

DEVELOPMENTAL BIOLOGY

CHAPTER – III

PATTERNS OF DEVELOPMENT

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Fusion of male and female gametes i.e. fertilisation result in the formation of zygote which carries the maternal and paternal genes, undergoes cytoplasmic reorganisation and nuclear activation. It is the first step in the development of embryo formation. The journey from zygote to a normal embryo is not easy and passes through important steps of **cleavage, blastula, gastrula, neurula and organogenesis**. The variety of egg types result in different patterns of development so that variation is also seen in the developmental steps of cleavage, blastula, gastrula, neurula and organogenesis.

Cleavage; Fertilisation is followed by a series of mitotic divisions in a rapid succession called **cleavage or segmentation**. The cleavage phase arbitrarily ends with the formation of group of cells called **blastula**. The cells, called blastomeres, are the building blocks of future organs of developing embryo. As there is no growth phase during cleavage, so the size of the blastomeres decreases. The location and orientation of zygote nucleus in the egg affects patterns and planes of cleavage as cleavage furrow is determined by the position of mitotic apparatus (Fig.3.1). The position of first cleavage is also specified by the point of sperm entry and subsequent rotation of egg cytoplasm.

Planes, Patterns and Laws of Cleavage

Planes of cleavage Cleavage occurs at different planes, as a result cleavage furrows formed are at animal vegetal axis or perpendicular to it. Following planes of cleavage occur in the animal egg;

1. **Meridional:** Cleavage furrow passes through the centre of animal vegetal axis bisecting the egg into two halves, e.g. the cleavage in Amphioxus and frog.
2. **Vertical:** Cleavage furrow passes parallel to the meridional plane, e.g. 3rd cleavage in chick.
3. **Equatorial:** Cleavage furrow is right angle to the meridional plane, e.g. 3rd cleavage of frog.
4. **Latitudinal or transverse:** Cleavage is parallel to the equatorial plane, e.g. 5th cleavage of frog.

Patterns of cleavage: Depending on the formation of cleavage furrows, different types of cleavage patterns are recognized (Fig 3.2) as:

1. **Radial:** Blastomeres of upper tier lie on the corresponding blastomeres of lower tier symmetrically arranged around the polar axis, e.g. in echinoderms.
2. **Bilateral:** Cleavage results in difference in size of blastomeres giving bilateral symmetry.
3. **Biradial:** First two cleavage planes are meridional and third is vertical giving a plane of cleavage which can be divided into two halves from any angle as in ctenophores.
4. **Spiral:** The mitotic spindle lies obliquely with respect to animal vegetal axis, so the upper tier of cells is slightly displaced in clockwise (dextral) or anticlockwise (sinistral) direction (nemerteans, annelids, molluscs). The cells touch each other more than in radial pattern and they make a thermodynamically stable packing arrangement. Spirally cleaving embryos undergo less divisions before gastrulation than radial cleaving embryos.

Based on the potency or capability of blastomeres two main types of cleavage are classified as:

1. **Determinate cleavage: Where the fate of each blastomeres is predetermined as in nematodes, annelids, molluscs, ascidian and cephalochordate Amphioxus. These eggs are also called mosaic eggs or plastic eggs.**
2. **Indeterminate cleavage: Where the blastomeres have more than one potencies as in echinoderm and all the vertebrate eggs. This type of cleavage occurs in the eggs which are without predetermined regions. These type of eggs are called regulative eggs.**

The determinate and indeterminate cleavage has also been explained by the fact that there are two types of specifications in the egg one is autonomous specification and other is conditional specification. Autonomous specification is seen in determinate eggs as the morphogenetic determinants, distributed in the cytoplasm of mature eggs, which are passed to the cleaving cells. Morphogenetic determinants specify the cell types so that if certain group of blastomeres is lacking or destroyed, the embryo does not form the structures which were assigned to the lost blastomeres. Thus, the embryo formed is not complete. Second type of specification is seen in early development of regulative eggs where the fate of the cells is not rigidly fixed but can change depending upon the interaction with the neighbouring blastomeres.

Based on the amount and distribution of yolk there are different patterns of cleavage as:

Holoblastic: It is total cleavage of the entire egg. It can be equal holoblastic when blastomeres are of equal size (Amphioxus, mammals) or unequal holoblastic when blastomeres are of unequal size (fish, amphibians).

Mammalian eggs are very small in size and difficult to procure, so mammalian cleavage has been studied with difficulty. The mammalian cleavage is slow and the zygote shows **rotational cleavage** where first cleavage is meridional but the second division is rotational as one of the two blastomere divide meridionally and other equatorially. Moreover, there is marked asynchrony in the early divisions of embryo. The blastomeres then huddle together and form gap junctions for passage of small molecules and thus form group of cells of morula by **compaction** which later on differentiate as **inner cell mass** and **trophoderm or trophoblast**.

Meroblastic is the incomplete cleavage due to inert dense inactive yolk in the **telolecithal** egg which exerts local retarding effect by mechanical impediment process. It is of two types **discoidal** (fishes, reptiles, birds and monotremata) where the cleavage is restricted to a small discoidal area over yolk free cytoplasm. **Superficial meroblastic** cleavage is in **centrolecithal** eggs as of insects where the cleavage is confined to peripheral cytoplasm of egg and does not extend to central yolk.

Similarities in cleavage patterns in different animal groups provide evidence for the evolutionary relationships as the cleavage patterns in reptiles, birds and later development similarities in mammals point to their close ancestral relationships.

Laws of cleavage

Cleavage also follows certain laws which are described as follow:

- **Sach's law:** Cells divide into two equal halves and each new plane intersect the preceding one at right angle.

Hertwig's Law: As the mitotic spindle lies in the longest axis of protoplasmic mass, the divisions cut the protoplasmic mass at right angles.

- **Belfaur's Law:** Rate of cleavage is inversely proportional to the amount of yolk present.
- **Pfulger's law:** The spindle axis elongates in the direction of least resistance.

Cleavage mechanism

Cleavage is the result of two co-ordinated cyclical processes, the first one is **karyokinesis** which means the mitotic division of the nucleus due to the formation of mitotic spindle. The karyokinesis is followed by the cytokinesis which is the cytoplasmic division. In insects, the karyokinesis is not immediately followed by the cytokinesis but is delayed.

