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Alternative sources of pluripotent stem cells: altered nuclear transfer

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Abstract. Altered nuclear transfer (ANT) is one of several methods that have been suggested for obtaining pluripotent stem cells without destroying human embryos. ANT proposes to alter the nucleus of a somatic cell and/or the cytoplasm of an enucleated oocyte such that when the two are combined, they *do not* produce a zygote, but rather they form a cell capable of producing pluripotent stem cells *without* being an embryo. The ANT proposal raises the serious question of whether it is possible to know with confidence that this procedure generates a non-embryo, rather than merely an embryo with a deficiency. Here I address the question of how embryos can be distinguished from non-embryos using scientific criteria and apply these criteria to the two forms of ANT proposed thus far: ANT combined with oocyte-assisted reprogramming (ANT-OAR) or with gene deletion (ANT-GD). I propose that the first globally coordinated event in human development, the formation of trophoblast and inner cell mass (ICM) lineages via Cdx2-Oct3/4 mutual cross-repression, is the earliest act of the embryo *qua* embryo; it is an operation intrinsic to an embryo as such, and entities lacking the power (*potentia*) for such an act cannot be considered embryos. Thus, I will argue that formation of trophoblast-ICM lineages is a both necessary and sufficient criterion for determining whether ANT produces an embryo or a non-embryonic entity.

INTRODUCTION

Current procedures for isolating human embryonic stem cells vary somewhat in their methodological detail, yet all involve destruction of the embryo. A number of scientific proposals have been put forward to explore possible alternative sources of pluripotent stem cells – sources that do not require the generation and subsequent destruction of human embryos. These proposals include obtaining stem cells from embryos that are clinically dead (Landry & Zucker 2004; Zhang *et al*. 2006b), removing stem cells (blastomere biopsy) without harming the embryo (Chung *et al*. 2006; Klimanskaya *et al*. 2006), creating non-embryonic entities that produce usable stem cells (Hurlbut 2004; Arkes *et al*. 2005; Condic 2005), converting adult cells

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into pluripotent stem cells by a process of epigenetic reprogramming (Cowan *et al*. 2005; Strelchenko *et al*. 2006; Tada & Tada 2006; Takahashi & Yamanaka 2006), and obtaining pluripotent stem cells from non-embryonic tissue (multiple laboratories are currently investigating stem cells from a wide range of postnatal tissues). While none of these approaches has been rigorously proven to be feasible thus far, the feasibility of using pluripotent stem cells derived from embryos to treat medical conditions has also not been rigorously proven for any animal model of human disease or injury. All of the alternative approaches are based on solid scientific premises and all are the subject of active research.

The call for discussion and development of alternative methods of obtaining pluripotent stem cells has been met with considerable support from those concerned about the social, political and ethical impact of destructive human embryo research. In the USA, the President's Council on Bioethics has recommended the proposed alternatives receive further discussion (Kass *et al*. 2005). Religious and political leaders, while not endorsing specific approaches, have seconded the recommendation that alternatives be given thorough consideration.

Support from the scientific community has been somewhat more qualified, with most scientific organizations expressing some degree of concern that efforts to research alternatives to destructive embryo research could delay the development of stem cell-based therapies. Thus, the world's largest professional society of stem cell researchers (the International Society for Stem Cell Research) notes in a published statement entitled 'Alternatives to Human Pluripotent Stem Cell Derivation':

> While it is commendable to seek ways to pursue human embryonic stem cell research without the destruction of embryos ... would it not be more ethical towards the 100 million Americans that could potentially benefit from therapies emerging from stem cell research to step up efforts to bring hESC [human embryonic stem cells] successfully into the clinic, rather than to waste precious time and resources? (available at http://www.isscr.org/press_releases/alternatives_statement.htm)

Despite concerns of some scientists that alternatives may delay advances in development of stem cell-based therapies research, the controversy surrounding destructive embryo research is not likely to evaporate. In a recent poll of attitudes towards stem cell research in the USA, approximately 61% of respondents characterized their position as 'Supporting research using adult stem cells and other alternatives, to see if there is no need to destroy human embryos for research', while only 23% identified themselves as 'Supporting all methods, including those that require destroying human embryos, to see which will be most successful' (survey results available at http://www.usccb.org/comm/archives/2004/04-163.htm). Should there be an alternative method of obtaining stem cells that does not require destroying human embryos and still provides cells that exhibit the properties of embryonic stem cells (i.e. pluripotency), the majority of Americans would clearly welcome such an alternative.

