Chapter 1
Stem Cells: Principles and Applications

Ágatha Oliveira, Juliana da Cruz Corrêa-Velloso, Talita Glaser, and Henning Ulrich

Abstract Stem cell research is a promising and markedly emerging area of investigation concerning basic and clinical research. Since the 50s, the understanding that undifferentiated cells are able to originate different cell types held great promise for regenerative medicine, making until today this field to one of intense and growing research. The possibility to artificially replace damaged tissue unlocked new possibilities for clinical treatment of so far incurable diseases. This chapter highlights basic concepts about stem cells, as well as their current and potential future applications. Moreover, it brings an overview of important historical facts of the path taken by science to get to the current status of this research field.

Keywords Stem cells • Differentiation • Therapeutic use

1.1 Historical Remarks

Stem cell history began far ago in the 1950s, when researchers first isolated embryonal carcinoma cells (ECCs) from teratocarcinomas (Yu and Thomson 2008; Stevens and Little 1954). These cells could differentiated into all thee germ layers and, in 1964, Kleinsmith and Pierce (1964) showed that a single ECC could undergo unlimited self-renewal and multi-lineage differentiation, defining the existence of a pluripotent stem cell and thus providing the intellectual framework for mouse and human embryonic stem cells (ESCs). In the earlies 1970s, ECCs were stably propagated *in vitro* and studied as "an *in vitro* model of development (Kahan and Ephussi 1970)" due to their properties, many research groups started to search for an *in vivo* counterpart of these cells.

During the embryonic development, as the zygote embryo divides, it forms a morula and the first differentiation occurs: cells from the outer layer differentiate to originate the trophectoderm and to form the blastocyst. The inner cell mass of the blastocyst (ICM) gives rise to all cells of the adult body, while the trophectoderm

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differentiates into the placenta. In 1980, it was found that the cells from the ICM are the counterpart of ECCs (Martin 1980). Differently from cells from the ICM, most ECC lines have limited potential of differentiation, are highly aneuploid and poorly contribute to chimeric mice (Atkin et al. 1974), which limits their utility as an *in vitro* model for development, favoring the use of ICM cells.

The first mouse ESC lines were derived from the ICM of mouse blastocysts and maintained in culture in the presence of fibroblast feeder layers and serum, as previously used for mouse ECCs (Martin 1981; Evans and Kaufman 1981). In 1988, it was found that a cytokine, the leukemia inhibitory factor (LIF), was the element secreted by the feeder layer responsible for sustaining ESCs in an undifferentiated state (Smith et al. 1988; Williams et al. 1988).

Human (hESC) derivation was achieved in 1998 (Thomson et al. 1998). These cells are karyotypically normal and differentiate into all three germ layers (Amit et al. 2000). In contrast to mouse ESCs, hESCs or nonhuman primate ESCs do not maintain pluripotency in the presence of LIF and its related cytokines in serum-containing media (Dahéron et al. 2004; Thomson et al. 1998; Humphey et al. 2004).

Due to many ethical issues related to the use of human embryos for obtaining hESC, a new model with similar characteristics was necessary. In this context, the reprogramming of mouse somatic cells into a pluripotent state by transfection with specific pluripotency-coding vectors was successfully conducted by Yamanaka's group (Takahashi and Yamanaka 2006), giving rise to induced pluripotent stem cells (iPSCs). Shortly after, this technique was applied to human cells (Takahashi et al. 2007; Yu et al. 2007; Lowry et al. 2008).

In 1976, during the same period when ICM cells had been shown to be pluripotent, Friedenstein and colleagues placed a whole bone marrow in plastic dishes and, after removal of the non-adherent hematopoietic cells, they found that the adherent cells could differentiate into all bone cell subtypes, such as osteoblasts, chondrocytes, adipocytes, and even myoblasts, defining the multipotency (Friedenstein et al. 1976; reviewed by Chamberlain et al. 2007). These cells were referred to as mesenchymal stem cells (MSCs), once they differentiate into mesenchymal-type cells, or as marrow stromal cells (Prockop 1997) due to the complex array found in the marrow from which they derive (Ashton et al. 1980; Bab et al. 1986; Castro-Malaspina et al. 1980). In summary, along the last six decades, stem cells have become an expanding research field that promises to strongly contribute to the advancement of basic and clinical sciences (Fig. 1.1).