Cleavage is a series of cell divisions forming multicellular structure from the unicellular fertilised egg. The divisions are never accompanied by growth and general shape of the embryo does not change. There are limited qualitative changes in the chemical composition of the embryo. The cytoplasmic constituents are not displaced during these divisions. The ratio of nucleus to cytoplasm becomes equal to that of the ordinary somatic cells. The rhythm of cleavage which is modulated by the time interval between two consecutive divisions varies in different animals. Mammalian eggs cleave slowly than eggs of other animals.

During the cleavage, there is an increase in the nuclear material at the expense of cytoplasm. The number of nuclei are doubled with each division of blastomeres. In Sea urchin, it is found that cytoplasmic ribonucleic acid changes to deoxyribonucleic acid as ribonucleotides are converted to deoxyribonucleotides by enzyme ribonucleotide reductase. As the nucleolus is lacking during cleavage, the production of ribonucleic acids is limited. Very little of rRNA is synthesised but it increases in gastrulation. There is increase in rRNA and tRNA during later stage of cleavage. The new proteins synthesised during cleavage are nuclear histones, tubulins and enzyme ribonucleotide reductase which are directly involved in the process of cell multiplication. Nuclear histones are produced for chromosomal replication. Tubulin is synthesised for its utilisation in forming microtubules. DNA polymerase and ribonucleotide reductase are for chromosomal replication. There is no growth phase so the successive divisions result in decreasing the size of blastomeres. It is followed by the second phase that is **cytokinesis**, division of cell cytoplasm. The contractile ring of actin microfilaments helps in this division. The mitotic spindle and the contractile ring is formed perpendicular to each other. The contractile ring bisects the mitotic plane by forming cleavage furrow. Cortical cytoplasm of egg has actin microfilaments which tighten to form a contractile ring and thus create a force for dividing the fertilised egg into blastomeres. In the insect egg, karyokinesis occurs several times before cytokinesis that is why several nuclei are seen in the periphery of insect blastula. Drug cytochalasin B inhibits the formation and organisation of microfilaments in the egg. As the cytoskeletal apparatus is busy during cleavage, therefore no morphogenetic movement begins.

Mitosis Promoting Factor, MPF, is important during cleavage as the blastomeres pass through a cell cycle consisting of two steps i.e. mitotic phase (M) and DNA synthesis phase (S). MPF has a large subunit called cyclin B which undergoes periodic synthesis and degradation. The rapid and synchronous divisions during the early cleavage are regulated by stored cytoplasmic regulators of cyclin B. In later stages, they are produced by the nucleus. One of the reasons for loss of synchronisation in the later stages of cleavage is the synthesis of these regulators by the different cells. Rhythm of cell division is under the control of the cyclin production and degradation. Calcium has important role in the degradation of cyclin

(Watanabe *et al*, 1991). Cyclin is also encoded in the m-RNA of oocyte cytoplasm (Minshull *et al*, 1989).Cyclin regulates the kinase dependent activity of the small subunit of MPF.

Morula: Cell divisions do not stop but the development events as assortment and reaggregation of the cell overshadow cleavage *per se*. The period of cleavage ends with the establishment of morula and then blastula. Morula is derived from the Latin word ‘morum’ which means mulberry to which they resemble structurally. Morula is succeeded by blastula where the blastomeres arrange themselves into epithelium, called **blastoderm** with the appearance of specialised cell adhesion mechanisms. One of the important cell adhesion molecules is EP cadherin. mRNA for this protein is in the form of informosome in the oocyte cytoplasm (Heasman *et al*, 1994) because if the informosome is destroyed by injecting antisense oligonucleotide complement to m-RNA, cell adhesion is reduced. A cavity also appears underlying blastoderm which is called **blastocoel** or segmentation cavity. It is important as it provides space for the morphogenetic movements.

Blastula:It is normally spherical in shape. As variety of egg types result in variation in cleavage patterns so the different forms of blastula are also expected (Fig. 3.3). Whatever be the variation in the gross form, all have a common cause of constructing a stratified embryo called **gastrula** (gaster-stomach) which is the next step in development process.

Types of blastula

1. **Coeloblastula** : Hollow spherical blastula surrounded by single layer of blastoderm (echinoderm , Amphioxus)
2. **Stereoblastula** : Solid blastula with unequal sized blastomeres that is micromeres and macromeres as in annelids, molluscs , nemerteans and Planaria.
3. **Periblastula** : Superficially cleaving egg of insect with peripheral blastomeres and yolk filled central blastocoel.
4. **Discoblastula** : Multi layered flat disc separated from yolk by subgerminal cavity in macrolecithal egg of fishes, reptiles and birds. Due to the presence of yolk, blastoderm becomes arched and the floor is flattened.
5. **Amphiblastula** : Blastoderm is of unequal thickness made of micromeres and macromeres with an eccentric blastocoel as in amphibia.
6. **Blastocyst** : In mammals, the blastula has the blastoderm which draws food for the yolk less embryo from the uterine wall. The blastula is called a blastocyst. It has a group of embryo formative cells called **inner cell mass** with small fluid filled spaces representing a place for food in the form of yolk in the ancestors. Blastoderm is called Trophoderm or Trophoblast as it helps in embedding of embryo into uterus. Blastoderm is the embryo formative region in invertebrates and in most of the chordates, but it is the inner cell mass (embryonic knob) in mammals. Inner cell mass is later on converted into bi-layered **embryonic disc** made of **upper epiblast** and **lower hypoblast**. With each successive development stage, there is narrowing down of potency of blastomeres. Zygote is totipotent, blastomeres are pluripotent and later on become unipotent.

Fate map: Diagrammatic representation of fate of each cell of blastula is prepared by various methods to understand gastrulation movements. Fate map is only a pictorial representation but of importance in understanding morphogenetic movements. The different methods which have been used to construct these fate maps are as follows:

Natural markers: Certain eggs have natural markers of different coloured granules as in amphibian egg, animal pole is pigmented and vegetal pole is unpigmented but gray crescent zone is the middle zone below the equatorial region.

Artificial markers: Vogt in (1925) used vital dyes like Nile Blue Sulphate, Neutral Red and Bismark Brown to stain early blastula and to study the fate of marked blastomeres. Carbon particle method was used by Spratt where the blastomeres were marked when the carbon particles adhered to them.