Given the many recent successes in developing therapies based on stem cells derived from non-embryonic tissues (including bone marrow, fat, nasal epithelium, placenta and umbilical cord blood) and the very significant scientific and medical problems associated with using embryonic stem cells for treatment of human disease (Condic 2002, 2007a, 2007b), it is reasonable to ask why should embryonic stem cells (ESCs) or cells with ESC-like properties be studied at all? There are several compelling reasons why pursuing a socially and morally acceptable source of cells with ESC-like properties is a worthwhile endeavour. First, pluripotent stem cells from different sources (and from different species) are not identical, and they may prove to have very different scientific and medical utility. Thus, stem cells from non-embryonic sources or from animal embryos cannot stand in as scientific or medical substitutes for evaluating the biologic

properties and therapeutic potential of human ESCs. If cells with the same properties as human ESCs could be obtained from non-embryonic sources, these properties could be studied without social controversy or moral compromise. Second, human ESCs have unique scientific interest, due to their role in human development, and are worthy of study in their own right. Human development presents questions of profound importance that are unlikely to be addressed by the study of stem cells from non-embryonic sources. Again, a morally permissible way of obtaining cells with the properties of human ESCs would allow this important area of scientific investigation to proceed. Third, basic scientific research does not directly address medical conditions, but rather is attempting to discover new information. Discoveries, by their very nature, cannot be predicted; they emerge from the study of interesting or important processes and advance our understanding of the natural world in unexpected and novel directions. Basic scientific study of ESCs is highly likely to yield important discoveries that have powerful, although indirect medical benefits. The availability of ethically obtained cells with the properties of human ESCs would enable any discoveries with possible medical applications to be translated efficiently to human patients.

Finally, there are significant pragmatic reasons to pursue an ethical source of human ESC-like cells. In the USA, the public has been led to believe that ESCs will provide near miraculous cures for a wide range of devastating medical conditions. Despite very real medical advances in the development of non-embryonic stem cell therapies, these treatments do not currently live up to the unrealistic expectations of 'stem cell cures' the public has been promised from ESCs and are unlikely to live up to such promises in the future. The fact that non-embryonic stem cell therapies are not a medical panacea will fuel an ever increasing outcry that ESCs be 'given a chance' to provide the magical cures the public expects. In the face of strong public demand, barriers against destructive embryo research will fall if ethical alternative sources of pluripotent stem cells are not available. Allowing unrestricted research on human embryos would establish a clear precedent that it is acceptable to use one class of human beings (in this case, humans at an embryonic stage) solely as a means to benefit the interests of another class of persons. Not since slavery has such instrumentalization of human beings been seriously considered as a morally acceptable course of action.

Altered nuclear transfer and somatic cell nuclear transfer

One of the proposals for obtaining pluripotent stem cells without destroying embryos is altered nuclear transfer (ANT). In contrast to several of the proposed alternatives, ANT is a conceptual outline rather than a specific methodological procedure. The ANT proposal is designed to address the following question: is it possible to generate cells with the properties of embryonic stem cells (pluripotent stem cells) from something that is not an embryo? To answer this question, ANT proposes to conduct research, using animal cells exclusively, until it can be determined whether (or not) it is possible to accomplish the goals of ANT and with what degree of certainty and reproducibility. ANT proposes to use a process similar to somatic cell nuclear transfer (SCNT, or cloning) to generate a non-embryonic entity from which stem cells can be obtained. In SCNT, the nucleus of an oocyte is removed to generate an enucleated oocyte or ooplast. A mature body cell (i.e. a somatic cell) is then fused to the ooplast, generating a hybrid cell that contains the genetic information of the somatic cell and (predominantly) the cytoplasm of the oocyte. Following this fusion, the somatic nucleus is 'reprogrammed' from its differentiated state to a state that is able (in rare cases) to support embryonic development.

The concept of nuclear reprogramming requires careful explanation. With few exceptions, all human cells contain a complete human genome, and the specific nature of each cell is due to its epigenetic state, that is, precisely which genes are expressed and which are not, due to

modifications and factors that do not affect the DNA sequence itself. Epigenetic factors include chemical modifications to DNA or to proteins associated with DNA, that can alter gene expression in critical ways. Epigenetic factors also include components of the cell, such as RNA and proteins, that influence which genes are expressed and which are not. In order for a somatic nucleus to participate in embryonic development, it must be 'reprogrammed' to a state that is capable of responding to the normal cascade of developmental events that are both initiated and subsequently directed by such epigenetic factors in the oocyte cytoplasm.

