A study of the prospects of Regenerative medicine highlighting some established protocols to generate organ-specific cells

A project submitted

by

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This work is dedicated to my parents and friends for their love and constant support…

Certification statement

This is to certify that this project titled “A study of the prospects of Regenerative medicine highlighting some established protocols to generate organ-specific cells” submitted for the partial fulfillment of the requirements for the degree Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Mr. Ashis Kumar Podder, Senior Lecturer, Department of Pharmacy, BRAC University and appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the supervisor,

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Abstract:

Regenerative medicine is a prominent field of research with a potential to substitute the current treatment options. From the replacement of damaged or injured tissue/organ to the changing direction of current medicinal practice, it can play a very impactful role. Tissue engineering is a sub-field of regenerative medicine that deals with the generation of body parts ex vivo, by seeding cells into a supporting scaffold. The basic components of regenerative medicine are cells and scaffolds, therefore, the current therapies include cell-based therapy, using biomaterials and implanting scaffolds seeded with cells. It is now possible to generate many cells or cells alike by guiding the differentiation of the stem cells. Various methods and protocols that are established through the research to get hepatocytes, cardiomyocytes and neural cells have been discussed in this project work. The study also highlighted the application of the above-mentioned cells in the treatment of many diseases.

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INTRODUCTION

Chapter 1: Introduction

In ancient Greek history, there was a tale of a titan Prometheus whom Zeus punished for giving fire to humankind. His liver was to be eaten by an eagle every day but surprisingly he survived because his liver regenerated itself. In the present time, the concept of regeneration is not a myth anymore, rather an emerging medical advancement. To restore or establish normal function of human body, the replacement or regeneration of human cells, tissue or organs is implicated as Regenerative Medicine (Sampogna, Guraya, & Forgione, 2015). Although it is a little-known field of research, this approach is beginning to represent a reliable choice of treatment for chronic diseases, acute injuries, and congenital malformations.

Regenerative medicine is a section of translational research in molecular biology and tissue engineering. It seeks to replace whether it is any tissue or organs that have been impaired by trauma, disease or inherited issues or it is the present clinical strategies focusing mostly on treating the symptoms. In fact, it has significant potential in circumstances where the body cannot heal itself, so, tissues and organs can be grown in the laboratory and implanted in the body. This is a promising field which is working to restore structure and function of damaged tissues and organs with a goal to find approaches to cure earlier untreatable injuries and ailments.

1.1 History of Regenerative Medicine:

The concept of regenerative medicine was not developed in a day. It has a long history that began way back in the 15th century. Just like an infant, it experienced many development, arguments, and researches and grew step by step forward to embrace adulthood. In the present time, it is a vast area with great potential.

Descartes (1596–1650) was the first to challenge a divine significance in biological phenomena. According to him, the physical and chemical properties can interpret the function of the human body (Polykandriotis, Popescu, & Horch, 2010). Due to numerous controversies and arguments, it took almost mid of the 18th century to generate the concept of regeneration. On one hand, the theory of generation and regeneration was thoroughly supported by the naturalists. On the other hand, Pre-formationists supported another idea. They believed that appendages were regenerated and organisms were born pre-existed as miniature (Polykandriotis et al., 2010) However, Aristotelean thesis gave an opposing supposition. It implied that giving rise to life was possible

for the undifferentiated matter. William Harvey (1578–1657) named it 'Epigenesis' after later researches. Abraham Trembley (1710–1784) created numerous publications on the regenerative phenomena on freshwater polyps (Dinsmore, 1991). At the end of 18th century, a revolution in natural sciences occurred when epigenesis gained acceptance setting aside philosophical and religious debate connected to science.

The 19th century brought the succession of development. In 1838–1839, the cell theory was described by Schleiden and Schwann. In 1858, Rudolf L. K. Virchow inaugurated the idea that cells are the basic units of life and can divisionally replicate themselves. In 1867, Julius Cohnheim, the well-known German pathologist gave 'Cohnheim hypothesis' which proposed that the reparative cells participating in the regeneration of injuries originate the bloodstream as in, the bone-marrow (Polykandriotis et al., 2010). Finally, this century ended with Barth's observation of hounds. He discovered dying vast majority of cells upon autologous bone transplantation and leaving a scaffolding behind. These scaffolds are gradually repopulated by new host cells and a suitable neovascular network.

In 1912, Alexis Carrel, a French scientist started an experiment. He took a small piece of heart muscle from a chicken embryo and cultured it which sustained for several decades surviving more than the average chicken's lifetime. So, it was assumed to be inherently immortal cells. Though this belief ruled the 20th century, in the 1960s, an opposing theory of this dogma, 'Hayflick limit' was proposed by Leonard Hayflick and Paul Moorhead. This theory implies that replication of differentiated cells in culture is not possible for more than 40–60 times and will certainly exhibit signs of senescence with consecutive passages. By the end of the 1960s, the introduction of the idea of pluripotency of germinal cells was established (Polykandriotis et al., 2010). In the 1970s, there was a considerable expansion of research with embryonal carcinoma stem cell.

The term 'tissue engineering' was officially introduced in 1987. The first conference on the 'Engineering of living tissue' was held in 1988 at Lake Tahoe, California and a year later, the proceedings of this conference were published as a book titled 'Tissue Engineering' (Viola, Lal, & Grad, 2004). Tissue engineering brought a new arena in the field of regenerative medicine bringing both challenges and developments. Gene technology, drug delivery systems, nanotechnology, tumor research and many other doors opened. Although financial resource

remains a significant barrier, this sector is the future of medical therapy. A new direction was found when niches of putative cardiac stem cells were identified in the murine and human heart lately. The basic research of regenerative medicine has become the necessity to discover the natural microenvironment and consequently, to engineer the artificial support essential for stem cells to act. It unlocked the idea that other organs can be regenerated just like liver. New tools to regenerative reprogramming of lost organ functions could be revealed by further molecular and cellular research into the functions of telocytes.

1.2 Relationship between Regenerative Medicine (RM) and Tissue Engineering (TE):

As we discussed earlier, to restore or establish normal function of human body, the replacement or regeneration of human cells, tissue or organs is implicated as Regenerative Medicine (RM) (Sampogna et al., 2015).

Tissue Engineering is a subfield of regenerative medicine, which is narrower in scope and strictly defined as the manufacturing of body parts ex vivo, by seeding cells into a supporting scaffold (Katari, Peloso, & Orlando, 2015). They depend on patient's own cell and so they are devoid of transplant rejection.

So, Regenerative Medicine is more of a therapy for the patient while Tissue Engineering is more of a capacity to build biologic material both in vivo and in vitro. However, RM and TE are often used interchangeably. On the other hand, some experts disagree saying that they represent different conceptual entities.

1.3 Elements of Regenerative Medicine and Tissue Engineering:

The two functional elements that have become the main focus of tissue engineering and regenerative medicines are – Cells and Scaffolds.

1.3.1. Cells:

The requirements must be followed by cells applied in tissue engineering and regenerative medicines are-

- Strong ability of differentiation
- Stable passage
- Be in response to stimuli signals
- Easily be harvested
- Seeded onto the extracellular matrix (ECM)

Primary cells are taken from the same organism which is going to accept implantation (Li et al., 2016). Although the differentiation rate is relatively slow, primary cells are still desirable with regard to lower immunological response and pathogen transmission. The ease of isolation of primary cells from the certain tissues enormously supports patient-specific therapy, especially for skin and auricles (Li et al., 2016). On one hand, some primary cells are replaceable for tissue formation in case a direct biopsy is not feasible, for example, peripheral vein cells are suitable for making heart valves. On the other hand, a number of tissues, such as spinal cord, peripheral nerve, etc., are too specific to be regenerated based on the primary cells because of their difficulties for direct biopsy as well as their lack of replaceable primary cells. Either way, stem cells are verified as an ideal source for regeneration.

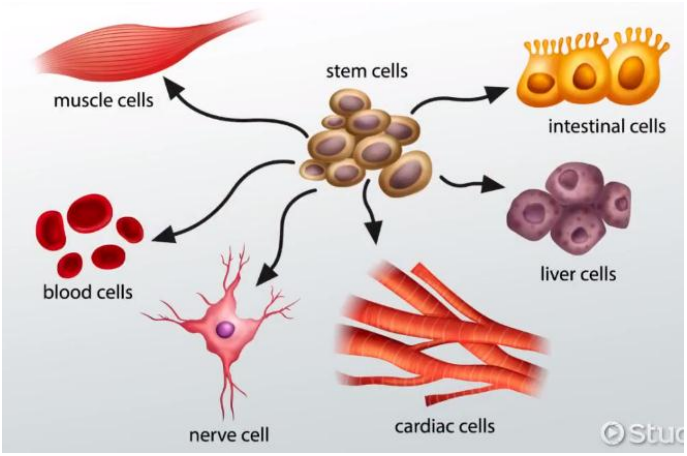

Fig 1.1: Organ-specific cells differentiated from stem cells

Classification of stem cells:

Stem cells can be classified based on the stage-

- **Embryonic stem cells (ESCs)**: These are most frequently used cell type derived from an early stage of the embryo. ESC can differentiate into various tissues like heart, liver,

nerve, kidney, bone/cartilage, lung, skin, and ovary with the structural support by scaffolds.

