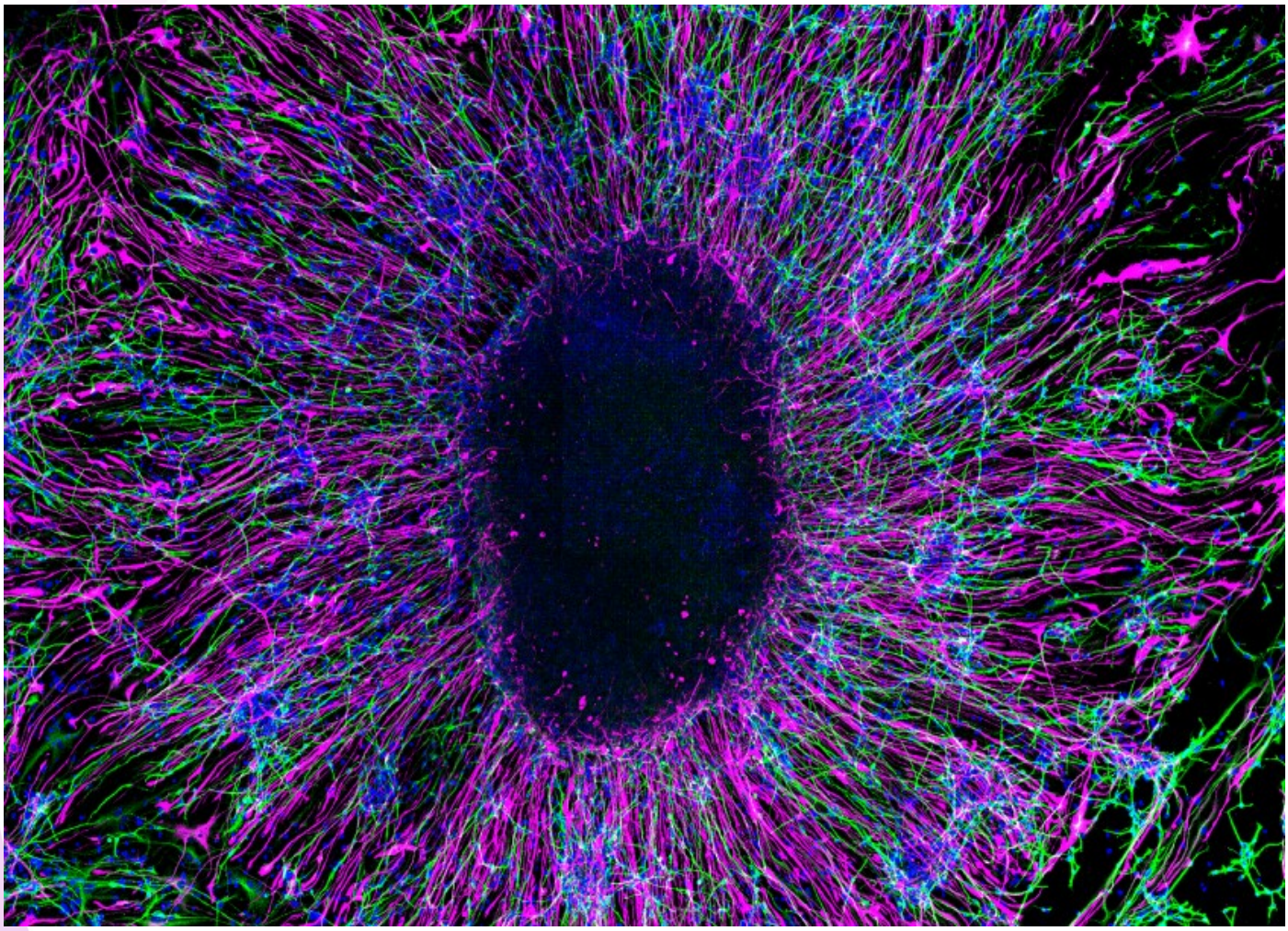
Growth and Applications of Cell-specific Organoids



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1.Preface

Amidst the rising demand for better healthcare options, organoids can be seen as a profound well brimming with a broad range of uses, thus sparking immense interest within the medical and biological scientific community. For 30 years regenerative medicine has not been able to treat irreparable damages to organs leaving only transplantation of livers, hearts, kidneys etc. from human to human as a viable option. The idea of growing functional organs or organ sections from a donor's stem cells for clinical use has been a great ambition since the very beginning of stem cell research. Organoids allow for the study of human tissues at the equal level of scientific scrutiny with an amount of reproducibility as well as profundity, that could only be reached with non-human model organisms. One of the ethically most disputed biomedical advance, the concept of biobanks, is deeply rooted with the uses that organoids offer. Personalized disease models, organoids generated from an individual's own stem cells with a physiological function that is identical to their corresponding body part and even the ability to trace back the beginnings of human brain development, their similarities and differences compared to our ancestors and closely related species. Organoids can answer more questions of the past and provide more solutions for the future than any other biotechnological application. But at the same time not only the complexity of physiological networks of the human body, the missing definition of body axes, the lack of blood vessels and a cardiac system, within an organoid, but also the linked ethical uncertainty and public concerns have to be overcome to realise the theoretical potential of the novel organoids. This paper will discuss the process of generating specific organoids, their corresponding applications, as well as hopes for further uses in the future. It will also debate the ethical complications of organoids and finally provide the opinion of the researcher Dr. Christian Kurt Hirt on several questions about organoids.

2.Introduction

2.1.What are Organoids?

