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From Stem Cells to IPS Cells. A Passionate Journey

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There is no doubt that this scientific finding could have potential clinical use, and as such it has been met with great hope, not only by medical science but also by the general public.

In addition to the unquestionable scientific interest in this finding is the fact that obtaining the aforementioned cells, which are similar to embryonic cells, does not require the destruction of human embryos, thus avoiding the serious ethical difficulty that the use of embryonic stem cells entails; it is well known that the human embryos from which they originate must be created and destroyed to obtain them.

This triple aspect – scientific interest, possible clinical usefulness and ethical goodness – has meant that as previously mentioned, the work by the Japanese and American researchers has been deserving of significant media attention.

However, the current study, conducted using human skin cells, has had its experimental prologue with animals and even its theoretical formulation stage.

In reality, it all originated when a few years ago, the dilemma of obtaining cells similar to embryonic cells by ethically correct procedures, i.e. by mechanisms which do not require the destruction of human embryos, was raised, since many researchers consider that embryonic stem cells constitute valuable biological material for important fundamental biomedical research[1]. Thus, the search for alternative sources of stem cells became a prime research objective.

Before using the reprogramming of adult human tissue cells to obtain cells similar to embryonic cells, other avenues were explored with the same end and are briefly summarised below.

The first possibility proposed was to obtain stem cells from embryonic blastomeres generated by in vitro fertilisation. In fact, if one cell is extracted from a 4 or 8 cell embryo, it can be cultured to generate stem cells and the embryo, even with one cell less, can survive if implanted in the uterus.

This technique was first used in 2004 by Strelchenko et al from the Reproductive Genetics Institute, Chicago, directed by Y Verlinsky[2]; they managed to obtain various cell lines from one pluripotent cell extracted from a 4-day old embryo (of 60 to 70 cells) generated by in vitro fertilisation, i.e. immediately before it reached the blastocyst developmental stage, which as mentioned, is achieved when the embryo is approximately five days old. When this technique is used, most of the time extraction of the cell to be used to generate the stem cells does not result in death of the embryo. However, to ethically legitimise this technique, it would have to be guaranteed that the embryo from which the blastomere used to generate the stem cells was extracted was subsequently implanted to prevent its destruction, something which in our opinion is difficult to ensure.

A step forward in the same direction was subsequently taken when Chung and a group of researchers from the Californian

company ACT (Advanced Cell Technology), led by Robert Lanza, undoubtedly one of the pioneers in this type of research, managed to obtain different tissue cell lines, such as bone, cartilage, neural tissue and respiratory epithelial cells, from embryo blastomeres of only eight cells[3].

Nevertheless, even then these experiments gave rise to undeniable therapeutic expectations, it did not appear that the cells thus obtained could be used for therapeutic purposes as they came from an individual other than the person requiring the cell transplant, which could lead to immunological rejection problems, similar to that which occurs with donor organ transplants.

The second attempt was to propose the creation of pseudo-embryonic biological structures generated by a technique called altered nuclear transfer (ANT), suggested recently by William B Hurlbut[4] of Stanford University in California. This author proposed genetic modification of the transferred somatic nucleus so that a human embryo could never develop from the biological entity generated, although cell lines similar to embryonic stem cells, and therefore useful for biomedical experiments, could be obtained; however, in the opinion of DA Melton from the Harvard Stem Cell Institute[5], this option had fundamental errors which somehow devalued the results obtained with it.

According to Hurlbut, ANT is carried out in three steps[6]. In the first, a somatic cell is taken from an adult subject and the chromatin structure of its nucleus is modified so that when this modified nucleus is transferred to the enucleated oocyte, it can never result in an embryo which is capable of developing normally. Subsequently, the pseudo-embryo thus generated is appropriately stimulated so that it can develop to give rise to a pseudo-blastocyst, which is theoretically incapable of generating a normal embryo, but from which cells similar to human embryonic stem cells can be extracted; hypothetically, these could be used for biomedical research.

