On recent advances in human engineering

Provocative trends in embryology, genetics, and regenerative medicine

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ABSTRACT. **Advances in embryology, genetics, and regenerative medicine regularly attract attention from scientists, scholars, journalists, and policymakers, yet implications of these advances may be broader than commonly supposed. Laboratories culturing human embryos, editing human genes, and creating human-animal chimeras have been working along lines that are now becoming intertwined. Embryogenic methods are weaving traditional *in vivo* and *in vitro* distinctions into a new "*in vivitro*" (in life in glass) fabric. These and other methods known to be in use or thought to be in development promise soon to bring society to startling choices and discomfiting predicaments, all in a global effort to supply reliably rejuvenating stem cells, to grow immunologically non-provocative replacement organs, and to prevent, treat, cure, or even someday eradicate diseases having genetic or epigenetic mechanisms. With humanity's human-engineering era now begun, procedural prohibitions, funding restrictions, institutional controls, and transparency rules are proving ineffective, and business incentives are migrating into the most basic life-sciences inquiries, wherein lie huge biomedical potentials and bioethical risks. Rights, health, and heritage are coming into play with bioethical presumptions and formal protections urgently needing reassessment.**

Key words: Bioethics, CRISPR/Cas9, embryogenic methods, embryology, gene editing, genetics, human-animal chimeras, human engineering, *in vitro*, *in vivo*, regenerative medicine

On May 4, 2016, in two online-before-print articles, research groups led by embryologists Ali H. Brivanlou in the United States and Magdalena Zernicka-Goetz in the United Kingdom reported the success of their joint efforts to culture human embryos beyond the implantation stage while working *in vitro* (in glass), not *in vivo* (in life), using artificial laboratory sheaths.[1,2] This achievement was a milestone in human embryo culture technology, in human embryology basic research, and in the rational design of human embryonic stem cell (hESC) differentiation protocols, including drug-discovery protocols. Milestone or not, the scope and potential of the methods employed were not fully explicated; implications for rights, biomedicinl technology, bioethics, health, and heritage were not much explored; and general discussion did not follow. Further interpretation is indicated.

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Starting with well described mouse embryo culture methods,[3] the researchers devised a new *in vitro* protocol for the culturing of human embryos using an implantation matrix — a "matrigel" with extracellular matrix proteins and growth factor media.[1,2] This matrigel could fix a blastocyst by surrounding it physically and biochemically in a fashion simulating implantation, with success promoted by integrin $\beta 1$ signaling from within the matrigel itself, thus resembling natural cell-matrix communication.[3] Unexpectedly, the crux of the matter mainly seemed to be this complex protein mixture, the matrigel, a simple commercially available substance that had been widely used in stem-cell research for decades. The authors acknowledged maintaining autonomous human embryonic development *in vitro* until Day 13. They also affirmed that cultured early human embryos up to Carnegie stage 5d/6a (Day 14), displayed all natural features of embryonic and extra-embryonic lineages.

Both studies reported an unexpected discovery: "self-organization" of the human embryo.[1,2] This self-organization implied the ability "to recapitulate many

key features of *in vivo* development, surprisingly independently of maternal input at least up to d.p.f. [day post fertilization] 12."[1] This required conditions closely resembling those *in vivo*, and these conditions had sufficiently been approximated to interest attentive life scientists, but few others. Over time, investors, funders, regulators, bioethicists, legislators, and ultimately even diplomats may also become interested.

Might this self-organizing ability have been expected? Or, if not expected, expectable? Maternal structures would surely play a supporting role at some time after Day 14, but that role could neither easily nor ethically be assessed in women *in vivo*. Such later maternal conditions might be mimicked in laboratories of the future, with *in vitro* pregnancy a distant and disconcerting prospect. Indeed, the observed self-organizing ability involved both an embryonic lineage, the embryoblast, and an extra-embryonic lineage, the trophoblast — no maternal tissue needed.[1,2]

Embryonic and extra-embryonic tissues must act in concert for embryogenesis to proceed. Coordination is complex. Embryonic inner cellular mass (ICM) cells are informationally connected via gap junctions, and trophectodermal (TE) cells are structurally connected via tight junctions. TE cells and primitive endodermal (PE) cells give rise to extra-embryonic tissues that form the yolk sac, the visceral endoderm (VE), and the placenta, an organ that interconnects with maternal tissues to supply the embryo but does not itself give rise to maternal tissues. The blastocyst, a TE hollow sphere bearing ICM and PE cells and blastocoel fluid, is surrounded by the zona pellucida, a clear protective glycomatrix and structural layer originating from the oocyte. While embryonic, extra-embryonic, and maternal tissues must act in concert for embryogenesis to proceed, these two studies reporting self-organization succeeded while simulating only early maternal tissues and simple stationary signaling.[1,2]

Embryologists have not ordinarily attempted to substitute *in vitro* for the roles played by these extra-embryonic lineages *in vivo*. Rather, the central object of embryologists' descriptive and experimental attentions has heretofore almost always been the embryo itself. Embryo development was being studied always *in vivo* and never early, making extra-embryonic lineages hard to assess. hESC researchers, on the other hand, were studying differentiation into embryonic lineages always *in vitro* and starting without the extra-embryonic lineages required to assure both self-organization and pluripotency.[4,5] If these lineages could complement each other quasi-naturally *in vitro*, then self-organization might be expected. For example, embryonic stem cells (ESCs) of many species have been described for decades as pluripotent (able to develop into most body cell types) and are known to initiate an early auto-pattern formation program that partially resembles *in vivo* the self-organization of germ layers that give rise to all primordial organ tissues *in vitro*.[4] Absent an extra-embryonic influence, ESC differentiation *in vitro* shows much quantitative, qualitative, and structural variation.[4,5] Self-patterning and self-organization were also recently affirmed by Brivanlou's laboratory while studying hESC auto-patterning *in vitro*; geometrical discs resembling blastocyst structures[5] could qualitatively, and somewhat quantitatively, improve self-patterning and self-organizing. TE and VE requirements would still need to be met, and replacing extra-embryonic lineages — harder to replace than early maternal tissues — could not yet be achieved.

Self-organization was expectable. Our understanding of embryogenesis from practical experience with mammalian ESCs and from human monozygotic twinning had long implied embryonic self-organization, which lurked as a natural fact awaiting explicit demonstration.

