The biological basis of the use of human embryonic stem cells for *in vitro* test systems

Introduction to the roundtable "Embryonic or adult stem cells: scientific and ethical considerations"

Marcel Leist[1], *Hannes Hentze*[2] *and Suzanne Kadereit*[1]
[1] Doerenkamp-Zbinden Chair for Alternative *in vitro* Methods, University of Konstanz, Germany; [2] S*BIO Pte Ltd, Singapore

Human embryonic stem cells (hESC) are presently being cultured in many laboratories, and differentiation protocols are available for a large variety of cell types. The most immediate use of these cells may not be therapeutic applications, but more immediately, the design of test systems for toxicological and pharmacological research (UKTI, 2005; Bremer and Hartung, 2004; Vogel, 2005). The use of such human-based test systems would contribute to a bottom-up test strategy for new chemicals, where initially the mode of action is explored, and animal experiments are only used as a last resort and in special situations (Leist et al., 2008a). One obstacle to the broad use of hESC for experimental test systems are ethical issues that have different legal implications in different countries (MBBNET, 2008).

What are embryonic stem cells? Before entering a bioethical debate it is important to create a common platform of biological facts important for such a debate. We will first take a look at natural conception and embryo development as basis for the overall understanding of the technology. After fertilization of the *oocyte* ("egg") in the oviduct by a *sperm*, a *zygote* is formed. This cell, which contains genetic information from two parental gametes (*i.e.* the oocyte and sperm) starts dividing while migrating down the oviduct, and gives rise to a tiny (less than 0.1 mm), blackberry-shaped, compact cell clump termed the *morula*, which eventually enters the uterus at around day 4. By day 5, a cavity is formed in the "ball" and the resulting structure of embryonic cells is now termed *early blastocyst*. The blastocyst is initially surrounded by a translucent structure (the *zona pellucida*) that protects it and prevents it from attaching to incorrect structures. In the uterus, the *blastocyst* begins to form different cell types - the outer cells (about 200), which will later become the placenta, and the inner cell mass (ICM) of about 30 cells which will later develop the embryo. Eventually it "hatches" *i.e.* it breaks out of the zona pellucida and attaches to the wall of the uterus. This process of "*implantation*" of the late blastocyst occurs around day 9 post-fertilization and represents the first physical connection between the early embryo and the fertilized woman. This step, also termed "*nidation*", is crucial for the development of embryo polarity (body axes/up and down-definition). Finally, at day 12-14, a dramatic morphological restructuring occurs when the embryonic cells form a double-layered structure – the *gastrula*. This is the start of primitive tissue formation (*primitive streak*) in the *embryo proper* (the real embryo). At day 14, therefore, pregnancy is established, the embryo has a close connection to the womb, it has developed polarity as well as "inside" and "outside" directionality, and, importantly, the three germ layers (primordial tissues) begin to form early organs such as the primitive gut and neural system. In parallel the placenta forms from formerly external cells of the blastocyst.

Couples facing problems in conceiving naturally (in some countries also women that can get anonymous sperm) now have the option to increase their chances of pregnancy by undergoing an *in vitro* fertilization (IVF) procedure. This process differs from a naturally-occurring pregnancy only in the initiation phase where sperm and oocyte meet. Oocytes are harvested from the woman, and this process is facilitated by ovarian stimulation with hormones. Then they are fertilized with a sperm sample from the potential father to form zygotes. Typically, 10-15 early pre-implantation embryos are generated in such a process and propagated *in vitro* up to the blastocyst stage (day 5). In some countries (e.g. Germany), national regulations forbid selection of the embryo after nuclear fusion and preclude the cultivation of more than three embryos at a time (Zollner et al., 2003). After positive selection of the best blastocysts, 1-3 (depending on the country) are re-implanted directly into the uterus, where they have the chance to attach to the uterine wall and form an embryo just as it occurs in a natural conception. IVF procedures result in a successful pregnancy in about 15-25% of the procedures. If the first attempt is unsuccessful, a second and third round of re-implantation of blastocysts may be initiated, since the initially superfluous blastocysts are typically cryopreserved, *i.e.* stored in a liquid nitrogen tank where they can be maintained for several years. It is not known when a blastocyst under conditions of cryopreservation loses its potential to form an embryo. Albeit one case study reported a successful pregnancy after implantation of a blastocyst stored for 12 years (Revel et al., 2004), it is generally observed that the quality is strongly falling after 5-10 years. Many supernumerary blastocysts accumulate in fertility clinics and will be ultimately destroyed. It is estimated that 400,000 fertilized oocytes were stored in 450 fertility clinics in the USA alone in 2003. Tens of thousands are also frozen in Germany,