Radioactive markers: C_{14} , P_{22} , tritiated thymidine were used to label the nucleus. It is done by immersing a piece of blastoderm in medium of tritiated thymidine which is incorporated into its DNA within three to eight hours of incubation. It is transplanted into the embryo of same age. The labeled cells are studied by preparing the auto radiogram. As this method needs incubation of the part of the embryo in the radioactive medium, so it can not be used to study early stages of development. Fate map of bird epiblast was prepared using this method (Rosenquist 1966, Nicolet, 1970).

Vital dyes and radioactive labels were diluted with each cell division but **fluorescent dyes** stain intensely and once injected in the blastomeres can be detected in the progeny of cells, many divisions later also. More recently, dil, a powerful fluorescent molecule is incorporated into the lipid membranes and fate of the cell is studied. Histochemical staining methods for enzyme specific staining of different cell types is also used to mark the cells of interest.

Genetic markers: They are the permanent way of marking the cells by creating mosaic embryos or chimaeric embryos of different genetic constitution but similar development patterns. Retrovirus marking is also used to study the fate of presumptive neural tissue. Retrovirus engineered to contain a reporter gene is incorporated into DNA of cells of host and reporter gene is expressed. Gene product is then demonstrated by histochemical methods.

Xenoplastic transplantations: Where quail blastomeres are transplanted into the blastomeres of chick as quail's cells show heterochromatin in the nucleus concentrated around the nucleoli and there are quail cell specific antigens to distinguish from chick so they can be identified from chick cells and fate of the region where cells are transplanted is studied. Fate maps besides throwing light on the destiny of parts of early embryo also help to understand the mechanism of morphogenetic movements.

Gastrulation movement is also called the morphogenetic movement. The space for the mass movement is provided by the blastocoel. To understand the difference in the gastrulation in the animals, the study of fate maps of blastula of different animals is important

Fatemap of Amphioxus. (Fig. 3.4) In Amphioxus the presumptive organ forming areas can be marked in the eight cell stage. Tung, Wu, Tung (1962) applied the vital stain marking method for the construction of fate map in Amphioxus embryo. They found that presumptive mesodermal area is crescentic cytoplasm extending farther around equator. A zone on opposite side forms notochord, crescentic area above the presumptive notochord develops into nervous system. The granular cytoplasm around the vegetal pole develops into the endodermal lining of alimentary canal.

Fate map of amphibian (Fig 3.4)

Wather Vogt (1925) used vital staining method for the construction of fate maps of amphibians. Vital stains do not interfere with the normal processes. A piece of agar or cellophane (stain carrier) is used and is pressed against the chosen area of blastula for a short

period. Cellophane is better than agar as it can be cut easily into desired size and shape . The stain does not diffuse into the neighboring cells.

The blastula of amphibian embryo is round and has three distinct regions:

1. The vegetal region is the pigment free macromere region. It represents presumptive endoderm and contains the material for the formation of midgut and hindgut of embryo.
2. Second region is that of animal pole of egg which consists of micromeres. It gives rise to future ectoderm of the animal and forms two main regions:
 - a. Region of prospective ectoderm which develops into the epidermis of skin.
 - b. Region of prospective central nervous system which forms brain, spinal cord and sense organs.
3. Third region is the marginal region of gray crescent. It forms the presumptive mesodermal cells. It consists of the following subregions:
 - a. Presumptive notochordal region which is present on the dorsal side and gives rise to notochord.
 - b. Below the notochordal area is the portion which forms the part of foregut.
 - c. Region of presumptive somites which develops on both the sides of notochordal area.
 - d. Ventrolateral mesodermal area which lies on lateral and ventral part of marginal zone and forms the mesodermal lining of the body cavity, kidney and reproductive organs.

Fate map of chick (Fig 3.4)

Spratt in 1946 used carbon particle method for making the fate map of chick. This method used carbon particles which stick to the surface of the cells, thus enabling to follow the movements of cells and draw the fate map. The fate map is of the epiblast as it contributes to the most of the embryonic structures.

Epiblast consists of the following regions:

1. Anterior half of epiblast area forms the prospective epidermis. It also contains the region for extra embryonic membranes.
2. Below the epidermis is the area of presumptive neural tissue.
3. Behind neural tissue is material for the presumptive notochord.
4. Presumptive mesoderm is situated in the midline behind the notochordal area and most of the posterior half of area pellucida forms the mesodermal lining of the body cavity.

Fate map of mammal

It is difficult to prepare the fate map of man's blastula due to the uterine development though it is being done using the embryos of mouse, rabbit, pig and monkey . Due to the similar reptilian and bird development, it is assumed that hypoblast forms the presumptive extra embryonic structures. The epiblast forms the presumptive neural tissue, epidermis, notochord and mesodermal tissues and endodermal structures.

Morphogenetic movements: Gastrulation is the compilation of different types of cellular movements which creates a trilaminar gastrula exhibiting variation in blastulation. These formative or morphogenetic movements may express themselves in various ways. The basic movement is of **spreading and stretching** of cells and most of the technical jargon is due to variations in these two movements. They are:

- **Epiboly** means spreading of micromeres over macromeres or movement of presumptive ectodermal cells by stretching and spreading.
- **Emboly** is inward movement of cells which is of various types as :
- **Invagination** means infolding or insinking of egg surface followed by migration of cells .
- **Involution** is the inturning or rotation of cellular materials upon itself so that it is directed inside.
- **Infiltration** is movement of cells to form a new layer, hypoblast.
- **Delamination** is separation of cells by splitting of layer.
- **Convergence** is movement of cells towards midline to form axial structures.
- **Divergence** : As the cells after involution do not remain as heap of cells but move to future position so it is called divergence
- **Extension** is the elongation of gastrula along anterior posterior axis.
- **Ingression** is movement of individual cells or small group of cells into the blastocoel.
- **Constriction** is reduction in size of blastopore .

The net result of all these movements is the formation of gastrula. **Embolic** movements result in relocation of the cells of the external surfaces to the interior and expansion of remaining cells to replace the parts moved to interior. The gastrulation is a result of integrated cell motility, specific migrational tendencies and selective adhesion of cells resulting in directional and co-ordinated movements.

The gastrulation has two fold significance, that is **rearrangement of cells** which later on help in **neurogenesis**. In some amphibian eggs, if the outer protective membranes are removed the egg forms **exogastrula** instead of normal gastrula where endoderm and mesoderm cells evaginate. The endodermal and mesodermal organs develop to a great extent but no nervous structures are formed. The gastrulation thus helps in inaugurating inductive interactions for neurogenesis.

