PART I. HUMAN STEM CELL TECHNOLOGY

This first part offers common definitions and explanations of key concepts about stem cells in order to assist the reader in understanding the key events in stem cell technology and the subject matter for which patent protection is claimed.

CHAPTER 1. BASIC CONCEPTS

A. Definition

A stem cell is an (1) undifferentiated cell (2) that can divide without limit and (3) whose progeny includes both further stem cells or cells destined to differentiate [3]. In other words, stem cells are (1) unspecialised cells (2) that can divide continuously (3) to produce cells like themselves (self-renewal), or cells of one or several specific differentiated types [4]. Several different sorts of differentiated cells can be formed by the progeny of a stem cell under appropriate conditions [5].

B. Types of stem cells

A distinction can be made between pluripotent and multipotent stem cells.

1. Pluripotent stem cells

Stem cells are called pluripotent when (1) they can develop into *any* cell type in the body that develops from the three germ layers (mesoderm, endoderm and ectoderm) from which all the cells of the body arise [6] and when (2) they can proliferate – in the laboratory (*in vitro*) indeed – *indefinitely*.
In other words, stem cells can be cultured indefinitely in an undifferentiated state, yet retain the ability to be differentiated into a variety of cell and tissue types, yet have the potential to develop almost all of the more than 200 different known cell types [7].

Stem cells with this unique property come from embryos and foetal tissue.

a. Embryonic stem cells

Pluripotent stem cells that are isolated from the inner cell mass of blastocyst-stage embryo (4- to 5-day embryo) are termed *embryonic stem cells ("ES cells")* [8].

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[3] FUCHS, E. & SEGRE, J.A., 100 *Cell*, 2000, 143-155; SLACK, J., *Essential Developmental Biology*, Blackwell Science, 2001.

[4] Cf. EUROPEAN GROUP ON ETHICS (EGE), *Opinion on Ethical Aspects of Human Stem Cell Research and Use*, Paris, 14 November 2000 (abbr. EGE, 2000), 5; NATIONAL INSTITUTES OF HEALTH (NIH), *Stem Cells: Scientific Progress And Future Research Directions*, June 2001 (abbr. NIH, 2001), p. 24; p. ES-2; THOMSON, J., 'Primate Embryonic Stem Cells – US patent 6.200.806, column 1.

[5] SLACK, J., 'Skinny Dipping for Stem Cells', *Nature Cell Biology*, 2001.

[6] NIH, 2001, ES-2.

[7] GEARHART et al., 'Human Embryonic Pluripotent Germ Cells' – US patent 6.090.622, column 4.

[8] EVANS & KAUFMAN, 'Establishment in Culture of Pluripotential Cells from Mouse Embryos', 292, *Nature*, 1981, 154-156; SCOTT, G.F., *Developmental Biology* (Sixth edition), Sunderland – Massachusetts, Sinauer Associates Inc., 2000, 98 and 592. Cf. EGE, 2000, 5; NIH, 2001, ES-2.

Embryos could be produced either by in vitro fertilisation (IVF) or by transfer of an adult nucleus to an enucleated egg cell or oocyte (somatic cell nuclear transfer, SCNT) [9].

Embryonic stem cells are pluripotent, not totipotent. At the blastocyst stage they can no longer develop into an embryo of their own. Evidence is emerging that these cells do not behave in the laboratory as they would in the developing embryo. If they are transferred to the uterus, they would neither implant nor develop into an embryo [10].

b. Embryonic germ cells

Pluripotent stem cells, which are isolated from the primordial germ cells located in the genital ridges/gonadal ridge of the 5- to 10-week foetus, are called *embryonic germ cells ("EG cells")* [11].

Embryonic stem cells and embryonic germ cells are both pluripotent, but they are not identical in their properties and characteristics [12].

