PhD Thesis

Engineering 3D pancreatic tissues for regenerative medicine

(再生医療に向けた立体膵組織の作製技術に関する研究)

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CHAPTER 1

1. INTRODUCTION

Every molecule in our body is a "tiny chemical factory"[1] that requires a constant supply of energy to keep working properly. After proteins, lipids, and polysaccharides that we are receiving with food are broken down by enzymes to amino acids, fatty acids, glycerol, and sugars, they are used by cells as an energy source or as building blocks for the production of other molecules. Sugars, and glucose among them, are an essential fuel for our body that is used for ATP production during glycolysis (sugar oxidation). Some amount of received sugars is immediately used by cells, the remaining is stored in the form of small granules of glycogen in the cell cytoplasm (e.g. in liver and muscles) or used to synthesise fats and then stored.

Contrary to most of the cells, nerve cells almost do not have glycogen or fatty acids storage and constantly require glucose supply[1]. In a healthy person, blood glucose level (the amount of glucose present in blood) is 4–6 mM and is tightly regulated. If for some reason, the body cannot restore this level and develops long-term high (hyperglycemia) or low (hypoglycemia) blood glucose, there is a need for medical intervention. Untreated chronic hyperglycemia leads to ketoacidosis – life-threatening condition, which is characterised by high ketone concentration, a product of the breakdown of fatty acids and the deamination of amino acids. The most common reason for high blood glucose levels is a group of metabolic diseases that are called Diabetes Mellitus. Hypoglycemia is usually caused by antidiabetic drugs, and is potentially lethal, especially because reoccurring hypoglycemia leads to biased judgement of oneself body condition and consequent person's unawareness of his/her critical blood glucose level[2]. Untreated hypoglycemia is even more dangerous, as it has a direct effect on our brain that requires

constant glucose supply, and so it may lead to central nervous system complications and damage resulting in person's life-long inability to maintain an active life[2].

1.1. PANCREAS – THE KEY PLAYER IN KEEPING NORMOGLYCEMIA

How exactly is the process of keeping blood glucose level normal (normoglycemia) working? What is going wrong when a person develops diabetes? What can we do to improve the diabetes situation?

Maintaining normoglycemia which allows all cells in our body function properly, is a complicated process, that involves the work of brain, liver, intestine and pancreas[3] that secrete different active molecules (e.g. catecholamines[4]) and most importantly pancreatic hormones, Fig. 1.

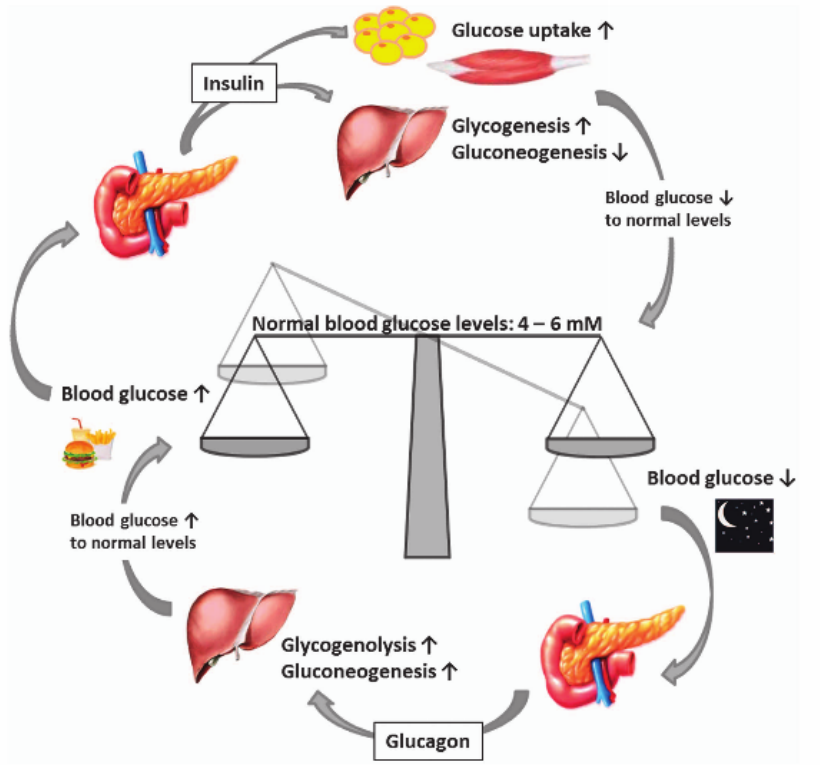


Fig. 1. Schematic representation of blood glucose homeostasis[3].

The pancreas is a relatively small organ (14-20 cm long, weight 50-125 g) that combines exocrine and endocrine gland functions (Fig. 2). The exocrine part, mostly comprised from acinar and duct cells, is part of the gastrointestinal system and secrete digestive enzymes into

the intestine, although latest research shows that they also play a role in the regulation of glucose homeostasis[3]. The endocrine part, so-called pancreatic islets of Langerhans (hereafter referred as pancreatic islets), secretes hormones directly into the blood flow, managing energy metabolism and storage in our body. 96-99% of pancreas amounts for exocrine acinar cells. The remaining 1-4% of pancreas corresponds to the endocrine cells in the form of ~ 3 million pancreatic islets. These small clusters of cells, usually of spherical shape have an average size of ~ 109 μm. Each of these islets is composed of at least five different cell types. The most abundant is β-cells, which comprises ~60% of islets. These cells secrete insulin, the hormone that plays an important role in the metabolism of carbohydrates, e.g. glucose, and is involved in the metabolism of fats and proteins. Insulin inhibits glucose release by the liver and promotes its absorption from the blood into liver, fat and skeletal muscles[4]. Next to β-cells are α-cells that comprise ~30% of islets. They secrete glucagon, which function is opposite to insulin, i.e. the promotion of glucose release into bloodstream and inhibition of insulin[3]. Remaining 10% are divided between somatostatin secreting γ-cells, pancreatic peptide secreting δ-cells and ghrelin secreting ε-cells[5], which also play an important role in hormone regulation of glucose homeostasis but the explanation of their functions is omitted as it is out of this research scope.

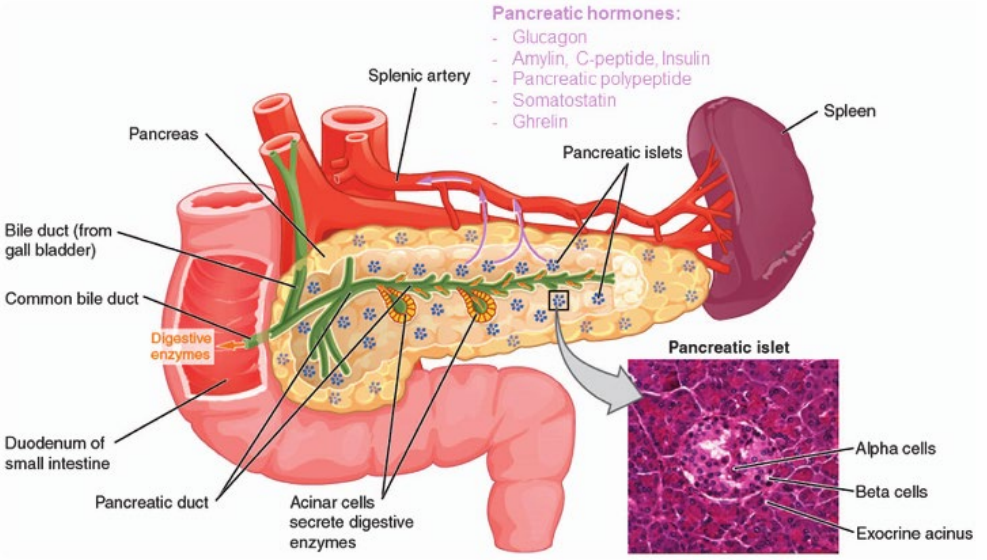


Fig. 2. Anatomical organisation of pancreas[3]. The micrograph initially was provided to the authors of the cited paper by the Regents of University of Michigan Medical School © 2012. The scheme was initially adapted from Human Anatomy and Physiology, OpenStax College resource.

1.2. DISTURBED GLUCOSE HOMEOSTASIS – DIABETES MELLITUS

The most critical pancreatic hormone is insulin, in its absence, the glucose uptake by liver and other tissues is drastically decreased that leads to hyperglycemia and may result in diabetes. Several types of this disease, although different in nature, result in abnormal glycemia, and impaired insulin secretion/sensing, and disturbed homeostasis of energy in the organism. Although diabetes is a metabolic disorder and not a virus or bacterial infection, it is often addressed as an "epidemic" because of rocketing up numbers of cases in the last two decades (Fig. 3). According to the International Diabetes Federation, Diabetes Mellitus effected an estimated 451 million people worldwide in 2017, that is approximately 8% of world population[6] and is the seventh leading cause of death in the United States[7].

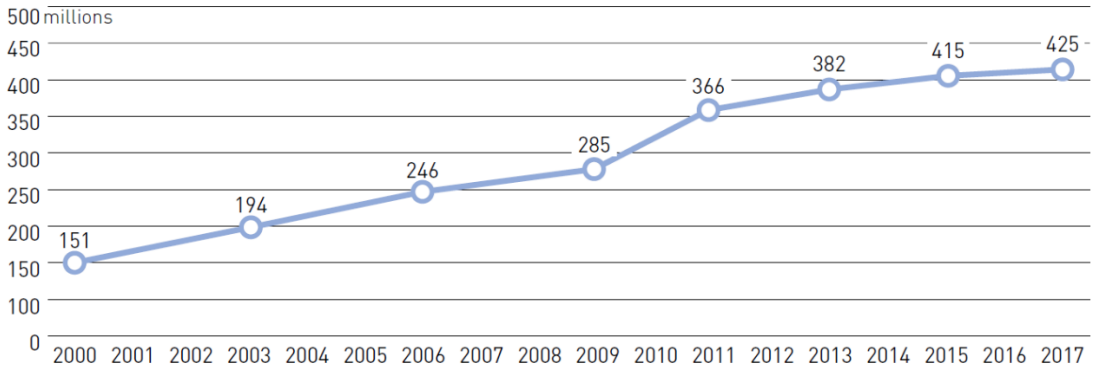


Fig. 3. Change of the number of adults (20-79 years) with diabetes worldwide[3].

Three main types of Diabetes are type 1, type 2 and gestational diabetes. Type 1 is an autoimmune disorder that results in insulin-secreting β-cell destruction and body inability to produce insulin, that requires compensation by life-long daily checks of blood glucose level and injections of exogenous insulin or pancreas transplantation in severe cases[8]. Among possible causes of type 2 are certain DNA genotypes and environmental factors. Type 2 is characterised by insulin resistance of tissues at early stages, that usually do not require serious medical intervention and can be corrected by changes in lifestyle and oral drug prescriptions. As disease progressing, it leads to gradual β-cell loss and need of exogenous insulin or surgical treatment (in rare cases) similarly to type 1[9,10]. The causes of type 2 are mainly genetics and lifestyle, with many cases caused by obesity (high waist-hip ratio), lack of physical activity and poor diet. Gestational diabetes is a metabolic disorder in pregnant women, it can be cured medically, but

requires careful supervision of a doctor[2]. Other types of diabetes are rare and usually caused by single gene mutations[2].

1.3. ISLET TRANSPLANTATION – A NOVEL WAY TO CURE DIABETES

The necessity to check blood glucose level and inject insulin to keep it stable several times a day makes diabetes management a tedious process. As it was already mentioned, repeating periods of low blood glucose levels lead to clouded judgement and unawareness of hypoglycemia with an increased risk of acute hypoglycemia that requires the assistance of another person for recovery. Despite many efforts made towards the search for the treatment of diabetes, there is still no perfect way to control blood glucose level. With time patients develop different complications, to name few nephropathy, retinopathy and vascular problems, that not only put financial pressure on patients but greatly decrease the quality of their everyday life and life expectancy[2] (Fig. 4).

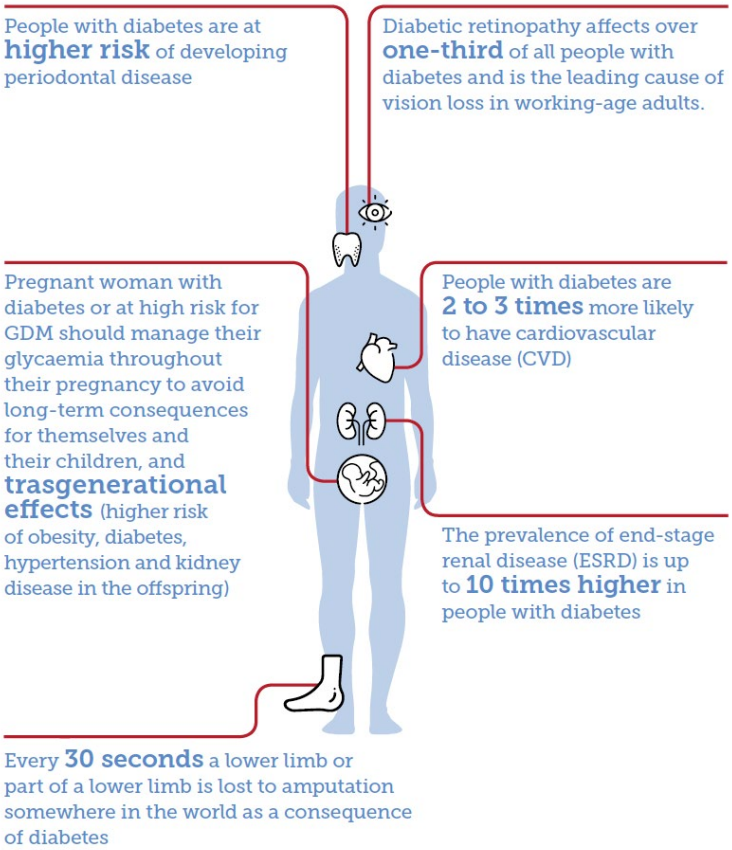


Fig. 4. Organs that are usually affected by long-term diabetes and the risks of the development of the corresponding complications by diabetes patients[2].

At some point, the degree of disease and caused complications may require medical intervention. Until recently, the only option for diabetic patients was whole pancreas transplantation (most usually it was performed along with kidneys transplantation[11]). Despite the high success rate, shortage of donors and the complications connected with required life-long immunosuppression therapy was limiting the approach application, and only 2% of all patients with type 1 diabetes could receive pancreas transplantation. With cases of diabetes were continuing to grow, researches were in the search for new therapies. The first step forward with diabetes treatment was islet transplantation (Fig. 5), that was first successfully performed in 1972 by Drs. Ballinger and Lacy, and later improved by Dr Shapiro[12] in 1990. Nowadays, the transplantation of allogeneic islets is considered to be more appropriate as it is safer, less complicated and less invasive than whole pancreas transplantation[12,13]. However, islet transplantation does not solve a problem with a shortage of donors, and life-long immunosuppression therapy. Consequently, islet transplantation is considered only in the case a patient has a high death risk unless it is done, similarly to pancreas transplantation. Thus, the procedure that logically has to be a cure for diabetes, in reality, is the last resort surgery. Thus, researches concentrated their efforts on improving islet transplantation process, so it would become available for every diabetes patient and will have more advantages than disadvantages.

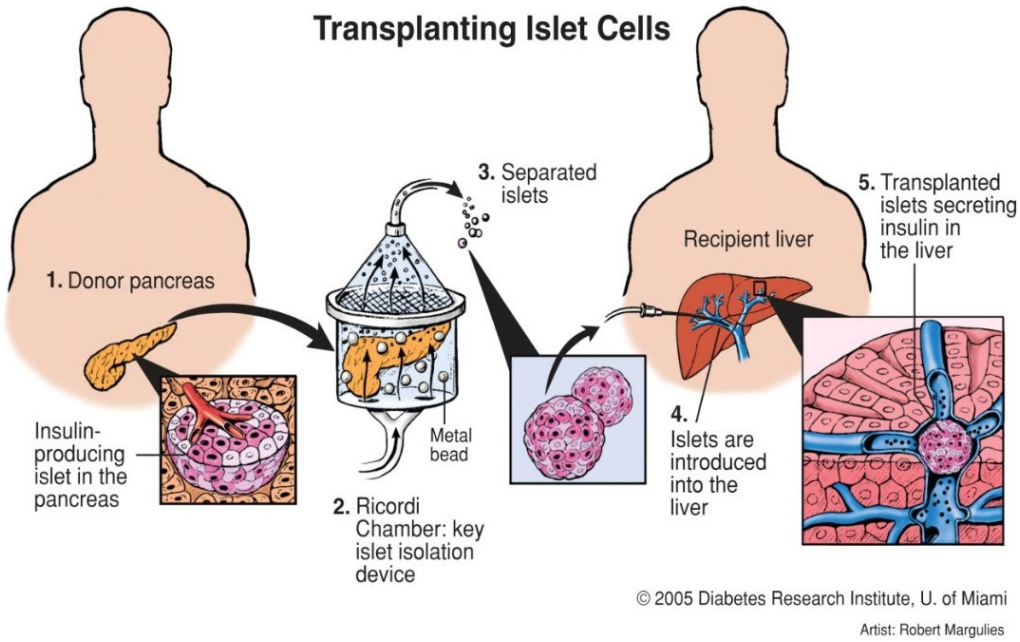


Fig. 5. A schematic representation of islet transplantation.

As a main theme for this research I chose islet transplantation, so I will explain different problems and ways to solve them in more detail. It seems that three main problems are 1) the isolation process and subsequent survival rate, 2) the source of islets and connected with a shortage of donors, and 3) the need for life-long immune suppression therapy.

The isolation procedure is critical, as it takes approximately 5-7 h to complete and during this multi-step process, islets are being torn apart, ripped off of their natural environment and vasculature, and after that are expected to act as an analogue of native pancreatic islets. It is not a surprise that some pancreatic islets (or some parts of them) are dying in the process, either due to hypoxia or effect of enzymes that are used for pancreatic islets purification from acinar cells. The isolation process has a vast effect on islets survival after transplantation, as any remaining dead or apoptotic cells or their by-products will lead to nonspecific inflammation and possible graft failure[13]

Another problem is the shortage of donors, or if rephrased, the source of pancreatic islets. At present, the only source of transplantable islets is deceased donors. Without considering tissue engineering approaches, which will be described in the next sub-chapter, the only possible alternative is the usage of animal pancreatic islets. Lately, extensive research is being conducted in the xenotransplantation field, especially in connection with porcine islets[2,14]. One of the reasons why porcine islets were considered as a solution to the problem of donor shortage is that pigs are already utilised for food and prosthetic components in surgical procedures (e.g. heart valves), and as big animals they have considerably large amounts of islets that can be isolated. However, the study shows that the isolation process is difficult and expensive. In addition, in the case of genetic modifications, which in theory should allow resolving the problem of the immune response, it would take a longer time before the isolation process because of the time required for the breeding. Thus, it is considered to be easier to use embryonic and fetal islets. Unfortunately, such islets are immature and require some time after transplantation to start working properly and usually induce higher immune response than adult ones[14]. Overall, I believe, that ethical concerns of this procedure will eventually lead to the limited usage of porcine islets (and not only islets) as an emergency or last resort method.

The last, but not least is an immune response. In the case of both allogeneic and xenogeneic islet transplantation, the immune system of recipient considers a transplant as a foreign body and activates protection mechanism that results in rejection and graft failure. Unfortunately,

this process is permanent and can only be prevented by life-long immunosuppressive therapy that leads to reduced quality of life and is avoided until there is no other way. The immune response is the major reason why both islet and pancreas transplantation are not used more ubiquitous and would limit transplantation even if the problem of donor shortage would be solved.

1.4. TISSUE ENGINEERING ROLE IN SEARCH OF DIABETES CURE – BIOARTIFICIAL ISLETS

In accordance with described above problems, one of the most important directions in pancreatic islets transplantation research is finding a source that will allow mass-production of islets that do not cause immune rejection or are protected from it. The most logical way seems to be fabrication of bioartificial islets using different cell sources of cells that can differentiate or be programmed to be β-cells: embryonic pluripotent stem cells (ES), induced pluripotent stem cells (iPS), mesenchymal adult stem cells (MS) or reprogrammed/transdifferentiated non-β cell types (e.g. acinar cells, enteroendocrine cells). Another interesting approach is finding a way of inducing proliferation of existing β-cells (normally, primary β-cells do not proliferate in culture)[4]. In this work, neither of this cell sources were used. However I am planning to use one of them in the future, so I will shortly describe them in next sub-chapter.

1.4.1. CELL SOURCE

ES are derived from the inner mass of the blastocyst phase of embryonic development that is achieved around 5th day after fertilisation (Fig. 6). The blastocyst is composed of around 30-34 cells that are called pluripotent as they can differentiate in any type of cells in our body. Protocols of ES differentiation into β-cells usually choose to follow biological signalling pathways and reproduce endocrine development of pancreas *in vitro*[4]. Recently several papers reported protocols that allow differentiation *in vitro* of pluripotent cell to β-cells that are glucose responsive and express mature genes. However, these protocols still require improvement. Most importantly, the therapeutic benefits of transplantation of differentiated ES cells still need to be proved[4], as it was proved that not fully matured ES can cause teratoma upon transplantation[15]. Moreover, the use of ES cells has several undecided problems, that are, obviously, ethical concerns; and graft-versus-host disease that results from body rejection of implanted cells, even though they are of human origin.

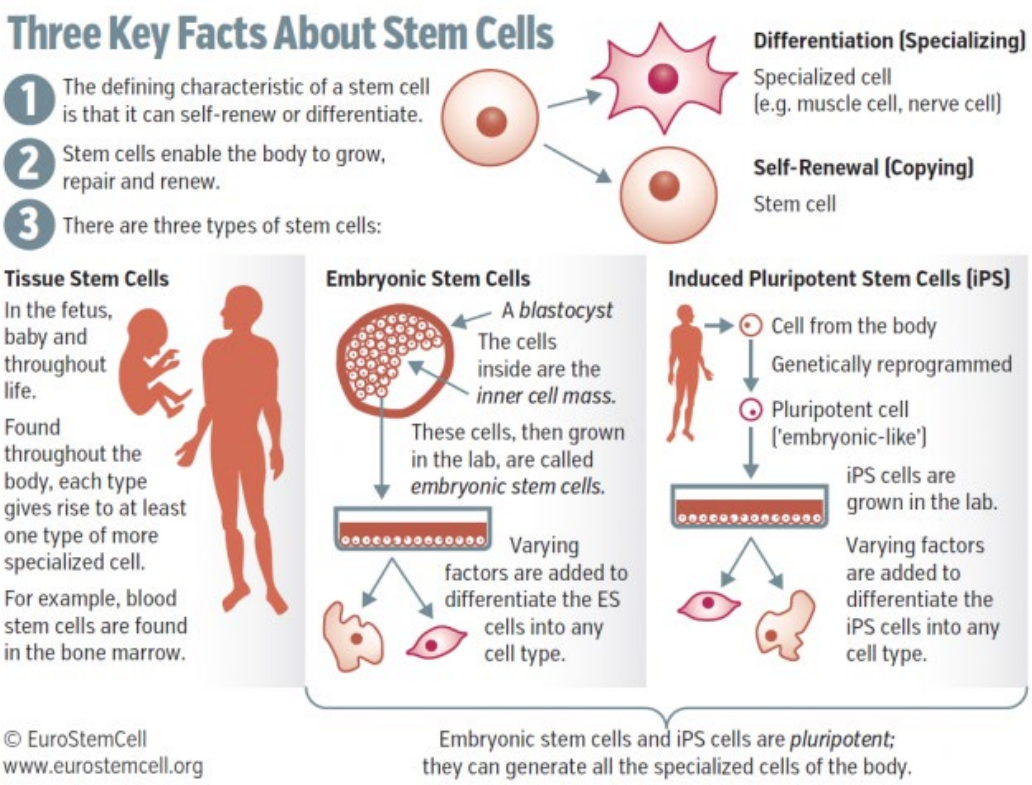


Fig. 6. Types and characteristics of stem cells[16].

Although from an ethical point of view, it would be preferable to use MS – stem cells, derived from an adult person (e.g. bone marrow) – as a source of mature β-cells, unfortunately, no preclinical study proved the feasibility of this approach[4]. One more source of pluripotent cells is iPS cell. iPS cells can be received from adult cells by introducing four specific genes that convert them to pluripotent stem cell state[17]. iPS cells are a desirable source of cells, as they allow avoiding ethical problem of ES cells, and are more flexible than MS cells. Moreover, theoretically, it should be possible to receive fully mature cells that will be ready for the right-away transplantation. However, as it is known that type 1 diabetes is an autoimmune disease, there is a possibility that patient induced pluripotent cells will retain abnormal characteristics that led to the disease in the first place[4]. Another approach is to induce differentiation to pancreatic progenitor cells *in vitro* with following implantation and further differentiation *in vivo.* Despite long time (2-3 months) required for the cell maturation, this approach will soon proceed to human trial testings[4].

The last approach that should be mentioned is inducing human β-cell replication. Research data shows that replication rate of primary insulin secreting β-cells both *in vivo* and *in vitro* is

very slow and equals ~0.2% of β-cell/24h[18]. Even embryonic β-cells have lower replication rate than spleen cells or basal keratinocytes that equals ~2-4% of β-cell/24h. Although some mitogens, growth factors and nutrients were successfully used for inducing rodent β-cell replication, they were proved to be of limited effect for human β-cells[18]. The latter can be connected with several differences in cell-cycle roadmaps in human and rodent β-cell discussed in detail by R.N. Kulkarni and A.F. Stewart et. al[18,19].

1.4.2. SPHEROID FABRICATION

Although there are still problems to decide with described cell sources, there is no doubt that the great number of researchers' efforts will be paid back in the nearest future. Thus, I can move on to the next step of bioartificial islet fabrication – fabrication of islet-like structures from successfully differentiated MS, ES or iPS cells[4,20,21]. Not only pluripotent cells derived β-cells but also isolated pancreatic islets require incubation for 24-72 h. It should be done, as it was already mentioned above, for the assessment of islets quality and increasing purification, as culture allows reducing the amount of dead, apoptotic cells and their by-products, which in its turn minimise non-specific inflammation response after transplantation. Apart from this, there are such down-to-earth reasons as the time required for a patient to arrive at the transplantation centre or the transplant be sent to the required location and to start immunosuppressive treatment for a recipient of islet transplant[13,22]. As *in vitro* culture seems to be an inevitable step in islet fabrication/transplantation, it is important to provide an optimal culturing environment.

First, let me describe some important facts about the formation of islet-like structures (or pseudoislets as they are called in some references), as it is an important step for bioartificial islets fabrication. β-cells, both primary and immortalised/cancer ones, are known to easily form islet-like structure or pancreatic spheroids – dense, spherical aggregates of pancreatic cells. Fig. 7 summarises the most popular ways of spheroid fabrication.

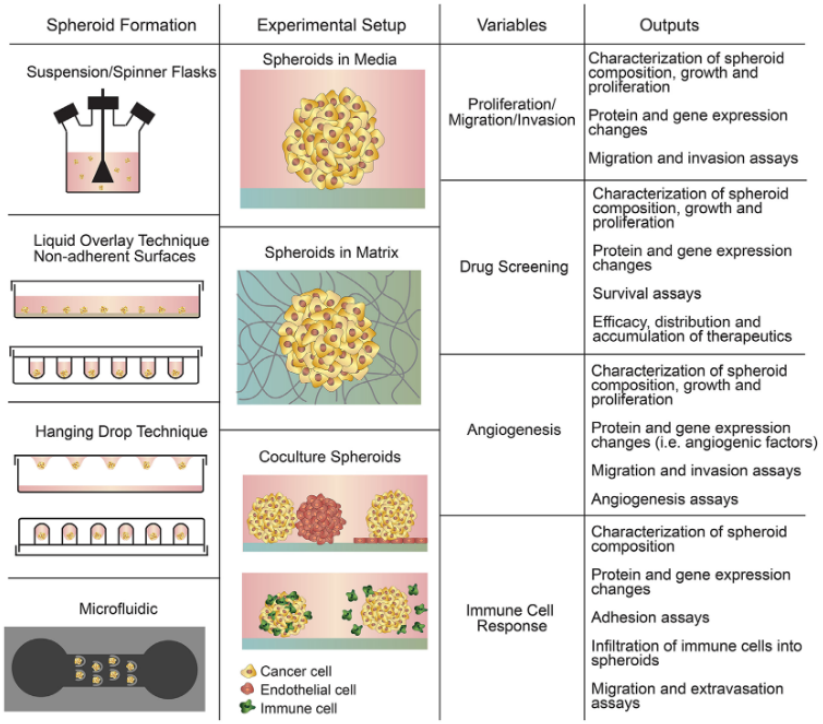


Fig. 7. Summary of spheroid assays including spheroid formation techniques, experimental setups, variables to study, and experimental outputs[23].

First methods of spheroid fabrication were very simple: culturing cells on non-adherent dishes under static conditions[24], but gradually more efficient ways of fabrication evolved. Roughly they could be divided for the following categories: hanging-drop technique[25]; culture on a dish, coated with a polymer (usually gelatin, collagen or some synthetic polymer gel)[24,25]; using multi-well dishes (96-well plates, plates with microwells)[25]; culturing in non-static conditions (shaking or spinner flasks)[23]; microfluidic devices[25] or use of special approaches (layer-by-layer cell coating, specific surfaces or dielectrophoresis)[24].

Although culturing on gelatin is the conventional method of fabrication it is still used by researches[24]. More often, culturing on modified surfaces is considered as a way of elucidating of relations between ECM and β-cells characteristics and behaviour. When cultured on coated dishes, β-cells have their preferences, showing good attachment to fibronectin, laminins and RGD (Arg-Gly-Asp sequence that is recognized by integrins and promotes cell binding) containing peptides, whereas they do not attach and form islet-like structures on low-fouling carboxymethyl-dextran coated and RGE (Arg-Gly-Glu, the mutant of RGD sequence that due

to change in 1 amino acid is not recognizable by the integrins) coated dishes[26]. The more advanced way of culturing spheroids on a gel is the usage of micropatterned surfaces, i.e. using PEGylated plates. As PEG is cytophobic, parts of a plate covered with PEG are preventing cell attachment, and cells concentrate on parts without PEG treatment, which allows size-controllable spheroid formation. Another version of this approach uses PEG-treated plates prepared by methanol/water co-casting that is cytophilic or poly(M-isopropylacrylamide) that allows cell attachment at 37 °C and at lower temperatures become hydrophilic and allows spheroid detachment[25].

One of the alternative approaches is the hanging-drop technique. In its original form, it is quite tedious, as it requires hand-made drops of single-cell suspension for each spheroid[25,27], and does not allow fabrication of uniform spheroids of the desired size. Lately, the design of a 384-well hanging-drop plate was reported. It consists of the mesh-like upper part with uniform holes (at latest version with the addition of micro-ring structure to stabilise the drops). The plate allows stabilising hanging drops that makes possible fabricating spheroids of uniform size. However, each drop still should be made by hand. Thus, the process is still time consuming[25].

One of the interesting recent approaches of pancreatic spheroid fabrication is based on layer-by-layer cell coating technique. In this approach, β-cells are coated with several nanometer-scale layers of fibronectin-gelatin mixture and then are seeded on the porous membrane. The coating facilitates spheroid formation and improves their insulin content and secretion compared with monolayer culture or spheroids formed without coating[28].