This reprogramming occurs in two major ways. First, reprogramming involves removal of chemical modifications that have been made to the somatic cell's DNA during development. Enzymes in the ooplast remove methyl groups that have been added to the DNA of the somatic cell. The presence of methyl molecules is known to prevent a gene from being expressed, and the attachment of methyl molecules to DNA is part of the normal developmental process that restricts cells to expressing only those genes that are appropriate for a specific differentiated state. Second, reprogramming involves the 'repackaging' of the DNA. Normally, DNA is associated with protein complexes consisting largely of histone molecules, and together the combination of DNA and associated protein forms the macro-molecular structure, chromatin. These histone-containing chromatin complexes restrict which regions of DNA are available for transcription by regulating its three-dimensional organization. There are many varieties of histone, and these proteins can also be modified in a number of ways (acetylation, methylation, phosphorylation and association with further proteins). In reprogramming, the histones proper to a somatic cell are replaced and/or chemically modified by the ooplast, and this alters structural organization of the somatic DNA such that it becomes more amenable to a wide range of gene expression.

Epigenetic reprogramming is neither specific to the ooplast (reprogramming can also occur following fusion of a somatic cell to an ESC or to a teratocarcinoma cell) nor is it a process that rewinds the somatic cell nucleus back through successively more primitive stages, ultimately culminating in the production of a zygote. Rather, the ooplast simply strips the chemical and structural modifications imposed upon DNA by development, thereby conditioning it to a state that is more neutral, or 'receptive' to whichever epigenetic factors it encounters in its new cellular environment. In other words, the reprogramming itself does not directly determine the subsequent developmental pattern the cell produced by SCNT will follow; rather, reprogramming allows the nucleus to respond to molecular factors present in the ooplast that drive zygote-specific patterns of development. If the molecular composition of the ooplast should be altered, the response of the reprogrammed somatic nucleus would also be altered. Similarly, if an ooplast-reprogrammed nucleus were immediately transferred to a different kind of cytoplasmic environment, an enucleated muscle cell for example, it would follow the developmental pattern specified by its new environment, and differentiate as a muscle cell, not as an embryo.

Reprogramming that occurs following SCNT is both uncontrolled and highly variable, thus, the outcomes of SCNT are unpredictable. In rare cases, generally 0.1–1% of all attempts (Paterson 2002), ooplast reprogramming during SCNT creates a sufficiently neutral nucleus that it responds to factors in the ooplast with a zygote-like pattern of gene expression. In these cases, SCNT produces an embryo that is sufficiently normal that it can survive to live birth following implantation into a uterus. In most cases, however, ooplast reprogramming is not sufficient to enable this. Thus, in the vast majority of attempts, SCNT is unsuccessful in this outcome and produces something that does not survive beyond a few days in culture.

While many consider *any* product of SCNT to be an embryo, this assumption is not necessarily valid. The difficulty in interpreting the products of SCNT is that this procedure can produce at least three conceptually distinct entities: a normal embryo (one that is normal enough to survive to live birth), a damaged embryo that does not proceed through entire development due to

defects in its epigenetic state, and a non-embryonic entity that is different from both the somatic cell originally used for SCNT and from a zygote. The criteria by which these three types of outcome can be scientifically distinguished are discussed in detail below.

Similar to SCNT, ANT would also involve the transfer of an adult somatic nucleus to an enucleated oocyte. The key difference between ANT and SCNT is that prior to transfer of the somatic nucleus, either the nucleus or the oocyte cytoplasm or both would be 'altered' in such a way that following fusion, a hybrid cell is generated that categorically cannot be an embryo, yet is capable of generating pluripotent stem cells. This could be accomplished by introducing modifications to the somatic DNA or the ooplast that prevent or circumvent reprogramming, or via modifications that change the nature of the developmental program the ooplast specifies. ANT would thus produce an entity whose composition would be limited by the manipulations performed prior to transfer of the somatic nucleus. ANT is likely to be much more successful in producing stem cells than SCNT is in producing zygotes as the developmental demands on ANT are far less stringent. ANT does not need to produce (indeed, is designed *not* to produce) a cell that is capable of all the complex interactions required for embryonic development, but rather to produce only a cell that generates pluripotent stem cells.

Distinguishing embryos from non-embryonic entities

The central moral and scientific issue with the ANT proposal is: how can we know with confidence that the product of ANT is not an embryo? Put in a slightly different manner, this question can also be stated as: how will the products of ANT be evaluated to determine whether they are embryos, non-embryonic entities, or damaged embryos? To address this question, it is important to establish criteria for robustly distinguishing between embryos and non-embryonic entities.

It has previously been proposed (Condic & Condic 2005) that embryos can be reliably distinguished from non-embryonic entities by examining their pattern of development. Briefly, this proposal argues that we are able to identify what something is by observing both its structure and its functions. Thus, we can identify something as a human being because it is composed of certain characteristic human parts and has the characteristic human act of rationality. For developing human embryos, the actions and structures characteristic of an adult are not yet fully manifest (embryos neither look like mature human beings nor are they capable of reason). However, developing human beings are composed of characteristic human parts (they are derived from human gametes, contain human DNA and other human molecules) and they have characteristic human behaviour (they exhibit a *human* pattern of development). The fundamental feature of this human pattern of development is its organization towards production of a mature human body. Human embryos operate as a whole, with all the cells acting in an orchestrated fashion to generate the structures and relationships required for the body to develop to its mature state. Human cells *in vitro*, in contrast, are composed of human DNA and other human molecules, but they show no global organization. A skin cell isolated from a mature body will divide many times in culture, to produce a large mass of cells, but it will not re-establish the whole organism from which it was removed; it will not regenerate an entire human body in culture.