- **Adult stem cells (ASCs):** Compared to ESC, these have less ethical issues and controversies which can be easily and directly generated from some organs or tissues. One of the most impactful ASC is Adipose-derived stem cells due to their prospective ability to differentiate into adipocytes, chondrocytes, myocytes, neuronal cells and osteoblasts. ASCs have wide applications in adipose tissue formation, lung repair, wound healing, neurogenesis, osteogenic and chondrogenic differentiation, cardiomyogenic and differentiation.

Stem cells can also be categorized based on the potential function of differentiation as-

- **Totipotent stem cells**: A single cell can produce all cells.
- **Pluripotent stem cells**: It is the ideal cell type that can differentiate into cells of all three germ layers.
- **Multipotent stem cells**: These differentiate into multiple, but limited cell types due to gene activation.
- **Unipotent stem cells**: These differentiate into one single cell type.

1.3.2. Scaffolds:

Scaffolds provide a three-dimensional (3D) structure for cell differentiation into a tissue or an organ (Li et al., 2016). Scaffolds for Tissue engineering and regenerative medicine must possess some properties.

- Appropriate physical, chemical, and mechanical properties to enable cell adhesion and proliferation.
- Good bio-compatibility to avoid immunogenic response of a cell or related tissue.
- Bio-degradability as scaffolds ought to be degraded during or after healing and the products of the degradation must be non-cytotoxic.
- Detailed requirements and necessity may vary with different tissues.

There are several methods for fabricating scaffolds like-

- Fiber bonding
- Phase separation or freeze dryingSolvent casting or particulate leaching
- Gas foaming
- Rapid prototyping
- Electrospinning

1.4 Current strategies used in regenerative medicine:

There are three approaches which are currently in practice-

1.4.1. Cell-based therapy:

Cell-based therapy which is one of the foundation concepts of regenerative medicine. The direct contribution to the structure and function of new tissues is the delivery of therapeutic cells. It can be called the principle paradigm of cell therapy to date. Depending on the particular circumstances, with various pros and cons, it can be both on already differentiated cells and undifferentiated stem cells.

Differentiated endogenous primary cells can be advantageous as they can be immediately implanted without further manipulation as well as they are not prone to rejection and inflammatory responses. On the other hand, they can be disadvantageous because they have difficulty in getting a considerable number of cells in vitro, also for organs with a great replication potential in vivo, as the cells lose the usual microenvironment needed to proliferate.

Conversely, undifferentiated or stem cells can be autologous, allogeneic and xenogeneic. They have the potential to self-renew and proliferate at the same time, they can sustain their undifferentiated condition until they are induced to differentiate into a particular cell type (Ilic & Polak, 2011).

Fig 1.2: Cell-based therapy

Some kinds of stem cells, their use, advantages, disadvantages etc. are discussed below-

- From nearly all human adult body tissues, Adult stem cells (ASCs) had been isolated. After minor injuries, restoring original tissue function is the goal of ASCs (Sampogna et al., 2015). Among these cells, using culture protocols, bone-marrow derived mesenchymal stem cells (MSCs) can differentiate into many kinds of cells. It is beneficial to treat bone, cartilage, nervous, muscle, cardiovascular, blood, gastrointestinal diseases. It also has some disadvantages like a low number in each tissue and difficulty in vitro expression without differentiation (Barrilleaux, Phinney, Prockop, & O'connor, 2006).
- Embryonic stem cells (ESCs) can be obtained by either aspirating the inner cell mass from an embryo during the blastocyst stage or therapeutic cloning or somatic cell nuclear transfer (SCNT). Maintaining its pluripotent state until they are induced to differentiate into one kind of cells, it can proliferate extensively. However, it interferes with ethical and moral issues as well as needs feeder cells for trophic support. It can also have issues like rejection as they are allogeneic but immune responses can be avoided with some new developing technologies, like therapeutic cloning and adult cell reprogramming (Sampogna et al., 2015)
- Induced pluripotent stem cells (iPSCs) could replace the controversial use of ESCs as they have the same potency. It can be obtained through a reprogramming of adult cells. However, this strategy could provoke insertional mutagenesis and oncogene activation.

So, it should be substituted by non-viral-based methods or by the adenovirus-based transient transfection without genomic integration (Sampogna et al., 2015),

- From amniotic fluid and placenta by amniocentesis or chorionic villus sampling in the developing fetus or from the placenta at birth, Amniotic fluid-derived stem cells (AFSCs) can be obtained. Although no human clinical trials have been performed, these cells are multipotent (De Coppi et al., 2007).

1.4.2. Use of Biomaterials:

Any material which has been engineered to interact with biological systems for a medical purpose, whether therapeutic or diagnostic and are non- viable materials that can be implanted to replace or repair missing tissue, can be called biomaterial (F.-M. Chen & Liu, 2016).

Biomaterials can be categorized as-

- Natural
- Synthetic

Natural materials can be integrated perfectly and furnished by other living organisms. However, according to food manufacturing practice, it cannot be produced easily in large quantity. On the other hand, synthetic materials can be identically reproduced in large quantity but it lacks biologic recognition (Sampogna et al., 2015)

The ideal biomaterial should be biocompatible, biodegradable and have a certain range of porosity. Porosity allows the exchange of nutrients and wastes but this property is extremely difficult to achieve successfully. However, 3D bioprinters which are a gift of dynamic optical projection stereolithography seems to be the perfect solution to this problem.

1.4.3. Implantation of scaffolds seeded with cells:

This approach is a combination of the cell-based therapy and use of biomaterials. By merging cells from the body with highly porous scaffold biomaterials, it aims to regenerate damaged tissues. This combination works as templates for tissue regeneration and guides the development

of new tissue (O'Brien, 2011). In 2006, it was reported that patients who were suffering from myelomeningocele and needed augmentation cystoplasty could use autologous engineered bladder constructs (Atala, Bauer, Soker, Yoo, & Retik, 2006).

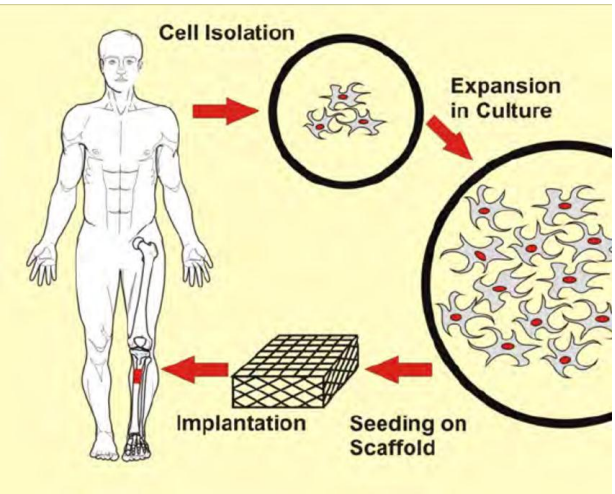

Fig 1.3: Scheme of bone tissue engineering

1.5. Therapies at the Preclinical Phase and in Clinical Testing

The above-described strategies are overviewed by breaking down into three broad categories-

1.5.1. Recapitulation of Tissue and Organ Structure:

In engineered tissue, organ structure and material composition can be captured by decellularizing organ and then recellularizing before transplantation. In fact, decellularized tissue without recellularization step has reached the market as a medical device and it is used in a human patient who to repair large muscle defect (Dahl et al., 2011). The engineering of blood vessels in vitro and their following decellularization before installment in patients who require kidney dialysis is a variation of this approach (Mao & Mooney, 2015). However, this approach faces several challenges. If the biological process that drives and directs self-assembly is understood, the challenges can be turned into advantages.

1.5.2. Integration of Graft Tissue by Vascularization and Innervation Induction:

The body needs to be properly integrated with the implanted grafts to contribute functionally and structurally. Presentation of angiogenic growth factors such as vascular endothelial growth factor (VEGF), angiopoietin (Ang), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) can exploit body's angiogenic response to vascularize engineered tissues (Mao & Mooney, 2015). On the other hand, without proper delivery of modality, application of growth factor may not be effective because of their short half-life in vivo, systemic effects of bolus delivery and potential toxicity. Moreover, there is another approach implying to pre-vascularize the graft or target site before implementation and it can also promote graft vascularization at the target site

For full integration and proper function of many tissues, the host cell requires to innervate. To be more specific, where motor control (skeletal tissue) or sensation (epidermis) provides the key function, innervation is particularly important. Growth factors may induce the innervation of engineered tissue (Mao & Mooney, 2015)

1.5.3. Alteration of the Host Environment: Modulating the Immune System and Cell infusion:

Indirect means of administration of cells such as interaction with host cells and secretion of growth factors can induce a therapeutic response. However, it does not require incorporation of the cells prominently and transplantation of cells from a bulk tissue (Forbes & Rosenthal, 2014). Additionally, to control the diseased or injured environment, altering the extra-cellular matrices and improving tissue regeneration via this mechanism, transplanted cells can play a vital role. In current clinical trials, cells are typically infused intravenously but rapid clearance often occurs in the administered cells, in this case, resulting in limited efficacy.