As it is described in recent review[25], microwell arrays are a simple and cost-effective way of spheroid formation and were successfully used for fabricating spheroids from hepatocytes, stem cells, pancreatic cells and fabricating of embryonic bodies[25]. Fig. 8 shows the schematic representation of several methods of microwell array fabrication. Usually, a milling technique or photo lithography (Fig. 8 A, B) are used for fabrication of replica that after can be used multiple times for fabrication of the microwell array. Some systems, as on Fig.8 C allow co-culture of two type of cells, widening possible applications of such arrays. One of the appealing points of microwell arrays is that they are completely tunable. Such parameters, as the well diameter, depth, arrangement and distance between them can be chosen and optimised according to the experiment needs. Moreover, in the case of methods showed in Fig. 8 C and D, the

material of the final array also can be varied. Thus, for my research I chose this method of spheroid plate fabrication, the details will be described in the Materials and Methods section.

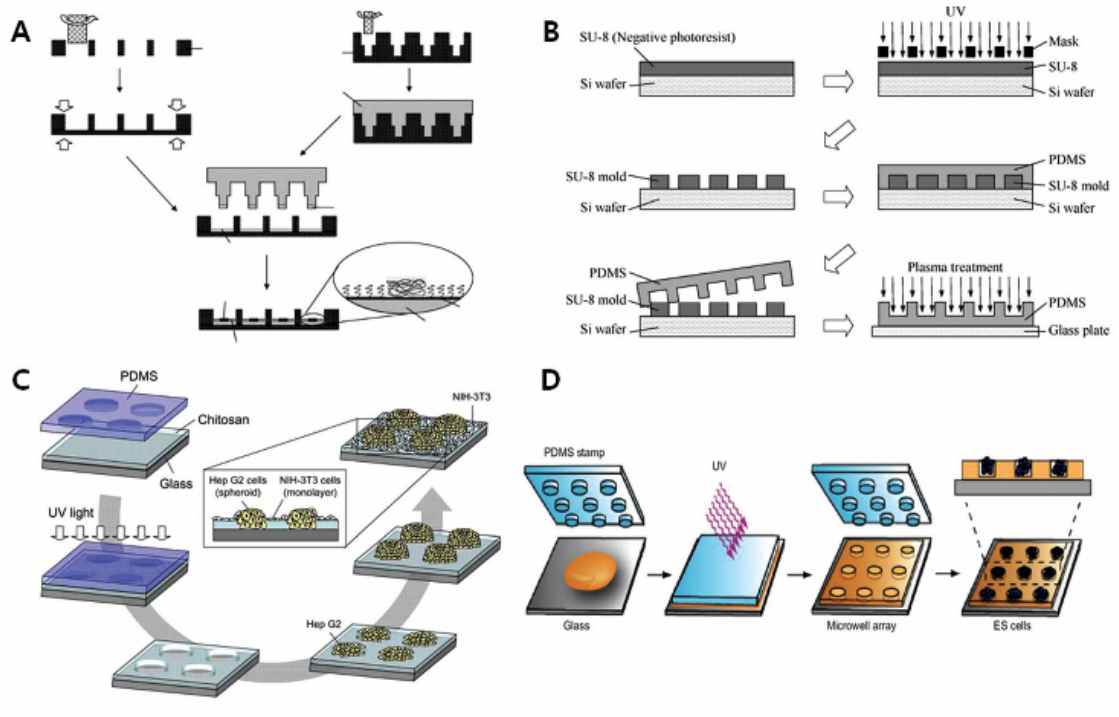


Fig. 8. Several methods of microwell array fabrication. A) PMMA replica is fabricated by the milling process. B) SU-8 replica is fabricated by photolithography. C, D) PDMS replica is fabricated by micromolding[25].

One of the more recent ways to fabricate spheroids that deserves mentioning is the use of microfluidic platforms. Microfluidic devices allow controlling the speed of the culture flow and thus creating more *in vivo*-like culturing conditions for cells. Microfluidic devices can incorporate microwell arrays (Fig. 9 A, F) and some mechanism to control the culture medium flow. The simplest microfluidic devices use gravitation force for inducing flow, and more advanced ones are using different types of pumps. One of the fields that microfluidic devices have an obvious advantage is drug testing, as it requires a flow rather than static conditions. The only disadvantage of these systems is a difficult production process that leads to their small size and consequent production of only limited quantities of spheroids. Overall, microfluidic systems are a promising way of cell culturing and will be more widely used in the future, as they allow recreation of cell microenvironment[25].

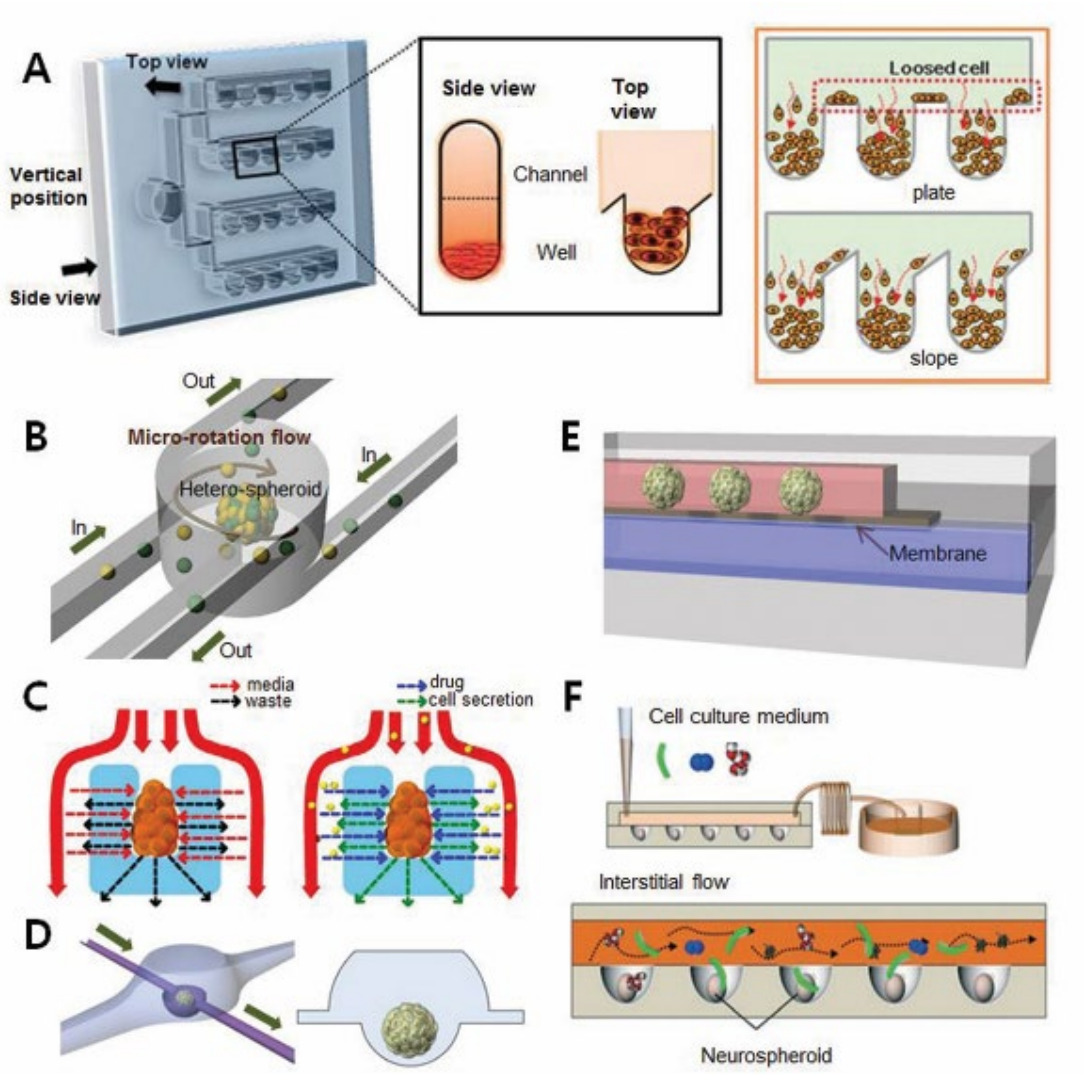


Fig. 9. Schematic representation of different microfluidic systems. A, F) Microfluidic chips with concave microwells, similar to the microwell arrays. B) Schematic representation of spheroid formation in a microfluidic device. C) A microfluidic device based on hydrodynamic trapping of cells in U-shaped geometries. D) A multilayer microfluidic device for culturing embryonic bodies. E) A microfluidic device based on semi-porous polycarbonate membrane[25].

Kind of an extension of microfluidic devices is the use of the encapsulation technique for forming small 3D tissue constructs. A variety of polymers are used in this approach (alginate, agarose, chitosan, PEG, etc.) for the production of fibres or microspheres with encapsulated cells. Although, of course, it is possible to produce these shapes without microfluidics, the latter one allows miniaturising the scale of produced carriers. Smaller sizes are advantageous in case of cell research[25], as it usually allows better nutrient and oxygen supply both *in vitro* and *in vivo*. The encapsulation provides a physical separation between cells and environment that can be desired in some cases (e.g. pancreatic islets) for protection of encapsulated cells

from the immune response, for example. However, in other cases, the addition of a physical barrier leads to a problem with the survival of cells inside due to lack of nutrients and oxygen. It leads to a search for optimal encapsulation material, both natural and synthetic. Moreover, it seems that there should be some effect of the material of encapsulation on cells that should depend on cell type. Although this is a very interesting area of research, it is still not well-known and lies out of the scope of this work. The clear advantage of encapsulation is that it allows easy moulding of small tissue pieces into bigger specific shapes[25].

1.4.3. BIOPRINTING AND SCAFFOLDS

The next logical step in the fabrication of bioartificial organs including pancreas is their incorporation in scaffolds, natural or bioprinted. Bioprinting and scaffolds can also be used as an alternative way of fabrication of small 3D tissues. Bioprinting is a fascinating approach, which allows printing 3D tissues with precise control over placement of cells and bioactive molecules, or everything else that is desired to be incorporated inside the scaffold[29]. However, the great advantage comes with great difficulties – the choice of printable material, i.e. bioink. The problem is similar to the mentioned above encapsulation. In addition to the requirement of being diffusible to nutrients and oxygen, several limitations coming from the way bioprinting is working. Different types of bioprinters have different requirements for the bioink that are summarised on the Fig. 10.

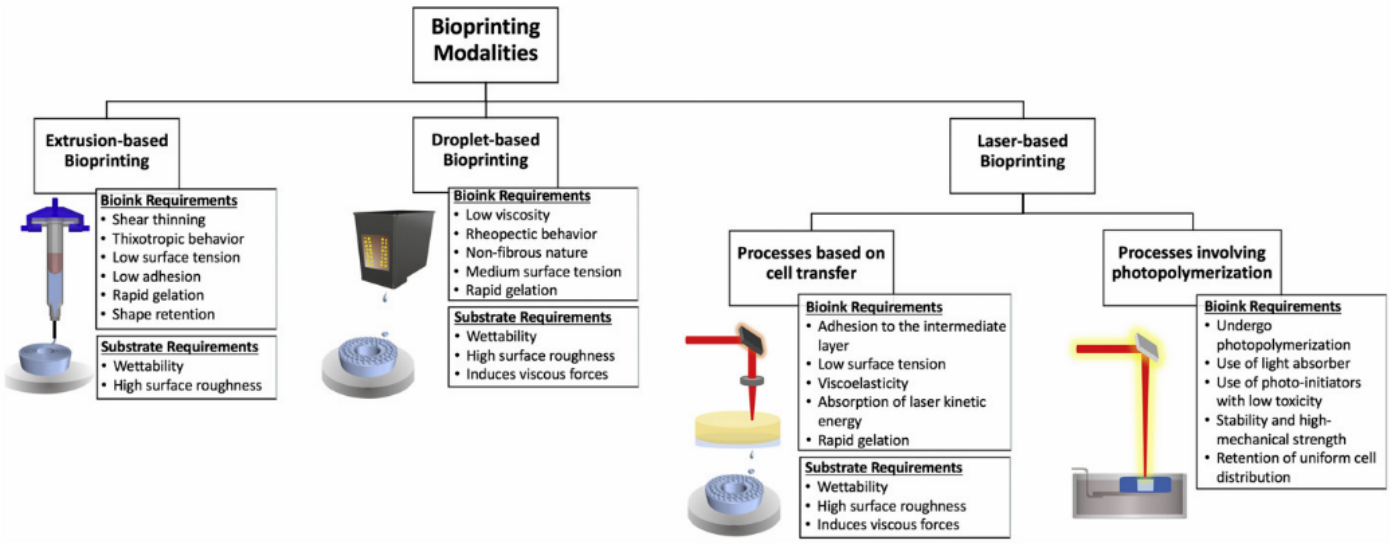


Fig. 10. Different types of bioprinters and requirements for utilised bioink[30].

In addition, as a polymer has a limited diffusion rate, it usually leads to limited amounts of cells that could be incorporated in the volume unit of bioink without cells undergoing apoptosis. Usually, the density is lower than required for the dense tissue fabrication. Of course, this problem can be solved by incorporation of the vasculature. However, this logical answer is still not an easy task to do. Overall, I believe that bioprinting is a promising way of tissue fabrication, although now several problems still require more in-depth research[30].

Apart from bioprinted scaffolds, decellularised organs are now being used to cover them with cells once again. Decellularisation destroys organ cells, living ECM and vascular ways intact. There are several ways to decellularize the organ, which include agitation in solution, manual disruption and several others[31]. However, they are not standardised and vary across papers a lot. The approach is tempting, as it allows preserving vascular ways and thus there is no need in the *de novo* fabrication of vasculature. At present, most of the successful experiments were done in small animals (it is true for pancreas too[32]), so there is still a long way until this method could be possibly used in humans.

1.4.4. Culturing microenvironment

As it was mentioned above, recent approaches for spheroid fabrication pay more attention to the cell microenvironment and try to recreate conditions similar to *in vivo*. Why do we need to care about cell microenvironment in case of pancreatic islets? Current isolation process includes purification step with the use of collagenase, which affects a wide range of laminins and collagens and destructs ECM and microvasculature (including the basement membrane) that results in anoikis, necroptosis and necrosis[33,34]. It was shown that both isolation and following *in vitro* culture of islets lead to upregulation of genes connected with inflammation, apoptosis, oxidative stress, cell-growth and angiogenesis, and downregulation of genes associated with the mature phenotype of islet β-cells, suggesting that these procedures subject islets to various stress-stimuli and trigger their dedifferentiation[35]. Moreover, islets cultured for three days showed downregulation of genes connected with insulin secretion and glucose sensing. One of the most upregulated genes was IL-8, which is known to be connected with poor outcomes of liver, lung and kidney transplantations[35]. These results suggest that culture conditions for pancreatic islets should be carefully considered and monitored.

What are the ideal culture conditions for pancreatic islets? Of course, it would be better to create the environment as much as possible close to *in vivo*. It seems that two main points should be taken into consideration – the 1) oxygen and nutrient supply and 2) ECM. The details of these two points are described in more detail below.

The nutrients supply. Role of culture medium additives on pancreatic islets functioning. As it was said above, the *in vitro* culture is required for both native and bioartificial pancreatic islets. In case of primary pancreatic islets at first, the Edmonton protocol required transfusion of islets no longer than 4 h after isolation. However, nowadays, *in vitro* culture is an inevitable step, as islets should be accessed for their quality to ensure a positive outcome of transplantation, and a patient needs time to arrive at the transplantation centre and start immunosuppressive therapy[2,36]. However, it was shown that after already 20 h of culture the islet-equivalent yield decreases by ~10% (up to 50% in different reports)[36]. Hence, comes the necessity of optimising culture medium conditions. Several limitations that are applied to the culture medium for human islets:

1. Culture media should not contain xenoproteins, as it increases the risk of xenozoonotic infection, thus, ideally, culture media for islets should be serum free[22]. There is a way to overcome this obstacle by using human serum, however, usage of whole serum has several disadvantages, such as possibility of introduction of immunogenic materials, difficulty in obtaining large amounts of human AB serum (the one of the lowest immunogenicity)[36] and variations in its composition, as it depends on a condition of a specific donor.

2. Culture medium should contain only supplements that are tested to be of low or no risk to human. This rule limits the addition of synthetic additives and growth factors or hormones, as they can have an advert effect on the patient because they usually have a weakened immune system. To date, such additives as Vitamin B, C and E, metal ions: Se, Ca, Zn, some binding proteins (transferrin and albumin) and amino acids (L-leucine, cysteine) were proved to be beneficial for islets culture (to a different extent depending on the source of islets)[22].

3. Culture medium should contain low concentration or no antibiotics. This rule comes first from the fact that addition of high concentration of antibiotics hinder the presence of resistant strains of bacteria and promote their survival. And second, using antibiotics, one should be careful about possible patient allergies to substances like penicillin, which are widely used in the research[22]. Although these limitations not necessarily should be applied in model research

in tissue engineering, they should be kept in mind, as an important factor for the translational research.

One more important point that is connected not so much with additives, but with culture media itself and oxygen supply, is maintenance temperature. Several reports showed that culturing islets at lower temperatures (22°C and even 4°C) may be superior to usual 37°C conditions, as they allow decreasing central necrosis and loss in islet mass, as well as preserving high glucose-stimulated insulin secretion (GSIS). It should be noticed though that the latter can be explained by the oxygen solubility in water solutions, as in the case of the same salinity and applied pressure, the cooler water solution contains more oxygen. Thus, islets are more oxygenated in cooler culture media that leads to described above positive effects. When they are returned to normal temperatures (i.e. transplanted) and if they have access to nutrients from the blood (at least provided by diffusion) they function better than partially compromised islets cultured at 37°C. The same explanation can be applied to the fact that islets preserved at 22°C or 37°C usually decrease in their diameter, while 4°C cultured ones preserve the initial diameter after isolation[36].

Role of extracellular matrix on pancreatic islet functioning. Pancreatic β-cells are of epithelial origin and without attachment to ECM undergo anoikis (programmed cell death in anchorage-dependent cells in case of their detachment from ECM). β1 integrins that are expressed on β-cells, and connect to such ECM components as collagen, fibronectin, laminin-1 and -5, are thought to play a role in morphogenesis, secretory cell differentiation, the establishment of polarity, regulation of cell proliferation and cell survival. Therefore, it is not a surprise, that β-cells were found to have better functions (i.e. insulin secretion) if cultured on collagen or Matrigel against untreated culture dishes[37], or reduced apoptosis when cultured on laminin-rich coating[38]. Moreover, the formation of spheroids followed by ECM secretion was linked to an increase in insulin secretion[39]. Although culturing in ECM may seem advantageous, in the application for the transplantation, this method has its limitations. Islets usually stick to the ECM, which contains polymers of animal origin, and so should be purified before transplantation that possibly will reduce the positive effect of preculture in ECM and will inflict stress on islets. This should not discourage researches from using ECM matrixes for culturing islets, as

it is very important to understand interactions between the two of them. Furthermore, the problem could be solved by using new types of materials or advanced dissociation protocols that will allow detaching islets from ECM without compromising their viability.

The co-culturing of islets with other types of cells is a close to ECM culture approach. Its advantage is the dynamic signalling between islets and other types of cells, which helps them to survive and receive necessary hormones for their functioning[22]. However, transplantation of cocultured islets seems to be complicated, as additional research should be done to prove that added cells are safe for the patient and do not cause any abnormalities.

1.5. THE GOAL OF THIS RESEARCH

As was described above, the islet transplantation and fabrication of bioartificial islets is an important direction of research in regenerative medicine, which is directed on the better understanding and search for a cure of Diabetes Mellitus. Although the idea of bioartificial islets at first was simple: fabrication of small spherical organelles comprised of β-cells, lately more and more researchers find evidence that not only the composition but the architecture, the spatial arrangement of different types of cells, as well as signalling from non-pancreatic cells influence islets functioning. Thus, fabrication of islets with architecture close to the native ones, including vasculature or several types of pancreatic cells, as well as use of some signaling molecules of non-pancreatic cells or other additives naturally existing in our body during culture *in vitro* seems to be an interesting research direction, which also should benefit our understanding of islet functioning. Of course, described research field is wide enough to be done by a group of researchers, so in this work, I concentrated on several points that seemed the most important to start such a broad investigation.

The first problem addressed in this work is the oxygen supply in 3D culture systems. From the literature research, I concluded that one of the main problems with culturing both native and bioartificial islets *in vitro* lies with the fact that most of the culturing techniques available nowadays are still strongly connected with monolayer culture. Most importantly, it is a problem of oxygen supply. It reflects, first of all, in use of culture media that initially were designed for the use in 2D culture and thus are not optimised for the requirements of 3D tissues. And secondly, the culturing techniques themselves, as a lot of them, although allow fabrication of 3D structures, do not consider the fact that 3D tissues require higher oxygenation than 2D cultures

due to diffusion limitations. The gap between our ability to culture 3D tissue *in vitro* and *in vivo* conditions is best illustrated by the fact that survival rate and functioning of islets after immediate transplantation is usually much better than after culture *in vitro*. Recently, of course, there is a change in this trend, and such platforms as microfluidic devices or oxygen permeable and oxygen generating spheroid plates are thoroughly investigated. Considering this, in this research I concentrated on improving the culturing technique of bioartificial islets, using mouse insulinoma β-cell lines MIN6 and MIN6-m9 as models for bioartificial islets fabrication.

The second problem addressed in this work is a fabrication of vascularized bioartificial islets. Improved *in vitro* culture conditions are important, however, even if bioartificial islets will not experience hypoxia during culture, they will still be subjected to hypoxic conditions after transplantation due to lack of vasculature. As it was described in the introduction, death due to hypoxia is one of the leading reasons for islet failure after transplantation. Thus, I investigated the possibility of an *in vitro* fabrication of bioartificial islets with vascular-like structures.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. PREPARATION OF OXYGEN PERMEABLE DEVICE

The fabrication of oxygen permeable polydimethylsiloxane (PDMS) culture device (PDMS-chip) was done in 4 steps (Fig. 11). 1) Drawing the design using Adobe® Illustrator software. The parameters of the mould were set to be: diameter = 500/1,000 μm, interspace = 50 μm. Wells were arranged in a triangular manner. Creating the drilling pathway using VCarve Pro 6.5. The depth of the wells was set to be 800 μm, 2) Fabricating the negative mould by drilling into the circular piece (diameter 40 mm, thickness 2 mm) of polystyrene plastic using a milling machine (Model A proll MDX-540, Roland, Japan; Fig.S1B) according to the design. 3) Making the replica mould by pouring an epoxy resin solution consisting of 2 : 1 (w/w) of resin : hardener (Crystal Resin NEO, NISSIN RESIN, Japan) onto the negative mould placed into a container with the same diameter (Fig. S1c). The container with epoxy resin was left for 48 h at room temperature to cure, and after that, the replica mould was separated from the negative mould. 4) Fabricating of a PDMS-chip by pouring PDMS solution consisting of 10 : 1 (w/w) of prepolymer : catalyst (Shin-Etsu silicone, Shin-Etsu Chemical, Japan) onto the replica mould, deaerating it in a desiccator connected with vacuum pump (ULVAC KIKO Inc., Japan) and heating for 30 min at 80°C. The resulted chip was peeled off from the replica mould, autoclaved (Labo Autoclave, SANYO, Japan) and treated with 4% Pluronic solution (Pluronic F-127, Sigma, Japan) in Milli-Q water (Milli-Q Advantage, Merk Millipore, Japan) to prevent cell attachment during culturing.

2.2. PREPARATION OF OXYGEN IMPERMEABLE DEVICE

Oxygen impermeable spheroid culture device was made from polymethylmethacrylate (PMMA). PMMA-chip consisted of two parts (Fig. 11): PMMA backbone and a PDMS insert with wells. PMMA backbone consisted from the wide ring (outside diameter 53 mm; inside diameter 40mm) that was cut from 1 cm thickness PMMA plate (Hazaiya, Japan); and the bottom part that was cut from 2 mm thickness plate (Comnet, Japan) on laser cutter (LaserPro,

Comnet, Japan). These two were glued with adhesive (Acrysunday Adhesive, ACRYSUN-DAY Co., Ltd.). The PDMS insert with wells was cut from PDMS-chip made as was described above, autoclaved and inserted into PMMA-chip. After that, it was treated with the Pluronic solution to prevent cell attachment during culturing.

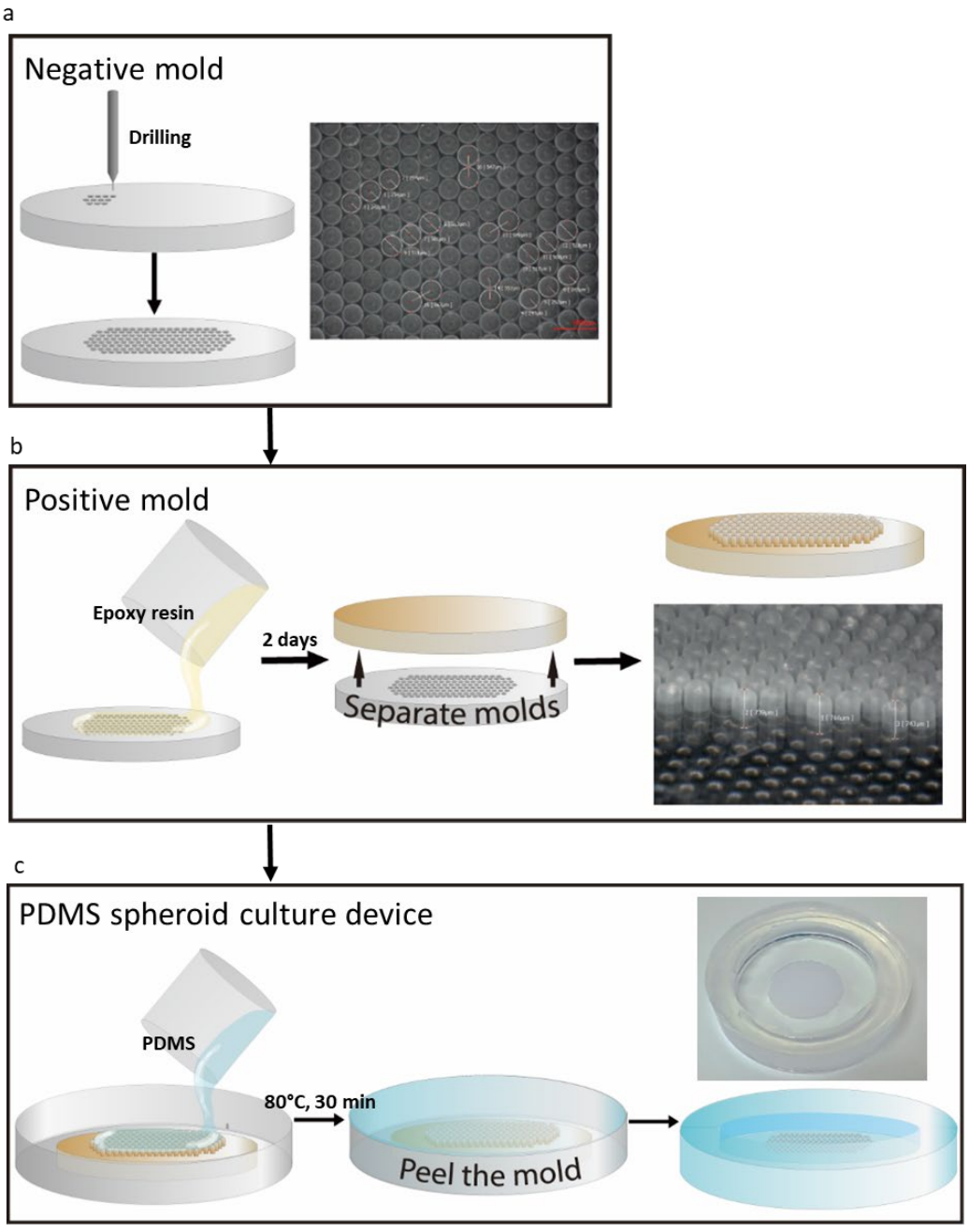


Fig. 11. Fabrication of PDMS spheroid plate. a: Fabrication of a negative mould by drilling wells in polystyrene circle and the photo of the resulted mould. **b**: fabrication of a positive mould by pouring epoxy resin solution onto the negative mould and the photo of the resulted mould. **c**: fabrication of PDMS-chip by pouring PDMS solution on positive mould and the photo of the final chip.

2.3. CELL CULTURE

Mouse insulinoma β-cell lines MIN6 (gift from Prof. Miyazaki, Osaka University, Osaka, Japan)[19] and MIN6-m9 (gift from prof. Seino, Kobe University, Kobe, Japan)[20] both were cultured in DMEM culture medium (4.5 g/L glucose, Sigma, Japan) supplemented with 10% fetal bovine serum (Biowest, Japan), 1% penicillin/streptomycin (Gibco, Japan), and 5×10^{-4} % β-mercaptoethanol (Sigma-Aldrich, Japan). All cell cultures were incubated at 37°C in 5% CO_2 (CO_2 incubator, SANYO, Japan). Culture medium was changed every two days.

Before seeding in spheroid culture devices and as a control, both cell types were cultured in monolayer and were passaged by trypsinisation (0.25% Trypsin-EDTA, Gibco, Japan) once a week with a split ratio 1:4. Seeding cell density for both PDMS- and PMMA- chips was varied in a range from 500 to 3,000 cells/well during optimisation, and 1,000 cells/well was chosen for further experiments.

For the experiment with the flatbed *epi*-relief contrast cellular monitoring system human cell line derived from pancreatic cancer PANC-1 (Cell Bank, Rikem, Japan) was used as a control of cancer cee line. The cells was cultured in RPMI1640 culture medium (Gibco, Japan) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. Cell cultures were incubated at 37°C in 5% CO_2. Culture medium was changed every two days.

For the experiment with antioxidants: L-Ascorbic Acid Phosphate Magnesium Salt n-Hydrate (AA2P, Wako, Japan) concentration in culture medium was in a range of 0.04-0.5 mM; N-Acetyl-L-cysteine (NAC, Sigma-Aldrich, Japan) concentration in culture medium was in a range of 1-10 mM; and dithiothreitol (DTT, Funakoshi, Japan) concentration in culture medium was in a range of 0.1-1 mM.

Pancreatic microvascular endothelial cells – PMVCs (Angio-Proteomie, Boston, MA, USA) of human origin, were cultured in EGM-2 culture medium (Lonza; EBM-2, CC-3256 culture medium supplemented with SingleQuot CC-4176).

Red fluorescent protein-expressing human neonatal dermal fibroblasts – RFP-HNFFs (Angio-Proteomie) were cultured in DMEM culture medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin.

Endothelial cells and fibroblasts were incubated at 37°C in 5% CO_2. Culture medium was changed on the next day after seeding and then every two days. Cells were passaged once

before using them for further experiments with 0.25% trypsin-EDTA at 80% confluency and re-seeded at ~ 500 000 cells/dish density.

When co-culture spheroids were seeded the total amount of cells in 1 well was kept ~1000 cells, as it was determined as an optimal for β-cells that comprised the major part of spheroids. Spheroids with different cell ratios were fabricated, and different types of culture media were used for spheroid maintenance, that is described in more detail in Chapter 4.

