A Review on Successful Approaches of Converting Adult Somatic Cells into Induced Pluripotent Stem Cells (iPSCs)

A project submitted

by

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Dedicated to my parents for their unconditional love and support

Certification Statement

This is to certify that the project titled "A Review on the Successful Approaches of **Dr Mesbah Talukder** Converting Adult Somatic Cells into Induced Pluripotent Stem Cells (iPSCs)" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of, Associate Professor, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writing of another.

Signed,

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Counter signed by the supervisor,

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Acknowledgement

At first, I would like be grateful to the Almighty for giving me the strength and endurance for finishing my work appropriately.

During the course of my work, several people have supported me to complete this paper and I cannot be more appreciative of them. I am truly indebted and thankful to be my supervisor **Dr Mesbah Talukder,** Associate Professor of the Department of Pharmacy, BRAC University, who has constantly supported and guided me and encouraged me to do my work accurately. He also inspired me and helped me to resolve any kind of inaccuracy that was present in my work. Besides, I must mention **Dr. Eva Rahman Kabir**, Chairperson of the Department of Pharmacy, BRAC University, who has been a continuous inspiration to me and has guided me to develop and grow as a better student and a person over the past years. I am also thankful to all the faculty members of the Pharmacy Department of BRAC University, without whom I would not be the improved student that I am.

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Abstract

The induced pluripotent stem cells (iPSCs) are referred to as patient-specific equivalents of embryonic stem cells for the reason that they originate from somatic cells once the forced expression of pluripotency reprogramming factors Oct4, Sox2, Klf4 and c-Myc are introduced. Moreover, iPSCs present exceptional opportunity for personalized cell therapies and thus play a major role in the field of regenerative medicine. In current years, iPSC technology has undergone significant development to conquer time-consuming and ineffective “reprogramming protocols” and to guarantee clinical-grade iPSCs and their functional derivatives. Latest improvements in iPSC technology incorporate better reprogramming methods utilizing novel delivery systems such as non-integrating viral and non-viral vectors, using mRNAs of defined factors, using microRNAs, cell extracts and recently the ground-breaking discovery of the method in which the cells activate their own genes to revert back to pluripotency. At the same time, as small chemical molecules, inhibitors of specific signaling or epigenetic regulators have become fundamental to iPSC reprogramming; they have the capability to substitute recognized reprogramming factors and further advance the reprogramming processes. Additionally, characterization of iPSCs has also been mentioned in this review for assessing the effectiveness and accuracy of the discussed reprogramming methods. Hence, in this piece of writing, we review the most up to date inventions of techniques in the iPSC field, a summary of all the various methods that had been employed by scientists over the past years for attaining iPSCs and the probable application of iPSCs, in the fields of cell therapy and tissue engineering.

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List of acronyms

ESCs = Embryonic stem cells.

MES cells = Mouse embryonic stem cells.

LIF = Leukemia inhibitory factor.

hESCs = Human embryonic stem cells.

ASCs = Adult stem cells.

HSCs = Hematopoietic stem cells.

NSCs = Neural stem cells.

iPSCs = Induced pluripotent stem cells.

hiPSCs = Human induced pluripotent cells.

1. Introduction:

The **stem cells** are undifferentiated cells that can segregate towards the production of several progeny cells which persist as stem cells and a number of cells which are intended to differentiate, that is develop into specific cells. Hence, stem cells are a continuous resource of the differentiated cells that construct the tissues and organs of animals and plants. Therefore, great importance is present in stem cells for the reason that they have huge ability in the advancement of therapies meant for substituting faulty or injured cells which may result from a range of diseases and inherited disorders, for example Parkinson disease, heart diseases, and diabetes. Thus, there are two main kinds of stem cells and they are embryonic stem cells and adult stem cells, which are also called tissue stem cells (Slack, May 2011).

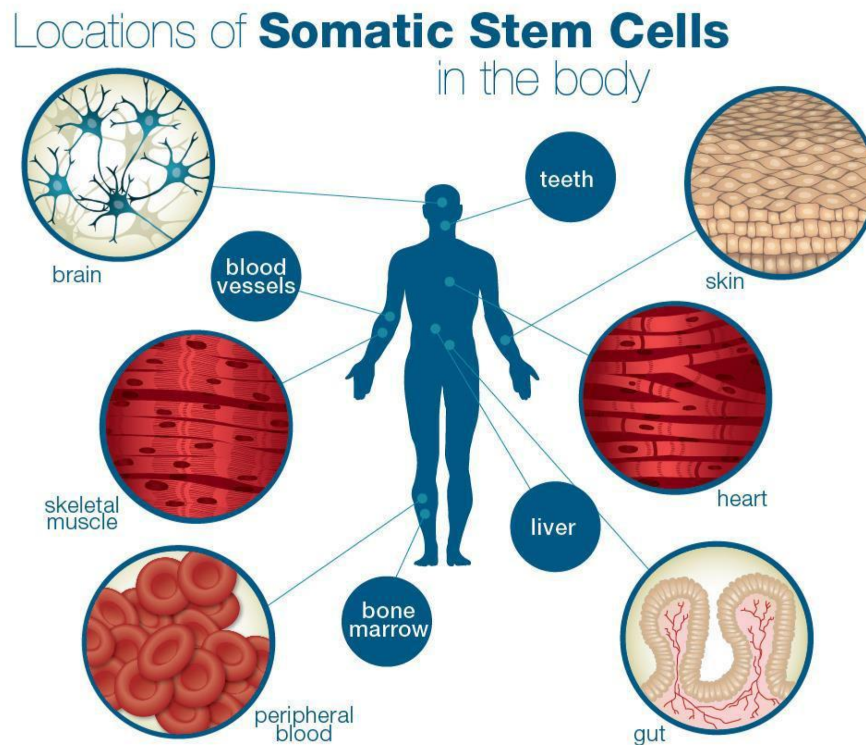

Fig.1.1: Illustration of the locations of “adult somatic stem cells” in the human body (Slack, May, 2018).

1.1 Embryonic Stem Cells (ESCs)

The **embryonic stem cells**, also called ES cells, are cells which are derivative of the interior collection of cells of a mammalian embryo, at a very premature point of growth, which are created from a "hollow sphere of dividing cells", also called a blastocyst. Hence, embryonic stem cells which are derivative of human embryos and from embryos of other mammalian species can be developed completely in culture mediums (Cyranoski, April 2018).

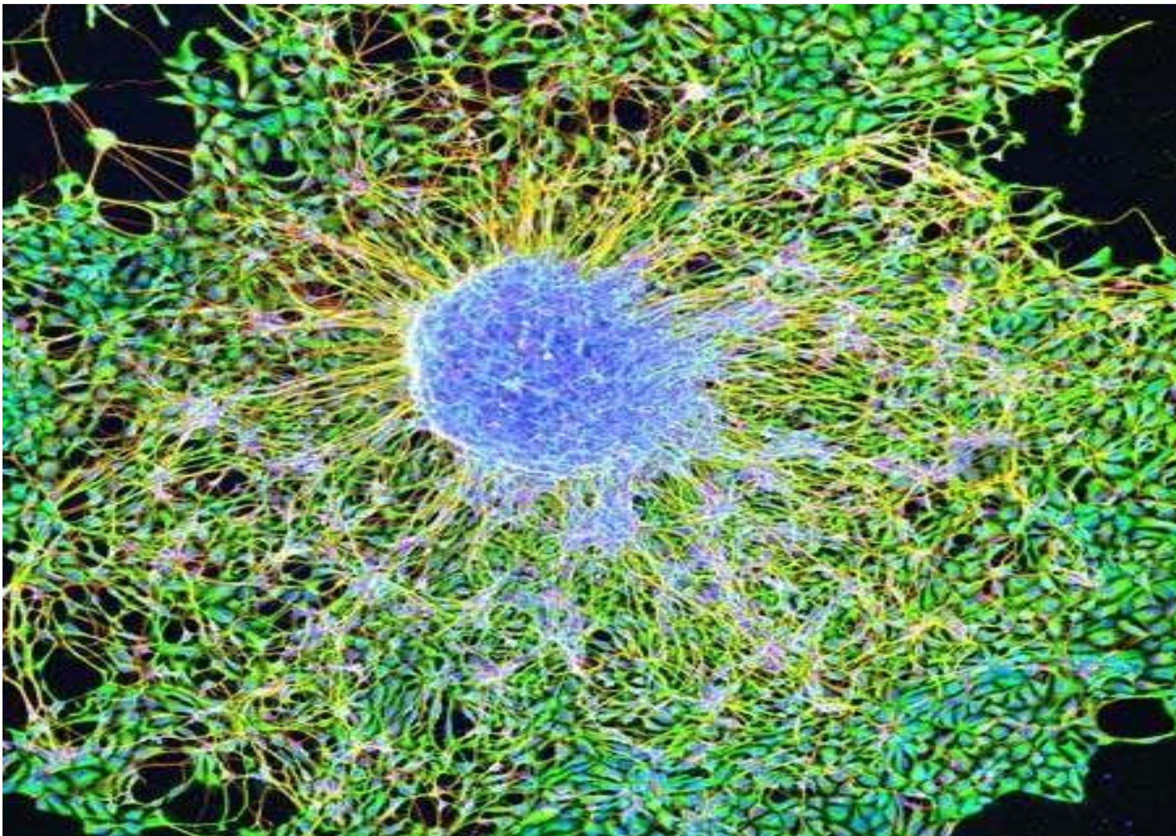

Fig.1.2: ES cells differentiating into neurons (Cyranoski, April 2018).

1.2 Mouse Embryonic Stem Cells (MES cells)

The most-examined embryonic stem cells must be the **“mouse embryonic stem cells”**, which were initially discovered in 1981. This sort of “stem cell” is capable of being cultivated for an indefinite time in the existence of a “glycoprotein cytokine”- leukemia inhibitory factor (LIF)”. If cultivated “mouse embryonic stem cells” are inserted at the “blastocyst stage”, into a premature “mouse embryo”, the cells will become incorporated within the embryo and generate cells that segregate into all or most of the kinds of tissues that consequently grow. This capability to proliferate “mouse embryos” is the essential aspect of “embryonic stem cells”, and this is the reason they are termed to be pluripotent—that is, capable of growing into any type of cells of the adult creature. In situations where embryonic stem cells were incorporated within an adult mouse, the consequence will be the development of a form of tumor referred to as a “teratoma” that consists of a mixture of differentiated or proliferated tissue types (Cyranoski, April 2018).

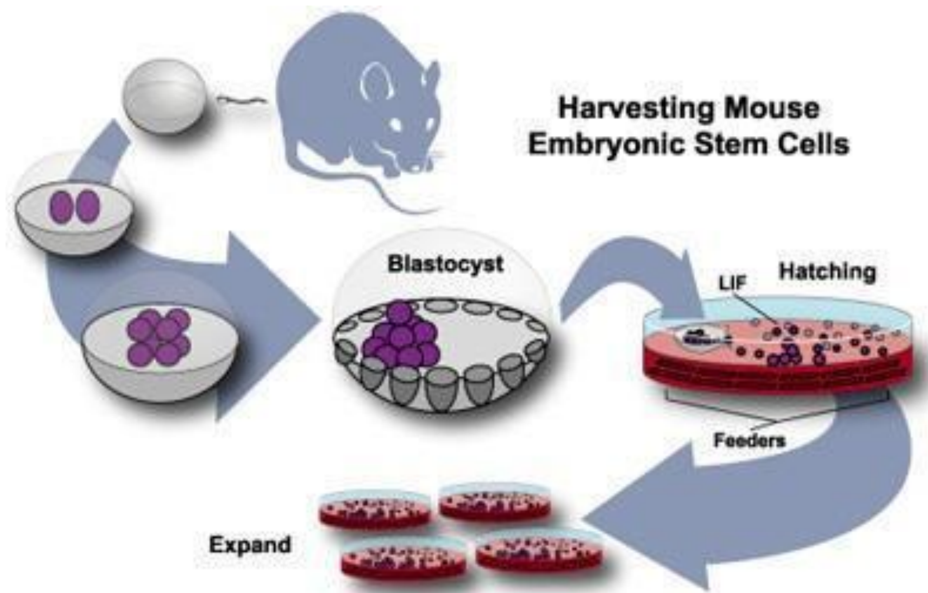

Fig.1.3: The process of harvesting Mouse embryonic stem cells (MES cells) (Whitehead Institute for Biomedical Research, December 2007).

