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The use of induced pluripotent stem cells in regenerative medicine

by

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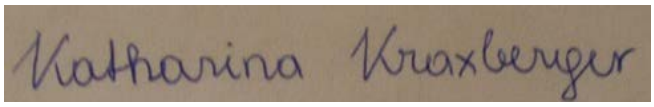

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ABSTRACT

Two characteristics define stem cells. They have either the ability of self-renewal or they can differentiate in different cell types. All tissues of the body are build out of Stem cells. Embryonic stem cells are pluripotent stem cells from the human blastocyst. To be a pluripotent stem cell, means that the cells can differentiate into all cell types of the body. The first successfully isolation of embryonic stem cells from human by Thomson, et al. (1998) appeared to offer unlimited facilities in term to understand early human development, tissue formation and differentiation *in-vitro*. Further it seemed to offer unlimited facilities to model diseases, discover disease mechanisms and use cell therapy for untreatable disease conditions. But research on embryonic stem cells was shortly afterwards claimed as not ethically and therefore different laws, which prohibited research on embryonic stem cells, had been established.

To overcome these drawbacks, Takashi and Yamanaka (2006) created new methods in basic research, disease modeling and regenerative medicine. They established the induced pluripotent stem cells by the use of only four transcription factors. Induced pluripotent cells are adult somatic cells which are reprogrammed back to the pluripotent state. Therefore, induced pluripotent stem cells show similar properties to embryonic stem cells, maybe overcome the ethical question and provide an unlimited tool for disease modeling and drug screening. Patients who suffer from degenerative diseases like Diabetes Mellitus, Myocardial infarction, Age related degeneration or Alzheimer could be treated with the use of induced pluripotent stem cells.

The aim of this thesis is to present in detail what induced pluripotent stem cells are, how they are derived, how they differ from embryonic stem cells and how they can be used in regenerative medicine. To understand these, also the explanation of some important transcription factors, genes and signaling pathways is necessary. Another aim of this thesis is to present the current research status in the use of induced pluripotent stem cells to treat Diabetes Mellitus, Retinal regeneration (Age related macular degeneration) and the heart for example after a myocardial infarction.

Finally the aim of this thesis is to present basic data about the Ethical question – also in induced pluripotent stem cells, and to compare the law of stem cells in the USA and in Europe.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF ABBREVIATIONS

45CFR46	title 45 in part 46 of the *Code of Federal Regulations*
AMD	age-related macular degeneration
BM	bone marrow
BMP	bone morphogenic protein
BMPs	bone morphogenetic proteins
CFR	*Code of Federal Regulations*
CMs	cardiomyocytes
CNV	copy number variant
CNVs	copy number variants
CoMiP	codon-optimized 4-in-1 minicircle
CRISPR	clustered regularly interspaced short palindromic repeats
CVD	cardiovascular disease
DM	Diabetes mellitus
DMR	differentially methylated regions
E	embryonic day
EB	embryoid body
EBNA	Epstein-Barr virus-encoded nuclear antigen-1
EGA	embryonic genome activation
ESCs	embryonic stem cells
FDA	Food and Drug Administration
FGFs	fibroblast growth factors
fRPE	fetal human retinal pigment epithelium
GSK	glycogen synthase kinase
hESCs	human embryonic stem cells
iPSCs	induced pluripotent stem cells
LIF	leukemia inhibitory factor
MEFs	mouse embryonic fibroblasts

MSCs	mesenchymal stem cells
NIH	National Institutes of Health
PBMCs	peripheral blood mononuclear cells
PDGF	platelet-derived growth factor
PH	polyhormonal
PSC	pluripotent stem cell
PSCs	pluripotent stem cells
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
SC	stem cell
SCs	stem cells
STGD	stargardt´s disease
T1DM	Diabetes mellitus Typ 1
T2DM	Diabetes mellitus Typ 2
TALEN	activator-like effector nuclease
TF	tanscription factor
VEGF	vascular endothelial growth factor
WNTs	wingless/INT proteins
ZFN	zinc finger nuclease

1. INTRODUCTION

1.1. Introduction in Cell Biology

All living things are built out of cells. The human body is for example build out of trillions of cells. The cells take in nutrients from food, convert nutrients into energy, provide structure to the body and carry out specific functions.
Cells also contain the body´s hereditary material in the DNA which is stored in the nucleus. The cell can make copies of their DNA. The DNA is stored in the nucleus because cells have many parts with different functions. The parts, which perform certain tasks within a cell, are called organelles. The basic organelles in a human cell are: Mitochondrium, Cytoskeleton, Cytoplasm, Endoplasmic reticulum, Golgi apparatus, Lysosomes, Peroxisomes, Nucleus, Plasma membrane and Ribosomes (Genetics Home Reference, 2014, p.4,5).
For the understanding of the molecular biology of cells, there is a lot of research going on at the moment, which is not only fundamental to all the biological sciences, it is also important for the growing numbers of applications in biotechnology, agriculture, biomedical engineering and medicine. Because the human genome is already sequenced completely, the progress in cells and molecular biology opens a new horizon in the practice of medicine. Examples are the identification of genes, which account to a variety of common diseases such as diabetes or heart diseases. Also the development of new drugs and the potential use of stem cells (SCs) to replace damaged tissues to treat patients who suffer from illnesses like diabetes, Parkinson´s disease, Alzheimer´s disease or spinal cord injuries are examples for the practice of medicine (Cooper and Hausman, 2014, p.3).

1.2. Introduction in SCs

SCs are cells with two defining properties. They have the capacity to self-renew and the ability to generate differentiated cells, which is referred as Potency. This means, that SCs are neither terminally differentiated nor the end of a differentiation pathway. They are able to divide indefinitely, because they are single cells with the capacity to create more stem cells.

If a Stem Cell (SC) divides the two developing daughter cells have either the choice to remain stem cells, or one of them can go into differentiation (Lanza and Atala, 2013, p.5,6). This can be seen in figure 1.

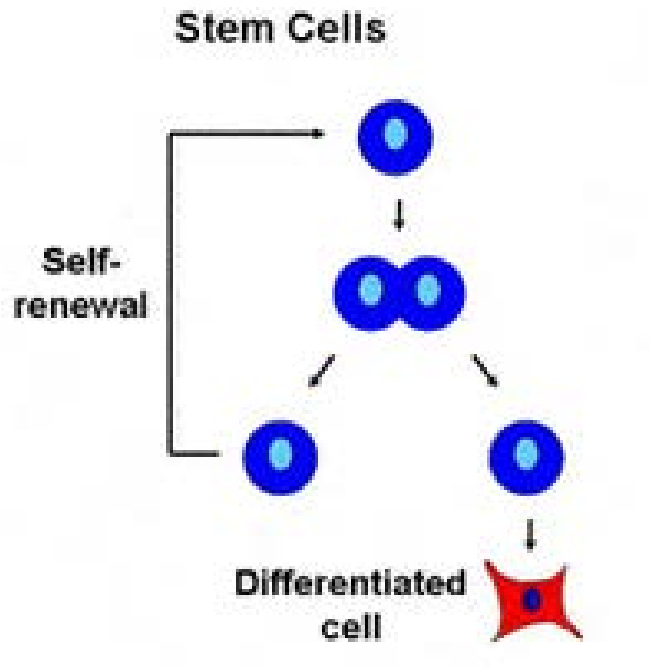

Figure 1: The two defining properties of SCs:
The capacity to self-renew and the ability to generate differentiated cells

Source: Massachusetts General Hospital, 2014. [image online] Available at: < http://www.massgeneral.org/regenmed/stemcells/whatarestemcells.aspx> [Accessed 21 January 2015]

1.2.1. Potency

As mentioned above, potency is an important property for defining SCs (Lanza and Atala, 2013, p.5,6). Potency is the skill of a SC to differentiate into a specialized cell type and further the ability to give rise to more mature cell types. SCs have different potentials to differentiate into different cell types (Hima, Bindu and Srilatha, 2011). These differentiation potentials are now explained.

Totipotent SCs:
Totipotent SCs are cells which are produced by the fusion of an egg and a sperm. They can form all embryonic and extraembryonic cell types including primitive germ-line stem cells and can in this way construct a whole viable

organism. They only exist in the fertilized egg and in the cells of the early embryo which result from the first cell divisions (Hima, Bindu and Srilatha, 2011).

Pluripotent SCs (PSCs):
PSCs can give rise to most cell types, which means that they form both, somatic cells and germ cells. They are characterized by a differentiation potential to all cell types of the adult organism and to self-renewal (Hima, Bindu and Srilatha, 2011). There are two types of PSCs. The induced pluripotent stem cells (iPSCs) (Takashi and Yamanaka, 2006) and embryonic stem cells (ESCs) (Gail, 1981).

Multipotent SCs:
Multipotent SCs can give rise to several cell types but only to cell types, which are closely related. Due this fact, they can only differentiate into a small number of cell types.
An example will be the cells in the bone marrow, which are able to give rise to blood cells, but are not able to give rise to any other cell types (Hima Bindu and Srilatha, 2011).

Oligopotent SCs:
Oligopotnet SCs can only differentiate into few cells. Examples for these SCs are lymphoid or myeloid SC (Hima Bindu and Srilatha, 2011).

Unipotent SCs:
Unipotent SCs can differentiate only in one cell type, but have also the property of self-renewal. Examples for these SC are muscle stem cells (Hima Bindu and Srilatha, 2011).

1.2.2. Self-Renewal

Self-renewal means that the potency of cells is obtained during cell division (Lanza, R. ed., 2009, p.73). Cell division is in this context also called mitosis. Mitosis is a conservative process, with the objective to produce two identical daughter cells. In Mitosis, the cell divides only once and the chromosome number is maintained as 2N. In mitosis, no pairing or crossing over of homologous chromosomes takes place (Cooper and Hausman, 2014, p.669).

Self-renewal means that in minimum one daughter cell must have the same capacity of self-renewal and differentiation than the parent cell. There exist two ways of self-renewal: the symmetric cell division and the asymmetric cell division (Lanza, R. ed., 2009, p.73).
During the symmetric cell division, two SCs with the capacity of differentiation and self-renewal are generated. This is often the case in transient stem cells during early embryonic development to increase body size.
During the asymmetric cell division, one daughter cell remains a SC, and the other daughter cell becomes a differentiated progeny cell or a SC with a limited capacity of differentiation. This is often the case in permanent stem cells. Permanent stem cells appear during later stages of development in embryos and in adults to maintain the homeostasis (Lanza, R. ed., 2009, p.73). This is important because some cells are lost in the adult organism due to cell death. The cell death is balanced by cell proliferation. This is important to maintain a constant number of cells in adult tissues and organs. Due to this fact, most tissues include cells, which are able to proliferate in order to replace dead cells and to ensure homeostasis (Cooper and Hausman, 2014, p.693).

It is already known, that self-renewal is regulated by extrinsic factors.
These factors control gene expression by regulating transcription factors. OCT3/4, for example, is a transcription factor which is vital for self-renewal or differentiation. However the processes, which accomplish self-renewal, are still not defined. This is because the mechanisms of apoptosis, cell cycle regulation and telomerase activity have not been fully elucidated (Lanza, R. ed., 2009, p.73).

1.2.3. Proliferation of Differentiated Cells (specific: Fibroblasts, Endothelial and Epithelial Cells)

The most of the differentiated cells in adult animals are not able to proliferate. If differentiated cells are lost, they must be replaced by the proliferation of less differentiated cells which are derived from self-renewing SCs.
But some types of the differentiated cells keep the ability to proliferate. So these cells are able to repair damaged tissue constant through the life of the organism

(Cooper and Hausman, 2014, p.693). These types of cells are able to resume proliferation as needed to replace cells which have been injured or died. Fibroblasts, (Fuchs and Horsley, 2008) endothelial cells (Carmeliet, 2003) and epithelial cells (Blanpain and Fuchs, 2009) are examples of cells which remain capable for proliferation throughout the life. How these three examples remain capable for proliferation is now explained.

Fibroblasts are dispersed in connective tissues where they have the importance to secrete collagen. Skin fibroblasts for example are normally arrested in the G0 stage of the cell cycle, but if needed the skin fibroblasts can rapidly proliferate to repair damaged tissue, which results from a cut or from a wound. After a cut the release of platelet-derived growth factor (PDGF) results in blood clotting at the site of the wound and it activates a receptor protein-tyrosine kinase. The protein-tyrosine kinase stimulates the proliferation of fibroblasts and their migration into the wound. In the wound the fibroblasts proliferate and secrete collagen which contributes to repair and regrowth the damaged tissue (Fuchs and Horsley, 2008).

Endothelial cells, which line blood vessels, are able to form new blood vessels if they are needed for repair, and regrowth of damaged tissue. The proliferation of these cells is triggered by the vascular endothelial growth factor (VEGF). The VEGF is a factor which is produced by cells of the tissue that the new capillaries will invade. The manufacturing of VEGF is triggered by a lack of oxygen. Therefore it results is a regulatory system. Tissues with a low oxygen supply, which results from insufficient circulation, promote endothelial cell proliferation and recruit new capillaries (Carmeliet, 2003).

Epithelial cells, which lie for example in liver cells and some other internal organs, are also able to proliferate. If for example a huge number of liver cells are damaged due a surgical removal of a part of the liver, the remaining cells are able to proliferate to replace the missing tissue (Blanpain and Fuchs, 2009). This ability to proliferate was for example tested in the liver of rats. Two-thirds of a rat-liver was removed surgical and the remaining cells really regenerated the entire liver in a few days (Taub, 2004).

However, most of the other differentiated cells like cardiac muscle cells are no longer able to undergo cell division which means that the cells are not able to proliferate. But they can be replaced by the proliferation of SCs which are present in most adult tissues (Wu, Chien and Mummery, 2008). Especially cells with a short life span like blood cells, sperms and epithelial cells of the skin and the digestive tract must be replaced continually in adult animals. They must be replaced continually because they are fully differentiated and are therefore not able to proliferate (Cooper and Hausman, 2014, p.694).

1.3. Types of SCs in Mammalian

We can distinguish between two types of stem cells in mammalian. The ESCs and the adult stem cells, which are also called somatic stem cells (National Institutes of Health).

1.3.1. ESCs

1.3.1.1. History of ESCs

Gail (1981) cultured the first ESCs from mouse embryos, exactly from the inner cell masses of blastocysts. He obtained that the cells from the inner cell mass are able to propagate indefinitely in culture. He also obtained that the cells are able to give rise to all tissue cells if they are reintroduced into early embryos. Due this fact, it could be concluded that cells from the inner cell masses of blastocysts are pluripotent cells, which means, as previously described, that they can give rise to all different types of cells in adult organs and tissues. These pluripotent cells can also be induced in culture to differentiate into a lot of different types of cells (Gail, 1981).

Due the foundation from Stevens (1970) that early mouse embryos quite often develop into tumors if they were removed from the uterus and transplanted to an abnormal site, it can be concluded that embryonic carcinoma cells were derived from normal embryonic cells.

Based on this precondition, Gail (1981) tried to culture cells from normal mouse blastocysts. In her study she isolated four colonies of growing cells after a week of culture from 30 embryos. She showed that the cells were able to repeatedly passage into mass cultures. She also showed that new cell lines were reproducibly derived by the repetition of the experiment with additional mouse embryos. She further showed that the ESCs, which were derived from normal embryos, share similarities with the embryonic carcinoma cells which were derived from tumors. Another finding of her study is that the ESCs are able to differentiate in culture into a lot of cell types, like endodermal cells, neuron-like cells or cartilage. She also injected the ESCs into a mouse, and showed the formation of tumors which contains multiple differentiated cell types. She concluded that ESCs could be introduced in culture of normal mouse embryos. This offers a new possibility for the treatment of a variety of human diseases (Gail, 1981).
The experiments form Gail (1981) and similar work from Evans and Kaufman (1981) demonstrated that ESCs can be cultured directly from normal mouse embryos.

1.3.1.2. human ESCs

Thomson, et al. (1998) indicated the first lines of human embryonic stem cells (hESCs) from human embryos. HESCs may provide a big advantage for the treatment of a lot of diseases in the future because of their differentiative and proliferative capacity. HESCs supply a model system for studying the molecular and cellular events which are connected with embryonic cell differentiation. But a lot of technical problems and ethical concerns still remain (Thomson, et al., 1998).

The hESCs are derived from embryos. These embryos have been developed from eggs, which have been fertilized *in-vitro* (in i*n-vitro* fertilization clinics). This means, that the hESCs are not obtained from eggs which were fertilized in a human, female body. They are obtained from a four to five old day old blastocyst. A blastocyst is a hollow ball which consists of three structures. This is the trophectoderm (surrounding layer), the blastocoels (cavity inside) and the

inner cell mass (approximately 30 cells). The embryonic stem cells are isolated from the inner cell mass (National Institutes of Health).

There exist also differences between the ESCs from mice and the hESC. Mouse ESCs can only be cultured in the presence of the growth factor LIF (leukemia inhibitory factor). This growth factor is important because it signals through the JAK/STAT pathway, and maintains the ESCs in their undifferentiated state. If LIF would be removed from the medium, the cells would firstly pile up into structures which are equal to embryos (also called embryoid bodies) and would then differentiate into a wide range of cell types like beating heart muscle cells, neurons or epithelial cells. In contrast hESCs do not need LIF but needs other growth factors to keep in the undifferentiated state (Thomson, et al., 1998). If particular growth factors or small molecules like retinoic acid are added, the ESCs are able to differentiate along specific pathways (Strickland and Mahdavi, 1978).

Due that fact, it is possible to obtain population specific types of cells for transplantation therapy. A lot of current research focuses on the development of protocols which provide efficient differentiation of ESCs along specific pathways with the aim to produce differentiated cells. The differentiated cells could further be used as transplants for a lot of different diseases (Cooper and Hausman, 2014, p.703).