As a tangible demonstration of the creative capacity of those who research in the biomedical field, Hurlbut's theoretical proposal was almost immediately put into practice by Meissner and Jaenisch[7]; the latter is known to be one of the most qualified experts in stem cell cloning and experimental techniques. These authors were able to create pseudo-embryos from a type of adult somatic cell, fibroblasts, the genomic material of which was modified so that it could not express Cdx2, a gene necessary for the proper development of the trophoblast, which is known to be essential for embryo implantation[8] Thus, the pseudo-embryos generated by this technique would be non-viable, as they would be unable to implant in the uterus, although they could constitute a source of stem cells similar to human pluripotential embryonic stem cells[9].

Many ways have being explored: extracting some cells form the embryo without destroing it; Altered Nucear Transfer; Oocite Assisted Reprogramming; the fusion of genetically modified somatic cells with embryonic stem cells…

However, from a bioethical point of view, this technique has objective difficulties since, although an altered blastocyst incapable of implanting in the uterus can be produced, it is not possible to rule out that the embryonic entity thus generated, at some stage in its development, has not had the characteristics of a viable human embryo[10], a fact which is experimentally difficult to verify at present.

Furthermore, it seems obvious that although these embryos may not be viable due to their altered genetic nature, they nevertheless continue to be human beings in the embryonic phase which have been unnaturally manipulated. Moreover, the fact of producing an embryo which is incapable of surviving would be tantamount to the creation of defective human lives, something which is difficult to justify ethically.

The third possibility was the creation of pseudo-embryonic biological structures for altered nuclear transfer with oocyte assisted reprogramming (ANT-OAR).

This technique attempted to reprogram stem cells from adult tissues to convert them into pluripotent stem cells, from which cells of all tissue types could be obtained, but without

the reprogramming ever converting them to totipotent stem cells from which a complete human embryo could develop.

When somatic nuclear transfer is used to generate clone embryos, the enucleated oocyte cytoplasm has the ability to reprogram the genome of the adult cell. To that end, in ANT-OAR, the genetically modified nucleus of the adult cell is activated by factors in the cytoplasm of the oocyte to which it is transferred. From this, a biological entity is formed from which pluripotent cells which can be used for biomedical experiments can be obtained, but which will never generate an embryo.

This technique, from an ethical point of view does not appear to offer objective difficulties and so has been endorsed by a significant number of prestigious scientists and bioethicists in a document entitled Production of Pluripotent Stem Cell by Oocyte Assisted Reprogramming http://www.eppc.org/publications/pubID.2374/pub_detail.asp).

However, there is serious difficulty in the fact that human oocytes are required to carry out the procedure, which means using a large number of oocyte donors, something which is not easy to achieve, particularly due to the risk posed to each of these women by the significant hormone stimulation which they undergo and which occasionally may result in severe ovarian hyperstimulation syndrome.

The fourth possibility is the creation of pseudo-embryonic biological structures by fusion of the genetically modified adult somatic cells with embryonic stem cells.

To resolve the serious problem of the use of human oocytes that ANT-OAR entails, it was proposed to fuse the nucleus of the genetically modified adult somatic cells with embryonic stem cells instead of doing so with oocytes, since the embryonic stem cells produce the same reprogramming effect in the genome of the adult somatic cell as the cytoplasm of the oocytes in somatic nuclear transfer. According to M Azim Surani[11], it is even possible that the embryonic stem cells are more efficient at reprogramming the chromosome material of the adult somatic cells than the cytoplasm of the oocytes itself. In this way, the resulting adult somatic cells, called cybrids by some[12], may attain a state of genomic undifferentiation similar to that of pluripotent cells, to thus be able to derive stem cells similar to embryonic stem cells from them.

This hypothetical possibility, which had already been proposed by M Tada et al[13] was put into practice by Cowan et al[14], who showed that if somatic cells are fused with embryonic stem cells, reprogramming of the chromosome material of the adult cells could be achieved up to the stage of pluripotent undifferentiated cells.