In addition, species diversity and the body-plan pattern-formation ability of the entire mammalian phylum presupposed self-organization. Pluripotency, a novelty around 200 million years ago, greatly accelerated evolution. Newly evolving mammalian body plans did not have to be reinvented but could incrementally, and on a geological time scale rather quickly, be modified. The ICM and ESCs have proved to be flexible regulative starting materials easily shaped, formed, and fine-tuned in molecular-genetic fashion throughout natural history. This self-patterning self-organization is the basis for the pluripotency of hESCs and, thus, for all blastocyst-based *in vitro* differentiation in regenerative medicine's embryogenic methods. Without self-organization, early external maternal induction could not by itself yield identical twins from one epiblast (the first tissue differentiated from the ICM) prior to differentiation into ectoderm and mesoderm. Self-patterning and self-organization had long been studied in mice, but investigators' interests originally were quite disparate and usually excluded early lethality, a process whose complexity, when finally attracting systematic attention, was to prove daunting.

How would two identical embryos (i.e., monozygotic twins) develop if both were self-organizing normally but

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one of them had to depend on maternal spatiotemporal signaling that was arising externally? They probably would not develop in an unbiased way, as the externally directed embryo's primitive streak would likely be patterned imprecisely. Maternal induction and control of embryonic development had long seemed highly improbable both *in vitro* and *in vivo*. Maternal tissues and extra-embryonic lineages were known to be required for fetal growth in earlier and later development alike, but maternal structures were not thought necessary for an embryo's self-organization (which was expected to proceed independently) but only for implantation, supply, and growth. Using the proper matrix showed that maternal tissues could effectively be mimicked biochemically, but extra-embryonic lineages still could not be mimicked, and, indeed, these lineages do now seem to be required for proper differentiation and full expression of pluripotency.[1,2] This being the case, the entire field of regenerative medicine may now shift toward differentiation protocols using an extra-embryonic sheath. Such methods are "embryogenic," their setting "*in vivitro,*" which is to say "in life in glass."

Self-organization is common to embryogenesis of all species, assuming permissive environmental conditions. Even a bird's egg, once outside its mother's body, still needs the right temperature, a condition easily mimicked by an incubator. While such biophysical environments might be developed for human embryos, the bioethical risks would be very high, with many experimental failures — dead or living but malformed — before any *in vitro* pregnancy would result in a healthy live baby. In the two self-organization reports,[1,2] maternal tissue conditions and an implantation event were physiologically and biochemically simulated to deliver a complex mixture of extracellular matrix (ECM) signaling molecules and proteins. These extrinsic cues also seem to be required for intrinsic autonomous self-patterning of the extra-embryonic layer, and thus, the embryonic lineages. No direct or focal cell contacts could mediate further maternal tissue patterning with the embryo proper. Mainly ECM and integrin $\beta 1$ signaling[3] and the biophysical properties of a steadily fixed implanted embryo seem to be required in these early embryonic stages. Still, the recent self-organization findings are important as they help close an evidence gap, enable a powerful but also ethically highly controversial method, and further illuminate early human embryogenesis.[1,2]

In summary, these seminal works[1,2,3] show how to simulate *in vivo* conditions needed by the blastocyst-stage embryo developing *in vitro* and thereby allow culture beyond the implantation stage — and maybe further still. The simplicity of the method suggests that embryologists may already know how to extend the embryonic stage — and its usefulness — in this easy-to-handle human embryo culture. Are human embryos already being cultured to higher stages somewhere today? "No" is likely the wrong guess; simply forgetting to discard an embryo culture for a day or two would yield higher stages.

Self-organization could not have been wholly unexpected. Nor could it have been wholly independent of researchers' actions, as it followed conscious provision of extra-embryonic lineages and maternal-like signaling cues. The main achievement reported was technical facilitation allowing better study of human embryogenesis and more rational design of hESC strategies.[1,2,3] Furthermore, a rather different conclusion could have been drawn: Embryonic stem cells, hESCs, and the corresponding pluripotent ICM cells of the blastocyst embryo are not independently pluripotent and autonomously self-organizing in the regulative patterning sense, as is widely believed and routinely described in introductions to embryonic stem-cell research papers up until the present. To the contrary, ESCs reach complete pluripotency only within an implanting, or artificially implanting, blastocyst in the context of naturally co-developing extra-embryonic lineages (TE and PE) that receive implantation signals from the mother's reception-ready endometrium.

The self-organization experiments officially had to stop at Day 14; how late human embryos develop under such conditions *in vitro* is neither said nor sayable by scientists, but development presumably could progress this way for several more weeks. Human ICM cells and hESCs are equivalents; cells of either type can give rise to an embryo if injected into blastocoels, the interiors of blastocysts, and we now know that embryos made in these ways can be brought to Day 14, if not to a much later day. Embryogenic methods have led us here, to perilous opportunities.

Life *in vitro*

Recent achievements and all the big trends — the induction of stem cells to pluripotency via the culturing of embryos, the adventurous culturing of embryos *in vitro* using embryogenic methods, the editing of genes[6,7,8,9] — herald an era of "human engineering," both belatedly and prematurely. Regenerative medicine, a "repair" field, already exists. Human design, a "make" field, does not yet officially exist, but it could, whether we

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want it or not, as investigators are openly assembling "make-for-repair" techniques.

The blastocyst is a powerful and broadly compatible embryogenic-methods vehicle, easily altered genetically and cellularly, as shown by thousands of *intra*species chimeric descendants, mainly rodents, and by far fewer *inter*species chimeras — cross-species cells, ICM/ESCs, including half-human-half-animal embryonic cells — which are not now allowed to develop. The self-patterning and self-organizing ability of ICM/ESCs is unique to mammalian evolution. This ability now acquires two unevolved traits, extreme promise and extreme risk, which societies individually and global society as a whole must modulate by laws, conventions, and regulations.

Combinations of cells from different species in the mammalian phylum can self-pattern and self-organize together because self-regulatory communication, or cell-to-cell signaling, has been subject to evolutionary conservation at very high levels. For example, in C57BL/6J, a common inbred strain of laboratory mouse, 99 percent of all genes have direct analogues in the human genome, while in chimpanzees up to 99 percent of the entire genome sequence is identical to that of humans. This combination of evolutionary conservation and embryo self-regulation is a bioethically highly explosive mixture that could lead to critical ICM experiments yielding hybrid embryos and even live-born human-animal chimeras (see Figure 1).

Interspecies chimera formation is already routine. It is used, officially or not and risks aside, to assess the pluripotency of hESCs. Chimera formation shows high efficiency in early embryos in most reports. Quite aside from ICM/ESC-based transplantation medicine, in which "self" organs ideal for rejection-free implantation might be grown expressly for particular patients in nonhuman animals, such as pigs, *in vitro* embryonic self-organization may portend live-born human-animal hybrids — and, with them, moral calamity and legal consequence.

