Chapter 2
Early Embryo Development

2.1 Syngamy and Spindle Formation

Following the entry of the spermatozoon, the oocyte undergoes the activation process that stimulates the second polar body extrusion. At this stage the female pronucleus, containing a haploid set of chromosomes, is formed. Its male counterpart, the male pronucleus, increases its volume due to chromatin decondensation. This event involves a reduction of disulphite cross-links, made by glutathione, and a replacement of sperm polyamines with histones from the oocyte. Finally, male and female pronuclei approach each other, thanks to the male pronucleus association with the sperm centrosome. The latter organizes the aster that captures the female pronucleus, drawing it toward the male pronucleus, and moves both of them toward the center of the oocyte. At the time of nuclear envelope dismantling, pronuclei are fused and syngamy takes place. The diploid genome of the zygote is thus created (Fig. 2.1).

To better understand the importance of the role of the sperm centrosome during fertilization, it is necessary to take a step back and carefully consider the gamete maturation process. The centrosome, approximately 1 μm in size, is a non-membrane-bound cytoplasmatic organelle composed by several protein complexes (Fig. 2.2). Its main function is the organization of interphase microtubule arrays and mitotic/meiotic spindles.

Originally, spermatids and primary oocytes display a typical centrosome organization with a pair of centrioles surrounded by pericentriolar material, in common with somatic cells. These structures undergo extensive modification and/or degeneration during gametogenesis. In non-rodent species, spermatozoa preserve centrioles but loose most of the pericentriolar centrosomal proteins, whereas oocytes waste centrioles and retain only a stockpile of centrosomal proteins. This reciprocal reduction of centrosomal constituents makes sperm and oocyte complementary to each other. In this way they become able to form a functional centrosome in the zygote only after fertilization.

In non-rodent species, centrosomes are reduced during spermiogenesis. Their mature spermatozoa have intact proximal centrioles, whereas the distal centrioles

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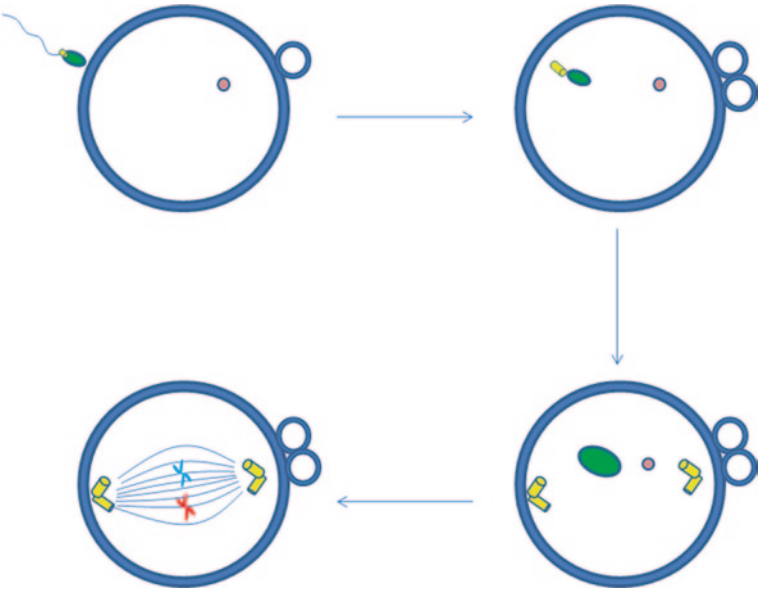

Fig. 2.1 The process of syngamy. After sperm entry, the oocyte extrudes the second polar body. The male pronucleus increases its volume due to chromatin decondensation and approaches the female pronucleus, moving toward the center of the oocyte. At the time of nuclear envelope dismantling, pronuclei are fused and syngamy takes place. The diploid genome distinctive of the zygote is created

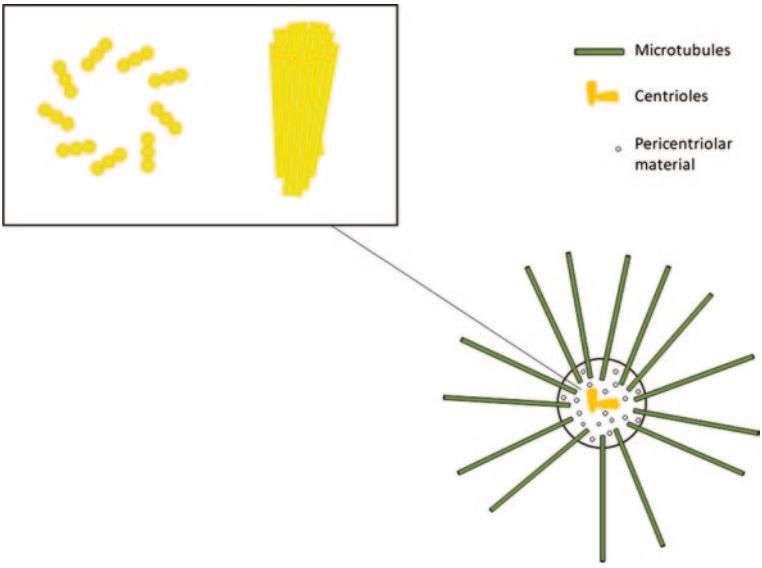

Fig. 2.2 Centrosome structure. The centrosome consists of a pair of orthogonally arranged cylinder-shaped centrioles (*yellow*) surrounded by an amorphous matrix of electron dense proteins referred to as pericentriolar material (*white circle*). Centrioles show the classic 9 + 0 pattern of nine triplet microtubules and no central pair of microtubules and contain centrin, cenexin, and tektin. The pericentriolar material contains a complex of proteins responsible for microtubule (*green*) nucleation