Defining human embryos by the ability to orchestrate development indicates that all embryos, including those that suffer from a defect, nonetheless exhibit a clear pattern of globally coordinated interactions; that is, they exhibit a 'wholeness' that can be observed even at very early stages of development. Defective embryos may show agenesis of parts or malformation of parts, but these defects occur in the context of a globally coordinated pattern of development. Depending on the precise nature of the defect afflicting an embryo, the developmental consequences may be mild or serious, with defects that are manifest earlier in development often having the most severe effects on subsequent formation of bodily structures. However, even in cases of

severe defect (such as failure to form a head, anencephaly), the embryo nonetheless exhibits a globally coordinated and uniquely human pattern of development. Thus, regardless of which parts of the embryo are malformed or fail to form, if the embryo exhibits global integration of parts to compose the whole, it is manifestly an embryo.

In contrast, non-embryonic entities fail to exhibit global integration of parts, and instead behave much like isolated cells; they act merely as components of an organism, rather than as a whole. Some types of human tumour cell (as in teratomas) have the ability to generate multiple mature phenotypes and even complex tissues such as muscle, teeth or hair, yet such tumours still behave as independent conglomerations and exhibit only a chaotic pattern of uncoordinated development. Such tumours are fully capable of forming all the cell types found in the mature body, yet they are incapable of organizing them along a single, integrated mode of development. Precisely because they lack self-directed, global coordination, such tumours are not defective embryos, they are non-embryonic entities.

Chaotic patterns of cell division and cell differentiation can also arise as a consequence of abnormal fertilization events, for example, fusion between grossly abnormal gametes or fusion of normal gametes in a grossly abnormal manner. Naturally occurring phenomena of this type are medically known as failures of fertilization. This is said to occur when fertilization produces a cell with defects so profound and global that an embryonic pattern of development is precluded; no embryo is produced when the sperm and oocyte have fused, rather a distorted human cell that proceeds to divide in a non-embryonic manner. An example of such a failure of fertilization is the formation of a tumour known as a complete hydatidiform mole. The most common way in which these are produced is when a normal sperm fertilizes an oocyte that has abnormally lost its own genetic material. Such moles grow quite rapidly and simulate a pregnancy (often referred to as molar pregnancy), yet they grow as a mass of disorganized cells and tissues, all of which are unrelated to each other or to anything resembling an embryo. In some cases, such tumour cells are multipotent, and generate foetal as well as placental cell types (Szulman & Surti 1978; Fisher *et al*. 1997; Paradinas *et al*. 1997; Weaver *et al*. 2000). Despite that moles are generated from human gametes in a process that mimics conception, the mole does not exhibit an embryonic pattern of organization and, therefore, it is a tumour, and not a defective or damaged embryo.

Thus, an organized pattern of development defines an embryo. Damaged or defective embryos show agenesis of parts or malformation of parts against a background of global organization. Even when a defect is severe, if global organization is present, an embryo is present. In contrast, non-embryonic entities show mere cellular organization; they are composed of living human cells, but these neither communicate in a coordinated manner nor organize themselves in into an integrated being of any kind. If we maintain that there is a real and substantial difference between a human *somatic cell* (a skin cell, for example) and a one cell human *zygote*, the only observational basis for this difference is the level of tissue organization these two entities exhibit; skin cells display cellular organization, while zygotes initiate and orchestrate a global pattern of integrated development that concludes in the production of the mature body. Although skin cells can be experimentally converted into zygotes by the process of SCNT, this does not mean that all skin cells are actual zygotes. Regardless of how a human zygote is formed (natural fertilization or SCNT), it is defined as a zygote by virtue of its capacity to self-organize the characteristically human pattern of embryonic development.

In evaluating the products of ANT, it will be essential to determine whether global organization is present at any time following transfer of the somatic nucleus to the ooplast. Should a cell produced by ANT show *any* form of developmental coordination (formation of a recognizable tissue or structure, for example) it is critical to determine whether ANT has generated something

that can be considered a defective embryo. The methods and criteria for evaluating this are presented below.

