Stem Cell Technology and Other Innovative Therapies
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INTRODUCTION

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Since the discovery of the chemical nature of the DNA molecule, unprecedented progress has been made in the Biological Sciences during the past 50 years. One can imagine that this period is only a beginning and that the Golden Age of Biology will continue for a long period of time. The increase in our knowledge is due, in large part, to the advent of molecular biology and the ability to clone and manipulate genes with the goal of exploring their function and mode of action. These new scientific developments made it possible to approach the central question of what characterizes living organisms and how they function. Thus, considerable advances have been made in our understanding of the biology of the cell which is the universal constitutive unit of all organisms, of developmental biology (that is, how a complex organism is built up from a single cell, the fertilized egg), as well as all branches of physiology such as immunology and neuroscience.

All these advances have *benefited medicine* in a major way. I would like to highlight organ transplantation as an example which is particularly relevant to this symposium on stem cells. To replace an organ, or a part of the body which is deteriorated or does not function, with a new one removed from another human being, has been a dream of humanity for a long time. The scene represented in Figure 1 (see page 137), the painting of Fernando del Rincon (1450-1517), illustrates Saint Come and Saint Damien who had exchanged during his sleep the leg (that was being destroyed by gangrene) of one of their servants with the leg of a Maure that had just died.

Unfortunately, at that time, transplantation was only a myth. Now it is possible to graft organs safely, thanks to the discovery of the immunosuppressive effect of a natural compound (cyclosporine), produced by a fungus. For a long time, organ transplantation was considered as unacceptable and unethical, but it is now performed routinely and it has saved many lives.

The aim of the Symposium that Prof. Thierry Boon and I have organized, concerns the possibility that novel therapies may be foreseen due to the remarkable progress that has been made both in developmental and cell biology (see section I of the program), and in immunology and genetics (see section II of the program).

I would like to introduce the topic of Stem Cell Biology that was discussed by experts in the field. First, I wish to thank them warmly for accepting the invitation of the Pontifical Academy of Sciences to participate in this symposium. Since they are among the most renowned leaders in this very active field, they are much solicited. Thus I appreciate their efforts to attend this event. I also want to thank the President and the Chancellor of the Academy who have welcomed our proposition to discuss these topics at the Vatican.

The issue of stem cells has attracted a great deal of interest during the last few years because it provides the hope that stem cells may be used in the future for replacing cells which are deficient or which have been subjected to abnormal and massive death, as it is the case in some degenerative diseases. Grafting cells for therapeutic purposes has been ongoing for a long time in the case of bone marrow or skin transplantation. *But now we are faced with the exciting prospect that cells with large differentiation potential, that are maintained in vitro in a normal proliferative state, could provide a variety of cell types for transplantation and therefore help cure diseases for which there is as yet no other treatment.*

I first discuss how the concept of the stem cell has emerged and second, the different types and sources of stem cells that have been described.

The Stem Cells Concept

The current definition of the term *stem cell* is that of a cell which is pluripotent and, on the one hand yields cells that differentiate into various phenotypes, while on the other hand, it produces cells that do not differentiate, remain pluripotent and similar to the mother cell.

Therefore, stem cells undergo asymmetrical divisions which not only allow them to self renew but also to replace differentiated cells that have died. Stem cells themselves divide slowly but they yield a pool of rapidly proliferating derivatives or tissue-specific precursors.

The first experimental demonstration that stem cells exist goes back to the early 1960s when the hematopoietic system was shown to harbor single cells responsible for the renewal of the circulating blood.

Earlier, Wilson (1925) (1) had introduced the term of stem cells in his description of the development of the *Ascaris* worm. In Wilson's view, the early steps of egg segmentation generate stem cells which undergo asymetric divisions, so that each time they divide, they give rise to both a cell similar to themselves and a cell with a different fate.

