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Review

"Evolution" of Embryogenesis: Complexity of the Early Developmental Stages in the Animal Kingdom

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Abstract

Multicellularity has gained the advantage over the unicellular world by expanding the number of increasingly more complex tissues that achieve advanced and specific functions. This same event gave rise to the most evolved group of organisms, namely mammals. Even though the complexity of multicellular organisms does not necessarily provide them with excellent adaptation modes performed by unicellular and prokaryotic organisms, this complexity *per se* stands as one of the greatest phenomena in biology. However, there is an inherent set of biochemical and physiological programs that all animals share and tend to execute in similar manners. Most of these processes are evolutionarily conserved and often arise several times with different phylogenetic origins; this implies their importance and universalism in the Animal kingdom. This review summarizes the most significant embryological mechanisms described in many model organisms of echinoderms, nematodes, insects, chordates, and mammals including humans. Although complex and diverse, most of the mechanisms share an outstanding number of similarities that lead researchers to find the answer as to how and in which way the mechanisms had succeeded to help develop complex life.

Keywords

Developmental biology; molecular embryology; gene regulatory network; developmental fields; cellular differentiation

1. Introduction

Developmental biology started its existence with August Weismann's discovery of germ cells being the hereditary agents in all multicellular eukaryotes. Later, the field gained momentum with the experiments of Wilhelm Roux, Hans Driesch, and Thomas Morgan in the late 19th century. It was not until the second half of the 20th century when molecular bases had replaced their theoretical stands. With the discovery of DNA and the launch of genomics, new horizons appeared [1].

Processes of independent distribution of chromosomes [2], transcription factors networking and cooperativity [3-5], morphogens activity [6-8], and establishing embryonic cues with different competence and fates [9, 10] are now better understood and pinned to the enhancing field of molecular embryology and developmental biology. Today, we know much about intercellular communication, cellular signaling, and external molecular cues affecting the developing embryo with downstream effects leading to genomic reprogramming and developmental determinants distribution. The recent research in the cross-talk between developmental biology and other molecular fields has helped establish correlation between cellular differentiation, growth, and senescence which gives insight into cancerogenesis, bodily ageing, and congenital disorders. And yet, the mechanisms of fertilization, embryonic nuclear organization, zygotic genome restart, cellular differentiation, and the embryonic (developmental) fields formation still remain recondite.

At what stage does the first process of cellular specification and competence start? Which mechanisms are responsible for the first cue of differentiation, axes formation, tissue organizers, and direction of growth and development? Do these processes differ among different phyla in the Animal kingdom? If so, to what degree do they differ? Does a common denominator of early development exist? These questions remain unanswered, although the recent advancement in developmental biology and related fields brings us closer to the better understanding of the complexity of embryogenesis.

This review focuses on biomolecular mechanisms of the early embryo development stages presented on the examples of seven different metazoans. The last chapter shifts the focus onto humans with similarities and differences in embryological events compared to other animals. Surprisingly, these are discrete genomic differences found in the agents of embryogenesis that play the major role in defining the supreme developmental process of humans over the rest of animals.

2. Fertilization and Cleavage

The *C. elegans* or *D. rero* embryos are optically transparent which facilitates their examination; similarly, sea urchin's fertilization takes place in water and proper laboratory conditions can be set up to follow the mechanism. Fertilization in higher vertebrates is more difficult to follow, since the process takes place inside the female's reproductive tract and the imaging techniques require a more advanced and skilled approach. However, even though the specific steps of fertilization and the cleavage divisions vary among different animals, the preliminary course of action remains very much alike.

During insemination, the chemoattraction mechanisms facilitate the sperm's entry into the egg [11]. In sea urchins, resact was isolated from the egg's vitelline layer as a chemoattractant for sperm [12]. Bindin was isolated as an insoluble protein on the sperm's acrosome vesicle that binds ERB1 on the egg and facilitates the acrosome reaction [13, 14]. In mammals, a number of molecules from sperm have been recognized to act as chemoattraction substances, e.g. Sp56 (acrosomal matrix glycoprotein), galactosyltransferase and the ADAM (a disintegrin and metalloproteinase) family, which interact with the egg's zona pellucida markers, especially ZP1 [15, 16].

Once the sperm enters the egg, the two pronuclei (condensed haploid chromatin) fuse and form the zygote. The exact mechanism of cortical (egg) and acrosomal (sperm) reactions and the pronuclei fusion differ among animals, but the fundamental principles remain the same; including in humans [17-19]. As shown in their elegant experiment, McCulloh and Chambers (1992) reputed that electrophysiological changes during the gametes fusion can lead to speculative sperm receptor kinases and the PLCγ activation, followed by the intracellular IP_3-driven signaling cascade [20]. Calcium ions play a central role in fertilization by activating a plethora of calcium-dependent intracellular signals, which mediate further rearrangements and early zygote processes, including microtubules nucleation, centrosomes and mitotic spindle positioning, chromatin remodeling, and cytoskeleton rearrangements [21-24]. These processes precede the next major event of embryo development, the cleavage.

Embryo cleavage is defined as the first rounds of cellular division with little to no growth of the dividing cells. This brings the blastula stage, where an embryo solely consists of equally sized blastomeres (the focus is shifted to producing new genetic material necessary for further specification stages). Either through the early genome activation (*Xenopus*, zebrafish) or maternal mRNA transcripts (mouse, human), the embryo depends on newly synthesized proteins that are essential in cleavage initiation [25-27]. Based on this process, animal embryos can be divided into two groups: the autologous embryos (depending on the maternal mRNA transcripts) and the conditional embryos (depending mostly on their own genome activation). Cyclins, cyclin-dependent kinases (cdk), and microtubules, among others, contribute to the mitotic spindle formation which divides each blastomere perpendicularly to the center position of the nucleus (Hartwig's rule). Blastomeres divide into identical daughter cells with a cleavage furrow cutting the previous cell at the right angle (Sach's rule). The rate of cleavage (and the pace of embryo development) is inversely proportional to the amount of yolk in the egg (Balfour's rule).

Classification of embryos can also be based on their cleavage type. In holoblastic cleavage, the whole zygote is completely dichotomized with the evenly deposited yolk. In meroblastic cleavage, the egg is divided incompletely with a portion of the yolk always remaining (Figure 1). According to Collazo et al., meroblastic cleavage appears to have arisen independently five times in different phyla, while holoblastic cleavage is a relatively new evolutionary tool occurring in more developed organisms [28]. Apart from the cleavage types, most animals exhibit a similar pattern of the first cellular division: first division at a longitudinal plane, another one at the angle of 90 degrees to the plane of the first, and the third division being perpendicular to the first two [29]. In echinoderms, the first two divisions are meridional, the third is equatorial, and the consecutive ones are either meridional or equatorial. In tunicates, the first two divisions are meridional. In nematodes, the first division is meridional, whereas the second is either equatorial (for AB cell) or meridional (for

P1 cell). In mammals, the first division is meridional and the second division can be either meridional or equatorial [29].