1.3 Human Embryonic Stem Cells (hESCs)

General practice of scientists with mouse embryonic stem cells made them able to develop "**human embryonic stem cells"** (HES cells) extracted from premature "human embryos", and earliest "human stem cell lines" were produced during 1998.Thus "human embryonic stem cells" are comparable to "mouse embryonic stem cells" in numerous behaviors, nevertheless "LIF" is not needed for their preservation. The HES cells create an extensive range of separated or proliferated tissues in vitro, and they produce "teratomas" as they are injected into mice which are "immunosuppressed". It is still not sure whether if the cells are able to take over the "entire tissues of a human embryo", although it usually is assumed from their additional characteristics that they are in fact "pluripotent cells" and for that reason are considered as a probable cause of separated or proliferated cells for "cell therapy"— which is a method of the substitution of malfunctioning cell type of individuals with healthy cells (Wert and Mummery, April 2003).

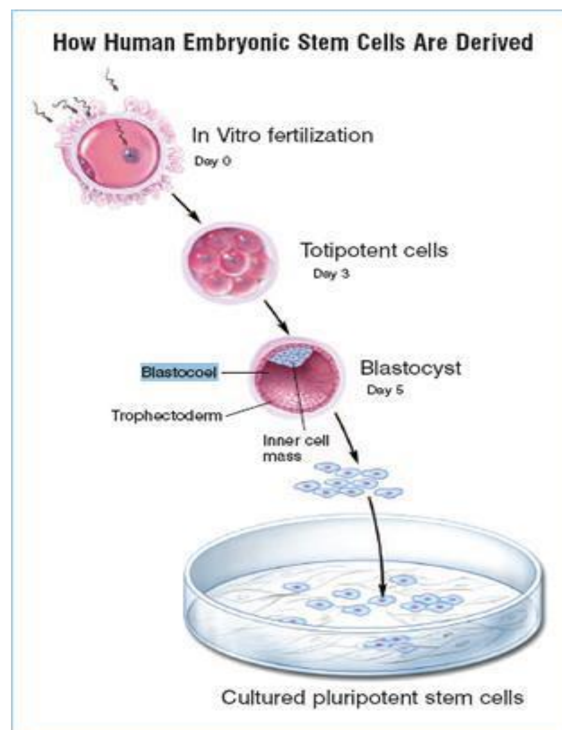

Fig.1.4: The process of extraction of Human Embryonic Stem Cells (HES cells) (Yu & Thomson, 2007).

Huge amounts of cells are capable of being created from "embryonic stem cells" for cell transplantation, such as neurons which secrete dopamine for healing of Parkinson disease and beta cells of the pancreas which secrete insulin for the healing of diabetes (Wert & Mummery, April 2003).

The application of human embryonic stem cells raise moral concerns, since the "blastocyst-stage embryos" are damaged during the course of acquiring the stem cells. The stem cells which are acquired from the embryos are created through the process of "in vitro fertilization"; moreover, people who regard "preimplantation-human embryos" to be human beings usually think that this sort of experiment is ethically incorrect. Although another group of people understand it for the reason that they consider the "blastocysts" to be merely "balls of cells", moreover "human cells" which are experimented in research laboratories have not formerly been awarded any individual ethical or authorized rank. Furthermore, it is well-known that not any of the cells of the internal "cell accumulation/mass" are entirely predestined to turn out to be part of the embryo itself—that is, every one of the cells attach to several or all of their cell produce to the placenta that has not been also awarded any individual official/authorized rank. The difference of observation going on this subject is showed by the information that the application of "human embryonic stem cells" is permissible in some nations and in others it is still illegal (Wert & Mummery, April 2003).

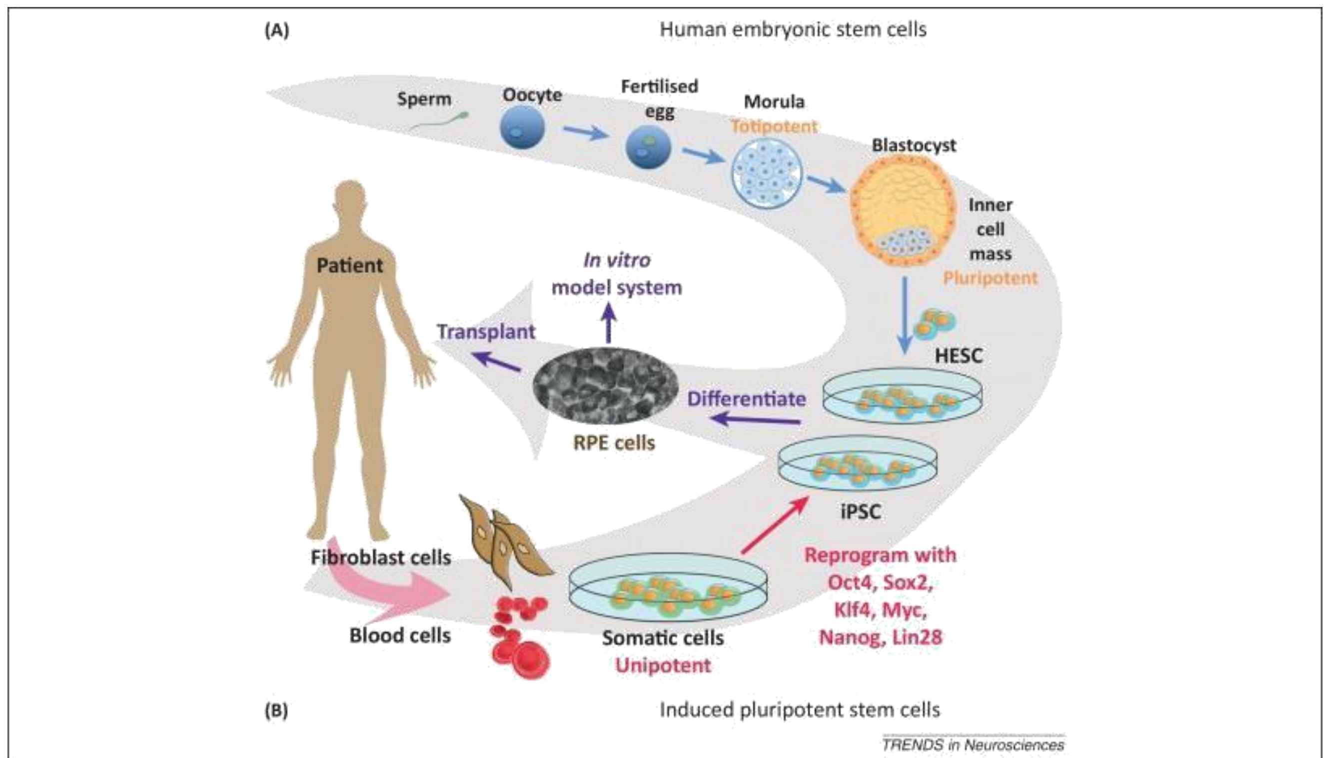

Fig.1.5: Schematic diagram illustrating how "human embryonic stem cells' and "induced pluripotent stem cells" are cultured in vitro and again transferred to the patient body for treating specific diseases (Liang & Y. Zhang, 2013).

1.4 Adult Stem Cells (ASCs)

There are several tissues in the fully developed human body, for example the "epidermis layer of the skin", "the internal lining of the small intestine" and the "the bone marrow" go through constant "cellular reproduction". They consist of stem cells which are known as "**adult stem cells"**, that continue indefinitely, and a large extent of "transit amplifying cells," that begin from the stem cells and segregate for a limited amount of time until they turn into distinguished cells. A number of kinds of tissue, for instance liver tissue, demonstrate minimum cell

division or go through division only once damaged. In these sorts of tissues, possibly there are no unique "stem-cell populations", as any cell knows how to contribute in tissue restoration whenever necessary (Slack, May 2018).

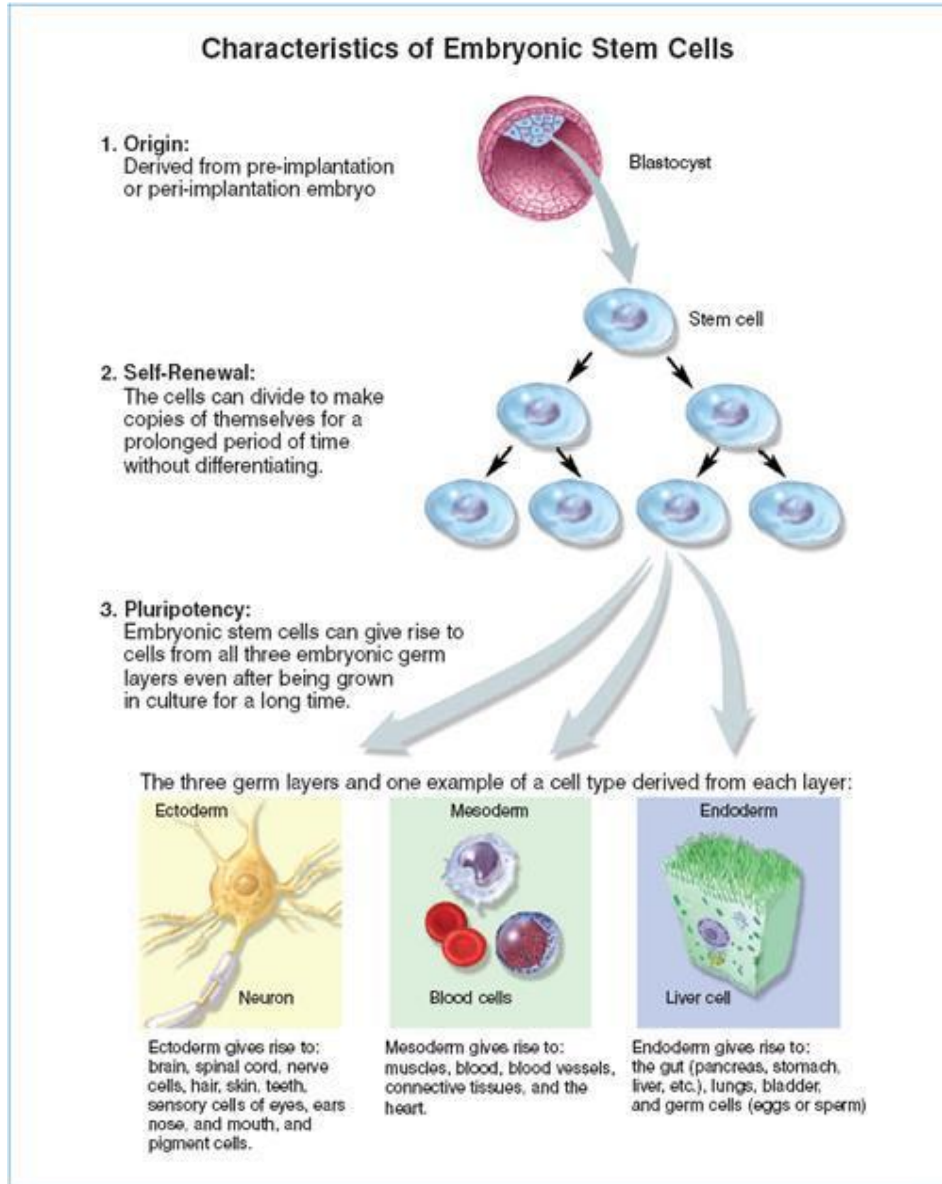

Fig.1.6: Schematic diagram showing the "characteristics of embryonic stem cells": its origin, self-renewal properties and its "pluripotency" (Yu & Thomson, 2007).

1.5 Epithelial Stem Cells

The top layer of the skin, also known as the epidermis of the skin consists of layer of cells labeled as “keratinocytes”. The basal layer that is placed after the “dermis” includes cells which divide. An amount of these cells are called “**epithelial stem cells”**, although most of them are “transit amplifying cells”. These “keratinocytes” gradually travel outward all the way throughout the “epidermis” as they become adult and they ultimately expire and wore off at the exterior surface of the skin (Blanpain, C., Horsley V., & Fuchs E., 2007).

