Embryos and Integration

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Summary
Our understanding of when human life both begins and ends depends critically on the distinction between a living human being and living human cells. Human beings are multicellular organisms that autonomously integrate the biological activities required for continued health and survival of the organism as a whole. While aggregates of cells can have complex properties, they do not show such global integration of function. All living organisms are capable of integration, but the mechanism by which it is accomplished depends on both the nature of the organism itself, on the complexity of the biological niche it has evolved to occupy and on the stage of development the organism has attained. The distinction between integration (an intrinsic organismal function) and coordination (a function that is observed in both organisms and aggregates of cells) discriminates between human beings and human cells at all stages of life.

Keywords: Organism, determination of death, beginning of life, integration

Integration versus coordination

A distinguishing feature of an organism is the capacity for integration, in contrast to mere coordination.[1] In a biological system, integration is defined as[2] the ability to compile information from diverse sources and generate a response that 1) is multifaceted, 2) is context dependent and 3) promotes the continued health and function of the body as a whole. Integration is a global response, and during postnatal stages of human life, it is uniquely accomplished by the brain (Figure 1A).

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[1]See Condic, M.L. (2016). Determination of Death: A scientific perspective on biological integration**. Journal of Medicine and Philosophy**. *(in press)*

[2]Merriam-Webster (http://www.merriam-webster.com/; Accessed 11/26/14) defines 'integrate' as, "to combine two or more things to form or create something; to form, coordinate or blend into a functioning, unified whole," with a synonym being "unite." In contrast, 'coordinate' is defined as, "to bring into a common action, movement, or condition; to act or work together properly and well," with a synonym being "harmonize." Thus integration combines two or more elements to result in a single, unified whole, whereas coordination simply involves communication of parts in order to achieve an effective outcome.

In contrast, living cells and cell aggregates are only capable of coordinated activity. Coordination is defined as the ability to bring cells, tissues or organs into a common action or condition in response to a signaling molecule that is released by a specific stimulus. Coordination can reflect either 1) a single type of response that occurs simultaneously in multiple cells or 2) a set of synchronous, but cell-type specific responses (Figure 1B).

Although integrated and coordinated functions can appear similar in many ways, they are distinguished by two important features. First, integration requires the compilation of multiple sources of information. When a process can be fully explained by a single molecular signal, it is not an integrated process, regardless of the number or complexity of the downstream events that this signal may initiate. Second, integration results in a multifaceted response, with different components of the system being regulated appropriately to reflect the function of the system as a whole. This differential regulation is not simply a matter of different responding cells having different intrinsic properties, and therefore different responses to a common signal; if multiple cell types respond in different ways to the same signal, this is a coordinated, but not an integrated response (Figure 1B). Rather, integration requires different responding tissues to be regulated differently; some activated, some inhibited, and perhaps to different extents, depending on context (Figure 1A).

Integration in non-human organisms

To appreciate the critical role of integration for all living things and the distinction between integration and coordination, it is instructive to compare the requirements for organismal function across the major divisions of living entities that occupy diverse environmental niches; i.e. microbes (bacteria, archaebacteria and protists), plants/fungi and animals (in particular, humans; Table 1). The four types of integration (cellular, signal-mediated, structural and organ-based) are discussed in greater detail below.

Table 1: Integration and Coordination in diverse types of living organisms.

Entity	Cell signaling (coordination)	Cellular integration	Signal-mediated integration	Structural integration	Integrating organ
Microbes	Yes	Yes	n/a	n/a	n/a
Plants and Fungi	Yes	Yes	Early development	Yes	No
Human-embryo	Yes	Yes	Early development	Early development	Placenta/brain
Human-postnatal	Yes	Yes	No	No	Brain
Human-brain death	Yes	Yes	No	No	No

Cellular integration: the capacity of individual cells to integrate information and construct a unified response that maintains the life and health of the cell as a whole. **Cell-signaling:** communication between cells that depends on diffusible or cell-surface signaling molecules. **Cell-contact dependent integration:** the capacity to integrate information from the body as a whole, based on cell-cell contacts and short range signaling between cells; requires the entire organism to have very small physical dimensions. **Structural integration:** the production and maintenance of cells with specific properties in an ordered manner such that the resulting structures autonomously support the continued life and health of the organism as a whole, based on cellular integration and the ordered relationships between cells of different types. **Integrating organ:** a required organ/structure that integrates information from the entire body to craft a response that promotes the health and life of the body.

Cellular Integration: To remain alive, all organisms must be capable of *integration*; i.e. they must maintain a balanced response to changing environmental conditions and to differing intracellular states. For single-cell organisms, the "organ" of integration is the cell itself. All unicellular organisms integrate information from the outside world via molecules and structures present at the cell surface. This information is relayed to diverse organelles and molecular structures throughout the cell. The balance of molecular signaling a cell experiences in response to changing environmental stimuli or to differing intracellular states results in an *integrated* response; i.e. graded changes in the internal processes of the cell that are appropriate to the context and that promote the continued life and health of the cell as a whole. Such "cellular integration" (Table 1) based on diffusion of molecular signals within the cell is possible due to the very small dimensions of cells and the efficient mechanisms for targeting those signals to appropriate intracellular structures. Cellular integration seen in all living organisms, including multicellular organisms such as plants and animals (see below).

Cell signaling: Unicellular organisms are also capable of cell-cell communication that results in coordinated behavior among cells of the same type. For example, chemical signals released by food or by other protists can result in "chemotaxis" or migration of cells towards the source of the signal. Thus, even microbes communicate with each other in ways that result in groups of cells responding in the same manner to a specific molecular signal. Importantly, although cell-cell signaling can be the basis for integration in multicellular animals (see below) for unicellular organisms, this kind of communication can only mediate *coordination*. Many amoebae converging at a food source may does not mean the collected amoebae constitute a single living being. They are independent organisms responding independently to a specific signal. Just as a flock of birds or a school of fish can independently respond to the same environmental signals and thereby appear to be acting as a unified whole, a collection of cells independently responding to a common molecular signal can also appear to be a "whole." Yet in all such cases, coordinated behavior does not constitute the integration characteristic of a single living organism because it serves no higher level of function beyond the function of the individual cells themselves.

In contrast to microbes, *multicellular* organisms require more complex forms of integration that incorporate the intrinsic capabilities of different cell types and knit these cellular responses into a unified whole. There are three types of integration in multicellular organisms; cell-contact based integration, structural integration and organ-mediated integration. Both plants and animals use the first two, whereas organ-mediated integration is unique to the animal kingdom.

Structural integration in plants and fungi: Plants, like all multicellular organisms, establish the structures required for global integration during embryonic development. As a plant matures it sequentially generates cells and structures (leaves, roots, vasculature, flower buds) in an ordered manner so that the health and function of the organism as a whole is maintained. Although the developmental mechanisms used by plants differ somewhat from those used by animals (see below), the result of development is the same: production of cells with specific properties that are appropriately ordered into structures and systems that are able to maintain the life of the organism (i.e. integrated, or globally unified structures).

Because plants are much simpler than animals, with far fewer specialized structures and far fewer constraints on the precise spatial relationships between these structures, the developmental

processes used by plants are also relatively simple. Thus, molecular mechanisms exist to insure that leaves are formed only on the sunlight-facing side of a plant, yet whether a particular shoot has leaves or a flower bud is not a matter of great consequence for the function of the organism as a whole, and is therefore not tightly regulated; e.g. two plants of the same species are structurally *similar*, but do not have identical patterns of branching and/or flower placement.