Switzerland, Austria, the UK and many European and Asian countries. Thus, it is reasonable to assume that by now over one million pre-implantation embryos are stored in the USA and elsewhere. A very small minority of these (less than 1%) is typically donated for research purposes, including hESC generation.

The *in vitro* generation of hESC starts with thawing and propagating a superfluous donated day five pre-implantation blastocyst. The ICM is isolated and placed in growth medium. These cells can be cultured *in vitro*, but they require a supportive "feeder" cell layer to provide them with nutrients and hormonal signals. Typically, mouse or human fibroblasts (connective tissue cells) are used as feeders. If the procedure is successful, the cellular outgrowth of this ICM will form the "passage 1" of a new hESC line, and once the cell culture dish is densely grown with hESC, the cells will be harvested and transferred to five fresh cell culture dishes. There they grow again to cover the whole dish (passage 2), and be transferred to five new dishes, and this procedure can be repeated continuously to generate more cells. It is now possible, in principle, to expand hESC indefinitely, and hESC have been cultured as stable cell lines up to passage numbers far beyond 150 using methods developed originally in 1998 in the laboratory of James Thomson (Thomson et al., 1998). All hESC harbour the potential to form a diversity of different cells – theoretically any cell type of the about 200 existing in the human body. Therefore, they are called "pluripotent" cells. Pluripotent means that the cells can form any known cell type, but they cannot generate a whole organism, because they are lacking the capacity for implantation and formation of a placenta. This capacity is only found in the zygote and the cells up to the 8-cell stage of the morula, and these cells are called "totipotent". It is important to emphasize here that hESC themselves are not totipotent and they are therefore unable to generate a new embryo.

The use of hESC in research requires the generation of hESC lines. The lines currently used were mostly produced from fertilized oocytes that had undergone about 7-8 divisions. The major objection to the use of ESC is that their generation is purported to involve an 'act of killing'. To evaluate this point of view it is best to look at a typical example of cell line generation: A couple wishing to have children decides to try *in vitro* fertilization (IVF) – oocytes are removed from the woman, fertilized with sperm, and two embryos are implanted into the woman. The remaining embryos are stored, frozen in liquid nitrogen, and the woman is lucky to become pregnant on the first attempt – possibly even giving birth to twins. Life goes on and the couple is content with their two kids. After more than five years of storage, the quality of the stored blastocysts starts to deteriorate, and the storage is expensive as well. Presently, at least half a million such left-over zygotes are stored in the US alone. None of these blastocysts has any potential at all to ever develop to a human being without a foster mother. After parental consent of our happy couple, the left-over material will either be destroyed (moved to a trash can), or it may be donated for research purposes (only in some countries). Embryo adoption schemes as an alternative to discarding them were discussed, but this obviously does not appear to be a realistic option for all stored blastocysts at annual storage costs of several hundred million $. In the case of research donation for hESC research, the blastocyst will be allowed to be thawed, and hESC will be generated. When the procedure is successful, this will result in the generation of a single continuously growing hESC line that can provide pluripotent cells indefinitely. For instance the first such cell line, generated around 1998 (Thomson et al., 1998) has now been spread to hundreds of laboratories worldwide; its usage has generated a tremendous amount of novel scientific knowledge which may enable future therapies, and it is still being used extensively today. Most laboratories working with hESC nowadays use such cell lines for their research, but were never involved in the generation of cell lines, *i.e.* the use of any blastocyst.

Concerning the issue of the "use of human beings for research", the definition of "human personhood" is the most crucial issue, and a wide range of viewpoints exists:

• On one side of the spectrum one can find views that human life and personality begin and reach their full extent within a single moment, when the nuclei and chromosomes of the gametes combine.

• On the other side of the spectrum one finds views that the first days after the zygote formation merely result in the formation of a "pile of cells" (morula/blastocyst stage) which cannot yet be assigned full human rights and therefore does not deserve the specific ethical consideration of a person (Schuklenk, 2008).

