The role of organoids in Cystic Fibrosis research

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Index

ABSTRACT

Cystic Fibrosis (CF) is a hereditary disease, caused by a mutation in the CFTR channel resulting in CFTR dysfunction. CFTR modulators are medicine that will attempt to restore the CFTR function. To assist CF patients better, it is important to know the individual response on CFTR modulators, striving to personalized medicine. Therefore, researchers are currently performing research on new methods using an organoid model.

Organoids are 3D structures, like 'mini-organs' and useful because of their characteristics, such as self-organization and multicellularity. Intestinal organoids were highlighted, because the intestine is also affected in CF. The aim of this thesis is to get a clear image of the role of organoids in study and treatment of Cystic Fibrosis.

Two methods will be highlighted: Forskolin Induced Swelling (FIS) assay and the CRISP/Cas9 technology. Both methods are using an organoid model and are hopeful approaches to realizing personalized medicine and develop the knowledge about CF.

INTRODUCTION

Cystic Fibrosis (CF) is the most common hereditary disease in the Netherlands, with more than 1500 patients [1]. This disease is correlated with reduced life expectancy and quality of life [2]. CF is an autosomal recessive disease which is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, a chloride channel at the cell membrane [2]. It is a monogenetic disease because a single gene is affected. There are currently almost 2000 CFTR mutations known that will cause CF. The most common (70% of CF patients) is deltaF508 [3]. This mutation causes a three base-pair deletion to occur that results in a deletion of phenylalanine at position 508. During translation there will be an abnormal folding of the CFTR protein caused by the absence of the phenylalanine and leads to accumulation inside the cell [4]. This results in a dysfunction of the CFTR protein. The dysfunction causes thickened secretion of mucus in different organs and an increase in salt concentration in sweat [2]. The effect of the protein dysfunction is not only present in the lung but also in other epithelia, such as the intestine [5].

CFTR modulators are drugs that target the CFTR protein. They can potentiate or correct the CFTR function. Potentiators improve the activity of the channel and correctors assist the protein to traffic to the cell surface. To examine the efficiency of CFTR modulators, CFTR activity needs to be measured. In clinical trials, efficiency of CFTR modulators can be predicted for a group, but less on individual basis [6]. Clinical trials to drug response for patients with mutations other than deltaF508 are therefore expensive and time-consuming. In recent years, much research on new cost-effective methods has been done to develop individual treatment for patients with rare CFTR mutations [3].

Animal models are badly represented in CF research, because there is no suitable comparison between animal and human CFTR functions. With an overexpression of other chloride channels in mice the effect is sufficient to overcome the dysfunctional CFTR channel. Because the lack of good comparable animal models it remains essential to study CF mainly in patients [7]. Through genetic screening the mutations causing CF are known, however measuring the individual response to CFTR modulators is not possible with that technique. Therefore, new methodology to study and potentially treat this disease are necessary. A new insight and treatment with organoids has proven to be helpful in this process.

The aim of this thesis is to get a clear image of the role of organoids in study and treatment of Cystic Fibrosis. First it will take a closer look at the concept of organoids and their applications and secondly, it focusses deeper on researches that have made a combination with an organoid model and CF. At last, it will show what the advantages, limitations and the future ideas are with the use of organoids in CF treatment.

ORGANOIDS

WHAT ARE ORGANOIDS?

In 1975, Rheinwald and Green, described the first long-term cultures of human cells. They were able to get 3D tissue structures from normal human cells. After these pioneering studies, the organoid model become more popular, namely to describe organogenesis [8]. However, the past decade organoids are seen in a slight new perspective. Organoids are now defined as 3D structures which will grow from stem cells to organ-specific cells [8]. They are easy to manipulate genetically, which makes them a beneficial tool for research development processes and tissue maintenance [9]. Furthermore, organoids are like mini-organs and have three main characteristics: self-organization, multicellularity and functionality. Through self-organization the cell becomes a 3D structure. The structure contains different cellular types and it has also some similar functions as a normal organ [5]. To grow organoids stem cells are valuable cells to start with, because of their important characteristics. Stem cells have capacity for clonal expansion and their daughter cells can differentiate into multiple stem cell types. So, stem cells are capable of self-renewal and multipotency [5]. In addition, organoids are able to expand indefinitely which is a valuable property [10].