A subcategory of human engineering is "blastocyst engineering," through which cross-species can be composed and semi-artificial early embryos can be genetically engineered or otherwise modified (see Figure 1). Blastocysts can be transformed by natural and artificial embryonic and extra-embryonic cells, enabling chimeras and genetic modifications of many new sorts for various purposes. Blastocyst engineering also includes the injection of induced pluripotent stem cells (iPSCs) and hESCs. iPSCs are derived by reprogramming adult somatic cells to become pluripotent hESCs or ICM cells,[4,9] but they cannot be programmed to an earlier *totipotent* state that would give rise to TE cells, as totipotency is determined by the zygote and asymmetric cell division and not by employing an easy-to-use self-stabilizing transcription network. Reprogrammed human iPSCs can be produced for any patient or person. Procedures are quickly becoming safer.[9] Blastocyst engineering is also advancing, its goal being to engineer a functioning embryo "at once" with any modification, including gene editing or culturing of ICM or embryo.

Such engineered early or late blastocyst-embryos can now be grown *in vitro* or *in vivo* for a range of new research strategies in an increasingly ambitious regenerative-medicine field, whose leaders are already making human-animal chimeric embryos (see Figure 1). Thus, a bioethical crevasse opens as intact maternal structures become optional, for some purposes even obsolete, for growth production to Day 14 and beyond. Very early human or chimeric embryos would bear differentiated precursor cells soon to become human stem and precursor cells and then early human organs. These early human organs would need to be harvested before a chimeric embryo had developed to a point at which its partial humanity had to be invested with some subset of human rights. "Harvesting" here would mean removing early human organs to be grown *in vitro* — or more likely *in vivo* in other animals, immunologically receptive ones, used as "secondary chimeras." When the human-derived organs were mature enough for transplantation into their intended recipients, who would have been the donors of the hESCs used to make the human portions of the original "primary chimeras," these secondary chimeras would be sacrificed. Unclear is whether the cross-species prefix "xeno-" would continue to precede "transplantation" when describing such sequential movement of donor-recipients' cells and the organs arising from them.

Secondary chimeras would be less emotive bioethically, as they would be, by definition, later-stage or adult animals transplanted with embryonic human organs — other than brain — to grow *in vivo* for transplantation. *In vitro* organ incubation is a prospect but still a distant one. Experimental protocols sufficiently detailed to pass peer review are available to any sophisticated reader,[1,2,3] although protocols are often not detailed enough for reproducibility to be tested with confidence. Nowadays, competing investigators are usually left guessing.

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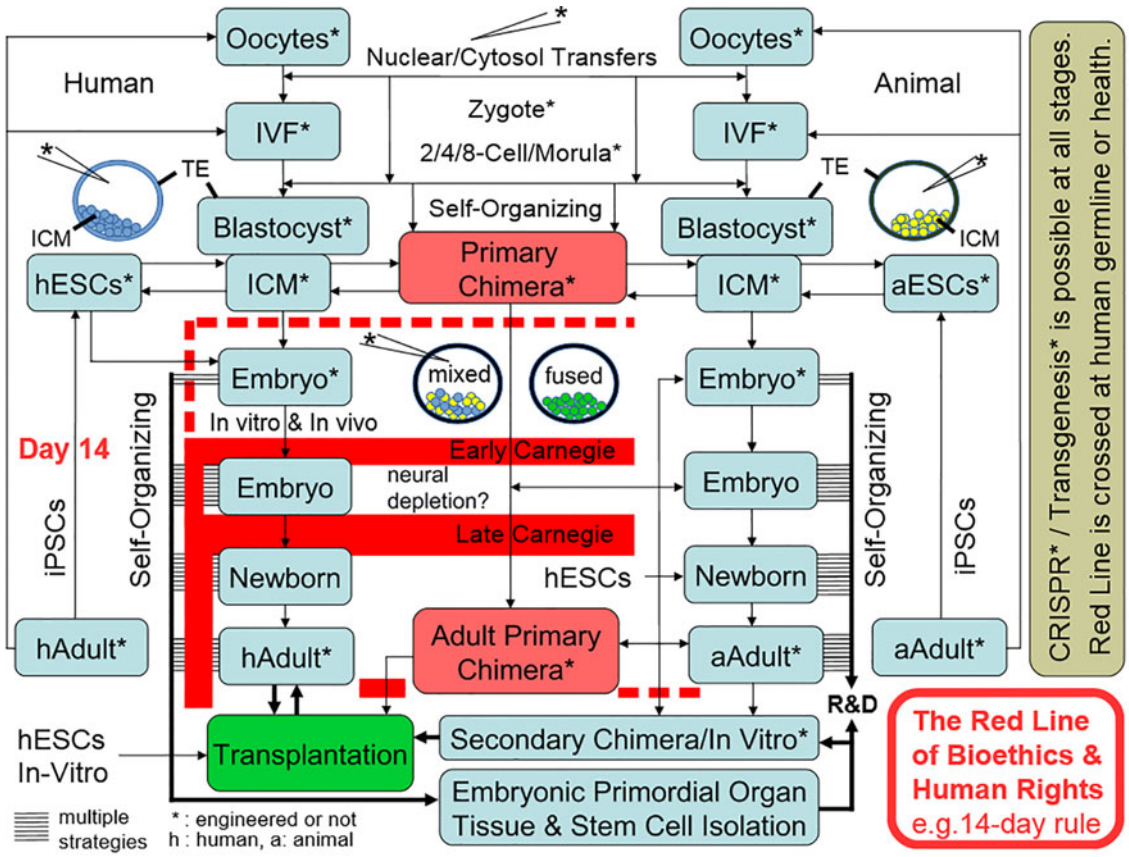

Figure 1. Human embryo culturing, primary and secondary chimeras, and human gene editing. Biomedical potential is constrained by bioethical barriers (red in online edition, gray in print edition) of various type and quality. Image by author. *Key*: * = "engineered or not." Research strategies may or may not incorporate engineering methods like CRISPR/Cas9, embryonic stem cells (ESCs), or other modifications. a = animal. h = human. ICM = inner cell mass. The blastocyst, a spherical structure, contains an inner cell mass that subsequently forms the embryo. iPSCs = induced pluripotent stem cells. IVF = *in vitro* fertilization. multiple lines = multiple strategies available. R&D = research and development. TE = trophectodermal cells. The trophectoderm, also called the *trophoblast,* is the outer layer of the blastocyst. Transgenesis = the introduction of an exogenous gene (a transgene) into a living organism, where it may be expressed; transgenesis is either natural or, as with CRISPR methods, engineered.