Likewise, because plants are capable of detecting only a limited number of environmental stimuli (light, gravity, heat, water and some molecular signaling agents) and can respond to environmental changes in only a limited number of ways, the ongoing integration required to maintain the health of the organism is also relatively simple. The primary "adaptive" response of plants to changing circumstances is differential growth or cell division. For example, roots will extend towards a source of water and shoots will extend towards a source of light. These responses do not require sophisticated systems for detecting subtle changes in the physiology of the plant as a whole or in the environment. Rather, the integrated function of the plant is accomplished by virtue of fact that cells with different properties (roots versus shoots) are generated in appropriate general locations within the body and are able to initiate cell division in response to specific signals.

Thus for plants, integration of the organism as a whole is largely accomplished during development, by production of correctly ordered structures (Table 1, "Structural integration"). In the mature state, a small number of chemical signals that are carried through the vascular system of the plant (e.g. auxins, cytokinins, gibberellins and abscisic acid) or through the atmosphere (ethylene) regulate both cell metabolism and a differential growth response to promote the life and health of the plant as a whole. This kind of "structural integration" only accommodates a limited range of variation in environmental conditions; i.e. plants do not respond adaptively to predators or hurricanes[3] because of their limited capacity for detecting and responding to changes in the outside world.

Importantly, reliance on structural integration also greatly restricts the environments plants are able to accommodate. Mangoes cannot survive in Minneapolis or saguaros in Savannah. Plants require specific environmental conditions, and (in most cases) will tolerate only a narrow range of variation in moisture, temperature and ambient light. The limited adaptability of plants reflects the limits of structural integration; once a structure has been assembled to accommodate a particular set of environmental conditions it cannot be reconfigured to accommodate a new set of conditions without disassembling what was built initially.

Integration in animals: In contrast to plants, animals are able to detect a much wider range of environmental information and react in diverse ways. Moreover, such diverse responses also produce very different metabolic states for the organism as a whole; e.g. running from a predator versus falling asleep on a sunny rock will place very different demands on the physiology of the same organism. Because animals must balance complex and rapidly changing sensory information with widely varying metabolic function of diverse body systems (respiratory,

[3] The evolutionary adaptation of plants to predators or environmental conditions is distinct from the ability of a specific plant to respond adaptively to life-threatening circumstances.

circulatory, hormonal, digestive, muscular, etc.), they require a means of integration that is far more complex and responsive than the integration seen in plants. As discussed in detail elsewhere[4], at postnatal stages of life, the nervous system is uniquely required to gather information from the diverse body systems as well as from the environment and to compile this information into a unified representation of the situation as a whole, so as to craft an integrated response that promotes the continued life and health of the animal as a whole.

Yet, if integration is required for a human to be a living organism and integration at postnatal stages critically requires a functioning nervous system, how are we to view the human embryo prior to formation of the brain? There is clear scientific evidence that mammalian embryos function as organisms from the moment of sperm-egg fusion onward[5], yet what is responsible for the global integration of function observed in the embryo prior to formation of the nervous system? Put in another way, how is a human embryo different from a mere collection of human cells?

Aristotelian view of the human soul

Aristotle held that the substantial form or soul is the organizing principle of the body. For an organizing principle to be present in the body, there must be a suitable structure or organ through which the soul can function; i.e. "Plainly those principles whose activity is bodily cannot exist without a body, e.g. walking cannot exist without feet."[6] In postnatal stages of life, the brain is required for both the rational functions of the human organizing principle and for organismal integration of bodily functions. Yet in considering the early stages of human development, this raises an important question: What are the earliest organic structures required for operation of the human organizing principle?

As discussed in detail elsewhere,[7] Aristotle does not require the human body or the human brain to be fully mature in order for a human organizing principle to be present, but only that the embryo must have a "principle of movement and of generation,"[8] that is directed towards production of the mature human form. Moreover, the embryonic structures required for this

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[4] See Condic, M.L. (2016). Determination of Death: A scientific perspective on biological integration**. Journal of Medicine and Philosophy**. *(in press)*

[5] Reviewed in: Condic, M.L. (2014). When does human life begin? The scientific evidence and terminology revisited. Journal of Law and Public Policy. Vol. 8 No. 1: 44-81; 18. Condic, M.L. (2008). When does human life begin? A scientific perspective. Westchester Institute White Paper 1, 1-18. Westchester Institute for Ethics & the Human Person, Thornwood, NY. (available at: http://www.bdfund.org/whitepapers). [Reprinted in: Natl Cathol Bioeth Quart. 9, 127-208.]

[6]Revised Oxford Translation, Jonathan Barnes, ed., The Complete Works of Aristotle: The Revised Oxford Translation (Princeton: Princeton University Press, 1984). Generatione animalium, ii,3, 736b22–25.

[7] Condic, M.L. and Flannery, K. (2014). A contemporary Aristotelian Embryology. Nova et Vetera 12(2): 495-508.

[8] GA, ii,6, 742a27-32

"principle of movement and of generation" must necessarily exist prior to the actual formation of mature structures, since, "all the organic parts whose nature is to bring others into being must always themselves exist before them."[9] Thus, something that is present at the very beginning of embryonic life must be responsible for the integrated, organismal function of the embryo and produce the "movement" of development that ultimately results in the formation of mature structures, including the brain. But what is it, precisely, that accomplishes this global integration in the early human embryo?

I propose that prior to the earliest stages of brain development (approximately the first four weeks of human life), a number of key structures/functions are responsible for global integration (i.e. organismal function), including a complex developmental program, cell-cell communication, diffusible signaling molecules and developmental cell movements. Together, these elements produce a unified (i.e. integrated) developmental sequence that autonomously generates the tissues, structures and organs required for the continued life and health of the embryo as a whole. Surprisingly, in later embryos and fetuses, even after the nervous system has formed and begun to function, the brain is not a required organ for integration. Rather, the vital functions uniquely accomplished by the brain in postnatal life are accomplished prenatally by the placenta.

Integration in early embryogenesis

The developmental program

At the very beginning of human life, the one-cell embryo, or zygote, possesses a complex molecular program that produces and directs subsequent generation of more mature human cells, organs and structures. This program consists of three, interacting elements. First, the zygote has a large number of specific molecules (transcription factors, enzymes, DNA binding proteins, microRNAs, etc.), many of which are provided to the embryo by the egg at sperm-egg fusion. These factors are critical components of totipotency, or the ability of the embryo to both produce and to organize all of the structures of the mature body through an orderly process of development.[10]

Second, the early embryo has uniquely modified DNA and associated proteins (i.e., a specific epigenetic state) that is not found in other cell types. This state is in part contributed by the DNA of the sperm and the egg, and in part actively produced in the first hours and days following sperm-egg fusion.

Finally, the DNA derived from sperm and egg carries a particular pattern of maternal and paternal "imprinting" (i.e., chemical alterations to a limited number of specific genes that regulate how they are used). During formation of the gametes, genes are imprinted in either a male or a female pattern, such that when sperm and egg fuse, the zygote has an equal balance of

[9] GA ii,6,742b3-6

[10] Condic, M.L. (2014). Totipotency: What it is and what it is not. Stem Cells and Development 23, 796-812.

both patterns. This state is largely retained into adulthood and allows for normal function of all tissues. When imprinting is not equally balanced between a male and female pattern (for example, when an egg containing only female-imprinted DNA is stimulated to begin cell division or when a zygote abnormally loses the maternally-derived pro-nucleus, retaining only paternally-derived DNA), normal development does not occur.

