CHAPTER

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Stem Cell Culture and Potential Applications

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Stem cells are the body's "master" cells because they give rise to all other tissues, organs, and systems in the body. Each tissue within the body contains unique type of stem cells that renew and replace that particular tissue (e.g. nerve, brain, cartilage, blood) when needed, due to damage or wear and tear. Stem cells have the remarkable potential to develop into many different cell types in the body. Basically, these cells serve as a sort of repair system for the body; they can theoretically divide without limit to replenish other cells as long as the person or any other living being is still alive. When a stem cell divides, each new cell has the potential to either remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell. The classical definition of a stem cell requires that it possess the following two properties :

Self-renewal - the ability to go through numerous cycles of cell division while maintaining the undifferentiated states

PLuripotency - the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either **totipotent** or **pluripotent** - to be able to give rise to any mature cell type, although **multipotent** or **unipotent** progenitor cells are sometimes referred to as stem cells

Totipotent cells are considered the "master" cells of the body because they contain all the genetic information needed to create all the cells of the body plus the placenta, which nourishes the embryo. Human cells have this capacity only during the first few divisions of a fertilized egg. After 3 - 4 divisions of totipotent cells, there follows a series of stages in which the cells become increasingly specialized. The next stage of division results in pluripotent cells, which are highly versatile and can give rise to any cell type except the cells of the placenta. At the next stage, cells become multipotent, meaning they can give rise to several other cell types, but those types are limited in number. An example of multipotent cells is hematopoietic cells—blood stem cells that can develop into several types of blood cells, but cannot develop into brain cells. At the end of the long chain of cell divisions that make up the embryo are "terminally differentiated" cells (cells that are considered to be permanently committed to a specific function).

Pluripotent stem cells are isolated from embryos that are a few days old. Cells from these embryos can be used to create pluripotent stem cell "lines" —cell cultures that can be grown indefinitely in the laboratory.

The two broad types of vertebrate stem cells are:

E**mbryonic stem cells** that are found in blastocysts and **Adult stem cells** that are found in adult tissues.

In a developing embryo, stem cells can differentiate into all of the specialized embryonic tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues.

As stem cells can be grown and transformed into specialized cells with characteristics consistent with cells of various tissues such as muscles or nerves through cell culture, their use in medical therapies has been proposed. In particular, embryonic stem cell lines, autologous embryonic stem cells generated through therapeutic cloning, and highly plastic adult stem cells from the umbilical cord blood or bone marrow are considered as promising candidates.

Identifying Stem Cells

The practical definition of a stem cell is the functional definition - the ability to regenerate tissue over a lifetime. For example, the gold standard test for a bone marrow or hematopoietic stem cell (HSC) is the ability to transplant one cell and save an individual without HSCs. In this case, a stem cell must be able to produce new blood cells and immune cells over a long term, demonstrating potency. It should also be possible to isolate stem cells from the transplanted individual, which can themselves be transplanted into another individual without HSCs, demonstrating that the stem cell was able to self-renew.

Properties of stem cells can be illustrated *in vitro*, using methods such as clonogenic assays, where single cells are characterized by their ability to differentiate and self-renew. As well, stem cells can be isolated based on a distinctive set of cell surface markers. However, *in vitro* culture conditions can alter the behavior of cells, making it unclear whether the cells will behave in a similar manner *in vivo*. Considerable debate exists whether some proposed adult cell populations are truly stem cells.

Embryonic stem cell lines (ES cell lines) are cultures of cells derived from the epiblast tissue of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryos. ES cells are pluripotent and during development, give rise to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. They do not contribute to the extra-embryonic membranes or the placenta.

A human embryonic stem cell is also defined by the presence of several transcription factors and cell surface proteins. The transcription factors Oct-4, Nanog, and Sox2 form the core regulatory network that ensures the suppression of genes that lead to differentiation and the maintenance of pluripotency. The cell surface antigens most commonly used to identify hES cells are the glycolipids SSEA3 and SSEA4 and the keratan sulfate antigens Tra-1-60 and Tra-1-81. The molecular definition of a stem cell includes many more proteins and continues to be a topic of research.

