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Human Embryonic Stem Cells: The Real Challenge for Research as well as for Bioethics Is Still ahead of Us

An Editorial

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Embryonic stem cells · Stem cells · Tetraploid complementation · Alternative sources · Bioethics

Abstract
Research on human embryonic stem cells (ESCs) has aroused a lot of controversy for years. Stimulated by recent work on mammalian embryology and new developments in stem cell research, an International Symposium entitled 'Stem Cell Research: A Challenge for Embryology, Regenerative Medicine and Bioethics' was held in Bonn (Germany) in 2006, bringing together embryologists, stem cell researchers and ethicists interested in human ESC research and the ensuing ethical debate. Two contributions to this Symposium are being published in *Cells Tissues Organs*, and the present paper aims to provide an introduction to these as well as personal impressions of the author about the perspectives that surfaced at the meeting, confronting them with relevant reports about stem cell research published recently. This paper highlights discussions about the mechanisms of specification of the main body axes during development, the role of extrinsic or intrinsic signals, and about the remarkable potential of ESCs to develop a basic body plan (individuation capacity) resembling properties of early embryonic cells (as shown by the formation of embryoid bodies and entire embryos if tetraploid complementation is performed). Another topic is 'alternative sources for human ESCs' recently proposed by the US President's Council on Bioethics ('organismically dead embryos', biopsied blastomeres or 'biological artifacts', e.g. created by 'altered nuclear transfer' and reprogramming of somatic cells). The possibility to rescue such (epi)genetically handicapped cells shows that this is not a way leading out of the ethical cul-de-sac. Recent reports about reprogramming somatic cells (fibroblasts) to gain ES-like potential highlight again the importance of focusing on the developmental potentiality as the major challenge for ethical considerations. Such a change of focus may be the only way out of the ethical impasse.

Introduction

Research on human embryonic stem cells (ESCs) has aroused a lot of controversy and became a topic of never-ending ethical debates in spite of the fact that expectations are high with respect to possible spin-offs for basic research as well as to applications in cell/tissue replacement

Abbreviations used in this paper

ANT	altered nuclear transfer
EB	embryoid body
ES	embryonic stem
ESCs	embryonic stem cells
TC	tetraploid complementation

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therapy. Many attempts have been made by ethicists, philosophers and politicians to clarify whether the problem of sacrificing early embryos (e.g. supernumerary ones) for the derivation of the ESCs can be weighed against the prospects of new therapies; why have all these efforts not been able to really clarify the situation and to calm the moods? It has been argued that this is due to a new wave of irrationality and fundamentalism which has started to increasingly overshadow any rational discourses with the result that the gap widens between groups whose beliefs and philosophies are incompatible. Is this really the end of the story about the ethical impasse? Are there points that have been overlooked or at least inadequately treated in the previous disputes, and considering these, could it be that new avenues may open up for making progress in this field?

Taking a closer look at the piles of ethical tractates that have been published since the first description of human ESCs by Thomson et al. [1998], it does indeed appear that certain aspects of actual studies on mammalian embryology have been largely omitted from the discussion. This is an oversight that urgently needs to be corrected. Recent publications have shown that it seems to be possible to turn somatic cells, mouse and human fibroblasts, into pluripotent (ES-like) cells [Takahashi and Yamanaka, 2006; Nakagawa et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007]. Consequently, experts who are familiar with the front of research in mammalian embryology have to be involved in this discussion, in order to be able to solve very basic questions about the concepts and definitions we are dealing with here. For example, what exactly is an early mammalian embryo, e.g. a morula: is it only a ball of pluripotent/omnipotent/totipotent cells or not? This question is recently being debated vividly by embryologists and it is at the same time very relevant for the ongoing bioethical disputes on normative aspects [for a discussion on the embryological terminology, see Denker, 2002, 2004, 2006]. Does an evaluation of recent embryological work on the nature and the development of organismic wholeness [Gilbert and Sarkar, 2000; see also Denker, 2004] help here? Likewise, a discussion on the ethical implications of the developmental potential of cells is largely missing in the literature, i.e. on the one hand those cells that can be taken from early embryos and on the other hand the ESCs that may be derived from these cells. For many years, it is, for example, an established fact that mouse ESCs offer the unique possibility to directly clone normal individuals using the method of tetraploid complementation (TC) [Nagy et al., 1990, 1993], a procedure that works without nuclear transfer to an oocyte (the only method of cloning that is discussed in nearly all ethical publications). Worldwide, the TC method is extensively used in many laboratories for mouse cloning, and there is no doubt among specialists that it could also be successfully applied to human ESCs if ever attempted. Obviously, donors of human embryos should at least get this information during the process of obtaining informed consent (since TC would allow to clone individuals from the donated embryos even after the derived ESCs have possibly been spread all over the world), but this information is so far not being transmitted [Denker and Denker, 2005]. In fact, implications of the availability of TC technology have been nearly totally omitted from the ethical literature with very few exceptions, perhaps because ethicists simply did not know about it [Denker, 1999, 2002; Deutscher Bundestag, 2003; Denker, 2006].

