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A Quest for Refocussing Stem Cell Induction Strategies: How to Deal with Ethical Objections and Patenting Problems

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Abstract

A recent ruling of the European Court of Justice (EU-CJ) in Luxembourg (18 October 2011) on ES cell patenting has renewed the interest in addressing so-far unsolved ethical problems of stem cell research. In this contribution I will outline ethical and patenting problems that arise when working with pluripotent stem cells, specifically in the modern field of induced pluripotent stem cell (iPSC) technology. The focus will be on stem cell potentiality, and I will argue that potentiality rather than the act of sacrificing embryos will have to be a central point of concern in stem cell ethics and patenting in the future. Possible solutions will be discussed.

When somatic cells are reprogrammed to gain "full" pluripotency, they acquire (so to say as a by-product) the capability to form viable embryos if tetraploid complementation (TC) is performed (termed "gold standard" by some authors). I argue that any human cells possessing this capability cannot be patented. In analogy to the arguments used by the EU-CJ, this must apply not only to patenting cell lines themselves but also to patenting technologies using these cells. The patenting problem is more than an obstacle for researchers and

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companies: It points to an ethical problem behind. The fact that the problem is being created by the process of iPSC induction asks for alternative strategies of stem cell derivation as well as for stringent criteria how to define and to test pluripotency vs. lower levels of potentiality. It will have to be discussed which genes should be seen here as crucial (e.g. genes involved in early embryonic pattern formation / self-organization processes). For ethical reasons it cannot be defended to use TC as a test for "full" pluripotency with human cells. It is thus necessary to discuss alternative test criteria.

Recent reports suggest that it may indeed be possible to directly induce lower degrees of potentiality (e.g. multipotency) while bypassing a pluripotent state, thus avoiding the addressed problems. It appears timely and prudent, therefore, to redefine goals and to refocus strategies for stem cell derivation, in addition to stem cell quality testing criteria, in order to avoid the ethical and patenting dilemma.

Keywords: iPSC, potentiality, pluripotency, multipotency, derivation strategies, bypassing pluripotency, direct reprogramming, tetraploid complementation, patenting, ethics.

9.1 Introduction

A recent ruling (of October 18, 2011) of the European Court of Justice in Luxembourg (EU-CJ) has renewed the interest of researchers as well as of the broader public in addressing so-far unsolved ethical problems of stem cell research [1, 2]. The Court ruled that human embryonic stem cells (hESCs) are to be excluded from patentability due to the fact that their derivation involves sacrificing embryos, an act that is considered contrary to *ordre public* and morality. Remarkably, the Court, by addressing definitions of the term "embryo", specifies that its rulings include products of artificial activation of eggs, i.e. parthenotes, and products of nuclear transfer to oocytes. This is in contrast to arguments that had been put forward by some authors before, i.e. that such artificially created embryos would not deserve the same moral status. Thus the EU-CJ puts emphasis on potentiality as an ethical argument, which is indeed a new focus and is in contrast to the circumstantial arguments used before by others (e.g. putting weight on the question where and how an embryo had been created, *in vivo* or in the lab, by sperm penetration or by nuclear transfer, etc.) which had led them to different conclusions.

I will argue in this contribution that considering the potentiality of cells is very timely and needs to be pursued seriously in stem cell ethics, in the

future. This argument is based not only on the ruling of the EU-CJ but even more so on the fact that most stem cell laboratories now focus on actively changing cell potentiality, specifically when deriving induced pluripotent stem cells (iPSCs). By endowing cells with properties they did not have before researchers unintentionally create at the same time an ethical problem: When cells reach pluripotency they acquire the ability to enable direct cloning by tetraploid complementation (TC). Acquisition of this new property, I will argue, must indeed preclude patentability, a consideration that illuminates the ethical ambiguity of inducing pluripotency.

For many researchers this warning may come as a surprise. Indeed, human iPSCs (hiPSCs) are so far most often addressed as ethically non-problematical since, in contrast to ESCs, iPSC derivation does not involve sacrificing embryos. Examples for such statements can be found often in the literature, e.g.: *"Direct reprogramming through the ectopic expression of defined transcription factors... represents a simple way to obtain pluripotent stem-cell lines from almost any somatic tissue and mammalian species. The use of such cells also circumvents the ethical issues associated with human cells"* [3]. However, there are also contrasting statements but they are so far found more rarely in the literature, e.g.: *"There would be severe ethical problems associated with using tetraploid complementation technology [with iPSCs] in humans, even without the intention of implanting the resulting artificially created embryos into a uterus (see, for example [4]). The issues are similar to those that have arisen over embryonic stem cells and include aspects of patentability." [5].* Likewise: *"The use of iPSCs and tetraploid complementation for human reproductive cloning would raise profound ethical objections. Professional standards and laws that ban human reproductive cloning by somatic cell nuclear transfer should be revised to also forbid it by other methods, such as iPSCs via tetraploid complementation."* [6].