Organoids can be divide in two different subtypes. Organoids derived from tissue-restricted adult stem cells (ASC) and pluripotent stem cells (PSC). PSC can be split in two groups: embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) [11]**.** An advantage of ASC is that they are already tissue-specific and patient-specific, however they contain only epithelial cells. On the other hand, organoids derived from PSCs can contain cells from of all three germ layers, depending on the tissue and the protocol they are using [11]. Induced pluripotent stem cells can be directly generated from for example skin fibroblasts and blood cells. Therefore, IPSC are already patient-specific and can also be a valuable resource for inaccessible cell types, because their ability to differentiate into any type of cells which is relevant by diseases [12]**.** The ability to work with organoids makes it effortless to work with human derived tissue from patients.

INTESTINAL ORGANOIDS

Our body contains different organs originating from different germ layers (endo, meso and ectoderm). Over the past few years organoids can now be made from all different layers. Consequently, most organs can be developed into organoids [5]. This thesis focusses on the endodermal organoids. The endoderm leads to the development of digestive and respiratory tracts, including organs such as lung, liver, pancreas and intestines. Much CF research is done with intestinal organoids [5]. For this reason, the mechanism of intestinal organoids is highlighted in the next section.

The gastrointestinal tract is the first part in the body that is affected by CF, due to the high expression of the CFTR channel in the intestine channel. Because of the dysfunction of CFTR, the gastric acid will not be neutralized in the intestine and this results to poor digestive function in the intestine [13]. Through the involvement of the intestine in CF, research to intestinal organoids could provide a powerful tool to improve CF treatment. However, one should not forget that CF remains a multiorgan involved disease [14].

The epithelium of the small intestine has a turn over time of approximately 5 days, which is extremely short [8]. Due to the short turn over time an organoid can be quickly grown. The epithelium contains two major components: the villus and the crypts. Cellular division occur in adults' tissue only in the crypts and not in the villus.

Crypt based columnar cells (CBC) are one of the stem cell populations in the crypt, which are closely associated with Paneth cells at the crypt bottoms. Together the Paneth and CBC cells form the stem cell zone in the crypt, see figure 1. Surface protein Lgr5+ (a Wnt target gene) makes it possible that single CBC cells regenerate an entire crypt-villus axis. The renewal of all the cells go from the bottom of the crypt to the top of the villus [9]. However, differentiated cells can also express Lrg5+, so additional factors are also significant for regeneration [15].

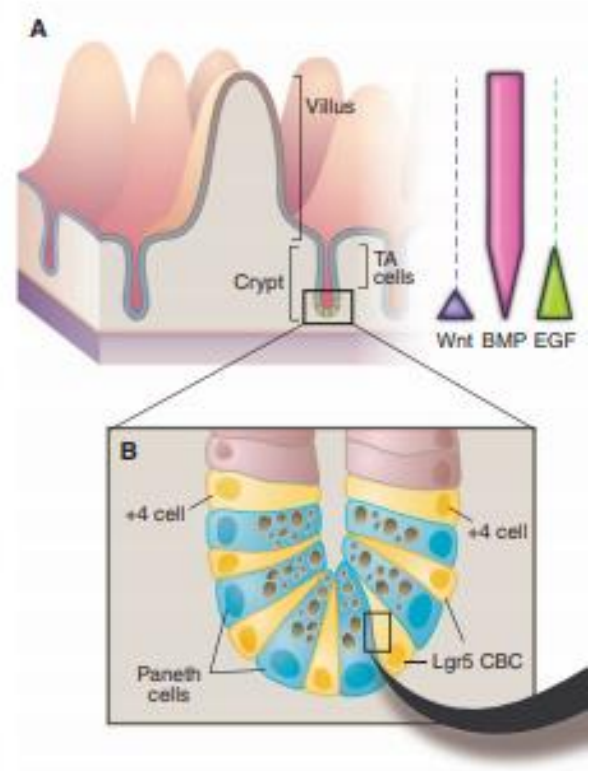

Figure 1 – Intestine epithelium structure and their molecular gradients [15]

For growing intestinal organoids and making the epithelium, three molecular gradients (additional factors) are essential [15]. Epidermal growth factor (EGF), Wnt factor and the villus bone morphogenetic protein (BMP) are playing an active role [5]. While the cells move from the crypt to the villus they are exposed to a Wnt gradient. Stem cells get Wnt mediators to move and therefore Wnt is important for proliferation and the stem cell state. The amount of Wnt molecules and their receptors decreases as they leave the stem cell zone and move away from Paneth cells, due to turnover by cellular division.

