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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – Concepts in Stem Cell Research – SBT1403

1. General introduction

Stem cells are undifferentiated cells that are present in the embryonic, fetal, and adult stages of life and give rise to differentiated cells that are building blocks of tissue and organs. In the post-natal and adult stages of life, tissue-specific stem cells are found in differentiated organs and are instrumental in repair following injury to the organ. The major characteristics of stem cells are: (a) self-renewal (the ability to extensively proliferate), (b) clonality (usually arising from a single cell), and (c) potency (the ability to differentiate into different cell types). These properties may differ between various stem cells. For example, embryonic stem cells (ESCs) derived from the blastocyst have a greater ability for self-renewal and potency while stem cells found in adult tissue have limited self-renewal since they would not proliferate extensively and can only differentiate into tissue-specific cells.

The human body develops from the zygote and blastocyst from which ESCs are derived into the germ layers endoderm, mesoderm, and ectoderm. Specific organs arise from the germ layers. Some of the progenitor cells that have contributed to organ formation do not terminally differentiate but are retained as tissue stem cells and can be found in bone marrow, bone, blood, muscle, liver, brain, adipose tissue, skin, and the gastrointestinal tract. The tissue stem cells may be called progenitor cells since they give rise to terminally differentiated and specialized cells of the tissue or organ. These cells may be dormant within tissue but would proliferate under circumstances of injury and repair. The dynamics of tissue stem cells or progenitor cells varies from tissue to tissue; for example, in bone marrow, liver, lung, and gut, stem cells regularly proliferate to supplement cells during normal turnover or injury, while in the pancreas, the heart, or the nervous system they proliferate to replace damaged cells following injury.

The idea of wound repair and organ regeneration is as old as humanity and is reflected in the ancient Greek myth of Prometheus, the Greek titan punished by Zeus for his disobedience in introducing fire and knowledge to human beings. In this myth, Prometheus is tied to a rock and everyday an eagle eats part of his liver which then regenerates. In modern-day medicine, work involving stem cells and organ regeneration commenced with the first attempts at bone marrow transplantation in animal models during the 1950s. These pioneering studies paved the way for human bone marrow transplantation, a therapy now widely used in various blood disorders. This new

therapeutic strategy revealed the existence of stem cells that regenerated adult tissue. Presently, regenerative medicine is a major focus of research not only to find therapies but also to understand basic biology and the pathogenesis of disease. Although a number of ethical issues have arisen in stem cell research, recent advances in stem cell isolation and development have helped scientists to identify and culture specific cell types for regeneration of tissue in various disorders such as Parkinson's, Alzheimer's, or diseases of the heart, muscles, lung, liver, and other organs.

Stem Cell Classification Based on Differentiation Potential

The ability to differentiate, one of the two main characteristics of stem cells, varies between stem cells depending on their origin and their derivation (fig. 1). All stem cells can be categorized according to their differentiation potential into 5 groups: totipotent or omnipotent, pluripotent, multipotent, oligopotent, and unipotent (table 1).

Table 1

Stem cell classification according to their differentiation potential and origin

Differentiation potential	Origin
Totipotent or omnipotent	
Pluripotent	ESCs, iPSCs
Multipotent	Fetal stem cells
Oligopotent	Adult or somatic stem cells
Unipotent	

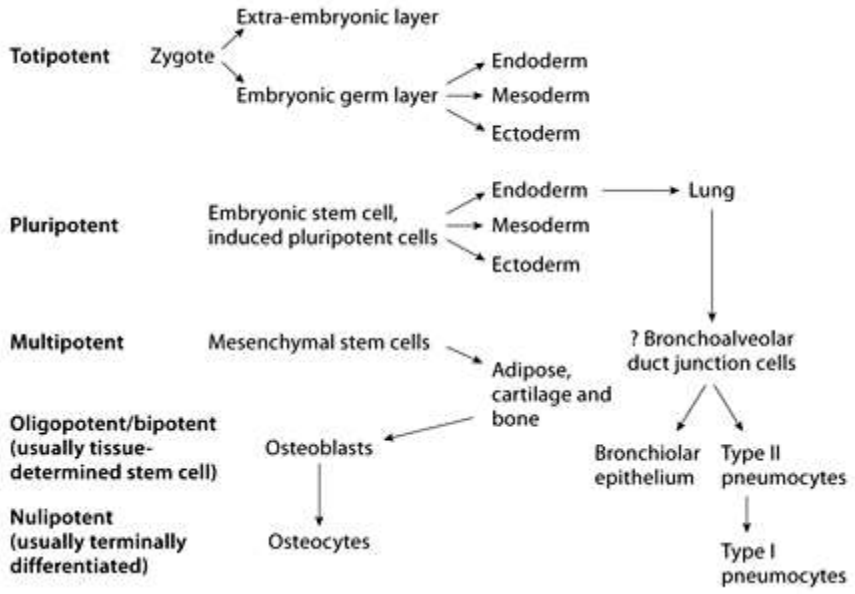


Fig. 1

The hierarchy of stem cells. Totipotent cells form embryonic and extra-embryonic tissue. Pluripotent cells form all 3 germ layers while multipotent cells generate cells limited to 1 germ layer. Bronchoalveolar duct junction cells in the lung may be multipotent while type II pneumocytes are oligopotent and differentiate into type I pneumocytes of the alveoli.

Totipotent Cells

Totipotent or omnipotent cells are the most undifferentiated cells and are found in early development. A fertilized oocyte and the cells of the first two divisions are totipotent cells, as they differentiate into both embryonic and extraembryonic tissues, thereby forming the embryo and the placenta.

Pluripotent Cells

Pluripotent stem cells are able to differentiate into cells that arise from the 3 germ layers – ectoderm, endoderm, and mesoderm – from which all tissues and organs develop. Pluripotent stem cells called ESCs were first derived from the inner cell mass of the blastocyst. Recently, Takahashi and Yamanaka generated pluripotent cells by reprogramming somatic cells. These cells are called induced pluripotent stem cells (iPSCs) and share similar characteristics with ESCs. Notably, there has been no pluripotent cell population isolated from the lung.

Multipotent Cells

Multipotent stem cells are found in most tissues and differentiate into cells from a single germ layer. Mesenchymal stem cells (MSCs) are the most recognized multipotent cell. They can be derived from a variety of tissue including bone marrow, adipose tissue, bone, Wharton's jelly, umbilical cord blood, and peripheral blood. MSCs are adherent to cell culture dishes and are characterized by specific surface cell markers. These cells can differentiate into mesoderm-derived tissue such as adipose tissue, bone, cartilage, and muscle. Recently, MSCs were differentiated into neuronal tissue which is derived from the ectoderm. This is an example of transdifferentiation, i.e. when a cell from one germ layer (mesoderm) differentiates into neuronal tissue (ectoderm). Tissue-resident MSCs have been isolated from the lung; however, no other multipotent cell has been isolated to date.

Oligopotent Cells

Oligopotent stem cells are able to self-renew and form 2 or more lineages within a specific tissue; for example, the ocular surface of the pig, including the cornea, has been reported to contain oligopotent stem cells that generate individual colonies of corneal and conjunctival cells. Hematopoietic stem cells are a typical example of oligopotent stem cells, as they can differentiate into both myeloid and lymphoid lineages. In the lung, studies suggest that bronchoalveolar duct junction cells may give rise to bronchiolar epithelium and alveolar epithelium.

Unipotent Cells

Unipotent stem cells can self-renew and differentiate into only one specific cell type and form a single lineage such as muscle stem cells, giving rise to mature muscle cells and not any other cells . In the lung, type II pneumocytes of the alveoli give rise to type I pneumocytes.

Stem Cell Classification Based on Origin

Stem cells can be grouped into 4 broad categories based on their origin: ESCs, fetal and adult stem cells, and iPSCs (table 1). In general, ESCs and iPSCs are pluripotent, whereas adult stem cells are oligopotent or unipotent.

Embryonic Stem Cells

ESCs are pluripotent, derived from the inner cell mass of the blastocyst, a stage of the pre-implantation embryo, 5–6 days post-fertilization. These cells can differentiate into tissue of the 3 primary germ layers but can also be maintained in an undifferentiated state for a prolonged period in culture. The blastocyst has 2 layers of cells, i.e. the inner cell mass, which will form the embryo, and the outer cell mass, called trophoblasts, that will form the placenta. Cells from the inner cell layer are separated from trophoblasts and transferred to a culture dish under very specific conditions to develop ESC lines. ESCs are identified by the presence of transcription factors such as Nanog and Oct4. These factors maintain the stem cells in an undifferentiated state, capable of self-renewal. ESCs that have been cultured in an undifferentiated state with no genetic abnormalities are propagated as an ESC line. These cells could be frozen and thawed for further cultures and experimentation. Culture conditions are critical in maintaining ESCs in an undifferentiated state. A feeder layer of embryonic fibroblast cells (MEFCs) or medium that contains the anti-differentiation cytokine leukemia inhibitory factor (LIF) are used. Withdrawal of LIF from the medium or removal of the ESCs from the feeder layer results in the formation of 'embryoid bodies', in which all 3 germ layers (endoderm, mesoderm, and ectoderm) are present.

Adult Stem Cells

Adult stem cells are derived from adult tissue. Examples include MSCs as well as stem cells derived from placental tissue such as human amnion epithelial cells. These cells have been shown to be anti-inflammatory and augment repair of animal models of injury. They have limited differentiation capacity although these cells have been differentiated into tissue from different germ cell layers in vitro.

Adult stem cells are of advantage since autologous cells do not raise issues of rejection or ethical controversies. Adult stem cells could be obtained from all tissues of the 3 germ layers as well as placenta. Several studies have demonstrated that transplantation of adult stem cells restores damaged organs in vivo, such as bone tissue repair and revascularization of the ischemic cardiac tissue via stem cell differentiation and generation of new specialized cells. Other studies have shown that cultured adult stem cells secrete various molecular mediators with anti-apoptotic, immunomodulatory, angiogenic, and chemoattractant properties that promote repair.

Tissue-Resident Stem Cells

The ability of some tissues and organs in the adult to renew and repair following injury is critically dependent on tissue-resident stem cells that generate tissue-specific, terminally differentiated cells. Studies suggest that these cells originate during ontogenesis and remain in a quiescent state till local stimuli activate their proliferation, differentiation or migration.

Tissue-resident stem cells reside in a ‘stem cell niche’. The stem cell niche is a microenvironment that controls the self-renewal and differentiation of these cells. There is a growing body of evidence that stem cell function is critically influenced by extrinsic signals from the microenvironment; therefore, the niche plays a crucial role in stem cell homeostasis and tissue repair. The majority of tissue-resident stem cells are dormant but are activated by specific signals during injury and repair. This dormancy of tissue-resident stem cells is not well understood but is likely influenced by the niche environment. This property is critical to maintaining a population of cells that do not perform other functions apart from generating tissue-specific cells during repair. The niche environment consists of various signals from extracellular matrix and soluble mediators that mediate cell signaling and gene expression, thereby regulating stem cell proliferation, migration, differentiation, or apoptosis. We still need to elucidate what the triggers are for stem cells to move from a self-renewal state and proliferation to differentiation and whether these signals are tissue specific.

Furthermore, the types of cell division that a stem cell undergoes determine the cells the type of cells generates. Symmetrical cell division by a stem cell results in identical daughter cells, which provides new cells to restitute damaged cells following injury (fig. 2). It is important to note that an uncontrolled increase in stem cell proliferation could lead to stem cell hyperplasia and/or carcinogenesis while a reduction in stem cells would impair organ repair; thus, balance in stem cell homeostasis is very important.

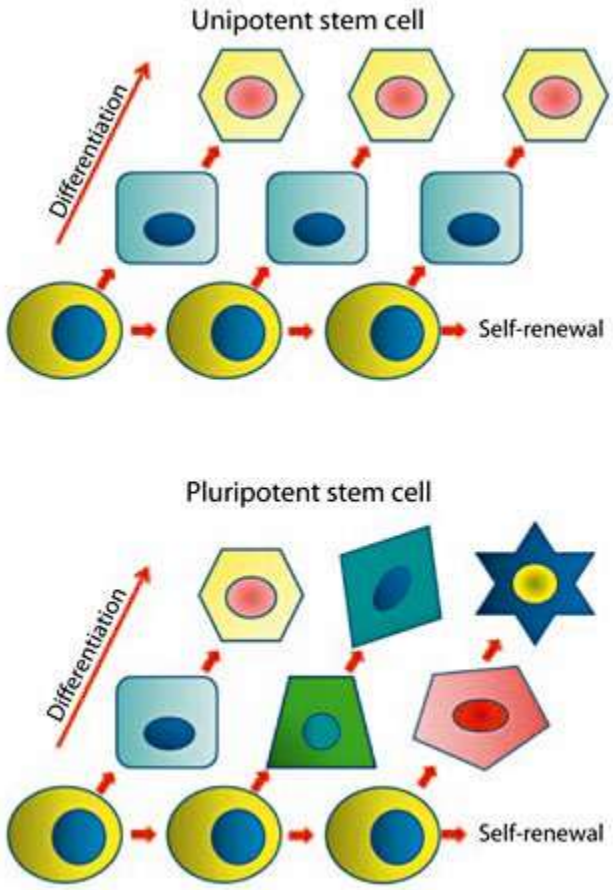


Fig. 2

Stem cells are characterized by their ability to self-renew and differentiate. Each individual daughter cell could go further in a symmetric division giving more stem cells or differentiate into one or more specialized cells, depending in the pluripotency of the stem cell, maintaining the population of tissue cells.

Asymmetric division occurs when a stem cell generates an identical daughter cell and a second differentiated daughter cell. This process allows for organ repair and regeneration while maintaining a population of stem cells.

Induced Pluripotent Stem Cells

iPSCs are produced from adult somatic cells that are genetically reprogrammed to an 'ESC-like state'. Mouse iPSCs were reported for the first time by Takahashi and Yamanaka [33] in 2006 by transducing mouse fibroblasts with 4 genes encoding the following transcription factors: octamer-binding transcription factor 3/4 (OCT3/4), SRY-related high-mobility group box protein-2 (SOX2), the oncoprotein c-MYC, and Kruppel-like factor 4 (KLF4). A year later, in 2007, Yamanaka and

collegues described the generation of human iPSCs from adult human dermal fibroblasts with the same 4 factors: Oct3/4, Sox2, Klf4, and c-Myc. They demonstrated that these cells were similar to human ESCs in terms of morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity, and they could differentiate into cell types of the 3 germ layers in vitro. iPSCs are currently useful tools for drug development, modeling of diseases, and regenerative medicine, but although these cells express identical characteristics of pluripotent stem cells it is not yet known if iPSCs and ESCs would significantly differ in clinical practice.

Retroviral vectors, used to introduce the reprogramming factors into adult cells, and oncogenes like c-Myc limit the use of iPSCs in a clinical study since the vectors used to introduce transcription factors to adult cells can cause cancers. Researchers are currently investigating new methods to generate safe iPSCs without genomic manipulation. New techniques have been described, using several types of mouse and human adult somatic cells. To avoid the use of oncoproteins c-MYC and KLF4 they have used one factor (OCT3/4 or KLF4) or they have substituted them with combinations of other factors [95,96], including the use of non-retroviral vector approaches, such as chemical compounds, plasmids, adenovirus, and transposons.

Despite the safety issues, this innovative discovery has created a powerful tool to reprogram somatic adult cells ‘sending them back’ to earlier undifferentiated stages and generating iPSCs, thereby creating an identical match to the cell donor and thus avoiding issues of rejection.

II. Unique Properties of Stem Cells

Stem cells have the ability to self-renew.

Unlike muscle cells, blood cells, or nerve cells—which do not normally replicate— stem cells may replicate many times. When a stem cell divides, the resulting two daughter cells may be: 1) both stem cells, 2) a stem cell and a one more differentiated cell, or 3) both more differentiated cells. What controls the balance between these types of divisions to maintain stem cells at an appropriate level within a given tissue is not yet well known.

Discovering the mechanism behind self-renewal may make it possible to understand how cell fate (stem vs. non-stem) is regulated during normal embryonic development and post-natally, or misregulated as during aging, or even in the development of cancer. Such information may also enable scientists to grow stem cells more efficiently in the laboratory. The specific factors and conditions that allow pluripotent stem cells to remain undifferentiated are of great interest to scientists. It has taken many years of trial and error to learn to derive and maintain pluripotent stem cells in the laboratory without the cells spontaneously differentiating into specific cell types.

Stem cells have the ability to recreate functional tissues.

Pluripotent stem cells are undifferentiated; they do not have any tissue-specific characteristics (such as morphology or gene expression pattern) that allow them to perform specialized functions. Yet they can give rise to all of the differentiated cells in the body, such as heart muscle cells, blood cells, and nerve cells. On the other hand, adult stem cells differentiate to yield the specialized cell types of the tissue or organ in which they reside, and may have defining morphological features and patterns of gene expression reflective of that tissue.

Different types of stems cells have varying degrees of potency; that is, the number of different cell types that they can form. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are beginning to understand the signals that trigger each step of the differentiation process. Signals for cell differentiation include factors secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment.

III. Properties of Stem Cells

Pluripotency

Pluripotent stem cells are master cells. They're able to make cells from all three basic body layers, so they can potentially produce any cell or tissue the body needs to repair itself. This "master" property is called pluripotency. Like all stem cells, pluripotent stem cells are also able to self-renew, meaning they can perpetually create more copies of themselves.

There are several types of pluripotent stem cells, including embryonic stem cells. At Children's Hospital Boston, we use the broader term because pluripotent stem cells can come from different sources, and each method creates a cell with slightly different properties.

But all of them are able to differentiate, or mature, into the three primary groups of cells that form a human being:

1. ectoderm — giving rise to the skin and nervous system
2. endoderm — forming the gastrointestinal and respiratory tracts, endocrine glands, liver, and pancreas
3. mesoderm — forming bone, cartilage, most of the circulatory system, muscles, connective tissue, and more

Right now, it's not clear which type or types of pluripotent stem cells will ultimately be used to create cells for treatment, but all of them are valuable for research purposes, and each type has unique lessons to teach scientists. Scientists are just beginning to understand the subtle differences between the different kinds of pluripotent stem cells, and studying all of them offers the greatest chance of success in using them to help patients.

Types of pluripotent stem cells:

Induced pluripotent cells (iPS cells):

Scientists have discovered ways to take an ordinary cell, such as a skin cell, and "reprogram" it by introducing several genes that convert it into a pluripotent cell. These genetically reprogrammed cells are known as induced pluripotent cells, or iPS cells. The Stem Cell Program at Children's Hospital

Boston was one of the first three labs to do this in human cells, an accomplishment cited as the Breakthrough of the Year in 2008 by the journal Science.

iPS cells offer great therapeutic potential. Because they come from a patient's own cells, they are genetically matched to that patient, so they can eliminate tissue matching and tissue rejection problems that currently hinder successful cell and tissue transplantation. iPS cells are also a valuable research tool for understanding how different diseases develop.

Because iPS cells are derived from skin or other body cells, some people feel that genetic reprogramming is more ethical than deriving embryonic stem cells from embryos or eggs. However, this process must be carefully controlled and tested for safety before it's used to create treatments. In animal studies, some of the genes and the viruses used to introduce them have been observed to cause cancer. More research is also needed to make the process of creating iPS cells more efficient.

iPS cells are of great interest at Children's, and the lab of George Q. Daley, MD, PhD, Director of Stem Cell Transplantation Program, reported creating 10 disease-specific iPS lines, the start of a growing repository of iPS cell lines.

Embryonic stem cells:

Scientists use "embryonic stem cell" as a general term for pluripotent stem cells that are made using embryos or eggs, rather than for cells genetically reprogrammed from the body. There are several types of embryonic stem cells:

1. "True" embryonic stem cell (ES cells)

These are perhaps the best-known type of pluripotent stem cell, made from unused embryos that are donated by couples who have undergone in vitro fertilization (IVF). The IVF process, in which the egg and sperm are brought together in a lab dish, frequently generates more embryos than a couple needs to achieve a pregnancy.

These unused embryos are sometimes frozen for future use, sometimes made available to other couples undergoing fertility treatment, and sometimes simply discarded, but some couples choose to donate them to science. For details on how they're turned into stem cells, visit our page How do we get pluripotent stem cells?

Pluripotent stem cells made from embryos are “generic” and aren’t genetically matched to a particular patient, so are unlikely to be used to create cells for treatment. Instead, they are used to advance our knowledge of how stem cells behave and differentiate.

2. Stem cells made by somatic cell nuclear transfer (ntES cells)

The term somatic cell nuclear transfer (SCNT) means, literally, transferring the nucleus (which contains all of a cell’s genetic instructions) from a somatic cell—any cell of the body—to another cell, in this case an egg cell. This type of pluripotent stem cell, sometimes called an ntES cell, has only been made successfully in lower animals. To make ntES cells in human patients, an egg donor would be needed, as well as a cell from the patient (typically a skin cell).

The process of transferring a different nucleus into the egg “reprograms” it to a pluripotent state, reactivating the full set of genes for making all the tissues of the body. The egg is then allowed to develop in the lab for several days, and pluripotent stem cells are derived from it. (Read more in How do we get pluripotent stem cells?)

Like iPS cells, ntES cells match the patient genetically. If created successfully in humans, and if proven safe, ntES cells could completely eliminate tissue matching and tissue rejection problems. For this reason, they are actively being researched at Children’s.

3. Stem cells from unfertilized eggs (parthenogenetic embryonic stem cells)

Through chemical treatments, unfertilized eggs can be “tricked” into developing into embryos without being fertilized by sperm, a process called parthenogenesis. The embryos are allowed to develop in the lab for several days, and then pluripotent stem cells can be derived from them (for more, see How do we get pluripotent stem cells?)

If this technique is proven safe, a woman might be able to donate her own eggs to create pluripotent stem cells matching her genetically that in turn could be used to make cells that wouldn’t be rejected by her immune system.

Through careful genetic typing, it might also be possible to use pES cells to create treatments for patients beyond the egg donor herself, by creating “master banks” of cells matched to different tissue types. In 2006, working with mice, Children’s researchers were the first to demonstrate the potential feasibility of this approach. (For details, see Turning pluripotent stem cells into treatment).

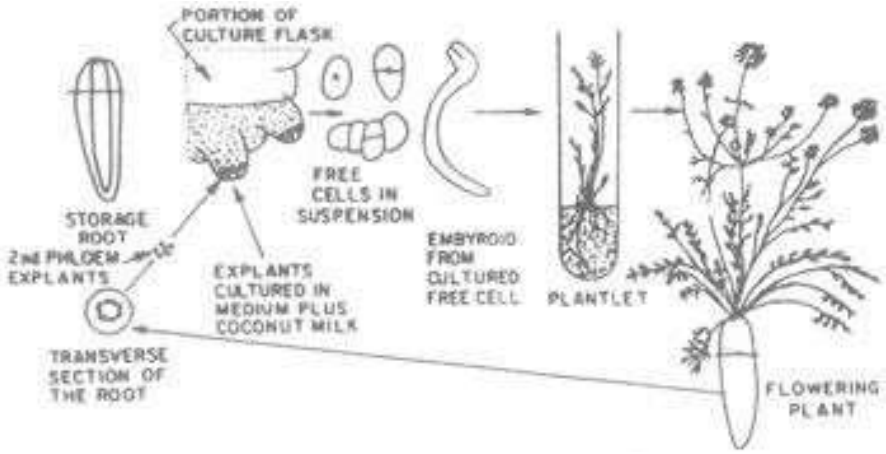
Because pES cells can be made more easily and more efficiently than ntES cells, they could potentially be ready for clinical use sooner. However, more needs to be known about their safety. Concerns have been raised that tissues derived from them might not function normally.

Totipotency

The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular totipotency. This is a capacity which is retained even after a cell has undergone final differentiation in the plant body. In plants, even highly mature and differentiated cells retain the ability to regenerate to a meristematic state as long as they have an intact membrane system and a viable nucleus. This is contradicting to animals, where differentiation is generally irreversible.

For a differentiated cell, to express its totipotency, it first undergoes dedifferentiation followed by redifferentiation. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed ‘dedifferentiation’. The phenomenon of conversion of component cells of callus tissue to whole plant or plant organs is called as ‘redifferentiation’.

The phenomenon of totipotency is demonstrated with the following experiment. Slices of the carrot root (shown on the left) were cut and small pieces of tissues were taken from the phloem region. These were inoculated into a liquid medium in special flasks, which were rotated slowly. The tissue grew actively and single cells and small cell aggregates dissociated into the medium (a single cell and some cell aggregates are drawn near the flask). Some of the cell clumps developed roots, and, when transferred to a semi-solid medium, these rooted nodules formed shoots. These plants could be transferred to soil where they developed into flowering plants. Phloem tissues taken from the roots of these plants could be used to repeat the cycle.



The term “totipotent” has two basically different interpretations: (i) capable of developing into a complete organism or (ii) capable of differentiating into any cell types of an organism (Condic, 2014). In the first and stricter sense, only zygotes or one-celled embryos are totipotent. In the second and wider sense cells which can develop to all the various cell types of an organism but under different condition each, are also totipotent. Based on this second definition, embryonic animal stem cells that can produce a wide range (but not all!) cell types are often considered to be totipotent (Condic, 2014). Since these definitions describe two significantly different developmental potencies, Condic recommended using the term “omnipotent” to suit to the second definition (Condic, 2014).

One can often meet the overstatement, even in university textbooks, that “all/most plant cells are totipotent.” This is based on the erroneous belief that if we can regenerate a whole plant from a cell/explant that evidences cellular totipotency. However, plant regeneration from a totipotent cell must fulfill two main criteria: (i) it must be initiated in an individual cell since totipotency is a cellular term (Condic, 2014); (ii) it must proceed autonomously as a single process (Verdeil et al., 2007).

Whole plants are regenerated from in vitro cultured plant cells either directly or indirectly (intervened by callus formation) via organogenesis or somatic embryogenesis. These processes are not autonomous but needs to be induced! Therefore, one could say, at best, that plant cells can (re)gain totipotency but they are not totipotent per se. Plant regeneration via several steps obviously does not fulfill the criterium of autonomous development. For example, plant regeneration via organogenesis includes at least two stages: either shoot or root is regenerated from the initial cell and a second induction step is required to regenerate the missing plant part. Not the same cell is forming the shoot and the root! In these processes, the initial cells of root/shoot development can be considered as pluripotent cells. Furthermore, auxin-induced organ regeneration (including callus formation) was

shown to initiate in “pericycle-like stem cells” in various tissues and not in somatic cells (Sugimoto et al., 2010). The direct de novo formation of stem cells from single differentiated somatic cells is widely believed to take place but hardly evidenced (Gaillochet and Lohmann, 2015; Perez-Garcia and Moreno-Risueno, 2018). Root formation on leaf explants detached from Arabidopsis plants might represent an example (Liu et al., 2014). However, endogenous callus formation initiated with the division of “pericycle-like stem cells” has recently been associated with this regeneration pathway as well (Bustillo-Avendaño et al., 2018). The capability for de novo meristem formation is mostly confined to callus tissues (Perez-Garcia and Moreno-Risueno, 2018). During these regeneration processes, appropriate hormonal gradients are established in the callus tissue leading to stem cell niche formation and stem cell differentiation (Perez-Garcia and Moreno-Risueno, 2018). Therefore, the new meristem does not have a clear single cell origin. Moreover, only the newly formed stem cells but not all cells of the callus tissue can be regarded as pluripotent.

