of peroxisome-proliferator-activated receptor γ in C2C12 myotubes. *Biochemical Journal* **433**, 155-161, doi:10.1042/bj20100749 (2011).
119 Koo, Y. D. *et al.* SUMO-Specific Protease 2 (SENP2) Is an Important Regulator of Fatty Acid Metabolism in Skeletal Muscle. *Diabetes* **64**, 2420-2431, doi:10.2337/db15-0115 (2015).
120 Pelleymounter, M. A. *et al.* Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* **269**, 540-543, doi:10.1126/science.7624776 (1995).
121 Zhang, Y. *et al.* Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432, doi:10.1038/372425a0 (1994).

122 Koo, Y. D. *et al.* SUMO-specific protease 2 mediates leptin-induced fatty acid oxidation in skeletal muscle. *Metabolism* **95**, 27-35, doi:https://doi.org/10.1016/j.metabol.2019.03.004 (2019).
123 Perry, R. J. *et al.* Leptin Mediates a Glucose-Fatty Acid Cycle to Maintain Glucose Homeostasis in Starvation. *Cell* **172**, 234-248.e217, doi:10.1016/j.cell.2017.12.001 (2018).
124 Martí, A., Berraondo, B. & Martínez, J. A. Leptin: physiological actions. *J Physiol Biochem* **55**, 43-49 (1999).
125 Minokoshi, Y. *et al.* Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* **415**, 339-343, doi:10.1038/415339a (2002).
126 Chung, S. S. *et al.* Control of Adipogenesis by the SUMO-Specific Protease SENP2. *Molecular and Cellular Biology* **30**, 2135-2146, doi:10.1128/MCB.00852-09 (2010).
127 Lee, J. S. *et al.* SUMO-specific protease 2 (SENP2) suppresses browning of white adipose tissue through C/EBPβ modulation. *bioRxiv*, 2020.2012.2016.422969, doi:10.1101/2020.12.16.422969 (2020).
128 Zheng, Q. *et al.* Senp2 regulates adipose lipid storage by de-SUMOylation of Setdb1. *Journal of Molecular Cell Biology* **10**, 258-266, doi:10.1093/jmcb/mjx055 (2018).
129 Röder, P. V., Wu, B., Liu, Y. & Han, W. Pancreatic regulation of glucose homeostasis. *Exp Mol Med* **48**, e219-e219, doi:10.1038/emm.2016.6 (2016).
130 Brissova, M. *et al.* Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem* **53**, 1087-1097, doi:10.1369/jhc.5C6684.2005 (2005).
131 Katsuura, G., Asakawa, A. & Inui, A. Roles of pancreatic polypeptide in regulation of food intake. *Peptides* **23**, 323-329, doi:10.1016/s0196-9781(01)00604-0 (2002).
132 Freychet, L. *et al.* Effect of intranasal glucagon on blood glucose levels in healthy subjects and hypoglycaemic patients with insulin-dependent diabetes. *Lancet* **1**, 1364-1366, doi:10.1016/s0140-6736(88)92181-2 (1988).
133 Komatsu, M., Takei, M., Ishii, H. & Sato, Y. Glucose-stimulated insulin secretion: A newer perspective. *J Diabetes Investig* **4**, 511-516, doi:10.1111/jdi.12094 (2013).
134 Sibrowski, W. & Seitz, H. J. Rapid action of insulin and cyclic AMP in the regulation of functional messenger RNA coding for glucokinase in rat liver. *J Biol Chem* **259**, 343-346 (1984).
135 Walton, P. E. & Etherton, T. D. Stimulation of lipogenesis by insulin in swine adipose tissue: antagonism by porcine growth hormone. *J Anim Sci* **62**, 1584-1595, doi:10.2527/jas1986.6261584x (1986).
136 Biolo, G., Declan Fleming, R. Y. & Wolfe, R. R. Physiologic hyperinsulinemia stimulates protein synthesis and enhances transport of selected amino acids in human skeletal muscle. *The Journal of clinical investigation* **95**, 811-819, doi:10.1172/JCI117731 (1995).
137 Kahn, B. B. Adipose Tissue, Inter-Organ Communication, and the Path to Type 2 Diabetes: The 2016 Banting Medal for Scientific Achievement Lecture. *Diabetes* **68**, 3-14, doi:10.2337/dbi18-0035 (2019).
138 Pandiri, A. R. Overview of exocrine pancreatic pathobiology. *Toxicol Pathol* **42**, 207-216, doi:10.1177/0192623313509907 (2014).
139 Karpińska, M. & Czauderna, M. Pancreas-Its Functions, Disorders, and Physiological Impact on the Mammals' Organism. *Front Physiol* **13**, 807632, doi:10.3389/fphys.2022.807632 (2022).

140 Adamska, A., Domenichini, A. & Falasca, M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. *Int J Mol Sci* **18**, 1338, doi:10.3390/ijms18071338 (2017).
141 World health organization (WHO). *Cancer*, <https://www.who.int/news-room/fact-sheets/detail/cancer> (2022).
142 Giovannucci, E. *et al.* Diabetes and cancer: a consensus report. *Diabetes care* **33**, 1674-1685, doi:10.2337/dc10-0666 (2010).
143 Cannata, D., Fierz, Y., Vijayakumar, A. & LeRoith, D. Type 2 diabetes and cancer: what is the connection? *The Mount Sinai journal of medicine, New York* **77**, 197-213, doi:10.1002/msj.20167 (2010).
144 Huxley, R., Ansary-Moghaddam, A., Berrington de González, A., Barzi, F. & Woodward, M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. *British Journal of Cancer* **92**, 2076-2083, doi:10.1038/sj.bjc.6602619 (2005).
145 Ben, Q. *et al.* Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. *European Journal of Cancer* **47**, 1928-1937, doi:10.1016/j.ejca.2011.03.003 (2011).
146 Perreault, L., Skyler, J. S. & Rosenstock, J. Novel therapies with precision mechanisms for type 2 diabetes mellitus. *Nat Rev Endocrinol* **17**, 364-377, doi:10.1038/s41574-021-00489-y (2021).
147 Targher, G., Corey, K. E., Byrne, C. D. & Roden, M. The complex link between NAFLD and type 2 diabetes mellitus — mechanisms and treatments. *Nature Reviews Gastroenterology & Hepatology* **18**, 599-612, doi:10.1038/s41575-021-00448-y (2021).
148 Wang, F., Herrington, M., Larsson, J. & Permert, J. The relationship between diabetes and pancreatic cancer. *Molecular Cancer* **2**, 4, doi:10.1186/1476-4598-2-4 (2003).
149 Basso, D. *et al.* Altered glucose metabolism and proteolysis in pancreatic cancer cell conditioned myoblasts: searching for a gene expression pattern with a microarray analysis of 5000 skeletal muscle genes. *Gut* **53**, 1159-1166, doi:10.1136/gut.2003.024471 (2004).
150 Permert, J. *et al.* Is profound peripheral insulin resistance in patients with pancreatic cancer caused by a tumor-associated factor? *The American Journal of Surgery* **165**, 61-67, doi:https://doi.org/10.1016/S0002-9610(05)80405-2 (1993).
151 Liu, J. *et al.* The Intracellular Mechanism of Insulin Resistance in Pancreatic Cancer Patients*. *The Journal of Clinical Endocrinology & Metabolism* **85**, 1232-1238, doi:10.1210/jcem.85.3.6400 (2000).
152 Adami, H. O. *et al.* Cancer risk in patients with diabetes mellitus. *Cancer Causes Control* **2**, 307-314, doi:10.1007/bf00051670 (1991).
153 Gullo, L., Pezzilli, R. & Morselli-Labate, A. M. Diabetes and the risk of pancreatic cancer. *N Engl J Med* **331**, 81-84, doi:10.1056/nejm199407143310203 (1994).
154 Huxley, R., Ansary-Moghaddam, A., Berrington de González, A., Barzi, F. & Woodward, M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. *Br J Cancer* **92**, 2076-2083, doi:10.1038/sj.bjc.6602619 (2005).
155 Li, Y., Bian, X., Wei, S., He, M. & Yang, Y. The relationship between pancreatic cancer and type 2 diabetes: cause and consequence. *Cancer Manag Res* **11**, 8257-8268, doi:10.2147/CMAR.S211972 (2019).
156 Pannala, R. *et al.* Prevalence and clinical profile of pancreatic cancer-associated diabetes mellitus. *Gastroenterology* **134**, 981-987, doi:10.1053/j.gastro.2008.01.039 (2008).

157 Goodwin, P. J. Insulin in the adjuvant breast cancer setting: a novel therapeutic target for lifestyle and pharmacologic interventions? *J Clin Oncol* **26**, 833-834, doi:10.1200/jco.2007.14.7132 (2008).
158 Renehan, A. G. *et al.* Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* **363**, 1346-1353, doi:10.1016/s0140-6736(04)16044-3 (2004).
159 Mittelbrunn, M. & Sánchez-Madrid, F. Intercellular communication: diverse structures for exchange of genetic information. *Nat Rev Mol Cell Biol* **13**, 328-335, doi:10.1038/nrm3335 (2012).
160 Pedersen, B. K. *et al.* Searching for the exercise factor: is IL-6 a candidate? *J Muscle Res Cell Motil* **24**, 113-119, doi:10.1023/a:1026070911202 (2003).
161 Pedersen, L. *et al.* Exercise-induced liver chemokine CXCL-1 expression is linked to muscle-derived interleukin-6 expression. *J Physiol* **589**, 1409-1420, doi:10.1113/jphysiol.2010.200733 (2011).
162 Pedersen, B. K., Akerstrom, T. C., Nielsen, A. R. & Fischer, C. P. Role of myokines in exercise and metabolism. *J Appl Physiol (1985)* **103**, 1093-1098, doi:10.1152/japplphysiol.00080.2007 (2007).
163 Catoire, M. & Kersten, S. The search for exercise factors in humans. *The FASEB Journal* **29**, 1615-1628, doi:10.1096/fj.14-263699 (2015).
164 Sandri, M. Protein breakdown in cancer cachexia. *Seminars in Cell & Developmental Biology* **54**, 11-19, doi:https://doi.org/10.1016/j.semcdb.2015.11.002 (2016).
165 Rohm, M., Zeigerer, A., Machado, J. & Herzig, S. Energy metabolism in cachexia. *EMBO Rep* **20**, e47258, doi:10.15252/embr.201847258 (2019).
166 Siddiqui, J. A., Pothuraju, R., Jain, M., Batra, S. K. & Nasser, M. W. Advances in cancer cachexia: Intersection between affected organs, mediators, and pharmacological interventions. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1873**, 188359, doi:https://doi.org/10.1016/j.bbcan.2020.188359 (2020).
167 Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *The Lancet Oncology* **12**, 489-495, doi:https://doi.org/10.1016/S1470-2045(10)70218-7 (2011).
168 María-Teresa Arango, P. Q.-R., John Castiblanco, and Gladis Montoya-Ortíz. in *Cell culture and cell analysis* (ed Shoenfeld Y Anaya JM, Rojas-Villarraga A,) Ch. Chapter 45, (El Rosario University Press, 2013).
169 Timmers, S., Schrauwen, P. & de Vogel, J. Muscular diacylglycerol metabolism and insulin resistance. *Physiol Behav* **94**, 242-251, doi:10.1016/j.physbeh.2007.12.002 (2008).
170 Kelley, D. E., Goodpaster, B., Wing, R. R. & Simoneau, J. A. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* **277**, E1130-1141, doi:10.1152/ajpendo.1999.277.6.E1130 (1999).
171 Henry, R. R., Abrams, L., Nikoulina, S. & Ciaraldi, T. P. Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects. Comparison using human skeletal muscle cell cultures. *Diabetes* **44**, 936-946, doi:10.2337/diab.44.8.936 (1995).
172 Blaak, E. E., van Aggel-Leijssen, D. P., Wagenmakers, A. J., Saris, W. H. & van Baak, M. A. Impaired oxidation of plasma-derived fatty acids in type 2 diabetic subjects during moderate-intensity exercise. *Diabetes* **49**, 2102-2107, doi:10.2337/diabetes.49.12.2102 (2000).

173 Gaster, M., Rustan, A. C., Aas, V. & Beck-Nielsen, H. Reduced Lipid Oxidation in Skeletal Muscle From Type 2 Diabetic Subjects May Be of Genetic Origin. *Diabetes* **53**, 542-548 (2004).
174 Al-Khalili, L. *et al.* Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. *Cell Mol Life Sci* **60**, 991-998, doi:10.1007/s00018-003-3001-3 (2003).
175 Muoio, D. M. *et al.* Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. *Diabetes* **51**, 901-909, doi:10.2337/diabetes.51.4.901 (2002).
176 Berggren, J. R., Tanner, C. J. & Houmard, J. A. Primary cell cultures in the study of human muscle metabolism. *Exerc Sport Sci Rev* **35**, 56-61, doi:10.1249/JES.0b013e31803eae63 (2007).
177 Eckardt, K., Taube, A. & Eckel, J. Obesity-associated insulin resistance in skeletal muscle: role of lipid accumulation and physical inactivity. *Rev Endocr Metab Disord* **12**, 163-172, doi:10.1007/s11154-011-9168-2 (2011).
178 Sarabia, V., Lam, L., Burdett, E., Leiter, L. A. & Klip, A. Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin. *J Clin Invest* **90**, 1386-1395, doi:10.1172/jci116005 (1992).
179 Regitz-Zagrosek, V., Lehmkuhl, E. & Weickert, M. O. Gender differences in the metabolic syndrome and their role for cardiovascular disease. *Clin Res Cardiol* **95**, 136-147, doi:10.1007/s00392-006-0351-5 (2006).
180 Aas, V., Torblå, S., Andersen, M. H., Jensen, J. & Rustan, A. C. Electrical stimulation improves insulin responses in a human skeletal muscle cell model of hyperglycemia. *Ann N Y Acad Sci* **967**, 506-515, doi:10.1111/j.1749-6632.2002.tb04309.x (2002).
181 Gaster, M., Petersen, I., Højlund, K., Poulsen, P. & Beck-Nielsen, H. The diabetic phenotype is conserved in myotubes established from diabetic subjects: evidence for primary defects in glucose transport and glycogen synthase activity. *Diabetes* **51**, 921-927, doi:10.2337/diabetes.51.4.921 (2002).
182 Henry, R. R. *et al.* Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects. Biochemical and molecular mechanisms. *The Journal of Clinical Investigation* **98**, 1231-1236, doi:10.1172/JCI118906 (1996).
183 Bourlier, V. *et al.* Enhanced glucose metabolism is preserved in cultured primary myotubes from obese donors in response to exercise training. *J Clin Endocrinol Metab* **98**, 3739-3747, doi:10.1210/jc.2013-1727 (2013).
184 Wensaas, A. J. *et al.* Fatty acid incubation of myotubes from humans with type 2 diabetes leads to enhanced release of beta-oxidation products because of impaired fatty acid oxidation: effects of tetradecylthioacetic acid and eicosapentaenoic acid. *Diabetes* **58**, 527-535, doi:10.2337/db08-1043 (2009).
185 Corpeleijn, E. *et al.* Oxidation of intramyocellular lipids is dependent on mitochondrial function and the availability of extracellular fatty acids. *American Journal of Physiology-Endocrinology and Metabolism* **299**, E14-E22 (2010).
186 Ukropcova, B. *et al.* Dynamic changes in fat oxidation in human primary myocytes mirror metabolic characteristics of the donor. *Journal of Clinical Investigation* **115**, 1934 (2005).

187 Bonavaud, S. *et al.* A discrepancy resolved: human satellite cells are not preprogrammed to fast and slow lineages. *Neuromuscul Disord* **11**, 747-752, doi:10.1016/s0960-8966(01)00222-x (2001).
188 Aas, V. *et al.* Are cultured human myotubes far from home? *Cell and tissue research* **354**, 671-682 (2013).
189 Nehlin, J. O., Just, M., Rustan, A. C. & Gaster, M. Human myotubes from myoblast cultures undergoing senescence exhibit defects in glucose and lipid metabolism. *Biogerontology* **12**, 349-365, doi:10.1007/s10522-011-9336-5 (2011).
190 Gaster, M., Kristensen, S. R., Beck-Nielsen, H. & Schroder, H. D. A cellular model system of differentiated human myotubes. *Apmis* **109**, 735-744, doi:10.1034/j.1600-0463.2001.d01-140.x (2001).
191 GREGOIRE, F. M., SMAS, C. M. & SUL, H. S. Understanding Adipocyte Differentiation. *Physiological Reviews* **78**, 783-809, doi:10.1152/physrev.1998.78.3.783 (1998).
192 Lee, M.-J., Wu, Y. & Fried, S. K. A Modified Protocol to Maximize Differentiation of Human Preadipocytes and Improve Metabolic Phenotypes. *Obesity* **20**, 2334-2340, doi:https://doi.org/10.1038/oby.2012.116 (2012).
193 Ntambi, J. M. & Young-Cheul, K. Adipocyte Differentiation and Gene Expression. *The Journal of Nutrition* **130**, 3122S-3126S, doi:10.1093/jn/130.12.3122S (2000).
194 Obregon, M. J. Thyroid hormone and adipocyte differentiation. *Thyroid* **18**, 185-195, doi:10.1089/thy.2007.0254 (2008).
195 Nie, T. *et al.* Reprogramming mature terminally differentiated adipocytes to induced pluripotent stem cells. *Science Bulletin* **60**, 1752-1758, doi:https://doi.org/10.1007/s11434-015-0796-x (2015).
196 Flynn, L. & Woodhouse, K. A. Adipose tissue engineering with cells in engineered matrices. *Organogenesis* **4**, 228-235, doi:10.4161/org.4.4.7082 (2008).
197 Harms, M. J. *et al.* Mature Human White Adipocytes Cultured under Membranes Maintain Identity, Function, and Can Transdifferentiate into Brown-like Adipocytes. *Cell Reports* **27**, 213-225.e215, doi:https://doi.org/10.1016/j.celrep.2019.03.026 (2019).
198 Fernyhough, M. E., Hausman, G. J. & Dodson, M. V. Progeny from Dedifferentiated Bovine Adipocytes Display Protracted Adipogenesis. *Cells Tissues Organs* **188**, 359-372, doi:10.1159/000134007 (2008).
199 Hausman, G. J. *et al.* BOARD-INVITED REVIEW: The biology and regulation of preadipocytes and adipocytes in meat animals1,2. *Journal of Animal Science* **87**, 1218-1246, doi:10.2527/jas.2008-1427 (2009).
200 Hausman, G. J., Wright, J. T. & Richardson, R. L. The influence of extracellular matrix substrata on preadipocyte development in serum-free cultures of stromal-vascular cells. *J Anim Sci* **74**, 2117-2128, doi:10.2527/1996.7492117x (1996).
201 Poulos, S. P., Dodson, M. V. & Hausman, G. J. Cell line models for differentiation: preadipocytes and adipocytes. *Experimental Biology and Medicine* **235**, 1185-1193, doi:10.1258/ebm.2010.010063 (2010).
202 Zvonic, S. *et al.* Secretome of Primary Cultures of Human Adipose-derived Stem Cells: Modulation of Serpins by Adipogenesis * S. *Molecular & Cellular Proteomics* **6**, 18-28, doi:10.1074/mcp.M600217-MCP200 (2007).
203 Dufau, J. *et al.* In vitro and ex vivo models of adipocytes. *American Journal of Physiology-Cell Physiology* **320**, C822-C841, doi:10.1152/ajpcell.00519.2020 (2021).

204 Mieczkowska, A. *et al.* Immunophenotyping and transcriptional profiling of in vitro cultured human adipose tissue derived stem cells. *Scientific Reports* **8**, 11339, doi:10.1038/s41598-018-29477-5 (2018).
205 Aas, V., Thoresen, G. H., Rustan, A. C. & Lund, J. Substrate oxidation in primary human skeletal muscle cells is influenced by donor age. *Cell Tissue Res* **382**, 599-608, doi:10.1007/s00441-020-03275-w (2020).
206 Lanza, I. R. & Nair, K. S. Muscle mitochondrial changes with aging and exercise. *Am J Clin Nutr* **89**, 467s-471s, doi:10.3945/ajcn.2008.26717D (2009).
207 Johannsen, D. L. *et al.* Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *J Clin Endocrinol Metab* **97**, 242-250, doi:10.1210/jc.2011-1798 (2012).
208 Crane, J. D., Devries, M. C., Safdar, A., Hamadeh, M. J. & Tarnopolsky, M. A. The effect of aging on human skeletal muscle mitochondrial and intramyocellular lipid ultrastructure. *J Gerontol A Biol Sci Med Sci* **65**, 119-128, doi:10.1093/gerona/glp179 (2010).
209 Jana, B. A., Chintamaneni, P. K., Krishnamurthy, P. T., Wadhwani, A. & Mohankumar, S. K. Cytosolic lipid excess-induced mitochondrial dysfunction is the cause or effect of high fat diet-induced skeletal muscle insulin resistance: a molecular insight. *Molecular Biology Reports* **46**, 957-963, doi:10.1007/s11033-018-4551-7 (2019).
210 Feng, Y. Z. *et al.* Myotubes from lean and severely obese subjects with and without type 2 diabetes respond differently to an in vitro model of exercise. *Am J Physiol Cell Physiol* **308**, C548-556, doi:10.1152/ajpcell.00314.2014 (2015).
211 Hellström, L. & Reynisdottir, S. Influence of heredity for obesity on adipocyte lipolysis in lean and obese subjects. *International Journal of Obesity* **24**, 340-344, doi:10.1038/sj.ijo.0801134 (2000).
212 Guilherme, A., Virbasius, J. V., Puri, V. & Czech, M. P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* **9**, 367-377, doi:10.1038/nrm2391 (2008).
213 Keuper, M. *et al.* Preadipocytes of obese humans display gender-specific bioenergetic responses to glucose and insulin. *Molecular metabolism* **20**, 28-37, doi:10.1016/j.molmet.2018.11.006 (2019).
214 Wang, Z. *et al.* Evaluation of specific metabolic rates of major organs and tissues: comparison between men and women. *Am J Hum Biol* **23**, 333-338, doi:10.1002/ajhb.21137 (2011).
215 Salehzadeh, F., Rune, A., Osler, M. & Al-Khalili, L. Testosterone or 17β-estradiol exposure reveals sex-specific effects on glucose and lipid metabolism in human myotubes. *Journal of Endocrinology* **210**, 219-229, doi:10.1530/JOE-10-0497 (2011).
216 Garrido, P., Salehzadeh, F., Duque-Guimaraes, D. E. & Al-Khalili, L. Negative regulation of glucose metabolism in human myotubes by supraphysiological doses of 17β-estradiol or testosterone. *Metabolism* **63**, 1178-1187, doi:10.1016/j.metabol.2014.06.003 (2014).
217 Lieber, M., Mazzetta, J., Nelson-Rees, W., Kaplan, M. & Todaro, G. Establishment of a continuous tumor-cell line (PANC-1) from a human carcinoma of the exocrine pancreas. *International Journal of Cancer* **15**, 741-747, doi:https://doi.org/10.1002/ijc.2910150505 (1975).
218 Deer, E. L. *et al.* Phenotype and genotype of pancreatic cancer cell lines. *Pancreas* **39**, 425-435, doi:10.1097/MPA.0b013e3181c15963 (2010).

219 Mirabelli, P., Coppola, L. & Salvatore, M. Cancer Cell Lines Are Useful Model Systems for Medical Research. *Cancers (Basel)* **11**, doi:10.3390/cancers11081098 (2019).
220 Gillet, J. P., Varma, S. & Gottesman, M. M. The clinical relevance of cancer cell lines. *J Natl Cancer Inst* **105**, 452-458, doi:10.1093/jnci/djt007 (2013).
221 Deer, E. L. *et al.* Phenotype and genotype of pancreatic cancer cell lines. *Pancreas* **39**, 425-435, doi:10.1097/MPA.0b013e3181c15963 (2010).
222 Oda, D., Savard, C. E., Nguyen, T. D., Swenson, E. R. & Lee, S. P. Culture of human main pancreatic duct epithelial cells. *In Vitro Cell Dev Biol Anim* **34**, 211-216, doi:10.1007/s11626-998-0126-6 (1998).
223 Ouyang, H. *et al.* Immortal Human Pancreatic Duct Epithelial Cell Lines with Near Normal Genotype and Phenotype. *The American Journal of Pathology* **157**, 1623-1631, doi:https://doi.org/10.1016/S0002-9440(10)64800-6 (2000).
224 Liu, N., Furukawa, T., Kobari, M. & Tsao, M.-S. Comparative Phenotypic Studies of Duct Epithelial Cell Lines Derived from Normal Human Pancreas and Pancreatic Carcinoma. *The American Journal of Pathology* **153**, 263-269, doi:https://doi.org/10.1016/S0002-9440(10)65567-8 (1998).
225 Pastor, D. M. *et al.* Primary cell lines: false representation or model system? a comparison of four human colorectal tumors and their coordinately established cell lines. *Int J Clin Exp Med* **3**, 69-83 (2010).
226 Borawski, J., Lindeman, A., Buxton, F., Labow, M. & Gaither, L. A. Optimization procedure for small interfering RNA transfection in a 384-well format. *J Biomol Screen* **12**, 546-559, doi:10.1177/1087057107300172 (2007).
227 Yamano, S., Dai, J. & Moursi, A. M. Comparison of transfection efficiency of nonviral gene transfer reagents. *Mol Biotechnol* **46**, 287-300, doi:10.1007/s12033-010-9302-5 (2010).
228 Shi, B. *et al.* An improved method for increasing the efficiency of gene transfection and transduction. *Int J Physiol Pathophysiol Pharmacol* **10**, 95-104 (2018).
229 Kim, T. K. & Eberwine, J. H. Mammalian cell transfection: the present and the future. *Anal Bioanal Chem* **397**, 3173-3178, doi:10.1007/s00216-010-3821-6 (2010).
230 Lufino, M. M., Edser, P. A. & Wade-Martins, R. Advances in high-capacity extrachromosomal vector technology: episomal maintenance, vector delivery, and transgene expression. *Mol Ther* **16**, 1525-1538, doi:10.1038/mt.2008.156 (2008).
231 Fus-Kujawa, A. *et al.* An Overview of Methods and Tools for Transfection of Eukaryotic Cells in vitro. *Frontiers in Bioengineering and Biotechnology* **9**, doi:10.3389/fbioe.2021.701031 (2021).
232 Han, H. RNA Interference to Knock Down Gene Expression. *Methods in molecular biology (Clifton, N.J.)* **1706**, 293-302, doi:10.1007/978-1-4939-7471-9_16 (2018).
233 Moore, C. B., Guthrie, E. H., Huang, M. T. & Taxman, D. J. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol* **629**, 141-158, doi:10.1007/978-1-60761-657-3_10 (2010).
234 Lund, J. *et al.* Exercise in vivo marks human myotubes in vitro: Training-induced increase in lipid metabolism. *PloS one* **12**, e0175441 (2017).
235 Wensaas, A. *et al.* Cell-based multiwell assays for the detection of substrate accumulation and oxidation. *Journal of lipid research* **48**, 961-967 (2007).