2. Multipotent stem cells

Stem cells are called multipotent when (1) they can give rise to *several terminally differentiated* cell types constituting a specific tissue or organ and (2) usually have a *limited time span*. One example are skin stem cells which give rise to epidermal cells, sebaceous glands and hair follicles. Another example are haematopoietic stem cells, which give rise to all the diverse blood cells (erythrocytes, lymphocytes, antibody-producing cells, etc.). Yet another example are neural stem cells, which give rise to all the cell types in the nervous system including glia (sheath cells) and the many different types of neurons [13].

Multipotent stem cells can be of foetal or adult origin.

a. Foetal stem cells

Multipotent stem cells like neural stem cells can be isolated from foetal neural tissue and multiplied in culture [14]. Multipotent stem cells like haematopoietic stem cells can be retrieved from the umbilical cord blood [15].

b. Adult stem cells (Tissue stem cells)

For some photographs of mice embryo's in the blastocyst phase, see SCOTT, 2000, Figure 11.22.F. (p. 356) and Figure 11.25 (p. 357). For some schemes, see SCOTT, 2000, Figure 11.32 (p. 363).

9 EGE, 2000, 6.

10 EGE, 2000, 6; NIH, 2001, ES-2. Cf. THOMSON, J., 'Primate Embryonic Stem Cells – US patent 6.200.806, column 1 : "Embryonic stem cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ or tissue type or, at least potentially, *into a complete embryo*".

11 MATSUI et al., 353, *Nature*, 750-751; SCOTT, G.F., *Developmental Biology* (Sixth edition), Sunderland – Massachusetts, Sinauer Associates Inc., 2000, 592; RESNICK et al., 'Long-term proliferation of mouse primordial germ cells in culture', 359, *Nature*, 1992, October 8, 550-551. Cf. EGE, 2000, 5; NIH, 2001, ES-2.

12 NIH, 2001, ES-2.

13 EGE, 2000, 5.

14 EGE, 2000, 6.

15 EGE, 2000, 6.

Multipotent stem cells present in adults are called *adult stem cells* or *tissue stem cells*. An adult stem cell is an undifferentiated cell that is found in a differentiated (specialised) tissue in the adult. It can yield the specialised cell types of the tissue from which it originated. In the body, it too, can renew itself [16].
Mammals appear to contain some 20 major types of somatic stem cells that can generate liver, pancreas, bone and cartilage. The prototype is the well-know haematopoietic stem cell, which resides in the bone marrow. Another example are neural stem cells.

Adult stem cells usually divide to generate *progenitor* or *precursor* cells [17]. Progenitor cells can be distinguished from adult stem cells in the following way: when a *stem cell* divides, one of the two new cells is often a stem cell capable of replicating itself again. In contrast, when a *progenitor cell* divides, it can form more progenitor cells or it can form two specialised cells, neither of which is capable of replicating itself [18]. Progenitor cells differentiate or develop into 'mature' cell types that have the characteristic shapes and specialised functions, e.g. muscle cell contraction or nerve cell signalling [19].
One example are epidermal stem cells or spermatoginial stem cells which can differentiate respectively into only keratinocyctes and spermatozoa [20].
Progenitor cells occur in adult or foetal tissue.

CHAPTER 2. RESEARCH RESULTS OF STEM CELLS

A. Animals

1. Pluripotent stem cells

Much basic understanding about embryonic stem cells has come from animal research, more in particular research using mice. Scientists have been working with mouse embryonic stem cells in vitro for more than 20 years.
Subsequent to the work with mouse embryos, several groups have attempted to develop stem cell lines from sheep, pig and cow. A cell line with embryonic stem cell-like appearance has reportedly been cultured from porcine embryos using culture conditions similar to mouse. Other groups have developed avian stem cell lines from chickens [21].