The very first question about the definition of organoids varies from source to source and has already seen prior use in 1946 in reference to a tumour case study (Smith and Cochrane, 1946). Over time its meaning has evolved substantially from certain histological features of tumours to today's generally accepted definition:

"Organoids are a collection of organ-specific cell types that develops from stem cells or organ progenitors and self-organizes through cell sorting and spatially restricted lineage commitment in a manner similar to in vivo." (Lancaster and Knoblich, 2014)

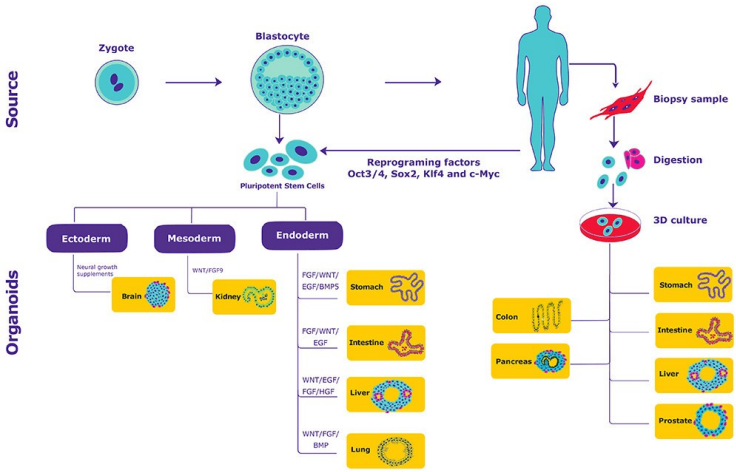

Fig.1 - Organoid generation from primary tissues and pluripotent stem cells by Merck, 2020

This further implies that:
1. The cell structure can differentiate into multiple organ-specific cell types.
2. Organoids are capable of recapitulating, in other words fulfilling a set of specific functions of the organ e.g. excretion or filtration, neural activity etc.
3. The cells within an organoid are able to group together and organize themselves into a spacial arrangement similar to the target organ. They must be able to recapitulate both major processes of self-organization when in the developing phase i. e. cell sorting out and spatially restricted lineage commitment.

2.2.Recent Scientific History

In the past months the first cardiac stem cell transplantations have faced first clinical testing in Japan. Using induced plutipotent stem cells (iPSCs) researchers have grown and transplanted tiny cardic muscle tissues into specific heart reagions of patients with serious heart malfunctions caused by ischemic cardiomyopathy. The tissues are arranged into thin sheets and attached to the surface of the hearts. When attached they will begin to grow and over time secrete proteins that are able to regenerate blood vessels resulting in an improved cardiac function. (Yoshiki et al. 2020)
The ability to grow cerebral organoids from basic progenitor cells allows for scrutiny within each brain development step of all mammals, such as great apes, mice, humans etc and has already uncovered human-specific features of brain development, when compared to our closest living relatives, the chimpanzees (Treutlein et al. 2019). Early neurodevelopmental stages can be modelled within controlled laboratory conditions. First comparative studies between human and nonhuman primate-induced pluripotent stem cells have now revealed unexpected differential regulation of L1 (long interspersed element-1) endogenous retrotranspons. These elements can be found in ~20% of all mammalian genomes and when mobilized they can impact the human genome, thus causing several human disorders.
One of the most notable achievments of cerebral organoids was the major role they played in pointing out the link between the Brazilian Zika virus and the resulting microcephaly outbreak. Again organoids grown from infected children could be isolated and the relatively slow neurodevelopment allowed investigators to cunclude how Zika could infect neural progenitor cells leading to severe defects in the cortical plate.
These listed accomplishments owing to organoids are just barely scratching the surface of the practical potential that is already in use, not the mention all the theoretical applications that we can hope to see in the near future.
With a history barely reaching a decade organoid technology as a whole is still in its infancy. Current protocols for the initiaition and growth of brain organoids can mimic cellular and molecular composition of human fetal brains around the second trimester. The limitations of organoids are mostly by the lack of blood vessels, therefore it relies on simple diffusion from the culture medium and cannot grow past the current maximum length of 5 millimeters. When cells are cultured over a longer period of time, a substantial amount of cells undergo apotosis due to the increasing inefficiency of nutrient trasportation with bigger size.

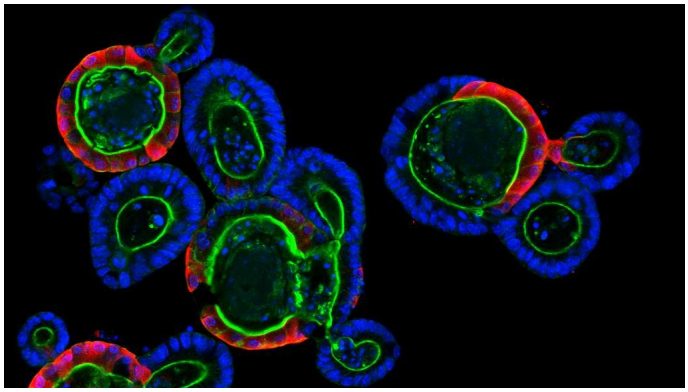

Fig.2 - Coloured intestinal organoid culture
by Sandy Evangelista, 2018

2.3.Fundamentals of Organoids: Stem Cells

Since organoids can not exist without the concept of stem cells, this section will give a short introduction to the types of stem cells that are directly involved in the field of organoid research only.
Stem cells are precursor cells that can self-renew indefinitely to generate multiple differentiated mature cell types. They can be retieved from embyonic and adult organisms, but their properties differ when an embryo reaches adulthood.
1.Embryonic stem cells (ESCs): are cells found in the inner cell mass of blastocysts. ESCs are plutipotent and can form all three derivatives of germ layers: extoderm, endoderm and mesoderm. This means that embryonic stem cells can differentiate into each of the +200 cell types of the adult body. Molecular characterization of th ESCs is well known and they are known to express surface markers. These include the surface markers CD9[A], CD24[A], alkaline phosphatase and several genes that are connected to pluripotence e.g. Oct-4[A], Rex-1[A], SOX-2[A], Nanog[A] etc.

appendix A?