Somatic embryogenesis is believed to be the definitive proof for the totipotency of somatic plant cells. Indeed, single cells forming embryos (embryogenic cells) are totipotent by definition since embryos can autonomously develop to whole plants. If all plant cells are totipotent, all plant cells could be able to form somatic embryos. This is obviously not the case. Although somatic embryogenesis is prevalent, it is confined to defined genotypes, developmental states, and explants.

Similarly, to organogenesis, somatic embryogenesis needs induction. This means that although certain somatic cells might (re)gain totipotency under appropriate conditions, they are not totipotent per se. Furthermore, somatic embryo formation not necessarily involves neither dedifferentiated somatic nor totipotent cells. Such as callus formation and organogenesis, initiation of embryos from cells surrounding the veins (often referred as procambial cells) was frequently observed (Guzzo et al., 1994; Rose et al., 2010; de Almeida et al., 2012). Whether in these cases embryogenesis shares the initial steps of lateral root/callus formation from “pericycle-like stem cells” still needs to be experimentally addressed. In carrot, somatic embryo formation could be tracked back to single cells or small cell clusters of perivascular origin in the fresh liquid culture of hypocotyl explants (Schmidt et al., 1997). In the presence of auxin (2,4-D), these cells form proembryogenic cell masses (PEMs) as a transitional stage toward embryogenesis. It is a second signal, the removal of auxin, that triggers embryo formation from PEMs (de Vries et al., 1988; Rose et al., 2010). These series of events question the direct autonomous development of somatic embryos from the single embryogenic cells. However, PEMs themselves might be regarded as overproliferating somatic embryos losing their organization (for review, Dudits et al., 1995).

Recent observations indicate that indirect embryogenesis progresses on surfaces of embryogenic calli via the reorganization of cell clusters instead of developing from single totipotent cells (for review, Su and Zhang, 2014). Several cellular and molecular steps of embryo formation have been revealed in the case of embryogenic Arabidopsis calli (Su et al., 2009, 2015; for review, Fehér et al., 2016). The following model could be established using fluorescent gene expression markers and confocal laser scanning microscopy (Su et al., 2009, 2015; Bai et al., 2013; Figure 3). Embryogenic calli form in the 2,4-D-containing culture medium. Moving them to auxin free conditions, the endogenous synthesis of auxin is induced. The synthesis takes place in the peripheral region of the calli via the expression of YUCCA (YUC) genes (Bai et al., 2013). Subsequently, the synthesis of the PINFORMED1 (PIN1) auxin transport proteins is induced. Their organized orientation results in auxin accumulations in peripheral cell clusters (Su et al., 2009). In between, in the regions exhibiting auxin minima, the gene coding for the WUSCHEL (WUS) meristem identity regulator starts to be expressed (Su et al., 2009). At this early state, the expression of WUS-RELATED HOMEOBOX 5 (WOX5), a master regulator of root meristem organization, partly overlaps with that of WUS (Su et al., 2015). At the next step, cotyledon primordia get organized at the places of auxin maxima on the callus periphery. At this time, cytokinin accumulation can be detected below the WUS expression domain and WOX5 expression is confined to this cytokinin-rich region. The site of WOX5 expression relates with root meristem emergence. In this way, the apical basal axis of the embryo is established before somatic embryos are even visible (Su et al., 2009, 2015; Bai et al., 2013). The above experimental observations indicate the formation (organization) of somatic embryos from groups instead of single callus cells. The induction and reorganization of hormone synthesis and distribution within the callus tissue results in the parallel formation of shoot and root meristems that is followed by the development of an embryo-like structure. This model argues that the regression to a fully dedifferentiated (totipotent) cellular state is not an absolute prerequisite for embryo regeneration from plant tissues.

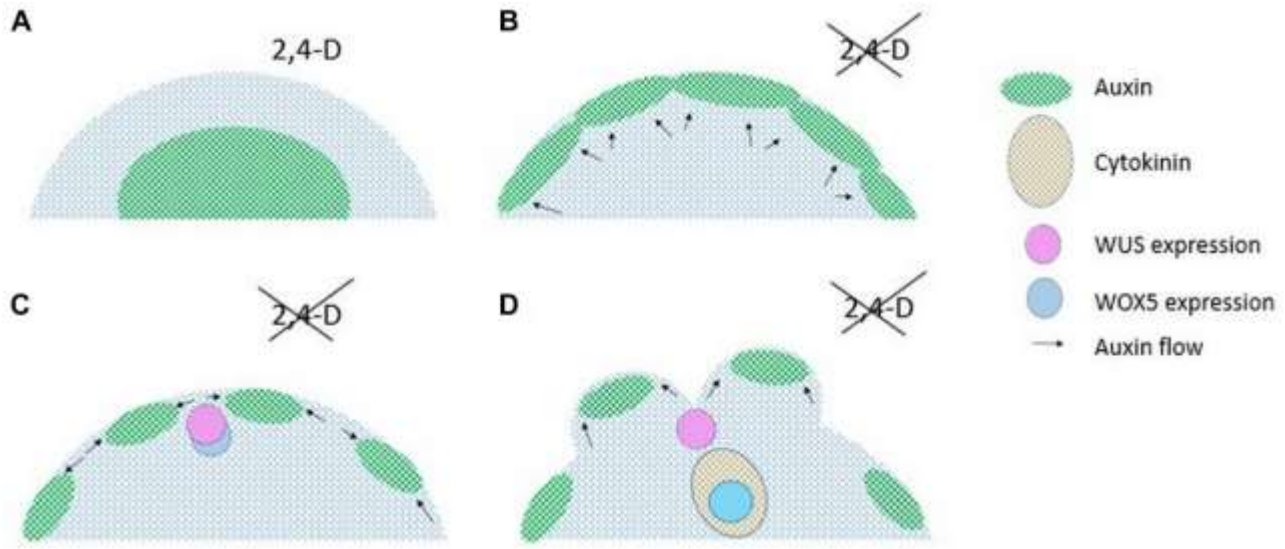


Figure 3. The schematic process of the early steps of multicellular somatic embryo formation on the surface of embryogenic calli. Embryogenic calli formed in the presence of 2,4-dichloro-phenoxyacetic acid (2,4-D) are blocked in development until the removal of this exogenous artificial auxin (A). Following 2,4-D removal, endogenous auxin starts to get produced and start to accumulate at the periphery due to directional auxin transport mediated by the PIN1 auxin efflux carrier proteins (B). Changes in PIN1 orientation result in auxin accumulation in patches at the callus surface. In between, at auxin minima, the expression the WUS transcription factor appears and that partly overlaps with that of WOX5 (C). Cotyledon primordia get organized at auxin maxima and the organizing center of the shoot meristem forms from the cells expressing WUS. The root meristem develops from the region accumulating cytokinin and expressing WOX5 (D). Based on the experiments described in Su et al. (2009, 2015) and Bai et al. (2013). Note that PIN1 is not shown for simplicity.

What is the Meaning of Totipotency?

Totipotency is the genetic potential of a plant cell to produce the entire plant. In other words, totipotency is the cell characteristic in which the potential for forming all the cell types in the adult organism is retained.

Expression of Totipotency in Culture:

The basis of tissue culture is to grow large number of cells in a sterile controlled environ-ment. The cells are obtained from stem, root or other plant parts and are allowed to grow in culture medium containing mineral nutrients, vi-tamins and hormones to encourage cell division and growth. As a result, the cells in culture will produce an unorganised proliferative mass of cells which is known as callus tissue.

The cells that comprise the callus mass are totipo-tent. Thus a callus tissue may be in a broader sense totipotent, i.e., it may be able to regen-erated back to normal plant given certain ma-nipulations of the medium and the cultural en-vironment. Truly speaking, totipotency of the cell is manifested through the process of differ-entiation and the hormones in this process play the major role than any other manipulations.

In the fifties, F. Skoog and C.O. Miller of U.S.A. discovered a new plant growth hormone kinetin from herring sperm DNA. With a correct concen-tration ratio of auxin and cytokinin in tobacco cultures, Skoog was able to demonstrate the role of kinetin in organogenesis. When the ratio of kinetin to auxin was higher, only shoot devel-oped. This is known as caulogenesis. But when the ratio was lower, only roots were formed. This is known as rhizogenesis.

Around the same pe-riod, F. C. Steward and his colleagues at Cornell University of USA, devised a method for growing carrot tissue by excising small disc, from the sec-ondary phloem region of carrot root and placing them in a moving liquid medium under aseptic conditions. In presence of coconut milk in the medium, the phloem tissue began to the grow actively.

In moving liquid medium some single cells and small groups of cells were loosened from the surface of growing tissue. When these iso-lated cells were grown separately it was found that some single cells developed somatic embryos or embryoids by a process that occurs in nor-mal zygotic embryo.

It is also observed in some experiment that cells of some callus mass fre-quently differentiate into vascular elements such as xylem and phloem without forming any plant organs or embryoids. This process is known as histogenesis or Cyto-differentiation. Thus the totipotent cells may express themselves in dif-ferent way on the basis of differentiation process and manipulation.

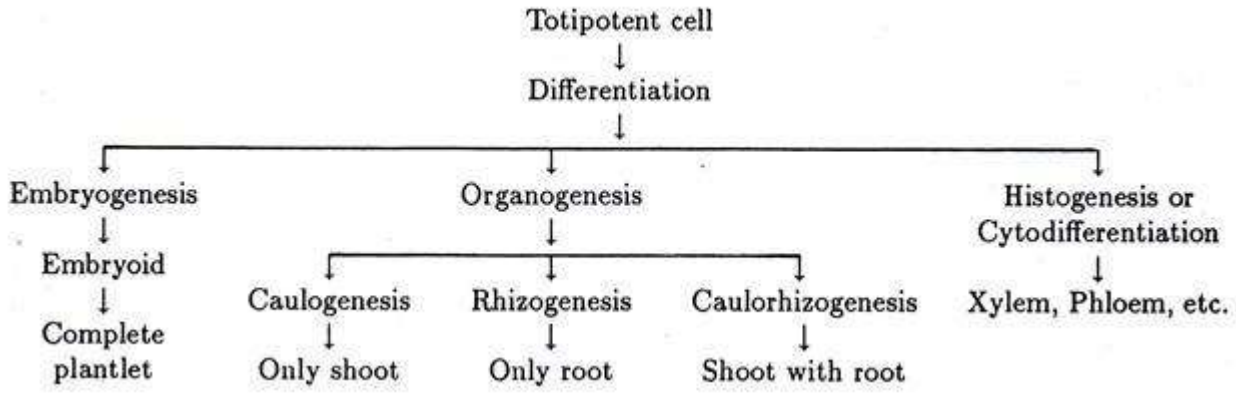
Where the totipotent cells are partially ex-pressed or not expressed, it is obvious that the limitation on its capacity for development must be imposed by the microenvironments. The

toti-potency of cells in the callus tissue may be re-tained for a longer period through several sub-cultures.

Practically, it is observed that the ex- plant first forms the callus tissue in the callus inducing medium and such callus tissue is main-tained through some subcultures. After then it is generally transferred to another medium which is expected to be favourable for the expression of totipotent cells. Actually, the regeneration medium is standardized by trial-and-error method.

In more or less suitable medium, the totipotent cells of the callus tissue give rise to meristematic nodules or meristemoids by repeated cell divi-sion. This may subsequently give rise to vascu-lar differentiation or it may form a primordium capable of giving rise to a shoot or root. Some-times the totipotent cell may produce embryoids through sequential stages of development such as globular stage, heart shaped stage and torpedo stage etc.

After prolonged culture, it has been obser-ved that calluses in some species (e.g. Nicotiana tabacum, Citrus aurantifolia etc.) maybe- come habituated. This means that they are now able to grow on a standard maintenance medium which is devoid of growth hormones. The cells of habituated callus also remain totipotent and are capable to regenerate a plant without any major manipulation.



A typical crown gall tumour cell has the ca-pacity for unlimited growth independent of ex-ogenous hormones. It shows totally lack of organ genic differentiation. So such tissue is consid-ered to have permanently lost the totipotentiality of the parent cells.

In some plant species, the crown gall bacterium (Agrobactenum tumefaciens) induces a special type of tumour, called teratomas, the cells of which possess the capac-ity to differentiate shoot buds and leaves when they are grown in culture for unlimited periods. Thus it is clear that the mode of expression of totipotency of plant cell in culture varies from plant to plant and also helps us to understand the process of differentiation in vitro.

Importance of Totipotency in Plant Science:

The ultimate objective in plant protoplast, cell and tissue culture is the reconstruction of plants from the totipotent cell. Although the process of differentiation is still mysterious in general, the expression of totipotent cell in cul-ture has provided a lot of information's.

On the other hand, the totipotentiality of somatic cells has been exploited in vegetative propagation of many economical, medicinal as well as agricul-turally important plant species. Therefore, from fundamental to applied aspect of plant biology, cellular totipotency is highly important.

Recent trends of plant tissue culture include genetic modification of plants, production of homozy-gous diploid plants through haploid cell culture, somatic hybridization, mutation etc. The suc-cess of all these studies depends upon the expres-sion of totipotency. In many cases, successful and exciting results have been obtained.

Plant breeders, horticulturists and commercial plant growers are now more interested in plant tis-sue culture only for the exploitation of totipo-tent cells in culture according to their desirable requirement. Totipotent cells within a bit of cal-lus tissue can be stored in liquid nitrogen for a long period. Therefore, for germplasm preser-vation of endangered plant species, totipotency can be utilized successfully.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – II – Concepts in Stem Cell Research – SBT1403

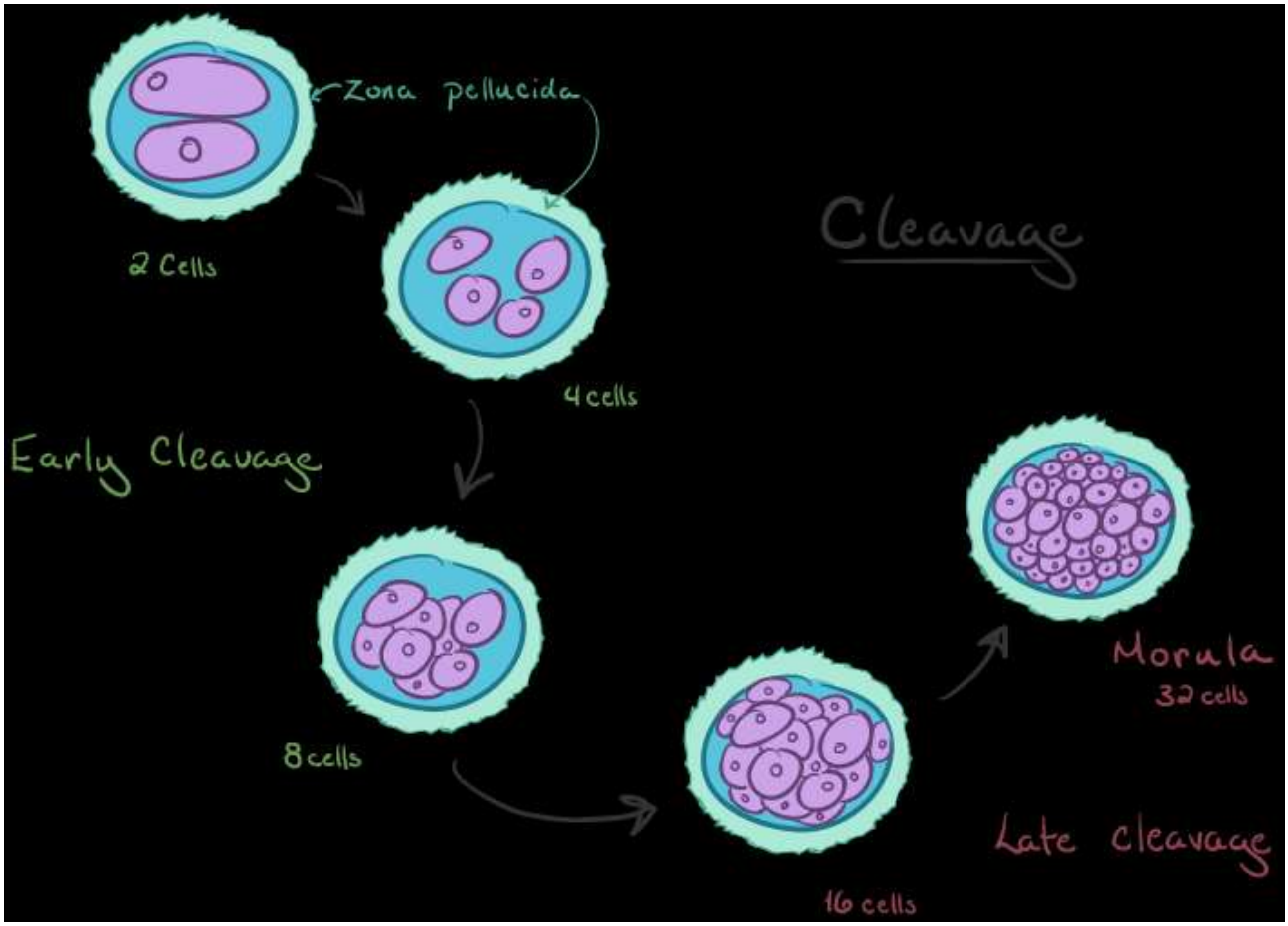
1. Embryogenesis

Embryogenesis, the first eight weeks of development after fertilization, is an incredibly complicated process. It's amazing that in eight weeks we're transforming from a single cell to an organism with a multi-level body plan. The circulatory, excretory, and neurologic systems all begin to develop during this stage. Luckily, like with many complex biological concepts, fertilization can be broken down into smaller, simpler ideas. The big idea of embryogenesis is going from a single cell to a ball of cells to a set of tubes.

Let's Start at the Very Beginning

01. Step 1: a zygote is the single cell formed when an egg and a sperm cell fuse; the fusion is known as fertilization
02. Step 2: the first 12-to 24-hours after a zygote is formed are spent in cleavage – very rapid cell division

The zygote's first priority is dividing to make lots of new cells, so it's first few days are spent in rapid mitotic division. With each round of division, it doubles in cell number, so the cell number is increasing at an exponential rate! This division is taking place so quickly that the cells don't have time to grow, so the 32 cell stage known as the morula is the same size as the zygote. At this point, the zona pellucida (a protective membrane of glycoproteins that had surrounded the egg cell) is still intact, which also limits how big it can grow.

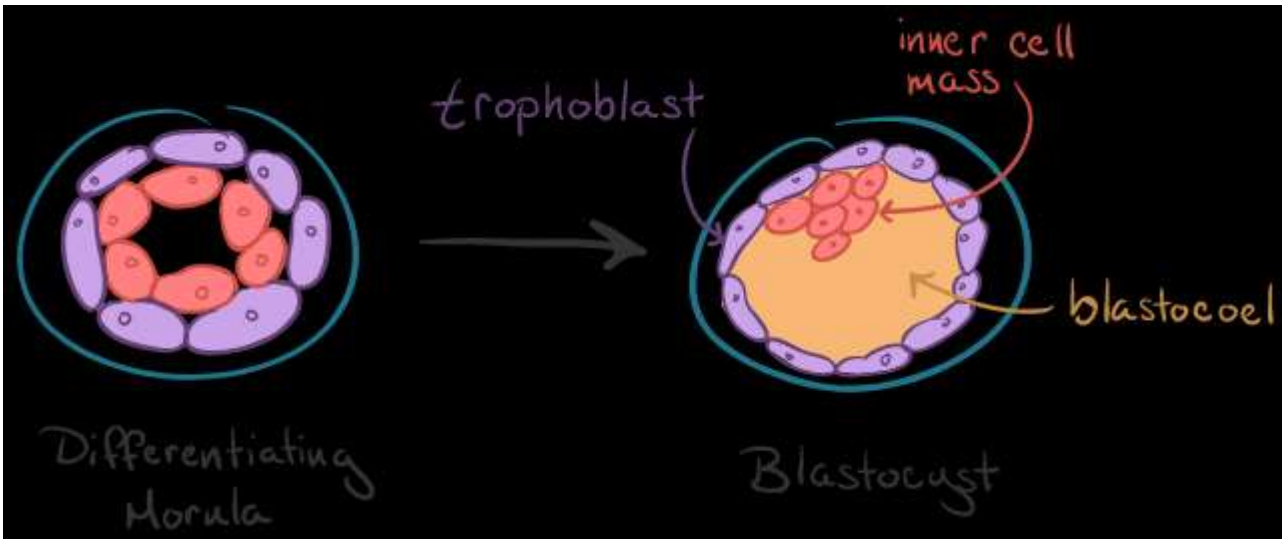


Blastulation and Cell Differentiation

03. Step 3: during blastulation, the mass of cells forms a hollow ball
04. Step 4: cells begin to differentiate, and form cavities

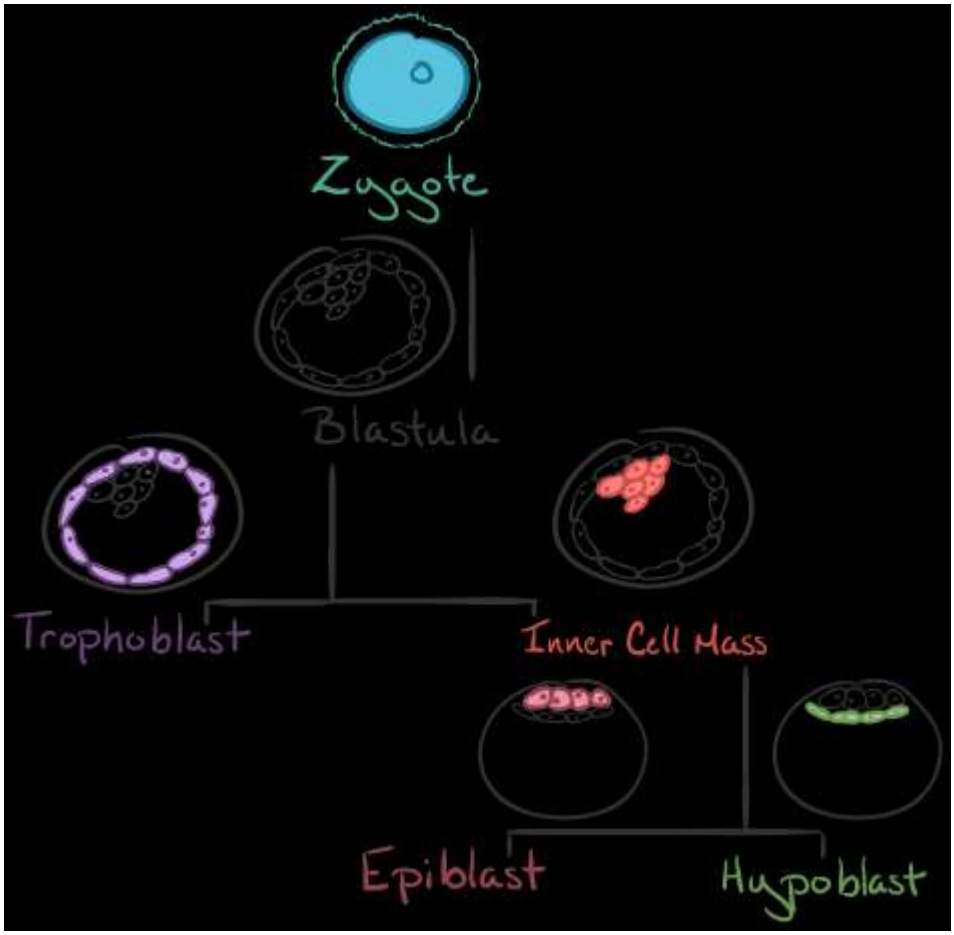
Around day 4, cells continue to divide, but they also begin to differentiate and develop more specific forms and functions. When a cell differentiates, it moves down a certain path toward being a specific type of cell (e.g. an ear cell or a kidney cell), and this process (99% of the time) only goes in one direction. Two layers develop: an outer shell layer known as the trophoblast, and an inner collection of cells called the inner cell mass. Rather than being arranged in a solid sphere of cells, the inner cell mass is pushed off to one side of the sphere formed by the trophoblast. The rest of the fluid-filled cavity is called the blastocoel, and the whole setup resembles a snow globe. The outer trophoblast will develop into structures that help the growing embryo implant in the mother's uterus. The inner cell mass will continue to differentiate and parts of it will eventually become the embryo, so it is sometimes called the embryoblast (the suffix "blast" means "to make"). This is also the time when the zona pellucida begins to disappear, allowing the ball of cells, now called a blastocyst, to

grow and change shape. In non-mammal animals, the term for this stage is “blastula”, but we will stick with terms that apply to human development for the purposes of this discussion.

