REVIEW SUMMARY

DEVELOPMENTAL BIOLOGY

The primitive streak and cellular principles of building an amniote body through gastrulation

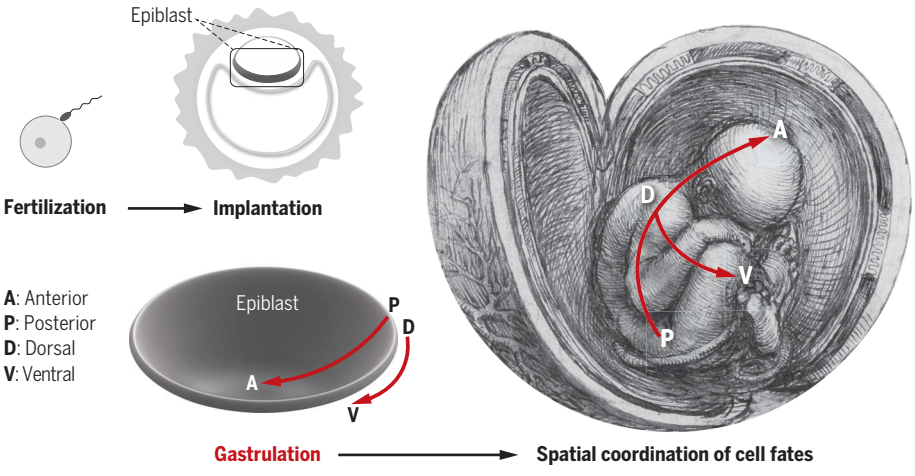
Guojun Sheng*, Alfonso Martinez Arias*, Ann Sutherland*

BACKGROUND: Pluripotent cells are generated by embryonic divisions that occur shortly after fertilization. These cells are transformed into the recognizable outline of an organism through the process of gastrulation, which endows them with lineage and spatial identities in the context of an emerging coordinate system. In many amniote embryos (such as those of reptiles, birds, and mammals), gastrulation has been associated with a transient structure called the primitive streak. Human development also follows this pattern. In humans, the primitive streak forms ~14 days after fertilization. The appearance of the primitive streak breaks the radial symmetry of the epiblast (a sheet of epithelialized pluripotent cells) and has been suggested to symbolize the emergence of human individuality. As such, many countries have established a legal limit of 14 days for the in vitro culture of fertilized human eggs—this is known as the "14-day rule." In recent years, pluripotent stem cells have become a promising in vitro model for studying the cellular and molecular mechanisms associated with early human development. Interpretation of developmental features observed in these in vitro models requires proper understanding of animal gastrulation in general and of the amniote primitive streak in particular.

ADVANCES: In this Review, we offer a phylogenetic and ontogenetic overview of the primitive streak and its role in mediating amniote gastrulation, and we discuss the implications of embryonic stem cell–based models of early mammalian embryogenesis on the function of this iconic structure. We provide evidence that the primitive streak is not a conserved feature in amniote development and that the mammalian and avian primitive streaks have evolved independently through different supracellular mechanisms that led to their morphological emergence. We argue that, in addition to mediating the emergence of germ layers from the pluripotent epiblast, gastrulation is principally a process in which an embryo acquires a coordinate system to organize its primary cell fates and the primordia of organs and tissues relative to each other in space. We highlight that in amniotes this process is regulated by a set of conserved signaling and transcriptional networks through a small collection of cellular behaviors, the tissue-level effects of which are governed by boundary conditions. We suggest that changing boundary conditions, in the form of evolution of extraembryonic lineages such as the trophectoderm and primitive endoderm, have played a key role in the transformation of the blastopore, characteristic of anamniote embryos, into the primitive streak. Variability in the organization of these tissues and the demarcation of embryonic and extraembryonic territories underpins the observed variation in the morphological appearance of the primitive streak in mammals and birds and of primitive streak–related structures in reptiles. Over the past few years, embryonic stem cells have been used as models to recapitulate several aspects of early mammalian embryogenesis. These studies have revealed that the germ layers, and even a rudimentary body plan, can form in the absence of a primitive streak.

OUTLOOK: Our model predicts that the most fundamental feature of a primitive streak–like structure in early amniote development is not its morphological manifestation but rather its capacity to mediate coordinated cell fate specification events in space. Our model also suggests that cell fate specification and tissue-level morphogenesis are regulated independently during gastrulation and then coordinated during embryonic development in vivo. In developmental models in vitro, these two processes can be uncoupled and have been shown to be influenced by different types of biomechanical parameters that mediate their coordination. This modularity leads us to suggest that the in vitro models are useful for studying gastrulation because their use without necessarily having to recapitulate embryonic structures. Future analyses of early amniote development, both in vivo and in vitro, would benefit from putting less emphasis on the primitive streak as a distinct embryological structure and more on its roles as a conduit for symmetry breaking and coordinated germ-layer differentiation.

Research into human development has direct societal and ethical impacts. Current ethical oversight in human embryo research, the 14-day rule, is effective in many countries and reflects an interdisciplinary consensus drawn somewhat arbitrarily to determine the legal rights of a human embryo. Our observations suggest that use of the primitive streak as a key developmental landmark for limiting ex vivo culture of human embryos should be reassessed. An alternative landmark, necessary for exerting ethical oversight in human-related developmental and stem cell biology research, should be selected through a consensual discussion between different stakeholders to ensure scientific and ethical rigor. ■



Schematic view of how a human embryo acquires spatial coordination of its primary cell fates through gastrulation. After fertilization, a human embryo implants into the uterus (between days 6 and 12) and gastrulation begins soon afterward, with the primitive streak emerging at day 14. Gastrulation allocates cell fates and spatial coordinates to epiblast cells, laying down the foundation of a human body.

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REVIEW

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The primitive streak and cellular principles of building an amniote body through gastrulation

Guojun Sheng[1]*, Alfonso Martinez Arias[2]*, Ann Sutherland[3]*

The primitive streak, a transient embryonic structure, marks bilateral symmetry in mammalian and avian embryos and helps confer anterior-posterior and dorsal-ventral spatial information to early differentiating cells during gastrulation. Its recapitulation in vitro may facilitate derivation of tissues and organs with in vivo–like complexity. Proper understanding of the primitive streak and what it entails in human development is key to achieving such research objectives. Here we provide an overview of the primitive streak and conclude that this structure is neither conserved nor necessary for gastrulation or early lineage diversification. We offer a model in which the primitive streak is viewed as part of a morphologically diverse yet molecularly conserved process of spatial coordinate acquisition. We predict that recapitulation of the primitive streak is dispensable for development in vitro.

Gastrulation is a stage of animal development that transforms the mass of cells from early postfertilization cell divisions into the recognizable outline of an organism. The term "gastrulation" was coined by the German embryologist Ernst Haeckel when discussing the development of sponges (*1*, *2*). Later, combined with the germ-layer theory, it acquired its common meaning as a phylogenetically conserved stage during which the primary germ layers are defined and organized. Scrutinized at the cellular level, gastrulation was revealed to be associated with species-specific cell movements that, for vertebrates, lead to a conserved pharyngula stage when embryos from different phylogenetic groups resemble each other the most and when organogenesis begins. Over the years, the term gastrulation has come to refer to a set of morphogenetic movements that serve to organize the three germ layers in animal development.