Though avoiding rejection of the host immune tissue has been the goal of regenerative medicine for a long time, the immune system plays an essential role in modulating regeneration. It includes both damaging and contributing to the curative process and engraftment. In fact,

immune rejection is a grave barrier to the integration of grafts formed with allogeneic cells. To induce allograft tolerance, immune engineering approaches have shown potential (Mao & Mooney, 2015). Inflammation associated with implantation of a foreign object can be reduced if the properties of implanted scaffolds are changed. Hence, active promotion of regeneration may be allowed by engineering a local immune response.

1.6. Applications of Regenerative Medicine:

1.6.1. Cell therapy:

- Adult stem cells (ASC), specifically, mesenchymal stem cells (MSC) can be differentiated into other cells by using different protocols and is useful in treatment of muscle, bone, cartilage, nervous, blood, gastrointestinal and cardiovascular diseases. FDA has approved a number of therapies which are commercially available. The first FDA permitted the biologic product in the orthopedic ground, Carticel, uses autologous chondrocytes. Harvesting from articular cartilage then expanded ex vivo and finally implanting at the injury spot effectively treated focal articular cartilage defects (Dewan, Gibson, Elisseeff, & Trice, 2014). Some other examples can be given such as-

  laViv, which improves the appearance of nasolabial fold wrinkles by injection of autologous fibroblasts.

  Celution is a medical device. From adipose tissue which was derived from liposuction, this device extracts cell.

  Epicel is autologous keratinocytes. It is used for severe burn wounds (Mao & Mooney, 2015)
- Mesenchymal stem cell therapy, Neural stem cell therapy, Hematopoietic stem cell transplantation showed the promising result in cases of myeloid malignancies, Hurler's Syndrome patients, osteogenesis imperfect and other blood cell diseases (Jain & Bansal, 2015)
- Embryonic stem cells (ESC) aim to regenerate functional myocardium as these have the potential to afford an infinite supply of cardiomyocytes for cell therapy

procedures. Moreover, blastocyst complementation system can generate definite human organs from many Pluripotent Stem Cells (PSCs) injected into xenogeneic sources and provide a novel approach to organ supply. In 2009, the first case of receiving these cells was a spinal cord injury in a car accident who received the injection of oligodendrocytes obtained from ESC (Tabakow & Raisman, 2014).

- Induced pluripotent stem cells (iPSCs) have been used in the generation of disease-specific lines, For example- Alzheimer's disease, Parkinson's disease, diabetes mellitus type I.
- Amniotic fluid-derived stem cells (AFSCs) from cell banks can represent a lifelong autologous source for heart-valve replacements (Sampogna et al., 2015).

1.6.2. Matrix bioscaffolds decellularization and then recellularization with stem cells:

- Using this approach, in 2008, the first trachea transplant developed from adult stem cell was performed and in the present time, this tactic has been extended to even tracheal cancer (Macchiarini et al., 2008)
- On a decellularized liver matrix, human hepatocyte progenitors were colonized to generate human liver cells. Even, with induced pluripotent stem cell (iPSC) which are derived cardiovascular progenitor cells were able to seed decellularized mouse heart as scaffolds (Jain & Bansal, 2015)
- Besides, decellularized scaffolds for tissue-engineering of the urethra, urinary bladder, lung and blood vessel were some successful utilization. This approach can also be utilized for digestive tract replacement and regeneration, healing of epithelial injury of the esophagus and even intestine transplantation.
- Currently, extracellular matrix scaffolds are used for heart valves, arterial grafts, dura mater grafts, urinary tract reconstitution, skin reconstruction, and orthopedic applications (Jain & Bansal, 2015).

1.6.3. Tissue engineering:

- By in vivo grafting of organoid units, Tissue-engineered small intestine (TESI) was produced. These are multicellular bunches of epithelium and mesenchyme and harvested from the native intestine.
- Various natural and synthetic biomaterials are used for bladder regeneration or developing a bladder from autologous stem cells seeded on a bladder-shaped scaffold.

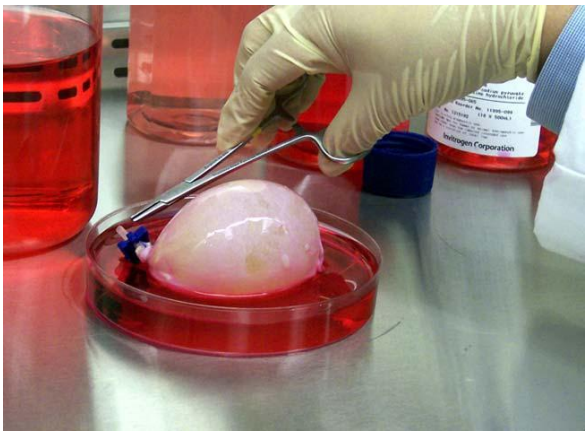

Fig 1.4: Bio-engineered Bladder

- Trachea regeneration can be possible too and does not need a donor. In June 2011, successful implantation of an artificial trachea in a 36-year-old patient who had late-stage tracheal cancer was performed. To perform this transplantation, stem cells were taken from the patient's hip, then these cells were treated with growth factors and incubated on a plastic replica of his natural trachea (Jain & Bansal, 2015).

Fig 1.5: Trachea Regeneration

- In case of the heart, stem cell therapies were improved by tissue-like cellular patches using bio-materials has been developed that act as a delivery platform. Besides, more strategies have been developed like the incorporation of micro templating or electrospinning to create a scaffold. On the other hand, researchers are exploring the option to generate cardiomyocyte by reprogramming endogenous non-myocytes. But before clinical trials, it requires validation (Garbern & Lee, 2013)
- Successful production of a renal assist device was led by bio-engineering with human cells. It was productively used in an extracorporeal setting for humans (Jain & Bansal, 2015). For kidney regeneration, the precise cellular signaling has been pinpointed and exposed the multi-layered characteristic of kidney growth. This study opened the pathway for achieving human kidney regeneration.
- For successful clinical translation of liver, the "cell sheet" technology has great potential (Yang et al., 2007).
- With self-assembled micro-tissues, a precursory human ovary has been developed. An artificial ovary has been bio-engineered by the US researchers that makes the same proportions of sex hormones as a healthy one which can be considered more natural option than hormone replacement therapy for women (Jain & Bansal, 2015).

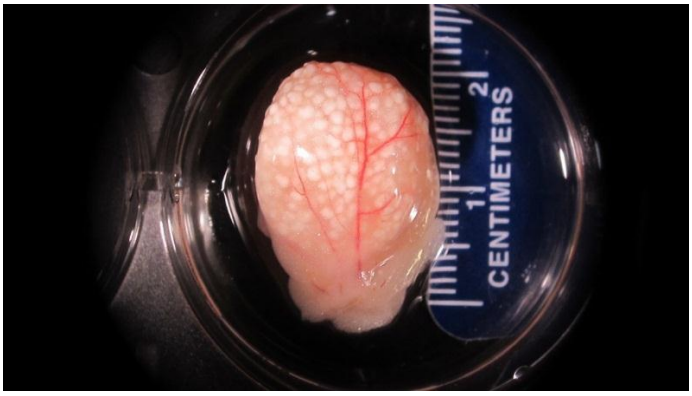

Fig 1.6: Bio-engineered Ovary

- A fully involute aged thymus has been successfully rejuvenated. It is quite similar with juvenile thymus in gene expression profile and architecture.
- For the severe epithelial injury treatment, an artificial complete skin model including dermis and epidermis has been developed (Jain & Bansal, 2015).

1.6.4. Organ Printing:

A new evolving technology in tissue engineering, a suitable other option for the solid scaffold-based usual biodegradable approach is Organ Printing. 3D functional living organ constructs and macro-tissues are layer-by-layer bio fabricated and used spheroids as building blocks in this process (Mironov et al., 2009). Three sequential steps comprise organ printing-

Preprocessing- A blueprint for organ in which a natural organ or tissue is gained in digitized image reconstruction form.

Processing- Cells or cell aggregates are placed layer-by-layer into a 3D environment for actual organ printing.

Post-processing- The printed organ is perfused and organ maturation is accelerated.

- This technique has a wide range of implication including printing heart tissue, blood vessels, skin tissue as well as the production of soft tissues and artificial bones.

- Researchers are currently working in developing bladders, kidneys, and hearts using this technique. Though they were not capable to regenerate a completely functional organ yet, they succeeded in generating a small model of human heart and kidney (Jain & Bansal, 2015). However, this technique has a major drawback of being the limited lifespan of days rather than years.