In the case of collagen culture, the collagen gel culture kit with cellMatrix Type I-A collagen from porcine tendon (Nitta Gelatin, Japan) was used, the gel was prepared according to manufacturer protocol. The density of HUVECs and PMVCs was 2×10^6 cells/ml; the culture medium was changed every two days. In the case of collagen coculture of endothelial cells and spheroids, the density of endothelial cells was 1×10^6 cells/ml, and the spheroid density was 1000 spheroids/ml.

2.4. MONITORING CELLS' CONDITION BY FLATBED *EPI* RELIEF-CONTRAST CELLULAR MONITORING SYSTEM

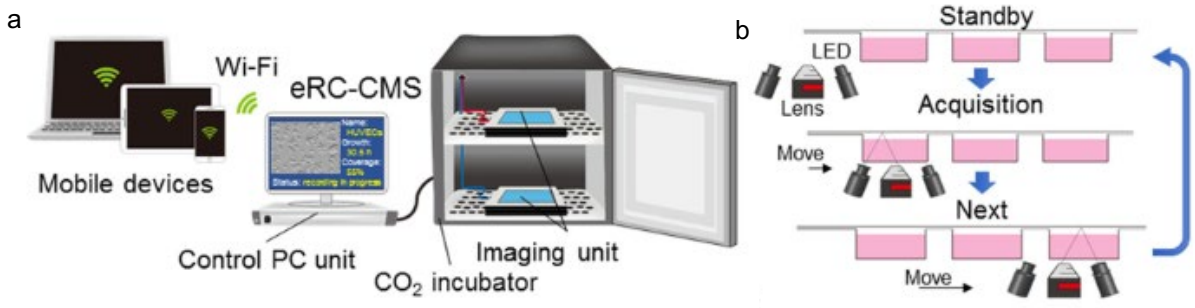


Fig. 12. Flatbed *epi*-relief contrast cellular monitoring system (eRC-CMS). a: the configuration of eRC-CMS, from right to left: the incubator for cell culturing equipped with objective lens and LEDs for *epi* relief-contrast imaging; the controlling PC with software that allows continuous monitoring of cells cultured in the incubator and sending collected data to another device by the network. **b**: the scheme of image acquisition.

Three cell lines: MIN6, MIN6-m9 and PANC1 were cultured in eRC-CMS for 6 days without culture medium exchange to ensure that photos are taken at the same position. Cell images were taken every 10 min at 3 different not overlapping positions of one PDMS- or PMMA-chip. In more details, the experiment is explained in chapter 3.3.2.

2.5. IMMUNOFLUORESCENCE STAINING FOR INSULIN, GLUCAGON AND VE-CADHERIN

Immunofluorescence staining was performed for MIN6, MIN6-m9 and different coculture 3D spheroids and frozen sections of spheroids.

Insulin staining. For direct staining of spheroids, they were fixed with 4% paraformaldehyde in PBS solution (Wako, Japan) for 30 min at 4°C, then washed with PBS (Gibco, Japan) 3 times. After blocking with blocking buffer (10% BSA Diluent/ Blocking solution kit, KPL, USA) for 1 h, mixture of primary antibodies for insulin (Insulin Antibody H-86, rabbit polyclonal, Santa Cruz Biotechnology, USA) and glucagon (Glucagon Antibody C-18, goat polyclonal, Santa Cruz Biotechnology, USA) was added (1:2,000 dilution rate) to spheroids, and they were kept overnight at 4°C. Next, spheroids were washed with 0.1% PBS-Tween 20 (Sigma-Aldrich, Japan) 3 times, and secondary antibodies (Alexa Fluor 488 Donkey Anti-Rabbit IgG H&L and Alexa Fluor 555 Donkey Anti-Goat IgG H&L, Abcam, Japan) were added (1:400 dilution rate) and kept for 2 h at room temperature. After that, spheroids were washed with PBS 3 times, treated with DAPI (1:1,000 dilution rate; Sigma, Japan) for 10 min, washed with PBS 2 additional times and observed under a confocal microscope (LSM 700, ZEISS, Japan).

In cases of frozen sectioning, spheroids were fixed with 4% paraformaldehyde for 30 min at 4°C, rinsed with PBS 3 times, successively submerged into 10%, 20% and 30% sucrose solutions (Wako, Japan) for 1 h respectively, at room temperature. After that, spheroids were transferred to a cryo dish (SHOEI, Japan), the sucrose solution was carefully aspirated so that only spheroids would be left, and the cryo dish was then filled with O.C.T. compound (Tissue-Tek O.C.T. compound, Sakura Finetek, Japan). The resulted sample was first snap-frozen with liquid nitrogen, and then kept at -80°C until cutting. Sections were cut at 10 μm thickness using a microtome (Bright Instruments, UK), placed on a micro slide glass (76×26 mm, Matsunami, Japan) and then stained in the same way as described above for 3D spheroids.

VE-cadherin staining. The procedure was the same with the described above insulin staining for both 3D spheroids and their frozen sections except for the dilution of primary antibody (Anti-VE cadherin antibody – intercellular junction marker, ab33168, Abcam) that was changed to 1:200. As the secondary antibodies for the VE-cadherin staining, Alexa Fluor 555 Goat Anti-Rabbit IgG H&L were used.

2.6. IMMUNOHISTOCHEMICAL STAINING FOR HIF1A, CD31, CD34 AND H&E STAINING

HIF1α staining. MIN6 and MIN6-m9 spheroids were stained with Hypoxyprobe-1 (Hypoxyprobe-1 OMNI Kit, CosmoBio, Co., Ltd, Japan) according to manufacturer protocol. After that, spheroids were fixed as it is described above, and the frozen sections were observed under the confocal microscope.

H&E staining. The H&E staining was done for the frozen sections of co-culture spheroids. For the rehydration, the slides with frozen sections were dipped into 100% ethanol twice, each time 5 min, then 90% ethanol and 70% ethanol, each 5 min. Followed by washing with miliQ water for 3 min. For hematoxylin staining, the slides were dipped into a hematoxylin solution (Wako) for 7 min and then rinsed with running water for 13 min. For eosin staining, slides were dipped into eosin solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan; 1% of eosin solution was diluted by 3 times in 70% ethanol) containing 0.1% of acetic acid for 3 min. After that, samples were dehydrated by the following sequence of washings: 90% ethnol for 30 s, 90% ethanol and 100% ethanol for 1 min, 100% ethanol twice for 5 min, xylene (Wako) twice for 5 min. The photos were taken with the microscope without the phase-contrast option.

CD31 and CD34 staining. The PMVC and HNDF coculture spheroids were fixed, and then their frozen sections were cut. The samples were sent to the Morpho Technology company, which performed the staining.

2.7. SCANNING ELECTRON MICROSCOPY

For scanning electron microscopy images, the culture medium was aspirated from PDMS-chip and spheroids were washed with PBS 3 times. Then they were fixed with a mixture of 2.5% glutaraldehyde (Wako, Japan) and 2% formaldehyde for 1 h at room temperature and washed with PBS one more time. Fixed spheroids were treated with 1% Osmium Tetroxide (OsO_4) solution (Electron Microscopy, Germany) in PBS for 1 h at 4°C, washed with Mili-Q water and dehydrated by successive treatment with: 30%, 50%, 70%, 90% ethanol solution (Wako, Japan), each time for 5 min and 100% ethanol solution 3 times, 5 min each, at room temperature. After that, ethanol was substituted to 100% t-butanol (Sigma-Aldrich, Japan) by washing spheroids with it 2 times. The samples were first kept at 4°C (Medicool, Sanyo, Japan)

for 1 h, then transferred to -30°C (Biomedical Freezer, Sanyo, Japan) for 1 h and finally lyophilized in a freeze dryer (FDU-1200, EYELA, Japan) overnight (pressure 10 Pa, temperature -45°C). The SEM pictures were taken by scanning electron microscope (Miniscope TM-1,000, Hitachi, Japan).

2.8. CELL VIABILITY

To determine cell viability the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Japan) was used. The procedure was according to the manufacturer protocol. Cell densities for calibration curve were 10,000; 20,000; 30,000; 40,000; 50,000 cells per well in case of monolayer and 500; 1,000; 1,500 cells per well in case of spheroids. In case of monolayer cells were treated with the reagent for 4 h, and in case of spheroids for 5 h.

2.9. RT-PCR

Total RNA was extracted from cultured monolayer/spheroids by using the RNeasy kit (QIAGEN, Japan) according to the manufacturer protocol. An additional step was added to break the spheroids before the extraction – spheroids were added into tubes with beads (BioMaster, Nippi, Japan) shacked for 5 sec, and after that, the total RNA was extracted according to the manufacturer protocol. On the day of extraction, recovered total RNA was reverse transcripted to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO, Japan), according to the manufacturer protocol. cDNA samples were kept at -80°C (Refrigerant, Nihon Freezer, Japan). RT-PCR was performed on a StepOne Real-time PCR system (Applied Biosystems, Japan). As components of the reaction, the SYBR Green Premix Ex Taq II (Clontech, Japan) was used, and the following primers were acquired from TAKARA Bio, Japan as a special order, see the list in table 1.

Table 1. The primer sequences for the RT-PCR analysis that were used in this work.

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
EEF2	CATGTTTGTGGTCAAGGCATAC	TTGTCAAAAGGATCCCCAGG
β-actin	GGCTGTATTCCCCTCCATCG	TGTAGACCATGTAGTTGAGGTCA
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Tbp	ACCCTTCACCAATGACTCCTATG	ACTTCGTGCCAGAAATGCTGA
INS1	CCCTTAGTGACCAGTATAATCAGAGA	ACCACAAAGATGCTGTTTGACAA
INS2	GCAGCACCTTTGTGGTTCCC	TGCAGTAGTTCTCCAGCTGG
GLUT1	GCTGTGCTTATGGGCTTCTC	CACATACATGGGCACAAAGC
GLUT2	TCAGAAGACAAGATCACCGGA	GCTGGTGTGACTGTAAGTGGG
Aldob	GCTATCTACCGTTGCCCTCTAC	TGGGTTGCCTTCTTGTTTGC
PDX1	GAACCCGAGGAAAACAAGAGG	GTTCAACATCACTGCCAGCTC
ZnT8	GGTGGACATGTTGCTGGGAG	CACCAGTCACCACCCAGATG
Trib3	TCTTTGGCAAGATCCGTAGAGG	ACCAGGGATGTAGCAGGATTC
Casp4	GGCAAAGAGTTCCTTACAGAGTACC	TGTCACTGCGTTCAGCATTGTTA
Hmox1	ACAGCATGTCCCAGGATTTGTC	AAGGAGGCCATCACCAGCTT

The details of genes choice and explanation of their function is written in detail in Chapter 3. mRNA of eukaryotic translation elongation factor 2 (EEF2) was used as the internal standard in all experiments. The RT-PCR experiment was repeated at least 2 times for cDNA prepared from at least 4 batches.

2.10.ELISA

C-peptide ELISA was performed to access the insulin secretion rate. First, cultured mono-layer or spheroids were washed with PBS and treated with no glucose containing DMEM (Gibco, Japan) for 2 h. After that, cells were washed with PBS again and treated with 4.5 g/L glucose DMEM for 20 min. The samples size of 500 μL were collected and frozen immediately until the procedure. Mouse C-peptide ELISA KIT (U-type, Shibayagi, Japan) was used for ELISA assay. Measurements were made in accordance with manufacturer protocol. The optical absorption was measured by a plate reader (Sunrise Rainbow, Thermo Tecan, Japan) at 450 nm (reference wavelength 620 nm).

2.11. FLUOROMETRIC DETECTION OF REACTIVE OXYGEN SPECIES

The monolayer was cultured for 4 days in 96-well plate, spheroids were cultured for 4 days in PDMS-chip, and 200 µL aliquots were transferred to 96-well plate just before measurements as the optimal pH for the reaction lies in the range pH 5-7, we did not use any special buffer solution and conducted the reaction directly in the culture medium. The concentrations of reaction components were as follows: pyronin B (Sigma-Aldrich, Japan) 2 µM, $MnCl_2$ (Sigma-Aldrich, Japan) 0.1 mM, sodium dodecyl sulfate (Wako, Japan) 0.1 mM. In case of fabrication of cellulose beads, we mixed reagents as follows: micro cellulose (Sigma-Aldrich, Japan) 0.440 g, pyronin B 3 mg, $MnCl_2$ 2 mg, sodium dodecyl sulfate 3.5 mg, 1-butyl-3-methylimidazolium chloride (Sigma-Aldrich, Japan) 11 g. The reagents were well mixed and heated at 85ºC for 7 h. After that, the hot cellulose solution was formed into beads of about 50 µL volume by using the pipette.

2.12. FLOW-CYTOMETRY

Spheroids and monolayer were cultured for 4 days, then washed with PBS and treated with dye DCFDA (Sigma-Aldrich, Japan) for 1h to stain ROS positive cells. After that, monolayer cells were trypsinised and collected in PBS. Spheroids were washed with PBS, then treated with Accutase (Innovative Cell Technologies, Japan) for 20 min in the incubator, centrifuged at 6,000 rpm for 30 s and resuspended in PBS. Before measuring the amount of ROS positive cells, all samples were treated with live/dead cell discriminator 7-AAD Viability Dye (Beckman coulter, Japan). Cells were counted with flow cytometer MoFLo astrios (Beckman coulter, Japan). We used unstained cells as a negative control and cell treated with 0.1 mM H_2O_2 as a positive control. See more details in supplementary Fig. 13.

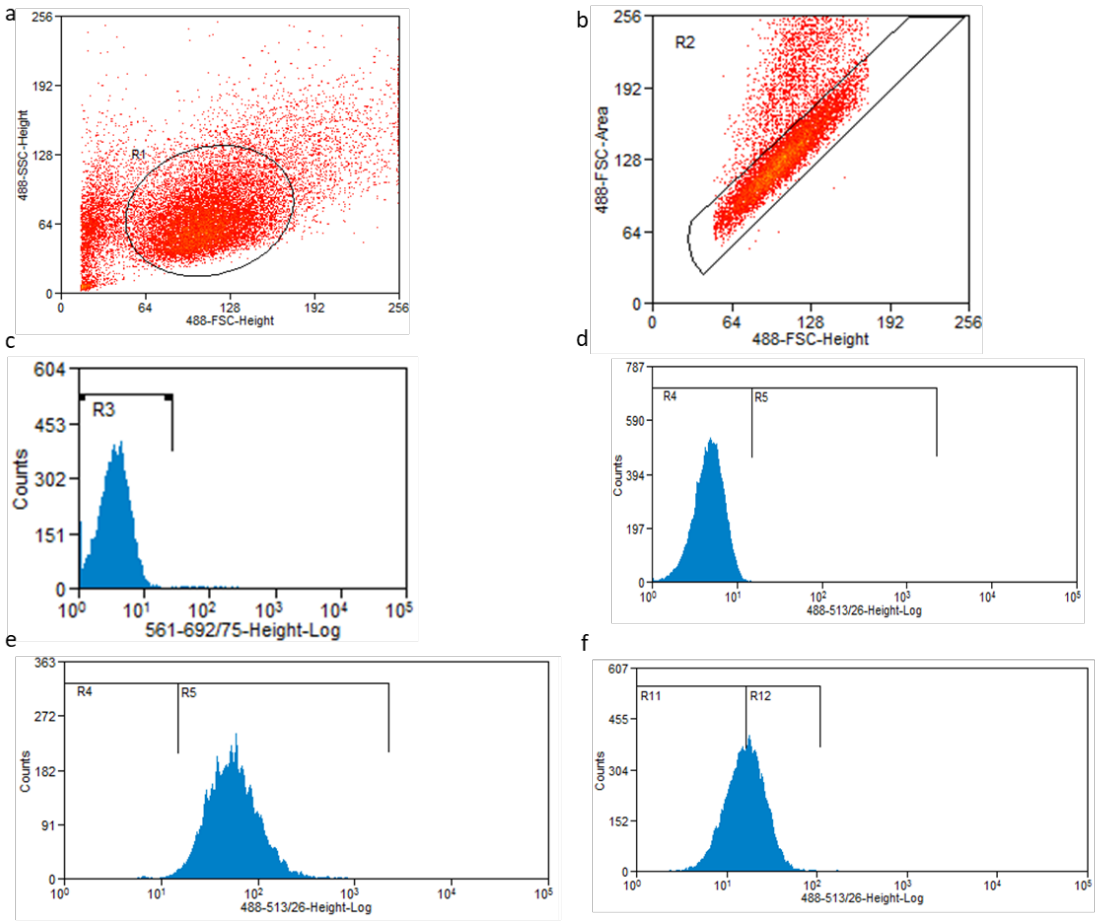


Fig. 13. The gating tree for flow cytometry experiment. Chosen laser line: 488 nm. Used emission filters: 692/75 for 7-AAD Viability Dye and 513/26 for DCFDA. **a**: FSC/SSC to **b**: SSC/pulse width to exclude events that could represent more than 1 cell to **c**: live gate to **d**: DCFDA negative to **e**: DCFDA positive. f: the example of a sample result with the set gates. A total of ~10,000 cells were analysed for each sample.

2.13. STATISTICAL ANALYSIS

The data of spheroid size measurements, insulin content, cell viability and gene expression are expressed as mean value ± SE, and one-way ANOVA with Turkey test (HSD) was performed. Tests were performed using XLSTAT software (Addinsoft SARL). Some of the gene expressions are represented as box-and-whisker plots, the lower and upper limits of the boxes are 1st and 3rd quartile, and whiskers represent the minimum and maximum of all the data.

CHAPTER 3

3. FABRICATION OF PANCREATIC SPHEROIDS AND OPTIMISATION OF THEIR CULTURE CONDITIONS

3.1. OXYGEN SUPPLY IN 3D TISSUE ENGINEERING

Culturing bioartificial islets as well as any 3D thick tissues is a complicated process where a lot of small details should be thoroughly considered and optimised. I already described in Chapter 1 that such points as culture medium additives and ECM environment are important for culturing islets and preserving their functions. In this chapter, I want to address the problem of oxygen supply, the stumbling stones of tissue engineering, in more detail. Researches learnt how to fabricate layers, spheroids and even more difficult structures from cells, but it is still difficult to provide them with enough oxygen to survive. When our tissues are formed during embryonic development, the process is harmonised, and all the tissues are formed together, so all organs have vasculature, neuron innervation and lymphatic system. When scientists are trying to recreate the tissue, they often choose to fabricate mature tissues and organs. The main reason is that one of the main goals of tissue engineering is to reduce the shortage of donors for organ transplantation.

Consequently, it is important to fabricate mature organs in the shortest time possible. This sets scientist with a difficult problem of fabricating the ready-to-use organ from scratch. In our body tissues are highly vascularized. Usually, all the cells are situated about 100 μm and not father than 200 μm[40] from the nearest blood vessel that supplies them with oxygen and nutrients. Thus, to succeed in whole bioartificial organ engineering, there is a need to find a way of creating this kind of structures.

For the purpose of the experiment explanation, I will divide the problem of oxygen supply for two parts. In this chapter, I will discuss the problem of oxygen supply to *in vitro* fabricated tissues, and in the next chapter, I will discuss the fabrication of vascularized tissues *in vitro*.

The problems that often arise when culturing spheroids *in vitro* is that they are depleted from the vasculature, and logically spheroids of size greater than ~ 150 μm experience core hypoxia that possibly leads to cell death[41]. The required rate of oxygen supply varies depending on cell

type. In case of pancreatic islets, native ones have a high demand for oxygen supply considering the fact that they make up no more than 2% of pancreatic volume and receive around 10% of pancreatic blood flow[42]. Oxygen tension decrease in isolated islets and even mild hypoxia during the culturing period leads to impaired GSIS[43,44]. It is expectable that the same applies to *in vitro* cultured bioartificial pancreatic islets (from now on pancreatic spheroids).

In the case of pancreatic islets/spheroids, several approaches were developed to address this issue. The most simple and direct one is to maintain pancreatic islets under hyperoxia conditions (35-50% oxygen)[45]. However, this approach only allowed to reduce islet mass loss but could not prevent it. Partly it may be explained by some approximations made in the paper. For example, for the evaluation of their results, the authors of cited paper used average oxygen levels throughout the culture dishes that equalled the partial pressure in % recalculated to mmHg. However, both computer simulations and experimental data show that the real oxygen partial concentration in the bottom layers of a culture medium is lower than near the air-liquid surface. Also, measurements show that the real oxygen partial pressure in culture dish kept in the humidified incubator is lower than calculated ideal from % content[40]. In another approach, oxygen tension in the culture medium was increased by placing PDMS rings with incorporated CaO_2 or beads of the same composition that gradually generated oxygen under contact with a culture medium[46]. This approach led to increased insulin secretion in monolayer culture of MIN6 cells. However, a potential drawback is that the oxygen release depends on the geometry of the PDMS insert and may create oxygen gradient, exposing adjacent cells to a higher oxygen tension than cells that are farther from it. The last approach uses PDMS as an oxygen permeable material for fabrication of spheroid culture device[41,47–50]. This kind of devices allows spatial separation of cultured spheroids and provide uniform oxygen conditions.

Moreover, comparing to other spheroid fabrication approaches, such as hanging drop technique[51], this method allows more straightforward and large-scale preparation of spheroids. As it was shown previously on HepG2 and MIN6-m9 cell lines, improved oxygen supply allowed reducing hypoxia and increasing cell growth rate and functioning (albumin[41] and insulin[47,49] secretion for HepG2 and Min6-m9/primary islet β-cells, respectively). Although improving oxygen supply is important, it should be kept in mind that too much oxygen is also harmful to

cells because of accumulated reactive oxygen species (ROS)[52–54]. Thus, oxygen should be provided in a controllable manner, or cells should be provided with some ROS scavengers.

3.2. PANCREATIC SPHEROID FABRICATION

3.2.1. CHOICE OF CELL LINES

One of the goals of our research was to fabricate a culture vessel that will provide oxygen supply for culturing pancreatic spheroids and prove that improved oxygen supply has a beneficial effect on spheroids' characteristics. Thus, I optimised culture conditions for the culture approach. By this, I wanted to show that it is important not only culture cells in 3D but also think about how the culture environment should be changed to match the changes in dimensions. Thus, for my purpose the cell line with β cell-like behaviour was required, in particular, I wanted to work with a cell line that can secrete insulin in response to changing glucose concentration (GSIS). Unfortunately, most of the commercially available β-cell lines do not secrete insulin or are not sensitive to changes in glucose concentration. The two most famous cell lines that were suitable for my experiment are INS1(and several followed clone cell lines with improved characteristics) and MIN6. Both of them are derived from insulinoma pancreas; the first one is derived from a rat and the second – from a mouse. Both these cell lines are not commercially available and can be received on the personal request to the professors of the laboratories that established the cell line. The INS1 is of American origin, and MIN6 was established in Japan. Apart from rodent origin cell lines, there are only two human cell lines that were proved to have GSIS. Both of them are young cell lines, with little publications and characterisations. Another drawback is that they require a special costly culture medium and some experience in handling, as researchers feedback shows that they are sometimes difficult to culture. iPS cells should be another good option, but for now, the highest success rate of differentiation of them to β-cells is not higher than 30%. Additionally, differentiated cells often have considerable numbers of cells expressing multiple hormones and lacking expression of mature genes[55]. Thus, considering that I was not experienced with pancreatic cell culture, at the beginning of the experiments, I chose to work with available in Japan and an easy-to-handle MIN6 cell line that was established by prof. Miyazaki[56]. However, for my future research, I am considering working with one of the human cell lines, or iPS cells.

Apart from MIN6 cell line, I decided to use its clone line – MIN6-m9 established by prof. Seino[57]. This line was cloned from a MIN6 cell with the highest insulin secretion rate, so I expected it to be the improved version of MIN6 cell line.

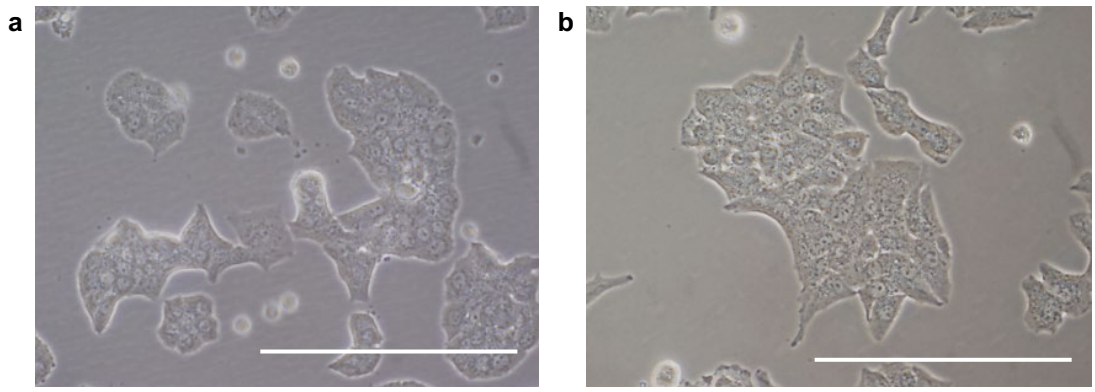


Fig. 14. The phase-contrast photos of cell morphology. a: MIN6; b: MIN6-m9. Scale bar 100 μm.

As it can be seen from the Fig. 14, two cell lines have different morphology. MIN6 has cells predominantly with a rounded shape and few with star-like shape; while MIN6-m9 mostly has star-like shaped cells. According to the prof. Seino paper[57], this difference in morphology has a connection with the ability of cells to secrete insulin, as star-like shaped cells had higher insulin secretion ability than rounder ones in his experiment.

Here I should mention, that I made several changes to the recommended culture conditions. According to the provided protocol, I should have used heat-inactivated FBS. However, it seems that heat inactivation was done back in1990-s to get read of mycoplasma contamination of FBS, that now is usually eliminated by manufactures by filtering through 0.1 μm filter. One more reason could be an inactivation of complement that is part of the immune system that reacts to the presence of bacteria. However, as nowadays conditions of raising cattle are controlled better

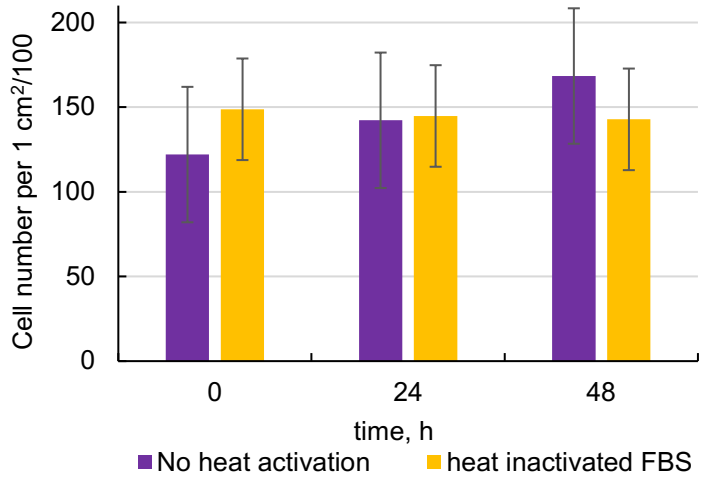


Fig. 15. Growth rate of MIN6 cells in culture media with heat inactivated and not heat inactivated FBS during 2 days after seeding.

than back in 1990-s, the possibility of complement contamination is much lower. Moreover, if not done properly heat inactivation may destroy vitamins and growth factors that will have an advert effect on the cell growth[58]. It was shown by the Coriell University that most cell lines do not grow better in heat-inactivated serum containing culture media. Thus, it is recommended to check if heat inactivation is necessary. After I checked cell growth rate (Fig. 15) in a culture medium with normal FBS or heat-inactivated FBS and found no differences both in cell growth and morphology, I concluded that heat inactivation is not necessary for my experiment.

Another thing that I have changed was the preservation protocol. In the case of MIN6, the use of CELLBANKER (Takara Bio Inc, Japan) solution was recommended, while in case of MIN6-m9 it was recommended to use a mixture of 20% DMSO and 80% FBS. I checked the viability of cells after thawing, and it was 76% for cells stored in the mixture of DMSO & FBS, and 81% for cell stored in CELLBANKER. Thus, I used the CELLBANKER to store both cell lines. I also preferred to use the same preservation protocol to reduce artefacts during culture period and viability measurements, to ensure faithful comparison.

3.2.2. PDMS MICROWELL ARRAY FABRICATION

Native pancreatic islets have the nearly spherical shape that is the same as the shape of a spheroid. Thus it was logical that I chose to fabricate pancreatic β-cells spheroids as a model of pancreatic islets. However, I should mention, that spheroid shape is often chosen even if it is not close to the real shape of an organ. The main reason to fabricate spheroids is that it is the simplest way to culture cells in 3D culture. As I already described in chapter 1, there are several ways to fabricate spheroids, from which in my case I decided to use microwell array devices. The main reason behind this decision is that, apart from the design, I also can choose the material of the devices to suit my goals.

In this work, I decided to use microwell array made from oxygen permeable polydimethylsiloxane (PDMS) for a spheroid formation that can provide enough oxygen supply to cells. Microwell arrays are the dish inserts with hundreds of wells of several hundred μm diameter. The possibility to regulate the diameter, depth and distance between wells allows flexible adjustment to the purposes of the experiment. The process of the PDMS microwell array is described in chapter 2 (Fig. 11).

The design for the microwell array was drawn in Adobe Illustrator by simply arranging circles of desired size in the desired pattern. This design, saved as PDF, then was transferred to

Vcarve Pro software, which creates a toolpath for a drilling machine. One of the things that can be changed here is the depth of wells. In my case, I chose the depth that was bigger than the diameter of the wells to secure spheroids inside them and reduce the chance of aspirating spheroids during culture medium exchange. Apart from this, another point to be careful about is the type of a driller and its speed. In my work I tried two types of drillers, one was made for working with plastic and another with metal. Surprisingly, the one designated for working with metals (Misumi, XAL-BEM2S0.25) allowed me producing smoother wells that is important, as the rough surface can lead to unnecessary cell attachment. Another important factor is the speed of the driller. The Roland milling machine allows changing x-y, z and rotational speed. The right combination for the mould preparation should be chosen by the try-and-error method. One of the things to keep in mind is that the acrylic plate, which I used in my experiment, is weak to heat, so high rotation speed coupled with high 3D speeds may lead to clumping of wells. However, too slow rotation speed also may result in clumped wells (Fig. 16). In my case reducing x-y speed to 30% and adding some Triton X-100 solution onto the surface of the plastic (to cool down both the plastic and the driller) allowed fabrication of smooth and clear wells.

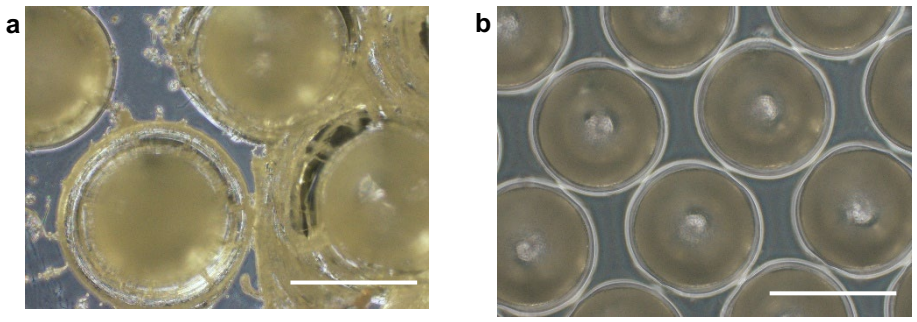


Fig. 16. The examples of acrylic moulds of the same design made with the different speed parameters of a drill. a) x-y speed – 20%, z speed – 80%, rotation speed – 60%. b) x-y speed – 30%, z speed – 100%, rotation speed – 100%. Scale bar 500 μm.