1.2 Stem Cell Characteristics and Potency Concepts

Stem cells have the remarkable potential to differentiate into more than 200 cell types found in an adult body. Throughout life, they give rise to cells that can become highly specialized and replace injured tissues, or participate in normal tissue regeneration. The classical definition of stem cells, which distinguishes them from other cell types, is determined by two key properties: first, stem cells have the ability to

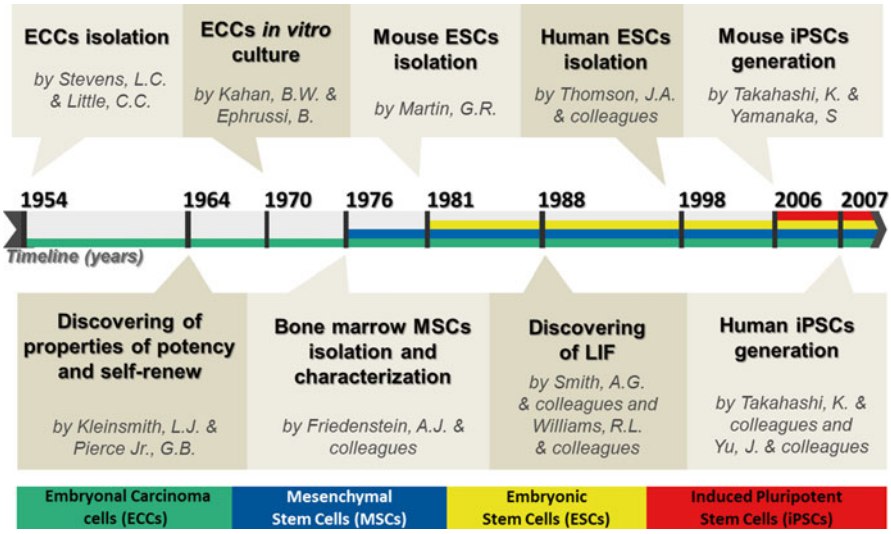

Fig. 1.1 Schematic timeline showing the most important historical milestones in stem cell research since the isolation of ECC in 1954, until the development of human iPSC in 2007. *ECCs* embryonal carcinoma cells, *MSCs* mesenchymal stem cells, *ESCs* embryonic stem cells, *iPSCs* induced pluripotent stem cells

self-renew, dividing in a way that generates copies of themselves; second, under specific physiologic or experimental conditions, they are able to differentiate, giving rise to mature types of cells that constitute distinct organs and tissues (Potten and Loeffler 1990).

The developmental stage of a stem cell defines its potential of differentiation. At the beginning of development, just after the fertilization, cells within the first few rounds of cell division are the only ones defined as totipotent. Under the right conditions, totipotent cells can generate not only a whole viable embryo, but also temporary support tissues and structures, including the placenta and the umbilical cord (Brook and Gardner 1997). The totipotency of these cells lasts until the blastomeric stage, approximately 4 days after fertilization, when cells start to specialize and originate pluripotent cells, as the inner cell mass within the blastocyst (Thomson et al. 1998; Reubinoff et al. 2000). Pluripotent stem cells can differentiate into cells derived from the three germ layers, generating any tissue type present in the organism, but they lose the ability to form the placenta or other extraembryonic tissues (Smith 2012). During embryonic maturation and tissue formation, when stimulated by transcriptional and epigenetic signals affecting gene expression, pluripotent stem cells can also give rise to multipotent stem cells. These cells are capable to differentiate into only a few different cell types originating or repairing a given tissue (Spangrude et al. 1988; Slack 2000). When the organism is completely formed and progenitor cells are committed to their differentiation fate, these lose their potency and are no longer able to change their phenotype determination.

