PERSPECTIVE

Human Cloning — The Science and Ethics of Nuclear Transplantation

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In addition to the moral argument against the use of somatic-cell nuclear transfer for the creation of a child ("reproductive cloning"), there are overwhelming scientific reasons to oppose this practice. In contrast, many believe that the practice of somatic-cell nuclear transfer with the goal of generating an embryonic stem-cell line (sometimes referred to as "therapeutic cloning") is justified, because it holds the promise of yielding new ways of studying and treating a number of diseases. Once isolated from a patient, an embryonic stem cell thus derived would be "customized" to the needs of the patient who had served as the nuclear donor and thus would obviate the need for immunosuppressive treatment as part of a therapeutic application. In addition, because embryonic stem cells can generate most, if not all, types of cells in vitro, a stem cell isolated from a patient with a complex genetic disease could be used to study the pathogenesis of the disease in culture. In Figure 1, the generation of a mouse by sexual reproduction is juxtaposed with its generation by nuclear cloning and the derivation of an embryonic stem-cell line by means of nuclear transfer from a patient's cell.

In contrast to an embryo derived by in vitro fertilization, a cloned embryo has little, if any, potential to develop into a normal human being. By circumventing the normal processes of gametogenesis and fertilization, nuclear cloning prevents the proper reprogramming of the clone's genome (described below), which is a prerequisite for the development of an embryo into a normal organism. It is unlikely that these biologic barriers to normal development can be overcome in the foreseeable future. However, embryonic stem cells derived from a cloned embryo are functionally indistinguishable from those that have been generated from embryos derived through in vitro ferti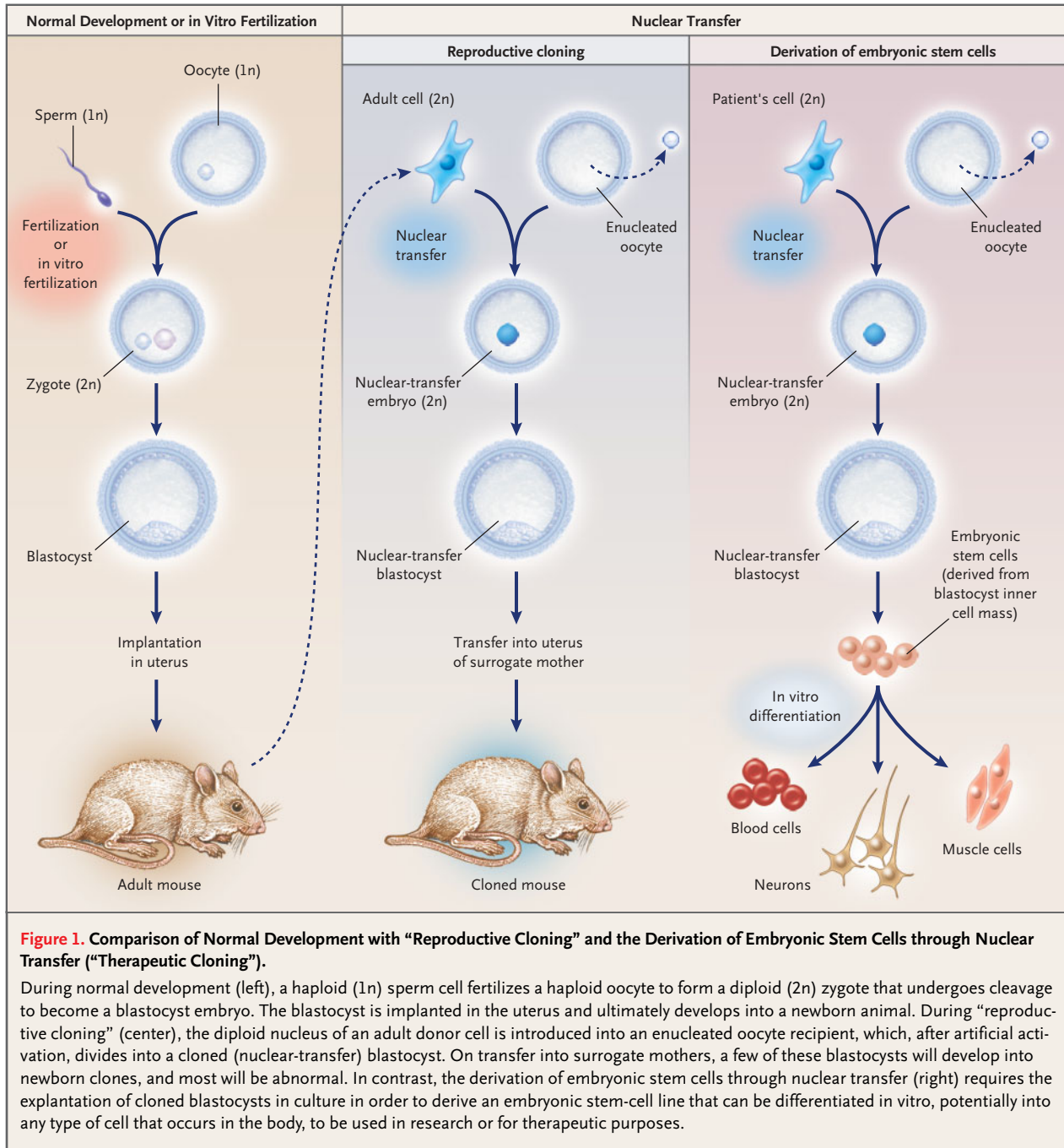lization. Both have an identical potential to serve as a source for cells that may prove useful for research or therapy.[1]

