NETAJI SUBHAS OPEN UNIVERSITY

STUDY MATERIAL

POST GRADUATE ZOOLOGY

Paper : 7

Group : A

Developmental Biology

[illegible]

PREFACE

In the auricular structure introduced by this University for students of Post- Graduate degree programme, the opportunity to pursue Post-Graduate course in Subject introduced by this University is equally ava.. ble to all learners. Instead of being guided by any presumption about ability level, it would perhaps stand to reason if receptivity of a learner is judged in the course of the learning process. That would be entirely in keeping with the objectives of open education which does not believe in artificial differentiation.

Keeping this in view, study materials of the Post-Graduate level in different subjects are being prepared on the basis of a well laid-out syllabus. The course structure combines the best elements in the approved syllabi of Central and State Universities in respective subjects. It has been so designed as to be upgradable with the addition of new information as well as results of fresh thinking and analysis.

The accepted methodology of distance education has been followed in the preparation of these study materials. Co-operation in every form of experienced scholars is indispensable for a work of this kind. We, therefore, owe an enormous debt of gratitude to everyone whose tireless efforts went into the writing, editing and devising of a proper lay-out of the materials. Practically speaking, their role amounts to an involvement in invisible teaching. For, whoever makes use of these study materials would virtually derive the benefit of learning under their collective care without each being seen by the other.

The more a learner would seriously pursue these study materials the easier it will be for him or her to reach out to larger horizons of a subject. Care has also been taken to make the language lucid and presentation attractive so mat they may be rated as quality self-learning materials. If anything remains still obscure or difficult to follow, arrangements are there to come to terms with them through the counselling sessions regularly available at the network of study centres set up by the University.

Needless to add, a great deal of these efforts is still experimental—in fact, pioneering in certain areas. Naturally, there is every possibility of some lapse or deficiency here and there. However, these do admit of rectification and further improvement in due course. On the whole, therefore, these study materials are expected to evoke wider appreciation the more they receive serious attention of all concerned.

Professor (Dr.) Subha Sankar Sarkar
Vice-Chancellor

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POST GRADUATE ZOOLOGY

[M.Sc]

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PGZO - 7 : A

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NETAJI SUBHAS OPEN UNIVERSITY

PGZO - 7
Developmental Biology and Endocrinology, Cell & Tissue structure, Function

Group A

Developmental Biology

Unit 1 ❐ Differentiation of primordial germ cell and structure of mature gamate in *Drosophila*, Role of poleplasm, influence of oskar gene, effect of grand childness mutation

Structure

1.1 Germ cells

Gametogenesis is the process by which the sperm and the egg are formed from primordial germ cells that provide the continuty of life between generations. In many animals such as insects, round worms and the vertebrates, there is a clear and early separation of germ cells, from somatic cell types. In several animal phyla these divisions are not well established. In these species such as cnidarians, flat worms and tunicates somatic cells can readily become germ cell, even in adult organism the zooids, buds and the polyps of many invertebrate phyla testify the ability of somactic cells to give rise to new individuals.

In this chapter we will discuss the process of origin of germ cells and the process of differentiation and the factors that regulate the differentiation of germ cell in the context of *Drosophila.*

A. Germ cell migration in *Drosophila*

The organisms where there is an established germ line, the germ cell donot arises within the gonad itself. Rather, their precursor primordial germ cells (PGC) migrate into the developing gonads, the first step in gametogenesis. Then, involves forming the PGC and in the genital ridge as the gonad is forming.

The *Drosophila* germ cells arise form the posterior pole of the zygote. The first step is passive phase wherein the germ cells are displaced by the movement of embryonic cells during gastrulation. The differentiation of the endoderm triggers active amoeboid movement in the primordial germ cells, they travel through the gut endothelium and migrate to the mesoderm. The PGCs then split into 2 groups, each of which become associated with the developing gonadal primordium.

The migration of the PGCs from endoderm to mesoderm is dependent on the

expression of the gene called *wunen*. The product of *wunen* gene is a protein which is expressed in the endoderm immidiately before PGC migration and it repels the PGCs. A mutant of their gene shows the PGCs wonder randomly instead of their normal fate within the developing gonad (Fig. 1.1).

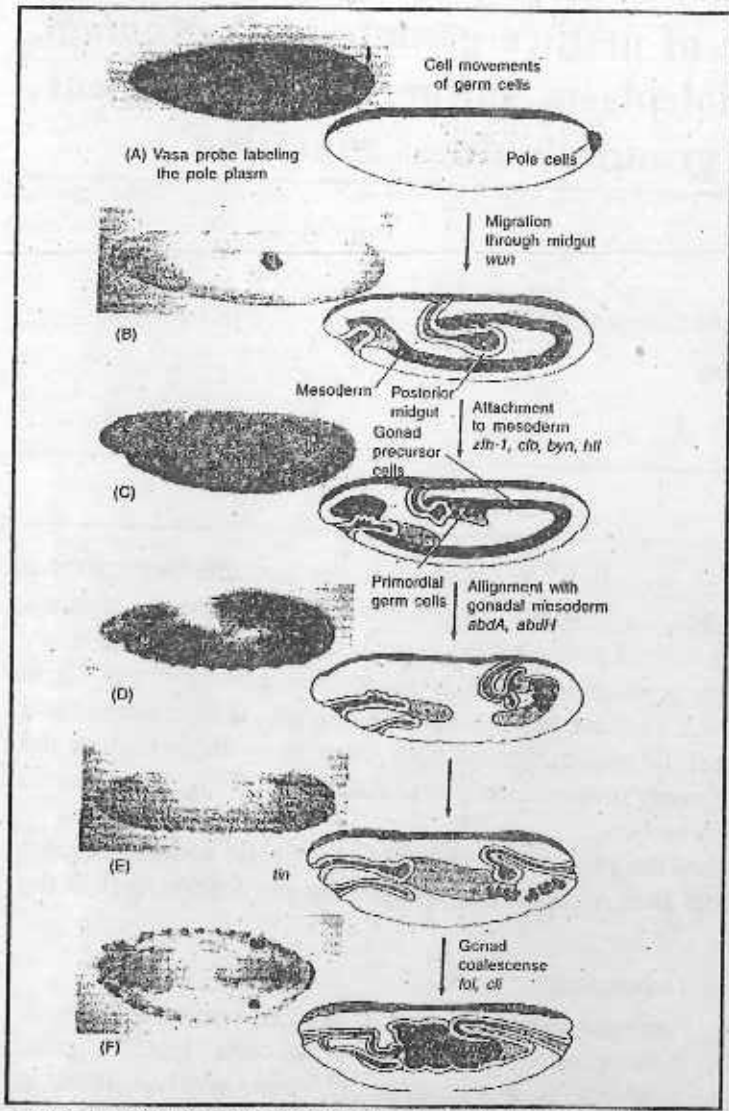


Fig 1.1 : Migration of germ cells in the *Drosophila* embryo. The left column shows the germ plasm as stained by antibodies to Vasa, a protein component of the germ plasm (D has been counterstained with antibodies to Engrailed protein to show the segmentation, and E and F are dorsal views.) The right column diagrams the movements of the germ cells. (A) The germ cells originate from the pole plasm at the posterior end of the egg. (B) Passive movements cary the PGCs into the posterior midgut. (C) The PGCs move through the endoderm and into the caudal visceral mesoderm by diapedesis. The *wunen (wun)* gene product expressed in the caudal mesoderm attracts them. (D-F) The movements of the mesoderm bring the PGCs into the region of the tenth through twelfth segments, where the mesoderm coelesces around them to form the gonads. (Photographs from Warrior et al. 1994, courtesy of R. Warrior ; diagrams after Howard 1998.)

B. Production of sperm or egg

The PGCs that migrating into the gonad are bipotential and can be differentiated into sperm or ova, depending on their gonadal environment. This fate of PGCs into the gonads has been observed in a number of animals including eligans, house fly, mouse and other animals.

In *Drosophila* the germ cells are instructed to differentiate either into sperm or eggs by the gonad cells. Female gonad cell make a product that is received by germ cells and which activates a series of proteins whose activity is critical for the early transcription of the germ cell sxl gene. The proper X-chromosome : autosome (A) ratio is also needed. By these mechanism the fliés get to make eggs while the XY flies make sperm.

C. Spermatogenesis

Spermatogenesis is the process of production of sperm from primordial germ cells. The primordial germ cells arrive at the genital ridge of male embryos then they become incorporated into the sex cord. They remain there until maturity and the PGC differentiated into spermatogenic cells. They remain associated with Sertoli cells and the Sertoli cells nourish and protect the developing sperm cells.

The spermatogenic cells divide mitotically to give rise a clone of sper-matogonia.

At maturity sper-matogonial cells divide to generate the primary spar-matocytes that enter into meiosis.

Each primary sperma-tocyte undergoes the 1st meiotic division to yield a pair of secondary sparmatocyte which complete the 2nd division of meiosis. The haploid cells are called spermatids.

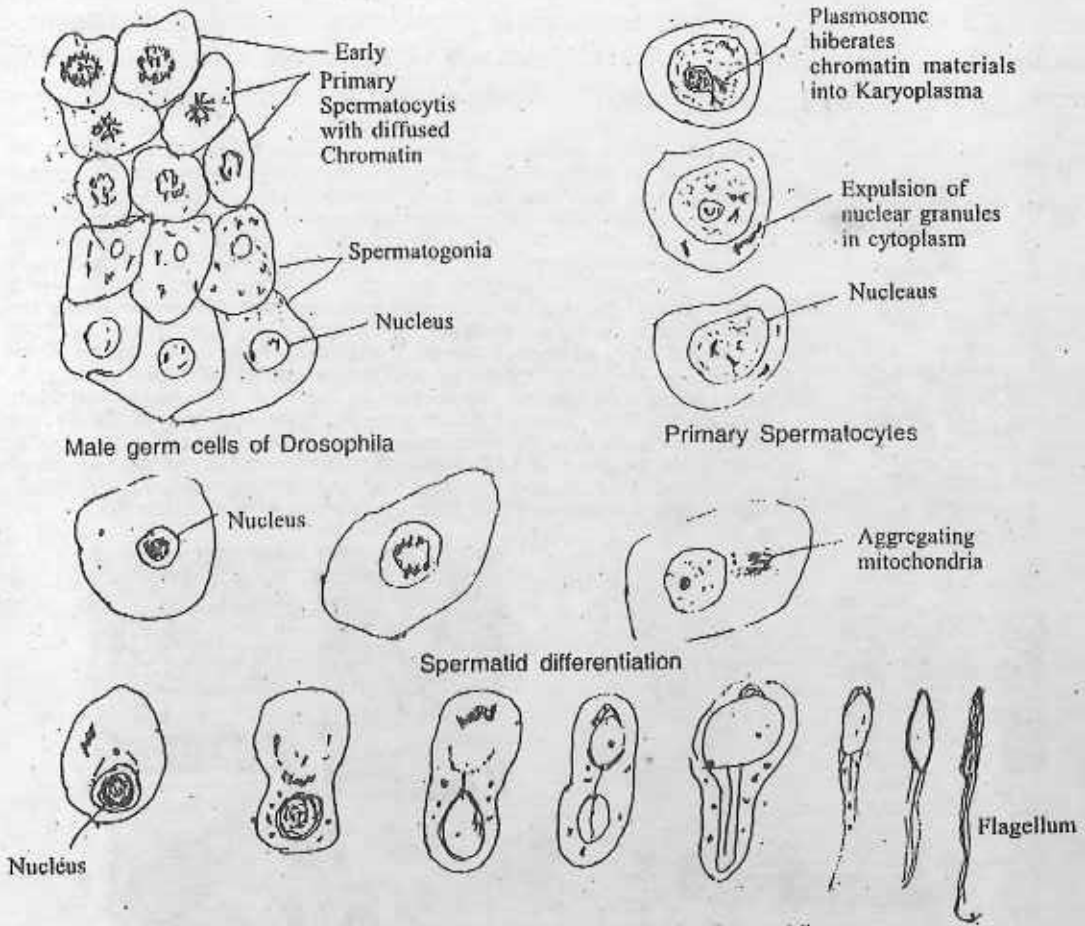


Fig 1.2 : Stages of Spermiogenesis in *Drosophila*

A haploid spermatid undergoes maturation by a process called spermiogenesis or spermateliosis.

During spermiogenesis spermatids undergo several morphological changes viz. the formation of acrosome from Golgi apparatus, condensation of neclear apparatus, formation of flagellum from centriole and extrudation of the remaining cyloplasm as cyloplasmic droplets.