Gastrulation in Amphioxus, frog, chick and mammal

Gastrulation in Amphioxus: The gastrulation in Amphioxus (Fig 3.5) is an example of simplest morphogenetic movement. The early development in Amphioxus (cephalochordate) resemble that of invertebrates as of echinodermata and at the same time are representative of vertebrate development in its simplest form. It would be difficult to understand the complex development of higher vertebrates without studying simple embryological development of Amphioxus.

After fertilisation, the cleavage starts about one hour later. Cleavage is holoblastic due to small amount of yolk, but the division is not absolutely equal. The two blastomeres establish the bilateral symmetry of the adult. At about seventh cleavage, synchronous divisions decline, therefore the progression in numbers of blastomeres become arithmetical rather than geometrical. Accumulation of semifluid material in the centre of mass of cells pushes the

blastomeres outward so that they are arranged in a single layer called blastoderm which encloses a central cavity forming a blastocoel and hollow sphere of cells is called **coeloblastula**. Fate map studies depict the prospective fate of different regions of germ layers.

The gastrulation process begins with the flattening of area around the prospective endoderm. This endodermal plate gradually invaginates or folds inwardly into the blastocoel. The new cavity created by invagination is **archenteron** or gastrocoel which opens to the exterior by **blastopore**. The circular rim of blastopore is termed the lip of blastopore. The primary factor for the gastrulation is the invagination and rapid proliferation of cells at the rim of blastopore is **epiboly**. The blastopore constricts, stops taking the cells inside the growing embryo and also shifts its position. Due to inward movement of the cells, the archenteron enlarges that obliterates the original blastocoel. Later on the gastrula elongates and the blastopore narrows down. Further growth and differentiation of gastrula involves the establishment of three important organ/ systems, viz. nervous system, mesoderm and notochord. The promordia of these systems arise simultaneously and their differentiation is termed as **neurogenesis, mesogenesis and notogenesis** respectively. The important steps in Amphioxus gastrulation are:

1. Gastrulation is initiated by simple invagination at prospective endoderm.
2. After invagination, the cells automatically involute as they are present on the margin.
3. A new cavity called archenteron is formed and blastocoel is reduced. The archenteron opens outside through blastopore which is a continuously changing entity.
4. Epiboly movements occur simultaneously to surround the embolic cells.
5. Contraction of blastopore completes gastrulation.
6. It is the simplest gastrulation movement due to simple blastula with single layered blastoderm and large blastocoel.

Gastrulation in Amphibia (Frog): Mature egg of frog is 2.0-3.0 mm in diameter and is mesolecithal. It is radially symmetrical prior to fertilisation but later on develops dorsoventral symmetry due to internal cytoplasmic rearrangement that forms gray crescent (dorsal side) a forerunner of notochord. The cleavage is holoblastic and for the first two divisions it is complete but later on becomes unequal due to yolk. Egg yolk reduces the rate of cleavage therefore, vegetal blastomeres divide slowly and with lesser frequency. At about fourth or fifth cleavage, a small space called blastocoel appears which increases rapidly. (Fig 3.6). Thus, there is formation of blastula which is a hollow ball, with a thin multi-layered roof of animal cells and floor of large yolky blastomeres so called micromeres and macromeres respectively. The fate map of frog blastula has been prepared by vital dyes which gives the description of what the particular area is destined to become later. Gastrulation is inaugurated in frog (Fig 3.6) by the deformation of certain prospective endodermal cells below the gray crescent area. The cells in this region assume bottle shape and move towards the interior. The neck of these cells remain attached to the surface of blastula and a pull is exerted on the attenuated necks resulting in indentation on the surface. With the multiplication and attenuation of bottle cells the indentation which may be called **invagination** deepens. The cleft formed by invagination (19 hours after fertilisation) is the

dorsal lip of blastopore. Cells begin to move internally and form a new cavity called **gastrocoel** or **archenteron**. With continued migration of cell, it increases in size. As more and more cells become involved in the process of gastrulation, the blastopore lips extend laterally and ventrally to form a complete ring of blastopore. Accordingly, the cells bounding the blastopore on lateral sides form the lateral lip and those on the ventral side as ventral lip. Blastopore becomes crescentic, horse shoe shaped and finally circular. At the beginning, the blastopore lies in the pigment free prospective endoderm and by the time it forms a complete circle, dark, pigmented cells of animal hemisphere moves into the interior except for the large group of yolk cells crowded at the blastopore called **yolk plug**. The size of the archenteron increases obliterating the original blastocoel. The lips of blastopore or rim of blastopore are not an anatomical entity but only constantly changing structural expression of turning of surface cells inside. The cells of the blastopore do not simply pile up but move through the blastopore lips to the interior and are redistributed. Thus, the cells at the rim of blastopore undergo **invagination**.

Once inside, the cells take different routes such as the notochordal and somatic mesoderm cells stream upward and stretch out along the longitudinal axis; and the lateral and ventral mesoderm spreads out in forward and downward direction between ectoderm and endoderm. The prospective notochord and mesodermal cells move as a unit i.e. is **mantle**. It has been found that the fibronectin lattice secreted by the cells of blastocoel roof helps in the attachment of mesodermal cells as mesodermal mantle or mesodermal sheet. It also directs cell movement.

The side, where the sperm enters the egg, marks the future ventral surface of embryo whereas the opposite side where the gastrulation occurs is the dorsal surface of embryo. The cortical rotation enables the vegetal blastomeres (opposite point of sperm entry) to induce the cells below the grey crescent to inaugurate gastrulation.

The dorsal lip of blastopore is the most important and powerful self differentiating group of cells or **organiser** in the amphibian gastrula. Throughout the gastrulation, the embryo retains its uniform size and shape because as soon as the prospective endoderm and mesodermal cells move from the surface, their place is taken over by ectoderm cells which undergo **epiboly** or stretching. So there is pronounced thinning and stretching of ectodermal cells and to compensate for the increased surface area the blastoderm changes from thick layer to a thin layer. Because of rapid epiboly by prospective ectoderm cells and NIMZ (Non involuting marginal zone) blastopore is shifted posteriorly. The contracting blastopore withdraws the yolk plug completely into the interior which marks the end of gastrulation movement. It is reduced to a small slit called proctodeum which joins the endodermal outgrowth to form a cloacal aperture. It is called anus in higher vertebrates. The archenteron forms pharynx and oral evagination extends from it towards epidermis. An ingrowth of epidermis referred to **stomodeum** fuses with the oral evagination to form oral plate. This oral plate is perforated to form mouth. The fate of blastoderm into anal or oral apertures is also one of the criteria for classifying the animals into **protostomes (mostly invertebrates) and deuterostomes** (echinoderms, chordates.)