236 Skrede, S., Bremer, J., Berge, R. & Rustan, A. Stimulation of fatty acid oxidation by a 3-thia fatty acid reduces triacylglycerol secretion in cultured rat hepatocytes. *Journal of lipid research* **35**, 1395-1404 (1994).
237 Bakke, S. S. *et al.* Palmitic acid follows a different metabolic pathway than oleic acid in human skeletal muscle cells; lower lipolysis rate despite an increased level of adipose triglyceride lipase. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1821**, 1323-1333 (2012).
238 Folch, J., Lees, M. & Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497-509 (1957).
239 Hessvik, N. P. *et al.* Metabolic switching of human myotubes is improved by n-3 fatty acids. *Journal of lipid research* **51**, 2090-2104 (2010).
240 Arya, M. *et al.* Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn* **5**, 209-219, doi:10.1586/14737159.5.2.209 (2005).
241 Smith, C. J. & Osborn, A. M. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol* **67**, 6-20, doi:10.1111/j.1574-6941.2008.00629.x (2009).
242 Adams, G. A beginner's guide to RT-PCR, qPCR and RT-qPCR. *The Biochemist* **42**, 48-53, doi:10.1042/bio20200034 (2020).
243 Tajadini, M., Panjehpour, M. & Javanmard, S. H. Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Adv Biomed Res* **3**, 85-85, doi:10.4103/2277-9175.127998 (2014).
244 Mishra, M., Tiwari, S. & Gomes, A. V. Protein purification and analysis: next generation Western blotting techniques. *Expert Rev Proteomics* **14**, 1037-1053, doi:10.1080/14789450.2017.1388167 (2017).
245 Aebersold, R. & Mann, M. Mass spectrometry-based proteomics. *Nature* **422**, 198-207, doi:10.1038/nature01511 (2003).
246 Aslam, B., Basit, M., Nisar, M. A., Khurshid, M. & Rasool, M. H. Proteomics: Technologies and Their Applications. *Journal of Chromatographic Science* **55**, 182-196, doi:10.1093/chromsci/bmw167 (2017).
247 Deracinois, B., Flahaut, C., Duban-Deweer, S. & Karamanos, Y. Comparative and Quantitative Global Proteomics Approaches: An Overview. *Proteomes* **1**, 180-218, doi:10.3390/proteomes1030180 (2013).
248 Gupta, A. & Kumar, A. in *Biomedical Journal* Vol. 37 163 (2014).
249 Angel, T. E. *et al.* Mass spectrometry-based proteomics: existing capabilities and future directions. *Chem Soc Rev* **41**, 3912-3928, doi:10.1039/c2cs15331a (2012).
250 Paré, B., Deschênes, L. T., Pouliot, R., Dupré, N. & Gros-Louis, F. An Optimized Approach to Recover Secreted Proteins from Fibroblast Conditioned-Media for Secretomic Analysis. *Frontiers in Cellular Neuroscience* **10**, doi:10.3389/fncel.2016.00070 (2016).
251 Scicchitano, B. M., Dobrowolny, G., Sica, G. & Musarò, A. Molecular Insights into Muscle Homeostasis, Atrophy and Wasting. *Curr Genomics* **19**, 356-369, doi:10.2174/1389202919666180101153911 (2018).
252 Mengeste, A. M., Rustan, A. C. & Lund, J. Skeletal muscle energy metabolism in obesity. *Obesity* **29**, 1582-1595, doi:https://doi.org/10.1002/oby.23227 (2021).
253 Hargreaves, M. & Spriet, L. L. Skeletal muscle energy metabolism during exercise. *Nature Metabolism* **2**, 817-828, doi:10.1038/s42255-020-0251-4 (2020).

254 Kopecký, J. *et al.* Energy metabolism of adipose tissue--physiological aspects and target in obesity treatment. *Physiol Res* **53 Suppl 1**, S225-232 (2004).
255 Luo, L. & Liu, M. Adipose tissue in control of metabolism. *Journal of Endocrinology* **231**, R77-R99, doi:10.1530/JOE-16-0211 (2016).
256 DeFronzo, R. A. & Tripathy, D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes care* **32 Suppl 2**, S157-S163, doi:10.2337/dc09-S302 (2009).
257 Sylow, L., Tokarz, V. L., Richter, E. A. & Klip, A. The many actions of insulin in skeletal muscle, the paramount tissue determining glycemia. *Cell Metabolism* **33**, 758-780, doi:https://doi.org/10.1016/j.cmet.2021.03.020 (2021).
258 Liang, Q. *et al.* SENP2 Suppresses Necdin Expression to Promote Brown Adipocyte Differentiation. *Cell Reports* **28**, 2004-2011.e2004, doi:https://doi.org/10.1016/j.celrep.2019.07.083 (2019).
259 Chinwalla, A. T. *et al.* Initial sequencing and comparative analysis of the mouse genome. *Nature* **420**, 520-562, doi:10.1038/nature01262 (2002).
260 Perlman, R. L. Mouse models of human disease: An evolutionary perspective. *Evol Med Public Health* **2016**, 170-176, doi:10.1093/emph/eow014 (2016).
261 Brown, S. D. M. & Hancock, J. M. The mouse genome. *Genome Dyn* **2**, 33-45, doi:10.1159/000095091 (2006).
262 Zhang, Q., Zeng, Y., Zhang, T. & Yang, T. Comparison Between Human and Rodent Neurons for Persistent Activity Performance: A Biologically Plausible Computational Investigation. *Frontiers in Systems Neuroscience* **15**, doi:10.3389/fnsys.2021.628839 (2021).
263 Dolenšek, J., Rupnik, M. S. & Stožer, A. Structural similarities and differences between the human and the mouse pancreas. *Islets* **7**, e1024405, doi:10.1080/19382014.2015.1024405 (2015).
264 Eizirik, D. L. *et al.* Major species differences between humans and rodents in the susceptibility to pancreatic beta-cell injury. *Proc Natl Acad Sci U S A* **91**, 9253-9256, doi:10.1073/pnas.91.20.9253 (1994).
265 Wanner, S. P. *et al.* Thermoregulatory responses in exercising rats: methodological aspects and relevance to human physiology. *Temperature (Austin)* **2**, 457-475, doi:10.1080/23328940.2015.1119615 (2015).
266 DeFronzo, R. A., Gunnarsson, R., Björkman, O., Olsson, M. & Wahren, J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest* **76**, 149-155, doi:10.1172/jci111938 (1985).
267 Kahn, B. B. Lilly lecture 1995. Glucose transport: pivotal step in insulin action. *Diabetes* **45**, 1644-1654, doi:10.2337/diab.45.11.1644 (1996).
268 White, U. A., Coulter, A. A., Miles, T. K. & Stephens, J. M. The STAT5A-Mediated Induction of Pyruvate Dehydrogenase Kinase 4 Expression by Prolactin or Growth Hormone in Adipocytes. *Diabetes* **56**, 1623-1629, doi:10.2337/db06-1286 (2007).
269 Pettersen, I. *et al.* Increased PDK4 mRNA expression is a sensitive marker of upregulated fatty acid oxidation. *Mitochondrion* **49**, doi:10.1016/j.mito.2019.07.009 (2019).
270 Virtanen, K. *et al.* Increased Fat Mass Compensates for Insulin Resistance in Abdominal Obesity and Type 2 Diabetes A Positron-Emitting Tomography Study. *Diabetes* **54**, 2720-2726, doi:10.2337/diabetes.54.9.2720 (2005).
271 Lawrence, J. C., Jr & Roach, P. J. New Insights Into the Role and Mechanism of Glycogen Synthase Activation by Insulin. *Diabetes* **46**, 541-547, doi:10.2337/diab.46.4.541 (1997).

272 Hemmings, B. A. & Restuccia, D. F. PI3K-PKB/Akt pathway. *Cold Spring Harb Perspect Biol* **4**, a011189, doi:10.1101/cshperspect.a011189 (2012).
273 Crosson, S. M., Khan, A., Printen, J., Pessin, J. E. & Saltiel, A. R. PTG gene deletion causes impaired glycogen synthesis and developmental insulin resistance. *J Clin Invest* **111**, 1423-1432, doi:10.1172/jci17975 (2003).
274 Kannan, R., Palmquist, D. L. & Baker, N. Contribution of intermuscular fat to lipogenesis from dietary glucose carbon in mice. *Biochim Biophys Acta* **431**, 225-232, doi:10.1016/0005-2760(76)90142-9 (1976).
275 Lee, J. S. *et al.* SENP2 suppresses browning of white adipose tissues by de-conjugating SUMO from C/EBPβ. *Cell Reports* **38**, 110408, doi:https://doi.org/10.1016/j.celrep.2022.110408 (2022).
276 Liu, Y. *et al.* Hepatic Small Ubiquitin-Related Modifier (SUMO)-Specific Protease 2 Controls Systemic Metabolism Through SUMOylation-Dependent Regulation of Liver-Adipose Tissue Crosstalk. *Hepatology* **74**, 1864-1883, doi:10.1002/hep.31881 (2021).
277 Hulver, M. W. *et al.* Skeletal muscle lipid metabolism with obesity. *Am J Physiol Endocrinol Metab* **284**, E741-747, doi:10.1152/ajpendo.00514.2002 (2003).
278 Kim, J. Y., Hickner, R. C., Cortright, R. L., Dohm, G. L. & Houmard, J. A. Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* **279**, E1039-1044, doi:10.1152/ajpendo.2000.279.5.E1039 (2000).
279 Malandrino, M. I. *et al.* Enhanced fatty acid oxidation in adipocytes and macrophages reduces lipid-induced triglyceride accumulation and inflammation. *American Journal of Physiology-Endocrinology and Metabolism* **308**, E756-E769, doi:10.1152/ajpendo.00362.2014 (2015).
280 Smith, R. L., Soeters, M. R., Wüst, R. C. I. & Houtkooper, R. H. Metabolic Flexibility as an Adaptation to Energy Resources and Requirements in Health and Disease. *Endocr Rev* **39**, 489-517, doi:10.1210/er.2017-00211 (2018).
281 Björntorp, P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.* **10**, 493-496, doi:doi:10.1161/01.ATV.10.4.493 (1990).
282 Pereira, M. J. *et al.* Impaired adipose tissue lipid storage, but not altered lipolysis, contributes to elevated levels of NEFA in type 2 diabetes. Degree of hyperglycemia and adiposity are important factors. *Metabolism* **65**, 1768-1780, doi:https://doi.org/10.1016/j.metabol.2016.09.008 (2016).
283 Pajvani, U. B. *et al.* Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy. *Nature Medicine* **11**, 797-803, doi:10.1038/nm1262 (2005).
284 Gorden, P. & Gavrilova, O. The clinical uses of leptin. *Current Opinion in Pharmacology* **3**, 655-659, doi:https://doi.org/10.1016/j.coph.2003.06.006 (2003).
285 Nawrocki, A. R. & Scherer, P. E. Keynote review: The adipocyte as a drug discovery target. *Drug Discovery Today* **10**, 1219-1230, doi:https://doi.org/10.1016/S1359-6446(05)03569-5 (2005).
286 Rydén, M. & Arner, P. Fat loss in cachexia--is there a role for adipocyte lipolysis? *Clin Nutr* **26**, 1-6, doi:10.1016/j.clnu.2006.09.009 (2007).
287 Pei, H. *et al.* SUMO-specific protease 2 (SENP2) functions as a tumor suppressor in osteosarcoma via SOX9 degradation. *Exp Ther Med* **16**, 5359-5365, doi:10.3892/etm.2018.6838 (2018).

288 Chen, X.-L. *et al.* SENP2 exerts an anti-tumor effect on chronic lymphocytic leukemia cells through the inhibition of the Notch and NF-κB signaling pathways. *International journal of oncology* **54**, 455-466, doi:10.3892/ijo.2018.4635 (2019).
289 Tokarz, P. & Woźniak, K. SENP Proteases as Potential Targets for Cancer Therapy. *Cancers* **13**, 2059, doi:10.3390/cancers13092059 (2021).
290 Shen, H. J., Zhu, H. Y., Yang, C. & Ji, F. SENP2 regulates hepatocellular carcinoma cell growth by modulating the stability of β-catenin. *Asian Pac J Cancer Prev* **13**, 3583-3587, doi:10.7314/apjcp.2012.13.8.3583 (2012).
291 Jiang, Q. F., Tian, Y. W., Shen, Q., Xue, H. Z. & Li, K. SENP2 regulated the stability of β-catenin through WWOX in hepatocellular carcinoma cell. *Tumour Biol* **35**, 9677-9682, doi:10.1007/s13277-014-2239-8 (2014).
292 Nan, J. *et al.* SENP2 regulates mitochondrial function and insulin secretion in pancreatic β cells. *Exp Mol Med* **54**, 72-80, doi:10.1038/s12276-021-00723-7 (2022).
293 Jung, H. S. *et al.* Senp2 expression was induced by chronic glucose stimulation in INS1 cells, and it was required for the associated induction of Ccnd1 and Mafa. *Islets* **8**, 207-216, doi:10.1080/19382014.2016.1235677 (2016).
294 Ryan, D. P., Hong, T. S. & Bardeesy, N. Pancreatic adenocarcinoma. *N Engl J Med* **371**, 1039-1049, doi:10.1056/NEJMra1404198 (2014).
295 Singhi, A. D., Koay, E. J., Chari, S. T. & Maitra, A. Early Detection of Pancreatic Cancer: Opportunities and Challenges. *Gastroenterology* **156**, 2024-2040, doi:10.1053/j.gastro.2019.01.259 (2019).
296 Maertin, S. *et al.* Roles of autophagy and metabolism in pancreatic cancer cell adaptation to environmental challenges. *Am J Physiol Gastrointest Liver Physiol* **313**, G524-g536, doi:10.1152/ajpgi.00138.2017 (2017).
297 Cheng, G. *et al.* Profiling and targeting of cellular bioenergetics: inhibition of pancreatic cancer cell proliferation. *British Journal of Cancer* **111**, 85-93, doi:10.1038/bjc.2014.272 (2014).
298 Hsu, P. P. & Sabatini, D. M. Cancer Cell Metabolism: Warburg and Beyond. *Cell* **134**, 703-707, doi:https://doi.org/10.1016/j.cell.2008.08.021 (2008).
299 Hanahan, D. & Weinberg, Robert A. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646-674, doi:https://doi.org/10.1016/j.cell.2011.02.013 (2011).
300 White, J. P. *et al.* Muscle oxidative capacity during IL-6-dependent cancer cachexia. *Am J Physiol Regul Integr Comp Physiol* **300**, R201-211, doi:10.1152/ajpregu.00300.2010 (2011).
301 Chacon-Cabrera, A. *et al.* Role of PARP activity in lung cancer-induced cachexia: Effects on muscle oxidative stress, proteolysis, anabolic markers, and phenotype. *J Cell Physiol* **232**, 3744-3761, doi:10.1002/jcp.25851 (2017).
302 Fontes-Oliveira, C. C. *et al.* Mitochondrial and sarcoplasmic reticulum abnormalities in cancer cachexia: altered energetic efficiency? *Biochim Biophys Acta* **1830**, 2770-2778, doi:10.1016/j.bbagen.2012.11.009 (2013).
303 White, J. P. *et al.* IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+mouse. *Skeletal Muscle* **2**, 14, doi:10.1186/2044-5040-2-14 (2012).
304 Tzika, A. A. *et al.* Skeletal muscle mitochondrial uncoupling in a murine cancer cachexia model. *Int J Oncol* **43**, 886-894, doi:10.3892/ijo.2013.1998 (2013).

305 Murphy, K. T., Struk, A., Malcontenti-Wilson, C., Christophi, C. & Lynch, G. S. Physiological characterization of a mouse model of cachexia in colorectal liver metastases. *Am J Physiol Regul Integr Comp Physiol* **304**, R854-864, doi:10.1152/ajpregu.00057.2013 (2013).
306 Gilliam, L. A. A. *et al.* Targeted overexpression of mitochondrial catalase protects against cancer chemotherapy-induced skeletal muscle dysfunction. *American Journal of Physiology-Endocrinology and Metabolism* **311**, E293-E301, doi:10.1152/ajpendo.00540.2015 (2016).
307 Ushmorov, A., Hack, V. & Dröge, W. Differential reconstitution of mitochondrial respiratory chain activity and plasma redox state by cysteine and ornithine in a model of cancer cachexia. *Cancer Res* **59**, 3527-3534 (1999).
308 Padrão, A. I. *et al.* Bladder cancer-induced skeletal muscle wasting: disclosing the role of mitochondria plasticity. *Int J Biochem Cell Biol* **45**, 1399-1409, doi:10.1016/j.biocel.2013.04.014 (2013).
309 Permert, J. *et al.* Pancreatic cancer is associated with impaired glucose metabolism. *Eur J Surg* **159**, 101-107 (1993).
310 Agustsson, T., D'souza, M. A., Nowak, G. & Isaksson, B. Mechanisms for skeletal muscle insulin resistance in patients with pancreatic ductal adenocarcinoma. *Nutrition* **27**, 796-801, doi:https://doi.org/10.1016/j.nut.2010.08.022 (2011).
311 Isaksson, B. *et al.* Impaired Insulin Action on Phosphatidylinositol 3-Kinase Activity and Glucose Transport in Skeletal Muscle of Pancreatic Cancer Patients. *Pancreas* **26** (2003).
312 Wigand, J. P. & Blackard, W. G. Downregulation of insulin receptors in obese man. *Diabetes* **28**, 287-291 (1979).
313 Perdomo, G. *et al.* Increased beta-oxidation in muscle cells enhances insulin-stimulated glucose metabolism and protects against fatty acid-induced insulin resistance despite intramyocellular lipid accumulation. *J Biol Chem* **279**, 27177-27186, doi:10.1074/jbc.M403566200 (2004).
314 Choi, C. S. *et al.* Continuous fat oxidation in acetyl-CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. *Proc Natl Acad Sci U S A* **104**, 16480-16485, doi:10.1073/pnas.0706794104 (2007).
315 Bruce, C. R. *et al.* Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance. *Diabetes* **58**, 550-558, doi:10.2337/db08-1078 (2009).
316 Rydén, M. *et al.* Lipolysis--not inflammation, cell death, or lipogenesis--is involved in adipose tissue loss in cancer cachexia. *Cancer* **113**, 1695-1704, doi:10.1002/cncr.23802 (2008).
317 Sauer, L. A., Nagel, W. O., Dauchy, R. T., Miceli, L. A. & Austin, J. E. Stimulation of tumor growth in adult rats in vivo during an acute fast. *Cancer Res* **46**, 3469-3475 (1986).
318 Sun, X. *et al.* Fat Wasting Is Damaging: Role of Adipose Tissue in Cancer-Associated Cachexia. *Front Cell Dev Biol* **8**, 33-33, doi:10.3389/fcell.2020.00033 (2020).

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SENP2 is vital for optimal insulin signaling and insulin-stimulated glycogen synthesis in human skeletal muscle cells

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ABSTRACT

Sentrin-specific protease (SENP) 2 has been suggested as a possible novel drug target for the treatment of obesity and type 2 diabetes mellitus after observations of a palmitate-induced increase in SENP2 that lead to increased fatty acid oxidation and improved insulin sensitivity in skeletal muscle cells from mice. However, no precedent research has examined the role of SENP2 in human skeletal muscle cells. In the present work, we have investigated the impact of SENP2 on fatty acid and glucose metabolism as well as insulin sensitivity in human skeletal muscle using cultured primary human myotubes. Acute (4 h) oleic acid oxidation was reduced in SENP2-knockdown (SENP2-KD) cells compared to control cells, with no difference in uptake. After prelabeling (24 h) with oleic acid, total lipid content and incorporation into triacylglycerol was decreased, while incorporation into other lipids, as well as complete oxidation and β-oxidation was increased in SENP2-KD cells. Basal glucose uptake (i.e., not under insulin-stimulated conditions) was higher in SENP2-KD cells, whereas oxidation was similar to control myotubes. Further, basal glycogen synthesis was not different in SENP2-KD myotubes, but both insulin-stimulated glycogen synthesis and Akt^{Ser473} phosphorylation was completely blunted in SENP2-KD cells. In conclusion, SENP2 plays an important role in fatty acid and glucose metabolism in human myotubes. Interestingly, it also appears to have a pivotal role in regulating myotube insulin sensitivity. Future studies should examine the role of SENP2 in regulation of insulin sensitivity in other tissues and *in vivo*, defining the potential for SENP2 as a drug target.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a significant contributor to morbidity and mortality in the population worldwide. T2DM develops due to insulin resistance and is characterized by impaired insulin-dependent glucose metabolism in metabolic important tissues, such as skeletal muscles, liver and adipose tissue (reviewed in (Czech, 2017)). Obesity is a strong risk factor for development of insulin resistance. Under circumstances where access to fatty acids exceeds oxidative capacity, as typically seen in obesity, lipid accumulates in other organs than adipose tissue (Boren et al., 2013). Thus, increasing fatty acid oxidation and/or decreasing lipid storage in skeletal muscles can reverse such insulin resistance (Perdomo et al., 2004; Choi et al., 2007; Feige et al., 2008; Bruce et al., 2009). Ectopic storage of fatty acids (mainly triacylglycerols (TAG)) correlates with decreased insulin sensitivity (reviewed in (Kelley and Goodpaster, 2001)). This can change insulin-signaling pathways and reduce insulin-stimulated uptake of glucose (Sugden and Holness, 2006; Holloway, 2009). Skeletal muscle cells from individuals with T2DM have shown a reduced ability to oxidize fatty acids, an increased storage of intracellular lipids, reduced insulin-stimulated glycogen synthesis and reduced Akt phosphorylation (Gaster et al., 2004; Kase et al. 2005, 2015; Corpeleijn et al., 2010).

The mechanisms underlying insulin resistance are not fully elucidated; however, increased levels of plasma free fatty acids and TAG, as well as hyperglycemia are suggested as causal factors (Tomás et al., 2002; Aas et al. 2004, 2005, 2006, 2011; Petersen and Shulman, 2006).

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Moreover, abnormal lipid metabolism with accumulation of lipotoxic intermediates (including DAG) and TAG and mitochondrial dysfunction in skeletal muscle have been linked to insulin resistance and development of T2DM (Martins et al., 2012). Furthermore, intracellular localization affects how these lipids affect muscle insulin sensitivity (Perreault et al., 2018). Blunted effects of insulin on insulin-dependent glucose uptake and changed insulin signaling has previously been reported in myotubes from individuals with T2DM (Kase et al., 2015). The insulin resistance in myotubes is thought to be caused by defects in the insulin signal transduction cascade: insulin receptor, insulin receptor substrate 1, phosphoinositide 3-kinase, and Akt activity (Petersen and Shulman, 2018).

Peroxisome proliferator-activated receptors (PPARs) are members of a nuclear receptor family known to serve key roles in regulation of energy metabolism in important metabolic organs (skeletal muscle, adipose tissue and liver). PPARγ is mainly expressed in adipose tissue, but also found in muscle and liver, and has an important role in lipid metabolism and increasing insulin sensitivity in these tissues (Desvergne and Wahli, 1999). However, PPARδ is the main subtype in skeletal muscle (Desvergne and Wahli, 1999). PPARs are known targets for SUMOylation (SUMO = small ubiquitin-related modifier) (Yamashita et al., 2004; Rytinki and Palvimo, 2009; Do Koo et al., 2015), a post-translational protein modification system with reversible activity (Hay, 2005). SUMOylation has an inhibitory effect on PPARγ and PPARδ translational activity and consequently for their target genes (Chung et al., 2010; Do Koo et al., 2015). SUMOylated PPARs are activated when deSUMOylated by a SUMO specific protease (SENP) (Huang et al., 2009). The SENPs are a family of cysteine proteases that deSUMOylates different target proteins (Nayak and Müller, 2014). SENP2 plays an important role in numerous processes in the body by maturing and deSUMOylating SUMOs (Reverter and Lima, 2006; Mikolajczyk et al., 2007; Békés et al., 2011). It has been shown in mice cells (C2C12 myotubes and 3T3-L1 preadipocytes) that SENP2 increases the activity of PPARδ and PPARγ by deSUMOylation (Chung et al., 2011; Do Koo et al., 2015). Further, studies have shown that SENP2 is induced by leptin and that it is an important regulator of fatty acid metabolism in skeletal muscle of mice (Do Koo et al. 2015, 2019).

The role of SENP2 in human skeletal muscle has not previously been explored. However, studies in mice have suggested a regulatory role on fatty acid metabolism in skeletal muscle (Do Koo et al., 2015). In addition, as SENP2s role in skeletal muscle glucose metabolism and insulin resistance has not yet been explored. The main purpose of this study was to elucidate how knockdown of SENP2 (SENP2-KD) in human skeletal muscle cells would influence basal (i.e., not insulin-stimulated) lipid and glucose metabolism. Further, we wanted to examine how SENP2-KD affected insulin sensitivity as measured by insulin response on glycogen synthesis and Akt phosphorylation.

2. Materials and methods

2.1. Materials

Corning® CellBIND® tissue culture plates were from Corning (Schiphol-Rijk, the Netherlands). Nunc™ Cell Culture Treated Flasks with Filter Caps, Nunc™ 96-MicroWell™ plates, DMEM-Glutamax™ low glucose with sodium pyruvate, human epidermal growth factor (hEGF), PBS, FBS, trypsin-EDTA, Pierce™ BCA Protein Assay Kit, Super Signal™ West Femto Maximum Sensitivity substrate, GeneJET Plasmid Maxiprep Kit, High-Capacity cDNA Reverse Transcription Kit, TaqMan reverse transcription kit reagents, MicroAmp® Optical 96-well Reaction Plate, MicroAmp® Optical Adhesive Film, Power SYBR® Green PCR Master Mix, and primers for TaqMan PCR were from Thermo Fisher Scientific (Waltham, MA, US). Insulin (Actrapid® Penfill® 100 IE/ml) was from Novo Nordisk (Bagsvaerd, Denmark). PromoCell®, dexamethasone, HEPES, 2-[2-[4-(trifluoromethoxy)phenyl]hydrazinylidene]-propanedinitrile (FCCP), D-glucose, oleic acid (18:1, n-9), BSA (essentially fatty acid-free), L-carnitine, glycogen (bovine), perchloric acid, β-mercaptoethanol, phosphatase inhibitor, and protease inhibitor were from Sigma-Aldrich (St. Louis, MO, US). RNeasy Mini Kit was from QIAGEN (Venlo, the Netherlands). D-[^{14}C(U)]glucose (111 and 10175 MBq/mmol) and [1-^{14}C]oleic acid (2183 MBq/mmol) were from PerkinElmer NEN® (Boston, MA, US). Ultima Gold™ XR, Pico Prias 6 ml PE vials, 96-well Isoplate®, Unifilter®-96 GF/B, and TopSeal®-A transparent film were from PerkinElmer (Shelton, CT, US). Tris-HCl, glycerol and thin layer chromatography (TLC) silica gel 60 plates were from Merck (Darmstadt, Germany). Free fatty acids (FFA, 2 mg/ml), cholesterol ester (CE, 2 mg/ml) and mono-, di-, triglyceride mix (4 mg/ml) were from Supelco (Bellefonte, PA, US). Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Precision Plus Protein™ Dual Color standard, bromophenol blue, Trans-Blot® Turbo™ Mini-size Transfer nitrocellulose membranes, Trans-Blot® Turbo™ Mini-size Transfer Stacks, Clarity Western ECL substrates, Tris/glycine/SDS buffer, and Tween 20 were from Bio-Rad (Copenhagen, Denmark). Anti-rabbit and anti-mouse IgG (HRP linked) antibodies, antibodies against myosin heavy chain (MHC) IIa (3403S), β-actin (4970) and α-tubulin (2144), and antibodies against total and phosphorylated Akt at Ser473 (9272 and 9271S, respectively) were from Cell Signaling Technology® Inc. (Beverly, MA, US). Antibody against SENP2 (ab124724) was from Abcam (Cambridge, UK). Antibody against MHCI (MAB1628) was from Millipore (Temecula, MA, US). pMD2.G and psPAX2 were generously provided by Addgene's non-profit repository (Watertown, MA, US) after deposition by Didier Trono on behalf of his lab. GIPZ human shRNA for SENP2-KD (RHS4430-200215 612, clone-ID V2LHS_201 229) and GIPZ shRNA empty vector control (RHS4349) were purchased as glycerol stocks from Horizon Discovery (Cambridge, UK).

2.2. Ethical approvals

Biopsies were obtained after informed written consent and approval by Regional Committees for Medical and Health Research Ethics (REK) North, Tromsø, Norway (ref. no. 2011/882). The study adhered to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3. Production of lentivirus particles

Glycerol stocks were prepared of the pMD2.G envelope plasmid and psPAX2 packaging plasmid. Plasmid DNA was isolated from pMD2.G, psPAX2, SENP2, and SCR shRNAs. Lentivirus particles containing human SENP2 shRNA or control (scrambled (SCR)) shRNA were generated through transient transfection of HEK293T cells as previously described (Barde et al., 2010).

2.4. Transduction of cultured primary human myoblasts

Satellite cells were established from biopsies of *musculus vastus lateralis* and a cell bank was established as described previously (Lund et al., 2017). A mixture of cells from six different donors (in passage 2) was cultured in 75 cm^2 Nunc flasks until 40% confluence. Then, the cells were transduced (MOI = 0.5) with shRNA viral particles targeting SENP2 or control. Polybrene (8 μg/ml) was used as transfection reagent. Transduced cells were selected with puromycin (0.5 μg/ml) 72 h after viral infection and cultured to approximately 90% confluence before frozen down to create a cell bank with transduced myoblasts (passage 3). There were no visual differences to be observed in the microscope after transducing the cells with lentivirus (except the GFP tag). The cells divided as normal and as explained below their differentiation ability was intact. This indicates no loss in cell viability.