Gastrulation in amniotic vertebrates (reptiles, birds, and mammals; see Fig. 1A for phylogenetic relationships among major animal groups) is thought to involve the primitive streak, a transient structure that forms along the midline of the epiblast (the pregastrulation ectoderm), first observed in a chick embryo by Russian embryologist Christian Pander in 1817 (*3*). The emergence of the primitive streak breaks an early morphologically radial symmetry, outlines the anterior-posterior axis of the embryo, and serves as a channel for the continuous passage of mesenchymal cells toward the interior of the embryo, where they are further assigned an endodermal or mesodermal fate. Bioethicists and government regulatory bodies have associated the primitive streak with human individuality and, as such, have established a legal limit of 14 days—approximately when the primitive streak appears in a human embryo—for the in vitro culture of fertilized human eggs (known as the "14-day rule") (*4*, *5*). Until recently (May 2021), the International Society for Stem Cell Research (ISSCR) (www.isscr.org) has stated its commitment to this 14-day rule, with updated guidelines recommending case-by-case review by national and institutional review boards (*6*). Placing such an emphasis on the primitive streak for the emergence of a human being raises questions about its role in the establishment of the human body plan and whether it represents a stereotyped and generalizable structure. To address these questions, the definition (or lack thereof) of gastrulation must be revisited.

William Ballard, an American embryologist who coined the word "pharyngula" as a phylotypic stage for all vertebrate animals, stated in a discussion on gastrulation that "real problems that require new observation and experimental proof are being glossed over" and that "there has been no progress at all since the 1930's in defining what gastrulation is or when it begins or ends" (*7*). Ballard also quoted Jean Pasteels (*8*) "in that the vertebrate gastrula does not have a definitive form. It is just an abstract collective term for individuals undergoing the movements of gastrulation." We believe that these statements reflect a situation that has persisted to this day. Genetic studies have identified a conserved network of signaling molecules and transcription factors associated with gastrulation and have revealed many common features of cell biology in different species that bridge the gap between unicellular and supracellular behaviors (*9*, *10*). Furthermore, pluripotent stem cells (PSCs) represent a model system for the study of early mammalian development (*11*, *12*), and the interpretation of such experiments raises questions about the nature of gastrulation and, more importantly, whether the behavior of PSCs in vitro can identify fundamental mechanisms underlying the events happening in vivo. Progress in human PSC research has prompted discussions of whether PSC-derived structures should be considered within the same ethical realm as embryos, and the conventional wisdom is converging toward drawing a similar line on the basis of whether these structures assemble a primitive streak and undergo gastrulation. All of these issues suggest that it is time to reassess gastrulation in light of data from more recent experimental techniques and models, from which general rules may emerge, with potential ethical implications.

Here we focus on the primitive streak in amniotic vertebrates and argue that gastrulation is not only a stage but, principally, a process whereby an embryo acquires a coordinate system to organize primary cell fates in space. We highlight that this process is regulated by a set of conserved signaling and transcriptional networks through a small set of cell behaviors, the tissue-level effects of which are governed by boundary conditions. This discussion leads us to suggest that global control by boundary conditions underpins the variability of the gastrulation process in different amniotic clades and to propose a relationship between gastrulation modes observed in amniotic and anamniotic (nonamniotic) vertebrates. Later, the process of organogenesis can also lead to similar conclusions, as supported by data from PSC models.

An organismal view of gastrulation

Gastrulation (literally "formation of a small gut") and its embryological connotation were introduced by Ernst Haeckel in the context of his gastraea theory (*1*), as part of his biogenetic law that lent embryological support to the Darwinian notion of common descent of all living organisms. A gastraea is a hypothetical ancestral organism characteristic of the gastrula stage that all metazoans presumably go through ontogenetically, and gastrulation was the process encompassing this developmental stage as a universal "rite of passage" in Haeckel's recapitulation theory. A key piece of evidence for Haeckel's theory was the presence of an endoderm-like, lineage-restricted cell layer during embryogenesis of sponges (Porifera; a phylum of diploblastic metazoans; Fig. 1A)—hence the names "gastraea" and "gastrulation" [from "gaster" (which means "stomach"), an endoderm derivative]. Recently, this description has been disputed in light of data from morphological and lineage-tracing analyses (*13*, *14*), suggesting that sponges do not undergo

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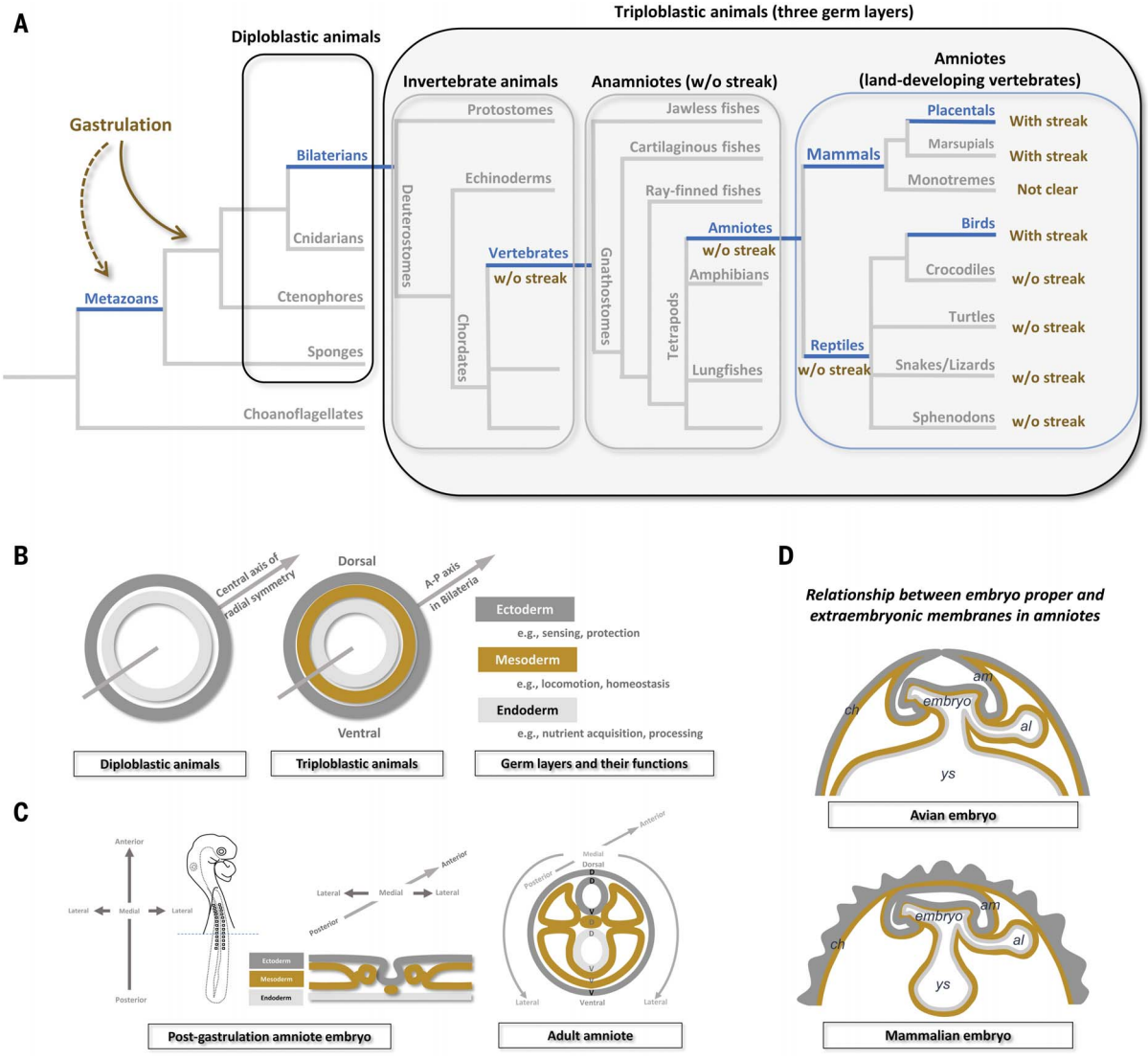