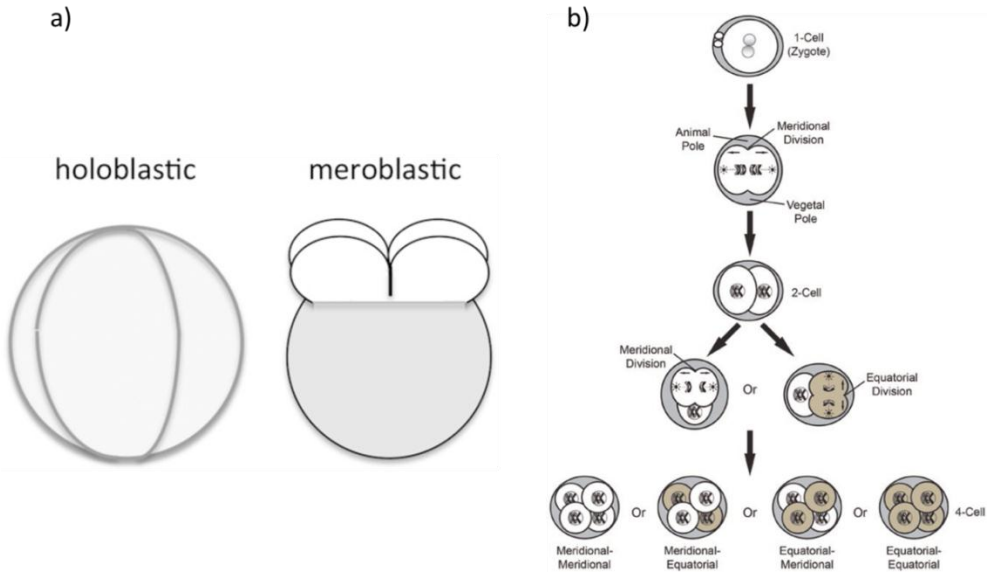

Figure 1 Cleavage types and holoblastic rotational cleavage of mammals. (a) Cleavage in animals is characterized as either holoblastic or meroblastic. In holoblastic cleavage, the yolk content is distributed evenly (equal cleavage) to every daughter cell or the distribution becomes uneven (unequal distribution) with the first division being usually along meridional plane. The yolk content remains intact at the vegetal pole of the egg, so that only blastomeres of the animal pole of the dividing embryo encompass the cleavage site. (b) Mammals represent the holoblastic (equal) rotational cleavage, where the upper tier of cells arrange rotationally over the bottom tier of embryo cells. The first division is meridional (M) and the second division is either meridional (M) or equatorial (E). It has been shown that variants MM or EE have more chances to survive because of even distribution of maternal constituents. Adapted from [29].

It is still uncertain as to how the axis of cleavage is determined by the zygote and its daughter cells. One hypothesis posits that the first division proceeds in an axis perpendicular to the main mass center of the cell [30]. Another one presumes the early fate constituents (autologous embryo type) determining the polarity axis along which the mitotic spindle is formed [31]. For instance, in *C. elegans*, the PAR-6/PAR-3/PKC-3 and PAR-2/PAR-1 complexes establish the primordial anterior-posterior axis along which the division takes place [32]. The question of the primordial cleavage axis in mammals has not yet been answered. Piotrowska and Zernicka-Goetz (2001) posited that the primordial embryo axis is formed and aided after fertilization provoked by the sperm's entry into the egg [33]. Gardner and Davies (2003) pointed out that the animal-vegetal axis of the zygote must be specified before fertilization [34]. Hiiragi and Solter (2004) performed a series of elegant experiments by removing either maternal or parental pronuclei from the gametes and showed that the primary axis in the mouse egg is not predetermined, but established by the spatial arrangement of the two opposing pronuclei [35]. Thus, the primordial cleavage axis in mammalian embryos is determined through the mechanisms triggered by fertilization.

3. The Early Genome Activation

In animals, maternal transcripts generated by the oocyte control the earliest zygotic developmental processes; with time, the quiescent zygotic genome becomes activated and the maternal determinants clear, which is known in the literature as the maternal-to-zygotic transition (MZT). During the MZT, an embryo becomes independent from maternal products and activates its own mRNA synthesis. Another term, the zygotic genome activation (ZGA) refers to the second event of the MZT which is the zygote's genome activation; although different organisms exhibit their genome expression at miscellaneous cell cycles, hence the most preferable term is the early genome activation (EGA). The MZT and EGA are very much conserved in all animals, therefore it is essential to recognize the species-specific developmental programs employed by different organisms [36].

In fast-developing vertebrates (almost all anamniotes), embryos are characterized by expeditious cell divisions following fertilization, lately occurring MZT, and accelerated growth; slow-developing amniotes are marked by much slower embryonic development and the early MZT [37] (Figure 2). In *Xenopus*, zebrafish, and *Drosophila* (anamniotes), the maternal factors (mostly the oocyte's mRNA molecules, including mi-RNA and piwi-RNA) are sufficient to guide the first cells through the earliest biological development [38-42]. In amniotes (including mammals), where the dependence on maternal constituents has not been objectively defined, many signaling molecules activate the zygotic genome (whose first products maintain the blastomeres homeostasis), regulate the chromatin permissiveness and its protein content, and control the cell cycle [43, 44]. Levels of early accessible transcription factors are also tightly regulated through miscellaneous mechanisms which include sequestration and phosphorylation [45, 46], *trans*-acting inhibitory factors [47], specialized promoters transactivation [48], and switching from TATA-containing to TATA-less promoter activation [49, 50].

The embryo genome is activated stepwise or by the so-called waves. The waves of newly transcribed genes appear because the early genes may be deleterious at the later stages for the embryo development, and thus the complex transcription machinery must decide of singular gene permissiveness timing as well as the spatial determination [51]. There are three possibilities of how the waves occur in the early embryos: clock-like zygote's genome activation (gene cluster A, then gene cluster B, etc.), cell count-dependent mechanism (a number of blastomeres impose the chromatin remodeling), and stepwise cycling (maternal factors trigger the EGA, which leads to further cellular growth and specialization) [51].

Researchers share different ideas on which mechanisms are critical for the embryo to switch from maternal to its own genome activity. Maternal factors may deposit in the dividing cells and initiate the "domino effect" for singular *loci* activation if the threshold for the maternal factors concentration is reached [36]. Also, the nuclear to cytoplasmic ratio (N:C) plays a significant role in the EGA [52]; with every cell division, 2- fold decrease of cytoplasm volume leads to doubling the N:C ratio, which might lead to the maternal mRNA transcripts titration, including the proteins involved in the ZGA inhibition [53]. Similarly to this hypothesis, another one proposes that free histones and transcription factors compete for specific genes *loci*, and that reducing the histones concentration by increasing the N:C ratio advances the transcription onset of the particular genes that had been repressed prior to the embryo cells cleavage [54].

Multiple genes have been correlated with the EGA in animals and their relatedness to the underlying molecular processes in the blastomeres have been recognized. For instance, in zebrafish Nanog, SoxB1, and Pouf5f3 (mammalian *Oct4* homolog) are critical to initiate approx.75% of the first ZGA stage genes transcription [55]. In mammals, Nanog, Oct4, TIF1α, DUX, and HERVL (endogenous retroviral element) support the early (2-, 4-, and 8-cell stage) embryo genome activation [56, 57].Understanding how the MZT is initiated, maintained, and controlled over the early course of embryogenesis is essential to understanding the developmental cues of embryogenesis.