These three components of the developmental program (required cytoplasmic factors, correct epigenetic state and correctly imprinted DNA) work together in complex ways to generate all of the mature systems required for life, including the nervous system. Consequently, the unique molecular configuration that constitutes the developmental program of the zygote is the physical structure Aristotle requires for the presence of a human organizing principle or soul.[11]

The critical nature of this developmental program can be better appreciated by considering how somatic cell nuclear transfer (SCNT or cloning) produces a living organism. In SCNT, a normal adult body cell is transferred to an egg cell that has had its own genetic material removed. Because the adult body cell largely retains a balanced pattern of maternal and paternal imprinting, it brings with it this critical aspect of the program that is required for totipotency. Following transfer, the egg-derived factors that normally establish an epigenetic state appropriate to a zygote are, in rare cases (typically one out of several hundred attempts), able to reconfigure the adult DNA to the epigenetic state of a zygote (the second component of the program), and thus make the DNA competent to drive subsequent development of the embryo. If reprogramming is successful, the transferred nucleus is then able to work with the cytoplasmic factors of the egg (the third component of the program) to produce an orderly developmental sequence. In the cloning process, the factors that are normally supplied by the sperm to initiate development must be artificially replicated by the experimenter, and in most cases, development does not proceed normally, but the fact that it proceeds at all indicates that the three major elements of the developmental program have been artificially constituted by the cloning procedure.

Yet <u>how</u> does this molecular program produce an integrated, organismal pattern of cell behavior? At postnatal stages, the nervous system is uniquely suited to collect global information from the body as a whole and subsequently craft an integrated response. How is it possible for the same kind of integration to occur in the absence of the nervous system? Cytoplasmic factors and correctly configured DNA are not intrinsically capable of gathering information from diverse sources and crafting a response that is appropriate to the organism as a whole. Yet clearly, human development proceeds in just such a globally integrated manner. Unlike plants, individual members of the human species have remarkably similar anatomy. Therefore random events cannot be responsible for the consistent placement of specific body structures and organs. How does the developing embryo determine the relative position of various body parts so as to correctly place the eyes in the head and the heart in the chest?

I propose that for animal embryos, in addition to the developmental program that drives the production of specific cell types in a specific sequence (just as it does in plants), several elements

[11] Condic, M.L. and Flannery, K. *op cit*.

that are uniquely dependent on the small dimensions of the early embryo enable global integration to occur in the absence of a functioning nervous system. These elements are: contact-mediated communication, gradients of signaling molecules and cell movements.

Contact-mediated communication

Although human cells are rightly considered parts of the human body at all stages of life, individual cells are also semi-autonomous living units that are capable of an impressive degree of intrinsic integration to maintain the life and health of the cell as a singular living entity. When cells are removed from the body and placed in laboratory culture, they continue to function in an organismal, integrated manner, independent of the body as a whole. Similar to free-living microbes, human cells possess complex systems for detecting diverse environmental information and are able to craft an integrated response that reflects their overall cellular status. The specific nature of the response depends on the nature of the cell that in turn, depends on the precise molecular state the cell possesses.

At early stages of development (Figure 2), the specific nature of each of the cells comprising the human body is controlled by the developmental program that drives embryonic development. Moreover, up through the early blastocyst stage, all cells of the embryo are in direct contact with each other, and can communicate via cell surface proteins and signaling molecules that diffuse over short distances. Consequently, the molecular program inherited by early cells derived from the zygote (i.e. the early "blastomeres") determines how each cell will respond to specific signals from its neighbors. By controlling the nature of the cells, the developmental program effectively determines both the nature of the signals and the nature of the response—while the proximity of the cells to each other enables global communication of cells within the body as a whole. Thus the program results not merely in production of different types of cells in a particular sequence (as it does in the development of plants), it also allows for body-wide *integrated* communication among cells that results in appropriate developmental behavior of the whole organism.

Substantial data from mammalian species[12] indicates that cells begin to become different from each other in a predictable pattern as early as the four-cell stage, and that communication between cells at the 8-16 cell stage establishes the first two definitive cell types of the embryo; trophectoderm (TE) and inner cell mass (ICM) (Figure 2A). Importantly, these early events depend on the molecular program of the embryo being played out in each of the early blastomeres and on cell-cell communication between blastomeres that are directly in contact with one another. Thus, global integration up to the early blastocyst stage (approximately 32 cells, and three days of development in humans) depends on both the ordered production of cells under the control of the developmental program and on the intimate forms of cell communication made possible by the very small dimensions of the embryo.

Importantly, the program directing early development does not merely produce *coordinated* activity among cells of the embryo, but rather is responsible for true organismal integration.

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[12] Reviewed in: Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. Zernicka-Goetz M, Morris SA, Bruce AW. Nat Rev Genet. 2009 Jul;10(7):467-77.

This is evident based on the fact that the embryo responds in an adaptive and context-dependent manner to altered circumstances in order to maintain the health and continued development of the organism as a whole. For example, if one cell is removed from an embryo at the 8-cell stage, the loss of this cell is detected by the 7 remaining cells, and the embryo responds in a global manner to compensate. The precise cellular mechanisms by which this compensation is achieved are not known, but clearly it is not a generic "wound response" designed only to repair damage; the fact that normal human individuals can develop after removal of one eighth of the body at this early stage indicates that the embryo has adaptively replaced the missing body parts so as to restore the normal pattern. Many aspects of the biologic state of the whole embryo must change to compensate for such a catastrophic loss and re-establish the original developmental trajectory, indicating that this is an integrated response, reflecting the status of the embryo as a whole.

Diffusible signaling molecules

As the embryo grows in size to the expanded blastocyst stage between days 3-5 of development (Figure 2B), contact-dependent cell communication continues to provide global integration between parts of the embryo that must act in a coordinated manner and respond adaptively to changes in circumstance. Thus, within the ICM, cells begin to differentiate into two distinct cell types, epiblast and hypoblast, beginning at about the seventh day of development in humans. Formation of these two new cell types depends on both cell-contacts and on diffusible signals.[13]

Similarly, cells of the epiblast are in intimate contact with the polar TE, and signals from the epiblast maintain polar TE in a proliferative state that allows them to subsequently generate the placenta,[14] while signals from the TE are required both for formation of primordial germ cells[15] and for initiation and maintenance of gastrulation[16] in the epiblast.

At the opposite side of the embryo (Figure 2B), cells of the mural TE interpret the lack of cell neighbors and epiblast-derived chemical signals to enter into a specific pattern of development appropriate to their unique location. Mural TE cells make relatively minor contributions to placenta formation, but are critical for implantation of the embryo.[17] Correspondingly, TE cells

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[13] Cell fate decisions and axis determination in the early mouse embryo. Takaoka K, Hamada H. Development. 2012 Jan;139(1):3-14.

[14] M. Murohashi, T. Nakamura, S. Tanaka, T. Ichise, N. Yoshida, T. Yamamoto, M. Shibuya, J. Schlessinger, N. Gotoh An FGF4-FRS2alpha-Cdx2 axis in trophoblast stem cells induces Bmp4 to regulate proper growth of early mouse embryos Stem Cells, 28 (2010), pp. 113–121.

[15] Mouse epiblasts change responsiveness to BMP4 signal required for PGC formation through functions of extraembryonic ectoderm. Okamura D, Hayashi K, Matsui Y. Mol Reprod Dev. 2005 Jan;70(1):20-9.