Fig. 1. Animal phylogeny and amniote body plan. (**A**) Phylogenetic relationships among major animal groups. Gastrulation is traditionally viewed as a conserved process of achieving cell lineage diversification in metazoans (animals). The relationship between gastrulation in sponges and that in the rest of the metazoans awaits further clarification (dashed arrow). Diploblastic indicates two germ layers (ectoderm and endoderm); triploblastic indicates three germ layers (ectoderm, mesoderm, and endoderm). Bilaterians are animals with bilateral body symmetry. The presence of a primitive streak–like structure during gastrulation is not conserved among amniotes (land-developing vertebrates, including extant mammals, birds, and reptiles). The term anamniotes collectively denotes vertebrate groups other than the amniotes. w/o, without. (**B**) Germ layers and their biological functions. (Left) Diploblastic animals have two germ layers and one major axis (the central axis of radial symmetry). (Middle) Triploblastic animals have three germ layers and two major axes [the anterior-posterior (A-P) and dorsal-ventral axes]. (Right) Simplified view of cellular functions of each germ layer. This overall functional assignment is conserved in all triploblastic animals. In diploblastic animals, some cellular functions that resemble those of the mesoderm are performed by either ectoderm or endoderm cells. (**C**) Basic 3D organization of a postgastrulation amniote embryo (left), with relationship of three germ layers and spatial coordinates in the anterior-posterior and medial-lateral axes shown in transverse-section view (middle). The medial-lateral axis in early development is transformed into the dorsal (D)–ventral (V) axis in the adult (right). (**D**) Schematic view of the relationship between the embryo and extraembryonic tissues in amniotes. am, amnion; ch, chorion; ys, yolk sac; al, allantois. Dark gray, ectoderm; light gray, endoderm; gold, mesoderm.

gastrulation as commonly seen in triploblastic animals. Nevertheless, comparative genomics and molecular phylogeny studies have shown that most of the genes involved in epithelial organization are present in sponge genomes (*15*, *16*), as are some of the genes involved in germ-layer formation in Bilateria (the protostomes and deuterostomes, including all vertebrate species) (*14*) and in the epithelial-to-mesenchymal transition (EMT) (*17*). In Cnidaria, a phylum of diploblastic animals most closely related to triploblastic Bilateria (Fig. 1, A and B), molecular tool kits for epithelial organization and EMT are present in genomes, and gastrulation generates mesendoderm-like cells that express mesoderm transcriptional regulators and mesoderm-specific lineage markers, despite their lack of a genuine mesoderm germ layer (*18*).

These seemingly contradictory observations highlighted the need for a conceptual framework for understanding the relationship between the primitive streak and amniote gastrulation from the perspective of metazoan phylogeny. The gastraea theory posited that

functional diversification is fundamental to multicellularity and that specification of cells dedicated to nutritional acquisition (i.e., the endoderm) is a conserved process among all metazoans (Fig. 1B). With this came notions of conservation of gastrulation at the genetic and morphogenetic levels, revealed in modern biological terms as molecular regulatory networks and dynamic changes in supracellular organization, respectively. Gastrulation, therefore, can be practically defined by traits that describe cell fates (e.g., neurons, notochord cells, and enterocytes) and shapes (e.g., epithelium, mesenchyme, migration, and EMT), as well as by their associated molecular characteristics (e.g., *Brachyury* for mesoderm and endoderm progenitors and *Nanog* for undifferentiated pluripotent cells). Depending on the choice of experimental models and investigative tools, the particular aspect of gastrulation to emphasize has varied since Haeckel's time. The core, unchanged, and unifying concept of gastrulation, in our view, is for it to serve as a conduit for diversification of cellular lineages and acquisition of a spatial coordinate system for subsequent functional integration in later development.

Central to our current understanding of gastrulation is the primitive streak, which is associated with a spatially oriented, dynamic sequence of individual cells leaving the surface layer in the process of ingression, through the EMT. The primitive streak has been viewed as iconic for gastrulation, although it is present only in specific amniotic vertebrates, such as birds and mammals (*19*) (Figs. 1 and 2). In reptiles (nonavian reptiles, in the context of this article), gastrulation is associated with involution (i.e., rolling of a sheet of cells over a horseshoe-shaped blastopore, an orifice characteristic of amphibian gastrulation) accompanied by ingression in the blastoporal (primitive) plate located posteriorly to the blastopore (*19*–*22*) (Fig. 3). Thus, all functional cell types shared by amniotic vertebrates can be generated regardless of the presence or absence of a primitive streak, leading to the question of whether gastrulation-related morphological features are conserved among all amniotes. Furthermore, relevant to the effort to recapitulate all, or part of, mammalian development in vitro is the question of whether a conserved morphogenetic process similar to the appearance of a primitive streak or a blastopore is required for the functional diversification of cell lineages.