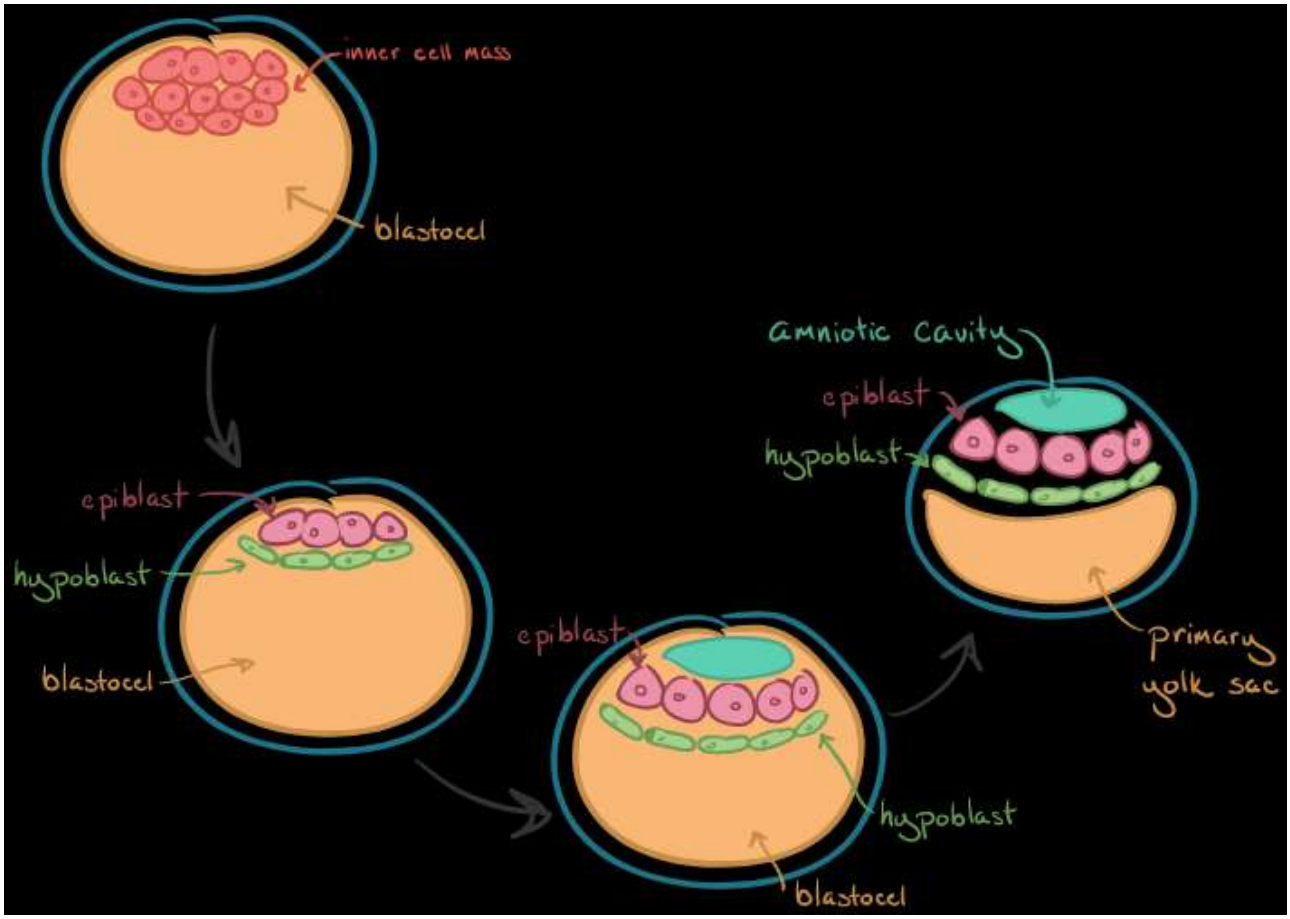


At this point, cells in the inner cell mass are pluripotent, meaning they can eventually turn into the cells of any body tissue (muscle, brain, bone, etc). During the second week, these cells differentiate further into the epiblast and the hypoblast, which are the two layers of the bilaminar disc. This disc is a flat slice across the developing sphere, and splits the environment into two cavities. The hypoblast is the layer facing the blastocoel, while the epiblast is on the other side. Let’s imagine each of these layers as a flat balloon. The balloons expand to fill the space, and become the two new cavities: the primitive yolk sac on the side of the hypoblast and the amniotic cavity on the side of epiblast. The amniotic cavity will eventually surround the fetus.

Quick recap: the outermost layer of the sphere is the trophoblast. Inside the sphere are two spaces that are each lined by either the hypoblast or the epiblast. The point where the epiblast and hypoblast press up against each other is known as the bilaminar disc, and this disk is what splits the sphere to make the two cavities.



The hypoblast does not contribute to the embryo, so we will now turn our focus solely on the epiblast.



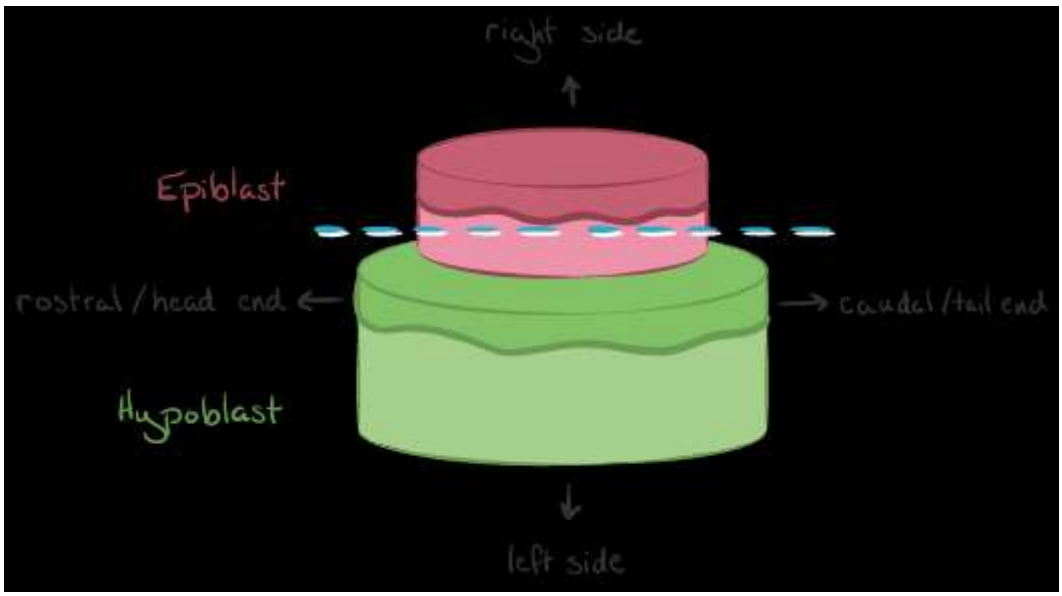
Making Tubes

05. Step 5: During gastrulation the three germ layers form; the cell mass is now known as a gastrula
    a. Step 5a: The primitive streak forms
06. Step 6: The notochord is formed

Week 3 of development is the week of gastrulation. A germ layer is a layer of cells that will go on to form one of our organizational tubes. Our anatomy can really be boiled down to an inner tube (our digestive tract), and a series of tubes that wrap around it. The three germ layers that will translate into these tubes are the ectoderm, the mesoderm, and the endoderm.

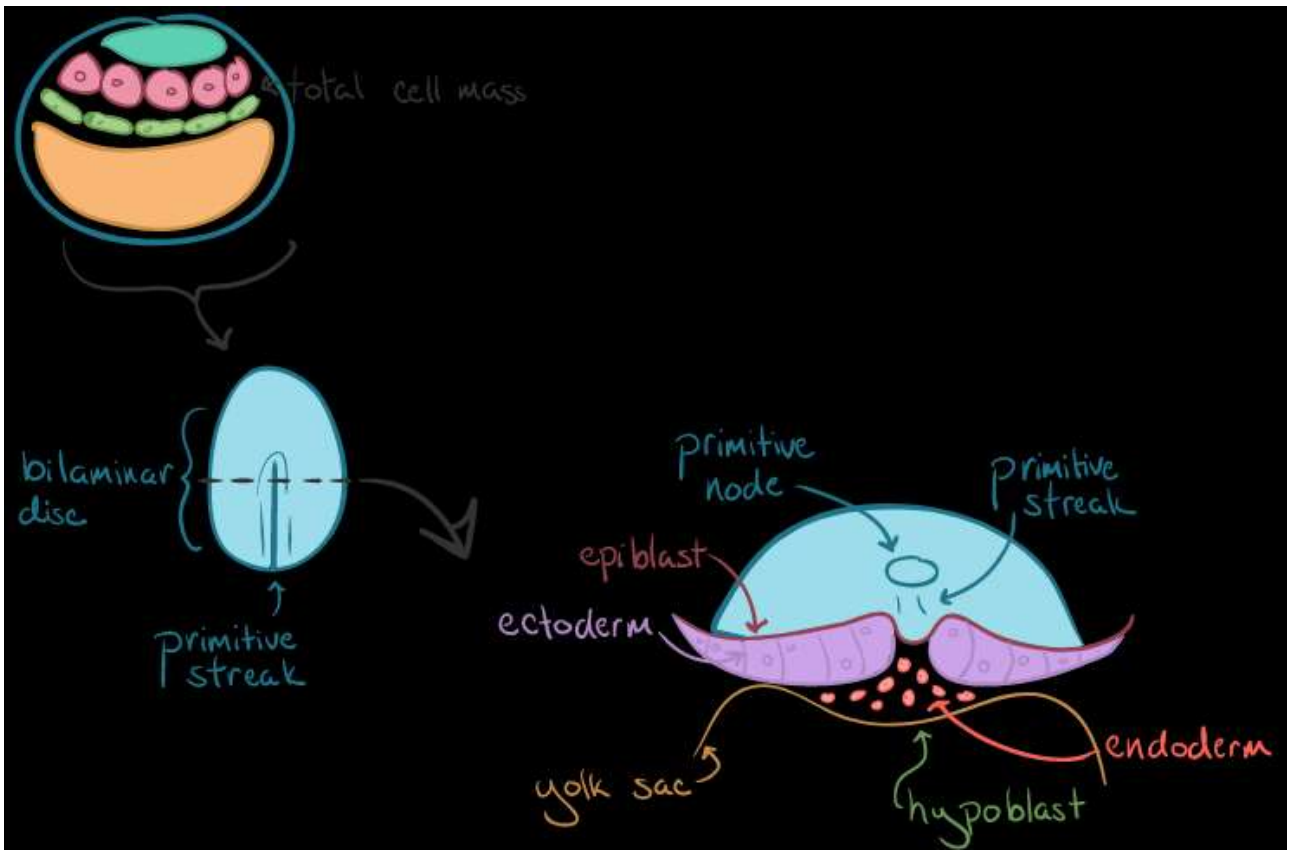
Germ Layer	What does the prefix mean?	Goes on to form:
Ectoderm	Outer, external	Epidermis (outer layer of skin), hair, nails, brain, spinal cord, peripheral nervous system
Mesoderm	Middle	Muscle, bone, connective tissue, notochord, kidney, gonads, circulatory system
Endoderm	Within	Epithelial lining of the digestive tract; Stomach, colon, liver, pancreas, bladder, lung

The first step of gastrulation is the formation of the primitive streak (~ day 16). Let's imagine the bilaminar disc as two tier cake. Imagine taking a knife and cutting into just the top layer (the epiblast) like you're going to cut a slice.



This cut is the primitive streak, and it cuts from the caudal (anus) end in toward the end that will eventually become the head (the rostral end). This streak determines the midline of the body, and separates the left and right sides. Like all deuterostomes, humans have bilateral symmetry, which means that there is a single across which we can split ourselves to make mirror images. What we are actually seeing when we look at a primitive streak are moving cells. They are going from the epiblast and moving down so they end up between the original epiblast layer and the hypoblast. I've always imagined the motion like water falling down a waterfall. The first layer to invaginate dives the deepest

and ends up closest to the hypoblast – this is the endoderm. The next layers will become the mesoderm, and the cells of the epiblast that continue to border the amniotic cavity are the ectoderm. We now have three germ layers, all of which will contribute to the developing embryo. In the picture below, the anus end is facing us.



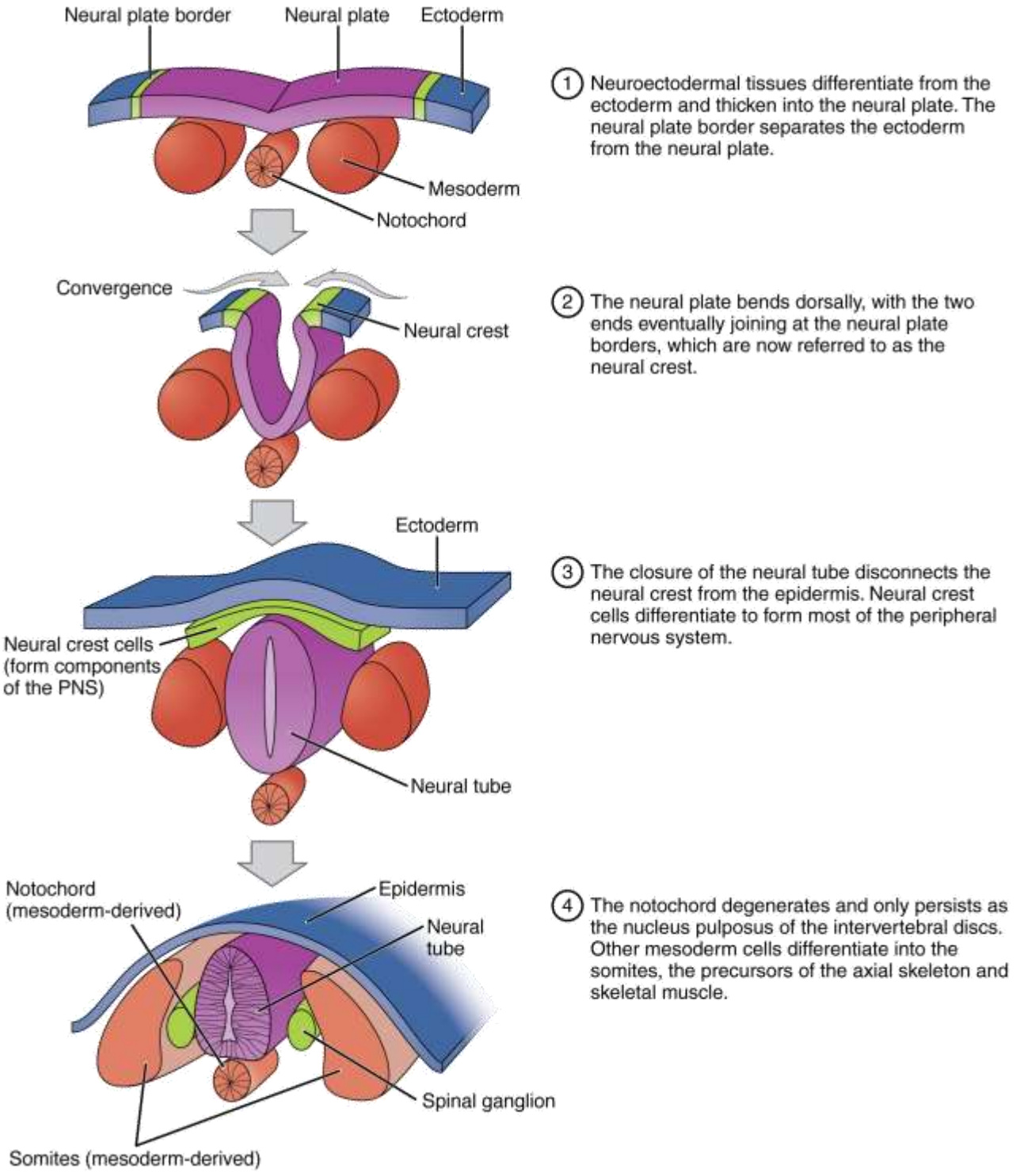
Directly beneath the primitive streak the mesoderm (the middle germ layer) forms a thin rod of cells known as the notochord. The notochord helps define the major axis of our bodies, and is important in inducing the next step of embryogenesis, when we finally start to make our tubes! The notochord is a defining feature of the Chordate phylum, and will eventually become our intervertebral discs.

Neurulation

06. Step 6: Tubes form, making a neurula
    a. Step 6a: The notochord induces the formation of the neural plate
    b. Step 6b: The neural plate folds in on itself to make the neural tube and neural crest
07. Step 7: The mesoderm has five distinct categories

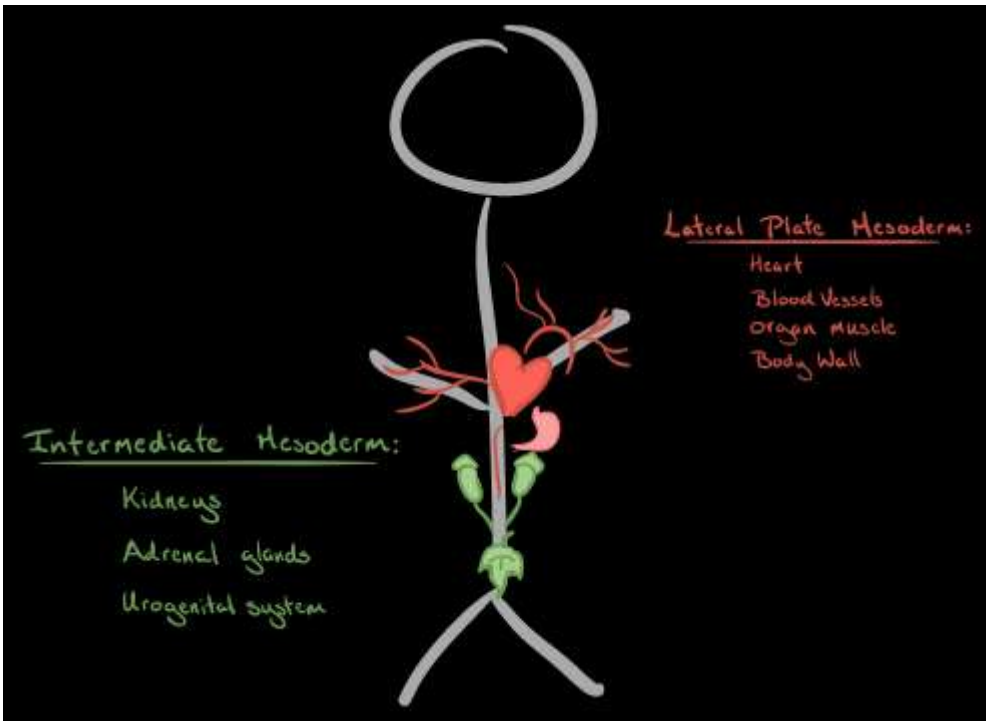
All this and we still haven't made tubes! Now that we have successfully made the cell layers, we have to create the final 3D product. The first step in this rolling is the creation of the notochord. The notochord causes the ectoderm above it to form a thick flat plate of cells called the neural plate. The neural plate extends the length of the rostral-caudal axis. The neural plate then bends back on itself and seals itself into a tube known as the neural tube that fits underneath the ectoderm. The borders of where the neural plate had been get pulled under with it, and become the neural crest. The neural tube will become the brain and spinal cord.

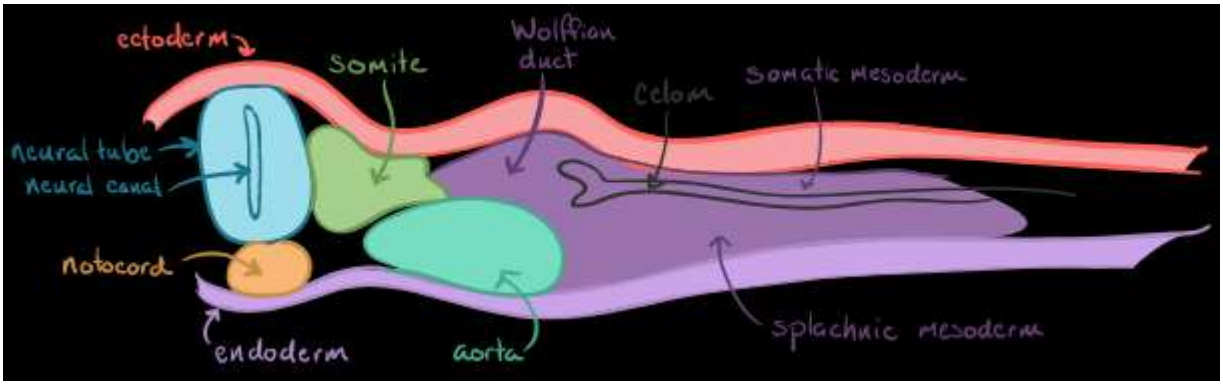
The neural crest is sometimes called the fourth germ layer, because the cells that become the sympathetic and parasympathetic nervous systems, melanocytes, Schwann cells, even some of the bones and connective tissue of the face.



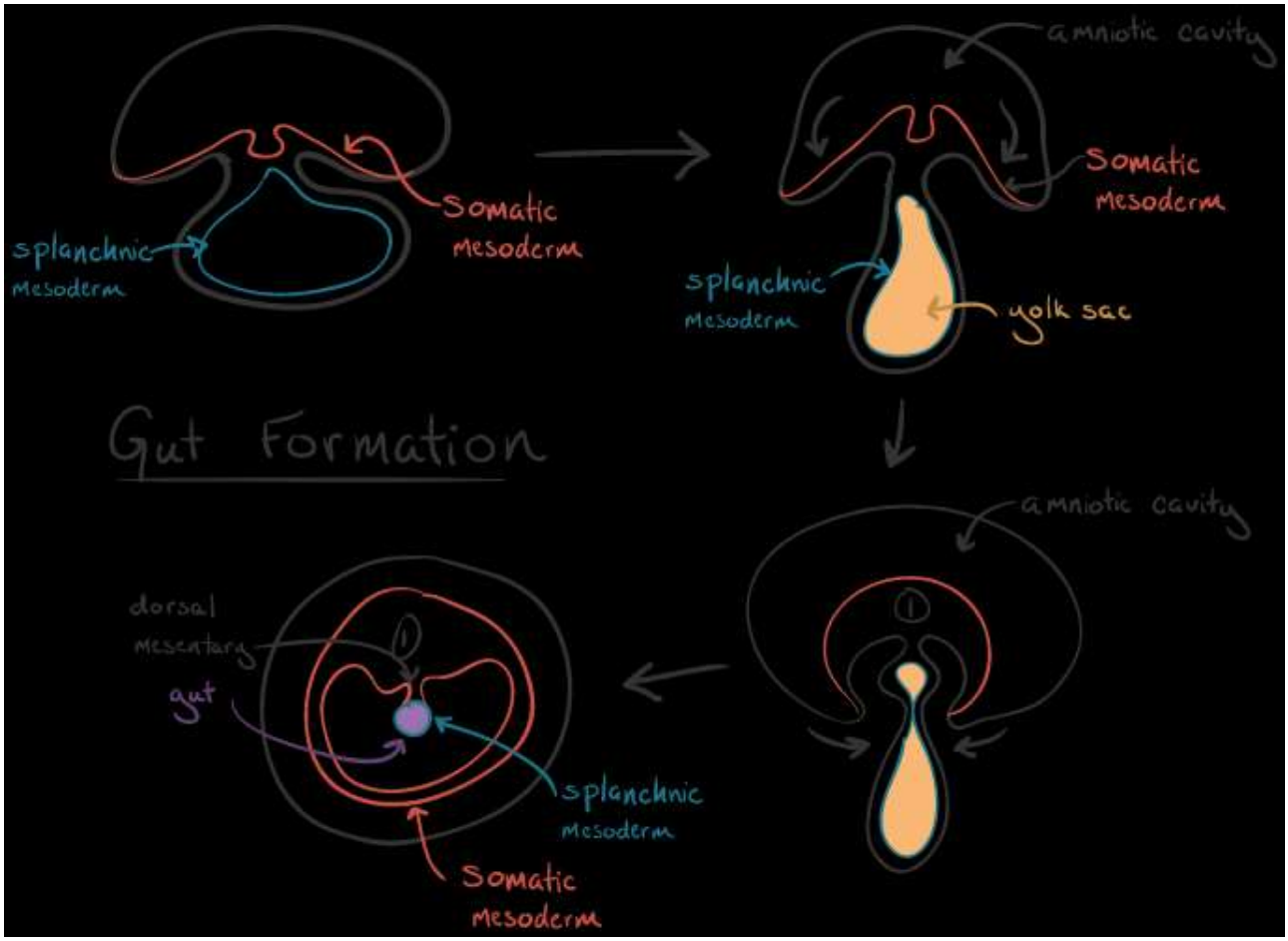
Meanwhile, the mesoderm can be subdivided into the axial, paraxial, intermediate, and lateral plate mesoderms. The notochord came from the axial mesoderm. The paraxial mesoderm will give rise to somites, which will differentiate into muscle, cartilage, bone, and dermis. Somite derivatives create a segmented body plan (see right). The intermediate mesoderm is the origin of our urogenital system – our kidneys, gonads, adrenal glands, and the ducts that connect them. The lateral plate

mesoderm will give rise to the heart (the first organ to develop!), blood vessels, the body wall, and the muscle in our organs.





Also at the same time, the endoderm is rolling into a tube as well – the digestive tract. The digestive tract is subdivided into the foregut, midgut, and hindgut. Each subdivision has its own nerve and blood supply. Organs related to the GI tract actually start off as outpouchings of this tube. The foregut gives rise to the esophagus, stomach, part of the duodenum, and the respiratory bud, which will eventually develop into the lungs. The second half of the duodenum through to the transverse colon arise from the midgut. The remainder of the GI tract, including the rest of the transverse colon, the descending colon, the sigmoid colon, and the rectum are formed from the hindgut.



That's what is going in with each of the three layers. While this is happening, the mesodermal layers are circling around the endoderm, and the part of the ectoderm that will become the skin is circling around both of the other layers. Some tubes, likes the neural tube, are closing, while the gut tube is connecting to the ectoderm to form the mouth and the anus. By the time eight weeks have passed, all of our tubes are in order, the primitive heart has been beating for almost five weeks, and development is well on its way!

II. In Vitro Fertilization

In vitro fertilisation (IVF) is a process by which egg cells are fertilised by sperm outside the body: in vitro. IVF is a major treatment in infertility when other methods of assisted reproductive technology have failed. The process involves hormonally controlling the ovulatory process, removing ova (eggs) from the woman's ovaries and letting sperm fertilise them in a fluid medium. The fertilised egg (zygote) is then transferred to the patient's uterus with the intent to establish a successful pregnancy. The first successful birth of a "test tube baby", Louise Brown, occurred in 1978. Robert G. Edwards, the physiologist who developed the treatment, was awarded the Nobel Prize in Physiology or Medicine in 2010.The term in vitro, from the Latin root meaning in glass, is used, because early biological experiments involving cultivation of tissues outside the living organism from which they came, were carried out in glass containers such as beakers, test tubes, or petri dishes. Today, the term in vitro is used to refer to any biological procedure that is performed outside the organism it would normally be occurring in, to distinguish it from an in vivo procedure, where the tissue remains inside the living organism within which it is normally found. A colloquial term for babies conceived as the result of IVF, "test tube babies", refers to the tube-shaped containers of glass or plastic resin, called test tubes, that are commonly used in chemistry labs and biology labs. However, in vitro fertilisation is usually performed in the shallower containers called Petri dishes. One IVF method, Autologous Endometrial Coculture, is actually performed on organic material, but is still considered in vitro.

Implications

IVF may be used to overcome female infertility in the woman due to problems of the fallopian tube, making fertilisation in vivo difficult. It may also assist in male infertility, where there is defect sperm quality, and in such cases intracytoplasmic sperm injection (ICSI) may be used, where a sperm cell is injected directly into the egg cell. This is used when sperm have difficulty penetrating the egg, and in these cases the partner's or a donor's sperm may be used. ICSI is also used when sperm numbers are very low. ICSI results in success rates equal to those of IVF. For IVF to be successful it typically requires healthy ova, sperm that can fertilise, and a uterus that can maintain a pregnancy. Due to the costs of the procedure, IVF is generally attempted only after less expensive options have failed.IVF can also be used with egg donation or surrogacy where the woman providing the egg isn't the same

who will carry the pregnancy to term. This means that IVF can be used for females who have already gone through menopause. The donated oocyte can be fertilised in a crucible. If the fertilisation is successful, the embryo will be transferred into the uterus, within which it may implant.IVF can also be combined with preimplantation genetic diagnosis (PGD) to rule out presence of genetic disorders. A similar but more general test has been developed called Preimplantation Genetic Haplotyping

Method

Theoretically, in vitro fertilisation could be performed by collecting the contents from a woman's fallopian tubes or uterus after natural ovulation, mixing it with semen, and reinserting into the uterus. However, without additional techniques, the chances of pregnancy would be extremely small. Such additional techniques that are routinely used in IVF include ovarian hyperstimulation to retrieve multiple eggs, ultrasound-guided transvaginal oocyte retrieval directly from the ovaries, egg and sperm preparation, as well as culture and selection of resultant embryos before embryo transfer back into the uterus.