[16] Ets2-dependent trophoblast signalling is required for gastrulation progression after primitive streak initiation. Polydorou C, Georgiades P. Nat Commun. 2013;4:1658.

[17] P. B. Sesagiri et al., Cellular and Molecular Regulation of Mammalian Blastocyst Hatching, 83 J. REPROD. IMMUNOLOGY 79 (2009); Y. P. Cheon et al., Role of Actin Filaments in the Hatching Process of Mouse Blastocyst, 7 ZYGOTE 123 (1999); S. Niimura et al., Time-lapse Videomicrographic Observations of Blastocyst Hatching in Cattle, 56 J. REPROD. DEV. 649

in this region have a unique pattern of gene expression, compared to polar TE and ICM.[18] Although the dimensions of the embryo have increased, the cell-intrinsic molecular program inherited from the zygote, together with the information provided by direct cell-contacts and by signaling molecules that are able to diffuse over many cell diameters enables cells to interpret their location within the body and respond in an integrated manner to continue an orderly process of development.

Importantly, integration persists at this stage, in part due to cell-cell contacts and longer-range molecular signaling (Table 1; "signal-mediated integration"), but also due to the cumulative effect of the developmental program that has produced cells of specific types in specific locations (Table 1; "structural integration"). Thus, if an embryo is damaged at the expanded blastocyst stage (Figure 3), it will compensate for this injury to re-establish a normal developmental trajectory. At this stage, the regenerative or compensatory response reflects the properties of specific cell types and cell communication in addition to the ordered relationships between cells.

Cell movements during embryogenesis

After the epiblast and hypoblast have been established, the next major events in development are notable in that they involve extensive cell movements that alter the relative positions of cells within the embryo. Gastrulation, neurulation and body folding occur between days 12-24 of human development, and all involve significant rearrangements of individual cells and groups of cells that bring them into proximity of new neighbors. During gastrulation, cells of the epiblast move to the future midline of the body and then dive into deeper positions within the embryo, before migrating out to their final locations. During neurulation, a portion of the surface layer of the embryo rolls up into a tube and is sealed inside the body to form the primitive nervous system, an event that brings tissue from lateral positions of the body together at the future midline of the back. During body folding, the originally sheet-like or planar embryo rolls up in three dimensions to generate a tube-shaped body, a dramatic remodeling that moves the heart from its initial position above the head down into the chest cavity where it ultimately belongs.

Why the embryo undergoes such extensive cellular gymnastics has always been something of a mystery. It seems unnecessary for cells to travel such large distances and for entire blocks of tissue to move around in such radical ways. Although it seems plausible that an embryo could develop without cells exchanging positions (i.e. that the original zygote could simply cleave, to

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(2010).; Rosario M. Perona & Paul W. Wasserman, Mouse Blastocysts Hatch in Vitro by Using a Trypsin-Like Proteinase Associated with Cells of Mural Trophectoderm, 114 DEV. BIOLOGY 42 (1986); G. V. Sireesha et al., Role of Cathepsins in Blastocyst Hatching in the Golden Hamster, 14 MOLECULAR HUMAN REPROD. 337 (2008).; N. Sharma et al., Implantation Serine Proteinases Heterodimerize and are Critical in Hatching and Implantation, 11 BMC DEV. BIOLOGY 61 (2006).

[18] Dissecting the first transcriptional divergence during human embryonic development. Bai Q, Assou S, Haouzi D, Ramirez JM, Monzo C, Becker F, Gerbal-Chaloin S, Hamamah S, De Vos J. Stem Cell Rev. 2012 Mar;8(1):150-62.

give rise to blastomeres that would go on to differentiate into tissues and organs appropriate to their overall location in the body), this has not been observed in any animal organism studied to date. In animals as diverse as worms, sea urchins, flies, fish and humans, cell movements (most especially, the cell movements of gastrulation) are a critical component of development. Across enormous evolutionary distances (the ancestors of humans and the nematode worm *C. elegans* diverged over 600 million years ago) and over widely varying patterns of development, it is universally the case that cells exchange neighbors as an early, required step towards segregating blastomeres into distinct developmental pathways. Consequently, cell rearrangement appears to be as close to a "rule" as exists in developmental biology of animal species. This strongly suggests that cell movement serves a required purpose for assembling even simple animals, regardless of the specific developmental mechanisms a species employs.

I propose that one possible explanation for cell rearrangements during development is the requirement for global integration of the body as a whole prior to the formation of the nervous system. There are likely to be physical/molecular constraints on the complexity of the "pre-pattern" that can exist within an oocyte (Figure 4). And constraints on the complexity of the egg cytoplasm will subsequently restrict the range of cell types that can be specified based entirely on factors inherited from the oocyte. This is particularly true when cells with very different developmental paths must combine to generate a specific structure or tissue. It is difficult to imagine, for example, how factors that specifically drive formation of the diverse tissues of the head (e.g. muscles, bones, skin, teeth, neural tissue and many others) could all be localized in a single presumptive "head" region of the oocyte.

Similarly, there may be limits on determining specific cell types in specific locations based on cell-cell communication (Figure 4). While cell communication clearly makes major contributions to mammalian development, such events are "random"; i.e. two or more cells that are equally capable of entering a particular developmental path both attempt to take this path while inhibiting the others from doing so, until one emerges the victor. Clearly, such random events have only limited ability to direct the formation of specific cell types in specific spatial locations. Cell movement may be required to adjust the final positions of cells that have arisen as a consequence of random cell-cell interactions.

For complex structures like the head to be produced, cells need to either 1) gain information about where they are situated within body and use this information to enter into specific developmental pathways that are appropriate for those locations, or 2) change positions to occupy the location appropriate to the kind of cell they already are. And both of these possibilities require cells to have access to a broad range of spatial information. The cell movements of gastrulation, neurulation and body folding allow cells to gain access to a much wider biological context while still relying primarily on direct cell-cell communication and diffusible signaling molecules as the means of obtaining information. As a cell is migrating, for example, it physically encounters other cells with specific properties, and therefore the moving cell can read-out a wide range of information regarding its location in the body as a whole.

Finally, cell movements and tissue rearrangements also serve to bring tissues with different properties together so that they can influence each other's development. For example, formation of the head requires molecular signals from a primitive gut-derived tissue known as the

prechordal plate, that is brought into proximity of the developing brain through the cell movements of gastrulation. Similarly, the cell movements of neurulation position the developing nervous system between two important sources of diffusible signaling molecules (the roofplate and the notochord) that subsequently act to establish the proper cell types within the nervous system. These signaling centers were initially quite distant from each other and from the future nervous system and can only assume their proper function once they have been relocated to their final position in the embryo. Indeed, the vast majority of structures in the body require multiple tissues to interact in complex and often transient ways during embryonic development. And many of these interactions involve cells that are brought into proximity by the cell movements of mid-embryogenesis.

The cellular and molecular mechanisms controlling cell movement during development are only poorly understood, but they clearly originate as a consequence of the developmental program initiated by the zygote and rely on cell-cell contacts and diffusible molecules to establish an integrated pattern of development that promotes the life and continued maturation of the embryo.

Integration in late embryogenesis and throughout fetogenesis

After the completion of gastrulation, neurulation and body folding, the embryo enters into a period during which the organs and organ systems are produced. At this time, all of the basic relationships in the body have been established and generation of specific organs proceeds through local signaling mechanisms. The circulatory and placental systems have been established, and the nervous system is beginning to connect various parts of the body to the brain. The embryo has become too large for individual cells to gain information about the body as a whole through any of the mechanisms already discussed (cell contacts, diffusible molecules or cell movements). Similarly, the "whole" also cannot gain information about the function of individual cells or tissues by these means. What, then, accomplishes organismal integration during the period of late embryogenesis and throughout the period of fetal growth up until birth?