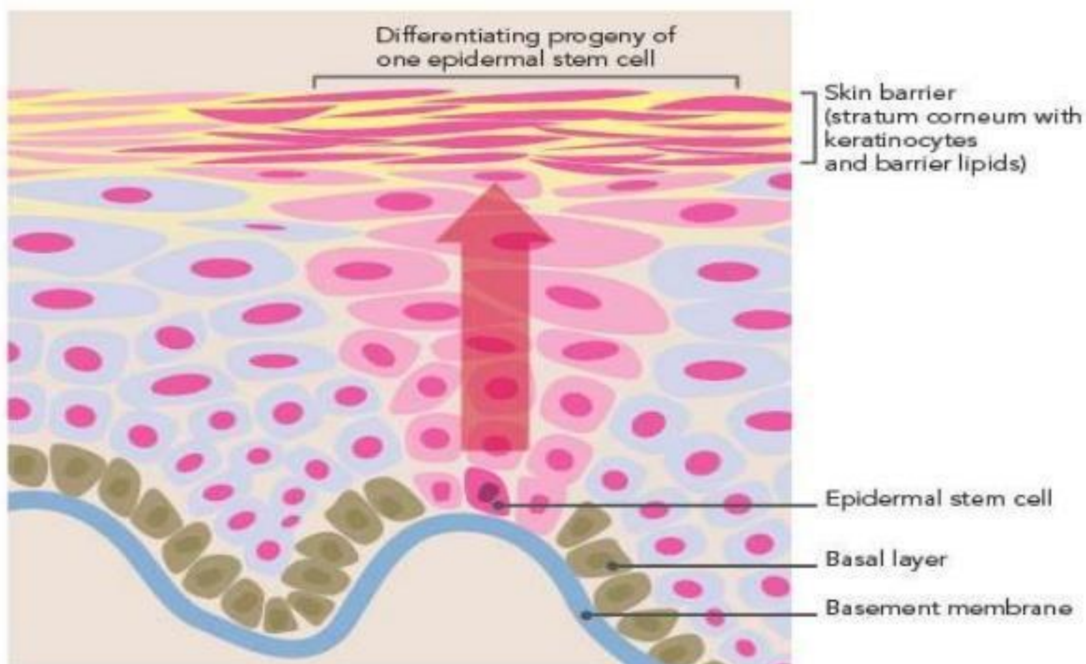

Fig.1.7: Diagram illustrating the function of “epithelial stem cells” in the renewal of skin cells and thus restoring the skin to act as the primary defense of the body (Kaufman, December 2017).

1.6 Hematopoietic Stem Cells (HSCs)

The bone marrow has cells labeled as "**hematopoietic stem cells"** that produce every cell kind of the "blood and immune system". The "hematopoietic stem cells" can too be found in little quantities in "peripheral blood" and in bigger quantities in the "umbilical cord blood". Within the "bone marrow", "hematopoietic stem cells" are attached to "osteoblasts" of the "trabecular bone" and to also to the "blood vessels". They produce offspring which are able to grow to be "lymphocytes", "granulocytes", "red blood cells", and specific other classes of cells, relying on the equilibrium of "growth factors" in their direct surroundings (Ashley Ng. & Warren Alexander, February 2017).

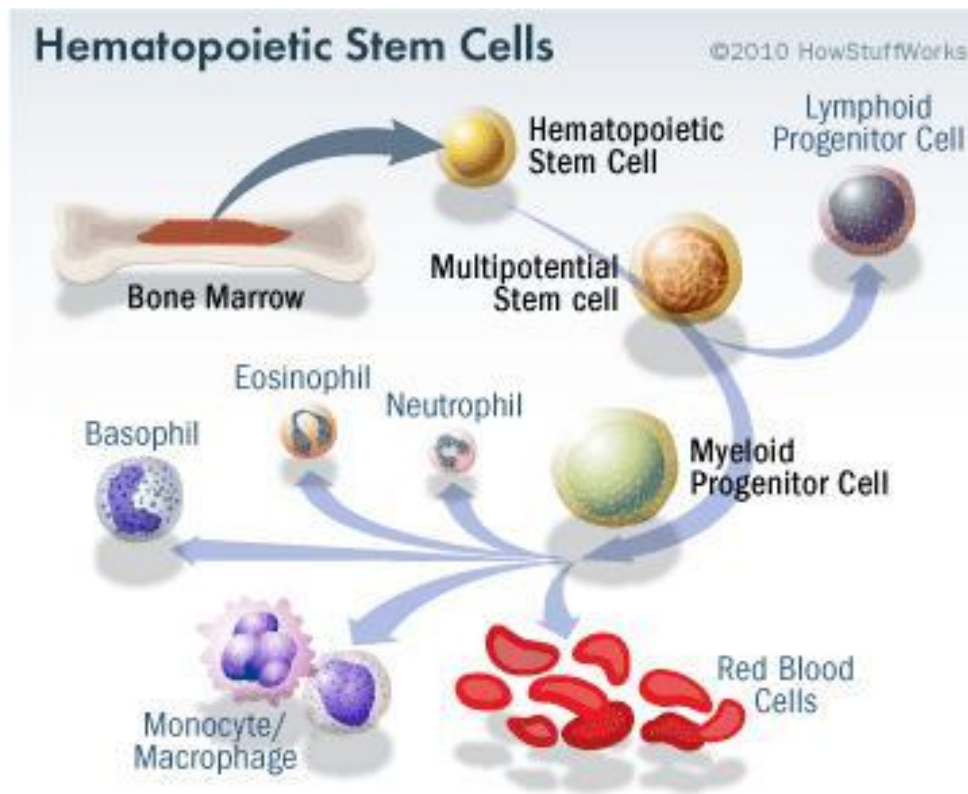

Fig.1.8: Schematic diagram showing the origin of "hematopoietic stem cells" and its "differentiated cell products" (Wagers & Weissman, 2006).

1.7 Neural Stem Cells (NSCs)

Earlier researches have revealed that the “stem cells” are also present in the brain. Usually in the case of “mammals” little amount of new “neurons” are produced following birth, nevertheless a number of “neurons” in the “olfactory bulbs” and also in the “hippocampus” are constantly produced. In general, these “neurons” occur from “**neural stem cells”**, that are able to be cultivated “in vitro” in the structure of “neurospheres”—that can be usually seen as tiny “cell clusters” which consists of “stem cells” and a number of their offspring. Nonetheless, this sort of stem cell is still being investigated for application in “cell therapy” to cure “Parkinson disease” and additional classes of “neurodegeneration” or “traumatic” injury to the “central nervous system” (Gage, Fred H. et al., 2013).

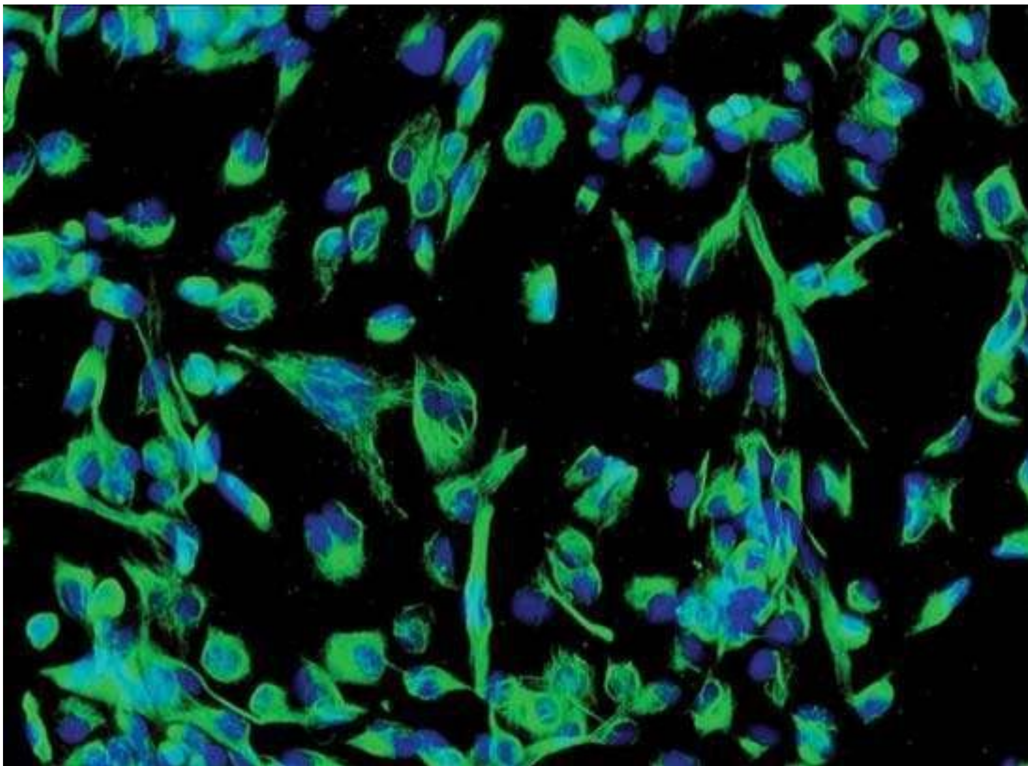

Fig.1.9: “Human neural stem cells” (cell nucleus illustrated in blue) (Kurtzman, June 2015).

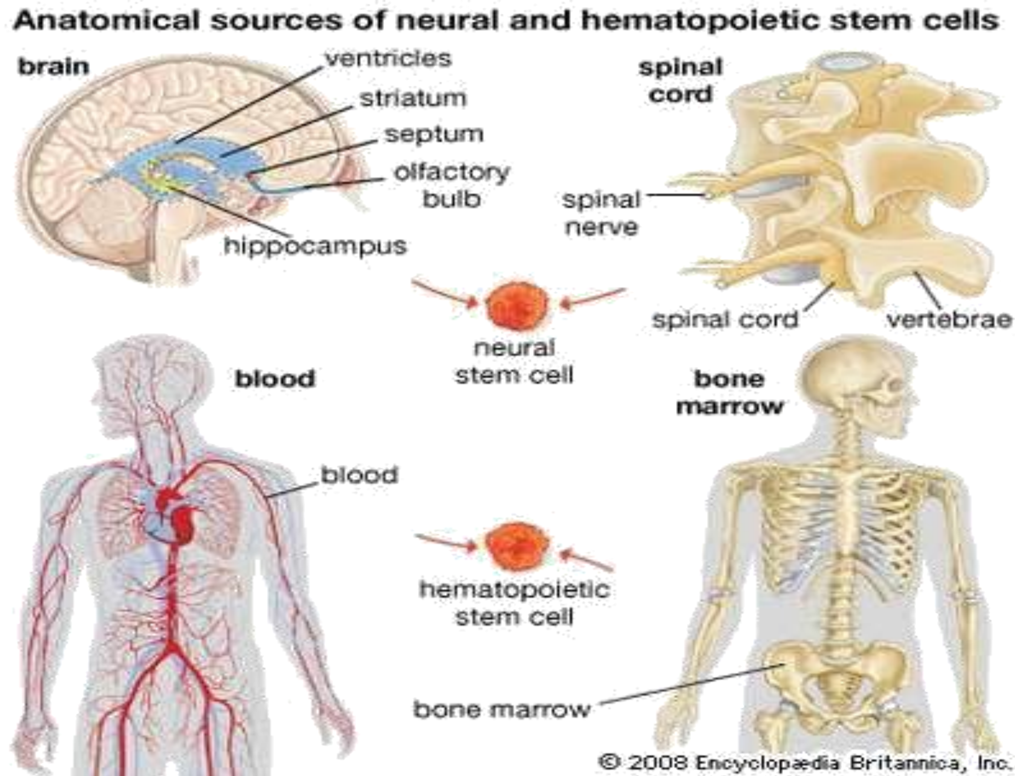

Fig.1.10: The hematopoietic and neural stem cells have incredible capability in improvement of treatments used for specific diseases, for example Parkinson disease and in some cases of diabetes. The "neural stem cells" arise within certain regions of brain and in the spinal cord, and "hematopoietic stem cells" arise in the blood and in the bone marrow (Rugnetta, 2008).