However, according to an editorial by E Phimister in the New England Journal of Medicine[15], despite this encouraging possibility, Kevin Eggan, one of the members of Cowan's own group and also co-author of the abovementioned paper, showed that it had still not been possible to fine tune the methodology necessary to generate stem cells similar to those obtained from blastocysts, although their studies may be the basis for future experiments which would allow this objective to be met.

In fact, the main biological disadvantage of this technique is that as the new cell derives from two cells, fibroblast and embryonic stem cell, which have a diploid nucleus (46 chromosome nucleus), the resulting cell will have double the chromosome content of normal adult cells, i.e. it will be a tetraploid cell with 92 chromosomes. Although the behaviour of tetraploid cells thus obtained is very similar to that of embryonic stem cells, they have practically no therapeutic potential and so can only be used for biomedical experimental objectives but never for therapeutic purposes. Consequently, as the authors themselves state[16], and also mentioned in a JAMA editorial[17], in order to make these techniques therapeutically useful, a method would have to be developed to remove the excess DNA contributed by the embryonic stem cell, to thus convert the tetraploid cell obtained into diploid, something which as Eggan himself recognises,

seems to be technically difficult to achieve at present.

From an ethical point of view, there is one problem, in my opinion insurmountable, in that in order to obtain this type of tetraploid cell, embryonic stem cells must be used; these are obtained from human embryos which have to be destroyed and therefore this technique would not resolve the ethical difficulty that the use of embryonic stem cells entails, since obtaining them requires ending the life of the donor embryo.

The fifth possibility is obtaining them from pseudo-embryos. As is known, normal zygotes have two pro-nuclei, one from the father and the other from the mother. However, after in vitro fertilisation, zygotes containing one or three pronuclei can be accidentally obtained; these zygotes are called aneuploids and are generally non-viable. It has been shown that normal embryonic stem cells which may be used for biomedical research can be obtained from the blastocyts of aneuploid embryos[18].

However, positive ethical evaluation of this technique must be made prudently, as it has been previously shown[19]y[20] that between 10% and 30% of aneuploid zygotes obtained by in vitro fertilisation produce viable blastocysts which may give rise to normal embryos. The consequences that the hypothetical transplants carried out with a type of cells which have an unbalanced genetic load may have for the recipient cannot be predicted either.

The sixth possibility is to obtain stem cells similar to embryonic stem cells from testicular stem cells which are pluripotent and which can behave as embryonic stem cells. This has been achieved by Guan et al[21] on confirming the pluripotentiality and plasticity of the immature masculine germ cells of adult mice; they found that using proper culture conditions, they could acquire biological properties similar to those of embryonic stem cells. The authors of the paper called these cells "multipotent adult germline stem cells" or maGSCs.

Stem cells similar to embryonic stem cells are obtained from maGSC cells from which nerve, heart, liver or intestinal cells can be derived.

However, at present, this technique can only be applied for therapeutic purposes to males, which means a significant limitation that will undoubtedly have to be resolved in the near future.

Expanding on the experiments by Guan et al, M Seandel et al[22] showed that spermatogonia progenitor cells (SPCs) can be obtained from testicular stroma; multipotent adult stem cells (MACs) can be derived from the SPCs and then the MACs are used to develop contractile cardiac cells "in vitro", like functional blood vessels "in vivo". Thus, the authors believe that MACs may be used for genetic studies, to promote tissue regeneration and for the recovery of ischaemic organs. This is unquestionably a great and encouraging scientific advance.

The seventh possibility is to obtain stem cells similar to embryonic stem cells from unactivated oocytes (unfertilised or unactive by somatic nuclear transfer). These oocytes cannot give rise to a viable embryo, so in principle, their cells could be used without any ethical problems to generate cell lines similar to human embryonic cell lines.