ES cells, being totipotent cells, require specific signals for correct differentiation - if injected directly into the body, ES cells will differentiate into many different types of cells, causing a teratoma. Differentiating ES cells into usable cells while avoiding transplant rejection are just a few of the hurdles that embryonic stem cell researchers still face. Many nations currently have moratoria on

either ES cell research or the production of new ES cell lines. Because of their combined abilities of unlimited expansion and pluripotency, embryonic stem cells remain a theoretically potential source for regenerative medicine and tissue replacement after injury or disease.

Adult stem cells

The term **adult stem cell** refers to any cell which is found in a developed organism that has two properties: the ability to divide and create another cell like itself and also divide and create a cell more differentiated than itself. Pluripotent adult stem cells are rare and generally small in number but can be found in a number of tissues including umbilical cord blood. Most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin (mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, etc.)

A great deal of adult stem cell research has focused on clarifying their capacity to divide or self-renew indefinitely and their differentiation potential. In mice, pluripotent stem cells are directly generated from adult fibroblast cultures.

While embryonic stem cell potential remains untested, adult stem cell treatments have been used for many years to treat successfully leukemia and related bone/blood cancers through bone marrow transplants. The use of adult stem cells in research and therapy is not as controversial as embryonic stem cells, because the production of adult stem cells does not require the destruction of an embryo. Consequently, more US government funding is being provided for adult stem cell research.

Lineage

To ensure self-renewal, stem cells undergo two types of cell division. Symmetric division gives rise to two identical daughter cells both endowed with stem cell properties. Asymmetric division, on the other hand, produces only one stem cell and a progenitor cell with limited self-renewal potential. Progenitors can go through several rounds of cell division before terminally differentiating into a mature cell. It is possible that the molecular distinction between symmetric and asymmetric divisions lies in differential segregation of cell membrane proteins (such as receptors) between the daughter cells. An alternative theory is that stem cells remain undifferentiated due to environmental cues in their particular niche. The signals that lead to reprogramming of cells to an embryonic-like state are also being investigated. These signal pathways include several transcription factors including the oncogene c-Myc. Initial studies indicate that transformation of mice cells with a combination of these anti-differentiation signals can reverse differentiation and may allow adult cells to become pluripotent. However, the need to transform these cells with an oncogene may prevent the use of this approach in therapy.

Applications of Stem Cells

Stems cells – whether cord blood, adult or embryonic – have numerous applications in the areas of scientific research and cell therapy. For researchers, stem cells are the key to understanding how humans develop the way they do. Hopefully, the study of stem cells will unravel the mystery of how an undifferentiated cell is able to differentiate, and will also determine what is the signal that triggers the sequence. The greater understanding, and possibly even control, of cell division and

differentiation is a significant strategy in the battle against dreaded illnesses such as cancer, which is basically the continuous multiplication of abnormal cells.

The use of stem cells for the testing of new medicines is another highly-anticipated application. Although certain cells are already utilized for this purpose – cancer cells, for example, are used to tests anti-tumor drugs – testing on pluripotent cells would open up this field to a much broader number of cell types.

The third, and possibly most important, application is cell therapy, which is the use of stem cells to produce the cells and tissues required for the renewal or repair of body organs that have been damaged by debilitating and mortal diseases such as cancer, spinal cord injuries, glaucoma and Parkinson's disease.

Stem cells provide the opportunity to study the growth and differentiation of individual cells into tissues. Understanding these processes could provide insights into the causes of birth defects, genetic abnormalities, and other disease states. If normal development were better understood, it might be possible to prevent or correct some of these conditions. Stem cells could be used to produce large amounts of one cell type to test new drugs for effectiveness and chemicals for toxicity. Stem cells might be transplanted into the body to treat disease (diabetes, Parkinson's disease) or injury (e.g., spinal cord). The damaging side effects of medical treatments might be repaired with stem cell treatment. For example, cancer chemotherapy destroys immune cells in patients, decreasing their ability to fight off a broad range of diseases; correcting this adverse effect would be a major advance.