1.3.1.3. The Tumor Forming Potential of ESCs

Stevens (1970) found out, that early mouse embryos quite often develop into tumors when they are removed from the uterus and are transplanted to an abnormal site. These developing tumors are called teratocarcinomas. Stevens (1970) analyzed the teratocarcinomas and found out that the contained cells are able to form numerous different tissues if they grew in an animal. He isolated cells from teratocarcinomas and obtained that they are able to grow in tissue culture. These cells could be differentiated into a lot of different cell types in culture. The injection of some of these teratocarcinomas cells into mouse blastocysts and the implantation of the blastocyst in a foster mother showed that some of these carcinoma cells take part of the normal development of a mouse.

Therefore, the differentiation ability of the embryonic carcinoma cells and the participation in normal mouse development advised that theses tumor-derived cells maybe closely related to normal ESCs. But the processes of development during the establishment of teratocarcinomas in mice were not known yet (Stevens, 1970).
Gail (1981) assumed later, that these embryonic carcinoma cells from the teratocarcinomas were essential for normal embryo cells.

1.3.2. Adult/Somatic Stem Cells

The adult SCs are also called somatic SCs. These are undifferentiated cells, which are found in differentiated tissues or organs of an adult mammalian. Their function is to maintain and repair tissues in living organism. The origin of these cells is still not known, but they are found in a lot of different tissues (National Institutes of Health).
Adult SCs occur in local tissue microenvironments, which are called SC niches. Niches provide environmental signals, which help these cells to remain SCs by controlling the balance between their self-renewal and their differentiation. Although SCs are rare in adult mammalian tissues, the SC niche provides a request for SC biology. The existence of SCs in the niche is the key for the regulation of the homeostasis of cells and account for aging and tumor genesis during adulthood (Morrison and Spradling, 2008). Adult SCs have the ability to repair damaged tissue. Therefore they have a great potential for the use of clinical medicine. If it is possible to isolate and culture these cells, they may be used to replace damaged tissue. With Adult SCs in culture, a lot of different disorders like diabetes, or degenerative diseases like Alzheimer´s could be treated. This is may be an optimal procedure, but the ESCs and the iPSCs provide a more multifunctional treatment for more different diseases.
However the most established clinical application of adult SCs is the haematopoietic stem cell transplantation (bone marrow transplantation). The bone marrow transplantation plays a major role in the treatment of a diversity of cancers (Cooper and Hausman, 2014, pp.701).

1.4. Somatic Cell Nuclear Transfer–Cloning

1.4.1. History of Somatic Cell Nuclear Transfer

After the isolation of the first hESC from Thomson, et al. (1998), demonstrations were published that a nucleus of an adult mammalian cell is able to differentiate into a whole, viable cloned animal.
Wilmut, et al. (1997) successfully cloned the sheep Dolly and induced with this ability a new field of regenerative medicine. They took a nucleus of a mammalian epithelial cell and transplanted it into an unfertilized, enucleated egg and obtained Dolly (Wilmut, et al., 1997). This process is called somatic cell nuclear transfer (Wilmut, et al., 2002). Since this initial success, also other animals like goats (Baguisi, et al., 1999), pigs (Betthauser, et al., 2000) or dogs (Lee, et al., 2005) have been cloned successfully.
It is interesting that this type of experiment was first carried out already in the 1950s with frogs (Gurdon, 1962). It took over 40 years to clone successfully, which let conclude, that there are still technical difficulties in the procedure (Wilmut, et al., 2002).

Cloning with the use of somatic cell nuclear transfer in mammals is with only 1-2% efficiency a very inefficient method. The small number of surviving animals after birth often shows a variety of abnormities which result in a limited lifespan. Also Dolly the sheep lived only 6 years, which is a limited lifespan, because a normal sheep lives around 12 years. These facts reflect the difficulties of completely reprogramming of the epigenetic state of an adult nucleus, including the difficulty of the reversal of DNA methylation (Rhind, et al., 2003).

1.4.2. Therapeutic and Reproductive Cloning

In general there exist two types of cloning. These are the therapeutic cloning and the reproductive cloning. Both of these types use the method of somatic cell nuclear transfer.
In the reproductive cloning, an adult somatic egg is transferred into the nucleus of the enucleated egg. Then the cells are cultured to an early embryo. After doing so the Blastocyst is transplanted into a foster mother and the foster mother

gives further birth to a clone. Dolly the sheep was for example cloned by the use of the reproductive cloning method (Wilmut, et al., 2002).

In the therapeutic cloning a nucleus from an adult human cell is also transferred into an enucleated egg. This egg would then produce an early embryo in culture and the so produced ESCs could then be used to generate different cells for transplantation and therapy This method would have advantages because the ESCs, which could be derived by this method, would be genetically identical to the recipient of the transplant and would due that fact avoiding immune rejections after transplantation (Thompson, et al., 1998).
But some barriers need to be overcome before therapeutic cloning could be applied to humans. The low efficiency, with which embryos are generated, should be improved. Also ethical concerns arise because of the damage of embryos which serve as a source of ESCs. Advancements in reprogramming of somatic cells to a pluripotent state, which are equal to ESCs may overcome this concerns (Rhind, et al., 2003).

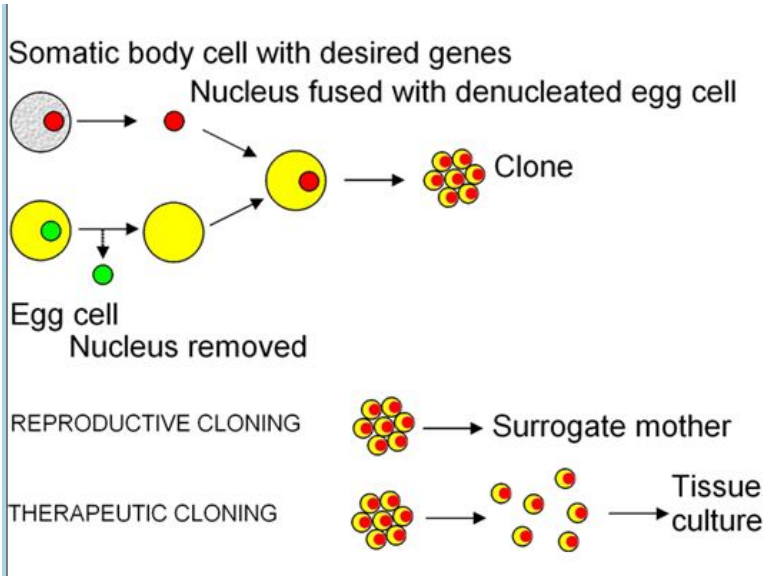

Figure 2: Therapeutic and reproductive cloning:

The two methods for cloning. An adult somatic egg is transferred into the nucleus of an enucleated egg. Then an early embryo is developed. After doing so, the embryo can be transferred into a surrogate mother, for doing reproductive cloning. For therapeutic cloning the cell could then be used to generate different cells for transplantation and therapy.

Source: Arnold, P., 2010. Reproductive cloning. [image online] Available at: < http://www.brighthub.com/science/genetics/articles/63436.aspx# > [Accessed 21 January 2015]

1.5. Differentiation in Early Development

The development of the human embryo starts with an oocyte to embryo transition. This means the stage of development in which the molecular programs of the embryo are activated and the molecular programs of the oocyte are degraded. The oocyte to embryo transition takes from the embryonic day (E) 0 to 3 and includes the fusion of the egg and sperm, the migration and fusion of the germ cell pronuclei and genetic and epigenetic reprogramming. Also a series of cleavage divisions through mitosis, in which the net size of the embryo stays the same starts in this stage of development. In human there exist three cleavage divisions. These are divisions from the 1 cell state to the 2 cells state, from 2 cells to 4 cells and from 4 cells to 8 cells. Between the 4 to 8 cells cleavage the embryonic genome activation (EGA) starts (Niakan, et al., 2012). The EGA differs in time from other species. In the mouse for example the EGA occurs during the 1-to 2-cell stage (Wang, et al., 2004).
After the EGA the embryo undergoes compaction. This means the formation of a cluster of cells, which is also called the morula. The morula is the first morphological induction which does not have radial symmetry. Following cell divisions develops a blastocyst. A blastocyst consists of three layers. These are a cavity, an inner cell mass and a trophectoderm. The cavity is fluid- filled and surrounded with the inner cell mass and the inner cell mass is surrounded by the trophectoderm which consists of extra-embryonic cells. The inner cell mass consists of pluripotent cells, which are able to give rise to all cells of a fetus. The extra-embryonic cells of the trophectoderm give later rise to the placental cytotrophoblast, syncytiotrophoblast and extravillous trophoblast (Niakan, et al., 2012).
The inner cell mass splits itself into early epiblast, which contain pluripotent cells, and into primitive endoderm, which contain cells which are able to form the York sac. This splitting happens before the blastocyst is able to implant into the uterine wall. The implantation into the uterine wall, which happens in humans around day 7, is necessary for further embryo development (Niakan, et al., 2012).

Once the embryo has implanted *in-vitro* into a host blastocyst to give rise to a chimera, the embryonic cells lose their ability to assist to the embryo. But when

the cells are implanted into genetically identical adult organism, the epiblast cells can generate teratocarcinomas, which are tumors that contain tissues from the three germ layers and embryonic carcinoma stem cell population. The three germ layers are endoderm, mesoderm and ectoderm. The embryonic carcinoma stem cells are able to form mouse chimeras. Some studies also evidence the ability of primordial germ cells to become embryonic germ cells in culture and are able to regain pluripotency (Lanza, R. ed., 2009, p.119-122).

A survey of cell lineage relationships in the early mouse shows the figure below (Lanza, R. ed., 2009, p.121).

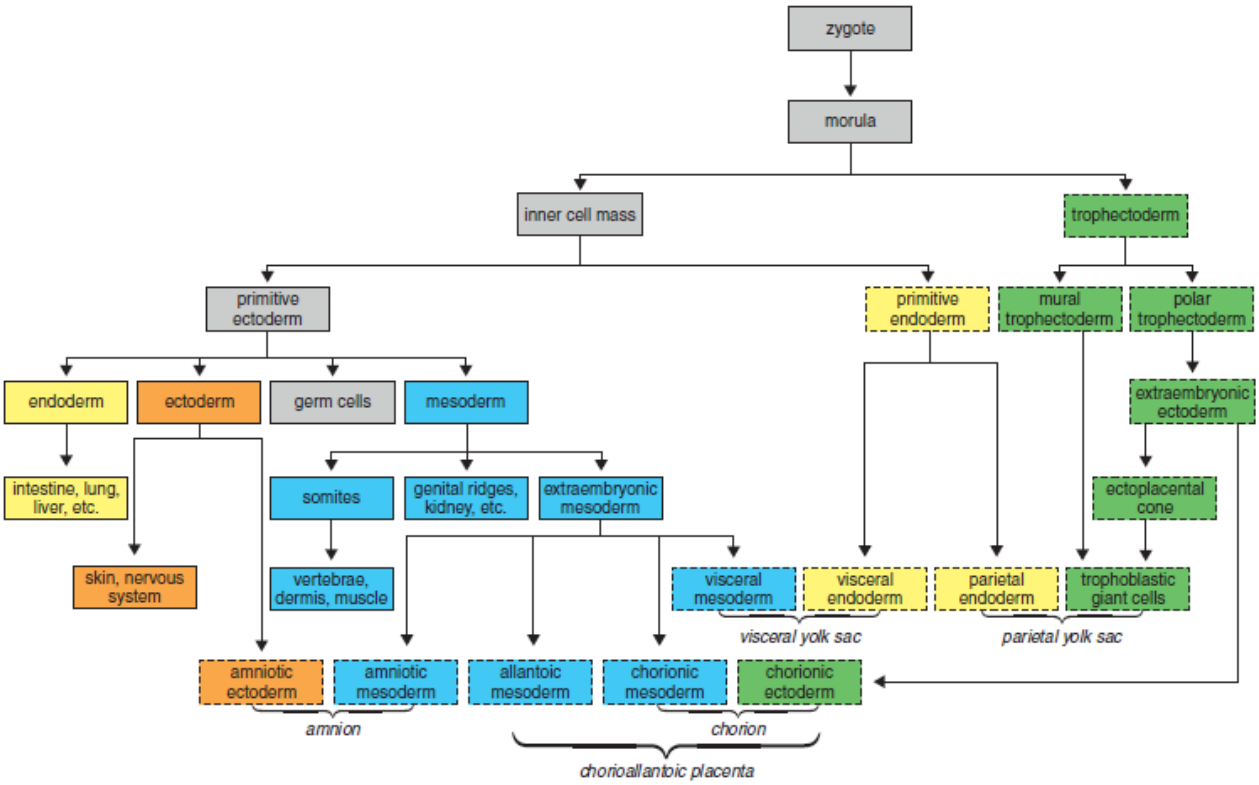

Figure 3: The lineages of the cell in the development of mice:

The three germ layers are ectoderm, endoderm and mesoderm. The tissues which are derived from the trophectoderm are green, from the endoderm are yellow, from the ectoderm are orange and from the mesoderm are blue painted. Pluripotent cells are gray painted.

Source: Lanza, R. ed., 2010. *Cell lineages in mouse development*. [image p.121]

1.6. Introduction into Induced Pluripotent Stem Cells

1.6.1. The Feature of Pluripotency

As mentioned above, a pluripotent Stem Cell (PSC) can give rise to an entire organism and can generate every cell type in an organism. This means, that pluripotent cells are able to form each of the three embryonic germ layers: the endoderm, ectoderm and mesoderm (Robinton and Daley, 2012).
In 1891, the characteristic of pluripotency was first reported by Driesch. He isolated two cells of a sea urchin blastocyst and observed the development of two complete sea urchins (Dreisch, 1891). In the 1960s and 1970s, Mintz and colleagues (Dewey, et al., 1977), Brinster (Brinster, 1974) and Gardner (Gardner, 1968) published studies about embryo aggregation and blastocyst chimaerism in mice and solidified the idea, that the cells of the inner cell mass of the mouse blastocyst are pluripotent cells.
In 1981, Evans and Kaufman (Evans and Kaufmann, 1981) and Martin (Martin, 1981) opened up the field to culture PSCs in a dish. They isolated mouse teratocarcinoma SC and native ESC. In 1998, Thomson and colleagues isolated successfully the first human ESC (Thomson, et al., 1998).

1.6.2. Definition of IPSCs

IPSCs are differentiated, adult cells, which are reprogrammed “back” to SC like characteristics. This is done by forcing the cells to express genes and factors, which are important for maintaining the defining properties of ESCs. IPSC are characterized as PSCs. It is not clear till now, if the iPSCs and the hESCs differ in significant ways clinically (National Institutes of Health, 2009).

1.6.3. Discovery of IPSCs

Although iPSCs are a relatively new field of research, the foundations of this field go back over 50 years.
In 1957, Conrad Waddington described that mammalian development is unidirectional, which means that embryonic stem cells develop into a more mature differentiated state. He explained that pluripotent stem cells are the top of a mountain and that they roll down like marbles becoming more differentiated

cells (Waddington, 1957). At this time, it was believed that cells become specialized by deleting or inactivating unnecessary genetic information (Weismann, 1893).

In 1962, John B. Gurdon showed for the first time by nuclear reprogramming that adult somatic cells can resort back into PSCs. He transferred a nucleus of a tadpole´s somatic cell into an oocyte and succeeded in obtaining a cloned frog (Gurdon, 1962).

In 1987, Davis et al. made an experiment that first demonstrated direct cell fate conversion by a defined transcription factor. In this study, they performed complementary DNA subtractions and converts thereby fibroblasts from a mouse into myoblasts. By screening the myocyte complementary DNA library, they selected and characterized three complementary DNAs. They found out, that one of these three complimentary DNA is sufficient to convert the fibroblasts into myoblasts. This complementary DNA encodes for a short protein segment which is similar to a sequence present in the MYC protein family (Davis, Weintraub and Lassar, 1987).

In 2006, Takashi and Yamanaka created new avenues in basic research, disease modeling and regenerative medicine. The question was, if it would be possible to reprogram or dedifferentiate somatic cells rather than an egg/oocyte by the use of defined factors. They choose 24 factors for the testing, which all were highly expressed in ESCs and, therefore may be important for keeping cells “ESC like”. By combining the four selected transcription factors (TF) OCT3/4, SOX2, *KLF4* and *C-MYC* they were able to generate iPSC directly from mouse embryonic or adult fibroblast cultures by retroviral introduction of the four genes. This happened through multiple events like silencing and activation of various pluripotent genes. The original mouse iPSCs were selected for the expression of the marker FBOX15. This is a marker of undifferentiated ESCs. Finally they could show that these cells can differentiate into various types of cells from all 3 germ layers (Takahashi and Yamanaka, 2006).

1.7. Reprogramming of Cells

1.7.1. Explanation of the Most Important TF

TF are necessary to control the expression of genes which are important to induce or maintain pluripotency.

To generate iPSCs and further clinically relevant cell types it is important to understand the TF which regulate cell differentiation. The generated, clinically relevant cell types can then be used in regenerative medicine (Chambers, et al., 2003).

1.7.1.1. OCT3/4

The mammalian POU TF OCT3/4 is expressed in the pluripotent early embryo cells and in germ cells. The expression of OCT3/4 in pluripotent cell populations is essential. In embryos where the OCT3/4 is not expressed, the cells of the inner cell mass in the blastocyst are not pluripotent. If there is no true inner cell mass, trophoblast proliferation is not possible. Therefore OCT3/4 determines growth factor signaling from SCs to the trophectoderm (Nichols, et al., 1998).

Another research group found out, that OCT3/4 is necessary to regulate the three fates of pluripotent cells. It increases differentiation into primitive endoderm and mesoderm. But they also found out, that repression of OCT3/4 leads to a loss of pluripotency and dedifferentiation to trophectoderm. Therefore OCT3/4 is a master regulator of pluripotency which is required to obtain self-renewal and to regulate developmental programmes (Niwa, Miyazaki and Smith, 2000).