Spermiogenesis is completed into production of mature male sex cells or sperm.

D. Genetic events during spermatogenesis

Like formation of PGC, their migration and localization in the gonad and the process of spermatogenesis is under the control of several genes. It has been shown that like mammalian DAZ gene the *Drosophila* gene RB97D and BOULE are both essential for spermatogenesis. Spermatogonia degenerate in male flies, deficient in RB97D, while the germ cells of male flies lacking the BOULE gene do not enter meiosis. Similarly ROUGHEX gene transcribe by premeiotic *Drosophila* spramatogonia control the number of meiotic division. Males lacking functional copy of ROUGHEX gene, undergo an extra meiotic metaphase in addition to the normal one. Increase in

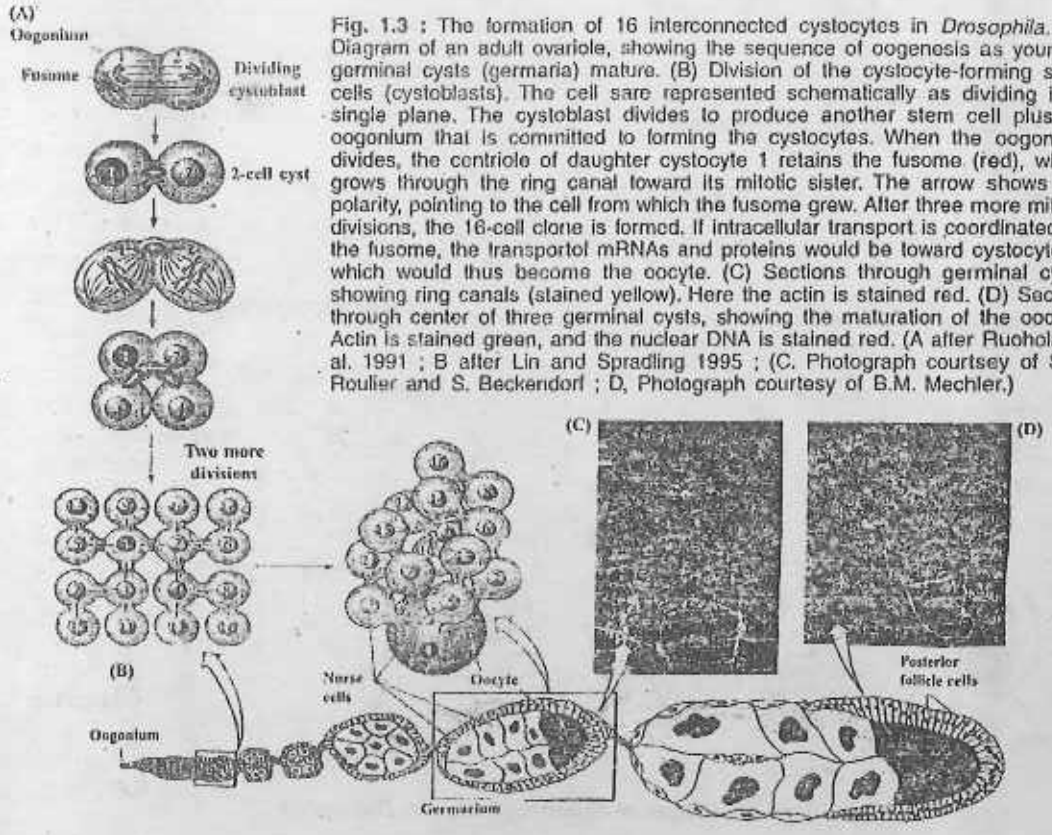


Fig. 1.3 : The formation of 16 interconnected cystocytes in *Drosophila*. (A) Diagram of an adult ovariole, showing the sequence of oogenesis as younger germinal cysts (germaria) mature. (B) Division of the cystocyte-forming stem cells (cystoblasts). The cell sare represented schematically as dividing in a single plane. The cystoblast divides to produce another stem cell plus an oogonium that is committed to forming the cystocytes. When the oogonium divides, the centriole of daughter cystocyte 1 retains the fusome (red), which grows through the ring canal toward its mitotic sister. The arrow shows the polarity, pointing to the cell from which the fusome grew. After three more mitotic divisions, the 16-cell clone is formed. If intracellular transport is coordinated by the fusome, the transportol mRNAs and proteins would be toward cystocyte 1, which would thus become the oocyte. (C) Sections through germinal cysts showing ring canals (stained yellow). Here the actin is stained red. (D) Section through center of three germinal cysts, showing the maturation of the oocyte. Actin is stained green, and the nuclear DNA is stained red. (A after Ruohola et al. 1991 ; B after Lin and Spradling 1995 ; (C. Photograph courtsey of S.E. Roulier and S. Beckendorf ; D, Photograph courtesy of B.M. Mechler.)

the concentration of ROUGHE gene result in the failure to executed meiosis II (Fig. 1.2).

During sparmatogenesis some sperm specific genes are transcribed. In *Drosophila melanogaster* the sperm specific genes transcribed is for β2 tubulin. This isoform of β2 tubulin is seen only during sparmatogenesis and is responsible for formation of meiotic spindles, the axonym and the microtubules. It is to be noted that a sperm axonym in *Drosophila* is a large undertaking. The sperm tail is 2mm long as long as the entire male fly. The sperm of related species of *D. biphorka* is approximately 20 times longer than the flies producing them.

E. Oogenesis

Oogenesis, a process of differentiation of the ovum differs from sparmatogenesis in several ways. The gamete produced by spermatogenesis is essentially a motile nucleus whereas gamate formed by oogenesis contains all the factors needed to initiate and maintain metabolism and development, in addition to forming a haploid nucleus. Oogenesis also builds up to store of cyloplasmic factors such as enzymes, mRNAs, organelles and metabolic substances. The sperm differentiate for motility, the oocyte develops a remarkable complete complex cytoplasm (Fig. 1.3).

In *Drosophila* and other insects the Oogenesis takes place by meroistic type of oogenesis. In meroistic oogenesis cytoplasmic connection remains between the cells produced by the organism. In *Drosophila*, each oogonium divides 4 times to produce a clone of 16 cells connected to each other through ring canals. These interconnected cells are called cystocytes and involves a highly order array of cell divisions. Only these 2 cells having 4 interconnections are capable of developing into oocyte and of these two, only one becomes egg. The other begins meiosis but does not complete it. Thus only one of the cystocytes become an ovum and all the other cells become nurse cells.

F. Transport of RNA from nurse cell to oocyte

The oocytes of meroistic insects do not pass through a transcriptionally active stage have no lampbrush chromosome. Autoradiography suggests that RNA synthesis is largely confined to nurse cells and that RNA made by these cells is actively transcript in oocyte of cytoplasm. When the egg chamber of a house fly is incubated in radioactive cytosine, the nuclei of nurse cells show intense labelling. When the labelling is stopped and the nurse cell is incubated for 5 more hours in non-radioactive medium, the RNA is seen entirely in the oocyte of nurse cells. Oogenesis takes place in only 12 days so that the nurse cells are metabolically active this time. As polytene chromosome present in *Drosophila* their enhanced RNA synthesis activity in nurse cells is attributed to a mechanism in which all the products of 15 nurse cells are known to pass ribosomal and massenger RNAs into the oocyte *cytoplasm* as a result

enhance proteins along with ribosomes are aggrigated in the anterior end of oocyte cytoplasm. Such a condition is called polyribosome or polysome.

1.2 Spermatogenesis in *Drosophila*

The detailed study on *Drosophila* spermatogenesis is difficult to unravel. Their chromosome number being only eight but their size and behaviour are somehow advantageous to study in the critical periods of spermatogenesis. The classical cytological study in this aspect was made as early as in 1929 by La Cellule, Metz in 1926 and Huettmer in 1930. They used routine fixatives and stains as former-alcohol-acitic acid and hematoxylin. Later, molecular and genetic studies of *Drosophila* spermatogenesis were done by Henning in 1996, Hales and Fuller in 1997 and Tates in 1971.

The gonads are the only organs that retain their identity throughout the entire development. The testes can be found from early larva to mature fly.

In earlier stage, they are located in the posterior third of the larva, and are first spherical and later ovoid in shape. At pupal stage, they begin to elongate, coiling and twisting and at the time of emergence, they are fully grown and elongate tubular organs.

Germ cells

The early testes of the very early larva contain only *spermatogonia*. They are small rounded cells, The testis attain an ovoid shape when the larva increases in size, and the growth period of spermatocytes begins followed by meiosis. Larvae, on way to become pupae, contain all the stages of spermotogenic germ cells excepting mature spermo. Pupal larger testes contain more spermatocytes and almost mature spermatozon. The testis of adult fly is almost filled with mature sperms.

The structure of the testis is unique in lacking well-defined cysts as formed in other insects. Certain stages of spermatogenesis have a tendency to be grouped together and such groups shrunk away from other groups of germ cells in different stages. But in later stages, larvae and pupa become intermingted with all the types of germ cells.

Spermatogonia and primary spermatocytes

Spermatogonia are comparatively smaller in size with heavily stained chromatin. When the testis elongates and increases in size, the spermatogonia are restricted to a small part in the distal end of the testis and continue to remain there of the adult fly. Dipteran homologous chromosomes have a tendency to occur in pairs in the gonodial divisions but in *Drosophila* male, these are seldom found.

By slight gradations, spermatogonia develop into spermatocytes by gradual

dissolution of the chromatin of spermatogonia as they enter growth period. In the spermatogonia chromatin is heavier and mere condensed. Whereas, in the early spermatocytes, the chromatin becomes loose and flocculent with a round vesicular body appearing in the nucleus.

The large plasmosome in the nucleus of early spormatocyte gradually disintegrates when the primary spermatocyte is ready to enter leptotene stage and now nucleouls also disappears.

Synapsis and diakinasis

Previous observations on their dipteraus have indicated that meiotic stages as leptotene, pachytene diplotene are observe and not usually found in males and chromosome pairing is also absent. But in Drosophila such conditions do not appear. All pre-dialinetic stages are extremely short in duration. The condensation of chromosomes is absent except in late diakinetic stage. The chromosomes conjugates in pairs but crossing over is absent in male. The partial synopsis occurs and is swiftly followed by diakinesis. The pairing is soon followed by tetrad formation.

After this stage, chromosomes intract into masses to assemble into four independent groups in every spermatocyte.

Subsequently, the nucleus elongates, its membrane disappears, the asters are formed and the chromosomes enter the 1st maturation division with XY combination lagging to appear at one side of the other *tetrads* of *dyads* which move to their respective poles.

Spermatials : After second maturation division, four spermatids are produced, which are small round germ cells and the small nucleus of such spermatids increases rapidly in size prior to its transformation.

Mitochondria : In the growing spermatocyte, mitochondria are situtated as a spheroid mass in localised area in the cytoplasm near the nucleus.

During diakinesis the large mitochondrial mass moves toward nucleus surronding it in the form of a ring. At 1st meiosis. it divided into two masses that move to respective poles.

During the second meiosis, the mitochondria surround the entire spindle and divide again into two equal masses in the formation of spermatids.

Spormiogenesis : Spermatial centriole is single and is located close to nuclear membrane. Later, when axial filament grows out from ,the centriole, it is becomes double by proximal division. Nucleus is greatly elongated and narrowed to from the head in spermatozoa. During this progressive transformation of the spermatid, the fibrillar dictyosomes disappear gradually and are replaced by ring-like structures.

The acroblast moves toward opposite side of the nucleus. The two separate masses eventually unite, forming a cap on the nucleus and forms the ascrosome of the sperms.

"Nebenkern" : The **Nebenkern** is a remarkable mitochondrial formation in the spirm of insect as *Drosophila.* Just after meiosis is completed mitochrondia of the spermatid are collected on one side of the haploid nucleus and fuse together into *two giant aggregates.* These aggregates this wrap around one another to produce the spherical Nebenkern. Fuller, 1993 and Tates, 1971revealed EM studies of nebenkern that it resembles an *onion slice* and this early stage of spermiogenesis is called "onion stage". When their flagellum elongates, the two mitochondrial regions of the Nebenkern unfold and elongate down the sides of the axonen (Fig 1.4).