In this respect, some time ago two attempts were made to obtain human cell lines similar to embryonic cell lines from parthenotes (parthogenetically activated ova), one by JB Cibelli's team[23] and the other by H Lin's group [24], but without achieving specific results. Now, it appears that a team of Russian and American researchers have managed it[25]. In fact, Revazova's group have obtained pluripotent cell lines from parthenogenetically generated blastocysts. The cells thus produced have morphology similar to human embryonic stem cells, express specific markers of these cells, have a high level of alkaline phosphatase and telomerases and express a normal 46 chromosome karyotype. In other words, they are cells which are similar to human embryonic stem cells, which can be used to generate different tissue cells and which may potentially be used for therapeutic purposes. These cells have been able to be cultured over 21 to 35 passes. However, the

use of these cells has several limitations. First, is that to obtain them requires the use of a large number of human oocytes, which as previously mentioned, has unquestionable ethical objections, as it is not easy acceptable for women to be used as a source of oocytes. Second, and contrary to the procedure proposed by Guan et al[26] which could only be applied to males, is that the cells obtained could only be used to treat the women who donate their ova. In this case, as this is an autologous transplant, immunological rejection is avoided, as has been already demonstrated in mice[27].

In any case, Revazova et al[28] are convinced that they have developed a method to "pathenogenetically create human embryonic stem cells and of having shown that these cells can differentiate into functional cells which may be of great value in the future to treat human degenerative diseases as well as for biomedical research".

The last possibility is by direct reprogramming of adult somatic cells

Along the same line as Revazova et al, a group of researchers from the University of Milan state that they have produced embryonic stem cells pathenogenetically, although their results have still not been published, as mentioned in Nature[29].

In any case, it is worth considering that although it is generally stated that parthenotes are not viable, there are species of American lizards, genus Cnemidophorus, from the family Teiidal, which naturally reproduce parthenogenetically, generating colonies of perfectly viable females. Therefore, in our opinion, it cannot be completely excluded that human parthenotes could not in some measure be equally viable.

The last possibility of which we are aware obtained cells similar to embryonic stem cells by direct reprogramming of adult somatic cells, proposed by K Takahashi and S Yamanaka[30]. This Japanese team analysed which factors present in human oocytes or in embryonic stem cells induced reprogramming of adult somatic cells, and identified several of them, using four: Oct3/4, Sox2, c-Myc and Klf4. These four genes code four specific proteins, known as transcription factors, which are those which are transferred to the somatic cell. These proteins induce the expression of other genes which reprogram the somatic cells to a state of pluripotentiality. Using these four genes, they managed to reprogram mouse adult somatic cells which express the pluripotentiality marker Fbx15, from which cells of all tissue types can be directly obtained without having to destroy any embryos. This is because at no time in the induced reprogramming are true embryonic cells generated, as the reprogramming process is always stopped in the evolutionary stage of the pluripotent cell. These cells were called induced pluripotent stem cells or iPS cells. However, the iPS cells generated differ from embryonic stem cells in their gene expression and in the DNA methylation patterns. When the iPS cells thus formed are injected into the blastocysts of normal animals, they do not produce viable chimeras.

These results were expanded and confirmed in a subsequent study by the same group[31], in which they managed to generate germline-competent iPS cells with genetic expression and DNA methylation patterns comparable to those of embryonic stem cells from iPS cells by controlling the expression of Nanog and Oct3/4. Likewise, they managed to obtain mouse adult chimeras which can transmit their genetic characteristics to the following generation if they injected them in murine blastocysts. However, approximately 20% of the mice generated developed tumours, possibly due to the use of c-Myc, which as previously mentioned is an oncogene. R Jaenisch also showed that some chimeras generated with iPS cells developed tumours [32].

In other words, the iPS cells obtained from the murine fibroblasts may generate chimeras with the ability to transmit their gene characteristics to the following generation.