OCT3/4 plays indirectly a role in regulating the *FGF4* expression. This is important for the differentiation and maintenance of extraembryonic endoderm from the trophectoderm (Avilion, et al., 2003).

1.7.1.2. SOX2

Avilion, et al. (1995) found out that SOX2 plays an early role in the inner cell mass, where it is masked by maternal proteins. A lack of SOX2 is critical in E 7.5. SOX2 is a TF which also influences the *FGF4* expression. This TF is necessary for the epiblast and extraembryonic ectoderm. The same research

group also made the hypothesis, that SOX2 is important in establishing early cell fate decisions, and in combination with OCT3/4 for the specification of the first three germ lines (Avilion, et al., 2003).

1.7.1.3. NANOG

The mRNA of the TF NANOG is present in pluripotent cells of human and mouse cell lines and is not present in differentiated cells. Therefore NANOG is a protein which helps to propagate ESCs. With the cytokine stimulation of *STAT3* NANOG is able to drive ESCs to self- renewal (Chambers, et al., 2003).

1.7.2. Explanations of the Most Important Genes

A couple of genes, which are commonly upregulated in tumors, have been shown to account for the long-term maintenance or proliferation of pluripotent cells.

1.7.2.1. STAT3

Pluripotent cells can be received in the presence of the leukemia inhibitory factor (LIF). LIF sorts by receptor complexes composed of a low affinity LIF receptor (LIFRβ) and *GP130. GP130* plays a basic role in self-renewal of pluripotent cells. A study found out, that the tyrosine residue of the cytoplasm domain from *GP130* is appropriate for *STAT3* activation, which is necessary for self-renewal. This study also found out, that the Stat3 gene is important to maintain cells in an undifferentiated state (Matsuda, et al., 1999).

1.7.2.2. KLF4

A study reported that the krüppel-like factor 4 was overexpressed in pluripotent cells which had a greater capacity to self-renew. They tested in this study the potency to self-renew on the basis of Embryoid body (EB) formation. They also found out, that *KLF4* overexpressed cells show higher levels of OCT3/4 with the conception that *KLF4* promotes self-renewal (Li, et al., 2005).

1.7.2.3. C-MYC

C-MYC is a gene which is often overexpressed in cancers. It is an oncogene which is a target gene in a signaling pathway. Expression of *C-MYC* can activate *β-CATENIN* (He, 1998). *C-MYC* plays an important role in cell growth, differentiation, proliferation and also self-renewal of SCs (Lewitzky and Yamanaka, 2007).

1.7.2.4. β-CATENIN

The *β-CATENIN* or *CATANB* is a membrane-bound gene and is induced by the Wnt-signal-transduction pathway, which plays a key role in cell-fate determination. Uncontrolled accumulation of *β-CATENIN* can result in developmental defects and tumor genesis (Kielman, et al., 2002).

1.7.3. Methods of Reprogramming Cells into IPSCs and the Perfect Donor Cells

Reprogramming of cells means the manipulation of cell fates. This opened the field for better fundamental ideas about stability of cell identity, tissue differentiation *in-vitro*, cellular transdifferentiation and new directions in research into human disease modeling (Robinton and Daley, 2012).

The generation of customized, personalized iPSCs is necessary because hESCs are present in limited numbers, their isolation is not ethical and they are not isolated from disease specific embryos. IPSCs would provide cells, which have limited immune rejection in patients because they are patient´s own cells (Robinton and Daley, 2012).

To produce clinically useful iPSCs it is important to choose the correct donor cell type and the best reprogramming method. Fibroblasts, keratinocytes and peripheral blood mononuclear cells (PBMCs) are favored cell types for the induction of pluripotency because they are from the patient own tissue, easy to obtain and easy to reprogram. Studies to establish large-scale iPSCs-biobanks have concentrated also on PBMCs because they are easy accessible and robustly reprogrammable (Hayden, 2011).

The successful generation of iPSCs by Takahashi and Yamanaka showed how ecotopic co-expression of transcription factors created new cell fates (Takahashi and Yamanaka, 2006). They found out that mouse fibroblasts could be reprogrammed into cells which resemble ESCs, cells called iPSCs, by the action of only four TF introduced by retroviral gene transfer. They used the four TF OCT3/4, SOX2, *KLF4* and *C-MYC*. These four reprogramming factors were identified as the most important after the screening of 24 genes, which were virally overexpressed in mouse embryonic fibroblasts. This study also shows that the iPSCs are able to differentiate into cell types of all three germ layers when they are introduced into immunocompromised mice (Takahashi and Yamanaka, 2006).

Further research has shown that human adult fibroblasts can be reprogrammed to pluripotent cells by a similar procedure. Due these findings, it is now possible to convert for example skin cells from a patient directly to iPSCs in culture and provide thereby a new route to the derivation of PSCs for the use in transplantation therapy (Takahashi, et al., 2007).

This leads to a lot of follow-up studies with the successful reprogramming (Robinton and Daley, 2012). The reprogramming of fibroblasts to iPSCs can also be induced by several other combinations of TF. Different combinations of TF induce pluripotency by activating a transcriptional program which is also expressed in ESCs and keeps the cells in the pluripotent state (Cohen and Melton, 2011).

Further research has demonstrated a wide variety of other cell types which are able to alter cellular identity. These are for example pancreatic β- cells (Stadtfeld, Brennand and Hochedlinger, 2008) and neural stem cells (Eminli, et al., 2008).

The core TF OCT3/4, SOX2 and NANOG plays central roles in reprogramming. They play important roles because these factors form an autoregulatory loop, in which they act together to regulate each other´s expression. These three factors also activate expression of other genes which maintain the pluripotent state and repress genes which allow differentiation along specific cell lineages. This autoregulation allows the cells to undergo differentiation in response to appropriate signals, and otherwise they maintain pluripotency. The maintenance of this autoregulatory pluripotency has allowed to overcome a major obstacle to

the potential therapeutic use of iPSCs. The TF *C-MYC* was initially used to reprogram fibroblasts into iPSCs but *C-MYC* is now not longer necessary. Due that fact, a main obstacle can be overcome because *C-MYC* can act as an oncogene after transplantation of the iPSCs which are produced with the use of *C-MYC*. An oncogene results in a high risk of cancer. Also the retroviral vectors used from Takashi and Yamanaka (2006) to introduce the genes into the fibroblasts can themselves cause harmful mutations which can lead to cancer (Kashyap, et al., 2009).

To overcome the drawbacks, which result from the use of retroviral vectors, multiple no integrating reprogramming techniques have been developed. These techniques produce iPSCs by transient expression of the reprogramming factors without the need of integration of viral genomes or foreign genes. This should avoid the risk of spontaneous tumor formation and should improve the quality of the generated iPSCs (Stadtfeld and Hochedlinger, 2010). Another method is the complete removal of the integrated viral DNA (Stadtfeld, et al., 2008).
Following the recent advances in reprogramming technology for the derivation of iPSCs are summarized.

1.7.3.1. Reprogramming with the Use of the Sendai Virus

The Sendai virus is a single-stranded RNA virus which is able to replicate in the cytoplasm of infected cells. But there is no stable integration into the target cell genome and there are no DNA intermediates (Fusaki, et al., 2009).
Reprogramming with the use of the Sendai virus is the most used integration-free method of iPSCs production available (Fusaki, et al., 2009). It was previously used to effectively reprogram fibroblasts and PBMCs (Dowey, et al., 2012).
But there are also some drawbacks. The reprogramming with the Sendai virus is very expensive and there is stable residual viral material. The fact, that there is stable residual viral material, requires an extended period about 10 to 20 passages of tissue culture to receive virus-free iPSC lines for further downstream analysis and differentiation experiments (Kretsovali, Hadjimichael and Charmpilas, 2012).

1.7.3.2. DNA-Based Episomal Reprogramming

The use of non-replicating or replicating episomal vectors for reprogramming is attractive because they reduce the biosafety concerns which are involved in the production and transduction of viral particles. But the reprogramming efficiency of non-replicating vectors is very low, which means that they require multiple transfections of the target cells (Okita, et al., 2008). This can be due the fact that the transfection efficiency of large polycistronic reprogramming plasmids is very low and the addition of the transgenic silencing mechanism of plasmid-based vectors in mammalian cells (Lu, et al., 2012).

To improve the transfection efficiency and expression rate Jia and colleagues (2010) developed minicircle vectors as a shuttle system for the reprogramming vectors. Minicircle vectors are smaller in size than standard plasmids because they consist of a special episomal vector, which do not contain any bacterial plasmid (Jia, et al., 2010).

Due the fact, that the minicircle vectors have still a quiet low efficiency and a high production and purification methodology (Narsinh, et al., 2011), a novel single plasmid reprogramming system has been developed. This system is called codon-optimized 4-in-1 minicircle (CoMiP) and carries the codon-optimized sequences of the four reprogramming factors from Yamanaka and a short hairpin RNA against the p53 tumor suppressor (Okita, et al., 2008). The CoMiP is highly efficient, integration-free, cost-effective and applicable to a wide variety of cell types, including fibroblasts and PBMCs (Lu, Zhang and Kay, 2013).

Human iPSCs can also be derivated by the binding of Epstein-Barr virus-encoded nuclear antigen-1 (EBNA-1) to a cis-acting viral DNA element called oriP. The EBNA-1/oriP association allows the stability of plasmids in actively dividing human cells as multicopy episomes that attach to chromosomes during mitosis (Hung, Kang and Kieff, 2001). After a lot of cell divisions, oriP/EBNA-based vectors are gradual lost from the target host cell. Through the prolonged expression of transgenes within the transfected cell, these vector systems increase the reprogramming efficiency (Okita, Yamakawa and Matsumura, 2013).

1.7.3.3. Reprogramming via mRNA

The transfection of modified mRNAs into parental cells is a good approach for induction of pluripotency without integration. So this method is independent of a DNA intermediate which leads to time-consuming screening experiments. But modified mRNA transfection still requires pre-treatment of target cells with the expensive interferon alpha antagonist, B1&R and a laborious series of mRNA transfection (Mandal and Rossi, 2013).

A limitation of mRNA-mediated reprogramming for clinical approaches is that it requires a feeder cell layer and a feeder cell-derived conditioned media. These both can increase the risk of transmitting undetected human pathogens to the host (Amit, et al., 2005).

1.7.3.4. Specific Editing of the iPSC Genome

The zinc finger nuclease (ZFN) system, the transcription activator-like effector nuclease (TALEN) system and the clustered regularly interspaced short palindromic repeats (CRISPR) system are the three main technologies which are used to target and correct mutations in the iPSCs. ZFNs (Hockemeyer, et al., 2009) and TALENs (Ding, et al., 2013) make use of sequence-specific DNA-binding domains linked to the nonspecific DNA cleavage domain Fok1, to form a functional dimeric nuclease. CRISPR make use of the RNA-guided Cas9 nuclease to generate directed double-stranded DNA breaks (Horii, et al., 2013). Which of these three genome editing methods has the highest cutting efficiency without additional off-target effects is still not clear (Kim and Kim, 2014).

Genome editing is correlated with cellular responses to DNA damage. Due that fact, the induction of double-strand DNA breaks triggers for example either error-prone, nonhomologous and joining or homology-directed repair at specific genomic locations. This leads to small insertions/deletions at the target site or introduction of homologous donor DNA template (Kim and Kim, 2014).

With the repair mechanisms homozygous or heterozygous knockout cell lines to introduce or correct specific gene mutations can be derived. The right correction or introduction of mutations in the same genetic background allows a better way for disease modeling. These cell lines are then called isogenic cell lines and are the foundation to elucidate the underlying molecular mechanism of a disease (Diecke, Jung, Lee and Ju, 2014).

It can be concluded, that a lot of integration-fee reprogramming technologies and genome engineering methods are in research at the moment.

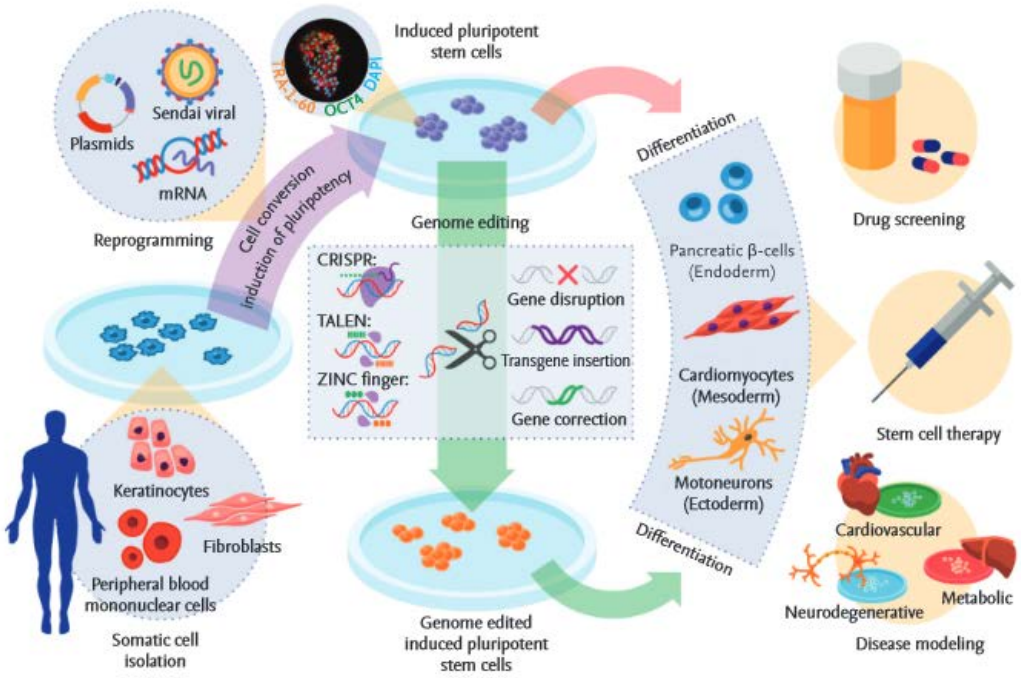

Figure 4: The generation, editing and use of human iPSCs in regenerative medicine:

Isolated fibroblasts, keratinocytes or PBMCs can be reprogrammed into iPSCs with the use of Plasmids, mRNA or Sendai virus. Then it is possible to edit the iPSCs with the use of CRISP, TALEN of ZINC finger. After doing so the iPSCs can be differentiated in tissues from all of the three germ layers like pancreatic β-cells. Then these cells can be used for disease modeling, stem cell therapy or drug screening.

Source: Diecke, Jung, Lee and Ju, 2014. *Figure 1*. [image]

It does not matter which method of derivation was applied, the iPSCs have the key features of ESCs. So they have the abilty to propagate indefinitely in culture and they are able to generate cells from each of the three embryonic germ layers (Hanna, Saha and Jaenisch, 2010).

1.8. Identification of Pluripotency

If cells are fully reprogrammed, they express a network of pluripotency genes which include OCT3/4, SOX2 and NANOG in a level which is comparable to ESCs. Fully reprogrammed cells also reactivate the telomerase gene expression, down-regulate THY1 and up-regulate SSEA1 (Stadtfeld, et al., 2008).
The positive staining for alkaline phosphatase activity has been broadly used as a marker of pluripotency. But recently published data have shown that this is an inadequate test for true iPSCs. It has been shown as insufficient because intermediate reprogrammed cells also stain positively (Chan, et al., 2009). The article from Chan, et al. (2009) also explains that iPSCs, which are reprogrammed by the use of virus-mediated reprogramming, silence proviral genes when the endogenous pluripotency genes are activated. This article shows further, that this event is paired with the expression of the embryonic antigens SSEA2, TRA-1-60, TRA-1-81, DNA methyltransferase 3 β and REX1 (Chan, et al., 2009).
Important for deriving fully reprogrammed cells is genome-wide epigenetic reprogramming. This degree of success is measured by evaluating the methylation status at the promoters of the genes which are necessary for maintaining pluripotency and the genes which are important for driving differentiation (Payer, Lee and Namekawa, 2011).
A very important process in the epigenetic reprogramming is the revival of the silent X chromosome. This occurs late in reprogramming and is a hallmark of ground-state pluripotency (Payer, Lee and Namekawa., 2011).

1.8.1. Functional Assays of Pluripotency – EB and two-dimensional directed differentiation in a culture dish

When cells are defined as fully reprogrammed cells they are typically also evaluated in functional assays. This characterization begins with the *in-vitro* differentiation. There are two methods for the *in-vitro* differentiation. One method is the differentiation of the cells as EB. These are compact balls of loosely organized tissue which are similar the gastrulating embryo. The other method is the two-dimensional directed differentiation in a culture dish. Both of these

cultures can then be evaluated for markers of each of the three germ layers (Stadtfeld, et al., 2010).

When mouse cells are analyzed for pluripotency scientists often analyze a developing chimaera. This analysis is able to evaluate the potential of iPSCs to contribute to the normal development of adult tissues after injection into the blastocyst (Boland, et al., 2009).

Tetraploid complementation is another test for mouse iPSCs. The iPSCs get injected into tetraploid blastocysts to measure the ability of the iPSCs to direct the normal development of a whole organism (Zhao, et al., 2009).

The teratoma forming potential is the most important test for human iPSC. For this assay iPSC were injected subcutaneously or intramuscularly into immunodeficient mice and the *in-vivo* differentiation potential of human iPSC is measured. Well differentiated tumors which contain cells of all the three germ layers reveal that cells are pluripotent and that they have a spontan differentiation potential. But this assay is not able to tell if the cells can produce all the cell types of the human body and is not able to tell something about the contribution of the iPSCs to germ line (Lensch, et al., 2007)

There are contrarieties to all of these functional assays and so the use of iPSCs for therapy is debated (Daley, et al., 2009). It will be important for the future to assume a consistent set of standards that can be applied uniformly worldwide (Robinton and Daley, 2012).