After gastrulation, the blastopore of protostomes (first mouth) divides to form both mouth and anal opening whereas in deuterostomes, the mouth arises at a different place away from site of blastopore and the anus forms at the site of blastopore. The protostomes division of metazoans embraces flat worms, annelids, molluscs and arthropods. The deuterostome division of metazoans includes echinoderms and vertebrates.

The steps in the amphibian gastrulation are:

- Invagination by change in shape of the cells which is apparent as a small slit in beginning below the grey crescent area.
- It is accompanied by involution.
- Blastopore changes shape and its final form is a circle.
- Epiboly occurs simultaneously with emboly.
- The blastocoel is obliterated by archenteron. The chordamesodermal cells move as a mass called chordamesodermal mantle.
- The termination of gastrulation is by contraction of blastopore and its subsequent shift to posterior position.

The difference in amphibian gastrulation from Amphioxus is because of 2 – 3 layered blastoderm and eccentric blastocoel .

Gastrulation in chick: (Fig 3.7) Avian egg is a telolecithal egg. Fertilised ovum passes down the oviduct acquiring en route layers of albumen, shell membranes and shell. The oviducal journey takes 15-20 hours and during this time cleavage takes place and germ layer formation is initiated. Cleavage is confined to the **blastodisc** and is called **meroblastic**. The blastula is called **discoblastula** which has blastoderm differentiated into **area pellucida** and **area opaca**. Area opaca separated from marginal zone is in contact with yolk and helps in dissolving yolk granules and forms extra embryonic structures. Area pellucida has subgerminal cavity below because of which it is not opaque. This space is created due to absorption of water from albumen by blastoderm cells which later on secrete the fluids between themselves and yolk. The blastoderm splits by the **delamination** forming upper epiblast which encompasses prospective ectoderm, mesoderm and endoderm, and the lower hypoblast layer which lines subgerminal cavity that forms extra embryonic structures. Thus, the avian blastoderm is bilaminar. The hypoblast is formed by the disconnected clusters of delaminated cells which form primary hypoblast. It is joined by the sheet of cells from posterior margin of blastoderm called **Koller's sickle** (a local thickening) which migrates anteriorly pushing primary hypoblast closer thus forming a complete secondary hypoblast (Eyal Giladi et al, 1992, Bertocchini and Stern, 2002). The space between the hypoblast and epiblast is called **blastocoel**. The two layers are joined at the marginal zone of area opaca. Epiblast forms complete embryonic structures whereas hypoblast forms extra embryonic membranes. The hypoblast provides the substratum for the oriented deployment of prospective mesoderm and endoderm.

Few hours after the incubation, there appears a thickening in the posterior marginal zone (PMZ) just anterior to Koller's sickle which extends to three fifth of entire length of area pellucida. This thickened strip is called **primitive streak**. However, the cells converging on the primitive streak do not pile up indefinitely rather they turn downward through the streak by **ingression and involution**. The first cells to ingress are endodermal precursors from epiblast and then the mesodermal cells which spread anterolaterally direction as a separate sheet of cells between hypoblast and epiblast. Gastrulation thus follows the process of **convergence, ingression, involution** and **elongation**. The elongation of primitive streak is directed by hypoblast layer. Primitive streak is broad initially and its edges are vaguely indicated, later on it elongates and contracts in transverse direction to form **definitive primitive streak**. The cells move singly but in a coordinated fashion. A structural expression of this movement through the streak is a depression along the length of streak i.e. **primitive groove** and **primitive folds** that flank the groove. The primitive streak, its groove

and folds correspond to the amphibian blastopore and its lips except that the avian blastopore is elongated rather than circular. Continued convergence of more material results in anterior elongation of the streak followed by called forward streaming or stretching movement. Due to this a thickening appears at the anterior end of streak called **primitive node or Henson's node**. Just posterior to the node is a pit called **primitive pit** and posteriorly from the pit extending full length of streak is primitive groove which is flanked by primitive ridges. Mid region of the streak is light due to the groove. The prospective notochordal tissue converges on the **Henson's node**, sinks through it, and then passes directly forward as a tongue of tissue called **head process**. The early head process is a thick aggregation of cells projecting forward from primordium of notochord and vertebral column but not the head. The establishment of head process marks the completion of elongation of the primitive streak. The streak then shortens and reduces to a fragment in the tail bud and finally disappears signifying the deployment of mesoderm is complete. The entire embryo develops anterior to the streak. When the node and streak moves posteriorly, the head process and prechordal mesoderm stretches out anteriorly.

Shortly after the completion of primitive streak, the area around it is called **embryonal area** and it gives rise to embryo proper whereas other area forms the extra embryonic structures. The primitive streak is equivalent to blastopore as it is a mass of moving cells.

The important differences in blastopore of amphibia with primitive streak of chick are

- In the primitive streak there is mixing of cells which may cross from one side to other and there is never a true cavity called archenteron.
- Embolic movement in chick through lateral lips precedes the embolic movement through dorsal lip of blastopore whereas it is opposite in amphibia.
- Henson's node is equivalent to the dorsal lip of blastopore of amphibia.
- In chick, there is literally no ventral lip although original posterior streak is considered as its functional homologue. No new archenteron develops but original cavity is replaced by endodermal cells.
- The movement of presumptive mesodermal cells is singly and not as a sheet of cells as in amphibia.

When primitive streak regresses, epiblast is composed entirely of presumptive ectodermal cells which proliferate, stretch and spread to surround the yolk completely. It is difficult and takes longer time than the embolic movements. It occurs by the multiplication and migration of presumptive ectodermal cells. The marginal cells of area opaca attach firmly to the vitelline envelope. These cells are different from other blastoderm cells as they send cytoplasmic processes on the vitelline envelope and pull the ectodermal cells around the yolk. They bind to the laminar proteins of the vitelline envelope. The cells migrating through the Henson's pit forms the head mesoderm and notochord. **Regression of primitive streak** marks the end of gastrulation in chick. After regression of streak, the remaining material is transformed into a tail bud. The termination of avian gastrulation is marked by the formation of three germ layers-- the ectoderm surrounding the yolk and endoderm replacing the hypoblast whereas mesoderm is positioned between the two layers.

