Open Peer Review on Qeios

Bioethical Assessment of Research with Humanoid or Humanized Biological Entities with Uncertain Moral Status

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Funding: No specific funding was received for this work.

Potential competing interests: No potential competing interests to declare.

Abstract

A number of studies have been conducted in recent years with humanoid or humanized biological entities such as hybrids or mixtures of species that involve a human contribution to the resulting individual. These include embryos whose genetic makeup is exclusively human but which do not come from the fertilization of gametes, and embryoid models obtained from stem cells, all of them with an uncertain moral status. Both conceptual and empirical limitations make it difficult to classify such entities within the pre-established biological categories, resulting in uncertainty as to the moral licitness of generating such entities and using them in experimentation.

In the question of chimera research, the state of science currently permits a very low human contribution to the animal individual, so there are no doubts about the biological nature and moral status of the chimeras that can now be generated. However, the possibility of further refinement of the technique requires us to consider what limits to set in this regard.

Given the inability to establish clear boundaries to systematize the degree of differences that should distinguish zygotes and embryos obtained by fertilization from those obtained by cloning, parthenogenesis or induced cellular dedifferentiation, the principle of bioethical prudence should be applied to current research with a view to preventing them from threatening human lives which, even in a very genetically imperfect state, should continue to be regarded as such.

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Keywords: humanoid, humanized biological entities, moral status, bioethics, hybrids.

Introduction

A number of studies have been conducted in recent years with humanoid or humanized biological entities whose moral status is uncertain. Both conceptual and empirical limitations make it difficult to classify such entities within the pre-established biological categories, resulting in uncertainty as to the moral licitness of generating such entities and using them in experimentation.

These entities can be divided into three major groups: 1) hybrids or mixtures of species involving a human contribution to the resulting individual; 2) embryos whose genetic makeup is exclusively human but which are not derived from the fertilization of gametes; and 3) embryoid models obtained from stem cells.

Among the different types of human-animal hybrids that science can construct are human-animal chimeras, obtained by introducing human stem cells into early animal embryos, so that the animal individual that develops contains human cells, and can form human tissues or organs. Other possibilities are the introduction of animal genes into humans or vice versa (transgenesis), the introduction of a human cell nucleus into the cytoplasm of an enucleated animal oocyte (cybrids), or fertilization between a human gamete and an animal gamete (hybridization).

The second major group includes those embryos obtained by cloning or parthenogenesis, which do not come from the fertilization of gametes and from which a live human being has never been derived, among other reasons because their implantation in the uterus for gestation is not legally permitted today anywhere in the world.

Finally, embryoids are models of embryonic regions obtained from stem cells in vitro. The emergence of this field of research is very recent, but it is moving rapidly, so that models increasingly resemble ordinary embryos.

This paper outlines the relevant scientific evidence on the biological nature of the different entities identified as problematic, and discusses the ethical assessment of their production and use.

Human-animal chimeras

The shortage of viable organs for transplantation hampers the replacement of damaged or dysfunctional vital organs. As a result, thousands of patients die every year while waiting for an organ transplant, a situation that is exacerbated in aging societies in the developed world, where cases of organ failure are greater. Given this situation, the growth of human organs in non-human animals is presented as a strategy to increase the number of organs available for transplantation.

Method of production

This strategy would make use of the development program of the host animal, such as a pig or sheep, to induce the development of human organs from human stem cells introduced into the animal embryo. Ideally, these organs will be functional and transplantable. These biological entities are called human-animal interspecies chimeras.

A chimera is an organism composed of a mixture of different cell populations derived from more than one individual, as is the case in question. There are different methods for obtaining chimeras, such as mixing early embryos or grafting tissues from different stages of development; the characteristics of the resulting chimeras will depend on the process used to generate them. The method used to produce chimeras capable of developing whole organs from the cells of another organism — or even from another species, as in the case of human-animal chimeras in which a human organ would develop in the animal — is called "blastocyst complementation". It is so called because the stage of embryonic development in which the cell transplantation occurs is that of the blastocyst, and because the recipient blastocyst has been genetically engineered to lack the desired organ of the other species, with the donor cells complementing this genetic deficiency. Thus, the donor pluripotent stem cells (PSCs) fill the empty niche of the recipient, resulting in an organ composed mainly of donor cells (Wu et al. 2016b, 18-24).

Blastocyst complementation was first introduced in 1993 in a study that used wild-type mouse embryonic stem cells (ESCs) to complement the blastocysts of recipient mice deficient in the Rag-2 gene, which prevents them from developing T and B lymphocytes. The resulting chimeric mice produced T and B lymphocytes that came exclusively from donor mouse cells (Chen et al. 1993, 4528-32).

Organic structures (ocular crystalline lens) were later produced by this method, also using mouse ESCs (Liégeois, Horner, and DePinho 1996, 1303-7). The generation of whole organs by blastocyst complementation was initially achieved in 2007, when wild-type mouse ESCs were used to successfully complement mouse blastocysts deficient in the Pdx1 gene, key to pancreatic development (Stanger, Tanaka, and Melton 2007). Subsequent studies (Espejel et al. 2010, 3120-6). have shown that blastocyst complementation can also be successful using induced pluripotent stem cells (iPSCs) instead of ESCs, which in humans has the medical advantage that the cells of the organ to be transplanted are recipient's own cells, and the ethical advantage of not requiring the destruction of human embryos to obtain them. The generation of interspecies chimeras by blastocyst complementation was first published in 2010 (Kobayashi et al. 2010, 787-799). In this paper, mouse blastocysts deficient in the Pdx1 gene were complemented with rat PSCs, and the resulting rat-mouse interspecies chimeras possessed an entirely rat pancreas.

Adapting blastocyst complementation for human organ production requires the use of appropriate host animals. Pigs and sheep seem particularly suitable for this purpose because of their similarity with humans in relation to organ size, as well as other advantages, such as breeding potential, period to reproductive maturity, and number of offspring and cost of maintenance, unlike non-human primates (Cooper 2012, 49-57).

The first case of blastocyst complementation in these types of larger animals was illustrated in a study in which

apancreatic pigs were complemented with ESCs, also porcine. The resulting chimeras had a pancreas entirely derived from the donor (Matsunari et al. 2013, 4557-62).

The next step, which is the generation of chimeras between humans and these types of large animals, presents a significant technical challenge.

Another fundamental aspect is obtaining human PSCs capable of contributing to the early development of the host animal for the generation of chimeric embryos. Cell culture conditions dictate the state of the PSCs, so different trials are testing different formulae, although the possibility of obtaining PSCs in a state analogous to that achieved in mice is uncertain (Wu et al. 2016a, 51-59).

Other key points to be resolved are the need to match the developmental stage of donor and recipient embryos, which is complex in different species (Cohen, Markoulaki, and Jaenisch 2018, 1445-52), as well as the identification of other unknown parameters (species barriers) that interfere in the development of interspecies chimeras (De Los Angeles, Pho, and Redmond 2018, 333-342). and the targeting of multiple lineages to achieve the production of human organs with no nerves or blood vessels derived from the host animal.

In this context, the new genome editing tools are going to be of great help in the rapid and easy generation of *knockout* embryos, personalized hosts. Especially promising is the CRISPR/Cas9 technique, which has already allowed the production of *knockout* animals in both pigs (Wu et al. 2017b, 473-486), (Watanabe et al. 2019, 8016) and sheep (Vilarino et al. 2017, 17472), both considered as desirable hosts for the production of human organs. In addition, multiplexed gene editing may allow the inhibition of multiple genes in the host (De Los Angeles, Pho, and Redmond 2018, 333-342).