In considering organismal integration during this period of life, it is important to appreciate the extremely controlled nature of the uterine environment and how this limits the requirements for integration. During prenatal life, the environmental conditions are remarkably constant (Figure 5) and integration within to this narrow range of variation is primarily accomplished by the placenta, and (to a limited extent) by the developing nervous system of the embryo/fetus.

The role of the placenta: Many of the functions that will be integrated by the brain following birth are accomplished prior to birth by the placenta. The placenta functions essentially as a passive filter for the transfer of oxygen, nutrients and other blood-borne factors from the mother to the embryo/fetus and the transfer of metabolites and carbon dioxide in the opposite direction. Differences in concentration of specific factors between the two circulatory systems are balanced by the placenta to provide for the diverse metabolic needs of the embryo/fetus. For example, after birth, the brain controls respiration, altering the rate of breathing to compensate for increased oxygen demands due to activity. Yet prenatally, oxygen is supplied and carbon dioxide is removed from the fetal bloodstream via the placenta. Gas exchange by the placenta is adaptive to the needs of the embryo/fetus, adjusting for increased activity and other metabolic states, such that the oxygen saturation of the embryonic/fetal blood remains relatively constant.

Thus, the integration of body's demands and oxygen supply is accomplished not by brain-mediated changes in behavior (e.g. increased breathing rate), but by adaptive oxygen transfer through the placenta.

Similarly, during prenatal life both digestion and elimination are largely accomplished by the placenta. Nutrients from the mother's bloodstream are transferred through the placenta to the blood of the embryo/fetus and conversely, waste products are removed.

Compared to the sophisticated information-processing accomplished by the brain, the placenta is a relatively simple organ. However, similar to the brain and unlike other organs in the body, the placenta receives chemical information from the body as a whole and balances it to reflect the overall needs of the embryo/fetus. The "response" of the placenta is not unitary, but rather context-dependent, multifaceted and designed to promote the health of the body as a whole. It performs an *integrating* function for the embryo/fetus, not merely a *coordinating* function (Figure 1).

The role of the restricted conditions provided by the uterine environment: The placenta does not acquire information from the body or craft an integrated response with anywhere near the accuracy and sophistication of the nervous system. How is it possible that the placenta is the primary organ of bodily integration during prenatal life?

Part of the reason the embryo/fetus does not require the detailed integration of bodily systems provided by the nervous system after birth is because it relies on the brain-mediated integration occurring in the mother (e.g. the relatively constant levels of oxygen and glucose in the maternal blood stream) to provide a stable environment that promotes the survival, health and maturation of both the embryo/fetus and the mother (Figure 5).[19] Within this environment, the placenta provides the required body-wide integration by adjusting the transfer of maternal and fetal components to accommodate the conditions the embryo/fetus is experiencing. Although the systems required after birth are established during prenatal life, they are not required to maintain organismal function prenatally, and are used very inefficiently during the prenatal period.

A second reason for the fact that the embryo/fetus does not require a fully developed nervous system for bodily integration lies in the restricted conditions encountered during prenatal life and the corresponding restriction in the demands on bodily integration (Figure 5). The uterine environment is not only *controlled* (thereby reducing the range of conditions the integrating systems of the embryo/fetus must accommodate) it is also quite *limited*. And these limitations reduce the requirements for integration in a number of important ways.

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[19] Interestingly, in rare cases where a fetus has been gestated to term following a brain-death diagnosis of the mother, this integration is provided by external medical interventions that maintain constant levels of oxygen and nutrients in the maternal blood supply. For review, see: One life ends, another begins: Management of a brain-dead pregnant mother-A systematic review. Esmaeilzadeh M, Dictus C, Kayvanpour E, Sedaghat-Hamedani F, Eichbaum M, Hofer S, Engelmann G, Fonouni H, Golriz M, Schmidt J, Unterberg A, Mehrabi A, Ahmadi R. BMC Med. 2010 Nov 18;8:74.

Due to the protected uterine environment, the embryo/fetus does not typically encounter painful or other noxious stimuli that would elicit an avoidance response. Sensory input the fetus does receive is limited and generally does not require any form of coordinated response. Moreover, for the latter half of gestation (during which the fetus becomes capable of volitional movement), the uterus is a physically confined space. Consequently, motor activity (both volitional and spontaneous) is limited, and therefore variation in metabolic load stays within a narrow range that does not require significant modulation of the body's compensatory systems (Figure 5; dotted lines). Similarly, the embryo/fetus does not experience significant immune challenges, due to protection by maternal antibodies. And, as noted earlier, temperature, nutritional status and blood oxygen levels are all maintained in an optimal range by a combination of maternal and placental integration. Within this stable and restricted environment, integrating information about the thermal, metabolic and immune state of the body is largely not required, and the integration that is required is accomplished by the placenta.

The role of the nervous system: Although brain-mediated processes contribute in limited ways to bodily integration for much of the prenatal period, it is important to appreciate that brain development is a very long process. The formation of the brain begins with the induction of neural tissue and the cell movements of neurulation during the third week post sperm-egg fusion. At this time, the nervous system is already patterned into broad regions, corresponding to different parts of the mature brain and spinal cord. Neurons are born beginning during the 4th week.[20] Synapses, or the molecular structures required for brain cells to communicate with each other, are detected in the cortex by the seventh week.[21] The nervous system begins simple processing of sensory information, such as pain, during the eighth week.[22] Yet building the complex system required to process and integrate sensory information from the environment as

[20] Tangential networks of precocious neurons and early axonal outgrowth in the embryonic human forebrain. Bystron I, Molnár Z, Otellin V, Blakemore C. J Neurosci. 2005;25:2781-92.; ApoER2 and VLDLR in the developing human telencephalon. Cheng L, Tian Z, Sun R, Wang Z, Shen J, Shan Z, Jin L, Lei L. Eur J Paediatr Neurol. 2011;15:361-7.; The first neurons of the human cerebral cortex. Bystron I, Rakic P, Molnár Z, Blakemore C. Nat Neurosci. 2006;9:880-6. Epub 2006 Jun 18.; Development of the human cerebral cortex: Boulder Committee revisited. Bystron I, Blakemore C, Rakic P. Nat Rev Neurosci. 2008;9:110-22.

[21] Synaptogenesis in layer I of the human cerebral cortex in the first half of gestation. Zecevic N. Cereb Cortex. 1998;8:245-52.

[22] Synaptogenesis in the cervical cord of the human embryo: sequence of synapse formation in a spinal reflex pathway. Okado N, Kakimi S, Kojima T. J Comp Neurol. 1979;184:491-518.; Onset of synapse formation in the human spinal cord. Okado N. J Comp Neurol. 1981;201:211-9.; The fine structure of the spinal cord in human embryos and early fetuses. Wozniak W, O'Rahilly R, Olszewska B. J Hirnforsch. 1980;21:101-24.; Early synaptogenesis in the spinal cord of human embryos. Milokhin AA. Acta Biol Hung. 1983;34:231-45.; Development of pain mechanisms. Fitzgerald M. Br Med Bull. 1991;47:667-75.

well as diverse information from the body takes an extraordinarily long time, with recent evidence indicating that the brain is not fully mature until approximately 25 years after birth.[23]

Thus, the brain begins forming relatively early in prenatal life, but most brain functions that are vital after birth are accomplished prenatally by the placenta. Importantly, both prior to and after the formation of the nervous system, the embryo is *autonomously* responsible for integration of bodily function. The body simply uses different means to accomplish integration at different periods of the life cycle.