Ovarian hyperstimulation

There are two main protocols for stimulating the ovaries for IVF treatment. The long protocol involves downregulation (suppression or exhaustion) of the pituitary ovarian axis by the prolonged use of a GnRH agonist. Subsequent ovarian hyperstimulation, typically using follicle stimulating hormone (FSH), starts once the process of downregulation is complete, generally after 10 to 14 days.The short protocol skips the downregulation part, and consists of a regimen of fertility medications to stimulate the development of multiple follicles of the ovaries. In most patients, injectable gonadotropins (usually FSH analogues) are used under close monitoring. Such monitoring frequently checks the estradiol level and, by means of gynecologic ultrasonography, follicular growth. Typically approximately 10 days of injections will be necessary. Spontaneous ovulation during the cycle is typically prevented by the use of GnRH antagonists that are used just during the last days of stimulation to block the natural surge of luteinising hormone (LH) and allow the physician to start the ovulation process by using medication, usually injectable human chorionic gonadotropins. Ovarian stimulation carries the risk of excessive stimulation. This complication is life-threatening and ovarian stimulation using gonadotropins must only be carried out under strict medical supervision.

Egg retrieval

When the ovarian follicles have reached a certain degree of development, final maturation is induced, generally by an injection of human chorionic gonadotropin (hCG). Commonly, this is known as the "trigger shot."[1] hCG acts as an analogue of luteinising hormone, and ovulation would occur between 38 and 40 hours after a single HCG injection,[2] but the egg retrieval is performed at a time usually between 34 and 36 hours after hCG injection, that is, just prior to when the follicles would rupture. This avails for scheduling the egg retrieval procedure at a time where the eggs are fully mature. HCG injection confers a risk of ovarian hyperstimulation syndrome. Using a GnRH agonist instead of hCG eliminates the risk of ovarian hyperstimulation syndrome, but with a delivery rate of approximately 6% less than with hCG.The eggs are retrieved from the patient using a transvaginal technique called transvaginal oocyte retrieval, involving an ultrasound-guided needle piercing the vaginal wall to reach the ovaries. Through this needle follicles can be aspirated, and the follicular fluid is handed to the IVF laboratory to identify ova. It is common to remove between ten and thirty eggs. The retrieval procedure takes about 20 minutes and is usually done under conscious sedation or general anaesthesia.

Egg and sperm preparation

In the laboratory, the identified eggs are stripped of surrounding cells and prepared for fertilisation. An oocyte selection may be performed prior to fertilisation to select eggs with optimal chances of successful pregnancy. In the meantime, semen is prepared for fertilisation by removing inactive cells and seminal fluid in a process called sperm washing. If semen is being provided by a sperm donor, it will usually have been prepared for treatment before being frozen and quarantined, and it will be thawed ready for use.

Fertilisation

The sperm and the egg are incubated together at a ratio of about 75,000:1 in the culture media for about 18 hours. In most cases, the egg will be fertilised by that time and the fertilised egg will show two pronuclei. In certain situations, such as low sperm count or motility, a single sperm may be injected directly into the egg using intracytoplasmic sperm injection (ICSI). The fertilised egg is passed to a special growth medium and left for about 48 hours until the egg consists of six to eight cells.In gamete intrafallopian transfer, eggs are removed from the woman and placed in one of the

fallopian tubes, along with the man's sperm. This allows fertilisation to take place inside the woman's body. Therefore, this variation is actually an in vivo fertilisation, not an in vitro fertilisation.

Embryo culture

Typically, embryos are cultured until having reached the 6–8 cell stage three days after retrieval. In many Canadian, American and Australian programmes, however, embryos are placed into an extended culture system with a transfer done at the blastocyst stage at around five days after retrieval, especially if many good-quality embryos are still available on day 3. Blastocyst stage transfers have been shown to result in higher pregnancy rates.[4] In Europe, transfers after 2 days are common. Culture of embryos can either be performed in an artificial culture medium or in an autologous endometrial coculture (on top of a layer of cells from the woman's own uterine lining). With artificial culture medium, there can either be the same culture medium throughout the period, or a sequential system can be used, in which the embryo is sequentially placed in different media. For example, when culturing to the blastocyst stage, one medium may be used for culture to day 3, and a second medium is used for culture thereafter.[5] Single or sequential medium are equally effective for the culture of human embryos to the blastocyst stage.[6] Artificial embryo culture media basically contain glucose, pyruvate, and energy-providing components, but addition of amino acids, nucleotides, vitamins, and cholesterol improve the performance of embryonic growth and development.[7] Methods to permit dynamic embryo culture with fluid flow and embryo movement are also available.[8] A new method in development uses the uterus as an incubator and the naturally occurring intrauterine fluids as culture medium by encapsulating the embryos in permeable intrauterine vessel.[9]

Embryo selection

Laboatories have developed grading methods to judge oocyte and embryo quality. In order to optimise pregnancy rates, there is significant evidence that a morphological scoring system is the best strategy for the selection of embryos.[10] However, presence of soluble HLA-G might be considered as a second parameter if a choice has to be made between embryos of morphologically equal quality.[10] Also, two-pronuclear zygotes (2PN) transitioning through 1PN or 3PN states tend to develop into poorer-quality embryos than those that constantly remain 2PN.More advanced methods

of embryo profiling may also be performed in order to optimise embryo selection, as further described in the "expansions"-section below.

Embryo transfer

Embryos are failed by the embryologist based on the amount of cells, evenness of growth and degree of fragmentation. The number to be transferred depends on the number available, the age of the woman and other health and diagnostic factors. In countries such as Canada, the UK, Australia and New Zealand, a maximum of two embryos are transferred except in unusual circumstances. In the UK and according to HFEA regulations, a woman over 40 may have up to three embryos transferred, whereas in the USA, younger women may have many embryos transferred based on individual fertility diagnosis. Most clinics and country regulatory bodies seek to minimise the risk of pregnancies carrying multiples. As it is not uncommon for more implantations to take than desired, the next step faced by the expectant mother is that of selective abortion. The embryos judged to be the "best" are transferred to the patient's uterus through a thin, plastic catheter, which goes through her vagina and cervix. Several embryos may be passed into the uterus to improve chances of implantation and pregnancy.

Acupuncture

An increasing number of fertility specialists and centers offer acupuncture as a part of their IVF protocol. Limited but supportive evidence from clinical trials and case series suggests that acupuncture may improve the success rate of IVF and the quality of life of patients undergoing IVF and that it is a safe adjunct therapy.[22] A systematic review and meta-analysis published in the British Medical Journal found that complementing the embryo transfer process with acupuncture was associated with significant and clinically relevant improvements in clinical pregnancy (where the expected number of patients needed to be treated to produce 1 additional pregnancy was 10), ongoing pregnancy (NNT 9), and live birth (NNT 9).

Acupuncture mechanisms

Four mechanisms by which it has been suggested that acupuncture may improve IVF outcomes are[22]

01. Neuroendocrinological modulations
02. Increased blood flow to uterus and ovaries
03. Modulation in cytokines
04. Reduction of stress, anxiety, and depression

In vitro fertilization broadly deals with the removal of eggs from a women, fertilizing them in the laboratory, and then transferring the fertilized eggs (zygotes) into the uterus a few days later.

Indications for IVF:

Infertility due to the following causes may be considered for IVF.

01. Failed ovulation induction
02. Tubal diseases
03. Cervical hostility
04. Endometriosis
05. Idiopathic infertility (in men and women).

Choice of Embryos:

- Around 2 or 3 days before the embryo transfer, the doctor will choose the best eggs to transfer to the womb.
- There are many processes available to aid selection, though non-invasive methods such as metabolomic profiling are being tested.
- Metabolomic profiling is the process of selecting the most beneficial eggs based on a number of different factors.

Fertilization and Culture:

- These eggs will then be fertilized in a lab and left to culture for 1-2 days.
- If many good quality embryos develop, the ones that are not going to be transferred can be frozen.

The Transfer:

Embryo transfer is a 15-minute procedure accomplished by inserting a catheter (preloaded with embryos) into the uterine cavity.

- The embryo transfer process is similar to the process for a pap smear.
- The doctor will insert a speculum into the woman's vagina to keep the vaginal walls open.
- Using ultrasound for accuracy, the doctor will then pass a catheter through the cervix and into the womb.
- From there, the embryos are passed through the tube and into the womb.
- The process is usually pain-free and rarely requires any sedatives.

Types of Embryo Transfer

Once fertilization has occurred, there are a few different options available for embryo transfer:

- Fresh embryo transfer: Once eggs have been fertilized, they are cultured for 1-2 days. The best embryos are chosen to transfer directly to the woman's uterus.
- Frozen embryo transfer: Any healthy embryos that were not used in the first transfer can be frozen and stored for future use. These can be thawed and transferred to the uterus.
- Blastocyst embryo transfer: If many healthy embryos develop after the fertilization, it is common to wait to see if the embryos develop into blastocysts. Blastocyst embryo transfer has a higher success rate than the standard embryo transfer on day 3. However, recent studies suggest that it may pose risks later in pregnancy and should not always be recommended.
- Assisted hatching (AH): A study in the Reproductive Biomedicine Online found that the process of assisted hatching – weakening the outer layer of the embryo before it is transferred to the uterus – does not improve pregnancy and implantation rates in women who are having fresh embryos transferred. The researchers noted, however, that women having frozen 1embryos implanted do benefit from having their embryos treated in this way.

Conditions for Embryo Transfer

Embryo transfer is needed in cases where natural fertilization is not an option or has difficulty occurring. There are many reasons for embryo transfer, including:

- Ovulation disorders: If ovulation is infrequent, fewer eggs are available for successful fertilization.
- Damage to Fallopian tubes: The Fallopian tubes are the passageway through which the embryos travel to reach the uterus. If the tubes become damaged or scarred, it is difficult for fertilized eggs to safely reach the womb.
- Endometriosis: When tissue from the uterus implants and grows outside of the uterus. This can affect how the female reproductive system works.
- Premature ovarian failure: If the ovaries fail, they do not produce normal amounts of estrogen or release eggs regularly.
- Uterine fibroids: Fibroids are small, benign tumors on the walls of the uterus. They can interfere with an egg's ability to plant itself in the uterus, preventing pregnancy.
- Genetic disorders: Some genetic disorders are known to prevent pregnancy from occurring.
- Impaired sperm production: In men, low sperm production, poor movement of the sperm, damage to the testes, or semen abnormalities are all reasons natural fertilization may fail.

Risks and Precautions of embryo transfers

- The risks of embryo transfers themselves are very low.
- These risks are mostly related to increased hormonal stimulation, causing an increased risk such as a blood clot blocking a blood vessel.
- The woman can also experience bleeding, changes in her vaginal discharge, infections, and complications of anesthesia if it is used. The risk of a miscarriage is about the same as in natural conception.
- The greatest risk of embryo transfer is the chance of multiple pregnancies. This occurs when multiple separate embryos attach to the uterus.
- This may increase the risk of stillbirth and children born with disabilities and is more common in pregnancies due to IVF than natural conception.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – III – Concepts in Stem Cell Research – SBT1403

1. Somatic Stem Cells

Adult stem cells, like all stem cells, share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies (shapes) and specialized functions. Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiating along a particular cellular development pathway, although this characteristic may not be as definitive as once thought [82] (see Figure 4.1. Distinguishing Features of Progenitor/Precursor Cells and Stem Cells).

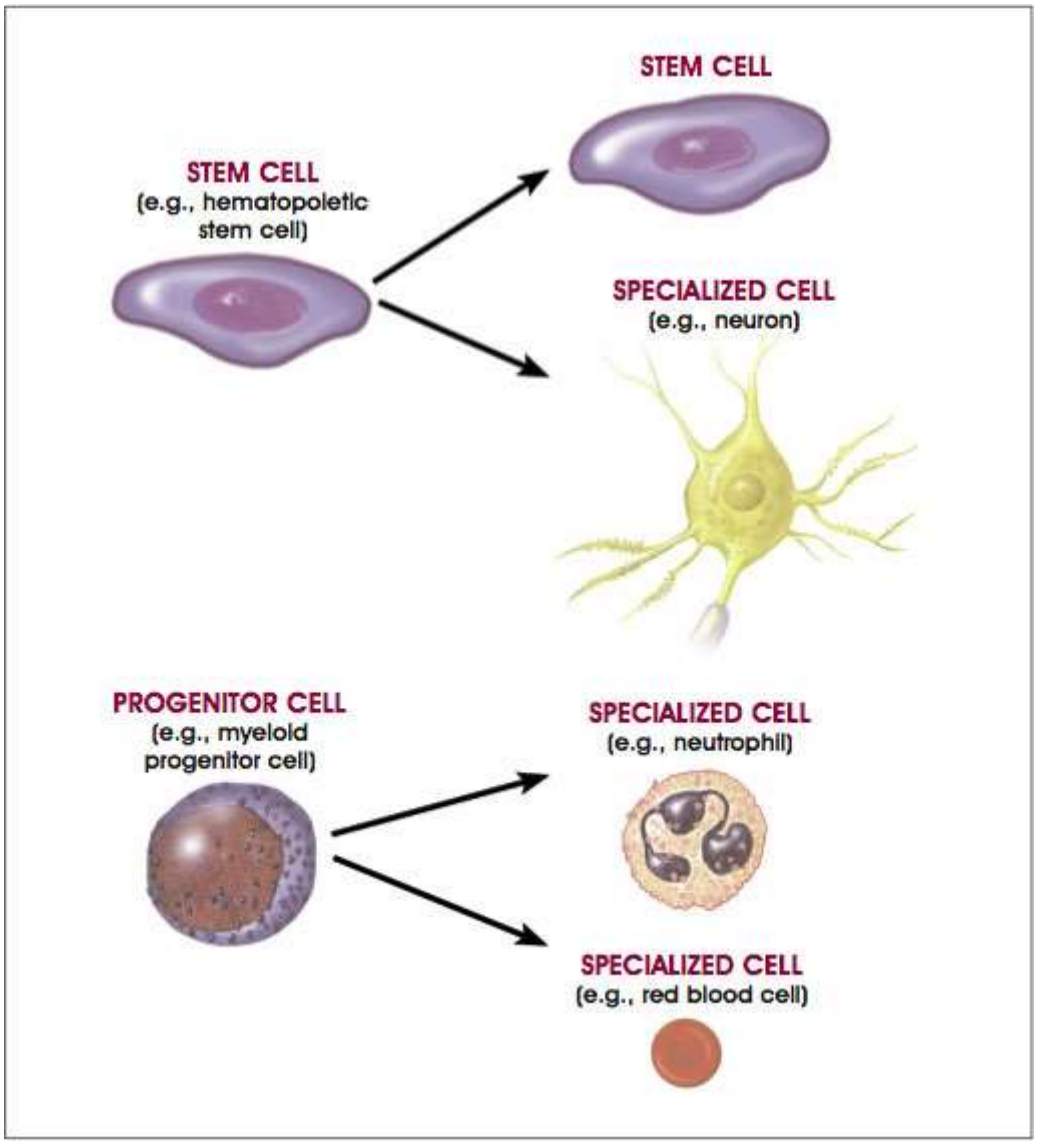

Figure 4.1. Distinguishing Features of Progenitor/Precursor Cells and Stem Cells. A stem cell is an unspecialized cell that is capable of replicating or self renewing itself and developing into specialized cells of a variety of cell types. The product of a stem cell undergoing division is at least one additional stem cell that has the same capabilities of the originating cell. Shown here is an example of a hematopoietic stem cell producing a second generation stem cell and a neuron. A progenitor cell (also known as a precursor cell) is unspecialized or has partial characteristics of a specialized cell that is capable of undergoing cell division and yielding two specialized cells. Shown here is an example of a myeloid progenitor/precursor undergoing cell division to yield two specialized cells (a neutrophil and a red blood cell).

Adult stem cells are rare. Their primary functions are to maintain the steady state functioning of a cell—called homeostasis—and, with limitations, to replace cells that die because of injury or disease [44, 58]. For example, only an estimated 1 in 10,000 to 15,000 cells in the bone marrow is a hematopoietic (bloodforming) stem cell (HSC) [105]. Furthermore, adult stem cells are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment. For example, HSCs are constantly being generated in the bone marrow where they differentiate into mature types of blood cells. Indeed, the primary role of HSCs is to replace blood cells [26] (see Chapter 5. Hematopoietic Stem Cells). In contrast, stem cells in the small intestine are stationary, and are physically separated from the mature cell types they generate. Gut epithelial stem cells (or precursors) occur at the bases of crypts—deep invaginations between the mature, differentiated epithelial cells that line the lumen of the intestine. These epithelial crypt cells divide fairly often, but remain part of the stationary group of cells they generate [93].

Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyst), adult stem cells share no such definitive means of characterization. In fact, no one knows the origin of adult stem cells in any mature tissue. Some have proposed that stem cells are somehow set aside during fetal development and restrained from differentiating. Definitions of adult stem cells vary in the scientific literature range from a simple description of the cells to a rigorous set of experimental criteria that must be met before characterizing a particular cell as an adult stem cell. Most of the information about adult stem cells comes from studies of mice. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.

In order to be classified as an adult stem cell, the cell should be capable of self-renewal for the lifetime of the organism. This criterion, although fundamental to the nature of a stem cell, is difficult to prove in vivo. It is nearly impossible, in an organism as complex as a human, to design an experiment that will allow the fate of candidate adult stem cells to be identified in vivo and tracked over an individual's entire lifetime.

Ideally, adult stem cells should also be clonogenic. In other words, a single adult stem cell should be able to generate a line of genetically identical cells, which then gives rise to all the appropriate, differentiated cell types of the tissue in which it resides. Again, this property is difficult to demonstrate in vivo; in practice, scientists show either that a stem cell is clonogenic in vitro, or that a purified population of candidate stem cells can repopulate the tissue.

An adult stem cell should also be able to give rise to fully differentiated cells that have mature phenotypes, are fully integrated into the tissue, and are capable of specialized functions that are appropriate for the tissue. The term phenotype refers to all the observable characteristics of a cell (or organism); its shape (morphology); interactions with other cells and the non-cellular environment (also called the extracellular matrix); proteins that appear on the cell surface (surface markers); and the cell's behavior (e.g., secretion, contraction, synaptic transmission).

The majority of researchers who lay claim to having identified adult stem cells rely on two of these characteristics—appropriate cell morphology, and the demonstration that the resulting, differentiated cell types display surface markers that identify them as belonging to the tissue. Some studies demonstrate that the differentiated cells that are derived from adult stem cells are truly functional, and a few studies show that cells are integrated into the differentiated tissue in vivo and that they interact appropriately with neighboring cells. At present, there is, however, a paucity of research, with a few notable exceptions, in which researchers were able to conduct studies of genetically identical (clonal) stem cells. In order to fully characterize the regenerating and self-renewal capabilities of the adult stem cell, and therefore to truly harness its potential, it will be important to demonstrate that a single adult stem cell can, indeed, generate a line of genetically identical cells, which then gives rise to all the appropriate, differentiated cell types of the tissue in which it resides.

Evidence for the Presence of Adult Stem Cells

Adult stem cells have been identified in many animal and human tissues. In general, three methods are used to determine whether candidate adult stem cells give rise to specialized cells. Adult stem cells can be labeled in vivo and then they can be tracked. Candidate adult stem cells can also be isolated and labeled and then transplanted back into the organism to determine what becomes of them. Finally, candidate adult stem cells can be isolated, grown in vitro and manipulated, by adding growth factors or introducing genes that help determine what differentiated cells types they will yield. For example, currently, scientists believe that stem cells in the fetal and adult brain divide and give rise to more stem cells or to several types of precursor cells, which give rise to nerve cells (neurons), of which there are many types.

It is often difficult—if not impossible—to distinguish adult, tissue-specific stem cells from progenitor cells, which are found in fetal or adult tissues and are partly differentiated cells that divide and give rise to differentiated cells. These are cells found in many organs that are generally thought to be present to replace cells and maintain the integrity of the tissue. Progenitor cells give rise to certain types of cells—such as the blood cells known as T lymphocytes, B lymphocytes, and natural killer cells—but are not thought to be capable of developing into all the cell types of a tissue and as such are not truly stem cells. The current wave of excitement over the existence of stem cells in many adult tissues is perhaps fueling claims that progenitor or precursor cells in those tissues are instead stem cells. Thus, there are reports of endothelial progenitor cells, skeletal muscle stem cells, epithelial precursors in the skin and digestive system, as well as some reports of progenitors or stem cells in the pancreas and liver.

Adult Stem Cell Plasticity

It was not until recently that anyone seriously considered the possibility that stem cells in adult tissues could generate the specialized cell types of another type of tissue from which they normally reside—either a tissue derived from the same embryonic germ layer or from a different germ layer. For example, studies have shown that blood stem cells (derived from mesoderm) may be able to generate both skeletal muscle (also derived from mesoderm) and neurons (derived from ectoderm). That realization has been triggered by a flurry of papers reporting that stem cells derived from one adult tissue can change their appearance and assume characteristics that resemble those of differentiated cells from other tissues.

The term plasticity, as used in this report, means that a stem cell from one adult tissue can generate the differentiated cell types of another tissue. At this time, there is no formally accepted name for this phenomenon in the scientific literature. It is variously referred to as "plasticity" [15, 52], "unorthodox differentiation" [10] or "transdifferentiation" [7, 54].

Approaches for Demonstrating Adult Stem Cell Plasticity

To be able to claim that adult stem cells demonstrate plasticity, it is first important to show that a cell population exists in the starting tissue that has the identifying features of stem cells. Then, it is necessary to show that the adult stem cells give rise to cell types that normally occur in a different tissue. Neither of these criteria is easily met. Simply proving the existence of an adult stem cell population in a differentiated tissue is a laborious process. It requires that the candidate stem cells are shown to be self-renewing, and that they can give rise to the differentiated cell types that are characteristic of that tissue.

To show that the adult stem cells can generate other cell types requires them to be tracked in their new environment, whether it is in vitro or in vivo. In general, this has been accomplished by obtaining the stem cells from a mouse that has been genetically engineered to express a molecular tag in all its cells. It is then necessary to show that the labeled adult stem cells have adopted key structural and biochemical characteristics of the new tissue they are claimed to have generated. Ultimately—and most importantly—it is necessary to demonstrate that the cells can integrate into their new tissue environment, survive in the tissue, and function like the mature cells of the tissue.

In the experiments reported to date, adult stem cells may assume the characteristics of cells that have developed from the same primary germ layer or a different germ layer (see Figure 4.2. Preliminary Evidence of Plasticity Among Nonhuman Adult Stem Cells). For example, many plasticity experiments involve stem cells derived from bone marrow, which is a mesodermal derivative. The bone marrow stem cells may then differentiate into another mesodermally derived tissue such as skeletal muscle [28, 43], cardiac muscle [51, 71] or liver [4, 54, 97].

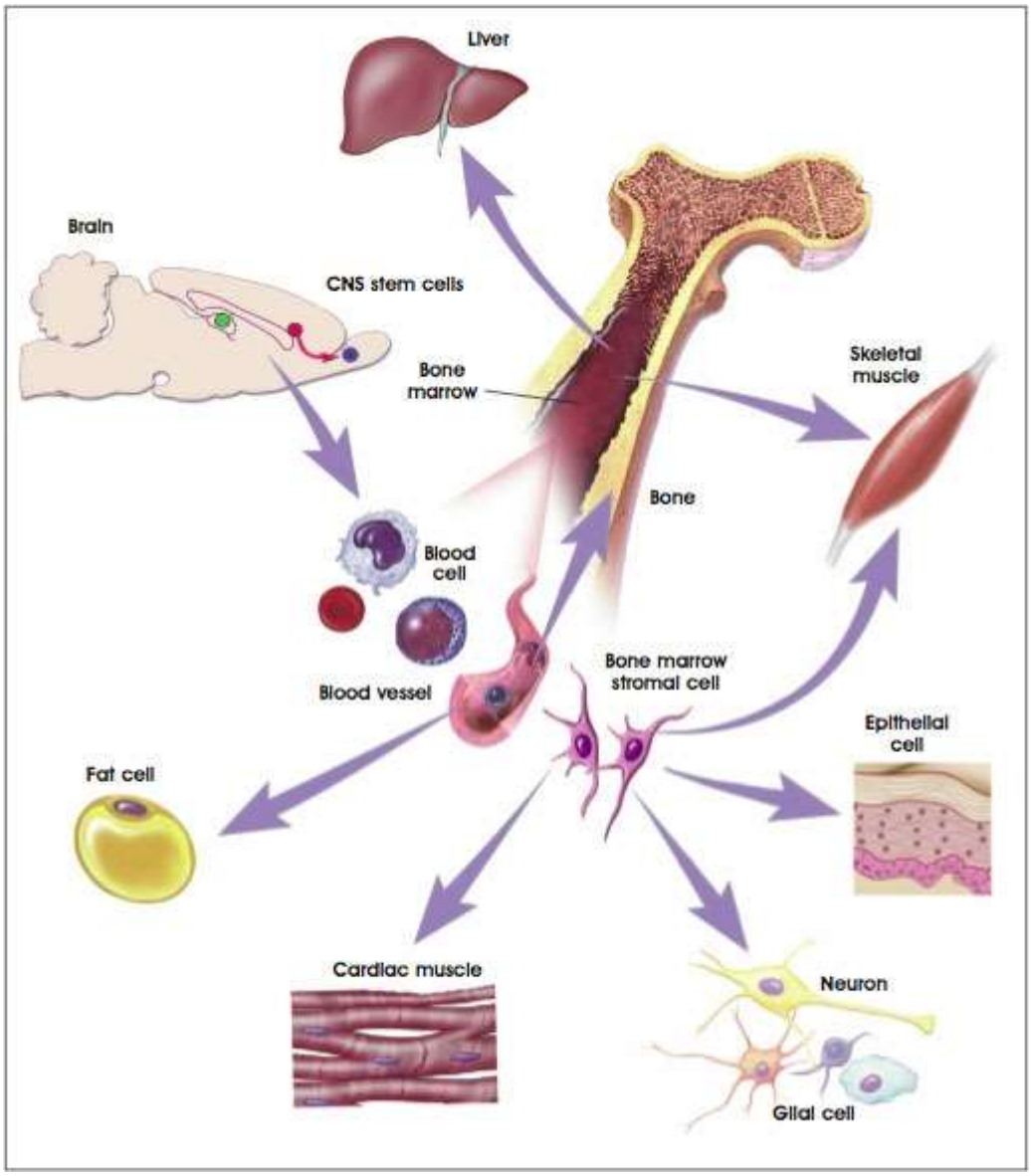

Figure 4.2. Preliminary Evidence of Plasticity Among Nonhuman Adult Stem Cells

Alternatively, adult stem cells may differentiate into a tissue that—during normal embryonic development—would arise from a different germ layer. For example, bone marrow-derived cells may differentiate into neural tissue, which is derived from embryonic ectoderm [15, 65]. And—reciprocally—neural stem cell lines cultured from adult brain tissue may differentiate to form hematopoietic cells [13], or even give rise to many different cell types in a chimeric embryo [17]. In both cases cited above, the cells would be deemed to show plasticity, but in the case of bone marrow stem cells generating brain cells, the finding is less predictable.