ANT-OAR

One proposed form of ANT (Arkes *et al*. 2005) is ANT in combination with oocyte-assisted reprogramming (ANT-OAR). The goal of ANT-OAR is to generate a cell with properties that are characteristic of an ESC but distinct from those of a zygote. Thus, if ANT-OAR was successful, it would generate a cell that would not develop to produce a stem cell, but would simply *be* a stem cell. Like any somatic cell, the cell produced by ANT-OAR would divide in culture to produce copies of itself. Like ESCs derived from embryos, the ANT-OAR cell could (in theory) be induced to differentiate into all cell types of the mature body.

How would it be possible to determine whether ANT-OAR indeed generates a stem cell and not a zygote? It is important to note that the molecular composition of ESCs has been described with a high degree of resolution. Recent studies have identified thousands of genes characteristically expressed in ESCs compared to other closely related cell types (Brandenberger *et al*. 2004; Boheler & Tarasov 2006; Li *et al*. 2006) and advanced gene profiling promises to provide a complete catalogue of all the genes that are expressed or not expressed in ESCs. Functional analysis of specific genes has determined that only a small handful of them are required to maintain the ESC state (Palmqvist *et al*. 2005; Pritsker *et al*. 2006). Thus, we are increasingly able to both provide a high-resolution molecular description of the characteristic components of an ESC and to define which of these components are required for a cell to exhibit ESC properties. It will be possible to determine the molecular nature of any cell generated by ANT-OAR and to compare it both to a zygote produced by fertilization of an oocyte and to ESCs that have been isolated from embryos. Until perfected, all experiments proposed to test ANT-OAR will be conducted in animal cell models, where efficacy of the procedures will be evaluated and refined prior to any discussion of proceeding to human cells. Moral issues associated with using information obtained from the study of human ESCs are not addressed here. Let it suffice that information regarding gene expression patterns of human ESCs is available in the literature and ANT-OAR would not require independent confirmation of this information.

How would it be possible to induce a somatic cell to change directly into a stem cell, without first generating a zygote? For many cell types, the specific pattern of genes they express is determined by a small number of master controls; that is, transcription factors that bind to DNA and initiate the complex pattern of gene activation and repression characteristic of a particular cell type. Several of these required for ESC function have been described, and it is likely that only a small number of such factors will be found to be sufficient to initiate an ESC-like pattern of gene expression. Indeed, recent work has indicated that as few as four of them may induce a somatic cell to assume an ESC-like state (Takahashi & Yamanaka 2006; Maherlie *et al*. 2007; Okita *et al*. 2007; Wernig *et al*. 2007). Thus, it may prove possible to directly convert a somatic cell into an ESC-like cell by providing these regulatory factors.

However, a significant problem with directly making somatic cells change into pluripotent stem cells by providing ESC-associated transcription factors is that the epigenetic state of the somatic cell (DNA methylation and chromatin structure) precludes full access of the ESC-associated factors to the DNA. Thus, attempts to directly reprogram somatic cells are often unsuccessful, or only partially successful, as the epigenetic state of the somatic cell DNA is non-responsive to the factors that have been experimentally introduced. ANT-OAR proposes to use the reprogramming capability of the ooplast to circumvent this non-responsive nature of somatic cell DNA. First, the somatic nucleus needs to be altered to express ESC-associated transcription factors at high levels. This manipulation could also be performed in combination

with manipulation of the ooplast, so that these same ESC-associated factors would be present in the ooplast at high concentrations prior to the fusion. As a further safeguard against possible generation of a zygote following fusion, factors that are associated with (or required for) the zygote could be eliminated from the ooplast prior to fusion (ANT-GD, below). Thus, when the somatic cell and the ooplast fuse, the somatic nucleus would be reprogrammed by the ooplast, and would respond to the ESC-associated transcription factors present in the newly formed cell. The new cell with its reprogrammed nucleus would immediately and only act in ways characteristic of an ESC. Moreover, as an ESC-like cell, the ANT-OAR product would divide in culture to generate more cells with the same ESC-like properties.

Based on the organismal criteria outlined above, the product of ANT-OAR would be a non-embryo; it would result in a cell with properties and behaviour of an ESC. The ANT-OAR cell would not be predicted to undergo any form of development or to generate cells that differed from itself, so long as it was maintained in culture under conditions that promote proliferation of ESCs, and not their differentiation into more mature cell types. If the ANT-OAR cell *did* undergo some form of development (its progeny were different in molecular composition or behaviour from the original cell produced following fusion with the ooplast), the precise nature of this development would need to be carefully examined. Specifically, it would be essential to determine whether the progeny of the ANT-OAR cell exhibited the random, chaotic interactions characteristic of tumours (indeed, characteristic of ESCs themselves, which robustly produce teratomas) or whether it proceeded along an organized pattern of development characteristic of a zygote. Importantly, it will be possible to determine the molecular composition of the ANT-OAR cell and its progeny with great precision and to compare this composition to that of a zygote, to the immediate progeny of a zygote and to bona fide ESCs.