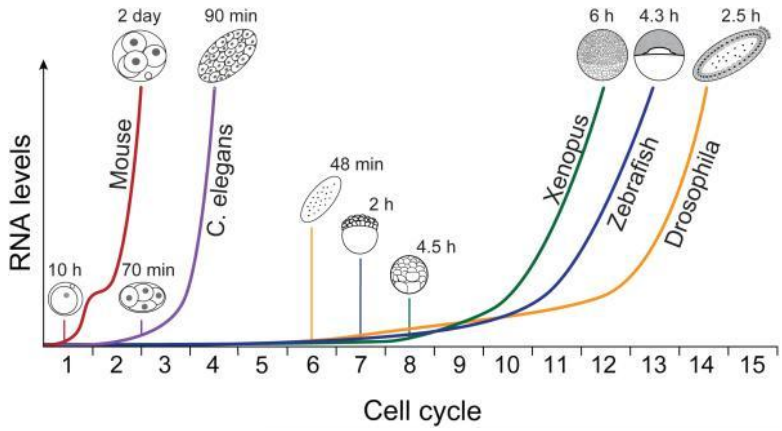

Figure 2 Timing of increase in the cytosolic mRNA level transcripts in different model organisms. The EGA is one of the two most relevant events of the maternal-to-zygotic (MZT) episode in most animals. Fast-developing animals express their own genetic material after many completed cell cycles (rapid growth – late MZT), whereas slow-developing animals usually do not depend on any maternal effect and hence activate their genome after 1-2 first cleavage divisions (slow growth – early MZT). Notice that the *fast* and *slow* development refers to the time interval between subsequent cell cycles in developing animal embryo. Mouse is an example of slow-developing animal; nematodes, amphibians, fish, and arthropods represent fast-developing animals. Adapted from [36].

4. Maternal Effect

The maternal effect is responsible for the autonomous embryonic specification through the early oocyte mRNA transcripts not depending on the EGA. This embryonic effect is mainly observed in the fast-developing animals that depend on the maternal factors synthesized in the egg. Maternal mRNA transcripts, depending on the species, control meiotic egg maturation, early anterior/posterior (A/P), dorsal-ventral (D/V), and left-right (L/R) axes formation, the germ cells region and lineage development, and gastrulation fate causes establishment [58, 59]. These pre-fertilization egg's cues containing maternal mRNA transcripts in different gradients found in different cell's compartments contribute to the phenomenon called the maternal anisotropy [76].

For instance, Bicoid and Nanos are the abundant axes and segmentation determinants in *Drosophila* (Table 1). During early developmental stages, *bicoid* and *nanos* primordial mRNA transcripts are transported to the anterior and posterior egg poles, respectively [60, 61]; Bicoid represses the caudal mRNA transcripts (Nanos, Caudal, Oskar) in the anterior pole establishing the

A/P axis of the oocyte [62]. Bicoid also enhances the anterior gap genes, whose varying concentrations lead to the pair-rule genes expression which establish the A/P axis perpendicular to periodic band units [62]. Another example of the maternal effect is the Balbiani body found in many vertebrates. The Balbiani body is a transient collection of organelles, inclusions, and molecules that assemble adjacent to the nucleus of primary oocytes [63]. Dosch et al. discovered that zebrafish' *Bucky ball* mediates the Balbiani body localization in the vegetal pole of the oocyte and thus overrides the default animal pole establishing the earliest A/P axis [64].

Table 1 Diversity in the onset and execution of developmental programs in Animalia.

Organism	Development type	EGA timing	Cleavage type	Early axes established in the oocyte	Autonomously specified cell lineages	Maternal effect
Sea urchin (echinoderms)	Slow	18-22 h	*Holoblastic* radial	An/Vg[1]	Mesenchyme	β-catenin/Tcf, Otx
C. elegans (nematodes)	Fast	90 min	*Holoblastic* rotational	A/P	Mesenchyme, germ line, gut	PAR-6, PAR-3
Drosophila (arthropods)	Fast	2.5 h	*Meroblastic* superficial	A/P, D/V	Pole cells, major pattern segments	Bicoid, Nanos, Caudal
Zebrafish (chordates)	Fast	4.3 h	*Meroblastic* discoidal	An/Vg	---	radar, yobo, janus, foxH1
Xenopus (chordates)	Fast	6 h	*Holoblastic* radial	An/Vg	Gut endoderm, ciliated ectoderm	VegT, Xbrachyury, Antipodean
Mus (mammals)	Slow	1-2 d	*Holoblastic* rotational	---	---	DICER1, Ago2, HR6A, Zar1
Human (mammals)	Slow	4-8 d	*Holoblastic* rotational	---	---	Mater, ZAR1

According to Davidson et al. [10], there are three types of embryos. Type 1 (echinodermates, nematodes) represents all metazoans expect for arthropods and vertebrates with both autonomous and conditional specification, rapid development, and lineage-specific activation of gene batteries. Type 2 (chordates, mammals) embryos encompass all vertebrates which are characterized by mostly conditional specification, slow or fast cleavage rate, and late embryo axis formation with early regional body plan execution. Type 3 refers to Arthropoda that exhibit the very unique cellularization process (cellular division with no plasma membrane formation) synchronous with rapid cleavage and prespecification of A/P and D/V axes in the egg. Cleavage in mammals is symmetrical and there is no experimentally determined early axis formation until the blastocyst stage (128-cell stage) which coalesces with their only conditional (indeterminate) specification; even though the maternal effect is majorly found in animals with autonomous specification, it has been show that maternal transcripts can be used by the egg and the early embryo in more contextual framework (see the text). [1] animal/vegetal axis.

It has been commonly regarded that the maternal control does not occur in slow-developing

animals, especially in vertebrates [65]; however, recent results have proven the notion wrong [65-70]. Among others, Zernicka-Goetz & Piotrowska and Gardner & Davies have shown that the dorsal-ventral axis formation in the mouse embryo relies on the maternal gene products [71, 72]. Since then, a great number of studies have shown that the maternal effect does indeed take place in mammals as well [70, 73-75].

5. Signaling Pathways

The cell's ability to receive and respond to both extracellular and intracellular signaling molecules decides about its survival and functionality. Signaling pathways are indispensable for interblastomeric communication, placenta-embryo cross-talk, and navigation of the molecular events inside the embryo cells. Dozens of signaling pathways have been described in literature, but some of the most critical are FGF, Notch, Hedgehog, Src, EGFR, BMP, and Wnt. Responding to hormones and morphogens in different concentrations, a signaling pathway may activate or repress the same gene or a group of genes and interact with each other stimulating and modeling the signal. A great deal of signaling molecules also act as the short- and long-range morphogens, including hedgehog ligands in limb formation [77], Wingless proteins in body patterning [78], and the TGF-β pathways in establishing the imaginal discs in insects [79].

A description of all or even a portion of the signaling pathways engaged in the embryo morphogenesis in animals exceeds the scope of this review. However, it will expand on the significance of extracellular signaling in the example of the peculiar Hedgehog signaling pathway (Figure 3).

Hedgehog proteins are conserved across a plethora of animals, especially the Hedgehog ligand whose Hedge domain is represented in protozoa and metazoa [80]. Starting from choanoflagellates, to sponges, to cnidarians, to mammals, Hedgehog directs the primordial body patterning and tissue development [81]. By its unusual covalent coupling of cholesterol at the C-terminus and the N-terminal palmitoylation, and the conserved nature in most Bilateria, Hedgehog and other proteins related to the pathway seem to be at the odds with other signaling pathways [82].