In the same edition of Nature[33], Wernig et al, from the Jaenisch group, also achieved the in vitro reprogramming of fibroblasts to pluripotent cells, using the same reprogram-

ming genes, Oct4 (also called Oct3/4 or Pou 5f1), Sox2, c-Myc and Klf4, verifying that the DNA methylation pattern, gene expression and the chromatin state of the pluripotent cells generated are similar to those of embryonic stem cells. Likewise, they managed to form chimeras, from which live term embryos developed, if injected in murine blastocysts.

Similarly, Maherali et al[34] and Daley et al[35]have managed to reprogram fibroblasts into induced pluripotent cells (iPS) and are the first to generate viable chimeras.

Everything up to this point has been carried out prior to the publication of the recent experiments of the US and Japanese groups which have been the reason for this review. However, the step which must be taken to transfer these experiments to humans does not appear to be easy or close at hand. Thus, asked Janet Rossant last July in Nature[36]: "Would the same magic molecular factors be efficient in generating iPS cells in humans? Various groups are trying to do so, but transferring these tests to humans has many difficulties".

In fact, the great advance which has now been achieved by Shinya Yamanaka's group from Kyoto University and James Thomson's group from the University of Wisconsin, is that the previously mentioned experiments by Takahashi and Yamanaka, conducted with murine fibroblasts[37], have now been carried out using human skin cells as the cell material to be reprogrammed. Thus, in view of its possible clinical use, a fundamental step forward has been taken, and so interest has been aroused in the experiments that we are discussing.

Thomson's team have published their experiments in Science[38]; as is well-known, Thomson was the researcher who in 1998[39] first managed to culture human embryonic cells. In order to achieve reprogramming of skin cells, these authors used a lentivirus as a vector to introduce the 4 genes which are used to reprogram the fibroblasts. The reprogramming genes used were Oct3/4, Sox2, Lin28 and Nanog.

For this procedure, the US researchers obtained 8 iPS cell lines, similar to the embryonic cell lines, allowing some of them to culture for 22 weeks. Finally, they managed to generate one iPS cell from every 10,000 reprogrammed somatic cells. The sources of the skin cells used were the foreskin of a newborn and the skin of a foetus.

Takahasi and Yamanaka used the same system as the Americans[40], but using a retrovirus to transfer the reprogramming genes; furthermore, these were not the same as those used by Thomson, since they used Oct3/4, Sox2, c-Myc and Klf4. These were the same as they had already used in their previous experiments with mice[41], aided in this case with a protein receptor SLc7a1, to improve the efficiency of the technique. Using this experimental method, they obtained one iPS cell per 5000 reprogrammed somatic cells, i.e. they managed to double the efficiency of Thomson's team. This means that ten centimetres of cultured skin could produce several iPS cell lines.

The iPS cells showed characteristics of embryonic cells, both in morphological appearance and in their multiplication in culture, similar functionality and ability to produce teratomas.

However, the use of c-Myc, an oncogene, by Takahashi and Yamanaka added a serious difficulty to their method in order for the cells obtained from the iPS to be used in human clinical treatment, since in this case it could favour tumour development in the hypothetical transplanted patients. Nevertheless, in a subsequent study[42], the same group achieved similar effects, both in humans (using skin cells from a 36-year old adult) and in mice when c-Myc was not used, i.e. using only the other three reprogramming genes, with the result that none of the 26 animals to which iPS cells obtained without using c-Myc developed tumours, while 6 of 37 animals transferred with cells which used c-Myc produced them.

In the Japanese experiments, skin from the face of a 36-year old woman and joint synovial tissue from a 69-year old male were used as a source of adult cells.

The iPS cells obtained by the Yamanaka group showed characteristics of embryonic cells, both in morphological appearance and in their multiplication in culture, similar functionality, ability to produce teratomas, and particularly the same genetic markers, although the genetic expression of the iPS cells and the DNA methylation patterns were different and especially failed in the production of live chimeras.

From the iPS cells thus obtained, they were able to achieve biological structures with the three germinal layers, from which all the cells in our body are derived, but in addition, when cultivated properly, they also managed to generate neuron and cardiac cells, with the peculiarity that the latter began to beat after a few days culture.