2.5. Cell culturing

For proliferation of transduced myoblasts, a DMEM-Glutamax™ (5.5 mmol/l glucose) medium supplemented with 10% FBS, 10 ng/ml

hEGF, 0.39 μg/ml dexamethasone, and 0.05% BSA. At approximately 80% confluence the medium was changed to DMEM-Glutamax™ (5.5 mmol/l glucose) supplemented with 2% FBS and 25 pmol/l insulin to initiate differentiation into multinucleated myotubes. The cells were allowed to differentiate for 7 days. The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C, and medium was changed every 2-3 days. Experiments were performed on cells from passage 3.

2.6. Acute substrate oxidation assay and measurement of acid-soluble metabolites (ASM)

Skeletal muscle cells (7000 cells/well) were cultured on 96-well CellBIND® microplates. Substrate, D-[^{14}C(U)]glucose (18.5 kBq/ml, 200 μmol/l) or [1-^{14}C]oleic acid (18.5 kBq/ml, 100 μmol/l) was given acutely during 4 h CO_2 trapping as described previously (Wensaas et al., 2007). CO_2 production (complete oxidation) and cell-associated radioactivity (CA) were assessed using a PerkinElmer 2450 MicroBeta^2 scintillation counter (PerkinElmer). Protein content in each well was determined with the Bio-Rad protein assay using a VICTOR™ *X*4 Multilabel Plate Reader (PerkinElmer). Measurement of acid-soluble metabolites (ASM), reflecting incomplete fatty acid oxidation (β-oxidation) and mainly consists of tricarboxylic acid cycle metabolites, was performed as previously described by Skrede et al., (1994) and modified by Bakke et al., (2012). In short, 100 μl of the radiolabeled medium was transferred to an Eppendorf tube and precipitated with 300 μl cold $HClO_4$ (1 mol/l) and 30 μl BSA (6%). Thereafter, the tube was centrifuged at 10 000 rpm/10 min/4 °C before 200 μl of the supernatant was counted by liquid scintillation of a Pacard Tri-Carb 1900 TR (PerkinElmer). The sum of $^{14}CO_2$ and CA was considered as total substrate uptake of glucose, whereas the sum of $^{14}CO_2$, ASM and CA was considered as total substrate uptake of oleic acid.

2.7. Glycogen synthesis

Cells were cultured and differentiated as described in subsection 2.5. Once fully differentiated, myotubes were starved for 1.5 h in glucose-free medium before exposed to serum-free DMEM (5.5 mmol/l glucose) supplemented with D-[^{14}C(U)]glucose (18.5 kBq/ml, 0.67 mmol/l), in absence or presence of 20 nmol/l or 100 nmol/l insulin for 3 h to measure glycogen synthesis. The cells were washed twice with PBS and harvested in 1 mol/l KOH. Protein content was determined by the Pierce BCA Protein Assay Kit before 20 mg/ml glycogen and more KOH (final concentration 4 mol/l) were added to the samples. Then, D-[^{14}C(U)]glucose incorporated into glycogen was measured as previously described (Hessvik et al., 2010).

2.8. Prelabeling with oleic acid to study lipid distribution and oxidation

Myotubes were incubated with [1-^{14}C]oleic acid (18.5 kBq/ml, 100 μM) for the last 24 h of the differentiation period. After prelabeling, parts of the radiolabeled medium were transferred to a 96-well plate (100 μl radiolabeled medium/well) and the rest to Eppendorf tubes and immediately frozen down at −20 °C for later measurement of complete oxidation (CO_2) and ASM, respectively. The cells were then washed twice with PBS and harvested in 250 μl 0.1% SDS. Cellular lipids were isolated as previously described (Gaster et al., 2004) by extraction of the homogenized cell fraction, separation of lipids by thin layer chromatography (TLC) and quantification by liquid scintillation (Tri-Carb, 1900; PerkinElmer). A non-polar solvent mixture of hexane:diethyl ether:acetic acid (65:35:1) was used to separate the lipids. The amount of lipids was related to total cell protein concentration determined by the Pierce protein assay.

For measurement of complete oxidation (CO_2) after prelabeling, 40 μl perchloric acid (1 mol/l) was added to the frozen radiolabeled medium before mounted in the appliance described by Wensaas et al., (2007). ASM after prelabeling with oleic acid was measured as described in subsection 2.6.

2.9. RNA isolation and analysis of gene expression by qPCR

Transduced primary human skeletal muscle cells were cultured in 6-well CellBIND® tissue culture plates as described in subsection 2.5. Total RNA was isolated using QIAGEN RNeasy Mini Kit according to the supplier's protocol. Total RNA was reversely transcribed using a High-Capacity cDNA Reverse Transcription Kit and TaqMan Reverse Transcription Reagents using a PerkinElmer 2720 Thermal Cycler (25 °C for 10 min, 37 °C for 80 min and 85 °C for 5 min). Primers were designed using Primer Express® (Thermo Fisher Scientific). qPCR was performed using a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Target genes were quantified in duplicates carried out in a 25 μl reaction volume according to the supplier's protocol. All assays were run for 44 cycles (95 °C for 15 s followed by 60 °C for 60 s). Expression levels were normalized to the housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was also analyzed; there were no differences between normalizing for *RPLP0* or *GAPDH*. Forward and reverse primers were used at a concentration of 30 μmol/l. Primer sequences and accession numbers are presented in Table 1.

2.10. Immunoblotting

Myotubes were harvested in Laemmli buffer (0.5 mol/l Tris-HCl, 10% SDS, 20% glycerol, 10% β-mercaptoethanol, and 5% bromophenol blue). The proteins were electrophoretically separated on 4–20% Mini-Protean TGX™ gels with Tris/glycine buffer (pH 8.3) followed by blotting to nitrocellulose membranes and incubation with antibodies. Immunoreactive bands were visualized with enhanced chemiluminescence (Chemidoc XRS, Bio-Rad) and quantified with Image Lab (version 6.0.1) software. Expression of SENP2 was normalized to expression of the housekeeping protein β-actin. Expressions of MHCI and MHCIIa were normalized to expression of the housekeeping protein α-tubulin. Phosphorylation of Akt at Ser473 was normalized for total Akt expression. Immunoblots with merging of protein of interest with protein standard are presented as supplementary material.

2.11. Presentation of data and statistics

All values are reported as mean ± SEM. The value *n* represents the number of individual experiments, each with at least duplicate measurements. Statistical analyses were performed using GraphPad Prism 8.3.0 for Windows (GraphPad Software Inc., La Jolla, CA, US) and SPSS version 27 (IBM® SPSS® Statistics for Windows, Armonk, NY, US).

Table 1
Description of primers.

Gene	Acc.no.	Forward sequence	Reverse sequence
GAPDH	NM002046	TGC ACC ACC ACC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG
GLUT1	K03195	CAG CAG CCC TAA GGA TCT CTC A	CCG GCT CGG CTG ACA TC
IRS1	NM_005544.2	CTA CTC AAA AGG GAG CGG AGA TAA	AAT AAC GGA CAC TGC ACA ACA GTC T
MYH2	C5814	AAG GTC GGC AAT GAG TAT GTC A	CAA CCA TCC ACA GGA ACA TCT TC
MYH7	NM_000257.2	CTC TGC ACA GGG AAA ATC TGA A	CCC CTG GAC TTT GTC TCA TT
PDK4	BC040239	TTT CCA GAA CCA ACC AAT TCA CA	TGC CCG CAT TGC ATT CTT A
RPLP0	M17885	CCA TTC TAT CAT CAA CGG GTA CAA	AGC AAG TGG GAA GGT GTA ATC C
SENP2	NM_021627.2	CTG AGG CGT CCC CAT TGT	CTT TCG GTA CTT CTC TCT TTC CTC TT

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *GLUT1*, glucose transporter 1; *IRS1*, insulin receptor substrate 1; *MYH*, myosin heavy chain; *PDK4*, pyruvate dehydrogenase kinase 4; *RPLP0*, ribosomal protein lateral stalk subunit P0; SENP2, SUMO specific protease 2.

Paired *t*-test or mixed-model analysis was used to evaluate the knockdown effect. Linear mixed-model analysis was used to compare differences between conditions with within-donor variation and simultaneously compare differences between groups with between-donor variation. The linear mixed-model analysis includes all observations in the statistical analyses and takes into account that not all observations are independent. A p value ≤ 0.05 was considered significant.

3. Results

3.1. Gene expression of SENP2 and fiber type markers increase with differentiation

To determine optimal time point for knockdown of the SENP2 gene in primary human skeletal muscle cells, we analyzed mRNA expression of *SENP2* as well as the differentiation markers *MYH7* and *MYH2* at initiation of differentiation (0 h) and thereafter every 2 h for the first 12 h of differentiation, followed by harvesting 1 day after differentiation and every second day until the final seventh day of differentiation. As expected, gene expression of *MYH7* (Fig. 1A), *MYH2* (Fig. 1B) and *SENP2* (Fig. 1C) increased with time of differentiation. Therefore, we decided to transduce the cells while they were myoblasts and then differentiate the transduced cells. SENP2-KD was confirmed by mRNA expression and immunoblotting (Fig. 2A and B/C, respectively). A non-transduced cell sample was also included to ensure that the scrambled virus did not affect the cells; no differences were observed (supplementary material). As SENP2-KD was performed prior to cell differentiation, protein expressions of MHCI and MHCIIa were measured to ensure that the ability of the myoblasts to differentiate into myotubes was not affected by SENP2-KD. Protein expressions of MHCI and MHCIIa were not affected by SENP2-KD (Fig. 3).

3.2. SENP2-KD in human myotubes reduced oxidation and oxidative reserve capacity after acute oleic acid incubation

To examine the role of SENP2 in fatty acid metabolism (Fig. 4), cell-associated oleic acid, complete oleic acid oxidation (CO_2) and acid-soluble metabolites (ASM) reflecting β-oxidation of oleic acid were measured in SENP2-KD myotubes and compared with control myotubes after 4 h. Total uptake and fractional oxidation of oleic acid were also calculated. SENP2-KD resulted in decreased complete oxidation (Fig. 4B) and fractional oxidation (Fig. 4E), i.e., oxidation relative to total uptake of oleic acid, as well as reduced oxidative reserve capacity (Fig. 4F). There were not observed any differences in accumulation (Fig. 4A) and uptake (Fig. 4D) of oleic acid, or incomplete fatty acid β-oxidation (Fig. 4C).

3.3. SENP2-KD in human myotubes reduced oleic acid incorporation into cellular lipids and increased oxidation of prelabeled oleic acid

To further examine changes in oleic acid metabolism we wanted to examine how SENP2-KD affected fatty acid distribution into complex lipids (Fig. 5). The myotubes were prelabeled with oleic acid for 24 h and incorporation into different lipid classes was measured by thin layer chromatography. SENP2-KD reduced the incorporation of oleic acid into total lipids, phospholipids (PL), diacylglycerol (DAG), TAG, and CE, whereas the level of unesterified oleic acid (FFA) was slightly increased (Fig. 5A). When adjusting the results for the much lower oleic acid incorporation in the knockdown cells it was observed that SENP2-KD in fact increased the relative incorporation into all lipid classes except for TAG, which remained reduced (Fig. 5B). In addition, oxidation (CO_2) and β-oxidation (ASM) after prelabelling with oleic acid for 24 h were markedly increased after SENP2-KD in myotubes (Fig. 6A and B, respectively).

3.4. SENP2-KD in human myotubes increased glucose uptake

To examine the role of SENP2 in glucose metabolism (Fig. 7), oxidation and cell-associated glucose were measured in SENP2-KD myotubes and compared with control myotubes. Total uptake and fractional oxidation of glucose were also calculated. SENP2-KD resulted in increased accumulation of glucose as measured by cell-associated radioactivity (Fig. 7A) as well as increased total glucose uptake (Fig. 7C), despite no change in glucose oxidation (Fig. 7B). However, fractional glucose oxidation, i.e., oxidation relative to total glucose uptake, was significantly reduced in SENP2-KD cells (Fig. 7D).

3.5. SENP2-KD in human myotubes blunted insulin-stimulated glycogen synthesis

In order to further analyze the role of SENP2 in glucose metabolism and its role on insulin sensitivity, basal and insulin-stimulated glycogen synthesis were examined (Fig. 8A). Basal glycogen synthesis was not different between control and SENP2-KD myotubes. Treatment with 100 nmol/l insulin increased glycogen synthesis in control myotubes, but not in SENP2-KD myotubes. To examine the mechanism behind this result, the response to 100 nmol/l insulin was evaluated by assessment of Akt phosphorylation at serine 473 (Fig. 8B and C). No changes in the basal level of pAkt/total Akt ratio were observed between cells from control and SENP2-KD. In line with the blunted insulin-stimulated glycogen synthesis, no effect of insulin on pAkt/total Akt ratio were observed in myotubes with downregulated SENP2 expression, whereas the control myotubes showed the expected increased pAkt/total Akt ratio after insulin stimulation (Fig. 8C). However, total Akt protein expression

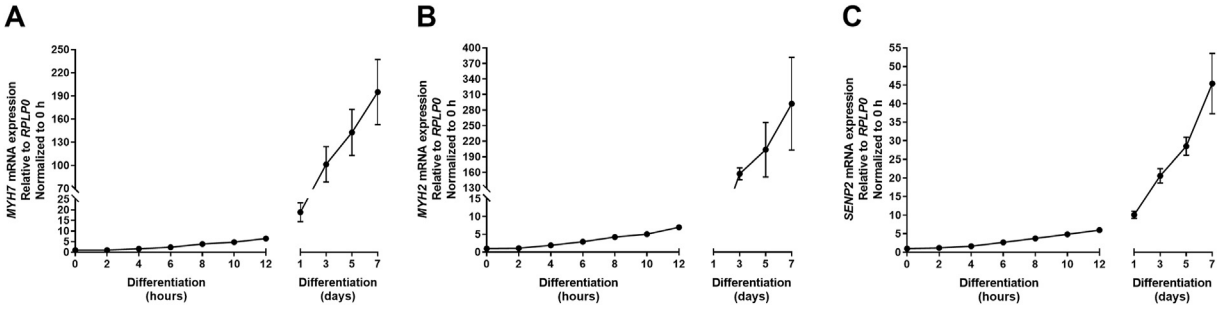

Fig. 1. Gene expression of fiber type markers and SENP2 throughout muscle cell differentiation. Cells were harvested every 2 h for the first 12 h of the differentiation period, then at day 1 of differentiation and every 2 days until day 7 of differentiation. RNA was harvested and expression of myosin heavy chain (*MYH*) *7* (**A**), *MYH2* (**B**) and SUMO specific peptidase (*SENP*) *2* (**C**) was analyzed by qPCR. Results are shown as mean ± SEM relative to the housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*) and normalized to the time point where differentiation was initiated (0 h), from three individual experiments ($n = 3$).

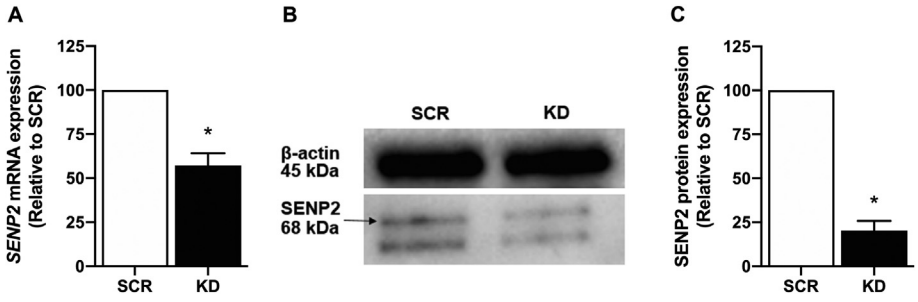

Fig. 2. Confirmation of SENP2-KD. Cells were harvested after seven days of differentiation. (**A**) RNA was harvested and expression of SUMO-specific peptidase 2 (SENP2) was analyzed by qPCR. Results are shown as mean ± SEM relative to the housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*) and normalized to SCR cells, from six individual experiments ($n = 6$). (**B and C**) Protein expressions of SUMO-specific peptidase 2 (SENP2) and β-actin were analyzed by immunoblotting of protein isolated from cell lysates. A non-transduced cell sample was also included to ensure that the scrambled virus did not affect the cells; no differences were observed (data not shown). Binding of SENP2 occurs at about 68 kDa whereas β-actin binds at approximately 45 kDa. B, One representative immunoblot. C, Quantified immunoblot of three individual experiments ($n = 3$). *Statistically significant versus SCR cells ($p \leq 0.05$, paired *t*-test). SCR, control cells, i.e., scrambled vector; KD, knockdown cells, i.e., vector for SENP2 knockdown.

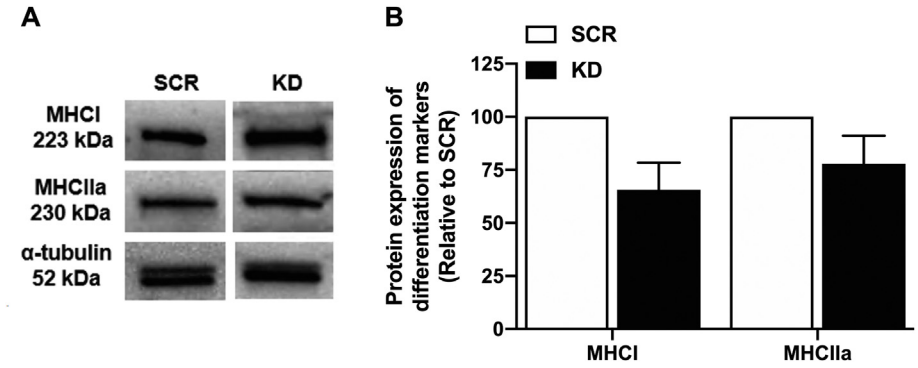

Fig. 3. Myotube differentiation was not affected SENP2-KD. Cells were harvested after seven days of differentiation. Protein expressions of the markers of differentiation myosin heavy chain (MHC) I and IIa and the housekeeping protein α-tubulin were analyzed by immunoblotting of protein isolated from cell lysates. Binding of MHCI occurs at about 223 kDa, MHCIIa at about 230 kDa, and α-tubulin at approximately 52 kDa. (**A**) One representative immunoblot. (**B**) Quantified immunoblot of three individual experiments ($n = 3$). SCR, control cells, i.e., scrambled vector; KD, knockdown cells, i.e., vector for SENP2 knockdown.

was lower in the SENP2-KD myotubes compared to control myotubes (Fig. 8D). In order to study the differences in basal glucose metabolism and the blunted insulin response in SENP2-KD myotubes further, we analyzed the mRNA expression of selected genes (Fig. 8E). mRNA expression of glucose transporter (*GLUT*) *1* and insulin receptor substrate (*IRS*) *1* was not different between SENP2-KD and control myotubes. There was a non-significant trend ($p = 0.09$) towards increased mRNA expression of pyruvate dehydrogenase kinase (*PDK*) *4* in the myotubes with downregulated SENP2.

4. Discussion

In this study, we demonstrate that SENP2-KD had a major impact on lipid metabolism, glucose metabolism and insulin-stimulated glycogen synthesis. SENP2-KD reduced acute (4 h incubation) oxidation of oleic acid and oxidative reserve capacity, incorporation of oleic acid into cellular lipids and promoted a higher oxidation of prelabeled (24 h incubation) oleic acid, whereas for glucose metabolism we observed an increased uptake of glucose and a blunted insulin-stimulated glycogen synthesis. This blunted effect of insulin was confirmed by a lack of insulin-stimulated Akt phosphorylation in SENP2-KD myotubes. In line with this, total Akt protein expression was lower in SENP2-KD myotubes.

Myotubes proliferate as myoblasts before they fuse into multinucleated myotubes. To establish if we could transduce the cells during proliferation, we needed to establish when expression of SENP2 was highest in the muscle cells and thus most likely important for myocyte function. Expression of SENP2 first started to increase after initiation of differentiation and continued to increase throughout differentiation. Therefore, we decided to transduce during proliferation. Visual examination in the microscope during cell proliferation and differentiation did not identify any morphological changes induced by SENP2-KD (data not shown). Protein expression of the differentiation markers MHCI and MHCIIa was not affected by SENP2-KD either. Combined these data suggests that SENP2-KD did not alter any major myocyte characteristics.

It has previously been shown that SENP2 is an important regulator of fatty acid metabolism in skeletal muscle of mice. Palmitic acid activated SENP2 in C2C12 myotubes and further increased expressions of genes involved in fatty acid oxidation (Do Koo et al., 2015). Treatment of our primary human myotubes with palmitic acid did not affect uptake or oxidation of oleic acid or mRNA expression of *SENP2* (data not shown). Furthermore, the previous work with murine cells showed that downregulation of SENP2 suppressed the palmitic acid-induced increase in gene expressions and thereby abolished fatty acid oxidation (Do Koo et al., 2015). We observed both a reduced fatty acid oxidation and reduced oxidative reserve capacity from acutely available oleic acid after SENP2-KD in human myotubes. After prelabeling the cells with oleic acid for 24 h, myotubes with and without SENP2-KD handled the oleic acid differently. Compared to control cells, SENP2-KD reduced the total incorporation of oleic acid into cellular lipids and the distribution of complex lipids changed in the myotubes: less was incorporated into TAG and relatively more into the other complex lipids (PL, DAG, FFA, and CE). FFA and DAG are intermediates for TAG synthesis at the same time as they are degradation products from lipolysis. DAG can be converted to either TAG or PL. Thus, it seems that SENP2-KD promoted PL synthesis to a greater extent than TAG synthesis. Increased proportion of intermediates in addition to reduced proportion of TAG may suggest an increased lipolysis after SENP2-KD in human myotubes. However, this needs to be further elucidated. Furthermore, in line with the reduced

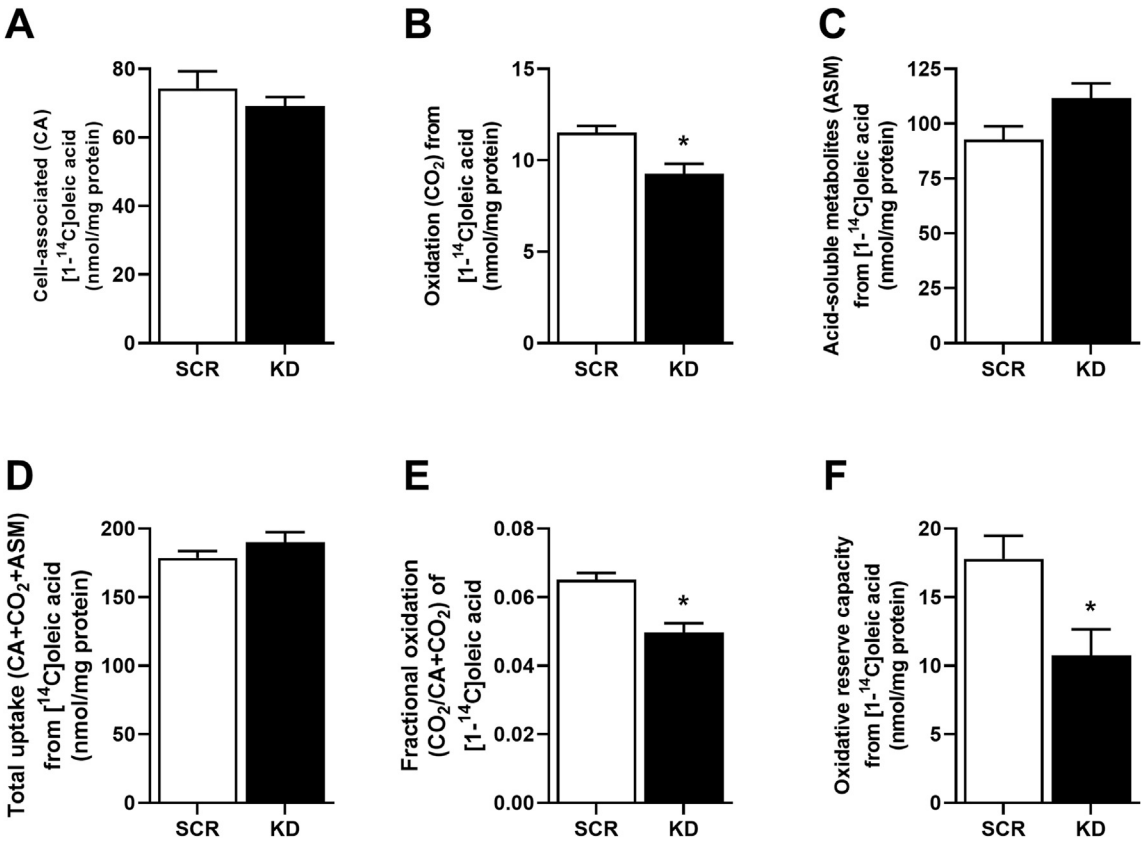

Fig. 4. Role of SENP2 for metabolism of fatty acids after acute incubation in human myotubes. Human myotubes were incubated with 100 μM [1-^{14}C]oleic acid (18.5 kBq/ml) for 4 h. Cell-associated (CA) radioactivity (**A**), complete oxidation measured as trapped CO_2 (**B**) and acid-soluble metabolites (ASM) (**C**) were analyzed. The combination of CA, CO_2 and ASM was taken as a measurement of total cellular oleic acid uptake (**D**). Fractional oleic acid oxidation was calculated as CO_2/CA + CO_2+ASM (**E**). As some parallel wells were treated with the mitochondrial uncoupler FCCP, oxidative reserve capacity was calculated as CO_2 from FCCP-treated cells − CO_2 from untreated cells (**F**). Results are shown as mean ± SEM in absolute values, nmol/mg protein from four individual experiments ($n = 4$) with eight replicates. *Statistically significant versus SCR cells ($p \leq 0.05$, mixed-model analysis). KD, cells with SENP2 knockdown; SCR, control cells i.e., with scrambled vector.

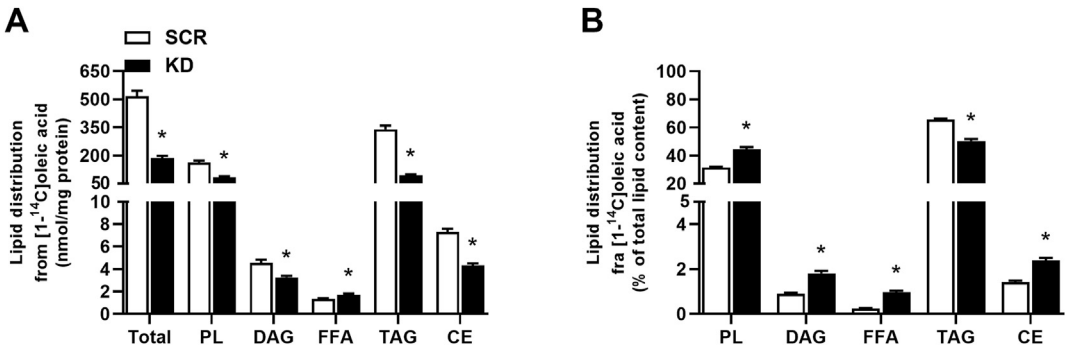

Fig. 5. Role of SENP2 for lipid distribution of oleic acid in human myotubes. Human myotubes were on day six of differentiation incubated with 100 μM [1-^{14}C] oleic acid (18.5 kBq/ml) for 24 h. Lipids were separated by thin layer chromatography and quantified by liquid scintillation. Results are shown as mean ± SEM for absolute values, nmol/mg protein (**A**) and related to total lipid content, % (**B**) from four individual experiments ($n = 4$) with three replicates. *Statistically significant versus SCR cells ($p \leq 0.05$, paired *t*-test). CE, cholesteryl ester; DAG, diacylglycerol; FFA, free fatty acids; KD, cells with SENP2 knockdown; PL, phospholipid; SCR, control cells i.e., with scrambled vector; TAG, triacylglycerol.

incorporation into lipids from prelabeled oleic acid in SENP2-KD myotubes, fatty acid oxidation was increased in these cells compared to control cells, both complete (measured as CO_2) and incomplete oxidation (measured as ASMs). This corresponds with recent findings from mice with liver-specific knockout of Senp2 where Senp2 deficiency upregulated genes involved in fatty acid oxidation and downregulated genes involved in lipogenesis (Liu et al., 2021). Further, it seems like the uptake of oleic acid, by looking at the sum of total oleic acid incorporation and total oxidized lipids (complete and incomplete), is somewhat lower (approximately 20%) in SENP2-KD cells compared to controls. This may explain some of the reduction in total lipid incorporation after SENP2-KD; however, as the relative increase in oxidation, particular incomplete oxidation (approximately 75%), is much higher than the apparent reduction in total uptake, we believe this to be the main reason behind the reduced total lipid incorporation.