The Hedgehog signaling has been extensively studied in *Drosophila melanogaster*, from which it had first been cloned [83]. Therefore, the following signal transduction pathway is based on the comprehensive analysis of the flies' mutations of the pathway's core components that are, in most cases, shared among vertebrates and invertebrates [80].

Located intracellularly, the Hedgehog signaling complex (HSC), composed of a few serine/threonine kinases, including protein kinase A (PKA) and glycogen synthase kinase 3 (GSK3). Phosphorylate Cubitus interruptus (CI), which is further truncated to its repressor form (CI-R) that moves to the nucleus and inhibits the set of genes that Hedgehog signaling controls [84, 85]. In the absence of Hedgehog, its transporter-like receptor, Patched, inhibits a G protein-coupled receptor (GPCR) family member, Smoothened (SMO) [86]. However, if the Hedgehog protein (ligand) binds Patched, SMO is released from inhibition and becomes phosphorylated by the kinases of the HSC. Once phosphorylated, SMO interacts with Costal 2 (COS2) and helps it become phosphorylated; phosphorylation of COS2 releases CI from the HSC [87, 88]. The full-length CI (CI-FL) migrates to the nucleus, but unlike its suppressor variant, it interacts with a vast array of genes by activating

their transcription [89]. The homologs to *Drosophila*'s CI proteins in vertebrates are GLI proteins (GLI1, GLI2, GLI3), which are the downstream effectors of the Hedgehog signaling [90].

In mammals, three isoforms of Hedgehog exist: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) [91]. Shh is involved in the notochord, neural tube, and limb formation [92]; Ihh signals in the embryonic bone and cartilage tissues [43], whereas Dhh is mainly active at the sites of the germ cell line development and peripheral nerves [94]. Although it must be remembered that the same signaling pathway can be present in multiple sites of the developing embryo demonstrating miscellaneous and very often contradicting functions in cell growth and tissue formation. More so, signaling pathways, such as Hedgehog, intertwine with many other signaling molecules and morphogens developing the molecular framework of activation and inhibition signals commanding the transcription changes in the given sets of genes.

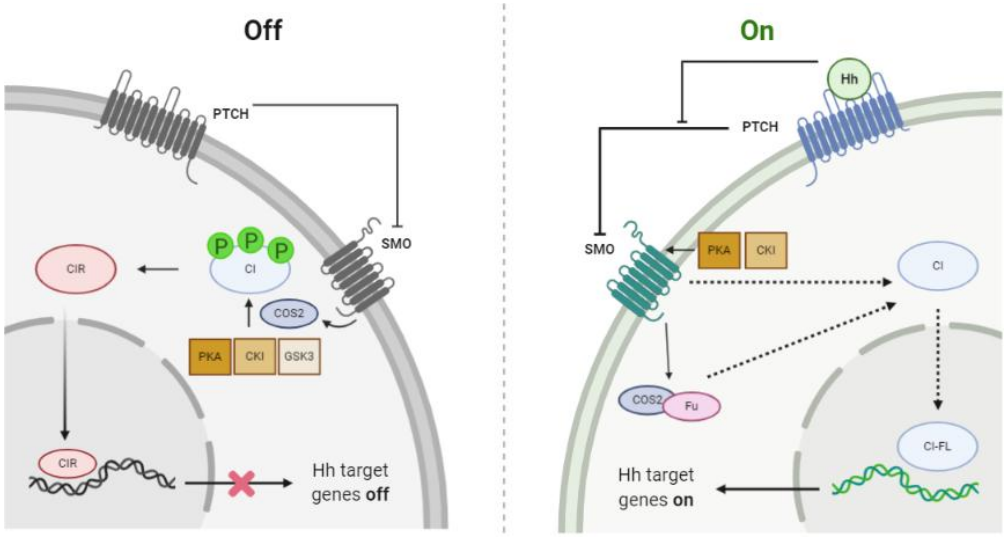

Figure 3 Hedgehog signaling pathway in *Drosophila melanogaster*. Hedgehog signaling portrays classical "on/off"" signaling pathway mode. In the absence of Hedgehog ligand (on the left), Patched (PTCH) inhibits Smoothed (SMO); the lack of SMO activity allows the Hedgehog signaling complex (HSC) kinases phosphorylate Slimb which mediates proteasome proteolysis of CI to its truncated repressor form, CIR. CIR is translocated to the nucleus and inhibits expression of all genes controlled by the Hedgehog signaling. Once Hh binds PTCH, inhibition of SMO is ceased, which becomes phosphorylated by PKA and CKI. Phosphorylated SMO recruits the COS2-Fused complex (Fused (Fu) is a Ser/Thr kinase) which otherwise would phosphorylate CI. Dissociated CI-COS2 complex frees CI and its full-length variant moves to the nucleus and activates the downstream Hedgehog-dependent genes.

6. Transcription Factors

These transcription changes are modulated by *trans*-acting molecules, transcription factors. Transcription factors (TFs) act as molecular switches turning the genes on or off and manipulating the genomic identity of specific tissue. In the developing embryo, morphogens and signaling pathways work together to act downstream on specific transcription factors whose DNA binding sites enable them to activate and/or deactivate a set of genes responsible for explicit functions of

the cell. TFs have binding sites for other proteins, too, and by reciprocal interactions they establish complex circuits that can be regulated and acted upon by signaling pathways, growth factors, mitogens, intercellular communication and environment sensing [95-97].

Transcription factors bind to or nearby the transcription start site (TSS) in the gene's promoter, where they regulate formation of the preinitiation complex (PIC) (the "core" PIC is composed of RNA polymerase II and the six transcription factors (A, B, D, E, F, H)) tethering to the consensus DNA *cis*- regulatory sequence, a TATA box [98]. The PIC factors control transcription of genes by regulating RNA polymerase engagement, its movement downstream the gene sequence, and the pace of mRNA polymerization; these proteins are conserved among different clades of animals and they are non-specific. However, if the promoter is activated by specific transcription factors with distinct binding sites at their domains, they can control spatial and temporal expression patterns of their target genes. Many transcription factors act upon the *cis*-regulatory elements (CRE) of a gene, such as enhancers, silencers, and insulators (distal regulation) to modulate the gene activity through chromatin remodeling, long-range looping, and non-coding RNA molecules [99-101]. CREs are the final target sites of often complex gene regulatory networks comprised of multiple functional motifs acting as genomic logical input gates, such as multi-input motif (MIM), dense overlapping region (DOR), or single-input motif (SIM) [98]. These high-leveled regulatory networks are especially present during the early gastrulation stages when the first progenitor fields and germ layer borders are established.

The large-scale meta-analysis of 119 transcription factors of 5 human cell lines were analyzed for their genome-wide binding profiles and hierarchy structure in building the genome regulatory network [95]. It was found that the human transcription factors establish a three-layered hierarchical network with the top (T) TFs regulating the lower orders of the network and co-associating with nc-RNAs and phosphorylome, the middle (M) TFs acting as the information-flow bottlenecks pipelining the communication between regulatory elements and the target genes, and the bottom (B) factors coupling with other "executive" TFs to change the state of expression of selected genes. This selectivity is controlled through a multilevel combinatorial and context-specific mode differentiating at specific genetic loci and being highly variable in the context of proximal (the core promoter site) and distal (intergenic, chromatin-related regulation) interactions. These findings overlap with the gene regulatory networks analyzed in other organisms [103-106].