BMP plays a role in differentiation and regulates stemness negatively. However, BMP antagonists prevent the BMP activity in the stem cell niche and so the antagonists are relevant for stem self-renewal. At last the growth factor EGF is expressed by the epithelium which induces cell proliferation and angiogenesis. Because of that the regulation of EGF is of great importance for stem cell homeostasis and regeneration [9].

Furthermore, in organoid models one must overcome the limited life span and the unnatural two-dimensional growth pattern in the intestinal cultures**.** Solving that problem, the epithelium must grow on an extracellular matrix (ECM), without the ECM organoids undergo cell death. For the ECM Matrigel is used what is produced from mouse sarcoma. It contains different ECM proteins and a variety of growth factors, such as EGF. EGF is meaningful for the long-term expansion of the organoids cultures [9]. Different molecular gradients and an extracellular matrix are essential for a working organoid model.

APPLICATION

Organoids allow researchers to study in vitro organogenesis and tissue homeostasis which is not possible in vivo using current techniques. The combination between in vitro and in vivo studies are useful to study gene functions and cellular processes. Organoids models showed to be a great promise for translational technology [8]. For example, it could be more relevant for in infectious and hereditary diseases, cancer, personalized drug treatment, drug discovery and toxicity tests and transplantation in the future [5]. Especially the personalized drug treatment will be a technological advance and a preferable outcome for the treatment of CF.

For personalized drug treatment Forskoline Induced Swelling (FIS) is used. FIS is an assay which measure the individual CFTR function and response to CFTR modulators in vitro [16]. FIS can make a distinction between the drug responses in individuals for different and even identical CFTR mutations. On other new upcoming method is CRISPR/Cas9 (short for Clustered regularly interspaced short palindromic repeats) genome editing using organoids. CRISPR/Cas 9 in organoids can restore mutations through NHEJ and HDR which results in genetic modifications [11]. This method is preferable for correcting the mutations in CFTR gene that cause CFTR dysfunction. Both methods will be explained more in detail, later in this thesis. Thus, organoids are a new approach which can be helpful in different fields of research, also in monogenetic diseases such as CF.

ORGANOIDS AS A TOOL FOR CF TREATMENT

In the previous section the mechanism and application of organoids were highlighted. This section focusses on the contribution of organoids in CF research. There are two main methods in CF research which using an organoid model. Firstly, the forskolin induced swelling assay which measured the effect of CFTR modulators on individual basis and secondly genome editing by CRISP/Cas9. Both methods will be explained here to get an idea of their contribution in CF research.

FORSKOLIN INDUCED SWELLING ASSAY

For the common F508delta mutation much research is performed with organoids and CFTR modulators. For example, Wong et al. were able to differentiate human pluripotent stem cells into CFTR-functional conducting airway epithelium. First, there was a bad functionality of CFTR in the lung organoids of the patient, but when the researchers treated the organoids with correctors they restored the CFTR functionality [17]. Moreover, illustrated by Ogawa et al. [18], lumacaftor which is an CFTR corrector was capable to correct folding defects of mutant CFTR protein. Resulting in the ability to correct the misfolding and translocation in patient-derived cholangiocyte organoids.

So, CFTR function can be restored by CFTR modulators. CFTR potentiator treatment in vivo is often variable and depends on patient-specific variability in mechanism such as absorption, excretion, metabolism and distribution. VX-770 (ivacaftor), a CFTR potentiator, had a different effect on individual patients with different mutations. Because of the different effects, the researchers wanted to characterize the pharmacokinetic details of VX-770 on individual basis. Dekker et al., used an assay to functionally measure VX-770 in plasma of healthy volunteers [19]. This assay is extensively written down by Boj et al. it's called the Forskolin Induced Swelling (FIS) assay. The swelling response of the organoids correlates to the clinical response to the drug. The main important property for this assay is the complete CFTR-dependence. The CFTR-dependence is showed through the analysis of intestinal organoids with CFTR -/- (knockout mice) or individuals with cystic fibrosis, where both groups give no swelling response [20].