Fig 1.7: Future of organ printing

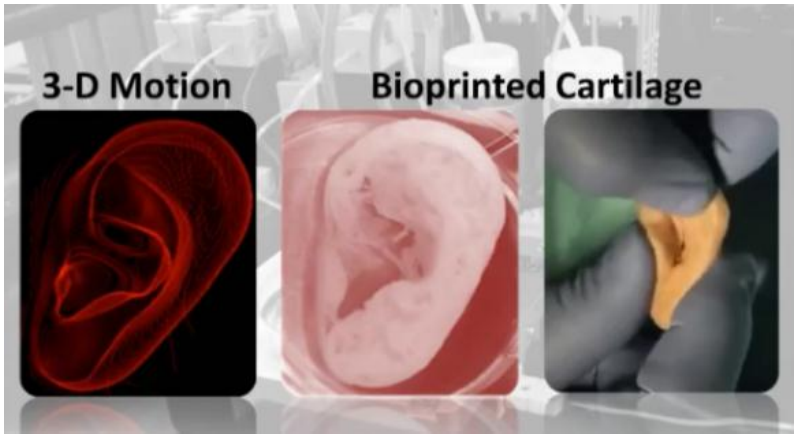

Fig 1.8: Bio-printed Cartilage

1.7. The scenario of Progress:

1.7.1. Progress in Regenerative Medicine:

- In 2009, the first case of receiving these cells was a spinal cord injury in a car accident who received the injection of oligodendrocytes obtained from ESC (Tabakow & Raisman, 2014)
- Secretory mammary glands were successfully generated by isolating single stem cell from adult mouse mammary glands and then by transplanting them into the fat-pad in mice.
- PSC derived rat pancreas and kidney was generated by using the concept of generation of organs using a blastocyst complementation system (Jain & Bansal, 2015)
- In 2008, trachea transplant grown from adult stem cell was performed. At present, this approach has been extended to even tracheal cancer (Macchiarini et al., 2008)
- One of the first successful reports was with human liver cells and it was performed on a decellularized liver matrix by colonizing human hepatocyte progenitors
- Besides, decellularized scaffolds for tissue-engineering of urinary bladder, lung, urethra and blood vessel were some successful utilization. This approach can also be utilized for digestive tract replacement and regeneration, healing of epithelial injury of the esophagus and even intestine transplantation.

1.7.2. Progress in Tissue Engineering:

- Various natural and synthetic biomaterials are used for bladder regeneration. It has also a role in growing bladder from autologous stem cells which were seeded on the scaffold of bladder shape. The experimental transplantation of bladder which was bio-engineered was first published in 2006 and appeared in Lancet (Atala et al., 2006)
- Tissue-like cellular patches using bio-materials has been developed that act as a delivery platform, incorporation of micro templating or electrospinning to create scaffold etc. are some potential progress in case of heart. (Jain & Bansal, 2015)
- Renal assist device with human cells, progress in human kidney regeneration concept.
- For successful clinical translation of liver, the potential of “cell sheet” technology (Yang et al., 2007)

- A precursory human ovary with self-assembled micro-tissues, an artificial ovary that makes sex hormones which can be considered more natural option than hormone replacement therapy for women.
- A fully involute aged thymus has been successfully rejuvenated which is quite similar to juvenile thymus in gene expression profile and architecture.
- Developing an artificial complete skin model including dermis and epidermis for treating severe epithelial injuries.
- Using a biodegradable collagen scaffold, successful reconstruction of the inner ear tympanic cavity and mastoid cavity. By shaping the structure of this scaffold, shape of the ear can be controlled and thus outer ear was successfully regrown (Jain & Bansal, 2015)
- Organ printing technology is progressing in printing heart tissue, blood vessels, skin tissue as well as the production of soft tissues and artificial bones. Currently, scientists are occupied on developing bladders, kidneys, and hearts using this technique. They succeeded in forming a small model of human heart and kidney.

1.8. Potential opportunities for Regenerative medicine:

Regenerative medicine can turn our practice of medicine in another direction. Regenerative medicine can repair unhealthy tissue or restore functions of the body by a 'once and for all' treatment which is very different from the present medical practice that uses surgical or pharmacological procedures.

- The patient likely requires a brief period of time or maybe forever with conventional pharmacological approaches. Cell therapy may seem a luxury to produce and/or administer but it aims to permanently restore the lost function of organ and tissue. Ultimately this is anticipated to be more cost-effective and advantageous than current medical practice (Polak, 2010). Moreover, regenerative medicines possess enormous opportunities in this ever-increasing aging population who are constantly suffering from various ailments. For instance, for gene therapy, cells can be used as vehicles and for studying in vitro, a particular disease development or for drug development, cultured

cells can be used. Also, in case of supporting the discovery of new drug and patient-specific cells for therapy, the discovery of iPSCs also have the potential to produce disease models (Polak, 2010).

- On the other hand, use of biomaterials has several prospects in the field of regeneration. One of these is the advent of nanotechnology which is a rather specific mode of action. It depends on the type of engineered material, its mechanical properties, its nano-modified surface and its molecular structure. Induction of a better cellular response can be carried out by appropriately nano-modified surfaces than untreated surfaces and a more robust, sustained and specific cell differentiation after cells have been placed in contact with these materials (Gentleman et al., 2009)

1.9. Major challenges in Regenerative medicine:

Regenerative Medicine may be a potential field of research but it faces many challenges that are slowing its development and growth. However, once regenerative tissue was thought to be impossible, but with time, the concept of regenerative medicine developed and showed wonders in many fields of research. It has now shifted the goal of medicine to a whole new dimension. Some of the challenges are-

Ethical issues are one of the major concerns that make the use of regenerative medicine controversial. The embryo-derived pluripotent stem cell lines are subject to human personhood and human reproduction, therefore, research on ESCs involves the destruction of human embryos. Use of iPSCs avoid this ethical debate as embryos are not used, rather, formed by reprogramming somatic stem cells. Though the use of induced pluripotent stem cells instead of embryonic stem cells solved this issue to some extent, this field of research is mostly ignored by bioethics.

- Ethical committees are mostly unfamiliar with regenerative medicine and so making an ethically acceptable design for clinical trials on regenerative medicine has become difficult.

- Although the principle of regenerative medicine is easy to explain as well as the benefits are easy to appraise, relatively few products have made it into clinical trials, and even fewer into therapy.

- Though the cell biologist and biomaterials experts have been the driving force for the development of regenerative medicine, the usual investors such as the big pharmaceutical companies that have the money, infrastructure, and clinical trial experience to bring a therapy to market are not pursuing regenerative medicines.
- Many people and organizations are involved in the handling and working regarding regenerative medicine. Also, it can be easily transferred between countries with different legal systems and ethical sensitivities. As these are human material and its derivative product, issues arise regarding who owns these products, what rights cell donors and scientists developing new products can or cannot assert and so on.
- Current regulation is addressing this field's potential more for curative purpose only, while it has the potential to be applied for preventing aging, for enhancement or cosmetic purpose (Trommelmans, 2010), in prophylactic strategies against cardiovascular chronic diseases (London, 2016), preventing osteoarthritis (De Bari, 2012) and liver failure etc.

1.10. Aim of the study:

As the stem cells are one of the key components of Regenerative medicine and Tissue Engineering, the present review work aims to study different established protocols to generate organ-specific cells.

1.11. Objectives of the study:

To reach the aim of the study, the research work was divided into some objectives that are listed below.

- To understand the difference in the concept of Regenerative Medicine and Tissue Engineering.
- To briefly study various protocols that are currently followed by researchers to differentiate stem cells into hepatocytes, cardiomyocytes, and neural cells.
- To focus on some applications and success where successful cell-therapy protocols and organ transplants were used.

CHAPTER: 2

SOME STEM CELLS DIFFERENTIATED IN VITRO

Chapter 2: Some Stem Cell Differentiated Cells in vitro

Stem cells like Adult Stem Cells (ASC), Embryonic Stem Cells (ESCs), induced Pluripotent Stem Cells can be differentiated in vitro by numerous protocols. Thus, they have the potential to generate cells of different parts of the human body such as hepatocytes, cardiomyocytes, neural cells, kidney cells, keratinocytes, erythrocytes etc. However, in this paper, hepatocytes, cardiomyocytes, and neural cells have been highlighted and discussed in details.

2.1. Hepatocyte:

The liver is the largest internal organ and its principle cell type is called hepatocyte which accounts for almost 70% of the mass of the adult organ. However, liver degradation, liver disease, and other liver-associated problems are very common. As an effective solution to these problems, researchers have been indulged themselves to generate hepatocytes from stem cells. Although the proper method to fully reproduce the characteristics of adult primary hepatocytes could not be established yet, approaches for differentiation to hepatocyte-like cells from stem cell was established.