A closer look at amniote embryos undergoing gastrulation, compared with embryos of anamniotes (nonamniote vertebrates), identifies three shared morphological features

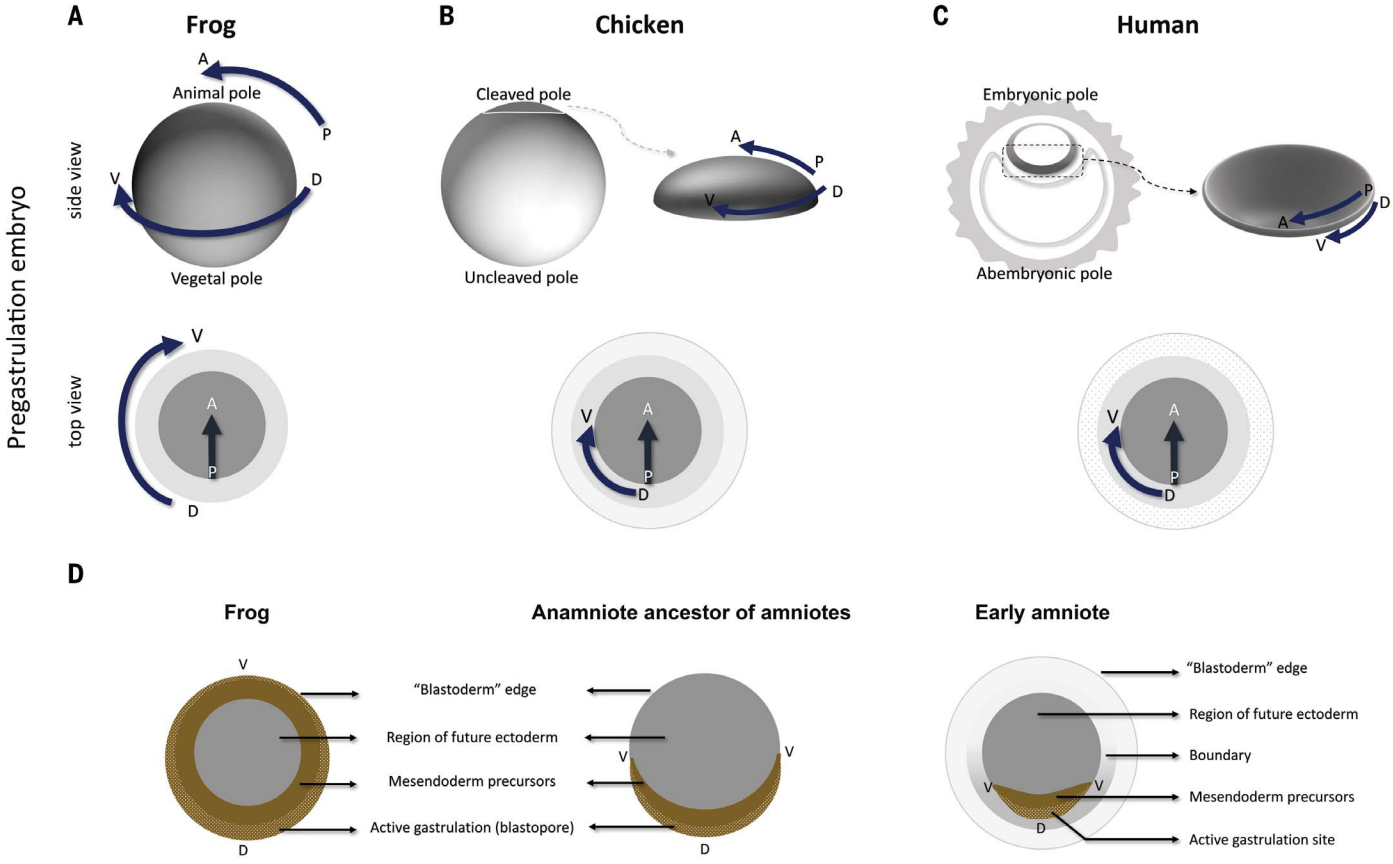

Fig. 2. Phylogenetic comparison of spatial organization in amphibian (frog), avian (chicken), and mammalian (human) embryos before gastrulation and hypothetical transitions in gastrulation morphogenesis during early amniote evolution. Future anterior-posterior and dorsal-ventral axes are indicated in pregastrulation embryos. (**A**) In the frog (*Xenopus*) embryo, the animal pole is where the oocyte nucleus is located before fertilization (it is also lighter because it has less yolk than the vegetal pole). (**B**) In the chicken embryo, the cleaved pole is where the oocyte nucleus is located and where cellularization of the fertilized egg takes place. The uncleaved pole is a part of the oocyte that does not contribute to the cellularized embryo and contains mostly nutritious materials. (**C**) In the human embryo, the embryonic pole is the side where the inner cell mass is located in a blastocyst. The abembryonic pole is the opposite side. The trophectoderm and primitive endoderm are drawn but not labeled. Epiblast cells form the proamniotic cavity, with upper cells giving rise to the amniotic ectoderm and lower cells (area represented by the dashed rectangle) giving rise to the major part of all three germ layers. In the lower panels, dark gray indicates cells that will contribute mainly to the ectoderm lineages; light gray denotes the marginal zone, which will control the location of internalization, contribute to mesendoderm cells, and mediate boundary biomechanical cross-talk—which, in amniotes, will influence the morphogenesis of primitive streak–like structures. (**D**) Hypothetical evolutionary transitions in gastrulation morphogenesis, leading to an amniote-specific mode of mesendoderm internalization.