In order to study plasticity within and across germ layer lines, the researcher must be sure that he/she is using only one kind of adult stem cell. The vast majority of experiments on plasticity have been conducted with adult stem cells derived either from the bone marrow or the brain. The bone marrow-derived cells are sometimes sorted—using a panel of surface markers—into populations of hematopoietic stem cells or bone marrow stromal cells [46, 54, 71]. The HSCs may be highly purified or partially purified, depending on the conditions used. Another way to separate population of bone marrow cells is by fractionation to yield cells that adhere to a growth substrate (stromal cells) or do not adhere (hematopoietic cells) [28].

To study plasticity of stem cells derived from the brain, the researcher must overcome several problems. Stem cells from the central nervous system (CNS), unlike bone marrow cells, do not occur in a single, accessible location. Instead, they are scattered in three places, at least in rodent brain—the tissue around the lateral ventricles in the forebrain, a migratory pathway for the cells that leads from the ventricles to the olfactory bulbs, and the hippocampus. Many of the experiments with CNS stem cells involve the formation of neurospheres, round aggregates of cells that are sometimes clonally derived. But it is not possible to observe cells in the center of a neurosphere, so to study plasticity in vitro, the cells are usually dissociated and plated in monolayers. To study plasticity in vivo, the cells may be dissociated before injection into the circulatory system of the recipient animal [13], or injected as neurospheres [17].

What is the Evidence for Plasticity?

The differentiated cell types that result from plasticity are usually reported to have the morphological characteristics of the differentiated cells and to display their characteristic surface markers. In reports that transplanted adult stem cells show plasticity in vivo, the stem cells typically are shown to have integrated into a mature host tissue and assumed at least some of its characteristics [15, 28, 51, 65, 71]. Many plasticity experiments involve injury to a particular tissue, which is intended to model a particular human disease or injury [13, 54, 71]. However, there is limited evidence to date that such adult stem cells can generate mature, fully functional cells or that the cells have restored lost function in vivo [54]. Most of the studies that show the plasticity of adult stem cells involve cells that are derived from the bone marrow [15, 28, 54, 65, 77] or brain [13, 17]. To date, adult stem cells are best characterized in these two tissues, which may account for the greater number of plasticity studies based on bone marrow and brain. Collectively, studies on plasticity suggest that stem cell populations in adult mammals are not fixed entities, and that after exposure to

a new environment, they may be able to populate other tissues and possibly differentiate into other cell types.

It is not yet possible to say whether plasticity occurs normally in vivo. Some scientists think it may [14, 64], but as yet there is no evidence to prove it. Also, it is not yet clear to what extent plasticity can occur in experimental settings, and how—or whether—the phenomenon can be harnessed to generate tissues that may be useful for therapeutic transplantation. If the phenomenon of plasticity is to be used as a basis for generating tissue for transplantation, the techniques for doing it will need to be reproducible and reliable. In some cases, debate continues about observations that adult stem cells yield cells of tissue types different than those from which they were obtained [7, 68].

Experimental Evidence of Adult Stem Cells and Plasticity

Adult Stem Cells of the Nervous System

More than 30 years ago, Altman and Das showed that two regions of the postnatal rat brain, the hippocampus and the olfactory bulb, contain dividing cells that become neurons [5, 6]. Despite these reports, the prevailing view at the time was that nerve cells in the adult brain do not divide. In fact, the notion that stem cells in the adult brain can generate its three major cell types—astrocytes and oligodendrocytes, as well as neurons—was not accepted until far more recently. Within the past five years, a series of studies has shown that stem cells occur in the adult mammalian brain and that these cells can generate its three major cell lineages [35, 48, 63, 66, 90, 96, 104] (see Chapter 8. Rebuilding the Nervous System with Stem Cells).

Today, scientists believe that stem cells in the fetal and adult brain divide and give rise to more stem cells or to several types of precursor cells. Neuronal precursors (also called neuroblasts) divide and give rise to nerve cells (neurons), of which there are many types. Glial precursors give rise to astrocytes or oligodendrocytes. Astrocytes are a kind of glial cell, which lend both mechanical and metabolic support for neurons; they make up 70 to 80 percent of the cells of the adult brain. Oligodendrocytes make myelin, the fatty material that ensheathes nerve cell axons and speeds nerve transmission. Under normal, in vivo conditions, neuronal precursors do not give rise to glial cells, and glial precursors do not give rise to neurons. In contrast, a fetal or adult CNS (central nervous system—the brain and spinal cord) stem cell may give rise to neurons, astrocytes, or oligodendrocytes, depending on the signals it receives and its three-dimensional environment within

the brain tissue. There is now widespread consensus that the adult mammalian brain does contain stem cells. However, there is no consensus about how many populations of CNS stem cells exist, how they may be related, and how they function in vivo. Because there are no markers currently available to identify the cells in vivo, the only method for testing whether a given population of CNS cells contains stem cells is to isolate the cells and manipulate them in vitro, a process that may change their intrinsic properties [67].

Despite these barriers, three groups of CNS stem cells have been reported to date. All occur in the adult rodent brain and preliminary evidence indicates they also occur in the adult human brain. One group occupies the brain tissue next to the ventricles, regions known as the ventricular zone and the sub-ventricular zone (see discussion below). The ventricles are spaces in the brain filled with cerebrospinal fluid. During fetal development, the tissue adjacent to the ventricles is a prominent region of actively dividing cells. By adulthood, however, this tissue is much smaller, although it still appears to contain stem cells [70].

A second group of adult CNS stem cells, described in mice but not in humans, occurs in a streak of tissue that connects the lateral ventricle and the olfactory bulb, which receives odor signals from the nose. In rodents, olfactory bulb neurons are constantly being replenished via this pathway [59, 61]. A third possible location for stem cells in adult mouse and human brain occurs in the hippocampus, a part of the brain thought to play a role in the formation of certain kinds of memory [27, 34].

Central Nervous System Stem Cells in the Subventricular Zone. CNS stem cells found in the forebrain that surrounds the lateral ventricles are heterogeneous and can be distinguished morphologically. Ependymal cells, which are ciliated, line the ventricles. Adjacent to the ependymal cell layer, in a region sometimes designated as the subependymal or subventricular zone, is a mixed cell population that consists of neuroblasts (immature neurons) that migrate to the olfactory bulb, precursor cells, and astrocytes. Some of the cells divide rapidly, while others divide slowly. The astrocyte-like cells can be identified because they contain glial fibrillary acidic protein (GFAP), whereas the ependymal cells stain positive for nestin, which is regarded as a marker of neural stem cells. Which of these cells best qualifies as a CNS stem cell is a matter of debate [76].

A recent report indicates that the astrocytes that occur in the subventricular zone of the rodent brain act as neural stem cells. The cells with astrocyte markers appear to generate neurons in vivo, as identified by their expression of specific neuronal markers. The in vitro assay to demonstrate that these astrocytes are, in fact, stem cells involves their ability to form neurospheres—groupings of undifferentiated cells that can be dissociated and coaxed to differentiate into neurons or glial cells [25]. Traditionally, these astrocytes have been regarded as differentiated cells, not as stem cells and so their designation as stem cells is not universally accepted.

A series of similar in vitro studies based on the formation of neurospheres was used to identify the subependymal zone as a source of adult rodent CNS stem cells. In these experiments, single, candidate stem cells derived from the subependymal zone are induced to give rise to neurospheres in the presence of mitogens—either epidermal growth factor (EGF) or fibroblast growth factor-2 (FGF-2). The neurospheres are dissociated and passaged. As long as a mitogen is present in the culture medium, the cells continue forming neurospheres without differentiating. Some populations of CNS cells are more responsive to EGF, others to FGF [100]. To induce differentiation into neurons or glia, cells are dissociated from the neurospheres and grown on an adherent surface in serum-free medium that contains specific growth factors. Collectively, the studies demonstrate that a population of cells derived from the adult rodent brain can self-renew and differentiate to yield the three major cell types of the CNS cells [41, 69, 74, 102].

Central Nervous System Stem Cells in the Ventricular Zone. Another group of potential CNS stem cells in the adult rodent brain may consist of the ependymal cells themselves [47]. Ependymal cells, which are ciliated, line the lateral ventricles. They have been described as non-dividing cells [24] that function as part of the blood-brain barrier [22]. The suggestion that ependymal cells from the ventricular zone of the adult rodent CNS may be stem cells is therefore unexpected. However, in a recent study, in which two molecular tags—the fluorescent marker Dil, and an adenovirus vector carrying lacZ tags—were used to label the ependymal cells that line the entire CNS ventricular system of adult rats, it was shown that these cells could, indeed, act as stem cells. A few days after labeling, fluorescent or lacZ+ cells were observed in the rostral migratory stream (which leads from the lateral ventricle to the olfactory bulb), and then in the olfactory bulb itself. The labeled cells in the olfactory bulb also stained for the neuronal markers βIII tubulin and Map2, which indicated that ependymal

cells from the ventricular zone of the adult rat brain had migrated along the rostral migratory stream to generate olfactory bulb neurons in vivo [47].

To show that Dil+ cells were neural stem cells and could generate astrocytes and oligodendrocytes as well as neurons, a neurosphere assay was performed in vitro. Dil-labeled cells were dissociated from the ventricular system and cultured in the presence of mitogen to generate neurospheres. Most of the neurospheres were Dil+; they could self-renew and generate neurons, astrocytes, and oligodendrocytes when induced to differentiate. Single, Dil+ ependymal cells isolated from the ventricular zone could also generate self-renewing neurospheres and differentiate into neurons and glia.

To show that ependymal cells can also divide in vivo, bromodeoxyuridine (BrdU) was administered in the drinking water to rats for a 2- to 6-week period. Bromodeoxyuridine (BrdU) is a DNA precursor that is only incorporated into dividing cells. Through a series of experiments, it was shown that ependymal cells divide slowly in vivo and give rise to a population of progenitor cells in the subventricular zone [47]. A different pattern of scattered BrdU-labeled cells was observed in the spinal cord, which suggested that ependymal cells along the central canal of the cord occasionally divide and give rise to nearby ependymal cells, but do not migrate away from the canal.

Collectively, the data suggest that CNS ependymal cells in adult rodents can function as stem cells. The cells can self-renew, and most proliferate via asymmetrical division. Many of the CNS ependymal cells are not actively dividing (quiescent), but they can be stimulated to do so in vitro (with mitogens) or in vivo (in response to injury). After injury, the ependymal cells in the spinal cord only give rise to astrocytes, not to neurons. How and whether ependymal cells from the ventricular zone are related to other candidate populations of CNS stem cells, such as those identified in the hippocampus [34], is not known.

Are ventricular and subventricular zone CNS stem cells the same population? These studies and other leave open the question of whether cells that directly line the ventricles—those in the ventricular zone—or cells that are at least a layer removed from this zone—in the subventricular zone

are the same population of CNS stem cells. A new study, based on the finding that they express different genes, confirms earlier reports that the ventricular and subventricular zone cell populations are distinct. The new research utilizes a technique called representational difference analysis, together with cDNA microarray analysis, to monitor the patterns of gene expression in the complex tissue of the developing and postnatal mouse brain. The study revealed the expression of a panel of genes known to be important in CNS development, such as L3-PSP (which encodes a phosphoserine phosphatase important in cell signaling), cyclin D2 (a cell cycle gene), and ERCC-1 (which is important in DNA excision repair). All of these genes in the recent study were expressed in cultured neurospheres, as well as the ventricular zone, the subventricular zone, and a brain area outside those germinal zones. This analysis also revealed the expression of novel genes such as A16F10, which is similar to a gene in an embryonic cancer cell line. A16F10 was expressed in neurospheres and at high levels in the subventricular zone, but not significantly in the ventricular zone. Interestingly, several of the genes identified in cultured neurospheres were also expressed in hematopoietic cells, suggesting that neural stem cells and blood-forming cells may share aspects of their genetic programs or signaling systems [38]. This finding may help explain recent reports that CNS stem cells derived from mouse brain can give rise to hematopoietic cells after injection into irradiated mice [13].

Central Nervous System Stem Cells in the Hippocampus. The hippocampus is one of the oldest parts of the cerebral cortex, in evolutionary terms, and is thought to play an important role in certain forms of memory. The region of the hippocampus in which stem cells apparently exist in mouse and human brains is the subgranular zone of the dentate gyrus. In mice, when BrdU is used to label dividing cells in this region, about 50% of the labeled cells differentiate into cells that appear to be dentate gyrus granule neurons, and 15% become glial cells. The rest of the BrdU-labeled cells do not have a recognizable phenotype [90]. Interestingly, many, if not all the BrdU-labeled cells in the adult rodent hippocampus occur next to blood vessels [33].

In the human dentate gyrus, some BrdU-labeled cells express NeuN, neuron-specific enolase, or calbindin, all of which are neuronal markers. The labeled neuron-like cells resemble dentate gyrus granule cells, in terms of their morphology (as they did in mice). Other BrdU-labeled cells express glial fibrillary acidic protein (GFAP) an astrocyte marker. The study involved autopsy material, obtained with family consent, from five cancer patients who had been injected with BrdU dissolved in saline prior to their death for diagnostic purposes. The patients ranged in age from 57 to 72 years.

The greatest number of BrdU-labeled cells were identified in the oldest patient, suggesting that new neuron formation in the hippocampus can continue late in life [27].

Fetal Central Nervous System Stem Cells. Not surprisingly, fetal stem cells are numerous in fetal tissues, where they are assumed to play an important role in the expansion and differentiation of all tissues of the developing organism. Depending on the developmental stage of an animal, fetal stem cells and precursor cells—which arise from stem cells—may make up the bulk of a tissue. This is certainly true in the brain [48], although it has not been demonstrated experimentally in many tissues.

It may seem obvious that the fetal brain contains stem cells that can generate all the types of neurons in the brain as well as astrocytes and oligodendrocytes, but it was not until fairly recently that the concept was proven experimentally. There has been a long-standing question as to whether or not the same cell type gives rise to both neurons and glia. In studies of the developing rodent brain, it has now been shown that all the major cell types in the fetal brain arise from a common population of progenitor cells [20, 34, 48, 80, 108].

Neural stem cells in the mammalian fetal brain are concentrated in seven major areas: olfactory bulb, ependymal (ventricular) zone of the lateral ventricles (which lie in the forebrain), subventricular zone (next to the ependymal zone), hippocampus, spinal cord, cerebellum (part of the hindbrain), and the cerebral cortex. Their number and pattern of development vary in different species. These cells appear to represent different stem cell populations, rather than a single population of stem cells that is dispersed in multiple sites. The normal development of the brain depends not only on the proliferation and differentiation of these fetal stem cells, but also on a genetically programmed process of selective cell death called apoptosis [76].

Little is known about stem cells in the human fetal brain. In one study, however, investigators derived clonal cell lines from CNS stem cells isolated from the diencephalon and cortex of human fetuses, 10.5 weeks post-conception [103]. The study is unusual, not only because it involves human CNS stem cells obtained from fetal tissue, but also because the cells were used to generate clonal cell lines of CNS stem cells that generated neurons, astrocytes, and oligodendrocytes, as determined on the basis of expressed markers. In a few experiments described as "preliminary," the human CNS stem cells were injected into the brains of immunosuppressed rats where they apparently differentiated into neuron-like cells or glial cells.

In a 1999 study, a serum-free growth medium that included EGF and FGF2 was devised to grow the human fetal CNS stem cells. Although most of the cells died, occasionally, single CNS stem cells survived, divided, and ultimately formed neurospheres after one to two weeks in culture. The neurospheres could be dissociated and individual cells replated. The cells resumed proliferation and formed new neurospheres, thus establishing an in vitro system that (like the system established for mouse CNS neurospheres) could be maintained up to 2 years. Depending on the culture conditions, the cells in the neurospheres could be maintained in an undifferentiated dividing state (in the presence of mitogen), or dissociated and induced to differentiate (after the removal of mitogen and the addition of specific growth factors to the culture medium). The differentiated cells consisted mostly of astrocytes (75%), some neurons (13%) and rare oligodendrocytes (1.2%). The neurons generated under these conditions expressed markers indicating they were GABAergic, [the major type of inhibitory neuron in the mammalian CNS responsive to the amino acid neurotransmitter, gammaaminobutyric acid (GABA)]. However, catecholamine-like cells that express tyrosine hydroxylase (TH, a critical enzyme in the dopamine-synthesis pathway) could be generated, if the culture conditions were altered to include different medium conditioned by a rat glioma line (BB49). Thus, the report indicates that human CNS stem cells obtained from early fetuses can be maintained in vitro for a long time without differentiating, induced to differentiate into the three major lineages of the CNS (and possibly two kinds of neurons, GABAergic and TH-positive), and engraft (in rats) in vivo [103].

Central Nervous System Neural Crest Stem Cells. Neural crest cells differ markedly from fetal or adult neural stem cells. During fetal development, neural crest cells migrate from the sides of the neural tube as it closes. The cells differentiate into a range of tissues, not all of which are part of the nervous system [56, 57, 91]. Neural crest cells form the sympathetic and parasympathetic components of the peripheral nervous system (PNS), including the network of nerves that innervate the heart and the gut, all the sensory ganglia (groups of neurons that occur in pairs along the dorsal surface of the spinal cord), and Schwann cells, which (like oligodendrocytes in the CNS) make myelin in the PNS. The non-neural tissues that arise from the neural crest are diverse. They populate certain hormone-secreting glands—including the adrenal medulla and Type I cells in the carotid body—pigment cells of the skin (melanocytes), cartilage and bone in the face and skull, and connective tissue in many parts of the body [76].

Thus, neural crest cells migrate far more extensively than other fetal neural stem cells during development, form mesenchymal tissues, most of which develop from embryonic mesoderm as well as the components of the CNS and PNS which arises from embryonic ectoderm. This close link, in

neural crest development, between ectodermally derived tissues and mesodermally derived tissues accounts in part for the interest in neural crest cells as a kind of stem cell. In fact, neural crest cells meet several criteria of stem cells. They can self-renew (at least in the fetus) and can differentiate into multiple cells types, which include cells derived from two of the three embryonic germ layers [76].

Recent studies indicate that neural crest cells persist late into gestation and can be isolated from E14.5 rat sciatic nerve, a peripheral nerve in the hindlimb. The cells incorporate BrdU, indicating that they are dividing in vivo. When transplanted into chick embryos, the rat neural crest cells develop into neurons and glia, an indication of their stem cell-like properties [67]. However, the ability of rat E14.5 neural crest cells taken from sciatic nerve to generate nerve and glial cells in chick is more limited than neural crest cells derived from younger, E10.5 rat embryos. At the earlier stage of development, the neural tube has formed, but neural crest cells have not yet migrated to their final destinations. Neural crest cells from early developmental stages are more sensitive to bone morphogenetic protein 2 (BMP2) signaling, which may help explain their greater differentiation potential [106].

Stem Cells in the Bone Marrow and Blood

The notion that the bone marrow contains stem cells is not new. One population of bone marrow cells, the hematopoietic stem cells (HSCs), is responsible for forming all of the types of blood cells in the body. HSCs were recognized as a stem cells more than 40 years ago [9, 99]. Bone marrow stromal cells—a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, and the reticular network that supports blood cell formation—were described shortly after the discovery of HSCs [30, 32, 73]. The mesenchymal stem cells of the bone marrow also give rise to these tissues, and may constitute the same population of cells as the bone marrow stromal cells [78]. Recently, a population of progenitor cells that differentiates into endothelial cells, a type of cell that lines the blood vessels, was isolated from circulating blood [8] and identified as originating in bone marrow [89]. Whether these endothelial progenitor cells, which resemble the angioblasts that give rise to blood vessels during embryonic development, represent a bona fide population of adult bone marrow stem cells remains uncertain. Thus, the bone marrow appears to contain three stem cell populations—hematopoietic stem cells, stromal cells, and (possibly) endothelial progenitor cells (see Figure 4.3. Hematopoietic and Stromal Stem Cell Differentiation).

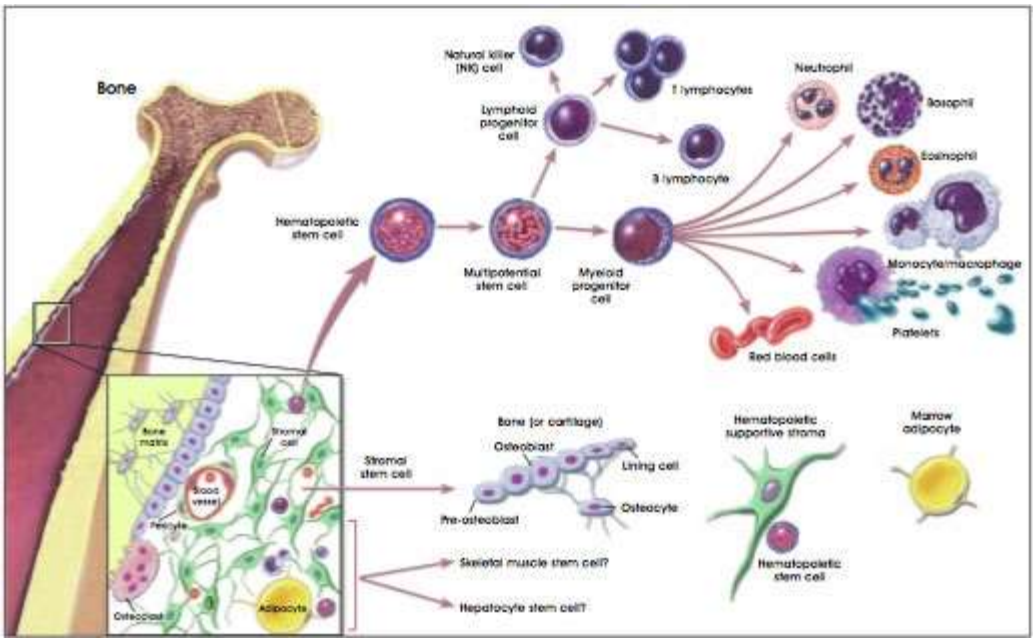

Figure 4.3. Hematopoietic and Stromal Stem Cell Differentiation

Two more apparent stem cell types have been reported in circulating blood, but have not been shown to originate from the bone marrow. One population, called pericytes, may be closely related to bone marrow stromal cells, although their origin remains elusive [12]. The second population of blood-born stem cells, which occur in four species of animals tested—guinea pigs, mice, rabbits, and humans—resemble stromal cells in that they can generate bone and fat [53].

Hematopoietic Stem Cells. Of all the cell types in the body, those that survive for the shortest period of time are blood cells and certain kinds of epithelial cells. For example, red blood cells (erythrocytes), which lack a nucleus, live for approximately 120 days in the bloodstream. The life of an animal literally depends on the ability of these and other blood cells to be replenished continuously. This replenishment process occurs largely in the bone marrow, where HSCs reside, divide, and differentiate into all the blood cell types. Both HSCs and differentiated blood cells cycle from the bone marrow to the blood and back again, under the influence of a barrage of secreted factors that regulate cell proliferation, differentiation, and migration (see Chapter 5. Hematopoietic Stem Cells).

HSCs can reconstitute the hematopoietic system of mice that have been subjected to lethal doses of radiation to destroy their own hematopoietic systems. This test, the rescue of lethally irradiated mice, has become a standard by which other candidate stem cells are measured because it shows, without question, that HSCs can regenerate an entire tissue system—in this case, the blood

[9, 99]. HSCs were first proven to be blood-forming stem cells in a series of experiments in mice; similar blood-forming stem cells occur in humans. HSCs are defined by their ability to self-renew and to give rise to all the kinds of blood cells in the body. This means that a single HSC is capable of regenerating the entire hematopoietic system, although this has been demonstrated only a few times in mice [72].

Over the years, many combinations of surface markers have been used to identify, isolate, and purify HSCs derived from bone marrow and blood. Undifferentiated HSCs and hematopoietic progenitor cells express c-kit, CD34, and H-2K. These cells usually lack the lineage marker Lin, or express it at very low levels (Lin-/low). And for transplant purposes, cells that are CD34+ Thy1+ Lin- are most likely to contain stem cells and result in engraftment.

Two kinds of HSCs have been defined. Long-term HSCs proliferate for the lifetime of an animal. In young adult mice, an estimated 8 to 10 % of long-term HSCs enter the cell cycle and divide each day. Short-term HSCs proliferate for a limited time, possibly a few months. Long-term HSCs have high levels of telomerase activity. Telomerase is an enzyme that helps maintain the length of the ends of chromosomes, called telomeres, by adding on nucleotides. Active telomerase is a characteristic of undifferentiated, dividing cells and cancer cells. Differentiated, human somatic cells do not show telomerase activity. In adult humans, HSCs occur in the bone marrow, blood, liver, and spleen, but are extremely rare in any of these tissues. In mice, only 1 in 10,000 to 15,000 bone marrow cells is a long-term HSC [105].

Short-term HSCs differentiate into lymphoid and myeloid precursors, the two classes of precursors for the two major lineages of blood cells. Lymphoid precursors differentiate into T cells, B cells, and natural killer cells. The mechanisms and pathways that lead to their differentiation are still being investigated [1, 2]. Myeloid precursors differentiate into monocytes and macrophages, neutrophils, eosinophils, basophils, megakaryocytes, and erythrocytes [3]. In vivo, bone marrow HSCs differentiate into mature, specialized blood cells that cycle constantly from the bone marrow to the blood, and back to the bone marrow [26]. A recent study showed that short-term HSCs are a heterogeneous population that differ significantly in terms of their ability to self-renew and repopulate the hematopoietic system [42].

Attempts to induce HSC to proliferate in vitro—on many substrates, including those intended to mimic conditions in the stroma—have frustrated scientists for many years. Although HSCs proliferate readily in vivo, they usually differentiate or die in vitro [26]. Thus, much of the research on HSCs has been focused on understanding the factors, cell-cell interactions, and cell-matrix

interactions that control their proliferation and differentiation in vivo, with the hope that similar conditions could be replicated in vitro. Many of the soluble factors that regulate HSC differentiation in vivo are cytokines, which are made by different cell types and are then concentrated in the bone marrow by the extracellular matrix of stromal cells—the sites of blood formation [45, 107]. Two of the most-studied cytokines are granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) [40, 81].

Also important to HSC proliferation and differentiation are interactions of the cells with adhesion molecules in the extracellular matrix of the bone marrow stroma [83, 101, 110].

Bone Marrow Stromal Cells. Bone marrow (BM) stromal cells have long been recognized for playing an important role in the differentiation of mature blood cells from HSCs (see Figure 4.3. Hematopoietic and Stromal Stem Cell Differentiation). But stromal cells also have other important functions [30, 31]. In addition to providing the physical environment in which HSCs differentiate, BM stromal cells generate cartilage, bone, and fat. Whether stromal cells are best classified as stem cells or progenitor cells for these tissues is still in question. There is also a question as to whether BM stromal cells and so-called mesenchymal stem cells are the same population [78].