2.1.1. General Concept of hepatocyte differentiation from stem cells:

The general concept to produce hepatocyte-like cells from both human ESCs and iPSCs includes mainly three consecutive steps-

1. Differentiation to definitive endoderm-like cells
2. Developmental induction of hepatic progenitor cells or hepatoblasts.
3. Induction of hepatocyte maturation

At the time of gastrulation, the endoderm germ layer is formed developing a primitive gut tube. This primitive gut tube is then split into foregut, hindgut and midgut regions. In mammalian embryos, liver originates from foregut region, to be more specific, ventral foregut endoderm (Zorn, 2008).

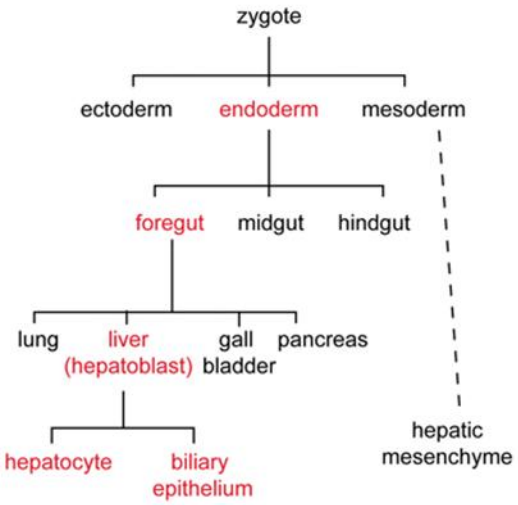

Fig 2.1: Origin of hepatocyte

For liver regeneration, the exposure of the transforming growth factor β (TGF-β) superfamily members activin A and bone morphogenic protein 4 (BMP4) is required for the stem cells. TGF-β activin A signaling initiates endoderm formation, specifically, higher doses induces endoderm and stimulates the expression of transcription factors. Shortly exposing to Wnt3a boosts the hepatic and definitive endodermis production. The expression of Wnt3a occurs at critical stages of human liver development, interacting particularly with activin A. Moreover, a combination of BMP4 and Fibroblast growth factors (FGF) has a contribution in definitive endoderm cell during the later stage of embryonic development by inducing hepatic fate in the ventral foregut endoderm. On the other hand, the effects of all these factors are inhibited if fetal bovine serum is present in the culture medium. During embryonic development, the specific marker genes of definitive endoderm whose expression specifies foregut endoderm are forkhead box A2 (Foxa2) and SRY (sex determining region Y)-box 17 (Sox17) (Zorn, 2008).

The next step is producing hepatoblast from definitive endoderm. To generate hepatoblast, specific growth factors are added in the culture media. Shortly after hepatic specification, liver genes including Albumin, Afp, Hnf4α begins to be expressed by the epithelium. In the developing hepatic diverticulum, Hepatocyte nuclear factor 4 alpha (HNF4α) is initially expressed and during liver development, it increases. Thus the differentiation toward hepatocyte

lineage is marked during in vitro differentiation. On the other hand, expression of albumin and Afp occurs in primitive hepatocytes (Zorn, 2008)

The concluding step is the induction of hepatocyte maturation. As we discussed earlier, any method to produce adult primary hepatocytes has not been established yet. So, inducing maturation to the cell is attempted to keep as similar as possible to primary hepatocyte. The physiological and molecular changes during neonatal and perinatal life are mimicked for this purpose. To proceed in this step, an interleukin-6 family cytokine, oncostatin M (OSM) and glucocorticoids are added in the culture media. Some marker genes are expressed at this stage like albumin, cytochrome p450 enzymes (CyP), CK18, α1-antitrypsin (ATT), C/EBPα, asialoglycoprotein receptor 1 (ASGPR), UGT1A1 and Prox1. Besides, the features of hepatocyte-like cells are further evaluated by some in vitro assays such as urea production, inducible cytochrome P450 activity, the uptake of indocyanine green and low- density lipoprotein (LDL) and secretion of albumin and alpha-1 antitrypsin into the medium. However, to maintain the hepatocyte-like cells in culture, endothelial and stromal cells are co-cultured for favoring hepatocyte-like cells maturation via paracrine factors or by cell-cell contact. This happens because of the aspects of the actual liver, as we know, hepatocytes form chords among themselves existing closely with non- parenchymal cells, such as stellate cells, hepatic sinusoidal endothelial cells and Kupffer cells (Zorn, 2008).

2.1.2. Methods of generation of hepatocytes from stem cells:

The present number of efficient hepatocyte differentiation procedures is the fruit of a decade of research (Wang et al., 2017). The scarce number and quality of available donor cell raised the need for alternative sources to produce fully functional hepatocytes. Cell transplantation is more beneficial than organ transplantation because cell transplantation is a less invasive process and several patients use one organ (Cameron et al., 2015). Although a method to fully reproduce the characteristics of adult primary hepatocytes could not be established yet, approaches for differentiation to hepatocyte-like cells (HLC) from stem cell were established. Some of those methods are discussed here.

2.1.2.1. Growth factor based protocol:

The growth factor approach is an adaptation of the protocol developed by Roelandt et al. Cells with pluripotent characteristics are differentiated to hepatocyte-like cells and thus mimics the liver development of the mammalian. It is a stepwise differentiation of ESC to cells with characteristics of primitive streak (PS)/ definitive endoderm (DE)/ mesendoderm (ME), hepatoblasts, and finally cells with functional and phenotypic characteristics of hepatocytes (Roelandt et al., 2010).

In 24 or 48 well plates pre-coated with Matrigel, hESC cell line was cultured, then incubated with dispase. Some cytokines and growth factors were added at various stages of the differentiation in this approach. At Day 0, Activin A and Wnt 3a were added that resulted in differentiation to definitive endoderm at Day 6. Here, the Nodal pathway is activated by the action of activin A. The efficient generation of Definitive endoderm from hESCs is promoted by a combined activation of Wnt-β catenin signaling and Nodal. For endoderm induction in iPSC and ESC cultures from human and mouse line, activin A is now utilized in high doses (S. Han, 2012). After differentiating to definitive endoderm, FGF-2 and/or BMP-4 and FGF-1/4/8 were added resulting in the differentiation to hepatoblasts at day 12-14. These growth factors are sequentially added and made cultures comprising of 70% albumin-positive cells which are the early hepatic cells from definitive endoderm cells (Cai et al., 2007). Increased expression of Albumin indicates greater generation of hepatocytes. Finally, the addition of HGF (Hepatocyte growth factor) and/or follistatin-288 generated matured hepatocyte-like cells on day 20. Hepatocyte growth factor (HGF) promotes hepatoblast proliferation, migration, and survival through its tyrosine kinase receptor c-Met and therefore, in PSC hepatic differentiation protocols, this growth factor is always used (S. Han, 2012).

2.1.2.2. Small molecule-based protocol:

This a more efficient and cost-effective approach than growth factor approach. This approach tried to avoid using growth factors by replacing those using small molecules. Small molecules

like IDE-2, BIO (bromo-indirubin-3'-oxime), sodium butyrate, SB431542 were added at various steps of differentiation media (Tasnim, Phan, & Yu, 2015)

BIO is a glycogen synthase kinase 3 (GSK-3) inhibitor that mimics Wnt signaling activation. On the other hand, LY294002 is a morpholine containing a chemical compound which has been testified to inhibit insulin growth factor, insulin and FGF-mediated phosphoinositide 3 kinase (PI3K)-AKT signaling pathway. This pathway acts to promote differentiation to DE and to maintain pluripotency. IDE-2 is a definitive endoderm inducer which is a small molecule agonist for Activin A. SB431542 is a drug candidate which is an inhibitor of the TGF-β/Activin/NODAL pathway but does not inhibit the BMP type. Sodium butyrate is a histone deacetylase inhibitor. It usually induces differentiation of many cells types (Tasnim et al., 2015)

Except for Activin A, replacement of other growth factors was possible. Combination of BIO and LY294002 could replace Wnt signaling while differentiating into definitive endoderm from hPSCs. In the second stage, while differentiating into hepatoblast, a mixture of sodium butyrate and DMSO was used. On the other hand, at the final stage of differentiating to hepatocytes, a combination of DMSO and SB431542 was used (Tasnim et al., 2015)

2.1.2.3. Chemically defined media based protocol:

This approach uses chemically defined media (CDM) devoid of serum and feeders or complex extra-cellular matrices such as Matrigel. This approach is advantageous because it is easier to transfer it to GMP conditions and avoid the presence of unknown factors which could interfere with molecular analyses. The coated tissue plates were aspirated, washed and then replaced with a collagenase: dispase solution to be incubated for less than 30 minutes. The obtained colonies were centrifuged and resuspended in CDM-PVA without cytokines. However, the medium was continuously replaced every day for 4 days with fresh CDM-PVA containing more or less Activin, basic fibroblast growth factor (bFGF), BMP-4, LY294002, and CHIR99021 and then incubated every day (Hannan, Segeritz, Touboul, & Vallier, 2013). From Day 5 to 12, the medium was replaced every day with RPMI-B27 medium containing more or less Activin A, BMP-4, and FGF-10 and then incubated. From day 12 onwards, the medium was replaced with the required amount of hepatocyte basal medium supplemented with OSM and HGF and then incubated which ultimately led to the maturation of hepatocyte-like cells. However, the

developmental time point of each stage could be determined by the distinct gene expression profile exhibited at each stage of the protocol.