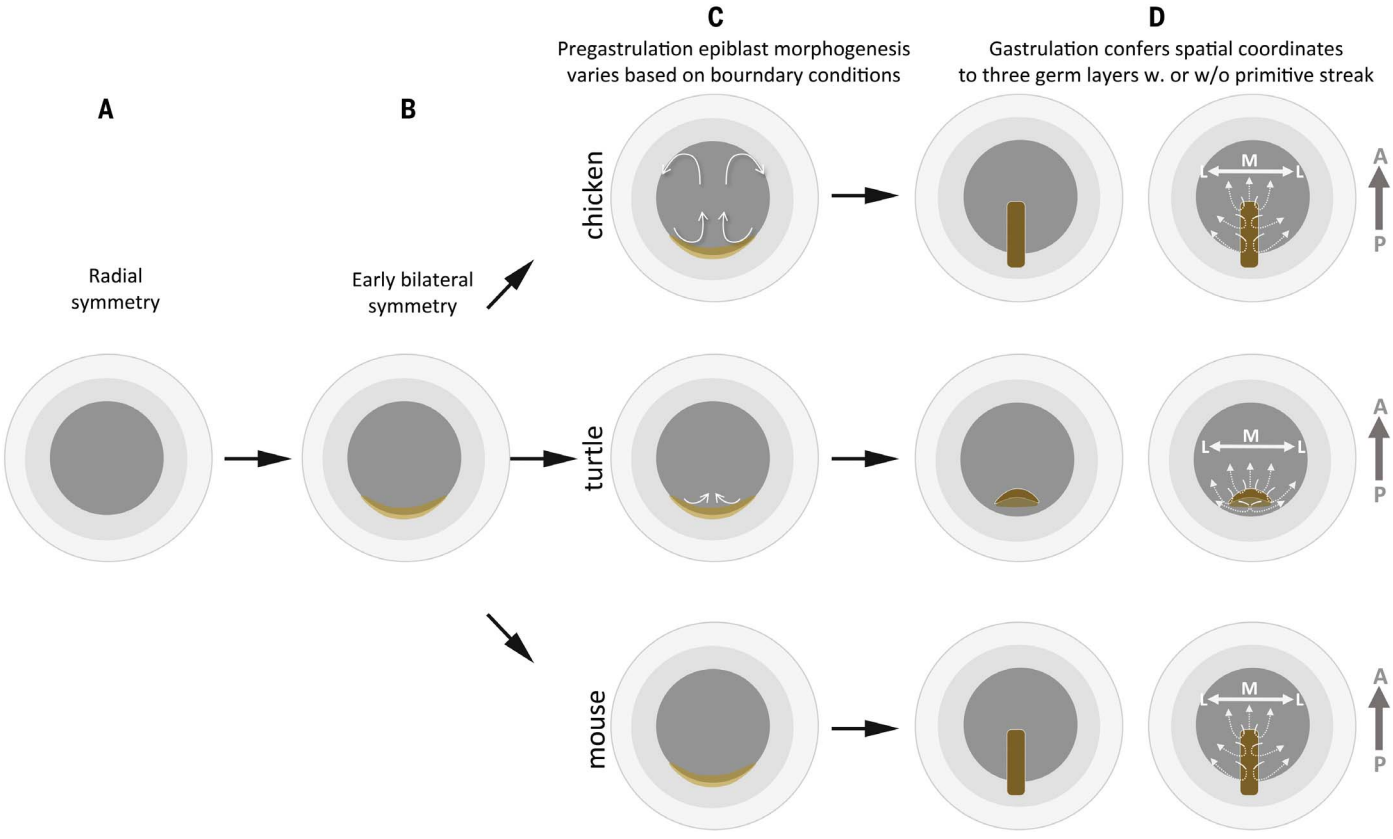

Fig. 3. The primitive streak as a morphogenetic consequence of variable boundary conditions in the pregastrulation epiblast of an amniote embryo. The primitive streak is neither conserved nor necessary for amniote gastrulation. Ontogenetically, in the pregastrulation embryo, radial symmetry (**A**) (similar to the top view in Fig. 2) is transformed into bilateral symmetry when mesoderm and endoderm precursors are induced asymmetrically in the marginal zone (**B**). Geometric organization of active internalization of mesendoderm cells is influenced by planar morphogenesis (white arrows) and cellular proliferation of the epiblast before onset of gastrulation and by boundary conditions reflected as embryo-specific biomechanical constraints on epiblast cells (**C**). In reptilian (e.g., turtle) embryos, such morphogenesis results in the formation of a blastopore as the active center of internalization, whereas in chick and mouse embryos, a primitive streak–like structure forms, with different morphogenetic origins. (**D**) Internalization of mesendoderm cells (small white arrows; the dotted parts indicate cell movement after internalization, contributing to the 3D organization of a postgastrulation embryo). M, medial; L, lateral.

among all amniote clades: (i) Internalization of mesoderm- and endoderm-fated cells takes place in a circumferentially restricted manner (compare an anamniote embryo in Fig. 2A with amniote embryos in Fig. 2, B and C). (ii) Pregastrulation ectoderm (epiblast) cells are organized as a single-cell–layered epithelium. (iii) The epiblast is divided into embryonic and extraembryonic territories, and gastrulation is initiated at their boundary (Fig. 2, B to D). To integrate these three features of amniote gastrulation in a simple model, the primitive streak in birds and therian mammals can be viewed as an independent morphogenetic adaptation of a basal, reptile-like bimodal form of involution and ingression (Figs. 1 to 3) (*19*, *20*). The presence of a neurenteric canal (i.e., an opening of the epiblast that connects the amniotic cavity and the yolk sac) in human and other primate embryos (*23*) and the rudimentary chordal canal associated with head process formation in the mouse embryo (*24*) can be considered to represent a residue of blastoporal involution that continues to play a role in axial mesendoderm internalization. These features and the proposed unifying model, however, highlight only morphogenetic constraints placed on amniote gastrulation, which by themselves may or may not be essential for fulfilling functional roles of gastrulation as a conduit for cell lineage diversification, spatial coordinate establishment, and inter– or intra–germ-layer coordination during tissue and organ formation.

The first two features conserved among amniote vertebrates are also observed in certain groups of anamniotes. For example, unlike *Xenopus*, the main amphibian model, all salamanders (Urodela) (*25*–*29*) and caecilians (Gymnophiona) (*30*, *31*) studied so far exhibit a gastrulation process that is restricted to or extremely biased toward the dorsal marginal zone. Similarly, such dorsally restricted gastrulation is also present in lungfish (the closest relatives of Tetrapoda) (*32*, *33*), dogfish (cartilaginous fish) (*34*), and lampreys (jawless vertebrates) (*35*), which suggests that the amniotes likely inherited this feature of gastrulation from an anamniote ancestor (see Fig. 1A for phylogenetic relationship). The second feature, a unilaminar, epithelialized pregastrulation ectoderm, is also present in certain anamniote groups (e.g., urodeles) (*36*), which suggests a preamniote origin. The third feature, that gastrulation is initiated at the boundary between intraembryonic and extraembryonic territories, is associated with the evolutionary invention of the amnion and chorion in ancestral amniotes (Fig. 1D), where internal fertilization, intrauterine early embryogenesis, and land-based fetal development necessitate the presence of these protective layers. The amnion, composed of ectoderm- and mesoderm-derived cells, provides a protective liquid-filled environment for the developing embryo and is the defining feature of amniotic vertebrates. The chorion, similarly composed of ectoderm and mesoderm cells, forms the external boundary of the embryo (including all intraembryonic and

extraembryonic cell lineages) and is the primary interface for fetal-environmental (including fetal-maternal) exchanges.

Except for birds, in which gastrulation and primitive streak formation takes place after oviposition (egg laying), all extant amniote embryos initiate gastrulation during their intrauterine development. Variations in amniote early development are manifested as differences in oocyte size (e.g., 30 mm in the chick versus 0.1 mm in the human), cleavage pattern (e.g., meroblastic cleavage with delayed cytokinesis and incomplete cytoplasm partitioning in the chick versus holoblastic cleavage with complete partitioning of zygotic cytoplasm in the human), cell cycle duration (e.g., a rapid-to-slow shift in birds versus a slow-to-rapid shift in mammals), and cell number at the onset of gastrulation (e.g., 100-fold difference between the chick epiblast and the mouse epiblast). These variables lead to diverse epiblast "landscapes" during the transition from pluripotency to a rudimentary embryonic architecture with three germ layers. Furthermore, these preconditions are met with the need to demarcate the pregastrulation epiblast into the intraembryonic and extraembryonic territories to facilitate amniogenesis, and as a consequence, internalization of mesoderm and endoderm cells is initiated at the intraembryonic-extraembryonic boundary. In embryos of eutherian animals (placental mammals, including mice and humans), the gastrulation process comes under the additional influences of implantation and placentation, both of which exhibit pronounced diversity and evolutionary adaptability. For example, a horse embryo initiates trophoblast-endometrium contact 1 month after fertilization, when the embryo has already reached organogenesis stages and placentation thereafter remains epitheliochorial (i.e., with superficial, noninvasive feto-maternal contacts). By contrast, a human embryo starts to breach the maternal endometrium soon after blastocyst hatching and completes the invasive implantation process by day 12 after fertilization, well before the initiation of gastrulation or primitive streak formation (*37*, *38*). Functional differentiation of the trophectoderm lineage becomes a crucial early event for eutherian mammals, and the onset and morphogenetic process of gastrulation are affected to a greater or lesser extent, reflecting physical and structural variations in eutherian feto-maternal interactions.