In the present contribution I will discuss in some detail what reasons are behind such warnings, focussing on the potentiality of ESCs and iPSCs. I will specifically address a peculiar property that pluripotent stem cells (ESCs and iPSCs) have, i.e. early embryonic pattern formation potential, which becomes particularly obvious when tetraploid complementation (TC) is performed. I will argue that this precludes patentability. Finally, I will put emphasis on alternative strategies for stem cell derivation, circumventing pluripotency, and I will argue that these strategies indeed appear successful and practical, which leads to the conclusion that their use is clearly preferable under ethical and patenting aspects.

9.2 Potential for Autonomous Pattern Formation: Embryoid Bodies

One of the remarkable biological properties of pluripotent cells is their ability to form, in suspension cultures, **"embryoid bodies" (EBs)**. What is most widely known about EBs is that formation of these embryo-like structures promotes the formation of germ layers. Much less intensely studied is the degree of order that the germ layers and their derivatives can attain in EBs, and how close their organisation can really come to the basic body plan of viable embryos. Surprisingly, this aspect appears to receive increased attention only recently.

A particularly remarkable observation on early embryonic pattern formation in EBs was published already in one of the pioneering papers on ESCs [7]. In this case the spontaneous formation of astonishingly embryo-like structures was observed in dense cultures of common marmoset ESCs (Callithrix jacchus, a South American primate): These structures were described to consist of a flat embryonic disc as typical for primates, with primitive ectoderm, primitive endoderm, an amnion with amniotic cavity, a yolk sac. Most remarkably, those authors also depicted and described, within this embryonic disc, an area of ordered ingression of cells, which they addressed as a primitive streak (PS). The PS is of utmost importance in vertebrate embryology, because it is the site where not only the formation of the definitive germ layers (in particular mesoderm and definitive endoderm) takes place but is also instrumental in individuation: The anterior part of the PS is the equivalent of Spemann's organizer which plays a central role in laying down the basic body plan, i.e. the ordered arrangement of germ layers and their derivatives according to the main body axes (dorso-ventral, anterior-posterior = cranio-caudal). The development of single or double organizers is decisive for the formation of a singlet vs. monozygotic twins (discussed in [8]).

While the structures formed spontaneously in the marmoset ESC cultures according to Thomson et al. [7] were remarkably embryo-like, and even appeared to show signs of incipient individuation (discussed in [8]) a comparable degree of order has never since been described to occur in ESC cultures in any other species, including the rhesus monkey and the mouse. Locally restricted gastrulation-like events have, however, been observed [9–12] . The degree of order attained appears to depend very much on physical conditions of culturing [9, 12], as was to be expected on the basis of what we know from developmental biology [8]. It is strongly influenced by other (non-stem) cells or matrix simultaneously present in the cultures. On the other

hand, such an early embryonic pattern-formation process has never been observed in comparable cultures of non-pluripotent cell types (e.g. fibroblasts) and thus obviously primarily depends on **intrinsic properties of pluripotent cells**. It was observed to occur either in suspension cultures, i.e. without the addition of other cell types and without attachment, or in dense flat cultures on feeder cells (fibroblasts) which likewise cannot be expected to provide specific pattern information, so that it is reasonable to assume that this pattern formation process is primarily **autonomous** and can be correctly addressed as a process of **self-organization** [9, 11]. The biological basis for these self-organization phenomena has been discussed earlier [8]. With respect to the question how close the observed *in-vitro* events may come to embryogenesis, ten Berge et al. [11] were astonished to observe a much higher degree of spatiotemporal order in gene activation cascades than they had expected to see in ESC cultures, specifically concerning *wnt* pathway-associated events, and that this was comparable to what is observed in mouse gastrulation *in vivo*.

What can EBs teach us with respect to ethical considerations? According to the described observations, pluripotent stem cells (PSCs) possess **gastrulation** potential and can show impressive early embryonic pattern formation (**self-organization**) potential *in vitro*. These processes are central elements of **basic body plan** formation and **individuation** during embryogenesis (for a review see [8]). Formation of EBs *in vitro* obviously can (depending on culturing conditions) come much closer to *in vivo* embryogenesis than originally thought by many. However, *in praxi* EBs rarely reach the high degree of order of a harmonious basic body plan. Therefore, depending on the degree of stringency one likes to use with respect to ethical norms, it could be argued that EB formation does or does not offer arguments with respect to ethical aspects of PSC use. With the recent focus of PSC research in mind we should not leave this discussion, however, without noticing that all these considerations do not only apply to ESCs but also to iPSCs because the latter cells basically show the same biological properties, including EB formation capacity, although the latter has so far been studied much less in detail with iPSCs than with ESCs.

9.3 Potential for Assisted Development: Tetraploid Complementation

Tetraploid complementation (TC) is a method for **cloning viable individuals from PSCs** (ESCs, but also iPSCs). We are discussing it here as a topic concerning stem cell quality testing strategies, ethics, legislation, and patenting regulations that is just beginning to be recognized.