Most cloned mammals derived by nuclear transfer die during gestation, and those that survive to birth frequently have the large offspring syndrome, a neonatal phenotype characterized by respiratory and metabolic abnormalities and an enlarged, dysfunctional placenta. In order for a donor nucleus to support development into a clone, it must be "reprogrammed" to a state compatible with embryonic development. Inadequate reprogramming of the donor nucleus is most likely the principal reason for the developmental failure of clones. The transferred nucleus must properly activate genes that are important for early embryonic development and must also suppress genes associated with differentiation that have been transcribed in the original donor cell.

However, gene-expression analyses indicate that 4 to 5 percent of the overall genome and 30 to 50 percent of imprinted genes (described below) are not correctly expressed in tissues of newborn cloned mice.[2] These data represent strong molecular evidence that cloned animals, even if they survive to birth, have serious gene-expression abnormalities. Moreover, as cloned mice age, severe pathological alterations in multiple organs and major metabolic disturbances that were not apparent at younger ages become manifest. A case in point is Dolly the sheep, the first mammal cloned from a somatic cell, which appeared healthy at a young age but died prematurely with numerous pathological abnormalities. These findings suggest that clones that survive to birth merely represent the least abnormal animals: subtle abnormalities that originate in faulty reprogramming may simply not be severe enough to interfere with their survival. Indeed, given the available evidence, it may be exceedingly difficult, if not impossible, to generate healthy cloned animals or humans.