The transition to a brain-based system of integration at birth

A number of dramatic and important changes occur during the transition from the fetus to the newborn. Many of the systems built during development that remain largely dormant in prenatal life are suddenly brought into play. At the first breath, the pulmonary system will begin acquiring oxygen for the newborn. Blood flow through the heart shifts within seconds of birth from a pattern appropriate to the fetus to the mature pattern seen in postnatal humans. This requires closing off of some vessels (for example, those leading from the placenta) to maintain a healthy pattern of circulation independent of the mother. Similarly, the digestive, hepatic and urinary systems are now fully responsible for acquiring nutrition and eliminating wastes. The metabolic demands of the infant will vary greatly, depending on wakefulness, activity and ambient temperature. Sensory information becomes far more acute and far more relevant to the physiologic needs of the infant; e.g. the newborn experiences both hunger and pain—and reacts to them so that they will be resolved. And the brain oversees all of these activities, both through direct neural connections and through modulation of endocrine and immune functions of the body. Although coordinated processes occur both before and after birth, in postnatal life the brain is uniquely responsible for integrating information to craft a unified response (Figure 1).

Why brain death is real death

For humans at postnatal stages of life, death of the brain eliminates the kind of integration characteristic of animal species, despite the persistence of specific cell types and structures built into the body during embryogenesis. Yet persistence of structures that support cell-communication and coordinated activity following death of the brain can be very confusing. Following death of the brain, a human body that is being supported by artificial interventions may appear to be alive because it maintains a kind of "structural integration," similar to that of plants—passively acquiring water and nutrition and reacting in limited ways to environmental stimuli (see Table 1). If this type of integration is sufficient for a plant to be alive, some find it reasonable to conclude that it should also be sufficient for a human to be alive, albeit in an impaired state.

[23] Gogtay N et al (2004) Dynamic mapping of human cortical development during childhood through early adulthood. Proc Natl Acad Sci USA 101:8174; Sowell ER et al (2003) Mapping cortical change across the human life span. Nat Neurosci 6:309

Yet this is a false conclusion. Plants *autonomously* function to support their own life and health; i.e. they are *organisms*. The type of integration seen in plants is simple compared to animals, but it is 1) generated by the plant itself and 2) naturally ordered to maintain the unified function of the plant as a whole. In contrast, following death of the brain a human body is incapable of autonomously supporting its own survival and therefore no longer functions as an organism; i.e. many of the "structures" required for survival are medical devices that replace the natural functions of the brain. The apparent integration seen in a human body being supported by medical interventions after death of the brain is not comparable to the autonomous structural integration that occurs in plants.

Moreover, even the simple kind of adaptation seen in plants (differential growth towards or away from nutrients, water or other environmental stimuli) does not occur in a human body after death of the brain because it is not the nature of humans to integrate information in this manner. Humans are animals, not plants. Thus, after the death of the brain, specifically human integration is lost, and therefore, a human organism no longer persists.

Interestingly, after death of the brain, the body enters a state of restricted metabolic function similar to that of the fetus (Figure 5). Mechanical interventions and activities performed by care-givers provide constant temperature, oxygen, nutrition, hydration and waste removal. Absence of volitional and spontaneous motor activity limits the metabolic variation the system encounters. Impaired immune function is compensated for by external interventions (antibiotics, sterile environment). Sensory information is limited to general sensation and does not require an integrated response. In many ways, the artificial interventions that sustain a body after death of the brain are similar to the functions provided prenatally by the placenta and the protective uterine environment. Yet there are two critical differences between the state of the body during prenatal life and the state of the body after death of the brain; the *source* of the integration and the environment in which the body functions.

In considering the parallels between the state of the body in prenatal life and the state of the body after death of the brain it is important to appreciate that *the placenta is an organ of the embryo*. Like all embryonic organs, the placenta is 1) generated by the embryo, 2) physically continuous with the embryo, 3) genetically identical to the embryo and 4) critically required for the function and survival of the embryo as a whole. The placenta is best considered a transient organ of the embryo and fetus; i.e. a bodily structure/organ that functions only during prenatal life, similar to the lungs that function only during postnatal life.

The fact that the placenta is an embryonic organ means that, just as is the case for plants and other living organisms, the integration seen in the embryo is *integration the embryo is accomplishing for itself*. Thus, during prenatal life, the body as a whole continuously functions as an organism, providing <u>*autonomous*</u> integration of bodily function. Following death of the brain, the metabolic state of the body is similar to the state it occupied in prenatal life, but the integration it requires is maintained *extrinsically*. The body no longer autonomously supplies the integration required to function as an organism.

Secondly, it is important to consider the difference in the natural environment between prenatal and postnatal life. Humans are animals; i.e. the kind of beings that sense, adapt and behave. We

are also rational; the kind of beings that understand, reason and choose. We have evolved to thrive in two different environments at pre- and postnatal stages of the life cycle. And after death of the brain, the body is manifestly incapable of functioning independently in the natural environment of a postnatal human organism. If the ability to survive in *any* environment is sufficient for a human to be considered alive, how are we to view human cells in the controlled environment of a laboratory culture, where all of their requirements are provided by the experimenter? The fact that cellular life can be maintained when integration is externally supplied does not provide evidence for the persistence of a human organism.

The capacity for integration distinguishes living human beings from dead human[24]. Living cells persist in the human body for some time following death of the brain and maintain their natural properties and relationships. Although communication between cells can provide a *coordinated* biologic response to specific signals, in the absence of brain function, the integration that is characteristic of a human organism is no longer possible. Bodily functions that persist after death of the brain reflect the properties of individual cells, functioning as autonomous cellular organisms within a system established by the human being during life. Coordination persists, but integration is lost. Therefore, death of the brain is a legitimate criteria for death of the human being, because at postnatal stages of life, organismal integration requires brain function.

Conclusions

Integration is a required feature for any organism to be alive. It is accomplished through a variety of mechanisms in diverse types of living entities. At the simplest level, single-cell organisms integrate information from the environment and from internal cellular processes to maintain a balance that is appropriate to their current situation. Plants largely accomplish integration by building bodily structures that allow for healthy function of the organism as a whole and by adapting to changing environmental circumstances via differential growth. In contrast, animals have far more complex physiology, and therefore require more sophisticated mechanisms for integration of bodily function that vary considerably over the life span.

In the early embryonic period, prior to formation of the brain, a sophisticated developmental program combined with a number of cellular mechanisms that depend on the small dimensions of the embryo (structural integration, cell-cell contacts, diffusible molecules and cell migration) provide body-wide integration of function. By mid-embryogenesis, the placenta and to a lesser extent, the nervous system, are responsible for integration. Yet following birth, the brain is uniquely required for bodily integration. Importantly, although integration is much simpler during prenatal life, the developing human continuously acts as an *organism* to autonomously sustain its own health and life.

Thus, integration is a common feature of all organisms, but the precise nature of this integration differs, depending on the nature of the organism itself and the demands imposed on it by the biological niche to which it has evolved. Mammals (including humans) have evolved to occupy

[24] See Condic, M.L. (2016). Determination of Death: A scientific perspective on biological integration**. Journal of Medicine and Philosophy**. *(in press)*

two very different biological niches with very different demands during prenatal and postnatal life, and therefore they have quite different strategies to accomplish integration during these two distinct periods.