Research has pointed out that mouse embryonic stem cells or mouse embryonic germ cells (and certain embryonic carcinoma cell lines) maintain an *undifferentiated* state in vitro when cultured on a feeder layer of fibroblasts (such as murine STO cells), when cultured in a medium conditioned by certain cells or by the exogenous addition

[16] NIH, 2001, ES-1.
[17] NIH, 2001, ES-2.
[18] NIH, 2001, ES-3. EGE in this respect erroneously speaks about progenitor *stem* cells (EGE, 2000, 5).
[19] NIH, 2001, ES-2.
[20] EGE, 2000, 5.
[21] GEARHART, J. & SHAMBLOTT, M.J., Human Embryonic Pluripotent Germ Cells – US patent 6.090.622, column 2 and the references cited there.

of Leukaemia Inhibitory Factor (LIF). Using the appropriate culture conditions, such cells can be grown relatively indefinitely.
Embryonic stem cells and embryonic germ cells can be induced to *differentiate* in vitro using retinoic acid or spontaneously by removal of the feeder layer conditioned media or exogenous LIF [22].

The research on mouse embryonic stem cells led to believe that mouse embryonic stem cells are fully pluripotent in the sense that they can colonise all tissues when transplanted into another pre-implantation embryo [23].
Other search on mouse embryonic stem cells has been focused on using these cells to create transgenic animals [24]. Such methods have a number of advantages as compared with more conventional techniques for introducing new genetic material into such animals, such as zygote injection and viral infection [25]. First, the gene of interest can be introduced and its integration and expression characterised in vitro. Second, the effect of the introduced gene on the embryonic stem cell or embryonic germ cell growth can be studied in vitro. Third, the characterised embryonic stem cells or embryonic germ cells having a novel inserted gene can be efficiently introduced into embryos by blastocyst injection or embryo aggregation and the consequences of the introduced gene on the development of the resulting transgenic chimeras monitored during prenatal or postnatal life. Fourth, the site in the embryonic stem cell or embryonic germ cell genome at which the introduced gene integrates can be specified permitting subsequent gene targeting and gene replacement [26].

2. Multipotent stem cells

Research is also carried out on mouse adult stem cells. While many scientists have assumed that these cells were programmed to produce specific tissues and were no longer able to produce other sorts of tissues, recent studies suggest that adult stem cells may be able to show more malleability than previously believed. For instance, it has been shown that mouse neural stem cells could give rise, in specific conditions of culture, to cells of other organs such as blood, muscle, intestine, liver and heart. Moreover, marrow stromal cells can generate astrocytes, a non-neuronal type of cells of the central nervous system and haematopoietic stem cells can give rise to myocytes [27].

B. Humans

1. Pluripotent stem cells

Although the study of mouse embryonic stem cells provided clues to understanding the differentiation of general mammalian tissues, dramatic differences in primate and mouse development of specific lineages limits the usefulness of mouse embryonic

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[22] GEARHART, J. & SHAMBLOTT, M.J., Human Embryonic Pluripotent Germ Cells – US patent 6.090.622, column 1-2 and the references cited there; THOMSON, J., 'Primate Embryonic Stem Cells – US patent 6.200.806, column 1 and the references cited there.
[23] SLACK, J., 'Skinny Dipping for Stem Cells', 3, *Nature Cell Biology,* September 2001, (205), 205.
[24] EGE, 2000, 7.
[25] US patent 6.090.622.
[26] THOMAS, K.R. & CAPECCI, M.R., 51 *Cell*, 1987, 503-512.
[27] EGE, 2000, 7.

stem cells as a model of human development [28]. Because humans are primates, and development is remarkably similar among primates, the interest of research shifted towards primate embryonic stem cells, hoping primate embryonic stem cells would provide a faithful model for understanding the differentiation of primate tissues in general and human tissues in particular [29].

In November 1998 James THOMSON and his team from the University of Wisconsin in Madison announced the isolation of cells from 14 human blastocysts, obtained from donated surplus embryos produced by in vitro fertilisation, and the development of the five human *embryonic stem* cell lines [30]. At the same time, John GEARHART and this team at the Johns Hopkins University in Baltimore reported the first derivation of human *embryonic germ* cells from an isolated population of cells in foetal gonadal tissue, known as the primordial germ cells [31].
From both of these sources, the scientists developed pluripotent stem cell "lines", which are capable of renewing themselves for long periods and giving rise to many types of human cells or tissues [32].