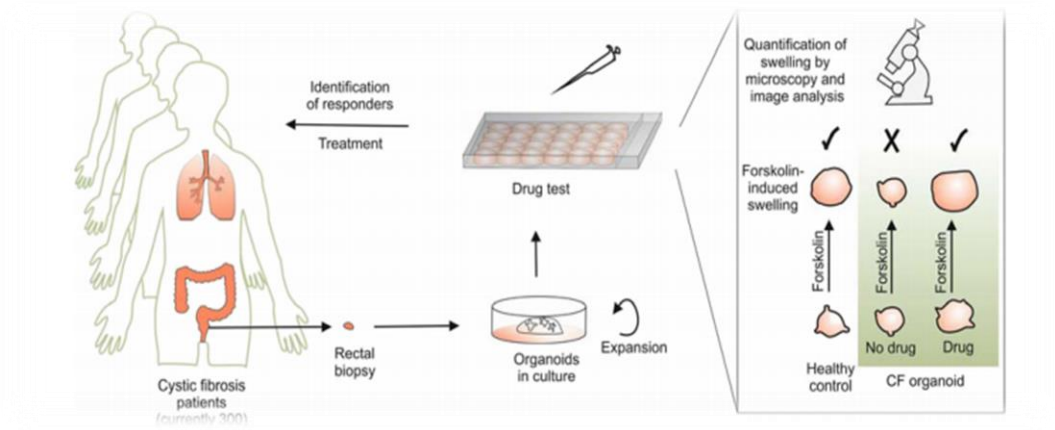

Figure 2 – Measurement of CFTR function in organoids through FIS [5]

In the figure above, Bartfield & Clevers [5] explain the process of measure the CFTR function in organoids through FIS. The quantificaton of swelling is done using microscopy and image analysis. Researchers add forskolin, which is an CFTR activator, because it raises intracellular cAMP. This results in activation and therefore opening of the CFTR channel, leading to ion uptake. Finally, it will lead to fluid transport to the organoid lumen [5].

Due to the prescribed mechanism forskolin stimulates organoid swelling. Potentiators (i.e. ivacaftor), that act upon the CFTR channel are added at the same time as the forskolin. On the other hand correcters (i.e. lumacaftor VX-809) are added 24 hours before the addition of forskolin, because they restore CFTR trafficking [16]. For the healty control, the addition of forskolin leads to CFTR-dependent swelling. In organoids from CF patients forskolin leads to impaired swelling (marked with an x in figure 2). However after the treament with a CFTR modulator or by CRISPR/Cas9 gene editing the impaired swelling can be restored.

Mainly for the remaining CF patients with other mutations than deltaF508 this assay is crucial, because it is able to match CFTR modulators to all types of CFTR mutations [16]. As a result, FIS is now a successful approach for individual treatment of patients with very rare CFTR mutations. Otherwise they had no access to the recently introduced CF drugs, because the lack of clinical research of drug response in rare mutations. Through the FIS assay, they may benefit from CFTR modulator therapy independent of their mutation [21]. Dekker et al., have also examined another assay, namely, Steady-state Lumen Area (SLA) in the absence of forskolin. With the SLA assay they can make a comparison of CFTR function among CF patients and healthy controls. SLA becomes quantified through measuring lumen area as a percentage of the total organoids area. As a result, SLA is a CFTR-dependent phenotype which compares non-CF controls with people with CF. So, FIS measurements can be compared between organoids from CF patients, but not between healthy controls and mutant CFTR organoids because the high SLA in healthy control cultures. Taken together SLA and FIS assays could help describe CFTR function and drug reaction on CFTR function for many CFTR mutations [21].

Upcoming treatment for CF consist of different correctors and potentiators which cause a different effect on individuals. Quantification of swelling in organoids toward CFTR modulators could help patients with rare mutations, because research has shown the individual effect on specific drugs. Therefore, this is a new methodology which assists in a better personalized treatment.