Gastrulation is followed by neurogenesis, notogenesis and mesogenesis as thickening of the median strip of ectoderm anterior to Henson's node forms neural plate which develops lateral folds called neural folds, finally fusing to form neural tube. **Fibroblast Growth Factor**, (FGF) synthesized in the Henson's node prior to gastrulation prepares the epiblast for neuronal phenotype (Streit *et al.* 2000). Then, appears the head fold, subcephalic pocket and

notochord. The mesoderm grows out laterally and posteriorly from the primitive streak. In early chick embryo stages, the area immediately anterior and beneath the head is free of mesoderm and is translucent called **proamnion**. After 21–22 hours of incubation, mesoderm on either side of notochord differentiates into mesodermal somites. At 21 hour of incubation, first somite is laid. Mesodermal somites are the accurate indicators of development stages of chick. One may compute the age of chick embryo by counting the number of its somites, since one somite is added every hour from twenty one hour till forty eight hours of incubation. It is not very clear what directs the differentiation of PMZ to initiate gastrulation. Recent studies have shown that *Vg1* gene plays a crucial role in forming the primitive streak (Skromme and Stern, 2002). The chordin and sonic hedgehog gene expression is for the formation of anterior portion of primitive streak that is Henson's node. The important steps in the chick development are:

- (1) Blastoderm is present as a small disc on the yolk. It splits to form epiblast and hypoblast where epiblast contributes to the complete embryo.
- (2) Embolic movements are different from that of lower vertebrates and they are mainly convergence, ingression and extension.
- (3) Primitive streak is a major characteristic structure of bird gastrulation which is initiated by PMZ.
- (4) No true archenteron is formed.
- (5) Epiboly is difficult and time consuming where the cells stretch and surround the yolk also. Primitive streak is like a moving escalator or conveyor belt which means a changing structure and is equivalent to blastopore functionally.
- (6) Termination of gastrulation is indicated by regression of streak which is equivalent to closure of blastopore.
- (7) The difference in morphogenetic movements as observed in birds is due to large yolk in eggs.

Gastrulation in mammal (man)(Fig.3.8 ,3.9): The mammals are viviparous compared to oviparous condition of lower vertebrates. It is difficult to obtain eggs and embryos in man though it can be obtained by sacrificing pregnant females in other mammals for in vitro studies. New techniques have developed for in vitro studies that has made astonishing advancements in mammalian embryology. Heavy doses of gonadotrophins are given to volunteer females that causes ripening of ovarian follicles. By a small incision, an aspirator and laparoscope is introduced into the abdomen and follicles are sucked up. They are grown and fertilised in vitro for developmental studies. After in vitro fertilisation, the egg has to be introduced into the uterus of female because of embryo's dependence on it. Reimplantation is done in pseudo pregnant uterus of female. This method of production of **test tube babies** was successfully done by Dr. Steptoe, Edwards and Bavister (1978) in England.

The ovum of placental mammals is alecithal (secondarily microlecithal) and undergoes holoblastic cleavage. The initial divisions occur as the ovum makes its way through the oviduct (Fig 3.8 & 3.9). The divisions are rapid so that by the end of third day, the number of blastomeres increase and it forms morula. In due course, the superficial cells form an epithelium called trophoblast and inner cluster of cells is called **inner cell mass** or **embryonic knob**. Zona pellucida disappears completely and blastocyst (mammalian blastula) attaches by the trophoblast to the uterine wall. The cells of inner cell mass are basophilic and give rise to the three germ layers so they are the formative cells. The cells of trophoblast or trophoderm multiply rapidly and push into the uterine wall. The cytolytic action of trophoblast breaks the uterine tissue which is used by the embryo as food till it develops placenta. This material is called and the nutrition is called embryotrophic. Sooner, a cavity appears in between inner cell mass and trophoblast The fluid is imbibed in this cavity and trophoblast is lifted off the egg but it remains attached from one side (called rauber's layer)

which corresponds to the dorsal side of embryo. The mammalian embryo is now called **blastocyst** or **blastodermic vesicle**. Hypoblast layer separates from inner cell mass and lines the cavity. In certain mammals like rabbit and insectivores, the layer of trophoblast over the blastodisc is lost so that it becomes superficial but in higher mammals it persists as such. The embryo now enters gastrulation which resembles that of birds as the primitive streak and Henson's node are formed but streak is smaller than that of birds. Striking differences in the development of birds and mammals specially gastrulation is the precocious formation of one of the fetal or extra embryonic membranes that is amnion. The chick embryo is in the advanced stage of development that is primitive streak is formed before the formation of amnion .

In man, first three days are spent by the fertilised egg in descending through the uterine tube during which time the cleavage starts. Cleavage is unique as it becomes asynchronous very rapidly . By 3rd day, that is about sixteen cell stage, morula is formed . At the eight cell stage, the blastomeres develop differential adhesion properties so, the outer cell surface becomes convex and inner surface becomes concave. This reorganisation is called **compaction** which involves the activity of cytoskeletal components and adhesion of blastomeres. The development of differential adhesion between blastomeres results in segregation of cells as **inner cell mass** and **outer cell mass**. Inner cell mass is also called **embryoblast** as it gives rise to the embryo proper . The outer cell mass is also called **trophoblast**. By 4th day, the cells begin to absorb fluids which is collected in the blastula so it is called blastocyst . By the 5th day the blastula hatches from the zona pellucida and later on embeds in uterine wall so called **implantation**. As long as zona pellucida is present the embryo cannot attach to the uterine wall.

Occasionally the blastocyst also implants in the peritoneal cavity or on the surface of ovary or on the other abnormal site. The epithelium at that region responds with the increased vascularity so that the blastocyst starts its development. This is called **ectopic pregnancy** which may sometimes threaten the life of mother as blood vessels at these sites are ruptured due to the growth of embryo.

The blastocyst hatches from the zona , by secreting protease enzyme trypsin which lyse the matrix of zona.. The trophoblast also secretes another set of enzymes as collagenase, stromelysin, plasminogen activator which digest the extracellular matrix of uterine tissue and enables it to bury in uterine wall. This is accompanied by the gastrular movements that is the formation of primitive streak and groove similar to that of birds. Twinning of embryos may sometimes result by :

- a) splitting of inner cell mass at an early stage (dizygotic twins)
- b) simultaneous ripening of two graafian follicles (monozygotic twins)
- c) Liberation of two ova for fertilisation .
- d) presence of two oocytes in one graafian follicle.