Finally, recent research suggests that the initial apoptosis is a significant barrier in interspecies chimerism using hPSCs. One paper shows that forced expression of factor BMI1 overcomes the apoptosis and enables hPSCs to integrate into mouse pre-implantation embryos and subsequently contribute to chimeras with both embryonic and extra-embryonic tissues (Huang et al. 2018, 4649). Another report states that human ESCs can contribute to chimeras in mouse embryos with the aid of BCL2-mediated anti-apoptosis (Wang et al. 2018, 126-9).

Relevant biological aspects

Thus, the derivation of chimera-competent PSCs in species other than rodents has yet to be achieved (Wu et al. 2016b, 18-24). In a 2015 study, culture parameters were modulated to obtain a stem-cell type with special spatial, molecular, and functional characteristics, designated as region-selective pluripotent stem cells (rsPSC). Human rsPSCs derived from human embryos, when injected into mouse embryos, showed the capacity to integrate, proliferate and differentiate into derivatives of all three embryonic germ layers (Wu et al. 2015, 316-21).

In another study in 2017, different types of human iPSCs (hiPSCs), obtained through the use of different in vitro culture methods, were tested to study their potential chimeric contribution in pigs and cattle pre-implantation blastocysts. Measurement of cell survival and the efficiency of integration into the inner cell mass of the blastocyst allowed the

researchers to identify the most promising cell types, as well as to determine that human cells thrived better in cattle embryos than in pig embryos. In light of the survival and chimeric efficiency findings, in a second phase of the study, they evaluated the chimeric potential of the selected hiPSCs in post-implantation pig embryos. After injection and implantation, embryos between 21-28 days gestation were collected; they found that some of the selected cells were inefficient for generating chimeras, while others differentiated to several cell types, although the levels of chimerism were very low. Moreover, the chimeric embryos had a developmental delay, suggesting that human cells interfered with normal pig development (Wu et al. 2017b, 473-486).

More recently, a study has been published in which human-mouse chimeras have been generated with the highest degree of human chimeric contribution achieved so far, obtaining up to 4% of human cells distributed in all three germ layers (Hu et al. 2020, eaaz0298). Advances such as this in obtaining a type of chimera-competent human stem cells are promising.

Finally, the first human-monkey chimeras have been produced (Tan et al. 2021, 2020-2032.e14). The importance of this study lies in the greater evolutionary closeness of the human being with the monkey than with other animals. The researchers hope that the knowledge that can be achieved in studies of this type can be used to improve chimerism with other species that are more distant evolutionarily but more convenient to use from an ethical point of view. In this research, the experiments were carried out ex vivo and researchers found that the human cells survived, proliferated, and generated several peri- and early post-implantation cell lineages inside monkey embryos. Nevertheless, transcriptomic differences between cells in human-monkey chimeric embryos when compared with normal embryos were found, which were related to altered cell-cell interactions between human and monkey cells and to some signaling pathways. The authors concluded that more studies are needed to improve interspecies chimerism.

However, there is a possibility that human cells may not be able to reach the required chimeric capacity. It has not been possible to culture pluripotent human cells that show a chimeric capacity like those of mice. On the contrary, recent experiments suggest that these cells cannot remain mitotically active during colonization (Aksoy et al. 2021, 56-74).

Therefore, the generation of whole functional human organs in animals is still far off. Other applications seem closer in time, like the study of human embryogenesis, testing of medicines in humanized animals, and research into the onset and progression of human diseases in an in-vivo environment (Wu et al. 2017a, 10487). Furthermore, for the organ transplant application, it would be necessary for both the organ in question and the surrounding endothelium to be of human origin, to avoid immune rejection. In this sense, an article has shown the obtaining of human endothelium in genetically modified pigs (Das et al. 2020, 297-302).

Bioethical assessment

Research with human-animal chimeras raises several ethical questions that we present below.

First, while obtaining a human pancreas in an animal, for example, does not seem to create any moral confusion about the status of that organism, the possibility of producing human-animal chimeras that are neither clearly non-human nor clearly human is worrying (Robert and Baylis 2003, 1-13), as creatures whose moral status is not defined could be generated.

The question of what proportion or type of human cells would make the organism in question a human or quasi-human being has not been resolved. Progress in this field needs to be monitored in order to anticipate the possibility that organisms of this type may be generated, and to define what limits to establish in research with these chimeras. We really do not know whether it is truly possible to generate a chimera whose resemblance to a human being could make it subject to a moral status equivalent to ours or at least superior to that of other non-human creatures, because currently the efficiency of the chimeric contribution of human cells to the animal embryo is extremely low (Wu et al. 2017a, 10487). In any case, this possibility does not seem to imply moral illicitness towards the production of any form of human-animal chimera.

Secondly, and in relation to the above, one wonders which chimeras would raise ethical concerns and which would not. Chimeras with a humanized appearance, gonads or brain have been identified as the most problematic (Bourret et al. 2016, 87), because these aspects are more strongly linked to our identity than other physiological attributes, and there is a broad consensus in avoiding their creation. Nevertheless, the potential for human cells implanted in the animal to colonize organs other than the one to be produced, and that such colonization could even reach its brain, is not yet fully controlled. If this happens, animals with a “humanized” brain could be generated to a greater or lesser extent, which could give the animal human or humanized cognitive qualities (Cabrera Trujillo and Engel-Glatter 2015, 595-617). Several authors argue that changes in cognitive abilities are morally relevant insofar as they increase the capacities that affect the moral status of any entity, including awareness, autonomy, and sociability (Porsdam Mann, Sun, and Hermerén 2019, 10), and their well-being (Hyun 2019, e103331; Counihan 2019, 195-203). The potential to colonize the reproductive organs is also worrisome; this could eventually result in the generation of human gametes in the animal, so that from two animals of a different sex, human gametes could be obtained and from these, a human being.

The ethical difficulty that this entails led the United States (US) National Institutes of Health (NIH) to announce in September 2015 that they would not fund research in which human pluripotent cells were introduced into non-human vertebrate animal embryos, while they considered a possible policy revision in this area (NIH 2015). However, a group of American researchers published a letter in which they stated their opposition to curbing these experiments (Sharma et al. 2015, 640). Subsequently, in August 2016, the NIH lifted the ban and proposed to allow the funding of studies involving chimeras, but with some caveats, such as not injecting human cells before the central nervous system begins to form and limiting the growth of chimeras, preventing their birth (Reardon 2016, 135). Notwithstanding, in March 2019, the ban limiting the growth of chimeric animal embryos beyond 14 days of development or the transplantation of such embryos into a surrogate uterus was lifted in Japan. That month, the Japanese Ministry for Education, Culture, Sports and Science (MEXT) issued new guidelines (Sawai, Hatta, and Fujita 2019, 513-4). that would allow human-animal embryos to be obtained, implanted into surrogate animals and brought to term (Cyranoski 2019), which has not occurred in any country to date.

Contrary to the condition of “no contribution to brain development,” it has been argued that it may preclude potential research applications with no good reason, since in animals such as pigs and mice, the human neural structures could never fully develop due to the longer gestation periods and larger skull size required (Dondorp and Johnson 2017, 341-2). This hypothesis would need to be tested with animal models, perhaps by performing chimerism experiments between

higher primates and other non-human animals.