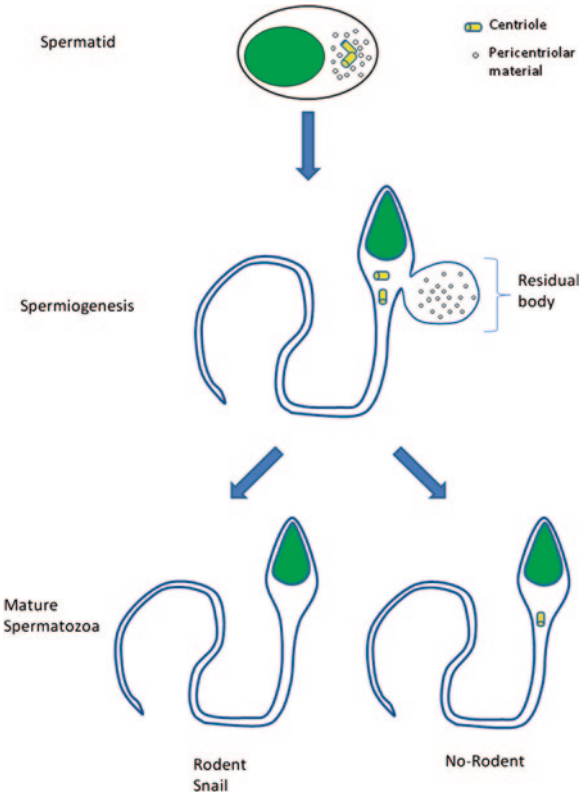

Fig. 2.3 Centrosome reduction during spermiogenesis. Male germ cells possess intact centrosomes containing centrioles and centrosomal proteins until the round spermatid stage. During spermiogenesis, centrosomal proteins are disjuncted from the centrioles and discarded with the residual bodies. Eventually, rodent and snail mature spermatozoa completely loose both centrioles, whereas non-rodent mammalian spermatozoa retain an intact proximal centriole but degenerate the distal one

are mostly disorganized or highly degenerated together with γ-tubulin and centrosomal proteins (Fig. 2.3).

By contrast, in rodents and snails, microtubules lose their nucleating function and centrosomal proteins are discarded during spermiation. The distal centriole degenerates during the testicular stage of spermiogenesis and the proximal centriole is lost during the epididymal stage (Fig. 2.3).

In female gametes, centrioles disappear during early oogenesis. Oogonia and fetal oocytes display normal centrioles until the pachytene stage, whereas these organelles are absent in mature oocytes (Fig. 2.4). This degenerative process has been demonstrated in many non-rodent species, including human, rhesus monkeys, rabbits, sheep, cow, and pig, as well as in lower species, such as sea urchins and Xenopus. Similarly, in rodents, centrioles completely degenerate during oogenesis and, at the time of germinal vesicle breakdown, multiple foci composed of perinuclear material, which gradually coalesce to form the poles of metaphase spindles, appear.

At fertilization, sperm and egg equally contribute haploid genomes as well as the relative centrosome components. Since in non-rodent mammals the MII oocyte centrosome is degraded and centrioles are absent, as previously described, early embryo development requires maternal and paternal