In order to differentiate cells into many mature cells types that are used for the potential treatment of diseases, ESCs are reprogrammed, in other words, induced to differentiate, creating various cell types that result in 3D cell aggregates, called emryoid bodies. Growth factors are mainly used to direct the differentiation process into forming the desired cell type.

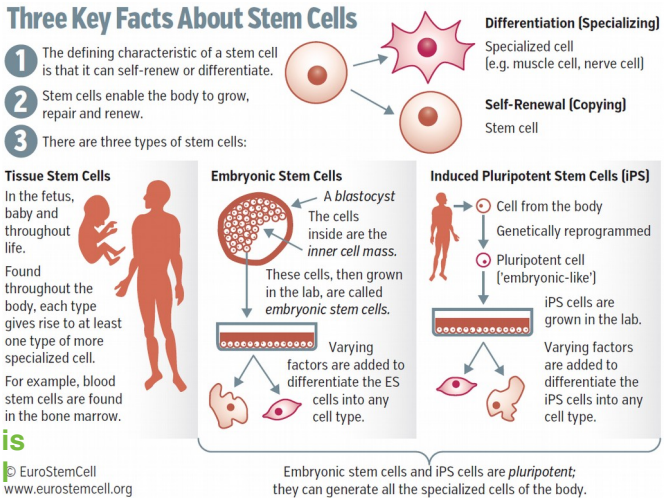

Fig.3 - Types of Stem cells relevant to organoid generation by Alexander van Servellen, 2014

2.Induced pluripotent stem cells (iPSCs):
are very recently, discovered, directly reprogrammed adult stem cells that produce the important induced pluripotent stem cells.
The reprogramming process allows mature cells from an individual to be treated in vitro with genes that can “dedifferentiate” into an ealier pluripotent stage that is very similar to the embryonic stem cells. This new approach allows the generation of cells of any lineage that are identical to the individuals donor cells, a breakthrough for the personalized medicine and biobanks.

3.Adult stem cells: also known as somatic stem cells, are cells that are not yet differentiated. After development they replace dead cells and regenerate damaged tissues, by cell division. In other words they can indefinetly self-renew the cell type of the organ they originate from, forming entire organoids from just few cells. Different variations of adult stem cells include: hematopoietic, epithelial, muscular and neural stem cells. Paticularly Mesenchymal stem cells (MSCs) of stromal origin are highly attractive for clinical usage, as they expand readily in vitro and are able to differentiate into a great spectrum of tissues, provide trophic support and modulate immune responses.

3.Stem Cell Organoid Engineering

Many different organoid generation protocols exist, depending on which specific organ the organoid is to be modelled after. Organoids can be grown from different types of cells, mainly from adult stem cells and from induced pluripotent stem cells (iPSCs), as well as embryonic stem cells (ESCs), donor tissue and progenitor organ cells (adult stem cells). It is important to note that many types organoids can only be produced with specific stem cells. The adult stem cells are immensely important for the generation of various types of pulmonary, neural and cancerous organoids. The induced pluripotent stem cells are irreplacable for the formation of brain cells, which is otherwise not feasible with adult stem cells. There are overlapping factors that allow for the growth of e.g. gut and liver organoids from either adult cells or iPSCs.
While stem cells proliferate to create the organoid system, the behaviour of stem cells is controlled by their surrounding microenvironment. Niche components are derived by either the stem cells themselves or exogenous addition to the system e.g. with small molecules and growth factors. If an organoid system for clear downstream applications is to be formed, further enhancement to control the growth of organoids must be considered. This is done by utilizing systematic engineering approaches to precisely manipulate each structural layer of each process during organoid formation. Most common methods include the modulation of the cells that have already been generated from controlling the stem cell’s self-renewal and differentiation, the direct modification of stem cells, or by manipulating the microenvironment. In this section a generalised method for producing intestinal organoids, developed by Hans Clevers, in many regards considered the father of organoids, will be presented, followed by a look in today’s advances in growing organoids within a novel defined hydrogel matrix, that will also be the a topic to discuss in the interview with Dr. Christian Kurt Hirt.

3.1 Mouse Intestinal Organoid Engineering Protocol

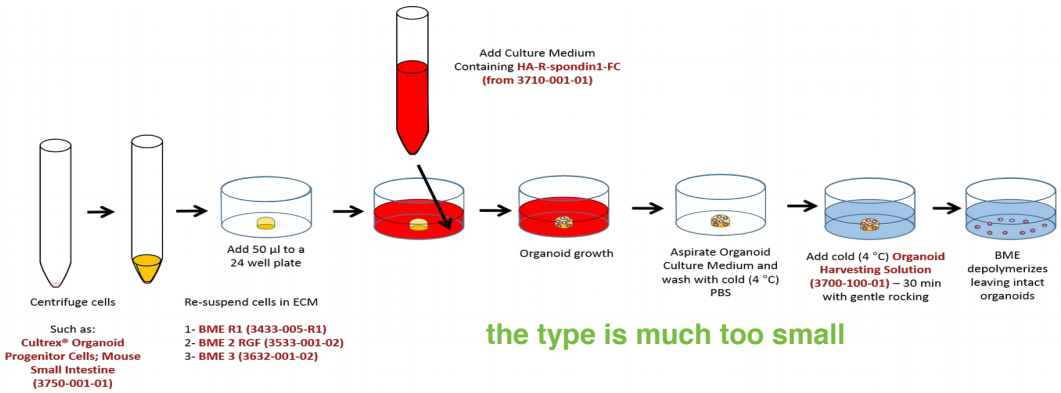

Fig.4 - Workflow of a submerged organoid culture and harvesting method by amsbio, n.d.