In an attempt to fill these gaps, an International Symposium entitled 'Stem Cell Research: A Challenge for Embryology, Regenerative Medicine and Bioethics' was held in Bonn (Germany) on May 12, 2006, bringing together embryologists, stem cell researchers and ethicists to discuss these topics. The symposium (organized by Hans-Werner Denker, Hans-Peter Hohn and Michael Thie) started with a session on 'Embryology/Stem Cell Research/Tissue Engineering Perspectives' with contributions by Davor Solter (Axis Development and Epigenetic Control of Preimplantation Mouse Development), Janet Rossant (Key Genes in the Earliest Differentiation Events in Mammalian Development, and Early Stem Cell Niches), Michael Thie (Gastrulation-Like Events in Primate ESC Colonies), and Charles J. Kirkpatrick (Perspectives of Tissue Engineering and Regenerative Medicine). It continued with a session on 'Biological Approaches to Resolve the Dilemma of Embryo Destruction' with contributions by Hans Schöler (Germ Line Development from Embryonic Stem Cells in vitro) and William Hurlbut (New Strategies to Overcome the Ethical Dilemma of Embryonic Stem Cell Research: The Border Line between 'Biological Artifacts' and Embryos). The Symposium ended with two podium discussions, a short one on 'Biological Definitions' (chaired by Janet Rossant and Hans-Werner Denker) and a very extensive discussion on 'Ethical Implications' (chaired by Ludger Honnefelder, with statements and discussion contributions by Thomas Heinemann, Søren Holm and William Hurlbut).

Two papers based on presentations given at that Symposium are being published now in *Cells Tissues Organs*. I am happy that Søren Holm and Thomas Heinemann have taken the time to update their statements given at the ethics part of the conference and to present their critical analysis [Holm, 2008; Heinemann, in preparation]. I

regret that time restraints have prevented the other speakers from contributing a paper here. After contemplating it I felt that it would certainly be too audacious for me even to attempt to sum up the top level biological presentations. The utmost I can do, I feel, is to try presenting a very personal impression of the perspectives as they surfaced for me at the Symposium, and to confront these with some very relevant information from the months after the meeting.