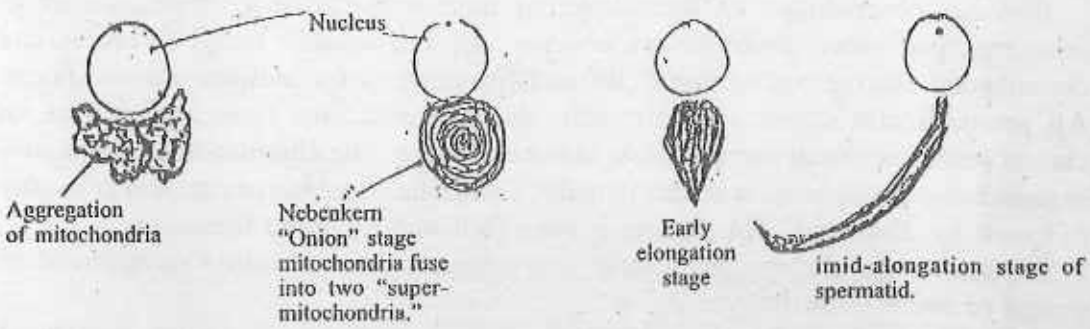


Fig 1.4 : Nebenkern formation in *Drosophila*

"Fuzzy Onions"

The mediator of mitochondrial fusion that regulates Nebenkern formation, is the product of the *fuzzy onionsgene* and appears to be a trans-membrane GTPase associated with the mitochondria during the time of fusion. Male flies with the mutation of fuzzy onion are sterile. They have defective Nebenkern due to the fact that mitochondria have failed to fuse into two giant "supermitochondria".

Hales and Fuller (1997) isolated and sequenced the wild-type allele of *Fuzzy onions* and showed that its product was a GTPase with transmembrane.

Using antibodies against the fuzzy onions GTPase, they showed that this protein is associated with mitochondria at the time of its fusion. It appears on the mitochondria just prior to fusion during the last stages of meiosis II and disappears soon after mitochondrial elogation.

Molecular and genetic aspects of *Drosophila* spermatogenesis

Hennig (1996) revealed that in Drosophila the regulation of sex determination of germ cells occurs relatively independent of that of somatic cells. The paternal and maternal genomes during the germ cell development receive different pattern of imprinting which results in a specific expression pattern of parental genes in the embryo.

During meiosis prophase, differentiation of male germ cells is initiated by re-organisation of chromatin, which leads to a higher packagin of chromatin required to accommodate the genoine in the small sperm head.

The genomic activity during spermatogenesis is found in cells upto the first meiotic division while the actual morphogenetic processes occur post miotically.

From the genetic point of view, it is to note that is *Drosophila* the number of genes giving male sterile phenotypes if mutated,is unexpectedly large. It is possible to assume that pleiotropic effects of many genes may affect sperm development. Another remarkable observation is that the no. of genes specifically and exclusively active during made from cell development is very small. Many genes required for sperm development. Another remarkable observation is that the no. of genes specifically and exclusively active during male germ cell development is very small. Many genes required for sperm development.' We also required for other cellular differentiation pathway. As for required for other cellular differentiation pathway. As for example, *Muscular myosin heavy chain,* though is expressed in testis (Miedema et al 1995), its mutation results embryonic or larval lethality.

Chromatin constitution in *Drosophila* male germ line

During sperm development the nuclear proteins undergo a transition resulting substitution of the normal chromosomal protein by another protein. There are 2 imp. aspects : (1) The normal *histone H1* absent in all shapes of spermatogenesis excepting stem cells. (2) During early postmeiotic development, the *chromatin* passes through a cycle of *condensation and decondensation* before it is finally packed and condensed into the sperm head.

This indicates that transcription is either very slow or fully absent in postmeiotic cells. The condensation-decondensation cycle is therefore related to the rearrangement of chromosomal proteins.

Normally, histone mRNAs are not polydenylated but polyadenylated variant forms of the mRNA for histone genes costing for the histones H2B, H3 & H4 exist in testis (Kremer, 1991). Both genes, **H3.3A** and **H3.3B** are expressed in testes and also in other tissues. However, H3.3A is more transcribed in testis while H3.3B is more strongly expressed in somatic cells.

Structure and function of Y-chromosomal male fertility gene

In 1961, Meyer et al. discovered that in Drosophila the activity of Y-chromosomal male fertility genes in the primary spermatocyte is accompanied by the formation of large lampbrush loops. These are the only genes in Drosophila forming such chromosomal structures contrary to amphibian oocyte.

Only few genes in Drosophila are active is the male germ line as : (i) B2-Tubulin (Kamphues et al. 1979), (ii) a group of seven genes of unknown function (Schafer

c.t. al. 1986), (iii) a history H5-like gene (Russel & Kaiser 1993), (iv)the stellage locus (Livak, 1990), (v) Janus B (Yanicostas, et al. 1989), (vi) a no. of Y-chromosomal fertility genes (Henning, 1988).

The nature of proteins bound to the Y-chromosomal lampbrush loops has been explored (Henmig 1996), one of the proteins, called tzf (testis zine finger appears as a typical nucleic acid binding protein, comparable to some *Transcription factors.*

Other genes active during *Drosophila* spermatogenesis

Henning (1996) identified several gene active in the male germ line. Two genes are single-copy gene functioning in somatic tissues. Some other genes have pleiotrop effects in Drosophila male germ line leading to male sterility one is the gene for the *Laminin B2 chain* (Wanpetel, 1992), and the other gene codes for the *muscular myosin heavy chain (mMHC)* (Miedema et al. 1995).

The *Laminin B2 gene* is expressed in the extracellular matrix of the testis envelope and also expressed in spermatocytes at the RNA level and its protein is expressed at the ultrastructural level of axoneme and the elongation of the nucleus.

The mMHC gene is transcribed in primary spermatocytes during meiotic prophase. The mMHC protein is found within differentiating Nebenkern derivatives. The myosin molecule may be constituents of the percrystalline material found in the tail of mature sperm. This protein may also control its final position in sperm alogation.

Both genes, laminin B2 and mMHC. Thus confirm that genes important in somatic tissues are also important in sperm differentiation.

1.3 Role of poleplasm

In *Drosophila* PGCs forms as a group of cells (Pole cells) at the posterior pole of the cellularizing blastoderm region. These nuclei migrate into the posterior region at the 9th nuclear division, and they become surrounded by the *Poleplasm,* a complex

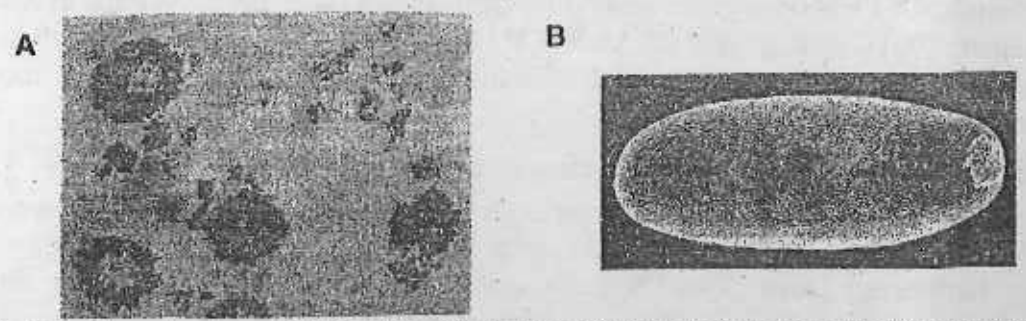


Fig 1.5 : The pole plasm of *Drosophila.* (A) Electron micrograph of polar granules from particulate fraction of *Drosophila* pole cells. (B) scanning electron micrograph of a *Drosophila* embryo just prior to completion of cleavage. The pole cells can be seen at the right of this picture. (Photograph courtesty of A.P. Mahowald.)

collection of mitochondria, fibrills and polar granules. If the pole cells nuclei are prevented from reaching the pole plasm, no germ cells will be made (Fig.1.5).

Nature has provided confirmation of the importance of both the poleplasm and its polar granules. One of the components of the poleplasm is the mRNA of the germ-cell-less gene (gcl). This gene was discovered by Jongens and his collegues (1992) when they mutated *Drosophila* and screened for females who did not have 'Grand offspring'. They assumed that if a female did not place functional poleplasm in her eggs, she could still have offspring, but those offspring would be sterile. This wild-type gcl gene is transcribed in the nurse cell of the fly's ovary, and its mRNA is transported into the egg. Once inside the egg, it is transported to the posterior most portion and resides within what will become the poleplasm. The gcl-encoded protein appears to enter the nucleus, and it is essential for pole cell production. Flies with mutations of this gene lack germ cells (Fig. 1.6).

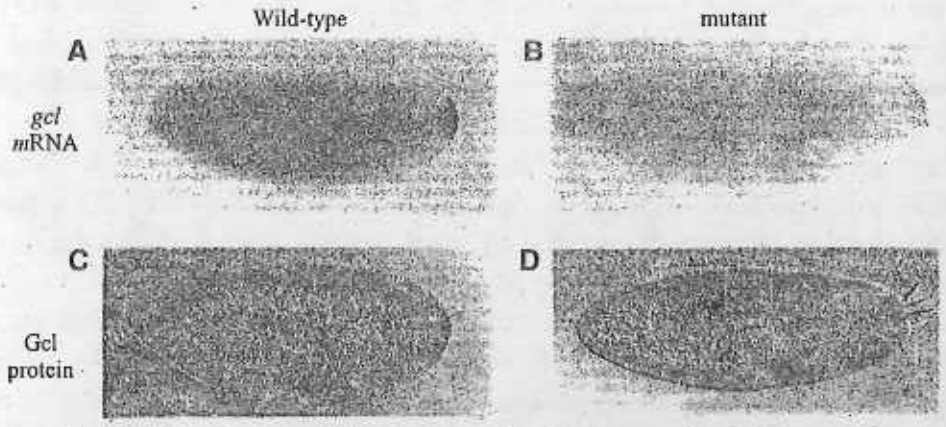


Fig 1.6 : Localization of *germ cell-less* gene products in the posterior of the egg and embryo. (A, B) The gel mRNA can be sen in the posterior pole of early-cleavage embryos produced by wild-type females (A), but not in embryos produced by *gcl*-deficient mutant females (B). (C,D) The protein encoded by the *gcl* gene can be detected in the germ cells at the cellular blastoderm stage of embryos produced by wild-type females (C), but not in embryos from mutant females (D). (From Jongens et al. 1992 ; (Photograph courtesy of T.A. Jongens.)

The posterior end of the egg is known to develop into sperm or egg cell. The posterior blastoderm cells are called poleplasm. The role of polar cytoplasm in *Drosophila melanogaster* in gamete formation can be established by different sets of experiments—

(a) The poleplasm can be stained specifically by using flourecent dyes and autoradiographic techniques. The results show that posterior blastoderm ie. polar cytoplasm has special staining specificity. Such syncytial blastoderm with posterior cells develops into normal flies.

(b) Irradiation of the end of the Syncytial blastoderm causes sterility in the fly that develops.

(c) The contents of the posterior end of a cellular preblastoderm can be removed and injected into an irradiated egg. Progametic cells can be found in the anterior end and the fly that develops is fertile.

1.4 Oskar gene

Cytoplasmic localization of mRNAs

Information required to start a fertilized animal egg on the path toward formation of an enbryo is laid down within the oocyte during oogenesis. We will briefly consider the fruit fly, whose egg, larval and adult stages are illustrated in figure.

The development of the anterior—posterior (head-abdomen) axis of a larval fly and subsequent adult is foreshadowed by localization of specific mRNAs along this same axis in the oocyte. For example mRNAs transcribed from the bicoid gene become preferentially localized at the anterior end of the oocyte, while mRNAs transcribed from the oscar gene become localized at the opposite end.

The mRNAs are subsequently translated at the site of localization. The protein encoded by bicoid mRNA plays a critical role in the development of the head and thorax, where as the protein encoded by oskar mRNA is required for the formation of germ cells, which develop at the posterior end of the larva.

The information that governs the cytoplasmic localization of a mRNA resides in the 3′ UTR (untranslated region) of either the bicoid or oskar mRNA. When the foreign gene is transcribed during oogenesis, the mRNA becomes localized in the site determined the 3′ UTR. Localization of mRNA is mediated by specific proteins that recognize localization sequences (called Zip codes) in this region of mRNA.

Microtubules, and the motor proteins that use them as tracks, play a key role in transporting mRNA containing particles to particular locations. The localization of oskar mRNAs in a fruit fly oocyte, for example, is disrupted by agents such as colchicine that depolymerize microtubules and by mutations that alter the activity of the Kinesis I motor protein.