1. A 24 well plate is warmed up to 37°C.
2. Stock solutions for small intestine organoid medium as well as 20ml of mouse small intestine medium are prepared.
3. The cryovials containing the small intestine progenitor cells are thawed in a 37°C water bath.
4. The contents of the cryovial are transferred to a 15 ml conical tube and 9ml of Advanced DMEM/F-12[A] cell culture medium is added.
5. The vial is put into a centrifuge at 500 x g for approximately 3 minutes in order to form pellets of the small intestine and the aspirate medium.
6. The organoid progenitor cells are resuspended in 160 µl of RGF BME-R1[A]. Then they are cautiously pippeted up and down for a total of 3 times (being mindful that no bubbles are introduced into the solution) to disperse the cells in the RGF BME-R1. Now 50µl of the RGF BME-R1 cell mixture is added into the center of three wells of the previously warmed 24 well plate. The cells within the hydrogel must not be in contact with the sides of the well.
7. The well plate is incubated for 25 minutes, so that the RGF BME-R1 hydrogel can polymerize.
8. 1.5 ml of the small intestine organoid starting medium are prepared and warmed up to 37°C for 5 minutes. Do not prolong this warming process in any way, as it may detrimentally affect the medium components.
9. 500µl of the heated small intestine starting and passaging medium is added to each well, containing a RGF BME-R1 dome. It should be pippeted into the corner of the well, facing away from the hydrogel, as to ensure the structural integrity of the hydrogel.
10. The plate is returned to the incubator to promote organoid growth.

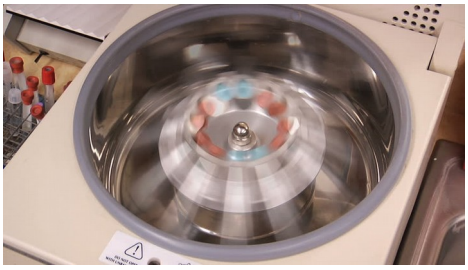

Fig.5 – Spinning centrifuge by Kevin McKeever, n.d.

Fig.6 – Incubator by Memmert, n.d.

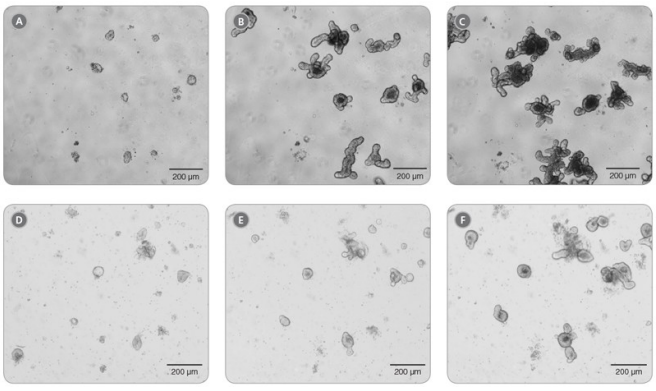

Fig.7 - Organoid Growth after (A) Day 1 (B) Day 5 and (C) Day 7, and colon at (D) Day 1 (E) Day 3 and (F) Day 7 by Stemcell Technologies, 2016

3.2.Epithelial Organoids in a Defined Hydrogel

Now that the generalised procedure of generating intestinal organoids from an organoid medium has been reviewed, a more in-depth look at one of the most modern cultivation methods (Christian Hirt et al.) will be taken. In his paper Growth of Epithelial Organoids in a Defined Hydrogel, Kurt and his colleagues seeked to develop a new protocol, that unlike the common methods, does not rely on an animal- and tumor-derived basement membrane ectract (BME) as a 3D scaffold, that limits theoretical applications in regenerative medicine.
Usually stem cells are firstly embedded into a BME (Matrigel or CultrexBME2) and then supplemented with a number of growth factors that reproduce the intestinal stem cell niche, such as R-spondin, noggin, EGF causing the $Lgr5^{+}$ stem cells to grow into organoids. During their first development phase, they form spherical cysts that predominantly consist of proliferating progenitor cells. After growing them for about 2-4 days in the incubator, they further transform into complex 3D structures with multiple, outward-pointing buds. These crypt-like areas hold the cell types that generally reside in small intestinal crypts, such as the $Lgr5^{+}$ stem cells and the inner core domain found in the organoid mainly contains cell types, which normally line the villi of the intestinal epithelium. Even after either forceful mechanical or enzymatic dissociation, the 3D system will always quickly regenerate and lead to seemingly indefinite expansion of intestinal stem cellts and the primary intestinal epithelium (in vitro). The utilized 3D scaffold BME consisted of a heterogenous mixture of extracellular matrix, abbreviated as ECM, proteins, protoglycans and growth factors secreted from Engelbreth–Holm–Swarm murine sarcomas. These substances contain xenogeneic components that together with batch to batch variations greatly reduce the use of cells of this matrix for clinical applications such as regenerative medicine and high-content screening. Furthermore, due to the heterogenous composition of the BME, precise modulation of physical and biochemical properties is impossible.

This led Hirt and his colleagues attempting to generate a defined fibrin-based hydrogel that supports long term expansion of epithelial organoids. Out of the four different scaffolds, that covered a wide range of biological, physical and chemical characteristics, soft fibrin-martices provide the best physical support and naturally occuring Arg-Gly-Asp adhesion domains on the scaffold and the constant supplementation of laminin-111[0], are best the most important factors for robust organoid formation and continous expansion. reference?
They further discovered that the transition from spherical organoids to budding oranoids was predominantly associated with a shift of force distribution that can be exerted on the matrix. Cystic organoids always apply uniform pressure on the matrix; in more differentiated organoids the pushing forces are primarily restricted to crypt-like domains and the core domains reacted correspondingly with an increase of internal pressure with increased contractility. Hirt and his colleagues essentially created a hydrogel that contains all necessary supplements within a scaffold that exhibits optimal consistency and stiffness, so that organoid expansion can proceed ideally.