In any case, when the time comes to circumvent these scenarios, a number of technical actions could be feasible, such as eliminating the genetic program that directs the neuronal development of stem cells before injecting them, or injection into the animal embryo of progenitor cells already committed to the target lineage at the right place and time (Wu et al. 2016b, 18-24). With regard to concerns about the production of human gametes in animals and their eventual fertilization, experimental data suggest that the formation of germ cells of one sex from exogenous cells is suppressed when the host embryo and the donor are of a different sex (Matsunari et al. 2013, 4557-62; McLaren 1975, 205-16; Nagashima et al. 2004, 702-7). Furthermore, sterilization of pigs with human organs has been proposed, or the simple physical separation of males and females, which would be sufficient to prevent their reproduction (Palacios-González 2017, 387-90).

A third ethical difficulty is that human ESCs are used in some of the experiments with human-animal chimeras. It would be ethically very positive if researchers were to stop using human ESCs in future experiments, and used iPS cells instead. Moreover, from a biomedical perspective, this would be much more useful, since the cells to be transplanted could come from the patient himself, potential problems with immune rejection would be drastically reduced.

Additional considerations can be made (Kwisda, White, and Hübner 2020, 24), such as the fact that the use of pigs as hosts raises safety concerns, given the possibility that pathogens found only in pigs may mutate and be passed to humans; or the necessity to cause the least harm possible to the animal, and to use chimeras with the lowest possible cognitive level.

Other human-animal mixtures

In addition to chimeras, there are other ways of obtaining biological organisms or entities that combine human and non-human biological material in one way or another. These are transgenesis, hybridization, and cybrid production.

Transgenesis

Transgenic or genetically modified organisms can be defined as any “organism that carries genes from another organism’s genome” (Fundación Española para la Ciencia y la Tecnología 2007). Thus, transgenesis does not always involve human genetic material. In fact, this is often not the case, as in transgenic crops for example, in which a given plant variety has been genetically improved by the incorporation of an exogenous gene from a non-human organism. Only those transgenic organisms that involve mixing human and non-human genetic material are of interest in this paper.

These types of organisms are generated to study the effect of a gene, such as mice carrying a human gene associated with a disease, or to obtain some valuable product, such as human insulin obtained in bacteria. These studies do not usually pose ethical problems, but this is not always the case.

Recent research has produced transgenic monkeys by introducing the human MCPH1 gene — which it seems is involved

in the evolution of our brain — into embryos of non-human primates. The research concluded that the transgenic monkeys "exhibited better short-term memory and shorter reaction time compared with the wild-type controls" (Shi et al. 2019, 480-93), which implies a cognitive improvement in the animals included in the study. Slower brain development was also observed, just as in humans, although the size of the animal brain did not change. The aim of this study was to acquire knowledge of the evolutionary process through which human intelligence has developed, or as the authors of the paper say, "to provide important [...] insights into the basic questions of what actually makes humans unique".

As regards the bioethical assessment, the wisdom of humanizing the animal brain is questionable, especially when it comes to humanizing animals very close to our species in evolutionary terms. The drawbacks, as has been pointed out in the case of chimeras, are the possibility of generating an organism of uncertain moral status and the risk of increasing the capacity of humanized animals to suffer.

Bing Su, lead author of the study, believes that introducing one or a few genes into the monkeys will not imply any significant change. Against this reasoning, however, the slippery slope argument warns of the risk of further progress in this type of research (Regalado 2019). Regardless of the potential progression of this line of research, the expected result in the humanized transgenic animal should be seriously considered, and if this expectation cannot be achieved with a considerable degree of certainty, the transgenic organism should not be created.

Hybridization

The ability to successfully carry out fertilization is a way of checking the functionality of a gamete, which can be useful, for example, in in vitro gametogenesis experiments (Hendriks et al. 2015, 285-96). In cases where this functionality needs to be tested in human gametes, one possibility is to fertilize it with an animal gamete of the opposite sex (Araki et al. 2004, 111-6), in order to avoid obtaining a human embryo, or, in the case of checking male functionality, to avoid wasting human eggs. These types of studies are also conducted to observe aspects of interest in the gametes, such as chromosomal analyses of human sperm performed by fertilization of mouse oocytes with this sperm (Araki, Yoshizawa, and Araki 2005, 1244-7).

Different studies have shown that fertile human sperm is capable of activating mouse oocytes after in vitro fertilization (Araki et al. 2004, 111-6; Mizuno, Hoshi and Huang 2002, 2350-5; Dozortsev et al. 1995, 403-7; Nikiforaki et al. 2014, 581-8), and the resulting entity develops normally, at least until the first mitosis (Rybouchkin et al. 1996, 2170-5).

Although there is no doubt that the entities resulting from these experiments are not living beings of our species (they are not human beings), the question remains as to how we might define them. To the best of our knowledge, the papers available in the literature do not perform an in-depth examination of the physiological characteristics of the resulting entities, their similarities and/or differences with the gamete donor species or their maximum viability. These are data that could shed light on these uncertainties, in the same way that the physiological study of the human embryo has allowed it to be identified as an autonomous organism from the very beginning of its development (Condic 2011, 25-43). Nevertheless, these are not the objective of the research carried out.

Apart from considerations on the nature of these entities, which will need to be resolved before the ethical licitness of their generation can be confirmed, other objections to these experiences may be found. These include the fact that, if it can be considered a reproductive act, it would involve the reproduction of a human being with a non-human animal, even if the resulting individual was only viable for a short period of time.

In conclusion, we believe the doubts raised are sufficient to avoid the conducting of these experiments on the basis of ethical criteria. Nevertheless, in-depth study of available empirical data could shed light on these issues. Given the uncertainty, this in-depth study should be carried out on the experiments already done, not by implementing new trials to this end.

Production of cybrids

Cybrids, or transmitocondrial cytoplasmic hybrids, are cells that contain the genetic nucleus of one individual and the mitochondria of another, usually obtained by isolating mitochondrial DNA (mtDNA)-containing cytoplasts and fusing these to cells lacking mtDNA from the same species (Bacman, Nissanka and Moraes 2020, 415-39). These types of cybrids are used in studies on mitochondrial dysfunction (Sazonova et al. 2018, 4647214; Swerdlow et al. 2017, 259-302).

However, a particular type of cybrid is the one obtained by introducing the genetic nucleus of a human somatic cell into an enucleated animal oocyte. This is somatic cell nuclear transfer (SCNT) (the process used in cloning), in which the recipient oocyte comes from a non-human animal and the nuclear genome is human. This would make it possible to do without human oocytes in stem cell research with the same nuclear DNA from people with diseases such as Alzheimer's, Parkinson's or similar, to see how such cells develop and thus study possible ways to prevent or mitigate these diseases.

Again, the question arises as to the nature of the resulting biological entity, which in this case contains human nuclear DNA and mtDNA of the animal concerned, as well as other cytoplasmic, non-human factors. Although the viability of these cybrids is also limited, they can develop to the morula state, albeit with incorrect genetic reprogramming (Chung et al. 2009, 213-23), which allows us to say that it is reasonable to think that the resulting entity is an organism. Moreover, most of its DNA is human, so its nature seems more inclined towards belonging to our species. This unresolved uncertainty makes it ethically wrong to undertake such experiments. In addition, from a biomedical point of view, the usefulness of these cybrids in stem cell research is called into question because of the genetic errors they experience (Chung et al. 2009, 213-23).