SENP1 has previously been shown to link glucose metabolism to amplification of insulin secretion and demonstrate that restoration of this axis rescues β-cell function in T2DM (Ferdaoussi et al., 2015), whereas

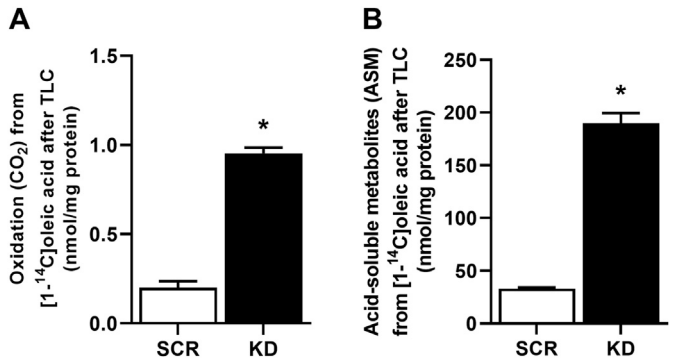

Fig. 6. Role of SENP2 in oxidation after prelabeling with oleic acid in human myotubes. Human myotubes were on day six of differentiation incubated with 100 μM [1-^{14}C]oleic acid (18.5 kBq/ml) for 24 h in order to measure distribution of oleic acid into complex lipids. Thereafter, the radiolabeled medium was saved and oxidation (CO_2) of oleic acid (**A**) and acid-soluble metabolites (ASM) from oleic acid (**B**) were measured. Results are shown as mean ± SEM in absolute values, nmol/mg protein, from four individual experiments ($n = 4$) with one (A) or three (B) replicates. *Statistically significant versus SCR cells ($p \leq 0.05$, paired *t*-test). KD, cells with SENP2 knockdown; SCR, control cells i.e., with scrambled vector; TLC, thin layer chromatography.

SENP2 has been suggested to play a role in enhancing β-cell mass in response to chronic high-glucose in pancreatic INS1-cells (Jung et al., 2016). Do Koo et al. observed that SENP2 overexpression in mice muscle alleviated HFD-induced obesity and insulin resistance, but it was unsure if these were direct SENP2 effects or secondary *in vivo* effects (Do Koo et al., 2015). In the cancerous MCF7 and MEF cells, it has been shown that SENP2 negatively regulated aerobic glycolysis. In MCF7 cells, overexpression of SENP2 led to reduced glucose uptake and production of lactate, inducing an increased ATP production (Tang et al., 2013). Furthermore, along with increased glucose oxidation, overexpression of SENP2 in both cell types caused reduced expression levels of key enzymes important for glycolysis indicating an inhibited glycolysis and increased oxidative phosphorylation. In contrast, knocking out SENP2 in MEF cells increased glucose uptake and lactate production but reduced ATP level (Tang et al., 2013). However, it is well known that metabolism in cancer cells is particular. Yet, the studies suggest that SENP2 might have a role in glucose metabolism, but the role of SENP2 in human skeletal muscle has not previously been examined. We observed that SENP2-KD in myotubes resulted in increased glucose uptake and an overall reduction in the proportion of glucose taken up that was oxidized (fractional oxidation). However, this was not explained by the expression of selected genes; neither mRNA expression of the basal glucose transporter, *GLUT1*, nor the metabolic regulator *PDK4* was significantly affected by down-regulation of SENP2.

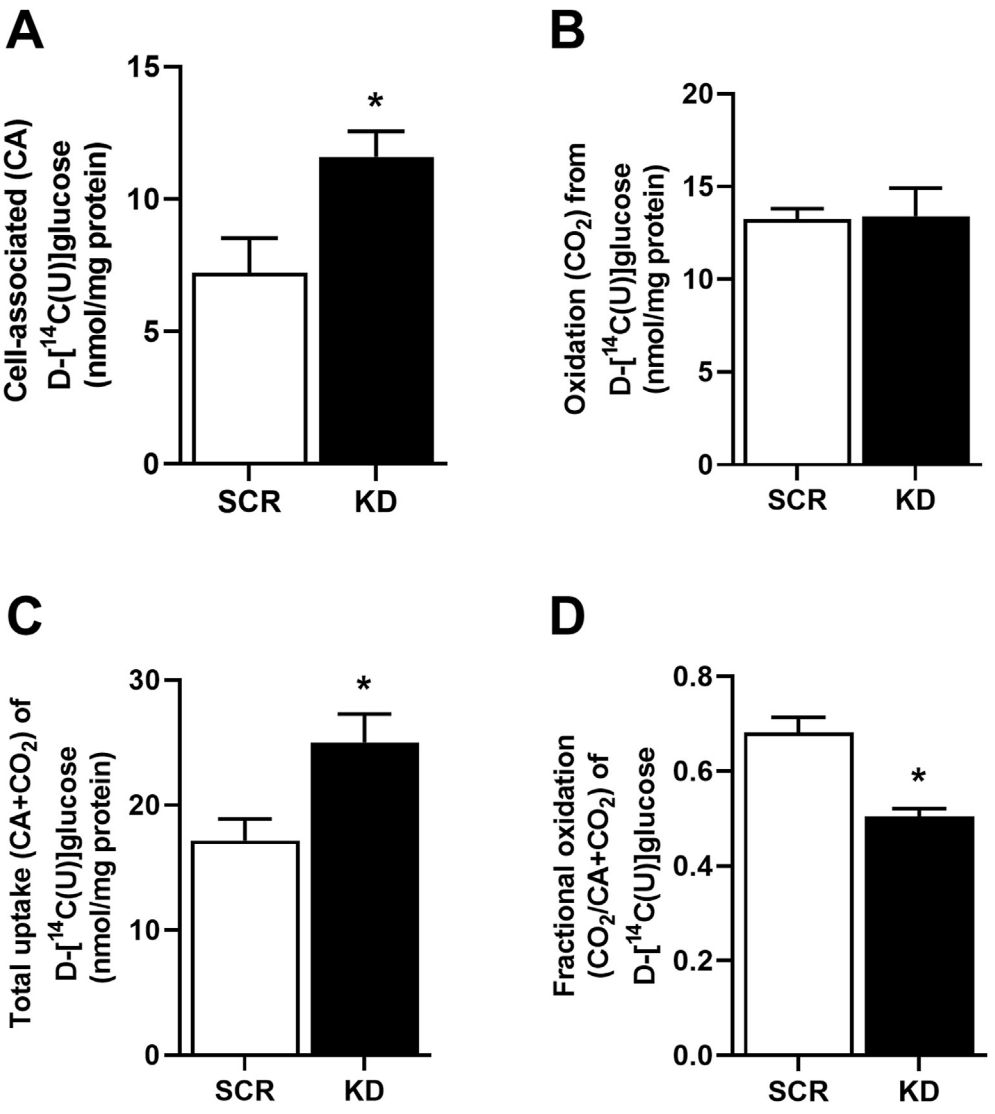

Fig. 7. Role of SENP2 for glucose metabolism in human myotubes. Human myotubes were incubated with 100 μM D-[^{14}C(U)]glucose (18.5 kBq/ml) for 4 h. Cell-associated (CA) radioactivity (**A**) and complete oxidation measured as trapped CO_2 (**B**) were analyzed. The combination of CA and CO_2 was taken as a measurement of total cellular glucose uptake (**C**). Fractional glucose oxidation was calculated as CO_2/CA + CO_2 (**D**). Results are shown as mean ± SEM in absolute values, nmol/mg protein from four individual experiments ($n = 4$) with eight replicates. *Statistically significant versus SCR cells ($p \leq 0.05$, mixed-model analysis). KD, cells with SENP2 knockdown; SCR, control cells i.e., with scrambled vector.

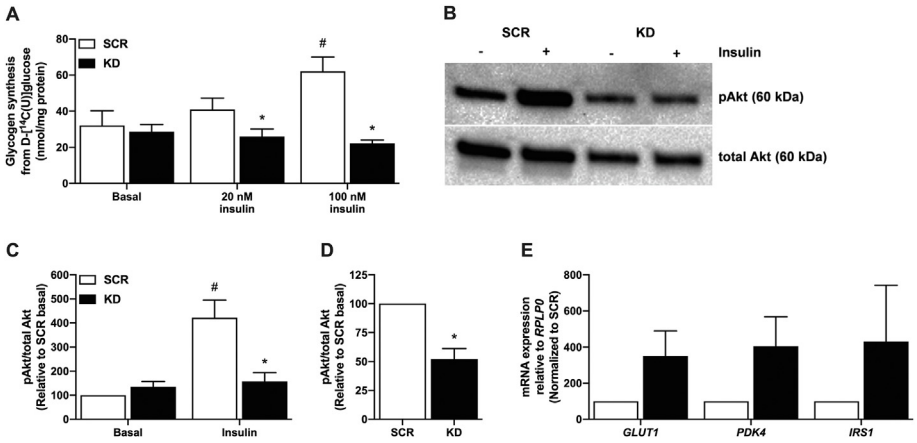

Fig. 8. Role of SENP2 for basal and insulin-stimulated glycogen synthesis and Akt phosphorylation in human myotubes. (**A**) Myotubes were starved for 1.5 h before incubated for 3 h with 5.5 mM D-[^{14}C(U)]glucose (37 kBq/ml) with no insulin (basal), 20 nM or 100 nM insulin. Results are shown as mean ± SEM in absolute values, nmol/mg protein, from four individual experiments ($n = 4$). (**B-D**) Akt phosphorylation and total Akt protein expression by immunoblotting. Myotubes were incubated for 15 min with 100 nM insulin before protein was isolated and the amount of total Akt and pAkt were assessed by immunoblotting. B, a representative immunoblot. C and D, quantified immunoblots relative to SCR. Results are shown as mean ± SEM from six individual experiments ($n = 6$). (**E**) mRNA expression of selected genes. RNA was harvested and expression of glucose transporter (*GLUT*) *1*, pyruvate dehydrogenase kinase (*PDK*) *4* and insulin receptor substrate (*IRS*) *1* were analyzed by qPCR. Results are shown as mean ± SEM relative to the housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*) and normalized to SCR cells, from six individual experiments ($n = 6$). *Statistically significant versus SCR cells ($p \leq 0.05$, paired *t*-test). #Statistically significant versus to SCR basal ($p \leq 0.05$, paired *t*-test). KD, cells with SENP2 knockdown; SCR, control cells i.e., with scrambled vector.

Reduced amount of SENP2 is thought to lead to a reduced activation of PPARs (Do Koo et al., 2015). PPARs are targets for thiazolidinediones (glitazones) that are known to improve insulin sensitivity in subjects with T2DM. Thus, the question was whether SENP2-KD and consequently reduced PPAR activity would reduce insulin sensitivity in our myotubes. We observed a marked reduction in insulin-stimulated glycogen synthesis in knockdown myotubes, accompanied by a blunted insulin-stimulated phosphorylation of Akt, showing clearly that SENP2 is important for insulin sensitivity in human skeletal muscle cells. Increased insulin-stimulated glycogen synthesis may be a result of the increased glucose uptake that insulin stimulated directly or through its effect on glycogen synthase (Lawrence and Roach, 1997). A previous study in L6 cells indicated that SUMOylation could lead to upregulation of GLUT4 (Giorgino et al., 2000). However, a recent study by Carmichael et al. contradicts the hypothesis of SUMOylation increasing GLUT4-mediated glucose uptake as SUMOylation did not affect insulin-dependent GLUT4 trafficking in L6 myocytes (Carmichael et al., 2019). The ratio of GLUT1 to GLUT4 is higher in human myotubes compared to adult muscle (Sarabia et al., 1992), resulting in lower insulin responsiveness of glucose transport (Sarabia et al., 1992; Al-Khalili et al., 2003). However, the molecular mechanisms of glucose transport remain the same (Al-Khalili et al., 2003). mRNA expression of *GLUT1* and *IRS1* was not different between control myotubes and SENP2-KD myotubes. Based on the results of Do Koo et al. on lipid metabolism, decreased deSUMOylation of PPAR (i.e., reduced PPAR activity) is hypothesized to be behind the found effect of SENP2 knockdown. A weakness of the present study is the lack of examination of PPAR SUMOylation. However, another target of SUMOylation include Akt itself (De la Cruz-Herrera et al., 2015). Examining total protein expression of Akt, SENP2-KD cells had significantly lower expression than control cells. Furthermore, previous data shows that although PPARγ is low expressed in muscle cells, PPARγ knockdown resulted in reduced insulin sensitivity in C2C12-cells (Verma et al., 2004). Hence, it seems likely that the SENP2 effect observed is secondary to reduced PPAR activity.

The results from the present study are somewhat contradictory to the findings by Do Koo et al. (Do Koo et al., 2015). Do Koo et al. examined the role of SENP2 in murine cells. Even though rodents have similarities to humans on several metabolic characteristics, metabolism of the two species are not identical. Due to smaller body weight, rodents have a higher basal metabolic rate, and main glucose uptake occurs in the liver of rodents but in skeletal muscle of humans. Therefore, direct transfer of findings from rodents to humans must be performed with caution (Kleinert et al., 2018). This may explain the different observations in lipid metabolism between our two studies, as well as different methodologies for the transgenic studies.

In conclusion, this work shows for the first time that SENP2 exerts a critical role for both glucose and lipid metabolism in human myotubes. Interestingly, both insulin-stimulated glycogen synthesis and Akt phosphorylation were blunted in SENP2-KD myotubes. Future studies should examine the role of SENP2 in regulation of insulin sensitivity, highlighting the potential of SENP2 as a novel target in the combat against the ever-increasing obesity and diabetes pandemic.

CRediT authorship contribution statement

Jenny Lund: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Validation, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Solveig A. Krapf:** Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Medina Sistek:** Investigation, Writing – review & editing. **Hege G. Bakke:** Methodology, Investigation, Writing – review & editing. **Stefano Bartesaghi:** Conceptualization, Methodology, Writing – review & editing. **Xiao-Rong Peng:** Conceptualization, Methodology, Writing – review & editing. **Arild C. Rustan:** Methodology, Funding acquisition, Resources, Writing – review & editing. **G. Hege Thoresen:** Conceptualization, Methodology, Funding acquisition, Resources, Writing – review & editing. **Eili T. Kase:** Conceptualization, Methodology, Validation, Project administration, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphar.2021.100061.

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References

Aas, V., Kase, E., Solberg, R., Jensen, J., Rustan, A., 2004. Chronic hyperglycaemia promotes lipogenesis and triacylglycerol accumulation in human skeletal muscle cells. Diabetologia 47, 1452–1461.

Aas, V., Rokling-Andersen, M., Wensaas, A., Thoresen, G., Kase, E., Rustan, A., 2005. Lipid metabolism in human skeletal muscle cells: effects of palmitate and chronic hyperglycaemia. Acta Physiol. Scand. 183, 31–41.

Aas, V., Rokling-Andersen, M.H., Kase, E.T., Thoresen, G.H., Rustan, A.C., 2006. Eicosapentaenoic acid (20: 5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. J. Lipid Res. 47, 366–374.

Aas, V., Hessvik, N.P., Wettergreen, M., Hvammen, A.W., Hallén, S., Thoresen, G.H., Rustan, A.C., 2011. Chronic hyperglycemia reduces substrate oxidation and impairs metabolic switching of human myotubes. BBA Mol. Basis Dis. 1812, 94–105.

Al-Khalili, L., Chibalin, A., Kannisto, K., Zhang, B., Permert, J., Holman, G., Ehrenborg, E., Ding, V., Zierath, J., Krook, A., 2003. Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. Cell. Mol. Life Sci. 60, 991–998.

Bakke, S.S., Moro, C., Nikolić, N., Hessvik, N.P., Badin, P.-M., Lauvhaug, L., Fredriksson, K., Hesselink, M.K., Boekschoten, M.V., Kersten, S., 2012. Palmitic acid follows a different metabolic pathway than oleic acid in human skeletal muscle cells; lower lipolysis rate despite an increased level of adipose triglyceride lipase. BBA Mol. Cell Biol. Lipids. 1821, 1323–1333.

Barde, I., Salmon, P., Trono, D., 2010. Production and titration of lentiviral vectors. Curr. Protoc. Neurosci. 53, 4.21. 21-24.21. 23.

Békés, M., Prudden, J., Srikumar, T., Raught, B., Boddy, M.N., Salvesen, G.S., 2011. The dynamics and mechanism of SUMO chain deconjugation by SUMO-specific proteases. J. Biol. Chem. 286, 10238–10247.

Boren, J., Taskinen, M.R., Olofsson, S.O., Levin, M., 2013. Ectopic lipid storage and insulin resistance: a harmful relationship. J. Intern. Med. 274, 25–40.

Bruce, C.R., Hoy, A.J., Turner, N., Watt, M.J., Allen, T.L., Carpenter, K., Cooney, G.J., Febbraio, M.A., Kraegen, E.W., 2009. Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet–induced insulin resistance. Diabetes 58, 550–558.

Carmichael, R.E., Wilkinson, K.A., Craig, T.J., 2019. Insulin-dependent GLUT4 trafficking is not regulated by protein SUMOylation in L6 myocytes. Sci. Rep. 9, 1–9.

Choi, C.S., Savage, D.B., Abu-Elheiga, L., Liu, Z.-X., Kim, S., Kulkarni, A., Distefano, A., Hwang, Y.-J., Reznick, R.M., Codella, R., 2007. Continuous fat oxidation in acetyl–CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. Proc. Natl. Acad. Sci. U.S.A 104, 16480–16485.

Chung, S.S., Ahn, B.Y., Kim, M., Choi, H.H., Park, H.S., Kang, S., Park, S.G., Kim, Y.-B., Cho, Y.M., Lee, H.K., 2010. Control of adipogenesis by the SUMO-specific protease SENP2. Mol. Cell Biol. 30, 2135–2146.

Chung, S.S., Ahn, B.Y., Kim, M., Kho, J.H., Jung, H.S., Park, K.S., 2011. SUMO modification selectively regulates transcriptional activity of peroxisome-proliferator-activated receptor γ in C2C12 myotubes. Biochem. J. 433, 155–161.

Corpeleijn, E., Hessvik, N.P., Bakke, S.S., Levin, K., Blaak, E.E., Thoresen, G.H., Gaster, M., Rustan, A.C., 2010. Oxidation of intramyocellular lipids is dependent on mitochondrial function and the availability of extracellular fatty acids. Am. J. Physiol. Endocrinol. Metab. 299, E14–E22.

De la Cruz-Herrera, C., Campagna, M., Lang, V., del Carmen González-Santamaría, J., Marcos-Villar, L., Rodríguez, M., Vidal, A., Collado, M., Rivas, C., 2015. SUMOylation regulates AKT1 activity. Oncogene 34, 1442–1450.

Czech, M.P., 2017. Insulin action and resistance in obesity and type 2 diabetes. Nat. Med. 23, 804.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20, 649–688.

Feige, J.N., Lagouge, M., Canto, C., Strehle, A., Houten, S.M., Milne, J.C., Lambert, P.D., Mataki, C., Elliott, P.J., Auwerx, J., 2008. Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell Metabol. 8, 347–358.

Ferdaoussi, M., Dai, X., Jensen, M.V., Wang, R., Peterson, B.S., Huang, C., Ilkayeva, O., Smith, N., Miller, N., Hajmrle, C., 2015. Isocitrate-to-SENP1 signaling amplifies insulin secretion and rescues dysfunctional β cells. J. Clin. Invest. 125, 3847–3860.

Gaster, M., Rustan, A.C., Aas, V., Beck-Nielsen, H., 2004. Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. Diabetes 53, 542–548.

Giorgino, F., De Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K.C., Smith, R.J., 2000. The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells. Proc. Natl. Acad. Sci. U.S.A 97, 1125–1130.

Hay, R.T., 2005. SUMO: a history of modification. Mol. Cell. 18, 1–12.

Hessvik, N.P., Bakke, S.S., Fredriksson, K., Boekschoten, M.V., Fj, A., Koster, G., Hesselink, M.K., Kersten, S., Kase, E.T., Rustan, A.C., 2010. Metabolic switching of human myotubes is improved by n-3 fatty acids. J. Lipid Res. 51, 2090–2104.

Holloway, G.P., 2009. Mitochondrial function and dysfunction in exercise and insulin resistance. Appl. Physiol. Nutr. Metabol. 34, 440–446.

Huang, C., Han, Y., Wang, Y., Sun, X., Yan, S., Yeh, E.T., Chen, Y., Cang, H., Li, H., Shi, G., 2009. SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. EMBO J. 28, 2748–2762.

Jung, H.S., Kang, Y.M., Park, H.S., Ahn, B.Y., Lee, H., Kim, M.J., Jang, J.Y., Kim, S.-W., 2016. Senp2 expression was induced by chronic glucose stimulation in INS1 cells, and it was required for the associated induction of Ccnd1 and Mafa. Islets 8, 207–216.

Kase, E.T., Wensaas, A.J., Aas, V., Højlund, K., Levin, K., Thoresen, G.H., Beck-Nielsen, H., Rustan, A.C., Gaster, M., 2005. Skeletal muscle lipid accumulation in type 2 diabetes may involve the liver X receptor pathway. Diabetes 54, 1108–1115.

Kase, E.T., Feng, Y.Z., Badin, P.-M., Bakke, S.S., Laurens, C., Coue, M., Langin, D., Gaster, M., Thoresen, G.H., Rustan, A.C., 2015. Primary defects in lipolysis and insulin action in skeletal muscle cells from type 2 diabetic individuals. BBA Mol. Cell Biol. Lipids 1851, 1194–1201.

Kelley, D.E., Goodpaster, B.H., 2001. Skeletal muscle triglyceride. An aspect of regional adiposity and insulin resistance. Clin. Diabetol. 2, 255–266.

Kleinert, M., Clemmensen, C., Hofmann, S.M., Moore, M.C., Renner, S., Woods, S.C., Huypens, P., Beckers, J., De Angelis, M.H., Schürmann, A., 2018. Animal models of obesity and diabetes mellitus. Nat. Rev. Endocrinol. 14, 140.

Do Koo, Y., Choi, J.W., Kim, M., Chae, S., Ahn, B.Y., Kim, M., Oh, B.C., Hwang, D., Seol, J.H., Kim, Y.-B., 2015. SUMO-specific protease 2 (SENP2) is an important regulator of fatty acid metabolism in skeletal muscle. Diabetes 64, 2420–2431.

Do Koo, Y., Lee, J.S., Lee, S.-A., Quaresma, P.G., Bhat, R., Haynes, W.G., Park, Y.J., Kim, Y.-B., Chung, S.S., Park, K.S., 2019. SUMO-specific protease 2 mediates leptin-induced fatty acid oxidation in skeletal muscle. Metabolism 95, 27–35.

Lawrence, J.C., Roach, P.J., 1997. New insights into the role and mechanism of glycogen synthase activation by insulin. Diabetes 46, 541–547.

Liu, Y., Dou, X., Zhou, W.y., Ding, M., Liu, L., Du, R.q., Guo, L., Qian, S.w., Tang, Y., Yang, Q.q., 2021. Hepatic SENP2 controls systemic metabolism via SUMOylation-dependent regulation of liver-adipose tissue crosstalk. Hepatology 74, 1864–1883.

Lund, J., Rustan, A.C., Løvsletten, N.G., Mudry, J.M., Langleite, T.M., Feng, Y.Z., Stensrud, C., Brubak, M.G., Drevon, C.A., Birkeland, K.I., 2017. Exercise in vivo marks human myotubes in vitro: training-induced increase in lipid metabolism. PloS One 12, e0175441.

Martins, A.R., Nachbar, R.T., Gorjao, R., Vinolo, M.A., Festuccia, W.T., Lambertucci, R.H., Cury-Boaventura, M.F., Silveira, L.R., Curi, R., Hirabara, S.M., 2012. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. Lipids Health Dis. 11, 1–11.

Mikolajczyk, J., Drag, M., Békés, M., Cao, J.T., Ronai, Z.e., Salvesen, G.S., 2007. Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. J. Biol. Chem. 282, 26217–26224.

Nayak, A., Müller, S., 2014. SUMO-specific proteases/isopeptidases: SENPs and beyond. Genome Biol. 15, 1–7.

Perdomo, G., Commerford, S.R., Richard, A.-M.T., Adams, S.H., Corkey, B.E., O'Doherty, R.M., Brown, N.F., 2004. Increased β-oxidation in muscle cells enhances insulin-stimulated glucose metabolism and protects against fatty acid-induced insulin resistance despite intramyocellular lipid accumulation. J. Biol. Chem. 279, 27177–27186.

Perreault, L., Newsom, S., Strauss, A., Kerege, A., Kahn, D., Harrison, K., Snell-Bergeon, J., Nemkov, T., D'Alessandro, A., Jackman, M., 2018. Intracellular localization of diacylglycerols and sphingolipids influences insulin sensitivity and mitochondrial function in human skeletal muscle. JCI Insight 3, 1–21.

Petersen, K.F., Shulman, G.I., 2006. Etiology of insulin resistance. Am. J. Med. 119, S10–S16.

Petersen, M.C., Shulman, G.I., 2018. Mechanisms of insulin action and insulin resistance. Physiol. Rev. 98, 2133–2223.

Reverter, D., Lima, C.D., 2006. Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates. Nat. Struct. Biol. 13, 1060–1068.

Rytinki, M.M., Palvimo, J.J., 2009. SUMOylation attenuates the function of PGC-1α. J. Biol. Chem. 284, 26184–26193.

Sarabia, V., Lam, L., Burdett, E., Leiter, L., Klip, A., 1992. Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin. J. Clin. Invest. 90, 1386–1395.

Skrede, S., Bremer, J., Berge, R., Rustan, A., 1994. Stimulation of fatty acid oxidation by a 3-thia fatty acid reduces triacylglycerol secretion in cultured rat hepatocytes. J. Lipid Res. 35, 1395–1404.

Sugden, M., Holness, M., 2006. Skeletal muscle lipid metabolism and the adipomuscular axis. Future Lipidol. 1, 153–163.

Tang, S., Huang, G., Tong, X., Xu, L., Cai, R., Li, J., Zhou, X., Song, S., Huang, C., Cheng, J., 2013. Role of SUMO-specific protease 2 in reprogramming cellular glucose metabolism. PloS One 8, e63965.

Tomás, E., Lin, Y.S., Dagher, Z., Saha, A., Luo, Z., Ido, Y., Ruderman, N.B., 2002. Hyperglycemia and insulin resistance: possible mechanisms. Ann. N. Y. Acad. Sci. 967, 43–51.

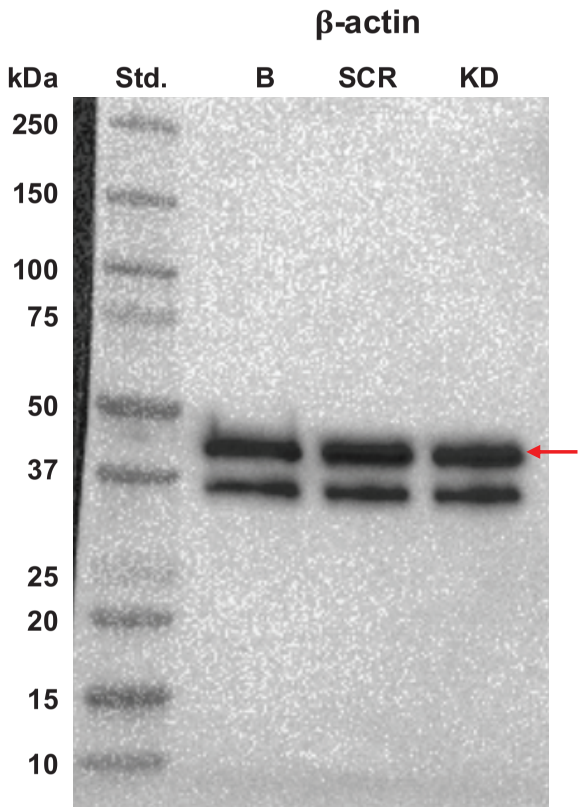
Verma, N.K., Singh, J., Dey, C.S., 2004. PPAR-γ expression modulates insulin sensitivity in C2C12 skeletal muscle cells. Br. J. Pharmacol. 143, 1006–1013.