Unit 2 ❐ Composition of semen, seminal protein and accessary reproductive structure of mammals

Structure

2.1 Semen

Semen or seminal fluid is the important organic substance which the male contributes during reproductive events the copulation. It is the product of the entire male reproductive system and is composed of *spermatozoa* and the *seminal plasmae.*

The Seminal plasma is a fluid and lymph like substance which contains many enzymes, nourishment in the form of fructose and proteins and contain those chemical molecules which protect the spermatozoa from other enviromental hazards. In many mammals, the semen tends to coagulate after its discharge from the penis. In the mouse, rat, gnineapegs, opossum etc. the semen coagualates into a second mass, The *vaginal plug,* once it reaches the vagina if the female. Coagulation of the semen also occurs in man, pig etc. but not in dog, bull and many other mammals. Human semen coagualtes immediately after discharge but liquifies a short time afterward due to the activity of two enzymes viz., *fibrinogenase* and *fibrinolysin,* both of which are synthesized in prostate gland. The liquification frees the sperm to make their long journey to the ova.

2.2 Composition of semen

During the process of ejaculation, sperm passes through the ejaculatory ducts and mixes with fluids from the seminal vesicles, the prostate, and the bulbourethral glands to form the semen. The seminal vesicles produce a yellowish viscous fluid rich in fructose and other substances that makes up about 70% of the human semen. The prostatic secretion, influenced by dihydrotestosterone, is a thin fluid containing

proteolytic enzymes, citric acid, acid phosphatase and lipids. The bulbourethral glands secrete a clear secretion into the lumen of the urethra to lubricate it.

Sertoli cells, which nurture and support developing spermatocytes, secrete a fluid into seminiferous tubules that helps transport of sperms to the genital ducts.

Seminal plasma of human contains a complex range of organic and inorganic constituents.

The components and contributions of semen are as follows :

Gland	Approximate%	Description
testes	2-5%	Approximately 200 to 500-million spermatozoa (also called *sperm* or *spermatozoons),* produced in the testes, are released per ejaculation.
seminal vesicle	65-75%	amino acids, citrate, enzymes, flavins, fructose (the main energy source of sperm cells, which rely entirely on sugars from the seminal plasma for energy), phosphorylcholine, prostaglandins (involved in suppressing an immune response by the female against the foreign semen), proteins, vitamin C.
prostate	25-30%	acid phosphatase, citric acid, fibrinolysin, prostate specific antigen, proteolytic enzymes, zinc (serves to help to stablize the DNA-containing chromatin in the sperm cells. A zinc deficiency may result in lowered fertility because of increased sperm fragility. Zinc deficiency can also adversely affect spermatogenesis).
bulbourethral glands	< 1%	galactose, mucus (serve to increase the mobility of sperm cells in the vagina and cervix by creating a less viscous channel for the sperm cells to swim through, and preventing their diffusion out of the semen. Contributes to the cohesive jelly-like texture of semen), pre-ejaculate, sialic acid.

A 1992 World Health Organization report described normal human semen as having a volume of 2 ml or greater, pH of 7.2 to 8.0, sperm concentration of 20×10^6 spermatozoa/ml or more, sperm count of 40×10^6 spermatozoa per ejaculate or more, and motility of 50% or more with forward progression (catagories a and b) of 25% or more with rapid progression (category a) within 60 minutes of ejaculation.

2.3 Semen protein

Semenogelin (Sg), the major protein of the human semen congulum, is present at high concentrations in seminal fluid vesicle secretions. It is degraded by the prostate specific antigen (PSA) to generate peptides of various biological activities. That were found on and inside spermotozoa. Lamirande et al (2009) experimentally proved that at concentrate of 0.1 to 1.0 mg/ml. Sg did not affect sperm motility but completely prevented capicitation induced by foetal cord serum ultrafiltrate; a partial inhibition of capacitation was noted with 0·03 mg Sg/ml.

Ribonuclease (RNase), which has as high as iso-electric point (PI-9.7) as Sg. (PI-9.5), also prevented sperm capacitation and O_2-related chemiluminscence but to a lower extent. Semenogelin is a potential scavenger for O_2-, but probably also affects the sperm oxidase. Spermatozoa rapidly processed Sg and a high proportion of Sg can be degraded after 15 minutes of incubation. The resulting polypeptide patterns were reminiscent of those obtained with PSA as a proteolytic enzyme. Therefore, semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process.

2.4 Accessory reproductive structures

More complex male accessory reproductive organs are found in those vertibrates where gamate union occurs within the protective structures of the maternal body (higher vertebrates). These are as follows :

Vasa efferentia and Vas deferens : The mature spermatozoa are collected by vasa efferintia which convey them to the main reproductive duct, the vas deferens.

Epididymis : The anterior portion of vas deferens becomes greatly lengthened, twisted or convoluted and highly coiled to form a compact structure called epididymis. The epididymis remains situtated at one cephalic end of a testis and it is a place of physiological maturation of sperm and also a place of storage of mature sperms.

Urethra and seminal vesicles : The posterior portion of each vas deferens remains less contorted and finally empties into the urinogenital sinus which is the male *urethra.* In same animals, vas deferens gives out some pouch-like, hollow glands called *seminal vesicles.* In most vertebrates, the seminal vesicle act as sperm storage organs during breeding season. In mammals it act as secretory glands which produce

a mucoid material containing structure and other nutrients and also large quantities of prostaglandins and fibrinogen.

Prostate gland : In metatherian and eutherian mammals, there are some more glands such as prostate glands and mucous glands. In man, prostate glands secrete a thin, milky, alkaline fluid containing citric acid, calciums, acid phosphatase, a clotting enzyme and profibrolysin.

Intromittent organ : When fertilization is internal, the male vertebrate usually develops intromittent or copulatory organs for introducing sperm into the reproductive tract of the female. Intromittent organs are particularly characteristics of reptiles and mammals. The intromittent organs of reptiles and mammals are of two types—paired hemipenis and penis. Snakes and lizards have a pair of stubby, grouped, sac-like hemipenes lying in pockets under the skin beside the cloaoa. These can be everted during sperm transfer. Male mammals exhibit a unpaired erectile penis.

Unit 3 ❐ *in vitro* and *in vivo* capacitation of mammalian sperm and role of fertilizin and ZP protein in fertilization

Structure

3.1 Contact and recognition between the gametes

Fertilization is the process whereby the sperm and the egg are fused together to begin the creation of a new individual whose genome is derived from both parents. It is a process, antithesis to meiotic division in respect of chromosomal numbers and number of genes in sexually reproducing organism.

Fertilization accomplishes two separate functions. The first function of fertilization is to transmit genes from parent to offspring and the second is to initiate in the egg cytoplasm, those reactions that permits development to proceed.

Although the details of fertilization vary from species to species, conception generally focused on four major events. In our discussion the content area will primarily be restricted to mammalian system and occasional references would be cited from other sources.

A complex dialogue exists between egg and sperm. The egg activates the sperm metabolism that is essential for fertilization and in turn the sperm reciprocates by activating the egg metabolism needed for the onset of development.

The structure of the gametes (the sperm and the egg) is organized in such a form that a sperm of a species structurally fits with the egg architecture. This fittings is not merely dependent on structural contributes but also on their chemical affinity.

The interaction by sperm and egg generally proceeds according to five basic steps– –

(i) Chemoattraction of the sperm to the egg by soluble molecules secreted by the egg.

(ii) The exocytosis of the acrosomal vesicle to release its enzymes.

(iii) The binding of sperm to the extracellular envelops of the egg.

(iv) Passage of the sperm through the extracellular envelope

(v) Fusion of egg and sperm cell membrane followed by gametic nuclei.

3.2 Sperm attraction

Species specific sperm attraction has been documented in numerous species including cnidarians, echinoderms, molluscs, urochordates, and vertebrates. In many species, sperm are attracted toward egg of their species by chemotaxis following a gradient of a chemical secreted by the egg. Miller (1978) first demonstrated that the eggs of cnidarians ***Orthopyxis caliculata*** not only secrete a chemostatic factor but also regulate the timing of its release. Miller, in his experiment, demonstrated that when sperm were added to oocytes that have not yet completed their second meiotic division, there was no attraction of sperm to egg. However, after the second meiotic division was finished and the eggs were ready to be fertilized, the sperm migrated towards them.

The mechanisms of chemotaxis differ among species and the chemotactic molecules are different in closely related species. One chemotactic molecule, a 14-amino acid peptide called *resact* has been isolted from the egg jelly of Sea urchin *Arbacia punctulata.* Resact is specific for *A. punctulata* and does not attract sperm of other species. *A. punctulata* sperm have receptors in their cell membranes, that binds resact and can swim up a concentration gradient of this compound until they reach the egg. The moleculer mechanism controlling the chemotaxis has recently been worked out. In Figure 6 the sperm chemotaxis in mammalian species has been summarized.

3.3 The acrosome reaction

A second interaction between sperm and egg is the acrosome reaction. In most marine invertebrates the acrosome reaction has two components—

(i) The fusion of the acrosomal vesicles with the sperm cell membrane in which an exocytosis that results in release of the content of the acrosome vehicle.

(ii) The extension of acrosomal process.

Summers and Hylander (1974) have extensively studied the events of acrosomal reactions in sea urchin *Stongylocentrotus purpuratus* in which the events have been sequentially summerized below (Fig. 3.1) :

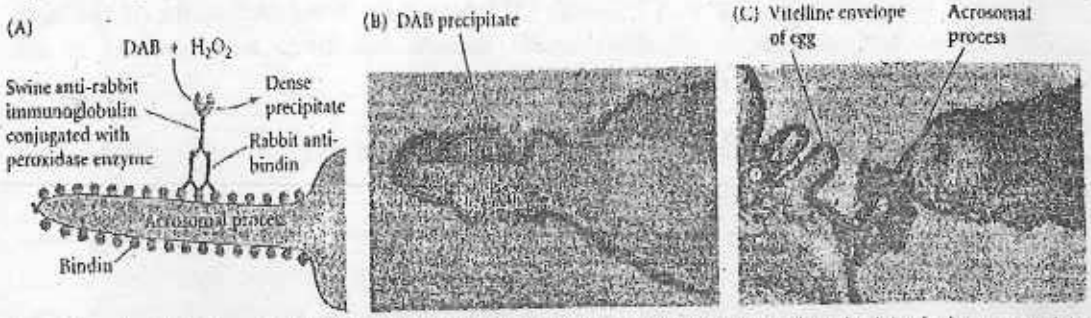


Fig. 3.1 : Localization of bindin on theacrosomal process. (A) Immuno-chemical technique used to localize bindin. Rabbit antibody was made to the bindin protein, and this antibody was incubated with sperm that had undergone the acrosome reaction. If bindin were present, the rabbit antibody would remain bound to the sperm. After any un-bound antibody was washed off, the sperm were treated with swine antibody that had been covalently linked to peroxidase enzymes. The swine antibody bound to the rabbit antibody, placing peroxidase molecules wherever bindin was present. Peroxidase catalyzes the formation of a dark precipitate from diaminobenzidine (DAB) and hydrogen peroxide. Thus, this percipitate formed only where bindin was present. (B) Localization of bindin to the acrosomal process after the acrosome reaction (33,200×). (C) Localization of bindin to the acrosomal process at the junction of the sperm and the egg. (B and C from Moy and Vacquier 1979 ; phtograph courtsey of V.D. Vacquier.)

Similarly in mammalian form, acrosomal reaction has been studied in golden hamster. Miezel (1984) has shown that during acrosomal reaction sperm cell membrane

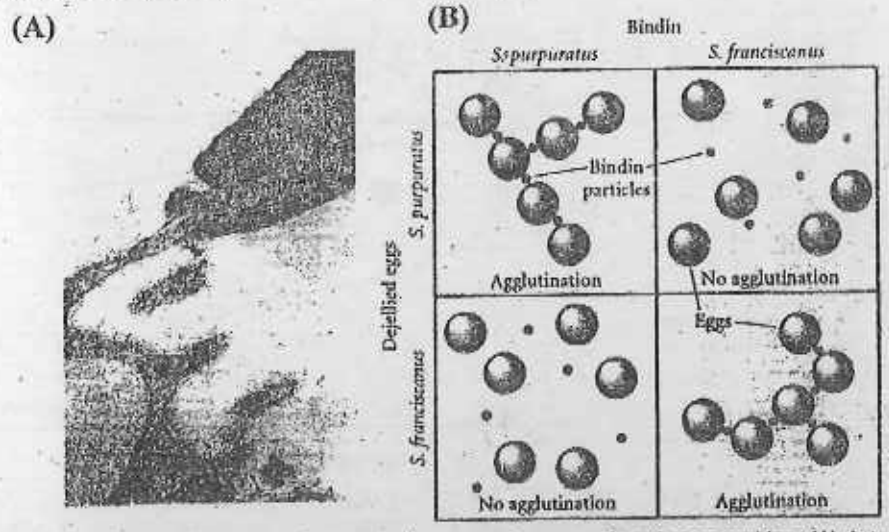


Fig. 3.2 : Species-specific binding of acrosomal process to egg surface in sea urchins. (A) Actual contact of a sea urchin sperm acrosomal process with an egg microvillus. (B) In vitro model of species-specific binding. The agglutinationof dejellied eggs by bindin was measured by adding bindin aggregates to a plastic well containing a suspension of eggs. After 2-5 minutes of gentle shaking, the wells were photographed. Each bindin bound to and agglutinated only eggs from its own species. (A from Epel 1977, photograph courtesy of F.D. Collins and D. Epel ; B based on photographs of Glabe and Vacquier 1977.)

and acrosomal membrane swell up and fusion between sperm cell membrane and adjuscent acrosomal membrane takes up at the tip of sperm nucleus and gradually glide down across the sperm membrane. The acrosomal reaction in mammalian form is intensely controlled by a protein called ZP3 reside on zona pellucida of the egg.