The hepatocytes like cells derived using this protocol are useful in many ways. These can model a wide number of inherited metabolic disorders in vitro. Moreover, these are useful in colonization of animal model's liver for 8 weeks while maintaining Albumin and ATT expression (Hannan et al., 2013).

2.1.2.4. Synthetic ECM based approach:

This approach is based on synthetic extra-cellular matrices human recombinant laminin in combination with serum-free differentiation process. Though the most extensively used ECM is mouse sarcoma-derived Matrigel, it has quite undefined characteristics and shows batch to batch inconsistency which results in complications to generate reproducible, reliable cultures of HLCs. Use of laminin could overcome these problems. Laminins (LNs) is heterotrimeric glycoproteins composed of one α, one β, and one γ chain as well as important extracellular matrix proteins which plays a role in cell adhesion, migration, proliferation, and differentiation. In this approach, hPSCs were passaged onto LN-521, differentiated to hepatocyte-like cells on recombinant laminin and then matured hepatocyte-like cells. Besides, some preparations were necessary for the continuation of this approach- Human Activin A stock solution, Mouse Wnt-3a stock solution, HGF stock solution, Oncostatin M stock solution, endoderm priming stock medium, KSR/DMSO differentiation medium and Hepatozyme maturation medium. This approach successfully generated hepatocyte-like cells which were almost alike in nature of adult human hepatocytes (Wang et al., 2017).

2.1.3. Role of supportive cells and matrices in hepatic differentiation protocols:

From our earlier discussion, we can demonstrate that cytokines, defined media and thus like supportive cells and matrices have a significant role in those hepatic differentiation protocols. To be more specific, their roles are illustrated below-

- According to some studies, in PSC hepatic differentiation cultures, the inductive signals established in liver embryogenesis is recapitulated by a using combination of cytokines or chemicals with co-cultures with supportive cells or cultures on definite matrices in the evolving liver.
- In several studies, Different sources such as 3T3 cells, STO feeder cells or ESC-derived fibroblast-like cells provided Fibroblast cells and these were utilized as supportive cells. These cells improved hepatic differentiation of hESC as well as primate iPSC and ESC. ESC-derived fibroblast secretes two factors- FGF2 and Activin A which were identified and these are essential for endoderm induction.
- mESC-Hep maturation can be improved by harvesting extra source of hepatocytes from rats and co-culturing them with mESC-derived cell cultures (Cho et al., 2008).
- Endothelial cells, kupffer cells, mesenchymal cells and stellate cells were reported to provide support for PSC-hep maturation and specification as well as constitute the microenvironment of the developing hepatocytes. It was demonstrated that endothelial cells improve mESC derived Hepatocyte generation, required for hepatic endoderm outgrowth and essential for inducing hepatic specification of endoderm (S. Han, 2012).
- A broad range of artificial materials and natural matrices such as collagen type I, polyurethane foam, vitrogen, matrigel, fibronectin, hollow fibers, poly-l-lactic acid plus polyglycolic acid, laminin, polyacrylamide, alginate microbeads, ultra web nanofibers, and also recombinant E-cadherin substratum were identified to improve both mouse and human PSC-Hep generation (S. Han, 2012).
- 3D scaffold system provides the physical support enabling mass cultivation of PSC-derived cells and spontaneous spheroid formation than 2D culture and this support of the scaffold and cytokines could lead generation of hepatocyte-like cells. Without cell isolation, it can obtain purity up to 98% (Haque et al., 2011)

2.1.4. Advantages of HLC over PHH:

In biomedical applications like cell therapy and drug studies, Primary Human Hepatocytes (PHH) are regularly used in the present time. Clinical trials have been performed with cost efficiency and simply doing cell administration by intravascular injection rather than surgery as

well as cryopreserved hepatocytes were used, even transplanted. However, these primary human hepatocytes have some inevitable drawbacks including-

- Lack of availability of healthy donors.
- Insufficient numbers and low viability of cells
- Difficulties in hepatocyte long-term maintenance.
- Immunological rejection for transplantation.
- Yield and quality of the isolated hepatocytes.

So, Researchers introduced another source of hepatocytes to overcome these limitations. This substitute is Hepatocyte-like cells (HLCs) which is produced in vitro. As we know, HLCs are usually derived from human pluripotent stem cell (hPSCs), including induced pluripotent stem cells (iPSCs), human embryonic stem cells (hESCs), gestational stem cells and mesenchymal stromal cells. This may be a new platform for liver cell therapy but no registered clinical trial data for this type of cell is available. However, it has potential advantages for Primary human hepatocytes (PHH).

- Potential of large-scale production of iPSC derived HLCs than PHH.
- Patient-specificity of iPSC-HLCs preventing transplanted cell immune rejection.
- The possibility of gene editing of autologous iPSC- HLCs with non-integrated tools. Inherited genetic liver diseases prior to transplantation and differentiation can be treated, especially with helping new tools such as the most widely used engineered CRISPR/ Cas system.

2.1.5. Application of differentiated hepatocytes:

HLCs have a wide area of application in research and regenerative medicine.

- In a mouse model, it has been shown that iPSCs-HLCs could efficiently be engrafted into the liver with normal function (Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, 2010).
- In non-obese diabetic severe combined immune-deficient mice, HLCs were applied in lethal fulminant hepatic failure and rescued them after cell therapy (Y.-F. Chen et al., 2012)

- Co-transplantation of iPSCs- HLCs, and MSCs could be a suitable option for end-stage liver disease treatment (Yu, Wang, & Nyberg, 2014).
- hESCs derived HLCs has a role in Liver disorders and metabolic liver disorder.
- HLCs can be used in generating disease modeling essential for screening alleviate compounds or gene therapy approaches by the possibility of 'gene correction' in iPSC technology for patients with monogenic inherited metabolic liver diseases, like Wilson's disease and Alpha-1 antitrypsin deficiency (Zakikhan et al., 2017).
- HLCs are important for metabolism of xenobiotics and drugs because these possess cytochrome P450 (CYPs) activity.
- HLCs can replace primary hepatocytes as a 'gold standard' for drug metabolism and drug toxicity test if researchers can generate HLCs with the ability of drug metabolism.
- HLCs were used in the discovery of new and safe drugs, small molecules and components to alleviate the respective property after the high-throughput screening. It can be the best way of "personalized drug administration" and contribute to the future direction of medicine.
- Recently bioengineering tools such as microfluidic-based cell culture device have been developed. It is a controlled system and it allows exact frequent and partial delivery of drugs, media and signaling factors to living cells. This system can open a new dimension to drug studies with HLCs, predict drug toxicity and feasible to study the candidate or new drug metabolism and drug-response screening in high-throughput testing.
- Achieving adequate and appropriate HLCs is an important route for cell therapy of patients with end-stage liver failure (Hannoun, Steichen, Dianat, Weber, & Dubart-Kupperschmitt, 2016).
- HLCs have been proved to be potent in vitro system to study patient-specific disease model and some human liver disease like viral hepatitis and Plasmodium infection as well as to drug study, especially with the help of modern techniques.

Research on the generation of iPSC derived hepatocytes has opened the door for many potential possibilities like a better understanding of inherited liver diseases, disease processes and expose new therapeutic target.

2.2. Cardiomyocyte:

One of the most important human organs is the heart. Throughout the body, our heart pumps blood via the circulatory system. Moreover, oxygen and nutrients are supplied to the tissue as well as also, carbon dioxide and other wastes are removed from the body as the heart pumps blood. But unfortunately, such important organ is least regenerative than most other organs in the body.

Cardiovascular diseases are very frequent, sudden and complex nowadays that it has become one of the principal cause of death around the world. So, it is very obvious to focus on generating cardiomyocyte from stem cell to have a reliable treatment option. As a result, numerous approaches have been developed.

2.2.1. Methods to produce cardiomyocytes from stem cells:

In most industrialized countries, Heart failure is the leading cause of death. Besides, in emerging countries, it is a growing cause of death (Moran, A. E., Forouzanfar, M. H., Roth, G. A., Mensah, G. A., Ezzati, M., Murray, C. J. L., Naghavi,M., 2014). Myocardium has a limited capacity for regeneration. So, in emerging therapies for cardiac muscle restoration, it is a primary challenge (Laflamme & Murry, 2014). Researchers have been investigating on producing cardiomyocytes in vitro to face this challenge. Some established methods are discussed here-

2.2.1.1. Embryoid Body (EB) based method:

Embryoid Body based method is derived from mouse cardiac differentiation protocols. To modulate cell signaling pathways as well as to direct PSCs into cardiomyocytes, a combination of physical and chemical cues is the driving force of this differentiation method (Batalov & Feinberg, 2015). This was a very initial approach where hESCs are differentiated into cardiomyocytes by employing Embryoid bodies. The medium in which these EB bodies are employed contains fetal calf serum. Cardiac differentiation in EB is further enhanced by co-

culturing PSCs with visceral endoderm-like cells (END-2). Some growth factors are essential for cardiovascular development if their temporal addition appropriately. These growth factors are Fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ) superfamily growth factors Activin A and BMP4 and the Wnt inhibitor DKK-1 and these also play a role in enhancing cardiac differentiation. Although this EB approach differentiated PSCs under the fully defined condition, its efficiency was lower than 1%. However, the efficiency was increased by the efforts of researchers from time to time (Batalov & Feinberg, 2015).