Clonotes

Method of production

In general, it can be said that cloning means obtaining more or less precise copies of a biological entity. More specifically, though, it can refer to three aspects: cloning genes, cloning cells, or cloning individuals, this latter possibility being the one we shall refer to in this text (Ayala 2015, 8879-86).

Cloning by SCNT starts from an oocyte and an adult cell of the individual to be cloned. First, the nucleus of the oocyte is extracted, and then replaced by the nucleus of an adult cell. This results in a hybrid zygote or clonote, composed of the nucleus of the adult cell and the mitochondria and cytoplasm of the oocyte used. More than 98% of the DNA content of this hybridized zygote is from the individual to be cloned and less than 2% is from the mtDNA of the oocyte.

After activation of this hybrid zygote, it begins to divide until it reaches the blastocyst stage; the blastocyst is an embryo of some 60-200 cells, which is essentially composed of the inner cell mass and other structures that will give rise to the placenta and extra-embryonic tissues.

The blastocyst produced can be used for two different purposes: reproductive or therapeutic. Reproductive cloning is defined as cloning in which the blastocyst is implanted in a female of its species, to continue the pregnancy until birth. Therapeutic cloning involves obtaining embryos that must be destroyed at a certain stage of development, to obtain stem cells with the genetic characteristics of the adult cell nucleus donor. This would imply the possibility of obtaining cells and tissues useful in regenerative medicine, which would be free of the problems of immune rejection. Few experts believe that the stem cell lines obtained from cloned embryos could become an important tool in biomedical research, as the technique itself is very expensive, difficult to perform, and presents undeniable ethical difficulties.

Relevant biological aspects

Successful reproductive cloning, i.e. the birth of cloned individuals, has already been achieved in the animal field, with the birth of the first mammal clone, Dolly the sheep, in 1997. This has never been accomplished in humans, because of both ethical and legal difficulties, since human reproductive cloning is not legal anywhere in the world.

Furthermore, the cloning process is not without technical difficulties. The efficiency of the technique, although it has improved over the years, remains low. Compared to natural breeding and assisted reproduction, reproductive cloning involves high embryonic and gestational losses. In addition, a relatively high proportion of clones also fail to survive once they are born, some with congenital defects. However, those clones that do manage to make it through the perinatal period are healthy, and appear to age normally (Sinclair et al. 2016, 12359).

The technical difficulties of the process have already been overcome — at least to a sufficient degree to achieve birth — in species close to humans, through the use of epigenetic modulators that have recently allowed the birth of primates (*Macaca fascicularis*) obtained by cloning (Liu et al. 2018, 881-7).

Bioethical assessment

Among the ethical disadvantages posed by cloning in humans is the fact that it does not seem reasonable to produce a human embryo destined to be destroyed, with the sole purpose of being a source for obtaining embryonic stem cells.

Another equally considerable added ethical difficulty is that to carry out these experiments and achieve the desired objectives requires the use of a high number of human oocytes, which in some circumstances may mean instrumental

manipulation of the donors (Baker 2014).

An additional possibility of SCNT in animals is to combine this with gene therapy, so that the genome of the somatic cell from which the nucleus is to be removed can be modified, to introduce genes into it that can be altered for a certain purpose. This modified genome can be transferred to the enucleated oocyte and thus produce a blastocyst with very specific genomic characteristics, which could certainly be very useful in the experimental field.

It is also thought that these genetic modifications could overcome the difficulties presented by human clonotes for a full-term pregnancy, derived from the complex epigenetic evolution of the nucleus from the adult cell to a sufficient degree to prevent its evolution until birth. Germline gene editing in human embryos presents serious ethical difficulties due both to its lack of safety and the possibility of obtaining designer individuals in which certain traits could be programmed.

If this technique were legalized, human reproductive cloning could become a reality that would be bioethically difficult to justify.

Parthenotes

Method of production

Parthenogenesis consists of obtaining “diploid” oocytes, which resemble human zygotes, from mammalian oocytes that can be activated to divide using a variety of stimuli that allow them to complete the second meiosis and eliminate the polar body, or keep it in addition to the other half of the genetic endowment of the egg as a pronucleus (Ozil 1990, 117-27; Vitullo and Ozil 1992, 128-36; Ozil and Huneau 2001, 917-28).

In certain conditions, this "diploid oocyte" or "parthenote" can start to divide, resulting in something very similar to an embryo ("embryoid"), from which it is distinguished by small but important changes in certain genes (López Moratalla 2004, 405-15).

These changes are due to the absence of the paternal genetic contribution, which provides the zygote with a genetic imprint that is essential for its subsequent evolution.

Relevant biological aspects

Parthenogenesis experiments have been conducted in animals, from which pluripotent stem cells have been derived. In various mammalian species, the parthenotes develop until the blastocyst stage, and their implantation has even been achieved in mice (Daughtry and Mitalipov 2014, 290-8). In humans too, PSCs have been derived from parthenotes (Revazova et al. 2007, 432-49), and have even been applied as treatment in animal models (Lee et al. 2019, 1029-46). One paper shows the production and analysis, for the first time, of a collection of parthenogenetic haploid human ESC lines (Sagi et al. 2016, 107-11).

Bioethical assessment

Genetic differences with respect to the zygote obtained by fertilization are sufficiently important as to prohibit its division beyond a limited number of cells, preventing its progression to birth. As in the case of cloning, we cannot dismiss the possibility that the genetic "repair" of these differences using the new editing tools will allow fetuses to be obtained or the birth of individuals from a parthenote (in this case of females only) in the not too distant future. The process would present the same ethical difficulties mentioned in the case of cloning.

Embryoids

In the field of organoid research, 3D tissues derived in vitro from stem cells that model cellular, anatomical and functional aspects of real organs on a micrometer to millimeter scale (Rossi, Manfrin and Lutolf 2018, 671-687; Xia and Izpisua Belmonte 2019, 877-94) progress in so-called “synthetic embryology” has soared in the last five years. Several studies have shown that stem cells from mice and humans can spontaneously organize themselves in vitro into 3D structures that are increasingly similar to embryos (Shahbazi and Zernicka-Goetz 2018, 878-887; Harrison et al. 2017, eaa11810; Rivron et al. 2018b, 183-185; Kime et al. 2019, 485-98; Beccari et al. 2018, 272-276; van den Brink et al. 2014, 4231-4242; Sozen et al. 2018, 979-989; Shao et al. 2017b, 208; Li et al. 2019, 687-702.e18; Biena et al. 2019, 127-41; Zhang et al. 2019, 496). These in vitro embryonic models are called “embryoids” (Simunovic and Brivanlou 2017, 976-985), “embryoid bodies” (Brickman and Serup 2017, e259), or “synthetic human entities with embryo-like features” (SHEEFs) (Aach et al. 2017, e20674).

The so-called embryonic organoid systems can be used to study various aspects of early embryo development in vitro, such as the establishment of body axes, gastrulation and neural tube development. While the classical organoids are typically composed of a restricted subset of cell types from a germ layer, in embryoids, cells of different germ layers coexist (as occurs in the embryo in vivo) (Harrison et al. 2017, eaa11810), the interaction of which can also be studied with these models (Rossi, Manfrin, and Lutolf 2018, 671-687).