1.8 Induced Pluripotent Stem Cells (iPSCs)

Since there are ethical and moral concerns regarding the application of embryonic stem cells, researchers have looked for several methods to reprogram mature somatic cells. Investigations of cell fusion, during which cases differentiated adult somatic cells, developed in culture media with embryonic stem cells, combine with the stem cells and attain characteristics like embryonic stem cells, directed to the thought that particular genes might

reprogram differentiated adult cells". A mentionable benefit of "cell fusion" is that it depends on present "embryonic stem cells" rather than on the "eggs'. On the other hand, "fused cells" triggered an immune reaction when it is transferred into humans, which results in "transplant rejection". Consequently, research has happened to be gradually more concentrated on the "genes and proteins" that are skilled for "reprogramming adult cells" to a "pluripotent state". With the aim of making adult cells "pluripotent" exclusive of "fusing" them to "embryonic stem cells", the "regulatory genes" which trigger pluripotency should be inserted inside the "nuclei of adult cells". For this to happen, adult cells are developed in cell medium, and particular mixture of "regulatory genes" are introduced into "retroviruses"- these are viruses that alter "RNA [ribonucleic acid]" into DNA) that are afterward placed back in the culture solution. These "retroviruses" carry the "RNA of the regulatory genes" inside the "nuclei of the adult cells", where finally the genes are subsequently incorporated within the DNA of the cells. Stastically, about "1 out of every 10,000 cells" attain "embryonic stem cell" characteristics. Even though the method is vague till now, it is obvious that a number of the genes offer "embryonic stem cell" characteristics by the way of the "regulation" of several other genes. Hence, adult cells that are converted into "reprogrammed" cells in this method are identified as "**induced pluripotent stem cells" (iPSCs)** (Megan Scudellari, **June** 2016).

Theoretically, like "embryonic stem cells", "induced pluripotent stem cells" are able to be triggered to divide into specific classes of cells that might be able to cure specific diseases. Furthermore, the reproduction of "induced pluripotent stem cells" from adult cells of individuals afflicted with "hereditary diseases" can be employed to signify the ailments in the research labs. For instance, during 2008, researchers extracted skin cells from a baby afflicted with a genetic "neurological disease" called "spinal muscular atrophy" and after that the extracted cells were "reprogrammed" into "induced pluripotent stem cells".

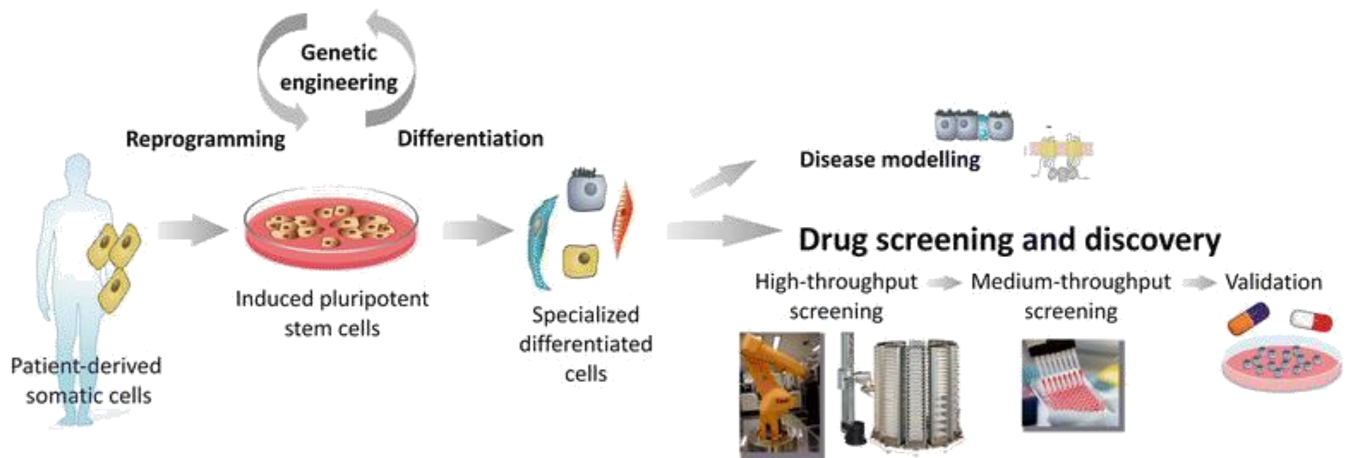

Fig.1.11: Illustration of the production of iPSCs and its application in regenerative medicine (Megan Scudellari, June 2016).

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The resultant "reprogrammed cells" possessed the "disease genotype" of adult cells and was triggered to divide into "motor neurons" that confirmed "functional deficiencies" linked with "spinal muscular atrophy". Consequently, as the disease was further investigated in the research lab, scientists were capable of investigating intimately the "cellular changes" that arise with the advancement of the disease. Representations like these secure does only expand scientist's knowledge about "genetic diseases" but it also assists in the improvement of latest "therapeutic" methods which are personalized to all sorts of genetic diseases (Megan Scudellari, June 2016).

Thus, "patient-specific induced pluripotent stem cells" are extremely important due to their therapeutic functions, for the reason that they are not as much expected to be "rejected by the immune system". On the other hand, prior to the use of "induced pluripotent stem cells" for the treatment of human diseases, researchers have got to discover a technique to insert the "active reprogramming genes" not including the involvement of "retroviruses", that are known to occur diseases in human beings such as "leukemia". A different promising substitute instead of the

usage of “retroviruses” for carrying “regulatory genes” inside the “nuclei of adult cells” is the appliance of “plasmids”, which are “tumourigenic” to a lesser amount compared to “viruses” (Singh V. K. & Kalsan, M., 2015).

2. Methodology of Research:

Thorough literature review was done to obtain all the information used in this review paper. The information was collected from various credible sources, including different peer-reviewed journals, online scholarly database, books, newspapers and magazines. Following are the list of some of the many journals that were searched extensively for the present study:

- The Journal of Regenerative Medicine..
- Cell death discovery.
- Oxford Academic.
- Frontiers in Cell and Developmental Biology.
- Nature Reviews Genetics.
- The Journal of Biological Chemistry.
- Cloning and Stem Cells.
- International Journal of Stem Cells

The aim of this review paper is to compile nearly all the methods and practices that have been discovered and used by scientists all around the world, over the past years, including the ones that have been invented recently. Hence, this review paper will help to look over all the advantages and disadvantages of the methods which had been discovered over the years and thus, enable scientists to invent new unique methods of reprogramming somatic cells, by picking up the successful advantages of the methods and leaving the disadvantages of those methods.

2.1 Transcription factors transduction by vectors:

The first iPSC reprogramming method used retroviral or lentiviral vectors for "expression" of OSKM "(Oct4, Sox2, Klf4, and cMyc)" factors. Even though these vectors ensure high reprogramming efficiency, they can cause insertional mutagenesis resulting in dangerous effects, such as tumour formation. Thus, for cell therapy purposes, alternative vectors were used to produce transgene integration-free iPSCs, including adenovirus, piggyBac transposon, episomal vectors, Sendai virus, plasmids, minicircle vectors, proteins, and synthetic RNAs. However, achieving full reprogramming by reactivation of key pluripotency markers is a more extensive process (Ichisaka & Yamanaka, 19 July 2007).

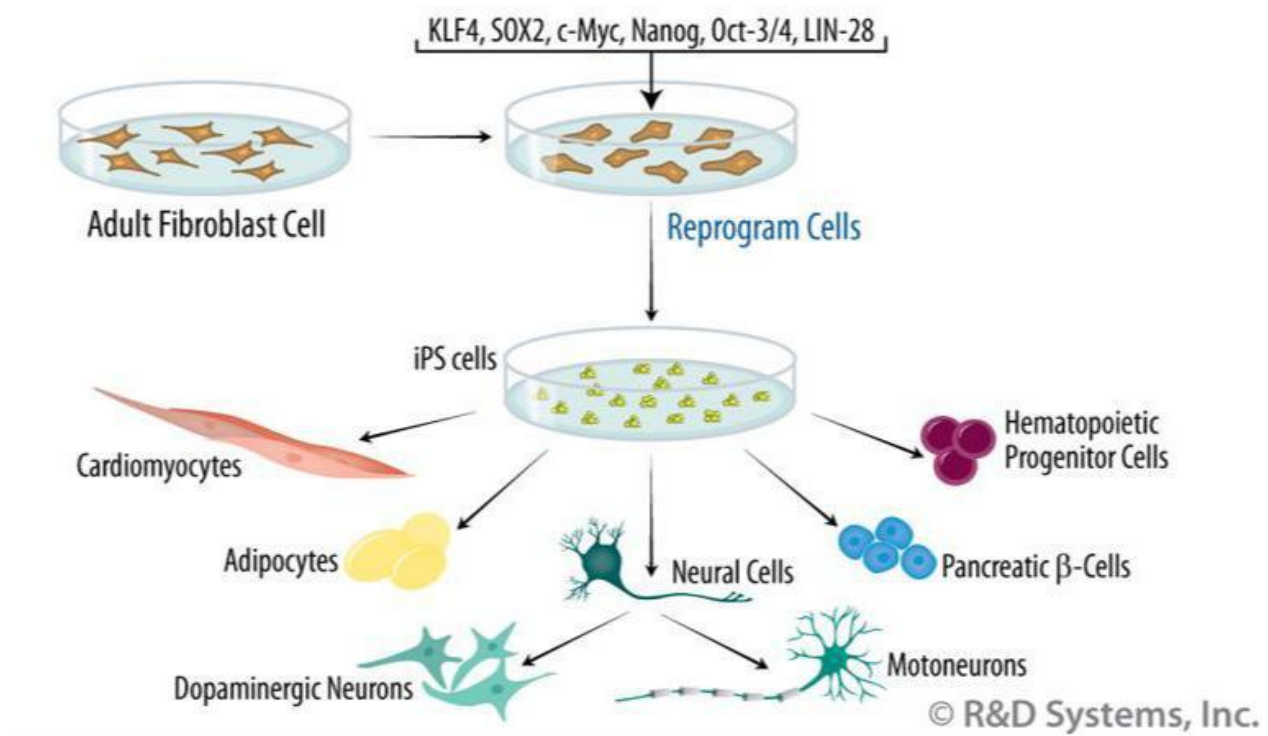

Fig.2.1: Illustration of the process of reprogramming of the adult fibroblast cells through the introduction of "transcription factors" (Ichisaka & Yamanaka, July2007).

Barriers to the Reprogramming Process: Although cell reprogramming is repeatable, the process is slow (around 2 weeks) and inefficient. Just a small portion of "transfected" cells (0.1–3%) become iPSCs, demonstrating that somatic cells must overcome barriers to revert to pluripotent state. Apoptosis and senescence are the eventual destiny of the majority of cells induced by OSKM (Apostolou & Hochedlinger, 24 October 2013).In spite of the success of adenovirus, piggyBac transposon, episomal vectors, Sendai virus, plasmids, minicircle vectors, proteins, and synthetic RNAs in producing iPSCs, regularly lacking a "genetic footprint", the application of DNA assembles still keeps the likelihood of "genomic integration of exogenous sequence". Further efforts to produce iPSCs by "non-integrating virus-mediated gene delivery" are not able to prevent the "safety concerns" that rise due to the use of viruses.