In recent months, other investigators have been successful in using somewhat different approaches to deriving human pluripotent stem cells. At least 5 other laboratories have been successful in deriving pluripotent stem cells from human embryos and one additional laboratory has created cell lines from foetal tissue.
In each case, the methods for deriving pluripotent stem cells from human embryos and embryonic germ cells from foetal tissue are similar, yet they *differ* in the isolation and culture conditions as initially described by THOMSON and GEARHART, respectively [33].

2. Multipotent stem cells

At present, there have been mulitple human *adult* stem cell lines that have been created. Few experiments have revealed that their plasticity was much higher than previously thought. Adult *neural* stem cells have been reported to give rise occasionally to other cell types including blood cells. A team at the University of Minnesota in Minneapolis has shown that cells isolated from the bone marrow of adults or children were able to become neural or muscle cells [34].
A major disease for which stem cells have already shown therapeutic potential is *cancer*. The transplantation of human *haematopoietic* stem cells is routinely used to restore the production of blood cells in patients affected by leukaemia or aplastic anaemia after chemotherapy. Adult stem cells, retrieved from the bone marrow of donors, as well as foetal stem cells, retrieved from the umbilical cord blood at birth, can be used as a source for haematopoietic stem cells [35].

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[28] The placenta provides just one example of the dramatic difference between mice and humans. Chorionic gonadotropin, expressed by the trophoblast, is an essential molecule involved in maternal recognition of pregnancy of all primates, including humans, see THOMSON, J., 'Primate Embryonic Stem Cells' – US patent 6.200.806.
[29] THOMSON, J., 'Primate Embryonic Stem Cells' – US patent 6.200.806.
[30] EGE, 2000, 8; NIH, 2001, ES-4. Also see infra.
[31] EGE, 2000, 8; NIH, 2001, ES-4. Also see infra.
[32] NIH, 2001, ES-4.
[33] NIH, 2001, ES-4.
[34] EGE, 2000, 8; NIH, 2001, ES-4.
[35] EGE, 2000, 8.

CHAPTER 3. APPLICATIONS AND PROSPECTS OF HUMAN STEM CELLS

A. Basic research

Culturing human embryonic stem cells will undoubtedly be key research tools for understanding fundamental events in embryonic development that one day may explain the causes of birth defects, infertility, pregnancy loss and approaches to correct or prevent it [36].

Another important area of research that links developmental biology and stem cell biology is understanding the genes and molecules, such as growth factors and nutrients, that function during the development of the embryo so that they can be used to grow stem cells in the laboratory and direct their development into specialised cell types [37].

B. Therapeutic potential

1. Cell and tissue replacement/transplantation (somatic cell therapy)

The recent interest in stem cells arises largely because it is thought that they might be of use in various incurable degenerative diseases or offer the prospect of tissue repair following trauma [38]. It is hoped that human embryonic *cells* could be grown in vitro and then caused to differentiate into the cell type required for transplantation by exposure to suitable culture conditions [39].

Fare more remote is the prospect of being able to grow whole *organs* in vitro. If tissues for the repair of organs become available, it would relieve the existing unsatisfied demand for donated organs for transplantation [40].

a. Diseases

Stem cells may hold the key to replacing cells lost in many devastating diseases. There is little doubt that this potential benefit underpins the vast interest about stem cell research. Research is being carried out, aiming at directing the differentiation of *pluripotent* stem cells to produce pure populations of particular cell types to be sued for the repair of diseased or damaged tissues.

A major focus of research is the use of stem cells to generate replacement tissues for treating neurological diseases. For many diseases, there are no effective treatments but the goal is to find a way to replace what natural processes have taken away. *Parkinson's disease and Alzheimer's disease* are among those diseases for which the

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[36] EGE, 2000, 9; NIH, 2001, ES-5.
[37] NIH, 2001, ES-5.
[38] SLACK, J., 'Skinny Dipping for Stem Cells', 3, *Nature Cell Biology*, September 2001, 205-206.
[39] SLACK, 2001, (205), 205. For some clarifying figures, see SCOTT, G.F., *Developmental Biology* (Sixth edition), Sunderland – Massachusetts, Sinauer Associates Inc., 2000, Figure 4.22 A & B, p. 101.
[40] EGE, 2000, 9.

concept of replacing destroyed or dysfunctional cells in the brain or spinal cord is a practical goal [41].