Before stem cells can be applied to human medical problems, substantial advances in basic cell biology and clinical technique are required. In addition, very challenging regulatory decisions will be required on the individually created tissue-based therapies resulting from stem cell research. Such decisions would likely be made by the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA). The potential benefits mentioned above would be likely only after many more years of research. Technical hurdles include developing the ability to control the differentiation of stem cells into a desired cell type (like a heart or nerve cell) and to ensure that uncontrolled development, such as a cancerous tumor, does not occur. Some experiments may involve the creation of a chimera, an organism that contains two or more genetically distinct cell types, from the same species or different species. If stem cells are to be used for transplantation, the problem of immune rejection must also be overcome. It is hoped that the creation of many more embryonic stem cell lines will eventually account for all the various immunological types needed for use in tissue transplantation therapy. Others envision the eventual development of a "universal donor" type of stem cell tissue, analogous to a universal blood donor. However, if the Somatic Cell Nuclear Transfer (SCNT) technique/cloning was employed using a cell nucleus from the patient, stem cells created via this method would be genetically identical to the patient, would presumably be recognized by the patient's immune system, and thus would avoid any tissue rejection problems that could occur in other stem cell therapeutic approaches. Because of this, many scientists believe that the SCNT technique may provide the best hope of eventually treating patients using stem cells for tissue transplantation.

ES cell cultures from piscine species

Embryonic stem (ES) cells are undifferentiated cells derived from early developing embryos of animals, characterised by their capacity for self-renewal and pluripotency. These cells retain their pluripotency after long-term cultivation *in vitro* and can be induced to differentiate into a variety of cell types. When introduced into host embryo, the ES cells can participate in normal development and contribute to several tissues of the host, including cells of the germ line. These characteristics make ES cells ideal experimental systems for *in vitro* studies of embryonic cell development and differentiation and a vector for the efficient transfer of foreign DNA into the germ line of an organism. In addition, ES cells provide an attractive strategy for the preservation of biodiversity (Hong *et al*., 1996). These cells have the potential to produce any type of cell of the body and can be propagated in unlimited quantities, which led to the importance of human ES cells (hESCs) in regenerative medicine and treatment of a variety of diseases.

In spite of the discouraging situation encountered in other animal species, fish are especially suited for developing ES cell technology for two reasons *viz.*, piscine species are of considerable interest for both basic studies in molecular, cellular, and developmental biology as well as for commercial interest. As model vertebrate organisms, zebrafish and medaka are competitive with mouse for the analysis of gene functions relevant to humans. In the aquaculture activity, there is an increasing interest for incorporating new fish species for diversification. Fish have several technical advantages over other vertebrates such as high fecundity, large transparent embryos, and rapid development. Fish embryos often develop outside the mother, which is a big advantage for initiating embryonic stem cell cultures from fish compared to mouse. The starting point is the mid-blastula (MB) embryo. The first goal in developing fish ES cells was to establish conditions that supported growth of the embryonic stem cells while avoiding spontaneous differentiation. ES cell-mediated gene transfer is a promising approach for producing site mutated transgenic fish with enhanced growth rates or disease resistance, as well as for analyzing functions of fish genes (Melamed *et al.*, 2002). ES cells along with other cellular based strategies, such as primordial germ cells and nuclear transfer, allow selecting the desired transformation events before transferring the transgene to the whole animal.

Another important aspect of piscine cells is their lowest position in the ladder of evolution, which makes them suitable for xenotransplantation in mammals (Wright and Yang, 1997; Laue *et al*., 2001; Wright and Pohajdak, 2001). The islet tissue in certain teleost fish like tilapia called Brockmann bodies has been shown to restore normoglycemia on transplantation into diabetic nude mice (Wright and Pohajdak, 2001). Similarly, reversal of streptozotocin-diabetes has been achieved after transplantation of piscine islets to nude mice (Laue et al., 2001). These reports indicate potential of piscine cells for clinical applications.