TC was developed as a variant of chimera formation in the mouse. It provides a method to produce viable embryos and even offspring derived entirely from PSCs that had been propagated before *in vitro* [13–15]. The method relies on the combination of the pluripotent cells with tetraploidized blastomeres or, alternatively, on the injection into tetraploidized blastocysts. Remarkably, this method of cloning viable individuals is successful not only with ESCs but also with iPSCs. In the latter case the term **"all-iPSC mice"** has become popular for the products of this type of cloning [16–20].

Cloning by TC is widely used in experimental research as some type of quality control for PSCs (ESCs and iPSCs) in the mouse. Testing cells for TC capability is being addressed by some authors as the "gold standard" of pluripotency and its use is being advocated in a way that might suggest using it also with human cells [16, 18].

Why would anyone possibly be interested in applying TC with **human** cells? It may appear improbable that TC will be used in the near future for **reproductive cloning** in the human since so far there is widespread consensus to consider this unethical. However, there appears to be reason to question whether this consensus can be expected to hold for long and worldwide. In the Western world, it has already been proposed to consider using TC technology in order to increase success rates in *in-vitro* fertilization/embryo transfer (IVF-ET) clinics [21]. The idea is to derive ESCs from human IVF embryos, expand them *in vitro*, and (re-)construct from them a (larger) number of embryos by TC which can then be used for embryo transfer (while aliquots of the ESCs as well as some of the numerous identical embryos produced could of course be stored in liquid nitrogen to be available for later use in repeated attempts) [21]. Since this means cloning, and since reproductive cloning in the human is considered illegal at least in a number of countries, one may be skeptical whether this technique will ever be applied in Western world countries. However, it cannot be excluded that legislation may develop in a different direction in other cultural environments. As an example, Buddhist authorities have expressed that they would consider embryo destruction in the course of "therapeutic cloning" (for the production of ESCs) unethical, but not so the (re-)construction of embryos in the course of reproductive cloning (for literature see [4]).

While any application of TC for reproductive cloning in the human may appear improbable at the moment its use for **research** and **quality testing** purposes has indeed been proposed frequently, in the mouse, and with respect to human cells at least indirectly. This notion is found in the literature particularly often since the advent of iPSC research. First of all TC is

recommended as the most rigorous pluripotency test ("gold standard") for iPS cells in the mouse (*"We therefore consider the tetraploid complementation as the state-of-the-art technique to assess the pluripotency of a given cell line"* [22]; *"This study underscores the intrinsic qualitative differences between iPS cells generated by different methods and highlights the need to rigorously characterize iPS cells beyond in vitro studies."* [23]). Likewise in the first reports on the generation of viable mice from iPSCs it had already been suggested indirectly to apply TC technology for iPSC quality testing also in the human, for the reason that this is considered the most rigorous pluripotency test [16, 18]. Remarkably, it was felt necessary, therefore, to publish a comment on these papers (in the same journal) clarifying that for ethical reasons it cannot be defended to follow this (implicit) recommendation, i.e. to use the technique for iPSC quality testing in the human [5].

Temptations may indeed be high to consider applying TC technology with human pluripotent cells, in spite of these warnings. Why? Recent literature is full of data asking for stringent quality testing in iPSC research. Individual iPSC lines are observed, in the mouse as well as in the human, to vary with respect to differentiation capacities, gene expression patterns and epigenetic marks/memory [24–28]. Stadtfeldt et al. [20] provided a typical and interesting example. They observed that transcripts encoded within the imprinted Dlk1-Dio3 gene cluster were aberrantly silenced in most of the iPSC clones, and that these clones failed to support the development of entirely iPSC-derived animals ("all-iPSC mice") when TC was performed thus revealing a lack of "complete pluripotency". This failure could, however, be corrected by a treatment with a histone deacetylation inhibitor which reactivated the locus. It is clear that investigators wish to have a test available to monitor the success of this type of cell quality improvement. In addition to epigenetic peculiarities of PSC lines (as compared to early embryonic cells) even chromosomal aberrations and gene deletions have been observed in some cases [29, 30].

Such observations obviously could be seen as a strong argument for using the most stringent pluripotency test (TC) also with human iPSCs in order to select "optimal" cell lines and/or stem cell derivation protocols. This logic may be particularly obvious if cells are to be used for **therapeutic purposes** (cell and tissue replacement) in the human, and likewise whenever iPSCs are used for **disease modelling**. In cell and tissue replacement, the concern is that transplanted cells should be genetically and epigenetically as "normal" as possible in order to minimize risks e.g. with respect to tumor formation. In disease modelling with genetic/epigenetic focus, experiments are usually done in the first place in the mouse model, sometimes including TC technology