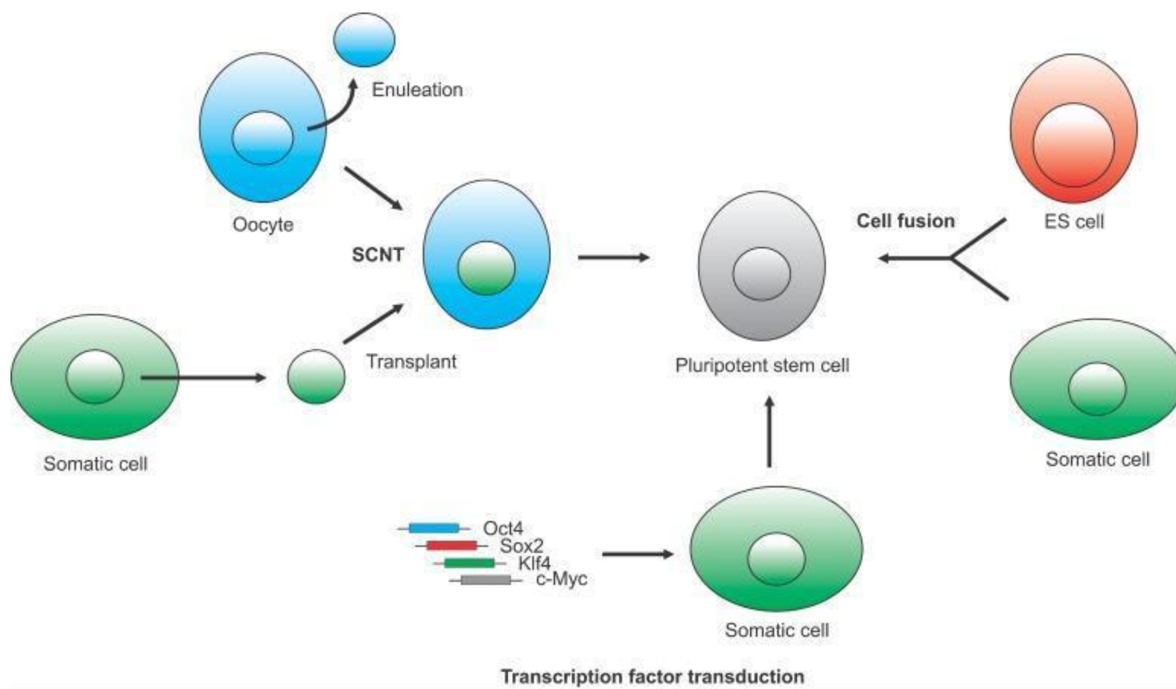

Figure 2.2: Three different pathways to create pluripotent stem cells from somatic stem cells. Pluripotent stem cells can be created by somatic cell nuclear transfer, cell fusion and transduction of transcription factors (Kim & Choi, 2011).

2.2 Somatic cell nuclear transfer:

This method comprises of extracting "the nucleus of a diploid cell" (full set of paired chromosomes) and then transferring it to an enucleated (a cell with no nucleus) "oocyte" (egg cell). The restructurized cells are stimulated to grow into embryos. Stimulation, or activation, is accomplished by a momentary "increase in the intracellular-free calcium concentration" provoked either by "electrical pulse" or by "chemical stimulation". The "preimplantation-stage embryos" are then preserved inside a "sequential culture media" and the developed embryos are transported and implanted in a "foster mother" (Fulka J Jr & Loi P, June 2004).

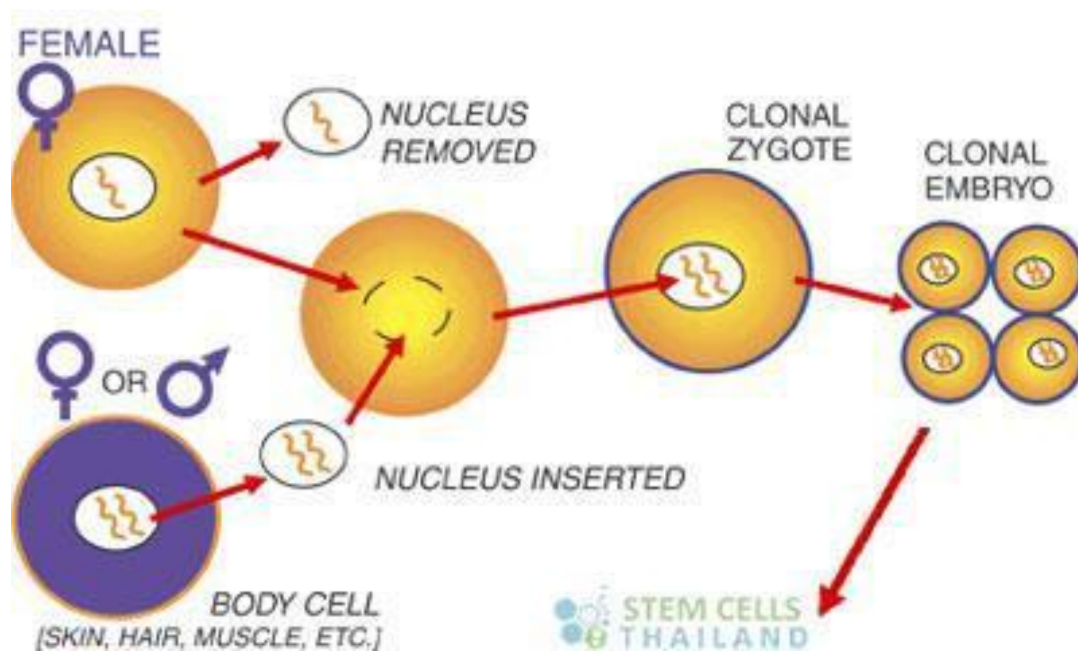

Fig.2.3: Demonstration of the method of somatic cell nuclear transfer and how it is performed (Fulka J Jr & Loi P, June 2004).

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Unfortunately, SCNT shows very little success rates for "full-term cloning". The effectiveness of "nuclear transfer" rely on an amount of vital scientific and "biological variables" such as "oocyte quality, enucleation, cell transfer procedures and oocyte activation". Furthermore, cloned mice showed many deformities, including abnormal gene expression patterns, abnormal placentas, and early death (Bortvin A & Eggan K, April 2003). Wakayama and colleagues discovered that the effectiveness of "mouse cloning" could be improved by "up to five-fold through the addition of the histone deacetylation inhibitor trichostatin A (TSA) into the oocyte activation medium" (Kishigami S & Mizutani E, February 2006). Thus, the successful reprogramming by SCNT must involve the exact epigenetic change by reprogramming factors of the oocyte, which bring on all the "epigenetic changes" that follow "down-regulation" of somatic genes and "up-regulation" of embryonic genes. (Kim & Choi, 2011). Furthermore, SCNT is unsuccessful to generate "phenotypically homogeneous clones" because of the mitochondrial DNA remained in the enucleated oocytes, as a result this leads to a variety of cell function irregularity and "phenotype change" in the consequential progeny. Thus, reprogramming by SCNT should be replaced by other safe methods such as fusion with ES cells or direct reprogramming by transduction of transcription factors.

2.2 Cell fusion-induced reprogramming:

Several groups have shown that pluripotent stem cells have a fundamental ability for pluripotential "nuclear reprogramming" of somatic cells by cell fusion. Therefore, somatic cells can attain a "pluripotent state" after being fused with pluripotent stem cells such as "embryonic stem cells, embryonic germ (EG), and embryonal carcinoma (EC) cells" (Tada M & Takahama Y, 2011). Tada and colleagues were the first and foremost to show "pluripotential nuclear reprogramming" of somatic cells by cell-cell fusion. They "fused" female EG cells, which are pluripotent cells derivative of "primordial germ cells", with "thymocytes" from adult mice (Tada M & Lefebvre L, 1997). These "fused tetraploid" cells were pluripotent and could "contribute to the all three germ layers in chimaeric embryos". Additionally, Tada and colleagues also demonstrated that somatic cells can achieve pluripotency after being fused with ES cells (Tada

M & Takahama Y, 2001).

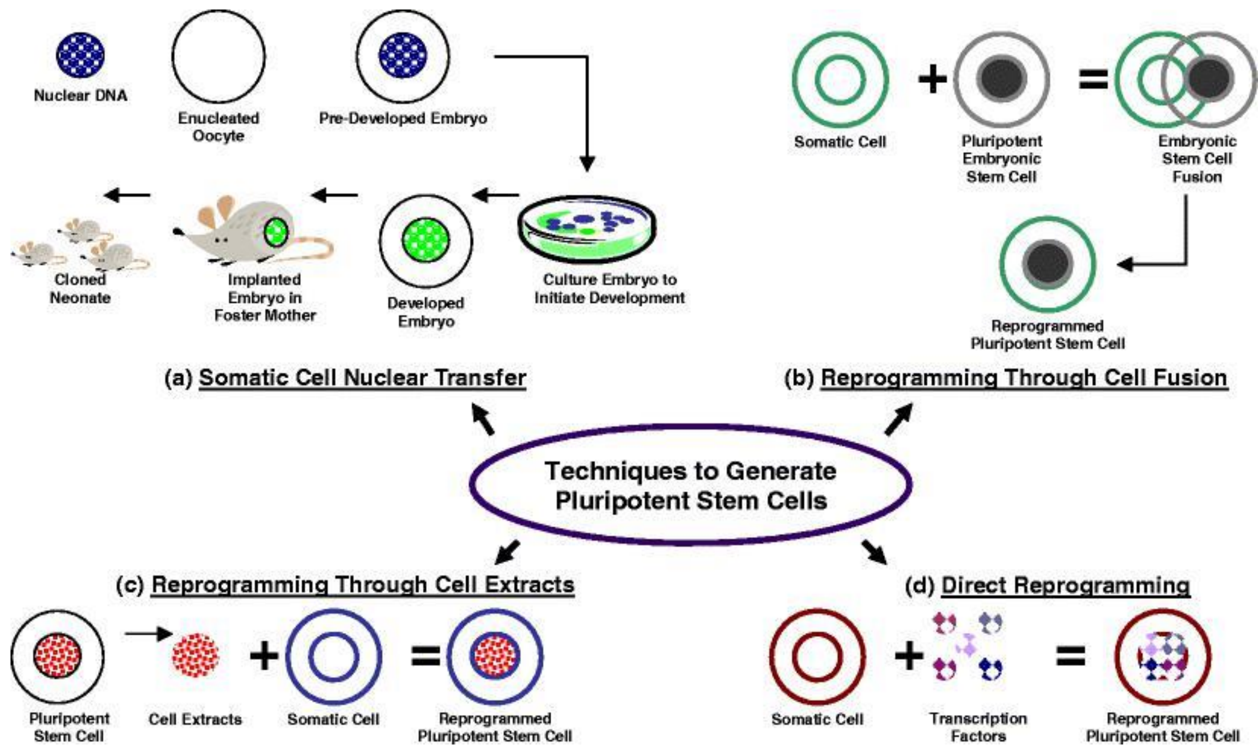

Fig.2.4: The four techniques to produce “Pluripotent stem cells” (Tada M & Takahama, 2001).

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While the fusion hybrid cells show pluripotential characteristics, the “fusion hybrid cells” are not similar to “the pluripotent fusion partner cells”. The fusion hybrid cells can form chimera but not contribute to germline. Although fusion-induced reprogramming is very efficient (about 95%), but the resultant hybrid cells lack therapeutic properties because of their “tetraploidy” and the existence of exogenous genes from the pluripotent fusion partner cells. (Kim & Choi, 2011).

2.3 Reprogramming through Cell Extracts:

This method is useful to comprehend whether and to what level “soluble factors of the embryonic stem cell” affects the “nuclear reprogramming” of the somatic cell. In this procedure “cell extracts” are obtained from pluripotent stem cell including “embryonic stem cells, embryonic germ cells or embryonic stem cell -like cells” and this “crude extract” is inserted into somatic cells (Patel M., & Yang, 2010). In particular, chemicals are used to “permeabilize” somatic cell membranes which are “incubated with cell extracts isolated from embryonic stem cells”. These cells are then able to “differentiate towards multiple cell lineages”.

Since the removed extract is composed of pluripotent “reprogramming factors”, hence it is used to help convert somatic cells reverse to its pluripotent position. One of the “factors Brg1” present in the extract has been shown to carry out a function in “nuclear reprogramming”. Moreover, other cell extracts including “Oct4, Utf1, Oxt2, Rex1 and Nanog” are also being recognized that in the future possibly will remove the requirement for “oocytes” to achieve “nuclear reprogramming”.

Therefore, “reprogramming through cell extracts” has been useful for human cells; though they were just incompletely “reprogrammed” to “pluripotency” and hence did not exhibit “in vivo differentiation”.