CRISPR/CAS9 TECHNOLOGY

In the previous paragraph it was presented that research can measure the effect from different modulators on CF patients using organoids. Another method that has been developed during the last decade is genome editing which is focused on gene correction and manipulation. The problem in CF is the mutation in the CFTR gene, correcting that gene will solve the problem in CF: the dysfunction of the CFTR protein.

Gene correction is done by CRISPR/Cas technology, which will later be explained in more detail. Combining CRISPR/Cas technology and organoids is a new research tool, which has shown efficiency for genome editing in many different organisms [7]. Because it is a recently new field of research it is still developing and there is a limited of research combining organoids and CRISPR/Cas technology. However, both methods combined ensure many new insights and hope for solving the cause of CF [11].

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a defense mechanism of bacteria and archaea against viral infections. A Cas gene is a CRISPR associated gen and can cause disease in bacteria strains through their rapidly exploited characteristic. After many researches this system has shown to be a model of nuclei-acid based immunity which is heritable, due to its stable integration into the bacterial genome [11].

For CRISPR systems different types are identified, this essay focuses on type II. Type II is being adapted as a genome editing tool. To understand the mechanism of the CRISPR/Cas system better the figure below will be explained in detail. The CRISPR system consists of fragments of foreign DNA (protospacers) which lies between repetitive DNA sequences (spacers) that are present in host DNA. If transcription takes place this results in formation of CRISPR RNA which contain both spacer and protospacer sequence. After that, the CRISPR RNA (cRNA) binds to transactivating CRISPR RNA (tracrRNA), which leads to a complex with Cas9 nuclease [11].

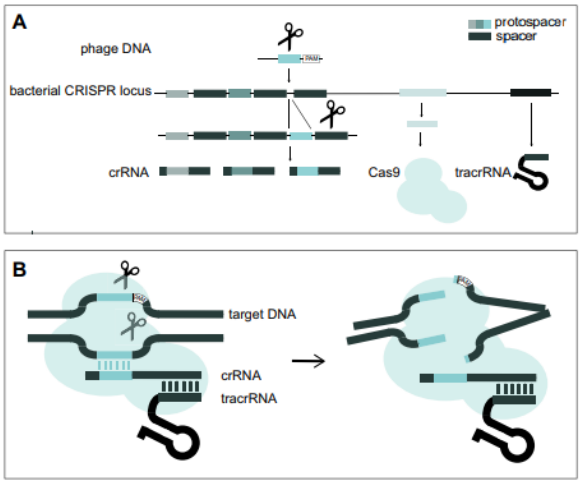

Figure 3 – The mechanism of the CRISPR/Cas9 system [11]

The spacer sequence (guide RNA) leads Cas9 to the complementary DNA sequences in the genome of the invading organism. Then, Cas9, a ribonucleoprotein endonuclease, can catalyze double strand breaks (DSB) at the target DNA sequence [7].

With the CRISPR mechanism research can bring a DSB at any desired location. Two pathways can repair a DSB: nonhomologous end joining (NHEJ) or homology directed repair (HDR), also named homologous recombination. There are two differences between HDR and NHEJ. Firstly, for HDR a template is required. Through the need of a template, the recombination can only happen during S/G2 phase of the cell cycle. Secondly, HDR can repair the DNA with more precision than NHEJ [11].

The mechanism of CRISPR/Cas9 ensures the possibility to correct genes. So, Driehuis and Clevers [11] wanted to confirm that the CFTR gene is the only impaired gene that cause the dysfunctional effect in CF. First, they used intestinal organoids and caused a frame shift in the two Apc alleles. Apc is a negative regulator of Wnt signaling and moreover a prominent tumor suppressor in intestinal cancer. Earlier in this thesis was stated that Wnt is very essential in formation of organoids. Through the frame shift in Apc organoids results in a Wnt-independent way**.** After sequencing the organoids NHEJ-based 'scars' were presented, concluding the feasibility of CRISPR in intestinal organoids. Furthermore, the same researchers examined that HDR can repair the CFTR gene. Because of the level of swelling after the gene correction was comparable with wild-type organoids [11].