A topic discussed intensely at this Symposium was the question whether we should see in an early mammalian embryo (a morula) more than just a cluster of cells, e.g. whether biology tells us that it is indeed pre-patterned in a cryptic way to make sure the basic body plan will subsequently develop in an ordered manner. A view previously held by some authors was that it is implantation in the uterus which provides information about the future body axes (a view to which I personally never subscribed), but this view is now being more and more abandoned due to results about relevant gene expression asymmetries (e.g. the gene lefty) that develop even in vitro, without a uterus [Takaoka et al., 2006; see also Torres-Padilla et al., 2007]. In contrast, work published in recent years by the groups of Richard Gardner and Magdalena Zernicka-Goetz [reviewed by Gardner, 2006; Zernicka-Goetz, 2006] suggests that specification of the main body axes during development starts long before implantation [these findings confirm certain conclusions drawn from classical histochemical observations, cited by Denker, 1976]. It is a topic of debate whether this axis specification starts already at oogenesis and/or sperm penetration into the oocyte, like in non-mammalian vertebrates, as data by Gardner [2006] and Zernicka-Goetz [2006] suggest. Methodological details may have influenced the outcome of the experiments done by various groups [Hiiragi and Solter, 2004, 2005; Gardner, 2006; Gardner and Davies, 2006; Zernicka-Goetz, 2006], but all authors agree that there is asymmetry in the cleavage stages and/or the blastocyst which most probably does play a role in specification of the future axes. A remaining question is how exactly this asymmetry arises. Unfortunately, Magdalena Zernicka-Goetz and Richard Gardner were unable to attend the Symposium, so only Davor Solter's view was presented that the asymmetries are imposed on the forming zygote and postzygotic stages not by oocyte asymmetry or by the point of sperm penetration but by the non-spherical outline of the zona pellucida. This view is, however, being refuted by Richard Gardner [Gardner, 2006a; Gardner and Davies, 2006] who concludes that not the shape of the zona pellucida but an asymmetry of the zygote cytoplasm is the first axis-determining factor (and that this asymmetry is derived from the oocyte, not the sperm entry point).

Any role of the shape of the zona pellucida, if at work here as proposed by Davor Solter [Hiiragi and Solter, 2004, 2005], may be of interest for the ethics debate insofar as it would seem to identify an extrinsic signal (mechanical constraint) which specifies axes. When we assume that on the contrary an asymmetry is imposed on the egg/zygote by sperm penetration and is instrumental in axis specification during normal development of the embryo (provided that it is somehow transmitted to the developing 'ball of cells' during cleavage), we have to expect that this kind of information about organismic organization would most probably be lost when ESCs are derived from this embryo and are constantly disaggregated during subculturing. So if the sperm penetration-derived asymmetry would be constitutive for individuation, we would have to assume that this incipient organismic wholeness of the morula and the blastocyst would be destroyed and could perhaps not be regained easily in cultured ESCs (it cannot be excluded, however, that other external asymmetry signals could replace this in vitro). If, on the other hand, physical constraints as provided by the asymmetry of the zona pellucida are normally significant in this context, i.e. if they impose the structural order necessary for axis development on the ball of blastomeres, they could most probably easily be replaced by other, even stochastically arising asymmetries of the cell culture conditions (e.g. the substratum) during ESC culture, and since ESCs maintain many of the early embryonic features including gene activation cascades necessary for early embryonic patterning, they might be able to translate the asymmetry signals into the development of a basic body plan. This would be of ethical concern when human ESCs are handled.

The biological basis for the remarkable potential of ESCs to develop a normal basic body pattern (realization of individuation capacity) that resembles the properties of early embryonic cells has been discussed before [Denker, 1999, 2004, 2006]. It can become apparent in two contexts: (i) formation of 'embryoid bodies' (EBs) in dense cultures of ESCs, and (ii) TC (already mentioned above). TC shows a potential not for autonomous but for (so to say) 'assisted' development since 'helper cells' are needed (e.g. artificially tetraploidized blastomeres whose developmental potential is restricted to an extraembryonic fate, specifically trophoblast). EB formation, on the other hand, shows that a self-structuring potential is present in ESCs and becomes apparent in dense cultures, and that this does not depend in this case on the addition of exter-