These variable and adaptable features of amniote early development at the organismal level challenge the notion that gastrulation is associated with a specific structure and underscore the importance of looking for components of the process that are more conserved across amniotes, potentially at the molecular and cellular levels. Genetic studies over the past 20 years have indeed revealed a conserved set of transcription factors and signaling molecules associated with gastrulation in both the amniotes and anamniotes. For example, interactions between bone morphogenetic protein (BMP), Nodal, and Wnt signaling lead to *Brachyury* expression at the onset of gastrulation and serve as a gateway for the specification of different organ and tissue primordia. However, this conservation at the molecular level contrasts with the morphogenetic-level variability (*39*) and leads us to search for common motifs at the level of cell behaviors as a way to understand the origin of the variety of gastrulation modes.

A cellular view of gastrulation

Vertebrate gastrulation is generally associated with the transformation of a cellular aggregate into a bilaterally elongated structure with spatial information conferred in all three germ layers (Figs. 1 to 3). This is accomplished in a species-specific manner. In amniotes, the initial step in this process is a mesenchymal-to-epithelial transition (MET) of the early cells to form the pluripotent epiblast epithelium (*40*), followed by ingression and concomitant specification of the endoderm and mesoderm, with an orderly process of EMT placing the three germ layers with respect to the anterior-posterior body axis (Fig. 1C). At the cellular level, the behaviors associated with EMT are quite well conserved between the mammalian and avian primitive streaks (*41*–*45*). The underlying basement membrane is degraded, and epiblast cells apically constrict to become flask-shaped and then detach from neighboring cells to exit the epithelium (*46*–*48*). At the molecular level, this involves down-regulation of E-cadherin expression (*49*) as well as decreased Rho activity and changes in organization of cytoskeletal components [particularly microtubules, (*41*, *50*) in the ingressing cell] without disturbing the overall epithelial nature of the tissue (*41*, *43*). These changes in cell behavior are associated with similar patterns of signaling and gene expression as well—including increases in Wnt, Nodal, and fibroblast growth factor (FGF) and FGF receptor 1 (FGFR1) signaling—that lead to up-regulated expression of *Brachyury*, *Snai1* and *Snai2*, and *FoxA2* transcription factors.

Similarly, at the cellular level, behaviors underlying formation and elongation of the primitive streak are fairly well conserved, including cell shape changes and polarized rearrangement of cells along the mediolateral axis. However, how these cellular level behaviors are deployed and choreographed to generate tissue shape changes are distinctly species specific, likely due to biomechanical constraints imposed by the particular embryonic environment. In birds, the mesendoderm precursors that will form the primitive streak are induced in a sickle-shaped region at the posterior margin of the area pellucida (*51*) and undergo extensive, polarized rearrangements that result in convergence on the midline and anterior extension (*52*–*54*). Epiblast cells in the area anterior to the forming primitive streak rearrange to extend perpendicular to the primitive streak, leading to anterior-posterior contraction and lateral elongation of this region and large-scale counterrotational flows of cells in regions of the epiblast lateral to the forming streak (*52*).

Studies of gastrulation in mammalian embryos reveal distinct tissue-level differences in primitive streak initiation and elongation from the model established in the avian embryo. The mouse embryo, with an elongated cup-shaped epiblast, has no morphological structure equivalent to the sickle-shaped mesendodermal precursor region of the chick, and the mouse primitive streak forms through progressive initiation of EMT rather than convergent extension of a precursor population (*43*). In discoid embryos (such as those of rabbits or pigs), in which the size and geometry of the epiblast are more similar to those in chick embryos, the posterior margin expands posteriorly and becomes less dense cellularly before primitive streak formation, in contrast to the convergent extension of mesendoderm precursors in the avian embryo (*55*, *56*). In addition, the pronounced counterrotational flows characteristic of the avian embryo are not seen in the rabbit embryo (*55*, *57*). Our knowledge of the primitive streak in human embryos, primarily on the basis of anatomical descriptions and comparisons with other primate embryos, reflects similarity to other mammalian embryos, although additional features (such as the neurenteric canal) have been discovered (*58*, *59*). The differences between avian and mammalian primitive streak formation reflect distinct patterns of biomechanical force generation, perhaps as a consequence of distinct biomechanical architecture at the border of intraembryonic and extraembryonic territories that influences tissue-level behaviors in these embryos. In addition to centrifugal tension exerted by expansion of the area opaca (the region of the epiblast that is physically attached to the yolk) in avian embryos (*60*, *61*), polarization and mediolateral intercalation of posterior marginal cells (*52*) initiate graded tangential forces along the margin of the epiblast that lead to cell shape changes in the marginal cells and formation of supracellular myosin cables in groups of 5 to 20 cells (*53*). The forces associated with this tensile ring are anisotropic, providing active tension posteriorly and passive tension anteriorly, and modelling shows that they are sufficient to drive the posterior epiblast cells forward (*53*). Anteriorly, the marginal tension may provide a boundary that directs the lateral movement of cells anterior to the streak to generate the counterrotational

flows of cells toward the posterior of the embryo (Fig. 4B). Furthermore, the marginal tensile ring may well act to regulate gene expression and to define the region of primitive streak formation (*62*).

It is tempting to speculate that such a tensile ring could similarly provide the boundary conditions to generate tissue-wide cell movement in mammalian embryos with discoid epiblasts, including human embryos. However, the posterior expansion and decrease in cellular density observed in some of these embryos (*56*, *63*), as well as the lack of strong posterior convergence movements, seem inconsistent with initiation of a tensile ring at the epiblast margin (Fig. 4D). Additionally, the effects on primitive streak formation of inhibiting myosin activation through Rho kinase inhibition differ substantially between rabbit and avian embryos (*52*, *57*), suggesting that the role of actomyosin contractility during primitive streak formation and gastrulation is not the same in these two embryo types (Fig. 4). Forces tangential to the margin of the epiblast are very unlikely to play a role in mouse embryo gastrulation, given the cuplike geometry of the epiblast. In fact, it may be that the absence of such tangential forces, as well as a less fluidlike epiblast, act as constraints for the behavior of epiblast cells in mouse embryos, leading to formation of the primitive streak without global cell movements. Additionally, although centrifugal forces on the epiblast may be generated through expansion of the fluid-filled proamniotic cavity (*64*), there is no tension generated by the extraembryonic tissues across the epiblast.