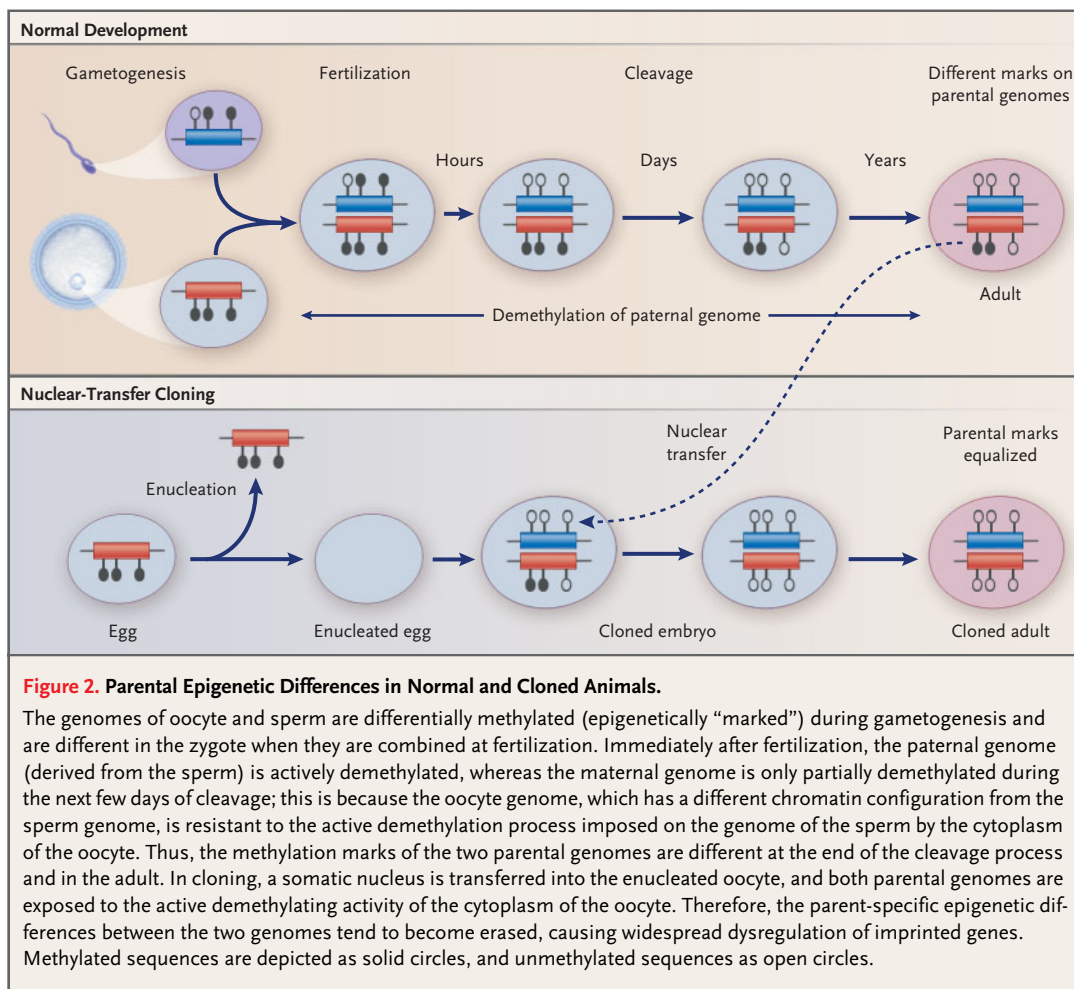
It is often argued that the technical problems associated with producing normal cloned mammals will be solved by scientific progress in the foreseeable future. But some of these problems may well prove to be insurmountable.

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Figure 1. Comparison of Normal Development with "Reproductive Cloning" and the Derivation of Embryonic Stem Cells through Nuclear Transfer ("Therapeutic Cloning").

During normal development (left), a haploid (1n) sperm cell fertilizes a haploid oocyte to form a diploid (2n) zygote that undergoes cleavage to become a blastocyst embryo. The blastocyst is implanted in the uterus and ultimately develops into a newborn animal. During "reproductive cloning" (center), the diploid nucleus of an adult donor cell is introduced into an enucleated oocyte recipient, which, after artificial activation, divides into a cloned (nuclear-transfer) blastocyst. On transfer into surrogate mothers, a few of these blastocysts will develop into newborn clones, and most will be abnormal. In contrast, the derivation of embryonic stem cells through nuclear transfer (right) requires the explantation of cloned blastocysts in culture in order to derive an embryonic stem-cell line that can be differentiated in vitro, potentially into any type of cell that occurs in the body, to be used in research or for therapeutic purposes.