nal structuring cues provided by the addition of 'helper cells'. Mouse EBs represent incomplete or malformed embryos: they lack trophoblast (the cell type needed for implantation) because the trophoblast differentiation potential of mouse ESCs is low (although not completely lacking) [Ralston and Rossant, 2005; Tolkunova et al., 2006; Xu, 2006; Li et al., 2007; Schenke-Layland et al., 2007]. Also, mouse EBs mostly show structural abnormalities with respect to the formation of a basic body plan, i.e. the degree of order attained (at their gastrulation-like epithelial-mesenchymal transition processes) is low. This, however, is not a fair argument against any such self-structuring potential that they might possess since normal embryos develop the same abnormalities under the same in vitro conditions [discussed by Denker, 2004]. So cautioning seems to be in place when thinking about differentiating human ESC colonies in vitro. In this context, it has to be stressed that human and non-human primate ESCs, in contrast to the mouse, do even have a pronounced trophoblast differentiation potential [reviewed by Denker, 2002; see also Thomson et al., 1996; Thomson and Marshall, 1998; Reubinoff et al., 2000; Gerami-Naini et al., 2004]. This means on the one hand that they differentiate into the cell type normally needed for implantation. Another aspect is that pattern formation in the epiblast (blastocyst and early post-blastocyst stages) seems to depend on a molecular cross-talk between trophoblast and embryoblast/epiblast [Rossant, 2004; Ralston and Rossant, 2005]. Not only the general differentiation of the embryoblast/epiblast might be regulated this way, but also instruction about anterior-posterior (craniocaudal) axis formation (positioning of the primitive streak) might be normally provided by asymmetrical growth of the trophoblast of the blastocyst [Gardner and Davies, 2002]. This could explain why mouse EBs (regularly lacking trophoblast) may be handicapped with respect to early embryonic pattern formation in vitro since, as mentioned above, mouse ESCs have only a very low trophoblast differentiation capacity. On the other hand, the very high degree of order described to develop in marmoset monkey EBs [Thomson et al., 1996] may be due to the presence of the trophoblast differentiation potential in primate (including human) ESCs, so that one should be aware of the possibility of a basic body plan development in human ESC cultures, an aspect of high ethical concern as discussed before [Denker, 1999, 2006].

Alternative sources for human ESCs were also intensely discussed at the Symposium [Hurlbut, 2005; The President's Council, 2005; Green, 2007]. The alternative sources proposed by the US President's Council are:

(a) 'organismically dead' embryos, 6- to 8-cell in vitro fertilization embryos which have ceased dividing;

(b) blastomeres obtained by 'non-harmful' biopsy of living embryos;

(c) so-called 'biological artifacts', i.e. genetically modified cells/embryos lacking certain properties needed for early development or implantation [method used: knockout or temporary knockdown of the respective gene, followed by 'altered nuclear transfer (ANT)' to an oocyte] (in the following we will mention the most often discussed example for this, i.e. the gene cdx2), and

(d) human somatic cells that have been reprogrammed by cytoplasmic factors or by direct genetic manipulation (i.e. without transferring nuclei to oocytes).

Discussions at the Symposium were controversial with respect to the question whether entities (a–c) are to be regarded as embryos, as (severely) compromised embryos or as sufficiently distinct from embryos so that they might be exempt from the ethical concerns connected with embryo research. Obviously this is a matter of definition which is always somewhat arbitrary. It was fortunate to have William Hurlbut at the Symposium, the proponent of the ANT concept [Hurlbut, 2005], so that the ethical reasons behind it could be discussed in detail. A critique of the ANT concept is presented by Søren Holm in this issue [Holm, 2008].

An important general aspect that surfaced at this Symposium was that with all these 'alternative sources' one ethical aspect remains the crux of the controversy: the aspect of potentiality, which applies for the developmental potential of the 'alternative sources' themselves, but even more so for the derived ESCs. As far as the 'alternative sources' are concerned, an intense discussion developed at the Symposium whether these sources should be seen as embryos (or embryo equivalents) or not. In case of the cdx2 knockout/knockdown (ANT) concept, this entity shows only incipient early epithelialization and blastocyst cavity formation, and a defect in further trophoblast differentiation. Some authors propose to address such an entity not as an embryo but as just a 'ball of (stem) cells'. An argument put forward against this view is that it appears possible to develop strategies to rescue 'organismically dead' embryos (alternative *a*, above) or blastomeres isolated by biopsy (*b*, above) e.g. by (re)combining them with the other cells, by gene therapy or by adding growth factors in order to stimulate their mitotic activity. What would that mean for their ethical status? Likewise, the entity created according to the ANT concept (alternative *c*; be it addressed as an embryo equivalent or an artifact) could be rescued by reactivating the gene that was

knocked out/knocked down. That this can in fact be done has already been shown by Meissner and Jaenisch [2005] in their 'proof-of-principle' experiment with respect to cdx2 in the mouse. Ethical aspects of this approach are discussed in this issue by Søren Holm [2008].