(which of course poses no ethical problem in the mouse) (as an example, see [22]: *"Genetic manipulation of iPS cells in combination with tetraploid embryo aggregation provides a practical and rapid approach to evaluate the efficacy of gene correction of human diseases in mouse models.").* But: How then could that be translated to human therapy without testing human iPSCs with comparable stringency, as it is being done with the mouse cells within the same experimental project? Would such considerations be a valid argument for the application of TC with human cells in order to use again the most stringent test? A simple and strict logic might suggest studying differentiation and gene expression in human TC embryos so produced *in vitro*. It should be seen, however, that even without transferring such human embryos created from iPSCs ("artificial" or "test" embryos) to a uterus such a procedure would (re-)create the problem of embryo destruction which the original idea of iPSC technology intends to eliminate. It would clearly be in conflict with e.g. the German embryo protection law (Embryonenschutzgesetz). Furthermore, any use or possible use of such a quality testing strategy involving cloning by TC would definitely need to be included in the catalogue of information to be given to cell donors when **informed consent** is obtained; so far cell donors are not informed about the possibility of cloning embryos from PSCs by TC at all. Indeed, the catalogue of information that is so far routinely given to cell donors must urgently be updated, in particular with regard to hiPSC derivation: It cannot be defended anymore to omit information that the gain of pluripotency implies the gain of cloning capability by TC. I will not expand on this important topic here because it has been addressed before [31] but it should be seen that appropriate regulations are still missing so tat the ethical problem remains unsolved.

9.4 Pluripotency, an Obstacle for Patenting

The recent ruling of the European Court of Justice (EU-CJ) mentioned in the Introduction is of interest not only with respect to embryo use for stem cell derivation, i.e. the point that received most of the attention of the broader public. This decision is also of interest with respect to consequences to be drawn from the developmental potential of pluripotent cells just discussed, in particular the TC capability. The court emphasized, in the definitions which it had been asked to provide of the term "embryo" in the context of the legal/patenting regulations in question, the aspect of **potentiality**. It was ruled that *"any human ovum after fertilisation, any non-fertilised human ovum into which the cell nucleus from a mature human cell has been transplanted,*

and any non-fertilised human ovum whose division and further development have been stimulated by parthenogenesis constitute a 'human embryo'; - it is for the referring court to ascertain, in the light of scientific developments, whether a stem cell obtained from a human embryo at the blastocyst stage constitutes a 'human embryo' within the meaning of Article 6(2)(c) of Directive 98/44." [1].

Previously a number of authors had argued that the degree of respect to be paid to human embryos (and the degree of legal protection given to them) should depend on the way how they had been created (natural fertilization vs. intracytoplasmic sperm injection or nuclear transfer technologies, artificial egg activation/parthenogenesis), on the actual location of the early embryo in question (within the female genital tract or *in vitro*) or whether the embryo is already implanted in the uterus or not yet. So for example Vrtovec and Vrtovec have argued with respect to ethical aspects of patenting of PSCs that *"the exclusion from patentability is probably not justifiable for human totipotent cells that are produced outside the human body by (. . .) 'techniques which human beings alone are capable of putting into practice and which nature is incapable of accomplishing by itself'"* ([32], p. 3028). This argument has immediately been rejected [33] but can still be heard. A logical consequence of the recent ruling of the EU-CJ, however, is that the potentiality of the cells (original embryonic cells or cells possessing equivalent potential, i.e. hESCs or hiPSCs) need to be seen as a major argument for ethical considerations and patenting. My prediction is, therefore, that future court decisions and legislative actions will have to use potentiality as a new focus, and that this will necessarily play a major role in future decisions specifically with respect to iPSCs, since the main point in the derivation of iPSCs is to endow originally ethically non-problematic cells with new potentiality. In case of PSCs these peculiar new properties include, as discussed above, early embryonic pattern formation (individuation) and TC capability, the main biological characteristics of early embryonic cells.

9.5 Alternative Approaches

Are there alternative strategies available for deriving stem and/or precursor cells while avoiding the ethical and patenting problems just discussed?

Recent literature suggests that it is indeed possible to choose alternative strategies for stem and precursor cell derivation bypassing pluripotency. Until recently it was assumed by most authors that somatic/adult stem cells are not sufficiently expandable *in vitro*, and that only pluripotent stem cells