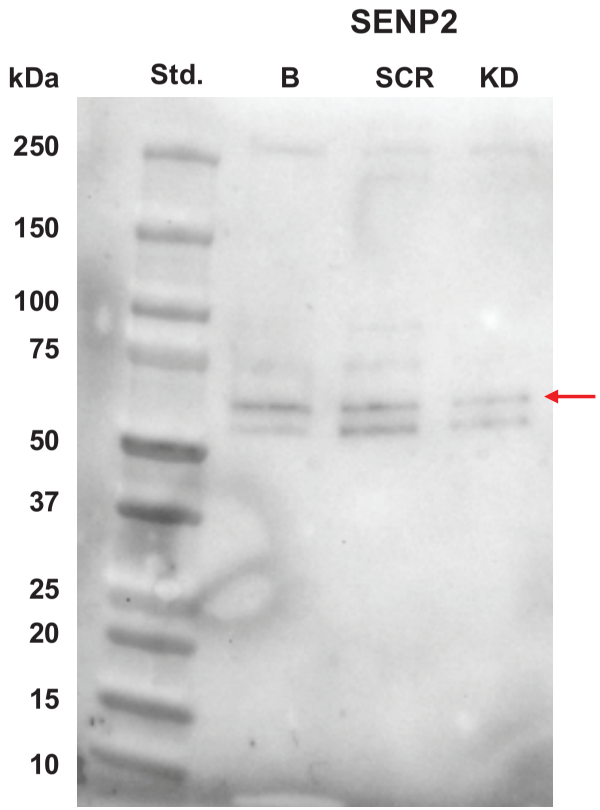
Wensaas, A., Rustan, A., Lövstedt, K., Kull, B., Wikström, S., Drevon, C., Hallen, S., 2007. Cell-based multiwell assays for the detection of substrate accumulation and oxidation. J. Lipid Res. 48, 961–967.

Yamashita, D., Yamaguchi, T., Shimizu, M., Nakata, N., Hirose, F., Osumi, T., 2004. The transactivating function of peroxisome proliferator-activated receptor γ is negatively regulated by SUMO conjugation in the amino-terminal domain. Gene Cell. 9, 1017–1029.

TO FIGURE 2

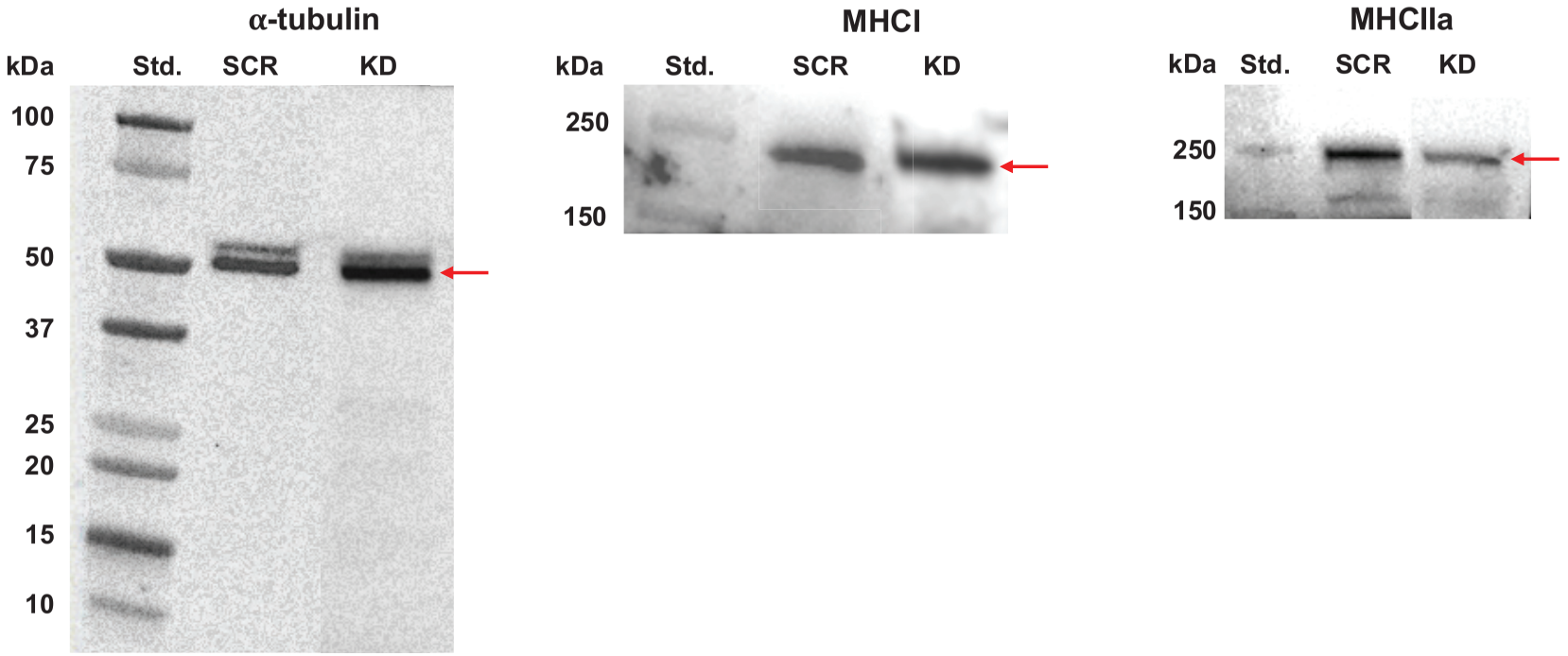
Std., protein standard; B, basal cells i.e. non-transduced; SCR, control cells i.e., with scrambled vector; KD, cells with SENP2 knockdown. Arrow shows protein of interest.





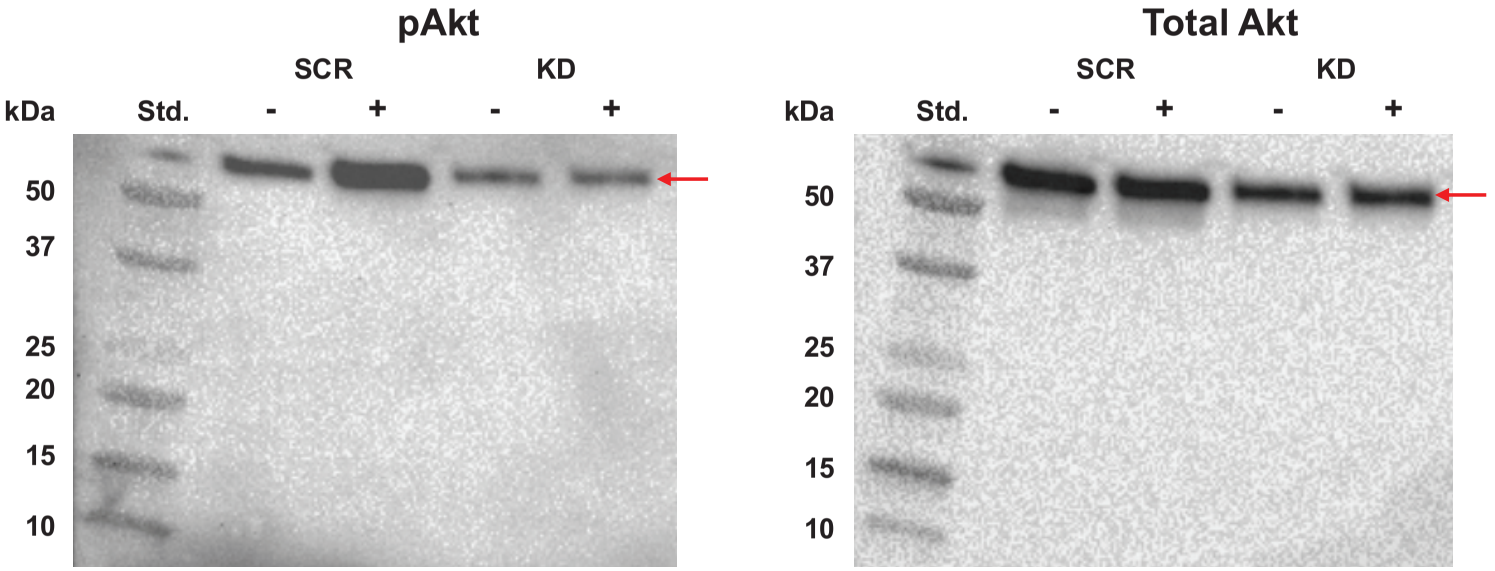
TO FIGURE 3

Std., protein standard; SCR, control cells i.e., with scrambled vector; KD, cells with SENP2 knockdown.
Arrow shows protein of interest.



TO FIGURE 8

Std., protein standard; SCR, control cells i.e., with scrambled vector; KD, cells with SENP2 knockdown; -/+, with or without insulin. Arrow shows protein of interest.



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Original Article

Pancreatic cancer cells show lower oleic acid oxidation and their conditioned medium inhibits oleic acid oxidation in human myotubes

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ABSTRACT

Background: /Objectives: We aimed to metabolically compare healthy primary human pancreatic epithelial cells (hPEC) to a pancreatic cancer cell line (PANC-1) and explore the effect on energy metabolism of exposing primary human myotubes to conditioned medium from hPEC and PANC-1 cells.
Methods: Differences in metabolism were examined with radiolabeled glucose, oleic acid and lactic acid, and by qPCR. Mass spectrometry-based proteomics was used to study global protein secretion from the two cell types. Pathway analyses were performed.
Results: PANC-1 cells tended to have higher glucose uptake, production of lactic acid, and glucose oxidation compared to hPEC cells. PANC-1 cells had higher uptake but lower oxidation of oleic acid, and mitochondrial reserve capacity from oleic acid was lower in PANC-1 cells. These differences in energy metabolism were reflected by differences in gene expressions and pathway analyses of the secretome. Conditioned medium from PANC-1 cells attenuated oleic acid oxidation in primary human myotubes.
Conclusions: Metabolic characterization of the PANC-1 cells revealed a glycolytic phenotype since they had an active glucose oxidation. Furthermore, PANC-1 cells showed a lower oleic acid oxidation and secreted a high amount of proteins into conditioned medium that also induced a reduced oleic acid oxidation in myotubes.

Introduction

Cancer is a major public health concern leading to morbidity and mortality. Pancreatic cancer has a 5-year survival rate of only 4.6% [1] and has also been linked to onset of diabetes [2]. It has been suggested that secreted peptides and proteins from pancreatic cancer cells can alter the metabolism in muscle and induce peripheral insulin resistance, but specific factors involved remains to be elucidated [3,4]. In muscle cells from mice treated with conditioned media (CM) from a variety of different pancreatic cell lines, numerous glucose metabolism-related genes were affected [3]. Glucose tolerance can be influenced by malignant disorders in general; however, frequency of impaired glucose tolerance and diabetes is higher in patients with pancreatic cancer than other types of cancer [4]. Skeletal muscles from patients with pancreatic ductal adenocarcinoma (PDAC) had decreased ATP production and glycogen content, and were also insulin resistant towards glucose uptake [5]. However, CM from human PDAC cells did not affect insulin action or glucose uptake in rat L6 myoblasts [5]. Another feature of cancer is cachexia, occurring in approximately 80% of pancreatic cancer patients [6]. Cachectic muscle has previously shown an impaired mitochondrial metabolism, with ineffective ATP generation and increased expression of mitochondrial uncoupling proteins [7].

The importance of energy consumption in cancer cells became evident with Warburg's observations [8]. He described that cancer cells had increased glucose consumption and that even with oxygen available, cancer cells tended to prefer using glucose for lactic acid production rather than oxidative phosphorylation (OXPHOS). As much as 66% of the available glucose was used for aerobic

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glycolysis [8], generally accompanied by increased glucose uptake through upregulation of glucose transporters (GLUTs), mainly GLUT1, to compensate for the low ATP production [9]. A recent study using different PDAC cell lines showed that these mainly used OXPHOS to obtain energy, leaving aerobic glycolysis to a lesser extent, and that amino acid supply and autophagy were important factors to sustain the cell growth [10]. However, Cheng et al. observed a great difference in preference to glycolysis amongst different PDAC cell lines [11].

Cancer cell alterations in fatty acid (FA) metabolism have been increasingly recognized, and studies on pancreatic cancer and breast cancer have shown that inhibition of FA uptake decreased cell migration and proliferation [12,13]. In breast cancer, a high expression of CD36 has been found, enhancing the uptake of free FAs into the cancer cell [13]. Furthermore, uptake of cholesterol through low-density lipoprotein receptor was higher in cancerous pancreas compared to healthy pancreas [14]. FA synthase, involved in lipid synthesis, was upregulated in PDAC biopsies [15].

In this study we compared non-malignant primary human pancreatic epithelial cells (hPEC) to a pancreatic cancer cell line (PANC-1)[5]. We studied differences in metabolism of glucose, oleic acid (OA) and lactic acid, as well as lactic acid production, and compared expression of selected metabolically relevant genes between hPEC and PANC-1 cells. Further, we wanted to study differences of the secretome of the pancreatic cells and examine whether CM could differently affect metabolism in primary human myotubes.

Methods

Cell culturing

PANC-1 cells

PANC-1 cells (ATCC® CRL-1469) were cultured in DMEM, high (25 mM) glucose supplemented with 10% FBS. Cells were kept in 75 cm^2 flasks before cultured for experiments. The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C, and medium was changed every 2–3 days.

hPEC cells

hPEC cells (Cell Biologics) were cultured in epithelial cell basal medium with supplements. Cells were kept in 75 cm^2 flasks before cultured for experiments (passage 6–8). The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C, and medium was changed every 2–3 days.

Skeletal muscle cells

Biopsies were obtained after informed written consent and approval by Regional Committee for Medical and Health Research Ethics North, Tromsø, Norway (2011/882). The study adhered to Declaration of Helsinki. Multinucleated human myotubes were established by activation and proliferation of satellite cells from *musculus vastus lateralis* from healthy donors as described [16]. Myoblasts (passage 3) were proliferated in DMEM-Glutamax™ (5.5 mM glucose) medium supplemented with 10% FBS, 10 ng/ml hEGF, 0.39 μg/ml dexamethasone, and 0.05% BSA. At approximately 80% confluence the medium was changed to DMEM-Glutamax™ (5.5 mM glucose) supplemented with 2% FBS and 25 pM insulin to initiate differentiation into multinucleated myotubes. The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C, medium was changed every 2–3 days and they differentiated for 7 days.

Harvesting of conditioned media from PANC-1 and hPEC cells and following treatment of myotubes

PANC-1 (1×10^6 cells/flask) and hPEC cells (2×10^6 cells/flask) were cultured in 75 cm^2 flasks in respective mediums for 24 h before washed and given DMEM, high glucose (PANC-1 medium without FBS, basal). After 24 and 48 h the medium was collected and spun down at 1000 rpm at 4 °C for 15 min before supernatants were frozen at −80 °C.

Human myoblasts were cultured and differentiated on 96-well CellBIND® microplates. The last 4 days of differentiation the cells were treated with medium collected from hPEC or PANC-1 cells. CM was added in a ratio of 50:50 with skeletal muscle cells differentiation medium.

Substrate oxidation assay

PANC-1 cells (10000 cells/well), hPEC cells (20000 cells/well) and myoblasts (7000 cells/well) were cultured on 96-well CellBIND® microplates. The last 24 h of culturing both cell types were in DMEM, high glucose. D-[^{14}C(U)]glucose (0.5 μCi/ml, 200 μM; PerkinElmer NEN®), [1−^{14}C]oleic acid ([^{14}C]OA, 0.5 μCi/ml, 100 μM; PerkinElmer NEN®) or L-[^{14}C(U)]lactic acid (1 μCi/ml, 200 μM; PerkinElmer NEN®) was given during 2 h CO_2 trapping as described [17]. CO_2 production (oxidation), in presence or absence of 1 μM FCCP, and cell-associated radioactivity (CA) were assessed using a PerkinElmer 2450 MicroBeta[2]2 scintillation counter. Protein content/well was determined with Bio-Rad protein assay using a VICTOR™ *X*4 Multilabel Plate Reader (PerkinElmer). Sum of $^{14}CO_2$ and CA: total substrate uptake.

Determination of lactate and glucose concentration in cell media

Each CM sample (20 μl) was added to a Biosen Eppendorf with glucose and lactate hemolyzing solution (EKF Diagnostics) before lactate and glucose concentration were determined using C-line glucose and lactate analyzer (EKF Diagnostics).

Proteomic analysis

PANC-1 (1×10^6 cells/flask) and hPEC cells (2×10^6 cells/flask) were cultured in 75 cm^2 flasks for 24 h in respective media before washed and given DMEM, high glucose without FBS. After 48 h culturing the media were collected and spun down at 1000 rpm at 4 °C for 15 min before supernatants were collected and frozen at −80 °C.

Proteins were reduced, alkylated and digested into peptides with trypsin. Resulting peptides were desalted and concentrated before mass spectrometry by the STAGE-TIP method using a 3 M Empore C18 resin disc. Each peptide mixture was analyzed by a nEASY-LC coupled to QExactive Plus with EASY Spray PepMap® RSLC column (C18, 2 μl, 100 Å, 75 μm × 25 cm) using 60 min LC separation gradient. Resulting MS raw files were submitted to the MaxQuant software version 1.6.1.0 for protein identification and label-free quantification. Carbamidomethyl (C) was set as a fixed modification and acetyl (protein N-term), carbamyl (N-term) and oxidation (M) were set as variable modifications. First search peptide tolerance of 20 ppm and main search error 4.5 ppm were used. Trypsin without proline restriction enzyme option was used, with two allowed miscleavages. The minimal unique + razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. Label-free quantitation was employed with default settings. UniProt database with 'human' entries (September 2018) was used for the database searches. Known contaminants as provided by MaxQuant and identified in

samples were excluded from further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository, dataset identifier PXD017613.

RNA isolation and analysis of gene expression by qPCR

PANC-1 and hPEC cells were cultured in 6-well CellBIND® plates. Total RNA was isolated using QIAGEN RNeasyMini Kit and reversely transcribed with High-Capacity cDNA Reverse Transcription Kit and TaqMan Reverse Transcription Reagents using a PerkinElmer 2720 Thermal Cycler (25 °C for 10 min, 37 °C for 80 min and 85 °C for 5 min). Primers were designed using Primer Express®. qPCR was performed using a StepOnePlus Real-Time PCR system. Target genes were quantified in duplicates carried out in a 25 μl reaction volume. All assays were run for 40 cycles (95 °C for 15 s before 60 °C for 60 s). Expression levels were normalized to the housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*). Forward and reverse primers (Supplementary Table 1) were used at a concentration of 30 μM.

Presentation of data and statistics

Values are reported as mean ± SEM; *n* represents number of individual experiments, each at least in duplicate. Statistical analyses were performed using GraphPad Prism 8 for Windows. Unpaired *t*-test was used to evaluate effects between cell types. Paired *t*-test was used to evaluate effects within a cell type. $p < 0.05$ was considered significant. For secretome analysis, four biological replicates from PANC-1 and hPEC cells were included. After protein identification and label-free quantification using MaxQuant-software, further data processing and statistical analysis (unpaired *t*-test with permutation-based FDR < 0.05) was done in Perseus software 1.6.1.3. Pathway analysis was done using Ingenuity Pathway Analysis (IPA).

Results

Differences in energy metabolism between hPEC and PANC-1 cells

We studied differences in metabolism between non-malignant hPEC cells and cancerous PANC-1 cells (Fig. 1). Glucose uptake was not significantly different between the two cell types (Fig. 1A), whereas oxidation of glucose was higher in PANC-1 compared to hPEC cells (Fig. 1B). Furthermore, glucose oxidation relative to uptake was higher in PANC-1 cells (data not shown). OA metabolism was also different between the two cell types, with higher uptake (Fig. 1C) and lower oxidation (Fig. 1D) of OA in PANC-1 compared to hPEC cells. No significant differences were observed in uptake or oxidation of lactic acid between the two cell types (Fig. 1E and F, respectively).

Lower mitochondrial reserve capacity in PANC-1 cells

OA metabolism differed more than glucose and lactate metabolism between the two cell types (Fig. 1). To examine the difference in oxidative capacity of OA even further, oxidation from [^{14}C]OA in presence and absence of the mitochondrial uncoupler FCCP was measured (Fig. 2). The capacity to oxidize [^{14}C]OA was lower in PANC-1 than hPEC cells both in the basal situation and when uncoupled by FCCP (Fig. 2). Mitochondrial reserve capacity was also lower in PANC-1 than hPEC cells (Fig. 2).

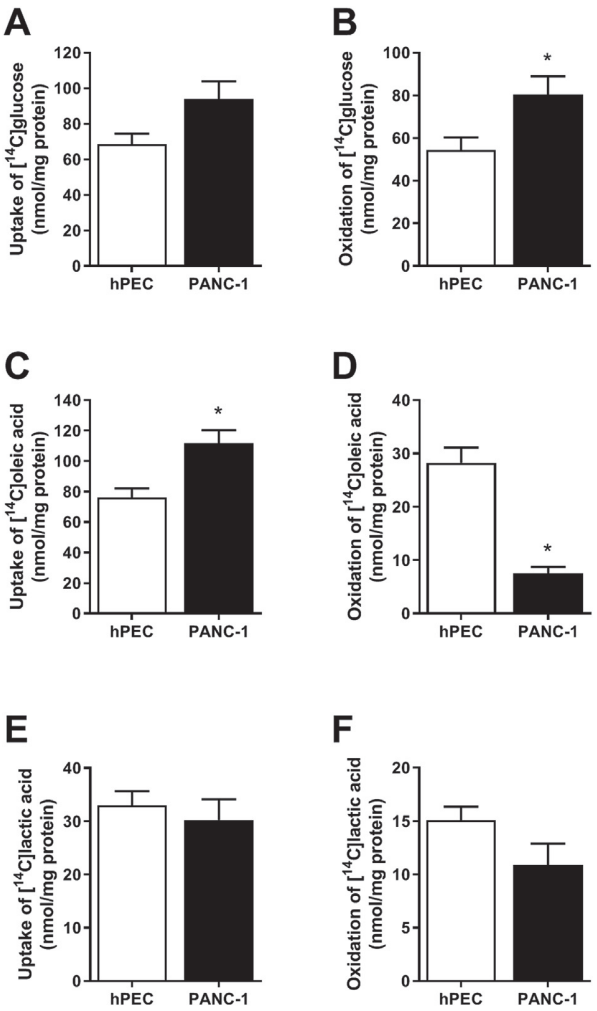

Fig. 1. Uptake and oxidation of glucose, oleic acid and lactic acid. A and B) Uptake (CO_2+CA), and oxidation (CO_2) of glucose. **C and D**) Uptake (CO_2+CA) and oxidation (CO_2) of oleic acid. **E and F**) Uptake (CO_2+CA) and oxidation (CO_2) of lactic acid. Data are presented as mean ± SEM, nmol/mg protein ($n = 4$). *Statistically significant compared to hPEC ($p < 0.05$, unpaired *t*-test).

Lower glucose and higher lactic acid concentration in media from PANC-1 cells

Generally, cancer cells will use more glucose and produce more lactic acid than non-malignant cells counterparts [8,18]. Therefore, media were harvested from the two cell types after 24 and 48 h culturing before glucose and lactic acid concentration were measured in both media as well as in the basal (0 h) medium. PANC-1 cells used more glucose than hPEC cells after 48 h culturing (Fig. 3A), and produced more lactic acid, already after 24 h culturing (Fig. 3B).

Different expression of genes involved energy metabolism between hPEC and PANC-1 cells

To further study the differences in energy metabolism between PANC-1 and hPEC cells, mRNA expression of selected substrate transporters (Fig. 4) and other genes involved in energy homeostasis (Fig. 5) were measured.

mRNA expression of FA transporter *CD36* was lower in PANC-1 compared to hPEC cells. The glucose transporters displayed

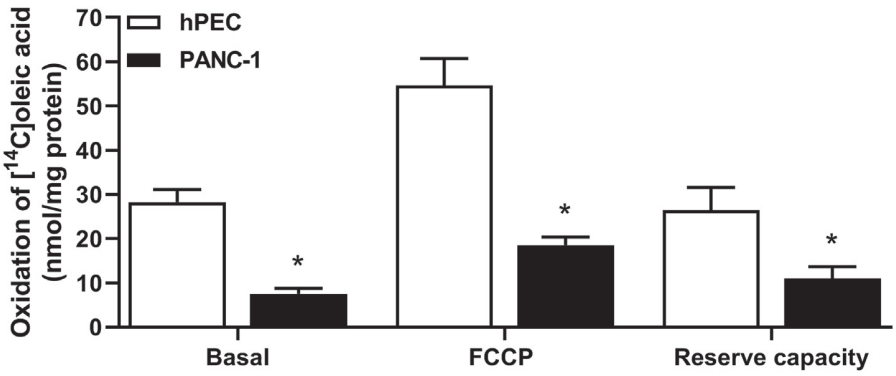

Fig. 2. Mitochondrial reserve capacity. Reserve capacity was calculated as oleic acid oxidation with FCCP present - oleic acid oxidation without FCCP present. Data are presented as mean ± SEM, nmol/mg protein ($n = 4$). *Statistically significant compared to hPEC ($p < 0.05$, unpaired t-test).

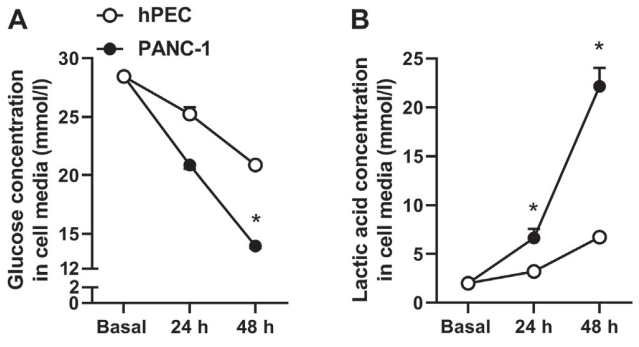

Fig. 3. Glucose and lactic acid concentration in media harvested from pancreatic hPEC and PANC-1 cells. A) Glucose concentration and **B)** lactic acid concentration in cell media. Data are presented as mean ± SEM, mM ($n = 4$). *Statistically significant compared to hPEC at same time point ($p < 0.05$, unpaired t-test).

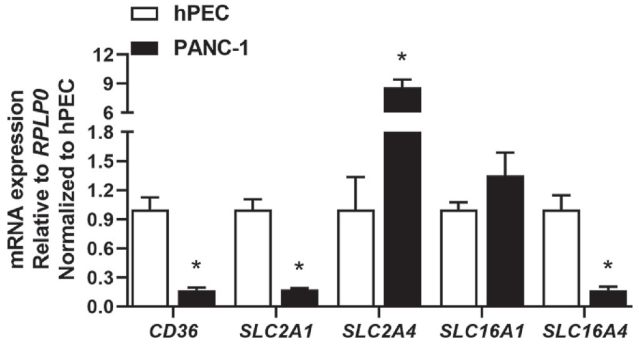

Fig. 4. Gene expression of glucose, fatty acid and lactic acid transporters. mRNA expressions of fatty acid transporter *CD36*, glucose transporters *SLC2A1* (GLUT1) and *SLC2A4* (GLUT4), and lactate transporters *SLC16A1* (MCT1) and *SLC16A4* (MCT4). Data are presented as mean ± SEM, normalized to hPEC cells ($n = 4$). *Statistically significant compared to hPEC ($p < 0.05$, unpaired t-test).

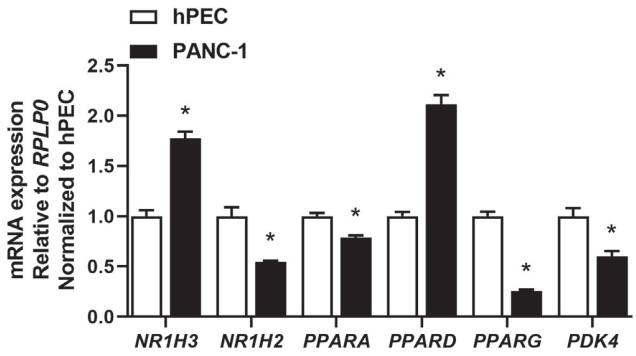

Fig. 5. Gene expression of metabolic nuclear receptors in hPEC and PANC-1 cells. mRNA expressions of nuclear receptor subfamily 1 group H member 3 (*NR1H3*, LXRα) and member 2 (*NR1H2*, LXRβ), peroxisome proliferator activated receptor alpha (*PPARA*), delta (*PPARD*) and gamma (*PPARG*, and pyruvate dehydrogenase kinase 4 (*PDK4*). Data are presented as mean ± SEM, normalized to hPEC cells ($n = 4$). *Statistically significant compared to hPEC ($p < 0.05$, unpaired t-test).

opposite expression patterns in the two cell types, with lower mRNA expression of the basal glucose transporter *SLC2A1* (GLUT1) and higher expression of the insulin-stimulated glucose transporter *SLC2A4* (GLUT4) in PANC-1 than hPEC cells. mRNA expression of lactate transporter *SLC16A1* (MCT1) was not different between the two cell types, whereas expression of lactate transporter *SLC16A4* (MCT4) was lower in PANC-1 than hPEC cells.