A principal biologic barrier to the creation of a normal animal through cloning is the epigenetic difference between the chromosomes inherited from the mother (the maternal genome) and those inherited from the father (the paternal genome). For example, parent-specific methylation marks are responsible for the expression of so-called imprinted genes and cause only one copy of such a gene, de-rived from either the sperm or the oocyte, to be active, whereas the other allele is inactive. The mono-allelic expression of imprinted genes is crucial for normal fetal development. When the genomes of the sperm and the oocyte are combined at fertilization, the parent-specific marks established during oogenesis and spermatogenesis persist in the ge-nome of the zygote (see Figure 2). Within hours

Figure 2. Parental Epigenetic Differences in Normal and Cloned Animals.

The genomes of oocyte and sperm are differentially methylated (epigenetically "marked") during gametogenesis and are different in the zygote when they are combined at fertilization. Immediately after fertilization, the paternal genome (derived from the sperm) is actively demethylated, whereas the maternal genome is only partially demethylated during the next few days of cleavage; this is because the oocyte genome, which has a different chromatin configuration from the sperm genome, is resistant to the active demethylation process imposed on the genome of the sperm by the cytoplasm of the oocyte. Thus, the methylation marks of the two parental genomes are different at the end of the cleavage process and in the adult. In cloning, a somatic nucleus is transferred into the enucleated oocyte, and both parental genomes are exposed to the active demethylating activity of the cytoplasm of the oocyte. Therefore, the parent-specific epigenetic differences between the two genomes tend to become erased, causing widespread dysregulation of imprinted genes. Methylated sequences are depicted as solid circles, and unmethylated sequences as open circles.

after fertilization, most of the global methylation marks, except those of imprinted genes, are stripped from the sperm genome; the oocyte genome, however, is in a chromatin state (inaccessible to the reprogramming factors) that renders it resistant to this process of active demethylation.

In contrast, after nuclear transfer, the epigenetic differences established during gametogenesis are subject to erasure, because both parental genomes of the somatic donor cell — not just the sperm genome, as in fertilization — are introduced into the oocyte from the outside and are thus equally exposed to the reprogramming activity of the oocyte cytoplasm. Therefore, in cloned animals, imprinted genes should be particularly vulnerable to inappropriate methylation, which causes abnormal expression — a prediction that has, as noted above, been verified experimentally. For cloning to be made safe, the two parental genomes of a somatic donor cell would need to be physically separated and individu-

ally treated in "oocyte-appropriate" and "sperm-appropriate" ways. At present, it seems that this is the only rational approach to guaranteeing the creation of the epigenetic differences that are normally established during gametogenesis. Such an approach is beyond our present abilities, implying that serious biologic barriers (rather than mere technical problems) hinder faithful reprogramming after nuclear transfer and thus preclude the use of nuclear cloning as a safe reproductive procedure.

The generation of embryonic stem cells by nuclear transplantation for use in therapeutic applications is another story. Embryonic stem cells are derived from the portion of the blastocyst termed the inner cell mass. The cells of the inner cell mass express "key" embryonic genes such as the transcription factor Oct-4. After explantation in culture, these cells extinguish Oct-4 expression and cease to proliferate. Only one or a few of the cells derived from the inner cell mass will eventually express Oct-4
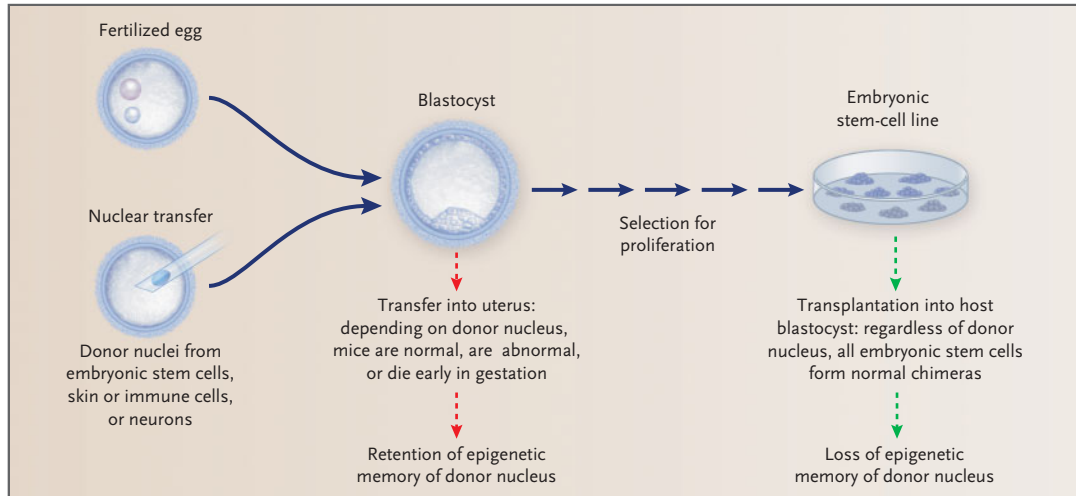
Figure 3. Retention of Epigenetic Memory in Blastocysts and Loss of Epigenetic Memory in Embryonic Stem Cells.