This scheme was not based on experimental evidence but on a conceptual view deduced from the careful analysis of the *Ascaris* cell lineage. Experimental data were first provided by studies carried out on hematopoiesis.

The Hematopoietic Stem Cell

It had long been known that cells present in the circulating blood have a short life span and are regularly renewed. The fact that the bone marrow contains a large number of cells that are immature forms of the different cell types present in the blood has designated this tissue as an important site for blood cell production.

During the first half of the 20th century, it was found that living cells are more vulnerable to X irradiation while they are dividing than during interphase. This explains why full body irradiation of mice with a sufficient dose of X-rays is lethal after a few days and that this death is due to the loss of blood cells and to aplasia of the hematopoietic tissues, namely the bone marrow and the spleen which becomes greatly reduced in volume. However, these mice can be rescued by injections of immunocompatible bone marrow cells, which reconstitute the pool of circulating cells as well as the population of precursors in the bone marrow. This process leads to long term reconstitution of the blood cells and restores the spleen to its normal size.

The fact that all types of blood cells can originate from a single cell which is endowed with self-renewal capacity was demonstrated by Till and McCulloch in 1961 (2).

Till and McCulloch rescued lethally irradiated mice with 10^5 bone marrow cells, instead of the usual millions of cells. In this case, the volume of the spleen did not increase globally, as in the previously performed experiments. In contrast, 8 to 10 bulges developed on its surface. These structures corresponded to colonies of hematopoietic cells (HC) that appeared to have developed independently from one another due to the rare seeding of the injected bone marrow cells. An analysis of each colony revealed that each contained all the blood cell types (except lymphocytes). Moreover, the new cells were shown to all be derived not from the host, but from the

injected donor bone marrow. To test if the founder cells were heterogeneous in nature, a few donor cells were labelled with a chromosomal marker and each colony was found to contain cells of one type or the other, but both cell types (with or without the nuclear marker) were never found in the same colony. This finding indicated that each colony was derived from one single progenitor that was designated a colony-forming unit-spleen: CFU-S. A clear demonstration that the CFU-S were cells endowed with self-renewal capacity derived from the following experiment: bone marrow cells were injected into a lethally irradiated mouse in which spleen colonies developed, as described above. These colonies were used as a source of cells to reconstitute a second lethally irradiated mouse. In this manner, the hypothesis was tested that long term reconstitution could occur only if the colony contained, not only dividing progenitors, but also stem cells capable of renewing these progenitors once they were differentiated. Indeed, this was exactly what was observed. The CFU-S had generated not only the differentiating precursors that had developed in the colonies but also another round of CFU-S that could form colonies in the spleen of a third lethally irradiated recipient. In addition, if the cells of these colonies were seeded at low density in a culture dish containing an appropriate culture medium, after 10 to 15 days, some of these cells generated colonies which contained several types of blood cells, as well as CFU-S capable of further propagations. These results were obtained by Don Metcalf in Australia and by Leo Sachs in Israel. Furthermore, the *in vitro* methods for growing HSC led to the discovery of growth factors that favor the differentiation of a given phenotypes of blood cells (3). For example, *erythropoietin* is a critical cytokine for red blood cell development.

After many decades of work the present view is that a pluripotent hematopoietic stem cell is able to generate i) *the stem cell* (SC) for the myeloid lineages: corresponding to the CFU-S which gives rise to erythrocytes and to other types of white blood cells, and ii) *the lymphoid SC* which generates the two types of lymphocytes, T and B.

The pluripotent SC are not actively dividing and, for this reason, they are radioresistant. They yield lineage restricted stem cells which divide actively under the influence of precise mixtures of growth and survival factors. This cellular compartment is destroyed by X-irradiation.

Progress has been made since these pioneering experiments, and although stem cells which divide slowly are rare, they are now well characterized particularly thanks to the work of Prof. Irving Weissman. For example, in the mouse, the HSC are recognizable because they express cell sur-

face molecules designated CD34, ckit, and sca-1 and, at the same time, are devoid of all the antigens that are specifically expressed by cells of the various differentiated blood cell lineages. A chapter by Prof. I. Weissman describes the hematopoietic and other stem cells in greater detail.