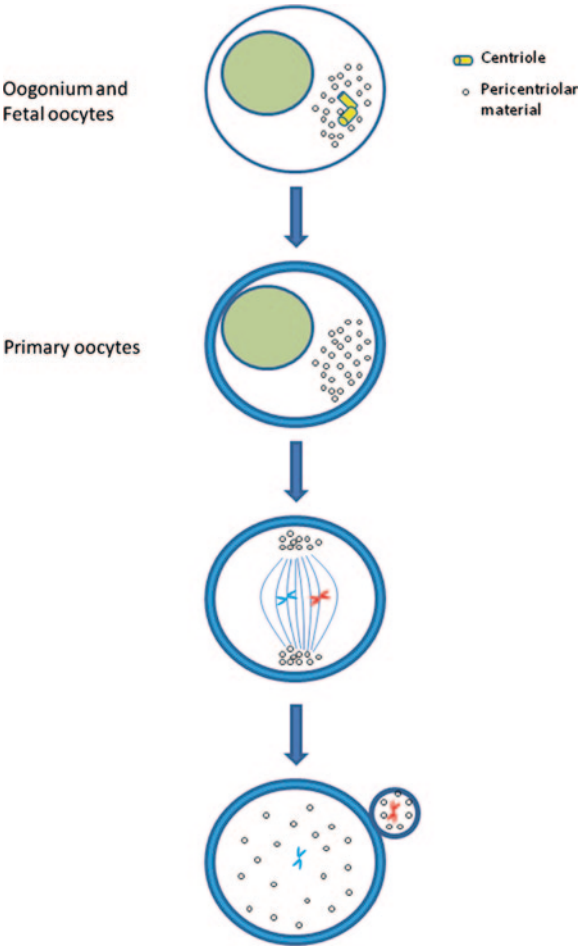

Fig. 2.4 Centrosome reduction during oogenesis. Oogonia possesses standard centrosomes containing centrioles and centrosomal proteins. Mammalian primary oocytes lose both centrioles completely, resulting in acentriolar and anastral poles during meiotic I and II divisions. The pericentriolar centrosomal proteins are dispersed in the oocyte cytoplasm during the non-dividing stages or distributed as concentric poles of the barrel-shaped spindles during dividing stages

contribution and, in particular, requires their elements to restore a normal and functional centrosome. Indeed, the sperm tail and its proximal centriole are introduced into the ooplasm together with the sperm head (Fig. 2.5). Paternal pronucleus decondenses in the ooplasm and the proximal centriole remains intact forming the aster, while most of the other sperm cytoplasmic structures including mitochondria, microtubule, and fibers are eliminated. The sperm aster enlarges and moves within cytoplasm, ensuring male and female pronuclei apposition. Although centrioles are paternally inherited, the formation of a single mitotic metaphase plate, with a bipolar spindle, requires the interaction between the sperm centriole and the with maternal pericentriolar proteins. In agreement with this, several studies have demonstrated that human sperm centrioles duplicate during the pronuclear stage, and at syngamy, centrioles are located at

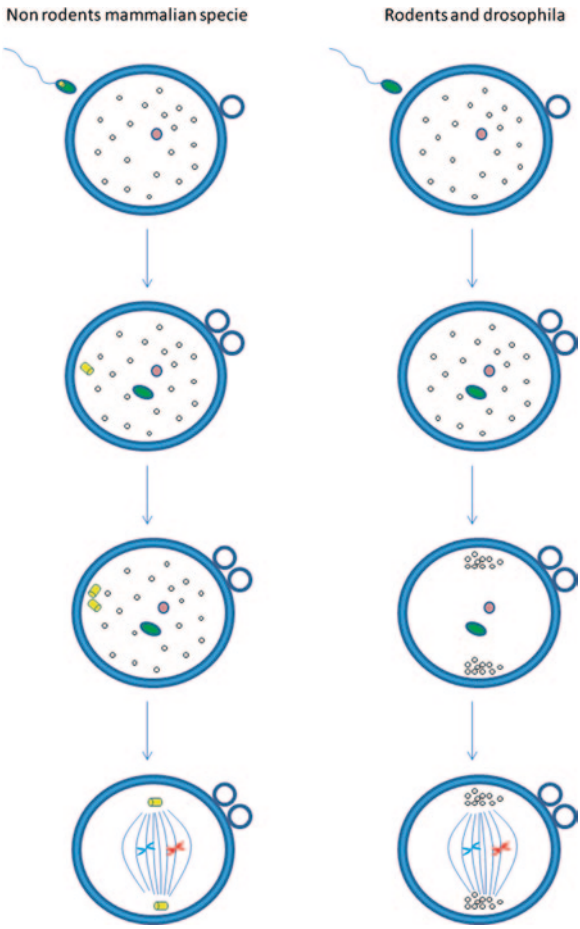

Fig. 2.5 Centrosome inheritance in mammalian species. In non-rodent mammalian species (*left panel*), sperm contains a proximal centriole before fertilization, while MII oocytes show a meiotic spindle with acentriolar centrosomes. The proximal centriole is introduced by the sperm and replicates forming the aster. At syngamy, it relocates to opposite poles to form the centers of the mitotic spindle poles and drives the first embryo mitotic duplication. By contrast, in rodents and in Drosophila, both sperm and oocyte lose their centrioles and only the centrosomal material is maternally inherited. This material remains dispersed in oocytes and after fertilization zygotes show centrosomal proteins aggregate forming aster-like structures

opposite poles of the first mitotic spindle, together with a surrounding pericentriolar material of the oocyte that nucleates microtubules. The same process was also described in sea urchin, rabbit, cattle, and sheep, whereby following insemination, microscope observations revealed one bipolar spindle displaying two centrioles at the opposite pole (first embryo cleavage). Together, these observations suggest that zygote centrosomes represent the origin of embryonic, fetal, and adult somatic cell centrosomes.

Conversely, in mouse and Drosophila, spermatozoa do not show the presence of centrioles and the centrosomes are maternally inherited, remaining dispersed in oocytes. Indeed, after sperm entry, typical centriole structures are absent in murine zygote, and early embryos show centrosomal proteins that aggregate in clusters forming small aster-like arrays of microtubules. Only at the blastocyst stage are centrosomes with double centrioles formed de novo.