After implantation, the development of the embryo depends strictly on the origin of the donor nucleus: blastocysts derived from a fertilized egg will develop with high efficiency into normal animals; those derived by nuclear transfer from an embryonic stem-cell donor will develop with high efficiency, and those derived by nuclear transfer from skin cells, immune cells, or neurons will develop with low efficiency, into abnormal animals. Thus, the blastocyst retains an epigenetic memory of the donor nucleus, which determines the phenotype of the animal, and gene expression in the blastocyst is influenced by the gene-expression pattern of the donor cell. In contrast, the process of deriving embryonic stem cells erases the epigenetic memory of the donor nucleus. This is because the cells of the inner cell mass are subject to strong selection for proliferation. In vitro selection for proliferation of embryonic stem cells, regardless of origin, to have an identical potential for in vitro differentiation and therapy.

again, and these few cells will resume rapid proliferation, yielding the cell populations that we call embryonic stem cells.[1] Thus, the propagation of blastocyst cells in vitro results in a rare population of surviving cells in which, as a consequence of the selection for proliferation in culture, the "epigenetic memory" of the donor nucleus has been erased (see Figure 3). This process ultimately results in embryonic stem cells that, regardless of the origin of their donor nucleus, have an identical potential for development. It is consistent with this notion that embryonic stem cells resulting from nuclear transfer generate chimeras with a phenotype identical to that of chimeras generated by embryonic stem cells derived from fertilized embryos. Thus, if "fertilized" embryonic stem cells are potentially useful for therapy, so are nuclear-transfer embryonic stem cells. Although careful studies of the safety and normality of differentiated products of these cells remain to be conducted before any clinical applications can be developed, 20 years of experience in generating mouse chimeras with embryonic stem cells has not revealed any obvious defects or tumor-forming potential of these cells.

Why is faulty reprogramming problematic for re-productive cloning but not for therapeutic applications? There are two main reasons for this seeming paradox. One is that a blastocyst retains an epigenetic memory of its donor nucleus, which strictly determines its potential for developing into a normal or abnormal fetus and postnatal animal (see Figure 3). Whereas a fertilized embryo develops normally, any embryo derived by somatic-cell nuclear transfer will be abnormal — although the efficiency with which a given clone develops to birth is strongly influenced by the state of differentiation of the donor cell. When derived from an embryonic donor, cloned embryos develop to birth with relatively high efficiency, but when derived from a somatic donor cell such as a fibroblast or immune cell, most die during gestation.

Second, in contrast to reproductive cloning, the therapeutic use of nuclear transfer does not require the formation of a fetus but relies, instead, on the direct differentiation of functional cells in culture. Because there is no requirement for the development of a fetus, the functionality of the differentiated cells that result from this process would not be expected to be affected by the disturbed imprinting that contributes substantially to the developmental failure

of clones. Because embryonic stem cells derived from fertilized embryos are able to participate in the generation of all normal embryonic tissues, embryonic stem cells generated through nuclear transfer should have a similar potential. And indeed, all the available evidence is consistent with this conclusion.