The moleculer mechanism of acrosomal reaction has been summarized in the following diagram (Fig. 3:2).

3.4 ZP proteins and binding of sperm to the extracellular envelop of the egg

Before the mammalian sperm can find the oocyte, its membrane first bind to and penetrate the egg's zona pellucida. The zona pellucida in mammals play a role analogous to that of vitelline envelope of invertebrates. However, the zona pellucida is more thicker and more dense structure than the vitelline envelope. The binding of sperm to zona pellucida is relatively species specific but not that of absolute level or category.

Zona pellucida is made of three major glycoproteins—ZP1, ZP2 and ZP3 [also called as zona proteins 1, 2 and 3]. Sperm binding to zona pellucida have been reviewed typically and the studies revealed that zona proteins interact with sperm in a sequencial way closely resembling an antigen-antibody reaction. However, it should be kept in mind that zona pellucida proteins are not only the exclusive architect of

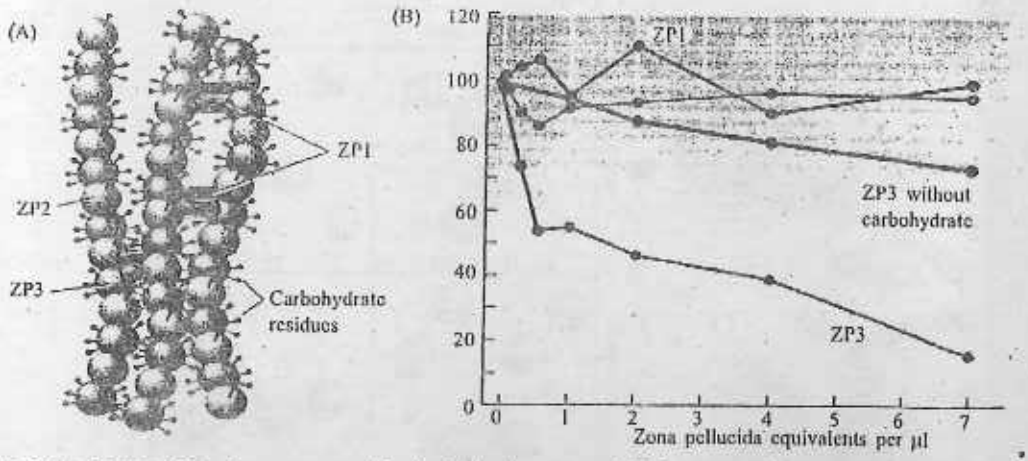


Fig 3.3 : Mouse ZP3, the zona protein that binds sperm. (A) Diagram of the fibrillar structure of the mouse zona pellucida. The major strands of the zona are composed of repeating dimers of proteins ZP2 and ZP3. These stands are occasionlly crosslinked by ZP1, forming a meshlike network. (B) Inhibition assay showing the specific decrease of mouse sperm binding to zonae pellucidae when sperm and zonaewere first indubated with increasingly large amounts of the glycoprotein ZP3. The importance of the carbohydrate portion of ZP3 is also indicated by this graph. (A after Wassarman 1989 ; B after Bleil and Wassarman 1980 and Florman and Wassarman 1985.)

gamete attraction or binding. Sperm specific proteins that express only in mammalian sperm during its maturation phase in epididymis facilitates egg-sperm attraction and binding. An inhibitor which blocks the expression of sperm specific protein in different parts of epididymis results in failure in fertilization process and to some extent infertility in man (Ray and Maiti, 1980) (Fig. 3.3).

3.5 Passage of the sperm through the extracellular envelop

The acrosome reaction releases engymes exocytotically. These proteolytic enzymes digest the egg protective coating, allowing the sperm to reach and fuse with the egg cell membrane.

The passage of sperm through egg envelope has been studied extensively in different organisms and the mechanism has been outlined as shown in the following diagram.

3.6 Fusion of egg and sperm cell membrane followed by gametic nuclei

The mammalian sperm that finally enters the egg carries its genetic contribution in a haploid pronucleus. In mammals, the process of pronuclear migration takes about 12 hours, compared with less than 1 hour in the sea urchin.

The mammalian sperm enterd almost tangentially to the surface of the egg rather than approaching perpendicularly and it fuses with numerous microvilli (Fig 3.4).

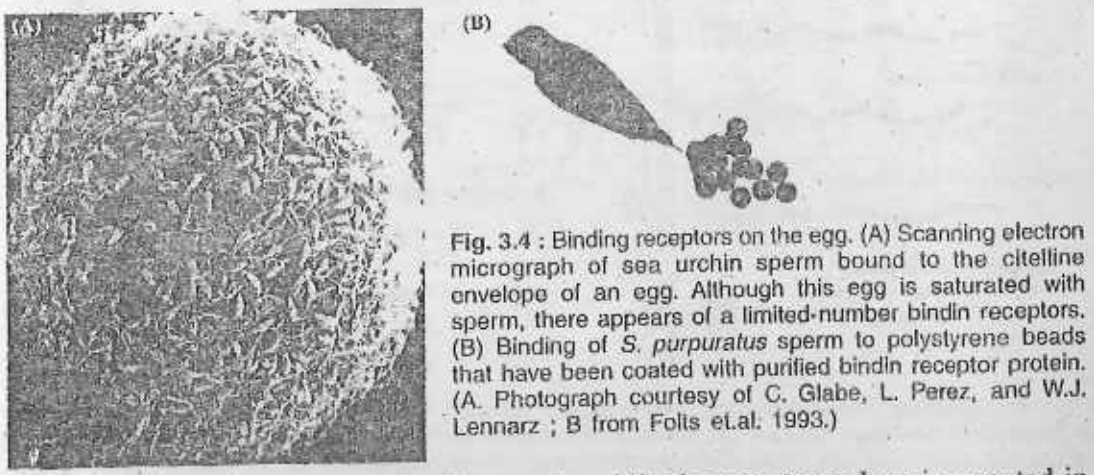


Fig. 3.4 : Binding receptors on the egg. (A) Scanning electron micrograph of sea urchin sperm bound to the citelline envelope of an egg. Although this egg is saturated with sperm, there appears of a limited-number bindin receptors. (B) Binding of *S. purpuratus* sperm to polystyrene beads that have been coated with purified bindin receptor protein. (A. Photograph courtesy of C. Glabe, L. Perez, and W.J. Lennarz ; B from Folts et.al. 1993.)

The mammalian sperms enters the oocyte while the oocyte nucleus is arrested in metaphase of its 2nd meiotic division. Through a series of chemical reactions the sperm pronucleus fuses with egg pronucleus. The division is actually contributed by microtubules joined the 2 pronuclei and enable them to migrate toward one another.

Upon meeting the two nuclear envelop breakdown. However, instead of producing

a common zygote nucleus as found in sea urchins, the chromatin condenses into chromosomes that orient themselves. Thus a true diploid nucleus in mammal is not seen first in the zygote but in the two cell stage.

Each sperm brings into the egg not only its pronucleus but also its mitochondria, its centriole and a small amount of cytoplasm. The sperm mitochondria and their DNA are degraded in the egg cytoplasm while the sperm centriole that survives during the process act as the organizing agent for making the new mitotic spindle.

Thus, the event of fertilization (Fig. 3.5) physically can be summerized as follows.

1. Fertilization accomplishes two separate activities and two separate activition i.e. sex and reproduction.
2. The events of fertilization substages are—
   (i) contact and recognision between sperm and egg.
   (ii) Regulation of sperm entry into the egg.
   (iii) Fusion of genetic material from the two gametes.
   (iv) Activation of egg metabolism to start development.

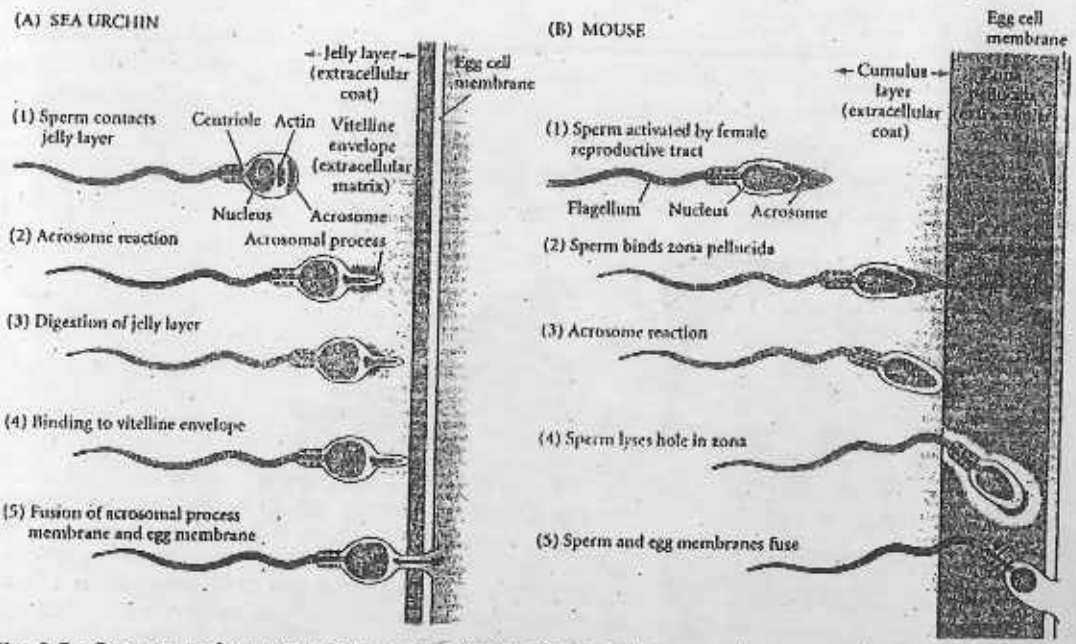


Fig. 3.5 : Summary of events leading to the fusion of egg and sperm plasma membrane in (A) the sea urchin and (B) the mouse. (A) Sea urchin fertilization is external. (1) The sperm is chemotactically attracted to and activated by the egg. (2, 3) Contact with the egg jelly triggers the acrosome reaction, allowing the acrosomal process to form and release proteolytic enzymes. (4) The sperm adheres to the vitelline envelope and lyses a hole in it. (5) The sperm adheres to the egg plasma membrane and fuses with it. The sperm pronucleus can now enter the egg cytoplasm. (B) Mammalian fertilization is internal. (1) The contents of the female reproductive tract capacitate, attract, and activate the sperm. (2) The acrosome-intact sperm binds to the zona pellucida, which is thicker than the vitelline envelope of sea urchins. (3) The acrosome reaction occurs on the zona pellucida. (4) The sperm digests a hole in the zona pellucida. (5) The sperm adhres to the egg, and their plasma membranes fuse.

3. When fertilization takes places, the egg secretes diffusible molecules that attract and activates the sperm.

4. Species specific chemotactic molecules secreted by the egg which attract sperm to enable fertilization.

5. The acrosome reaction releases enzymes exocytotically and such proteolytic enzymes digest the egg protective coating, allowing sperm to reach and fuse with the egg cell membrane.