Transcription factors receive signaling from the cellular environment that stimulates either one cell, a group of cells, or the entire embryo region. The Spemann-Mangold organizer in amphibians, which gives rise to the central nervous system, is induced by the dorsalizing Nieuwkoop center located in the dorsalmost vegetal cells [107, 108]. Formation of the Nieuwkoop center and the Spemann-Mangold organizer requires the maternal Wnt signaling. The organizer also needs the Wnt pathway to activate two key mesodermal transcription factors: *siamois* and *twin* [109]. These two proteins regulate the expression of an autoregulatory homeobox family transcription factor, Goosecoid, which directly controls the Spemann-Mangold organizer [110]. In this scenario, the *goosecoid* gene is the final target of a complex MIM regulatory network.

In the chicken embryo, Hensen's node is the analogous structure to the Nieuwkoop's center, which activates two sets of genes: the posterior marginal zone (PMZ) genes (*Vg1* and *nodal*) and the anterior border-setting genes (c*hordin* and *sonic hedgehog*) [111]. Chordin and Nodal are the bone morphogenetic protein (BMP) antagonists that dorsalize ectoderm and mesoderm, but they are insufficient for the nervous system induction [112]. In this transcription factors network, the

fibroblast growth signaling (FGF) pathway activates *brachyury* and *tbx6* to specify the mesoderm cells and antagonize BMP [113]. These transcription factors also activate *sip1* (intercellular mediator of the transforming growth factor-β signaling) which prevents primitive streak cell ingression and allows the neural plate cells to remain in the epiblast.

These are only a few examples of how transcription factors operate in developing embryos. Together with morphogens, transcription factors constitute the spatiotemporal cues of the developing embryo in animals.

7. Morphogens

One of the greatest findings in developmental biology was presented by a mathematician. Alan Turing, in his 1952's *Chemical Basis of Morphogenesis* was the first to coin the term "morphogen" [114]. By definition, a morphogen is a biologically active substance with heterogeneous spatial distribution in a developmental field. This chemical substance fits the reaction-diffusion biological model which corresponds to the spatial and temporal changes of its concentration and further diffusion over a function of distance and time [115]. Morphogens occur in tissues in the forms of "waves" with a departure from equilibrium as an oscillatory drift; these oscillations are the temporally and spatially concentration gradients that organize different cell types in a defined spatial array (Figure 4).

According to the iconic French flag model, differential cellular responses elicited by a morphogen are the direct read-outs of different threshold levels of the morphogen which a given cell is exposed to [6]. Over the spatially distributed gradient of a morphogen, the low-threshold responders (cells) are activated by small doses of the morphogen, while the high-threshold responders need much higher concentrations of the same molecule [116]. These observations lead to formulating three main properties of biologically active morphogens. First, a morphogen must be distributed with a concentration gradient across a tissue (biological field). Second, it must act over great distances, not only through paracrine secretion. Finally, a morphogen must act directly on the cell and change expression of the genes [7].

The first discovered morphogen was Bicoid in *Drosophila* [117]. Bicoid determines the anterior pole of the fly's embryo by segmental distribution and interactions with other proteins; the anterior gradient is regulated through the very low affinity of the target cells – only high concentrations of Bicoid protein change the target cell's genes expression [118]. Several years after the identification of Bicoid, another morphogen was described in *D. melanogaster*, Decapentaplegic (dpp) [119]. This member of the *Drosophila* TGF-β family defines the dorsal-ventral axis in the blastoderm and drives disparate cell lines' fates due to its concentration gradient [120]. To add to the complexity of how signaling pathways, morphogens and transcription factors dovetail on different molecular levels of all embryonic events, it was shown that the posterior pole cells (of the egg), which express a segment-polarity transcription factor Engrailed (en) that induce hedgehog expression; Engrailed acts here as a morphogen more than a protein of a signaling pathway. The hedgehog gradient finally instructs the neighboring cells to express *dpp*, *tolloid*, *shrew*, and *screw*, among others, which confine the dorsal part of the embryo while being repressed by the ventralizing factors such as *dorsal* and *pipe* [121, 122].

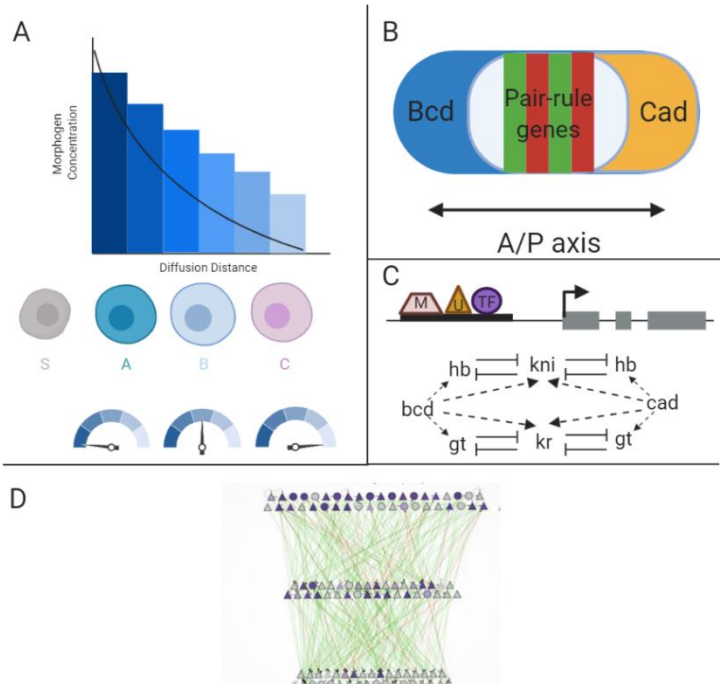

Figure 4 Morphogen gradient in a developing tissue and its biochemical activity toward the target transcription factors. (a) Long-range morphogens act in the gradient fashion by first regulating expression of the gene with the lowest affinity for that morphogen and regulating genes at further distances by a decrease in a morphogen's concentration. The differential response of cells to the morphogens gradients establishes differential genes patterning within the separate populations of cells which allows for the formation of developmental fields (*S,* source cell; *A, B, C*, different cell types). (b) The anterior/posterior (A/P) axis establishment in *D. melanogaster* through the action of different morphogens forming their own gradient niches across the maturing oocyte. Bicoid (bcd) and Huntchback (hb) form the anterior gradient in the oocyte preventing the *caudal* mRNA expression in the anterior part of the egg and "pushing" it back, where Caudal establish the posterior pole. Pair-rule genes divide the embryo into seven transverse bands with equal distribution of determinants and body segments polarity, further formulating the A/P axis. (c) Modular regulation of target genes by morphogens and activated transcription factors network. Morphogen effectors (M) integrate the patterning signals by binding to specific *cis*-regulatory elements (CREs) in the DNA and to multiple TFs, including uniformly expressed factors (U), regulatory factors, and repressors that comprise the morphogen-regulated transcriptional network. (d) Exemplary transcriptional network of DNA-binding and non-binding *trans*-regulatory elements and their intermodal hierarchical control (adapted from [95]).