Consider "maternal structures" generically. These seem to signal "implantation" (an ECM stimulus through integrin $\beta 1$[3]) to the extra-embryonic lineages (TE and PE), which then assist, likely via intra-blastocyst signaling, in a combined embryonic-extraembryonic, quasi-autonomous, and therefore expectable self-patterning type of self-organization. These extra-embryonic lineages seem to transmit a checkpoint transduction signal reporting implantation was successful and development can proceed; the extra-embryonic lineages later "need to know" when to build structures that bridge maternal tissues with the embryo proper and, in some species, when to exit the pluripotent diapause state of delayed differentiation. The diapause state could be the molecular-genetic ancestor of "stemness," the uncommitted quality of a predifferentiated and therefore still pluripotent cell. Stemness can be maintained indefinitely by signaling cascades that stabilize the evolutionarily conserved transcription networks of the stemness state. Which precise molecular TE-to-embryo signaling steps might be involved here is not known. Cell-to-cell signaling or secreted-protein signaling may be involved. Integrin $\beta 1$ likely initiates FAK/MAPK-mediated transcription of implantation factors that drive physical

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implantation and transcription and thereby override an implantation checkpoint. Knowing the answer would require further investigation, and that investigation would likely involve human embryo experiments currently regarded as unethical, if not illegal.

Nonmaternal extra-embryonic cell lineages are developmental derivatives of the embryo's totipotent zygote, and the subsequent 2 + 4 + 8-cell-stage embryos, which accordingly split into embryonic and extra-embryonic cell lineages. These cell lineages, which give rise to blastocysts, can be engineered and could technically be used for all embryo experiments and are known to be required for the development of a complete embryo both *in vivo* and now also *in vitro*. While it would seem to be of the utmost importance, the potential utility of these cell lineages is rarely noted in the literature, although blastocyst engineering vehicles are still today to be regarded as absolute requirements for the pluripotency of hESCs and ICM cells.

In no purely *in vitro* protocol, as attested throughout the literature, could hESCs give rise to all human cell types; this assumption must now be revised. A blastocyst attachment from the extra-embryonic cell lineage — specifically, a proteinogenic extracellular implantation matrix with the oocyte's zona pellucida and adult endometrium interface — seems to be prerequisite for an hESC's autonomous self-organization into a developing embryo and all the cell types, tissues, and organs of a future person.

Although maternal structures seem not to be required for embryonic self-organization,[1,2] the extra-embryonic surrounding layers still seem to have a major signaling, patterning, framing, or cueing function that is required to assemble and coordinate a natural implantation scenario for the hESCs, starting with extracellular outside-activated integrin $\beta 1$ signaling.[3] The nonmaternal extra-embryonic lineages are in direct adjacent signaling contact with the embryo via cell-to-cell adhesion and in contact with the maternal structures. A three-layered structure — embryo, extra-embryonic blastocyst structures, and maternal structures — is thus simulated by an extracellular matrix. One layer influences one or two adjacent layers; all three are needed for live birth, but only the inner two are needed for self-organization by Day 14.

In short, the embryo autonomously develops inside of, and depends on, the second TE (trophectodermal) layer of the extra-embryonic blastocyst, which at implantation undergoes a signaling transcription transition *in vitro* mediated by a stationary external extracellular matrix. A signaling event from the maternal matrix activates the extra-embryonic layers that also play a crucial but much less frequently mentioned and less well understood role to allow proper development of the embryo inside. Hence, the self-organizing ability of the human embryo and of hESCs always comprises a functional extra-embryonic codevelopment, the "instructive" second layer, which needs a third maternal-layer growth-stage transition stimulus at the implantation stage. This is a crucial limiting process profoundly important for research, for regenerative-medicine product development, and for bioethics. Embryogenic methods — "embryogenic" being a portmanteau combining "embryonic" and "genetic" — are now on the research-and-development table. Researchers who have shied away from this prospect must now be conversant about its bioethical as well as its biotechnical dimensions.

Science takes time to be appreciated. What embryologists have come to know incrementally, other scientists, ethics committees, governments, and the public may find shockingly new — when, or if, they learn its details. All parties will also find shocking, as the author has, the biomedical potential and bioethical peril presented by embryogenic methods. Both laboratories reporting self-organization described most of its methods diligently, but neither described its embryogenic methods directly; both, though, stressed only the benefits of rational hESC protocols that would also require embryo culture.[1,2] Soon, or already today, we will face real dilemmas long thought only hypothetical. To heal an ailing human child or adult may we, should we, must we destroy an embryonic life? At which stage should human embryonic life be protected? Before, at, or after Day 14?

The implantation stage — which commences around Day 6 or 7 — has been seen as a high technical barrier, even a natural border and sacrosanct limit, and has been accepted as such by scientists and expert observers worldwide; a dozen nations have established it as a formal stopping point for human embryo experiments, and other nations seem to honor it informally. The rule is accepted by the International Society for Stem Cell Research (ISSCR).[10] The implantation stage corresponds to Carnegie stage 4 (Day 6 or 7). Carnegie 5d/6a at Day 14 is the nominal limit, a post-implantation stage that is characterized by a primitive streak axis and differentiating germ layers: neuroectoderm, mesoderm, and future endoderm. If experiments are not stopped arbitrarily at Day 14 for "slower" (or "faster") embryos,

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the rule will have been broken "overnight" or "over the weekend." A developmental-stage rule, while it would seem to make more sense, could itself easily be bent by assessing morphological characteristics tendentiously. Bright lines may be drawn, but if embryogenic methods become the basis of a new intensely competitive industry, one hard to regulate either nationally or globally, then somewhere those lines would be crossed.