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2.2 Cleavage, Compaction, and Blastulation

The first cleavage after syngamy is usually completed within 24 h after ovulation. After the first mitotic event, the zygote is divided into two cells, known as blastomeres that contain a full copy of the new embryo genome. Subsequently, a series of successive mitotic divisions take place and the embryo remains surrounded by the zona pellucida for several days. During this developmental phase, the embryo maintains the same total volume since the original cytoplasm is split among the newly formed blastomeres that become smaller and smaller with each division (Fig. 2.6).

Furthermore, transcripts and proteins stored in the oocyte during its maturation are steadily used up and degraded starting from fertilization, and embryonic genome activation with new RNAs produced under the direct and exclusive control of the embryo transcriptional machinery is required to continue the development.

Embryonic cleavages occur when the embryo is transported along the maternal oviduct and enters the uterus to implant. Species-specific timing characterizes this event as described in Fig. 2.7.

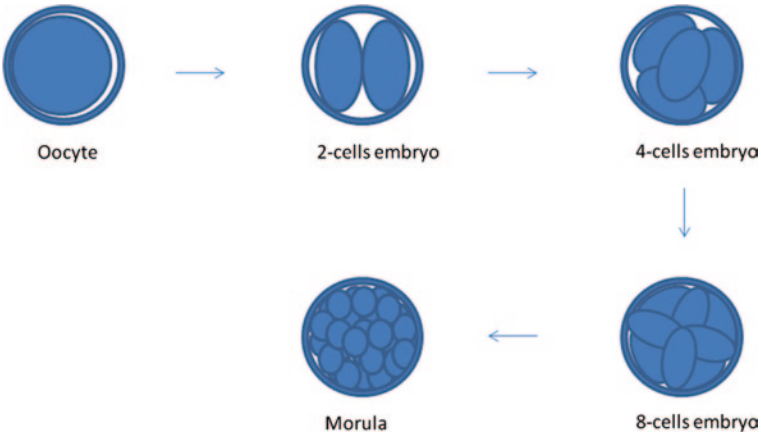

Fig. 2.6 Embryo cleavage. After fertilization the newly formed zygote remains surrounded by the zona pellucida and mitotically divides. The embryo maintains the same total volume and the original cytoplasm is split among newly formed blastomeres that become smaller and smaller

The following phase is characterized by the first cell differentiation regulated by the activation of specific transcription factors, namely CDX2 and EOMES (Fig. 2.8). In particular, morula cells, that are identical to each other, start to change and the outer cells differentiate in epithelium constituting the trophectoderm or trophoblast. This event is known as compaction and gives the embryo a smoother surface. Trophectoderm cells attach with neighboring cells and form tight junctions and desmosomes. These specialized intercellular structures contribute to intercellular sealing and tissue integrity, critical for vectorial transport and blastocoel cavity formation.

The step that follows morula compaction is known as blastulation phase, during which the embryo transforms into a blastocyst. The trophoblast cells secrete a fluid into the central cavity—the blastocyst cavity or blastocoel—lining the cavity. The inner cells move toward one pole, forming the inner cell mass (ICM). These cells maintain their pluripotent state and will form the embryo proper, while the trophectoderm cells will give rise to the embryonic placenta. The blastocoel continues to gradually increase its volume and the blastocyst expands. This process is made possible thanks to the activity of a sodium pump located in the cell membranes of the trophoblast cells that osmotically draws water into the central cavity. The zona pellucida is still present, but its components will be soon lysed by a blastocyst-secreted protease, known as strypsin, and by proteolytic enzymes produced by the endometrium. The process that leads to zona pellucida degradation is described as hatching and enable the trophoblast cells to directly bind the uterine cavity (Fig. 2.9).

During blastulation, ICM cells further differentiate into two cells population as described in Fig. 2.10:

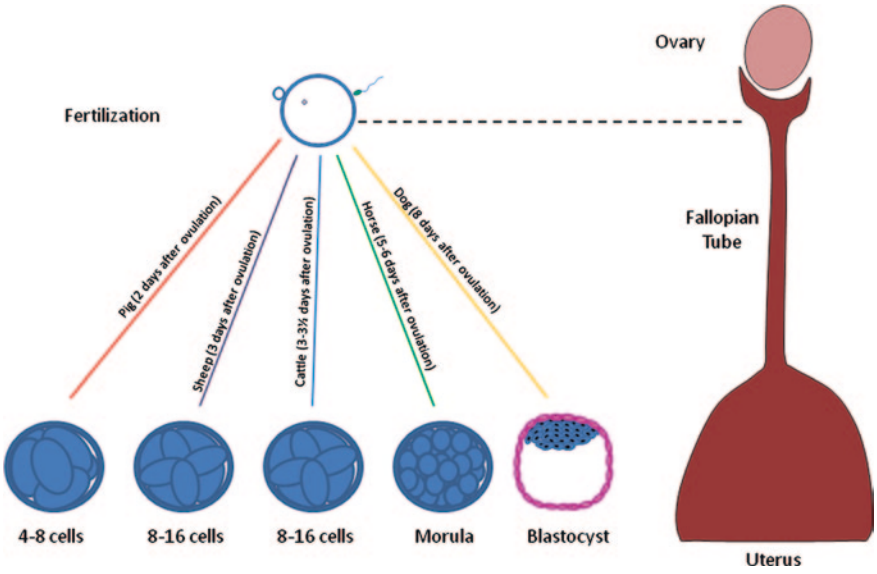

Fig. 2.7 Times and related stages of embryo passage from the oviduct into the uterus. The oocyte is fertilized in the ampulla of the uterine tube and the newly formed embryo mitotically divides during its transport along the oviduct. The time of entry in the uterus and the embryo stage are species-specific

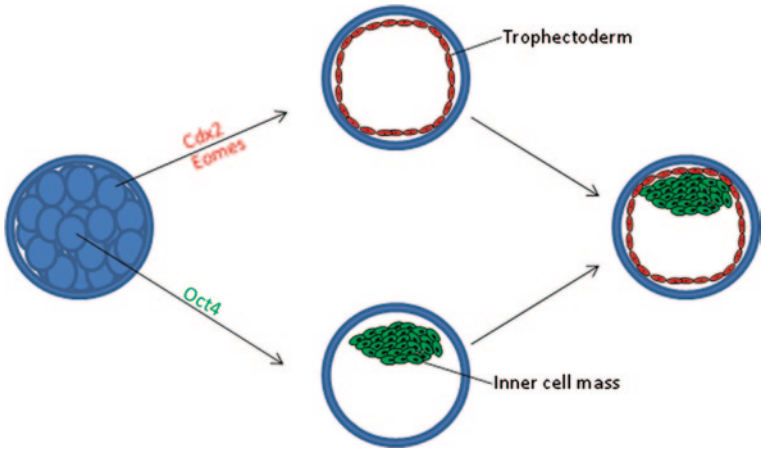

Fig. 2.8 Scheme of initial differentiation. After morula compaction, blastomeres begin to differentiate. Some cells activate the transcription of *CDX2* and *EOMES*, which induce trophectoderm cell formation. Others continue to express OCT4 that maintains the pluripotent status of inner cell mass cells

1. Hypoblast cells: small epithelial cuboidal cells closer to the blastocoel that will form the inner epithelium of the yolk sac. They need the activation of GATA-binding factor 6 (GATA-6), a fundamental transcription factor driving the formation of the primitive endoderm.

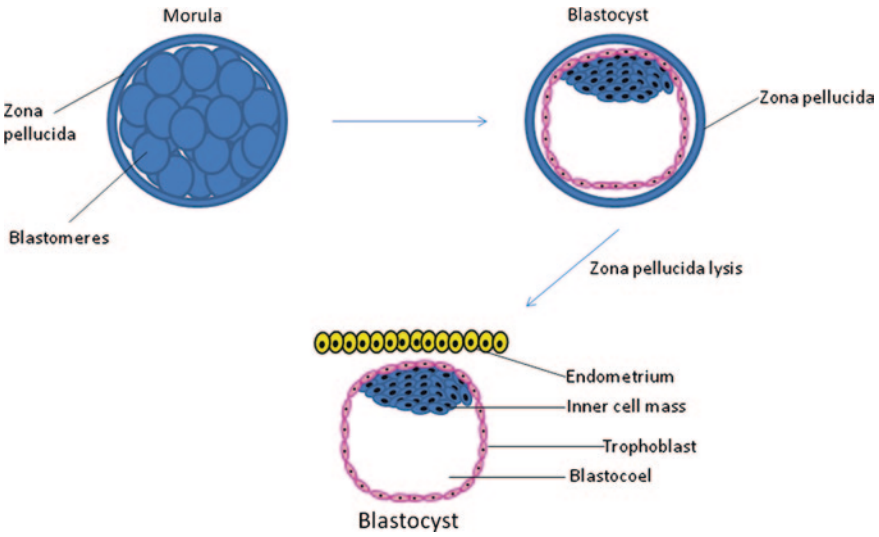

Fig. 2.9 Early embryo development and hatching. Trophoblast cells secrete a fluid into the central cavity, forming the blastocoel. The pluripotent inner cells move toward one pole, forming the inner cell mass. Finally, the zona pellucida is lysed by proteolytic enzymes (hatching), enabling the trophoblast cells to directly bind the uterine cavity (endometrium)