The PDMS-chip was designed to provide oxygen supply to the spheroids, so as a control I fabricated the PMMA-chip, oxygen non-permeable microwell array. The latter was fabricated mainly from acryl with only the well area cut from the PDMS-chip. As so, the area with spheroids was surrounded by oxygen impermeable material, and the only way to receive oxygen was through the gap between culture dish and its lead.

3.3. CHARACTERISATION OF PANCREATIC SPHEROIDS CULTURED IN THE PDMS-CHIP AND THE PMMA-CHIP

In this sub-chapter, I will compare spheroids cultured in two types of microwell arrays with different oxygen supply to prove the positive effect of oxygen supply on the functioning of pancreatic β-cells. Both types of spheroids were also compared with monolayer culture to show the advantages of culturing cells in a 3D structure. The experiment was done with MIN6 and MIN6-m9 cell lines to show the influence of oxygen supply on different cell lines.

3.3.1. INFLUENCE OF A WELL DIAMETER

First, I compared two well diameters: 500 and 1000 μm. Although the 500 μm is closer to the real pancreatic islet size, the 1000 μm well should have provided more space for cells to grow and more culture medium around. I also varied seeding density in the range of 500–3,000 cells/well (Fig. 17). I measured the spheroid size every day during 1 week of culture and found out that at first, spheroids shrank in size, but after day 4 of culture spheroids entered a phase of steady growth (Fig. 17). I chose the day 4 of culture as an observation point for the next experiment, so I can avoid any additional impacts from the process of spheroids formation that can interfere with the results. The morphology of spheroids was nearly spherical for all tested conditions (Fig. 17d). At lower cell seeding densities 500 cells/well and 1,000 cells/well, the PDMS-chip of ⌀500 μm wells allowed formation of spheroids with the average diameters of 160 ± 7 μm and 180 ± 10 μm respectively; while the same PMMA-chip produced spheroids with average diameters of 100 ± 7 μm and 120 ± 6 μm respectively (Fig. 17e). There was a significant 50-μm difference in size of spheroids cultured in the PMMA-chip and the PDMS-chips on the first day of culture, although the initial seeding density was the same. It possibly could be explained by cell death due to limited oxygen supply right after seeding that lead to smaller cell number in spheroids cultured in PMMA-chip [44]. Higher cell seeding densities in both of these 500 μm chips did not result in bigger spheroids because not all seeded cells were able to settle down into wells and floating cells were aspirated during culture medium exchanges. Because in the PDMS-chip with 1,000 μm wells the space for spheroid growth was bigger, it allowed the formation of spheroids with a bigger diameter in case of higher seeding densities. Surprisingly, the difference in spheroid sized that was obvious at first days of culture became less obvious after 1 week of culture, because, despite well diameter and seeding density

difference, spheroids seemed to stop growing after becoming approximately 200 µm in diameter.

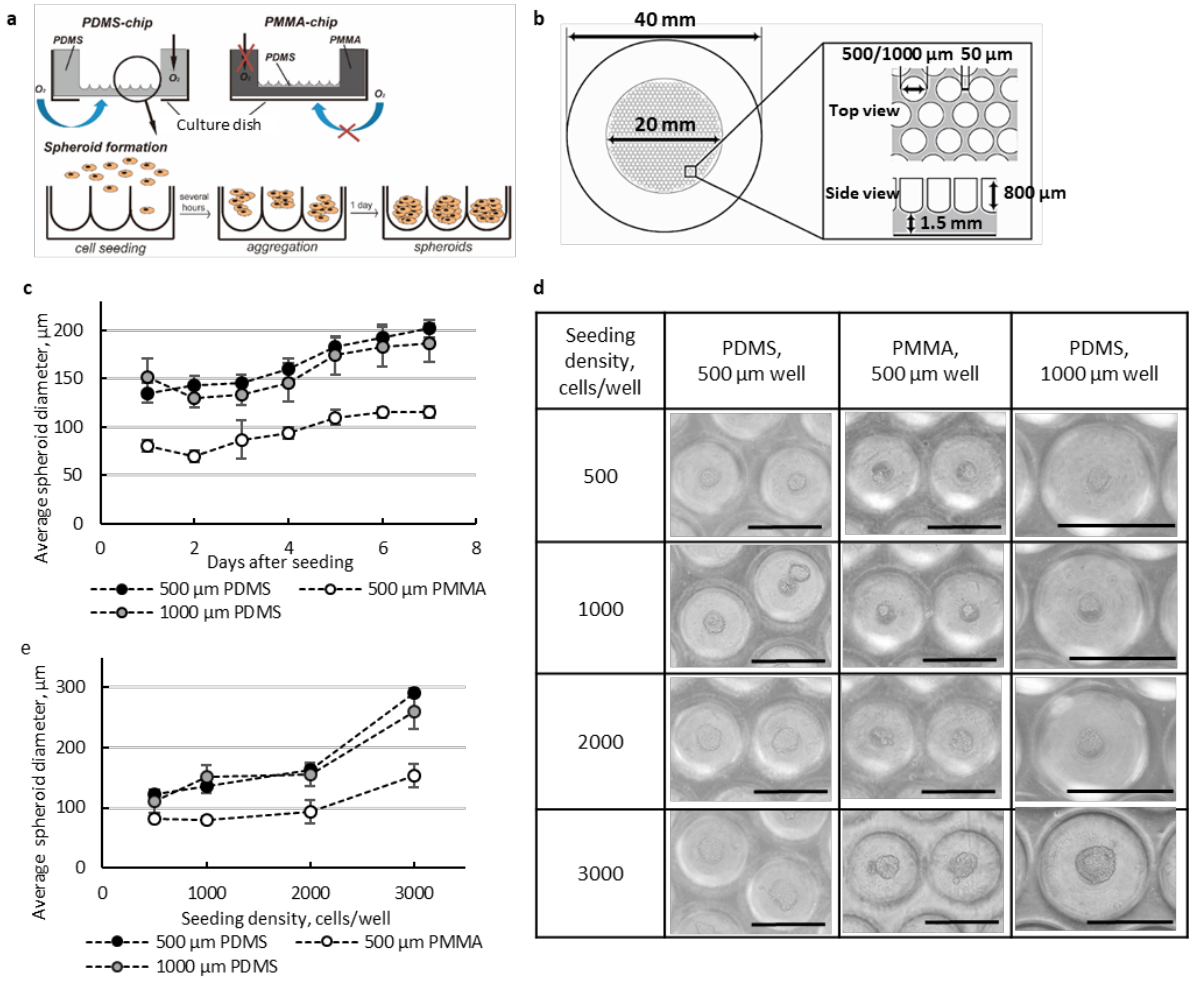


Fig. 17. Optimisation of spheroid fabrication conditions. a: Schematic representation of PDMS and PMMA spheroid culture devices and the spheroid formation process. **b:** Details of the spheroid culture device design. **c, d, e:** effects of well size and material of the device and seeding density of MIN6 cells. **c:** Changes in average spheroid diameter (for representative seeding density of 1000 cells/well) in different devices during 7 days of culture (n=10, p=0.05). **d:** Optical microscopy images of spheroids on Day 1 of culture. Scale bar of the first two columns: 500 µm; the third column: 1000 µm. **e:** Average size of spheroids on Day 1 of culture (n=10, p=0.05).

I need to conduct further research to elucidate the reason for this phenomenon, but it can be that after reaching a certain size, more and more cells become growth arrested that prevents spheroid enlargement. As an optimal design and seeding density, I chose condition with 500 µm and 1000 cell/well seeding density. The main reason for the decision was that usage of this combination resulted in nearly targeted 200 µm spheroids on day 4. As for the size of the well,

I decided to use the smaller one, as it allows culturing more spheroids at one time without loss in spheroids' quality.

Recently, I found some information that too much of free volume around pancreatic spheroids is not beneficial. It seems that around 30-40% of free space (volume) is the best condition for native pancreatic islets to keep functioning. More or less of free space results in different problems with islet functioning. I still have not done literature research on this statement, but I will do this in the nearest future.

3.3.2. EVALUATION OF SPHEROID FABRICATION BY THE FLATBED *EPI* RELIEF-CONTRAST CELLULAR MONITORING SYSTEM.

To investigate the process of spheroid formation in more detail, I used flatbed epi relief-contrast cellular monitoring system (eRC-CMS) to observe MIN6, MIN6-m9 and PANC1 cells forming spheroids in PDMS-chip with (oxy-chip) or without hole (non-oxy-chip) in the bottom. PANC-1 cell line was chosen as a control as it is a pancreatic cell line that cannot secrete insulin and is often used in cancer research. Contrary to the conventional way of cell observation under a microscope, when cells are observed only at some specific points of time, usually once a day, eRC-CMS allows continuous control of cell quality and behaviour. Thus, I decided to use the system to observe details of spheroid formation thinking that it may reveal some specifics of the process that are overlooked in the conventional observation method. Unlike the phase-contrast microscope, eRC-CMS is equipped with a LED light that is integrated into the incubator configuration together with the objective lens. The whole imagining unit is flat that allows to move it freely along the incubator shelf. The unit was designed in a way it allows cell is imagining by using light reflected by the surface of the culture vessel lid, i.e. oblique epi-illumination. The speed of the imagining unit was 9.0 mm/s (x-axis) and 12.5 mm/s (y-axis). In my experiment, I set the camera to take pictures every 10 min in 3 different, not overlapping positions that were not too far from each other (because of the speed limit). The camera was

controlled from outside of the incubator by PC that allows keeping the culture conditions stable during the experiment. Time-lapse imagining revealed the differences in spheroid formation between chosen cell lines and between 2 types of chips.

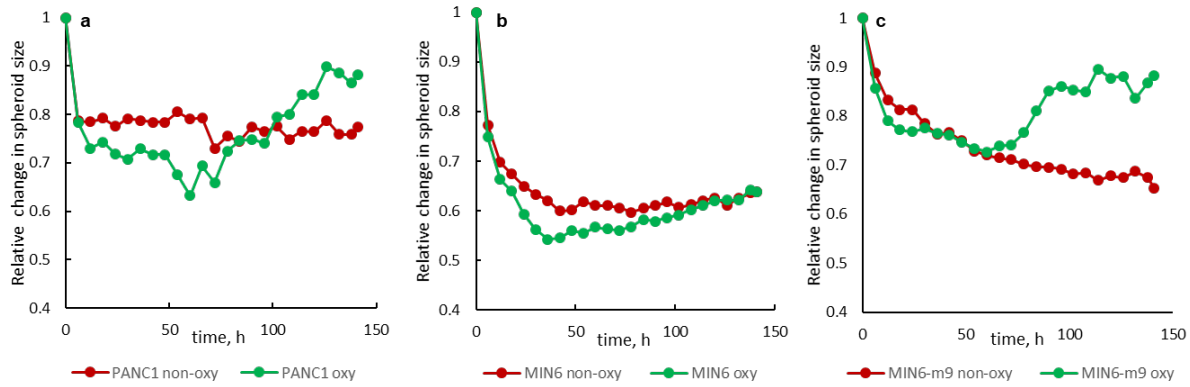


Fig. 18. Changes in relative spheroid size during 6 days of culture of different cell types in oxy and non-oxy chips. The relative size was calculated as the diameter of the spheroid at time x divided to the diameter of the spheroid at 0 h. **a:** PANC1. **b:** MIN6. **c:** MIN6-m9.

As can be seen from Fig.18 (a, b, c), all three cell types in oxy-chip had the first phase of decreasing in size that on the photos was confirmed to be a phase of aggregation. After 6 h, as can be seen from Fig. 19 (second row) MIN6 cell line formed dense aggregates, while PANC1 and MIN6-m9 were able to form only loose aggregates. After 24 h, MIN6 cells in a PDMS-chip formed well-defined spheroids, and after approximately 2 days in culture, the diameter of spheroids started gradually increasing. In case of MIN6-m9, the formation of dense aggregate but not spheroid took almost 2.5 days, after what we observed an increase in aggregate diameter that was connected with cell disaggregation (Fig. 19, last row). For PANC1 the formation of dense aggregate took the longest time, around 3 days, after what we once again observed the increase in diameter caused by cell disaggregation. As it can be seen from the series of images on Fig. 19 and from the graph on Fig. 18, MIN6-m9 and PANC1 aggregates decreased in size to a lesser extent than MIN6 aggregates. This fact shows that probably the heterogeneous nature of MIN6 cells plays an important role in spheroid formation.

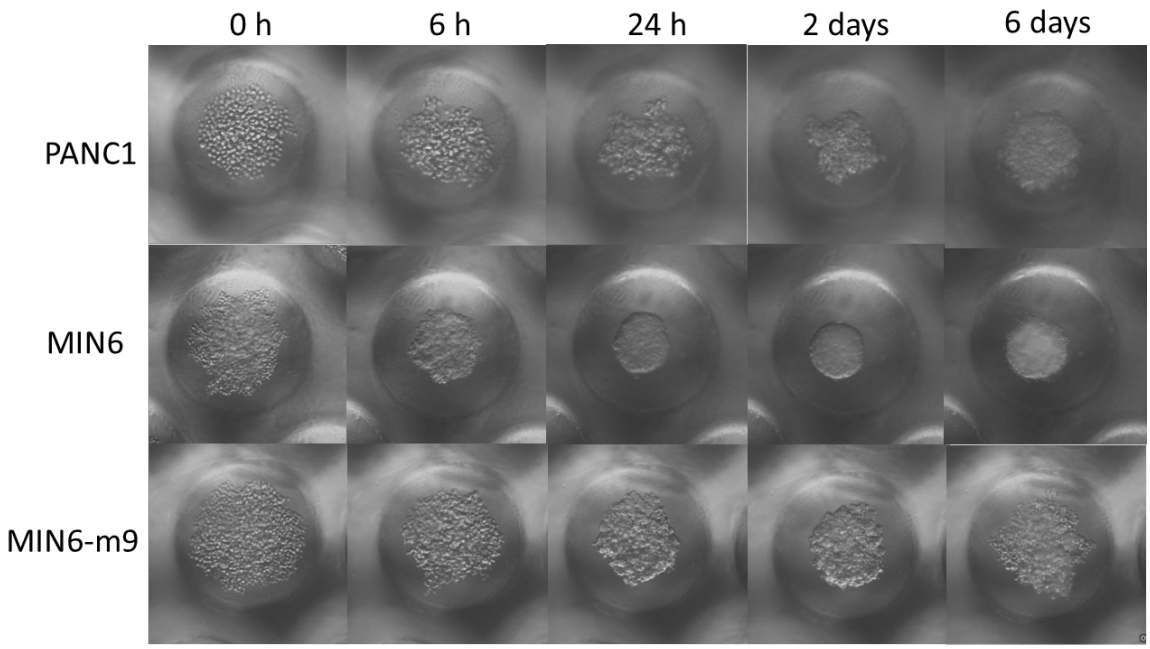


Fig. 19. Time-lapse images of spheroids cultured in oxy-chip. The spheroid formation of PANC1, MIN6 and MIN6-m9 was monitored by taking photos every 10 min. Only representative photos are shown in the image.

Similarly to oxy-chip, MIN6 cells cultured in nonoxy-chip (Fig. 20) were able to form spheroids in 1 day by aggregating and shrinking to the small size, and after 2 days they gradually increased in diameter. Judging from Fig. 18, in case of oxy-chip MIN6 cells, shrank more and then increased in diameter more than in case of nonoxy-chip. In the case of MIN6-m9 and PANC1 cell lines, I observed a very delayed formation of spheroid that took 6 days (the whole period of observation) that was not followed by the increase in diameter. We could not prolong the time of observation as at the moment the eRC-CMS does not allow culture medium exchange inside the incubator without moving the culture vessel. Thus, the culture medium was not exchanged for 6 days that may have had some bad effects on the spheroid formation.

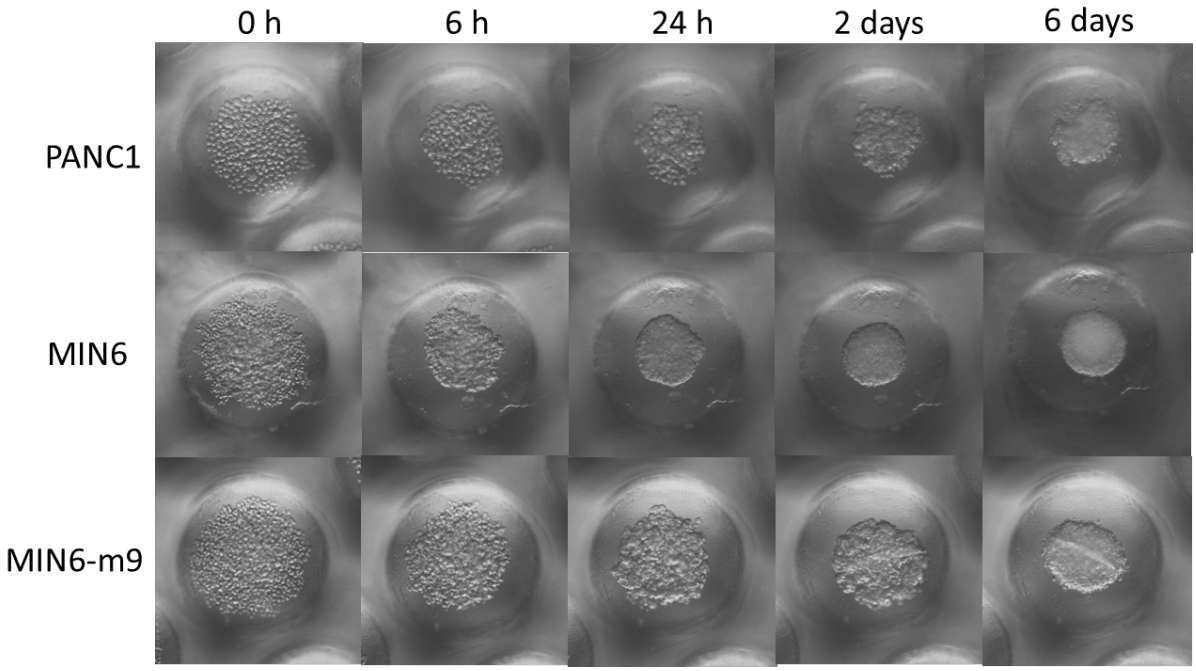


Fig. 20. Time-lapse images of spheroids cultured in PMMA-chip. The spheroid formation of PANC1, MIN6 and MIN6-m9 was monitored by taking photos every 10 min. Only representative photos are shown in the image.

These results show that both the cell nature and oxygen conditions play an important role in the spheroid formation, as for MIN6-m9 and PANC1 cell line less oxygen supply led to spheroids, but in case of MIN6, higher oxygen conditions were more favourable.

3.3.3. VISUAL CHARACTERISATION OF SPHEROIDS

To prove the benefits of using oxygen permeable PDMS-chips for β-cells functioning, I compared morphology and insulin secretion ability of spheroids formed from MIN6 and MIN6-m9 cultured in the PDMS- and the PMMA-chips. According to literature[59], MIN6 cell line is inhomogeneous and consists of cells with different responses to glucose in terms of insulin secretion. Moreover, this cell line consists of cells positive for insulin and glucagon, indicating a mixture of β- and α-cells[59]. MIN6-m9, on the other hand, was cloned from one MIN6 cell with high response to glucose and is a cell line with homogeneous characteristics. Considering this fact, I intended to used MIN6-m9 cell line in my experiment as a pure β-cell line. MIN6 and MIN6-m9 spheroids were cultured with the same seeding density.

Both cell lines formed spheroids in 1 day in PMMA-/ PDMS-chips. 4 days after seeding, I investigated hypoxia level in spheroids by staining for HIF-1α (hypoxia-inducible factor - 1α). For both MIN6 and MIN6-m9, the fluorescent intensity estimated (by ImageJ software) as the intensity of green colour of the photos (Fig. 21a) was 2 times lower for spheroids cultured in PDMS-chips, indicating that oxygen permeable devices indeed allowed decreasing hypoxia level of cells in the spheroid structure. To find out whether reduced hypoxia level affected cell functioning, I first characterised spheroids by staining for insulin and glucagon (Fig. 21b), the latter is typically secreted by pancreatic α-cells but was reported present in MIN6 cells as well[59]. Surprisingly, not only MIN6 but MIN6-m9 spheroids also had a small number of cells (~10%) positive both for insulin and for glucagon. After checking the same double staining for monolayer culture of both cell types (Fig. 22), I found that even when cultured as a monolayer, there was a fracture of cells positive for glucagon. In the case of MIN6 cells, the presence of glucagon positive cells can be explained by the initial composition of the cell line[59]. In case of MIN6-m9 cell line that should be homogeneous in characteristics, a possible explanation for heterogeneous characteristics of cells may be that β-cells dedifferentiate to α-cells, which was observed in native pancreatic islets cultured *in vitro* due to metabolic or oxidative stress[60]. Interestingly, in both types of spheroids, glucagon-positive cells were concentrated on the periphery of spheroids similar to the natural structure of mouse islets[61].

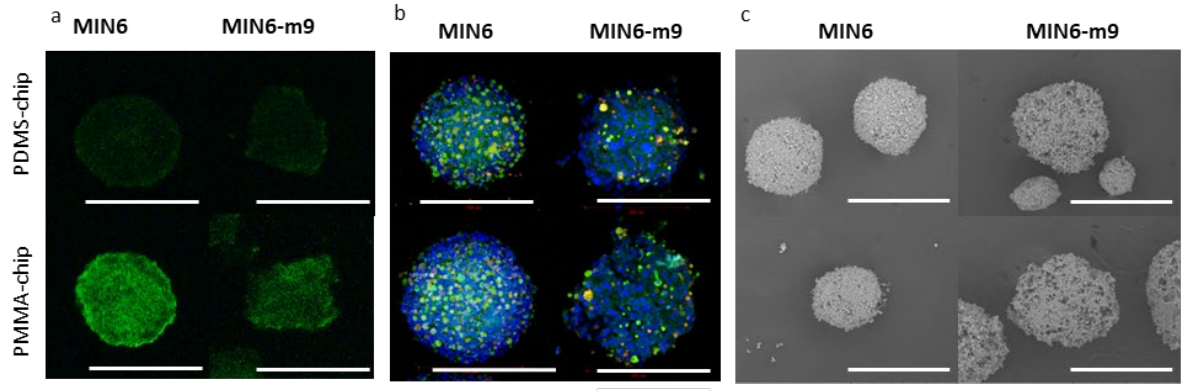


Fig. 21. **Qualitative characterisation of culture spheroids.** Immunofluorescent staining of MIN6 (left) and MIN6-m9 (right) spheroids (Day 4 after seeding) cultured in PDMS- (top) and PMMA-chips (bottom) for **a:** HIF-1α. Staining was performed on 12 µm spheroid frozen sections. Scale bar: 200 µm. **b:** Insulin (green), glucagon (red), and DAPI (blue). Staining was carried out for 3D spheroids. Scale bar: 200 µm. **c:** SEM images of 3D spheroids treated with OsO_4. Scale bar: 300 µm.

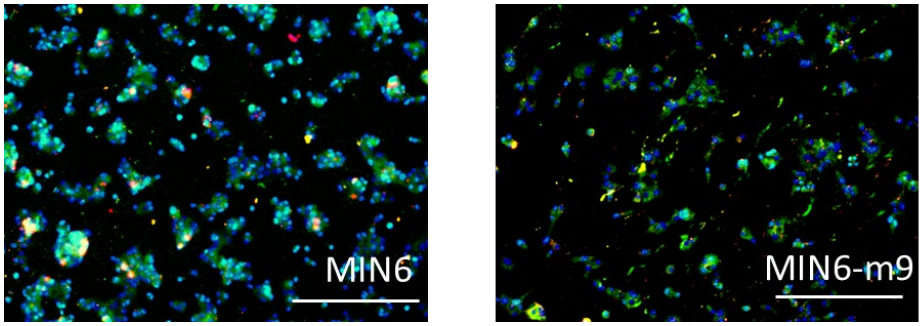


Fig. 22. Immunofluorescent staining of MIN6 (left) and MIN6-m9 (right) monolayers (day 2 after seeding) for insulin (green), glucagon (red) and DAPI (blue). Scale bar — 500 μm.

Scanning electron microscopy was performed to examine spheroids structures (Fig. 21c). MIN6-m9 spheroids had a less dense structure with many gaps between cells, while MIN6 spheroids were well-defined and tightly packed with cells. This is likely due to the weaker cell-cell attachment between MIN6-m9 cells comparing to MIN6 cells. Although an additional investigation should be conducted, I can suggest two possible explanations for observed results. The MIN6 cell line is known to consist of cells with heterogeneous characteristics: with different morphology and ability to secrete insulin[57,59]. It possibly contains cells that secrete different amounts of ECM proteins or cell-cell adhesion molecules. On the contrary, MIN6-m9 cell line, as a purified cell line, consists of more homogeneous cells in their characteristics and may have partial loss of ability to secrete ECM proteins or cell-cell adhesion molecules[62].

3.3.4. The characterisation of pancreatic spheroids by genes expression analysis and glucose-induced insulin secretion

The most important characteristic of pancreatic islets, considering their function in our body, is the ability to secrete insulin in response to changing glucose concentration. Taking into consideration that deprived oxygen supply leads to impaired insulin secretion[44], I hypothesised that improved oxygen supply *in vitro* would have a positive effect on the functionality of bioartificial pancreatic islets. To examine how different culture conditions influence cell characteristics, I compared expression of several genes characteristic for pancreatic cells and amounts of secreted insulin for MIN6 and MIN6-m9 cell lines cultured as monolayer or spheroids in different chips.

Let me describe in more detail chosen genes. One of the most important choices that should be done before starting RT-PCR is the choice of the house-keeping gene. The most two widely used ones both for rodents and humans are genes that codes *β-actin* and *GAPDH*. However, it is important to keep in mind, that housekeeping genes are not universal, and some may work better or worse in the specific conditions. For example, the *GAPDH* was found to change its expression depending on glucose concentration in culture medium in rat native islet cells and INS1 cell line[63]. Thus, it is important to find the optimal housekeeping gene for the experiment that will keep the expression on a relatively stable level. There is a software dedicated to the determination of optimal = most stable housekeeping gene named geNorm. Unfortunately, it is not available for free, so in this work, I simply tested several housekeeping genes known from the literature and checked how stable are their expressions in the conditions of my experiment. The housekeeping genes that I tested were as follows:

- *β-actin* is a gene that codes the protein with the same name. Actin is a highly preserved protein in vertebrates and has 6 isoforms. The β one is one of the two that are expressed ubiquitously throughout all the cells.
- *GAPDH* is a gene that codes the enzyme with the same name. This enzyme catalyses the sixth step of glycolysis and is the part of machinery breaking down the glucose. As this gene is an indispensable part of the cellular machinery, it is often used as a housekeeping gene. However, it is sensitive to the glucose concentration (that, of course, is obvious from the function of the enzyme it codes) and oxidative stress[64]. Thus, one should be careful when deciding to use this gene as a housekeeping one, as more often it would be better to check it as a target gene, to be sure that the cells are functioning properly.
- *eEF2* is a gene that codes the protein eukaryotic elongation factor 2. This protein catalyses the movement of the ribosome relative to the mRNA and is an inconsistent part of the elongation mechanism in most eukaryotes, as it is essential for protein synthesis. Thus, it is abundantly and highly expressed in tissues and was shown to be a housekeeping gene in a study with mice[65].
- *Tbp* is a gene that codes TATA box binding protein. TATA box is a sequence located in the regulatory part of DNA near the beginning of many genes. Thus, this protein helps to direct the process of DNA reading, showing where the reading of a gene should start[66].

In my experiment, the *GAPDH* was shown to have unstable expression and thus could not be used as a housekeeping gene. The *Tbp* and *β-actin* were not expressed at high levels, which was most certainly connected with the chosen condition for gene amplification. *eEF2* had high and stable expression throughout all experiments, so it was chosen as a housekeeping gene.

For the first part of the work, as target genes I chose the following ones:

- *PDX1* (pancreatic and duodenal homeobox 1) codes the nuclear protein that activates transcription of such genes as insulin, somatostatin, glucokinase, islet amyloid polypeptide and glucose transporter 2. It is known to be involved in pancreas maturation and glucose-dependent regulation of insulin gene expression and transcription. It is expressed in the whole pancreas and not only in β-cells. In latter, it is required for the preservation of their phenotype.
- *INS1* and *INS2* are the genes that code the two types of insulin peptide in rodents. I should notice here, that having 2 types of insulin is normal for rodents only, and this does not apply to humans. *INS1* and *INS2* have almost identical sequences, differing only by 6 amino acids and deletion of 2 amino acids in the C-peptide of the ins1 protein[67]. In native mouse pancreatic islets, the expression of two genes is approximately on the same level. MIN6 pancreatic spheroids exhibit the same behaviour when formed from cells of low passages. However, spheroids, formed from cells of passage $\geq$ 35, exhibit different ratio where *INS2* gene is expressed higher than *INS1*. This gene profile was linked to the MIN6 pancreatic spheroids losing their ability to secrete insulin in response to glucose[67]. In my work, I decided to check both genes to keep in touch with the cells' condition.
- *GLUT1* and *GLUT2* code glucose transporters 1 and 2. Both are cell membrane proteins that help to transport glucose across the plasma membrane of mammalian cells. *GLUT1* is responsible for the basal glucose uptake necessary for the cellular respiration. Its gene expression level increases with reduced glucose concentration and vice versa. *GLUT2* has high capacity but low affinity for glucose and plays a major role in glucose-sensing mechanism in rodents. However, it only plays a minor role in sensing glucose in humans. Its gene expression is increased with increasing of glucose concentration.

On Fig. 23 I showed the results of RT-PCR for the selected genes during a week of culture. As it can be seen from the Fig. 23a, *PDX1* expression was slightly upregulated in the first 4 days of culture for spheroids and the first day of culture for monolayer showing that β-cells maintained their level of maturity. Then gene expression was downregulated after 1 week of

culture for spheroids and monolayer that shows that culturing cells for prolonged time leads to cell dysfunction, that was reported in the literature (see Chapter 1). For MIN6 spheroids cultured in the PMMA-chip, I observed even greater upregulation of *PDX1* with sudden downregulation after 1 week. Here I should explain that *PDX1* although important for β-cell maturity, also is known to be upregulated in pancreatic cancer cells[68], as it is connected with the maintenance of cell proliferation and reduction of apoptosis in tumour cells[68]. Thus, moderate upregulation of *PDX1* is usually considered to be a sign of preservation of β-cell phenotype. However, strong upregulation can be a sign of cells turning cancerous. I need to conduct more experiment, probably including primary β-cells, to clarify this point and be able to answer if this upregulation is good or not.