Other Types of Stem Cells

Blood cells are not the only cells that are replaced throughout life and stem cells are present in many tissues such as skin, skeletal muscle and intestinal epithelium in which cells are constantly renewed. Their presence has been demonstrated also in the liver and even in the central and peripheral nervous systems (CNS, PNS).

Under normal circumstances, these stem cells give rise to a progeny which is restricted to the cell types of the tissue to which they belong.

Consequently, owing to the presence of their stem cells, mammals are endowed, at least to a certain extent, with tissue regenerative capacity.

Regeneration in the Animal Kingdom

The capacity to regenerate tissues and organs is widespread in the animal kingdom, since it exists in species belonging to virtually all groups of animals. Only in nematodes and cephalocordates has no case been reported, perhaps because the possibility has yet to be investigated.

The strategies developed by nature to regenerate tissues are of these different types. First, in certain animals, like Planaria, which are freshwater flat worms 3-4 cm long, the capacity to regenerate relies on the fact that they possess, even in the adult, pluripotent embryonic-like stem cells, designated *neoblasts*, which are dispersed throughout the body. When the differentiated cells composing their organs have exhausted their normal life span, they are replaced by the progeny of neoblasts (4). Moreover, if the worm is cut into several pieces, each can reconstitute a complete organism due to the capacity of the neoblasts to proliferate, to form a regeneration blastema at the site of wound, and to differentiate those parts of the worm which are lacking in the correct antero-posterior and ventro-dorsal orientation. This regenerative capacity has been termed a 'secondary embryogenesis'.

Second, certain vertebrates, such as Urodeles are also endowed with considerable potential to regenerate including limbs, the jaw, eyes and tail. When the limb of a salamander is cut, the epidermis covers the lesion and a bud, similar to the regeneration blastema of Planarians, forms at the site of

the cut. This blastema grows and is progressively patterned to regenerate the part of the limb that was removed. In constrast to the blastemas which grow in Planarian worms and which are formed by neoblasts that have migrated to the wound site, regeneration in Urodeles results from the dedifferentiation and growth of the tissues located at the site of the cut.

Third, in mammals, removal or destruction of tissues is merely followed by healing: the epidermis lining the lesion covers the exposed internal tissues but this healing process does not involve the replacement of the removed tissue. However, even in higher vertebrates, stem cells, distributed in numerous types of adult tissues, are able to ensure the replacement of differentiated cells when they have reached the end of their normal life span.

In summary, a brief review of the regeneration processes in animals indicates that they rely on three different strategies:

i) mobilisation of embryonic-like pluripotent stem cells (e.g. Planaria)

ii) dedifferentation of already differentiated cells which then re-differentiate (e.g. Urodeles)

iii) turn-over of the cells in differentiated adult tissues via stem cells whose differentiation capabilities are thought to be restricted to the cell phenotypes of the tissues to which they belong.

Plasticity of the Commitment of Adult Stem Cells

The dogma according to which developmental potential of adult stem cells is restricted was challenged during the past few years by several authors. A large amount of work has been done in order to determine if the SC from adults could be used in cellular therapy in mouse and subsequently in human. Despite early reports that stem cells from the adult CNS or skeletal muscle could reconstitute the hematopoietic system of a lethally irradiated mouse (5), these results have not been confirmed and may well have been due to contamination by blood cells within these tissues (6). On the other hand, reports about cells from the adult bone marrow, used in many transplantation experiments, seem to indicate that these cells are endowed with far more extensive differentiation capabilities than previously thought (e.g. 7-9).

Although some of the results that have been reported in the flurry of scientific literature (most often in high standard journals) during the past few years failed to be confirmed by other research groups, this phenomenon reflects the great significance and high expectations that this technology will aid human health.