Formation of the dorsoventral axis in *Xenopus* expresses the complexity of developmental field establishment and modeling. After the sperm entry, GSK3 is deactivated hence freeing β-catenin that accumulates in the dorsal portion of the amphibian embryo [124]. β-catenin and Tcf3 form a heterodimer that activates two transcription factors: Siamois and Twin. These two proteins interact with other transcription factors induced by TGF-β family members, including Vg1, Activin

and Brachyury, in order to upregulate the expression of a homeobox protein, Goosecoid [109, 125, 126]. Goosecoid is also a morphogen as it acts in the gradient manner and supports the organizer formation [127]. However, the BMP family members act counter to β-catenin, TGF-β family members and Goosecoid by guiding the organizer's neighboring ectodermal cells to become the epidermis (instead of the default neural tube tissue). The organizer center responds be secreting the BMP inhibitors: noggin, chordin and follistatin [128]. It also represses the *Wnt8* gene whose product ventralizes the amphibian embryo [129]. This example represents two significant features of the morphogen system: many signaling pathways act as morphogens and that inhibitors consistently produced by the neighboring cells form an opposing gradient repressing the antagonizing oscillatory wave [7, 123].

The complicated interplay between morphogens, signaling pathways and transcription factors allows for the precisely controlled mechanisms to navigate the formation and development of progenitor fields in the embryos. These mechanisms conclude in changing the cells' competence and what lineage they can differentiate into. Their state of lineage potency is usually determined by the fine balance between activation and repression of genes.

8. Cellular Differentiation

Differentiation is the final step in a cell's fate determination, where one cell lineage differentiates into another; this is the stage where the activity of morphogens, signaling pathways, and TFs conclude the cell fate determination. By the narrower definition, cellular differentiation is the committed and gradual change of the cell's potency into a specialized tissue cell which cannot differentiate any further (terminal differentiation). In order to develop into a specific cell lineage, the group of cells is first inducted by an external inducer that generates a concentration gradient of other inductive signals (the signaling cascade), which produces a sequence of differently induced cells. When this happens, these cells are *specified*, which means they are reversibly committed to develop into specific cell lineage and when additional extracellular signaling occurs (mostly through morphogens and direct intercellular communication), the specified cells commit irreversibly to their proper cell fate in the process of *determination* [130].

During the stage of specification, inducers make a given cell *competent* to respond to specific inductive signaling which either activates or deactivates a group of genes dedicated to only a few lineages. The best example is the mesodermal development in most vertebrates. Retinoic acid (RA) gradient in early mesodermal regions establishes the boundaries of Hoxb4, which subsequently makes the intermediate mesoderm cells competent to three paracrine transcription factors, Lim1, Pax2, and Pax8 which lead to the nephron tubules formation [131, 132].

One of the pioneering experiments in developmental biology that explain the mechanisms of early cell fate determination in animals was done on 8-cell tunicate embryos [133]. The yellow crescent cytoplasm was transferred from a B4.1 blastomere (developing further to endoderm and mesoderm, including skeletal myoblasts) to B4.2 cell (primarily fated as ectoderm), which induced it to develop into the skeletal muscle cell line. Competence prepares the cell to enter one of the given lineages, but a strong inducer can redefine the cell's specification and a cascade of determinants may lead to the ultimate differentiation. If the thoracic somite in a chick is transplanted to the cervical region, the animal will develop ribs in its neck [134]. This happens because the paraxial mesoderm cells are highly sensitive to the BMP gradients and the antagonists'

(Activin, Noggin), but also to the FGF and Wnt signaling pathways [135, 136]; the combinatorial effects of Wnt/β-catenin/FGF signaling opposing the high gradients of retinoic acid (RA) in the vertebrate somites is due to a molecular oscillator, the segmentation clock, which is another molecular rhythm device alike the one acting during the EGA that depends on the interplay between region-specific regulators and modifiers [137]. Henceforth, the introduction of the aforementioned thoracic somites to different morphogens and their gradients, signaling pathways, and paracrine factors specifies them and makes them develop into an entirely new cell line.

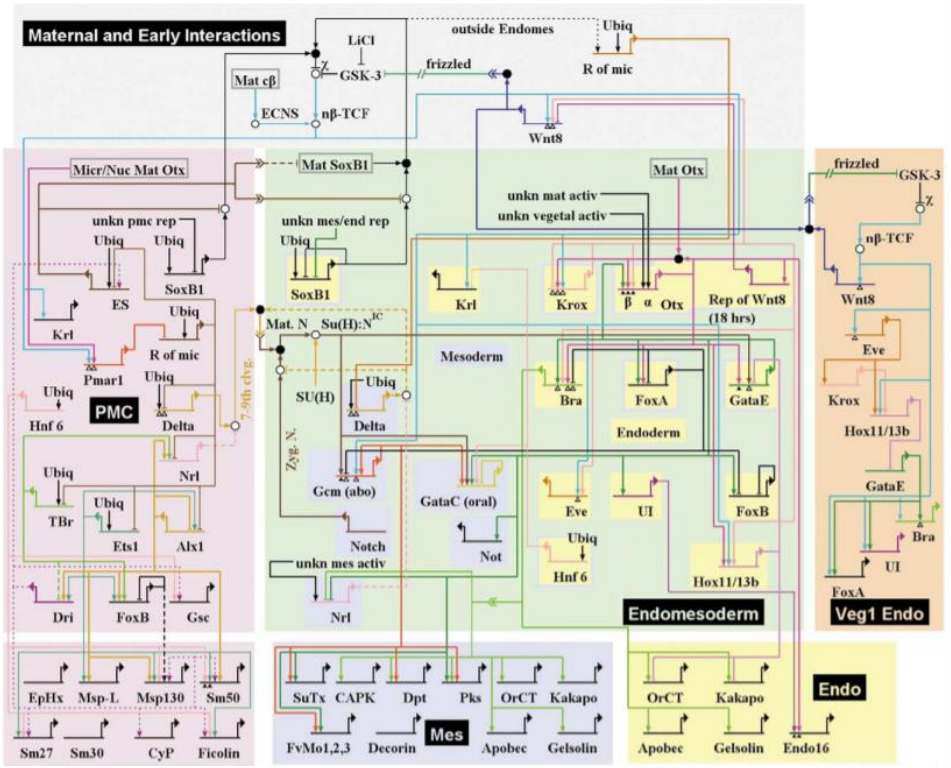

Figure 5 Exemplary gene regulatory network (GRN) of endomesoderm specification in sea urchin embryo. The interactions between *cis*- and *trans*-regulatory elements comprise the genetic modules or batteries controlled by specific binding sites of promoters and transcription factors changing the differential outcome of any given embryonic cell. The differential outcome varies at any given temporal and spatial configuration of the total set of transcription factors found at the differentiating cell. Each specified cell, in order to become fully determined into a tissue-specialized cell, must receive a distinct set of signals that will change its differentiating potential; this is achieved by the precise control of transcriptional network aggregated in separate *circuits* reciprocally affecting other circuits in a greater network of genetic regulation. Circuits compose the spatial domains (black boxes) which further build the bigger GRN that controls all embryonic events located in separate developmental fields. Short horizontal lines represent *cis*-regulatory elements; a bent arrow depicts transcription, while all the genes read at the bottom belong to the periphery of the GRN and are the ultimate target genes of higher (hierarchically) regulatory circuits. Black triangles represent the identified binding sites, while white triangles represent non-verified binding sites. Dotted lines indicate indirect relationships. Adapted from [96].