6. Fusion between sperm and egg is mediated by protein molecules.

7. The fusion of sperm and egg results in the activation of crutial metabolic reactions within the egg.

8. Genetic material is carried in a male and female pro-nucleus which migrate toward each other.

9. Fertilization takes place either internally (as in mammals) or externally (as in sea urcnin, cnidarians etc.)

3.7 Capacitation of sperm in mammals

The female reproductive tract in mammals is not a passive conduct through which sperm reach but a highly specialized tissues that actively regulates the transport and maturity of both gamets. Both the male and female gametes utilize a combination of

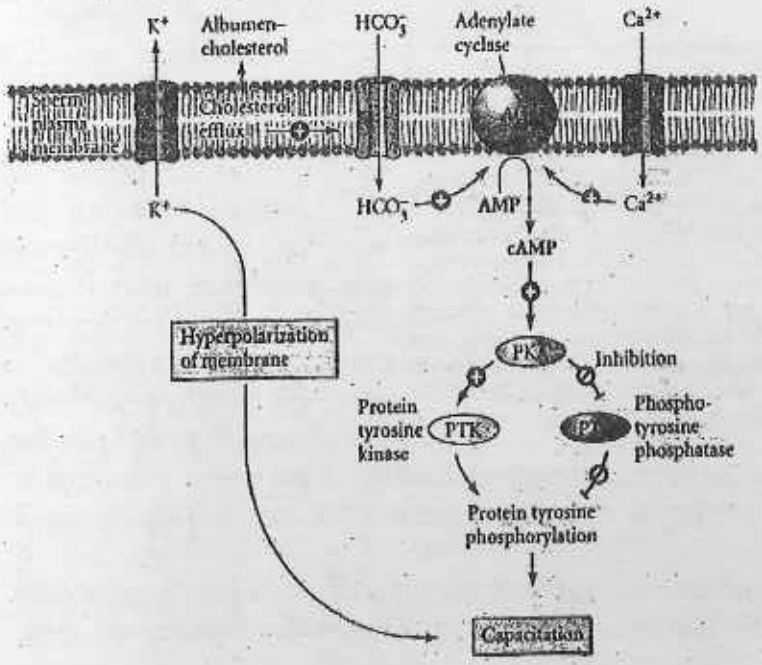


Fig. 3.6 : Hypothetical model for mammalian sperm capacitation. The efflux of potassium (whose cause we do not know) results in a change in the resting potential of the sperm cell membrane. The removal of cholesterol by albumin stimulates ion channels that enable calcium and bicarbonate ions to enter the sperm. These ions promote the activity of adenylate cyclase, which makes cAMP from AMP. The rise in cAMP activates protein kinase A, causing it to activate the protein tyrosine kinases (while inactivating the protein phosphateses). The kinases phosphorylate proteins that are essential for capacitation. (After Visconti and Kopf 1998.)

small scale biochemical interactions and large scale physical propulsion to get to the ampullae, the region of the oviduct where fertilization takes place.

Similarly, the sperm after its formation within the testis are released into epididymal part and migrate through differentially and selectively for maturation.

Newly ejaculated mammalian sperm are unable to undergo the acrocrome reaction until they have resided for sometime in the female reproductive tract the set of physiological changes by which the sperm become competent to fertilize the egg is called ***capacitation***. The sperm, that are not capacitated are 'held up' in the cumulus matrix and are unable to reach the egg.

The requirements for capacitation very from species to species. Capacitation can be **mismicker** in vitro by incubating sperm in a tissue culture medium containing calcium ions, bicarbonate and serum albumin or in fluid taken from the oviducts. The role of oviduct in capacitation has attracted the attention of the reproductive biologist to solve out the cases of infertility due to contribution of inactivated or ghost sperm from the male contributors. It has been found that it is compulsory for the sperm to reach the ampullae where they first acquire the competence for the fertilization. Subsequent competence achieved in the oviduct where small molecules enriched the sperm to encounter the external surface protectively covered by several membranes and envelopes.

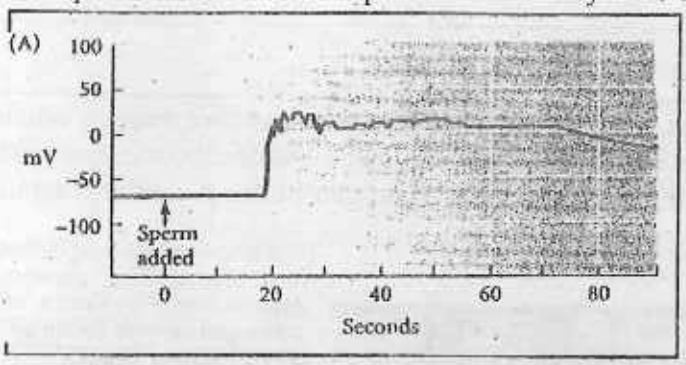


Fig. 3.7 : Membrane potential of sea urchin eggs before and after fertilisation. (A) Before the addition of sperm, the potential difference across the egg plasma membrane is about –70 mV. Within 1-3 seconds after the fertilizing sperm contacts the egg, the potential shifts in a positive direction. (B,C) *Lytechinus* eggs photographed during first cleavage. (B) Control eggs developing in 490 mM Na+. (C) Polyspermy in eggs fertilized in similarly high concentrations of sperm in 120mM Na+ (choline was substituted for sodium). (D) Table showing the rise of polyspermy with decreasing sodium ion concentration. (From Jaffe 1980 ; photographs courtsy of L.A. Jaffe).

There may be an important connection between sperm translocation and capacitation. Smith (1998), has documented that before entering the ampullae of the oviduct the uncapacitated sperm bind actively to the membranes of the oviduct cells in the isthmus part. This binding is temporary and appears to be broken when the sperm become capacitated through temporary binding of the sperm with ampullar cells is extremely significant for the expansion of sperm life span and its survival in the oviduct canal.

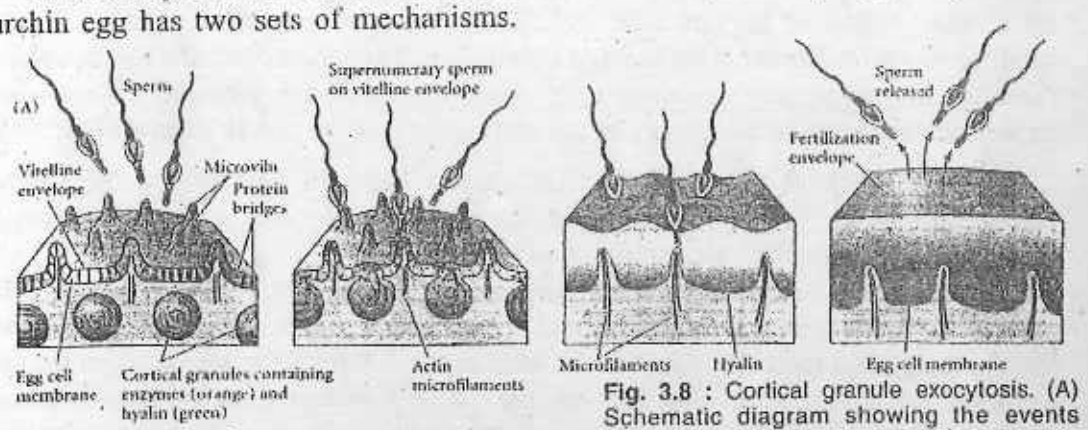
The moleculer mechanism that take place during capacitation is poorly understood. But very recently a model has been proposed to explain the mammalian sperm

capacitation, the diagram explains the mechanism of sperm capacitation (Fig. 3.6 & 3.7) as follows :

3.8 Polyspermy

In most animals, any sperm that enters the egg can provide a haploid nucleus & a centroile to the egg. But in normal, only one sperm enters the egg & a haploid sperm nucleus & a haploid egg nucleus combine to form diploid nucleus of fertilized egg & thus restores the chromosome number appropriate for the species. This event is called *monospermy*. However, a set of multiple sperm may enter the egg called *polyspermy* leads to disastrous consequences in most organisms. In nature and in experimental conditions such events have been studied and the mechanism by which the polyspermy is prevented has been worked out.

Different species have evolved various mechanism to prevent polyspermy. The sea urchin egg has two sets of mechanisms.



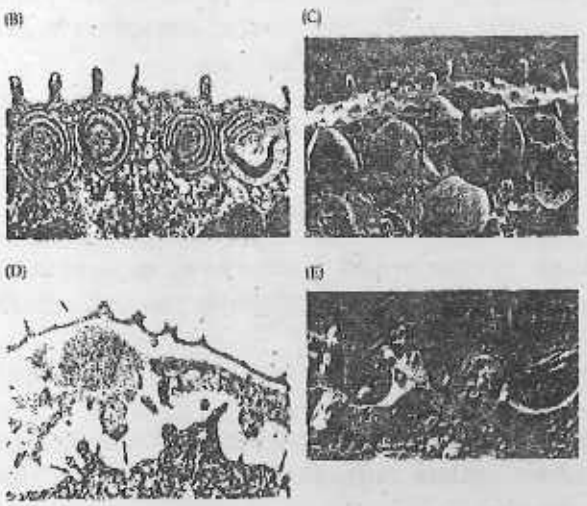


Fig. 3.8 : Cortical granule exocytosis. (A) Schematic diagram showing the events leading to teh formation of the fertilization envelope and the hyaline layer. As cortical granules undergo exocytosis, they release proteases that cleave the proteins linking the vitelline envelope to the cell membrane. Mucopolysaccharides released by the cortical granules form an osmotic gradient, thereby causing water to enter and swell the space between the vitelline envelope and the plasma membrane. Ohter enzymes released from the cortical granules harden the vitelline envelope (now the fertilization envelope) and release sperm bound to it. (B,C) Transmission and scanning electron micrographs of the same region of a recently fertilized egg, showing the raising of the fertilization envelope and the points at which the cortical granules have fused with the plasma membrane of the egg (arrows in D). (A after Austin 1965; B-E from Chandler and Heuser 1979, photographs courtsey of D.E. Chandler.)

A) Fast block to polyspermy : Accomplishing an electric change in the egg cell memebrane in a very fast reaction & mechanism is known as *the fast block to polyspermy.* A fast block to polyspermy is achieved by changing the electric potential of egg membrane. The egg membrane provides a selective barrier between the egg cytoplasm & outside environment, so that the ion concentration within the egg differs greatly from those of its surrounding. This concentration difference is especially significant for sodium & potassium ions. Sea water has a particularly high sodium ion concentration whereas egg cytoplasm contain relatively small Na+. The reverse is true for K+. This condition is maintained by the cell membrane which inhibits entry of Na+ into the oocyte & prevents K+ to leaking out into the environment. At this stage, resting membrane potential is generally about seventy millivolt (expressed as –70 mv) because the inside of the cell is negetively charged with respect to the exterior). Within 1 to 3 seconds after binding of sperm with egg cell membrane the resting potential shifted to a positive level, i.e. about +20 mv. This change is caused by a small influx of Na^+ into the egg. This change in electrical gradiant inhibits binding of sperm further with the egg membrane. Thereby no further sperm entry is possible through the egg membrane. The result of membrane potential change under experimental condition using eggs of sea urchin has been shown in diagram (Fig. 3.8).

B) Slow block of polyspermy : Unlike fast block to polyspermy the another mechanism which brings about prevention of further entry of sperm is known as *cortical granule reaction* or as *slow block to polyspermy.*

Beneath the sea urchin egg cell membrane, there are about 15,000 cortical granules, each about 1 mm in diameter, are found. Upon sperm enjoy these cortical granules fuse with egg cell membrane & release their controls into the space between the cell membrane & vitelline envelop fibrous mat. Several proteins are released by these cortical granules exocytosis which ultimately accumulate on the fibrous mat forming a secondary barrier. This cortical reaction is essential to prevent polyspermy & is achieved very rapidly within the perview of prevention of polyspermy.

The detailed mechanism of cortical granule reaction has been summarized in the following diagram.

3.9 Molecular mechanism of egg activation

Although fertilization is depicted as merely the means to merge two haploid nuclei but actually it is the event that initiate the activation of metabolic processes in the egg cytoplasm required for initiation of development.

Sea urchin is used as a classical model to study the events that undergo sequentially and in some, hand to hand.

Contact or fusion between sea urchin sperm & egg activates the two major blocks to polyspermy. The fast block initiated by sodium influx into the cell and the slow block initiated by the intracellular release of Ca^{++}.

The release of sodium as stated earlier is responsible for cortical grannule reaction and is also responsible for the reentry of the egg into the cell cyle and activation of egg protein synthesis.

Calcium release activates a whole series of metabolic reaction that initiates embroynic development such as the activation of NAD$^+$ kinase which converts NAD$^+$ to NADP, a key process for supply of oxygen in the developing organism.