Thus, coordinated cell behaviors, acting under the particular constraints of the embryonic environment, produce tissue-level deformations that generate a linear region of cell ingression. Are these morphogenic constraints a necessity for amniote gastrulation or merely a problem to be solved? The variations in the mode of gastrulation among amniote and anamniote vertebrates [as discussed above and in (*65*)] suggest the latter. In fact, recent data show that manipulation of the extent and cellular behaviors of the mesendoderm in the chick embryo can transform the primitive streak into gastrulation modes more similar to those of amphibian or reptile embryos (*62*, *66*). Data from the field of mammalian stem cell and regenerative biology appear to further support this line of argument.

in vitro models of mammalian gastrulation

Over the past few years, PSCs have been used to generate a number of models of early mammalian development. PSCs are clonal derivatives from mammalian blastocysts [embryonic stem cells (ESCs)] or reprogrammed adult cells (induced PSCs). PSCs can be maintained in culture for many generations without losing their ability to produce all cells of an organism, and they can be steered to differentiate into any cell type by controlling the culture conditions. This differentiation is asynchronous, exhibits large heterogeneities in gene expression (*67*, *68*), and reveals the existence of cell-intrinsic programs of gene expression associated with specific fates. In all cases, cells go through patterns of gene expression that mirror events observed in embryos and, early on, cell fate decisions in the early epiblast. Thus, during differentiation into endodermal and mesodermal derivatives in culture, cells go through a sequence of events similar to those of gastrulation—down-regulation of pluripotency genes, MET, activation of Wnt and Nodal signaling, transient expression of *Brachyury*, and engagement into an EMT (*69*)—before expressing specific fate determinants. However, these changes occur without any multicellular coordination or morphogenesis. Despite the sequential events of MET, expression of *Brachyury*, and EMT, no primitive streak–like structure is visible in the culture.

In an attempt to restrain the heterogeneities that arise in adherent culture, human and mouse PSCs have been cultured on two-dimensional (2D) micropatterned structures (*70*). Under these conditions, cells form tight epithelia, and exposure to BMP, Nodal, and Wnt signals results in the emergence of proportionate concentric rings of gene expression identified as the different germ layers and some of their derivatives during gastrulation (*70*–*72*). In these arrangements, also known as 2D gastruloids, radial symmetry can be broken only by spatially controlled asymmetric flow of signal agonists and antagonists (*73*). These experiments have provided insights into the mechanisms by which cells interpret and respond to morphogens in perigastrulation stages. However, likely because of the confinement of cells on the substrate, cellular growth and movement are impaired and patterns of gene expression do not exhibit the structural evolution that they do in the embryo. In the case of mouse PSCs, this experimental setting has been shown to recapitulate relationships between signals and fates in the embryo that are known from genetic studies, thus validating the patterns observed for human ESCs (*72*). On the basis of movements correlated with cells expressing *Brachyury*, it has been suggested that there is a circular

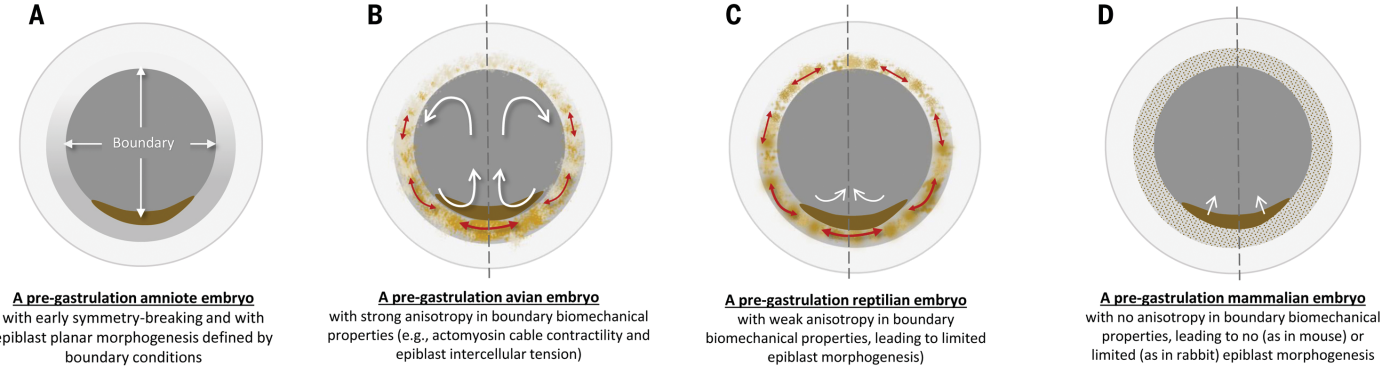

Fig. 4. Biomechanical properties at the embryonic-extraembryonic boundary can influence epiblast morphogenesis before gastrulation. (**A**) An amniote embryo after symmetry breaking but before gastrulation. Epiblast morphogenesis and, consequently, the morphology of a primitive streak–like structure are influenced by embryonic-extraembryonic boundary conditions. (**B**) The avian embryo has strong biomechanical anisotropy at the boundary and undergoes prominent planar rearrangement of epiblast cells, leading to primitive streak formation. (**C**) The reptilian embryo has weak biomechanical anisotropy at the boundary and undergoes limited, regional rearrangement, leading to blastopore formation. (**D**) The mammalian embryo has no or weak biomechanical anisotropy at the boundary and undergoes no (in mice) or limited (in rabbits) epiblast planar rearrangement. A primitive streak–like structure still forms in the mammalian embryo, likely due to directional EMT signal propagation and cell proliferation. In (B) to (D), the dashed line denotes the midline, and the white arrows indicate the direction of global movements of embryonic epiblast cells. In (B) and (C), the red arrows indicate the direction and strength of the intercellular tension force between epiblast cells located at the embryonic-extraembryonic boundary.

primitive streak in these 2D gastruloids (*74*). However, it is not clear that these movements are related to the presence of a primitive streak or whether they are likely to reflect the observation that *Brachyury* promotes cell movement (*69*, *75*). The primitive streak in vivo is a structure with a vectorial component that defines the anterior-posterior axis of the embryo, which is absent in the micropattern experiments. Nevertheless, together with the free adherent culture experiments, this work shows that a primitive streak is not necessary to implement the schedules of gene expression associated with the amniote body plan, as has been suggested by perturbation experiments of avian development in vivo, in which properly patterned mesoderm induction could be uncoupled from primitive streak formation (*76*).

The coordination of signal responses and proportionate patterning of gene expression in experimental systems in vitro highlights the role of cell collectives in cell fate decisions and provides an opportunity to explore the role of biomechanical signals during gastrulation. These arrangements provide a versatile device to address the role that mechanochemical signaling plays in the early stages of development. Thus, BMP and Nodal (*77*) as well as Wnt (*78*) signaling are influenced by the geometry and mechanics of the cellular environment (*77*). In particular, Wnt signaling and *Brachyury* expression, two key regulators of the onset and progression of primitive streak formation, appear to be mechanosensitive. *Brachyury* expression and polarized cell movements can be induced at points of high tension in 2D cultures of human PSCs on soft substrates by Wnt/β-catenin signaling (*78*). This relationship between mechanics, Wnt signaling, and *Brachyury* has been described in other systems (*79*) and suggests that β-catenin might act as a general mechanotransducer during gastrulation.