A curious and unexpected phenomenon was reported to occur when normal cells and Embryonic Stem cells (ES cells) were co-cultured *in vitro*: cell fusion. However, strong selective pressure led to the fusion of ES cells or hematopoietic stem cells with normal cells, thus generating rare aneuploid hybrids. Given the prior work of Harris, Ephrussi and Davidson, this was not surprising (e.g. 10). However, that fusion could also occur *in vivo* to form stable binucleate heterokaryons after injection of bone marrow cells was not anticipated. This finding was demonstrated by one of today's speakers, Prof. Helen Blau who will also give us an overview of the state of the art. In 1983, she first showed that previously silent genes could be activated in non dividing cell fusion products. This reprogramming of nuclear gene expression has now been shown by her and other groups to occur in heterokaryons that form *in vivo* during life (11-16). These and other findings suggest that adult stem cells hold promise for cellular therapy in certain human tissues based on currently available reliable experimental data.

Embryonic Stem Cells

The discovery of the embryonic stem cell constitutes one of the greatest achievements of modern biotechnologies. These cells are obtained from mammalian embryos at an early stage of development, when certain cells are still multipotent. Their history dates back to the 1950s (see 17 for references) when it became possible to fertilize the mammalian ovocyte *in vitro* and to culture the embryo up to the blastocyst stage. Once the blastocyst has 'hatched', i.e. has exited from the membrane (*zona pellucida*) that surrounds the egg, it can only survive and develop further if it becomes implanted into the uterine wall. At this stage it is a ball surrounded by a cell sheet that will become the placenta and is occupied by a cavity – the blastocoele – in which an '*inner cell mass*', will further develop into the embryo.

The potential to culture the mammalian embryo *in vitro*, an embryo which normally develops in the mother's womb, was an important advance: for the first time, the mammalian embryo could be subjected to close observation and experimentation. As an example, I wish to mention the remarkable historical experiments carried out independently by Andrzej Tarkowski in England and Beatrice Mintz in the US in the 1960s which demonstrated the high level of plasticity displayed by mouse embryos at early stages of their development. Two embryos were closely juxtaposed when the egg began to divide (*morula* stage), one derived from a strain with

white and the other with black fur. The two aggregated morulas were implanted into the womb of a foster mother where they developed into a single mouse which had stripes of black and white fur showing that cells from both embryos participated in the skin of the chimera. The same observation was made for virtually all tissues of the body.

These findings showed that at this early stage, the fate of embryonic cells is not yet determined and that the early mammalian embryo exhibits a large degree of plasticity. The demonstration that, at the blastocyst stage, each embryonic cell is essentially as totipotent as the egg itself was shown by an experiment in which a few cells of the *inner cell mass* were removed from a black strain embryo and introduced into the blastocoel of a white strain embryo. The injected cells adhered to the host's inner cell mass and participated in the formation of an embryo in which virtually all tissues were chimeric. The same result was obtained when one single cell was implanted: the tissues and organs of the recipient embryo were chimeric, showing the pluripotentiality of every single cell of the embryo at that stage (18).

In normal development, soon after the inner cell mass is formed in the embryo, decisive morphogenetic events take place: the cells become arranged into germ layers (ectoderm, mesoderm, endoderm) by the process called 'gastrulation' and they lose their totipotency to become restricted to definite fates. These experiments proved the existence of embryonic stem cells (below).

In 1981, Gail Martin in the US and Martin Evans in the UK (19-20) published articles reporting that the embryonic stem cells of the inner cell mass of the preimplantation embryo could be grown in tissue culture *in vitro* under conditions that allowed them to proliferate without differentiating. Cell lines of pluripotent embryonic stem cells (ES cells) could then be maintained in culture indefinitely. These results were striking because the embryonic stem cells obtained due to the pioneering work of Gail Martin and Martin Evans corresponded to the *in vitro 'capture and maintenance'* of a stage which in normal development is only transitory.