Epel, (1980) has summarised the molecular mechanism of activation of egg metabolism in sea urchin which is presented below (Fig. 3.9 and 3.10).

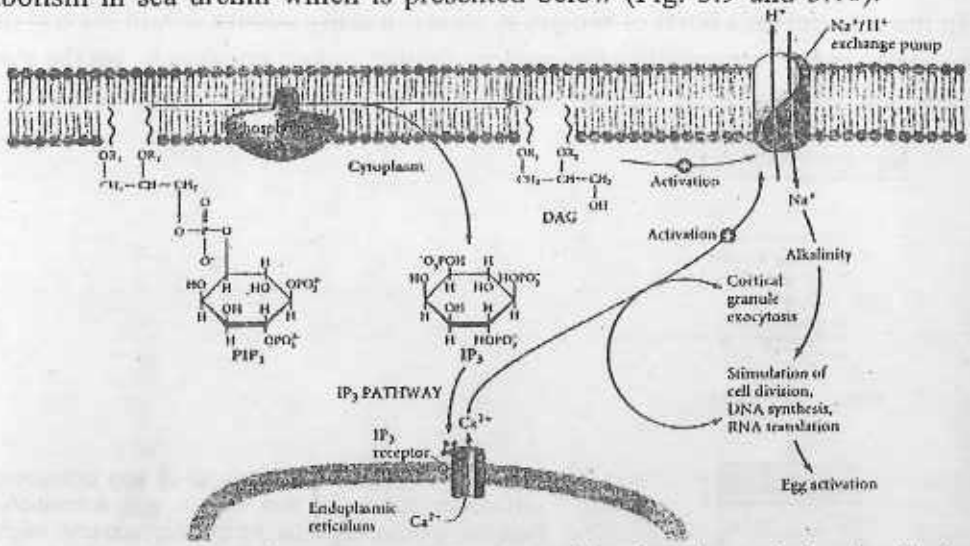


Fig. 3.9 : The roles of inositol phosphates in releasing calcium from the endoplasmic reticulum and the initiation of development. Phospholipase C splits PIP_2 into IP_3 and DAG. The IP_3 releases calcium from the endosplasmic reticulu, and the DAG, with assistance from the released Ca^{2+}, activates the sodium-hydrogen exchange pump in the membrane.

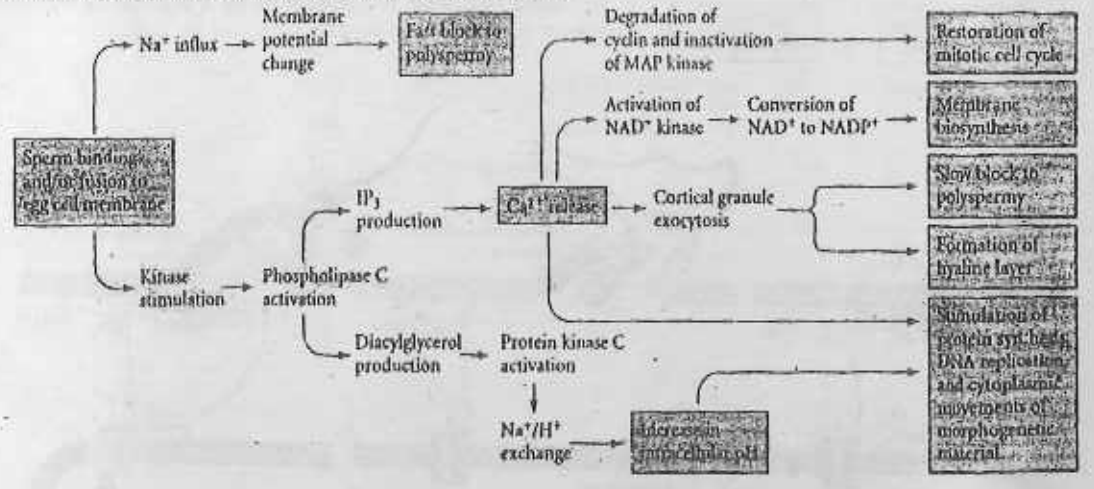


Fig. 3.10 : Model of egg activation in the sea wreliz (after Epel 1980)

On the other hand, the late responses of fertilization includes the activation of new burst of DNA & protein synthesis. The fusion of egg and sperm causes the intracellular pH to increase due to the production of diacylglycerol. This rise in intracellular pH begins with a second influx of Na^+ ions which causes 1:1 exchange between Na^+ from the sea water and H^+ from the egg. The loss of H^+ causes the pH of the egg to rise which consequently increases Ca^{++} elevation and together to stimulate new DNA synthesis.

In the sea urchin, a burst of protein synthesis usually occurs within several minutes after sperm entry. Interestingly this protein synthesis does not depend on the synthesis of new mRNAs, rather it utilizes mRNAs present in the oocyte cytoplasm.

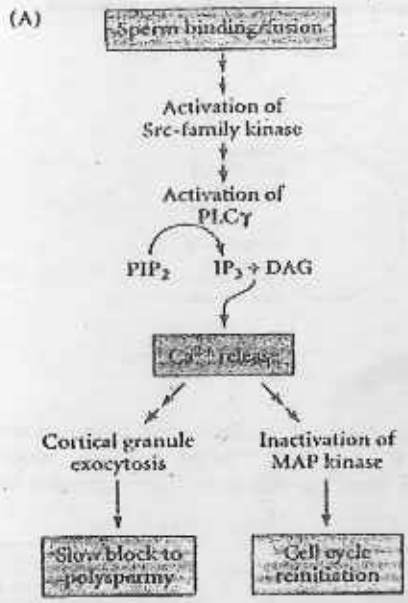
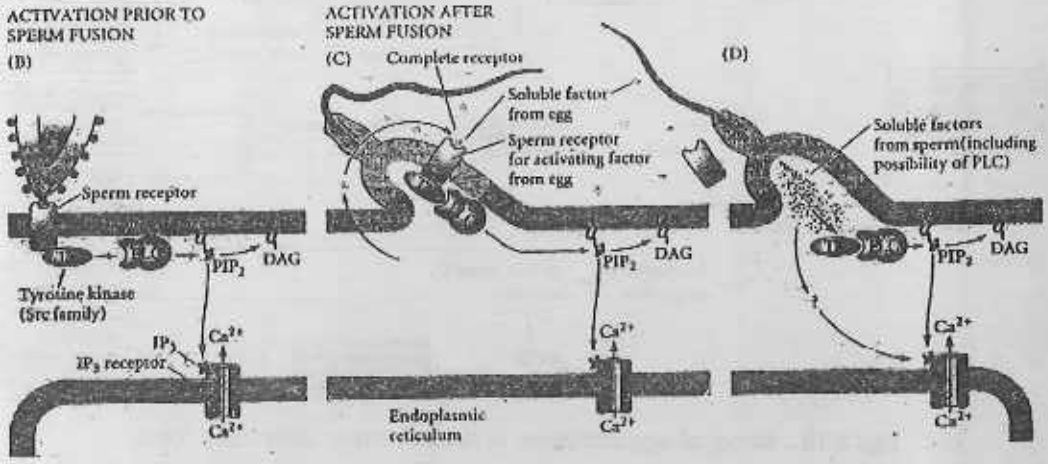


Fig. 3.11 : Possible mechanisms of egg activation. (A) A schematic outline of sea urchin egg activation. (B-D) Possible mechanisms by which this scheme might be a accomplished. (B) the bindin receptor activates a cytoplasmic Src kinase. (C) An activated Src kinase or PLC in the sperm plasma membrane activates the egg pathways. (D) Calcium release and egg activation by activated PLC from the sperm or by a substance from the sperm that activates egg PLC.



The mRNAs present in the egg cytoplasm in non-translated form meant to encode proteins such as listones, tubulish, actins & morphogenetic factors that are used during early development.

One mechanism for this global rise for the translation of messages stored in the oocyte appeares to be the release of inhibitors which musked the encoded RNAs. The removal of these mask proteins triggered by synthesis of activator proteins which degrades mask proteins such as 4E binding protein which ancode cyclin B. The cyclin B protein combines with Cdk 1 cyclin to create mitosis promoting factor (MPF) which is required to initiate cell division.

Thus fertilization activates pathways that target the translational inhibitory proteins for degradation and the newly accessible 5′ end of the mRNA can interact with those proteins that allow the messages to be translated. One of those mRNAs encodes a protein (CP) critical for cell division. In such a manner, fertilization can initiate mitosis and the sea urchin can begin to from a multi-cellular organism.

In recent years the molecular mechanism of egg activation has been further revisited. The mechanism suggests that sperm contact & fusion activates a special kind of protein called G protein which in turn in a cascade manner activates a series of protein channels and ultimately release Ca^{++} which is responsible for cortical grannule exocytosis and inactivation of MAP kinsese (Fig. 3.11).

3.10 *Invitro* fertilization

Infertility i.e. the inability to achieve or sustain pregnancy is not a disease in the usual sense of the word. It is not a symptom nor a condition that prevents the physical well being of the infertile individual or couple. However, since the desire to have children can be exceptionally strong for biological and social reasons, the search for alternative ways to have child is a pivotal issue in clinical research.

In vitro fertilization (IVF) is an assisted reproductive technology in which oocytes and sperms retrieved from the male and female partners and placed together in a petridish, where fertilization can take place. After the fertilized eggs have began dividing, they are transferred into the female partner uterus, where implantation and embryonic development can occur as in a typical pregnancy.

IVF was developed in the early 1970s and the first IVF baby Louise Brown was born in England in 1978. Since then the number of IVF procedures perform in each year has increased and their success rate has improved.

The IVF procedure has 4 basic steps—

Step-1 : Ovarian stimulation and monitering : In this step oocytes are stimulated and women are injected with gonadotropins or anti oestrogens over a period of days or weeks in order to hyperstimulate the ovaries to produce mature oocytes.

Step-2 : Egg retrieval : Once the follicle has matured (but not yet ruptured), the physician retrieves as many oocytes as possible. This is done surgically, guiding an aspiration pipette to each mature follicle and sucking up the oocyte. Once recovered, these oocytes that are mature and healthy are transferred to a sterile container to await fertilization in the laboratory.

Step-3 : Fertilization : A semen sample is collected from the male partner approximately 2 hours before the female partner's oocytes are retrieved. These sperm are processed by a procedure called sperm washing. Sperm washing capacitates the sperm and selects only the healthiest and most active sperm in the sample. The selected sperm are placed in a petridish with the oocytes, and the gametes are incubated at body temperature. In general, each oocyte is incubated for 12-18 hours with 50,000-100,000 motile sperm. If fertilization is successful, the eggs will begin to divide. The success rate for achieving fertilization in this way is between 50 to 70 percent.

Step-4 : Embryo transfer : Embryo transfer is not complicated and can be performed without anesthesia or surgery. The procedure is usually done 3 days after egg retrieval and fertilization. The healthy embroyos (those that have divided well and now contain 6-8 cells). The embryos are sucked into a tubular catheter and then transferred via the catheter direcetly to the uterus. Normal implantation and maturation of at least one embryo (Fig. 3.12) is required to achieve pregnancy.

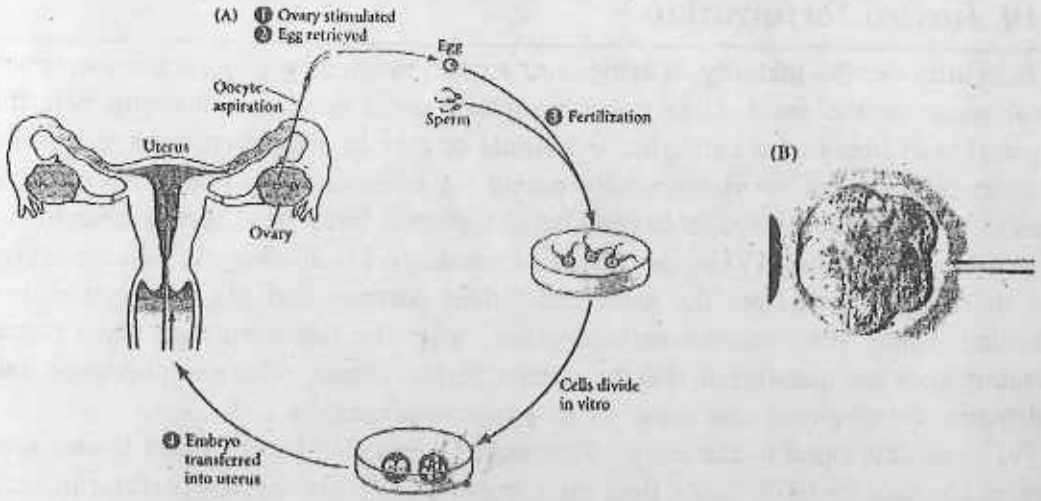


Fig. 3.12 : In vitro fertilization. (A) The IVF process can be divided into four basic steps : (1) ovarian stimulation, (2) egg retrieval ; (3) fertilization and (4) transfer of the embryo into the uterus. (B) Assisted hatching, whereby a hole is poked in the zona pellucida, is a procedure to help ensure the embryo implants in the uterus. (B, photograph courtesy of The Institute for Reproductive Medicine and Science of St. Barnabas, Livingstone, NJ.)