Scientists hope to derive different applications from these studies, such as infertility treatments (by allowing better understanding of gastrulation and implantation), improvements in vitro fertilization, design of new contraceptives, investigation of drugs to prevent diseases determined in the embryonic stage, as well as examining the influence of factors such as diet or improving organoid production (Rivron et al. 2018a, 106-111).

Method of production

To obtain these embryoids, an appropriate number of stem cell aggregates are exposed in vitro to different molecules or morphogens that, in the natural process of embryonic development in vivo, direct cell differentiation in the embryo, as well as the spatial organization of their cells (Pera et al. 2015, 917-919).

Relevant biological aspects

In recent years, several advances have been made in the generation of embryonic models, both mouse (Rivron et al. 2018b, 183-185; van den Brink et al. 2014, 4231-4242; Beccari et al. 2018, 272-276; Harrison et al. 2017, eaa11810; Sozen et al. 2018, 979-989) and human (Shao et al. 2017a, 419-425; Shao et al. 2017b, 208).

Among the most recent, researchers have managed to incorporate similar models of extra-embryonic structures such as precursors of the amniotic sac and the placenta, and these models have been introduced into the uterus of mouse females and have initiated the implantation process (Rivron et al. 2018b, 183-185; Zhang et al. 2019, 496).

In 2019 the in vitro generation of a “blastoid” in mice was reported; this is a model of the blastocyst-stage embryo, which can recapitulate events during preimplantation and early postimplantation development in vitro in the uterus (Li et al. 2019, 687-702.e18). In this paper, it should noted that the blastoids were obtained from stem cells derived from adult cells, so that virtually any cell can serve as a basis and it is not necessary to use cells from an embryo. This is one more step in the field of embryoid research.

Recently, gastrula-like and blastocyst-like structures have been obtained also from human cells (Moris et al. 2020, 410-415; Liu et al. 2021, 627-632; Yu et al. 2021, 620-626; Fan et al. 2021, 81). At present, embryoids stop developing after a few days of culture, and there are still many technical difficulties to resolve to improve embryonic models (Shahbazi and Zernicka-Goetz 2018, 878-887). Therefore, the likelihood that this technology will allow human beings to be obtained from stem cells is currently zero, so that for the moment there are no medical risks associated with these experiments. However, the latest advances in the human species show great similarities between embryonic models and normal embryos, both at a morphological and functional level (Moris et al. 2020, 410-415; Liu et al. 2021, 627-632; Yu et al. 2021, 620-626; Fan et al. 2021, 81), so it could be possible in the future that these models would be so perfected that they would come to be considered embryos.

Bioethical assessment

The prospect of generating human embryo-like structures in vitro is not without ethical concerns (Pera et al. 2015, 917-919; Hyun 2017, 718-20; Shen 2018, 19-22). From an ethical point of view, the ability to obtain in vitro models to study embryonic development without having to resort to a real embryo is extremely positive, and objective benefits may be derived from this research. However, the possibility that refinement of these models could give rise in the future to the generation of viable human embryos using such techniques is troubling. Because of the extent to which they resemble embryos, human gastruloids raise potential conceptual and ethical concerns related to the creation of early human life in vitro. If human gastruloids are considered functionally similar to human embryos, a number of ethical and regulatory concerns arise about the desirability of creating these PSC-derived constructs (Rivron et al. 2018b, 183-185; Aach et al. 2017, e20674; Munsie, Hyun and Sugarman 2017, 942-5). Although this is not the case at present, the state of science will have to be reviewed regularly to ensure that this remains so in the future (Rivron et al. 2018a, 106-111). In the above paper, some blastoids implanted in the uterus of mice and generated live, albeit disorganized, tissues. However, the

authors of the article themselves point out that their work “pave[s] the way to creating viable synthetic embryos by using cultured cells,” which, if applied in humans, would be ethically unacceptable. In this regard, uncertainty has been raised about “at what point a partial model contains enough material to ethically represent the whole” (Rivron et al. 2018b, 183-185). Animal models should establish how far one can go without incurring the production of a human being by this method.

An additional ethical problem is that ESCs are often used for these experiments. Using iPS cells instead would solve this ethical difficulty. Furthermore, embryoid research would also raise all the issues that have been associated with research with organoids (Bredenoord, Clevers and Knoblich 2017, eaaf9414) such as their legal status, the need to develop appropriate consent procedures (Boers et al. 2016, 938-41; Boers and Bredenoord 2018, 642-5), and the donor’s perspective.

Conclusion

In the question of research with chimeras, the state of science currently allows a very low human contribution to the animal individual, so there are no doubts about the biological nature and moral status of the chimeras that can now be generated. However, the possibility of further refinement of the technique requires us to consider what limits to set in this regard. As mentioned, obtaining certain human organs in host animals for transplantation, such as a pancreas or kidney, does not appear to generate conceptual or moral confusion. Nevertheless, the question of the proportion or type of human cells that would be ethically permissible has not yet been resolved, although human contributions to the animals’ appearance, brain or gonads seem to be the most problematic. As progress in this field allows us to glimpse the possible attainable scenarios, the bioethical debate and the appropriate regulations should seek to provide effective guidance to steer this development in accordance not only with biomedical, but also ethical, criteria.

As regards the other possible human-animal “mixtures”, ethical evaluation differs between transgenesis and the production of cybrids and hybrids. In the first case, certain applications of the technique do not pose bioethical drawbacks, such as the aforementioned production of human insulin from bacteria. However, other possibilities such as recent brain research in monkeys are more controversial. It can therefore be deduced that the ethical difference will be based on the type of change produced in the non-human individual, which in turn depends on the gene inserted and the species of the recipient, the future transgenic organism. As in the case of chimeras, the main objection lies in the possibility of endowing a trait that is too closely linked to human dignity to a living, non-human being, which would force us to rethink its moral status. It is such entities that we must avoid producing. In the second case, that of cybrids and hybrids, we are currently facing the impossible task of defining their moral status. In the case of cybrids, where, despite their non-viability, most of the genetic material is human, we could effectively find ourselves before a human being, which would make obtaining it for research purposes and its subsequent destruction ethically unacceptable. When in doubt, it is not right to produce such entities. As for hybrids, they raise no doubts about their possible human nature, since only half of their genomic endowment is human, although uncertainty about their nature remains. Furthermore, the possibility of considering their creation as a human-animal reproductive act involves another serious ethical objection.

Obtaining clonotes or parthenotes also poses the same ethical dilemmas as those raised by obtaining SHEEFs. All these "embryoids" resemble, in one way or another, human zygotes or blastocysts derived from fertilization of the male and female gametes, but have genetic differences in varying degrees that prevent their division and organized growth to the fetal state and subsequent birth. The magnitude of these genetic abnormalities is the argument that has led many scientists to consider them as non-human.

In favor of these techniques, it is claimed that the embryos obtained, because they cannot to be considered human embryos, given their genetic differences, could be used as a source of stem cells, as well as research material, whose necessary destruction would not entail the bioethical issues that are associated with the destruction of human embryos obtained by in vitro fertilization.