How do inducers change the fate of the cell on the genomic level? One of the finest approaches for explaining this phenomenon is seeing genes congregated as hardwired logical systems called batteries [76]. Each battery has a *cis*-regulatory module controlled by a very specific set of *trans*-acting modifiers (TFs). A node is a gene encoding one transcription factor and also having its own *cis*-regulatory module (sequence). Nodes cluster into batteries, and these construct the entire gene regulatory network (GRN) of an organism (Figure 5). By convention, repression is the dominant gene expression state, therefore inducers and *trans*-acting factors modulate the expression pattern of separate "batteries" of genes with a cumulative effect of changing the target cell's genomic identity. Ultimately, cells with similar lineage identity the make up the progenitor field, where the subcircuits of the GRN share the same clusters of transcription factors.

9. Intercellular Communication during Embryogenesis

The community effect is another course of action taken by the embryo cell through which the progenitor fields are established [138]. The competence for specification is also achieved through TF loops, chromatin remodeling, and autocrine synthesis of both a receptor and a ligand for signaling pathways, but it is the interblastomeric communication which maintains or changes the cell's differentiation state.

In Figure 6, the interactions between tissue A and tissue B can be either activating or inhibiting and these instructive interactions are carried by autocrine or juxtacrine (morphogens) signaling, or direct communication between cells in tissue A and tissue B; if another sequence of cells is engaged (tissue C), the cumulative effect of all three signaling sources can be diverse and reshape this specific progenitor field. There is a marvelous number of such interactions that define the community effect.

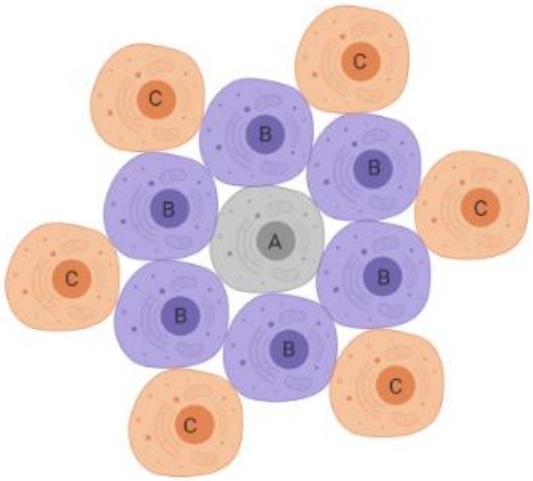

Figure 6 Community effect of blastomeres. Signaling is reciprocal and mixed, where the activation and inhibition inputs can affect the same cell and encode a combinatorial output. Interblastomeric communication is dynamic with the flow of differentiating information always adjusting to the nearby cell-to-cell communication and secreted signaling molecules diffusing over some distance of the cell community.

The sea urchin veg_2 cells (second vegetal tier's cells) produce and secrete Wnt8 that affects only these cells. At the 60-cell stage embryo, Wnt8 is localized in all veg_2 tier cells [139]. The posterior and anterior cells in the *Drosophila* embryo synthesize two transcription factors crucial for intrasegmental patterning: Wingless and Engrailed; Engrailed is required to maintain the Wingless expression, which in turn activates the expression of Engrailed in the nearby cells [140]. The Nodal and Shh's co-play determines the left-right axis asymmetry in different animals where the right side cells express Activin, Fgf8, and Snail to block the synthesis of Shh and Pitx2; the left side depends on the Nodal/Hedgehog signaling which either inhibits the BMP signaling or *snail* expression or upregulates the synthetic activity in cells producing Cerberus, Lefty-1, and Pitx2 [141-143]. These are only a few examples of how intercellular communication is important to maintain or modify the changes of differentiation state in developing cells.

During embryogenesis two events occur interchangeably: the mesenchymal-epithelial transition (MET) and the epithelial-mesenchymal transition (EMT). MET is characterized by the cell's loss of motility, multipolar morphology and mesenchymal markers, such as N-cadherin, Twist, or fibronectin [144-146]. EMT is the reverse process, where mesenchymal cells gain all the features of epithelium, including gap junction, tight and adhere junctions, apico-basal polarity, and molecular markers such as E-cadherin, claudins, and connexins [147, 148]. It is the mesenchymal's interactions with the extracellular matrix (ECM) and its proteins, and the epithelial cell-to-cell adhesion that largely drives embryogenesis in many animals. Many studies have tried to point at the earliest stage of the intercellular signaling occurrence and this matter has not yet been resolved. The research on *Cx43* gene, coding for connexin essential to the formation of gap junctions, showed that the gap junctional intercellular communication (GJIC) does not occur before the 8-cell stage mouse embryo [149]. In another study, blocking the *Cx43* expression in the pre-implantation mouse embryo led to downregulation of "stemness" factors (Nanog, ALP, Oct3/4) and drastic embryo development changes [150]. Those results demonstrated the opposite notion, where the GJIC does emerge at the very early embryonic stage.

Gap and tight junctions are only some of many examples how intercellular communication regulate cellular differentiation and establish numerous progenitor fields in the developing embryo. Direct determinants distribute between cells, juxtacrine and paracrine signaling through dissolved proteins, morphogens' gradients across different developmental fields, transcription factors looping – these mechanisms target chromatin and gene regulatory sequences that eventually concentrate on gene expression and the patterning of embryonic fields. Such mechanisms are observed in simple *Volvox* colonies and in the highly complex gene regulatory networks of human development [151].

10. Embryogenesis Mechanisms – Human Perspective

Humans share most embryological phenomena with other mammals which justifies the usage of experimental data from rodents, for instance, to explain the same molecular mechanisms in the human model. Major discrepancies occur in the earliest stages of cleavage and genome activation, and later during establishment of the specific developmental fields.

The human embryo represents the holoblastic rotational cleavage with equal distribution of yolk [29]. Humans are slow-developing animals with the EGA shift at the 4- to 8-cell embryo stage; however, there is a lack of consensus whether or not that human embryos depend on the

maternal effect [152]. Like in mice, human embryos undergo pronuclear fusion, temporally distinct cleavage phases, compaction, and cavitation [37]. Moreover, both humans and mice exhibit stage-specific gene activation during the EGA phase [153]. However, the major genome activation (major EGA) with a dramatic reprogramming of gene expression in mice starts at the 2-cell stage [51], whereas, the major clearance of maternal determinants and independent embryo gene expression begins at the 4-cell stage in humans [154] (Figure 7). Zernicka-Goetz (1998) applied microsurgery to separate the animal and vegetal poles of the mouse zygote (meridional cut) and showed those eggs successfully developed into morulae and blastocysts indicating no physiological relevance of the spatial patterning (maternal effect) [155]. Similar experimental approach had been taken in the past generating analogous results [156]. On the other hand, Antczak & van Blerkom (1997) had showed that leptin and STAT3, a cytokine and a signal transducer, are spatially polarized in mural and human oocytes proving the spatial patterning in mammals [157]. Recent data indicate that even slow-developing animals depend on the maternal determinants to some extent, including humans [158-160].