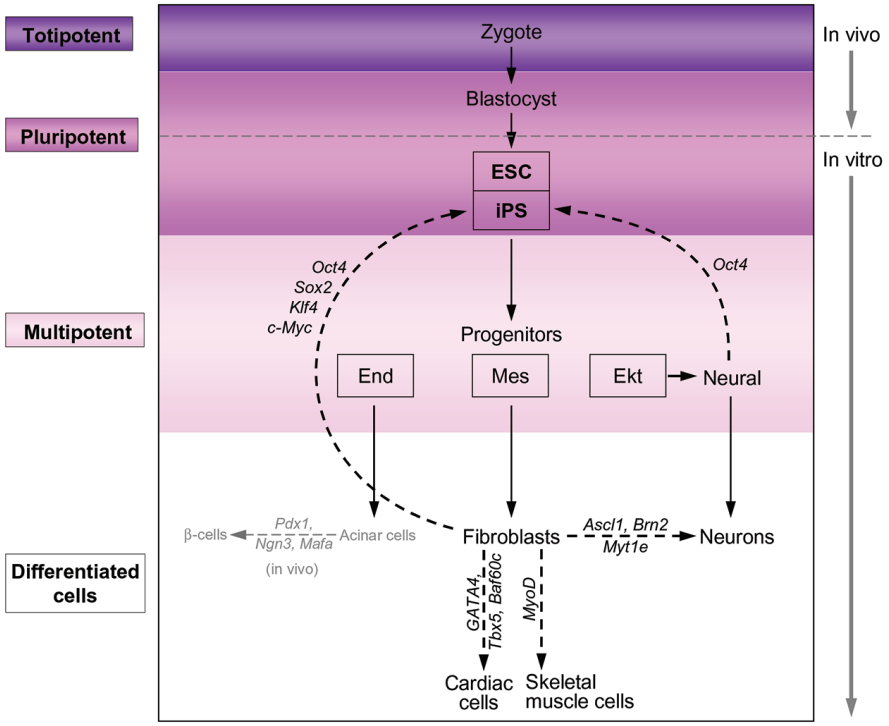

Figure 9.1 Strategies for stem and progenitor cell derivation and cell reprogramming. The *"traditional" strategy* includes a pluripotent state of cells (ESC, iPSC; above); from these pluripotent stem cells multipotent lineage-specific progenitors and finally the various differentiated cell types are derived. In case of iPSC, the cells of origin are differentiated cells (e.g. fibroblasts) which are reprogrammed by activation of the four pluripotency-associated genes Oct4, Sox2, Klf4 and c-Myc (Yamanaka factors; left part of diagram). The *alternative strategy* (lower part of the diagram) avoids the induction of the ethically problematic pluripotent state (*direct reprogramming, bypassing pluripotency*): In this strategy, transcription factor-induced lineage reprogramming results either in cells remaining within the same cell lineage (i.e. mesoderm), or may produce functional cells of other lineages (converting mesodermal fibroblasts into ectodermal neurons). This transcription factor-induced lineage reprogramming not only avoids the ethical problem posed by a self-organizing and cloning capability gained (e.g. TC capability) but possibly also reduces tumor-formation risks (from [48] with permission).

(ESCs and iPSCs) offer the advantage of growing well and of being able, in addition, to differentiate as desired for regenerative medicine. In the "classical" approach, iPSCs are created by transduction and overexpression or at least temporal activation of the "Yamanaka factor" genes Oct4, Sox2,

Klf4, c-Myc; for transplantation or for disease modelling experiments the pluripotent cells so created are subsequently converted into multipotent progenitor cells, and these to the various differentiated cell types of interest (Figure 9.1). This strategy has been more or less the same in all investigations involving the creation of PSCs, irrespective of the cell type of origin chosen (fibroblasts, epithelial cells, etc.), and also irrespective of the mode of derivation (transient or permanent genetic, or epigenetic modification). Since with this "classical" strategy the induction of pluripotency is the first goal (in case of iPSCs; in case of ESCs it is not the induction but the maintenance of it), this strategy unfortunately creates exactly the ethical problem discussed above.

According to recent literature it appears possible, however, to use **alternative strategies bypassing pluripotency**. This opens a chance to avoid the ethical (and patenting) problems posed by an early embryonic pattern formation/self-organization potential of cells. Direct reprogramming, not including a (transient) state of pluripotency, has now been described for the derivation of e.g. cardiomyocytes, blood progenitors and neuronal cells, and in some of these cases the derived cells were efficiently expandable *in vitro*, a property that many authors previously considered to be a specific characteristic and major advantage of PSCs only. Literature on this new line of research is very rapidly expanding at present. These alternative strategies are depicted graphically in the lower part of Figure 9.1. The cells of origin (e.g. fibroblasts) are in these cases directly reprogrammed to form stem and progenitor cells possessing a lower level potentiality (without passing through a pluripotent state), using combinations of transcription factors that are specific for the desired differentiation pathway (direct transduction or indirect epigenetic modification). Examples are given in the lower part of Figure 9.1 (for more examples see [34–44]).

9.6 Conclusions

That the developmental potential of pluripotent stem cells must be considered as an eminent aspect of the ethics of stem cell use (including aspects of patenting) has been pointed out and discussed already since many years [4, 45, 46]. In particular after the advent and widespread use of cell reprogramming technologies it must be seen as a logical necessity to discuss which avenues could be taken to develop strategies for stem cell derivation which avoid the ethical problem of pluripotency [4, 47]. While this argumentation had originally been based predominantly on theory and only on few experimental findings, the numerous recent publications on direct

reprogramming/bypassing pluripotency mentioned in the previous paragraph suggest that we have now arrived at a point at which such avenues become indeed a most attractive option. One may ask why such a redirection of focus (direct reprogramming) has not been searched for more actively already during previous years, although the ethical arguments why such efforts should appear necessary had already been published since years.