ANT-GD

A second form of ANT that has been proposed (Hurlbut 2004; Condic *et al*. 2005) is ANT in conjunction with gene deletion (ANT-GD). This form has the same goal as ANT-OAR: to alter the nucleus of a somatic cell and/or the cytoplasm of an ooplast such that once they are fused, no zygote is formed and yet the cell produced is capable of generating pluripotent stem cells. As discussed above, ANT-OAR proposes to accomplish this goal by producing a cell with positive characteristics that are different from a zygote and uniquely associated with ESCs. In contrast, ANT-GD proposes to accomplish the goal by removing factors from the somatic cell or ooplast that are critical for the formation of a zygote, and yet not required for the production of ESCs. The two proposed forms of ANT are not mutually exclusive, and could be used in combination, expression of ESC-associated factors together with deletion of zygote-associated factors. However, ANT-GD raises unique concerns that need to be addressed.

It is important to consider whether removal of required factors (ANT-GD) is conceptually and morally synonymous with addition of distinguishing factors (ANT-OAR). In the latter case, a cell is produced with properties that are not observed in a zygote but, rather, are uniquely associated with a pluripotent stem cell. In contrast, when genes are removed, one is left with the questions of how many must be removed before an embryo no longer exists, and is removal of a single gene sufficient to fundamentally alter the nature of the zygote? If so, what is the nature of the cell produced following this deletion? More importantly, how can one be confident that what remains is not a damaged or disabled embryo? In addressing such questions, it is important to consider the difference between structures and functions that are incidentally associated with a particular type of cell and those that are *characteristic* of a particular cell type. Zygotes, for example, are very large cells compared to ESCs, yet if ANT-OAR were merely to alter cell size so that a cell with characteristically ESC-like dimensions was generated, this positive effect

would not be sufficient to determine that the cell produced was indeed an ESC and not merely a very small zygote. What is key to the ANT-OAR proposal is that it should not target properties, such as cell size, that are incidental but rather properties that critically *define the nature* of the cell, factors that both promote the ESC-like state and simultaneously preclude a zygote-like state.

Gene deletions used for ANT-GD must be designed to preclude formation of a zygote and must target characteristics that are essential to the zygote. In selecting them, the nature of each gene must be considered carefully; its expression must be critical to the essential function of the zygote itself. Fundamental to ANT-GD is the assertion that if a cell cannot perform the essential acts of a zygote, it is not a zygote. The essential function is global, organized development towards formation of the mature body; this is the precise trait that distinguishes a zygote from a mature, differentiated cell. If the cell formed by ANT-GD specifically lacks the capacity for organismal function, it is not a zygote, but rather a different type of cell.

While a relatively large number of genes could be targeted for deletion in ANT-GD, one proposed target is the gene that codes for the transcription factor Cdx2. This gene is critically required for formation of one of the two earliest cell types of the embryo, that of the trophoblast. Trophoblast cells go on to produce the tissues of the amnion, chorion and placenta, while making minor contributions to the postnatal body of the developing embryo. Deletion of *Cdx2* (Chawengsaksophak *et al*. 2004; Strumpf *et al*. 2005) results in failure of trophoblast formation and no implantation of the embryo in the uterus (no clinical pregnancy is established), while over-expression induces trophoblast differentiation (Tolkunova *et al*. 2006). While it is clear that Cdx2 plays a crucial role in the formation of the trophoblast, its importance for organismal function of the zygote as a whole has only recently been appreciated. During mammalian development, each cell at the two-cell stage appears to have a distinct developmental bias, with one cell contributing primarily to the trophoblast and the other contributing primarily to the ICM (Zernicka-Goetz 2002; Gardner & Davies 2003; Stanton *et al*. 2003; Rossant & Tam 2004; Plusa *et al*. 2005). Although this bias can be detected at the two-cell stage, it is not entirely clear when cells become specified to enter into distinct developmental paths (are cells actually different from each other at the two cell stage, or do differences arise later?) and what genes regulate this decision. Recent studies suggest that it is made very early, and is likely to require expression of *Cdx2*. When somatic cells from mice are engineered to lack *Cdx2* and then are transferred to an ooplast using ANT-GD, the resulting entitles do not develop, but rather generate clusters of cells that have the properties of ESCs (Meissner & Jaenisch 2006). These findings strongly indicate that *Cdx2* is required for the self-organization that defines a zygote.