What, then, is the bioethical difficulty involved in obtaining these embryoids, including the aforementioned SHEEFs? In our view, it lies in the impossibility of setting clear limits on the magnitude of genetic differences with the human embryo obtained by fertilization, so that a real embryo can be clearly distinguished from an embryoid. As we know, from time to time, human embryos obtained by fertilization present genetic errors in varying degrees that can range from not affecting the phenotype at all, to making them non-viable, resulting in their death in the very early stages of embryonic life. Nevertheless, this does not stop us from considering that zygote or the early embryo that derives from it as human, even if the genetic defects involved make it incompatible with normal development. So, is it possible to determine the precise level of genetic alteration needed to consider these entities as a human being or a mere cell aggregate? Would we say that in previous experiments that led to the production of cloned macaque zygotes, which were unable to divide until birth, that they were not true macaque zygotes? What degree of possible genetic repair would be required to consider that an embryoid can be deemed an embryo? The answer is uncertain.

Given that it is impossible to establish clear boundaries to systematize the degree of differences that should distinguish zygotes and embryos obtained by fertilization from those obtained by cloning, parthenogenesis or induced cellular dedifferentiation, the principle of bioethical prudence should be applied to current research with a view to preventing them from threatening human lives that, even in a very genetically imperfect state, should continue to be regarded as such.

Best Practices

Based on the analysis carried out, different recommendations can be made for the different investigations with humanized entities that have been analyzed.

Regarding research with human-animal chimeras, current research is ethically acceptable, provided that animal welfare is safeguarded to the extent possible, in accordance with the ethics guidelines for animal research.

As for human-animal transgenesis, it should only be done in one direction, from human to animal and not vice versa. However, the function of the gene in question must be considered, so that the genetic change cannot lead to a variation in the moral status of the non-human animal.

In relation to human-animal hybrids and cybrids, ethical doubts about their nature persist, so it is recommended not to carry out this type of experiment.

As for parthenotes, and especially clonotes, doubts about their nature are even stronger, so experiments with these types of entities should not be carried out either.

Finally, embryoids today constitute only very partial models of embryonic development, so in their current state they do not pose the risk of being experimenting with embryonic human beings. On the contrary, their use can represent an ethical alternative for those who carry out research with human embryos in countries where this practice is legal.

Research Agenda

Regarding human-animal chimeras, research into technical means to ensure control of the fate of human stem cells in the animal organism must continue.

As for human animal hybrids, it would be interesting gaining knowledge about the physiological characteristics of the resulting entities, their similarities and/or differences with the gamete donor species or their maximum viability, but these studies should be carried out on the experiments already done, not by implementing new trials to this end, due to the ethical impediments to such investigations.

In the field of embryoids, the establishment of more complex embryonic models is desirable, since they would allow for more in-depth studies, but only up to a point, before such entities can come to be considered human embryos or raise doubts about their nature or moral status.

Educational Implications

Some of the research discussed in this article is very new, and progress in these fields is rapid. Efforts must be made from the scientific world to inform about these advances in a way that is affordable for the general public, so that citizens can participate in decision-making with a properly formed opinion.

Declaration of Conflicting Interests’

The authors declare that there is no conflict of interest.

References

- Aach, John, Jeantine Lunshof, Eswar Iyer, and George M. Church. 2017. “Addressing the ethical issues raised by synthetic human entities with embryo-like features”. *Elife,* 6: e20674.

- Aksoy, Irène, Cloé Rognard, Anaïs Moulin, et al. 2021. “Apoptosis, G1 Phase Stall, and Premature Differentiation Account for Low Chimeric Competence of Human and Rhesus Monkey Naive Pluripotent Stem Cells”. *Stem Cell Reports,* 16(1): 56-74.
- Araki, Yasuyuki, Midori Yoshizawa, Hiroyuki Abe, Yoshihiko Murase et al. 2004. “Use of mouse oocytes to evaluate the ability of human sperm to activate oocytes after failure of activation by intracytoplasmic sperm injection”. *Zygote* 12(2): 111-6.
- Araki, Yasuyuki, Midori Yoshizawa and Yasuhisa Araki. 2005. “A novel method for chromosome analysis of human sperm using enucleated mouse oocytes”. Hum Reprod, 20(5): 1244-7.
- Ayala, Francisco J. 2015. “Cloning humans? Biological, ethical, and social considerations”.*PNAS,* 112: 8879-86.
- Bacman, Sandra, Nadee Nissanka and Carlos T. Moraes. 2020. “Cybrid technology”.*Methods Cell Biol,* 155: 415-39.
- Baker, Monya. 2014. “Stem cells made by cloning adult humans”.*Nature,* DOI: https://doi.org/10.1038/nature.2014.15107
- Beccari, Leonardo, Naomi Moris, Mehmet Girgin, et al. 2018. “Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids”. *Nature,* 562(7726): 272-276.
- Boers, Sarah N., and Annalien Bredenoord. 2018. “Consent for governance in the ethical use of organoids”.*Nat Cell Biol,* 20(6): 642-5.
- Boers, Sarah N., Johannes JM van Delden, Hans Clevers, and Annalien L. Bredenoord. 2016. “Organoid biobanking: identifying the ethics”. *EMBO Rep, 17*(7): 938–41.
- Bourret, Rodolphe, Eric Martinez, François Vialla, et al. 2016. “Human-animal chimeras: ethical issues about farming chimeric animals bearing human organs”. *Stem Cell Res Ther, 7*(1): 87.
- Bredenoord, Annalien L., Hans Clevers, and Juergen A. Knoblich. 2017. “Human tissues in a dish: The research and ethical implications of organoid technology”. *Science, 355*(6322): eaaf9414.
- Brickman, Joshua M., and Palle Serup. 2017. “Properties of embryoid bodies”.*Wiley Interdiscip Rev Dev Biol,*6(2): e259.
- Cabrera Trujillo, Laura Yenisa, and Sabrina Engel-Glatter. 2015. “Human-animal chimera: a neuro driven discussion? Comparison of three leading European research countries”. *Sci Eng Ethics,*21(3): 595-617.
- Chen, Jianzhu, Russell Lansford, Valerie Stewart, et al. 1993. “RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development”. *Proc Natl Acad Sci USA, 90*(10): 4528-32.
- Chung, Young, Colin E. Bishop, Nathan R. Treff, et al. 2009. “Reprogramming of human somatic cells using human and animal oocytes”. *Cloning Stem Cells,* 11(2): 213-23.
- Cohen, Malkiel A., Styliani Markoulaki, and Rudolf Jaenisch. 2018. “Matched Developmental Timing of Donor Cells with the Host Is Crucial for Chimera Formation”. *Stem Cell Reports,* 10(5): 1445-52.
- Condic, Maureen L. 2011. “Preimplantation Stages of Human Development: The Biological and Moral Status of Early Embryos”. In *Is this Cell a Human Being? Exploring the Status of Embryos, Stem Cells and Human-Animal Hybrids* edited by Antoine Suarez and Joachim Huarte, 25-43. Berlin Heidelberg: Springer-Verlag.
- Cooper, David K. C. 2012. “A brief history of cross-species organ transplantation”.*Proc (Bayl Univ Med Cent), 25*(1): 49-57.