The ES cell lines have provided biologists with a remarkable tool with which to study the molecular mechanisms underlying the differentiation process. They have allowed gene targeting techniques to be developed in the mouse. Through this technique, the activity of virtually any gene of the mammalian genome can be either abolished or modified thus providing an experimental means to explore the role of these genes and their mode of action.

The ES cells can be maintained in an undifferentiated and proliferative state only if they are subjected to appropriate culture conditions involving factors which prevent them from differentiating. Otherwise,

they will give rise to a multitude of diverse cell types. If they are cultured in the usual type of medium, ES cells differentiate as they would in the course of normal embryogenesis. However, the ES cells are not able to give rise to an embryo within the culture dish and merely yield anarchically distributed differentiated tissues.

In the recent years, culture conditions that rely on the use of various cytokines and growth factors, have made it possible to induce the differentiation of a high proportion of ES cells into selected cell types such as neurons, pancreatic islet cells producing insulin, cardiomyocytes, etc… This has nurtured the hope of using these cells for therapy in order to replace cells that are deficient or have died in human tissues.

For many years, biotechnology was able to produce ES cells from only mouse embryos (and even exclusively from certain strains of mice such as the strain 129). This technology attracted more attention when in 1998, Thomson and his colleagues (21) published that they had been able to derive ES cells lines from human embryos. Moreover, it was shown that germ cells isolated from the gonads of older human embryos can also give rise to permanent lines of embryonic stem cells endowed with properties that are very close to those of the embryonic stem cells derived from the inner cell mass at the blastocyst stage. The stem cells that result from germ cell proliferation are designated as EG cells in order to distinguish them from the now classical ES cells (22).

Chapters in this book constitute symposium presentations by eminent specialists of the ES cells and germ cells. They discuss the possibilities that may be expected from these biotechnological advances to cure diseases for which there is currently no effective treatments: Prof. Ron McKay from the NIH, Prof. Rudolf Jaenisch from the MIT and Prof. Azim Surani from the University of Cambridge (UK).

These spectacular advances in cellular biotechnologies generate hope that human beings may be relieved from pain and disease. They, however, also raise undeniable ethical problems. The aim of this symposium volume is to inform as much as possible the members of the Pontifical Academy of the progress of science, as it stands at this moment, with the hope that these reports will stimulate future interest in following the progress that will certainly occur in this very rapidly moving field.

We, scientists, are convinced that we have the duty to provide everyone with all of the available information resulting from the advances of science, since we believe that knowledge is critical for every reasonable human to make informed ethical decision regarding medical interventions.