BM stromal cells have many features that distinguish them from HSCs. The two cell types are easy to separate in vitro. When bone marrow is dissociated, and the mixture of cells it contains is plated at low density, the stromal cells adhere to the surface of the culture dish, and the HSCs do not. Given specific in vitro conditions, BM stromal cells form colonies from a single cell called the colony forming unit-F (CFU-F). These colonies may then differentiate as adipocytes or myelosupportive stroma, a clonal assay that indicates the stem cell-like nature of stromal cells. Unlike HSCs, which do not divide in vitro (or proliferate only to a limited extent), BM stromal cells can proliferate for up to 35 population doublings in vitro [16]. They grow rapidly under the influence of such mitogens as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) [12].

To date, it has not been possible to isolate a population of pure stromal cells from bone marrow. Panels of markers used to identify the cells include receptors for certain cytokines (interleukin-1, 3, 4, 6, and 7) receptors for proteins in the extracellular matrix, (ICAM-1 and 2, VCAM-1, the alpha-1, 2, and 3 integrins, and the beta-1, 2, 3 and 4 integrins), etc. [64]. Despite the use of these markers and another stromal cell marker called Stro-1, the origin and specific identity of stromal cells have remained elusive. Like HSCs, BM stromal cells arise from embryonic mesoderm during development, although no specific precursor or stem cell for stromal cells has been isolated

and identified. One theory about their origin is that a common kind of progenitor cell—perhaps a primordial endothelial cell that lines embryonic blood vessels—gives rise to both HSCs and to mesodermal precursors. The latter may then differentiate into myogenic precursors (the satellite cells that are thought to function as stem cells in skeletal muscle), and the BM stromal cells [10].

In vivo, the differentiation of stromal cells into fat and bone is not straightforward. Bone marrow adipocytes and myelosupportive stromal cells—both of which are derived from BM stromal cells—may be regarded as interchangeable phenotypes [10, 11]. Adipocytes do not develop until postnatal life, as the bones enlarge and the marrow space increases to accommodate enhanced hematopoiesis. When the skeleton stops growing, and the mass of HSCs decreases in a normal, age-dependent fashion, BM stromal cells differentiate into adipocytes, which fill the extra space. New bone formation is obviously greater during skeletal growth, although bone "turns over" throughout life. Bone forming cells are osteoblasts, but their relationship to BM stromal cells is not clear. New trabecular bone, which is the inner region of bone next to the marrow, could logically develop from the action of BM stromal cells. But the outside surface of bone also turns over, as does bone next to the Haversian system (small canals that form concentric rings within bone). And neither of these surfaces is in contact with BM stromal cells [10, 11].

Adult Stem Cells in Other Tissues

It is often difficult—if not impossible—to distinguish adult, tissue-specific stem cells from progenitor cells. With that caveat in mind, the following summary identifies reports of stem cells in various adult tissues.

Endothelial Progenitor Cells. Endothelial cells line the inner surfaces of blood vessels throughout the body, and it has been difficult to identify specific endothelial stem cells in either the embryonic or the adult mammal. During embryonic development, just after gastrulation, a kind of cell called the hemangioblast, which is derived from mesoderm, is presumed to be the precursor of both the hematopoietic and endothelial cell lineages. The embryonic vasculature formed at this stage is transient and consists of blood islands in the yolk sac. But hemangioblasts, per se, have not been isolated from the embryo and their existence remains in question. The process of forming new blood vessels in the embryo is called vasculogenesis. In the adult, the process of forming blood vessels from pre-existing blood vessels is called angiogenesis [50].

Evidence that hemangioblasts do exist comes from studies of mouse embryonic stem cells that are directed to differentiate in vitro. These studies have shown that a precursor cell derived from mouse ES cells that express Flk-1 [the receptor for vascular endothelial growth factor (VEGF) in mice] can give rise to both blood cells and blood vessel cells [88, 109]. Both VEGF and fibroblast growth factor-2 (FGF-2) play critical roles in endothelial cell differentiation in vivo [79].

Several recent reports indicate that the bone marrow contains cells that can give rise to new blood vessels in tissues that are ischemic (damaged due to the deprivation of blood and oxygen) [8, 29, 49, 94]. But it is unclear from these studies what cell type(s) in the bone marrow induced angiogenesis. In a study which sought to address that question, researchers found that adult human bone marrow contains cells that resemble embryonic hemangioblasts, and may therefore be called endothelial stem cells.

In more recent experiments, human bone marrow-derived cells were injected into the tail veins of rats with induced cardiac ischemia. The human cells migrated to the rat heart where they generated new blood vessels in the infarcted muscle (a process akin to vasculogenesis), and also induced angiogenesis. The candidate endothelial stem cells are CD34+(a marker for HSCs), and they express the transcription factor GATA-2 [51]. A similar study using transgenic mice that express the gene for enhanced green fluorescent protein (which allows the cells to be tracked), showed that bone-marrow-derived cells could repopulate an area of infarcted heart muscle in mice, and generate not only blood vessels, but also cardiomyocytes that integrated into the host tissue [71].

And, in a series of experiments in adult mammals, progenitor endothelial cells were isolated from peripheral blood (of mice and humans) by using antibodies against CD34 and Flk-1, the receptor for VEGF. The cells were mononuclear blood cells (meaning they have a nucleus) and are referred to as MBCD34+ cells and MBFlk1+ cells. When plated in tissue-culture dishes, the cells attached to the substrate, became spindle-shaped, and formed tube-like structures that resemble blood vessels. When transplanted into mice of the same species (autologous transplants) with induced ischemia in one limb, the MBCD34+ cells promoted the formation of new blood vessels [8]. Although the adult MBCD34+ and MBFlk1+ cells function in some ways like stem cells, they are usually regarded as progenitor cells.

Skeletal Muscle Stem Cells. Skeletal muscle, like the cardiac muscle of the heart and the smooth muscle in the walls of blood vessels, the digestive system, and the respiratory system, is derived from embryonic mesoderm. To date, at least three populations of skeletal muscle stem cells

have been identified: satellite cells, cells in the wall of the dorsal aorta, and so-called "side population" cells.

Satellite cells in skeletal muscle were identified 40 years ago in frogs by electron microscopy [62], and thereafter in mammals [84]. Satellite cells occur on the surface of the basal lamina of a mature muscle cell, or myofiber. In adult mammals, satellite cells mediate muscle growth [85]. Although satellite cells are normally non-dividing, they can be triggered to proliferate as a result of injury, or weight-bearing exercise. Under either of these circumstances, muscle satellite cells give rise to myogenic precursor cells, which then differentiate into the myofibrils that typify skeletal muscle. A group of transcription factors called myogenic regulatory factors (MRFs) play important roles in these differentiation events. The so-called primary MRFs, MyoD and Myf5, help regulate myoblast formation during embryogenesis. The secondary MRFs, myogenin and MRF4, regulate the terminal differentiation of myofibrils [86].

With regard to satellite cells, scientists have been addressing two questions. Are skeletal muscle satellite cells true adult stem cells or are they instead precursor cells? Are satellite cells the only cell type that can regenerate skeletal muscle. For example, a recent report indicates that muscle stem cells may also occur in the dorsal aorta of mouse embryos, and constitute a cell type that gives rise both to muscle satellite cells and endothelial cells. Whether the dorsal aorta cells meet the criteria of a self-renewing muscle stem cell is a matter of debate [21].

Another report indicates that a different kind of stem cell, called an SP cell, can also regenerate skeletal muscle may be present in muscle and bone marrow. SP stands for a side population of cells that can be separated by fluorescence-activated cell sorting analysis. Intravenously injecting these muscle-derived stem cells restored the expression of dystrophin in mdx mice. Dystrophin is the protein that is defective in people with Duchenne's muscular dystrophy; mdx mice provide a model for the human disease. Dystrophin expression in the SP cell-treated mice was lower than would be needed for clinical benefit. Injection of bone marrow- or muscle-derived SP cells into the dystrophic muscle of the mice yielded equivocal results that the transplanted cells had integrated into the host tissue. The authors conclude that a similar population of SP stem cells can be derived from either adult mouse bone marrow or skeletal muscle, and suggest "there may be some direct relationship between bone marrow-derived stem cells and other tissue- or organ-specific cells" [43]. Thus, stem cell or progenitor cell types from various mesodermally-derived tissues may be able to generate skeletal muscle.

Epithelial Cell Precursors in the Skin and Digestive System. Epithelial cells, which constitute 60 percent of the differentiated cells in the body are responsible for covering the internal and external surfaces of the body, including the lining of vessels and other cavities. The epithelial cells in skin and the digestive tract are replaced constantly. Other epithelial cell populations—in the ducts of the liver or pancreas, for example—turn over more slowly. The cell population that renews the epithelium of the small intestine occurs in the intestinal crypts, deep invaginations in the lining of the gut. The crypt cells are often regarded as stem cells; one of them can give rise to an organized cluster of cells called a structural-proliferative unit [93].

The skin of mammals contains at least three populations of epithelial cells: epidermal cells, hair follicle cells, and glandular epithelial cells, such as those that make up the sweat glands. The replacement patterns for epithelial cells in these three compartments differ, and in all the compartments, a stem cell population has been postulated. For example, stem cells in the bulge region of the hair follicle appear to give rise to multiple cell types. Their progeny can migrate down to the base of the follicle where they become matrix cells, which may then give rise to different cell types in the hair follicle, of which there are seven [39]. The bulge stem cells of the follicle may also give rise to the epidermis of the skin [95].

Another population of stem cells in skin occurs in the basal layer of the epidermis. These stem cells proliferate in the basal region, and then differentiate as they move toward the outer surface of the skin. The keratinocytes in the outermost layer lack nuclei and act as a protective barrier. A dividing skin stem cell can divide asymmetrically to produce two kinds of daughter cells. One is another self-renewing stem cell. The second kind of daughter cell is an intermediate precursor cell which is then committed to replicate a few times before differentiating into keratinocytes. Self-renewing stem cells can be distinguished from this intermediate precusor cell by their higher level of β1 integrin expression, which signals keratinocytes to proliferate via a mitogen-activated protein (MAP) kinase [112]. Other signaling pathways include that triggered by -catenin, which helps maintain the stem-cell state [111], and the pathway regulated by the oncoprotein c-Myc, which triggers stem cells to give rise to transit amplifying cells [36].

Stem Cells in the Pancreas and Liver. The status of stem cells in the adult pancreas and liver is unclear. During embryonic development, both tissues arise from endoderm. A recent study indicates that a single precursor cell derived from embryonic endoderm may generate both the ventral pancreas and the liver [23]. In adult mammals, however, both the pancreas and the liver contain multiple kinds of differentiated cells that may be repopulated or regenerated by multiple types of

stem cells. In the pancreas, endocrine (hormone-producing) cells occur in the islets of Langerhans. They include the beta cells (which produce insulin), the alpha cells (which secrete glucagon), and cells that release the peptide hormones somatostatin and pancreatic polypeptide. Stem cells in the adult pancreas are postulated to occur in the pancreatic ducts or in the islets themselves. Several recent reports indicate that stem cells that express nestin—which is usually regarded as a marker of neural stem cells—can generate all of the cell types in the islets [60, 113].

The identity of stem cells that can repopulate the liver of adult mammals is also in question. Recent studies in rodents indicate that HSCs (derived from mesoderm) may be able to home to liver after it is damaged, and demonstrate plasticity in becoming into hepatocytes (usually derived from endoderm) [54, 77, 97]. But the question remains as to whether cells from the bone marrow normally generate hepatocytes in vivo. It is not known whether this kind of plasticity occurs without severe damage to the liver or whether HSCs from the bone marrow generate oval cells of the liver [18]. Although hepatic oval cells exist in the liver, it is not clear whether they actually generate new hepatocytes [87, 98]. Oval cells may arise from the portal tracts in liver and may give rise to either hepatocytes [19, 55] and to the epithelium of the bile ducts [37, 92]. Indeed, hepatocytes themselves, may be responsible for the well-know regenerative capacity of liver.

II. Haemopoietic Stem Cell

Hematopoietic stem cells (HSCs), also spelled Hæmatopoietic stem cells, are multipotent stem cells that give rise to all the blood cell types from the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells). The definition of haematopoietic stem cells has undergone considerable revision in the last two decades. The hematopoietic tissue contains cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent, and unipotent progenitors. HSCs constitute 1:10.000 of cells in myeloid tissue.HSCs are a heterogeneous population. Three classes of stem cells exist, distinguished by their ratio of lymphoid to myeloid progeny (L/M) in blood. Myeloid-biased (My-bi) HSC have low L/M ratio (>0, <3), whereas lymphoid-biased (Ly-bi) HSC show a large ratio (>10). The third category consists of the balanced (Bala) HSC for which $3 \leq L/M \leq 10$. Much work is currently being undertaken to investigate the properties of these different classes of HSCs, but it appears that only the myeloid-biased and -balanced HSCs have durable self-renewal properties. In addition, serial transplantation experiments have shown that each subtype preferentially re-creates its blood cell type distribution, suggesting an inherited epigenetic program for each subtype.

Source

Sketch of bone marrow and its cells

HSCs are found in the bone marrow of adults, which includes femurs, hip, ribs, sternum, and other bones. Cells can be obtained directly by removal from the hip using a needle and syringe, or from the blood following pre-treatment with cytokines, such as G-CSF (granulocyte colony-stimulating factors), that induce cells to be released from the bone marrow compartment. Other sources for clinical and scientific use include umbilical cord blood, peripheral blood a small number of stem and progenitor cells circulate in the bloodstream, in the past 10 years, researchers have found that they can coax the cells to migrate from marrow to blood in greater numbers by injecting the donor with a cytokine, such as granulocyte-colony stimulating factor (GCSF)and recent study shown that ex-vivo expansion of HSCs is possible in 3D bioreactor. Because HSCs are not generated in the adult but during the embryogenesis, many scientific groups are studying HSCs during the embryonic

development. It is now well described in mammalians that the first definitive HSCs are detected in the AGM (Aorta-gonad-mesonephros), and then massively expanded in the Fetal Liver prior to colonize before birth the bone marrow. Such fundamental research could help to understand the mechanisms that are responsible of HSCs generation and/or amplification, and to the discovery of new molecules that could eventually be used to maintain or expand HSCs in vitro.

Functional characteristics

Multipotency and self-renewal

As stem cells, HSC are defined by their ability to replenish all blood cell types (Multipotency) and their ability to self-renew.

It is known that a small number of HSCs can expand to generate a very large number of daughter HSCs. This phenomenon is used in bone marrow transplantation, when a small number of HSCs reconstitute the hematopoietic system. This indicates that, subsequent to bone marrow transplantation, symmetrical cell divisions into two daughter HSCs must occur.

Stem cell self-renewal is thought to occur in the stem cell niche in the bone marrow, and it is reasonable to assume that key signals present in this niche will be important in self-renewal. There is much interest in the environmental and molecular requirements for HSC self-renewal, as understanding the ability of HSC to replenish themselves will eventually allow the generation of expanded populations of HSC in vitro that can be used therapeutically.

Stem cell heterogeneity

It was originally believed that all HSC were alike in their self-renewal and differentiation abilities. This view was first challenged by the 2002 discovery by the Muller-Sieburg group in San Diego, who illustrated that different stem cells can show distinct repopulation patterns that are epigenetically predetermined intrinsic properties of clonal Thy-1lo SCA-1+ lin- c-kit+ HSC.[1][2][3] The results of these clonal studies led to the notion of lineage bias. Using the ratio $\rho = L / M$ of lymphoid (L) to myeloid (M) cells in blood as a quantitative marker, the stem cell compartment can be split into three categories of HSC. Balanced (Bala) HSC repopulate peripheral white blood cells in the same ratio of myeloid to lymphoid cells as seen in unmanipulated mice (on average about 15% myeloid and 85% lymphoid cells, or $3 \leq \rho \leq 10$). Myeloid-biased (My-bi) HSC give rise to too few

lymphocytes resulting in ratios $0<\rho<3$, while lymphoid-biased (Ly-bi) HSC generate too few myeloid cells, which results in lymphoid-to-myeloid ratios of $10<\rho$Interleukin 7 (IL-7).[2]

Subsequent to this, other groups confirmed and highlighted the original findings (refer to the excellent mini-review by Timm Schroeder[4]). For example, the Eaves group confirmed in 2007 that repopulation kinetics, long-term self-renewal capacity, and My-bi and Ly-bi are stably inherited intrinsic HSC properties.[5] In 2010, the Goodell group provided additional insights about the molecular basis of lineage bias in side population Side population (SP) SCA-1+ lin- c-kit+ HSC.[6] As previously shown for IL-7 signaling, it was found that a member of the transforming growth factor family (TGF-beta) induces and inhibits the proliferation of My-bi and Ly-bi HSC, respectively.

Functional assays

A cobblestone area-forming cell (CAFC) assay is a cell culture-based empirical assay. When plated onto a confluent culture of stromal feeder layer, a fraction of HSCs creep between the gaps (even though the stromal cells are touching each other) and eventually settle between the stromal cells and the substratum (here the dish surface) or trapped in the cellular processes between the stromal cells. Emperipolesis is the in vivo phenomenon in which one cell is completely engulfed into another (e.g., thymocytes into thymic nurse cells); on the other hand, when in vitro, lymphoid lineage cells creep beneath nurse-like cells, the process is called pseudoemperipolesis. This similar phenomenon is more commonly known in HSC field by the cell culture terminology cobble stone area-forming cells (CAFC), which means areas of cluster of cells that look dull cobblestone-like under phase contrast microscopy, compared to the other HSCs, which are refractile. This happens because the cells that are floating loosely on top of the stromal cells are spherical and thus refractile. However, the cells that creep beneath the stromal cells are flattened and, thus, not refractile. The mechanism of pseudoemperipolesis is only recently coming to light. It may be mediated by interaction through CXCR4 (CD184) the receptor for CXC Chemokines (e.g., SDF1) and $\alpha 4\beta 1$ integrins.[7]

Mobility

HSCs have a higher potential than other immature blood cells to pass the bone marrow barrier, and, thus, may travel in the blood from the bone marrow in one bone to another bone. If they settle in the thymus, they will develop into T cells. In the case of fetuses and other extramedullary hematopoiesis, HSCs may also settle in the liver or spleen and develop. This ability is the reason why HSCs may be harvested directly from the blood.

Physical characteristics

With regard to morphology, hematopoietic stem cells resemble lymphocytes. They are non-adherent, and rounded, with a rounded nucleus and low cytoplasm-to-nucleus ratio. Since PHSC cannot be isolated as a pure population, it is not possible to identify them in a microscope. The above description is based on the morphological characteristics of a heterogeneous population, of which PHSC are a component.

Markers

In reference to phenotype, hematopoeitic stem cells are identified by their small size, lack of lineage (lin) markers, low staining (side population) with vital dyes such as rhodamine 123 (rhodamineDULL, also called rholo) or Hoechst 33342, and presence of various antigenic markers on their surface.

Cluster of differentiation and other markers

Many of these markers belong to the cluster of differentiation series, like: CD34, CD38, CD90, CD133, CD105, CD45, and also c-kit, - the receptor for stem cell factor. The haematopoietic stem cells are negative for the markers that are used for detection of lineage commitment, and are, thus, called Lin-; and, during their purification by FACS, a bunch of up to 14 different mature blood-lineage marker, e.g., CD13 & CD33 for myeloid, CD71 for erythroid, CD19 for B cells, CD61 for megakaryocytic, etc. for humans; and, B220 (murine CD45) for B cells, Mac-1 (CD11b/CD18) for monocytes, Gr-1 for Granulocytes, Ter119 for erythroid cells, Il7Ra, CD3, CD4, CD5, CD8 for T cells, etc. (for mice) antibodies are used as a mixture to deplete the lin+ cells or late multipotent progenitors (MPP)s.

There are many differences between the human and mice hematopoietic cell markers for the commonly accepted type of haematopoietic stem cells.[1].

Mouse HSC : CD34lo/-, SCA-1+, Thy1.1+/lo, CD38+, C-kit+, lin-

Human HSC : CD34+, CD59+, Thy1/CD90+, CD38lo/-, C-kit/CD117+, lin-

However, not all stem cells are covered by these combinations that, nonetheless, have become popular. In fact, even in humans, there are hematopoietic stem cells that are CD34-/CD38-.[8][9] Also some later studies suggested that earliest stem cells may lack c-kit on the cell surface.[10] For human HSCs use of CD133 was one step ahead as both CD34+ and CD34- HSCs were CD133+.

Traditional purification method used to yield a reasonable purity level of mouse haematopoietic stem cells, in general, requires a large(~10-12) battery of markers, most of which were surrogate markers with little functional significance, and thus partial overlap with the stem cell populations and sometimes other closely related cells that are not stem cells. Also, some of these markers (e.g., Thy1) are not conserved across mouse species, and use of markers like CD34- for HSC purification requires mice to be at least 8 weeks old.

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UNIT – IV – Concepts in Stem Cell Research – SBT1403

1. Tissue engineering triad

With the increasing number of patients suffering from damaged or diseased organs and the shortage of organ donors, the need for methods to construct human tissues outside the body has arisen. Tissue engineering is a newly emerging biomedical technology and methodology which combines the disciplines of both the materials and life sciences to replace a diseased or damaged tissue or organ with a living, functional engineered substitute [1, 2]. The so-called triad in tissue engineering encompasses three basic components called scaffold, cell and signaling biomolecule.

Whatever the approach being used in tissue engineering, the critical issues to optimize any tissue engineering strategy toward producing a functional equivalent tissue are the source of the cells and substrate biomaterial to deliver the cells in particular anatomical sites where a regenerative process is required. Due to their unique properties, stem cells and polymeric biomaterials are key design options. Briefly, stem cells have the ability to self-renew and commit to specific cell lineages in response to appropriate stimuli, providing excellent regenerative potential that will most likely lead to functionality of the engineered tissue. Polymeric materials are biocompatible, degradable, and flexible in processing and property design. A major focus of tissue engineering, therefore, is to utilize functional polymers with appropriate characteristics, as a means of controlling stem cell function. Based on their differentiation potential, stem cells used for tissue engineering can be divided into two categories: pluripotent stem cells and multipotent stem cells. Pluripotent stem cells include embryonic stem cells (ESCs) as well as induced pluripotent stem cells (iPSCs). Because ESCs are isolated from the inner cell mass of the blastocyst during embryological development, their use in tissue engineering is controversial and more limited while more attention has been paid to adult stem cells, which are multipotent and have a larger capacity to differentiate into a limited number of cell types [3]. Adult stem cells can be found in many adult tissue types including bone marrow, peripheral blood, adipose tissues, nervous tissues, muscles, dermis, etc. For instance, mesenchymal stem cells (MSCs) which reside in the bone marrow can differentiate into bone (osteoblasts) [4], muscle (myoblasts) [5], fat (adipocytes) [6] and cartilage (chrondocytes) [3] cells, while neural stem cells (NSCs) either give rise to support cells in the nervous system of vertebrates (astrocytes and oligodendrocytes) or neurons [7]. In vivo, differentiation and self-renewal of stem cells are dominated by signals from their surrounding microenvironment [8]. This microenvironment or “niche” is composed of other cell types as well as numerous chemical, mechanical and topographical cues at micro- and nano-scales, which are believed to serve as signaling mechanisms to determine cell-

specific recruitment, migration, proliferation, differentiation as well as the production of numerous proteins required for hierarchical tissue organization [9].

In vivo, the cells are surrounded by a biological matrix comprising of tissue-specific combinations of insoluble proteins (e.g. collagens, laminins, and fibronectins), glycosaminoglycans (e.g. hyaluronan) and inorganic hydroxyapatite crystals (in bone) that are collectively referred to as the extracellular matrix (ECM). The varied composition of the ECM components not only contains a reservoir of cell-signaling motifs (ligands) and growth factors that guide cellular anchorage and behavior, but also provides physical architecture and mechanical strength to the tissue. The spatial distribution and concentration of ECM ligands, together with the tissue-specific topography and mechanical properties (in addition to signals from adjacent cells—juxtacrine signalling—and the surrounding fluid), provide signaling gradients that direct cell migration and cellular production of ECM constituents. In this dynamic environment, the bidirectional flow of information between the ECM and the cells mediates gene expression, ECM remodeling and ultimately tissue/organ function.

Native ECM exhibits macro- to nano-scale patterns of chemistry and topography [10]. Tissue stiffness is also known to vary depending on the organ type, disease state and aging process [11-13]. In tissue culture, stem cell differentiation has traditionally been controlled by the addition of soluble factors to the growth media [14]. However, most stem cell differentiation protocols yield heterogeneous cell types [15, 16]. Moreover, cells encounter very different, unfamiliar surfaces and environments when cultured in vitro or when materials are implanted into the body. Therefore, it is desirable to use more biomimetic in vitro culture conditions to regulate stem cell fate so as to advance clinical translation of stem cells through better expansion techniques and scaffolding for the regeneration of many tissues. Recent advances have facilitated further the creation of substrates with precise micro- and nano-cues, variable stiffness and chemical composition to better mimic the in vivo microenvironment [2, 17, and 18]. By employing various novel approaches, tissue engineers aim to incorporate topographical, mechanical and chemical cues into biomaterials to control stem cell fate decisions [2, 18, and 19].

The four fundamental aspects are: (1) Cell Sources and Culture (2) Cell Orientation (3) Cell Support Materials and (4) Design and Engineering of Tissues.

Tissue engineering (TE) refers to the application of the principles of engineering to cell culture for the construction of functional anatomical units (tissues/organs). The ultimate purpose of TE is to supply various body parts for the repair or replacement of damaged tissues or organs.

Tissue engineering may be regarded as the backbone of reconstructive surgery. It is possible to supply almost all surgical implants (skin, blood vessels, ligaments, heart valves, joint surfaces, nerves) through the developments in tissue engineering.

There are two schools of thought while dealing with tissue engineering techniques:

01. Some workers believe that the living cells possess an innate potential of biological regeneration. This implies that when suitable cells are allowed to grow on an appropriate support matrix, the cells proliferate, and ultimately result in an organized and functional tissue. This tissue resembles the original tissue in structure and function. This approach is very simple, and economical, although the success is limited.

02. According the second school of thought, there are several control processes to produce a new and functional tissue. Thus, tissue regeneration in vivo or tissue production in vitro are very complex. Therefore, tissue engineering is not a simple regeneration of cells, and it requires a comprehensive approach with a thorough understanding of cellular configuration, special arrangement and control process.

Tissue engineering is a complicated process. Some fundamental and basic aspects of TE with special reference to the following aspects are briefly described:

1. Cell Sources and Culture:

Adequate quantities of cells are required for tissue engineering. There are three types of cell sources- autologous, allogeneic and xenogeneic.

1.1 Autologous Cell Sources:

The cell source is said to be autologous when the patient's own cells are used in TE. This is a straight forward approach. A piece of desired tissue is taken by biopsy. It may be enzymatically digested or explant cultured, and the cells are grown to the required number.