The analysis of *GLUT1* (Fig. 23b) gene expression is a little bit trickier as it depends both on glucose concentration and hypoxia. I observed upregulation of the *GLUT1* on the first and fourth day of culture and a decrease in relative gene expression on day 7 for the monolayer culture and spheroids in the PMMA-chip. Taking into consideration that culture medium was changed every two days (on day 2,4 and 6 of culture), I may expect that slight *GLUT1* upregulation was induced by decreasing glucose concentration. The upregulation on the fourth day of culture is even greater. Thus I can conclude that cells experienced conditions with low glucose, which is logical as cells proliferate and require glucose for it. However, if I will compare it with the spheroids in the PDMS-chip, the latter one had almost the same expression of *GLUT1* on the first and the fourth days of culture. One possible explanation could be that upregulated *GLUT1* on the fourth day of culture for monolayer and spheroids in the PMMA-chip could be partially explained by cells experiencing hypoxic conditions[69] that also was showed by staining (Fig. 22). The results of day 7 are more difficult to explain; my guess is that hypoxia, glucose concentration and possibly cell apoptosis played a role in observed values of gene expression. The precise analysis could be done if I will have the gene expression profile for native mouse pancreatic islets and will check expressions of other genes connected with hypoxic conditions.

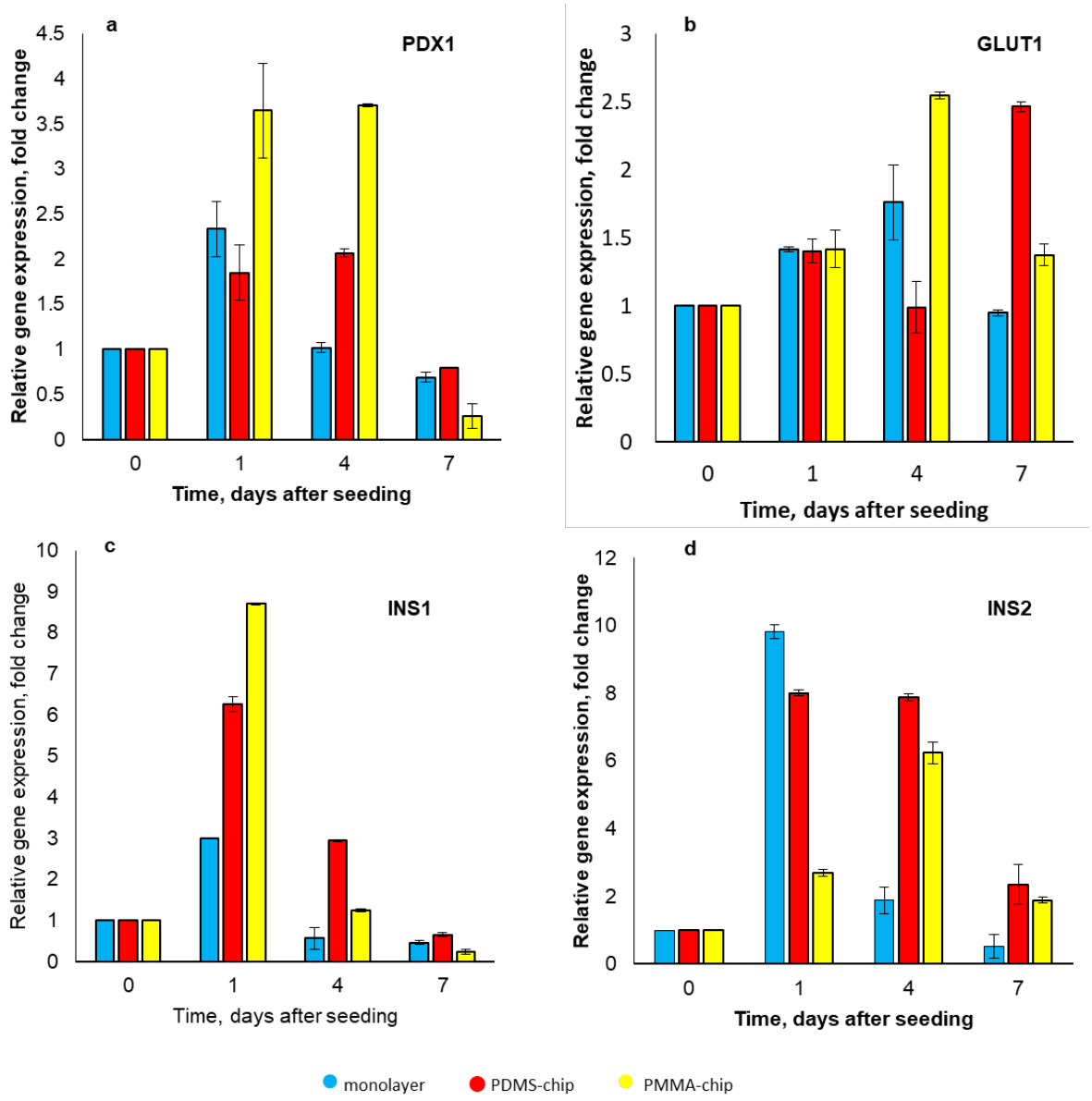


Fig. 23. Quantitative comparison of MIN6 spheroids and monolayer. RT-PCR results for PDX1 (a), GLUT1 (b), INS1 (c) and INS2 (d) expressions of MIN6 monolayer and spheroids in PDMS-chip and PMMA-chip. n=4, p=0.05.

On the first day after seeding, *INS1* gene expression was upregulated for all cells, for spheroids more than for monolayer culture, and surprisingly the expression was higher for spheroids in the PMMA-chip than in the PDMS-chip. I do not have an explanation for that, but I can speculate that it can be connected with oxidative stress of spheroids in the PDMS-chip that will be discussed in next sub-chapter. It is possible that in the first day the cell number was still small and lower oxygen concentration in the PMMA-chip did not result in hypoxic conditions and at the same time did not lead to the production of excessive amount of reactive oxygen

species. On day 4 and day 7 I observed a gradual decrease in *INS1* expression that is in agreement with previous literature results. Though, I should point out that *INS1* expression was always higher for spheroid culture showing the importance of cell-cell contact for insulin secretion[35]. Starting from the 4th day of culture *INS1* expression was higher for spheroids in the PDMS-chip than in the PMMA-chip showing the importance of oxygen supply in the long run.

Interesting results were observed for *INS2* expression. The *INS2* expression was greatly increased in monolayer culture comparing to the INS1 expression. For spheroids in PDMS-chip, *INS2* expression was increased and stayed stable until day 4, and then decreased on day 7 together with *INS1* expression. For spheroids in the PMMA-chip on the first day of culture, the *INS1* expression was upregulated more than *INS2* that should be a positive result. However, on day 4 and 7 I saw the reverse picture, with *INS2* expression being upregulated more than *INS1*. Thus, the *INS1*:*INS2* expression ratio was reversed in monolayer and spheroids in the PMMA-chip that possibly should have resulted in lower insulin secretion rate. The ratio was less disturbed for the spheroids in the PDMS-chip. However it was not perfect, and I may speculate that this also was a result of oxidative or metabolic stress.

However, I observed strikingly different results for MIN6-m9 cell line for all genes (Fig. 24). The *PDX1* gene was downregulated for all culture conditions at day 1 and 4 of culture that indicates the loss of β-cell phenotype. After 1 week of culture, I observed a moderate increase in gene expression for all cultures. However, the results were highly variable that can be seen from the error bars and so can hardly indicate a real improvement in the cells' condition. I believe that these results can be explained by the fact that selected culture conditions were not optimal for MIN6-m9 cell line due to high glucose concentration, and I will talk about it in later sub-chapters.

Not less strange results were observed for the *GLUT1*. For the monolayer and spheroids in the PMMA-chip, there was slight upregulation on day 1 of culture and strong downregulation on day 4 and 7. This most certainly shows the decrease in glucose uptake by cells and may be a sign of oxidative or metabolic stress. For spheroids in the PDMS-chip, the day 1 and 4 of culture were marked by the moderate upregulation of *GLUT1* that can be connected with glucose concentration in culture medium. However, on day 7 there was a strong downregulation similar to other culture types. Together with *PDX1* expression, the *GLUT1* expression most certainly indicates the changes in cell functioning and possibly phenotype during the culture period.

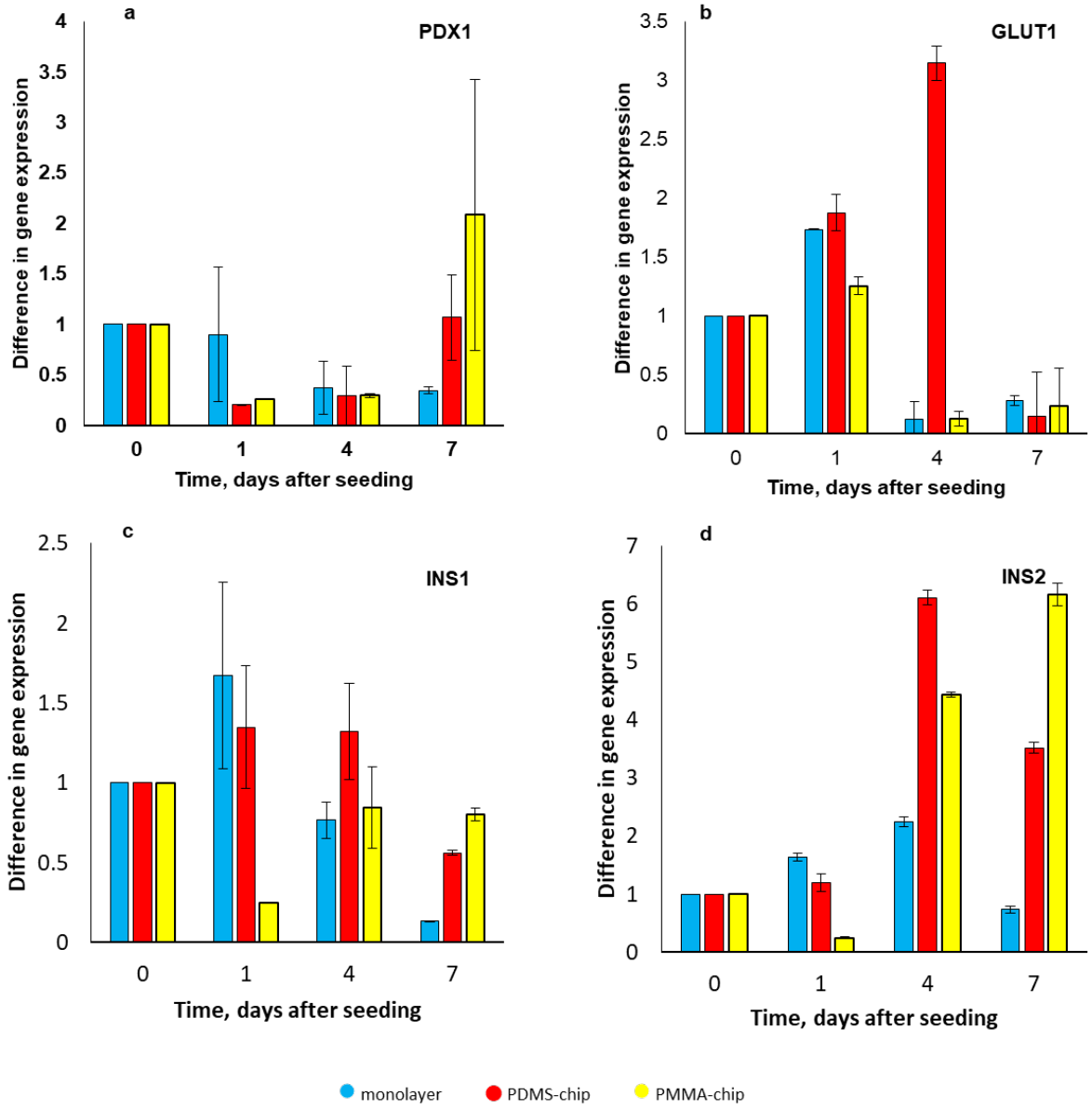


Fig. 24. Quantitative comparison of MIN6-m9 spheroids and monolayer. RT-PCR results for PDX1 (a), GLUT1 (b), INS1 (c) and INS2 (d) expressions of MIN6 monolayer and spheroids in PDMS-chip and PMMA-chip. n=4, p=0.05.

Although some upregulation in *INS1* expression was observed (Fig. 24c), the results were highly variable (can be seen from error bars) and in general the expression gradually decreased during the culture period. Moreover, there was no positive effect of the PDMS-chip as the *INS1* expression was almost the same for both types of spheroids and even monolayer. The *INS2* expression was greatly upregulated on day 4 and 7 of culture for both spheroid types (Fig. 24d) but stayed almost the same for monolayer culture. Further, if we will look into an *INS1*:*INS2*

ratio, it is obvious that it was disturbed even to a greater extent than in MIN6 after 1 week of culture, as *INS1* expression gradually goes down, and *INS2* expression gradually goes up. According to the literature[67], this is the sign of cells changing their phenotype and becoming unresponsive to glucose. The only reason that I can consider is the chosen culture conditions.

I should notice here, that although MIN6 did not show these drastic changes, it should not be interpreted as MIN6 is a better cell line for pancreatic islet model. As MIN6 is a non-homogeneous cell line, it can contain cells with different sensitivity to the components of the culture system, so that the negative effect is somewhat hindered. MIN6-m9 as a homogeneous cell line showed the changes in a clearer manner, highlighting the problem of culture conditions. As so, the problem should be elucidated to prevent cell damage in the long run.

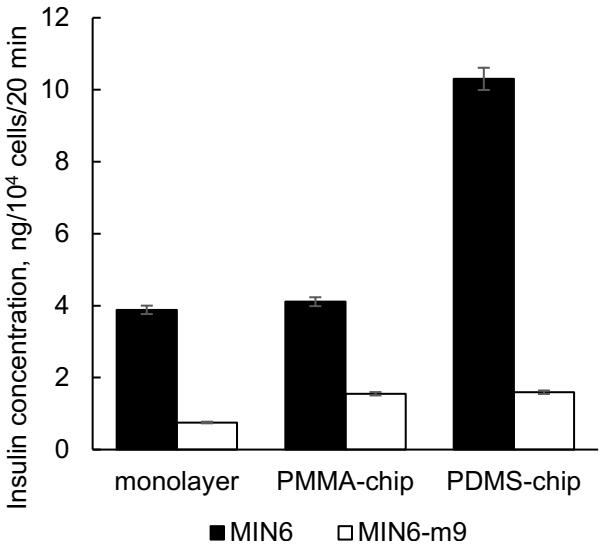


Fig. 25. ELISA results for insulin secretion rate for MIN6 and MIN6-m9 cultured for 4 days.

ELISA results were in accordance with RT-PCR results: for the MIN6 cell line, I observed increasing amounts of secreted insulin in the order of monolayer<PMMA-chip<PDMS-chip, but for MIN6-m9 cell line there was almost no difference in insulin concentration between all culture types (Fig. 25). From immunofluorescent staining images, I observed weaker insulin staining for insulin in MIN6-m9 cells (Fig. 21b), so I expected that both gene expression and insulin content would be less than in MIN6 cells. Possibly, the results can be explained by senescence of the cells[36].

3.4. Optimisation of culture conditions for pancreatic β-cell spheroids in PDMS-chip

3.4.1. Antioxidant influence on pancreatic β-cell monolayer and spheroid functioning

Judging from the described above results, I decided to use only MIN6 cell line for my further experiments as its gene expression profile seemed more understandable than for MIN6-m9. Because I observed signs of the harmful effect of oxy-chip on MIN6-m9 cells, I considered that one of the problems could be reactive oxygen species (ROS). Native pancreatic islets *in vitro* are cultured in a culture medium that has a complicated composition with a lot of antioxidants, vitamins and growth factors. However, in my experiment with MIN6 and MIN6-m9 pancreatic β-cell spheroids I used the culture protocol and the culture medium recommended for the monolayer culture. I considered that this could be my miss, as this protocol does not take into consideration the fact of cells being cultured in a 3D environment with increased oxygen concertation. Although there are several points to be checked, first, I decided to check the production of ROS and include an antioxidant, ROS scavenger, into the culture medium composition.

ROS appears to play an important role in insulin secretion[70–72] but chronic exposure to high concentrations of ROS is damaging for cells and is known to play a role in β-cell dysfunction in diabetes[73]. In the latter case, secretion of antioxidants by the cells may disrupt the normal insulin-secreting mechanism[70–72].

I first attempted to measure ROS production in cells by fluorimetry, using the oxidation reaction of fluorescent dye pyronin B by peroxide group-containing substances in the presence of the $MnCl_2$-SDS complex that mimics the action of peroxidases[74]. This system was convenient, as in solution it allows detection of nanomolar concentrations of hydrogen peroxide or peroxide group containing substances[74,75]. Moreover, the SDS, which is a part of the detection system, allows lysis of cells without additional steps. However, I was not able to get the repeatable results, which probably is connected with the interference of FBS components, such as BSA[74] in the culture medium. The interference of the BSA is most probably can be explained by the fact that BSA similar to SDS has surfactant properties that makes it possible for BSA to

interfere with the formation of $MnCl_2$-SDS complex. It is possible to remove the undesired components of FBS by sample pre-treatment, but it will greatly increase the time and efforts required for the experiment. As a possible way to solve the problem, I considered immobilisation of the system components in cellulose-ionic liquid hydrogel as it is shown on the scheme below (Fig. 26). The conditions for this experiment were optimised by the author of the thesis[75].

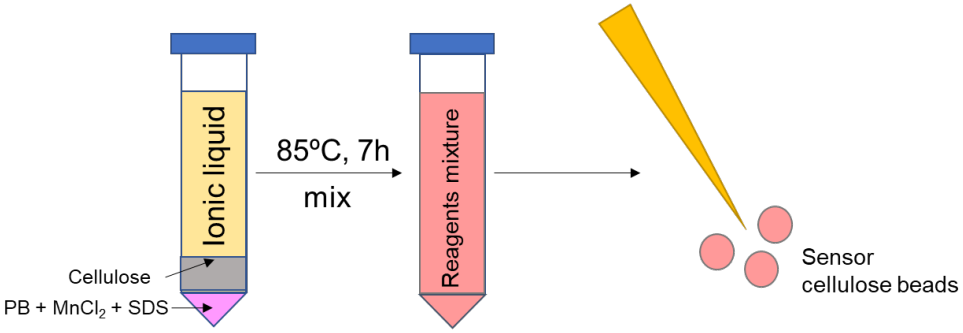


Fig. 26. Schematic representation of the fabrication of cellulose-ionic liquid hydrogel beads with immobilised reaction components for the determination of ROS.

I hypothesised that cellulose beads would decrease the interferences of the FBS components. Although I was able to successfully prepare small cellulose beads, in case of the measurement in the solution, the beads did not give reproducible signals that mainly should be connected with the scattering of light on the beads that interfered with the detection.

Eventually, I decided to measure the number of ROS-positive cells in spheroids cultured in different types of chips by flow cytometry. Spheroids cultured in the PDMS-chip had ~14% higher concentrations of intracellular ROS than those cultured in the PMMA-chip (Table 2).

Table 2. Relative amounts of live ROS positive (ROS+) and negative (ROS–) cells and median fluorescent intensity (MFI) of MIN6 cells cultured in different conditions, as assessed by flow cytometry of cells double-stained with DCFDA and 7-AAD viability dye. Unstained cells were used as a negative control. Cells treated with 0.1 mM H_2O_2 were used as a positive control. PDMS+AA2P = cells cultured in culture medium containing 0.08 mM AA2P. Similarly, PDMS+NAC = 5 mM NAC and PDMS+DTT = 0.1 mM DTT. PMMA and PDMS indicate cells cultured in PMMA- and PDMS-chips, respectively. All samples were taken on Day 4 of culture.

Sample	ROS –, %	ROS +, %	MFI
Negative control	99.95	0.05	4.86
Positive control	1.54	98.72	58.17
Monolayer	58.91	44.18	13.72
PMMA	56.82	50.12	15.01
PDMS	44.92	58.58	17.19
PDMS +AA2P	65.74	37.20	13.11
PDMS + DTT	62.33	40.83	13.11
PDMS + NAC	97.64	3.00	7.29

The elevated amount of ROS in the PDMS-chip may be associated with excessive oxygen concentration. I then studied the protective effects of antioxidants on β-cells from excessive ROS. Because L-ascorbic acid is unstable in water, I used its derivative ascorbic acid-2-phosphate (AA2P), which was reported to have the same antioxidant effect on fibroblasts as L-ascorbic acid[76,77], but the effect lasted up to 1 month in the culture medium[76]. I determined the optimal concentration of AA2P in culture medium (Fig. 27). Concentrations > 0.1 mM led to cell morphological changes including problems with cell attachment and death, which can be observed in the microscopy images (Fig. 27a, b). Lower concentrations had a positive effect on cell proliferation and *INS1* expression (Fig. 27a, b). I chose 0.08 mM to be the optimal concentration, as it induced the highest upregulation of *INS1* in both the monolayer and spheroid cultures (from now on I will mean spheroids in the PDMS-chip when I say spheroids) and did not cause cell detachment or inability to form spheroids. It should be noted that in the case

of spheroid culture, the toxic effect of AA2P at concentrations > 0.1 mM was even more obvious than in monolayer culture; major problems with spheroid formation were observed at 0.3 mM, while at the same concentration in the monolayer culture the cells appeared to be still not damaged by the antioxidant.

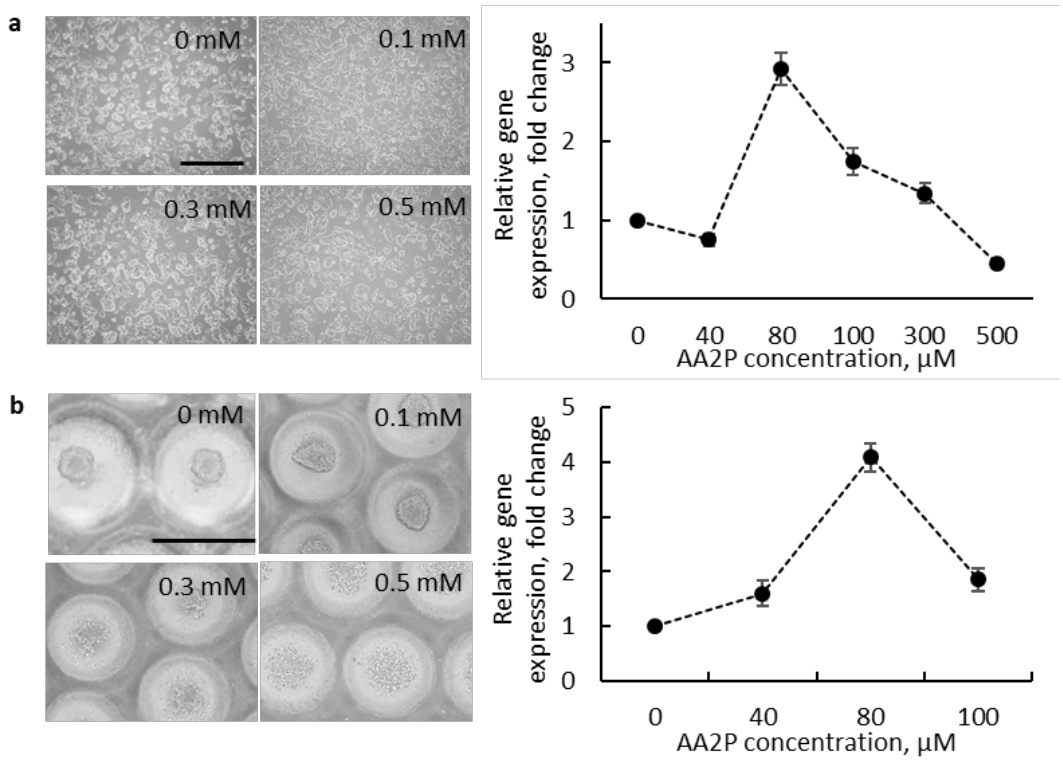


Fig.27. Influence of different concentrations of ascorbic acid-2-phosphate (AA2P) on morphology and INS1 gene expression of MIN6 cells cultured in monolayer and spheroid. **a**: optical microscope images of MIN6 monolayer cultured without and with the addition of 0.1, 0.3, and 0.5 mM of AA2P; and corresponding optimisation of AA2P concentration in culture medium judged by INS1 gene expression. **b**: optical microscope images of MIN6 spheroids cultured without and with the addition of 0.1, 0.3, and 0.5 mM of AA2P; and corresponding optimisation of AA2P concentration in culture medium judged by INS1 gene expression.

To prove that this effect was due to the antioxidant and not vitamin activity of AA2P, I compared the effect with that of N-Acetyl-L-cysteine (NAC), which has been shown to have a protective effect on MIN6 monolayer cells against aldosterone-induced oxidative stress[78], and with dithiothreitol (DTT), which has been shown to reduce cell apoptosis[79]. I tested several concentrations of NAC and DTT, based on concentrations used in previous studies[80]. As shown in the optical microscopy images (Fig. 28), both NAC and DTT had no negative effect on the

morphology of cells either in monolayer or spheroid culture at 1–5 mM and 0.1 mM concentrations, respectively. 10 mM NAC did not have any negative effect on monolayer culture but led to an inability to form spheroids when cells were cultured in the PDMS-chip. DTT concentrations > 0.1 mM led to changes in cell morphology, including their detachment and inability to form spheroids, similar to the effect observed for AA2P. Therefore, I decided to use NAC at 5 mM and DTT at 0.1 mM as controls for the flow cytometry experiments. All the antioxidants reduced ROS concentration, and the lowest ROS concentration was observed with NAC addition. The latter may be connected with the higher concentrations of NAC comparing to other antioxidants (Table 2). The addition of AA2P led to a ~21% decrease in ROS concentration in the PDMS-chip. Addition of NAC led to the almost complete disappearance of ROS, while the effect of DTT was similar to that of AA2P (~18% decrease in ROS concentration). I conclude that AA2P, similar to NAC and DTT, scavenges ROS in cells and hence exhibited antioxidant activity in the tested culture conditions.

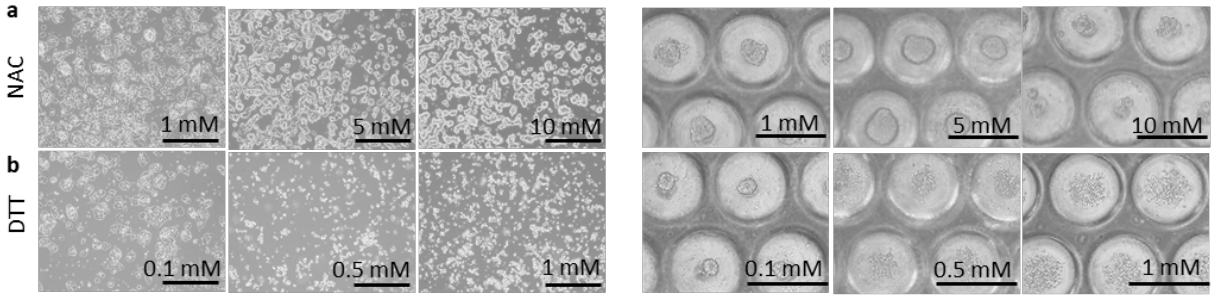


Fig.28. Influence of different concentrations of N-Acetyl-Cysteine (NAC) and dithiothreitol (DTT) on the morphology of MIN6 monolayer and spheroids. Optical microscope images of MIN6 monolayer (left) and spheroids (right) cultured with addition of different concentrations of NAC and DTT. **a**: 1, 5, and 10 mM of NAC. **b**: 0.1, 0.5, and 1 mM of DTT.

To show the influence of decreased ROS concentration on β-cell characteristics, I investigated the expression of *INS1* and *GLUT2*, and insulin secretion rate. All the antioxidants induced an increase in expression of both *INS1* and *GLUT2* (Fig. 29). The highest upregulation of both genes and an increase in insulin secretion (~ 5-fold) were observed in the presence of DTT. However, the concentration of NAC that led to the largest decrease in ROS concentration did not lead to an increase in insulin secretion, despite its upregulation of the selected genes. This may be due to the fact that although excessive amounts of ROS are harmful to cells, some ROS are still required for normal cell signalling. NAC may have been too effective as a scavenger of ROS and disrupted the normal functioning of cells[37]. The addition of AA2P showed a

similar decrease in ROS concentration as that for DTT, but it led to an increase in insulin secretion by 3-fold compared to spheroids cultured in the PDMS-chip without AA2P.

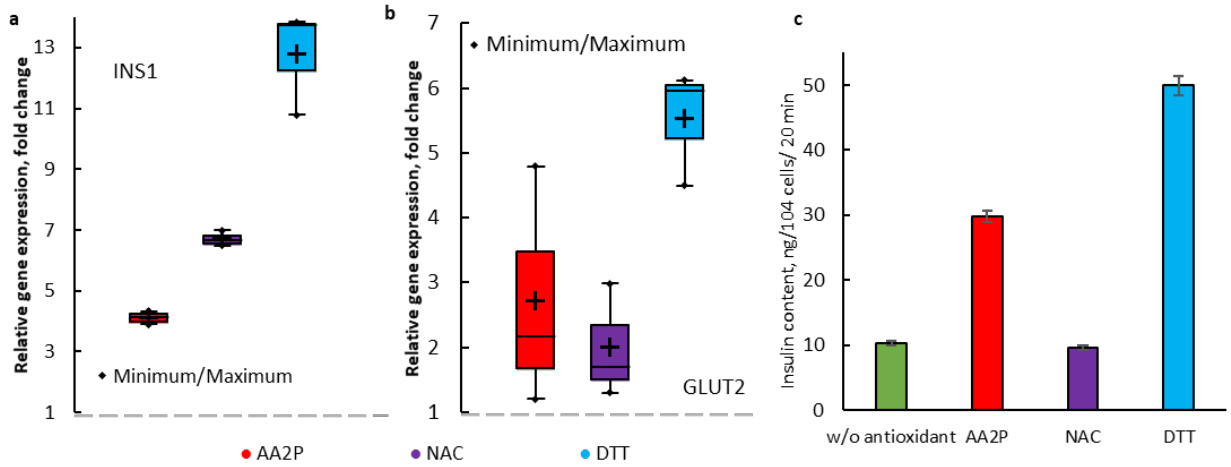


Fig.29. Comparison of the effect of different antioxidants added to culture medium on MIN6 spheroids. Influence of chosen AA2P concentration on INS1(**a**) and GLUT2 (**b**) genes expression of MIN6 spheroids cultured in the PDMS-chip for 4 days, n=4, p=0.05. **c**: Influence of antioxidants on insulin secretion rate of MIN6 spheroids cultured in the PDMS-chip for 4 days. + represents mean, error bars represent SE (n=3, p=0.05).

In this experiment, I showed that ROS generated as a result of chosen spheroid culture conditions could be harmful to cells and that addition of an antioxidant is necessary to protect cells from ROS. However, there are at least two possible reasons for ROS generation in my system, so to be sure what is the main one I still needed additional experiments. The first reason is that the rich oxygen conditions lead to higher ROS generation rates, so the amount of antioxidant in the culture medium is not enough and an additional amount of ROS scavenger is needed. The second possible reason lies with glucose concentration. I used a culture medium with 25 mM glucose, which is ~ 5-fold higher than normal blood glucose concentration for humans; thus, apart from excessive oxygen concentration, there was a possible risk of glucotoxicity in my system[81]. Elevated glucose concentrations can lead to formation and storage of increased amounts of advanced glycation products, which are known to be a source of ROS[73].

3.4.2. THE INFLUENCE OF GLUCOSE CONCENTRATION IN A CULTURE MEDIUM ON PANCREATIC B-CELL VIABILITY AND ABILITY TO FORM SPHEROIDS

I decided to check my hypothesis and investigate the influence of glucose concentration on the functioning of cells. First, I checked the viability of cells cultured as a monolayer. The method I chose is based on that only alive cells can convert the reagent without fluorescence to fluorescent one. Using different cell seeding densities, it is possible to generate a calibration curve that is used for the estimation of the viability of target samples of known seeding densities. Thus, if target samples have higher fluorescence than one would expect from their seeding density and calibration curve, the viability will be higher than 100%; and if it would be lower than expectable – then less. The difficult point of this method is that cells that do not proliferate fast enough have decreasing viability, which does not necessarily mean that they are dead.