2.2.1.2. PSC Monolayer-Based Differentiation Method:

This method is very much like EB based method except, PSCs were grown in 2D monolayer instead of 3D EB culture. A number of monolayer based methods had been established to increase the efficiency of cardiomyocyte differentiation. In 2007, Michael A Laflamme carried out an experiment to induce cardiac differentiation which showed efficiency above 30%. For 5 days, he sequentially hESCs with human recombinant Activin A and BMP4 in the RPMI-B27 medium. It was basically a monolayer culture system (Laflamme et al., 2007).

However, later, it was established that endogenous Wnt/β-catenin signaling controls the mechanism of the Activin/BMP4 differentiation. At the beginning of differentiation, Wnt/β-catenin signaling is particularly stimulated and then after the formation of mesoderm, this signaling is inhibited (Paige et al., 2010). So, Suppression or stimulation of this signaling at early phases after mesoderm formation reduces the cardiomyocyte yield.

Based on this understanding of Wnt/β-catenin signaling, a more modified protocol was established by Xiaojun Lian in 2012. In the beginning, he added GSK3-inhibitor CHIR99021 to stimulate Wnt/β-catenin signaling and after the mesoderm formation, he suppressed it chemically or by expressing β-catenin shRNA. This modification demonstrated the consistent generation of cardiomyocytes (Batalov & Feinberg, 2015). During the first 5 days of differentiation, cardiomyocytes are greatly inhibited by the presence of insulin present in the B27 supplement. Pre-treatment of undifferentiated hPSCs with the Gsk3 inhibitor is critical for robust cardiac differentiation, (Xiaojun Lian et al., 2013). Cardiomyocytes around 30% – 90% range were generated across these hPSC lines by this monolayer directed differentiation protocol that used

B27 supplement without insulin and pre-treated Gsk3 inhibitor in the Activin A and BMP4 (Hazeltine et al., 2012). They found that multiple hPSC lines to cardiomyocytes can be sufficiently controlled by sequentially activating canonical Wnt signaling by Gsk3 inhibitor treatment and inhibiting Wnt signaling by inducible expression of β-catenin shRNA (X. Lian et al., 2012).

2.2.1.3. Cocktail Method:

This method uses a cocktail of 5-azacytidine (5-aza), Salvianolic acid B (SalB) and Cardiomyocyte lysis medium (CLM) to obtain a higher differentiation rate of cardiomyocytes from Mesenchymal stem cells. 5-Aza is a chemical analog of cytidine. As it is an anticancer drug and also, can induce MSCs to differentiate into cardiomyocyte-like cells, it is generally known as a demethylation pharmaceutical. SalB is a monomer of Salvia miltiorrhiza radix and it can protect cardiomyocytes from apoptosis, restrict the poly (ADP-ribose) polymerase-1 pathway, and ensure the integrity of cardiac tissue's nuclei and mitochondria during acute myocardial infarction. Cardiomyocyte lysis medium (CLM) is another potential source of paracrine factor for triggering the differentiation of MSCs into cardiomyocyte-like cells. Experiments showed that cocktail of these three shows greater differentiation efficiency than using them individually (Gao et al., 2014).

MSCs are differentiated to cardiomyocytes through inhibition of the Wnt/Beta-Catenin Signaling Pathway and experiment involved 20 neonatal rats aged 0–2 days.

The cardiomyocytes were isolated, washed, treated with 0.0625% pancreatic enzyme solution and 0.1% collagenase II solution, incubated, centrifuged and finally cultured with complete medium (20% FBS, DF12 and 1% BrdU). By treating with 0.25% trypsin, well cultured MSCs were harvested and then incubated again (Gao et al., 2014). Based on different treatment conditions, MSCs were divided into eight groups to advance this process, - control group, 5-aza group, SalB group, 5-aza+SalB group, CLM group, 5-aza+CLM group, SalB+CLM group, 5-aza+SalB+CLM group. These groups were individually cultured for 2 weeks and induced with the above-mentioned pharmaceuticals.

2.2.2. Application of differentiated cardiomyocytes:

- To treat heart diseases, disease-specific, and patient-specific drug development, new cell sources, even, whole human heart engineering are some possible requirement as we know it. hiPSC derived cardiomyocytes have the features that can be advantageous in treating heart disease. Firstly, these cardiomyocytes are renewable. Besides, these are devoid of the secondary abnormalities resulting from long-term pharmaceutical therapy, end-stage diseases, and comorbidities. Though the availability of these cells is limited, still these cells are a major source of study as well as have the potential to produce patient-specific lineage for drug screening, disease modeling, drug safety testing and cell replacement therapy.
- By delivering these cardiomyocytes with pacemaking properties, "biological pacemakers' are formed which can be used in cardiac rhythm disturbances (Shiba, Hauch, & Laflamme, 2009).

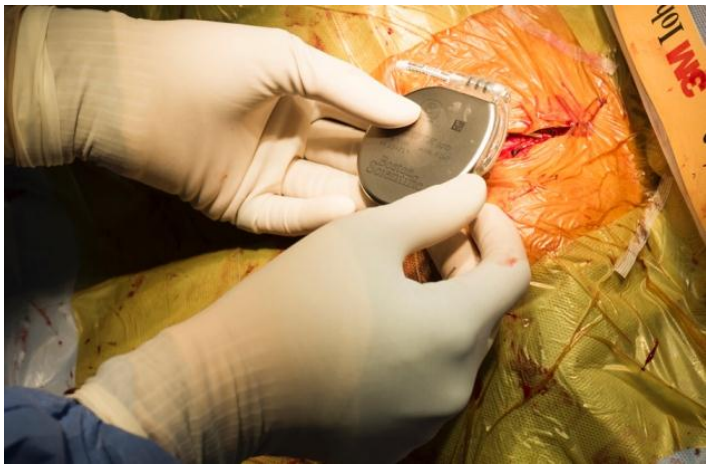

Fig 2.2: Biological Pacemaker

- Cardiomyocytes derived from iPSC has been utilized to model cardiac channelopathy and cardiac hypertrophy (Lu & Yang, 2011).
- As ESC or iPSC derived cardiomyocytes are scalable, originated from human as well as reproducible in vitro, these are used in drug safety testing as in vitro model.

- These cardiomyocytes play an important role in drug screening and drug safety testing by providing a unique disease-specific or patient-specific system (Braam et al., 2010).
- Early pre-clinical studies show that cardiomyocytes differentiated from neonatal and fetal sources were used in infract repair as cell-based therapy. Transplanting these differentiated cardiomyocytes into the rodent heart resulted in preserving left ventricular function.

2.3. Neuron:

Neuron or nerve cell is the fundamental unit, in another word, the basic building block of the nervous system. Neurons are one of the most important cell types as these are specialized in information transmission throughout the body, responsible for information communication in both electrical and chemical forms and keep a balanced network between body and brain. New nerve cells are formed throughout life in some parts of the brain by Neurogenesis process which is the process by which neurons are generated from neural stem cells. Neurons release a chemical called neurotransmitter that transmits signals from one neuron to another "target" neuron, gland cell or muscle cells across a chemical synapse, such as a neuromuscular junction.

Glia:

These are also known as glial cells or neuroglia and these are non-neuronal cells in the central nervous system including the brain and spinal cord and the peripheral nervous system forming myelin, maintaining homeostasis as well as providing support and protection for neurons.

2.3.1. Some methods regarding neuron:

A neuron is specialized cell type in our body and its most important part is the brain that controls the whole human body. However, people often face several neurological disorders like Alzheimer's disease, Parkinson’s disease, brain injuries, stupor, seizure, stroke, tumor etc. As the availability of living human brain tissue is limited, advancement in interpreting the molecular and cellular pathophysiology of neuropsychiatric disorders is slowed down. Researchers have been working on to progress in this area and established many protocols to overcome these barriers. Some of the protocols are illustrated here.

2.3.1.1. Neural Stem Cell production protocol:

Neural stem cells have the potential for cell therapy in neurological diseases such as stroke, Parkinson's disease, and spinal cord injury. So, its production optimization grabbed the attention of the researchers. This method was developed to obtain hNSCs from hES cells, by which abundant hNSCs could be harvested in a relatively short time. (X. Han et al., 2017). This protocol uses Mouse embryonic fibroblasts (MEF) cells treated with mitomycin C to block the MEF division and these MEF cells were obtained from 16-day pregnant mice and cultured in 10% fetal bovine serum (FBS) in high glucose DMEM. hES cells were cultured on MEF cells under certain conditions and seeded on 6-well plates. The plates were coated with Matrigel for neural stem cell induction and pre-covered with poly-L-ornithine and laminin for human NSC culture and passage. For Neural Stem Cell Induction, hES cells were digested by applying StemPro Accutase. The medium needed to be replaced in some steps. Noggin was also used in this protocol in the later steps for hES cell differentiation. This is also known as bone morphogenesis protein (BMP) inhibitor and it is a critical neural-inducing factor both in frog and mammalian.