The new strategies of inducing direct conversion of somatic cells to a stem/progenitor state, bypassing pluripotency, appear highly promising and recommendable. They are obviously preferable for ethical reasons because they avoid the problem created by inducing an embryo formation/cloning potential which fully pluripotent cells have. An additional advantage of these new strategies may be to reduce the risk of tumor formation after transplanting such cells because the tumor formation potential may be connected with the embryonic pattern formation/self-organization potential. A word of caution appears to be in place, nevertheless: In order for any such alternative strategy to be ethically acceptable, it must be made sure that it does not involve a transitory state of pluripotency that could remain undetected. Many of the induction protocols require very long culturing time periods, and we are far from understanding exactly what cascade of events takes place during this time period. Some of the protocols include while some omit Oct4, some use combinations of certain (but not all) of the Yamanaka factors while others do not. Which of the possible protocols will be safest in order to exclude TC capability as well as tumor formation risks? It will have to be discussed which genes should be seen here as crucial (e.g. genes involved in early embryonic pattern formation / self-organization processes) [4, 47]. This will be an important topic for future research. Strategies for testing this will need to be developed and discussed: For ethical reasons it cannot be defended to test human cells by cloning via TC [5]. It will thus be necessary to define appropriate combinations of *in vitro* gene expression profiling that may be useful instead in a first approximation, combined with *in vitro* culturing conditions that avoid the initiation of individuation processes. In any case it will be necessary to improve the catalogue of informations routinely given to cell donors: This information needs to include all aspects of the potentiality that the donated cells will or may acquire as a result of reprogramming, including TC capability, because this touches upon personal interests donors have (genetic identity and uniqueness). Implications of cell banking need to be included keeping in mind that ethical and legal standards may change and already differ in the various cultural environments. These aspects are

particularly relevant when long term storage and widespread use of the cells are envisaged.

Obviously this complex field of ethical problems can be avoided by circumventing any gain of pluripotency at all. A general recommendation for strategies of stem cell derivation would thus be to deviate from the widespread practice of activating the pluripotency program (creating iPSCs) and rather to rely on the alternative strategies bypassing pluripotency, i.e. to convert cells directly to multipotent stem/progenitor cells as in the examples given above.

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References

[1] EU-CJ, Judgment of the Court (Grand Chamber) of 18 October 2011(reference for a preliminary ruling from the Bundesgerichtshof - Germany) - Oliver Brüstle v Greenpeace e.V. (Case C-34/10) 1. EUGH Curia Europa(http://curia.europa.eu/juris/document/document.jsf?docid=115334&mode=lst&pageIndex=1&dir=&occ=first&part=1&text=&doclang=EN&cid=114275), 2011.

[2] Callaway E. European court bans patents based on embryonic stem cells. Final decision could stifle investments in developing therapies. Nature News (Published online 18 October 2011, doi:10.1038/news.2011.597), 2011

[3] González F., Boué S., Belmonte J. C. Methods for making induced pluripotent stem cells: reprogramming a la carte. Nat. Rev. Genet., 12: 231–242, 2011.

[4] Denker H.-W. Induced pluripotent stem cells: how to deal with the developmental potential. Reprod. Biomed. Online, 19 Suppl 1: 34–37, 2009.

[5] Denker H.-W. Ethical concerns over use of new cloning technique in humans. Nature, 461 (7262): 341, 2009.

[6] Lo B., Parham L., Alvarez-Buylla A., Cedars M., Conklin B., Fisher S., Gates E., Giudice L., Halme D. G., Hershon W., Kriegstein A., Kwok P. Y., Wagner R. Cloning mice and men: prohibiting the use of iPS cells for human reproductive cloning. Cell Stem Cell, 6 (1): 16–20, 2010.

[7] Thomson J. A., Kalishman J., Golos T. G., Durning M., Harris C. P., Hearn J. P. Pluripotent Cell Lines Derived from Common Marmoset (Callithrix jacchus) Blastocysts. Biol. Reprod., 55(2): 254–259, 1996.

[8] Denker H.-W. Early human development: new data raise important embryological and ethical questions relevant for stem cell research. Naturwissenschaften, 91 (1): 1–21, 2004.

[9] Fuchs C., Scheinast M., Pasteiner, W., Lagger S., Hofner, M., Hoellrigl A., Schultheis M., Weitzer, G. Self-Organization Phenomena in Embryonic Stem Cell-Derived Embryoid Bodies: Axis Formation and Breaking of Symmetry during Cardiomyogenesis. Cells Tissues Organs, 195(5): 377–391, 2012.

[10] Behr R., Heneweer C., Viebahn C., Denker H.-W., Thie M. Epithelial-mesenchymal transition in colonies of rhesus monkey embryonic stem cells: a model for processes involved in gastrulation. Stem Cells, 23 (6): 805–816, 2005.

[11] ten Berge D., Koole W., Fuerer C., Fish M., Eroglu E., Nusse R. Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell, 3 (5): 508–518, 2008.

[12] Maranca-Hüwel B., Denker H.-W. Epithelial-mesenchymal transition in rhesus monkey embryonic stem cell colonies: the role of culturing conditions. *In Vitro* Cell. Dev. Biol. Anim., 46 (6): 516–528, 2010.

[13] Nagy A., Gocza, E., Diaz E. M., Prideaux V. R., Ivanyi E., Markkula M., Rossant J. Embryonic stem cells alone are able to support fetal development in the mouse. Development, 110 (3): 815–821, 1990.