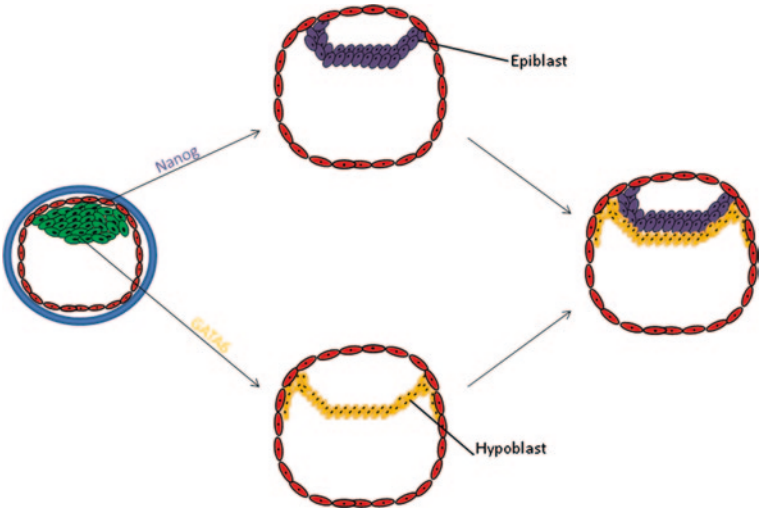

Fig. 2.10 ICM cell differentiation. During blastulation ICM pluripotent cells differentiate into epiblast and hypoblast cells, through the activation of *Nanog* and *GATA6* genes, respectively

2. Epiblast cells: pluripotent cells that express NANOG and form the primary ectoderm. They can also differentiate into all cells of the three layers during the gastrulation process, giving rise to the embryo proper and all the different tissue types that can be found in an organism.

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2.3 Cell Commitment and Waddington Model of Epigenetic Restriction: Asymmetric Imprinting

More than 230 different cell types are present in the adult mammalian body. Although they all derive from one single (zygote), they are able to differentiate in a tissue and time specific way and to respond to specific developmental cues. At the end of its differentiation process each of this type of cells is highly specialized and committed to a distinct determined fate. How a particular cell differentiates into its final cell type is still to be fully elucidated and represents a challenging goal of developmental biology. At present, four main processes are considered to play a key role and have been shown to be involved in cell commitment, specification, and determination. These processes are cell proliferation, cell movement, cell specialization, and cell interaction. It has also been demonstrated that each cell constituting an embryo is the target as well as the source of specific cues for its neighboring cells. Each cell retains a memory of its own cell proliferation history and its positional changes. These complex interactions have been shown to be regulated through differential gene expression and epigenetic restrictions that gradually limit cell potency to a more limited phenotype-related expression pattern. These concepts have been nicely depicted by Waddington in his very famous epigenetic landscape where a ball represents a cell of an embryo committing to a certain cell fate by rolling from a non-committed, pluripotent condition down a hill marked by slopes and valleys. In Waddington's metaphor, the hill represents the many different and complex process of the cell differentiation process. All those slopes and valleys eventually address the ball along a progressively more restricted potency pathway, toward a favored position at the bottom of the hill, where the

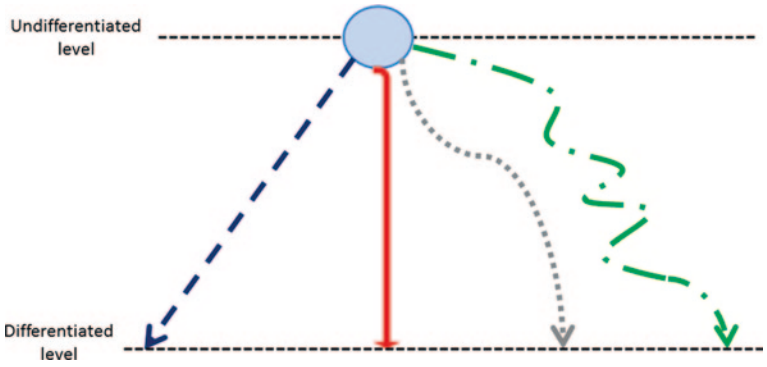

Fig. 2.11 The differentiation slope. According to the original model suggested by Waddington, different developmental canalizations (depicted in the figure as *blue*, *red*, *gray*, and *green lines*) along the differentiation slope, allow an organism to develop from the fertilized egg. The entire set of genes expressed by the differentiating organism and their interactions lead to the composition of a 'developmental system' which produces a phenotype. Interestingly, recent studies have shown that differentiated cells of an organism retain a memory of their own cell differentiation history and positional changes. They can thus be forced in an upstream, counter-current direction up the differentiation slope, along different states of increased potency

cell is unipotent and is characterized by a tissue-specific differentiated state. Many recent studies carried out in stem cells have demonstrated that differentiated cells of our organism can be forced in an upstream, counter-current direction up the differentiation hill, along different states of increased potency. However, it is important to remember that overexpression or activation of additional factors is needed in order to allow epigenetic reprograming events and reach a high plasticity state such as that obtained in induced pluripotent stem (iPS) cells (Fig. 2.11).