Whereas reproductive cloning is rejected almost unanimously, the use of embryos generated either by in vitro fertilization or by nuclear cloning for the purpose of generating embryonic stem cells remains controversial. How relevant to the public debate is the difference between the embryo created by in vitro fertilization and the cloned embryo? As discussed above, a cloned human embryo would have little, if any, potential to develop into a healthy human being, for it would lack attributes that are essential to the beginning of normal human life. Indeed, the blastocyst produced by somatic-cell nuclear transfer harbors fundamental biologic deficien-

cies that preclude its ever becoming a healthy human with any acceptable efficiency. Therefore, we may be justified in distinguishing the embryo produced by in vitro fertilization from the product of somatic-cell nuclear transfer. Following a different line of reasoning, McHugh has proposed calling the latter a "clonote" rather than a "zygote" or "embryo."[3] Such a distinction makes biologic sense, is consistent with the available evidence, and may contribute to a more rational discussion of nuclear-transfer technology.

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2. Hochedlinger K, Jaenisch R. Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. N Engl J Med 2003;349:275-86.
3. McHugh PR. Zygote and "clonote" — the ethical use of embryonic stem cells. N Engl J Med 2004;351:209-11.

Altered Nuclear Transfer in Stem-Cell Research — A Flawed Proposal

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The study of human embryonic stem cells is a matter of intense public debate, primarily because derivation of such cells requires the destruction of human blastocysts, a procedure that some find morally objectionable. William Hurlbut, M.D., of Stanford University and a member of the President's Council on Bioethics has recently proposed to the council an alternative way to derive embryonic stem cells that, he argues, circumvents this objection.[1] The chair of the council, Leon Kass, M.D., Ph.D., favors Hurlbut's proposal.[2] We believe that it is flawed.

Hurlbut's proposal is based on the observation that mouse embryos carrying a mutation in the *Cdx2* gene die at the blastocyst stage because they fail to form a trophectoderm (from which the placenta normally develops).[3] These embryos can still give rise to mouse embryonic stem cells, and Hurlbut argues that a human embryo with a similar mutation would lack the capacity to become a human being and would thus represent an ethically uncontroversial

source of human embryonic stem cells. He proposes that embryonic stem cells could be derived by a process he calls altered nuclear transfer, in which a *CDX2* mutation would be introduced in vitro into a human cell that would then be used as a nuclear donor to obtain embryonic stem cells by nuclear transfer.

There are several problems with this approach. First, it is not known whether human *CDX2*-deficient embryos die at the same stage as mice and whether they could be used to derive embryonic stem cells. To answer these questions would require a substantial research effort that would consume time and precious resources that we believe could be put to better use. Moreover, this research would itself require the use of human embryos, and it is therefore unlikely to quell the ethical debate.

Second, in mice, *Cdx2* is required not only for trophectoderm formation but also for the subsequent development of a normal embryo.[3] It is likely that human embryonic stem cells carrying a mutation in *CDX2* will be restricted in their developmental capacity

in ways that are impossible to predict but that will probably limit their usefulness in research and clinical applications. Hurlbut suggests that this problem could be circumvented by inactivating *CDX2* reversibly, perhaps by RNA interference. This adds another layer of complexity and would require further time-consuming experiments. Even if these extra manipulations proved technically feasible, it is not clear that reversible inactivation of *CDX2* is ethically distinct from destroying the embryo by the immunosurgical method that is routinely used to derive human embryonic stem cells.

In addition to these major technical obstacles, we believe that Hurlbut's argument for the ethical superiority of altered nuclear transfer rests on a flawed scientific assumption. He argues, on the basis of supposed insights from systems biology, that it is acceptable to destroy a *CDX2* mutant embryo but not a normal embryo, because the former has "no inherent principle of unity, no coherent drive in the direction of the mature human form." But these are ill-