Figure 1: Integration and Coordination. (A) Integration involves compilation of multiple signals to generate a response that is multifaceted and appropriate to the state of the body as a whole. The output of the integrating structure can be both qualitatively and quantitatively different for different responding structures; e.g. strongly activating (solid arrow), weakly activating (dashed arrow) and inhibitory (ball-end arrow). Altering the balance of input signals will alter the output of the integrating structure to reflect the specific circumstances. The output of responding structures reflects their specific nature and the type of signal received from the integrating structure. At postnatal stages of human life, the brain is uniquely capable of integration. (B) Coordination is initiated by a limited number of signals that result in the release of an output signaling molecule. The output molecule can affect multiple cell types, with the output of the responding structures being determined by their nature. Responses can be scaled to reflect the strength of signal received, but are qualitatively identical in all cases. Thus, the body's response to a coordinating signal may be complex, but it is nonetheless *unitary*; i.e. determined by a single molecular agent and therefore unable to adapt to altered environmental or bodily circumstances. Many different cells and structures participate in coordinated interactions, including cells in the brain.

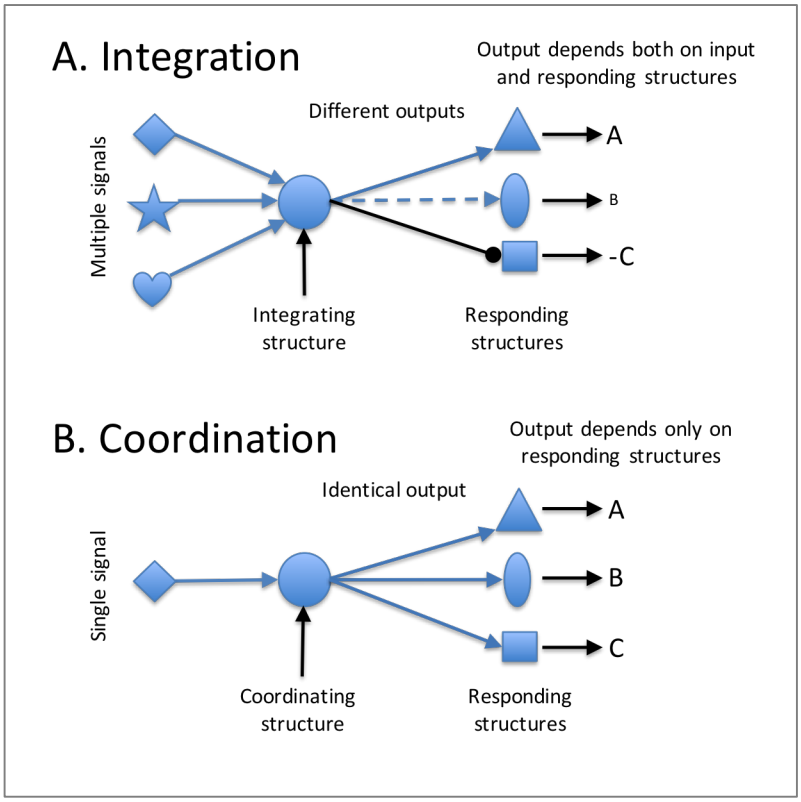

Figure 2: Early mammalian development. (**A**) Development is initiated at sperm-egg fusion, an event that forms the zygote or one-cell embryo. Cell division to produce two cells, or blastomeres, typically occurs within a day of sperm-egg fusion. The morula stage is reached typically by three days, with the expanded blastocyst stage being reached by 5-6 days. (**B**) Anatomy and cell types of the expanded blastocyst embryo (shown as a mid-sagittal section). The position of the inner cell mass (green and pink) defines the embryonic pole. The entire embryo is surrounded by an acellular protein layer known as the zona pellucida (grey). Together, epiblast and primitive endoderm constitute the inner cell mass. The blastocyst cavity is a fluid filled space. Polar (light blue) and mural (dark blue) trophectoderm have distinct molecular properties and distinct developmental functions.

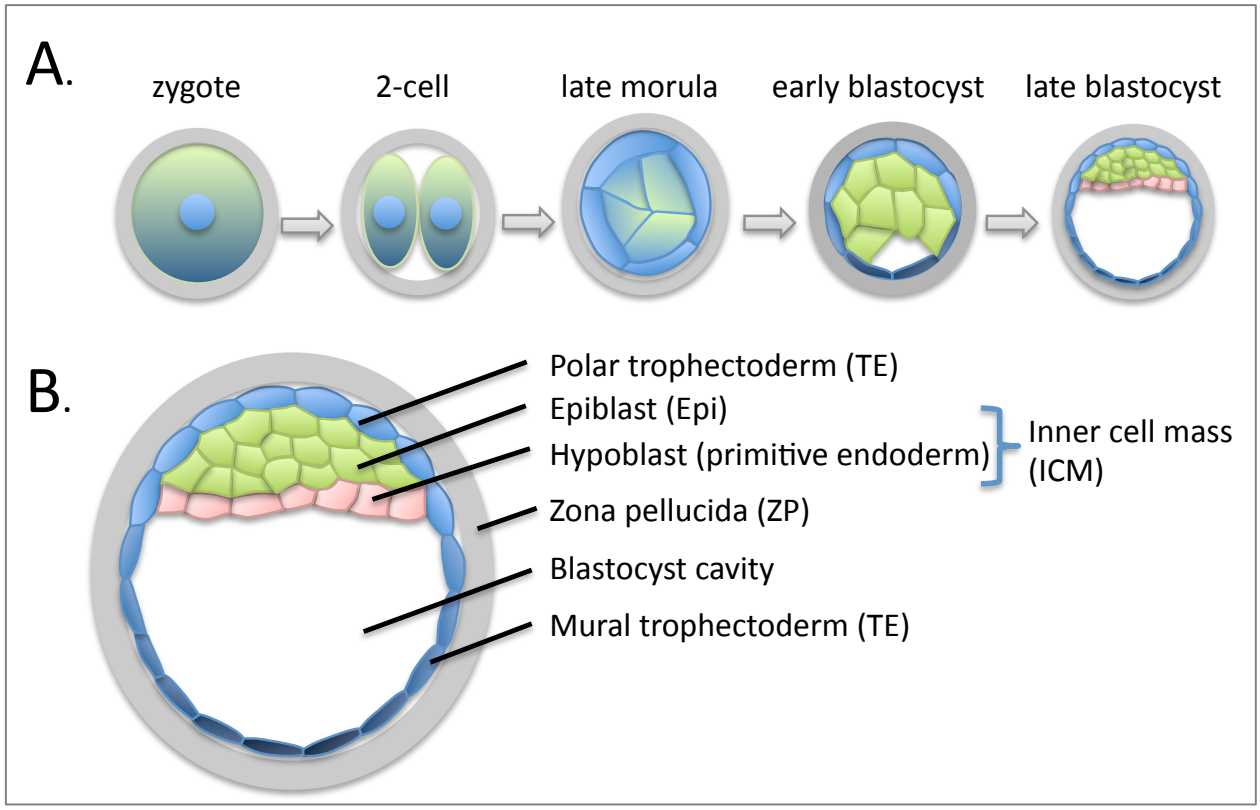

Figure 3: Twinning at the blastocyst stage. (**A**) Cells of the blastocyst have distinct molecular properties and restricted developmental capabilities. Subsequent panels show the result of splitting at the dotted line. (**B**) After splitting, a closed sphere rapidly reforms (curved grey arrows), and within the sphere, cells of each of the embryonic lineages replace cells within their own tissues (colored arrows). Cells within specific lineages (TE, epiblast or PE) are likely to assume a new positional identity that reflects their new location. There is no evidence for respecification across lineages contributing to regeneration of the blastocyst. (**C**) The smaller, "demi-embryos" resulting from splitting have approximately half the number of cells as the original blastocyst, and proceed from the blastocyst stage in synchrony with un-split sibling controls.