One argument that has been put forward in relation to this concept questions whether it should be seen as ethically acceptable to purposely deprive an entity (compromised embryo) of its possibility to implant in the uterus (by depriving it of trophoblast tissue, as in the cdx2 knockout proposal). I am reminded in this context of experiments I have done myself on biochemical factors involved in embryo implantation in earlier years. In that case, a detailed analysis of the involved proteinase system showed us a way how we could very specifically interfere with implantation initiation in the rabbit model by suppressing the key enzyme of the blastocyst (blastolemmase). When highly potent, non-toxic blastolemmase inhibitors were administered to the uterus, implantation was inhibited, but development of the blastocysts continued (although at a slightly reduced pace) for some days so that the non-implanted embryos finally possessed e.g. a neural tube, somites and a heart anlage [for an illustration, see Denker, 2000, fig. 1c]. Some of these embryos accomplished a delayed attachment to the uterine mucosa which, however, remained insufficient for the formation of a normal placenta, and, probably due to what could be called (somewhat superficially) a nutritional defect, they all succumbed and were finally resorbed. When I saw these results of my experiments I felt that there was reason for great concern here with respect to possible applications in the human and I concluded that it should be regarded as non-desirable to base contraception on interference with development at any stage beyond the formation of the zygote [Denker, 1977, p. 101]. The cdx2 knockout concept is certainly different from the just described scenario insofar as the process of trophoblast differentiation is already targeted (and not the later trophoblast functions during implantation). However, the ethical concerns that should come up are somewhat related in both cases.

Reprogramming of somatic cells in order to derive ESCs from them (alternative source *d*, see above) has become perhaps the most interesting alternative, as suggested by recent publications that have appeared after the Symposium [Takahashi and Yamanaka, 2006; Okita et al., 2007; Wernig et al., 2007]. These papers describe the successful generation of pluripotent/omnipotent stem cells from somatic cells, i.e. from mouse fibroblasts, by gene manipulation. More recently, the same was shown to be possible with human cells [Nakagawa et al., 2007; Takahashi et al., 2007; Yu et al., 2007]. This obviously opens up a way to avoid using (and sacrificing) embryos or oocytes during the process of ES-like cell generation. However, this brings us back to the other already mentioned important point discussed at the Symposium, the developmental potential of the derived ESCs (or ES-like cells), and to the method of TC. Wernig et al. [2007] have indeed shown that these engineered cells can ('re')constitute an embryo when TC is performed. Would they also be able to initiate basic body plan formation, an ordered gastrulation, not only under this but also under other conditions, e.g. autonomously in dense in vitro cultures like in Thomson's experiments in the marmoset monkey [Thomson et al., 1996]? But even if the addition of helper cells would be required as in TC: Imagine a patient for whom such ES-like cells are generated from his own fibroblasts, since he needs their derivatives for some type of cell replacement therapy. If he is willing to donate the surplus of these cells (after expansion in vitro), or if surplus cells are stored in liquid nitrogen for a possible later repeat of the therapy, would it not be imperative to inform him about the fact that embryos could be cloned from these cells by TC at any time, even after his death, and that these cells would be genetically identical to himself (except for the genetic modifications that have been induced during the cell line derivation process, if these are at all permanent modifications)? This is of course only one side of the coin, i.e. the view of the donor, not talking about the view of the cloned child. As mentioned in the beginning, patients or cell/embryo donors are at present not even being informed about TC when asked to give their 'informed' consent [Denker and Denker, 2005].