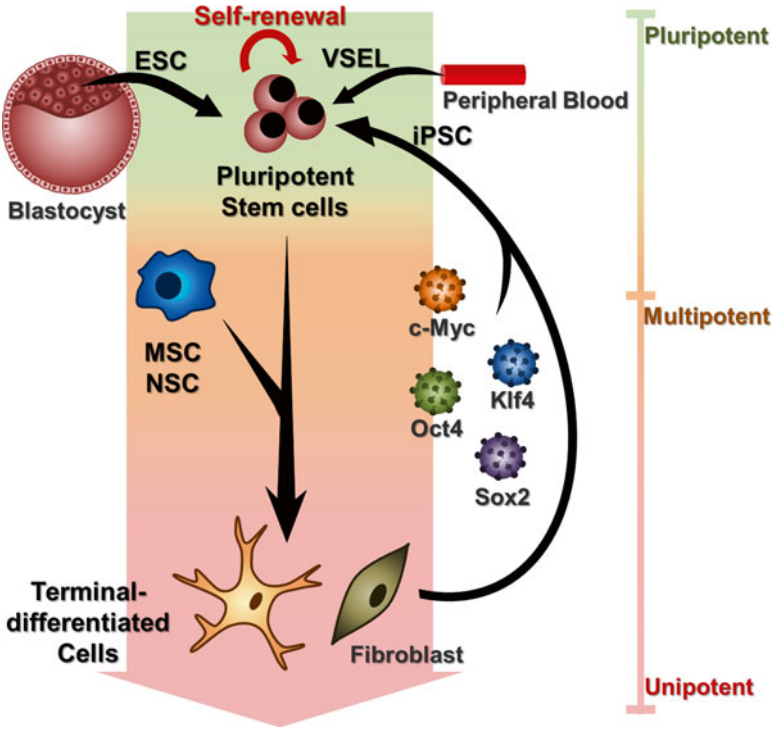

Fig. 1.2 Differentiation potential of pluripotent stem cells and their origins. Pluripotent stem cells can be extracted from a blastocyst under development and as Very Small Embryonic Like (VSEL) stem cells from adult tissues. Throughout development, pluripotent stem cells originate different cell lines, and their potency is lost by the time each cell line becomes committed to its own phenotype/fate. In this process, intermediate multipotent cells like MSCs and NSCs are also generated. *In vitro*, the overexpression of specific reprogramming factors [c-Myc, Oct4 (octamer binding transcription factor-4), Klf4 (Krüppel-Like Factor 4) and Sox2 (sex determining region Y, box 2)] induces pluripotency in specialized cells, such as fibroblasts, originated iPSCs. *ESC* embryonic stem cell, *VSEL* very small embryonic/epiblast-like stem cell, *MSC* mesenchymal stem cell, *NSC* neural stem cell, *iPSC* induced pluripotent stem cell

1.3 Stem Cell Origins

Pluri- and multi-potent stem cells can be obtained of embryos, some tissues from an adult individual, and also be generated through *in vitro* interventions (Fig. 1.2). The following items focus on the most prominent sources of stem cells.

1.3.1 Embryonic Stem Cells (ESCs)

ESCs are pluripotent stem cells obtained from the inner cell mass of the blastocyst, as mentioned before. Due to their capability to generate every adult tissue type, they provide a renewable resource for studying normal and disease development, besides their

potential therapeutic applications (Lerou and Daley 2005). As a matter of fact, the establishment and optimization of embryonic cell lineage protocols are crucial for improving knowledge about both physiological and pathological states.

Since mouse ESC isolation (Evans and Kaufman 1981), molecular mechanisms involved in the maintenance of self-renewing and pluripotency have been extensively studied. Among these molecular mechanisms are induction of conformational changes in chromatin by the epigenetic machinery, transcription factor networks and specific signaling pathways, which are able to orchestrate the pluripotency of ESCs (Marks and Stunnenberg 2014; Welling and Geijsen 2013). Several transcription factors have been shown to be indispensable in regulating the pluripotent state of ESCs *in vivo* and *in vitro* (Dunn et al. 2014; Takashima et al. 2014), including Oct4 and Nanog, being part of well-characterized core network factors with crucial roles in maintaining pluripotency (Boyer et al. 2014; Loh et al. 2006).

1.3.2 Induced Pluripotent Stem Cells (iPSCs)

The somatic cell nuclear transplantation (SCNT) technique was developed aiming to engineer cells with pluripotency properties. In this method, the nucleus of a differentiated cell is transferred to an enucleated oocyte, reaching nearly 100 % of transfection efficiency in mice (Wakayama et al. 1998). However, the method involves a range of ethical issues regarding human cells, once the resulting oocyte development, despite countless obstacles to bypass, could result in a cloned individual. Furthermore, these cells are inapt for cell transplantation due to the fact that they are triploid.