1.9. Transdifferentiation – are IPSCs Unnecessary?

Transdifferentiation means the differentiation of somatic cells into other types of differentiated cells without undergoing the iPSCs state.

Transdifferentiation was the first time established by Davis, Weintraub and Lassar (1987). They showed in their study that the TF MyoD was efficient to induce the differentiation of fibroblasts into muscle cell.

Due that fact, researchers have more recently investigated the possibility that fibroblasts can be direct differentiated into other cell types with the potential use in transplantation therapy. There has already been success obtained. For example mouse fibroblasts could be differentiated into heart muscle cells (Ieda, et al., 2010) and neurons (Vierbuchen, et al., 2010) with the combination of only

three TF. If this basic approach can be successfully applied to humans, these may provide an alternative direct route to generating differentiated cell populations without the need of iPSCs.

1.10. Differences of Mouse and Human iPSCs

Mouse iPSC and human iPSC look different. Mouse iPSC colonies appear rounder than human iPSC colonies. Human iPSC colonies are flatter than mouse iPSC. Human iPSC colonies are very similar to a definitely type of PSC which is derived from the epiblast of the early mouse embryo. This feature indicates, that mouse and human iPSCs probably reflect distinct developmental states (Tesar, et al., 2007).

The pluripotent state of mouse SCs is called a “naïve” state. This is because the pluripotent state is equal to the most primitive state of the mouse inner cell mass. The human stem cells are more “primed” because they proliferate in response to different cytokines which reflect the distinct developmental states of these populations (Wray, Kalkan and Smith, 2010).

1.11. Differences of IPSCs and ESCs–and How These Features Influence Disease Modeling and Therapeutics

Creating iPSCs and using them for treatment and regenerative medicine is a great idea, but the important question whether iPSCs and ESCs are analogous remains (Robinton and Daley, 2012).

1.11.1. Differentiation Abilities

Differences in their differentiation abilities concerning the *in-vitro* differentiation assays are already described between iPSCs and ESCs.

Some researchers think that iPSCs have a lower differentiation capacity than ESCs (Feng, et al., 2009). They think so because they observed that some mouse iPSCs had less ability to form teratomas than mouse ESCs (Miura, et al.,

2009). The iPSCs were also less able to differentiate into neuroepithelial (Hu, et al., 2010), haemotopoietic and neuronal lineages (Hu, et al., 2010) than the ESCs (Feng, et al., 2010).

Researchers think that the lower differentiation capacity of iPSCs is due to an influence of the cellular origin. This opinion was also affirmed by studies. It was, for example, discovered that IPSCs, which were derived from mouse bone-marrow and B-cells showed a better differentiation in the haemotopoietic lineage than IPSCs derived from neural-progenitor or fibroblast cells. They also found out, that these limitations are due to epigenetic modifications, because they treated the neural-progenitor IPSCs with trichostatin A (=histone-deacetylase inhibitor) and 5-azacytindine (=methylation – resistant cytosine analogue) and these cells increased their ability to become hematopoietic cells (Kim, et al., 2010).

Another research group derived iPSCs from human retinal-pigment epithelial cells and these cells showed a higher differentiation potential into retinal-pigment epithelial cells than ESCs or IPSCs from other tissues (Friedrich, et al., 2010).

Another research group derived iPSCs from human pancreatic islet β-cells and found out, that these cells showed a higher differentiation potential into insulin-producing cells *in-vitro* and *in-vivo* than ESCs or iPSCs from other tissues. This is because the derived iPSCs from human pancreatic islet β-cells maintain open chromatin at the loci of key β-cell genes (Bar-Nur, et al., 2011).

These findings can be used as differentiation and potency for disease modeling.

1.11.2. Genetic and Epigenetic

Genomic Sequencing and gene-expression analysis also shows differences between iPSCs and ESCs.

The DNA sequence shows genetic differences between iPSCs and ESCs. Chromosomal anomalies were defined as a common feature of stem-cell populations. IPSCs and ESCs have a tendency to increase chromosomes 12 and 17. But only iPSCs have a tendency to increase the ploidy of chromosomes 1 and 9 and only ESCs have the tendency to increase chromosomes 3 and 20 (Ben-David, Mayshar and Benvenisty, 2011).

Point mutations in iPSCs are also different than in ESCs. They occur particularly in the oncogenic pathways (Bar-Nur, et al., 2011).

A research group found out that more copy number variants (CNVs) are present in early passage human iPSCs than in intermediate-passage human iPSCs, fibroblasts or human ESCs. The same article published also, that CNVs confers a selective disadvantage. A control in this article shows, that the most of the CNVs were created *de-novo* in fragile regions of the genome (Hussein, et al., 2011).

When cells are reprogrammed to iPSCs, global epigenetic remodeling takes place. This introduces epigenetic changes which are necessary for reprogramming or are inadvertently. A failure of the cells to methylate pluripotency genes leads to partial reprogramming of the iPSCs. Studies show, that iPSCs closely resemble ESCs in the term of DNA methylosomes (Chan, et al., 2009) but there are differentially methylated regions (DMR). Some of these DMRs represent weak points and so failed epigenetic reprogramming has been identified (Lister, et al., 2011). The DMRs contain a lot of genes which are important for developmental processes (Laurent, et al., 2011). Due the reason that there are a lot of unique DMRs in iPSCs and not in ESCs it can be concluded, that these arise during reprogramming. Kim, et al. (2010) for example told that there are more DMRs in mouse iPSCs than in ESCs. But the DMRs are not present in specific loci and therefore there are no consistent differences between iPSCs and ESCs. These DMRs can therefore be more seen as technical limitations of reprogramming rather than as loci which let distinguish ESCs from iPSCs (Kim, et al., 2010).

Additionally the iPSCs specific methylation links the iPSCs to their tissue of origin and affects their differentiation grade (Kim, et al., 2010). That link can be so distinct that for example iPSCs from myeloid and lymphoid origins of blood-derived cells can be distinguished. IPSCs which are not derived from haematopoietic cells, for example iPSCs which are derived from fibroblasts, show often remaining repressive methylation at loci which are required for haematopoietic fates. This leads to the reduction of the blood forming potential *in-vitro* (Kim, et al., 2010).

During the normal development of female mammals one X chromosome is inactivated in each cell. Also one feature of epigenetic reprogramming is that the inactivated X chromosome must be reactivated. But the X-inactivation markers in pluripotent cells have poor fidelity, and so this is an area of debate (Shen, et al., 2008). Some studies have shown that the most human iPSCs do not reactivate the X chromosome (Tchieu, et al., 2010) but other studies have shown that some human iPSCs reactivate the X chromosome. They show the reactivation by the use of immunostaining. If the clones lose immunostaining for trimethylated H3K27 on the X chromosome, the X chromosome is reactivated (Marchetto, et al., 2010).
It should be considered that the published comparisons are from different laboratories which used different reprogramming methods. One article already proofed that there is a strong correlation between transcriptional signatures and specific laboratories for iPSCs and ESCs. So the produced data might be specific to the cells derived in a particular lab (Newman and Cooper, 2010).

It can be concluded, that there is no direct answer if iPSCs are equivalent to ESCs. IPSCs and ESCs are not identical and there are no distinct populations but they overlap each other with a greater variability inherent within each population than between the populations. IPSCs and ESCs are functionally equivalent, but in practice they show genetic and epigenetic differences that reflect their histories. So any given IPSC is not equivalent to the ideal ESC. Researchers are at the moment developing the necessary protocols to profit from the potential of iPSCs. It will be clear how to evaluate the genetic, epigenetic and functional status of different iPSCs protocols to accomplish the ambitious goals of the field will be created (Robinton and Daley, 2012).

1.12. Important Signalling Pathways which Underlie Pluripotency

1.12.1. LIF and BMP Signalling

ESCs from mice needs the LIF (Ying, et al., 2003) and the bone morphogenic proteins (BMP) (Qi, et al., 2004) to obtain their undifferentiated state.

LIF works as it is now explained. An activation of the LIF receptor leads to a dimerization of the receptor with gp130 subunits and subsequent tyrosine phyosphorylation and nuclear localization of the transcriptional activator *STAT3* (Heinrich, et al., 2003).
BMP4 are members of the TGFβ-family which bind to type1 TGFβ-receptors Alk1, Alk2, Alk3 or Alk6 and help to obtain pluripotency as followed explained. After ligation, type1 receptors form heterodimers with type 2 receptors. These type 2 receptors recruit and phosphorylate the receptor activated SMADS 1, 5 and 8. These SMADS are also called R-SMADS. After doing so, a phosohorylation of R-SMADS through serine/threonine forms a complex with co-SMAD4. This complex is able to accordingly enter the nucleus and initiate transcription (Shi and Massagué, 2003).

1.12.2. TGFβ and FGF Signaling

In contrast to ESCs from mice, ESCs from human requires TGFβ/ACTIVIN (James, et al., 2005) and FGF signaling (Vallier, et al., 2009) to remain in the undifferentiated state. TGFβ/ACTIVIN are members of the second branch of the TGFβ-family which bind to receptors Alk4, Alk5 or Alk7. This binding triggers serine/threonine phosphorylation of the C-terminal region of SMAD protein 2 and 3 which also dimerize with SMAD 4. This allows then nuclear entry and transcription (Shi and Massagué, 2003).
The growth factors from fibroblasts function through tyrosine receptor dimerization upon ligand binding and subsequent activation of phosphorylation events in the MAP kinase cascade (Chang and Karin, 2001).
Fascinating, FGF signaling is further able to phosphorylate BMP and TGFβ mediated R-SMADS at the “linker” domain of the proteins. This process has been associated as signal termination with linker phosphorylation (Pera, et al., 2003). It allows the recognition of SMAD proteins with the ubiquitin ligase *SMURF1* (Sapkota, et al., 2007). The polyubiquitation of the SMAD proteins by *SMURF1* then leads to a subsequent degradation of the SMAD proteins and further to a termination of the signal. Therefore there exists an intricate balance of antagonistic signaling inputs in the maintenance of human ESCs pluripotency. FGF signals may additionally act as inhibition from the differentiation among BMP signals in human ESCs because their definitive roles in proliferation and

survival. They promote thereby degradation of any active SMAD 1/5/8 proteins (Pera, et al., 2003).
Optional an excess of FGFβ/ACTIVIN signaling can lead to definitive endoderm formation of ESCs. FGF signals may also fine-tune the amount of active TGFβ-mediated SMAD 2/3 proteins to produce a suitable activity which is necessary to maintenance pluripotency (D´Amour, et al., 2005).

1.12.3. HEDGEHOG- and WNT-Pathways

The HEDGEHOG- and the WNT-pathways are closely connected signaling systems which play key roles during the embryonic development. These pathways were first described in Drosophila. They have also been found to control development in vertebrate and invertebrate embryos (Ingham, Nakano and Seger, 2011).
These both pathways are important in the development of limbs, nervous system, skeleton, lung, teeth, hair and gonads. They further play a major role in the regulation of the proliferation from SCs in adult tissues. WNT proteins are secreted growth factors, which are able to bind to a complex of the receptors Fizzeled and LRP families. These complex signals and this leads to stabilization of *β-CATENIN* which activates the WNT-pathway (Clevers, 2006).
Otherwise, when WNT signaling is absence, the *β-CATENIN* is phosphorylated by casein kinase-1 and GSK-3 in a complex with the proteins AXIN and APC. The phosphorilation of *β-CATENIN* follows its ubiquitation and degradation. Then the phosphorylation of the LRP is activated because the binding of WNT leads to the association of DISHEVELLED, which is a cytoplasmic protein, with FRIZZELED. This phosphorylation supplies binding sites for AXIN which leads to the association of AXIN with the receptor and a disruption of the AXIN, casein kinase-1, GSK-3 complex with *β-CATENIN*. This prevents the *β-CATENIN* degradation which leads to higher *β-CATENIN* levels. The role of *β-CATENIN* in WNT-signaling is to act as a direct regulator of gene expression by forming a complex with the members of the TCF-family, which are TF. When the *β-CATENIN* is not present, the TCF-family acts as repressors. But *β-CATENIN* converts the TCF-family members to activators, and this expresses target genes which code for other cell signaling molecules and TF which control the cell fate (Huang and He, 2008).

1.12.4. NOTCH-Pathway:

The NOTCH signaling pathway also controls cell fate during the animal development.

In general, NOTCH is a large protein with contains a single transmembrane domain which acts as a receptor for signaling by transmembrane proteins on the surface of adjacent cells. So this pathway acts as direct cell-cell interactions during development.

If the NOTCH gets activated, this initiates direct pathways of transcriptional activation. In detail the ligand binding leads to proteolytic cleavage of NOTCH by γ-secretase and the intracellular domain of NOTCH gets translocated into the nucleus. After doing so, the NOTCH intracellular domain interacts with TF, which are called CSL in mammals, and converts them from a repressor to an activator of its target genes. The NOTCH signaling pathway also targets, as the WNT signaling pathway, genes which encode other transcriptional regulatory proteins to determine the cell fate (Kopan and Ilagan. 2009).

1.13.Cell Culture Conditions

The iPSC generation is strongly influenced by the cell culture conditions and the cell signaling. Therefore, iPSCs should be cultured on media, which is optimized for ESCs. An important ingredient of the media for mouse and human ESCs is the LIF and the basic fibroblast growth factor. But the roles of these cytokines in the process are still unclear. The self-renewal potential of the ESCs is supported by the WNT-signaling (Ying et al., 2008).

Glycogen synthase kinase (GSK) 3-b mediates the Wnt3a signal. Without this signal, the GSK3-b inactivates target genes like C-MYC and *β-CATENIN*. This could be done by phosphorylation and proteasom mediated degradation. However, the GSK3-b can be inhibited with a chemical drug like CHIR99021 which results in activation of the WNT-signaling. Due that fact, addition of Wnt3a (Marson, et al., 2008) or CHIO99021 (Li, et al., 2009) improves the reprogramming efficiency.

If Vitamin C is added to the media, the iPSCs generation from mouse and human is enhanced. This is because Vitamin C is able to palliate cell senescence (Esteban, et al., 2009).

The presence of O_2 is also important for SC maintenance and differentiation. A low O_2 tension assists the viability of neural crest cells and hematopoietic SCs. It further prevents the differentiation of hESCs. Researchers found out, that the reprogramming efficiency is four times higher in hypoxic conditions (these are conditions with 5% O_2) in mouse and human fibroblasts than in low O_2 conditions (Yoshida, et al., 2009).

1.14. The Use of IPSCs in Regenerative Medicine

1.14.1. Cell Transplantation

There are two different cell sources for cell Transplantation. These are the autologous cell transplantation and the allogeneic cell transplantation. In the autologous cell transplantation the patient's own cells can be used for the generation of IPSCs. This would represent an advantage because the risks of rejection and infection could be diminished. But these autologous iPSCs are associated with high medical costs and with a period of time from more than three months to generate the iPSCs. These autografts also might have disease phenotypes. Therefore it is also necessary to generate iPSCs from allogeneic sources, which means, that the iPSCs are generated from multiple donors. In this method, it is important to match the three major types of human leukocyte antigen, which play an important role in the immune system, between the donor and the recipient (Inoue, et al., 2014).

New techniques like for example the structural self-formation of complex organs buds in 3D stem cells culture. These techniques include for example the generation of pancreatic tissue. In one study wild-type rat pluripotent SCs were injected into the blastocysts of *PDX-1*-deficient mice, which were unable to grow a pancreas. Following the injection, the generation of normally functioning pancreatic tissue could be observed (Kobayashi, et al., 2010). The production via Transdifferentiation, which means that the cells do not undergo the pluripotent state, plays also in the transplantation an important role. It has already been applied in the generation of β-cells (Zhou, et al., 2008) and cardiomyocytes (Qian, et al., 2012).

1.14.2. The Use of IPSCs in Disease Modeling and Drug Discovery

Although animal models have contributed to a better understanding of disease mechanisms, there are limitations concerning human diseases. There have already been drugs developed, which show therapeutic effects in rodent models, but are inefficient in human. An example for this is the development of the Amyotrophic lateral sclerosis. Therefore disease modeling with human cells is necessary (Desnuelle, et al., 2001).

Ebert et al. (2009 and 2012) reconstructed the first disease state of the spinal muscular atrophy successfully. They showed the possibility of using patient-derived iPSCs for reproduce disease phenotypes and their potential application in the use of these cells for drug screening.

Nowadays, a lot of patient specific iPSC lines have been successfully reconstructed and are used for disease modeling. These iPSC lines show particular use for rare diseases (Bellin, et al., 2012).

But the adequate control is also important to consider because there are differences between diseased cells and cells from healthy donors. Also the genetic differences between cells should be observed. Cells from healthy family members show a better source for disease modeling than cells from foreign persons. Todaym technologies like ZNF (Hockemeyer, et al., 2009) or TALEN (Hockemeyer, et al., 2011), which are genetic editing technologies, have made gene corrections in patient-specific iPSCs also possible.

1.14.3. Illnesses in which the iPSCs can be used

The possibility of an autologous transplantation can eliminate the need for taking the whole life drugs. IPSCs represent a limitless source of cells which can be used in cell replacement therapy and the pluripotency of this cells offers the possibility to form functional organ structures. However, ongoing and strict guidelines to decrease side effects and increase the patient safety are necessary (Diecke, Jung, Lee and Ju, 2014).

For diseases, which have limited treatment options, the need of iPSCs seems necessary. An example is the cardiac disease, because cardiomyocytes are not renewable. With the use of the self-renewing iPSCs, lost cardiomyocytes could

be replaced. The first clinical trail, in the treatment of age-related macular degeneration was already permitted in Japan (Diecke, Jung, Lee and Ju, 2014). This thesis will describe the use of iPSCs in the treatment of Diabetes Mellitus, Age-related macular degeneration and in the heart in detail.