4.Applications

Organoids offer a broad scope of promising experimental applications ranging from uncovering truths of human development to manufacturing clinical models for drug testing and creating new possibillities in regenerative medicine. All of those research fields profit greatly from the new prospects that organoids show. The constant drive in seeking out better healthcare is currently in highest demand and therefore most research about organoids is conducted within the biomedical community. However, with the emergence of self-organizing cell structures, the field of human brain research can now access the daunting complexity of the brain. Until now only post-mortem or surgically removed human brains were used for the study of development of this incredibly inctricate organ, that led to several disadvantages during the examination, such as variability in genetic and environmental background and inconsistency in tissue processing and preservation. Only fresh brain samples from

animal model organisms (mice) could be used for studying the principles behind brain development and function, which again differs in many regards from the human counterpart, such as the different location of the radial glial cells (RGCs) and primary neural stem cells, that are found in the ventricular zone of the mouse neocortex. These RGCs along with the outer radial glial cells (oRGCs) in the outer subventricular zone (OSVZ) have over time contributed to the expansion of the human neocortex and are therefore crucial for a better understanding of the human brain (Lui et al. 2011). This is exactly why the organoids may find even greater interest within the field of neurobiology.
This chapter is comprised of the current applications and future prospects of organoids within the medical and biohistoric research areas.

4.1.Clinical Applications

Organoids provide a special platform, that allows for identification of mechanisms in adult human diseases. Cellular organization can now be studied with a level of scrutiny that can lead to advances in functional genomics and protenomics, which includes the analysis of single cells and high-throughput transcriptonomics, proteonimics and a broad characterization of chromatin domains and transcription regulatoty elements. Furthermore, this research could further enhance their repute as models to study organ morphology, function and disease, opening up new possibilities for drug development and regenerative medicine.
Organoids grown from iPSCs have already proven to posess tremendous potential for applications in tissue engineering and regenerative medicine.
In vivo the organoids posess regenerative abilities equally potent to ESCs. Human induced plutipotent cells (hIPSCs)- derived intestinal organoids are comprised of both endoderm and mesoderm cells that are able to differentiate into guts, that are fully supplied with blood (vascularized), when transplanted into immuno-compromised mice. Very rare diseases and mutations can now be traced from the very beginning of mutation and treatments as well as drugs of the highest possible success rate can be deduced by growing and inspecting somatic stem cells from the patient, essentially opening a new realm of personalized medicine and biobanks.

4.2.Single cell genomic atlas uncovering human- specific features of brain development

In 2019, Gray Camp et al. were able to analyze stem cell-derived cerebral organoids using single-cell transcriptomics[B] and accessible chromatin profiling[B] to examine gene regulatory differences that are unique to humans. By inspecting all developmental stages of cerebral brain formation from pluripotency, to neuroectoderm and neuroepithelial stages, and concluded by divergence into neuronal fates in various different brain regions. Although brain region composition faces variation in organoids for different iPSC lines, regional gene expression patterns remain similar across individuals. It was found that human neural development proceeds at a slower pace relative to other primates. By temporal alignment of differentiation paths[B] in humans and, chimpanzees an d great apes, human-specific gene expression that had been determined to distinct cell states along progenitor to neuron lineages within the cortex were discovered.

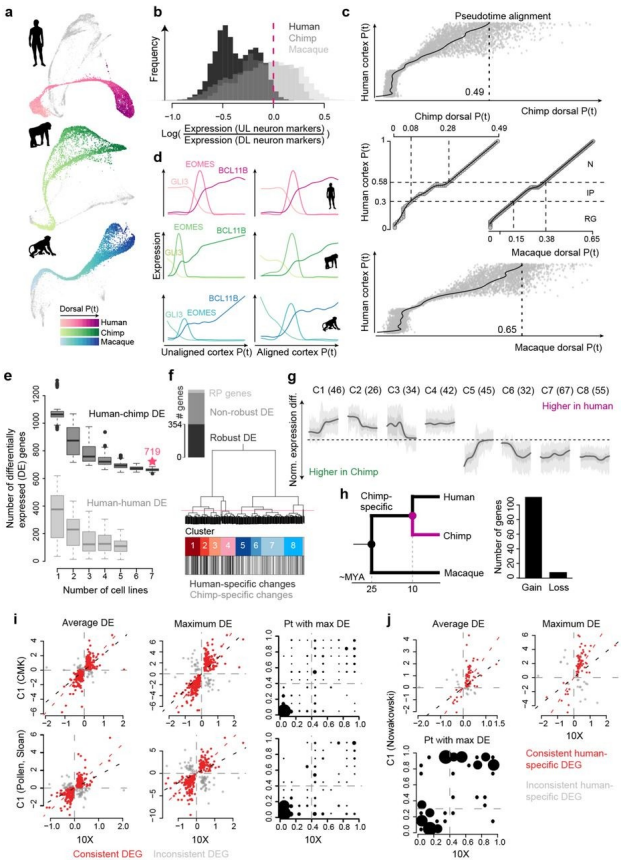

Fig.8 - Pseudotime alignment between primates and differential expression between human and chimpanzee by Sabrina Kanton, 2019

I am not sure I understand everything in this figure, and I definitely can not read it.

Most notable features of Fig.8 include the table (i), which compares the human-chimpanzee pseudotemporal differential expression (DE). It utilizes Fluidigm C1-based sickle-cell sequencing[B] data of human and chimpanzee organoids. The first two columns visualize the initially estimated human-chimpanzee DE directionality and magnitude. The red dots represent consistent DE genes and get bigger with a higher frequency. The two rows represent results derived from C1 data collected by the researchers.

5.Ethical Complications

Main ethical issues related to human cerebral organoids are also found in the research of gastruloids and in vitro fertilization. Although human medicine is sure to benefit greatly from derivation of PSCs and their use, privacy concerns remain popular. Given the relative lack of complexity within cerebral organoid constructions and the absence of mature neural networks, concerns about cognitive function or the ability to think are unfounded for now. But should more advanced bioengineering strategies be found to further mimic the functionality of human brains, through increased vascularization and perfusion, thus generating working neural networks a moral concerns are sure to be raised. The next uprising ambition, to link many human organoids into working systems may trigger even greater questions about the appropriateness of experimenting with cellular structures that so closely resemble a natural human brain.