2.4 Direct reprogramming:

This method divides “somatic cells” to cells of “target lineage” through “multipotent pathways”, avoiding the stage of pluripotency. For instance, adult stem cells are self-restorative cells that can divide to form some specific cell types (Ankshita Prasad, 2016). Therefore, it is a “cell lineage” transformation process that narrowly imitates the usual “iPSC reprogramming method” but go around the “pluripotency” phases. Despite the fact that iPSC initiation need 1 or 2 weeks of “transcription factor expression”, “direct reprogramming” employs “transient expression of the Yamanaka factors”, which needs no more than a week,

to drive the cells in the direction of an “epigenetically unstable-plastic state” (no pluripotency), prior to directing it toward the “target cell lineage”. “Direct reprogramming” as well as merges “transient expression of iPSC factors” with different “signaling environments” for example, the “growth factors” or “signaling pathway modulators” which encourage “cell lineage conversion” under suitable culture conditions (Ma T & Xie M, February 2013). Even though “direct reprogramming” circumvents pluripotency, the “differentiation” procedure could still require an intermediary “multipotency stage” (Prasad, Loong & Chua, February 2016).

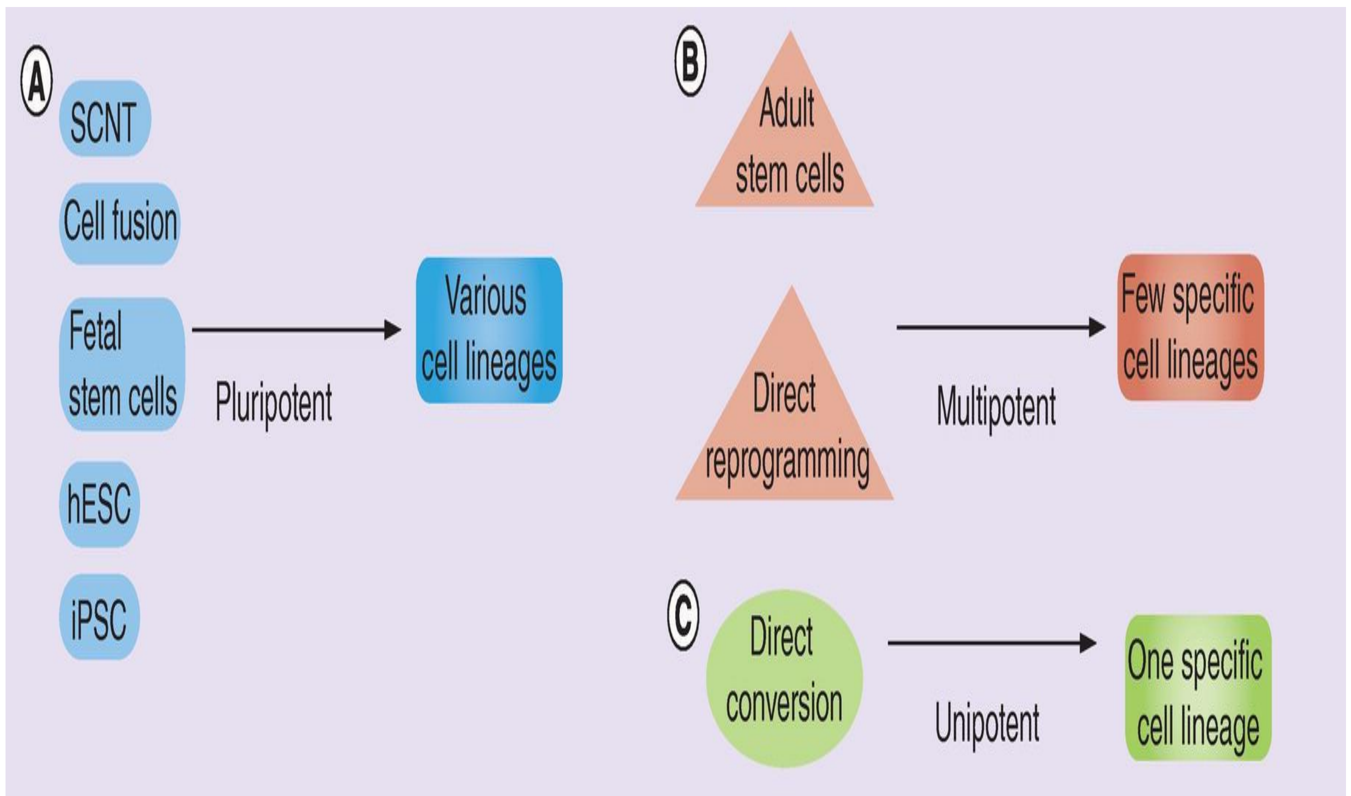

Fig.2.5: Various methods of obtaining cells of “target lineage” through “pluripotent, multipotent and unipotent pathways” (Prasad, Loong & Chua, February 2016).

2.5 Direct conversion:

This technique is established on the theory of the "dominant action of transcription factors" involved in the "transdifferentiation" process, where the cells go through a "lineage change" to predecessor cells of one particular "target lineage". This method is a substitute pathway that is used to produce "lineage-specific terminal cells". The procedure alters and assigns fully developed and fully divided cells into one exact "terminal cell type" avoiding the phase of pluripotency.

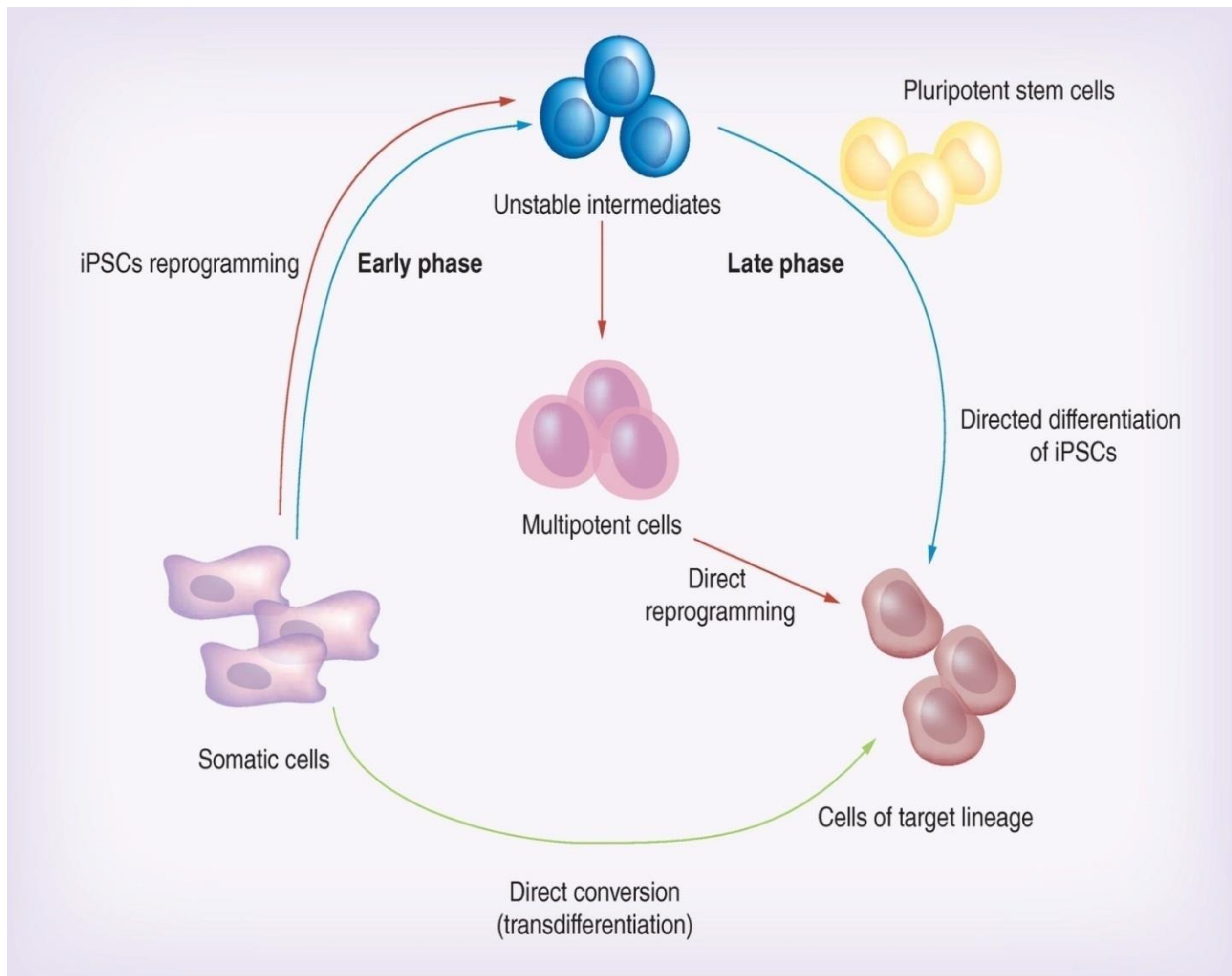

Fig.2.6: Demonstration of the "wave models of reprogramming" (Silva J & Smith A, 2008).

In preference to the “iPSC reprogramming technique”, in which the “epigenetic” information is wiped away to achieve a “pluripotent ground state”, “direct conversion” largely targets at initiating the “epigenetic features” of the most wanted cell form to attain alteration among two specifically isolated cell forms (Silva J & Smith A, February 2008).

2.6 Production of iPSCs using proteins or mRNAs of defined factors:

Even though iPSCs can be acquired by “removable transposon” or the “non-integration episomal system”, all of these processes still entail inserting unknown DNA into the cell so that the consequential cell lines require to be “sequenced” to validate that they are certainly free of “genome alteration” (Kaji, Woltjen & Yu, 2009). Therefore, a “reprogramming” pathway with the aim of not using any DNA is greatly advantageous. Hence, distribution of “reprogramming factors in their protein form” is the primary apparent option. During 2009, two groups illustrated that they were able to achieve “transgene-free iPSCs” with “proteins of reprogramming factors” (Kim & Zhou, 2009). During these experiments, “reprogramming factors” were combined with “cell penetrating peptides”. Later on, repetitive contact of “fibroblast cells to these proteins” resulted in them to be converted into an “ESC-like morphology”, “expressed undifferentiated cell markers”, and achieved “differentiation ability”. Nevertheless, these “protein-based reprogramming” experimentation has not been broadly conducted successfully (Wang & Jie Na, October 2011). Supposedly, mRNAs may probably be an ultimate medium for “reprogramming” due to a number of causes: they are a lot smaller than DNA, so they are able to penetrate cells with increased effectiveness; secondly, mRNAs will be “translated into proteins” by the host cell, therefore they are accurately “modified” and have elevated activity compared to proteins formed in “bacterial cells”. Additionally, mRNAs are simple to “multiplex” and the quantity of each “factor” can be accurately modulated. Consequently, mRNA can be a flexible tool equally for “reprogramming” and to study the “mechanism of this process” (Plews, 2010). Though, mRNA too has a quantity of severe shortcomings that is the “expression window of mRNA” is no more than 2–3 days and for this reason, “repeated transfection” is required to complete “reprogramming” and “innate immune response” should be “suppressed” along with “mRNA

transfection". While presently these problems make "mRNA reprogramming" researches costly and complicated to replicate, although with the invention of further "reprogramming factors" and tools to speed up this procedure, it may perhaps be possible to stimulate pluripotency within 2–3 days via an optimum mRNA and small molecule "cocktail." This kind of rate is alike the "reprogramming" rate of "somatic nuclear transfer", and as a result is supposed to be attainable in hypothesis by using "definite factors" (Wang & Jie Na, October 2011).

2.7 Producing iPSCs by "microRNAs":

Recently, three studies stated that "miRNAs" on their own are adequate to trigger "pluripotency" in human and mouse cells. The "miR302 cluster" is a straight goal of "Oct4 and Sox2" (Card & Suh, 2008) and are there in large quantities in mouse and human ES cells (Suh & Lin, 2004). During 2008, Lin and colleagues stated the conversion of "human skin cancer cells" into an "ES-like" condition by "overexpression of the miR302 cluster" (Lin & Card, 2008). During 2011, two separate teams declared extremely effective "generation of mouse and human iPSCs" either by "lentiviral delivery of miR302" into "fibroblast cells" or by "transfection of mature miR200c, miR302, and mir369" (Anokye, Danso & Miyoshi, 2011). These "microRNA iPSCs" were impossible to tell apart from "OSKM-iPSCs" in "pluripotency marker expression", "teratoma formation", and "germline transmission"- in rats. In contrast to "coding gene based reprogramming" techniques, "microRNA reprogramming" presents quite a few obvious advantages. It absolutely evades applying "oncogenic transcription factors" for example "cMyc and Oct4" and is not required to initiate "genetic changes" into "cell genomes". On the other hand, this novel "reprogramming" method needs to be effectively frequently performed by other researchers and the question about how the "molecular mechanism of miRNAs" turn on the whole "pluripotency network" is still to be discovered ((Wang & Jie Na, October 2011).