To develop ES cell lines and gene targeting techniques in fish, extensive studies have been done in small model fishes such as zebrafish and medaka, because they offer the possibility of combining embryological, genetic, and molecular analysis of vertebrate development. ES-like cell lines have been established in medaka (Wakamatsu *et al*., 1994; Hong *et al*., 1996) and zebrafish (Collodi *et al*., 1992; Sun *et al*., 1995). One medaka ES-like cell line, MES1, was shown to retain a diploid karyotype and the ability to form viable chimeras (Hong *et al*., 1998).

Development of ES cell technology in model fish prompted application of feeder-free cell culture conditions to commercial species. The main objective was to improve the productivity of farmed fish by targeting genes related to commercial traits on ES cells, such as growth, or disease resistance. A technical goal was to genetically modify fish by making germline chimeras or by nuclear transfer (Bejar *et al*., 1999). The first, long-term, stable cell line from a commercial species was SaBE-1, which was derived from the marine fish gilthead seabream (Bejar *et al*., 1999). From this cell line a clonal culture was derived (SaBE-1c) that was screened for pluripotency *in vitro* and *in vivo* (Bejar *et al.*, 2002). Cells were characterized for proliferation, chromosome complement, alkaline phosphatase staining, telomerase activity, and induction of cell differentiation. Chimeric fish have been made in which all three embryonic germ layers are mosaic, but the efficiency was low in terms of survival and contribution of the donor cells (Bejar *et. al*., 2002). Long-term embryonic cell lines have been subsequently derived from species of commercial relevance in Asia. The first two were SBES1 from red seabream, *Pagrus major* (Chen *et al.*, 2003a) and LJES1 from sea perch, *Lateolabrax japonicus* (Chen *et al.*, 2003b). These two lines have been maintained for more than 50 passages *in vitro* and characterized for traits of pluripotency. They have been induced to differentiate into various cell types after treatment with retinoic acid (RA). Holen and Hamre (2003) derived a long-term embryonic stem cell–like culture from the turbot, *Scophtalmus maximus* and these cells expressed Oct4 transcription factor. Two other cell lines, FEC from the flounder (Chen *et al*., 2004) and TEC from turbot, *Scophthalmus maximus* (Chen *et al.*, 2005), have been developed and partially characterized. More recently Chen *et al*. (2007) developed pluripotency and chimera competence of an embryonic stem cell line from the sea perch (*L. japonicus*).

In spite of the feasibility of ES cell line derivation in commercial species, a main obstacle is the production and evaluation of chimeric animals resulting from technical disadvantages when compared with model species, including difficulties in handling and rearing, as well as long generation times (2 to 3 years to reach sexual maturity). In this way only chimeras have been obtained so far in the gilthead seabream and with low efficiency (Bejar *et al*., 2002). Because of the expensive and time-consuming process, the methodology of chimera production must be optimized in model species before application to commercial fish.

Fish embryonic stem cells - applications

There is scope for application of embryonic stem cell technology to commercial fish species to improve productivity by transgenesis. Research efforts on development of transgenic fish with enhanced resistance to pathogens and better growth and breeding performance/colour manipulations in ornamental fishes will be beneficial to the development of aquaulture technologies. ES cells, have the intrinsic ability to self-renew and can be applied to biodiversity rescuing, gene-targeting and germ-line transmission. In fisheries, there is scope for application of induced pluripotent stem cells (iPSCs) which are produced by reprogramming adult somatic cells using pluripotency genes, especially in the case of difficult to breed large sized marine fish species as well as in the case of endangered species. In human medicine, stem cell research offers the possibility of curing fatal and debilitating diseases; in aquaculture, it may enhance fish production and reduce environmental risks. Stem cell lines that could potentially be used to modify the genetic traits of

any fish species are being developed in different parts of the world. Fish ES/iPS cell lines have many avenues for fish biology, functional genomics, molecular embryology and conservation of biodiversity.