• In between these extremes, there are also views that acknowledge the zygote as being unique (as opposed to other cells) in its natural, but theoretical, potentiality to become a human being, but that full human personhood and the rights associated to it are acquired gradually. Here, metaphors are often evoked of the embryo being a walnut, a cherry or a caterpillar, which may or may not become a walnut tree, a cherry tree or a butterfly – but they certainly are none of the latter, and therefore they deserve a different status. No one would, for instance, argue that each walnut deserves the same protection as a full-grown walnut tree.

We have no authority of deciding who is right in the above issue, but we can look at the basic concepts associated with these different views.

One line of thought builds on the concepts of continuity, identity and potentiality. Obviously, a zygote does not fit our intuitive and accustomed picture of a human being. Still, it clearly belongs to the human race (biologically). In order to assign the status of personhood to a microscopic pile of cells, is has to pass at least the test of the following characteristics:

• A defined succession of events leading **invariably** from the zygote to a human person (= continuity);

• A situation in which **one** zygote only becomes **one** person and **one** person can only be derived from one single zygote (= identity);

• The full capacity of a zygote to become a human person **without further manipulation or help** (= potentiality).

If one accepts that these three requirements are met, ***and*** if one assumes that fulfillment of these requirements is both necessary and sufficient for the personhood definition, then a zygote indeed ***is*** a human being.

However, biological research in mammals has shown that these assumptions

may not hold true: the continuity criterion is not fulfilled, because *most* zygotes (70%) never become persons (because of implantation failure), the identity criterion is not met, because one zygote can produce twins, and *vice versa* some persons are made up from two fused zygotes, and the full potentiality is not found in the zygote, as an embryo can never develop to a person in the absence of the mother's womb, *i.e.* without maternal signals received upon implantation. It may also be argued that these requirements are necessary, but not sufficient (e.g. time as additional factor, see: gradual acquisition of personhood).

It has been argued that hESC can by no means be used to substitute animal experiments. This would mean that experiments are instead performed on "humans". However, this argument contains a misconception. The testing of toxicity on human cells allows man to take responsibility for himself without relying on animals and their suffering. It would not be a substitute, but an entirely different, and possibly better approach. Human cell-based *in vitro* methods would fully correspond to the new vision of a mechanistically based toxicology (Leist et al., 2008b; Hartung and Leist, 2008) that implies higher financial efficiency of the procedure and improved safety for mankind. For instance the EU project ESNATS has been started with the goal to develop novel hESC-based alternative test systems for drug profiling. This is a logical consequence of a line of research that has shown that 3R (reduction, replacement, and refinement of animal experiments) methods have been astonishingly successful and can sometimes yield better data than animal based tests (Hartung, 2001, 2007a, 2007b, 2008; Leist et al., 2008b). hESC-based test systems could contribute to such a strategy of safety testing with fewer animals, and with data more relevant to humans, and more based on sound science. This concept should by no means be mistaken (or distorted) to imply that animal experiments would be substituted by embryo research. Typical examples for the application of hESC at the moment are in the field of developmental neurotoxicity. The thalidomide catastrophe showed that animal models can be very poor predictors of human developmental toxicity. Accordingly, a lot of focus has been on the development of ESC-based test systems, initially on murine cells, and in recent years also using human cells (Pellizer et al., 2005). Research on human neurons is extremely difficult due to the poor availability of the material. Therefore, most experiments to test toxicity have relied on animals or animal material, and this has sometimes led to detrimental erroneous conclusions (see thalidomide). Another illustrative example is cardiotoxicity (in particular toxicity of drugs that cause changes of the heart beat). This is frequently associated with drugs that interact with the so-called hERG potassium channel on the heart. It is likely that in the near future, hESC-derived cells may be substituting the current assays performed with primary porcine or rabbit cardiomyocytes (heart muscle cells). Such cells can now be generated from hESC with almost 100% purity, and these cells have been shown to function in the hERG test (Xu et al.).

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Correspondence to:

Dr. Suzanne Kadereit
University of Konstanz
Box M657
78457 Konstanz
Germany
e-mail:
Suzanne.kadereit@uni-konstanz.de
Tel.: +49-7531-885037
Fax: +49-7531-885039