2.8 Via chemical compounds:

Achieving iPSCs via chemical compound by itself would be the best suitable technique, given that they are effortless to use and this does not engage “genetic modification” at all. The primary composites used in “reprogramming” experimentations were “DNA methyltransferase inhibitor 5′-azacytidine” and the “HDAC inhibitor valproic acid (VPA)”, that elevated the “reprogramming” effectiveness by “five folds and more than a hundred folds” correspondingly (Huangfu & Mikkelsen, 2008). Several tiny molecules possibly could substitute particular “reprogramming factors”. For instance, the “G9a histone methyltransferase inhibitor- BIX-01294”, has been confirmed to be capable of replacing “cMyc” and develop the “reprogramming” effectiveness of “neural stem cells” by “Oct4 and Klf4” (Shi & Huangfu, 2008).

2.9 Reprogramming from human blood cells:

This process signifies a unique and innovative approach of creating iPS cells from ‘donor’ which need slight “manipulation time” in culture media. This capability to “reprogram cells from the human blood” will assist the progress of a dependable technique to produce “patient-specific” stem cells.

In short, moving “peripheral blood cells” are extracted from a 26-year-old male donor, and “CD34 cells (mPB014F)” were collected. Reproduction of “CD34 iPS cells” was carried out by means of a procedure changed from formerly published technique. Mobilized peripheral blood is the major supply of cells for “hematopoietic transplantation”, “immunotherapy” and “gene therapy” (Yuin-Han Lo, March 2018). This method illustrates a cutting-edge use for “mobilized peripheral blood” in the production of iPS cells. Additional results from this experiment also presented the first evidence of the theory that cells from the “human blood lineage” are open to “reprogramming”. Therefore, blood cells signify a source of cells that prevent the necessity for “skin biopsies” and involve minimum preservation in culture medium prior to “reprogramming”. Therefore, “reprogramming” of blood cells is a significant step in the direction of improvement

of more proficient and effective methods of producing “patient-specific pluripotent stem cells”. In addition, this research presents an approach for the production of pluripotent stem cells for diseases caused by “somatic genetic disorders” specific to the “hematopoietic system” that cannot be summarized by “conventional fibroblast reprogramming” (Yuin-Han Lo, March 2018).

2.10 By activating the cell's own genes:

Until now, “reprogramming” had been achievable only by introducing the significant genes for the transformation, known as “Yamanaka factors”, inserted into skin cells in laboratories, where they are not usually “active” at all.

Professor Timo Otonkoski at the University of Helsinki and Professor Juha Kere at Karolinska Institute and King's College London, in the company of their groups of researchers, have made it possible for the first time in transforming skin cells into pluripotent stem cells by “activating the cell's own genes”. This is accomplished by using “gene editing technology called CRISPRa” that can be aimed at activating genes. The technique employs a “blunted version of the Cas9 gene scissors” which does not slice DNA and can as a result be employed to stimulate “gene expression” without altering the “genome” (ScienceDaily, July 2018).

"CRISPR/Cas9” can be used to activate genes. Therefore, this is an attractive possibility for “cellular reprogramming” because numerous genes can be targeted at the same time. Hence, reprogramming on the basis of activation of “endogenous genes” rather than “overexpression of transgenes” is also hypothetically a more “physiological way” of controlling “cell fate” and might result in more normal cells. In this study, it was demonstrated that it is possible to engineer a “CRISPR activator system” that allows “robust reprogramming of iPSC," said Professor Otonkoski.

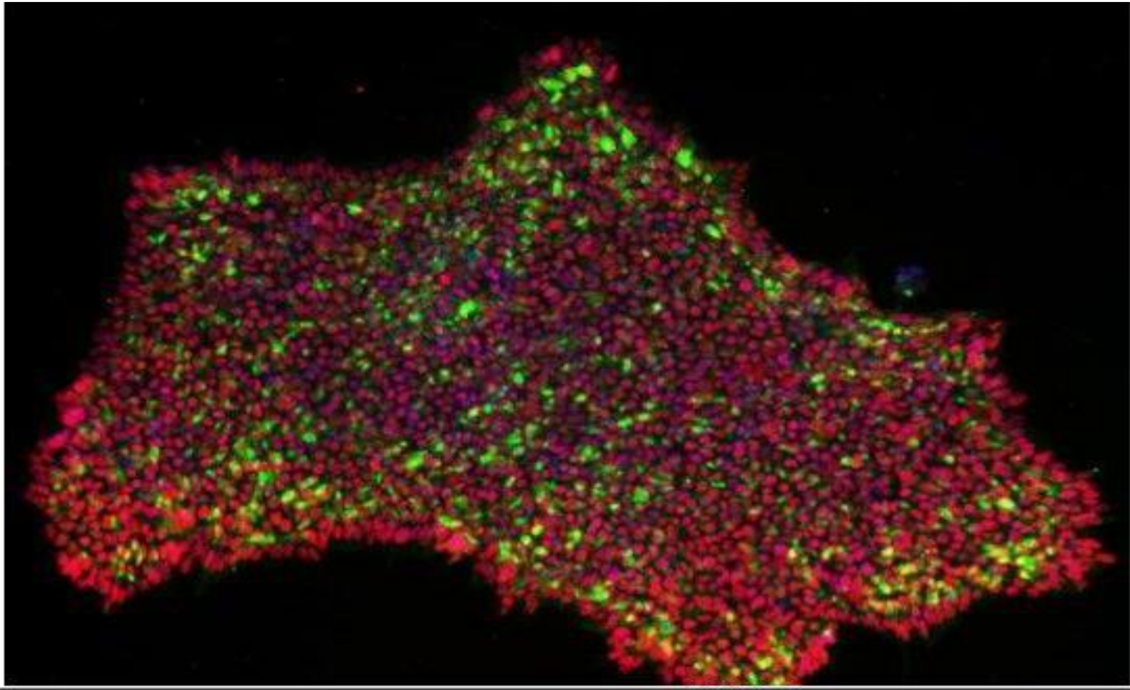

Fig.2.7: Photograph showing “CRISPRa reprogrammed induced pluripotent stem cell colonies” stained for “pluripotency marker expression” (ScienceDaily, July 2018).

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A vital part for the accomplishment was also turning on a significant “genetic element” that was previously found to control the initial steps of “human embryo development” following “fertilization”. Professor Kere said, "by means of this technology, pluripotent stem cells were obtained that resembled very closely typical early embryonal cells" (ScienceDaily, July 2018).

2.12 Characterization of iPSCs:

As iPSC “reprogramming efficiencies” are small and the quality of the newly produced iPSCs is altered by a number of factors, hence it is essential to cautiously “characterize” the iPSCs after completing the process of “reprogramming”. Different sorts of processes have been used to distinguish iPSCs. The “characteristic morphology” of iPSCs is frequently used as the earliest sign of iPSC development (Brouwer & Zhou, February 2016).

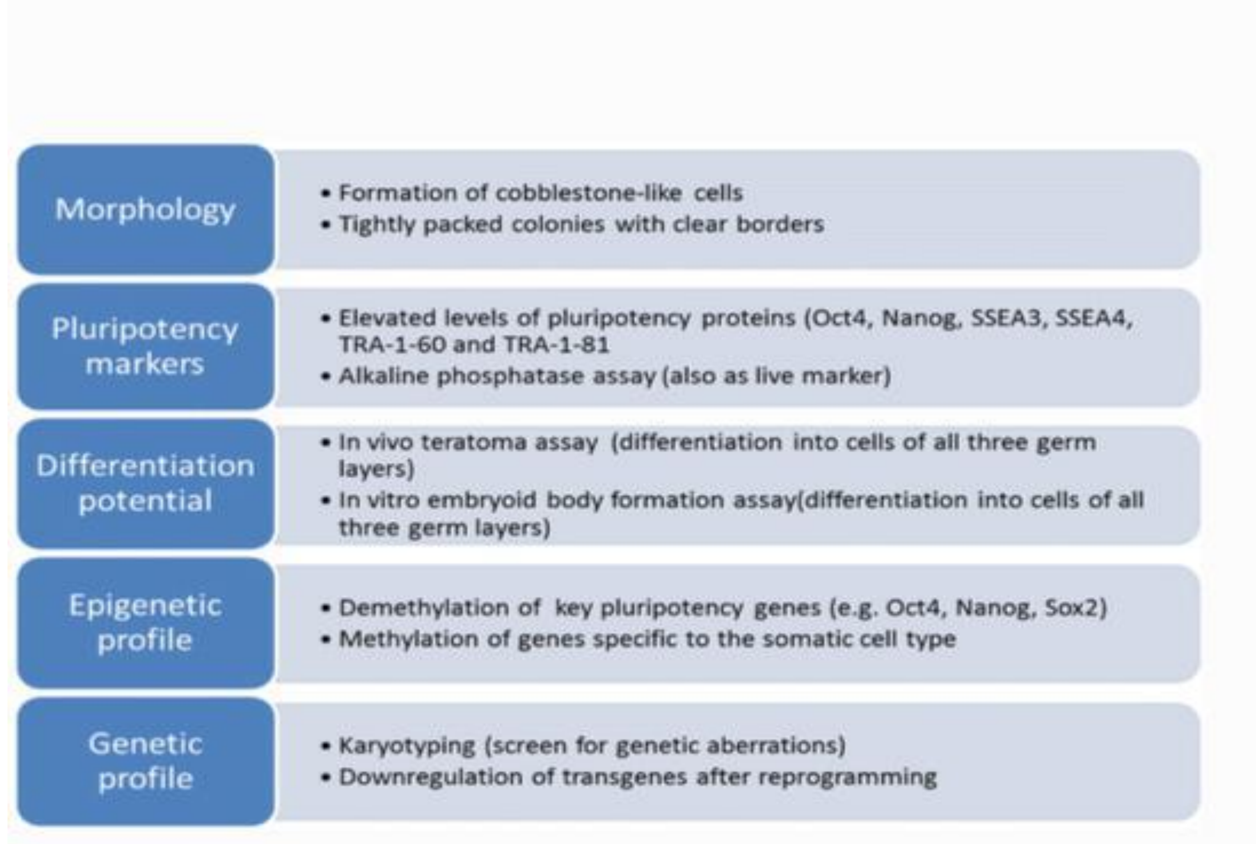

Fig.2.8: Outline of factors which must be assessed to “characterize” reprogrammed iPSCs. The iPSCs can be characterized on the basis of five different characteristics: “morphology, pluripotency markers, differentiation potential, epigenetic profile and genetic profile ”. For each feature, factors are specified which are important to evaluate the different characteristics (Brouwer & Zhou, February 2016).