1.15.Clinical Trail

Carrying out the series of steps, which are called phases of clinical trial, are necessary to bring a drug or a treatment method on the market. Each of the 4 phases is specific to a different research question. The 4 phases are explained as followed:

Phase 1: A new drug or a new treatment method is tested by researchers the first time in a small group of people. This is done to evaluate the safety, a safe dosage area and to identify the side effects.

Phase 2: The new drug or treatment method is tested in a larger group of people. This is done to further evaluate the safety and its effectiveness.

Phase 3: In this Phase the new drug or treatment method is tested in large groups of people. This is done to prove the effectiveness, to compare it to other drugs or treatments, to represent side effects and to collect information about the new drug or the new treatment method in terms of safety.

Phase 4: In the last Phase of clinical tail studies are done about the new drugs or treatment methods after they has been marketed concerning its effectiveness in different populations and its side effects connected with the long- term use (NIH, 2008).

2. INSULIN-PRODUCIN CELLS DERIVED FROM IPSCs: A POTENTIAL TREATMENT METHOD FOR DIABETES MELLITUS (DM)

2.1. Definitions

A β-cell is a cell with the phenotype of a mature insulin-producing cell, which can be found in pancreatic islets. β-cell precursors are insulin-producing cells that are not mature and therefore they do not have the complete phenotype of a true β-cell. The new insulin-producing cells always originate from precursor cells (Lanza, 2009, p.513).

2.2. Pancreatic and Islet Development

The Pancreas derives from the endoderm of the embryonic gut tube (Lanza, 2009, p.303).

Progenitor cells during embryonic development of the pancreas

Endocrine cells, exocrine cells, which secrete digestive enzymes, and epithelial cells, which flush these enzymes, exist within the adult pancreas (Lanza, 2009, p.299).

The endocrine cells are arranged in the islets of Langerhans and they compose around 1% of the cells in the adult pancreas. The different cell types of the endocrine cells are A, B, D and PP cells. The function of the A cells is to secrete glucagon, the function of the B cells is to secrete insulin, the function of the D cells is to secret somatostatin and the function of the PP cells is to secrete pancreatic polypeptide (Lanza, 2009, p.299).

In development, the gut tube generates a sheet of epithelial cells, which was shown to expresses the homeobox gene *PDX-1* around E 9 in mice. Around E12.5, a tubular structure is formed by budding of the epithelial cells from the gut

tube. From this tubular structure, the exocrine and endocrine tissues of the mature pancreas differentiate (Lanza, 2009, p.299).
The endocrine progenitors migrate from this tubular structure to form the pancreatic islets just before birth. During that period, differentiation is marked by several factors. NEUROGENIN3 expression marks, for example, progenitor cells for endocrine lineages (Lanza, 2009, p.299).

There exist two different explanations why pancreatic progenitors become endocrine cells. One is that the axis of mitosis in epithelial cells correlates with the identity of the daughter cells. This means, when the plane of division is perpendicular to the lumen in the pancreas, both cells remain epithelial, and when the plane of division is parallel to the lumen in the pancreas, one daughter cell detaches from the lumen and may become an endocrine progenitor cell.
The other explanation is that the cell-cell interactions by the NOTCH-pathway affect the differentiation. Due to this fact, mutations in the NOTCH-pathway genes are able to influence the fate of endocrine cells (Lanza, 2009, p.299).

Key Transcription Factors

The transcription factor *PDX-1* plays a key role in early pancreatic development. NGN3 is expressed in precursor cells, which later differentiate into islets. It is also thought, that transient NGN3 expression is the result of the inhibition of NOTCH-signaling and the stimulation of signaling by molecules of the TGF-β family. *PAX4* seems to play a key role in the post-NGN3 β-cell differentiation. Transcription factors, which appear to be important in the final stages of β cell differentiation, are BETA2/NEUROD, NKX2.2, NKX6.1 and MAFA (Lanza, 2009, p.514).

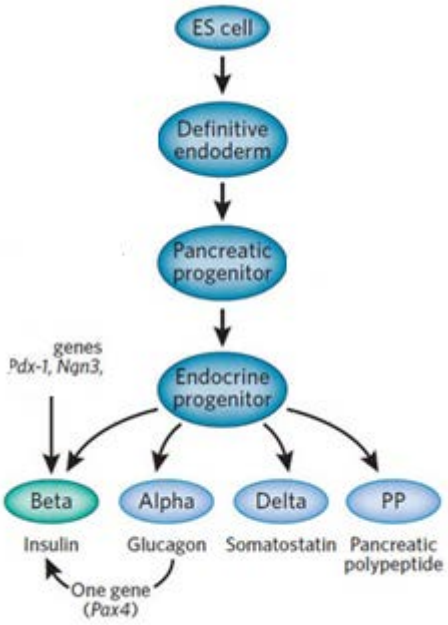

Figure 5: The pancreatic islet development:

The different cell types of the endocrine cells are A, B, D and PP cells. The function of the A cells is to secrete glucagon, the function of the B cells is to secrete insulin, the function of the D cells is to secret somatostatin and the function of the PP cells is to secrete pancreatic polypeptide The key transcription factors *PDX-1*, NGN3 and *PAX4* are also on the picture.

Source: cf: Wagner, B.K., 2010. *Transdifferentiation may help restore beta-cell mass lost in type 1 diabetes*. [image online] Available at: Nature Chemical Biology<http://www.nature.com/nchembio/journal/v6/n12/full/nchembio.472.html> [Accessed 28 January 2015]

In general, new cells are produced in the pancreas during adulthood but their origin is still not clear. It is also not known if the adult pancreas has true SCs and which role these cells play during pancreas regeneration and maintenance (Lanza, 2009, p.299).

2.3. Facts and Current Treatment Methods about DM

5% of the deaths in the world are caused through the disease DM or simply diabetes. It is an illness, which concerns developing and developed countries (Sheik Abdulazeez, 2014).

In 2012, an estimated 9.3% of the American population had DM, some fraction of that percentage was still undiagnosed. In 2010, DM was the 7th leading cause of death in the US (National Diabetes Statistic Report, 2014).

There exist two types of DM that are relevant for this thesis: Diabetes mellitus Type 1 (T1DM) and the Diabetes mellitus Type 2 (T2DM). In T1DM, the insulin-secreting β-cells, which are located in the pancreatic islets of Langerhans, are irreversibly destroyed, which leads to a lack of insulin production (Zaida, et al., 2010). In T2DM, the pancreas does not produce enough insulin. It can also be the case that the body gets resistant to insulin (Zaida, et al., 2010).

T1DM and T2DM result in a reduced glucose control in the body, which often causes other health problems (Sheik Abdulazeez, 2014). Insulin, which is produced within the β-cells in the pancreas, is required for life. It has the important role to regulate the glucose metabolism (Beta Cell Biology Consortium, 2002-2014) because it allows the glucose to enter the cells of the body from the bloodstream (Frank and Daneman, 2010).
Too high blood glucose level, as is the case in DM, can lead to clinical conditions which affect the heart, blood vessels, kidneys, nerves, teeth and eyes. This can lead to blindness, kidney failure, lower limb amputation and cardiovascular diseases. Also, bacterial infections are more likely to erupt (International Diabetes Federation, 2014).
Foot infections are the most common problems in persons with DM (Springer, 2006).

Current treatment methods of DM do not treat the causes of the disease. Lifelong exogenous insulin therapy is still necessary for the management of T1DM and T2DM (Author Manuscript, 2005). SCs are a great opportunity to treat diabetes. Scientists were already successful in producing insulin secreting cells from human iPSCs (Kunisada, et al., 2012).
This will be a great chance to decrease the increasing amount of population, which is suffering from DM. In the year 2012, 371 million people on the world had the disease.

In 2030, a number of 552 million cases worldwide are estimated (International Diabetes Federation, 2014).

2.3.1. Current Cellular Based Treatment Methods for DM

Pancreas transplantation or even just the transplantation of the pancreatic islet cells, which can be introduced into the liver through the portal vein of DM patients, depicts some possible cell therapy treatment methods for DM (Springer, 2006). Since 1978, more than 150 000 pancreas transplantations have been performed (Lanza, 2009, p.513). These are very promising techniques, but they are often not efficient because of immune rejections. It is also often a problem, that not enough donor organs are available for transplantation (Karniel, et al., 2007).
The limited supply of insulin-producing pancreatic β-cells for transplantation is due to the fact that transplants are currently only available from cadaver donors (Lanza, 2009, p.513).

This limitation makes it clear, that it is attractive to get insulin-secreting cells from the patient´s own tissue SCs. The bone marrow (BM) is an attractive donor of stem cells for the generation of alternative β-cells (Karniel, et al., 2007). This is because in the BM there are mesenchymal stem cells (MSCs), which can differentiate into many tissues from the three germ layers. There was already research done to treat T1DM with MSCs (Sordi, et al., 2010).
One research group did an experiment with mice. They showed the enlargement of BM-MSCs *in-vitro* and their differentiation to insulin-producing cells in mice. The cells could not express NEUROD, which is a key transcription factor in differentiated β-cells, *in-vitro*. But *in-vivo* (in streptozotocin-diabetic immunodeficient mice), the cells could express NEUROD, which resulted in a reduction of hyperglycemia (Karniel, 2007).
Another study showed that BM derived SCs are able to improve diabetes because they are more effective in enhancing regeneration and survival of existing β-cells than the differentiation into new β-cells (Sordi, et al., 2010). However, BM transplantations have problems such as low levels of insulin expression, total lack of or low insulin secretion and limited growth capacity (Urban, et al., 2008).

2.4. IPSCs in the Treatment of DM

Due to the problems of transplantations of β-cells (Urban, et al., 2008), the reprogramming of somatic cells into iPSCs, which have the same haplotype as the host and avoid immune rejections, is a good opportunity to treat DM (Zaida, et al., 2010).
The idea to provide glucose-responsive insulin producing cells with iPSCs can be an alternative to pancreatic and islet transplantation. ESCs as well as adult SCs have already shown an enormous potential as a source for insulin producing cells. Adult SCs have an advantage over ESCs because they are not ethically discussed. However, a disadvantage of them is their ability to form teratomas. Because SCs can create a renewable source of islets, thereby circumventing islet transplantation, SC research has become the centre of research for the treatment of DM (Mccall, et al., 2009).

One group (Kunisada, et al., 2012) generated a robust protocol for the generation of insulin-producing cells from human iPSCs *in-vitro*. They showed that improvements of individual cell lines might not be required by taking human iPSCs, which were generated from different people's fibroblasts by over expression of the transcription factors OCT3/4, SOX2 and *KLF4* (Kunisada, et al., 2012). They used these three TF for reprogramming because another research group found out, that iPSCs can be generated from patients with T1DM by reprogramming their adult fibroblast with these three factors (Maehr, et al., 2009).

The research group (Kunisada, et al., 2012) did their research in a four step method.
First, they looked at the differentiation of the human iPSCs into definitive endoderm. This is necessary because the germ layer endoderm gives rise to the insulin producing pancreas cells (Lanza, 2009, p.303).They checked the differentiation into definitive endoderm by the method of immunostaining for the transcription factors SOX17 and *FOXA2* and came to the result, that the combined treatment with ACTIVIN A and CHIR99021 leads to the most efficient differentiation from hips into definitive endoderm.

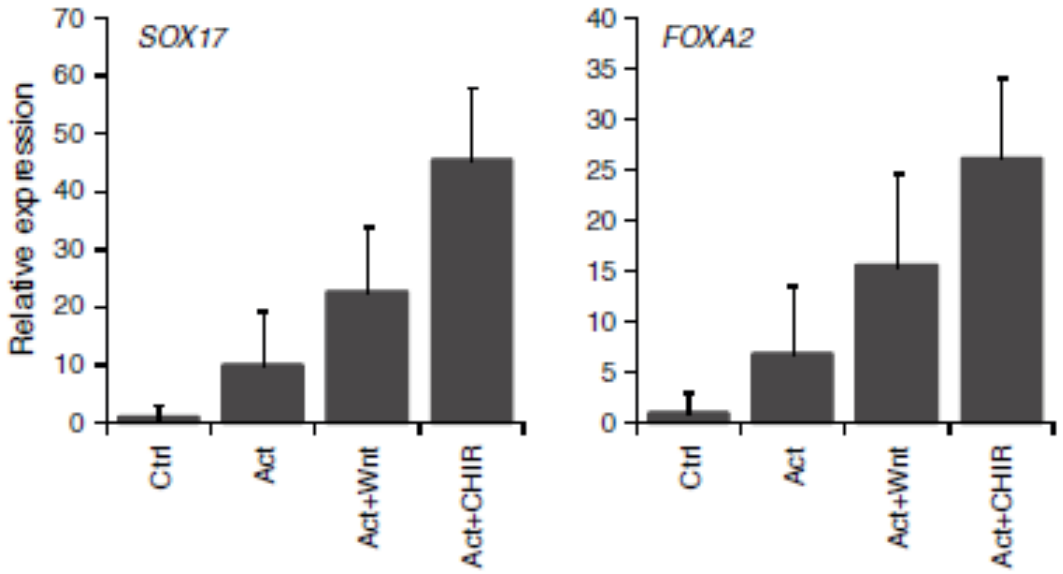

Figure 6: Differentiation potential of human iPSCs into definitive endoderm by looking at the TF SOX17 and *FOXA2:*

On the Y-axis the relative change of mRNA expression compared to the control cells can be seen. It can be seen that the combined treatment with ACTIVIN A and CHIR99021 shows the biggest differentiation potential. ACTIVIN A and the combination of ACTIVIN A with WNT was also tested, but shows not as a good result.

Source: Kunisada, Y., Tsubooka-Yamazoe, N., Shoji, M., Hosoya, M., 2011. *hips cells were treated with the indicated factors for three days as shown in A*. [figure 1B] Available at: Stem Cell Research< http://www.sciencedirect.com/science/article/pii/S1873506111001243> [Accessed 08 December 2014]

Second, they looked at the pancreatic differentiation from the definitive endoderm. They did this by analyzing for the presence of *PDX-1* positive cells (Kunisada, et al., 2012).

PDX-1, known as pancreatic and duodenal homeobox 1, encodes a protein, which plays a role in early development of the pancreas and plays a major role in glucose-dependent regulation of insulin gene expression (National Center for Biotechnology Information, 2014). Kunisada et al. (2012) came to the result that combined treatment with NOGGIN and Dorsmorphin yielded the same percentage of *PDX-1* positive cells than treatment with NOGGIN and retinoic acid and is more stable than the cell population. Due to these findings they used NOGGIN and Dorsmorphin for the following experiments.

The third step of this study was to investigate conditions that allow the effective generation of insulin-producing cells from *PDX-1* positive cells. They screened for small molecules that increased insulin expression by *PDX-1* positive pancreatic progenitor cells after day 10. Forskolin, Daxamethasone and Alk 5 inhibitor II were all found to increase insulin expression. To examine the effect of these three molecules on differentiation into insulin producing cells, the cells were cultured in combination with *PDX-1* and Nicotinamide, which was reported to induce differentiation. The results of these cultures clearly show that culturing the cells in the presence of all four genes in combination was similar to treating the cells with Forskolin alone. However the cultures where all four genes were used were better attached to the plates and grew at a larger density. Due this fact, they used this combination for the following experiment.
The last step in this study was the pharmacological stimulation of insulin secretion (Kunisada, et al., 2012). In this step, they are analyzing the C-peptide level. The C-peptide is also called connecting peptide and connects the insulin A chain to the B chain from the pro-insulin. It can be determined in the lab with an enzymatic assay and correlates to amount of secreted insulin (American Association for Clinical Chemistry. 2014).
In this step of the study, they measured the C-peptide level in the culture medium after incubation with glucose to examine the functionally of the human induced pluripotent-derived insulin-positive cells. They came to the result, that glucose alone did not alter insulin secretion. However, treatment with tolbutamine, carbachol, IBMX and potassium chloride yielded an increased c-peptide release. So they concluded that the cells were able to secrete c-peptide in response to different stimuli, but were not fully mature β-cells with the ability to secrete insulin in response to glucose stimulation (Kunisada, et al., 2012).

The four step method carried out by Kunisada et al. (2012) was effective to induce insulin-producing cells, but there are still several issues to be improved. The insulin-producing cells showed only little insulin secretion in response to glucose stimulation. This led to the conclusion that the culture conditions need to be further optimized because the cells were not fully functional mature β-cells. Another drawback is that they cultured the cells on mitomycin-C treated mouse fibroblasts. This is a drawback because animal-derived substances are

undesirable for clinical use in humans. Due to ethical concerns this study was not done with hESCs. It would also be necessary to compare this study with a lot of other protocols for improving the knowledge of human pancreatic development (Kunisada, et al., 2012).

Another research group (Zaida, et al., 2010) derived iPSCs from mouse skin fibroblast and showed that they can be differentiated into insulin-secreting β-like cells *in-vitro*. To reduce the possibility of graft rejection after transplantation the chosen mice were close relatives to the diabetic mouse models. They showed under pathological and physiological conditions, that these differentiated cells have the ability to respond to glucose simulation. These cells were injected into the liver of two different mouse systems. One mouse modeled T1DM and the other T2DM. The injected cells adjusted the hyperglycemic phenotype in both models. This study also shows that the transplanted cells were able to prevent insulin resistance and β-cell failure, which will be a key component for the treatment of T2DM.
Due to the fact that long-term improvement of hyperglycemia was achieved, the protocol represents a origin proof of principle for the potential clinical applications of reprogrammed somatic cells in the treatment of DM. But they also had some troubles like the loss of mice. So, this research group also concluded that further research is necessary (Zaida, et al., 2010).