The need to question the ethical implications of the TC technology in the human is not a far-fetched argument regarding the recent literature. A Belgian-British team has recently proposed to introduce TC into in vitro fertilization and embryo transfer protocols in the human [Devolder and Ward, 2007]. The proposed logic in their argument is that success rates using this procedure might be considerably increased if one does not directly transfer the in vitro produced embryo to a uterus but first creates ESCs from it; these ESCs are then expanded in vitro and transformed into (a larger number of) embryos by TC (using trophoblast cells differentiated from the ESCs as helper cells). This multiplication step (a cloning procedure, no doubt) increases the chance for finally obtaining the wanted child, those authors argue, since supernumerary embryos (of the same genotype) can of course be frozen and part of the ESCs can also be kept on stock in liquid nitrogen to repeat the procedure at any time point.

This proposal makes it very obvious that we have to think seriously about the developmental potential of cells we are handling in the laboratory and the clinics. According to the recent state of knowledge (confirmed by the experts present at the Symposium in Bonn), cells taken from early embryos (blastomeres) and ES(-like) cells are the only cell types that have the potential to (re)constitute an embryo at TC [Denker, 2006]. The possibility to apply TC in the human (as already proposed since it is successful in the mouse [Devolder and Ward, 2007]) forces us to give high priority to the potentiality argument in ethical discussions. Will human fibroblasts, when transformed into pluripotent cells by genetic manipulations, also gain the ability to constitute a viable embryo at TC? This was the case in the mouse as recently shown by Wernig et al. [2007]!

Is there an escape from the ethical dilemma? After the problem of sacrificing embryos (and oocytes) has possibly been solved by the prospect to use genetically modified somatic cells (fibroblasts), the remaining potentiality problem still needs to be tackled. After having discussed ethical problems with the cdx2 knockout/knockdown concept (see above) at the Symposium, the question arose whether other genes, such as those critically involved in gastrulation/basic body pattern formation [Tam and Loebel, 2007], could be ethically more acceptable targets. This idea was a logical consequence of the discussions about the pattern formation capabilities that had been described for marmoset monkey ESCs [Thomson et al., 1996, see above] as well as the (more limited) gastrulation-like (epithelial-mesenchymal transition) events in rhesus monkey ESCs presented by Michael Thie [Behr et al., 2005], and the established fact that ESCs of course perform an ordered gastrulation after TC. To knock out/down a single gene playing a role in gastrulation would possibly not suffice to eliminate the gastrulation potential completely since this is a complex process involving the cooperation of many genes. Of course, the fact that all genes known to be essential in early development also seem to play a role somewhere in other tissues in adult life has to be taken into consideration. However, an appropriate combination of a few key genes for axis formation and gastrulation (including the events involved in morphogenetic movements and induction for example) could possibly be efficient enough. Would such genetically or epigenetically engineered cells, e.g. when generated from fibroblasts, be ethically acceptable under potentiality aspects, and how can we make sure they do not have gastrulation/individuation capacity at TC? If future strategies will focus on interference with gastrulation genes, it would become imperative to test that this has indeed been achieved. Pluripotent/omnipotent cell lines which possess tetraploid complementability have to be considered ethically non-acceptable. How can the (lack of) gastrulation potential be tested in an ethically sound way, since TC (being clearly a technology for reproductive cloning) is certainly ethically not acceptable in the human? Years ago we had already proposed to develop an in vitro test system that would allow to check whether cells do or do not possess this unwanted, ethically problematical potential, and to do this under conditions under which formation of a basic body plan is impossible. The criteria would be the gene expression patterns of the cells under scrutiny; the selection of the specific set of genes and the conditions of in vitro culturing would have to be defined [Denker, 2003; Thie et al., 2003; Behr et al., 2005]. On the basis of the discussions held at the Symposium in Bonn, and underlined by recent reports on the generation of ES-like cells from fibroblasts [Takahashi and Yamanaka, 2006; Nakagawa et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007], it appears to me that it has now indeed become inevitable to invest time and money into such research. It is time to face the central problem, the problem posed by the developmental potential of the cells. Research concepts that are based on these considerations may be the only way really leading out of the ethical impasse.

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