Schwank et al [14], were able to get a functional CFTR gene correction from the disease-causing mutation in CF patients. That makes them the first which illustrating CRISPR in adult intestinal organoids. They used CRISPR/Cas9 to make a correction on the CFTR gene by homologous recombination in intestinal stem cells of CF patients with a F508del mutation. With forskolin treatment they observed expansion of the organoid surface area in the corrected organoids, where swelling in the non-transfected control organoids was still absent. They were able to correct the mutant F508 del allele in intestinal organoids, so the CFTR protein became fully functional. However, the adult corrected intestinal stem cells cannot be used to rescue the pathophysiology in the lung. Moreover, Firth et al were able to correct iPSC from a CF patient with F508del differentiated to mature airway epithelial cells to get the normal CFTR function and expression back with CRISPR/Cas9 [7].

Until now the CRISPR/Cas9 technology is only able and used to correct the F508del mutation. Despite the positive results in this new field it is not directly applicable for clinical applications [11]. Besides that, in the future this new methodology will potentially lead to better treatment of the disease.

CONCLUSION/DISCUSSION

This thesis focused on the role of organoids in study and treatment of Cystic Fibrosis. Therefore, it has examined the concept of organoids and their applications. Besides that, it has mentioned various methods that made a combination with an organoid model and CF. It has highlighted the method FIS and the CRISPR/Cas9 technology, which have both their own contribution to the research to CF treatment.

In conclusion, organoids have tree main characteristics, such as: self-organization, multicellularity and functionality. Because of that organoids can be helpful in different fields of research, also in a monogenetic disease such as CF. Intestinal organoids play a key role in CF research. They are made from the endoderm to grow a 'mini-gut', for which different molecular gradients are required.

Furthermore, treatment for CF consists of different correctors and potentiators which cause a different effect on individual level. Using the forskolin induced swelling test researchers examine the effect of the modulators for individual patients. The CRISPR/Cas9 technology is until now only used to correct the F508del mutation, nonetheless it is successful in this mutation.

Not only for personalized medicine, but also for drug development, toxicology, regenerative medicine organoids are an excellent upcoming tool [5]. Besides that, using organoids to gain knowledge about CF is an excellent alternative to animal testing [9]. Moreover, advantages of using an organoid model is that you can look at many different types of cells and even at the development of embryo and adult tissue. However, an important and main limitation of organoids is the absence of immune cells, lack of innervation from the brain and lack of blood vessels [8].

An advantage of the forskolin induced swelling test compared to the CFTR readout in 2D airway cells is the rate of processing the swelling assay. So, with less technical variability the swelling test gives more accurate measurements of the CFTR function. Through this precise measurement researchers are able to pick up genetic background effects between patients with the same CFTR mutations [16]. Nevertheless, FIS is an indirect result of measuring the CFTR function, by coupling ion transport to fluid transport. Whereas readout in 2D cells give a direct CFTR function, because it allows to select stimulation of apical and basolateral compartments [16].

The positive effect of combining organoids and gene correction is the possibility of clonal expansion of stem cells. Additionally, you can obtain a selection of recombinant clonal organoid cultures with the desired change in the gene [14]. All studies using CRISPR/Cas9, to correct the CFTR gene, were able to restore F508del mutation in organoids. Remarkable is the fact that they only restore the common mutation delta F508 and no other mutations. Therefore CRISPR/Cas9 is still only helpful for patients with the F508 delta mutation. Although, gene correction at this monogenetic disease looks successful, it is still not available in clinical setting.

But where in this are the patients? Where do we stand now? Organoids models are an excellent tool to test the reaction of modulators on patients with rare mutations, besides that it is an animal saving solution. Hopefully the next years it will finally lead to better personalized medicine treatment, which can help the patients more.

Although the CRISPR/Cas9 is a promising method to correct the CFTR deletion and therefore the CFTR function in patients, it is still a long journey to get this in clinical settings. When future research makes it possible to use these methods in a clinical setting the overall quality of life could be greatly improved for CF patients.

For the first time in the treatment of CF the true cause of the disease could be cured using gene correction. As this is still a future technology current research will focus on finding a personal treatment for individuals using in vitro samples of every patient. Every new breakthrough results in an improvement of quality of life for CF patients.

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