Another, more recent research article shows the creation of fully functional pancreatic β-cells out of human PSCs, which may be used in the future for implantation into diabetic patients for regenerative treatment and for drug discovery (Pagliuca, et al., 2014). In their differentiation protocol, they also run a control, the so-called polyhormonal (PH) cells. PH cells are *in-vitro* differentiated hPSCs, which resemble human fetal β-cells more than adult β-cells. They also tested different variables to verify the similarity of the SC-β cells to primary human β-cells, which were isolated from human cadavers. For doing so they tested for changes in calcium ions to indicate the detection of changes in glucose concentrations by the SC-β cells, and they observed the changes in calcium influx by using the Fluo-4AM dye, which is a fluorescent calcium indicator dye.

Using fluorescent microscopy with this dye allows analyzing calcium flux at the population- and single-cell level. The population-cell analysis showed, that the cells respond to sequential glucose challenges. Also the most single-cells show in the single-cell analysis that they respond to glucose by fluxing calcium. Therefore, it could be concluded that the SC-β cells at population- and single-cell level function *in-vitro* because they flux calcium likewise to human islet cells. Further they also tested for the genetic similarity by global gene expression analysis, which was measured by a transcriptional microarray that was sorted for insulin expression. The cadaveric islet β-cells and the SC-β cells express the TF *PDX-1*, which is characteristic for human β-cells.

Afterwards, they tested for proper insulin packaging by the use of electron microscopy imaging of insulin packaging granules to compare their structure to adult β-cells. They came to the result that also this key feature of adult human β cells exists in SC-β cells.

They transplanted the SC derived β cells into the kidney capsule of immunocompromised mice to test their function *in-vivo.* They observed that the SC-β cells function i*n-vivo* after transplantation.

They also transplanted the SC derived β cells into the kidney capsule of Akita mice and tested thereby the control of hyperglycemia as a potential treatment method of DM. This transplantation rapidly reversed the worsening hyperglycemia in the mice. The measurement of the fasting blood glucose averaged <200 mg/dl. They also transplanted the control cells, the PH cells, into the kidney capsule of Akita mice, but these mice showed stepwise higher blood glucose levels which approached 600 mg/dl. (Pagliuca, et al., 2014). They used Akita mice in this test because they are a useful diabetes mouse model. They have a mutation in the insulin gene, which leads to protein misfolding, irreversible β-cell failure and depict hyperglycemia (Yoshioka, et al., 1997).

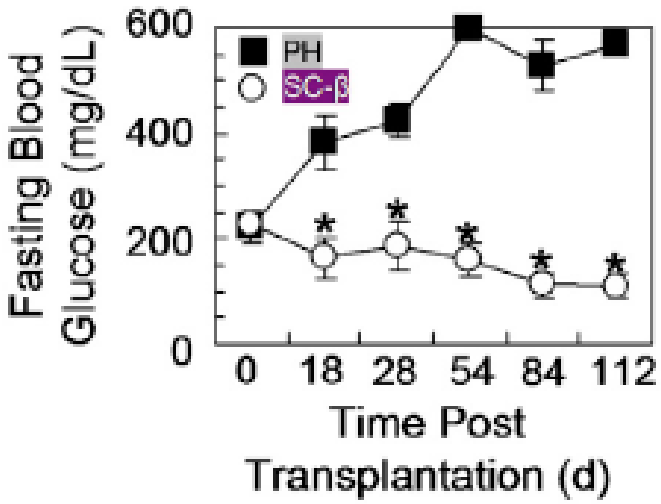

Figure 7: The Utility of SC-cells compared to PH cells for treating DM in Akita mice:

The measurement of the fasting blood glucose by the use of the SC-β cells averaged <200 mg/dl and the use of PH cells showed stepwise higher blood glucose levels which approached 600 mg/dl.

Source: Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., Melton, D.A., 2014. *Generation of Functional Human Pancreatic β Cells In Vitro* [Figure 6A: Transplanted SC-β Cells Rapidly Ameliorate Hyperglycemia in Diabetic Mice], Cell 159, 428-439

Pagliuca, et al. (2014) came to the conclusions, that functional human SC-β cells can be generated from human PSCs *in-vitro*. They also showed that the cells are able to function similarly to primary human β-cells *in-vitro* and *in-vivo* post transplantation. They also make clear that the cells can be generated without genetic modifications and in large numbers.

The work from Paglica, et al. generated millions of glucose-responsive β-cells from human PSCs *in-vitro*, which would be a great step to supply an all-time cell source for drug discovery and cell transplantation therapy in DM. But for the use of these cells in a clinical setting, more detailed analysis of the gene expression differences between primary human β-cells and SC-β cells will be necessary. Also the production of other endocrine hormone cells will be necessary to use these generated cells for testing of pharmaceuticals (Pagliuca, et al., 2014).

Due to the fact, that the pancreatic islets are highly-organized micro-organs, it is an important to respect these complexities by the derivation of strategies for the transplantation of islets from iPSCs (Lanza, 2009, p.514).

Most studies, in which SCs are used for β-cell replacement include hepatic stem cells, pancreatic stem cells, MSCs and embryonic stem cells (ESCs) (XioFang, et al., 2013).

Despite the promising results of these studies about the use of iPSCs in the treatment of DM, these studies are still in the early stages. The exact mechanism of the direct differentiation of pancreatic endocrine cells and their further differentiation into functional islets is still unclear. There is also a lack of monitoring the safety and the long-term efficacy of the iPSCs in the treatments of DM. This implies that a large amount of research must still be carried out before human clinical trails can be performed (XioFang, et al., 2013).

3. RETINAL REGENERATION-AGE RELATED MACULAR DEGENERATION AND THE FIRST CLINICAL TRAIL USING IPSCs IN HUMAN

3.1. The Structure of the Eye

In the human eye, the outer region, the middle layer and the inner layer can be distinguished.

Cornea and sclera are part of the outer region of the eye. The function of the cornea is to refract and transmit light to the lens and the retina. Another function of the cornea is to protect the eye against structural damages and infections.

The function of the sclera is to maintain the shape of the eye by forming a connective tissue coat. The sclera has also a vision part, which is coated by the conjunctiva. The conjunctiva is a transparent mucous membrane.

Iris, ciliar body and choroid are parts of the middle layer of the eye. The function of the iris is to control the size of the pupil and following the amount of light which reaches the retina. The function of the ciliar body is to control the power and the shape of the lens. The ciliar body produces the aqueous. The role of the choroid is to provide oxygen and nutrients to the outer retinal layers.

The retina is a complex, layered structure of neurons and also known as the inner layer of the eye (Willoughby, et al., 2010).

3.2. Facts about the Retina and Possible Treatment Methods of Illnesses Concerning the Retina

The retina is a complex multilayered tissue in the eye with the function to convert the information from light energy into electrical energy. The electrical energy is than transmitted to the brain, where it creates an image of the visual environment.

A lot of illnesses of the outer retina are caused by the degeneration of a relatively simple epithelial monolayer which is called retinal pigment epithelium (RPE).

The RPE is not a defined fraction of the retina because it lies juxtaposed against the outermost layer of the retina. But the RPE plays an important role as

supporting tissue in retinol cycling, nutrient transport, phagocytosis of the fragile photoreceptor outer segments and growth factor production. That is why in the past there was much research done which focused on the replacement of this monolayer by using autologous cell sources. Quiet recent research studies using both, ESCs and iPSCs. A couple of research groups have already successfully differentiated cells. These cells show a lot of similarities to RPE cells concerning characteristics and morphological characteristics.
Stem Cell derived RPE cells will be ideal candidates for transplantation into retina for several, currently not treatable, illnesses like the age-related macular degeneration, Stargardt´s disease (STGD) and retinitis pigmentosa (RP).
Because of the promising potential of SC-based therapies to treat retinal diseases, there are at the moment several phase I clinical trails going on. This leads to the conclusion that the eye is one of the first organs which will be targeted by SCs in regenerative medicine. This is reinforced due the fact, that the eye is easily accessible and because there is a lot of surgical expertise in this topic. In consideration of these facts, documentation with photography and other imaging modalities is possible (Ramsden, et al., 2013).

3.3. Age related macular degeneration (AMD) and Possible Treatment Methods

The AMD is the leading cause of vision loss worldwide (Lim, et al., 2012).
This disease affects the retina by damaging the macular area and leads due to this damage to a loss of the central visual field (Ramsden, et al., 2013).
Firstly there is a formation of drusen which means that deposits are forming between the RPE and the Bruch´s membrane. Secondly there is a loss of high-acuity vision due to dysfunction or death of RPE cells and loss of photoreceptors (Strauss, 2005).
This visual loss occurs either due to neovascular age-related macular degeneration or due to geographic atrophy. The neovascular age-related macular degeneration is also referred as “wet” age-related macular degeneration because in this process choroidal neovascularisation breaks through to the neural retina which leads to leaking fluid, lipids, blood and finally to fibrous scarring.

The geographic atrophy is also referred as “late dry” because in this process the RPE, the choriocapillaris and the photoreceptors are progressively atrophied (Lim, et al., 2012).

Ten years ago, there existed treatment method for the AMD. Nowadays there are already pharmaceuticals which try to suppress VEGF, and so they are referred as anti-VEGF therapy (Lim, et al., 2012). These pharmaceuticals are able to slow the wet age-related macular degeneration. But these pharmaceuticals are only corresponding for 5% of patients which suffer from AMD. For the dry age-related macular degeneration there exists to date no effective treatment option (Gehrs, et al., 2006). Possible treatment methods for the wet AMD are also laser photocoagulation, photodynamic therapy and visual rehabilitation (Lim, et al., 2012). A new research area is the treatment is the intraocular transplantation of SCs and further differentiation of them into photoreceptors and the hope for the re-establishment of the vision (Lim, et al., 2012).
Schwartz, et al. (2012) already published a paper in which PRE cells derived form hESCs were transplanted into a patient with dry AMD. Firstly they had 99% pure differentiation efficiency from the hESCs into the RPE cells. Finally they looked at the cells 4 months after rejection and showed that the hESCs-derived RPE cells exhibited no signs of tumorigenicity, hyperopoliferation, ecotopic tissue formation or apparent rejection This report was the first description were hESCs-derived cells were transplanted into human patient (Schwartz, et al., 2012).

3.3.1. The treatment of AMD with IPSCs

The use of iPSCs in the treatment of AMD starts with the successful generation of RPE from the iPSCs. Then the RPE have to be transplanted into the recipient.

3.3.1.1. The Successful Differentiation of IPSCs into RPE

There already exist protocols, which describe a successful differentiation of RPE from iPSCs. Some of these are described as followed.
Hirami, et al. (2009) reported that iPSCs can differentiate into ocular cells including PRE cells by treating them with WNT and NODAL antagonists and

culturing them with the expression markers of retinal progenitor cells. Hirami, et al. (2009) studied they used human iPSCs which were induced from human dermal fibroblast and reprogrammed by the use of the TF OCT3/4, SOX2, *KLF4* and *C-MYC*. They also showed in one human cell line that the treatment with retinoic acid and taurine can generate cells which are positive for photoreceptor markers (Hirami, et al., 2009).
Another report showed that iPSCs which were reprogrammed by the use of OCT3/4, SOX2, NANOG and LIN28 could also differentiate to RPE cells. They showed differentiation into RPE by removing bFGF from the medium which allows the cells to overgrow and allows them spontaneously differentiation (Buchholz, et al., 2009). They used this technique, because another article showed that under these culture conditions hESCs were also differentiated into RPE (Klimanskaya, et al., 2004).

The article from Buchholz, et al. (2009) also shows that iPSCs which were derived from fetal lung fibroblasts pigmented 1-3 weeks earlier than iPSCs which were derived from postnatal foreskin fibroblast. But the article also shows that the iPSCs derived from fetal lung fibroblast expanded rarely to a size which would be enough for enrichment. Due to the early pigmentation, fetal lung fibroblasts (expanded and exhibited an epithelial phenotype in the 1-3 weeks) were used in this study. They also showed after isolation of the cells that the cells are able to form highly differentiated RPE monolayers by culturing.
They further showed that these iPSCs derived RPE share similarities to fetal human RPE (fRPE) and RPE derived from hESC. They came to these results because the iPSC derived RPE expression of the marker mRNA was quantitatively similar to that of the hESC derived RPE and the fRPE. Further the marker proteins were adequately expressed and localized in polarized monolayers. They also showed that the level of phagocytosis by iPSC derived RPE is similar to the level of fRPE and hESC derived RPE (Buchholz, et al., 2009).

Creating RPE cells from human iPSC is, as described above, possible (Buchholz, et al., 2009 and Klimanskaya, et al., 2004) but these findings gives no

reference, that those cells have specific RPE functions which fully distinguish them from other types of cells(Kokkinaki, Sahibzada and Golestaneh, 2011).
A Research group published a paper which proofs the similarity of human iPSCs derived RPE and native RPE. They generated the human iPSCs with the use of the TF OCT34, SOX2, NANOG and LIN28. They explain in the paper that human iPSC derived RPE asset ion transport, membrane potential, polarized VEGF secretion and gene expression profile similar to those of native RPE under specific conditions. They tested these properties by the use of whole-cell electrophysiology. But they also found out, that the iPSC derived cells show rapid telomere shortening, DNA chromosomal damage and heightened p21 expression (Kokkinaki, Sahibzada and Golestaneh, 2011). P21 is also known as the cyclin-dependent kinase inhibitor and blocks phosphorylation of the retinoblastoma protein by cyclinE/cdk2 which prevents the cell from entering the S phase in the cell cycle. This happens due the fact, that the retinoblastoma protein is the master switch which controls the entry of cells into the cell cycle. (Cooper and Hausman, 2014, pp.644).
The described characteristics of the cells in the study from Kokkinaki, Sahibzada and Golestaneh (2011) might have an influence on the survival of the transplanted cells *in-vivo*. So this study concluded that further research needs to be done which will focus specifically on the rapid senescence of human iPSC derived cells (Kokkinaki, Sahibzada and Golestaneh, 2011).

3.3.1.2. Human IPSC derived RPE and their Transplantation

The feature of the iPSCs to generate functional RPE cells (Buchholz, et al., 2009) made this derived cell type to a best-known candidate for transplantation. There exist already some, but not enough, data for effectiveness of human iPSC derived RPE in animal models.
One model system that was used to test the use of human iPSC derived RPE cells in transplantation is the Royal College of Surgeons rat. This is a dystrophic rat, with a mutation in a gen which is deciding for photoreceptor outer segment phagocytosis. This mutation results in a loss of RPE cells and further in photoreceptor degradation. In one study they injected the human iPSC derived RPE into the subretinal space of RCS. They did this to show that human iPSC derived RPE cells are not only capable for phagocytosing photoreceptor material

in-vitro, they assort also *in-vivo.* The results show, that the rats resulted in long-term photoreceptor survival and maintenance visual function (Carr, et al., 2009).

In 2013 a trail using human iPSC derived RPE to treat the wet type of AMD has been advertised. Masayo Takahashi, who is an ophthalmologist at the RISKEN Center for Developmental Biology in Kobe Japan, and her team will show to recruit patients which suffer from AMD. Masayo Takahashi plans to reprogram skin samples with proteins into iPSCs and will further transform the iPSCs with other factors into retinal cells. Then she will place a small sheet of cells under the damaged area of the retina. If all works without a mistake, the cells will grow and repair the damaged RPE. This clinical trail will use monolayer sheets of cells, which provides safety of appropriate RPE layer organization prior to implantation (Cyranoski, 2014).
They used monolayer sheets due the fact that cell polarization and thigh junction formation are necessary for a lot of RPE functions. This also shows a lack of teratoma and any epitopic tissue formation in the implanted rat bodies with respect to safety in humans (Diniz, et al., 2013).
The main goal of this trail will be to see, if the cells are safe meaning that they do not trigger an immune reaction and do not form tumors (Cyranoski, 2013).
There exist already a study, in which the safety of their derived human iPSC derived RPE is tested. This study shows that the created human iPSC derived RPE meets clinical use in the terms of quality, quantity, consistency and safety. Their created monolayers of cells also show the ability to express typical RPE markers. The study also shows that after autologous transplantation of nonhuman iPSC derived RPE cell sheets no immune rejection or tumor formation could be observed (Kamao, et al., 2014).
There exists also a study, which reported that the tumor genetic potential of the iPSC derived RPE cells, tested in rats, is negligible (Kanemura, 2014).
The study of transplanting iPSC derived RPE cells in human will be possible to be done with human because Takahashi will carry out a “clinical” study, which is in Japan tightly regulated than a clinical trail in the USA (Cyranoski, 2013).

In July 2013 Japan´s regulatory authorities gave the team from Masayo Takahashi the permission to collect cells for the use of clinical iPSCs studies.

Skin cells from a 70 year old woman, which was suffering from AMD were taken and reprogrammed into iPSCs. These cells became later retinal tissue (Reardon and Cyranoski, 2014). Then the cells were transplanted into the eye of the 70 year old women in a two hour procedure at the Institute for Biomedical Research and Innovation. The women showed no errors like bleeding after the surgery (Cyranoski, 2014). The clinical trail succeeded. Masayo Takahashi demonstrates that these cells are safe in the use in patients and this will maybe change the mind of the regulatory agencies like the Food and Drug Administration (FDA) and the European Medicines Agency.
One researcher in the USA is also working on research in macular degeneration similar to Takahashi´s. He applied to the FDA and hopes that he can began with clinical trails in 2017 (Reardon and Cyranoski, 2014).

The tail from Masayo Takahashi can be seen as a start point, which should be acquired to as many patients as possible (Cyranoski, 2014).
Masayo Takahashi is, due to her great succeed in using iPSC clinical study in humans the first time, the winner of the 2014 Stem Cell Person of the Year award.

4. IPSCs AND THE REGENERATING HEART

4.1. Heart Failure, Myocardial Infarction and their Need of IPSCs

The most significant cause of mortality in the US is a heart disease. It accounts for more than 8000,000 deaths per year, which is equivalent to 1 death, every 39 persons (Roger, et al., 2011).