2.3.1.2. Protocol for Differentiation of hiPSC into the mixed culture of neurons and glia:

This protocol differentiates hiPSCs into neural and glial cells and serves as a system for neurotoxicity testing. Firstly, hiPSC colony fragments were plated in Petri dishes pre-coated with hESC-qualified basement membrane matrix, also called "qualified matrix" or any other suitable protein substrate and then transferred to a qualified matrix-DMEM/F12-coated plate filled with complete hiPSC medium. After going through some processes, Embryoid bodies were generated and transferred to coated dishes to generate neuroepithelial aggregates or rosettes whose dissociation results in neuronal differentiation later. On day 10, the medium was changed with neuronal differentiation medium (ND) and refreshing it twice a week resulted in neuronal/glial cell derivatives on day 28. So, cells begins to differentiate into mixed cultures of neurons and glia as well as forms clusters of neuronal cell bodies progressively which are linked by bundles of neuritis upon dissociation of rosette and in the presence of complete ND medium, replating

onto laminin- or standard matrix-coated dishes or plates (Pistollato, Canovas-Jorda, Zagoura, & Price, 2017).

2.3.1.3. Protocol for differentiating hiPSCs to electrophysiologically mature neuronal networks:

In case of neural differentiation of iPSCs, to establish optimized and standardized methods is the functional maturity of the resulting neuronal networks is marked as one of the most questions (Gunhanlar et al., 2017). This is a simplified differentiation protocol concentrated on yielding iPSC-derived cortical lineage neuronal networks that are electrophysiologically mature. Here, electrophysiological properties act as the defining property of neuronal maturation. hiPSC lines were firstly dissociated from mouse embryonic fibroblasts with collagenase, then transferred to non-adherent plates in hESC medium to generate EB bodies and changed into neural induction medium after 2 days. These EBs undergoes dissociation and then in neural induction medium, plated onto laminin-coated dishes resulting in pre-NPCs. These pre-NPCs again undergoes dissociation by collagenase and in NPC medium containing basic fibroblast growth factor, replated onto laminin-coated dishes to generate mature NPCs. Plating onto poly-L-ornithine/laminin-coated coverslips initiates neural differentiation of these NPCs. This plating is done in neural differentiation medium which is supplemented with growth factors, brain-derived neurotrophic factor, dibutyryl cyclic adenosine monophosphate, ascorbic acid and glial cell-derived neurotrophic factor.

However, co-culturing with astrocyte might be more a flexible alternative. Whether it is a matter of guiding neuronal precursors or during development or increasing the neuronal fiber projections length, astrocytes are involved. Besides, astrocytes modulate synaptic transmission, develops the functional maturation of hPSC derived neurons substantially and have the potential for introduction of the variable source, especially regarding species differences. On the other hand, the above-mentioned protocol generated electrophysiologically mature iPSC derived neuronal networks devoid of astrocyte co-culture or specialized media**.**

2.3.2. Application of Neural Stem cells:

- In the treatment of damaged brain including possible therapeutic targets like Parkinson's disease, spinal cord injury, brain ischemia and Huntington's disease, as a cell

transplantation therapy, adult, and embryonic neural stem cells can be a tremendous source. However, it is only possible if there is any way to control differentiation of neural stem cells in vitro and in vivo and neuron and glia of specific type can be obtained.

- Neural stem cells can play a role in the ideal treatment of restoring lost CNS functions and reconstructing damaged neuronal circuit (Shimazaki, 2003).
- Neurological disorders of some host animal model were functionally recovered to some extent by the role of neural stem cells (Bjorklund & Lindvall, 2000).
- A potential approach for CNS repair can be obtained by mobilizing endogenous neural stem cells that can also result in significantly recovering memory and learning function.

CHAPTER: 3

DISCUSSION

Chapter 3: Discussion

Regenerative medicine is mostly based on the concept to repair or replace any damaged tissue or organs. The beginning of this field was way back to the ancient time, almost 600 years ago and this field progressed step by step towards successes. There has been a wide range application of regenerative medicine in healthcare system ranging from transplantation or replacement to cardiology, cosmetology, neurology, hepatology, gynecology and many more. Cell-based therapy, using biomaterials and implanting scaffolds seeded with cells are the usual strategies to apply for regenerative medicine.

Regenerative medicine has made it possible to successfully generate different types of cells by differentiating stem cells. So far, a wide range of methods has been used to generate these cells. Some methods were successful, some were partially successful and on the contrary, some of them were unsuccessful too. In this paper, differentiation to hepatocytes, cardiomyocytes, and neural cells was discussed briefly. Here, some protocols or methods to generate these cells as well as some applications of these differentiated cells have been discussed.

In most cases, generation of hepatocytes from stem cells includes 3 steps- differentiating into endoderm, then differentiating to hepatic progenitor cell and finally differentiating to hepatocytes. Though researchers have not been able to generate fully grown hepatocytes yet, they were successful in generating hepatocyte-like cells (HLC). Different methods have been discussed using growth factors, small molecules, chemically defined media and synthetic extracellular matrices.

In each method, the differentiation steps were the same but more or less different compounds and medium were used to proceed in these steps. Activin A was the common factor found in each of these methods of differentiation into hepatocyte-like cells. More specifically, each method required activin A to differentiate hPSC into definitive endoderm. Growth factor-based method used Activin A and Wnt3a. Small molecules based method replaced the use of all growth factors except for Activin A. Besides Activin A, this method used small molecules like LY294002, BIO in this step. The chemically defined media based protocol used CDM-PVA containing Activin A and bFGF while Synthetic Extracellular matrices based method used Endoderm priming media supplemented with Activin A & Wnt 3a. An overview is given below-

Table 1: An overview of the vital factors used in the methods to generate hepatocyte-like cells from stem cells.

Growth Factor based protocol	**Small molecule-based protocol**	**Steps of differentiation**	**Chemically defined media based protocol**	**Synthetic Extra-cellular matrices based protocol**
Activin A, Wnt 3a	Activin A, LY294002, BIO	hPSC ⇩	CDM-PVA containing Activin A and bFGF	Endoderm priming media supplemented with Activin A & Wnt 3a
BMP-4, FGF	Sodium butyrate, DMSO	Definitive Endoderm ⇩	CDM-PVA containing Activin A, bFGF, BMP-4, LY294002 & CHIR99021.	KSR/DMSO differentiation medium
FGF, Follistatin	SB431542, DMSO	Hepatoblast ⇩ Hepatocytes	Oncostatin M & HGF	HepatoZYME maturation medium supplemented with HGF and OSM

To evaluate the properties of these hepatocyte-like cells, Primary Human Hepatocytes (PHH) are used as the standard. Some tests used for this evaluation are- qPCR, immunofluorescence, Albumin secretion, and urea production, Cytochrome P450 activity, Cell viability assay etc.

In case of cardiomyocytes, Embryoid-body based method, PSC monolayer method and cocktail method have been discussed. Among them, Embryoid-body based method can be called a classic method and its efficiency was lower than 1%. Later, researchers kept modifying this protocol and

came up with PSC monolayer based protocol. These modifications resulted in much higher efficiency, almost close to 90%. Embryoid-body based method used 3D Embryoid culture while PSC monolayer method used 2D monolayer.

Finally, some methods to create neuronal cells that can be beneficial in brain injuries, Alzheimer's disease, Parkinson's disease, seizures and other neural diseases have been discussed. Production of Neural Stem cells, differentiation of hiPSC into the mixed culture of neurons and glia and differentiation of hiPSCs to electrophysiologically mature neuronal networks were mainly focused.

Some factors were common in these protocols and mostly regulated the whole process. These are Activin A, Wnt signaling, Bone morphogenic protein, fibroblast growth factors, nodal signaling etc. because these have a significant role in embryonic development, cell proliferation, differentiation.

These differentiated cells have a wide range of application as well as have the potential to expand these applications.

CHAPTER: 4

CONCLUSION

Chapter 4: Conclusion

Regenerative medicine is a bright field of research which have the potential to direct the current medicinal strategies to a different light. It may be a new arena of research but it is developing rapidly with time as well as researchers are putting a lot of efforts towards this field. The field of regenerative medicine is full of potentials and possibilities. However, this field is facing some drawbacks like inadequate financial support and investments, ethical issues and acceptance. Overcoming these drawbacks may accelerate the progress of regenerative medicine. The challenges may be vast, but there is the possibility of enormous opportunities. The greatest developments in regenerative medicine are undoubtedly yet to come.

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