Embryogenic methods are predicated on the fact that extra-embryonic structures are always required to achieve the entire scope, range, and capacity of hESC pluripotency. Accordingly, such methods may offer the cheapest, most straightforward, and most reliable approach to regenerative medicine and organ transplantation. *In vitro* protocols may still be confounded by the complexity of embryonic differentiation, which would require orchestrated contextualization signals of higher complexity than conventional *in vitro* protocols can provide. Deriving cell lines from pure hESCs *in vitro* is much easier than finding, harvesting, transporting, and implanting viable organ grafts, but authentic high-quality transplantable cell lines to be used for regenerative purposes are still very difficult to generate starting only with hESCs. Many cell types are unavailable via solely *in vitro* protocols, and some that are available may not fulfill all functional requirements. Some day, biotechnologists may be able to culture all these cell types *in vitro*, but how to assemble them into functional organs will be a long-term quest. In an embryogenic world, *de novo* organs would self-organize and initiate their development, but they would have to grow to usefully implantable proportions. Their growth process would require techniques as yet unreported and presumably still uninvented, and these techniques would surely be challenged on bioethical and even common-decency grounds. *De novo* organs could, already today, be customized genetically so as to meet recipient-specific needs; instrumental here would be gene editing and adult somatic-cell reprogramming to create induced pluripotent stem cells (iPSCs).[6,7,8,9] With any identified genetic defect edited out, any organ derived from a patient's own somatic cells would be ideal for implantation — assuming, of course, that reasons for the original organ's failure had fully been understood and would with high confidence not endanger the replacement, and assuming also that the technical chasm separating a single cell and a mature organ could be bridged.

Engineering of oocytes, blastocysts, hESCs, or patient-specific iPSCs could facilitate the growth of histocompatible organs *in vitro* and, by so doing, prompt the rise of a powerful new industry needing to grow human or human-animal "laboratory embryos" beyond Day 14, when the human neural lineage starts to develop. With the central nervous system (CNS) growing normally, personhood rights of an engineered embryo would become harder and harder to dismiss. A rule obviating this dilemma would have to be established and observed.

Members of the two reporting laboratories have already proposed a lifting of the Day 14 rule,[1,2] presumably because they have figured from theory — or have learned from unpublished observations — that they could successfully culture human embryos beyond Day 14. They have not been alone.[11]

Could exceptions to the Day 14 rule be defended? The human nonchimeric embryo would present a straightforward dilemma, one entraining familiar reproductive-rights and human-rights disputes still unsettled in many countries. The primary human-animal chimeric embryo would introduce an especially volatile topic, since an unintended interspecies mixing of traits would be hard to detect early on but could become heartrendingly obvious later on. The secondary human-animal chimeric embryo would present a more intricate puzzle, since the survival of hESC-derived organs would be the paramount concern in any "make-to-repair" regenerative-medicine transplantation effort, and harvesting might be less likely to succeed within a Day 14 limit than beyond it.

Primary human-animal chimeras could suffer many health problems; if locked in research cages hidden from public view, they might become an ethical irritant even if only imagined to exhibit human traits. Were they in fact to exhibit human traits expressed in appearance or behavior or intellect then sociocultural and science-policy consequences would surely be severe, as might be legal consequences.

An embryogenic-methods culture that grows human organs[12] without a CNS could be a technical solution proposed in grants of the future, such as by knocking out CNS-differentiation genes (see Figure 1). Embryonic organs and adult stem cells derived *in vivitro* from cultured human embryos might be crafted and grown for xenotransplantation; all of this could potentially be done without involving any human neural lineages, since only specific organs and tissues would be selected for further development. Several recent experiments indicate that this approach could work.[12] It would be very different ethically from relying on primary human-swine and human-sheep chimeras, which

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have already been created — without U.S. government funding and despite the risk that human neural lineages would be expressed — at the University of California, Davis, (UCD) in collaboration with other institutions in California and outside the United States.[13] To pass ethical review, not just by governments and committees and activists but by scientists and graft recipients and their families, methods should neither include nor affect human neural tissues or brain or human cognition at any stage. Embryogenic methods in this regard show promise, as they can accurately stop embryo development before a bioethically sensitive day limit; primary animal chimera methods cannot.

The protection of early embryonic human life has been linked to an embryo's life potential, its neural development, motility, pain perception, and autonomous viability. The human neural plate begins to form from the neuroectoderm at Carnegie stage 8–9, becomes partly multilayered at Carnegie stage 10, and exhibits early precursor forebrain and midbrain demarcation at Carnegie stage 11–12, which occurs as early as Day 26; pain perception and cognition are generally believed to start later.[14,15] Neural development has become particularly important for legislators in nations that have adopted the Day 14 rule not just for embryo research but for legal pregnancy termination.[11] Very late gestational limits are found in some Common Law countries, such as the United Kingdom (Week 24, Abortion Act of 1967) or Singapore, as well as in Civil Code countries, such as Italy (*circa* Week 25), Spain, and the Netherlands (Week 22). In the U.S., legal restrictions on pregnancy termination remain highly variable (Weeks 6 to 28).[16] Relatively late limits (e.g., Week 22) were initially derived from traditional, even ancient, conventions about quickening as reported by mothers. Today, first fetal movements, which demonstrate CNS activity, can be detected as early as Week 12, though a more usual point of first detection would be Week 16. Quickening is therefore starting as many as 10 weeks earlier than had been expected prior to the availability of advanced ultrasound imaging. About a quarter of babies delivered at Week 23 in medically advanced nations can be resuscitated successfully, and some of these resuscitated babies survive to go home.[17]

Embryogenic methods may be employed in work that never gets reported. The only way these methods could be kept unused would be to end access to (1) human oocytes, (2) fertilized human zygotes, (3) early human embryos left over from *in vitro* fertilization (IVF) procedures, and (4) early human embryos engineered from hESCs.[18,19] Such measures would collaterally end programs and practices now widely accepted, while embryonic "contraband" could still easily be traded, stored, cultured indefinitely and uncontrollably, and shipped in a cryopreserved state. Suppressive efforts would bring significant risks and controversies, inevitably engendering new ethical dilemmas and legal challenges.

An edited human genome

Other recent far-reaching methodological breakthroughs have been seen in editing genomes and their related elements using CRISPR/Cas9 and similar tools.[20] CRISPR is popularly pronounced "crisper" and is professionally known to abbreviate "clustered regulatory interspaced short palindromic repeats." Cas9 stands for "CRISPR-associated protein 9," referring to an enzyme, an endonuclease, that catalyzes an editing reaction. In other words, CRISPR/Cas9 edits a DNA sequence using an RNA-sequence template, and it does so in a highly customizable way, changing any gene in any organism in any respect.