The piscine embryonic stem (ES) cells have attracted the attention of fish breeders and molecular biologists owing to its possible importance in producing transgenic fish with site-directed integration of foreign gene and in studying gene function in fish. ES cells provide unique tool for cell-mediated gene transfer and targeted gene mutations due to the possibility of *in vitro* selection of desired genotypes. When ES cells colonize germ cells in chimeras, transgenic animals with modified phenotypes are generated and used either for functional genomics studies or for improving productivity in commercial settings. Establishment of ES gene targeting techniques in cultured fish provides a novel approach for genetic improvements; developmental biology and analysis of gene function in fish.

Embryonic stem (ES) cells represent promising cellular vehicle for the production of genetically modified fish. ES cells provide unique tool for cell-mediated gene transfer and targeted gene mutations due to the possibility of *in vitro* selection of desired genotypes. When ES cells contribute to the germ line in chimaeric embryos, transgenic animals may be generated with modified genetic traits. Though the ES cell approach has up to now been limited to mice, there is an increasing interest to develop this technology in both model and commercial fish species, with so far promising results in the medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). ES cell lines have also potential application for colour manipulations in ornamental fish and also as model systems for therapeutic research in aquaculture.

Taking into consideration, the non-invasive and easy access to fish embryos due to its natural oviparous mode of reproduction as compared to the viviparous mode of mammalian reproduction, fish embryos neither evoke ethical issues nor involve invasive interventions. However, ethical issues involved in using human embryos for deriving ES cells have led to the development of induced pluripotent stem cells (iPSCs). iPSCs are produced by reprogramming somatic cells using pluripotency genes. iPSCs were first developed by Takahashi and Yamanaka (2006) from mouse fibroblasts and later from human somatic cells by several workers (Thompson *et al*., 2007; Okita *et al*., 2008; Woltjen *et al*., 2009). These pluripotent cells can differentiate into any type of cell in the body and proliferate indefinitely in culture. In fisheries, there is scope for application of iPS cell technology especially in the case of difficult to breed large sized marine fish species as well as in the case of endangered species. iPS cell lines developed by reprogramming primary fibroblasts from adult fish tissues could be attempted for deriving germ cells and subsequently for development of surrogate broodstock technology. Surrogate broodstocking could be made use of especially in the case of large aquaculture species or endangered species of fish by transplanting germ cells into small fish species that matures fast, and then use the eggs produced for larval production. This technology has already been attempted to produce eggs of endangered species of trout in salmon (Okutsu *et al*., 2009)

Laboratory fish species, in particular zebrafish and medaka, have been the focus of research towards stem cell cultures. Medaka is the second organism (next mouse) that generated ES cells and the first that gave rise to a spermatogonial stem cell line capable of test-tube sperm production. Most recently, the first haploid stem cells capable of producing whole animals have also been

generated from medaka. ES-like cells have also been reported in zebrafish and several marine species. Attempts for germline transmission of ES cell cultures and gene targeting have been reported in zebrafish. Recent years have witnessed the progress in markers and procedures for ES cell characterization. These include the identification of fish homologs/paralogs of mammalian pluripotency genes and parameters for optimal chimera formation. In addition, fish germ cell cultures and transplantation have attracted considerable interest for germline transmission and surrogate production. Haploid ES cell nuclear transfer has proven in medaka the feasibility of semi-cloning as a novel assisted reproductive technology. These pioneer experiments demonstrate the possibility of surrogate production of aquaculture broodstock by germ cell transplantation. This approach might be extended to propagate/restore a population of endangered species in conservation biology.

The derivation of germ cells from fish embryonic stem cells (ESCs) or induced pluripotent stem (iPSCs) cells represents a desirable experimental model and potential strategy for improving reproductive performance of commercially important difficult to breed fish species. Moreover, ES cells may be a method to preserve biodiversity in species for which embryo or gamete cryopreservation is not possible. Recent progress in fish stem cell culture and transplantation will provide valuable systems and tools for basic research and applications in sustainable aquaculture and fish biodiversity conservation

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