When PSCs are aggregated and grown in suspension, large-scale patterns emerge that are not predictable from adherent cultures. Thus, under controlled culture conditions PSCs form aggregates that, when exposed to Wnt signaling, undergo symmetry breaking, which is manifest in localized expression of *Brachyury* at one pole of the aggregate, global shape changes, and the expression of mesoderm- and endoderm-specific genes regionally organized with reference to an orthogonal coordinate system (*69*, *80*–*86*). These structures are called "gastruloids" (*80*), and despite the pronounced recapitulation of fate specification and multiaxial organization, they lack a morphologically recognizable primitive streak or any of the aforementioned morphological features associated with amniote gastrulation. Gastruloids emphasize the disconnect between gene expression and morphogenesis revealed in adherent cultures. They also extend the conclusion, derived from micropattern experiments, that multicellular ensembles can pattern gene expression in space and, when allowed to grow, create patterns of structured gene expression that mimic the situation in embryos. Gastruloids also highlight the importance of boundary conditions in early morphogenetic events. In this regard, a recent variation of the gastruloids demonstrated that spatial localization of a Wnt/Nodal signaling center to one end of an aggregate of naïve cells increases the spatial ordering of the fates emerging from the gastruloid (*87*). Mouse and human epiblasts are organized very differently, owing to the arrangement of the extraembryonic tissues, but when these constraints are removed by placing the PCS or epiblast derivatives in culture, both form very similar structures (*85*). This suggests that the primitive streak can be hypothesized as a mechanical response to the boundary conditions surrounding the embryo (Fig. 4). In support of this, when ESCs are placed in culture with trophoblast stem cells (TSCs), they form structures that resemble the early epiblast, in which a primitive streak–like structure can be observed to emerge at the interface between ESCs and TSCs (*88*, *89*). This is even more accentuated when the primitive endoderm is induced in these constructions (*90*). The initiation of gastrulation can also be observed in a human model of amniotic sac formation (*91*), supporting the notion that the primitive streak might be a consequence of mechanical constraints imposed on the epiblast.

Origin and function of the primitive streak: A look beyond

The PSC models of mammalian development provide some insights into the role that gastrulation and its associated structures play in laying down the amniote body plan. In particular, they call into question whether the primitive streak is the hallmark of gastrulation. First, these models reveal that fate-specific schedules of gene expression and morphogenesis, including the primitive streak, are two independent processes that are coordinated during embryonic development in vivo. Gastruloids, which lack a primitive streak**,** still exhibit polarized gene expression and are able to lay down a body plan, but their constituent cells are not spatially constrained at their boundaries, as seen in embryos (Fig. 4). By contrast, when the initial aggregates are 2D and epithelial, they require higher levels of Wnt signaling for patterning, facilitated by BMP from the trophectoderm (*88*), and exhibit EMT-like movements associated with *Brachyury* expression at the interface between the PSCs and TSCs, similar to what occurs in the embryo. This suggests that aggregates of PSCs have an intrinsic ability to break symmetry associated with polarized *Brachyury* expression, but the signaling threshold for these events is raised when the cells are epithelial, and interactions with the trophectoderm bias the onset of symmetry breaking and trigger the localized emergence of the primitive streak. We surmise that having an epithelial substrate for the initiation of the primitive streak in embryos (*40*, *92*) allows for controlled, precise patterning, which is absent in gastruloids. Although PSC models (gastruloids in particular) exhibit a well-organized outline of the primordia for tissues and organs, this outline lacks the precision and detailed patterning characteristic of embryos, which are necessary for the generation of a functional organism. Therefore, the primitive streak may represent a structural feature that allows alignment of the primordia and serves as a morphogenetic conduit for precision in spatial and temporal coordination of early development.

We would like to propose that the avian primitive streak, which has served as the reference structure for amniote gastrulation, is not a conserved structure but rather part of a continuum that spans species-specific structures seen in different amniote clades. It has arisen independently a number of times, as the common ancestor of birds and mammals likely lacked a primitive streak (Fig. 1). One may also view primitive streak–like structures—including avian primitive streaks, reptilian blastopores, and various types of mammalian primitive streaks—in a broader sense, as a manifestation of the midline that indicates the emergence of anterior-posterior polarity from the initial radial symmetry and serves as a reference point for gastrulation movement and precise coordination of three-germ-layer differentiation. In all amniotes, the primitive streak–like structure begins at a precise location that is not only the posterior but also the seed of the midline of the organism. Adopting such a view implies putting less emphasis on the primitive streak as a distinct embryological structure and more on its roles as a conduit for symmetry breaking and coordinated germ-layer differentiation. Furthermore, it supports the view of gastrulation as a process that may begin before the visualization of patterned cell movements. Recapitulation of these functions of the primitive streak in vitro, rather than of its morphological manifestation, will have profound implications in developmental and translational biology.

Our discussion supports the recent reassessment of the primitive streak as it pertains to ethical debates surrounding rules and regulations of in vitro studies of human development (*6*). Currently, as a key element of ethical oversight in human embryo research, the 14-day rule is effective in many countries, including the US, UK, Japan, China, and Spain. This rule reflects an interdisciplinary consensus in developmental and stem cell biology and assisted human reproduction and represents a somewhat arbitrary concept to determine the legal rights of a human embryo (*93*). The scientific

argument was that the primitive streak, formed after ~14 days of human development post-fertilization, represented a definitive sign of human individuality—i.e., from that point on, only one human individual would emerge, endowed with rights and sanctity that must be protected through ethical and legal means. We have argued that the primitive streak is neither conserved nor necessary for germ-layer formation or spatial coordinate acquisition, therefore emphasizing the arbitrariness of the original 14-day decision and supporting the new ISSCR guidelines. However, this line of reasoning does not dispute the necessity of exerting ethical oversight in human-related developmental and stem cell biology research, nor does it preclude defining an alternative developmental landmark as a limit for the culture of human embryos ex vivo. This should be done, as it was by the Warnock committee, through a consensual discussion among different stakeholders to ensure scientific and ethical rigor.

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The primitive streak and cellular principles of building an amniote body through gastrulation

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The nonconserved primitive streak

In human development, a linear structure called the primitive streak appears 14 days after fertilization. This structure marks the transition of the embryo from having radial to bilateral symmetry. The primitive streak also gives anterior-posterior and dorsal-ventral spatial information to cells undergoing gastrulation and forming the various body cell types. In a Review, Sheng *et al*. present a phylogenetic and ontogenetic overview of the primitive streak. They discuss organismal, cellular, and molecular features of the primitive streak and how it functions in amniote gastrulation. The observation that this structure is not conserved and is not required for development in vitro has implications for embryonic stem cell–based models and considerations about human development research. —BAP

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