- Counihan, Daniel. 2019. “Neurological Chimeras and the Moral Staircase”.*Methods Mol Biol.,* 2005: 195-203.
- Cyranoski, David. 2019. “Japan approves first human-animal embryo experiments”.*Nature*. DOI: 10.1038/d41586-019-02275-3
- Das, Satyabrata, Naoko Koyano-Nakagawa, Ohad Gafni et al. 2020. “Generation of human endothelium in pig embryos deficient in ETV2”. *Nat Biotechnol,* 38(3): 297-302.
- Daughtry, Brittany, and Shoukhrat Mitalipov. 2014. “Concise review: parthenote stem cells for regenerative medicine: genetic, epigenetic, and developmental features”. *Stem Cells Transl Med,* 3(3): 290-8.
- De Los Angeles, Alejandro, Nam Pho, and D. Eugene Redmond Jr. 2018. “Generating Human Organs via Interspecies Chimera Formation: Advances and Barriers”. *Yale J Biol Med, 91*(3): 333-342.
- Dondorp, Wybo, and Martin H. Johnson. 2017. “Human-animal chimeras: circumventing rather than discussing ethical concerns comes at a price”. *Reprod Biomed Online, 35*(4): 341-2.
- Dozortsev, Dmitri, Andrei Rybouchkin. Petra de Sutter, et al. 1995. “Fertilization and early embryology: Human oocyte activation following intracytoplasmic injection: the role of the sperm cell”. *Hum Reprod,* 10, 403-7.
- Espejel, Silvia, Garrett R. Roll, K. John McLaughlin, et al. 2010. “Induced pluripotent stem cell-derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice”. *J Clin Invest,* 120(9): 3120-6.
- Fan, Yon, Zheying Min, Samhan Alsolami, et al. 2021. “Generation of human blastocyst-like structures from pluripotent stem cells”. *Cell Discov,* 7: 81.
- Fundación Española para la Ciencia y la Tecnología. 2007.*Organismos modificados genéticamente en la agricultura y la alimentación*. https://www.fecyt.es/es/publicacion/organismos-modificados-geneticamente-en-la-agricultura-y-la-alimentacion Accessed March 20, 2022.
- Harrison, Sarah Ellys, Berna Sozen, Neophytos Christodoulou, et al. 2017. “Assembly of embryonic and extraembryonic stem cells to mimic embryogenesis in vitro”. *Science,* 356(6334): eaal1810.
- Hendriks, Saskia, Eline A. F. Dancet, Ans M. M. van Pelt, et al. 2015. “Artificial gametes: a systematic review of biological progress towards clinical application”. *Hum Reprod Update,* 21(3): 285–96.
- Hu, Zhixing, Hanqin Li, Houbo Jiang, et al. 2020. “Transient inhibition of mTOR in human pluripotent stem cells enables robust formation of mouse-human chimeric embryos”. *Science Advances,* 6(20): eaaz0298.
- Huang, Ke, Yanling Zhu, Yanlin Ma, et al. 2018. “BMI1 enables interspecies chimerism with human pluripotent stem cells”. *Nat Commun.,* 9(1): 4649.
- Hyun, Insoo. 2017. “Engineering Ethics and Self-Organizing Models of Human Development: Opportunities and Challenges”. *Cell Stem Cell,* 21(6): 718-20.
- Hyun, Insoo. 2019. “Ethical considerations for human-animal neurological chimera research: mouse models and beyond”. *EMBO J,* 38(21): e103331.
- Kime, Cody, Hiroshi Kiyonari, Satoshi Ohtsuka, et al. 2019. “Induced 2C expression and implantation-competent blastocyst-like cysts from primed pluripotent stem cells”. *Stem Cell Reports,* 13: 485–98.
- Kobayashi, Toshihiro, Tomoyuli Yamaguchi, Sanae Hamanaka, et al. 2010. “Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells”. *Cell, 142*(5): 787-799.
- Kwisda Koko, Luvie White, and Dietmar Hübner. 2020. “Ethical arguments concerning human-animal chimera

research: a systematic review". *BMC Med Ethics*, 21(1): 24.

- Lee, Jea-Young., Sandra Acosta, Julian P. Tuazon, et al. 2019. "Human parthenogenetic neural stem cell grafts promote multiple regenerative processes in a traumatic brain injury model". *Theranostics, 9*(4): 1029–46.
- Li, Ronghui., Cuiqing Zhong, Yang Yu, et al. 2019. "Generation of Blastocyst-like Structures from Mouse Embryonic and Adult Cell Cultures". *Cell,* 179(3): 687-702.e18.
- Liégeois, Nanette, James Horner, and Ronald DePinho. 1996. "Lens complementation system for the genetic analysis of growth, differentiation, and apoptosis in vivo". *Proc Natl Acad Sci USA,*93(3): 1303-7.
- Liu, Xiaodong, Jia Ping Tan, Jan Schröder, et al. 2021. "Modelling human blastocysts by reprogramming fibroblasts into iBlastoids". *Nature,* 591: 627–632.
- Liu, Zhen, Yijun Cai, Yan Wang, et al. 2018. "Cloning of Macaque Monkeys by Somatic Cell Nuclear Transfer".*Cell,* 172(4): 881-7.
- López Moratalla, Natalia. 2004. "La partenogénesis: sin el glamour de la clonación".*Cuadernos de Bioética,* 3: 405-15.
- Mathew, Biena, Silvia Muñoz-Descalzo, Elena Corujo-Simon, et al. 2019. "Mouse ICM Organoids Reveal Three-Dimensional Cell Fate Clustering". *Biophys J, 116*(1): 127-41.
- Matsunari, Hitomi, Hiroshi Nagashima, Masahito Watanabe and Hiromitsu Nakauchi. 2013. "Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs". *Proc Natl Acad Sci USA,*110(12): 4557-62.
- McLaren, Anne. 1975. "Sex chimaerism and germ cell distribution in a series of chimaeric mice".*J Embryol Exp Morphol,* 33(1): 205–16.
- Mizuno, Kaorulo, Kazuhiko Hoshi, and Thomas Huang. 2002. "Fertilization and embryo development in a mouse ICSI model using human and mouse sperm after immobilization in polyvinylpyrrolidone". *Hum Reprod,* 17(9): 2350–5.
- Moris, Naomi, Kerim Anlas, Susanne C. van den Brink, et al. 2020. "An in vitro model of early anteroposterior organization during human development". *Nature* 582: 410–415.
- Munsie, Megan, Insoo Hyun and Jeremy Sugarman. 2017. "Ethical issues in human organoid and gastruloid research". *Development,* 144(6): 942-5.
- Nagashima, Hiroshi, Christopher Giannakis, Rodney J. Ashman, and Mark B. Nottle. 2004. "Sex differentiation and germ cell production in chimeric pigs produced by inner cell mass injection into blastocysts". *Biol Reprod,* 70(3): 702–7.
- NIH. 2015. *NIH Research Involving Introduction of Human Pluripotent Cells into Non-Human Vertebrate Animal Pre-Gastrulation Embryos*. https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-158.html Accessed March 21, 2022.
- Nikiforaki, Dimitra, Frauke Vanden Meerschaut, Stefanie De Gheselle, et al. 2014. "Sperm involved in recurrent partial hydatidiform moles cannot induce the normal pattern of calcium oscillations". *Fertil Steril,* 102(2): 581-8.
- Ozil, Jean-Pierre. 1990. "The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation". *Development,* 109: 117-27.
- Ozil, Jean-Pierre, and Daniel Huneau. 2001. Activation of rabbit oocytes: the impact of the Ca2+ signal regime on development. *Development* 128; 917-28.
- Palacios-González, César. 2017. "Chimeras intended for human gamete production: an ethical alternative?"*Reprod Biomed Online,* 35(4): 387-90.