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The iPSCs can be usually seen as tiny cells with a large "nucleus/cytoplasm ratio" which produce dense "colonies" that are distinctly outlined by "clear borders". Besides "cell morphology", many other "cellular and molecular methods" are used. Hence, from one of these methods, it consists of the "assessment" of the existence of "pluripotency marker proteins" (that is, "Oct4, Nanog, SSEA3, SSEA4, TRA-1-60 and TRA-1-81"), that are "expressed in pluripotent stem cells" (Boulting G. L. & Kiskinis, 2011). In view of the fact that these "markers" are not essentially "specific" to pluripotent stem cells, therefore, the "expression" of several of the "markers" must be evaluated in "combination" to verify the occurrence of pluripotent stem cells. "Alkaline phosphatase assays" can also be used to identify iPSCs. This technique uses the "high enzymatic activity of phosphatases" in pluripotent stem cells to produce a "fluorescent signal" and can be employed as a "live marker" for iPSCs (Singh U. & Quintanilla, 2012). Additionally, to these techniques using "morphological characteristics" and "cell specific markers", functional assessment of the produced iPSCs can be carried out by evaluating the "differentiation potential" of the iPSCs. Therefore, iPSCs should be able to "terminally differentiate" into cells of all "three germ layers", which can be evaluated via "in vivo teratoma formation assays" or "in vitro differentiation" through "embryoid body (EB) formation" into cells of the three "germ layers". Moreover, as "reprogramming" manipulates the "genetic" and "epigenetic" composition of the cells, therefore iPSCs should be carefully portrayed for "genetic aberrations" and "epigenetic analyses" for example "gene expression" and "DNA methylation profiles". "Karyotyping" is frequently applied to assess genetic anomaly in iPSCs. On the other hand, if "transgenes" are used for reprogramming, it is also vital to assess if the "expression levels of the transgenes" are appropriately "down-regulated" after the iPSCs are produced. For carrying out assessment of the "epigenetic profile" of the iPSCs, "DNA methylation patterns" can be evaluated. Ever since "DNA methylation" provides the "silencing of genes", hence it is essential that the produced iPSCs demonstrate "DNA demethylation" at major "pluripotency genes", that is "Oct4, Nanog, Sox2", whereas genes "specific to the donor cell type" turn out to be "methylated and silenced". To end with, it is significant to note down that the methods used to "characterize" iPSCs referred above should be applied in "combination" instead of alone (Brouwer & Zhou, February 2016).

3. Pluripotent Stem Cells: Applications, Challenges and Future directions:

Self-regeneration and pluripotency are basic characteristics of ESCs and iPSCs, making them striking to the academic world and industry for their probable preclinical and clinical applications in the cure of an extensive variety of diseases and pathological conditions.

Regenerative medicine is a thrilling and rapid moving field of research with the ambitious aim of using stem cells to substitute tissues/organs injured by damage, disease, or other inherited defects. Because of their capability to divide into all the specific cell categories of grown, "pluripotent stem cells" (PSCs), including human ESCs and iPSCs, are a potential source for cell-based therapies (Tabar & Studer, February 2014). "Therapeutic potential" of pluripotent stem cells have been evaluated in preclinical studies, where PSCs transplantation has been useful to treat different diseases (Harding & Mirochnitchenko, February 2014). Strikingly, cell therapy into animal models has shown positive effects, such as restoration of locomotion after spinal cord injury with "hESC-derived oligodendrocyte" (Kriks, Shim & Piao, December 2011), improved vision with "hESC-derived retinal pigment epithelium" (RPE) in blindness representations (Lund, Wang & Klimanskaya, 2006), and improved cardiac function in a "porcine ischemic cardiomyopathy" model with iPSC-derived cardiomyocytes.

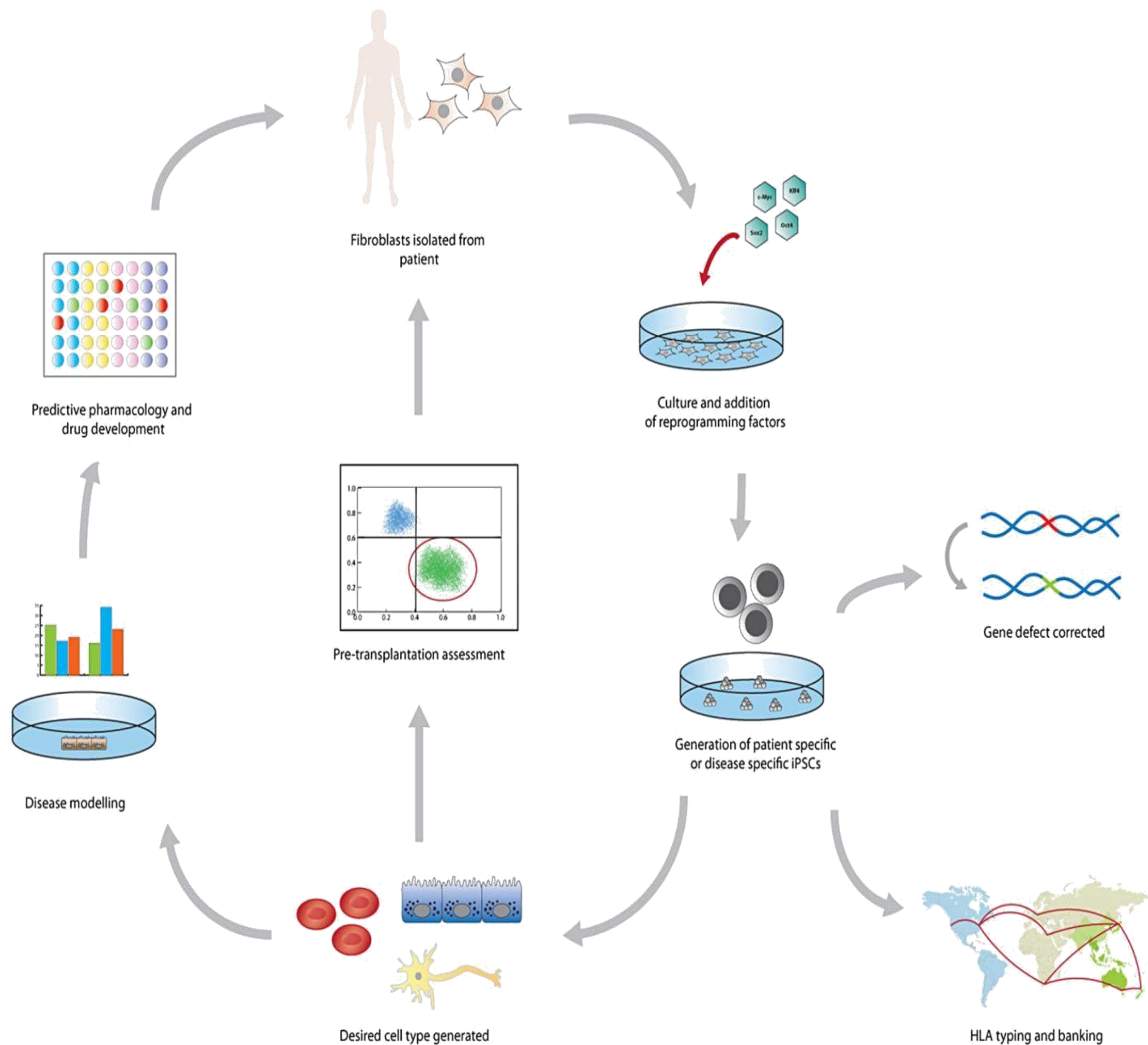

Fig.3.1: Schematic diagram of how "induced pluripotent stem cell (iPSC)" can be applied in a medical setting. "Fibroblasts" or other suitable cells types can be extracted from the patient and iPSCs can be generated (Tabar & Studer, February 2014).

Consequently, these iPSCs can be used for both individual and population requirements. In case of individuals, specialized cells can be derived for "cell replacement therapies" or "individualized pharmacokinetic studies". On the other hand, iPSCs can be used as a

representation of a combined disease. iPSCs can also be applied to “gene therapies” or be “human leukocyte antigen (HLA)” typed and stored for future use. (Sara D. Qi, 18 September 2013.)

The usage of “viral vectors” to transport “reprogramming factors”, which leads to everlasting “integration of oncogenes” and results in potentially harmful genomic alterations, is a serious concern (Higuchi, Ling & Kumar, January 2015). To overcome those issues, in mouse, a “protocol transgene-independent” that uses small molecules has been lately recognized, although the approach is less efficient than viral transduction with OSKM factors and the reprogramming process is a more extensive process (Hou, Li & Zhang, 2013). In human, the same objective has not been achieved yet and Oct4 transduction is required (Zhu, Li & Zhou, December 2010) suggesting the need to optimize the reprogramming strategies for the clinical safety of hiPSCs and develop the efficiency of the process.

Current studies have provided confirmation of genetic and epigenetic variations between different iPSC lines (G. Liang & Y. Zhang, August 2013). A number of the “variations” may perhaps be passed down from “donor somatic cells” or acquired during either the “reprogramming process” or “extensive culturing” (G. Liang & Y. Zhang, August 2013). Even though such genetic and epigenetic variation influence only a small portion of the genome, they might modify the characteristics of iPSCs and their subsequent derivatives, resulting in increased risk of “tumorigenicity”, altered differentiation potential of iPSCs, or distorted functional activity of iPSC derivatives (G. Liang and Y. Zhang, August 2013). Optimization of the reprogramming strategy and culture conditions may add in reducing or completely removing such variations (Carey, Markoulaki & Hanna, December 2011).

Another important issue is the establishment of quality controls to guarantee the safety of human iPSCs and their derivatives selected for downstream application (Jung, Bauer & Nolta, January 2012). Serum and mouse-derived feeder cells, used regularly to culture iPS cells, may pass on exogenous antigens or pathogens to reprogrammed cells, causing immune response or disease (Seki & K. Fukuda, January 2015). Thus, to produce clinically acceptable iPSCs, xeno-free cell culture systems should be used. Numerous investigations have been conducted and are still in progress, to create animal-product-free culture system decreasing the risks for patients (Seki & K. Fukuda, January 2015). This can be overcome by developing dependable and reproducible protocols for a direct and efficient differentiation of iPSCs in the preferred tissue.

Besides, pluripotent characteristics and developmental potential of iPSCs should be identified by teratoma formation (Wesselschmidt, 2011). iPSCs should also be differentiated in vitro to test their capability to generate the desired cell type. Evidence shows that epigenetic memories or partial reprogramming may affect differentiation properties of iPSCs (Kim, R. Zhao, A. Doi, December 2011) resulting either in a diverse population of differentiated cells or remaining undifferentiated cells that may be tumorigenic when transplanted in vitro.

Toxicity studies should also be performed on iPSC-derived products through the examination of major organs after transplantation of in vivo models.

Cell patient-derived hiPSCs signify an astonishing cellular model to study several genetic diseases. Consequently, in recent years, a large number of publications have reported that hiPSCs produced from patient with genetic disease, after differentiation, are able to repeat different aspects related with pathologies (Sterneckert, P. Reinhardt, and H. R. Schöler, Spetember 2014). Although cell patient-derived hiPSCs are constrained by the fact that they indicate a cellular model and they cannot replicate all the aspects of a disease and aspects, they remain a tremendously priceless means for the discovery of novel drugs with possible therapeutic applications and for toxicology studies (Maury, Gauthier, Peschanski, October 2012). Using hiPSCs for screening is a more cost-effective approach than animal testing. In addition, the chances of producing iPSCs from "healthy donors" and patients with "genetic" or "acquired diseases" offers a more accurate system to assess the exact effect of a drug in a more physiological condition compared to the current cellular models represented by immortalized human cell lines (Maury, Gauthier, Peschanski, October 2012). This advantage combined with their capability to divide into a broad range of specific cells will allow investigators to achieve targeted preclinical toxicological in vitro trials (Maury, Gauthier, Peschanski, October 2012). Although cell-based in vitro assays allow high-throughput and/or high-content screens, they do not imitate the complex setting in vivo. In vitro assays must be followed by animal model tests, the only way presently available to obtain a global understanding of crosstalk between different cell types and organs in a living organism, essential to recognize and characterize molecules and to allow their clinical transformation from bench to bedside (Fink Jr., June 2009). Moreover, the likelihood to produce cell patient-derived iPSCs provided a solution to overcome the strong ethical concerns and immunological rejection that are at present key obstacles to the clinical performance of hESCs.

4. Conclusion:

It is now indisputable that stem cell research is the way forward to tackle and possibly cure human diseases. Pluripotent stem cells, due to their capability of differentiating into a wide range cell types, are of most interest where functional "adult stem cell" types are difficult to access, enlarge or drive. In particular, hiPSCs represent an exciting alternative to embryonic cells, avoiding the ethical issues associated with their use and providing a better model for studying human diseases and possibly finding more effective therapies.

Although the progresses reached so far, further intensive investigations on the properties of human PSCs need to be performed equally to comprehend the fundamental biology of pluripotency and "cellular differentiation" and to work out all the different issues associated with therapeutic applications. In addition, improvement of the current technologies should be performed to achieve clinical-grade human PSCs for safe cell therapies.

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