Undoubtedly, the main advantage of the use of iPS cells is ethical, as it does not require the destruction of human embryos to obtain them. This has been recognised by a large number of bioethics experts, as well as by researchers who work in this passionate area.

Until the difficulties have been resolved to be able to use iPS cells for therapeutic ends in humans, these cells may be biological material of great interest for experimental objectives

However, they also have unquestionable biological advantages with respect to embryonic stem cells, if the aim of their use is therapeutic. In fact, if stem cells from embryos left over from in vitro fertilisation are used, as the embryo used is a human individual who is different from the person intended to benefit from the transplant, there is a high probability of inducing immunological rejection. As is obvious, this presents serious difficulty for the use of embryonic stem cells for therapeutic ends. This would be resolved with iPS cells, because as they come from the same individual requiring the cell transplant, rejection would not occur. However, it is still early to be optimistic when it comes to searching for a therapeutic goal, since the disadvantages to be overcome before being able to use iPS cells in humans for clinical purposes are now objectives.

The first of these is that in order to insert the four (or even only three) reprogramming regulatory genes (genes which produce proteins which in turn control the activity of other genes) viruses were used, retroviruses in the case of Takahashi and Yamanaka and lentiviruses in the case of Thomson. The genetic material of these viruses, which are potentially pathogenic, may insert itself in the DNA of the cell to be reprogrammed, and so could transmit viral diseases to the hypothetical recipient. In any case, this difficulty could be overcome using the homologous recombination presently used to produce "knocked-out" animals; as is well-known, these are normal except in the gene which has been specifically removed or inactivated. In other words, in this way, the four reprogramming genes can be inserted in a directed and innocuous manner, transfecting the fibroblasts with suitable constructions using electroporation.

The second difficulty is that since the iPS cells are very undifferentiated, although less so than the embryonic cells, like these they have the possibility of developing tumours in the potential recipients, although to a lesser degree as they are not as undifferentiated.

An additional difficulty may also be the intense genetic modification involved in the introduction of the four regulatory transcription genes, the genetic consequences of which are presently unpredictable.

In any case, a final comment seems appropriate. Until the aforementioned difficulties have been resolved to be able to use iPS cells for therapeutic ends in humans, these cells may be biological material of great interest for experimental objectives, those which they are now attempting to achieve using embryonic stem cells, without their use entailing any ethical difficulty. Thus, using iPS cells, it will be possible to continue researching the biological regulation of the first stages of human life, learning more about the pathogenic mechanism of many diseases and using them as a biological means to evaluate new drugs. However, surely one of the first practical applications of iPS cells could be the possibility of obtaining cell models of

human genetic diseases, deriving cell lines from patients who suffer them. In this way it would be possible to both deepen the understanding of their pathogenicity and to advance their treatment.

Outside these biomedical considerations, it seems interesting to consider that the production of iPS cells is technically more simple and consequently more economical that somatic nuclear transfer, so in theory, these procedures could be carried out in laboratories which do not have large technical resources.

Finally, it is worth noting that the importance of these findings may be inferred from the fact that several of the pioneers in the use of embryonic stem cells have shown their intention to stop using them to redirect their research with iPS cells. Among them, Robert Lanza, director of Advanced Cell Technology, one of the leading companies in experimentation with human embryos and Ian Wilmut, the "father" of Dolly the sheep, who after declaring that the research that may result from the use of iPS cells is "a hundred times more interesting" than that conducted with embryonic stem cells, have shown their intention to stop using embryonic stem cells in favour of using iPS cells[43]. This is undoubtedly a great experimental advance, which must be greeted as a great hope for finding ethical pathways which allow the development that reparatory and regenerative medicine requires. To that end, Thomson himself commented[44] that probably "a decade from now, stem cell wars will be just a funny historical footnote on a page in the history of science".

Note

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