In view of that, iPSCs were developed by reprogrammation of somatic cells into a pluripotent state, originating cells with morphology, self-renewal and pluripotency properties similar to ESCs. Since the pioneering work of Takahashi and Yamanaka in reprogramming mouse fibroblasts (Takahashi and Yamanaka 2006) and introducing the concept of iPSCs, many studies were conducted to refine the reprogramming procedure for increasing effectiveness and eliminating traces of the viral genome that could have been incorporated into the genome of the resulting iPSCs. That is because the initial reprogramming technique occurred by retroviral transduction of factors including Oct4 (octamer binding transcription factor-4), Sox2 (sex determining region Y, box 2), Klf4 (Krüppel-Like Factor 4) and c-Myc. Reprogramming of human cells was done by the same group (Takahashi et al. 2007) and, simultaneously, by Yu and colleagues (2007), with the latter research group introducing a reprogramming method based on the use of Nanog and Lin28 instead of Klf4 and c-Myc. In fact, the combination of these six transcription factors resulted in increased efficiency in reprogramming human fibroblast cells (Liao et al. 2008). Next, numerous reprogramming factors were found to interfere with the efficacy of pluripotency induction, including c-Myc, which seems to be dispensable (Wernig et al. 2008). As further improvement, combinations of these factors with proteins, peptides and RNA interference, among other mechanisms, gave rise to different protocols that do not necessarily involve viral infection, but transposon and nucleofection with plasmids (O'Malley et al. 2009; Malik and Rao 2013).

1.3.3 Very Small Embryonic/Epiblast-Like Stem Cells (VSELs)

Very small embryonic/epiblast-like stem cells (VSELs) are a developmentally early stem cell population that remains in an undifferentiated state and resides in adult tissues. They are rare and slightly smaller than red blood cells, and were first described in 2006 (Kucia et al. 2006).

VSELs keep circulating in the adult body during stress situations through peripheral blood and express markers of pluripotency, including Oct4, Nanog, and SSEA, and are able to differentiate into all three germ layers. These cells are Sca1+Lin−CD45− in mice and CD133+Lin−CD45− in humans, and their morphology is characterized by a high nuclear/cytoplasmic ratio and euchromatin content, which are typical for ESCs. VSELs are a promising source for future cell therapies (Ratajczak et al. 2012).

1.3.4 Mesenchymal Stem Cells (MSCs)

MSCs are non-hematopoietic stromal cells capable of differentiating into mesenchymal tissues, such as bone, cartilage, muscle, ligament, tendon, and adipose, contributing to the regeneration of these tissues (Chamberlain et al. 2007). They can be isolated from different sources including adipose tissue, bone marrow, amniotic fluid, umbilical cord, placenta, menstrual blood and even dental pulps (Portmann-Lanz et al. 2006; Musina et al. 2008; Tirino et al. 2011; Ma et al. 2014). In addition, these cells have the ability to self-renew and are identified by their phenotype, being positive for CD29, CD44, CD73 and CD90 cell surface markers, while negative for the hematopoietic markers CD34, CD45 and CD14. Moreover, MSCs contribute to cellular homeostasis maintenance and many physiological and pathological processes such as aging, tissue damage and inflammatory diseases (Prockop 1997; Sordi et al. 2005; Le Blanc et al. 2003).

When transplanted, MSCs are able to migrate to injury sites. This trafficking into and through tissue is a process that involves adhesion molecules, chemokine receptors and their ligands. Several studies conducted to elucidate the mechanisms underlying this process reported the functional expression of various chemokine receptors and adhesion molecules on human MSCs (Chamberlain et al. 2007). The differentiation potential of MSCs is limited in comparison to ESCs and iPSCs, characterizing them as multipotent cells, even though they are a great promise for clinical applications especially due to their immunoregulatory functions.

1.3.5 Neural Stem Cells (NSCs)

NSCs are multipotent stem cells capable to differentiate into many neural cell types from the central nervous system (CNS). They are found in both the developing and the adult brain, with some distinct properties. Basically, during early embryo development

the rearrangement of neuroepithelial cells leads to the neural tube formation. In the formed ventricular zone, these cells constantly proliferate to increase cell number and then migrate to form the CNS (Merkle and Alvarez-Buylla 2006). A niche of stem cells remains in the ventricular zone and gives rise to the radial glial cells, another NSC type that differentiate into distinct neural cell types. Moreover, NSCs seem to modify their morphology, gene expression profile and other properties throughout the embryonic development (reviewed by Götz et al. 2015), since they first originate a large amount of neurons and later start to produce more glial cells. In mammals, radial glial cells are no longer present in the after-birth brain, giving rise to multipotent adult NSCs (aNSCs) (Merkle et al. 2004).