Our understanding of the molecular mechanisms underlying early mammalian development strongly supports the conclusion that ANT-GD using *Cdx2* produces an ESC-like cell and not a zygote. Recent work has shown that the *Cdx2* protein product can interact with that of *Oct3/4* to regulate formation of the trophoblast and ICM (Niwa *et al*. 2005; Tolkunova *et al*. 2006; Zhang *et al*. 2006a). Cdx2 is principally required for trophoblast formation and Oct3/4 for ICM formation and for maintenance of a pluripotent stem cell state. Both of these are expressed in the early embryo prior to formation of trophoblast and ICM, and interactions between them direct cells into either trophoblast or ICM lineage. How might Cdx2 and Oct3/4 interact for this mutually exclusive purpose? Transcription factors that regulate specific cellular states often have dual functions: to promote patterns of gene expression that are characteristic of the phenotype and to repress expression of genes that are not associated with it. Thus, factors such as Cdx2 and Oct3/4 that promote the formation of contrasting cell types often regulate each other so that only one factor can be functional in any one cell at a specific time (Fig. 1a). Cdx2 and Oct3/4 proteins bind to their own genes and actively promote their own expression, such that cells expressing either of these two factors will subsequently produce more of them due to the

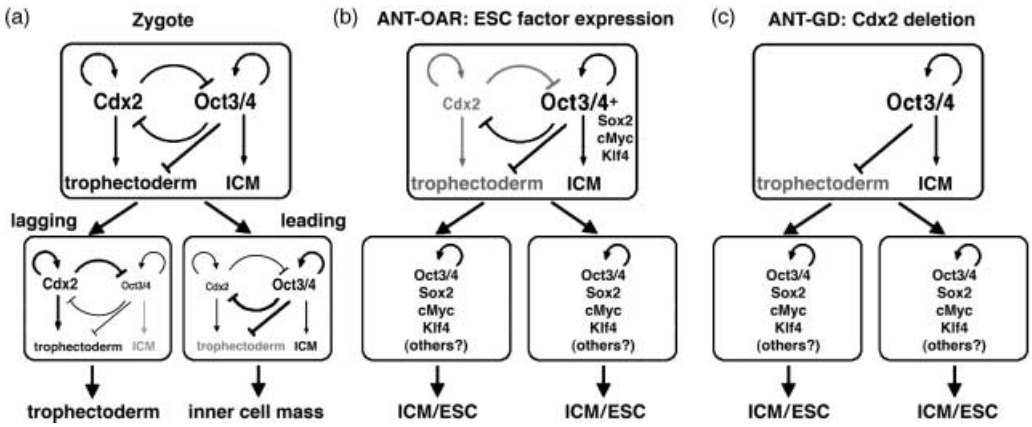

Figure 1. A schematic representation of normal development and possible ANT manipulations. (a) At the two cell stage, the blastomere that divides first (the leading blastomere) contributes predominantly to the ICM, while the blastomere that divides second (the lagging blastomere) predominantly generates trophectoderm (Zernicka-Goetz, 2002; Gardner and Davies, 2003; Stanton *et al.*, 2003; Rossant and Tam, 2004; Plusa *et al.*, 2005). By the eight-cell stage, Cdx2 and Oct3/4 mutual cross-repression establishes a clear molecular difference between these two lineages. (b) In ANT-OAR, over expression of factors that are required for ICM formation (for example, Oct3/4) and/or of later acting factors that are specific to Oct3/4 expressing ICM/ESCs [for example, Sox2, cMyc and K1f4; (Takahashi and Yamanaka, 2006; Maherali *et at.*, 2007; Okita *et al.*, 2007; Wernig *et al.*, 2007)], produces a cell that is not capable of the essential function of the zygote. The ANT-OAR cell immediately exhibits the properties of a pluripotent stem cell derived from the ICM (*cf.* the leading cell in Fig. 1a) (c) in ANT-GD, deletion of factors that are required for the essential function of the zygote (for example, Cdx2) accomplishes the same goals as overexpressing ESC-associated factors; the cell produced by Cdx2 deletion immediately has the properties of the ICM/ESC lineage (*cf.* the leading cell in Fig. 1a), and is not capable of the coordinated interactions that are essential to a zygote. Panel 1a adapted from (Niwa *et al.*, 2005).