Expressions of *PPARA* and *PPARG* were lower, whereas *PPARD* was higher expressed in PANC-1 than hPEC cells. LXRs also displayed opposite expression patterns in the two cell types: *NR1H3* (LXRα) was higher expressed whereas *NR1H2* (LXRβ) was lower expressed in PANC-1 than hPEC cells. mRNA expression of *PDK4*, a main regulator of substrate oxidation, was lower in PANC-1 than hPEC cells.

Conditioned medium from PANC-1 cells reduces oleic acid oxidation in cultured human myotubes

It has been suggested that secreted peptides from pancreatic cancer cells can alter the energy metabolism in muscle [3,4] and ultimately lead to cachexia [19]. Therefore, effect of treating primary human myotubes with CM from hPEC and PANC-1 cells on energy metabolism was examined. Glucose metabolism in myotubes was not affected by treatment with hPEC or PANC-1 CM (Fig. 6A and B). OA uptake was also not different in myotubes exposed to CM (Fig. 6C); however, OA oxidation was lower in myotubes exposed to PANC-1 CM compared to control (Fig. 6D).

Global secretome analysis reveals higher protein secretion from PANC-1 cells

Secretomes of hPEC and PANC-1 cells were examined using quantitative label-free proteomics and pathway analyses (Fig. 7 and supplementary file 1). In total, over 800 proteins were identified. Principal component analysis showed that the protein profiles in the two secretomes were clearly different (Fig. 7A), and volcano plot showed a higher amount of proteins important in regulation of

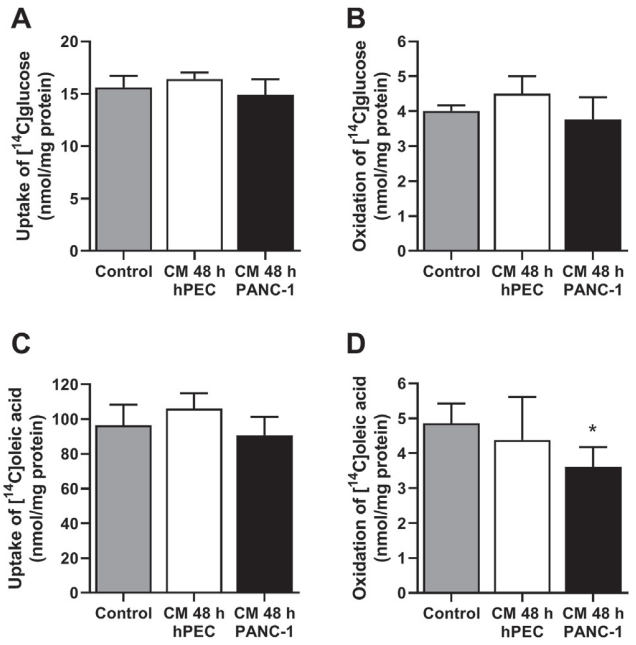

Fig. 6. Metabolism of glucose and oleic acid in myotubes treated with hPEC and PANC-1 media. PANC-1 and hPEC cells were cultured in respective media for 48 h before harvested. The last 4 days of myotube differentiation they were treated with these media or control medium (DMEM, high glucose with 10% FBS). **A and B**) Uptake and oxidation glucose. **C and D**) Uptake and oxidation of oleic acid. Data are presented as mean ± SEM, nmol/mg protein ($n = 4$). *Statistically significant compared to control ($p < 0.05$, paired t-test).

energy metabolism in the PANC-1 secretome (Fig. 7B). Pathway analysis of the secretome data showed 60 pathways to be significantly different between PANC-1 and hPEC cells. Five of these pathways were particularly interesting given the results from the functional metabolism studies; sirtuin signaling pathway, glycolysis, gluconeogenesis, pentose phosphate pathway, and FA biosynthesis initiation pathway were significantly different between PANC-1 and hPEC cells (Fig. 7C). The majority of regulated proteins in these pathways were upregulated in the cancerous PANC-1 cells (Fig. 7C and supplementary file 1).

Discussion

In this study, we wanted to explore metabolic differences between primary human pancreatic cells (hPEC) and a human pancreatic cancer cell line (PANC-1). Further, we wanted to study differences of the secretome of the pancreatic cells and examine whether CM could differently affect metabolism in primary human myotubes.

PDAC cell lines show differences in glycolytic preference [10,11]. PANC-1 cells exhibited a glycolytic phenotype. Although secreting more lactic acid and tending to take up more glucose than hPEC cells, glucose oxidation was also higher in PANC-1 cells than hPEC, possibly implying increased OXPHOS pathway. Furthermore, gene expression of *PDK4* was lower in PANC-1 than hPEC cells, consistent with findings in pancreatic cancer tumors compared to normal pancreatic tissue of the same person [20]. PDK4 inactivates the pyruvate dehydrogenase complex (PDC), which in turn favors the use of FAs rather than glucose [21]. As PANC-1 cells had lower *PDK4* mRNA expression than hPEC it indicates that the PDC is less inhibited, and that PANC-1 cells use glucose to greater extent than hPEC. This is supported by the glucose oxidation data, showing that PANC-1 cells oxidized more glucose than hPEC, and the fact that the PANC-1 cells produced more lactate.

Gene expression of the insulin-dependent GLUT4 transporter

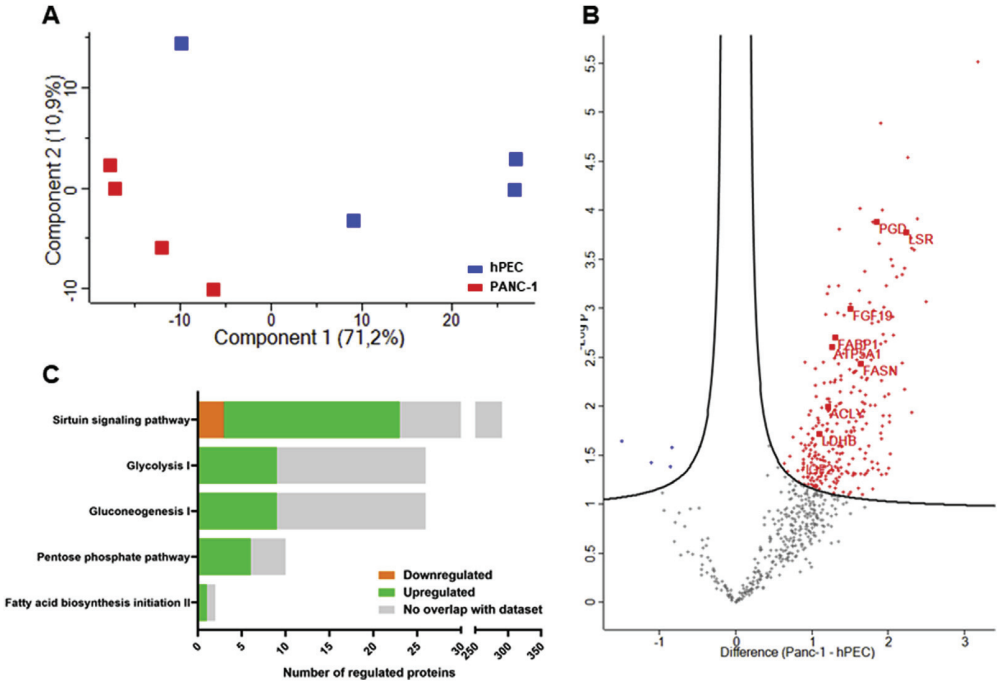

Fig. 7. Secretome analysis of hPEC and PANC-1 cells. A) Principal component analysis of the secretome data. **B)** Volcano plot showing differences in protein secretion between PANC-1 and hPEC cells. ACLY, ATP citrate lyase; FABP1, fatty acid binding protein 1; FASN, fatty acid synthase; FGF19, fibroblast growth factor 19; LDHB, lactate dehydrogenase B; LSR, lipolysis-stimulated lipoprotein receptor; PGD, phosphogluconate dehydrogenase. **C)** Selected differently regulated canonical pathways in secretome of PANC-1 compared to hPEC cells from IPA.

was higher, whereas mRNA expression of GLUT1 was lower in PANC-1 than hPEC cells. However, GLUT1 was expressed to a greater extent than GLUT4 in both cell types. These results are not typical in cancer cells, as they tend to increase expression of glucose transporters to compensate for low ATP production [18]. However, as PANC-1 cells displayed high activity in the OXPHOS pathway, compensation might not be needed and therefore no GLUT1 upregulation. Higher mRNA expression of GLUT4 in PANC-1 than hPEC cells indicates that it might be important in PANC-1 cancer cells. This is in line with the findings that cell-cycle regulatory protein human ecdysoneless (ECD) had increased expression in PDAC compared with minimal expression in non-neoplastic pancreatic ducts, and that ECD knockdown lead to GLUT4 down-regulation and decreased production of ATP and lactic acid in pancreatic cancer cells [22].

K-Ras mutation has been reported to enhance the glycolytic pathway in PDAC by increasing expression of GLUTs and rate-limiting enzymes of glycolysis. This enhanced glucose metabolism can promote tumorigenesis in PDAC; e.g. the oxidative pentose phosphate pathway has been shown to be activated by oncogenic K-Ras in order to promote cellular proliferation [23]. Interestingly, the pentose phosphate pathway and glycolytic pathways (glycolysis and gluconeogenesis) were found to be different between the secretome of PANC-1 and hPEC cells, with majority of proteins being upregulated in PANC-1. Looking at specific proteins secreted it was observed that e.g. phosphogluconate dehydrogenase (PGD), the second dehydrogenase in the pentose phosphate shunt, was secreted from PANC-1 cells only.

Lactate metabolism was not different between the two cell types, but lactate production was very high in PANC-1 cells as seen by the large accumulation in the medium. Furthermore, it was observed that PANC-1 cells secreted lactate dehydrogenase B (LDHB), an important protein for the fermentation of pyruvate to lactate. We found no difference in mRNA expression of MCT1 lactate transporter between the two cell types, but expression of the other main lactate transporter, MCT4, was lower in PANC-1 than hPEC cells. The former is typically involved in lactic acid import [24] and the latter in lactic acid export [25]. A previous study showed a general reduction of both MCT1 and MCT4 at mRNA level in the PDAC cell lines whereas MCT4 expression was similar between human pancreatic ductal epithelial and PANC-1 cells [26]. This contradicts with current findings but might be explained by different control cells of choice.

PANC-1 cells had lower OA oxidative capacity, despite higher OA uptake and lower mitochondrial reserve capacity than hPEC cells. This implies that primary hPEC cells are more efficient than PANC-1 cancer cells to metabolize FAs. This was partially supported by lower gene expression of the nuclear metabolic regulator *PPARG* in PANC-1 cells; however, gene expression of another metabolic nuclear regulator, LXRα, was higher. Furthermore, that PANC-1 cells had high OA uptake but low expression of *CD36* was contradictory, confirming that mRNA expression does not always correlate with functional data. Lipogenic protein ATP citrate lyase (ACLY) as well as the lipolysis-stimulated lipoprotein receptor (LSR) was found in the secretome from PANC-1 cells only, which may be part of the explanation of higher OA uptake in these cells.

Muscle cells seem to be specifically targeted in pancreatic cancer by factors released by the tumor cells leading to altered glucose metabolism and cachexia [19]. Therefore, impact of CM from the two pancreatic cell types on metabolic factors in primary human myotubes were explored, including an in-depth exploration of the potential factors secreted using proteomics. Others have shown that CM from pancreatic cancer cells (PANC-1 and Colo-320 cells) increased glucose uptake in GLUT4-transfected L6 myoblasts [27]. No effect of PANC-1 CM was observed on glucose metabolism in human myotubes. Reasons for this discrepancy could be the difference in metabolic capacity between L6 myotubes and primary human myotubes and that the L6 myotubes were GLUT4-transfected [28]. OA oxidation was lower in PANC-1 than hPEC cells. Interestingly, treating primary human myotubes with PANC-1 CM induced a reduction in OA oxidation in the myotubes. This suggests that FA metabolism was inhibited by some factors present in PANC-1 CM but not hPEC CM, possibly the same factors resulting in lower OA oxidation in PANC-1 than hPEC cells. The FA biosynthesis initiation pathway was different between the two cell types, with majority of proteins upregulated; e.g. FA synthase (FASN) and FA binding protein 1 (FABP1) was found to be part of the PANC-1 secretome only. The sirtuin signaling pathway was not surprisingly different between PANC-1 and hPEC cells, being a regulator of cancer cell growth and survival, and metabolic state of tumors [29]. Its role in mitochondrial function and glycolytic regulation is known, whereas the role in lipogenesis and FA oxidation is less explored [29]. PANC-1 cells had an altered lipid metabolism compared to hPEC cells, possibly a result of these two pathways being different in the two cell types.

In conclusion, PANC-1 cells displayed a more glycolytic phenotype compared to hPEC cells. Although tending to have higher glucose uptake and production of lactic acid, PANC-1 cells also displayed higher glucose oxidation than hPEC cells. Interestingly, PANC-1 cells had higher uptake but lower oxidation of OA and lower mitochondrial reserve capacity from OA. These changes in energy metabolism were reflected by differences in gene expressions and differential release of proteins, comprising different pathways. CM from PANC-1 cells reduced OA oxidation in primary human myotubes; further studies are needed to reveal the mediator(s) of this effect.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pan.2020.04.014.

References

[1] Decker GA, Batheja MJ, Collins JM, Silva AC, Mekeel KL, Moss AA, et al. Risk factors for pancreatic adenocarcinoma and prospects for screening. Gastroenterol Hepatol 2010;6:246.
[2] Wang F, Herrington M, Larsson J, Permert J. The relationship between diabetes and pancreatic cancer. Mol Canc 2003;2:4.
[3] Basso D, Millino C, Greco E, Romualdi C, Fogar P, Valerio A, et al. Altered glucose metabolism and proteolysis in pancreatic cancer cell conditioned myoblasts: searching for a gene expression pattern with a microarray analysis of 5000 skeletal muscle genes. Gut 2004;53:1159–66.
[4] Permert J, Adrian TE, Jacobsson P, Jorfelt L, Fruin AB, Larsson J. Is profound peripheral insulin resistance in patients with pancreatic cancer caused by a tumor-associated factor? Am J Surg 1993;165:61–7.
[5] Agustsson T, D'souza MA, Nowak G, Isaksson B. Mechanisms for skeletal muscle insulin resistance in patients with pancreatic ductal adenocarcinoma. Nutrition 2011;27:796–801.
[6] Fearon KC, Voss AC, Hustead DS. Definition of cancer cachexia: effect of weight loss, reduced food intake, and systemic inflammation on functional status and prognosis. Am J Clin Nutr 2006;83:1345–50.
[7] Porporato P. Understanding cachexia as a cancer metabolism syndrome. Oncogenesis 2016;5:e200.
[8] Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen

Physiol 1927;8:519.
[9] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
[10] Maertin S, Elperin JM, Lotshaw E, Sendler M, Speakman SD, Takakura K, et al. Roles of autophagy and metabolism in pancreatic cancer cell adaptation to environmental challenges. Am J Physiol Gastrointest Liver Physiol 2017;313:G524–36.
[11] Cheng G, Zielonka J, McAllister D, Tsai S, Dwinell MB, Kalyanaraman B. Profiling and targeting of cellular bioenergetics: inhibition of pancreatic cancer cell proliferation. Br J Canc 2014;111:85–93.
[12] Okumura T, Ohuchida K, Sada M, Abe T, Endo S, Koikawa K, et al. Extrapancreatic invasion induces lipolytic and fibrotic changes in the adipose microenvironment, with released fatty acids enhancing the invasiveness of pancreatic cancer cells. Oncotarget 2017;8:18280.
[13] Zhao J, Zhi Z, Wang C, Xing H, Song G, Yu X, et al. Exogenous lipids promote the growth of breast cancer cells via CD36. Oncol Rep 2017;38:2105–15.
[14] Guillaumond F, Bidaut G, Ouaissi M, Servais S, Gouirand V, Olivares O, et al. Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy for pancreatic adenocarcinoma. Proc Natl Acad Sci U S A 2015;112:2473–8.
[15] Alo PL, Amini M, Piro F, Pizzuti L, Sebastiani V, Botti C, et al. Immnunohistochemical expression and prognostic significance of fatty acid synthase in pancreatic carcinoma. Anticancer Res 2007;27:2523–7.
[16] Lund J, Rustan AC, Løvsletten NG, Mudry JM, Langleite TM, Feng YZ, et al. Exercise in vivo marks human myotubes in vitro: training-induced increase in lipid metabolism. PloS One 2017;12:e0175441.
[17] Wensaas A, Rustan A, Lövstedt K, Kull B, Wikström S, Drevon C, et al. Cell-based multiwell assays for the detection of substrate accumulation and oxidation. J Lipid Res 2007;48:961–7.
[18] Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell 2008;134:703–7.
[19] Tisdale MJ. Cachexia in cancer patients. Nat Rev Canc 2002;2:862.
[20] Li H, Wang X, Fang Y, Huo Z, Lu X, Zhan X, et al. Integrated expression profiles analysis reveals novel predictive biomarker in pancreatic ductal adenocarcinoma. Oncotarget 2017;8:52571–83.
[21] Cairns RA. Drivers of the Warburg phenotype. Cancer J 2015;21:56–61.
[22] Dey P, Rachagani S, Chakraborty S, Singh PK, Zhao X, Gurumurthy CB, et al. Overexpression of ecdysoneless in pancreatic cancer and its role in oncogenesis by regulating glycolysis. Clin Canc Res : an official journal of the American Association for Cancer Research 2012;18:6188–98.
[23] Yan L, Raj P, Yao W, Ying H. Glucose metabolism in pancreatic cancer. Cancers (Basel) 2019;11.
[24] Draoui N, Feron O. Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatments. Disease models & mechanisms. vol. 4; 2011. p. 727–32.
[25] Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 2000;350(Pt 1):219–27.
[26] Kong SC, Nøhr-Nielsen A, Zeeberg K, Reshkin SJ, Hoffmann EK, Novak I, et al. Monocarboxylate transporters MCT1 and MCT4 regulate migration and invasion of pancreatic ductal adenocarcinoma cells. Pancreas 2016;45:1036–47.
[27] Li J, Adrian TE. A factor from pancreatic and colonic cancer cells stimulates glucose uptake and lactate production in myoblasts. Biochem Biophys Res Commun 1999;260:626–33.
[28] Abdelmoez AM, Sardon Puig L, Smith JA, Gabriel BM, Savikj M, Dollet L, et al. Comparative profiling of skeletal muscle models reveals heterogeneity of transcriptome and metabolism. Am J Physiol Cell Physiol 2020;318:C615–26.
[29] German NJ, Haigis MC. Sirtuins and the metabolic hurdles in cancer. Curr Biol 2015;25:R569–83.

Supplementary table. Description of primers.

Gene	Acc.no.	Forward sequence	Reverse sequence
ABCA1	AF165281	GAA CAG CAG TTG GAT GGC TTA GA	ACA GAA CCA TTA CTG GAC TGG ACA T
ANGPTL4	NM139314	CCT CCG CAG GGA CAA GAA	GTG GGA TGG AGC GGA AGT ACT
CD36	L06850	AGT CAC TGC GAC ATG ATT AAT GGT	CTG CAA TAC CTG GCT TTT CTC AA
NR1H2	U07132	GGC GAG GGT GTC CAG CTA A	CGG AGA AGG AGC GTT TGT TG
NR1H3	U22662	GGA GGA GTG TGT CCT GTC AGA AG	CAT GAG CCT GTT CCT CCT CTT G
PDK4	BC040239	TTT CCA GAA CCA ACC AAT TCA CA	TGC CCG CAT TGC ATT CTT A
PPARA	L02932	TCC ACC TGC AGA GCA ACC A	CCG GAG GTC TGC CAT TTT T
PPARD	BC002715	AGC ATC CTC ACC GGC AAA	ATG TCT CGA TGT CGA TGT CGT GGA TCA C
PPARG	L40904	AGC CTG CGA AAG CCT TTT G	ATT CCA GTG CAT TGA ACT TCA CA
RPLP0	M17885	CCA TTC TAT CAT CAA CGG GTA CAA	AGC AAG TGG GAA GGT GTA ATC C
SLC2A1	K03195	CAG CAG CCC TAA GGA TCT CTC A	CCG GCT CGG CTG ACA TC
SLC2A4	M20747	ACC CTG GTC CTT GCT GTG TT	ACC CCA ATG TTG TAC CCA AAC T
SLC16A1	NM_003051	CCC TAA ACA AGA GAA ACG ATC AGT CT	AAC ATG ATC ACA TTT CCA GAG AGG TA
SLC16A4	NM_004696	CTT TCG TTG TTA CTG GTG GAT ATC TG	AAC CGG GTA GAA GTC CCA TAG TC

Primers were designed using Primer Express® (Applied Biosystems). ABCA1, ATP binding cassette subfamily A member 1; ANGPTL4, angiopoietin like 4; CD36, CD36 molecule; NR1H2/3, nuclear receptor subfamily 1 group H member 2/3; PDK4, pyruvate dehydrogenase kinase 4; PPARA/D/G, peroxisome proliferator activated receptor alpha/delta/gamma; RPLP0, ribosomal protein lateral stalk subunit P0; SLC2A1/4, solute carrier family 2 member 1/4; SLC16A1/4, solute carrier family 16 member 1/4.

Supplementary file 1

https://view.officeapps.live.com/op/view.aspx?src=https%3A%2F%2Fars.els-cdn.com%2Fcontent%2Fimage%2F1-s2.0-S1424390320301435-mmc1.xlsx&wdOrigin=BROWSELINK

IV

Pancreatic cancer cell conditioned primary myotubes display increased leucine turnover, increased lipid accumulation and reduced glucose uptake.

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Keywords: pancreatic cancer, cachexia, cell model, energy metabolism, protein metabolism.

Abstract

Background: Metabolic alterations in cancer cells are well-known. Metabolic changes have also been seen to occur in other tissues than the cancerous tissue. For instance, cachexia, peripheral insulin resistance or both is commonly seen in patients with cancer. We explored differences in leucine, glucose and oleic acid in myotubes conditioned with media from either a pancreatic cancer cell line, PANC-1, or primary human pancreatic cells, hPEC

Methods: For protein turnover we used scintillation proximity assay, while glucose and oleic acid handling was analyzed with substrate oxidation assay. We also performed qPCR to study gene expression, immunoblotting and proteomic analysis to study at protein expression.

Results: We found that the PANC-1-conditioned myotubes had an imbalance in protein turnover with decreased accumulation and increased decay, followed by decreased *MYH2* gene expression. Glucose uptake was decreased despite increased insulin-stimulated Akt phosphorylation. Fatty acid uptake was increased whereas fatty acid oxidation was unchanged, leading to accumulation of intracellular lipids (TAG) in myotubes conditioned with PANC-1 medium. These findings were supported by decreased gene expression of electron transport chain complex III, *UQCRB.* and increased gene expression of CD36. Secretome analyses revealed increased release of growth factors and growth factor receptor from PANC-1 cells potentially affecting muscle cell metabolism.

Conclusion: myotubes exposed to pancreatic cancer cell media display altered energy metabolism with increased protein/leucine turnover and lipid accumulation while glucose uptake and oxidation, Indicating a production and release of substances from pancreatic cancer cells affecting skeletal muscle energy metabolism and protein turnover.

Introduction

Cancer causes great morbidity and mortality and is a major public health concern worldwide. Pancreatic cancer, with pancreatic ductal adenocarcinoma (PDAC) being the most common type, is one of the cancers with poorest prognosis with a five-year survival rate of only 5% [1].

Metabolic alterations are well-known occurrences with cancer. Cancer cells have higher uptake of glucose and produce more lactic acid, even in the presence of oxygen, compared to normal cells [2], called the Warburg effect. Alterations in fatty acid metabolism in cancer cells are also increasingly more recognized [2]. Metabolic changes have, however, also been seen to occur in other tissues than the cancer tissue itself.

Skeletal muscle is one of the main regulators of glucose and lipids in the human body and a reservoir of amino acids with a profound impact on whole-body homeostasis. Cancer cachexia is a syndrome causing weight loss and muscle wasting, and is especially seen in striated muscle [3]. Cachexia is caused by factors secreted from (or other types of interactions with) the tumors, leading to increased basal metabolic rate and energy expenditure as well as loss of skeletal muscle due to an imbalance in protein synthesis and degradation [4]. Cachexia is found to occur in more than half of all cancer patients and responsible for more than 20% of cancer-related deaths [4]. In pancreatic cancer even higher incidence of cachexia is seen compared to other cancers [4]. Furthermore, mitochondrial dysfunction is often seen in cancer-cachexic muscle [5], associated with accumulation of lipid intermediates and potentially insulin resistance in skeletal muscle [6].

Additionally, glucose intolerance is more frequently seen in patients with pancreatic cancer. However, rather than being a risk factor for developing cancer, it is believed to be caused by the presence of the pancreatic tumor itself, as surgical removal of the cancer significant decrease in insulin resistance [7]. Insulin resistance in PDAC is manifested by impaired glucose transport, glucose oxidation and glycogen synthesis [7]. Dysregulation of lipid metabolism also plays a key role for cancer cells success. Cellular homeostasis is changed by alterations in fatty acid uptake, synthesis and hydrolysis [8], to support the rapid proliferation, survival, migration, invasion, and metastasis of cancer cells [8]. Dysregulation of lipid metabolism in pancreatic cancer cells can also be seen in light of peripheral insulin resistance often occurring in skeletal muscle of these patients [7], and its role in pathogenesis of cachexia [8]. For many patients with pancreatic cancer both cachexia and glucose intolerance are present at time of diagnosis [9].

Previously, we have compared non-malignant primary human pancreatic epithelial cells (hPEC) with a pancreatic cancer cell line (PANC-1) [10]. PANC-1 cells displayed a more glycolytic phenotype, though higher lactic acid production, also less efficient at metabolizing fatty acids compared to hPEC cells. This changes were reflected in gene expressions and protein secretion. Comparing energy metabolism in human myotubes conditioned with media from hPEC (chPEC-exposed), PANC-1 (cPANC-1-exposed) and non-conditioned cells, we found reduced oleic acid oxidation in cPANC-1-exposed compared to non-conditioned myotubes [10]. There is increasingly evident that pancreatic cancer has a great impact on muscle tissue already early in the disease development, and increasing our knowledge about this relationship can be of help in the treatment of pancreatic cancer. Herein, we wanted to further investigate the effects of cPANC-1 and chPEC on energy metabolism in primary human myotubes and to study possible differences in glucose, lipid and protein metabolism between the two conditioned myotube cultures.

Materials and methods

Materials

PANC-1 cells (ATCC® CRL-1469) were purchased from American Type Culture Collection (Manassas, VA, US). Human primary pancreatic epithelial cells (hPEC), human epithelial cell basal medium and epithelial cell medium supplement kit were purchased from Cell Biologics (Pelo Biotech, Chicago, IL, US). 6-well and 96-well Corning® CellBIND® tissue culture plates were from Corning (Schiphol-Rijk, the Netherlands). Nunc™ Cell Culture Treated Flasks with Filter Caps, Nunc™ 96-MicroWell™ plates, Dulbecco`s Modified Eagle's Medium (DMEM) high glucose with HEPES and L-glutamine, DMEM-Glutmax™ low glucose with sodium pyruvate, DPBS (without Mg^{2+} and Ca^{2+}), FBS, human epidermal growth factor (hEGF), trypsin-EDTA, penicillin-streptomycin (10000 IE/ml), amphotericin B, Countess™ counting chamber slides, Pierce™ BCA Protein Assay Kit, TaqMan reverse transcription kit reagents, High-Capacity cDNA Reverse Transcription Kit, Primers for TaqMan PCR, MicroAmp® Optical 96-well Reaction Plate, MicroAmp® Optical Adhesive Film, primers for qPCR, and Power SYBR® Green PCR Master Mix were from Thermo Fisher Scientific (Waltham, MA, US). Insulin (Actrapid® Penfill® 100 IE/ml) was from Novo Nordisk (Bagsvaerd, Denmark). Trypan blue 0.4% solution, DMSO, gentamicin, dexamethasone, L-glutamine, BSA (essentially fatty acid-free), L-carnitine, D-glucose, oleic acid (18:1, n-9), sodium lactic acid, carbonyl cyanide-*4*-(trifluoromethoxy)-phenylhydrazone (FCCP), and HEPES were from Sigma-Aldrich (St. Louis, MO, US). QIAshredder and RNeasy Mini Kit were from QIAGEN (Venlo, the Netherlands). Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad (Copenhagen, Denmark). D-[^{14}C(U)]glucose (18.5 kBq/ml), L-[14C(U)]leucine (37 kBq/ml) and [1-14C]oleic acid (18.5 kBq) were from PerkinElmer NEN® (Boston, MA, US). Ultima Gold™, 96-well Isoplate®, Unifilter®-96 GF/B, and TopSeal®-A transparent film were from PerkinElmer (Shelton, CT, US). Glucose/Lactate Hemolyzing Solution was purchased from EKF Diagnostics (Cardiff, UK).