This technique has been instrumentalized from prokaryotic — bacterial, cyanobacterial, archaeal — defenses against viruses, the best known group among these viruses being the bacteriophages. CRISPR/Cas9 using a "guide-RNA" (gRNA) has proved to be a flexible and highly, although not perfectly, reliable tool for manipulating and modifying genomic DNA sequences in bacteria, plants, cnidarians (hydra), fish, mammals, and other species, including humans. gRNA can be customized and then generated artificially and is commercially available to target particular point mutations — i.e., single-nucleotide polymorphisms (SNPs) — and also longer stretches of DNA, including whole genes and nongene regions, thus enabling customizable knock-in and knock-out interventions comprising anywhere from one nucleotide to thousands of nucleotides. Methods employing CRISPR/Cas9 are remarkable for their amenability, tractability, simplicity, and feasibility; most importantly, gRNAs can be designed *in silico* and generated *ex machina*.[6,7,8]

CRISPR/Cas9 may have further advantages over other more complicated, less efficient, older methods. It seems likely to prove more suitable for specific mutational goals. Being DNA-free, it obviates some, but not all, adverse and off-target effects. And, at least in theory, it should prove more efficient in editing both alleles of a bi-allelic gene-modification target,

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such as one to be altered when correcting a homozygous defect.[7,8,10] Many assumptions remain to be tested fully. So far, the best case for CRISPR/Cas9 is a composite of comparative specificity and comparative efficiency. Among risks yet to be clarified are unwanted off-target effects on DNA — meaning effects in the genome itself — and also on RNA, the importance of whose many recently discovered roles far exceeds prior appreciation even among life scientists. Further testing is needed using proteomics and "next-generation whole-genome sequencing" (NGS) of DNA and RNA.

Important aspects of CRISPR/Cas9 technology have been reviewed and are widely available.[6,20] Nevertheless, media opinion has failed to highlight both the entirety of its potential and the reality of its risks. CRISPR/Cas9 methods can now be employed cheaply in any ordinarily equipped molecular-genetics laboratory anywhere in the world; capital requirements and skill requirements are both low. Any biologist could use CRISPR/Cas9 effectively after only a few weeks of preparation and training. All necessary reagents and protocols are commercially available or, for academics, freely available. GeneScript, AddGene, Thermofisher, Sigma-Aldrich, GeneCopoeia, Labomics, OriGene, and AppliedStemCell are prominent sources, and peer-to-peer "technology transfers" and "R&D-result transfers" are common outside formal channels. In consequence, risks to genetic safety, to societal gene-pool security, and to health itself have begun to emerge. Primary editing — altering the germline — is supposedly off limits. Secondary editing — repairing somatic defects — is not off limits. A primary-secondary distinction in the chimera case is clear enough; such a distinction in the gene-editing case is a legalistic imposition on nature, and nature need not obey.

Primary gene editing might reasonably be attempted in an embryogenic-methods IVF context to repair a familial genetic defect, the goal being an embryo and thus a child, a procreating adult, and countless future generations all free of one specific risk. Even if that goal could be achieved, though, one or more new defects might be created by the method itself, and these would similarly apply to all future generations. Still, many families would accept this gamble: trading expungement of a serious known problem now for random unknown changes that might or might not prove to be problems later. Secondary gene editing would seem safer since *by definition* germline cells would not be effected, but primary editing could still occur inadvertently, irrespective of any "secondary" intention. If future refinements of method could somehow *guarantee* somatic-only or germline-only or somatic-plus-germline changes then wondrous visions would become tangible realities. At improvement's current pace, such refinements may await in a future not far distant.

A first generation of engineered humans may already exist following reputedly successful but still unreported, and therefore uncitable, efforts to cure genetic disease. Whether gene-edited patients could, ironically, prove to be the progenitors of transgenic diseases will depend on the accuracy, validity, and correctness of the gene-editing strategies used.

Human engineering's prospect, if not its reality, readily brings to mind dark fantasies, and some of these have long since found their way to print and screen. Tamer thoughts also warrant consideration. A private medical industry could arise to advance familial genetic endowments for rich clients willing to dismiss risk or quietly discard defective products. With oocytes and sperm commercially available today[21,22,23] and embryonic-genetic modifications now feasible, a human-engineering tourism industry could be thought a money-maker in loosely regulated economies. Even where laws more reliably rule, "patentable human bio-property" might be foreseen, as nonhuman antecedents already exist. Even if beneficence prevails, disappointment, repulsion, and regret must be anticipated. A human embryo was first reported to have been CRISPR-edited in 2015. The procedure used was described as having major drawbacks.[10]

Editing can be used to correct both germline and somatic mutations.[6,7,8] Not only the embryo[10] but also the human adult could be edited somatically to cure a disease,[6,7,8] all of which seems ethically benign at first sight. However, several patients have lost their lives in attempts at therapeutic secondary gene editing using prior state-of-the art methods — not CRISPR/Cas9 but not unrelated. Clinical studies worldwide are often not well thought through; reasons may be numerous, but among the reasons in rapidly advancing fields could be an insufficient presence of life scientists with a post-doctoral research-and-development background. While notably easy to learn and apply, CRISPR/Cas9 methods are not foolproof; if made in human stem cells, embryos, fetuses, neonates, infants, children, or adults, mistakes could not be discarded in a "just for practice" bin. The labor market for scientists with recent post-doctoral training is, anecdotally, neither free nor fluid, suggesting a bottleneck effect for staffing clinical trials planned under one nation's rules but performed in an-

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other nation where the rules are different. CRISPR/Cas9 methods could be used to advance selected features — intelligence, muscle type, muscle mass, body size, body shape, skin color, behavioral propensities — or to mute negative characteristics. Indeed, a set of "advancing and healing mutations" is already known and published, while genetic research data is accumulating unpublished in private hands, held tightly there by confidentiality agreements, some of them applying expressly to CRISPR research.[6,7,8,10] Whether this private knowledge has been used in human engineering experiments we have no way to say. Since human zygotes are already traded on markets worldwide,[21,22,23] private human engineering[10] may have already begun somewhere and may be ongoing there. Speculation is a poor foundation for science policy, but science policy must be mindful of the possible, not just the demonstrable.

Taken together, risks now appear unscalable in the sense that non-corrective genetic alterations and undirected changes made during either corrective or non-corrective interventions may radiate within our species permanently. While unwanted changes themselves may in future be corrected by methods similar to the ones that made them, new undirected changes may again be introduced. Can we estimate the ultimate scale of any and all such effects? No; they could just as plausibly be negligible as catastrophic — whence "unscalable." Human genetic security thus becomes a high-priority global policy topic. Oocyte markets may be good early targets for international regulation.

Within a decade of its first reported sequencing, the human genome became subject to manipulation. Just as the genome itself had been sequenced faster than expected, so the genome's manipulation may accelerate faster than expected. For example, today the "HGP-write" project concentrates on synthesizing a quasi-human genome in cells able to create an artificial quasi-human life form.[10,24] This project may fail for technical reasons, but its failure would itself inform any similar future attempt. Counting on failure is not a prudent strategy.