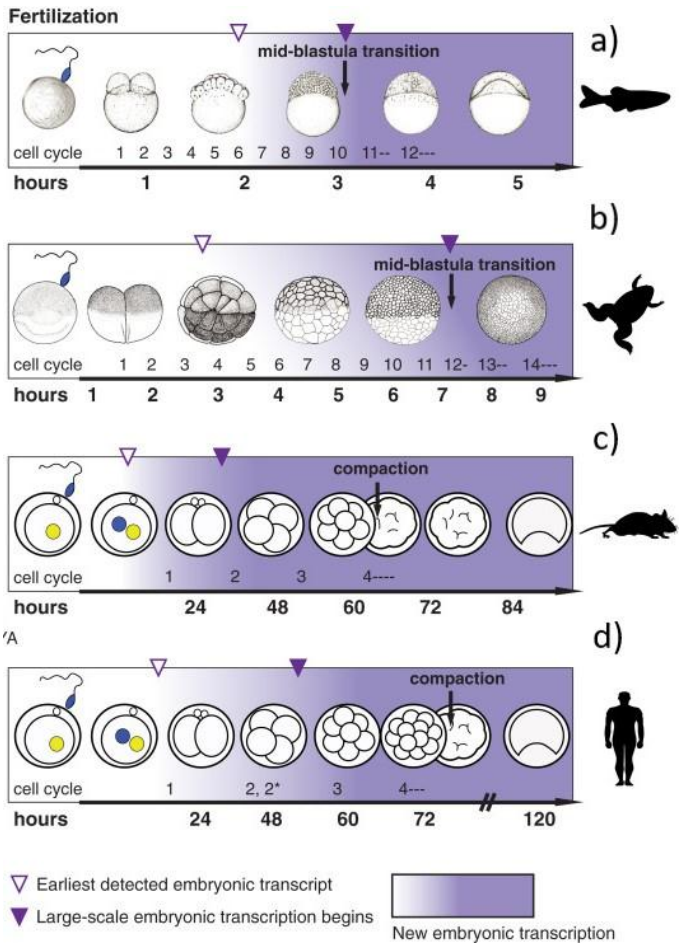

Figure 7 Early genome activation onset in fast- and slow-developing vertebrates. Fast-developing vertebrates (a, b) exhibit rapid cell division following fertilization with late onset of the EGA event. Slow-developing mammals (c, d) are mostly independent from maternal determinants and hence start producing their mRNA transcripts much earlier in the pre-gastrulation stage of development. Divisions *2* and *2** refer to the two asynchronous divisions in the second cleavage cycle. Adapted from [37].

Mammals represent the holoblastic, isolecithal cleavage with evenly distributed yolk with the first division being meridional (starting from the animal down to the vegetal pole) and the second being either meridional or equatorial [161]. In humans, the meridional cleavage generates two equally-sized cells, and the 2-cell embryo's cleavage furrow can produce four possible variants: one blastomere can divide meridionally and the other divides equatorially (ME variant), or the two blastomeres divide either through the equatorial (E variant) or the meridional furrow (M variant) [162]. During this time, the major genome reprogramming occurs, DNA methyltransferases catalyze *de novo* methylation patterning at CpG sites, methyl-CpG-binding proteins (MECPs) are inactivated, euchromatin is more abundant, and chromatin is found in the more relaxed state [163]. Morphogens act upon the early blastomeres through similar signaling pathways found in other vertebrates and invertebrates, activate the cascades of cellular signaling, and engage miscellaneous transcription factors to binding promoters and regulatory sites at the activated genes sites.

Most signaling pathways are conserved among animals during their development, however, some molecular and physiological differences count for the pathways' diversity and the scope of targets. For example, *Drosophila* codes 7 different genes belonging to the *Wingless* (Wnt) proteins family, whereas the human genome codes for as many as 19 Wnt proteins [164]. Even though the mode of action is conserved, the variety and heterogeneity of the human signaling pathways and morphogens promote a higher differentiation in the developmental fields.

Fibroblast Growth Factor (FGF) signaling is conserved among all vertebrates [165]. FGF ligands act in numerous stages and tissues during embryonic development, including embryo implantation, A/V axis patterning, and neural induction [166-168]. FGF ligands, especially the highly dynamic FGF8, are expressed in the primitive streak and the neuromesodermal progenitors (NMP) region where some cells develop into the spinal cord and others become the paraxial mesoderm cells (developing into somites) [169, 170]. FGFs are known as the "stemness" inducting factors promoting the NMP cells self-renewal and inhibiting their neural markers expression [171, 172]. However, it has been found that despite the conservation of FGF signaling in higher vertebrates, cell responses differ markedly between human and mouse in this instance. hESCs (human epiblast stem cells) have been recognized to respond to Nodal and Activin signaling by expressing Nanog; hESCs also act upon FGF2 induction, expressing Nanog and Oct4, whereas EpiSCs (mural epiblast stem cells) do not express *FGF2*. Thus, FGF signaling is limited early neurogenesis stage in mice [173].

Human developmental fields (progenitor fields) are formed as the resultants of the GRN acting through spatially and temporally well-defined waves of morphogens and signaling pathways that employ specific but overlapping sets of transcription factors modulating the gene batteries activity to hinder or support their expression profiles. Alike in other animals, human blastomeres communicate with each other to maintain tissue competence or change the state of their differentiation. Interblastomeric communication in humans is another developmental biology topic where the consensus has not been reached yet. In one study, tight and anchoring junctions have been shown to appear as early as at the 2- to 6-cell stage, while compared to the mural late junctions assembling occurring around the 8-cell stage [174]. Another study has shown that the electrical coupling (gap junctions) between early human blastomeres does not occur until the 8-cell stage [175]. Those uncertainties leave much room for more profound and sophisticated research that one day would bring a definite answer to the question: What cellular and

biochemical capacities allow for the formation of all human developmental fields derived from a single cell?

11. Conclusion

Since the emergence of multicellularity, which had occurred independently several times in the past, plants and animals have arisen employing different molecular tools to achieve the growing complexity of their tissues. Animals are the most complex multicellular eukaryotes on the planet; wisely adapting to the environment through tissue modification. Gene batteries and TF networks have consistently been evolving and increasing in complexity to help develop likewise complex and functional organs.

This is regulated through the well-tuned and synchronized cross-talk between soluble morphogens which induce the target cells' responses by concentration gradients and the highly conserved, yet species-specific signaling pathways regulating gene expression patterns through transcription factors. Even though some animals can be dependent on maternal constituents more than others, and hence go through the EGA later in the developmental process, every oocyte and every sperm cell contains embryological determinants that initiate first cascades of interconnected and often contradicting actions leading to axes formation, uneven gene regulators distribution, and interblastomeric connections establishment. Further molecular effects and developmental cues presence are aftermath of the organizing hierarchical structure of biochemical molecules.

However, none of those mechanisms, whether concerned separately or as a whole, explain *how* exactly a single cell, the zygote, gives rise to the unspeakable variety of cells across all multicellular organisms. No one can emphatically explain how the minute precision of morphogenesis is actually regulated and how the cellular cues control the cellular growth of every developmental niche. Why do the morphogenetic niches form in the first place and what evolutionary mechanisms have decided on employing specific genes orchestrating the complex intermodal networks and structural genes being orchestrated: developmental biology has not yet answered its major questions that had been asked since Aristotle.

However, the complexity of early developmental processes has been well studied, but many research results are contradicting and opposing to the already well-established facts which are taken for granted. Finding answers to the most frequently asked questions in developmental biology will one day bring this field closer to understanding the miracle of life.

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