6.Interview with Dr. Christian Kurt Hirt

Dr. Christian Kurt Hirt is a postdoctoral fellow at the ETH Zurich. Along with Prof. Dr. Gerald Schwank he studies organoids for medical applications and has developed novel hydrogel matrizes that now replace older BME scaffolds. Currently he works with pancreatic organoids derived from patients at the teaching hospital. This paragraph features some general questions regarding technologies and the potential of organoids as well as his paper about their new fibrin-based defined hydrogel.

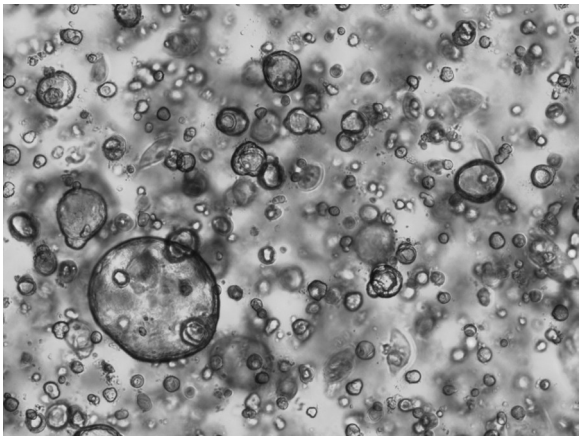

Fig.9 – Electron microscope image of pancreatic cancer organoids grown in a fibrin-based hydrogel by Christian Hirt, 2018

What would you consider to be most fascinating about organoids?

Hirt: That would be their innate ability to group together by themselves, forming a quite limited but eventually functional and intercommunicating cell mass. For that they only require certain growth factors and a fitting temperature.

Why did you choose to research organoids instead of other bioengineering methods when dealing with cancer?

Hirt: Currently there are not that many promising surefire solutions for curing cancer beside the usual surgical treatment or chemotherapies. Both of them do include a certain risk factor which depends on the patient. Instead we want to ensure safety and efficacy of our targeted drug therapies, which are adjusted to fit each patients medical background.

Does that mean there are no near future prospects for cancer treatment but organoids?

Hirt: There are heaps of clinical trials for more common carcinomas underway. This forces us to be reliant on patients that give their consent to experimental treatments and each new treatment must be done repeatedly with patients with the same type of cancer for a conclusive efficacy yield. This means that it is very difficult to again, develop drugs that can fight malignant cancer cells of the rare type. This makes organoids and their uses in personalized medicine especially attractive.

Why are iPSCs more frequently used than ESCs concerning the generation of organoids?

Hirt: Because the chromatin within embryonic stem cells is much more closely packed, which complicates the initiation and differentiation process.

Your Paper Growth of Epithelial Organoids suggests a fibrin/liamin as the best base for a new hydrogel. Why exactly this combination?

Hirt: Initially we attempted to closely imitate the BME scaffolds without including “mouse factors” that could lead to faulty organoid development down the line. At that time we also worked together with groups of researchers that had been experimenting with PEG-based hydrogels. But that proved difficult to finalise, because organoids need a very specific type of environment, one that is quite stiff and promotes cell expansion into spheroids and a fairly soft and hydrolyzing material that hinders cell proliferation at a certain point but still continous with cell differentiation. Fibrin was a long favourite of ours and we experimented with many different concentrations of fibrin, but with the inclusion of liamin, we could not only create a suitable consistency but also introduce factors that promote cell growth.

Are there already treatments that have been cleared for regular usage that based on organoid- testing?

Hirt: There are indeed already a lot of chemo- and anti cancer drugs that have been tested on the basis of organoids and approved of. The disease model system of organoids is after all one of its most promising features.

What do think about transplating (complete) pancreatic organoids?

Hirt: I believe they are very much possible from a theoretical standpoint. There have been experiments where biologists reprogrammed entire organoids, so that they join up with each other to form a mass larger than the upper limit of 5mm, but they do not resemble a real pancreas in structure and they can not exist in vivo without a fully vascularized system.

7.Summary

Although organoid research is still in its infancy compared to other genetic engineering methods, it is arguably one of the most promising discoveries of the past decade, albeit they face many limitations such as lack of developed blood vessels, a defined body axis that exactly mimics real organ areas, their ability to recapitulate cell-specific functions is nothing short of astounding. Modeling is an essential part of the modern scientific method. It enables us to deduce a set of basic principles from observation of complex phenomena. Cellular organoid models are especially ubiquitous due to their relative ease of generation and use.
Dr. Hirt regards them as more realistic disease models than generic 2D cultures and live mice models and in that he sees the primary use of organoids. This technology can contribute to human brain development discoveries, regenerative medecine, partial organ transplants. They provide an effective, less costly and time-consuming alternative to conventional patient drug testing, enable more precise toxicity screening and facilitate treatment of rare mutations and cancers. The next step in organoid research would be finding solutions to vascularize cerebral, cardiac and intestinal cell aggregates, or even combining intestinal, cerebral, liver or any other stem-cell derived organoid and reprogramming them to connect and intercommunicate with the layered combined organoid structures. If blood vessels can be introduced into organoids, then linked to masses of cardiac mucles, spherical organoids of greater sizes than the current limit of 5 mm are not impossible. If this barrier has been overcome, then disease modelling as a whole would be revamped. Complex structures such as brains would be possible to accurately configure for identical to life situations. Not only biological obstacles, but also the ethical and moral aspects of this ever so present technology must be carefully considered. For now organoids can only communicate in simple electrochemical signals, far distanced from real human thought, but the moment that a relatively functional brain is modelled, old controversy about animal experimentation will fade and emerging disputes about the adequacy of creating synthetic human life will be inevitable.