Another major discovery frontier for research on adult and embryonic stem cells is the development of transplantable pancreatic tissues that can be used to treat *diabetes.* Scientists in academic and industrial research are vigorously pursuing all possible avenues of research, including ways to direct the specialisation of adult and embryonic stem cells to become pancreatic islet-like cells that produce insulin and can be used to control blood glucose levels. Researchers have recently shown that human embryonic stem cells to be directly differentiated into cells that produce insulin [42].

b. Trauma

The transplantation of stem cells could also help to repair spinal cord damage which occurs frequently, mainly following trauma (for instance car accidents) and is responsible for paraplegia [43].

2. Gene delivery system (somatic cell gene therapy)

Stem cells are already being explored as a *vehicle (vector) for delivering genes* to specific tissues in the body [44].

One current application in clinical trials is the use of haematopoietic stem cells genetically modified to make them resistant to the HIV virus, which is responsible for *AIDS* [45].

Stem cell-based therapies are also a major area of investigation in *cancer* research. For many years, restoration of blood and immune system function has been used as a component in the care of cancer patients who have been treated with chemotherapeutic agents. Now, researchers are trying to devise more ways to use specialised cells derived from stem cells to target specific cancerous cells and directly deliver treatments that will destroy or modify them [46].

C. Toxicology testing

Another future use of human stem cells and their derivatives include the toxicology testing of candidate therapeutic drugs. Although animal model testing is a mainstay of pharmaceutical research, it cannot always predict the effects that a developmental drug may have on human cells. Stem cells might be used to develop specialised liver cells to evaluate drug detoxifying capabilities and. Stem cell technology represents a new type of early warning system to prevent adverse reactions in patients. Such rapid screening of large numbers of candidate drugs, is the most likely immediate biomedical application [47].

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41 EGE, 2000, 9; NIH, 2001-ES-4.
42 EGE, 2000, 9; NIH, 2001-ES-5.
43 EGE, 2000, 9.
44 EGE, 2000, 9; NIH, 2001, ES-5.
45 EGE, 2000, 9.
46 NIH, 2001, ES-5.
47 EGE, 2000, 9; NIH, 2001, ES-5.

Future uses of human pluripotent cell lines might include the exploration of the effects of chromosomal abnormalities in early development. This might include the ability to monitor the development of early childhood tumors, many of which are embryonic in origin [48].

D. Related technology. Somatic cell nuclear transfer

Another important aspect of developing therapies based on stem cells is devising ways to prevent the immune system of recipients from rejecting the donated cells and tissues that are derived from human pluripotent stem cells. In view of the avoidance of immune rejection of cells or tissues developed from embryonic stem cells, research is at present pursued in the field of somatic cell nuclear transfer (SCNT). Neural tissues can be sometimes transplanted from one individual to another without suffering immunological rejection, but it has been reported that for all other tissues, stem cell therapy would need to be accompanied by long-term treatments with immunosuppresive drugs, leading to increased susceptibility to infections and even to cancer [49].

SCNT comprises the transfer of nuclei from the patient's own body cells into donated human – or even animal – unfertilised eggs from which the nuclei have been removed, after which the reconstructed eggs are stimulated – with electricity for example – to develop the blastocyst stage. Pluripotent stem cells could then be derived, to form cells genetically identical to the patient. As a result, no rejection of any transplanted cells would take place [50].

(If the reconstructed embryos were transferred to a woman's uterus, this could lead to the cloning of human individuals).

[48] NIH, 2001, ES-5.
[49] EGE, 2000, 10.
[50] EGE, 2000, 10.