In my case, I gradually decreased glucose concentration of glucose in the culture medium, taking cells cultured in 25 mM glucose as a control; and cells cultured in culture media with 20 mM, 15 mM, 10 mM, 5 mM glucose as target samples. As it can be seen from Fig. 30 even after 1 week of culture, cells that were cultured in culture media with 10 and 5 mM glucose are not rounded and seem to be alive. It was confirmed by the measurements (Fig. 31e, f) that although fluorescence was not increasing it was not decreasing either. However, on the viability graph (Fig. 31a red and green lines) they seem to have near 0% viability. I assume that even in culture media with 5 and 10 mM glucose cells were not dead but entered a resting phase, although the more detailed investigation is needed to prove this argument. The viability of cells cultured in culture media with 20 and 15 mM glucose after the first several days of adaption period gradually increased to 100% and 80%. I believe that these results show that if cells were cultured in 20 or 15 mM glucose for a longer period of time, there would be no difference in their viability compared with cell cultured in 25 mM glucose.

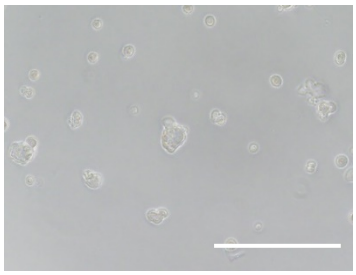
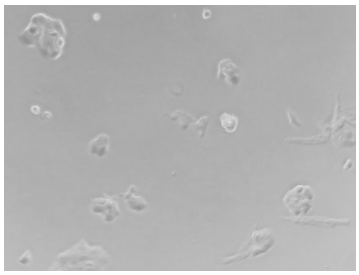
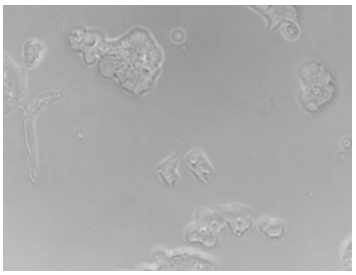
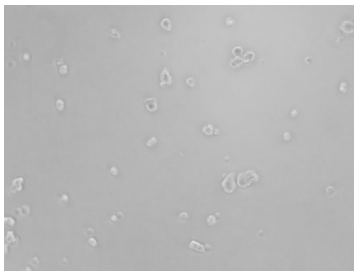
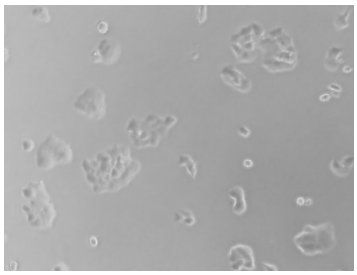
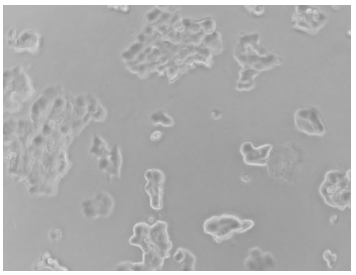
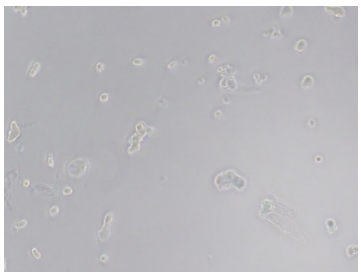
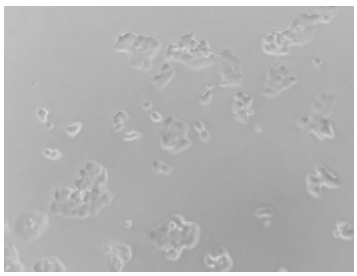
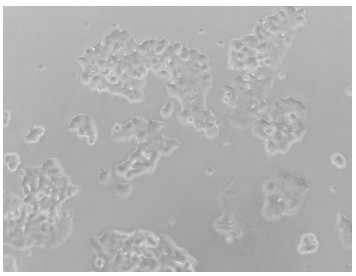
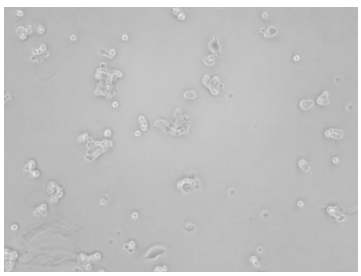
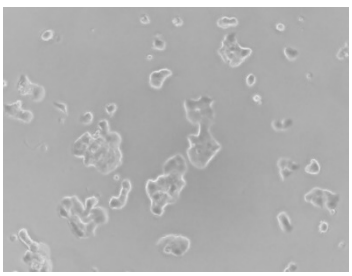
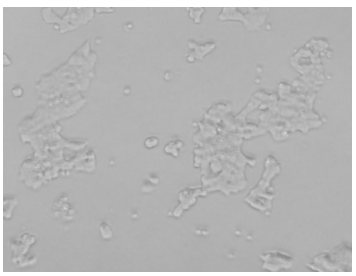
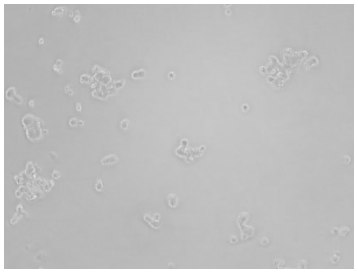
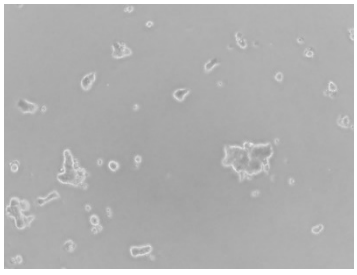
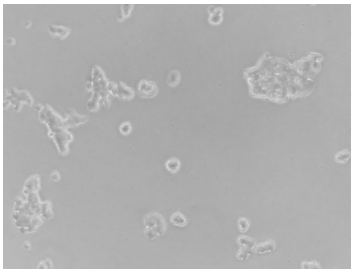
Glucose conc., mM	Day of culture		
	1	3	6
25			
20			
15			
10			
5			

Fig. 30. Phase-contrast optical images of MIN6 monolayer in culture media with different glucose concentrations. The scale is the same for all images, scale bar 100μm.

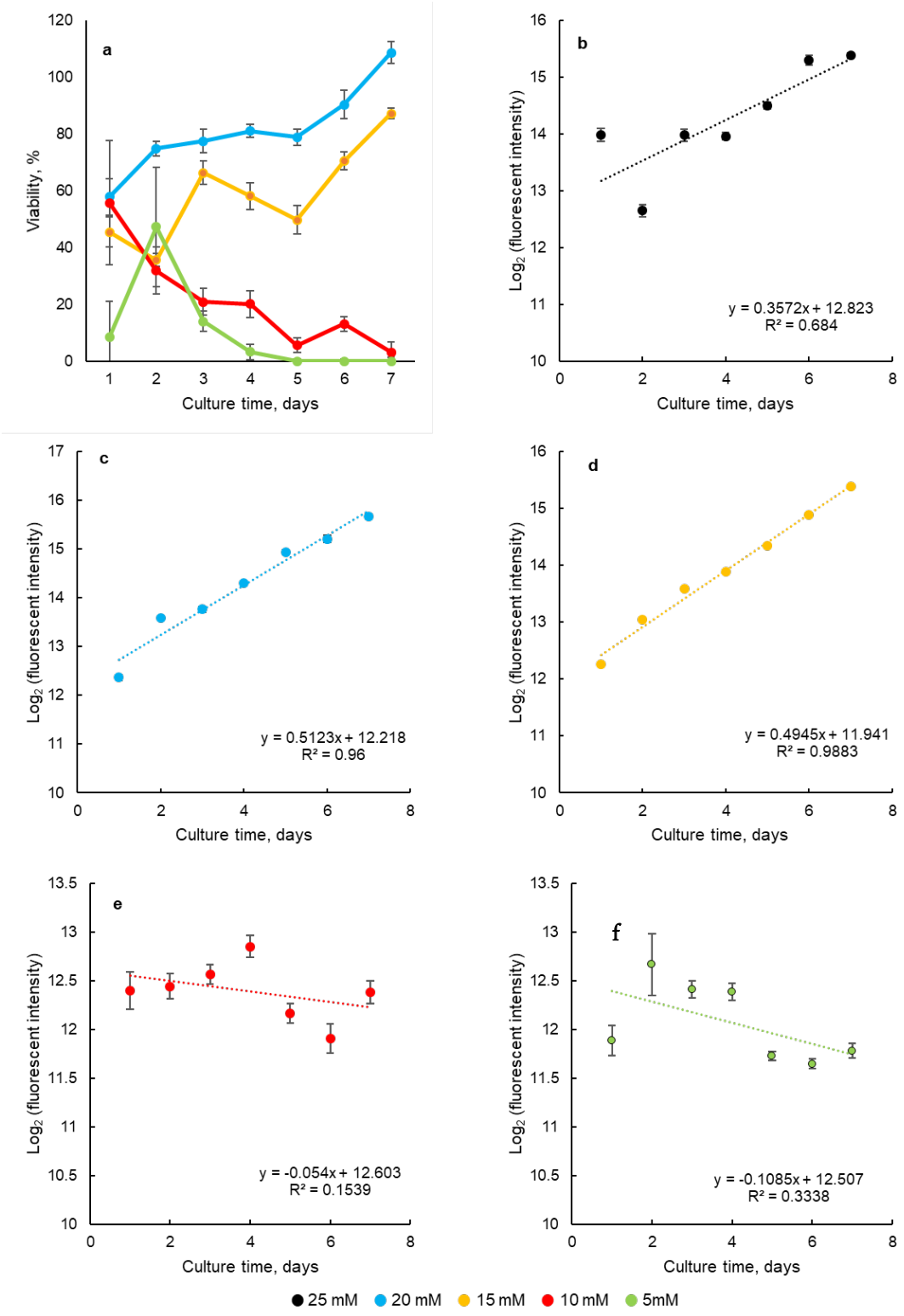


Fig. 31. The viability of MIN6 monolayer in culture media with different glucose concentration. a: the viability of MIN6 represented as percentage from viability of MIN6 cultured in 25 mM glucose culture medium. Cell division curves for MIN6 monolayer in culture medium with glucose concentration: 25 mM (b), 20 mM (c), 15 mM (e), 10 mM (d) and 5 mM (f). n=3, p=0.05.

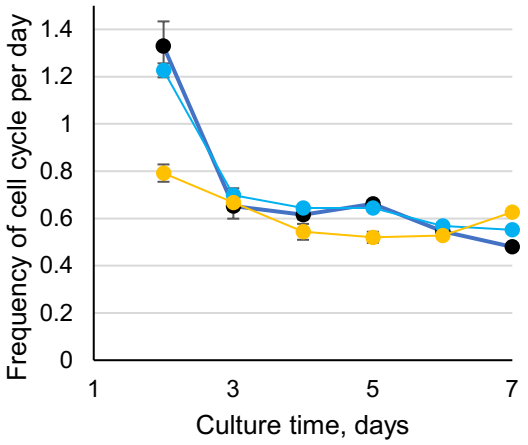


Fig. 32. Influence of glucose concentration in a culture medium on a frequency of cell cycle per day for MIN6 monolayer. n=3, p=0.05.

Next, I compared cell division curves for all investigated glucose concentrations using as an approximation the simple equation $N_t = N_0 2^{tf}$, where N_t is a number of cells at a given time, t; N_0 is a seeded number of cells; and f is a frequency of cell cycle per day. This equation does not take into consideration the possibility that not all cells proliferate but for my purpose of comparison, I considered it not critical. In my case instead of the real number of cells, I used the measured fluorescence intensity. For cells cultured in media with 25, 20, 15 mM glucose the curves almost perfectly fitted to the 2^x curve (Fig. 31b-d), but for 10 mM and 5 mM glucose – not (Fig. 31e, f), for the explained above reasons. Thus, I performed calculations only for the first three glucose concentrations. The resulted frequencies of cell cycle per day are represented on the Fig. 32. After first 2 days of adaption, starting from the 3rd day of culture, the number of cycles stabilized and was not higher but the same for all threae glucose concentrations that shows that both 20 mM and 15 mM glucose conditions are feasible for the maintanance of MIN6 in a monolayer culture.

I conducted the same viability test for MIN6-m9 monolayer (Fig. 33, 34), MIN6 spheroid (Fig. 35, 36) and MIN6-m9 spheroids (Fig. 37, 38). The results were similar to the MIN6 monolayer so I will give only some short comments to every picture before moving to the next step of this experiment.

The viability test for MIN6-m9 monolayer similarly to MIN6 monolayer showed that cells cultured in 20 mM and 15 mM glucose concentration have good viability of ~80% and ~60% of the cells cultured in 25 mM glucose (Fig. 34). For the MIN6-m9 line, contrary to MIN6 monolayer, first days were marked with higher viability than later in the week. This behaviour resembles primary pancreatic cells. Cells cultured in 10 mM glucose are represented by lower viability comparing to the cells in 25 mM glucose concentration. Judging from the phase-contrast microscopy images (Fig. 33) cells cultured in 5 mM glucose did not seem to die, but they were not proliferating or proliferating slowly. These observations seem to be interesting, so in

future, I will check the possibility of "wakening them up" by placing into culture medium with higher glucose concentration.

In the case of spheroids, I did not observe any problems in spheroid formation for both MIN6 and MIN6-m9 in culture media with all investigated glucose concentrations. For lower glucose concentrations the formation of spheroids was delayed, as can be seen from Fig. 35 and 37 but did not resulted in defected spheroids. Spheroids cultured in 20 mM and 15 mM glucose concentrations for both MIN6 and MIN6-m9 had comparable viability – ~100% (Fig. 36, 38), with spheroids cultured in 25 mM glucose concentration. Culturing spheroid for 3-4 days in both 20 mM and 15 mM glucose culture media resulted in more viable spheroids. The 10 mM and 5 mM conditions lead to decrease in MIN6 spheroids viability, but MIN6-m9 spheroids showed viability close to the control. On the phase-contrast photos (Fig. 35 and 37) ven spheroids that showed lower viability do not seem to be disaggregating, showing that similar to a monolayer culture, spheroids may also be in a "sleeping" phase.

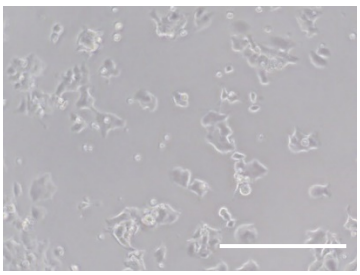
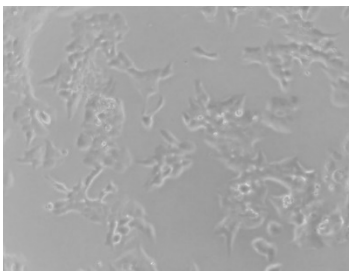
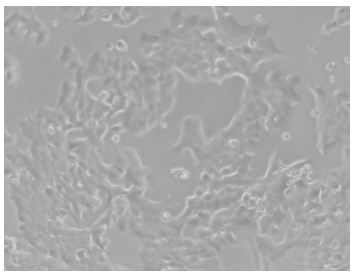
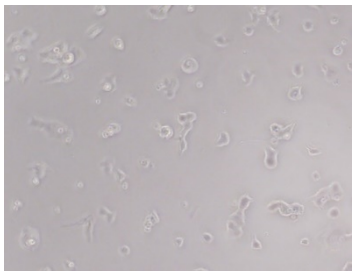
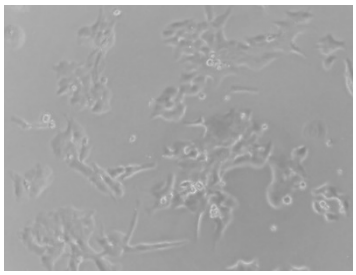
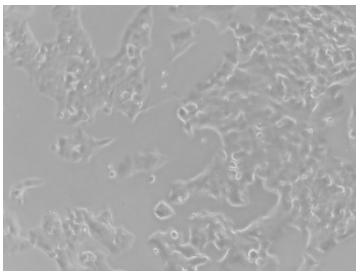
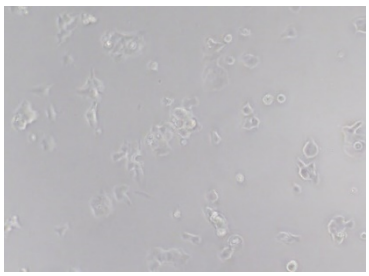
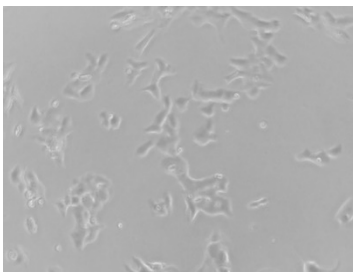
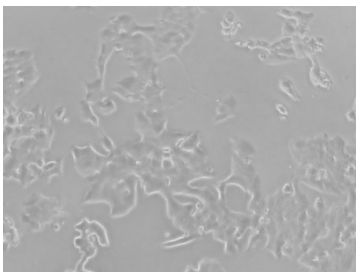
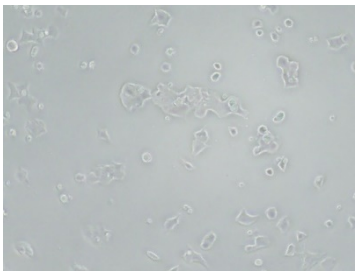
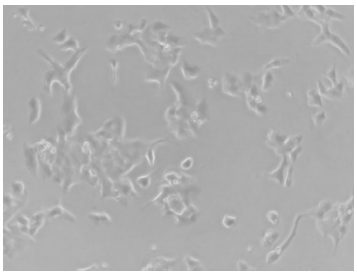
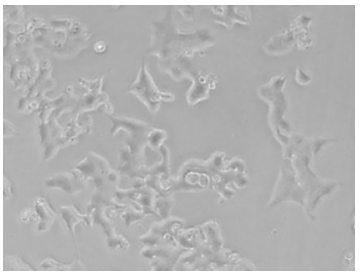
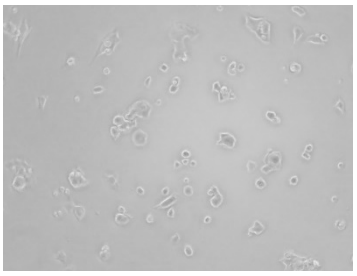
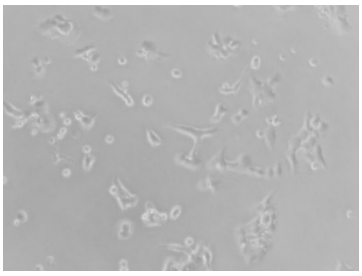
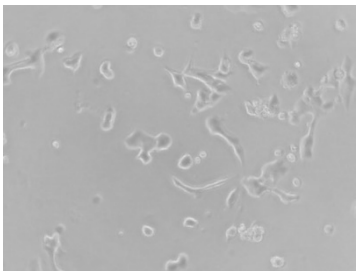
Glucose conc., mM	Day of culture		
	1	3	6
25			
20			
15			
10			
5			

Fig. 33. Phase-contrast optical images of the MIN6-m9 monolayer in culture media with different glucose concentrations. The scale is the same for all images, scale bar 100μm.

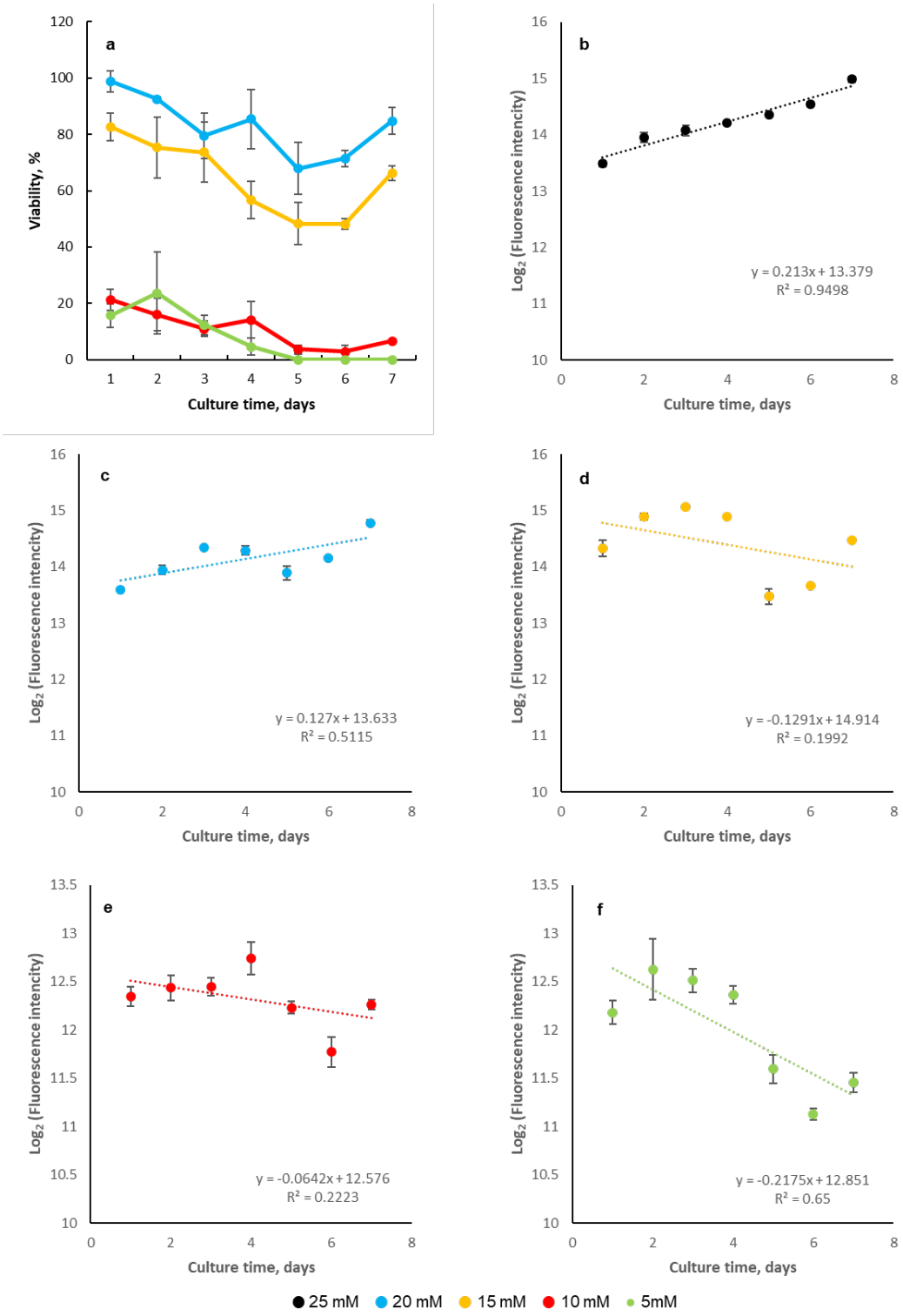


Fig. 34. The viability of MIN6-m9 monolayer in culture media with different glucose concentration. a: the viability of MIN6-m9 represented as percentage from viability of MIN6-m9 cultured in 25 mM glucose culture medium. Cell division curves for MIN6-m9 monolayer in culture medium with glucose concentration: 25 mM (b), 20 mM (c), 15 mM (e), 10 mM (d) and 5 mM (f). n=3, p=0.05.

Glucose conc., mM	Day of culture		
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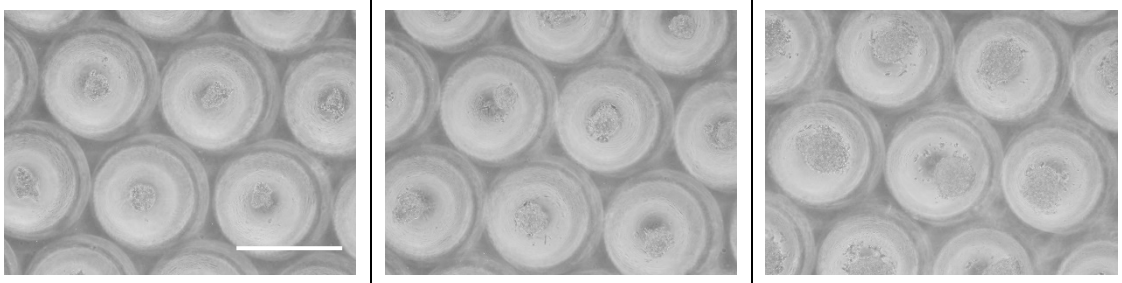
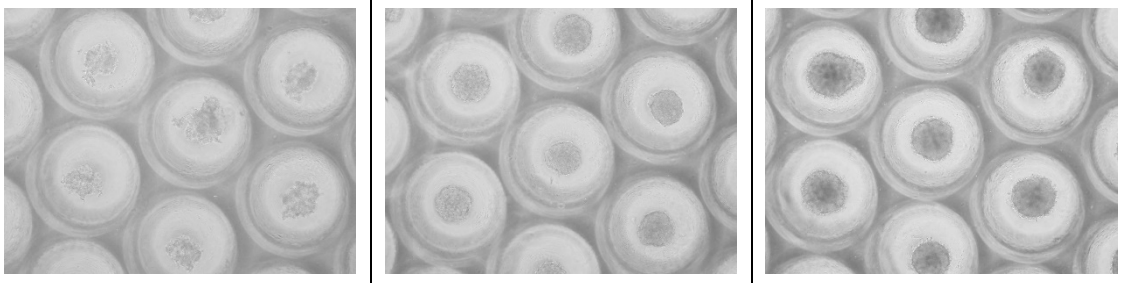
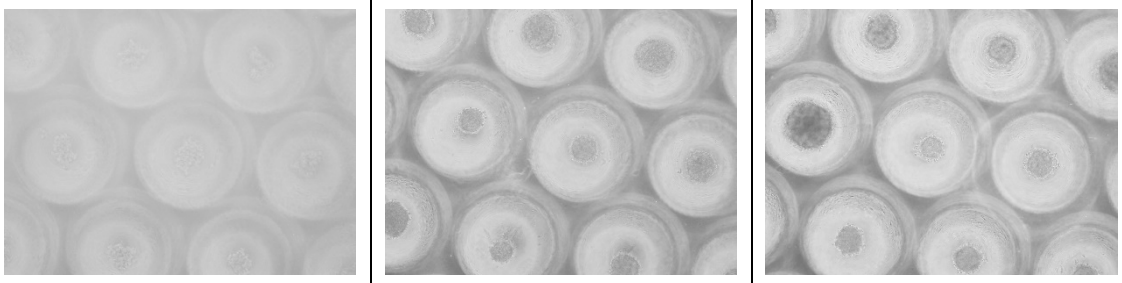
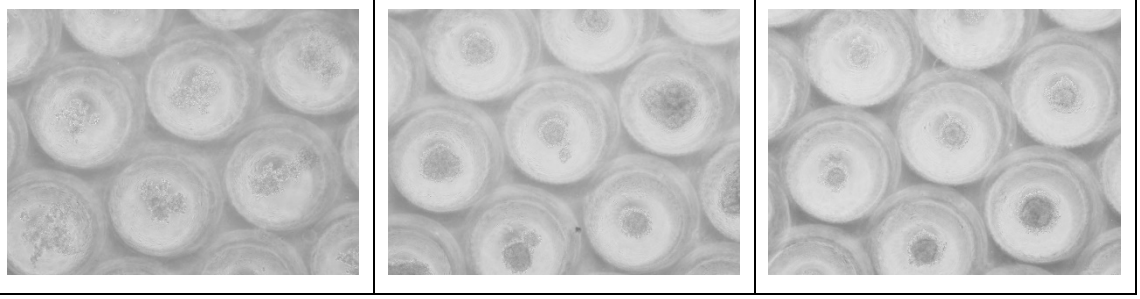
25			
20			
15			
10			
5			

Fig. 35. Phase-contrast optical images of MIN6 spheroids in culture media with different glucose concentrations. The scale is the same for all images, scale bar 500μm.

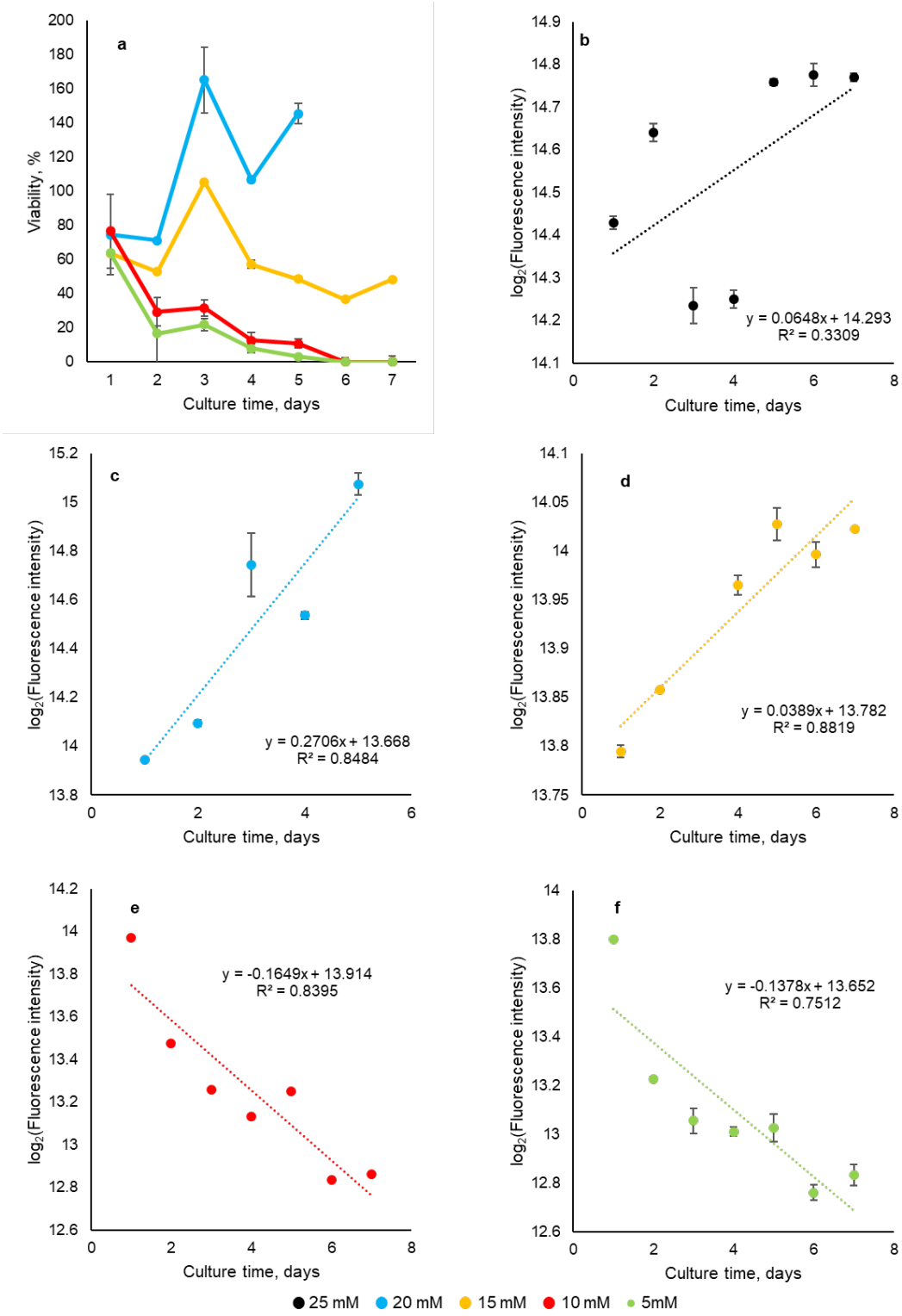


Fig. 36. The viability of MIN6 spheroids in culture media with different glucose concentration. a: the viability of MIN6 represented as percentage from viability of MIN6 cultured in 25 mM glucose culture medium. Cell division curves for MIN6 spheroids in culture medium with glucose concentration: 25 mM (b), 20 mM (c), 15 mM (e), 10 mM (d) and 5 mM (f). n=3, p=0.05.