- Pera, Martin F., Guido de Wert, Wybo. Dondorp, et al. 2015. “What if stem cells turn into embryos in a dish?”*Nat Methods,* 12(10): 917-919.
- Porsdam Mann, Sebastian, Rosa Sun, and Göran Hermerén. 2019. “A framework for the ethical assessment of chimeric animal research involving human neural tissue”. *BMC Med Ethics,* 20(1): 10.
- Reardon, Sara. 2016. “US agency to lift ban on funding human-animal hybrids”.*Nature, 536*(7615): 135.
- Regalado, A. 2019. “Chinese scientists have put human brain genes in monkeys—and yes, they may be smarter”.*MIT Technology Review,* April 10. Chinese scientists have put human brain genes in monkeys—and yes, they may be smarter | MIT Technology Review Accessed March 20, 2022.
- Revazova, Elena, Nikolay Turovets, O. Kochetkova, et al. 2007. Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells,* 9(3); 432-49.
- Rivron, Nicolas C., Javier Frias-Aldeguer, Erik J. Vrij, et al. 2018a. “Blastocyst-like structures generated solely from stem cells”. *Nature,* 557(7703): 106-111.
- Rivron, Nicolas, Martin Pera, Janet Rossant, et al. 2018b. “Debate ethics of embryo models from stem cells”.*Nature,* 564(7735): 183-185.
- Robert, Jason Scott, and François Baylis. 2003. “Crossing species boundaries”.*Am J Bioeth,* 3(3): 1-13.
- Rossi, Giuliana, Andrea Manfrin, and Matthias P. Lutolf. 2018. “Progress and potential in organoid research”.*Nat Rev Genet,* 19(11): 671-687.
- Rybouchkin, Andrei, Dmitri Dozortsev, Maria Josephina Pelinck, et al. 1996. “Andrology: Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes”. *Human reprod,* 11: 2170-5.
- Sagi, Ido, Gloryn Chia, Tamar Golan-Lev, et al. 2016. “Derivation and differentiation of haploid human embryonic stem cells”. *Nature,* 532: 107-11.
- Sawai, Tsutomu, Taichi Hatta, and Misao Fujita. 2019. “Japan Significantly Relaxes Its Human-Animal Chimeric Embryo Research Regulations”. *Cell Stem Cell.,* 24(4): 513-4.
- Sazonova, Margarita A, Vasily V. Sinyov, Anastasia I. Ryzhkova, et al. 2018. “Cybrid Models of Pathological Cell Processes in Different Diseases”. *Oxid Med Cell Longev,* 2018: 4647214.
- Shahbazi, Marta N., and Magdalena Zernicka-Goetz. 2018. “Deconstructing and reconstructing the mouse and human early embryo”. *Nat Cell Biol,* 20(8): 878-887.
- Shao, Yue, Kenichiro Taniguchi, Katherine Gurdziel, et al. 2017a. “Self-organized amniogenesis by human pluripotent stem cells in a biomimetic implantation-like niche”. *Nat Mater,* 16(4): 419-425.
- Shao, Yue., Kenichiro Taniguchi, Ryan F. Townshend, et al. 2017b. “A pluripotent stem cell-based model for post-implantation human amniotic sac development”. *Nat Commun,* 8(1): 208.
- Sharma, Arun, Vittorio Sebastiano, Christopher T. Scott, et al. 2015. Lift NIH restrictions on chimera research.*Science* 350(6261); 640.
- Shen, Helen. 2018. “The labs growing human embryos for longer than ever before”.*Nature,* 559(7712): 19-22.
- Shi, Lei, Xin Luo, Jin Jiang, et al. 2019. “Transgenic rhesus monkeys carrying the human MCPH1 gene copies show human-like neoteny of brain development”. *Natl Sci Rev Pages,*6(3): 480–93.

- Simunovic, Mijo, and Ali H. Brivanlou. 2017. “Embryoids, organoids and gastruloids: new approaches to understanding embryogenesis”. *Development,* 144(6): 976-985.
- Sinclair, Kevin D., S.A. Corr, Carlos G. Gutierrez, et al. 2016. Healthy ageing of cloned sheep*Nat Commun,* 7; 12359.
- Sozen, Berna, Gianluca Amadei, Andy. Cox, et al. 2018. “Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures”. *Nat Cell Biol,* 20(8): 979-989.
- Stanger, Ben, Akemi J. Tanaka, and Douglas A. Melton. 2007. “Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver”. *Nature,* 445(7130): 886-91.
- Swerdlow, R., S. Koppel, I. Weidling, et al. 2017. Mitochondria, Cybrids, Aging, and Alzheimer's Disease.*Prog Mol Biol Transl Sci* 146; 259-302.
- Tan, Tao, Jun Wu, Chenyang Si, et al. 2021. “Chimeric contribution of human extended pluripotent stem cells to monkey embryos ex vivo”. *Cell,* 184(8): 2020-2032.e14.
- van den Brink, Susanne, Peter Baillie-Johnson, Tina Balayo, et al. 2014. “Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells”. *Development,* 141(22): 4231-4242.
- Vilarino, Marcela, Sheikh Tamir Rashid, Fabian Patrick Suchy, et al. 2017. “CRISPR/Cas9 microinjection in oocytes disables pancreas development in sheep”. *Sci Rep,* 7(1): 17472.
- Vitullo, Alfredo, and Jean Pierre Ozil. 1992. “Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation”. *Developmental Biology,* 151: 128-36.
- Wang, Xuepeng, Tianda Li, Tongtong Cui, et al. 2018. “Human embryonic stem cells contribute to embryonic and extraembryonic lineages in mouse embryos upon inhibition of apoptosis”. *Cell Res,* 28(1): 126-9.
- Watanabe, Masahito, Kazuaki Nakano, Ayuko Uchikura, et al. 2019. “Anephrogenic phenotype induced by SALL1 gene knockout in pigs”. *Sci Rep,* 9(1): 8016.
- Wu, Jun, Daiji Okamura, Mo Li, et al. 2015. “An alternative pluripotent state confers interspecies chimaeric competency”. *Nature,* 521(7552): 316-21.
- Wu, Jun, Henry T. Greely, Rudolf Jaenisch, et al. 2016a. “Stem cells and interspecies chimaeras”.*Nature,* 540(7631): 51-59.
- Wu, Jun, Aida Platero Luengo, Maria Antonia Gil, et al. 2016b. “Generation of human organs in pigs via interspecies blastocyst complementation”. *Reprod Domest Anim,* 51(Suppl 2): 18-24.
- Wu, Jun, Aida Platero-Luengo, Masahiro Sakurai, et al. 2017a. “Interspecies Chimerism with Mammalian Pluripotent Stem Cells”. *Cell,* 168(3): 473-486.
- Wu, Jun, Marcela Vilarino, Keiichiro Suzuki, et al. 2017b. “CRISPR-Cas9 mediated one-step disabling of pancreatogenesis in pigs”. *Sci Rep,* 7(1): 10487.
- Xia, Yun, and Juan Carlos Izpisua Belmonte 2019. “Design Approaches for Generating Organ Constructs”.*Cell Stem Cell,* 24(6): 877-94.
- Yu, Lequian, Yulei Wei, Jialei Duan, et al. 2021. “Blastocyst-like structures generated from human pluripotent stem cells”. *Nature,* 591: 620–626.
- Zhang, Shaopeng, Tianzhi Chen, Naixin Chen, et al. 2019. “Implantation initiation of self-assembled embryo-like structures generated using three types of mouse blastocyst-derived stem cells”. *Nat Commun,* 10(1): 496.