Postface

Firstly I would like to thank the kind Dr. Christian Kurt Hirt for his willingness to participate in the interview and for the organoid images that he sent me. I especially want to express my gratitude that he was able to provide me with pictures of the electron microscopes of his workspace in the university, ableit at that time I had already finished the layout of this paper and sadly could not include it in the paper.

Unfortunately it was not possible for me to visit a research institute due to the current Covid-19 situation. The interview was conducted before the outbreak, but since Dr Hirt lives in Zurich, the questions about organoids had to be asked over the phone, therefore no self-made photos of the research institute were taken.

Write a review book instead ;-)

Initially I wanted to structure the paper “Growth and Applications of Cell-specific of Organoids” in a manner that covered most areas of the genetic engineering method in a detailed, but still intelligible way. At the end however, there was no more writing space left for the section about human-specific features of the brain, so that I could not elaborate in greater detail about the full scope of the research conducted bamp and his colleagues, as well as the many technical terms. Then again, after proof reading the paper several times I do not believe that I could have cut out any parts, since the first pages mainly dealt with the fundamentals of organoid technology. Later in the intestinal organoid generation chapters I did not feel like I should have removed those sections either, since the goal of the semester work was after all about the applications and the workings of genetic engineering. The article following the organoid generation protocol which explains the works of Dr. Hirt could not be removed as they provided necessary information for the research the interview partner is specialized on. Leaving me to believe that the section about human-specific brain features should have been replaced with information that is easier to grasp and explain. But eventually I decided to include a specific appendix for every sequencing technology mentioned in in the chapter and the more advanced methods Gray Camp and his colleagues used to visualize their data. This resulted in a paper structure that builds up in complexity from the fundamentals of organoids, that are thorougly explained, to more difficult and complex chapters at the end of the paper, that could be fit within 8 pages excluding title page, table of context, postface, references and appendices.

References

1. AMSBIO (2020) |
Organoid Culture Handbook (V 2.5)
http://www.amsbio.com/brochures/organoid-culture-handbook.pdf | Last retrieved: 2020, April 14th

2. AMSBIO (n.d.) |
Mouse Small Intestine Organoid Growth and Harvesting Protocols |
http://www.amsbio.com/mouse-intestine-organoids-protocol.aspx | Last retrieved: 2020, April 14th

3. Artegiani B. / Clevers H. (2018) | *Use and application of 3D-organoid technology* |
https://academic.oup.com/hmg/article/27/R2/R99/5001715 | Last retrieved : 2020, April 14th

4. Barbuzano J. (2017) | *Organoids: A new window into disease, development and discovery* |
https://hsci.harvard.edu/organoids | Last retrieved: 2020, April 10th 7

5. Bian S. / Repic M. / Guo Z. et al. (2018) |
Genetically engineered cerebral organoids model brain tumor formation |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6071863/ | Last retrieved: 2020, April 14th

6. Broguiere N. / Hirt C. / Isenmann L. et al. (2018) |
Growth of Epithelial Organoids in a Defined Hydrogel |
https://onlinelibrary.wiley.com/doi/full/10.1002/adma.201801621 |Last retrieved : 2020, April 14th

7. Chang E. / Jin S. / Nam M. et al. (2019) |
Human Induced Pluripotent Stem Cells : Clinical Significance and Applications in Neurologic Diseases |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6732359/ | Last retrieved: 2020, April 19th

8. Clevers H. (2020) |
Hans Clevers (Hubrecht I., UU) 2: Generating Epithelial Organoids from Adult Tissue |
https://www.youtube.com/watch?v=q9_K74L4Qvs | Last retrieved: 2020, April 14th

9. Clinton J. / McWilliams-Koeppen (2018) | *Initiation, Expansion, and Cryopreservation of Human Primary Tissue Derived Normal and Diseased Organoids in Embedded Three Dimensional Culture* |
https://currentprotocols.onlinelibrary.wiley.com/doi/full/10.1002/cpcb.66 | Last retrieved 2020, April 15th

10. Chagastelles P. C. / Nardi N. B. (2011) | *Biology of stem cells: an overview* |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4089750/ | Last retrieved: 2020, April 14th

11. Dahl-Jensen S. / Grapin-Botton A. (2017) |
The physics of organoids: a biophysical approach to understanding organogenesis |
https://dev.biologists.org/content/develop/144/6/946.full.pdf | Last retrieved: 2020, April 14th

12. Evangelista S. (2018) | *Organoids – mimicking our organs in the lab* |
https://actu.epfl.ch/news/organoids-mimicking-our-organs-in-the-lab/ | Last retrieved: 2020, April 14th

13. Hohwieler M. / Illing A. / Hermann P. C. et al. (2017) |
Human pluripotent stem cell-derived acinar/ductal organoids generate human pancreas upon orthotopic transplantation and allow disease modelling |
https://gut.bmj.com/content/66/3/473 | Last retrieved: 2020, April 14th

14. International Society for Stem Cell Reseatch (ISSCR) (2017) |
"Organoids: What is the Science and What are the Clinical Applications?" |
https://www.youtube.com/watch?v=SuthgBZqBk0 | Last retrieved: 2020, April 14th

15. Kanton S. / Boyle M. J. / Treutlein B. et al. (2019) |
Single-cell genomic atlas of great ape cerebral organoids uncovers human-specific features of brain development |
https://www.biorxiv.org/content/10.1101/685057v1.full | Last retrieved: 2020, April 14th

16. Koo B. / Choi B. / Park H. et al. (2019) |
Past, Present, and Future of Brain Organoid Technology |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6776157/ | Last retrieved: 2020, April 19th