Ethical approvals

Skeletal muscle biopsies were obtained after informed written consent and approval by Regional Committees for Medical and Health Research Ethics (REK) North, Tromsø, Norway (ref. no. 2011/882). The study adhered to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Cell culturing

PANC-1 cells

PANC-1 cells were cultured in DMEM high glucose (25 mmol/l) supplemented with 10% FBS (PANC-1 medium), according to manufacturer's instructions. Cells were kept in 75 cm^2 Nunc™ flasks before cultured for experiments and kept in a humidified 5% CO_2 atmosphere at 37°C, and the medium was changed every 2-3 days.

hPEC cells

hPEC cells were cultured in epithelial cell medium with supplements according to manufacturer's instructions (hPEC medium). Cells were kept in 75 cm^2 Nunc™ flasks before cultured for experiments and kept in a humidified 5% CO_2 atmosphere at 37°C, and the medium was changed every 2-3 days. Experiments were performed on cells in passage 6- 8.

Skeletal muscle cells

Biopsies were obtained from *musculus vastus lateralis* of healthy male donors after informed written consent and approval by the Regional Committee for Medical and Health Research Ethics North, Tromsø, Norway (ref. no. 2011/882), adhering to the Declaration of Helsinki. To establish multinucleated human myotubes satellite cells were first proliferated as myoblasts as previously described [10], in DMEM-Glutamax™ (5.5 mmol/l glucose) medium supplemented with 10% FBS, 10 ng/ml hEGF, 0.39 µg/ml dexamethasone, and 0.05% BSA. At approximately 80% confluence, the medium was changed to DMEM-Glutamax™ (5.5 mmol/l glucose) supplemented with 2% FBS and 25 pmol/l insulin to initiate differentiation into multinucleated myotubes for 7 days, in humidified 5% CO_2 atmosphere at 37°C, and medium change every 2-3 days. Experiments were performed on cells in passage 3-4.

PANC-1- and hPEC-conditioned myotubes

Due to different proliferation rate, unequal number of PANC-1 and hPEC cells were seeded to achieve approximately equal cell number at time of harvesting. PANC-1 (~1×10^6 cells/flask) and hPEC cells (~2×10^6 cells/flask) were cultured in respective media in 75 cm^2 flasks for 24 h. Cells were washed and both cell types were given DMEM medium high glucose (PANC-1 medium without FBS). After 48 h, medium was collected and spun down at 1000 rpm at 4°C for 15 min, supernatants were frozen at -80°C. Glucose and lactate levels were measured in collected media as described [10], and glucose concentration was adjusted to be the same in both media (approximately 22 mmol/l). Human skeletal muscle cells were cultured and differentiated in cell culture plates. The last 4 days of differentiation

cells were conditioned with medium collected from hPEC (chPEC) or PANC-1 (cPANC-1) cells. Conditioned medium was added in a ratio of 50:50 with DMEM-Glutamax™ (5.5 mmol/l glucose) supplemented with 2% FBS and 25 pmol/l insulin.

Scintillation proximity assay (SPA)

Myotubes were cultured in a 96-well ScintiPlate. After differentiation and conditioning, the myotubes were exposed to [^{14}C]leucine (37 kBq/ml, 0.8 mmol/l) followed by measurements of protein (leucine) accumulated in the cells by scintillation proximity assay (SPA) [11]. [^{14}C]leucine was monitored over the next 24 h (measurements at 0, 2, 4, 6, 8, and 24 h) using a 2450 MicroBeta^2 scintillation counter (PerkinElmer). Thereafter, the medium was changed to DMEM with 0.8 mmol/l leucine, 10 mmol/l HEPES, 0.5% BSA and 0.1 mmol/l glucose, and decay of [^{14}C]leucine was monitored over 6 h (measurements at 0, 2, 4, and 6 h). The amount of radioactivity in the cells was related to total cell protein content measured according to Bradford [12].

Substrate oxidation assay

Skeletal muscle cells were cultured on 96-well CellBIND® microplates and differentiated as described under "Cell Culturing". Substrate, D-[^{14}C(U)]glucose (18.5 kBq/ml, 200 µmol/l) or [1-^{14}C]oleic acid (18.5 kBq/ml, 100 µmol/l), was given during 4 h CO_2 trapping as described previously [11]. CO_2 production was measured in presence or absence of 1 µmol/l FCCP, 0.5 µmol/l rotenone in combination with 2,5 µmol/l antimycin A, or 1 µmol/l oligomycin. CO_2 production and cell-associated radioactivity (CA) were assessed using a 2450 MicroBeta^2 scintillation counter (PerkinElmer). Protein per well was determined with the Bio-Rad protein assay using a VICTOR™ *X*4 Multilabel Plate Reader (PerkinElmer) as described previously [12]. The sum of $^{14}CO_2$ and CA was considered as total substrate uptake.

Thin layer chromatography and measurement of acid-soluble metabolites (ASM)

Skeletal muscle cells were cultured on 12-well CellBIND® microplates and differentiated as described under "Cell Culturing", followed by incubated with [1-^{14}C]oleic acid (18.5 kBq/ml, 100 µmol/l) for 4 h. After incubation, the cells were washed twice with PBS and harvested with 250 µl 0.1% SDS per well. Cellular lipids were extracted as previously described by Gaster et al. by extraction of homogenized cell fraction, separation of lipids by thin layer chromatography and quantification by liquid scintillation [13]. A non-polar solvent mixture of hexane: ether: acetic

acid (65:35:1) was used to separate the lipids. The amount of neutral lipids was related to total cell protein concentration determined by Pierce protein assay.

Measurement of acid-soluble metabolites (ASM), reflecting incomplete fatty acid oxidation (β-oxidation) and mainly consists of tricarboxylic acid cycle metabolites, was performed as described and modified by Bakke et al. [14]. In short, 100 µl of the radiolabeled medium was transferred to an Eppendorf tube and precipitated with 300 µl cold $HClO_4$ (1 mol/l) and 30 µl BSA (6%). Thereafter, the tube was centrifuged at 10000 rpm/10 min/4°C before 200 µl of the supernatant was counted by liquid scintillation of a Packard Tri-Carb 1900 TR (PerkinElmer).

Glycogen synthesis

Cells were cultured and differentiated as described under "Cell Culturing". The cells were starved for 1.5 h in glucose-free DMEM medium before exposed to serum-free DMEM (5.5 mmol/l glucose) supplemented with D-[^{14}C(U)]glucose (18.5 kBq/ml, 0.67 mmol/l), in absence or presence of 20 nmol/l or 100 nmol/l insulin for 3 h. Cells were washed twice with PBS and harvested in 1 mol/l KOH. Protein content was determined by the Pierce BCA Protein Assay Kit before 20 mg/ml glycogen and more KOH (final concentration 4 mol/l) were added to the samples. Incorporated of D-[^{14}C(U)]glucose into glycogen was measured as previously described by Hessvik et al. [15].

Immunoblotting

Myotubes were harvested in Laemmli buffer (0.5 mol/l Tris-HCl, 10% SDS, 20% glycerol, 10% β-mercaptoethanol, and 5% bromophenol blue). Proteins were electrophoretically separated on 4-20% Mini-Protean TGX™ gels with Tris/glycine buffer (pH 8.3) followed by blotting to nitrocellulose membranes and incubation with antibodies. For total AKT and Akt phosphorylation immunoreactive bands were visualized with enhanced chemiluminescence (Chemidoc XRS, Bio-Rad) and quantified with Image Lab (version 6.0.1) software. The level of Akt phosphorylated at serine 473 was normalized for total Akt level. For total AMPK and AMPK phosphorylation immunoreactive bands were visualized with Image Studio Lite Ver. 5.2. The level of AMPK phosphorylated at threonine 172 was normalized for total AMPK level.

RNA isolation and analysis of gene expression by qPCR

Primary human myotubes were cultured in 6-well CellBIND® plates as described under "Cell Culturing". Total RNA was isolated using QIAGEN RNeasy Mini Kit according to the supplier`s protocol and reversely transcribed using a High-Capacity cDNA Reverse Transcription Kit and TaqMan Reverse Transcription Reagents using a

PerkinElmer 2720 Thermal Cycler (25°C for 10 min, 37°C for 80 min and 85°C for 5 min). Primers were designed using Primer Express® (Thermo Fisher Scientific, Waltham, MA, US). qPCR was performed using a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Target genes were quantified in duplicates carried out in a 25 µl reaction volume according to the supplier's protocol. All assays were run for 44 cycles (95°C for 15 s followed by 60°C for 60 s). Two housekeeping genes were examined, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*). Gene expression levels were comparable regardless of which housekeeping gene that was used; data shown are presented relative to *RPLP0*. Forward and reverse primers were used at a concentration of 30 µmol/l. Primers are presented in **Supplementary Table 1**.

Proteomic

Re-analysis of proteomic data from a previous analysis [10] was performed. Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository, dataset identifier PXD017613. STRING software, version 11.5) was applied to identify functional protein association networks using annotated key words: Growth factors, growth factor binding proteins, cytokines.

Presentation of data and statistics

All values are reported as means ± SEM. Value *n* represents the number of individual experiments, each with at least duplicate observations. Statistical analyses were performed using GraphPad Prism 8.3.0 (538) for Windows. Unpaired *t* test was used to evaluate the effects on myotubes conditioned with either hPEC or PANC-1. Paired t test was used to evaluate the effects of a treatment on myotubes conditioned with either of the two cell types. A p value < 0.05 was considered significant.

Results

Measurement of leucine incorporation in myotubes showed decreased protein synthesis and increased protein decay after conditioned with media from pancreatic cancer cells

As cancer-induced cachexia leads to increased degradation of protein in skeletal muscle [4], we wanted to examine protein content in myotubes after conditioning with media from hPEC (chPEC-exposed) or PANC-1 cells (cPANC-1-exposed). We found that the overall protein content was significantly decreased **after for 4 days conditioning with cPANC-1 and chPEC media (Figure 1A)**. To further explore the difference seen in protein content we traced cellular accumulation of [^{14}C]leucine as a measure of protein synthesis in myotubes and thereafter evaluated the decay of radioactivity from the cells. Accumulation of leucine over 24 h was significantly less for cPANC-1-exposed myotubes compared to chPEC-exposed cells **(Figures 1B and C)**. Further, the decay of radioactivity measured over 6 h was increased for cPANC-1-exposed myotubes compared to chPEC-exposed cells **(Figures 1D and E)**.

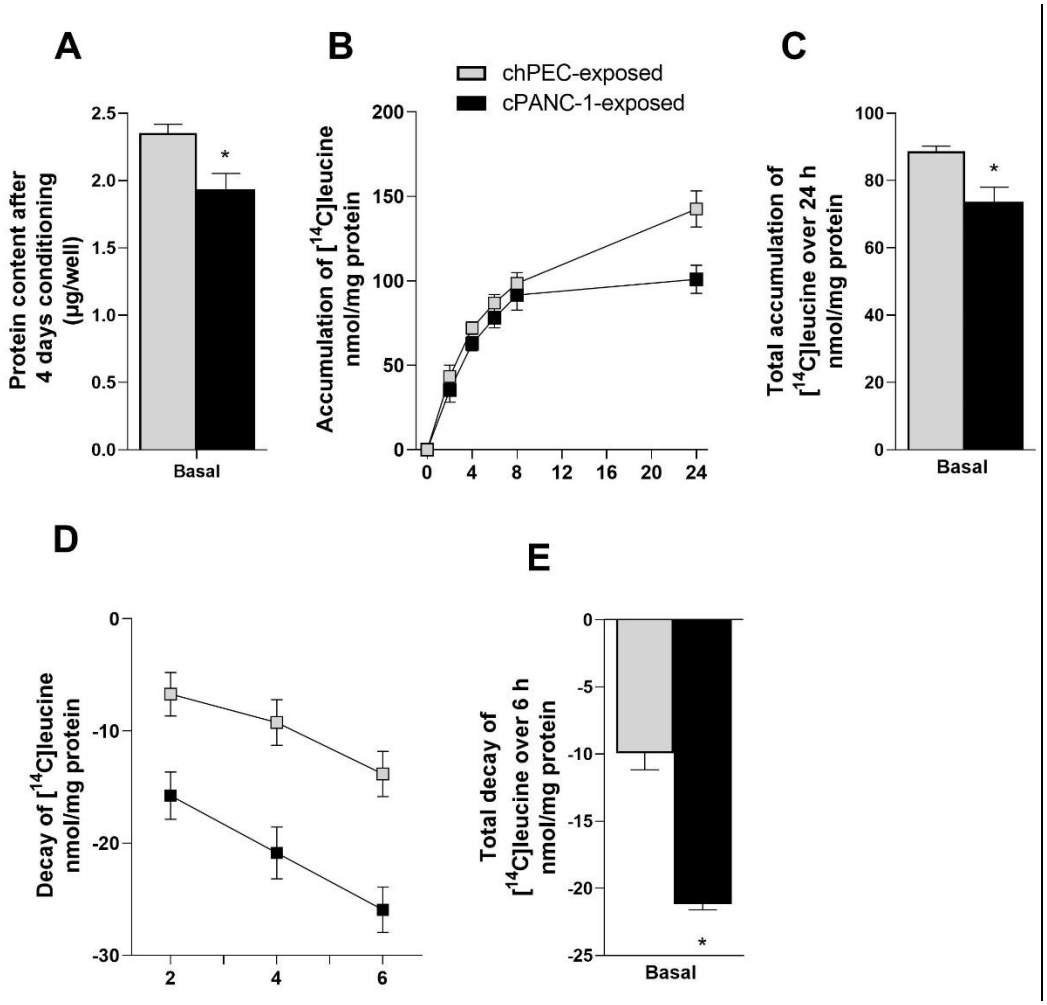

Fig. 1: Incorporation and decay of leucine in myotubes after conditioning with medium collected from hPEC or PANC- 1 cells

Myotubes were conditioned with media from human pancreatic epithelial cells (chPEC-exposed) or a human pancreatic cancer cell line (cPANC-1-exposed) during the last 4 days of differentiation. A) Protein content was measured with using Bradford protein assay. Muscle cells were incubated with [^{14}C]leucine (37 kBq, 0.8 mmol/l) and (B) cellular accumulation of leucine was measured over 24 h by scintillation proximity assay (SPA). (C) Overall accumulation of [^{14}C]leucine was calculated as area under the curve. (D) The medium was then changed to label-free medium and the decay of radioactivity was measured over 6 h by SPA. (E) Overall decay over 6 h was calculated as area under the curve. Data are presented from four individual experiments, each with three biological replicates (n = 12). *Statistically significant for cPANC-1-exposed myotubes versus chPEC-exposed myotubes ($p \leq 0.05$, SPSS linear mixed model analysis).

mRNA expression of selected genes important for protein synthesis and cachexia was also measured. We found that the mRNA expression of the ATP hydrolyzing motor protein, myosin heavy chain 2 (*MYH2*) was significantly reduced in cPANC-1-exposed myotubes compared to chPEC-exposed cells, while no changes were found for the serine-threonine kinase mechanistic target of rapamycin (*MTOR*), the mitogen-activated protein kinase 8 (*MAPK8)*, the myogenic regulatory factor myoblast determination protein 1 (*MYOD1)* or the immune response regulator nuclear factor kappa B (*NFκB*) **(Figure 2)**.

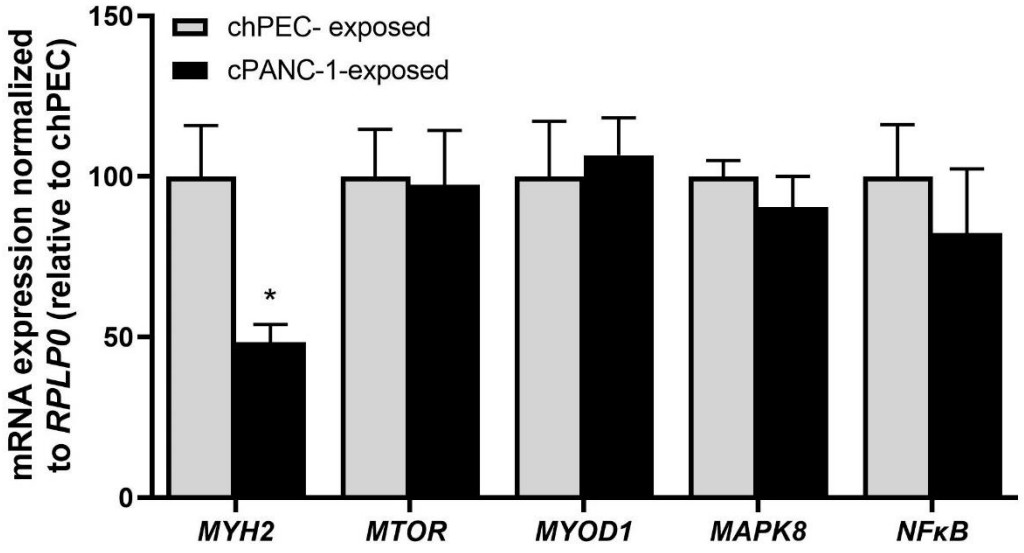

Fig. 2: Gene expression related to protein turnover in myotubes after conditioning with media collected from hPEC or PANC-1 cells.

Myotubes were conditioned with media from human pancreatic epithelial cells (chPEC-exposed) or a human pancreatic cancer cell line (cPANC-1-exposed) during the last 4 days of differentiation. mRNA was isolated and the expressions of myosin heavy chain 2 (*MYH2*), mechanistic target of rapamycin (*MTOR*), myoblast determination protein 1 (*MYOD1*), mitogen-activated protein kinase 8 (MAPK8), and nuclear factor kappa B (*NFκB*) were assessed. The expression of each gene was normalized for housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*). Data are presented from two individual experiments with three biological replicates each (n = 6) relative to expression in chPEC-exposed cells. *Statistically significant in cPANC-1-exposed myotubes versus chPEC-exposed myotubes ($p \leq 0.05$, unpaired t test).

Myotubes conditioned with PANC-1 culture medium had lower glucose uptake and oxidation compared to hPEC-conditioned myotubes

The link between pancreatic cancer and peripheral insulin resistance is not well understood. We wanted to explore whether conditioning skeletal muscle cells with media from hPEC and PANC-1 cells influenced glucose metabolism. We found that cPANC-1-exposed myotubes had lower uptake and oxidation of [^{14}C]glucose compared to cells exposed to chPEC-exposed medium **(Figures 3A and B)**.

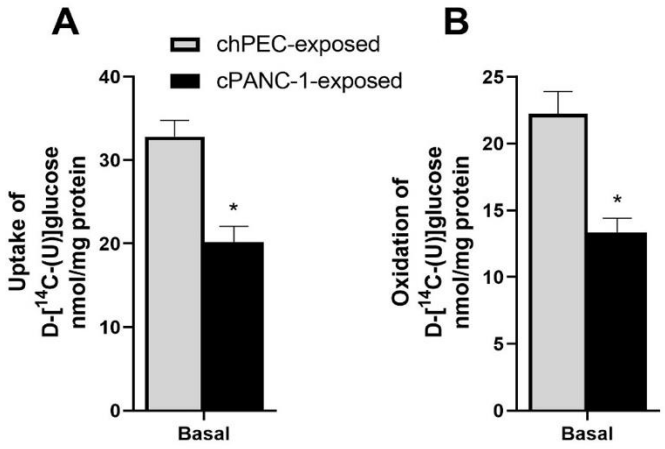

Fig. 3: Impact of conditioning myotubes with media from hPEC or PANC-1 cells on glucose metabolism. The last 4 days of differentiation, myotubes were conditioned with media from human pancreatic epithelial cells (chPEC-exposed) or a human pancreatic cancer cell line (cPANC-1-exposed). Thereafter, myotubes were incubated with 200 µmol/l D-[^{14}C(U)]glucose (18.5 kBq/ml) for 4 h before glucose uptake and oxidation were measured. (A) Uptake was calculated as CO_2 captured + cell associated (CA) of [^{14}C]glucose. Complete oxidation was measured as trapped CO_2 (B). Data are presented from 3 individual experiments, each with 8 biological replicates (n = 24). *Statistically significant in cPANC-1-exposed exposed myotubes versus chPEC-exposed exposed cells ($p \leq 0.05$, unpaired t test).

Akt phosphorylation was higher under basal conditions and when treated with insulin in myotubes conditioned with PANC-1 and hPEC culture media

Insulin increased glycogen synthesis in chPEC-exposed myotubes, while the insulin effect seemed to be lower in cPANC-1-exposed cells **(Figure 4A)**; however, the response to insulin was not significantly different between the two conditions. There was an insignificant reduction in the mRNA expression of the insulin-dependent glucose transporter *SLC2A4* (p=0.079) in cPANC-1-exposed cells compared to chPEC-exposed myotubes **(Figure 4B).** To further examine how insulin affected these cells, we assessed Akt phosphorylation at serine 473 in cells treated with or without insulin. Akt phosphorylation was higher in cPANC-1-exposed myotubes compared to chPEC-exposed myotubes, both at basal condition and after insulin treatment (**Figures 4C and D**).

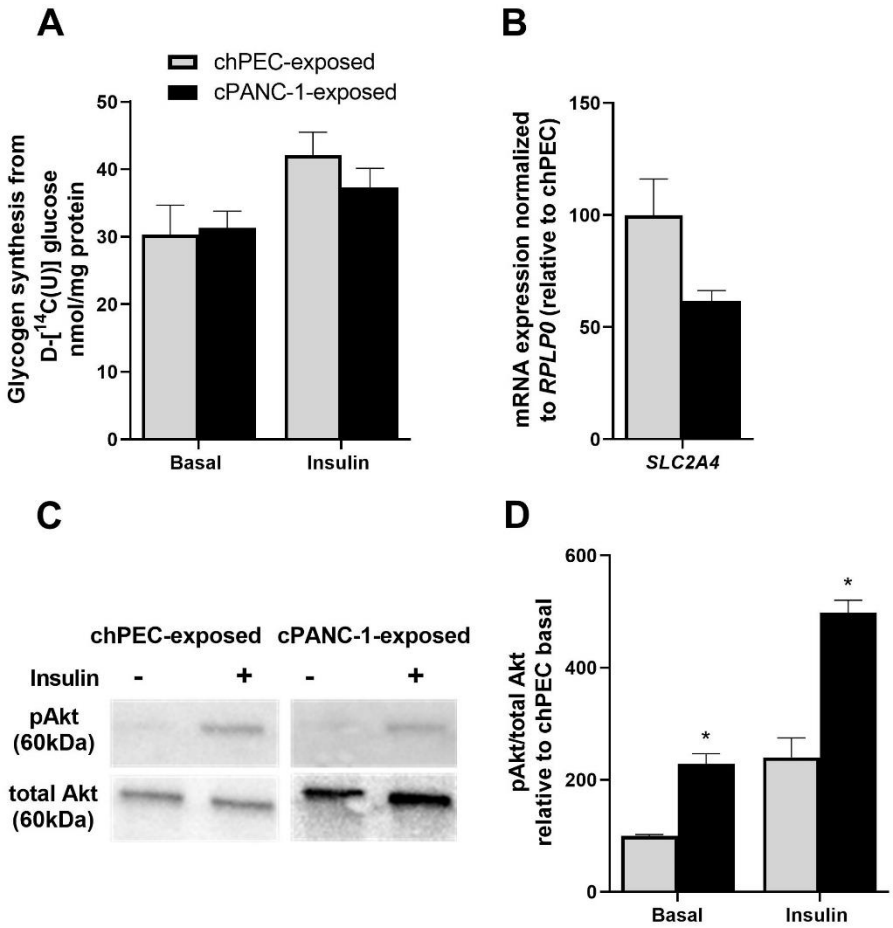

Fig. 4: Impact of conditioning myotubes with media from hPEC or PANC-1 cells on insulin responses. Myotubes were conditioned with media from human pancreatic epithelial cells (chPEC-exposed) or a human pancreatic cancer cell line (cPANC-1-exposed) the last 4 days of the differentiation period (A) After starving myotubes for 90 min in media containing no glucose, insulin-stimulated glycogen synthesis was measured as incorporation of D-[^{14}C(U)]glucose (18.5 kBq/ml, 0.67 mmol/l)) into glycogen in the presence or absence of 100 nmol/l insulin for 3 h. (B) SLC2A4, solute carrier family 2 member 4 mRNA expression was normalized for housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*) and expression in cPANC-1-exposed myotubes presented relative to chPEC-exposed cells (n = 3-6).(C) Protein was isolated and expressions of total Akt and Akt phosphorylated at serine 473 were measured by immunoblotting. One representative immunoblot is shown (D). Quantified immunoblot adjusted to internal control and total Akt (n = 3). *Statistically significant in cPANC-1-exposed myotubes versus chPEC-exposed myotubes ($p \leq 0.05$, unpaired t test), $^{\#}$statistically significant compared to basal ($p \leq 0.05$, paired t test).

Myotubes exposed to PANC-1-conditioned medium showed increased lipid accumulation and responded less to electron chain complex inhibitors compared to hPEC-conditioned myotubes.

We have previously shown that PANC-1 conditioned myotubes had reduced ability to oxidize energy substrates compared to nonconditioned myotubes [10]. In this study, we found no difference in incomplete fatty acid oxidation measured as ASM **(Figure 5A)** or basal complete fatty acid oxidation to CO_2 **(Figure 5B)** in cPANC-1-exposed and chPEC-exposed myotubes. Mitochondrial dysfunction is a common feature of cancer cachexia which occur when the mitochondria work less efficiently than they should due to another disease or condition, such as cachexia [4] or aging [16]. We wanted to explore the mitochondrial responsiveness by using electron transport chain (ETC) inhibitors. Treatment with oligomycin or a combination of rotenone and antimycin A revealed that cPANC-1-exposed myotubes had less response to the inhibitors, i.e. the oxidation after inhibitor exposure was higher in cPANC-1-exposed cells compared to chPEC-exposed **(Figure 5B).** Further, total lipid accumulation from oleic acid and level of free oleic acid (FFA) were increased in cPANC-1-exposed cells compared to chPEC-exposed cells **(Figure 5C)**. Incorporation of oleic acid into triacylglycerol (TAG) and cholesterol ester (CE) was also higher in cPANC-1-exposed exposed myotubes compared to chPEC-exposed exposed cells **(Figure 5D).**

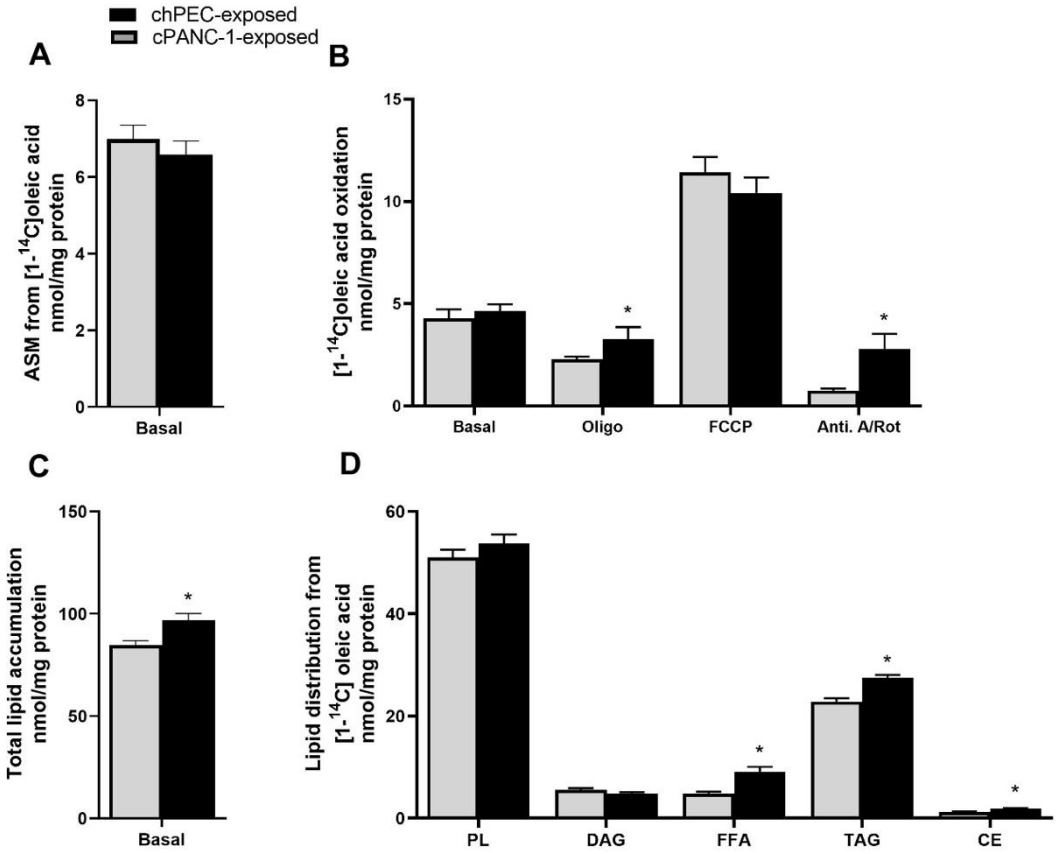

Fig. 5: Fatty acid metabolism in PANC-1 and hPEC conditioned myotubes. The last 4 days of differentiation, myotubes were conditioned with media from hPEC (chPEC-exposed) or PANC-1 (cPANC-1-exposed) cells. Thereafter, the muscle cells were incubated with [1-^{14}C]oleic acid (18.5 kBq, 100 µmol/l) in presence or absence of 1 µmol/l oligomycin (ETC complex V inhibitor), 1 µmol/l FCCP (mitochondrial uncoupler) or 2.5 µmol/l antimycin A (ETC complex III inhibitor) combined with 0.5 µmol/l rotenone (ETC complex I inhibitor) for 4 h. (A) Incomplete oxidation measured as acid soluble metabolites (ASM). (B) Complete oxidation measured as trapped CO_2. (C) Total lipid accumulation and (D) lipid distribution. Values are presented as mean ± SEM in absolute values as nmol/mg protein. Data are from three individual experiments, each with two biological replicates (n = 6). *Statistically significant in cPANC-1-exposed versus chPEC-exposed myotubes ($p \leq 0.05$, paired t test). CE, cholesteryl ester; DAG, diacylglycerol; FFA, free fatty acids; PL, phospholipid; TAG, triacylglycerol.