In the early embryo or blastocyst we can identify a group of cells, called the inner cell mass, the outer cells referred to as the trophoblast and the blastocoel, which is a fluid filled cavity. As already discussed, inner cell mass gives rise to the embryo proper and consists of cells that have the ability to differentiate in every tissue of the body. Because of this ability they are considered to be pluripotent. The inner cell mass soon develops into a bilaminar structure that comprises the epiblast and the hypoblast. Through the process of gastrulation the epiblast gives rise to all three somatic germ layers of the embryo, ectoderm, mesoderm, and endoderm, and allows for the differentiation of the primordial germ cells. The hypoblast, on the other hand, will lead to the formation of the extraembryonic structures (Fig. 2.12).

Cell specification and differentiation are made possible thanks to decisions driven by many complex process that result from cell-intrinsic properties but, at the same time, need inputs deriving from cell-extrinsic signals.

Cell-intrinsic properties control specification process through an asymmetric cleavage that leads to an unequal distribution of cytoplasmic determinants (proteins, mRNA, etc.) (Fig. 2.13). Once asymmetry is created from homogeneity, the daughter cells become very different in content and are determined to distinct fates. Cell-extrinsic specification is supported by cues deriving from interactions between cells

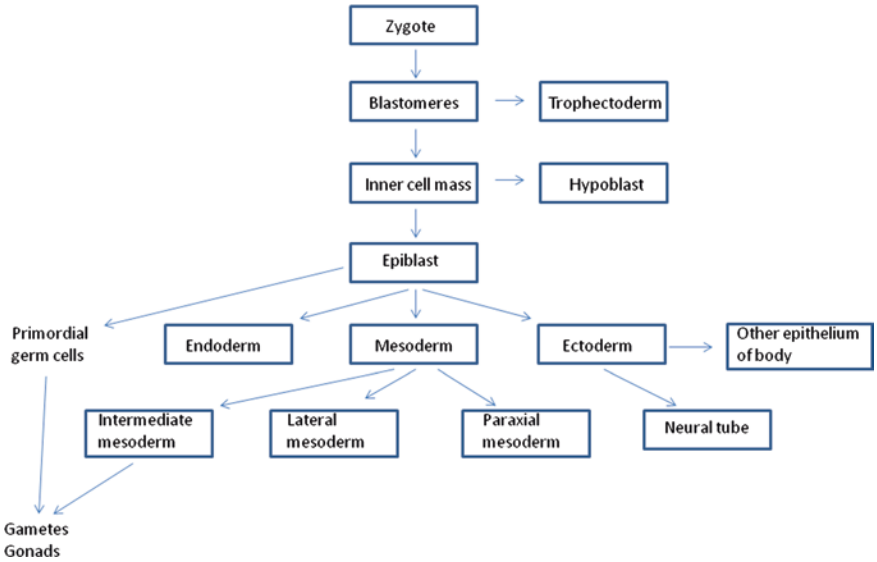

Fig. 2.12 Summary of the first steps of zygote differentiation

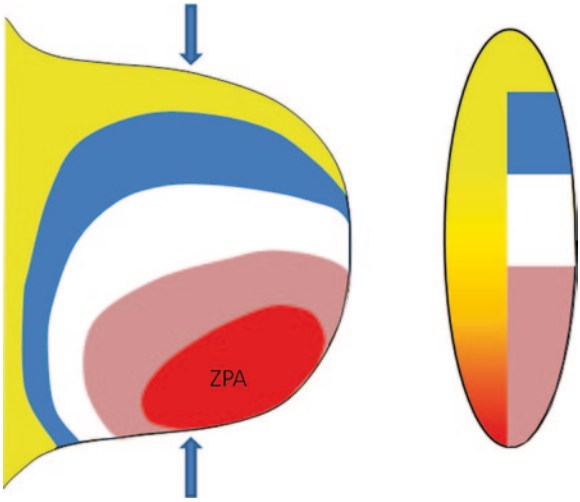

Fig. 2.13 The French flag model. Based on this model, the zone of polarizing activity (ZPA), produces a morphogen that diffuses across the nearby areas to generate a spatial gradient

as well as from soluble molecules that can diffuse and carry signals that control cell differentiation decisions in a concentration-dependent fashion, defined as morphogens and normally released in a paracrine fashion. Several major families of morphogens have been characterized and are known to be directly involved in cell induction toward a specific lineage. Many of these molecules are also involved in the formation of specific concentration gradient that drive cells to their correct spatial positioning, thus contributing to patterning and organization of the body axis (see Table 2.1).