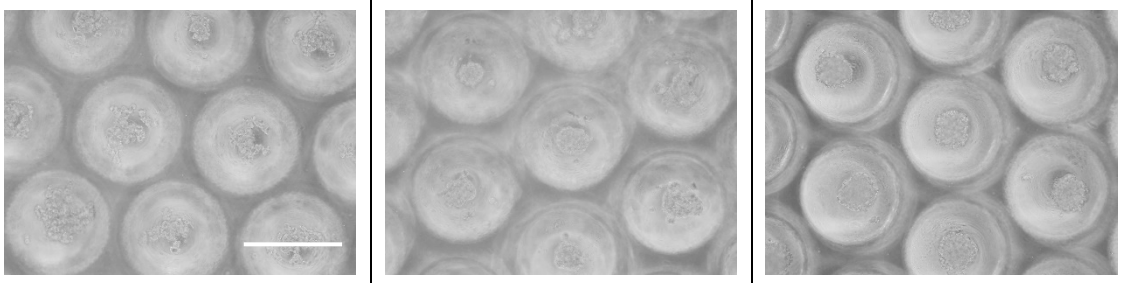
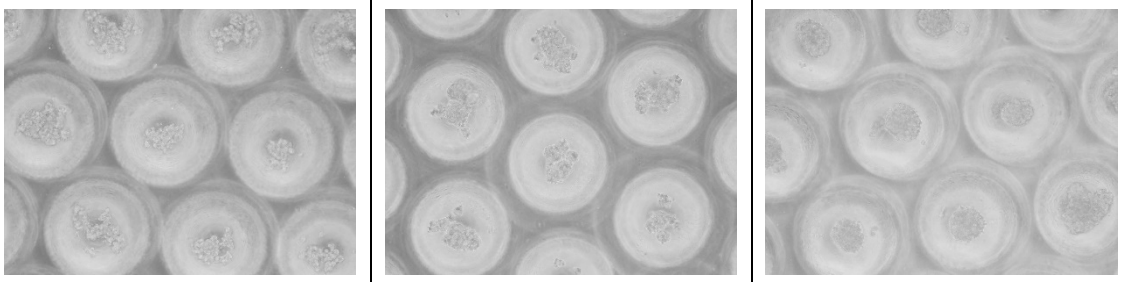
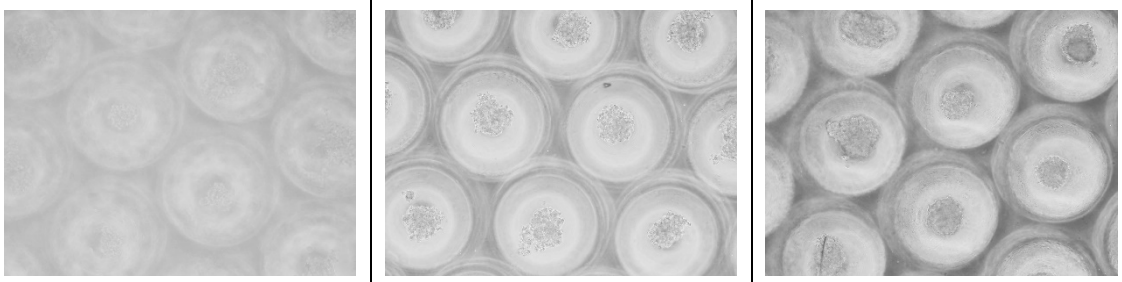
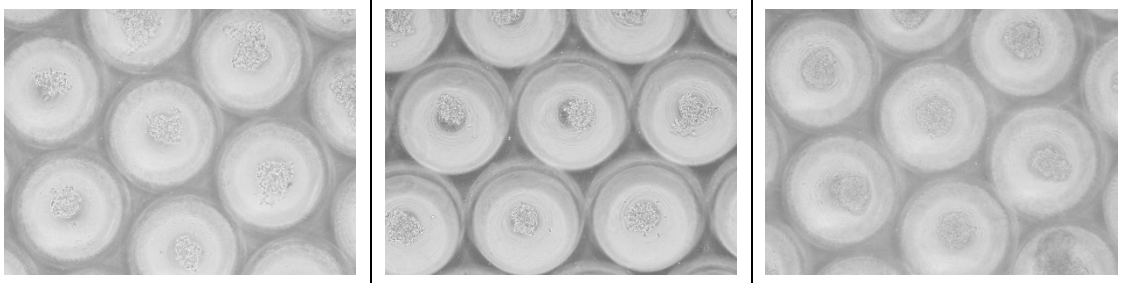
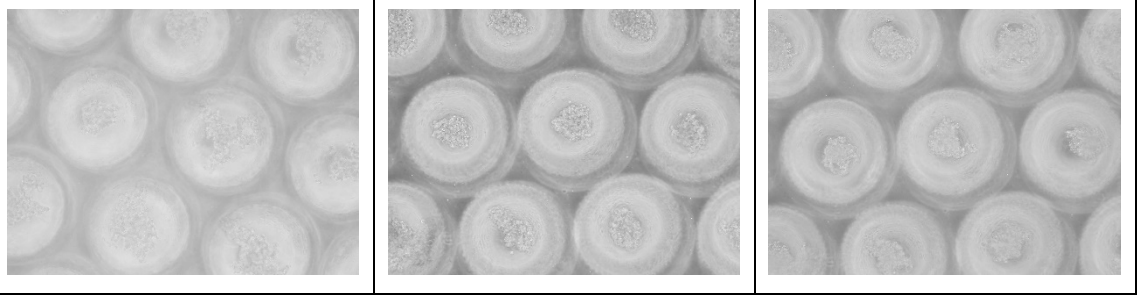
Glucose conc., mM	Day of culture		
	1	3	6
25			
20			
15			
10			
5			

Fig. 37. Phase-contrast optical images of MIN6-m9 spheroids in culture media with different glucose concentrations. The scale is the same for all images, scale bar 500μm.

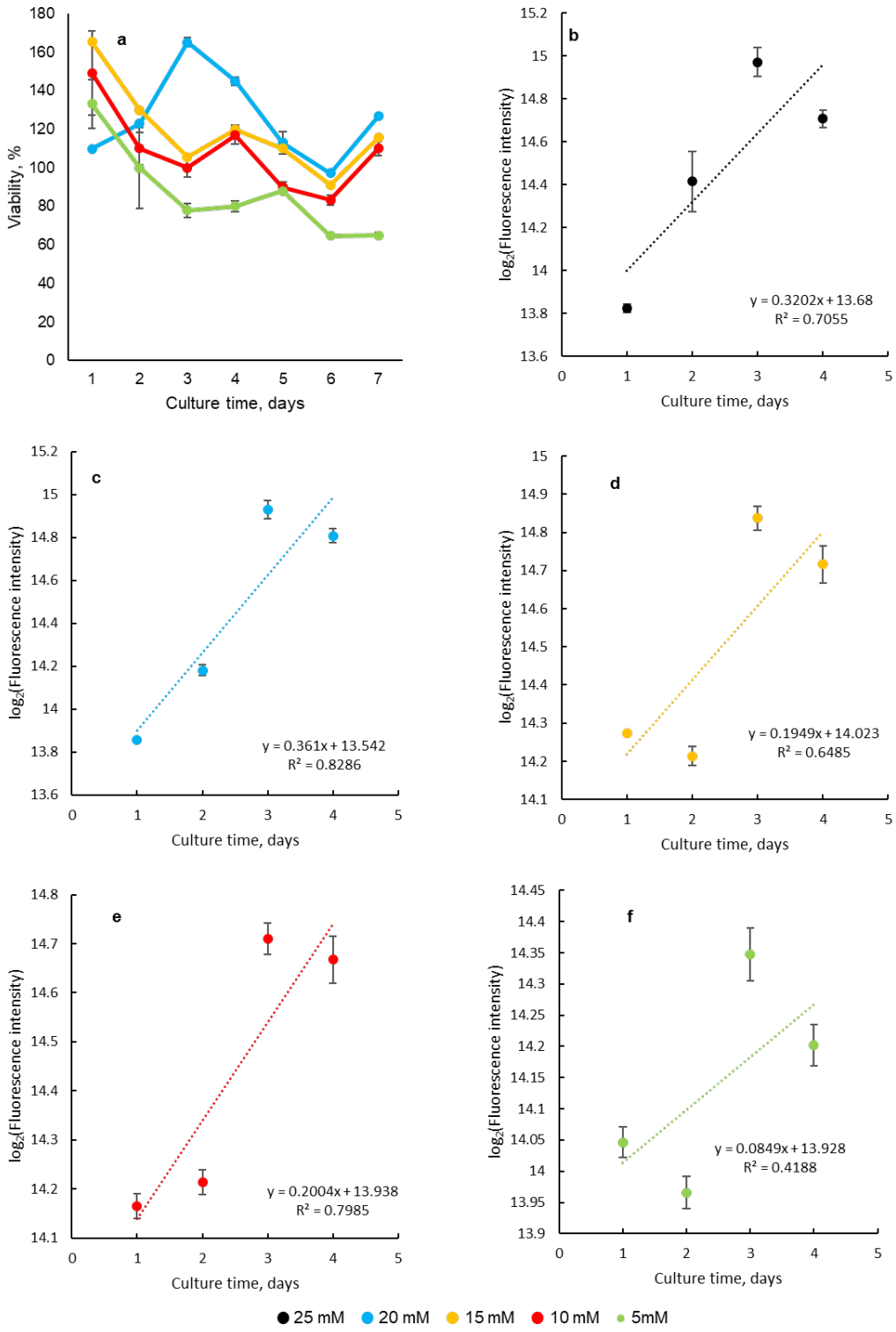


Fig. 38. The viability of MIN6-m9 spheroids in culture media with different glucose concentration. a: the viability of MIN6 represented as percentage from viability of MIN6 cultured in 25 mM glucose culture medium. Cell division curves for MIN6 spheroids in culture medium with glucose concentration: 25 mM (b), 20 mM (c), 15 mM (e), 10 mM (d) and 5 mM (f). n=3, p=0.05.

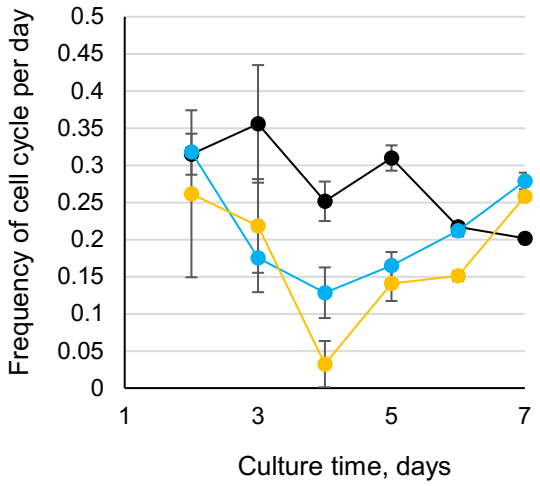


Fig. 39. Influence of glucose concentration in a culture medium on a frequency of cell cycle per day for MIN6-m9 monolayer. n=3, p=0.05.

For MIN6-m9 the adaptation period was longer than for MIN6 (Fig. 39), however, approximately after 5 days of culture cells showed similar frequency in cell cycle per day that became the same after one week of culture. Interestingly, MIN6-m9 showed lower values of the frequency of cell cycle per day than MIN6 that shows that MIN6 contains cells with different dividing frequencies. Overall, I believe that MIN6-m9 similarly to MIN6 may be maintained in 20 mM or 15 mM glucose culture media on a routine basis.

I did the same evaluation for the spheroid culture, although it may not be as relevant as analysis of monolayer results due to the fact that experiment was done with whole, not broken spheroids thus cells in different parts of spheroid could have been exposed to different amounts of reagent. The results for MIN6 spheroids are presented on the Fig. 40. I should notice right away that I have gotten strange results for the spheroids cultured in 25 mM glucose on days 3 and 4, I will repeat the experiment one more time to clarify the situation. Apart from that, I observed that culture in 20 mM glucose led to the higher frequency of cell cycle (the data for days 6 and 7 will be added later) during 5 days of culture. After the 5th day of culture, 25 mM and 15 mM glucose conditions led to the decreasing frequency of cell cycle. This phenomenon I also observed under a microscope, the MIN6 and MIN6-m9 spheroids tend to slow down their growth after half a week in culture. It seems to be an interesting observation, so I will investigate it in more detail in the future. The same analysis for MIN6-m9 will be added later.

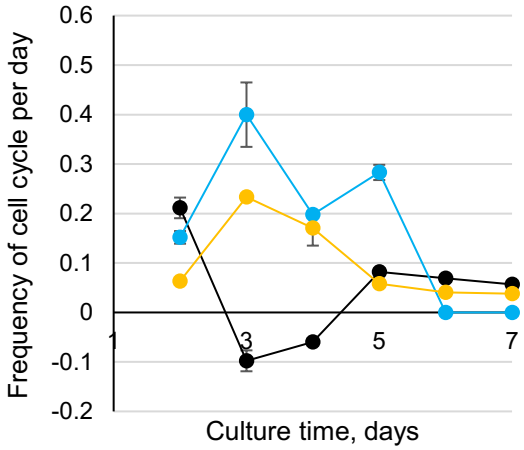


Fig. 40. Influence of glucose concentration in a culture medium on a frequency of cell cycle per day for MIN6 spheroid. n=3, p=0.05.

However, these viability tests were based on the principle of determination of cell metabolic activity that has several limitations. In clinical application, the detection of islet viability is an important step that helps to predict the outcome of the future transplantation. Usually, a combination of two dyes is used for staining. One method, which determines the integrity of the cell membrane, is the live/dead staining that utilises SYTO/SYBR and propidium iodide/ethidium bromide/ethidium homodimer-1 to stain alive and dead cells, correspondingly. The second method uses fluorescein diacetate instead of SYTO/SYBR and instead of membrane integrity, it detects the esterase activity inside the cell. The reagent that I used in my experiment also detects the esterase activity. It is an acetylated derivative of fluorescein (has green fluorescence). The attached acetyl group converts dye to a non-fluorescent one but allows it to penetrate through the cell membrane. Once it gets into the cell, non-specific esterases of cell cytoplasm de-acetylate the reagent molecule, and it becomes fluorescent once again. The fluorescent form of the dye is charged, so it remains inside the cell for some time that is specific to cell type and depends on culture conditions[82]. The use of fluorescent reagent makes it very important to precisely repeat the detection conditions every time the viability of cells assessed, as the dye concentration and used buffer system, the incubation and exposure time and even the time of contact with light before measuring have a potential influence on the fluorescent signal[82]. However, several pieces of research showed that even though islets subjected to different treatment had almost the same staining with, for example, FDA/PI, their functions were not the same. As one example, it was reported in one of the papers that control islets and islets kept under hypoxic conditions for 6 h were evaluated by FDA-like dye staining. Although both groups of islets showed similar staining pattern and intensity, subsequent transplantation showed that control islets reversed diabetes in diabetic rodents, while the hypoxic ones did not[82]. This shows that viability staining only valuable to some extent and cannot be used for the endpoint decision.

3.4.3. THE INFLUENCE OF GLUCOSE CONCENTRATION IN A CULTURE MEDIUM ON PANCREATIC CELLS/SPHEROIDS GENE PROFILE AND INSULIN SECRETION.

Thus, I decided to evaluate MIN6/MIN6-m9 cells cultured as monolayer/spheroid in conditions with different glucose concentrations by RT-PCR and ELISA. To assess the cells condition, I chose following genes based on the research of changes in a gene expression profile of native rat islets cultured in media with different glucose concentrations[63]:

- *Aldob* is the gene that codes aldolase B enzyme, which is primarily involved in the breakdown of fructose and to a lesser extent of glucose. Thus, it is a gene that is connected with cell metabolism and was expected to correlate with glucose concentration and be upregulated in conditions with higher glucose content.
- *ZnT8* is the gene that codes a zinc-efflux transporter, which promotes the accumulation of Zn in intracellular vesicles of pancreatic β-cells. Disruption of this gene expression is connected with Diabetes Mellitus and leads to reduced insulin content and GSIS.
- *Trib3* is the gene that codes tribbles homolog 3, a putative protein kinase that is part of the transcription factor NF-kappaB. Is known to be associated with Diabetes Mellitus and disrupts insulin signalling by binding to Akt kinases (responsible for cellular growth, glucose homeostasis and neuronal development) and blocking their activation. Its overexpression is connected with endoplasmic reticulum stress.
- *Hmox1* is the gene that codes heme oxygenase, a membrane-bound enzyme that helps to convert heme to biliverdin. Overexpression of this gene is linked to the oxidative stress.
- *Casp4* is the gene that codes one protein from a cysteine-aspartic acid protease family known to regulate cell apoptosis. Particular this one is known to be inflammatory caspase[83].

Apart from listed above genes, I also checked the *INS1* and *GLUT2*. As it can be seen from Fig. 41 and 42, glucose concentration affected the gene profile of MIN6 monolayer.

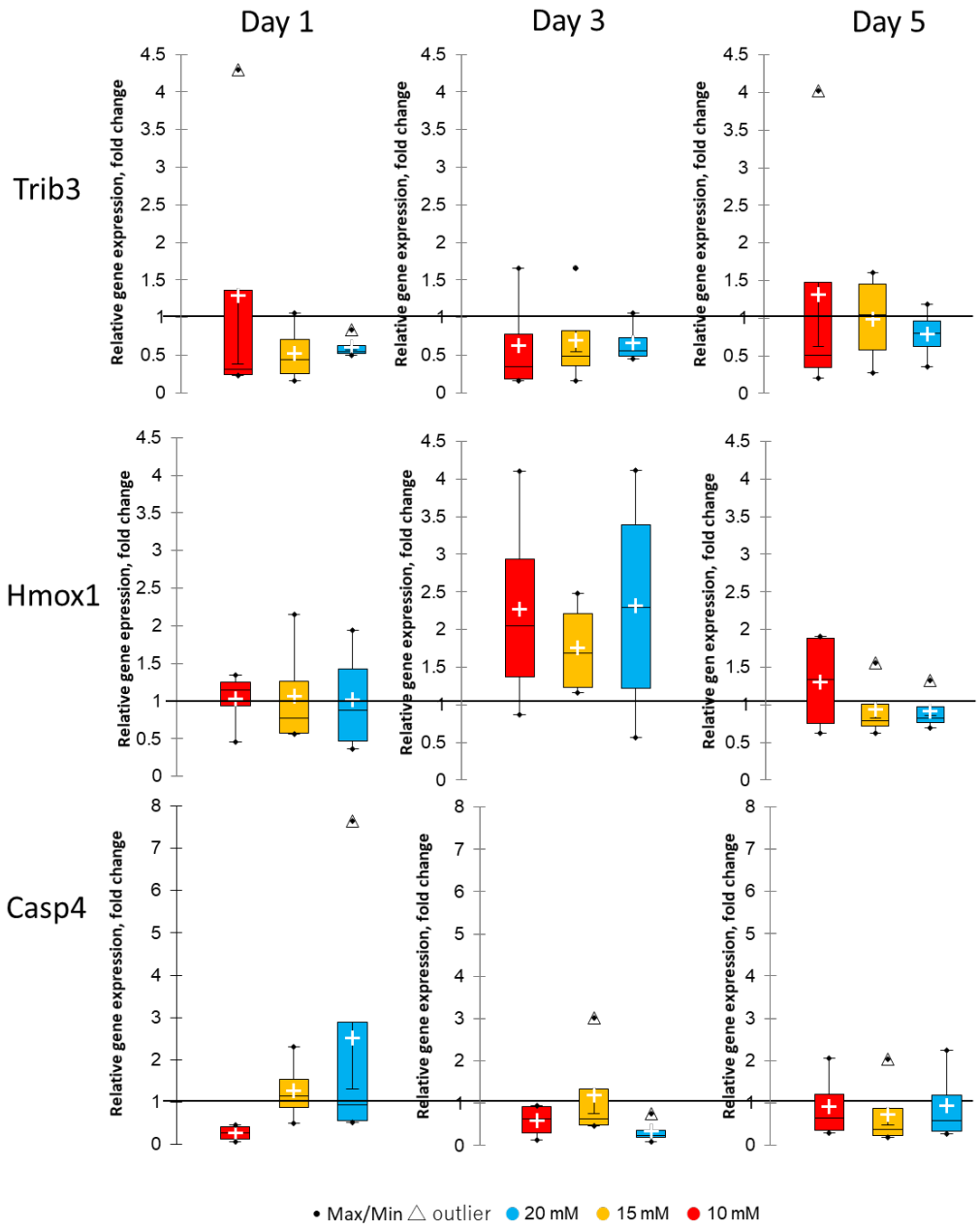


Fig.41. Influence of glucose concentration in culture medium on gene profile of MIN6 monolayer. The gene expression of MIN6 monolayer cultured in a culture medium with 25 mM glucose was taken as a control and is represented as a line y=1 on the graphs. n=4, p=0.05.

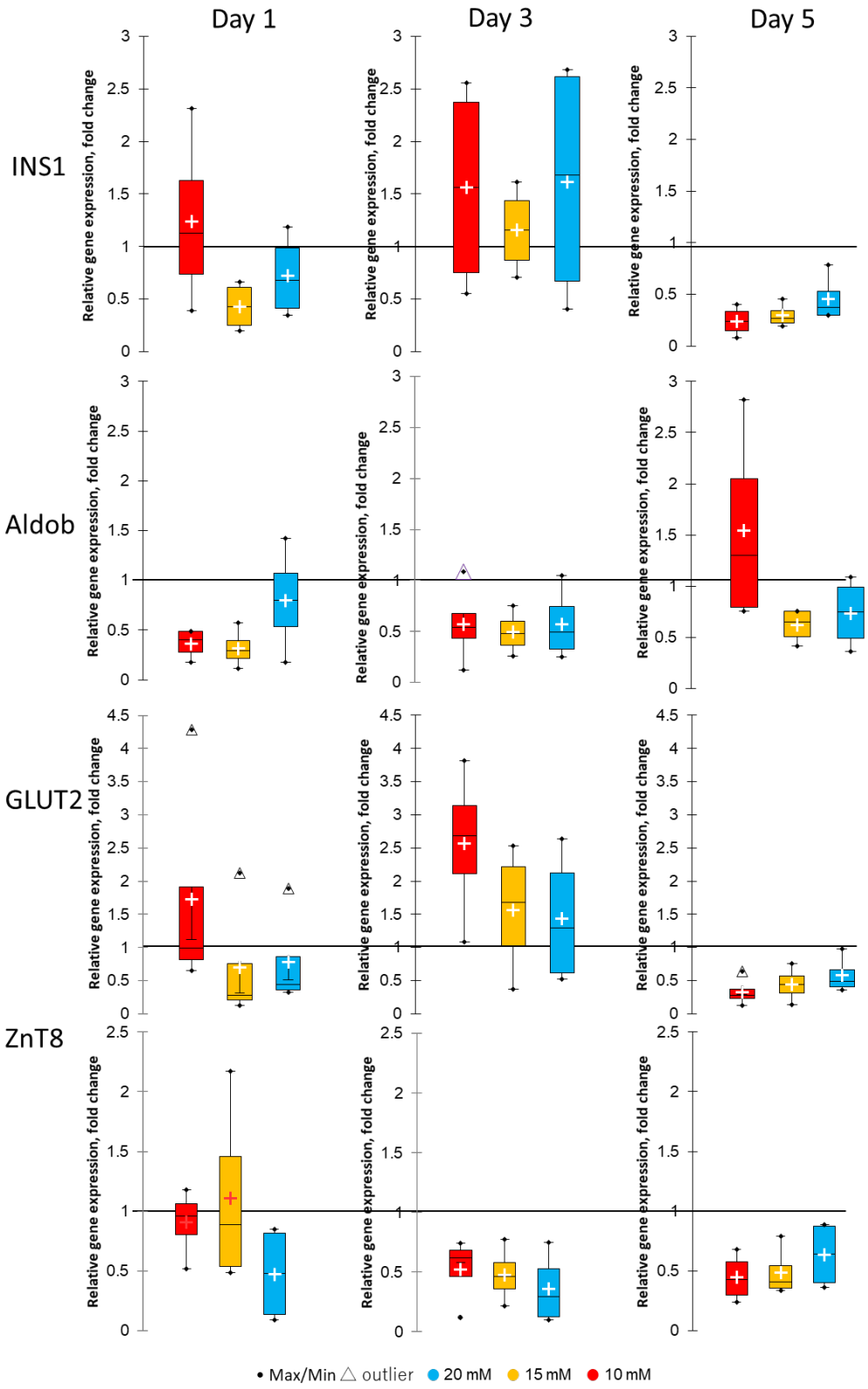


Fig. 42. Influence of glucose concentration in culture medium on gene profile of MIN6 monolayer. The gene expression of MIN6 monolayer cultured in a culture medium with 25 mM glucose was taken as a control and is represented as a line y=1 on the graphs. n=4, p=0.05.

Decrease of glucose to 20 mM led to moderate upregulation of *INS1* and *GLUT2* expression on day 3 of culture (Fig. 42). The subsequent downregulation, although, unfortunate, is the expected result as I showed previously that long culture of MIN6 leads to a digression in their functioning. Another gene connected with insulin secretion – *ZnT8* – was downregulated, however, to draw some conclusions I need to perform ELISA to connect gene expression with insulin secretion. Apart from *INS1* and *GLUT2, Hmox1*(Fig. 41) was also upregulated that can be connected with the increase of ROS in my system. I will confirm this statement in the nearest future by flow cytometry. *Aldob* was slightly downregulated that is in accordance with results of the viability tests. The *Trib3* also was downregulated showing the reduced endoplasmic reticulum stress. *Casp4* was slightly downregulated or almost the same, showing that reduced glucose did not lead to cell apoptosis.

The same results I observed for 15 mM and 10 mM culture conditions. The latter led to upregulation of *INS1* and *GLUT2* already on the first day of culture, *Aldob* on the fifth day of culture (in the case of 15 mM and 20 mM it was downregulated). The *ZnT8* was downregulated but to the lesser extent than in 20 mM and 15 mM glucose culture conditions. *Hmox1* was upregulated stronger than in other glucose culture conditions but not significantly. Overall, I can expect that 10 mM glucose culture conditions may be preferable for the monolayer MIN6 culture. This latter result seems particularly interesting in the light of viability tests. As I discussed above, according to the viability test 20 mM and 15 mM should have been preferable for maintaining MIN6. However, RT-PCR results are showing that functionally MIN6 cells cultured in 10 mM are superior to ones cultured in 25 mM, 20 mM, and 15 mM. Although it may seem surprising, the same results were received for primary rat islets and IS1 cell line[63]. To draw final conclusions I need to perform ELISA and flow cytometry, which will allow me to connect a gene profile with the insulin secretion rate and ROS production. Furthermore, I intend to conduct the same experiment for MIN6 spheroids and MIN6-m9 monolayer and spheroids.

To conclude this chapter, I showed that the PDMS-chip improved oxygen supply to pancreatic β-cell spheroids, decreasing hypoxia of cells in the core of spheroids and increasing insulin secretion in comparison to the PMMA-chip. However, rich oxygen conditions paired with high glucose concentration led to an increase in the concentration of intracellular ROS, which could

interfere with cell signalling and metabolism, including insulin secretion. Therefore, I examined neutralisation of the negative effect of excessive amounts of ROS by addition of antioxidants or culturing cells in lower glucose concentrations. Further, I demonstrated that addition of AA2P, as well as the addition of widely used antioxidants NAC and DTT, led to upregulation of *INS1* and *GLUT2* expression and increased insulin secretion in the spheroid culture of MIN6 pancreatic β-cells. Culturing MIN6 and MIN6-m9 in lower glucose concentration did not lead to a decrease in cell viability both in case of monolayer and spheroid culture and in case of MIN6 led to improvement in gene profile on the 3rd day of culture.

My results indicate that transfer cells from 2D to 3D culture require a thorough investigation and design of different environmental parameters such as culture medium composition, oxygen supply and geometric parameters of culture vessels. In future, I intend to continue this work and try to apply described results to human cell lines and iPS and primary human cells.

CHAPTER 4

4. FABRICATION OF A VASCULARISED PANCREATIC SPHEROID

4.1. STRATEGIES FOR TISSUE VASCULARISATION

As I already mentioned at the beginning of Chapter 3, oxygen and nutrient supply is an important point for 3D tissue fabrication. The PDMS-chip allows improved oxygen supply *in vitro*; however, it is still limited by diffusion through cell layers and does not allow fabrication of tissue on cm-level. If I want to take this research one step further, oxygen and nutrient delivery, as well as removal of waste materials (such as metabolites), should be preserved not only *in vitro* but also *in vivo* after transplantation. To ensure these functions fabrication of vascular structure seems to be an inevitable step. At present, there are two main concepts of vasculature fabrication: stimulation of angiogenesis *in vivo* after tissue transplantation or connection of *in vitro* preformed vasculature to the host blood vessel system.

The first approach requires stimulation of host vasculature by such growth factors as VEGF and FGF that can be produced either by cells themselves or be incorporated into transplanted scaffolds. Several approaches were developed to help host vasculature penetrate newly transplanted tissues. First, it was found that the chemical composition of the scaffold plays an important role, as some scaffolds (e.g. collagen-chitosan-hydroxyapatite) trigger a severe inflammatory response and prevent angiogenesis in transplanted tissues. Interestingly the best results for vascularisation were shown not by the highly biocompatible scaffolds, but by the scaffolds that induced light inflammatory response after transplantation[84]. Moreover, not only the content but also the pore size of utilized scaffolds is important, with pores of 250-300 μm size resulting in better penetration of host vasculature into a transplanted tissue. To further improve ingrowth of blood vessels, growth factors are incorporated into scaffolds by covalent immobilisation, collagen coating of the scaffold surface, loading of factor-containing gels into scaffold pores, encapsulation of them into small particles with a desired degradation rate or stimulating their local production by incorporating plasmid DNA. One more recent approach utilises different cell types, such as mesenchymal stem cells or genetically modified cells that

constantly secrete VEGF. The latter one, although tempting, seems difficult to realise in clinical application, as genetically modified cells are not easily proven to be safe for patients. Overall, the process of angiogenesis requires a coordinate sequence of humoral and cellular events and requires some time to be complete (the physiological speed of microvessel growth is estimated to be 5 μm/h), so in case of large cell constructs, it may not be the most effective way of vascularisation.