The aNSCs expresses glial fibrillary acidic protein (GFAP), an astrocyte marker, and are located in the subventricular zone (SVZ) of the lateral ventricle's wall, and in the subgranular zone (SGZ) of the hippocampus' dentate gyrus in the adult brain (Doetsch et al. 1999; Gage et al. 1998). In the SVZ, aNSCs are known as type B cells and their derived neural progenitors are type C cells, which can be identified by Mash1 gene expression. The later give rise to neuroblasts, some expressing Olig2 that generate oligodendrocytes (Parras et al. 2004). In summary, prenatal neuroephitelial cells originate radial glial cells that disappear after birth and give rise to astrocyte-like cells (Merkle and Alvarez-Buylla 2006). Since these GFAP-expressing cells are able to replenish the SVZ after ablation and differentiate into neurons (Doetsch et al. 1997), aNSC seems to be a promising cell type for cell therapy use.

1.4 Stem Cell Applications

Once the possibility of differentiating stem cells into specific phenotypes *in vitro*, had been established, a variety of methods emerged aiming to increase cell fate specificity and differentiation efficiency. This book provides several advanced protocols regarding stem cell differentiation (Fig. 1.3). Such approach allows science to advance in different areas of basic and applied research, facilitating the understanding of physiological and pathological processes, and enabling the advancement of medicine to control and/or cure several diseases by unraveling cellular and molecular mechanisms involved in each process.

ESCs originate embryoid bodies *in vitro*, which tend to spontaneously differentiate into distinct tissues, mimicking the embryonic development (Ling and Neben 1997). In the presence of specific growth factors and small molecules, ESCs can differentiate into particular cell types mimicking mechanisms underlying development of different organs/tissues, such as pancreas and liver (Zaret and Grompe 2008), muscles (Xie et al. 2011), the cardiovascular (Feraud and Vittet 2003; Winkler et al. 2004) and the nervous systems (Lupo et al. 2014). Furthermore, ESCs provide a suitable model to study the impact of genetic mutations and toxicity of diverse substances during early development. In combination with adult stem cells as further model systems, these can be employed for unraveling bases of differentiation, physiology, biochemistry and potential pathologic processes during embryonic development and adult cell differentiation.

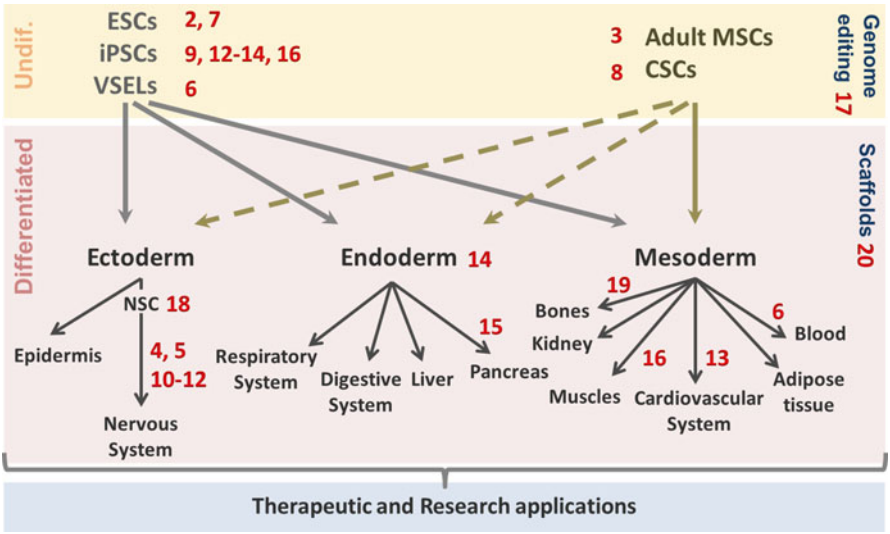

Fig. 1.3 Stem cell topics covered by this book. *ESCs* embryonic stem cells, *iPSCs* induced pluripotent stem cells, *VSELs* very small embryonic-like stem cells, *MSCs* mesenchymal stem cells, *CSCs* cancer stem cells, *NSC* neural stem cell