The still young CRISPR method can surely be further advanced, but needing solution are more problems than popularly supposed: specificity failure resulting in off-target effects; efficiency failure resulting in fewer edits than intended; unintended mutations in DNA or RNA; unintended alterations in epigenetic function and, thus, cellular complexity and disease risk; mosaicism, a condition in which an organism contains cells of two genetically different types simultaneously; hidden accumulation of non-specific mutations and off-target effects which over many generations may compromise chromosomal stability and overall structural genomic integrity.[6,7,8,10] In sum, CRISPR is not yet advanced enough for human engineering of any sort.

That said, new enzymes are being found that may improve performance. One is Cpf1 introduced in 2015 as a putative Class 2 CRISPR effector.[25] The future of all related trends will depend on research funding, and a CRISPR/Cas9 bias may persist, making newly introduced enzymes like Cpf1,[25] as well as other innovative toolsets and editing systems harder to study. Industry has already invested many hundreds of millions of dollars in CRISPR/Cas9, making the development, introduction, and optimization of additional methods and procedures less tempting. One might ask why industry is investing so much capital in a method likely to remain restricted in well regulated countries. CRISPR/Cas9 has approved uses in humans other than in embryos, and CRISPR/Cas9 remains totally unrestricted in less well regulated countries.

CRISPR/Cas9 may become reliable enough for strictly secondary editing tasks: somatic gene therapy and epigenetic modification.[10] Furthermore, it can be modularly combined and employed to alter gene expression rather than a gene sequence. For example, the CRISPR epigenetic activator enhances or blocks endogenous gene expression; epigenetic activators were also developed by Merck via a merger with MilliporeSigma.[26] These CRISPR epigenetic activators were listed sixth among the top ten innovations of 2015 by *The Scientist*.[27]

CRISPR/Cas9 in different settings has already been used for genome editing[6,7,8,20] and for epigenetic and other modifications of transcription.[20] One setting has been the editing of zygotes, including human tripronuclear (3PN) zygotes,[10,20] meaning the primary editing of the human embryo or germline, as in the first report of successful modification of human embryonic life with CRISPR.[10,20] The authors said they used "nonviable" 3PN embryos that could not give rise to a live birth,[16] which seems not entirely correct, as an implanted 3PN zygote can give rise to an embryo, although pregnancy and fetal health are unlikely because embryo quality is low.[28] "3PN" serves here only as a negative prognostic indicator. Hence, viability could have been retained *in vivo*, and *in vitro* culture methods for human embryos[1,2] might have been extended. A second setting has been somatic CRISPR/Cas9 editing *in vivo* to cure heritable and acquired diseases in local and organismal applications for tissues, adult stem cells, and

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adult organs. A third setting has been *ex vivo* (outside the body) editing of progenitor cells and stem cells before transplantation. Related strategies have been used in immune cells or immune stem cells (hematopoietic stem cells, HSCs) for reasons including cancer therapy and elimination of the human immunodeficiency virus genome from T-lymphoid cells *ex vivo*.[29] The failure of non-embryogenic HSCs reveals that embryogenic methods, including AGM-HSC derivates, might be essential, as researchers using hESCs *in vitro* have not reproducibly succeeded in generating high-quality HSCs with short and long term potential.[30] Subsequently, the HSCs generated by embryogenic methods could be further amplified, processed, and CRISPR-modified in many ways and for many therapies. Embryogenic methods, then, may hold their greatest promise when augmented by gene editing. Patient-specific therapies free of immune rejection could be possible for all cell types, all tissue types, all organ types. That said, none of this promise will be realized without reducing risks still to be characterized and solving bioethical problems entirely new to the human experience.

Engineering ourselves

Not much longer will we be writing science-fiction when describing genetically engineered humans or cultured human embryos or chimeras part human and part, or parts, nonhuman. These are just simple steps ahead or, if already taken in shadow, behind. If it wants to benefit from new embryogenic methods and from human engineering, global society must devise and enforce rules that direct technological progress wisely.

Research transparency — access to all risk-relevant information — should be the first goal. Weakening the assumption that priority is a prize prompting rapid and full disclosure is the fact, albeit by its nature hard to document, that key results today are often not published at all or not published in full. As explicated above, the self-organizing ability of human embryos *in vitro* and the researcher's ability to culture beyond the implantation stage would have been observed well before publication — and by two large teams whose senior scientists had carefully been coordinating their publication plans.[1,2] Sometimes innovative work is published after a strategic delay imposed by a senior author; motivations for delay may vary, but society at large and junior researchers in particular may be disadvantaged. While a rush to publication may be expected to increase, at least marginally, the overall incidence of retractions, a reluctance to publish may allow norm-bending work to proceed unrevealed and unhindered. Loosening laboratory hierarchy to allow junior scientists more say in research direction and publication could increase transparency, but the science culture of many nations would likely at least at first be resistant. Regulation to defend bioethical standards — which themselves must be adapted to rapidly changing realities — would have to reach into laboratories of any structure.

What about chimeras? They date from Beatrice Mintz in the 1960s when 8-cell-stage blastocysts were aggregated to make intraspecies — mouse-mouse — chimeras.[31] Now, decades later, mouse-rat[32] chimeras and many human-mouse early embryo chimeras have been made and described.[18,31] Yet if these early chimeras were not sacrificed or did not die naturally or if they were kept as breeding stock then human-mouse *adult* counterparts would still exist today. What would they look like? How would they behave? Would they be in cages? Would they have human rights or animal rights or both? Would they need our help? While these human-animal chimeras have effectively been censored from our awareness, other human-animal chimeras do exist now and more are being created.[13] Yet as we seem set to make more of them, we must take their roles in our future seriously — not only as investments, tools, and means to many ends but also as beings more like ourselves than nature had made them. On human-rights grounds, animal-rights grounds, and human-health-risk grounds the author opposes their creation and is not alone in this opposition.