The main advantages of autologous cells in TE are:

i. Avoidance of immune complications
ii. Reduction in the possible transfer of inherent infections.

There are certain disadvantages associated with autologous cells.

i. It is not always possible to obtain sufficient biopsy material from the patient.
ii. Disease state and age of the patient will be limiting factors.

Allogeneic Cell Sources:

If the cells are taken from a person other than the patient, the source is said to be allogeneic.

The advantages of allogeneic cell source are listed:

i. Obtained in good quantity from a healthy donor.
ii. Cells can be cultured in a large scale.
iii. Cost-effective with consistent quality.
iv. Available as and when required by a patient.

The major problem of allogeneic cell source is the immunological complications that may ultimately lead to graft rejection. The immune responses however, are variable depending on the type of cells used. For instance, endothelial cells are more immunogenic while fibroblasts and smooth muscle cells are less immunogenic. The age of the donor is another important factor that contributes to immunological complications. Thus, cells from adult donors are highly immunogenic while fetal or neonatal cells elicit little or no immune response.

Xenogeneic Cell Sources:

When the cells are taken from different species (e.g. pig source for humans) the source is said to be xenogeneic. This approach is not in common use due to immulogical complications.

Culture of Cells:

The methods adapted for culturing of cells required for tissue engineering depend on the type and functions of cells. For most of the cells, the conventional monolayer cultures serve the purpose. The major drawback of monolayer cultures is that cells may lose their morphology, functions and proliferative capacity after several generations. Some workers prefer three dimensional cultures for the cells to be used in tissue engineering. The nutrient and gaseous exchanges are the limiting factors in three dimensional cultures.

Genetic Alterations of Cultured Cells for Use in TE:

Gene therapy can be successfully employed in tissue engineering. This can be achieved by transferring the desired genes to cells in culture. The new genes may increase the production of an existing protein or may synthesize a new protein.

Some success has been achieved in this direction:

i. Genetically altered fibroblasts can produce transferrin, clotting factor VIII and clotting factor IX.
ii. Modified endothelial cells can synthesize tissue plasminogen activator.
iii. Genetically engineered keratinocytes can produce trans-glutaminase-l (This enzyme is lacking in patients suffering from a dermal disorder, lamellar icthyosis). The altered keratinocytes proved successful when transplanted in animal (rat) models of this disease.

2. Cell Orientation:

The orientation of cells with regard to specific shape and spatial arrangement is influenced by the following environmental factors:

Substrate Guidance:

The topographical features of the substrate determine the contact guidance. These features may be in the form of ridges, aligned fibers etc. It is possible to use differential attachment to substrates as a means of producing different alignment of cells. In recent years, synthetic polymer substrate collagen fibrils and fibronectin are used as bioresorbable templates for tissue engineering.

Chemical Gradients:

Development of chemical gradients is required for cellular orientation and for the stimulation of cellular functions. Certain growth factors and extracellular macromolecules are capable of creating chemical gradients e.g. vascular endothelial growth factor (VEGF), oligosaccharide fragments of hyaluronan, fibronectin, and collagen. There are certain practical difficulties in maintaining effective chemical gradients for the cells in three dimensional cultures. This is particularly the limiting factor when the cells become dense.

Mechanical Cues:

The response of the cells to mechanical signals is complex and this may result in any one or more of the following:

1. Changes in the cell alignment.
2. Deformation of cytoskeleton.
3. Altered matrix formation.
4. Synthesis of regulatory molecules (e.g growth factors, hormones).

There are mainly three mechanical cues governing cell populations:

a. Tensional forces.
b. Compressional forces.
c. Shear forces.

3. Cell Support Materials:

The support materials of cells largely determine the nature of adherent cells or cell types, and consequently tissue engineering.

There are a large number of support materials which may be broadly categorized as follows:

Traditional Abiotic Materials:

The traditional abiotic support materials include plastics, ceramics and metals. These materials cannot be resorbed or become biologically integrated into the tissues. Therefore, it is preferable to avoid the traditional materials in tissue engineering.

Bio-prosthesis Materials:

The natural materials modified to become biologically inert represent bio-prosthesis materials. They are formed by extensive chemical cross linking of natural tissues. For instance, the natural collagen-based connective tissue (e.g. porcine heart values) can be stabilized by treatment with glutaraldehyde.

The product formed is non-immunogenic that remains unchanged at the site of transplantation for several years. However, growth of some cells or even connective tissue can occur on bio-prosthesis materials. The design and fabrication of these materials is done in such a way that their functions are not affected by the surrounding host tissues.

Synthetic Materials:

A wide range of synthetic bioresorbable polymers are available as support materials. The most commonly used polymers in tissue engineering are poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and copolymer PLGA (poly (lactic-co-glycolic acid). The composition and dimensions of these polymers can be so adjusted to make them stable in vivo, besides supporting in vivo cell growth.

There are certain advantages in using synthetic polymers:

i. Production is easy and relatively cheap.

ii. Composition of polymers is reproducible even in large scale production.

There are however, some disadvantages also:

i. Compatibility with cells is not as good as natural polymers.

ii. On degradation, they may form some products which cause undesirable cellular effects.

Natural Polymers:

The most widely used natural polymer materials are collagen-chondroitin sulfate aggregates. These materials are commercially available with varying composition under the trade name Integra. The other natural polymers for cell support are usually obtained by their aggregation in culture as it occurs in vivo e.g. collagen gels, fibrin glue, Matrigel and some polysaccharides.

Among the polysaccharides, chitosan and hyaluronan are used as hydrated gels. The natural polymers mainly act on the principle of intermolecular interaction within the polymers to promote intimate molecular packing. The so formed molecules can effectively serve as support materials.

Semi-natural Materials:

Semi-natural materials are derived from the natural macromolecular polymers or whole tissues. They are the modified materials to achieve aggregation or stabilization.

Some examples of semi-natural materials are listed below:

i. Chemically cross-linked hyaluronan, stabilized by benzyl esterification.

ii. Collagen cross-linked with agents such as tannic acid or carbodiimide.

4. Design and Engineering of Tissues:

The following surgical criteria are taken into consideration while dealing with tissue engineering:

i. Rapid restoration of the desired function.

ii. Ease of fixing the tissue.

iii. Minimal patient discomfort.

For designing tissue engineering, the source of donor cells is very critical. Use of patients own cells (autologous cells) is favoured to avoid immunological complications. Allogeneic cells are also used, particularly when the TE construct is designed for temporary repair. It is observed that when the cells are cultured and/or preserved (i.e. cryopreservation), the antigenicity of allogeneic cells is reduced.

Another important criteria in TE is the support material, its degradation products, cell adhesion characteristics and mechanical cues. The design and tissue engineering with respect to skin, urothelium and peripheral nerve are briefly described hereunder.

Tissue Engineered Skin:

It was first demonstrated in 1975 that human keratinocytes could be grown in the laboratory in a form suitable for grafting. Many improvements have been made since then. It is now possible to grow epithelial cells to produce a continuous sheet which progresses to form carnified layers.

The major difficulty with TE skin is the dermal layer possessing blood capillaries, nerves, sweat glands and other accessory organs. Some developments have occurred in recent years to produce implantable skin substitutes which may be regarded as tissue engineering skin constructs.

Integra™:

This is a bio artificial material composed of collagen-glycosaminoglycan. Integra™ is not a true TE construct. It is mainly used to carry the seeded cells.

Dermagraft™:

This is composed of poly (glycolic acid) polymer mesh seeded with human dermal fibroblasts from neonatal foreskins.

Apligraf™:

This has human dermal fibroblasts seeded into collagen gel. A layer of human keratinocytes is then placed on the upper surface. The tissue constructs described above have limited shelf-life (about 5 days). However, they can integrate into the surrounding normal tissue and form a good skin cover. Further, there is no evidence of immunological complications with TE constructs.

Tissue Engineered Urothelium:

It is now possible to culture urothelial cells and bladder smooth muscle cells. This raises the hope that the construction of TE urothelium is possible. In fact, some success has been reported in the development of a functional bladder in dogs.

For this purpose, poly (glycolic acid) polymer base was shaped into a bladder and muscle cells were coated on the outer surface. The lumenal surface (i.e. inner surface) coated with pre-cultured urothelial cells. The bladder constructed in this way functioned almost like a normal one, and was maintained for about one year.

Tissue Engineered Peripheral Nerve Implants:

Peripheral nerve injury is a common occurrence of trauma and tumor resection surgery, often leading to irreversible muscle atrophy. Therefore, the repair of injured peripheral nerves assumes significance.

A diagrammatic representation of the basic design of a peripheral nerve implant is depicted in Fig 40.4.

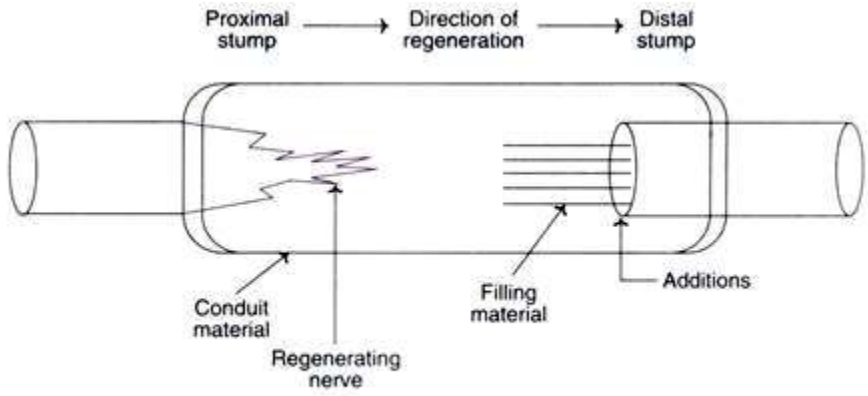

Fig. 40.4 : A diagrammatic representation of the basic design for peripheral nerve implant.

The regeneration of the injured nerve occurs from the proximal stump to rejoin at distal stump. The regeneration is guided by three types of substances.

Conduct material:

This is the outer layer and is the primary source of guidance. Conduct material is composed of collagen-glycosaminoglycan's, PLGA (poly lactic-co-glycolic acid), hyaluronan and fibronectin. All these are bioresorbable materials.

Filling material:

This supports the neural cells for regeneration, besides guiding the process of regeneration. Filling material contains collagen, fibrin, fibronectin and agarose.

Additives:

Additives include a large number of growth factors; neurotrophic factors (in different forms, combinations, ratios) e.g. fibroblast growth factor (FGF), nerve growth factor (NGF). Additions of Schwann cells or transfected fibroblasts promote nerve generation process.

Tissue Modeling:

Research is in progress to create tissue models in the form of artificial organs. Some of the recent development on experimental tissue modeling are briefly outlined.

Artificial liver:

Hepatocytes, cultured as spheroids or hepatocytes and fibroblasts cultured as hetero-spheroids can be used. They are held in the artificial support systems such as porous gelatin sponges, agarose or collagen. Addition of exogenous molecules is useful for the long – term culture of liver cells. Some progress has been reported in creating artificial liver as is evident from the hepatocytes three-dimensional structure and metabolic functions.

Artificial pancreas:

Spheroids of insulin secreting cells have been developed from mouse insulinoma beta cells. Some workers could implant fetal islet-like cell clusters under the kidneys of mice, although the functions were not encouraging due to limitation of oxygen supply.

Other Tissue Models:

Pituitary gland:

Multicellular spheroids could be created to study certain hormonal release e.g. luteinizing hormone (LH), following stimulation by luteinizing hormone releasing hormone (LHRH). Some success has also been achieved to create spheroids for the production of melatonin.

Thyroid gland:

Thyroid cell spheroids can be used for the study of cell adhesion, motility, and thyroid follicle biogenesis.

Brain cell cultures:

Three dimensional brain cell cultures have been used for the study of neural myelination and demyelination, neuronal regeneration, and neurotoxicity of lead. Aggregated brain cells are also used for the study of Alzheimer’s disease and Parkinson’s disease.

Heart cell cultures:

Aggregated heart cells have been used for the study of cardiac development and physiology.

II. Applications of Tissue Engineering

The basic model for treating dental diseases is the removal of destructed tissues and replacing that with synthetic materials. However, a few numbers of such materials have relatively the same physical and chemical properties of the natural tooth and often suffer from a mechanical fracture. Moreover, the biocompatibility of most of such materials is the subject of much controversy. Today, tissue engineering has made it clear that instead of using synthetic materials, we can utilize dental regeneration.[1] In fact, using this realm of study, in addition to atraumatic care and a treatment with greater longevity, we could replace the tissues, which are destroyed as a result of cancer and periodontal diseases or were absent congenitally.[2] In dentistry, we are looking forward to totally regenerate tissues such as alveolar bone, periodontal ligament, enamel, dentin, and even the whole tooth. Obviously, the first step is to be fully acquainted with the biology and embryonic development of desired tissues.[3]

Langer et al. defined the tissue engineering as an interdisciplinary field of study, which employs the principles of engineering and life science to develop the biological components, which need to be healed or improved.[4] Tissue engineering comprises three major components of biologic tissues, that is, adult stem cells, growth factors, and extracellular matrix scaffolds.[5]

In general, stem cells are those clonogenic cells capable of spontaneous division and distinction from various cell lines. Stem cells are classified into 2 groups of embryonic and adult cells. Adult stem cells are responsible for restoration and reconstruction of different tissues.[6] In tooth, in addition to the isolation of dental pulp stem cells (DPSCs), other sources of stem cells are also mentioned, which are human exfoliated deciduous teeth (SHEDs), apical papilla (SCAP), and DPSCs.[7-10] Recent studies have shown that the stem cells of other tissues, such as bone marrow, fat tissue, and endometrium are also able to differentiate odontoblasts or tooth-forming cells.[11-13] Such cells brought hope to the future of regenerative medicine, especially regenerative dentistry.[14] In addition to the stem cells, scaffolds and messenger molecules are 2 other key components of tissue engineering. Scaffolds, which are currently divided into 2 groups of natural and synthetic, are in fact playing the role of extracellular matrix and acting as a carrier for growth factors and depends on the type of tissue to be regenerated there are considerable difference among these factors.[15,16]

Periodontology

Periodontitis is an inflammatory disease which clinically causes the loss of a number of tissues, such as periodontal ligament and alveolar bone.[17] Surgery, grafts, growth factors, and membranes have been the common treatments for this disease, so far. Recently, a population of stem cells was identified in periodontal ligament.[18,19] Such cells, named periodontal ligament stem cells, were able to distinguish osteoblasts and cementoblasts in vitro condition and were also capable to convert to cementum and ligament tissue in vivo condition.[14,20] These cells were also detected in periodontal ligament of laboratory mice and sheep and their functionality were also assessed in neural cells.[20,21] Further, studies indicated that freezing has no impact on these cells, so banking them is counted as a clinical necessity.[22] One of the applications of tissue engineering in periodontal regeneration is the use of stem cells and signals on scaffolds and their implantation of the lesion area. Studies revealed that by implanting a ceramic scaffold containing mice periodontal ligament cells, a periodontal cementum, and ligament is formed.[22] Moreover, the transplantation of bone marrow stem cells in class III lesion area of a dog has led to the regeneration of ligament, cementum, and alveolar bone.[23] It is reported recently that the stem cells of root apical papilla area in combination with ligament stem cells, can form periodontal structures.[10] Several studies have assessed the possibility of using tissue engineering in treating periodontal diseases.[24] One of the other applicable methods of tissue engineering in periodontology is use of gene therapy.[25] Within this method, the stem cells were transfected by adenoviruses containing growth factor and placed in the lesion area.[26] Numerous studies have reported the application of gene therapy in the regeneration of periodontal.[27-29]

Certainly, having a general information of the cellular and molecular trend of the periodontal tissues is necessary for successful regeneration of periodontal using the tissue engineering. Further, the favorable mechanism of cell proliferation and their transfer to the scaffold, differentiation- inducing conditions, and gene mutation probability should be studied in long-term period.

Endodontics

Every year, millions of teeth are survived by endodontic treatments. Although modern therapies could guarantee a high rate of success in most of the cases, an ideal treatment would be based on regenerative approach, which after removing the necrotic pulp could replace the tooth with a fresh pulp and survive the tissue. Regenerative endodontics means generating tissue to replace the damaged pulp or necrotic tissue. Dental pulp tissue contains stem cells, which can differentiate odontobalsts

in response to various growth factors. There are several methods for pulp regeneration.[30] One of these methods is direct injection of stem cells into the disinfected channel, after opening of in vivo apex. One of the major problems of this method is how to find a source of stem cells capable of differentiating various types of cells existing in a pulp (fibroblast, odontoblast, endothelial cells). Furthermore, to prevent from cell movement toward other tissues use of scaffold seems necessary. Cordeiro et al. by direct injection of scaffolds containing teeth (SHED) stem cells and endothelial cells into the root channel managed to form a structure similar to dental pulp.[31]

The other method is called ex vivo. In this method, the stem cells are grown and differentiated on the scaffold in the presence of required signals, then will be implanted in the root channel. Each technique has some pros and cons, which should be defined through clinical research studies and basic sciences.

In dental pulp tissue engineering, soft scaffolding, such as hydrogels, can be used instead of natural and synthetic polymer scaffolds. Such scaffolds are in syringe type and are injectable in the root channel.[32] One of the other probable applications of stem cells in endodontics is in apexogenesis and apexification. Immature permanent teeth are usually rich sources of stem cells and blood vessels, which could be used in cells for apexogenesis.[33]

Regeneration of pulp vessels and nerve is one of the basic problems of dental pulp tissue engineering. The pulp is a tissue full of nerves, which enters the pulp through the apical hole along with blood vessels. These nerves have numerous roles and their regeneration is extremely vital in pulp. Recently, it is specified that some member of Bone morphogenetic protein family contribute to neurogenesis.[34] Moreover, the significance of endothelial cells and Vascular endothelial growth factor is confirmed in angiogenic.[35]

It is noteworthy that the success of regenerative endodontics treatments depends on the capability of scholars in creating a technique which allows the dentist to generate a pulp functional tissue in the disinfected and transformed channel.

Oral and Maxillofacial Surgery

The application of tissue engineering in oral surgery includes several branches, which studied in different articles.

Tissue engineering of temporomandibular joint

Temporomandibular joint is a complicated system, which could be interrupted due to trauma or inflammation. Common available treatments usually cause the formation of oscar fibrocartilage tissue, which lacks the mechanical properties of natural cartilage tissue. Tissue engineering of this joint calls for regeneration of 2 components of bone and cartilage. Cartilage generation, however, is more difficult than bone regeneration, in that in most studies although stem cells differentiation is to chondrocytes, the regenerate tissue is not like the natural tissue, structurally and functionally. Many growth factors were identified in this way, which could contribute to the joint regeneration.[38] According to studies conducted in 2004 and 2005 based on detailed computer images, a scaffold was designed of polylactic acid and hydroXyapatite (PLLA/HA), in which the chondrocyte cells were differentiated and the gingival fibroblasts cells, which under the influence of Ad.BMP7 were transferred to the scaffold. The scaffold was then implanted subcutaneously in the body of a laboratory mouse with no immune system. 4 weeks after, the bone and cartilage structures were observable with certain interface.[39] This study indicated that the tissue engineering of temporomandibular joint is possible though serious challenges lie in this method.

Tissue engineering of skin and oral mucosa

Regeneration of skin and oral mucosa in patients, who lost a part of their tissues due to burn, major surgeries, or trauma is the matter of the utmost significance. Previously, initial treatments were skin grafts, but gradually the use of cultured epithelial layers from small biopsies become increasingly common. Skin is the first engineered tissue, which is confirmed by the FDA for clinical applications. All FDA-certified regenerated skill products are composed of Foreskin neonatal-based cells. These cells are capable of proliferation and could be the generator of 80,000 meters of final skin product.[40]

Like skin, oral mucosa is formed of stratified squamous epithelium, which covers the lamina propria. At present, multilayer cultivation of gingival keratinocytes has resulted in some relative success and could be applied for biocompatibility tests and studies of oral biology.[41] Commercial products, such as skin Ethic’s gingival epithelium and Keratinized EpiOral squamous epithelial products are currently in stock.

Tissue engineering of salivary glands

Losing the function of salivary glands is reported following the adverse effect of medicines, radiotherapy, and autoimmune diseases, such as Sjogren's syndrome. Decreased saliva, which is known as gastrostomia, is often associated with caries, mucosal infections, and dysphasia. Salivary glands are composed of four certain types of epithelial cells (acini, ducts, basal, and myoeptithelial) and discovering a cell able to differentiate to these cells is a great challenge for tissue engineering. The epithelial cells of salivary gland ducts, which were cultivated on PLLA scaffold, were used in the initial studies.[42] However, it was gradually revealed that these cells are not efficient in tight junctions and therefore cannot stimulate the unilateral movement of liquid saliva.[43] Currently, the progenitor epithelial cells of salivary glands are detected and could be used in tissue engineering. The stem cells from mouse bone marrow could also differentiate to acini cells and express the alpha amylase.[44]

At present, gene therapy is used as a clinical trial for regeneration and stimulation of salivary tissues in patients with head and neck cancer, who have radiotherapy.[45]

Implant therapy

Today, the use of implant as a standard method of dentistry is fully accepted. The success of dental implants depends on their placement in a bone with sufficient density and volume. One of the major problems in implant surgery is that in most of thecases, surgeons are faced with considerable erosion of alveolar bone, where it is needed to increase the bone length. Techniques which are currently evaluated in different studies, include use of different kinds of membranes, Xenografts, and HA.[46]

Use of bone tissue engineering in bone defections is one of the alternative methods for grafts.[47] Several studies have used ceramic and fibrin scaffolds for bone formation. Due to their slow rate of erosion as well as low plasticity, porous ceramics have shown favorable results.[48] However, due to hemostatic property, angiogenic ability, faster healing, and bone formation process, as well as osteoconductive property, fibrin is a suitable material. This element is a combination of fibrinogen and thrombin, and in terms of biocompatibility, biodegradation, and appropriate cell band is approved.[49] Moreover, platelet-rich plasma is realized as one of the effective growth factors in bone tissue engineering.[50] Use of hydrogels for covering implants would make the culture of osteoblasts in implants and their better osteointegration possible.

One of the other effective methods for controlled release of the growth factors is the use of drug delivery systems by various microspheres.[51] By covering the implant surface, the polylactic microspheres, which contain growth factors such as transforming growth factor-beta or insulin-like growth factor-I, could yield successful results in increasing osteointegration.

Cleft palate

Cleft lip and palate is a common congenital disease, which is seen in almost one or 2 babies per 1000 birth. The current approach for treating cleft lip and palate is surgery and bone grafts, which have some limitations.[52] By regenerating bone as well as soft tissues using the stem cells, scaffolds, and growth factors, tissue engineering could be accepted as an alternative treatment. Currently, more studies are concentrated on the identification of molecular messaging system, which causes such disease and several growth factors were detected in this way, the most important of which is Wnt pathway.[53]

Since cleft lip and palate is a congenital defect, children are our target patients.Hence, we should study more precisely how to use tissue engineering and control it in children.

Restorative

The aim of restorative dentistry is to regenerate those dental structures which are lost due to decays. These structures include tooth enamel and dentin, which are currently replaced by composites, amalgams, or porcelain. The subject of replacing such damaged tissues with natural ones came up recently.[54] Many animal studies are carried out for dentin regeneration, so far and they were effective. In the study of Iohara et al., the transplantation of a pulp exposed with stem cells of dental pulp has led to dentin regeneration and the formation of a dentin bridge.[55] Use of different kinds of stem cells, including buds, milk teeth, permanent tooth pulp, and bone marrow could help dentin generation.[14] Regenerating enamel by tissue engineering is much harder than regenerating dentin, in that the ameloblastoma cells, which compose the enamel, will destroy after tooth growth. Therefore, in contrast to dentin, the damaged enamel is not able to repair itself. It is specified recently that under certain conditions, some of oral epithelial cells are able to differentiate to ameloblastoma cells. Harada et al. carried out a research on anterior teeth of mouse and by observing the permanent germination of these teeth and frequent generation of enamel during the lifetime of this animal, were managed to identify the epithelial stem cells in cervical area of these teeth.[56] Further, the molecular

messaging pathway, which causes the differentiation of stem cells to ameloblastoma, was also detected, which is called Notch pathway. The identification of this pathway could be helpful in inducing different stem cells to ameloblastoma. According to a study conducted in 2007 by Honda et al.,[57] enamel organ epithelial cells were isolated from the third molar of guinea pig and placed on collagen sponge along with dental pulp cells. 4 weeks after, the enamel and dentin structures were established. Moreover, within another study, Hu et al. could differentiate the stem cells of bone marrow to ameloblastoma.[58] According to this study, a combination of stem cells of bone marrow, epithelial dental embryonic cells, and dental mesenchyme was used.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT –V – Concepts in Stem Cell Research – SBT1403

1. Gene Therapy

01. Gene therapy (also called human gene transfer) is a medical field which focuses on the utilization of the therapeutic delivery of nucleic acids into a patient's cells as a drug to treat disease. The first attempt at modifying human DNA was performed in 1980 by Martin Cline, but the first successful nuclear gene transfer in humans, approved by the National Institutes of Health, was performed in May 1989. The first therapeutic use of gene transfer as well as the first direct insertion of human DNA into the nuclear genome was performed by French Anderson in a trial starting in September 1990. It is thought to be able to cure many genetic disorders or treat them over time.

02. Between 1989 and December 2018, over 2,900 clinical trials were conducted, with more than half of them in phase I. As of 2017, Spark Therapeutics' Luxturna (RPE65 mutation-induced blindness) and Novartis' Kymriah (Chimeric antigen receptor T cell therapy) are the FDA's first approved gene therapies to enter the market.

03. Since that time, drugs such as Novartis' Zolgensma and Alnylam's Patisiran have also received FDA approval, in addition to other companies' gene therapy drugs. Most of these approaches utilize adeno-associated viruses (AAVs) and lentiviruses for performing gene insertions, in vivo and ex vivo, respectively. ASO / siRNA approaches such as those conducted by Alnylam and Ionis Pharmaceuticals require non-viral delivery systems, and utilize alternative mechanisms for trafficking to liver cells by way of GalNAc transporters.

04. The concept of gene therapy is to fix a genetic problem at its source. If, for instance, in an (usually recessively) inherited disease a mutation in a certain gene results in the production of a dysfunctional protein, gene therapy could be used to deliver a copy of this gene that does not contain the deleterious mutation, and thereby produces a functional protein. This strategy is referred to as gene replacement therapy and is employed to treat inherited retinal diseases.

05. Not all medical procedures that introduce alterations to a patient's genetic makeup can be considered gene therapy. Bone marrow transplantation and organ transplants in general have been found to introduce foreign DNA into patients. Gene therapy is defined by the precision of the procedure and the intention of direct therapeutic effect.

06. Somatic cell gene therapy involves the transfer of gene to a diseased somatic cell either within the body or outside the body with the help of a viral or non viral gene therapy vector.

07. Ex vivo is any procedure accomplished outside. In gene therapy clinical trials cells are modified in a variety of ways to correct the gene. In ex vivo cells are modified outside the patient's body and the corrected version is transplanted back in to the patient. The cells are treated with either a viral or non viral gene therapy vector carrying the corrected copy of the gene.