Based on this principle, stem cells, and particularly iPSCs, have gained importance as simplified disease models. The use of patients' cells to generate iPSCs provides a more accurate understanding of how the genome contributes to erroneous or defective differentiation processes underlying neurodegenerative diseases. For instance, in Parkinson's disease, death of dopaminergic neurons in a specific stage of life may result from compromised development, whose etiology may involve genetic inheritance (reviewed by Badger et al. 2014). Thus, dopaminergic neurons differentiated from reprogrammed patients' fibroblasts may help to elucidate the participation of genetic preprogrammed mechanisms in the disease. Furthermore, the use of patients' cells enables the refinement of individualized treatment, since some interventions might lead to different effects depending on the individual background.

iPSC-based approaches may deflect issues involved in the development and application of human cell therapy in curing diseases, since sources of pluripotent human stem cells are scarce and comprises several ethical issues. Cell therapy is based on the transplantation of cells, whether differentiated or undifferentiated, to reverse the injury in the subject. For this purpose, stem cells from different sources are cultured *in vitro* and transplanted into animal models to assess their effectiveness. Many studies are being conducted in the hope for developing efficient therapies for so far irreversible conditions. Therefore, stem cells can be manipulated *in vitro* for effective differentiation and integration and survival in a living tissue without immunological rejection after transplantation.

One way to manipulate stem cells is by means of genome editing, as further discussed in Chap. 17. DNA modifications can be incorporated or excluded from the genome in knock-in and -out models, respectively. Moreover, gene expression

can also be modulated by using RNA interference (Martin and Caplen 2007), the Cre/loxP system (Van Duyne 2015) and other recombinase systems (Kilby et al. 1993; Gaj and Barbas 2014). Despite the available technologies, genetic modifications still need refinement and this intervention has opened a new range of research possibilities to analyze gene function, genetic diseases, mutation studies and pharmacological applications.

1.5 Stem Cell Research's Ethical Issues

Despite of its huge potential regarding regenerative medicine and contribution to basic science, stem cell research faces ethical and political challenges that delay the advancement of this field. Some sources of stem cells such as adult MSCs and VSELs do not bring up strong ethical concerns, while hESCs and iPSCs are constant subjects of discussions.

Currently, the extraction of hESCs requires the destruction of a human embryo, being the main reason why ESCs raise ethical discussions. There are two main positions in relation to embryos' use in research: (1) those who are strictly against embryo utilization for research purposes, because they consider the embryo morally equivalent to an adult human being; and (2) those who defend the utilization of embryos for therapeutic purposes in research, and also consider this as an obligation in view of the benefits for patients (Devolder and Savulescu 2006).

From a legal standpoint, these distinct ethical perspectives translate into stem cell legislations that greatly vary from country to country (for a review of different policies around the world, see Dhar and Ho 2009). Brazil was the pioneer country to develop a law regulating the use of ESCs in 2005 (Dhar and Ho 2009). In the United States, for example, embryos produced during *in vitro* fertilization, which eventually need to be discarded, can be used for scientific experiments, since they are not produced for research purposes (Green 2002). Stem cells produced by SCNT share the same ethical concerns of hESC, once this technique enables the development of a cloned embryo. Moreover, obtaining human oocytes involves trade, which in turn results in additional ethical problems (Alpers and Lo 1995).

Developed as a promising alternative for ESCs and aiming to bypass the ethical concerns that accompany these cells' use, iPSCs rapidly are controversely discussed. The central topic relies on the possibility that iPSCs could originate, accidentally or on purpose, a totipotent cell similar to oocyte, which raises ethical concerns similar to those of SCNT cells, such as cloning possibilities (de Miguel-Beriain 2015). Additionally, there are further questions regarding iPSCs research hovering over bioethics, since the manipulation of these cells could culminate in a range of unthinkable possibilities, including the generation of gametes *in vitro* and the creation of a human chimaera (Carvalho and Ramalho-Santos 2013).

At this moment, the scientific community has not yet reached a consensus on the use of stem cells and its ethical implications. Thus, highlighting the importance of the topic is each researcher's responsibility, who must be aware of the boundaries

set by the legislation in the country where the experiments are being conducted. In addition to legal considerations, it is noteworthy mentioning the need for a personal judgment in developing any stem cell research.

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