Zygotes or hESCs or human ICM cells or human blastocysts are still needed as embryogenic vehicles so as to unfold the potential of pluripotency. hESCs could be amplified and distributed like HeLa cells, immortal and ubiquitous in laboratories but originally from the lethal cervical cancer of one young woman, Henrietta Lacks (1920–1951). Tight regulations do, in theory, exist to prohibit a dissemination of hESCs in some countries but can only slow the trend toward human engineering, not stop it. Oocytes are available in clinical markets, controlled and uncontrolled, as well as in black markets. For example, in China, oocytes supposedly cost about $5 USD or 30 Yuan — affordable as well as available.[21,22,23]

Previous methods have been displaced in at least one line of work. The California Institute of Regenerative Medicine, established to bypass federal funding restrictions dating from the George W. Bush presidency, has

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been funding an effort to use pigs and sheep to develop human organs. Genes coding for targeted organs are knocked out in animal stem cells. But genes coding for the CNS are not knocked out in the human iPSCs used to make the chimera, as knocking out all genes coding for any and all parts of the brain would be prodigiously difficult. At the animal's blastocyst stage, these human iPSCs are added in the expectation that their genes will complement the animal genome: "embryo complementation" this phenomenon is called. Any viable animal would need to have adopted the human genes directing development of the organ that was not going to be grown at all without complementation. Pigs and sheep growing human organs and using them successfully would be animal-human chimeras liable — through cross-complementation and cross-species hybridization — to exhibit the occasional human trait, such as a behavioral trait, but fated nonetheless to be sacrificed as donors once they had grown to a targeted size. Recognizably human traits are said not to be expected in these animals, although hESCs would admittedly make contributions to the host animal's development, including its neurological development.[13]

Recognizably human traits are likewise said not to be expected in these human-animal chimeras, but what if expectations are sometimes wrong? Or always wrong? The right usage of these new technologies could indeed solve many problems, but what if the human iPSCs complementing the animal embryos contribute to brain development? A project principal and UCD veterinarian and developmental biologist, Pablo Ross, has answered this question forthrightly:

> We don't want to grow them [the chimeras] to stages we don't need to, since that would be more controversial[.] . . . My view is that the contribution of human cells is going to be minimal, maybe 3 percent, maybe 5 percent. But what if they contributed to 100 percent of the brain? What if the embryo that develops is mostly human? It's something that we don't expect, but no one has done this experiment, so we can't rule it out.[13]

Although technically and scientifically interesting, these experiments edge toward a moral abyss; they also violate the Day-14 rule[11] by creating primary chimeras. Inhibiting the formation of an animal organ so as to allow complementation means using CRISPR.[6,7,8,20] When intending to grow, say, a human pancreas, the formation of a swine pancreas would first have to be inhibited. Would the targeted genes be knocked out cleanly? If no, the harvested pancreas, once transplanted, would be rejected as foreign. Even if yes, the knock-out would still have added to the animal host some number of CRISPR-associated defects, genetic and epigenetic, and these might turn out to affect the function of the graft indirectly. Even if not, even if the human recipient were to accept the graft as "self" and so need no immunosuppression, the human-animal chimeric host would still have been sacrificed, perhaps with some degree of a human infant's situational awareness.

A sound bioethical requirement for knock-outs in chimera protocols would be assessment of a human iPSC's potential to differentiate toward and commit to a neural lineage; all neural potential in ICM/hESCs would have to be blocked with high confidence *before* introduction. We may assume that had it been feasible this step would have been taken and that as it has not been taken a decision has been made to pay for grown-to-order organs a moral price higher than society or potential organ recipients realize. Could we all become accustomed to this price and invent arguments to defend its ongoing and expanding payment? Yes. Our ancestors nimbly invented arguments to defend race slavery, which in its day was an innovation highly valued by its beneficiaries. Moral habituation to a vibrant human-animal chimera industry, if allowed to implant itself firmly, is easily imagined.

Where should society draw its "red line" when human neural cells are involved? Human-embryogenic-methods researchers should end their *in vivitro* work at a stage preceding any human brain development even if human fetuses growing *in utero* can legally be terminated well beyond that stage. Chimera researchers should make impossible — not just improbable — any human or part-human brain development. Primary human-animal chimeras should be forbidden because they may develop human-animal brains post Day 14.

Pandora's genetic toolbox has been opened by procedures now regulated in few countries and, if assuming a black market in oocytes, hard to regulate in any country. Biotechnology companies of the future may want to sell engineered human organs grown *in vitro* or in animals or both, ethics aside, if return on investment beckons.

In the United States, the National Institutes of Health (NIH) Guidelines for Human Embryonic Stem Cell Research, issued in July 2009, did not endorse chimera experimentation in any form involving neural lineages.[33] In September 2015, the NIH stopped all funding for

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chimera experiments,[34] but in mid-2016 the NIH lifted its moratorium.[32] The ISSCR continues to endorse the Day-14 rule but neither prohibits nor criticizes chimera experimentation generally; it should instead acknowledge that the Day-14 rule automatically prohibits the creation of primary human-animal chimeras and that embryogenic methods may be adapted to reach regenerative medicine's goals in a morally noncorrosive way. Approved now by the NIH is injection of pluripotent stem cells at an embryo's post-gastrulation phase, which phase is followed by organogenesis; excluding neural lineages from these injected cells would be difficult. Other researchers argue against the moral significance of implanting such lineages, contending that treatment of human-animal chimeras can well enough be modified case-by-case: "Because the transfer of human stem cells could have unpredicted effects on a chimaeric animal's capacity to suffer, it is crucial that qualified veterinary staff and researchers monitor experiments for deviations from normal behaviours and species-typical functioning, and use clear criteria for humane interventional euthanasia."[35] In other words, a chimera exhibiting human-like behavior — Ross's "more controversial" product whose creation could not be ruled out[13] — should simply be killed.

How to deploy new technologies to optimize benefit in an ethically sustainable way is biology's most pressing problem. While embryogenic methods are beginning to stand out as most versatile and least objectionable of these technologies, the problem overall is nowhere near solution and with time may become more complex, not less. Nonetheless, genetic diseases can now, presumably, be cured. Our task is to align our actions — our research, our funding, our regulations, our laws — toward safe and compassionate ends through safe and compassionate means.

Author Notes

Roman Anton was born in Darmstadt, Germany, and earned a diploma in biology (M.Sc., B.Sc.), with distinction, at the University of Technology in Darmstadt. He went on to the University of Ulm, where he studied molecular and cell biology and where in June 2008 — for research in signal transduction, pattern formation, and embryonic stem cell biology — he earned a Ph.D. in natural sciences (*Dr. rer. nat.*). In his postdoctoral work, in projects both academic and industrial, he concentrated on molecular and cellular screening and signal transduction in cancer biology, cardiovascular disease, and neurobiology. He has worked at the Max Planck Institute of Immunobiology and Epigenetics, Freiburg, at the New York University Cancer Institute, at the University of Bonn, at the Natural and Medical Science Institute (NMI), University of Tübingen, and at the University of California, San Francisco. He also holds an MBA with a focus on finance and sustainable strategy, has performed empirical research in financial markets and monetary policy, and has published two textbooks in these fields.

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