Table 2.1 Genes directly involved in cell commitment (morphogens) and their specific functions

Family	Members	Functions
Fibroblast growth factor (FGF)	*FGF1–FGF23*	Play important roles in neurogenesis, axon growth, and differentiation during development of the central nervous system. Promote endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures. They thus promote angiogenesis, the growth of new blood vessels from the pre-existing vasculature. Stimulate repair of injured skin and mucosal tissues by stimulating the proliferation, migration and differentiation of epithelial cells
Hedgehog	Sonic hedgehog (*Shh*), Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*)	Involved in the developmental pattern formation of various organs, such as the nervous system, muscle, the heart, and the lungs. Hedgehog signaling has also been implicated in the development of several human cancers
Wingless (WNT)	*WNT1–WNT11*, *WNT16*	Wnt signaling plays a significant role in both the cardiovascular and nervous systems during embryonic cell patterning, proliferation, differentiation, and orientation. Furthermore, modulation of Wnt signaling under specific cellular influences can either promote or prevent the early and late stages of apoptotic cellular injury in neurons, endothelial cells, vascular smooth muscle cells, and cardiomyocytes
Transforming growth factor-β (TGF-β)	*TGF*-β, *Activin*, bone morphogenetic protein (*BMP*), nodal, glial-derived neurotrophic factor (*GDNF*), inhibin, mullerian inhibitory substance (*MIS*)	The TGF-β family has different important functions related to osteoblast differentiation, neurogenesis, ventral mesoderm specification, angiogenesis, extracellular matrix neogenesis, immunosuppression, apoptosis induction, gonad growth, placenta formation, left–right axis determination

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2.4 Establishment of the Body Axis

Decisions taken in the very early embryo lead to cell commitment and the definition of the different lineages. Although a vast array of information is slowly accumulating, it is not fully understood as yet how multiple types of cells and polarities are generated in an embryo that originates from a single cell. Similarly, we still need to define the origin of the signals that activate cell differentiation and generate asymmetries. At present, early polarity is not thought to depend on asymmmetric localization of maternal determinants.

In the eight-cell mouse embryo, we have to focus on the process known as compaction that leads to the establishment of an "inside–outside polarity". Compaction lead to the accumulation of specific molecules, such as Par3 (partitioning defective 3), Par6 (partitioning defective 6), and atypical PKC (aPKC) to the apical side of the embryo, while localizes Par1 (partitioning defective 1) and E-cadherin to the basolateral side of the cells.

This established inside–outside polarity leads to the activation of a signaling loop, called Hippo in Drosophila and Stk3 (Ser/Thr kinase 3)/Mst in the mouse that inhibits proliferation in the inside cells, or inner cell mass (ICM) and downregulates expression of trophectoderm (TE)-specific genes such as caudal-related homeobox 2 (*Cdx2*). Two types of cells are therefore present in the blastocyst; TE and ICM cells and it is believed that the decision on one or the other cell fate may depend on epigenetic marks related to differences in histone acetylation and methylation among blastomeres.

The ICM is going to soon segregate two types of cells: the primitive endoderm (PrE) and the epiblast. The PrE is formed by a thin layer of cells located on the

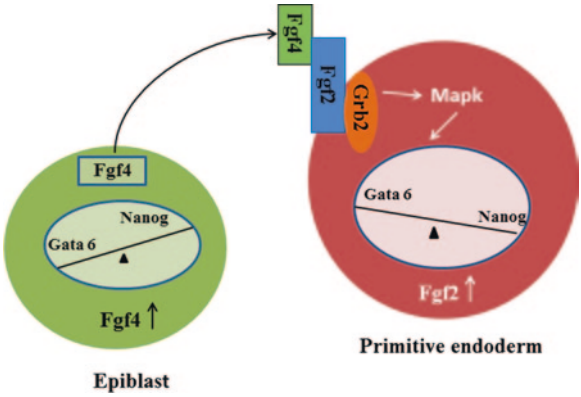

Fig. 2.14 Primitive endoderm formation. Epiblast cells increase the expression of *Fgf4*, that binds *Fgfr2* exhibited by primitive endoderm progenitor cells. This interaction cause the repression of *Nanog* expression and the activation of *Grb2* and *Mapk* genes, resulting in the induction of primitive endoderm specific genes, such as *Gata6*

surface of the ICM, facing the blastocoel cavity, and positive for members of the GATA family of transcription factors. The epiblast, on the other hand is inside the ICM and expresses Nanog and Oct3/4. The segregation of the epiblast and PrE lineages in the ICM is known to be regulated by fibroblast growth factor (FGF) and FGF receptor signaling and by the mitogen-activated protein kinase (Mapk) signaling pathway Inhibition of such signaling has been shown to address ICM cells to the epiblast fate (Fig. 2.14).

The formation of the ICM and establishment of the blastocoel cavity creates the embryonic–abembryonic axis, which is at 90° to the margin between the ICM and blastocoel. This axis is considered by several authors as the template for future dorsal–ventral polarity.

The anterior–posterior polarity of the embryo is established in response to signals produced by the visceral endoderm (VE), which is a layer of cells derived from the epiblast that covers the extraembryonic ectoderm and epiblast of the postimplantation embryo. Most VE becomes the endoderm of the extraembryonic yolk sac but a subset of VE cells differentiate into the endoderm of the embryo proper. Molecules produced by the VE act on the nearby epiblast and specify it to future anterior identity. Among them are Lefty 1 and Cer that are Nodal antagonists and specify the fate of the contiguous epiblast portion or anterior visceral endoderm (AVE). By contrast the epiblast region, which is located in a more distant position and is not reached by the AVE derived signals forms the primitive streak on the opposite side of the embryo.

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