17. Lancaster M. A. / Knoblich J. A. / Corsini N. S. et al (2017) |
Guided self-organization and cortical plate formation in human brain organoids |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5824977/ | Last retrieved: 2020, April 14th

18. Lehmann R. / Lee C. M. / Shugart E. C. (2019) |
Human organoids: a new dimension in cell biology |
https://www.molbiolcell.org/doi/full/10.1091/mbc.E19-03-0135 | Last retrieved: 2020, April 15th

19. Madeline A. L. / Meritxell H. (2019) |
Disease modelling in human organoids |
https://www.ncbi.nlm.nih.gov/pmc/artiles/PMC6679380/ | Last retrieved: 2020, April 10th

20. Munsie M. / Hyun I. / Sugarman J. (2017)
Ethical issues in human organoid and gastruloid research |
https://dev.biologists.org/content/144/6/942 | Last retrieved: 2020, April 15th

21. Muotri A. R. (2019) | *Brain organoids and insights on human evolution* |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6544132/ | Last retrieved: 2020, April 19th

22. Servellen A. v. / Oba I. (2014) |
Stem cell research: Trends in and perspectives on the evolving international landscape |
https://www.researchtrends.com/issue-36-march-2014/stem-cell-research/ |Last retrieved 2020, April 14th

23. Sinagoga K. L. / Wells J. M. (2015) |
Generating human intestinal tissues from pluripotent stem cells to study development and disease |
https://www.embopress.org/doi/10.15252/embj.201490686 | Last retrieved: 2020, April 14th

24. Takasato / Chiu / M. H. (2016) |
3D Organoid Culture: New In Vitro Models of Development and Disease |
https://www.sigmaaldrich.com/technical-documents/articles/biology/cell-culture/3d-organoid-culture.html |
Last retrieved: 2020, April 15th

25. Wei N. / Quan Z. / Tang H. et al. |
Three-Dimensional Organoid System Transplantation Technologies in Future Treatment of Central Nervous System Diseases |
https://www.hindawi.com/journals/sci/2017/5682354/ | Last retrieved: 2020, April 15th

26. Yin X. / Mead. B. E. / Safaee H. et al. | *Stem Cell Organoid Engineering* |
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4728053/ | Last retrieved: 2020, April 10th

27. Zimmer K. (2018) | *First iPS Cell Trial for Heart Disease Raises Excitement, Concern* |
https://www.the-scientist.com/news-opinion/first-ips-cell-trial-for-heart-disease-raises-excitement--concern-64743 | Last retrieved: 2020, April 19th

28. Zimmer K. (2018) | *Increasing Number of iPS Cell Therapies Tested in Clinical Trials* | *https://www.the-scientist.com/news-opinion/increasing-number-of-ips-cell-therapies-in-clinical-trials--65150* |
Last retrieved: 2020, April 14th

Appendices

Appendix A : List and explanation of surface markers and genes connected to pluripotence

Surface markers, or cell surface markers, are proteins and carbohydrates found within the lipid bylayer of cell membranes. They include antigens, receptors and other molecules and play a major role in intercellular communication and recognition (Don-Wook Shin and Ted Xiao, 2012).

Surface marker / gene	Function	Reference
CD-9	Immune cell marker, activates thrombocytes and realises fusion of egg and sperm cell	BD Biosciences, 2010
CD-24	Cell adehesion molecule, contributes to a variety of downstream applications and is irreplacable for neural development	
Oct-4	First master gene required for stemness properties of murine and primate embryonic stell cells (PESC)	Zeineddine et al., 2014
Rex-1	Marker for pluripotency, regulates pluripotent states	Naira C. Rezende et al., 2011
Sox-2	Maintainance and acquisition of pluripotency, also a cofactor of Oct-4	Frederick C.K. Wong et al., 2016
Nanog	Transcription factor involved in self-renewal of ESCs, maintanance of undifferentiated pluripotent cells	Natalia Gawlik-Rzemieniewska and Ilona Bednarek, 2015

Table 1 – List of surface markers and genes linked to pluripotent genes and funktions

Appendix B : Sequencing technologies and data visualization utilized in chapter 4.2.

Sequencing technology/ visualization method	Description	Reference
Single-cell transcriptonomics	Single-cell transcriptonomics examines gene expression of cells by comparing the messenger RNA concentration of genes.	Itamar Kanter and Tomer Kalinski, 2015
Accessible chromatin profiling (ATAC-seq)	Accessible chromatin profiling allows for determination of the degree that nuclear macromplecules are physically able to contact chromatinized DNA.	Sandy L.Klemm et al, 2019
(Pseudo)-Temporal analysis	Also known as trajectory inference is a technique used in single-cell transcriptonomics. It can visualize patterns of dynamics of a cell, i.e. the folding of proteins into distinct three dimensional structures, and arrange cells based on the pace and progression.	Christina Kendziorski,2016
Fluidigm C1	Novel automated solution for single-cell genomics research. It prepares sing-cell templates for mRNA and DNA sequencing, epigenetics or miRNA expression.	Fluidigm, 2015

Table 2 – List of various sequencing and visualization methods applied in chapter 4.2

Appendix C : In vitro fertilization and resulting ethical dispute

In vitro fertilization describes the procedure in which mature egg cells are fertilized with sperm cells outside the body and then placed into the uterus of another woman, so that gastation of an embryo inside an unralated woman occurs.
Since its initial development stages in vitro fertilization has been a sourve of moral, ethical and religious dispute. There has been an official doctrinal statement resisting IVF from the Roman Catholic church in 1987, that based their opposition on three reasons: the destruction of human embryos; the possibility of in vitro fertilization by a donor other than the married husband and severing the essential bond between the sexual act and procreation.
There have also been cases of increased percentages of twins with IVF and unresolved moral concerns regarding the cryopersavation of ovaries, eggs, sperm cells or embryos for future pregnancies (Editors of Encyclopedia Britannica, 2020)