REFERENCES

1. Wilson, E.B., *The Cell in Development and Heredity*. Third edition, Macmillan (New York, 1925).
2. Till, J. and McCulloch, E.A., 'A direct measurement of the radiation sensitivity of normal mouse bone marrow cells', *Rad. Res.*, 14, pp. 213-222 (1961).
3. Moore, M. and Metcalf, D., *Hemopoietic Cells*, North-Holland Publishers (Amsterdam, 1971).
4. Newmark, P. A. and Alvarado, A.S., 'Not your father's planarian: a classic model enters the era of functional genomics', *Nat. Rev. Genet.*, 3, pp. 210-219 (2002).
5. Bjornson, C.R., Rietze, R.L., Reynolds, B.A., Magli, M.C. and Vescovi, A.L., 'Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*', *Science,* 283, pp. 534-537 (1999).
6. Jackson, K.A., Mi, T. and Goodell, M.A., 'From the cover: hematopoietic potential of stem cells isolated from murine skeletal muscle', *Proc. Natl. Acad. Sci. USA*, 96, pp. 14482-14486 (1999).
   McKinney-Freeman, S.L., Jackson, K.A., Camargo, F.D., Ferrari, G., Mavillo, F. and Goodell, M.A., 'Muscle derived hematopoietic stem cells are hematopoietic in origin', *Proc. Natl. Acad. Sci. USA*, 99, pp. 1341-1346 (2002).
   Olmsted-Davis, E.A., Gugala, Z., Camargo, F., Gannon, F.H., Jackson, K., Anderson Kienstra, K.A., Shine, D.H., Lindsey, R.W., Hirshi, K.K., Goodell, M.A., Brenner, M.,K. and Davis, A.R., 'Primitive adult hematopoietic stem cells can function as osteoblast precursors', *Proc. Natl. Acad. Sci. USA*, 100, pp. 15877-15882 (2003).
7. Ferrari, G., Cusella-de Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Mavilio, F., 'Muscle regeneration by bone marrow-derived myogenic progenitors', *Science,* 279, pp. 1528-1530 (1998).
8. Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C. D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A. and Verfaillie, C.M., 'Pluripotency of mesenchymal stem cells derived from adult marrow', *Nature,* 418, pp. 41-49 (2002).
9. Petersen, B.E., Bowen, W.C., .Patrene, K.D., Mars, W.M., Sullivan, A.K., Murase, N., Boggs, S.S., Greenberger, J.S. and Goff, J.P., 'Bone marrow as a potential source of hepatic oval cells', *Science,* 284, pp. 1168-1170 (1999).
10. Harris, H., *Cell fusion*, Harris H. ed., Harvard University Press (Cambridge, Mass, 1970).

11. Blau, H.M., Chiu, C.P. and Webster, C., 'Cytoplasmic activation of human nuclear genes in stable heterocaryons', *Cell,* 32, pp. 1171-1180 (1983).
12. Priller, J., Persons, D.A., Klett, F.F., Kempermann, G., Kreutzberg, G. W. and Dirnagl, U., 'Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells *in vivo*', *J. Cell Biol.,* 155, pp. 733-738 (2001).
13. LaBarge, M.A. and Blau, H.M., 'Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury', *Cell,* 111, pp. 589-601 (2002).
14. Weimann, J.M., Johansson, C.B., Trejo, A. and Blau, H.M., 'Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant', *Nat. Cell Biol.*, 5, pp. 959-966 (2003).
15. Weimann, J.M., Charlton, C.A., Brazelton, T.R., Hackman, R.C. and Blau, H.M., 'Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains', *Proc. Natl. Acad. Sci. USA,* 100, pp. 2088-2093 (2003).
16. Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J.M., Fike, J.R., Lee, H.O., Pfeffer, K., Lois, C., Morrison, S.J. and Alvarez-Buylla, A., 'Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes', *Nature,* 425, pp. 968-973 (2003).
17. Le Douarin, N.M., *Des chimères, des clones et des gènes*, Ed. Odile Jacob (2000).
18. Papaioannou, V.E., Mcburney, M.W., Gardner, R.L. and Evans, M.J., 'Fate of teratocarcinoma cells injected into early mouse embryos', *Nature,* 258, pp. 70-73 (1975).
19. Martin, G.R., 'Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells', *Proc. Natl. Acad. Sci. USA,* 78, pp. 7634-7638 (1981).
20. Evans, M.J. and Kaufman, M.H., 'Establishment in culture of pluripotential cells from mouse embryos', *Nature*, 292, pp. 154-156 (1981).
21. Thomson, J.A., Itskovitz-eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M., 'Embryonic stem cell lines derived from human blastocysts' (published erratum appears in *Science* 1998, 282, 1827), *Science,* 282, pp. 1145-1147 (1998).
22. Shamblott, M.J., Axelman, J., Wang, S., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R., and Gearhart, J.D., 'Derivation of pluripotent stem cells from cultured human primordial germ cells', *Proc. Natl. Acad. Sci. USA,* 95, pp. 13726-13731 (1998).

Figure 1. *Miracle of Saint Côme and Saint Damien*. Fernando del Rincon (1450-1517), Museum of Prado, Madrid.