Cardiac muscle cells, which are also called cardiomyocytes (CMs), die due to a lack of oxygen when the blood supply to the heart is chocked for example due to a blood clot or due to thrombosis. The number of cells that die has been estimated to be up to a billion cells, depending on how long and how much the blood supply is reduced. There are a small number of adult SCs present in the heart, but this are not enough to repair the huge number of damaged cells.
The heart´s contractile muscle mass is reduced due to the loss of muscle cells, and so the wall of the heart thins locally at the site of the dead tissue. The dead tissue is replaced by scar tissue, which is also called fibrosis. Fibrosis is necessary because it prevents the heart rupturing under the pressure of the blood that it pumps. (Mummery, et al., 2011, pp.197).
But this fibroblastic scar initiates a lot of events which leads to remodeling, hypertrophy, cell death and heart failure. The formation of the scar tissue after myocardial infarction suggests that the heart has little or no capacity to generate new CMs (Pasumarthi and Field, 2002).

The loss of contractile muscle mass leads to the fact that less blood can be effectively pumped though the body. This phenomenon is known as heart failure, myocardial infarction or heart attack, which is a serious, often life-threatening condition. The heart fails to provide organs and tissues with enough blood, so they receive too little oxygen to sustain normal function.
If the heart fails to pump the blood into the aorta, the blood accumulates behind the left cardiac chamber and as a negative effect the pressure in the small blood vessels in the lung increases. The fluid may leak out into the lung tissue. Then the oxygen from the air, which is inhaled by the lungs, becomes lower than it

should and the amount of oxygen in the blood decreases. One consequence of this phenomenon is the shortness of breath, which is one of the most noticeable symptoms of heart failure (Mummery, et al., 2011, pp.197).

To palliate these symptoms from heart failure, a life-long use of drugs is required. The used drugs are not able to cure the disease. If a cardiac muscle cell could be filled with new healthy CMs after their loss, this would maybe increase the ability of the heart to pump blood around the body and the development of heart failure might be prevented. This method will also reduce or eliminate the need for drugs to increase the contractile force of the heart. Human iPSC lines obtained from cell banks would be a possible alternative, because the need of this CMs will be urgent, due the fact that time is too short to derive the patient´s own SCs and differentiate them to CMs (Mummery, et al., 2011, pp.197).
If this would be possible, the need for high–throughput production of *de-novo* CMs will be necessary because 1 billion cells would potentially needed to be replaced after a myocardial infarction (Laftlamme and Murry, 2005).
Due this fact, the engraftment of 3D engineered constructs, also called Embryoid Bodies (EB) would be the best approach for replacing scar tissue with new working myocardium. Concerns about cell survival, immune rejection, electrical maturation, electrical coupling and arrhythmia have to be mentioned (Zhao, et al., 2011).

4.2. The Embryonic Cardiac Development

To understand the *in-vitro* differentiation of iPSCs into CMs it is important to highlight some key steps and factors in cardiac development.

The heart forms soon after the gastrulation and is the earliest functional organ the vertebrate embryos. It forms from anterior migrating MSCs in the primitive streak, where heart forming or cardiac progenitor cells are mostly located in the mid-streak. Due the fact that the mesoderm lies between the ectoderm and the endoderm, signals from obvious cell populations promote the induction of cardiac

mesoderm. In particular, the endoderm seems to have a big influence on cardiogenesis (Brandt, 2003).

The early stages of mesoderm formation and with it the cardiogenesis is regulated by three families of protein growth factors. These are the bone morphogenetic proteins (BMPs), Wingless/INT proteins (WNTs), and the fibroblast growth factors (FGFs). The presence of these growth factors allows differentiation, and the presence of their inhibitors inhibits differentiation. Genetic disruption of these growth factors or their inhibitors has a great negative effect on cardiac development (Olson and Schneider, 2003).

BMP signaling proteins generally promotes cardiogenesis (Olson and Schneider, 2003). BMP2 is able to up regulate FGF8, and these both together can drive mesoderm cells into myocardial differentiation (Brandt, 2003). FGFR1 shows to play a role in cardiogenesis (Park, et al., 2008), because knockouts of FGF8 and FGFR1 lead to defects in cells to form the second heart field (Ilagan, et al., 2006). WNT proteins participate in the development of the heart and can therefore inhibit or support differentiation. They can inhibit the differentiation by activation of the canonical signaling pathway, which acts via *β-CATENIN*/GSK3. They can also promote the differentiation by activation of the non-canonical pathway, which acts via PKC/JKN (Olson and Schneider, 2003).
Research work in chick and zebrafish shows that the FGFs play also a cardioinductive role. It was shown in Drosophila, that FGFs provide positional cues for cell specification (Brandt, 2003).

Once the mesoderm migrated cells received determined signals, a highly conserved heart-specific combination of TF are switched on, which manufacture the cardiac transcriptional program.
To enter a precardic mesodermal stage of the development, the cells activate the TF MESP1 (Wu, 2008). The first step is the expression of the TF of the mesodermal precursor cells in the primitive streak. These are TF like T-BOX FACTOR BRACHYURY and MIX11 (Bondue, et al., 2011). Some MESP1 positive cells express the TF NKX2-5, TBX5, ISL1 and LIM, which are activated during the formation of the heart fields and so concerned as markers of the

cardiac lineage. NKX2-5 pointed out to play an essential role for the rating of pattering signals within the primitive heart tube in mice. NKX2-5 and TBX5 plays also a role in the activation of cardiac structural genes. For doing so, they associate with members of the GATA family (GATA4/5/6) and with serum response factors (Kuo, et al., 1997). TKX2-5 can also work together with TBX5 to activate protein junctions. GATA4 plays an important role in heart tube formation (Molkentin, et al., 1997), proepicardium formation (Watt, Battle and Duncan, 2004) and muscle development (Pu, et al., 2004). GATA6 plays also a role in myogenesis, but embryos with GATA 4 and 6 are not able to develop heart tissue (Zhao, et al., 2008).

TF of the myocyte enhancer factor 2 family (MEF2) plays an important role in regulating cardiac muscle structural genes and are thereby key factors in CMs differentiation.

In human iPSCs the control of the sequential activation of the TF, which drive the formation of mesoderm into cardiac cell fate, is much the same as in embryos. Due this assumption, many successful protocols which include the genesis of CMs show the activation or the inhibition of the above described signaling pathways (Martin-Puig, Wang and Chien, 2008).

4.3. Cell Types of the Heart

The heart is four-chambered and consists of a lot of different cell types. These are smooth muscle cells, endothelial cells, epicardium cells, fibroblasts, CMs, pacemaker cells and purkinje fibres. All cell types of the heart are essential to provide structural, biochemical, mechanical and electrical properties.

Smooth muscle cells form the coronary arteries, which regulate inflow and outflow from the vasculature. The interior lining of blood vessels and cardiac valves is called endocardium and is formed from endothelial cells. The epicardium differentiates into precursors of coronary vasculature and cardiac fibroblasts. More than 50% of the heart cells are cardiac fibroblasts. Atrial and ventricular CMs form the myocardium, which are the muscular walls of the heart. Purkinje fibres and pacemaker cells are specialized CMs which generate and conduct the electrical impulses. A group of pacemaker cells form the sinoatrail node which is able to generate impulses necessary for the initiate heart

contraction. The electrical impulse from the atria to the ventricles is conducted by the atrioventricular node which is located between the atria and ventricles (Xin, Olson and Bassel-Duby, 2013).

4.4. Functional CMs Derived from Human IPSCs

It is important to know, that the most research in generating CMS derived from human PSCs is done by the method of EB.
To obtain EB, the human PSCs are removed from conditions which support pluripotency and are plated on low-adherency plates or are suspended in droplets. If doing so, EB are forming. EB are cell aggregates which spontaneously differentiate into all 3 germ layers. A CM-like phenotype, which is able to contract spontaneously, will be developed by some of this EB (Itskovitz-Eldor, 2000).

A research group has already demonstrated that mouse iPSCs have similar development pathways than mouse ESCs. They showed that iPSCs are, like ESCs, able to differentiate into the three cardiovascular cell lines CMs, smooth muscle cells and endothelial cells. In their study they created iPSCs with the TF OCT3/4, SOX2, *C-MYC* and *KLF4*. They checked if the iPSCs are able to differentiate into FLK-1-positive progenitors and their mesoderm progeny, which include cells of the cardiovascular lineage. Immunostaining of mice tissues derived from iPSCs showed, that iPSCs are able to contribute to CMs, smooth muscle cells and endothelial cells *in-vivo*. To track the differentiation *in-vitro*, they looked at two Systems. In the first system they looked at the EB formation of each cell type. In the second system they cultured the cells on collagen type IV, which was reported for differentiation to mesoderm lineages. Both systems showed the differentiation of the iPSCs by expressing cardiovascular makers (Schenke-Layland, et al., 2008).

The ability to differentiate into these three cardiovascular cell types (CMs, smooth muscle cells and endothelial cells) has also been demonstrated in human iPSCs. In one study Zhang, et al. (2009) used human iPSCs which were reprogrammed by the lentiviral-mediated transduction of the TF OCT3/4, SOX2,

NANOG and *LIN28*. They used a subset of these iPSCs of fetal origin including IMR90 clone1, IMR90 clone 4. They also used cells from a newborn origin including Foreskin clone 1 and Foreskin clone 2. They further compared the differentiation potential form the iPSCs with the ESCs by using the EB method. For doing so the cells were maintained on irradiated mouse embryonic fibroblasts (MEFs) first at high density and prior to EB formation at lower density. So the cells were able to differentiate in contracting EBs from both cell types. But the derived EBs showed different percentages of effective contracting EBs. Each of the tested iPSC lines formed contracting outgrowths of the cells which suggested cardiac differentiation. But the efficiency of forming contracting EBs varied significant after 30 days in culture from less than 1% to 10%. IMR90 clone 4 showed a development of contracting EB from human IPSCs of around 9.5% and Foreskin clone 1 showed around 4% differentiation. So they used both of these (one fetal origin, one newborn origin) iPSC lines for the comparison with the human ESC lines, H1 and H9. In the first step they compared the *in-vitro* differentiation of the iPSCs and the ESCs by the contraction rates. In both cell lines the first contractions were monitored 8-9 days after EB formation but the efficiency varied from line to line. H9 ESCs showed the greatest contraction efficiency with 22% and the Foreskin clone 1 showed the lowest efficiency with 4.2%. IMR90 clone 4 and H1 showed nearly the same efficiencies with approximately 10%.
They also compared the cardiac gene expression patterns from the iPSCs and ESCs derived CMs with RT-PCR and showed similarity. So this study concluded that human iPSCs are able to differentiate into functional CMs and are a viable option as an autologous cell source for cardiac repair (Zhang, et al., 2009).

There exist are already differentiation protocols from fibroblast into iPSC and CMs without the use of the reprogramming factor *C-MYC.* These protocols bring the potential of iPSCs for clinical therapies closer to reality since *C-MYC* is an oncogene. In one article the researchers compare *C-MYC* free IPSC lines with cell lines differentiated with the use of *C-MYC*. They found out that the *C-MYC* fee iPSC lines demonstrated more efficient cardiogenesis and a better ability to integrate into the host heart tissue than the cell lines which were differentiated with the use of *C-MYC*. Further the CMs, which were created from the *C-MYC*

free iPSC lines, showed robust and sustained beating activity *in-vitro* (Martinez-Fernandez, et al., 2009).

The transplantation of CMs, derived from iPSCs which were obtained from mouse embryonic fibroblasts and reprogrammed with OCT3/4, SOX2, *KLF4* and *C-MYC*, showed already improvement of the left ventricular part of the heart after infraction in animal models (Nelson, et al., 2009).
In the first step of this study Nelson, et al. (2009) expanded the generated iPSCs in ESC-media and then they differentiated the iPSCs into EB. The expression of pre-cardiac mesoderm and cardiac differentiation markers was identified by the use of RT-PCR. For doing so, the RNA was extracted with an RNaeasy column and then transcribed into cDNA. 5-day-old EB showed up regulation of the pre-cardiac markers MESP1, TBX5, CXCR4 and FLK-1. After 12 days, the expression of the cardiac TF MEF2c, GSTA4 and MYOCARDIN showed the capacity for cardiac tissue maturation. To further check for the potential of differentiation, they labeled the iPSC progenitors with the *lacZ-Gene* and transferred the labeled cells a surrogate uterus. By tracking at the early stages of organogenesis, the transferred cells demonstrated contribution to the heart field, including cardiac inflow and outflow tracts and right ventricles of the embryonic heart parenchyma.
Myocardial ischemia was confirmed in mice by electrocardiography, echocardiography and color change of the ventricular wall. Then the iPSCs were transplanted. Therefore they injected 2.5 µl four times within 30 minutes after ligation. Injection into immunodeficient adult mice showed aggressive growth and tumor outgrowth between 2-4 weeks which compromised the safety. But transplantation of iPSCs into immunocompetent host showed an absence of tumor growth at 8 weeks followed-up. Intramyocardial transplantation of 200,000 iPSCs/heart shows stable engraftment without a detectable tumor formation. Due these findings it was possible to test the therapeutic potential of iPSCs in immunocompetent hosts. They showed that iPSCs intervention improved the functional performance after acute myocardial infarction because the iPSCs improved cardiac contractility, rebuilt the ventricular wall thickness and restored the electric stability. So the regeneration of cardiac, smooth muscle and endothelial tissue was shown (Nelson, et al., 2009).

The results of studies like explained above sound very promising, but the expansion of them into human would require the derivation of clone, lineage bound cardiac cells. Further the expansion into human would require efficient purification protocols to avoid the teratoma potential. The differentiation potential of the iPSCs is also affected by the variance of the epigenetic profiles because different donor cell types or different reprogramming methods are used to create the cells (Gosh, et al., 2010).
Due those facts, highly varied cardiac differentiation potentials from iPSCs have already been described (Zhang, et al., 2009 and Schenke-Layland, et al., 2008). Therefore the human iPSC lines must be characterized in detail for clinical use. Also their differential potential must be quantified before. Currently the process of differentiation is quite inefficient, and so more efficient differentiation protocols will be required. The created CMs are phenotypically and electrophysically quite heterogeneous and more similar to fetal cells than to adult cells, and so they act like immature CMs (Zwi, et al., 2009).

4.5. Challenges for Regenerative Medicine in the Use of IPSCs in the Heart

The use of human iPSCs would maybe someday create customized, patient specific cells for cardiac regenerative therapies and cardiovascular tissue engineering. But before, some hurdles must overcome.
The EB methodology must be adapted to a high-throughput methodology. This can be done by using flasks with rotating paddles or by the use of simple suspension cultures which produce human iPSC derived CMs in high quantities (Burridge, et al., 2012).
Also the scalability should be improved and the costs should be reduced. This could be done by the elimination of growth factors from the monolayer protocol and by the application of other low-molecular-weight compounds (Willems, et al., 2009).
Also the maturation of the human iPSC derived CMs must be improved. Although electrophysiological properties have been demonstrated over time in culture, a better Ca^{2+} machinery and better ion channels within cells *in-vitro* must

be developed. Ca^{2+} is very important for cardiac contractions, because the muscle needs Ca^{2+}, which binds to TROPONIN and allows the actin-myosin mediated cell shortening (Heuvel, et al., 2013).
The use of small molecules, such as the triiodothyronine binding thyroid hormone receptors, could be a possible technique to induce maturation (Lee, et al., 2010).
Also classifying the type of CMs and selecting the CMs into the three types, ventricular, atrial and nodal cardiomyocytes, will be important (Ma, et al., 2011).

If the new CMs get transplanted, they have to induce vascularization to keep alive and have to couple electrically with the existing heart muscle, to overcome the risk of fatal arrhythmias (Boudoulas and Hatzopouls, 2009).
Due the fact, that the PSCs, which will be still in the transplanted cell population, might be tumorgenic, the detection of the presence of these cells with suitable markers will be necessary (Burridge, et al., 2012).
All the work till date with human iPSCs derived CMs has been performed in animal models, hereby mostly in rodents. The rodent heart beats approximately 450 beats per minute, which is difficult for human cardiomyocytes to keep pace. The guinea pig model has with approximately 230– 380 beats per minute a more suitable heart rate. To date, no immunocompromised strains which are convenient for cardiac engraftment exist. Assessment of human-sized cardiac patches in larger animals like pigs will be required before human iPSCs derived CMs can be clinically used (Burridge, et al., 2012).

Zhou, et al. (2014) did already a study with Yorkshire domestic pigs. This study was the first in which allogeneic iPSCs were transplanted *in-vivo* of large animals. In this study, the iPSCs were derived from porcine myocardial fibroblasts with the hope that these cells are able to generate beating CMs, because previous studies which used skin fibroblasts showed no beating activity in the CMs. In this study the researchers wanted to test the effects of the created iPSCs after allogeneically transplantation into pig hearts to improve chronic myocardial ischema. Another aim of this study was to compare the tissue in which cells were injected with the tissue where no cells were injected.

In the first step they removed the tissue of the pig. For doing so, they took a 3 month old Yorkshire pig which was healthy and used a tissue explants method. In detail they obtained 1x1 cm ventricular tissue, washed it 3 times, cut it into 2mm dices and washed it twice again. These small tissue clumps were transferred into a culture dish and cultured. After 3 to 7 days fibroblasts began to migrate out of the tissue fragments into the surrounding area of the culture. These myocardial-derived fibroblasts were harvested by trypsinization and cultured. Then these myocardial-derived fibroblasts were reprogrammed into iPSCs by the use of lentiviral vectors which contained the 4 human TF OCT3/4, SOX2, *KLF4* and *C-MYC*. These colonies were picked about a week later, given into 24-well-plates and expanded following a standard human iPSC cultural protocol. After 12-17 days the pig iPSCs were used for the allogeneic transplantation. They created a chronic ischemic model in all pigs by the use of an ameroid constrictor placement (Zhou, et al., 2014). An ameroid constrictor is a ring of stainless steel, which contains inside a ring of casein. The swells as it absorb tissue fluid. So ameroid constrictors are equipment for gradual occlusion of the blood flow and to indicate an ischemic model (Farlex, Inc., 2014).
The next step of the study from Zhou, et al. (2014) was the allogneically transplantation of the iPSCs into the pig hearts. They were direct injected into ischemic myocardium of 10 pigs. From these 10 pigs, always 2 animals were sacrificed at a time of 2, 4, 6, 8 and 12 weeks after the injection. Then the hearts of these killed pigs were harvested and the differentiation of the injected cells was studied. In general, no immune reaction was shown after the transplantation. The allogeneically transplanted pig iPSCs proliferated and survived well in ischemic environment. The study shows also, that the transplanted iPSCs are proangiogenic, which means that they showed the ability to form new blood vessels. But the study also shows that the transplanted iPSCs formed tumors and the obtained CMs showed no beating activity. Maybe the use of more efficient, non-integrative vectors for reprogramming should be used for further cell-based therapies.