Phosphorylation of the AMP-activated protein kinase (AMPK) was also markedly decreased in cPANC-1-exposed myotubes compared to chPEC-exposed cells (**Figure 6A and B**). Lastly, mRNA expression of the fatty acid transporter (*CD36)* was increased while mRNA expressions of the ETC complex III (*UQCRB*) and the nuclear receptor *PPARA* were decreased in cPANC-1-exposed myotubes compared to chPEC-exposed cells **(Figure 6C).**

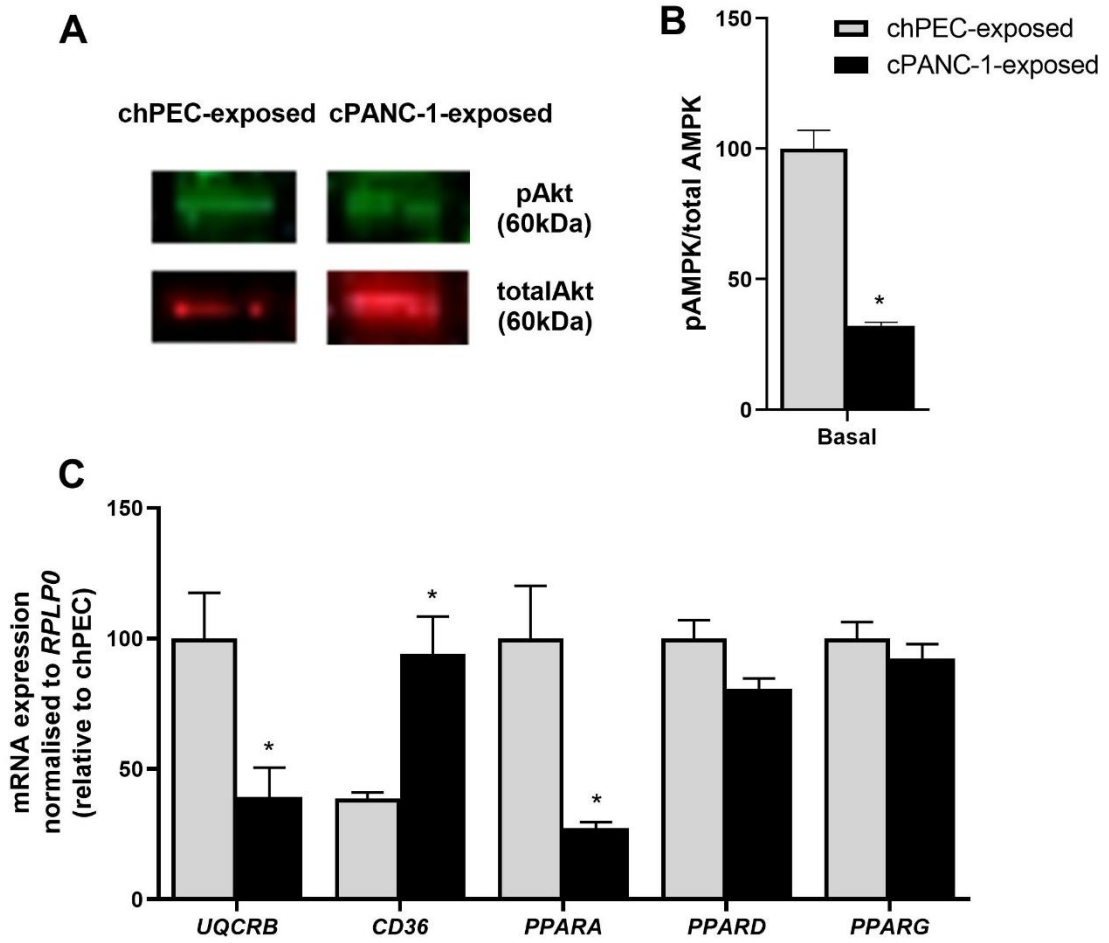

Fig. 6: AMPK phosphorylation and gene expression in PANC-1 and hPEC conditioned myotubes.

The last 4 days of differentiation, myotubes were conditioned with media from human primary pancreatic epithelial cells (chPEC-exposed) or a pancreatic cancer cell line (cPANC-1-exposed). Protein was isolated and total AMPK and pAMPK (Thr172) expression was measured by immunoblotting, one representative immunoblot is shown in (A). Quantified immunoblots adjusted to total AMPK (B). mRNA expressions of ETC complex III (*UQCRB*), fatty acid transporter (*CD36*) and peroxisome proliferator-activated receptor alpha (*PPARA*), delta (*PPARD*) and gamma (*PPARG*) (C). Values are presented as mean ± SEM in absolute values as nmol/mg protein. Data in B and C are from three individual experiments with two biological replicates (n = 6). *Statistically significant in cPANC-1-exposed versus chPEC-exposed myotubes ($p \leq 0.05$, paired t test).

Secretomes from hPEC and PANC-1 cells indicate an increased secretion of certain cytokines, growth factors and growth factor-binding proteins potentially affecting metabolism in skeletal muscle cells

Secretomes from hPEC and PANC-1 were examined using quantitative label-free proteomics (data previously published in Krapf et al.[10]). Now we searched for cytokines, growth factors and growth factor-binding proteins in the secretome using STRING software, and we found several proteins of interest **(Table 1)**. Most of these proteins were upregulated in PANC-1 media compared to hPEC media, including stem cell factor KIT ligand (KITLG), fibroblast growth factor 19 (FGF19), metallopeptidase inhibitor 1 (TIMP1), insulin-like growth factor 2 (IGF2) and insulin-like growth factor-binding protein 2 (IGFBP2), while transforming growth factor beta-2 (TGFB2) was downregulated **(Table 1).**

Table 1: Cytokines, growth factors and growth factor binding proteins differently secreted by PANC-1 and hPEC cells

Symbol	Protein	log10 Fold Change PANC-1 versus hPEC
VGF	Neurosecretory protein VGF	3.175
KITLG	Kit ligand	2.381
AREG	Amphiregulin	1.810
LTBP3	Latent-transforming growth factor beta-binding protein 3	1.779
IGFBP2	Insulin-like growth factor-binding protein 2	1.694
LTBP4	Latent-transforming growth factor beta-binding protein 4	1.594
FGF19	Fibroblast growth factor 19	1.510
TIMP1	Metalloproteinase inhibitor 1	1.392
GMFB	Glia maturation factor beta	1.338
BMP1	Bone morphogenetic protein 1	1.362
MIF	Macrophage migration inhibitory factor	0.887
IGF2	Insulin-like growth factor II	0.885
TGFB2	Transforming growth factor beta-2	-1.110

Color coding: Growth factors (annotated keywords in STRING), growth factor binding proteins (annotated keywords in STRING), cytokines.

Discussion

In this study we found that myotubes exposed to conditioned medium from PANC-1-cells had decreased protein content compared to myotubes exposed to hPEC medium. The PANC-1 medium exposed myotubes also showed decreased overall leucine accumulation, increased overall leucine decay, and decreased gene expression of *MYH2*. Further, glucose uptake and oxidation were decreased in cPANC-1-exposed cells. Moreover, we observed higher total cellular accumulation of oleic acid and higher amount of FFA, TAG and CE, supported by increased *CD36* gene expression in cPANC-1-exposed myotubes compared to chPEC-exposed cells. Basal fatty acid oxidation was equal in chPEC-exposed and cPANC-1-exposed myotubes; however, ETC complex inhibitors had less impact on cPANC-1-exposed cells and mRNA expression of the ETC complex III-related gene *UQCRB* was reduced in cPANC-1-exposed myotubes.

Imbalance in protein metabolism due to hyperactive catabolism of muscle protein and decreased muscle protein mass is a common hallmark of cancer cachexia, and is often already present at the time of diagnosis for many patients with pancreatic cancer [17]. When exploring protein content in PANC-1 exposed myotubes we found decrease in protein content. Therefore, we further explored leucine accumulation and decay in the cPANC-1-exposed myotubes. We found a decreased accumulation and an increased decay of radioactive leucine, suggesting an overall decrease of protein in these cells, this was also evident when comparing protein amount from the two different cells as protein content was lower in cPANC-1-exposed compared to chPEC-exposed myotubes (data not shown). Also, gene expression of *MYH2* was significantly lower in cPANC-1-exposed myotubes. MYH2 is one of three isoforms of myosine heavy chain, encoding a myosin heavy chain expressed in the fast twitch type 2A fibers [18], the others being MYH1, expressed in the fast twitch type 2X, and MYH7, expressed in the slow twitch type 1 [19]. Grown in culture, human satellite cells mature into myotubes that express all three isoforms [20]. Mice studies have uncovered selective atrophy in type II fiber types during cachexia [21]. Muscle wasting in cancer cachexia is linked to increased NFκB activation in muscle [22], however we observed no difference in NFκB gene expression between our conditioned myotubes. The serine-threonine kinase mTOR is ubiquitously expressed and part of two different protein complexes named mTORC1 and mTORC2 [23]. The role of mTOR in cancer-cachexia seem rather two-faced. On one hand, cachexic muscle has reduced gene expression of mTOR complex 1 (mTORC1), which results in decreased protein and lipid synthesis, on the other hand, inhibiting mTORC1 protects against cachexia by upregulating autophagy and

inhibiting pro-cachectic factors [23]. We observed no significant difference in *MTOR* mRNA expression between cPANC-1-exposed and chPEC-exposed myotubes.

Further, we found that cPANC-1-exposed myotubes had lower uptake and oxidation of glucose compared to chPEC-exposed myotubes. *Liu et al*. have shown that the diabetes typically found in pancreatic cancer patients is characterized by profound peripheral insulin resistance, which impairs skeletal muscle glycogen synthesis and storage, and is also associated with a post-insulin resistance defect, and no difference in *SLC2A4* gene expression [24]. As the insulin receptor is not thought to be affected in pancreatic cancer-related diabetes, Though, uptake and oxidation of glucose was lower, while glycogen synthesis was not affected by insulin, suggest that the defect might be further down the cascade towards glucose uptake and glycogen synthesis.

In our experiments total lipid accumulation was increased in cPANC-1-exposed compared to chPEC-exposed myotubes, and significantly more oleic acid was found as FFA or incorporated into TAG and CE, accompanied by increased gene expression of *CD36*. Tumor-bearing animals have been shown to have a 50% increase of CD36 in skeletal muscle [25]. Disruption of normal mitochondrial activity, leading to mitochondrial dysfunction, is associated with lipid metabolic disorders; however, increased lipid accumulation (i.e. TAG accumulation) can also lead to mitochondrial dysfunction [26]. The accumulation of cytosolic fatty acids will in turn increase production of reactive oxygen species, which then leads to decreased mitochondrial biogenesis, increased mutation rates and aging-like impaired mitochondrial function [27]. When comparing fatty acid oxidation in the conditioned myotubes, we observed decreased effects of mitochondrial inhibitors in the cPANC-1-exposed cells. These are similar findings as seen in mitochondria of aging cells [16]. Previous work comparing human satellite cells from old versus young individuals found that muscle cells from young subjects had a decrease in basal respiration after addition of oligomycin, while old cells were less responsive, indicating that old cells already were respiring at minimal capacity [28]. However, old cells remained responsive to FCCP, though to a lesser extent than the young cells [28]. This is similar to our results and indicates aging-like impaired mitochondrial function in myotubes after cPANC-1-exposure. Additionally, mitochondrial dysfunction in subjects with lipid storage diseases was associated with significant loss of activity in ETC complex I-IV compared to control [29]. Studies on rat muscle cells found that only small changes in complex III activity (approximately 5% inhibition) was sufficient to change respiration [30]. Similarly to these findings, we found

a lower mRNA level of the ETC complex III, *UQCRB*, in our cPANC-1-exposed myotubes compared to chPEC-exposed.

AMPK phosphorylation was significantly lower in cPANC-1-exposed myotubes. As a major cellular energy sensor, AMPK acts as a moderator of skeletal muscle lipid metabolism [31]. Activation of AMPK through exercise is found to be beneficial as it increases glucose uptake independent of insulin [31]. AMPK also increases oxidative phosphorylation in skeletal muscle through transcriptional regulation [31]. The phosphorylation of AMPKα is inversely correlated with lipid accumulation in murine skeletal muscle C2C12 cells [31]. This supports the finding of increased accumulation of lipids in our cell model after cPANC-1-exposure, also linked to the typically increased lipid accumulation seen in cells with mitochondrial dysfunction [27].

Investigating relevant genes, we found a significant decrease in *PPARA* gene expression in cPANC-1-exposed compared to chPEC-exposed myotubes. PPARs are important metabolic regulators in the body, with different expression pattern across metabolic tissues. They are also involved in tumor development, though expression levels varies between different tumor types and stages of cancer [32]. Although PPARα agonists have been reported to show antitumor effects in colon carcinogenesis, it is still disputed whether PPARα represses or promotes cancer [32]. In patients with cachexia due to chronic obstructive pulmonary disease (COPD), *PPARA* mRNA expression was decreased compared with non-cachectic COPD patients [33]. PPARα may play a role in glucose utilization in aged muscle and decreased muscle glycogen concentrations have been detected in aged PPARα-deficient mice [34]. This supports our current findings on mitochondria and glucose handling, implying an interference of the cancer cells secretome on myotube energy metabolism causing decreased mitochondrial function and thereof increased lipid accumulation.

Previously, we have shown the different metabolic profiles of hPEC and PANC-1 cells, where PANC-1 cells oxidized more glucose and less oleic acid compared to hPEC [10]. In addition, PANC-1 cells secreted a higher amount of proteins than hPEC [10]. Secreted substances from pancreatic cancer cells has been purposed to alter metabolism in skeletal muscle and induce peripheral insulin resistance and cachexia in skeletal muscle [7]. When studying the proteins secreted from PANC-1 and hPEC cells, we here found increased release of growth factors and growth factor-binding proteins. These included stem cell factor KITLG, often found to be upregulated in several types of cancers [35], fibroblast growth factor 19 (FGF19), which upregulation is linked to hepatocellular carcinoma and unfavorable

outcome of such [36], and tissue inhibitor of metalloproteinases-1 (TIMP1) which is typically upregulated in patients with PDAC [37]. TIMP1 is associated with poor clinical outcome for cancers [38] and suggested as a biomarker for cachexia [39]. Also, insulin-like growth factor and binding protein (IGF2 and IGFBP2) had increased secretion from PANC-1 compared to hPEC cells. These are tumor growth promotors, therefore often increased in different tumors [40]. Increased IGF2 has also been found to lead to activation of Akt [41]. This could potentially explain the higher basal level of Akt phosphorylation seen in our cPANC-1-exposed compared to chPEC-exposed exposed myotubes. Both increased and decreased Akt activity has been observed during cancer cachexia [23].

Taken together, our data supports the suggestion that cancer cells release peptides and proteins, potentially growth factors, that impact metabolism in skeletal muscle cells. However, it is important to take in to consideration that this is solemnly the results from one pancreatic cancer cell line, and the implications of conditioning primary human myotubes with another pancreatic cell line might give different results. Also important to consider is that there might be other factors in the conditioned media influencing the myotubes metabolism. To mention one, we have previously shown that glucose levels decrease significantly more in PANC-1 media compared to hPEC while lactate levels are significantly increased [10]. Although glucose levels were adjusted to be equal in the conditioned media in this study, lactate levels were not corrected.

In conclusion, our data showed that substances released from pancreatic cancer cells can induce changes in energy metabolism in primary human myotubes. Changes in leucine, glucose and oleic acid metabolism were affected in a manner similar as in cachexia. Our research contribute to pancreatic cancer research, however, further studies are needed to elucidate the mechanistic pathways leading to cancer-induced changes in skeletal muscle metabolism.

Authors' contribution

SAK, JL, AURS, HGB, ACR GHT, ETK; Investigation, Writing – review & editing. **SAK, JL, ACR, HGB, GHT, ETK:** Conceptualization, Formal analysis, Visualization, Validation, Methodology. **ACR GHT, ETK:** Funding acquisition, Resources. **SAK, JL, ETK**: Writing – original draft. **SAK, ETK**: Project administration, Supervision, Editing.

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Declaration of competing interest

None

References

1. Adamska, A., A. Domenichini, and M. Falasca, *Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies.* International journal of molecular sciences, 2017. **18**(7): p. 1338.
2. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
3. Sandri, M., *Protein breakdown in cancer cachexia.* Seminars in Cell & Developmental Biology, 2016. **54**: p. 11-19.
4. Siddiqui, J.A., et al., *Advances in cancer cachexia: Intersection between affected organs, mediators, and pharmacological interventions.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2020. **1873**(2): p. 188359.
5. Kunzke, T., et al., *Derangements of amino acids in cachectic skeletal muscle are caused by mitochondrial dysfunction.* J Cachexia Sarcopenia Muscle, 2020. **11**(1): p. 226-240.
6. Holloszy, J.O., *Skeletal muscle "mitochondrial deficiency" does not mediate insulin resistance.* The American Journal of Clinical Nutrition, 2008. **89**(1): p. 463S-466S.
7. Permert, J., et al., *Is profound peripheral insulin resistance in patients with pancreatic cancer caused by a tumor-associated factor?* The American Journal of Surgery, 1993. **165**(1): p. 61-67.
8. Röhrig, F. and A. Schulze, *The multifaceted roles of fatty acid synthesis in cancer.* Nature Reviews Cancer, 2016. **16**(11): p. 732-749.
9. Wang, F., et al., *The relationship between diabetes and pancreatic cancer*. Molecular Cancer, 2003. **2**(1): p. 4.
10. Krapf, S.A., et al., *Pancreatic cancer cells show lower oleic acid oxidation and their conditioned medium inhibits oleic acid oxidation in human myotubes.* Pancreatology, 2020. **20**(4): p. 676-682.
11. Wensaas, A.J., et al., *Cell-based multiwell assays for the detection of substrate accumulation and oxidation.* J Lipid Res, 2007. **48**(4): p. 961-7.
12. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical Biochemistry, 1976. **72**(1): p. 248-254.
13. Gaster, M., et al., *Reduced Lipid Oxidation in Skeletal Muscle From Type 2 Diabetic Subjects May Be of Genetic Origin.* Diabetes, 2004. **53**(3): p. 542-548.
14. Bakke, S.S., et al., *Palmitic acid follows a different metabolic pathway than oleic acid in human skeletal muscle cells; lower lipolysis rate despite an increased level of adipose triglyceride lipase.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2012. **1821**(10): p. 1323-1333.
15. Hessvik, N.P., et al., *Metabolic switching of human myotubes is improved by n-3 fatty acids.* J Lipid Res, 2010. **51**(8): p. 2090-104.
16. Srivastava, S., *The Mitochondrial Basis of Aging and Age-Related Disorders.* Genes, 2017. **8**(12): p. 398.
17. Poulia, K.A., et al., *Pancreatic Cancer and Cachexia-Metabolic Mechanisms and Novel Insights.* Nutrients, 2020. **12**(6): p. 1543.
18. Madigan, N.N., et al., *Filamentous tangles with nemaline rods in MYH2 myopathy: a novel phenotype.* Acta Neuropathologica Communications, 2021. **9**(1): p. 79.
19. Schiaffino, S. and C. Reggiani, *Fiber Types in Mammalian Skeletal Muscles.* Physiological Reviews, 2011. **91**(4): p. 1447-1531.
20. Nikolić, N., et al., *Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise.* PLoS One, 2012. **7**(3): p. e33203.
21. Acharyya, S., et al., *Dystrophin glycoprotein complex dysfunction: A regulatory link between muscular dystrophy and cancer cachexia.* Cancer Cell, 2005. **8**(5): p. 421-432.
22. Chen, M.-C., et al., *Combined administration of fucoidan ameliorates tumor and chemotherapy-induced skeletal muscle atrophy in bladder cancer-bearing mice.* Oncotarget, 2016. **7**(32): p. 51608-51618.

23. Duval, A.P., et al., *mTOR and Tumor Cachexia.* International Journal of Molecular Sciences, 2018. **19**(8): p. 2225.
24. Liu, J., et al., *The Intracellular Mechanism of Insulin Resistance in Pancreatic Cancer Patients*.* The Journal of Clinical Endocrinology & Metabolism, 2000. **85**(3): p. 1232-1238.
25. Fuster, G., et al., *Are Peroxisome Proliferator-Activated Receptors Involved in Skeletal Muscle Wasting during Experimental Cancer Cachexia? Role of β2-Adrenergic Agonists.* Cancer Research, 2007. **67**(13): p. 6512-6519.
26. Guo, C., et al., *Oxidative stress, mitochondrial damage and neurodegenerative diseases.* Neural regeneration research, 2013. **8**(21): p. 2003-2014.
27. Jana, B.A., et al., *Cytosolic lipid excess-induced mitochondrial dysfunction is the cause or effect of high fat diet-induced skeletal muscle insulin resistance: a molecular insight.* Molecular Biology Reports, 2019. **46**(1): p. 957-963.
28. Pala, F., et al., *Distinct metabolic states govern skeletal muscle stem cell fates during prenatal and postnatal myogenesis.* Journal of Cell Science, 2018. **131**(14).
29. Debashree, B., et al., *Mitochondrial dysfunction in human skeletal muscle biopsies of lipid storage disorder.* Journal of Neurochemistry, 2018. **145**(4): p. 323-341.
30. Taylor, R.W., et al., *The control of mitochondrial oxidations by complex III in rat muscle and liver mitochondria. Implications for our understanding of mitochondrial cytopathies in man.* Journal of Biological Chemistry, 1994. **269**(5): p. 3523-3528.
31. Jørgensen, S.B., E.A. Richter, and J.F.P. Wojtaszewski, *Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise.* The Journal of physiology, 2006. **574**(Pt 1): p. 17-31.
32. Takada, I. and M. Makishima, *Peroxisome proliferator-activated receptor agonists and antagonists: a patent review (2014-present).* Expert Opin Ther Pat, 2020. **30**(1): p. 1-13.
33. Remels, A.H., et al., *Peroxisome proliferator-activated receptor expression is reduced in skeletal muscle in COPD.* European Respiratory Journal, 2007. **30**(2): p. 245-252.
34. Atherton, H.J., et al., *Metabolomics of the interaction between PPAR-alpha and age in the PPAR-alpha-null mouse.* Mol Syst Biol, 2009. **5**: p. 259.
35. Yang, S., et al., *KITLG is a novel target of miR-34c that is associated with the inhibition of growth and invasion in colorectal cancer cells.* Journal of Cellular and Molecular Medicine, 2014. **18**(10): p. 2092-2102.
36. Miura, S., et al., *Fibroblast growth factor 19 expression correlates with tumor progression and poorer prognosis of hepatocellular carcinoma.* BMC Cancer, 2012. **12**: p. 56.
37. Pan, S., et al., *Protein alterations associated with pancreatic cancer and chronic pancreatitis found in human plasma using global quantitative proteomics profiling.* Journal of proteome research, 2011. **10**(5): p. 2359-2376.
38. Seubert, B., et al., *Tissue inhibitor of metalloproteinases (TIMP)-1 creates a premetastatic niche in the liver through SDF-1/CXCR4-dependent neutrophil recruitment in mice.* Hepatology, 2015. **61**(1): p. 238-48.
39. Prokopchuk, O., et al., *Elevated systemic levels of the matrix metalloproteinase inhibitor TIMP-1 correlate with clinical markers of cachexia in patients with chronic pancreatitis and pancreatic cancer.* BMC Cancer, 2018. **18**(1): p. 128.
40. Bergman, D., et al., *Insulin-Like Growth Factor 2 in Development and Disease: A Mini-Review.* Gerontology, 2013. **59**(3): p. 240-249.
41. Barton-Davis, E.R., D.I. Shoturma, and H.L. Sweeney, *Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle.* Acta Physiol Scand, 1999. **167**(4): p. 301-5.

Supplementary Table 1. Description of primers.

Gene	Acc.no.	Forward sequence	Reverse sequence
CD36	L06850	AGTCACTGCGACATGATTAATGGT	CTGCAATACCTGGCTTTTCTCAA
GAPDH	NM_002046	TGCACCACCACCTGCTTAGC	GGCATGGACTGTGGTCATGAG
SLC2A4	M20747	ACCCTGGTCCTTGCTGTGTT	ACCCCAATGTTGTACCCAAACT
MYH2	NM_005963	CCAGACTGTGTCTGCTCTCTTCAG	CAGGACAAGCTCATGCTCCAT
MTOR	NM_001386500	CCGAGCGACGAGAGATCATC	ACTTGAGCCAGGTTCTCATGTCT
MYOD1	BC064493	GCGCCCAAAAGATTGAACTTA	CCGCCTCTCCTACCTCAAGA
MAPK8	NM_001278547	CTGGTCAGCAGGGTGTCACA	TCGCAGAGGGAGAAAAGCAA
NFκB	NM001165412.1	ACGAGCTCCGAGACAGTGACA	GAGACTCGGTAAAGCTGAGTTTGC
PPARA	L02932	TCCACCTGCAGAGCAACCA	CCGGAGGTCTGCCATTTTT
PPARD	BC002715	AGCATCCTCACCGGCAAA	ATGTCTCGATGTCGATGTCGTGGATCAC
PPARG	L40904	AGCCTGCGAAAGCCTTTTG	ATTCCAGTGCATTGAACTTCACA
RPLP0	M17885	CCATTCTATCATCAACGGGTACAA	AGCAAGTGGGAAGGTGTAATCC
UQCRB	NM_006294.4	AAGGTCCAAGGTCTCCTCTCTTC	CATCTCCAGCAGGTACTTCACTCA

Primers were designed using Primer Express® (Applied Biosystems). *CD36*, cluster of differentiation 36; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; SLC2A4, solute carrier family 2 member 4; *MYH2*, myosin heavy chain 2; *MTOR*, mechanistic target of rapamycin kinase; *MYOD1*, myoblast determination protein 1;MAPK8, mitogen-activated protein kinase 8; *NFκB*, nuclear factor kappa B; *PPARA/D/G*, peroxisome proliferator-activated receptor alpha/delta/gamma; *RPLP0*, ribosomal protein lateral stalk subunit P0; *UQCRB*, ubiquinol-cytochrome c reductase binding protein.