positive feedback loop. Conversely, Cdx2 and Oct3/4 both repress the expression of the opposite factor such, that cells expressing Cdx2 will rapidly shut off expression of Oct3/4, and vice versa. This kind of interaction between two opposing genes is known as mutual cross-repression. Based on this, it appears that by the eight-cell stage (and possibly earlier), Cdx2 has effectively shut off expression of Oct3/4 in the cells that will go on to produce trophoblast and in those that will go on to produce the ICM, Oct3/4 has shut off expression of Cdx2. Independent lines of evidence support the conclusion that there are differences in developmental fate between blastomeres at the two-cell stage (Gardner 2001; Piotrowska *et al*. 2001; Fujimori *et al*. 2003; Piotrowska-Nitsche *et al*. 2005). At the four-cell stage, there are differences in epigenetic state (specifically histone H3 methylation) between blastomeres with different developmental fates (Torres-Padilla *et al*. 2007), although the mechanism responsible for establishing these differences and whether it depends on Cdx2–Oct3/4 interactions is as yet unknown. The precise timing of when these interactions occur is not clear, and indeed, it is likely to vary somewhat from individual to individual, due to the highly regulative nature of mammalian development. However, the precise timing does not alter the significance of these interactions for distinguishing embryos from non-embryonic entities. Mutual cross-repression between Cdx2 and Oct3/4 has the characteristics expected of an organism; it is a coordinated action that establishes two distinct, interacting cell types. These subsequently continue to communicate in an integrated manner to ultimately generate the mature structures of the body. Thus, the first global act of the embryo appears to be coordinated establishment of ICM and trophoblast precursors by a mechanism that is likely to involve mutual cross-repression of Cdx2 and Oct3/4 (Fig. 1A).

Based on these observations, elimination of the *Cdx2* gene appears to eliminate a critical intrinsic power (*potentia*) characteristic of a zygote: the ability to establish the first globally coordinated act of embryogenesis. Without this, the cell is not a zygote. Moreover, deletion of *Cdx2* appears to produce the same effect as direct induction of ESC-like cells using ANT-OAR. If factors such as Oct3/4 that promote the ESC-like state are over-expressed in ANT-OAR, the cell is driven into an ICM-like lineage (Fig. 1b). The ANT-OAR cell has properties that distinguish it from a zygote and these properties preclude the cell from functioning as a zygote. Similarly, without Cdx2, the Oct3/4 present in the ooplast appears to directly induce and maintain an ESC-like state, while simultaneously precluding the globally coordinated interactions required for embryogenesis (Fig. 1c). Thus, differentiation of cells destined to become trophoblast or to become ICM, via Cdx2-Oct3/4 mutual cross-repression, is the first globally coordinated act of the embryo. Any entity derived from ANT that possesses this power is an embryo, regardless of any defects that may affect subsequent development. Conversely, any entity produced by ANT that is unable to organize the first global interaction of embryogenesis lacks an *essential power* of a zygote, and is therefore not an embryo. This hypothesis can be empirically tested to determine whether manipulations of Cdx2 and/or Oct3/4 eliminate the globally coordinated interactions that characterize an embryo and critically distinguish an embryo from a non-embryonic entity.

Defining life

One of the difficult features of the ANT proposal is the concern that by attempting to experimentally determine the necessary conditions for the existence of a human embryo, science is attempting to reduce life to mere mechanism. Thus, if deleting a specific gene precludes an embryo from coming into existence following fertilization or transfer of a somatic nucleus to an ooplast, does this gene thereby embody what 'being human' is, substantially? Similarly, if manipulating a somatic nucleus to express a handful of ESC-associated factors can prevent formation of an embryo, does the absence of these factors constitute the substance of an embryo? Is science attempting to reduce the mystery of human life to a single gene or set of genes, the presence or absence of which constitute human nature, in its entirety?

In the light of these concerns, it is important to remember that regardless of any spiritual considerations, humans are naturally subject to material conditions. Babies do not spontaneously spring forth from breadboxes or hatch from chicken eggs, precisely because breadboxes and chicken eggs lack the necessary material conditions to support a human life. In more philosophical terms, not all matter is capable of being formed by a human soul. The matter that composes the human body must be of a certain, specific type in order for the body to be both alive and human. A human soul cannot inform a statue, because rock lacks the necessary material disposition to be animated by a human soul. Nor is simply being 'organic matter' or 'matter of a human' sufficient to support the life of a human. A human soul can no more animate the body of a tree or a dog or a human skin cell than it can a statue, because the matter of trees and dogs and skin cells lacks the structures required for the operation of those powers proper to the human soul. Matter must be properly disposed (be in proximate potency) to receive a form, and the form 'human being' is no exception. It is this precise requirement of proper material disposition that ANT seeks to understand and utilize. ANT does not attempt to *reduce* life to these material conditions but rather to *define* (at least some of) the conditions that are required for human life.

Just as we know that oxygen is required for life, and yet life does not reduce to the presence or absence of oxygen, ANT is attempting to determine what molecular constituents of a cell are required for organismal life, without making any claims that such molecules therefore constitute life. Just as we know with confidence that altering our atmosphere by removal of oxygen or addition of cyanide will rapidly preclude the continued existence of human life, we can know with

confidence that removal or addition of specific genes prior to the fusion of an ooplast and a somatic cell will pre-empt life from coming into existence. Such knowledge does not constitute a reductionist definition of human life but merely establishes some of the material conditions, at the molecular level, that are *necessary* for a living human cell to be a living human embryo.

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