[14] Nagy A., Rossant J., Nagy R., Abramow-Newerly W., Roder J. C. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl. Acad. Sci. USA, 90 (18): 8424–8428, 1993.

[15] Tam P. P. L., Rossant J. Mouse embryonic chimeras: tools for studying mammalian development. Development, 130 (25): 6155–6163, 2003.

[16] Boland M. J., Hazen J. L., Nazor K. L., Rodriguez A. R., Gifford W., Martin G., Kupriyanov S., Baldwin K. K. Adult mice generated from induced pluripotent stem cells. Nature, 461 (7260): 91–94, 2009.

[17] Kang L., Wang J., Zhang Y., Kou Z., Gao S. iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. Cell Stem Cell, 5 (2): 135–138, 2009.

[18] Zhao X.-y., Li W., Lv Z., Liu L., Tong M., Hai T., Hao J., Gou C.-l., Ma Q.-w., Wang L., Zeng F., Zhou Q. iPS cells produce viable mice through tetraploid complementation. Nature, 461: 86–90, 2009.

[19] Zhao X.-Y., Lv Z., Li W., Zeng F., Zhou Q. Production of mice using iPS cells and tetraploid complementation. Nat Protoc, 5 (5): 963–971, 2010.

[20] Stadtfeld M., Apostolou E., Akutsu H., Fukuda A., Follett P., Natesan, S., Kono T., Shioda T., Hochedlinger, K. Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. Nature, 465 (7295): 175–181, 2010.

[21] Devolder K., Ward C. M., Rescuing human embryonic stem cell research: The possibility of embryo reconstruction after stem cell derivation. Metaphilosophy, 38 (2): 245–263, 2007.

[22] Wu G., Liu N., Rittelmeyer I., Sharma A. D., Sgodda M., Zaehres H., Bleidissel M., Greber B., Gentile L., Han D. W., Rudolph C., Steinemann D., Schambach A., Ott M., Schöler H. R., Cantz T. Generation of Healthy Mice from Gene-Corrected Disease-Specific Induced Pluripotent Stem Cells. PLoS Biol., 9 (7): e1001099, 2011.

[23] Han J., Yuan P., Yang H., Zhang J., Soh B. S., Li P., Lim S. L., Cao S., Tay J., Orlov Y. L., Lufkin T., Ng H. H., Tam W. L., Lim B. Tbx3 improves the germ-line competency of induced pluripotent stem cells. Nature, 463: 1096–1100, 2010.

[24] Liu L., Luo G. Z., Yang W., Zhao X., Zheng Q., Lv Z., Li W., Wu H. J., Wang L., Wang X. J., Zhou Q. Activation of the imprinted Dlk1-Dio3 region correlates with pluripotency levels of mouse stem cells. J. Biol. Chem., 285 (25): 19483–19490, 2010.

[25] Kim K., Doi A., Wen B., Ng K., Zhao R., Cahan P., Kim J., Aryee M. J., Ji H., Ehrlich L. I., Yabuuchi A., Takeuchi A., Cunniff K. C., Hongguang H., McKinney-Freeman S., Naveiras O., Yoon T. J., Irizarry R. A., Jung N., Seita J., Hanna J., Murakami P., Jaenisch R., Weissleder R., Orkin S. H., Weissman I. L., Feinberg A. P., Daley G. Q. Epigenetic memory in induced pluripotent stem cells. Nature, 467 (7313): 285–290, 2010.

[26] Bar-Nur O., Russ H. A., Efrat S., Benvenisty N. Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell, 9 (1): 17–23, 2011.

[27] Lister R., Pelizzola M., Kida Y. S., Hawkins R. D., Nery J. R., Hon G., Antosiewicz-Bourget J., O'Malley R., Castanon R., Klugman S., Downes M., Yu R., Stewart R., Ren B., Thomson J. A., Evans R. M., Ecker J. R. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature, 471 (7336): 68–73, 2011.

[28] Nazor K. L., Altun G., Lynch C., Tran H., Harness J. V., Slavin I., Garitaonandia I., Muller F. J., Wang Y. C., Boscolo F. S., Fakunle E., Dumevska B., Lee S., Park H. S., Olee T., D'Lima D. D., Semechkin R., Parast M. M., Galat V., Laslett A. L., Schmidt U., Keirstead H. S., Loring J. F., Laurent L. C. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. Cell Stem Cell, 10 (5): 620–634, 2012.

[29] Mayshar Y., Ben-David U., Lavon N., Biancotti J. C., Yakir B., Clark A. T., Plath K., Lowry W. E., Benvenisty N. Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell, 7 (4): 521–531, 2010.

[30] Laurent L. C., Ulitsky I., Slavin I., Tran H., Schork A., Morey R., Lynch C., Harness J. V., Lee S., Barrero M. J., Ku S., Martynova M., Semechkin R., Galat V., Gottesfeld J., Izpisua Belmonte J. C., Murry C., Keirstead H. S., Park H. S., Schmidt U., Laslett A. L., Muller F. J., Nievergelt C. M., Shamir R., Loring J. F. Dynamic Changes in the Copy Number of Pluripotency and Cell Proliferation Genes in Human ESCs and iPSCs during Reprogramming and Time in Culture. Cell Stem Cell, 8 (1): 106–118, 2011.