When inner cell mass is isolated and grown under certain conditions, the cells remain undifferentiated and divide in culture (Martin, 1981). They are called **embryonic stem cells** (ESM), which are now days exploited for therapeutic use . They are called embryonic stem cells as each of them can generate all the cells of embryo when separated (Gardner , 1972).

Thus we see, that gastrulation is a very coordinated process giving rise to the new forms of the embryo. As exemplified from the study of protochordate (Amphioxus), amphibia (frog), birds (chick), mammal(man), we conclude that there is an increasing complexity in the gastrular movements of higher vertebrates . Comparative study of gastrulation in different animals enables us to understand thoroughly the complicated process. These movements depend on the type of egg, the type of blastula and mode of development. The similar development process in birds and mammals, though the differences exist in the egg types, throws light on the fact that both have descended from the common reptilian ancestors.

Whatever be the epibolic or embolic combination, the final aim of the rearrangement of cells of the embryo is achieved by gastrulation. By this type of mass movement of cells or gastrular movement, the final form is called **gastrula** which has external ectoderm, inner endoderm and mesoderm sandwiched between the two . It is the basis for inductive interactions with new neighbours of cells for organogenesis.

Studies on amphibian gastrulation have explained that one of the primary requirement for the inauguration of gastrulation is the expression of factors from specific region of oocyte called **Nieuwkoop center** (or PMZ of bird). The cytoplasm in this region contains mRNA previously transcribed by the maternal genes. It is likely that in mammals B - catenin, activin and BMP are important molecules for gastrulation. Knock out studies in mice have shown that both activin type- 1 and activin type – 2 receptors are required for the appropriate development and elongation of the primitive streak. The factors like B catenin, BMP, activin are first expressed in hypoblast which induces the epiblast to form the primitive streak. Later development of the primitive streak requires the expression of zygotic genes by the epiblast which is mainly the homeobox gene **goosecoid**. Primitive streak defines the left and right side of the embryo. The embolic cells when enter the primitive streak, the adjacent epiblast cells cease to express E-cadherin that holds the cells together so that they involute as single cells and move inside the streak.

Derrangements of gastrulation disturb the migration and differentiation of mesoderm cells causing array of defects like caudal dysplasia, sirenomelia (fusion of lower limb buds), etc. Mutation in genetic loci involved in gastrulation and notogenesis result in developmental defects.

Through out the gastrulation , the volume of embryo does not change but these movements require energy and so there is increase in oxidation processes. Compared to cleavage and blastula there is increase in oxygen consumption also. Histochemical methods have been employed to study source of energy during gastrulation and it is found that in dorsal lip of blastopore most of glycogen is used. Animal pole of gastrula shows high oxygen consumption compared to vegetal pole. Second peculiar metabolic feature is increase in protein synthesis during this period. It has been found that there is activation of certain genes during this period as mRNA present during gastrula are different from blastula . It also supplies adequate quantities of tRNA and rRNA. The successor of gastrula is neural stage where the next step of development begins that is **differentiation** . Mammalian gastrulation has some peculiar features :

1. It follows the similar gastrulation process as that of bird and reptile though it is having negligible amount of yolk in the egg. Small primitive streak is formed as compared to that of bird and reptile.
2. The blastoderm differentiates into trophoblast and inner cell mass.
3. Inner cell mass forms the complete embryo so the gastrulation is restricted in this region
4. Foetal membranes, a characteristic feature of amniotes, start forming before gastrulation because of the immediate requirement of food by the growing embryo. This is a prerequisite for the formation of placenta in viviparous mammals.

Fate of germ layers; Gastrulation is important in laying down the primary germ layers of the embryo. The simple organisation of embryo when it changes from monolayered to bilayered and trilayered is reminiscent of ancestral adult conditions that occur in primitive groups of

invertebrates. Cnidarians and ctenophores have only two germ layers that is outer ectoderm and inner endoderm so they are called **diploblastic** but the germ layers are called primary germ layers. In Bilateria which includes few invertebrates starting from platyhelminthes to all the chordates, three germ layers are laid and are called secondary germ layer. The relationship of these layers is also evident from their names, the outer layer ectoderm, the middle layer mesoderm and the endoderm as the inner layer. The germ layers are the starting point in the fabrication of variation which different classes of animals have developed but with the common lay out plan of general body structure of vertebrate group as a whole. It also marks the transition from simple increase in number of cells to developmental differentiation as within the layer localised group of cells with different development potentials become apparent. This is evident from the fact that gastrulation is followed by neurulation, notogenesis and mesogenesis.

As discussed earlier, the ectodermal cells in front of the blastopore or in front of the primitive streak in chick and mammals begin to divide rapidly to form thick plate of ectoderm followed by neural fold and neural tube Neural tube differentiates anteriorly as brain and posteriorly as spinal cord. Neural crest cells form few ganglion cells or pigment cells.

The development of notochord from chordamesoderm is called notogenesis and the separation of mesoderm is called mesogenesis. The cells of roof of archenteron form the notochord below the neural tube.

On the sides of the notochord, the mesoderm spreads out in the form of sheet. It grows down on either side of gut and meets in the midventral line. The mesodermal sheet splits into two layers, the outer layer near the ectoderm as **somatic mesoderm** and the inner layer close to endoderm is the **splanchnic mesoderm**. The space between the two is **coelom**. At the sametime, the sheet gets sub divided horizontally into three distinct regions.

1. Dorsal segment is **epimere**
2. Middle segment **mesomere**
3. Ventral segment is **hypomere**

The lateral wall of the epimere is called **dermatome**. It forms dermis and dermal derivatives, its dorsal median wall is called **sclerotome**. It forms the units of vertebral column. The ventro median wall is **myotome** that forms the skeletal muscles and appendicular skeleton. The fate of three germ layers is listed below:

A. **Ectoderm or (Neuroectoderm)**

1. **Somatic Ectoderm** – Epidermis, epidermal derivatives (scales, feathers, hair, nail, claws, glands, etc); olfactory organs, lens of eyes, auditory vesicles (labyrinth), anterior pituitary, lining of stomodaeum and proctodeum.
2. **Neural crest** – Ganglia; sensory nerves; branchial skeleton, adrenal medulla, pigment cells.
3. **Neural tube** – Brain, spinal cord, cranial and spinal motor nerves, retina, optic nerve and posterior pituitary.