08. Opposite of ex vivo is what we call in vivo where cells are treated inside the patient's body. The corrected copy of the genes is transferred into the body of the patient. The cells may be treated either with a viral or non viral vector carrying the corrected copy of the gene. If the patient is weak or the cell cannot be extracted out from the body, the gene is introduced directly into the body.

09. Gene therapy done in a restricted area or to a particular site is called in-situ. In situ gene therapy requires the vector to be placed directly into the affected tissues. In vivo gene therapy involves injecting the vector into the blood stream. The vector then must find the target tissue and deliver the therapeutic genes.

10. Methods of Gene Therapy:

    1. In situ gene therapy:

        1. In situ gene therapy comprises transfer of corrected copy of the gene into the targeted organ or tissue. The major concern of current time gene therapy protocol is the lack of efficient transduction of the targeted organ. The method is effectively used against cystic fibrosis, a disease of airway epithelium.

        2. The method is also explored for cancer gene therapy where the viral vector is engineered to contain the herpes simplex virus thymidine kinase gene. After injection of the viral vector the patient is treated with a prodrug such as Ganciclovir, which causes 75% reduction in the tumor cell population.

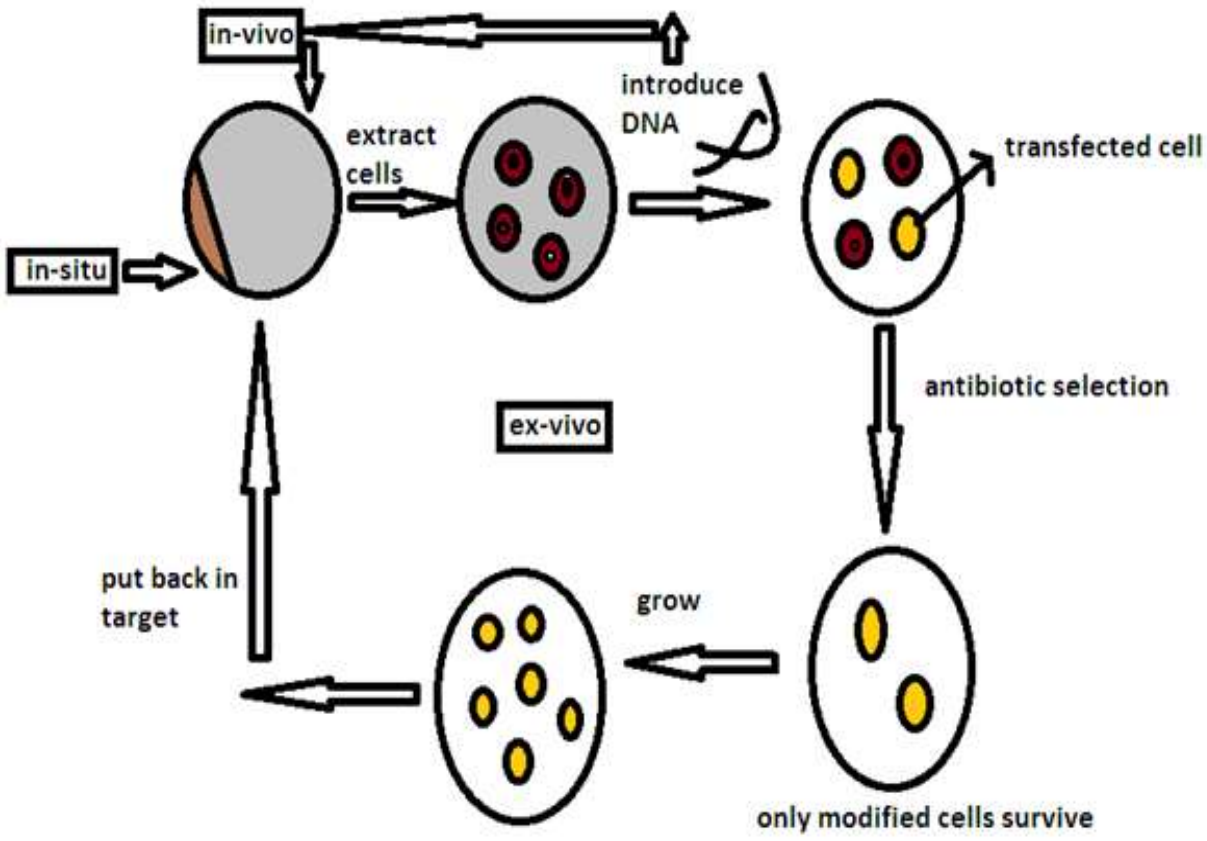
2. In vivo gene therapy:

    1. Delivery of corrected copy of the gene systemically through injection is a highly efficient way to transfer a transgene to the patient's body. The major problem of in vivo method is its inefficient targeting.

    2. The transgene delivered into the body by means of viral or non viral vector also evokes the immune response. The immune response against the vector leads to its clearance and only transient expression of transgene. The neutralizing antibody does not allow the second injection of the vector. Reducing the neutralizing antibody is the current area of research in order to improve the delivery of gene therapy vector.

    3. All gene therapy delivery protocols require the transgene to cross the plasma membrane and enter inside the nucleus. The major obstacle is still to deliver the transgene effectively to the intracellular compartment.

    4. Many modifications have been suggested into the viral vectors and also non viral vectors to target the gene to the tissue. VP22, a protein of herpes simplex virus has a property to spread from one cell to the other, and this property has been successfully implemented in designing the vectors.



Ex vivo, *in vivo*, and *in situ* gene therapy

II. Transgenic Animals

Definition

A transgenic animal is one whose genome has been altered by the transfer of a gene or genes from another species or breed.

The photo shows two transgenic mice positioned either side of a plain mouse. The transgenic mice have been genetically modified so that they carry a green fluorescent protein which glows green under blue light. Credit: Ingrid Moen et alet al., BMC Cancer, 12/21 (2012), 1-10.

Importance

Transgenic animals are routinely used in the laboratory as models in biomedical research. Over 95 per cent of those used are genetically modified rodents, predominantly mice. They are important tools

for researching human disease, being used to understand gene function in the context of disease susceptibility, progression and to determine responses to a therapeutic intervention.

Mice have also been genetically modified to naturally produce human antibodies for use as therapeutics. Seven out of the eleven monoclonal antibody drugs approved by the FDA between 2006 and 2011 were derived from transgenic mice.

Transgenic farm animals are also being explored as a means to produce large quantities of complex human proteins for the treatment of human disease. Such therapeutic proteins are currently produced in mammalian cell-based reactors, but this production process is expensive. In 2008, for example, the building of a new cell-based manufacturing facility for one therapeutic protein was estimated to cost over US$500 million. A cheaper option would be to develop a means to produce recombinant proteins in the milk, blood or eggs of transgenic animals. Progress in this area, however, has been slow to-date. Only two biomedical products have so far received regulatory approval. The first is human antithrombin III, a therapeutic protein produced in the milk of transgenic goats, which is used to prevent clots in patients with hereditary antithrombin deficiency receiving surgery or undergoing childbirth. A relatively small herd of goats (about 80) can supply enough human antithrombin III for all of Europe. The second product is a recombinant human C12 esterase inhibitior produced in the milk of transgenic rabbits. This is used to treat hereditary angiodema, a rare genetic disorder which causes blood vessels in the blood to expand and cause skin swellings.

Discovery

The ability to produce transgenic animals is reliant on a number of components. One of the first things needed to generate transgenic animals is the ability to transfer embryos. The first successful transfer of embryos was achieved by Walter Heape in Angora rabbits in 1891. Another important component is the ability to manipulate the embryo. In vitro manipulation of embryos in mice was first reported in the 1940s using a culture system. What is also vital is the ability to manipulate eggs. This was made possible through the efforts of Ralph Brinster, attached to the University of Pennsylvania, who in 1963 devised a reliable system to culture eggs, and that of Teh Ping Lin, based at the California School of Medicine, who in 1966 outlined a technique to micro-inject fertilised mouse eggs which enabled the accurate insertion of foreign DNA.

The first genetic modification of animals was reported in 1974 by the virologist Rudolph Jaenisch, then at the Salk Institute, and the mouse embryologist Beatrice Mintz at Fox Chase Cancer Center. They demonstrated the feasibility of modifying genes in mice by injecting the SV40 virus into early-stage mouse embryos. The resulting mice carried the modified gene in all their tissues. In 1976, Jaenisch reported that the Moloney Murine Leukemia Virus could also be passed on to offspring by infecting an embryo. Four years later, in 1980, Jon Gordon and George Scango together with Frank Ruddle, announced the birth of a mouse born with genetic material they had inserted into newly fertilised mouse eggs. By 1981 other scientists had reported the successful implantation of foreign DNA into mice, thereby altering the genetic makeup of the animals. This included Mintz with Tim Stewart and Erwin Wagner at the Fox Chase Cancer Center in Philadelphia; Brinster and Richard Palmiter at the University of Washington, Seattle; and Frank Costantini and Elizabeth Lacy at Oxford University.

Such work laid the basis for the creation of transgenic mice genetically modified to inherit particular forms of cancer. These mice were generated as a laboratory tool to better understand the onset and progression of cancer. The advantage of such mice is that they provide a model which closely mimics the human body. The mice not only provide a means to gain greater insight into cancer but also to test experimental drugs.

Application

Transgenic animals are animals (most commonly mice) that have had a foreign gene deliberately inserted into their genome. Such animals are most commonly created by the microinjection of DNA into the pronuclei of a fertilised egg which is subsequently implanted into the oviduct of a pseudopregnant surrogate mother. This results in the recipient animal giving birth to genetically modified offspring. The progeny are then bred with other transgenic offspring to establish a transgenic line. Transgenic animals can also be created by inserting DNA into embryonic stem cells which are then micro-injected into an embryo which has developed for five or six days after fertilisation, or infecting an embryo with viruses that carry a DNA of interest. This final method is commonly used to manipulate a single gene, in most cases this involves removing or 'knocking out' a target gene. The end result is what is known as a ‘knockout’ animal.

Since the mid-1980s transgenic mice have become a key model for investigating disease. Mice are the model of choice not only because there is extensive analysis of its completed genome sequence, but its genome is similar to the human. Moreover, physiologic and behavioural tests performed on mice can be extrapolated directly to human disease. Robust and sophisticated techniques are also easily available for the generic manipulation of mouse cells and embryos. Another advantage of mice is the fact that they have a short reproduction cycle. Other transgenic species, such as pig, sheep and rats are also used, but their use in pharmaceutical research has so far been limited due to technical constraints. Recent technological advances, however, are laying the foundation for wider adoption of the transgenic rat.

Transgenic rodents play a number of critical roles in drug discovery and development. Importantly, they enable scientists to study the function of specific genes at the level of the whole organism which has enhanced the study of physiology and disease biology and facilitated the identification of new drug targets. Due to their similarity in physiology and gene function between humans and rodents, transgenic rodents can be developed to mimic human disease. Indeed, an array of transgenic mice models have been produced for this purpose. Mice are being used as models, for example, to study obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, ageing, Alzheimer's disease and Parkinson's disease. They are also used to study different forms of cancer. In addition, transgenic pigs are being investigated as a source of organs for transplants, which if proven clinically safe could overcome some of the severe donor organ shortages. The development of transgenic animals has recently been transformed by the emergence of the new gene editing tool CRISPR which greatly reduced the number of steps involved in the creation of transgenic animals, making the whole process much faster and less costly.

This section on transgenic mice was jointly written by Lara Marks and Dmitriy Myelnikov. For more information see D. Myelnikov, 'Transforming mice: technique and communication in the making of transgenic animals, 1974-1988', unpublished PhD, Cambridge University, 2015.

Transgenic animals: timeline of key events

Date	Event	People	Places
23 Jun 1925	Oliver Smithies was born in Halifax, United Kingdom	Smithes	University of Washington, University of North Carolina
1929	Jackson Memorial Laboratories established to develop inbred strains of mice to study the genetics of cancer and other diseases		Jackson Memorial Laboratoroies
19 Aug 1929	Frank Ruddle was born in West New York, New Jersey	Ruddle	Yale University
18 Sep 1951	Anthony J Clark was born in Blackpool, UK	Anthony Clark	Roslin Institute
1974	First publication on inserting foreign DNA into mice	Jaenisch, Mintz	Salk Institute, Fox Chase Institute for Cancer Research
September 1980	Scientists reported the first successful development of transgenic mice	Barbosa, Gordon, Plotkin, Ruddle, Scangos	Yale University
November 1980	Technique published using fine glass micropipettes to inject DNA directly into the nuclei of cultured mammalian cells. High efficiency of the method enables investigators to generate transgenic mice containing random insertions of exogenous DNA.	Capecchi	University of Utah
5 Nov 1981	First successful transmission of foreign DNA into laboratory mice	Constantini, Lacy	Oxford University, Yale University
December 1982	Giant mice made with the injection of rat growth hormone	Brinster, Palmiter	University of Pennsylvania,

Date	Event	People	Places
			University of Washington Seattle
1983	Course started in the molecular embyology of mice	Costantini, Hogan, Lacy	Cold Spring Harbour Laboratory, NIMR, Sloan Kettering Cancer Research Center, Columbia University
1985	First transgenic mice created with with genes coding for both the heavy and light chain domains in an antibody.	Kohler, Rusconi	Max-Planck Institute
6 Nov 1987	Publication of gene targeting technique for targetting mutations in any gene	Thomas, Capecchi	University of Utah
1988	Patent application filed for a method to create transgenic mice for the production of human antibodies	Bruggeman, Caskey, Neuberger, Surani, Teale, Waldmann, Williams	Laboratory of Molecular Biology, Babraham Institute, Cambridge University
12 Apr 1988	OncoMouse patent granted	Leder, Stewart	Harvard University
12 Jun 1992	First transgenic mouse model created for studying link between DNA methylation and disease	Li, Bestor, Jaenisch	Whitehead Institute for Biomedical Research
1994	First transgenic mice strains reported for producing human monoclonal antibodies	Bruggemann, S.Green, Lonsberg, Neuberger	Cell Genesys, GenPharm, Laboratory of Molecular Biology
5 Jul 1996	Dolly the sheep, the first cloned mammal, was born	Wilmut, Campbell	Roslin Institute
9 Jul 1997	Birth of first sheep cloned with human genes	Schnieke, Kind, Ritchie, Mycock, Scott,	PPL Therapeutics, Roslin Institute

Date	Event	People	Places
		Wilmutt, Colman, Campbell	
14 Feb 2003	Dolly the sheep, the first cloned mammal, died	Wilmut	Roslin Institute
12 Aug 2004	Anthony J Clark died	Anthony Clark	Roslin Institute
September 2006	First fully human monoclonal antibody drug approved		Agensys, Amgen
2007	Nobel Prize for Physiology for Medicine awarded for discoveries enabling germline gene modification in mice using embryonic stem cells	Capecchi, Evans, Smithies	University of North Carolina, University of Utah
10 Mar 2013	Frank Ruddle died in New Haven, Connecticut	Ruddle	Yale University
26 Oct 2013	Michael Neuberger died	Neuberger	Laboratory of Molecular Biology
23 Sep 2015	Beijing Genomics Institute announced the sale of the first micropigs created with the help of the TALENs gene-editing technique		Beijing Genomics Institute
5 Oct 2015	CRISPR/Cas9 modified 60 genes in pig embryos in first step to create organs suitable for human transplants	Church	Harvard University
10 Jan 2017	Oliver Smithies died	Smithies	University of Washington, University of North Carolina
20 Apr 2017	Diabetes research using transgenic mice shows the protein P2X7R plays important role in inflammation and immune system offering new avenue for treating kidney disease	Menzies	University of Edinburgh, University College London, Imperial College

Date	Event	People	Places
23 Jan 2019	CRISPR-Cas9 used to control genetic inheritance in mice	Grunwald, Gntz, Poplawski, Xu, Bier, Cooper	University of California San Diego

The dependence of man on animals such as cattle, sheep, poultry, pig and fish for various purposes (milk, meat, eggs, wool etc.) is well known.

Improvement in the genetic characteristics of livestock and other domestic animals (e.g., high milk yield, weight gain, etc.), in the early days, was carried out by selective breeding methods.

This technique primarily involves a combination of mating and selection of animals with improved genetic traits. Although selective breeding is very time consuming and costly, it was the only method available, till some years ago, to enhance the genetic characteristics of animals.

For larger animals with long gestation period, it might take several decades to create a desired character by conventional breeding. With the advent of modern biotechnology, it is now possible to carry out manipulations at the genetic level to get the desired characteristics in animals.

Trans-genesis refers to the phenomenon of introduction of exogeneous DNA into the genome to create and maintain a stable heritable character. The foreign DNA that is introduced is called transgene. And the animal whose genome is altered by adding one or more transgenes is said to be transgenic.

The transgenes behave like other genes present in the animals' genome and are passed on to the offspring's. Thus, transgenic animals are genetically engineered or genetically modified organisms (GMOs) with a new heritable character. It was in 1980s, the genetic manipulation of animals by introducing genes into fertilized eggs became a reality.

Importance of Transgenic Animals-General:

Trans-genesis has now become a powerful tool for studying the gene expression and developmental processes in higher organisms, besides the improvement in their genetic characteristics. Transgenic animals serve as good models for understanding the human diseases.

Further, several proteins produced by transgenic animals are important for medical and pharmaceutical applications. Thus, the transgenic farm animals are a part of the lucrative world-wide biotechnology industry, with great benefits to mankind. Trans-genesis is important for improving the quality and quantity of milk, meat, eggs and wool production, besides creating drug resistant animals.

Milk as the Medium of Protein Production:

Milk is the secretion of mammary glands that can be collected frequently without causing any harm to the animal. Thus, milk from the transgenic animals can serve as a good and authenticated source of human proteins for a wide range of applications. Another advantage with milk is that it contains only a few proteins (casein, lactalbumin, immunoglobulin etc.) in the native state, therefore isolation and purification of a new protein from milk is easy.

Commonly used Animals for Trans-genesis:

The first animals used for trans-genesis was a mouse. The 'Super Mouse', was created by inserting a rat gene for growth hormone into the mouse genome. The offspring was much larger than the parents. Super Mouse attracted a lot of public attention, since it was a product of genetic manipulation rather than the normal route of sexual reproduction. Mouse continues to be an animal of choice for most transgenic experiments. The other animals used for trans-genesis include rat, rabbit, pig, cow, goat, sheep and fish.

Position Effects:

Position effect is the phenomenon of different levels of gene expression that is observed after insertion of a new gene at different position in the eukaryotic genome. This is commonly observed in transgenic animals as well as plants. These transgenic organisms show variable levels and patterns of transgene expression. In a majority of cases, position effects are dependent on the site of transgene integration. In general, the defective expression is due to the insertion of transgene into a region of highly packed chromatin. The transgene will be more active if inserted into an area of open chromatin.

The positional effects are overcome by a group of DNA sequences called insulators. The sequences referred to as specialized chromatin structure (SCS) are known to perform the functions of insulators. It has been demonstrated that the expression of the gene is appropriate if the transgene is flanked by insulators.

Animal Bioreactors:

Trans-genesis is wonderfully utilized for production proteins of pharmaceutical and medical use. In fact, any protein synthesized in the human body can be made in the transgenic animals, provided that the genes are correctly programmed. The advantage with transgenic animals is to produce scarce human proteins in huge quantities. Thus, the animals serving as factories for production of biologically important products are referred to as animal bioreactors or sometimes pharm animals. Frankly speaking, transgenic animals as bioreactors can be commercially exploited for the benefit of mankind.

Once developed, animal bioreactors are cost- effective for the production of large quantities of human proteins. Routine breeding and healthful living conditions are enough to maintain transgenic animals. A list of the therapeutically important proteins produced by animal bioreactors is given Table 41.2.

Transgenic Animals in Xenotransplantation:

Organ transplantation (kidney, liver, heart etc.) in humans has now become one of the advanced surgical practices to replace the defective, non-functional or severally damaged organs. The major limitation of transplantation is the shortage of organ donors. This often results in long waiting times and many unnecessary deaths of organ failure patients.

Xenotransplantation refers to the replacement of failed human organs by the functional animal organs. The major limitation of xenotransplantation is the phenomenon of hyper acute organ rejection due to host immune system.

The organ rejections is mainly due to the following two causes:

i. The antibodies raised against the foreign organ.
ii. Activation of host's complement system.

Pigs in Xenotransplantation?

Some workers are actively conducting research to utilize organs of pigs in xenotransplantation. It is now identified that the major reason for rejection of pig organs by primates is due to the presence of a special group of disaccharides (Gal-α 1, 3-Gal) in pigs, and not in primates.

The enzyme responsible for the synthesis of specific disaccharides in pigs has been identified. It is α 1, 3-galactosyltransferase, present in pigs and not in primates. Scientists are optimistic that knockout pigs lacking the gene encoding the enzyme α 1, 3-galactosyltransferase can be developed in the next few years. Another approach is to introduce genes in primates that can degrade or modify Gal-α 1, 3-Gal disaccharide groups (of pigs). This will reduce immunogenicity.

Besides the above, there are other strategies to avoid hyperactive organ rejection by the hosts in xenotransplantation.

i. Expression of antibodies against the pig disaccharides.
ii. Expression of complement— inactivating protein on the cell surfaces.

By the above approaches, it may be possible to overcome immediate hyperactive rejection of organs. The next problem is the delayed rejection which involves the macrophages and natural killer cells of the host.

Another concern of xenotransplantation is that the endogenous pig retroviruses could get activated after organ transplantation. This may lead to new genetic changes with unknown consequences.

The use of transgenic animals in xenotransplantation is only at the laboratory experimental stages, involving animals. It is doubtful whether this will become a reality in the near future.

There is a vigorous debate concerning the ethics of xenotransplantation and the majority of general public are against it.

Production of Transgenic Animals using Virus as a Vector

Rabbits:

Rabbits are used as experimental models in gene transfer experiments. In 1985, the successful production of transgenic rabbits was reported, for the first time, and included the growth hormone construct MT-hGH. The rate of degeneration of rabbit zygotes caused by injection was below 10%. The pre-implantation development capacity of injected zygotes is significantly lower compared with control embryos.

Pigs:

Pig zygotes must be centrifuged to show the pro-nucleus. Fifty percent of the centrifuged non-injected zygotes develop in vivo up to the morula or blastocyst stage. After microinjection, 10- 20% development to various stages of embryonic development occurs. Of the injected zygotes, 5.6% to 11% developed and led to the birth of piglets. The integration rate in pigs is approximately 10%. Growth-hormone constructs used in initial experiments led to an expression rate of 50%.

The production of transgenic F1 offspring is possible. In the authors' own experiments, inheritance of the trans-gene could be proved in two out of five animals. It is not necessary to centrifuge sheep embryos to make the pro-nucleus visible. According to "Nomarski optics", 80% of the pro-nuclei can be located if a microscope with interference contrast is available. The capacity for in vivo development of sheep zygotes with injection (26 per cent) and without (10 per cent) is half that of pig embryos after similar treatment.

Seven days after in vivo culture of non-treated and non-in vitro cultured sheep zygotes, Rexroad and Wall in 1987 observed a development rate of 86%. An in vitro culture of five hours' duration reduced this development rate to 65%, and after the injection of a buffer solution a reduction to 42 per cent was observed. 19% developed to the 32-cell stage after injection of DNA solution.

Sheep and Goats:

Until recently, the trans-genes introduced into sheep inserted randomly in the genome and often worked poorly. However, in July 2000, success at inserting a trans-gene into a specific gene locus was reported. The gene was the human gene for alpha 1-antitrypsin, and two of the animals expressed large quantities of the human protein in their milk.

(1) It was done as sheep fibroblasts (connective tissue cells) growing in tissue culture were treated with a vector that contained these segments of DNA
(2) Regions homologous to the sheep COL1 A1 gene. This gene encodes Type 1 collagen (Its absence in humans causes the inherited disease osteogenesis imperfecta). This locus was chosen because fibroblasts secrete large amounts of collagen and thus one would expect the gene to be easily accessible in the chromatin.

A neomycin-resistance gene to aid in isolating those cells that successfully incorporated the vector. The human gene encoding alphal-antitrypsin. Some people inherit two non- or poorly-functioning genes for this protein. Its resulting low level or absence produces the disease Alphal-Antitrypsin Deficiency (A1AD or Alphal).

The main symptoms are damage to the lungs (and sometimes to the liver).

(1) Promoter sites from the beta-Iactoglobulin gene. These promote hormone-driven gene expression in milk-producing cells.
(2) Binding sites for ribosome's for efficient translation of the mRNAs.

Successfully-transformed cells were then:

(a) Fused with enucleated sheep eggs and
(b) Implanted in the uterus of a ewe (female sheep),
(c) Several embryos survived until their birth, and two young lambs have now lived over a year, When treated with hormones, these two lambs secreted milk containing large amounts of alphal-antitrypsin (650 μg/ml; 50 times higher than previous results using random insertion of the transgene).

The work on transgenic milk production is expensive requiring large facilities for purifying the protein from sheep's milk. Purification is important because even when 99.9% pure, human patients can develop antibodies against the tiny amounts of sheep proteins that remain.

GTC Bio-therapeutics, won preliminary approval to market a human protein, anti-thrombin, in Europe. Their protein, the first made in a transgenic animal to receive regulatory approval for human therapy and was secreted in the milk of transgenic goats.

Chickens:

Chickens have several advantages over other farm animals:

(i) Grow faster than sheep and goats and large numbers can be grown in close quarters
(ii) Synthesize several grams of protein in the "white" of their eggs.

Two methods have succeeded in producing chickens carrying and expressing foreign genes:

1. Infecting embryos with a viral vector carrying the human gene for a therapeutic protein and promoter sequences that will respond to the signals for making proteins (e.g., lysozyme) in egg white.
2. Transforming rooster sperm with a human gene and the appropriate promoters and checking for any transgenic offspring.

Preliminary results from both methods indicate that it may be possible for chickens to produce as much as 0.1 g of human protein in each egg that they lay. Not only should this cost less than producing therapeutic proteins in culture vessels, but chickens will probably add the correct sugars to glycosylated proteins something that E. coli cannot do.

Transgenic Fish:

Aquatic animals are being engineered to increase aquaculture production, for medical and industrial research, and for ornamental reasons (Fig. 18.7). Genes inserted to promote disease resistance may allow transgenic fish to absorb higher levels of toxic substances, including heavy metals. In turn, consumers of these fish may be ingesting higher amounts of substances such as mercury and selenium.

Transgenic fish that have genes from species such as peanuts or shellfish that are common causes of allergic reactions in humans may prompt allergic reactions in an unsuspecting consumer. Transgenic species may behave much like invasive species when interacting with the natural environment.

They may compete with native species for resources and pose a threat to the genetic diversity of native populations, especially when genetic modifications such as a rapid growth rate offer advantages over slower-developing native species. Despite industry assurances that transgenic fish would be unable to naturally reproduce or significantly threaten the environment, some scientists are far more doubtful.