[31] Denker U., Denker H.-W. Embryonale Stammzellforschung: Aufklärung notwendig. Problematik der informierten Zustimmung der Spender. Deutsches Ärzteblatt, 102 (13): A892–A893, 2005.

[32] Vrtovec K. T., Vrtovec B. Is Totipotency of a Human Cell a Sufficient Reason to Exclude its Patentability under the European Law? Stem Cells, 25 (12): 3026–3028, 2007.

[33] Denker H.-W. Totipotency/pluripotency and patentability. Stem Cells, 26 (6): 1656–1657, 2008.

[34] Ieda M., Fu J. D., Delgado-Olguin P., Vedantham V., Hayashi Y., Bruneau B. G., Srivastava D. Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors. Cell, 142 (3): 375–386, 2010.

[35] Szabo E., Rampalli S., Risueno R. M., Schnerch A., Mitchell R., Fiebig-Comyn A., Levadoux-Martin M., Bhatia M. Direct conversion of human fibroblasts to multilineage blood progenitors. Nature, 468 (7323): 521–526, 2010.

[36] Vierbuchen T., Ostermeier A., Pang Z. P., Kokubu Y., Sudhof T. C., Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. Nature, 463 (7284): 1035–1041, 2010.

[37] Caiazzo M., Dell'anno M. T., Dvoretskova E., Lazarevic D., Taverna S., Leo D., Sotnikova T. D., Menegon A., Roncaglia P., Colciago G.,

Russo G., Carninci P., Pezzoli G., Gainetdinov, R. R., Gustincich S., Dityatev A., Broccoli V. Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature, 476 (7359): 224–227, 2011.

[38] Pfisterer U., Kirkeby A., Torper O., Wood J., Nelander J., Dufour A., Bjorklund A., Lindvall O., Jakobsson J., Parmar M. Direct conversion of human fibroblasts to dopaminergic neurons. Proc. Natl. Acad. Sci. USA, 108 (25): 10343–10348, 2011.

[39] Son E. Y., Ichida J. K., Wainger B. J., Toma J. S., Rafuse V. F., Woolf C. J., Eggan K. Conversion of Mouse and Human Fibroblasts into Functional Spinal Motor Neurons. Cell Stem Cell, 9 (3): 205–218, 2011.

[40] Qiang L., Fujita R., Yamashita T., Angulo S., Rhinn H., Rhee D., Doege C., Chau L., Aubry L., Vanti W. B., Moreno H., Abeliovich A. Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. Cell, 146 (3): 359–371, 2011.

[41] Yoo A. S., Sun A. X., Li L., Shcheglovitov A., Portmann T., Li Y., Lee-Messer C., Dolmetsch R. E., Tsien R. W., Crabtree G. R. MicroRNA-mediated conversion of human fibroblasts to neurons. Nature, 476 (7359): 228–231, 2011.

[42] Jayawardena T. M., Egemnazarov B., Finch E. A., Zhang L., Payne J. A., Pandya K., Zhang Z., Rosenberg P., Mirotsou M., Dzau V. J. MicroRNA-Mediated *In Vitro* and *In Vivo* Direct Reprogramming of Cardiac Fibroblasts to Cardiomyocytes. Circ. Res., 110 (11): 1465–1473, 2012.

[43] Thier M., Worsdorfer P., Lakes, Y. B., Gorris R., Herms S., Opitz T., Seiferling D., Quandel T., Hoffmann P., Nothen, M. M., Brüstle O., Edenhofer F. Direct Conversion of Fibroblasts into Stably Expandable Neural Stem Cells. Cell Stem Cell, 10 (4): 473–479, 2012.

[44] Han D. W., Tapia N., Hermann, A., Hemmer K., Hoing S., Arauzo-Bravo M. J., Zaehres H., Wu G., Frank S., Moritz S., Greber B., Yang J. H., Lee H. T., Schwamborn J. C., Storch A., Schöler H. R. Direct Reprogramming of Fibroblasts into Neural Stem Cells by Defined Factors. Cell Stem Cell, 10 (4): 465–472, 2012.

[45] Denker H.-W. Embryonic stem cells: An exciting field for basic research and tissue engineering, but also an ethical dilemma? Cells Tissues Organs, 165: 246–249, 1999.

[46] Denker, H.-W. Potentiality of embryonic stem cells: an ethical problem even with alternative stem cell sources. J. Med. Ethics, 32 (11): 665–671, 2006.

[47] Denker, H.-W. Human embryonic stem cells: the real challenge for research as well as for bioethics is still ahead of us. Cells Tissues Organs, 187 (4): 250–256, 2008.

[48] Wobus, A. M. The Janus face of pluripotent stem cells–connection between pluripotency and tumourigenicity. Bioessays, 32 (11): 993–1002, 2010.