B **Mesoderm (Chordamesoderm)**

1. **Notochord** – Persists as a rod in Amphioxus; in others replaced by vertebral column.
2. **Epimere** :
 - (a) Dermatome - Dermis
 - (b) Sclerotome – Vertebral column
 - (c) Myotome – Skeletal muscles and appendicular skeleton.

3. Mesomere :-

- (a) Excretory organs
- (b) Reproductive organs

4. Hypomere

- (a) Somatic layer – Parietal peritoneum
- (b) Splanchnic layer – Visceral peritoneum, mesenteries, heart, blood cells, blood vessels, gonads, muscles of viscera
- (c) Coelom – Body cavity (in protostomes)

C. Endoderm (Archenteron) :

Lining of digestive tract, liver, pancreas, respiratory system, urinary bladder, Endocrine glands (thyroid, parathyroid, thymus), lining of vagina, urethra, Reproductive glands, auditory tube and cavity of middle ear.

Somatopleure is the term for the somatic mesoderm and ectoderm where as splanchnic mesoderm and endoderm form **splanchnopleure** .

Mesodermal tissue is important as besides forming different organs it also forms the body cavity or coelom . The coelom is the cavity which encloses various visceral organs. These organs are held in position by the mesodermal fold called **peritoneum**. Coelom formation in, protostomes is by the splitting of mesodermal tissue which creates a space or cavity in which organs are placed . Lower deuterostomes on the other hand have different method of coelom formation. The archenteron evaginates to form enterocoelic pouches. The cavity of pouch becomes the coelom and the pouch moves down to meet mid ventrally and the wall forms the mesoderm. Similar coelom development in protochordates and the echinoderms explains the origin of this group from echinodermata. The flatworms lack body cavity and have solid body construction so they are called acoelomates. Rotifers and roundworms do not have true coelomic cavity as it is not lined by mesoderm. It is called **pseudocoel**. It is formed from the persistent blastocoel. Since it is not a true cavity so the viscera is not held by peritoneal cover but lies free in the cavity or space.

Neurulation The formation of neural tube is called neurulation and it causes the change from gastrula to neurula. (Fig 3.10 & Fig 3.11) During neurulation the embryo stretches to some extent. Neurulation is by two ways that is **primary neurulation** and **secondary neurulation**. Primary neurulation is by chordamesoderm which directs the overlying ectoderm to proliferate, invaginate and form neural tube by pinching off. In secondary neurulation neural tube is formed from the solid group of cells that sinks into the embryo and later on cavitates .(Schoenwolf , 1991). Secondary neurulation, is exclusively seen in fishes, whereas in amphibia, birds and mammals it is by both the methods. In amphibia, caudal neurulation is by secondary method but in man it is observed that secondary neurulation starts at thirty five somite stage. Neurulation occurs in the following stages.

- a. Neural plate stage(Medullary plate)
- b. Neural fold stage (Medullary fold)
- c. Neural tube stage.

The presumptive neural ectoderm region becomes distinct from the non nervous ectoderm when inductive signals are given by the underlying mesodermal cells on the ectoderm. Following these instructions, the cells become columnar neural plate cells. The shaping of

neural plate involves the forces intrinsic to neural plate cells. The midline neural plate cells also known as called Median Hinge Point (MHP) cells, (Catala *et al* , 1996) are the most important in shaping the neural plate. The bending of neural plate is by both the intrinsic and extrinsic factors. MHP cells are anchored to the notochord cells below and form a hinge which results in furrowing in the middorsal line of the neural plate . This is mainly due to the induction by the notochordal cells on the overlying cells to reduce the height of cells by changing them to wedge shape. The furrowing of neural plate facilitates the formation of neural folds which fuse at the dorsal midline to form the neural tube. The neural tube encloses a space called neural canal or **neurocoel** which opens anteriorly and by **neuropore** but the opening is closed posterirly due to the complete fusion of medullary folds. In certain vertebrates it may communicate with the archenteron as **neurenteric** canal .The cells which do not participate in the formation of neural tube, form neural crest cells. The formation of neural tube does not occur simultaneously throughout the ectoderm e.g. in the bird's neurulation starts forming at the anterior end earlier than that at caudal end. Unlike the neural tube closure in chicks, in mammals it is initiated at several places along the anterior posterior axis. Failure to close the neural tube in posterior region in man results in abnormal condition of spine called **spina bifida** or if it fails to close at anterior region it result in **anencephaly** The neural tube closure which is very important step of neurulation is a result of genetic and environmental factors. Certain genes as Pax 3 sonic hedgehog are essential for the neurulation . The role of dietary factors as cholesterol and folic acid for correct neurulation can not be ignored.

Neural tube thus formed separates from the surface ectoderm which is explained on the basis of difference in adhesivitiy of cells. It occurs due to change of E-cadherin adhesion molecule to N-cadherin type. One of the key proteins involved in activating neural phenotype in ectoderm is neurogenin (Ma *et al* ,1996) . In *Xenopus*, neural canal retains contact with the archenteron through blastopore and it is called neurenteric canal whereas in many animals blastopore is occluded.

Secondary neurulation is seen in the frog and in chick mainly in lumbar and caudal regions. Neurulation by primary method starts at the anterior region while the gastrulation movements are in full swing. As Henson's node recedes farther, the neural plate becomes differentiated and anterior part of neural plate proceed close to neural tube stage. Neural tissue differentiates from ectoderm after the closure of blastopore.

To summarise in the end of this chapter some of the important points listed are:

- Fertilisation is followed by cleavage which converts unicellular zygote to multicellular embryo called morula and then the blastula when the blastocoel appears.
- Due to differences in egg type, a variety is seen in cleavage patterns, blastulae formed and morphogenetic movements.
- Blastula is succeeded by stratification of embryo (three germ layers) called gastrula. The goal is successfully achieved by the blastulae using different methods of mass movements involving number of cell adhesion molecules.
- There is a transition in development process from simple multiplication (cleavage) of cells to that of differentiation (gastrulation , organ rudiment).
- Each step is important for complete development of embryo and no step can be by passed (Fertilisation, cleavage, morula , blastula,.gastrula , neurula).

- Gastrula is the source of different organ rudiments by inductive interactions and the fate of germ layers is uniformly same in all the animals.
- Embryonic development is a step wise complicated but sequential coordinated process.

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