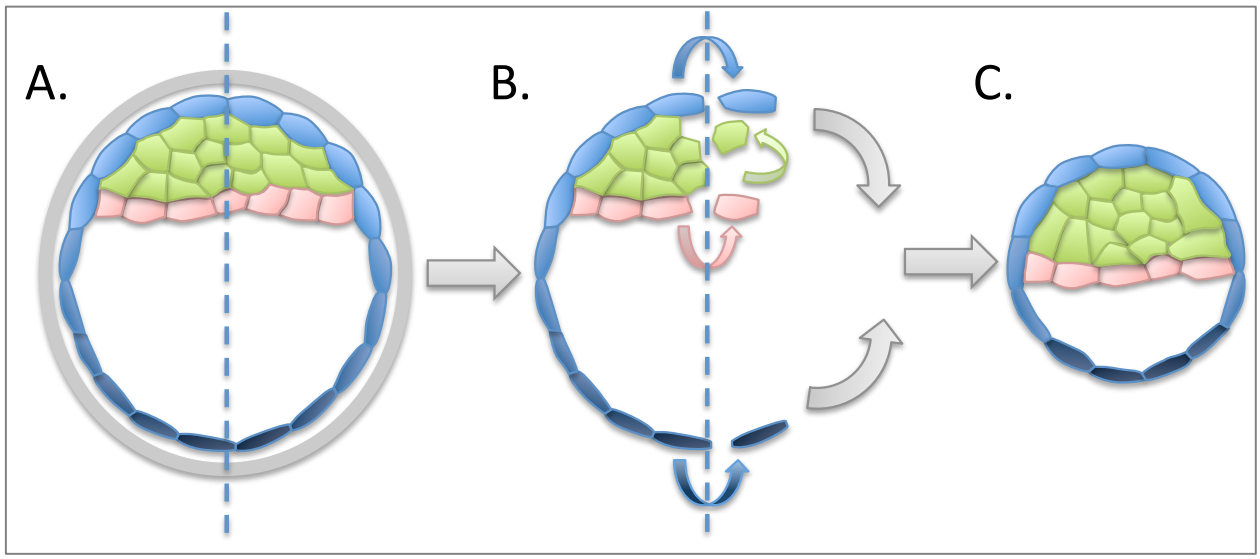

Figure 4: Complexity of the "pre-pattern" in the oocyte may limit the cells that can be directly formed without cell rearrangements. (**A**) If the egg has no molecular asymmetry, all differences between cell types must arise as a consequence of cell-cell interaction between neighboring cells (arrows). These interactions cannot be random, or no consistent pattern of development will result. (**B**) If there is a simple pre-pattern in the egg, cells with different properties are generated by cell division, with differences being enhanced by cell-cell interaction. (**C**) With a more complex pre-pattern in the egg, a larger number of cell types can be produced by cell division, with differences between cells being reinforced by cell-cell interaction. The limits of molecular gradations possible in the egg will limit the number of different cell types that can be specified in this manner, potentially requiring cells to rearrange so that new types of cell-cell interactions are possible.

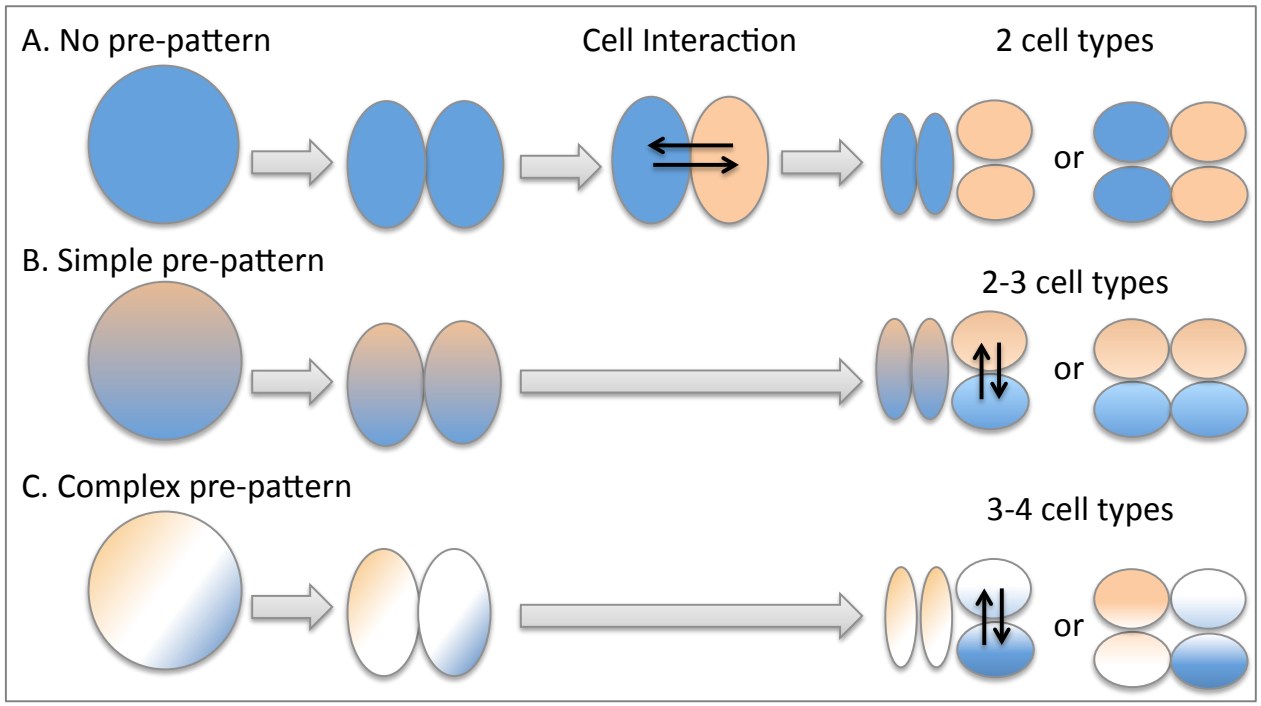

Figure 5: Homeostasis is mediated by the brain at postnatal stages. The embryo/fetus functions within a very narrow range of metabolic states (dotted lines), due to limited voluntary activity and the controlled uterine environment. For example, in postnatal life, body temperature is brought outside a healthy range by exercise, exposure or infection, and is brought back into a healthy range by brain-mediated processes (shivering, sweating, panting, vasoconstriction/dilation or alterations in activity). In contrast, the embryo/fetus does not experience large changes in activity or environmental temperature, and is only rarely exposed to infectious agents that are not effectively neutralized by the maternal immune system. Therefore, brain-mediated integration of the bodily systems that regulate temperature ia largely not required in prenatal life. Similarly, in postnatal life, fluctuations in blood chemistry are driven by exercise, feasting/fasting and dehydration. Such variations are not experienced prenatally, and therefore the brain-mediated adaptive responses (changes in eating, drinking and respiration) are not critically required. In this controlled environment, and the placenta is able to compensate for the small fluctuations in blood chemistry the embryo/fetus experiences.