The second approach relies on the pre-formed vasculature. The major advantage of the concept is that transplanted tissue possibly can be fully blood-perfused within a short period of time, as cells only need to connect with existing host vasculature and there is no need of angiogenesis. The major challenge of this approach is that capillary-like structures formed *in vitro* are often unstable as they lack the mature structure of host vasculature. Results of research show that scaffolds seeded with endothelial or endothelial progenitor cells are unstable. The use of genetically modified cells is limited as they pose risks of cancer. It was also shown that structure formed only by HUVEC showed minimum perfusion and after 60 days exhibited signs of deterioration, however the addition of mesenchymal precursor cells allowed stabilising such constructs for up to 1 year[84]. An interesting but tricky approach to fabricated mature vasculature is first to transplant the fabricated tissue into a well-vascularised site, wait until the transplant will be vascularised and then re-transplant it to the desired site. However, this approach is close to the previously described angiogenetic one and is complicated, as it requires time and would not apply to thick tissues.

To conclude, both approaches are still not perfect, and the desired vascularisation is usually delayed. Thus, to ensure the safety of the transplant during the first day after transplantation, which is usually marked by cell death due to hypoxia, inflammation etc., there is a need for further improvement of these methods, and maybe some new ideas and innovative approaches for vasculature fabrication.

4.2. The optimisation of cell ratio for the co-culture spheroids of MIN6, PMVC and fibroblasts.

In this work, I chose the second approach of fabricating pre-vascularized pancreatic islets as the next step toward fabrication of pancreatic tissue *in vitro*. From the literature, I knew that it is possible to form spheroids from MIN6 and aorta endothelial cells[85] or HUVECs[86]. Thus my

first step was to investigate the possibility to fabricate co-culture spheroids from MIN6 and pancreatic microvascular endothelial cells (PMVC). It worth mentioning, that I decided to use PMVC as my major cell line of interest instead of HUVEC, as it is known that pancreatic microvasculature has several specific characteristics compared to normal blood vessel, such as increased number of fenestrated capillaries (~10 times more compared to surrounding exocrine tissues) that allows more effective hormone exchange[87].

The first problem was the choice of culture medium. MIN6 cells are cultured in a MIN6 culture medium that is based on DMEM, whereas PMVC similarly to HUVEC should be cultured in EGM-2 to form vasculature. First, I tried co-culturing spheroids in the EGM-2 culture medium. I observed the formation of core spheroid with many single cells around that were not incorporated in the spheroid structure (Fig. 43a). Prolonged culture of these spheroids led to the formation of loosely aggregated cells that were easily destroyed by pipetting (Fig. 43b).

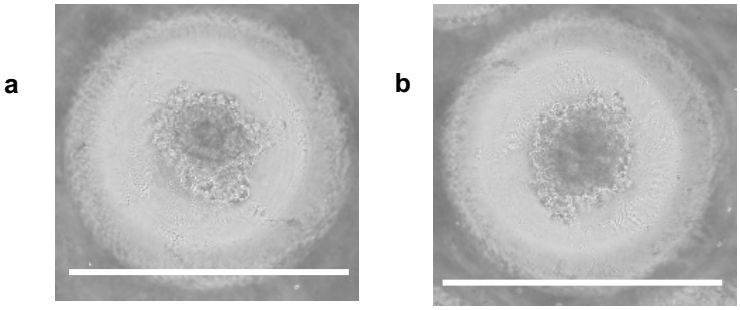


Fig. 43. Phase-contrast optical microscopy images of MIN6 and PMVC co-cultured spheroids in EGM-2 a: day 4, b: day 10. Scale bar 500 μm.

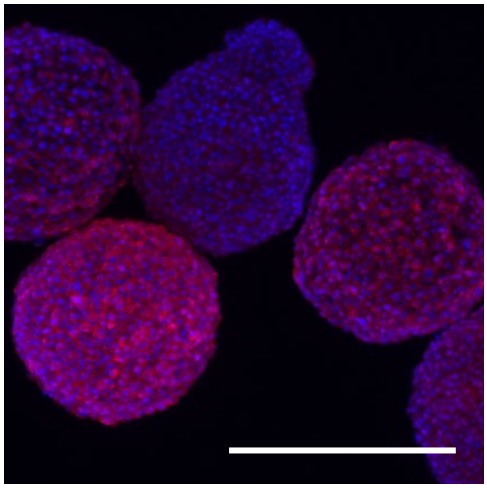


Fig. 44. Immunofluorescent staining of MIN6 and PMVC co-cultured spheroids for VE-cadherin. Scale bar 200 μm.

Although, this question requires further investigation, one of the possible reasons for cells' inability to form spheroids could be the smaller concentration of FBS in EGM-2. To facilitate the spheroid formation, I decided to use culture medium containing DMEM as a base and EGM-2 supplements that I will call PEGM-2 from now on. I also checked two approaches for co-culture spheroid formation: 1) mixture of 2 types of cells and 2) culture of MIN6 spheroid with the followed addition of PMVC. After 2-3 days of culture, cells in the first approach formed spheroids that I attempted to stain for VE-cadherin (Fig. 44). However, some of the spheroids

were not stained, whereas others were brightly stained as if they only consisted of PMVC. Prolonged culture of both types of spheroids resulted in the formation of weak spheroids (Fig. 45), easily destroyable by pipetting. There is a possibility that I need to work with the cell ratio more thoroughly to successfully form the MIN6 and PMVC co-culture spheroids.

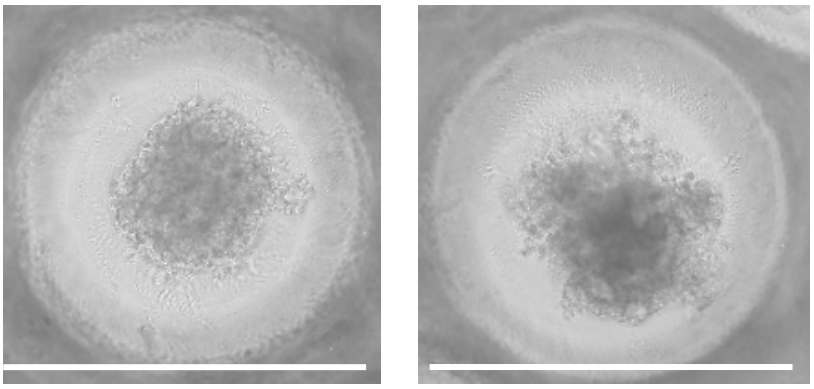


Fig. 45. Phase-contrast optical microscopy images of MIN6 and PMVC co-cultured spheroids in PEGM-2 a: MIN6 and PMVC were mixed and cultured for 5 days, b: MIN6 spheroids were formed, and after 1 day of culture PMVC were added, the resulted mixture was cultured for 5 days (overall 6^{th} day of culture). Scale bar 500 μm.

Judging from my results that not all spheroids were containing both types of cells and long culture led to deformation of spheroids I concluded that cells do not have a good attachment to each other. The first assumption was that cells lack common cadherin, as they represent two different cell types, and as so cannot attach to each other. Consequently, I decided to add fibroblast to co-cultured spheroid to facilitate their aggregation and vascular formation.

Co-culturing 3 types of cells may be a tricky procedure, as it is important to optimise cell ratio. The goal was to fabricate pancreatic spheroids, so I used an excess of MIN6 cells. Taking into consideration that contrary to PMVC and fibroblasts that have their doubling time at approximately 2 days, MIN6 double their population only in 4 days, I decided to use a 10-fold excess of MIN6. I optimised cell ratio in two steps. First, I fabricated fibroblasts : vascular endothelial cells (FV) spheroids to confirm the possibility of vascular formation in a spheroid shape. I chose three ratios of fibroblasts : PMVC = 5 : 1, 10 : 1 and 20 : 1. The possibility of vascular formation in spheroid shape in case of fibroblast and HUVEC co-culture spheroid is known from literature[88]. FV spheroids were easily formed in PEGM-2 culture medium (Fig. 46) that under fluorescent microscope looked uniformly red meaning cell dispersed uniformly. After 11 days of culture, I stained frozen sections for VE-cadherin (Fig. 46). I confirmed with

immunofluorescence staining that vascular endothelial cells prefer to stay in the outer layers of a spheroid and form circular patterns gradually mixing with fibroblasts. However, it was unclear if the vasculature was formed or it was just the disposition of cells.

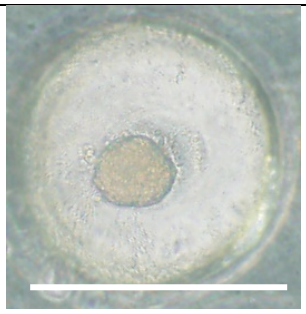
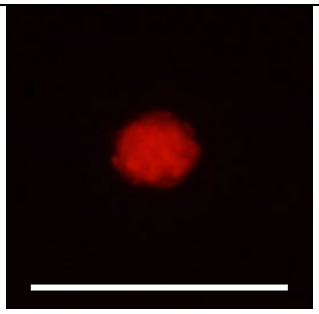
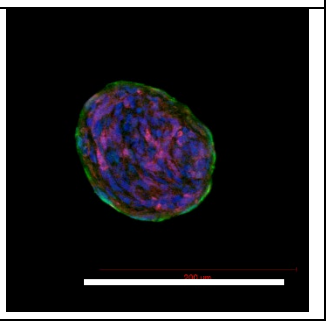
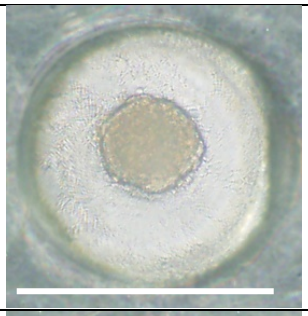
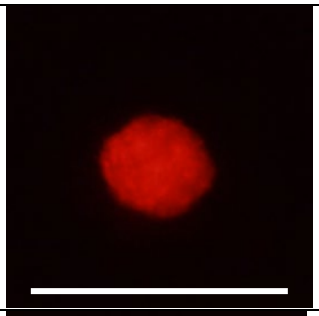
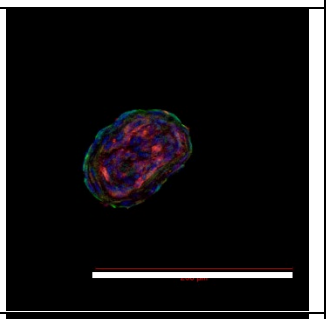
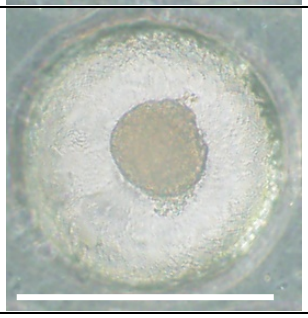
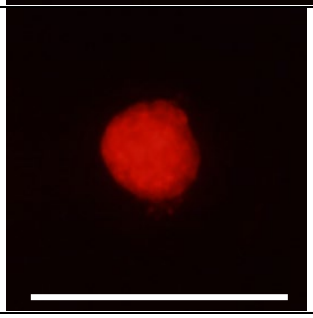
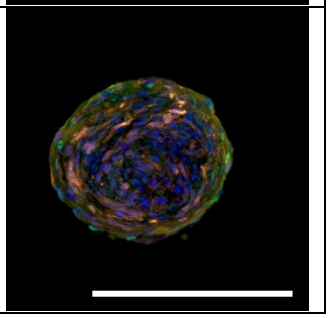
Fib : PMVC	Phase-contrast	Fluorescent	Confocal
5 : 1			
10 : 1			
20 : 1			

Fig. 46. Characterisation of FV spheroids with phase-contrast optical microscopy, fluorescent microscopy and confocal microscopy. For this experiment, fibroblasts with red fluorescent tag were used, and spheroids were stained for VE-cadherin (green). All pictures are taken after 11 days of culture. For the first two columns scale bar 500 μm; for the last – 200 μm.

To further investigate vascular-like structure formation in FV spheroids I conducted the HE, CD31 and CD34 immunostaining (Fig. 47). CD31 and CD34 are used in immunohistochemistry to stain blood vessels. I confirmed that all ratios resulted in spheroids with the collagen reach outer layer that was CD31 and CD34 positive and so consisted of PMVC. The inside part of spheroids with all ratios had weak staining for CD31 and CD34 with the formation of gaps between cells. I believe that I confirmed the formation of the vascular-like structure in FV

spheroid. However, it is possible that I need to culture spheroids for an additional amount of time to see more clear results.

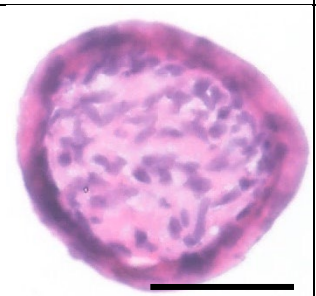
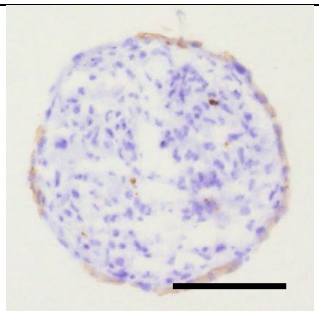
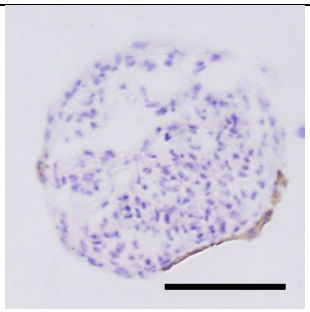
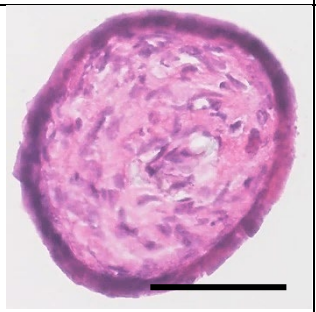
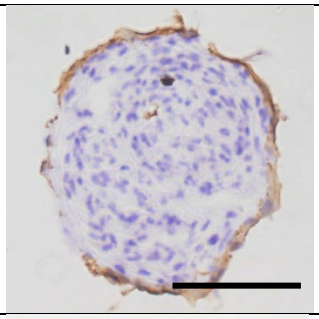
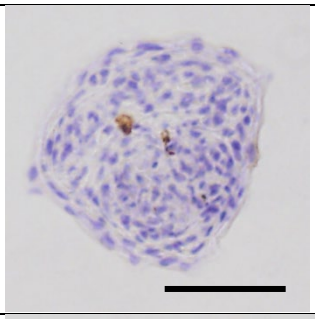
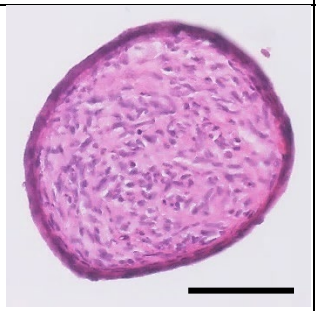
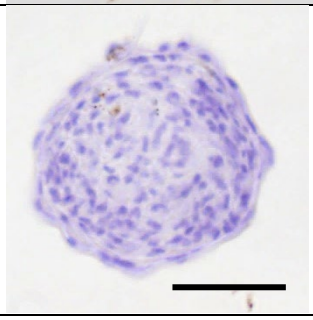
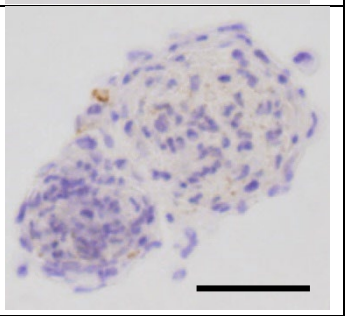
Fib : PMVC	HE	CD31	CD34
5 : 1			
10 : 1			
20 : 1			

Fig. 47. Characterisation of FV spheroids with HE, CD31 and CD34 staining. All pictures are taken after 11 days of culture. Scale bars 100 μm

To facilitate vasculature formation, I also may try reducing oxygenation of the spheroids or stabilising HIF1α, which is the marker of hypoxic conditions and induces VEGF secretion thus promoting vascularisation. Especially I am interested in the second approach as hypoxic conditions can be harmful to pancreatic β-cells, I would prefer to keep the oxygenation level but stabilise HIF1α in unnatural for this protein conditions. It seems that this is possible with the addition of Zn ion[80], although precautions should be taken as excessive amounts of Zn ion are toxic for cells. For now, I only did some pilot experiments in this direction, so I do not have any showable data. However, I will continue this research in the nearest future.

The second step of ratio optimisation for co-culture spheroids was an investigation of the structural changes in β-cells : fibroblasts (PF) spheroids depending on cell-to-cell ratio. For this experiment, I also chose the same three ratios 5:1, 10:1 and 20:1 (Fig. 48). Interestingly, I observed that 5:1 ratio resulted in spheroids with fibroblasts making a core surrounded by MIN6 cells, but ratios 10:1 and 20:1 resulted in MIN6 spheroids with fibroblasts dispersed through spheroids. As I intended to use fibroblasts only as "helpers" for the vasculature formation, I decided that the structure observed at 10:1 and 20:1 ratios would be the most desirable for my next experiment.

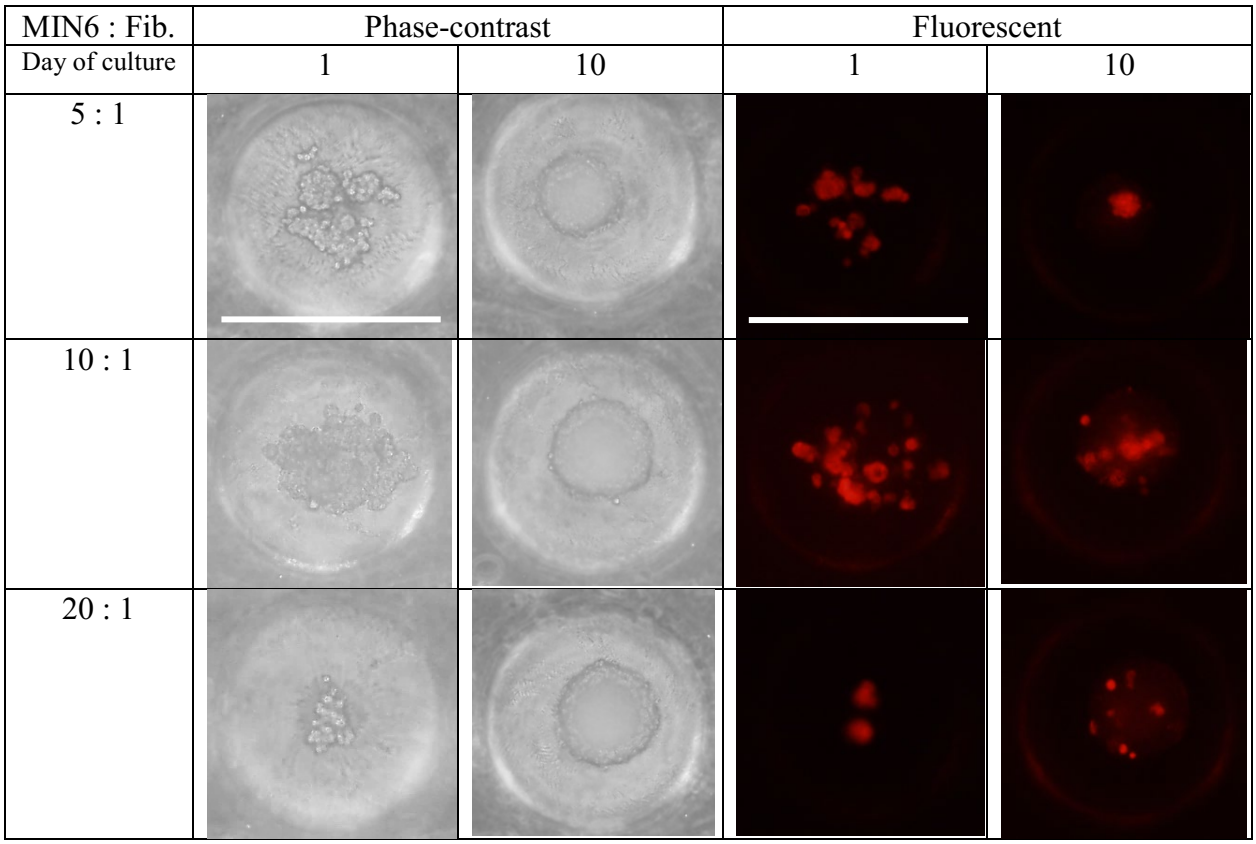


Fig. 48. Characterisation of PF spheroids with phase-contrast optical microscopy and fluorescent microscopy. For this experiment, fibroblasts with a red fluorescent tag were used. The scale is the same for all images, scale bar 500 μm.

Based on the described results I decide to investigate several ratios of a triple spheroid, fabricated from MIN6 : fibroblasts : vascular endothelial cells (PFV). I chose 10:1:0.5 and 10:1:1 ratios, so I kept the ratio of MIN6 : fibroblasts at the value I found led to the formation of the desired structure and used approximately 20:1 or 10:1 ratio of (MIN6+fibroblasts) : vascular endothelial cells. For now, I confirmed that spheroids are formed after 1 day of culture and keep their dense structure until the 5th day of culture. After that, cells are starting to break from

the dense sphere, and spheroid structure is degraded. Possibly it can be explained by the fast proliferation of vascular endothelial cells that are known to be unable to form strong spheroids (Fig. 49).

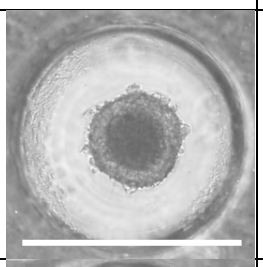
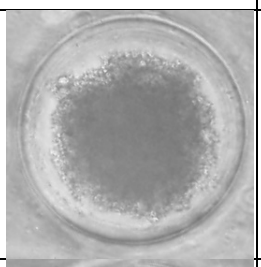
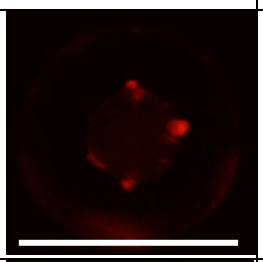
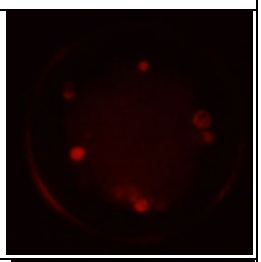
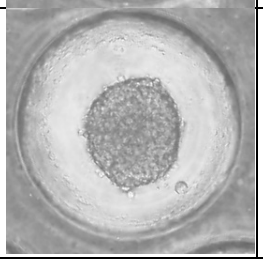
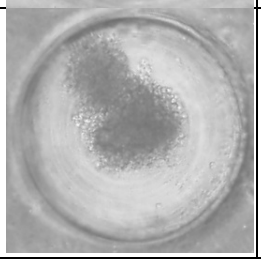
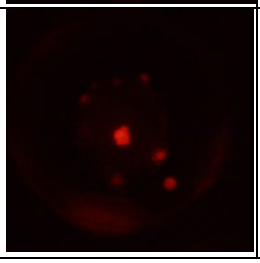
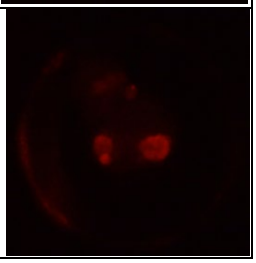
P:F:V	Phase-contrast		Fluorescent	
Day of culture	5	10	5	10
10:0.5:1				
10:1:1				

Fig. 49. Characterisation of PFV spheroids with phase-contrast optical microscopy and fluorescent microscopy. For this experiment, fibroblasts with a red fluorescent tag were used. The scale is the same for all images, scale bar 500 μm.

At the moment I am still carrying out the experiments with the PFV spheroids, as I only have taken the phase-contrast photos, and I need to perform immunofluorescence/immunohistochemical staining to confirm if there is vascular-like structure inside these spheroids. Further I tried to incorporate spheroids with different cell composition into a collagen gel and see if vasculature formation would be induced in the environment beneficial for vascular endothelial cells. The two methods that I used are shown on the schemes below (Fig. 50).

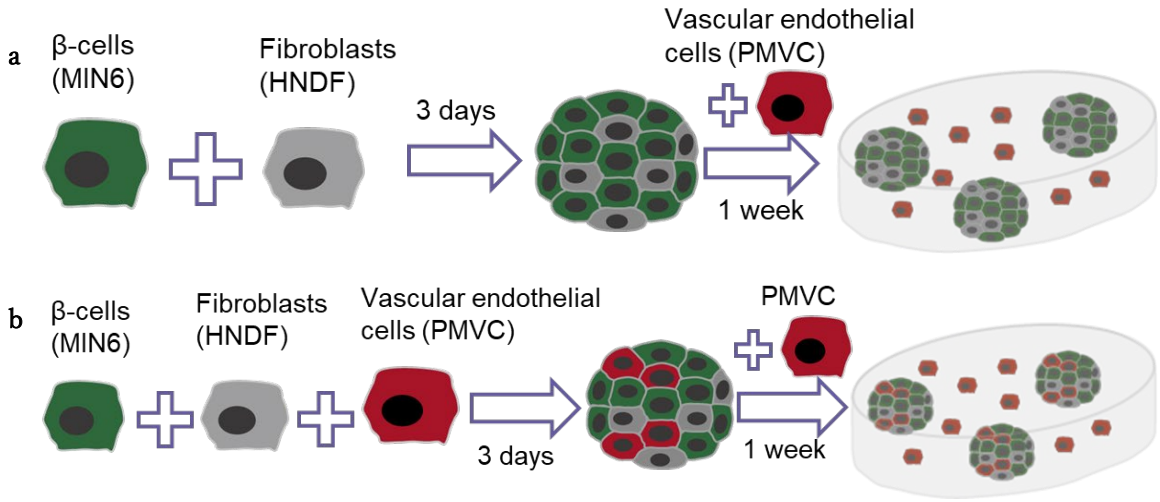


Fig. 50. Schematic representation of conducted vascular-like structure formation experiment. a: control experiment with PF spheroids and PMVC in collagen. b: PFV spheroids with PMVC in collagen.

As it can be seen the difference is in the composition of spheroids: in the first method there was no PMVC incorporated into spheroids, and they were dispersed only in a collagen gel; in the second method PMVC are both incorporated into spheroids composition and dispersed in a collagen gel. In the first case, after 1 week of culture PMVC formed vascular "beds" surrounding spheroids but did not penetrate inside them. The size of the vascular "beds" was around 200 μm, and their formation was observed only in the proximity of spheroids. In the areas without spheroids, only thin (~ 50 μm) vascular-like tubes were formed. It is possible that longer culture will lead to the penetration of vascular-like tubes into spheroids. However, I want to find the fastest possible way of fabrication of vascularized spheroids. In the second case, after 1 week of culture, PMVC connected with the vascular endothelial cells incorporated into spheroids, forming a penetrating network (Fig. 51). Resulted vascular-like tubes were thin with some of them as less as 20 μm. I should notice, however, that at the moment I cannot comment on the perfusability of formed vascular-like structures and this question will require some additional experiments that I will conduct in the nearest future.

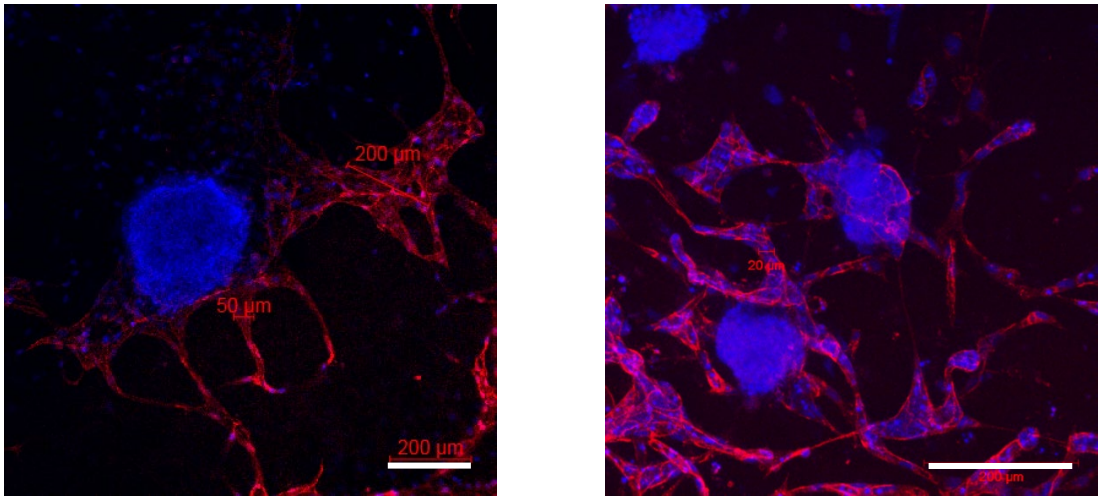


Fig. 51. Confocal microscopy images of PF spheroids (a) and PFV spheroids (b) collagen co-culture with PMVC. Scale bars 200 µm.

In this chapter, I considered the possibility of vascularised spheroids fabrication *in vitro* from MIN6, fibroblast and pancreatic microvascular endothelial cells. I confirmed that these three types of cell could form spheroids at a specific ratio and in the modified EGM-2 culture medium with a high content of FBS (20%). Further, these spheroids were cultured in a collagen gel with dispersed pancreatic microvascular endothelial cells and were shown to connect with each other after 1 week of culture to form a vascular-like network. Although this direction of my research is still on its early steps, I have some promising results that could be used for future experiments.

CHAPTER 5

5. THESIS CONCLUSIONS

1. In this work, I successfully fabricated oxygen permeable microwell array from PDMS (PDMS-chip) that allows decreasing hypoxia in pancreatic spheroids fabricated from MIN6 and MIN6-m9 in 2 times compared with spheroids cultured in the oxygen impermeable microwell array from PMMA (PMMA-chip). Thus, I proved that PDMS-chip indeed improves the oxygen supply for spheroids.
2. PDMS-chip allowed increasing *INS1* expression and led to 2.5 times higher insulin content in MIN6 pancreatic spheroids compared to monolayer and spheroids cultured in PMMA-chip. Thus, showing a clear advantage of culturing pancreatic MIN6 spheroids in conditions with higher oxygen supply.
3. I showed that not only MIN6, but also MIN6-m9 cell line has heterogeneous nature, and consists of cell positive for insulin, glucagon or both. I also showed that MIN6-m9 spheroids are mechanically less strong than MIN6 spheroids.
4. I optimized the amount of antioxidant ascorbic acid-2-phosphate (AA2P) that is required for culturing MIN6 spheroids in PDMS-chip and showed that its addition indeed leads to decreased production of reactive oxygen species. I showed that addition of AA2P allows 4 times upregulation of *INS1* and 2 times upregulation of *GLUT2*. The upregulation of the genes correlated with the 3 times increased insulin content in spheroids cultured with addition of AA2P compared to spheroids cultured without its addition.
5. I optimized the glucose concentration in culture medium used for culturing MIN6 and MIN6-m9 as monolayer and spheroids. I proved that standard conditions with 25 mM glucose concentration are not optimal for culturing MIN6 as monolayer and spheroids, and use of a culture medium with glucose concentrationin the range 10-20 mM would be advisable.
6. I optimized conditions for fabrication of co-culture spheroids from three types of cells {MIN6 : fibroblasts : pancreatic microvascular endothelial cells}, and showed that the

presence of vascular cells inside spheroids allowed the formation of penetrating vascular-like structure in collagen tissue model with impregnated vascular endothelial cells.

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