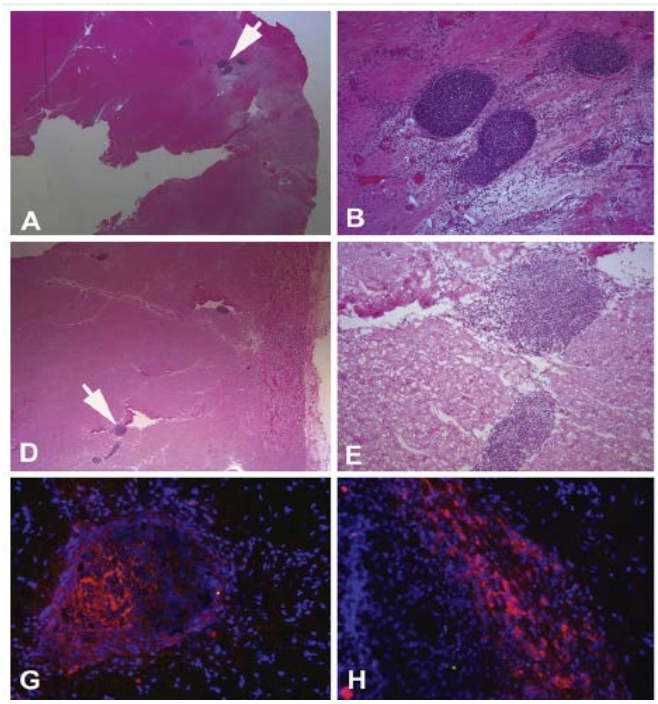

Figure 8: The results after Transplantation of the cells:

At 4 to 8 weeks the cells started already proliferation into small spherical shaped tumors, which are surrounded by thin capsules. On picture A the area with 3 spherical shaped tumors at 4 weeks after injection can be seen. This can also be seen at picture B to a higher magnification. At 6 weeks these cell clusters showed already decreasing size and number. This can be seen on picture D and E. The cells inside this tumor-mass were homogenous and showed no signs of differentiation into any specific lineage. This can be seen in the pictures G and H. It can also be seen that the most of these tumors were spherical shaped and some of them were irregular or elongated, but all showed thin layers of capsules. Multiple spherical shaped tumors were shown within an elongated cell cluster, which suggests variable growth speed within a single cell cluster.

Source: Zhou, Y., Wang, S., Yu, Z., Hoyt, R.F., Hunt, T., Kindzelski, B., Shou, D., Xie, W., Du, Y., Liu, C., Horvath, K.A., 2014. *Induced Pluripotent Stem Cell Transplantation in the Treatment of Porcine Chronic Myocardial Ischemia* [Figure], The Society of Thorancic Surgeons 0003-4975

The study from Zhou, et al. (2014) provided a very useful result concerning the survival and differentiation potential of the iPSCs *in-vivo*. But the results are only valid for three months post-transplantation. Long-term observations and analysis will be needed for the use in regenerative medicine. The study resulted also in three unexpected animal deaths, which were caused by surgical wound infection after the ameroid placement (Zhou, et al., 2014).

There exist already a lot to different differentiation methods and the core pathways which control CMs differentiation have already been identified. But there are still a lot of questions regarding the best differentiation methods and the securing normal physiological response of cells. Also many technical issues

are still unsolved. An example will be the question what would happen, if the cells successfully engraft and electrically couple, but do not respond normally to endogenous stimuli (Burridge, et al., 2012).

4.6. Human IPSC derived CMs for Disease Modeling

Cardiovascular disease (CVD) models based on the technology of iPSCs have the potential to analyze the molecular pathways which will be accountable for disease pathology and for a drug development.

The CVD model on the example of hypertrophic cardiomyopathy is now explained. Due to abnormal thickening of the ventricular wall, this disease is able to block the aortic outflow which can lead to heart failure and malignant ventricular arrhythmias. To supply better understanding of the disease pathophysiology and to find novel therapies for this disease, Lan, et al. (2013) developed a CVD model for hypertrophic cardiomyopathy. The cause of this disease has already been identified as a mutation in the cardiac sacromere but the pathways, which lead to the mutation, are still not discovered. This research group developed the CVD model from 5 related patients with a mutation in the beta-myosin heavy chain gene (MYH7). For doing so, the iPSCs from these patients were generated from primary fibroblasts through lentiviral infection with the TF OCT3/4, SOX2, *KLF4* and *C-MYC.* The iPSC derived CMs were generated by the use of EB differentiation protocols. 40 days after cardiac induction genetic analysis showed that hypertrophy-related genes were up-regulated and arrhythmogenic waveforms and abnormal Ca^{2+} transients were pointed out. In their results they also show that after 30 days post-induction the cytoplasm Ca^{2+} concentration was around 30% higher than in control subjects. To check the effectiveness of Caffeine, they added Caffeine to the CVD model and showed smaller Ca^{2+} release into the cytoplasm than control subjects. This result denoted that there was worse contractile function. Then they administrated calcineurin inhibitors, which block hypertrophy related signaling pathways, and showed reduced hypertrophy in the model. So this CDV model connected abnormal Ca^{2+} service as the central mechanism in the pathophysiology of hypertrophic cardiomyopathy (Lan, et al., 2013).

5. LAW CONCERNING SCs IN REGENERATIVE MEDICINE

5.1. Regulations of SCs in the USA

In January 2009 Barack Obama becomes President of the USA. He made changes in the support of research on human embryos. Until March 2009, no ESC lines could be supported with federal funds (University of Leicester, 2014). This includes frozen embryos, which were stored in IVF clinics, and embryos which were created with the use of somatic cell nuclear transfer (Fischbach and Fischbach, 2004).But private funding of ESC research in the USA has never been forbidden. Therefore this sector is much unregulated (University of Leicester, 2014). Also the funding obtained from business, private foundations or other humane sources was always possible (Fischbach and Fischbach, 2004). There existed only 61 SC lines in 2001, which researchers in the USA could work with publicly foundation, because also the deriving of new lines was embedded (University of Leicester, 2014).

Before, exactly in July 2006, President Bush allowed the use of public funds for projects which include the damage of human embryos. But with this regulation, the individual states have had always the capacity to pass laws to approve hESC research using state funds. According to that, some states like Connecticut, Massachusetts, California and Illinois have changed their legislation. Therefore, a private $3 billion Institute for Regenerative Medicine in California was established (University of Leicester, 2014).

But Barack Obama changed the funding situation for SC research fundamentally because he has made federal funding for research on new SC lines possible. His aim is to strongly support new research (University of Leicester, 2014).

There are some federal regulations (Fischbach and Fischbach, 2004) which were released by agencies of the federal government during History:

Code of Federal Regulations (CFR): This is a collection of regulations which were released in 1981 from agencies of the federal government. The title 45 in part 46 of this regulation (45CFR46) covers the research on human subjects.

The including Belmont Report is one of the most interacting regulations in the field of bioethics. This document defines the principles of respect for persons, justice and beneficence.

The coverage written in 45CFR46 were known as the Common Rule after 1991 by all federal agencies which did research with human subjects. The Common Rule has 4 subparts: A, B, C and D. Subpart A is about basic policies for human protection. Subpart B is about research on viable fetus, pregnant women and human IVF, whereby the dissociation of the IC; is legal. Subpart C is about studies which deal with prisoner. And Subpart D deals with special requirements for experiments which include children.

Appropriations Bill: This regulation was introduced in 1996 and influences the Subpart B from 45CFR46. It extends the protection to every organism which is derived by fertilization, parthenogenesis or cloning. In this regulation, also the preimplantation blastulae are included. President Clinton and President George W. Bush have signed these bills. This blocks the use of federal funds for new SC lines.

In the early 2000, Harriet Raab said that SCs are not organisms and so the 45CFR46 is not valid. Due that fact SCs, which were derived from embryos with private funds, could be supported by the government. Harold Varmus, which was then the Director of the National Institutes of Health (NIH), adopted this opinion. After doing so, the NIH formed a commission of scientist, patient advocates, lawyers and cleric people to look at the guidelines for the use of hESCs. Finally the NIH made a document, which provides that the following criteria are met. 1) The SCs were acquired from embryos which were produced in IVF clinics for reproductive aim. 2) The SCs were in excess of clinical need, which means that the donor is successfully pregnant or had decided to stop with the IVF. 3) The SCs were obtained from frozen embryos and here is enough time between the creating of the embryos and the decision of donate them. 4) Consent and Institutional approval is necessary. 5) There is no exchange of money, because this could influence.

With this regulation, it was possible to use as of March 2004 the around 400,000 frozen embryos of the IVF clinics among other things for medical research. President Clinton accepted this, but President George W. Bush rejected this NIH guidelines. George W. Bush said, that he would not accept the damage of more

embryos to create new hESC lines because he believed that there existed 62 hESC lines, but in reality there were fewer than 5. This is due the fact that most of the hESC lines were cultured with the contact of mouse cells and bovine serum. Some of them are also not well characterized in terms of viability and differentiation.
The number of available hESC lines is not sufficient to provide the genetic diversity (Fischbach and Fischbach, 2004).

In March 2009 President Barack Obama removed certain limitations on federal funding in the research which involves also the generation of new hESC lines. Before he signed the executive order, he said the following (Lee, 2009):
"Today, with the Executive Order I am about to sign, we will bring the change that so many scientists and researchers; doctors and innovators; patients and loved ones have hoped for, and fought for, these past eight years: we will lift the ban on federal funding for promising embryonic stem cell research. We will vigorously support scientists who pursue this research. And we will aim for America to lead the world in the discoveries it one day may yield (Lee, 2009)."

The regulations, which are written above are for hESC lines. Policy-makers are at the moment dealing with the question, if there should also be laws which govern other types of PSCs which are different from hESCs. They also speculate which new legislation is needed (University of Utah, 2015).

5.2. Regulations of SCs in Europe

The member states of the European Union have different regulations on hESCs research. This reflects the ethical, philosophical and religious diversity in Europe. Belgium and the United Kingdom allow for example the supply of hESCs from surplus IVF embryos and the creation of human embryos for the production of hESC in particular situations.
In Germany and Italy the production of hESCs from human embryos is totally forbidden.
Austria, Bulgaria, Ireland Slovakia, Poland and some other countries do not have legislation in the SC area at all.

In the table the legal use of SCs in the member states of the EU can be seen (University of Leicester, 2014).

	AT	BE	BG	CY	CZ	DE	DK	EE	EL	ES	FI	FR	HU	IE	IT*	LT	LU	LV	MT	NL	PL	PT	RO	SE	SI	SK	UK
Allowing procurement of hES cells from super-numary embryos by law		X			X		X		X	X	X	X								X		X		X			X
Specific legislation for human embryo research incl. supernumerary embryos but without specific reference to hES cells								X					X					X							X		
Prohibiting procurement of hES cells from human embryos but allowing importation of hES cell lines						X									X												
No specific legislation regarding hES cell research	X		X	X										X		X	X		X		X		X			X	
Allowing creation of human embryos for procurement of hES cells by law		X																						X			X
Prohibiting creation of human embryo for research purpose and for procurement of hES cells by law or by ratification of the Convention of the Council of Europe on Human rights and Biomedicine signed in Oviedo on 4 April 1997			X	X	X	X	X	X	X	X	X	X	X		X	X	X	X		X	X	X	X	X	X	X	

1) hES cells = human Embryonic Stem cells *IT has no law regarding the importation & IT scientists are working on imported hES cell lines

COUNTRY CODE KEY:

AT : Austria
BE : Belgium
BG: Bulgaria
CY : Cyprus
CZ : Cze
DE : Germany
DK : Denmark
EE : Estonia
EL : Greece
FI : Finland
FR : France
HU : Hungary
IE : Ireland
LT : Lithuania
LU : Luxembourg
LV : Latvia
MT : Malta
PL : Poland
PT : Portugal
RO: Romania
SE : Sweden
SK : Slovakia
UK : United Kingdom

Figure 9: Regulations regarding ESC research in the different EU States:

This table summarizes the legal use of SCs in research of 25 member states of the EU.

Source: University of Leicester, 2014. *Regulations in EU Member States regarding hES´ cell research*. [image online] Available at: <http://www2.le.ac.uk/departments/genetics/genie/gs/law/lawembryonic> [Accessed 16 January 2015]

5.3. Regulations of SCs in Austria

Imported ESC lines can be used for research in Austria because there is no Austrian law about it. But research on embryos and the derivation of ESC lines is not allowed. Embryos are in Austria only for reproduction. But if ESCs have already been established in a county, in which it is legal, they can be used also in Austria. This is due the fact that the Reproductive Medicine Act (Fortpflanzungsgesetz) 2004 is the only law which regulates stem cell research in Austria (Knoblich, 2013).

In 2001, the Austrian Bioethics Commission was established. This commission was formed to counsel the government in terms of arising issues in human medicine inclusive stem cell research. In 2009, there was a suggestion from a big group of this commission to cancel the ban on generating ESCs for research. A small group of this commission would have more restrictive legislation. These two groups did not find an agreement and so their suggestions have not been into law yet (Knoblich, 2013).

6. ETHICAL POINTS CONCERNING THE USE OF SCs IN REGENERATIVE MEDICINE

6.1. Ethical Points Concerning hESCs

HESCs preserve a big possibility for developing new treatment methods for a lot of serious and at the moment untreatable diseases. But the research of hESCs is ethically controversial, because it results in the demolition of human embryos. Therefore a lot of ethical questions have been raised and scientists, clinicians and the society must formulate answers to these questions (Lanza, R. ed., 2009, p.601). Following some of these questions would be elucidated.

The most important question is, if we are allowed to deliberately kill a developing human being with the hidden agenda to gain scientific knowledge and provide medical benefits. This question is still relevant, because existing hESC lines were created by demolition of embryos. There are still a lot of thousands embryos frozen, which could be used to create new lines (Weiss, 2003).
To answer the question, it is important to define when human life begins. Some people are thinking that human life begins at the conception. People with this view, think that an early embryo is morally the same as a child or an adult human being. Therefore, the embryo cannot be used in research without his/her approval (The Linacre Centre for Healthcare Ethics, 2001). Another group of people think that the embryo is not yet a fully human being because they hold a developmental view of the beginning of life. In this group of people, there is no denial that the embryo has the potential to become a human being, but they think that features which develop across the gestation like the ability to fell or to think are necessary (Ford, 1998, and Shannon, 2001). Another point why they think like this is because the embryo can still undergo twinning at the early stage and so the embryo lacks human individuality (Strain, et al., 1998). But a lot of people with this few do not agree to research which results in the destruction of embryos because they think that there should be respect in front of the nascent form of human life (Lebacqz, 2001).

In this question it is important to mention, that the American law does not credit the early embryo as a person (Lanza, R. ed., 2009, p.602).

Other important questions are the question if hESC research should be deferred and the question if we, as human beings, can benefit from others through the destruction of embryos (Lanza, R. ed., 2009, p.602).

Another more controversial territory question is if we are allowed to create an embryo to destroy it afterwards to produce SC lines. This was for example done at the Jones Institute in Norfolk, VA in 2002 (Kolata, 2001). They did it because they said that only with the production of new SC lines it will be possible in the future to develop SC lines with special properties like genetic matching. They further argue that it is better to use embryos which have been produced just for the purpose of SC line production than embryos which were originally created for a reproductive purpose (Kolata, 2001).

Another question concerning hESC research is if we should support cloning of human embryos. These question further leads to other questions like if the produced organism should be regarded as a human embryo (Lanza, R. ed., 2009, p.604)

6.2. Ethical Points Concerning iPSCs

At first, people thought that with iPSCs there would not be an ethical question, because for their derivation no embryo would be destroyed (Krauthammer, 2007). But with the easy, fast and cheap new techniques for the derivation of iPSCs ethic concerns have been obtained (Lehrman, 2010).
The human cloning from iPSCs would be possible, if the cells would be transformed sperm and egg cells (Lehrman, 2010). Also the control of the original tissue donation and the purpose to which it is applied are ethical concernes of iPSCs. One example is that it may be impossible to maintain donor privacy. If the cells of a person are used to study or treat diseases, it will always be important to know the health history from the donor and his personal information linked to the cells (Lehrman, 2010).

The scientific side arise the question if iPSCs would be appropriate for the use in human transplant and cell regeneration therapies because current iPSC lines have a big rate of tumorigenicity (Hyun, et al., 2007).

Another ethical question about iPSCs concerns the existing ethical norms. There is a norm which allows consent and withdrawal. But it is possible that a person donate tissue, and do not want to use it for studies which connect human and animal cells. How should examples like this be handled?
Another ethical concern is, that for example after a heart change, the cells of the patient maybe grow in labs all over the world. Is the person than even able to ask them to be destroyed (Lehrman, 2010)?
Also the money is an ethical concern. If a person donates skin cells, and they turn for example into pharmaceutical testing tools, models to study diseases or expensive treatment methods, should he become a part of the profit (Lehrman, 2010)?

7. LITERATURE

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