3.11 Success rate and complications : limitation of IVF

Inspite of it's novelty IVF is not free from ethical or surgical limitations. The success rate till today is 31 couples out of every 100 who try one retrieval with IVF and likely to achieve pregnancy and delivery. However, the success rate, drops to 25% or low according to the age of the mother. After 40 years of age the success rate is less than 5%. This decline may be due to the declining viability of eggs as women advance in age.

Another serious limitation is the rate of multiple births. It has been statistically proven that when three embryos were transferred, the multiple birth rate was 46% for women aged 20-24. The rate was 39% for women aged 40-44, when seven or more embroys were transferred. Therefore, the multiple birth poise severe health hazards to mother along with malformations, infant death, premature delivery, low birth rate etc. Moreover, multiple birth rate may increase the incidence of diseases (Fig. 3.13) like high blood pressure, diabetic etc.

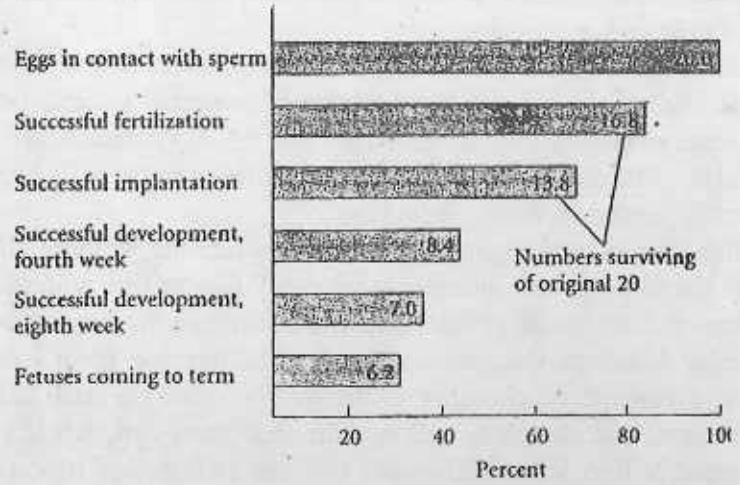


Fig. 3.13 : The fate of 20 hypotheitcal human eggs in the United States and western Europe. Under normal conditions, only 6.2, or fewer, of the original 20 eggs would be expected to develop successfully to term. (After Volpe 1987).

The wide spread practice of IVF has given rise numerous ethical and legal concerns over the safety of the techniques as well as availability of the technique to the greater mass of people. The economic inequality that occurs when only a portion of the population has financial access to a medical technology consider as a gift of medical wonder.

Unit 4 ❐ Role of nurse cell and follicular cell in yolk production in *Drosophila*

Structure

4.1 Nurse cell

The *Nurse cells* are found only in invertebrates such as annelids, cnidarians, molluscs and insects. They are originated from the same organiser that give rise to the oocyte. During the original divisions in the ovary, at some points a differential division separates cells destined to become oocytes from their sisters which develop into *nurse cells.*

The oocytes of *Drosophila* lack lampbrush chromosomes and do not synthesize RNA. In it the nurse cells are the chief source of maternal genetic information and ribosomes. Gene amplification mechanisms for RNA synthesis are found in the genomes of nurse cells, due to which the chromosomes of nurse cells became polytanic and metabolically active in RNA synthesis.

Thus, during oocyte vitellogenesis of *Drosophila,* the volume of a nurse cell nucleus, nucleoli and cytoplasm doubles once every four to five hours. As *Drosophila* nurse cells develop, they retain cytoplasmic connections with the oocyte forming ring canals, bridges or fusomes. Oocytes and nurse cells develop from stem cells within a germinarium, a constricted chamber in the ovaris. A single stem cell undergoes a differential division, one daughter cell remain as a stem cell, while the other cells ooginium, completes four mitotic divisions to form a cluster of 16 cystocytes one of which becomes an oocyte and next 15 become nurse cells. The follicle cells, surround all the cystocytes.

Functions : The principal role of nurse cells are :

1) Supply oocyte nutrient reserve during its growth.

2) acts as selective barrier between vascular system and the oocyte, transporting ascented molecules (Yolk precursers) into oocyte cytoplasm.

3) synsnsize accessory egg membrane.

4.2 Follicle cells

The follicle cells are the accessory cells of vertebrates and invertebrates. In

vertebrates, These cells are derived from the germinal epithelium and become organised as a single layer of epithelial cells surrouding the developing oocyte. Though they may synthesise same substances for oocyte storage. They act more as a selective barrier. Mediating transfer of materials (eg. yolk proteins) entering the oocyte from the blood stream, have to pass through the membranes of the follicle cells on the way.

To facilitate this transfer processes. These are thousands of cytoplasmic processes

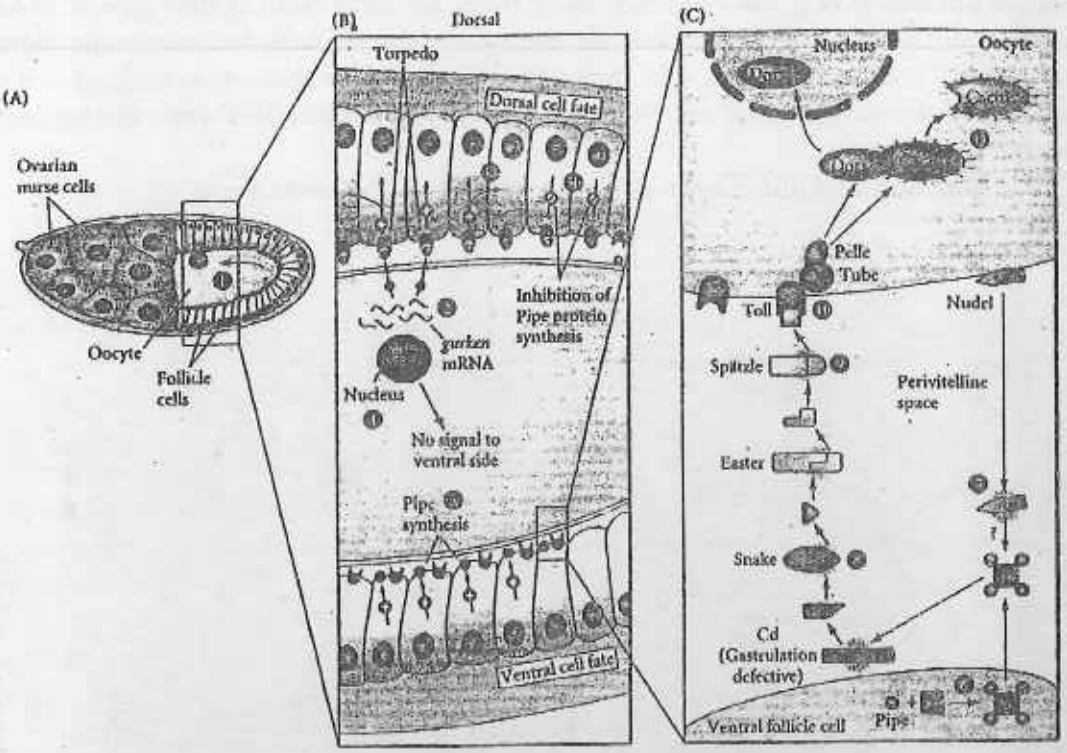


Fig. 4.1 :

(1) Oocyte nucleus travels to anterior dorsal side of cocyte. It synthesizes *gurken* mRNA, which remians between the nucleus and the follicle cells.
(2) *gurken* messages are translated. The Gurken protein is received by Torpedo proteins during mid-oogenesis.
(3a) Torpedo signal causes follicle cells to differntiate to a dorsal morphology.
(3b) Synthesis of pipe protein is inhibited in dorsal follicle cells.
(4) Gurken protein does not diffuse to ventral side.
(5) Ventral follicle cells synthesize Pipe protein.
(6) In ventral follicle cells, Pipe completes the modification of an unknown factor (x).
(7) Nudel and factor (x) interact to split the Gastrulation-deficient (Gd) protein.
(8) The activated Gd protein splits the Snake protein, and the activated Snake protein cleaves the Easter protein.
(9) The activated Easter protein splits Spatzle ; activated sparzle binds to Toll receptor protein.
(10) Toll activation activates Tube and Pelle, which phosphorylate the Cactus protein. Cactus is degraded, releasing it form Dorsal.
(11) Dorsal protein enters the nucleus and ventralize the cell.

called *microvilli,* reading out from the surfaces of book the oocytes and the follicle cells. In contrast with nurse cells, follicle cells do not arise from oogonia, are not in direct cytoplasmic communication with the oocyte and generally transport rather than synthesize oocyte—bound materials (Fig. 4.1).

In *Drosophila,* the follicular epithelium surrounding the developing oocyte is initially symmetrical but this symmetrical is broken by a signal from the oocyte nucleus. The oocyte nucleus is originally located, away from the nurse cells. It then moves to an anterior dorsal position and signals the overlying follicles cells to become the more columnar dorsal follicle cells. The dorsohizing signed from the oocyte nucleus is the product of the *gurken gene,* the only gene known to be transcribed from the haploid ooctye nucleus.

The function of follicle cells is similar to that of the nurse cells.

Unit 5 ❐ Teratogenesis – genetic & induced by drug thalidomide

Structure

5.1 Introduction

Teratology is the study of abnormalities of physiological development. It is often thought of as the study of **birth defects**, but it is much broader than that, taking in other developmental stages, such as **puberty**; and other life forms, such as plants. The term stems from the **Greek** (téras, **genitive** - tératos), meaning *monster*, or *marvel* and - lógos, meaning *speech* or, more loosely, *the study of*.

Teratology meaning *monster*, or *marvel* and as early as 17th century referred to a discourse on prodigies and marvels, of anything so extraordinary as to seem abnormal. In the 19th century, it acquired a meaning closer related to biological deformities, mostly in the field of botany. Currently, its most instrumental meaning is that of the medical study of teratogenesis, congenital malformations or grossly deformed individuals.

Teratogen & Teratogenesis : Any agent that can disturb the development of an embryo or foetus. Teratogens may cause a birth defect in the child. Or a teratogen may halt the pregnancy outright. Thus abnormal development or formation of a terata (individuals' shows gross deviation from the normal due to congenital malformations) is called teratogenesis. Since the development of a normal phenotype requires both a normal genotype and a favorable environment, therefore the teratogenesis can be due to either abnormal genotype or the environment.

5.2 Objectives

This unit of the module of Developmental Biology is meant for the study of teratogenesis that gives a concise idea about how the terata delopes. Finally after completion of the topic, the reader shall be able to understand the

- Basic idea of teratology
- How teratogenesis is related with genes
- How drugs involved in teratology
- Different types of drugs and their interaction
- Mechanism of teratogenesis and
- Wilson's 6 principle

5.3 Genetic teratogenesis

Genetic teratogenesis in human beings

Abnormal genes may be inherited from one or both parents and they may be dominant or recessive. In the majority of the badly affected individuals, however, there is no Mandelian pattern of inheritance. The abnormalities merely occur more frequently among relatives than in general population. In those occurring among relatives, there may be complex interplay between several genes.

Deformities due to abnormal dominant genes are rare. In most of these, the skeleton is affected and the deformities include **achondroplasia** i.e. insufficient growth of long bones, **arachnodactyly** i.e. abnormally long hand and foot bones , **craniocleidal dysostasis** i.e. absence of rudimentary development of clavicle and abnormal shape of skull, and **osteogenesis imperfecta** i.e. incomplete development or hypoplasia of osteoid tissue and collagen, resulting in bone fracture. Such conditions will appear in 50% of the offspring of the affected parent.

Abnormal recessive genes do not fid phenotypic expression unless inherited from both parents. In such cases the abnormality may be found in 25 % of the offspring. Ex. Cystic fibrosis, Sprengel's deformity of the shoulder etc.

5.4 Genetic teratogenesis in animals

1. **Gene-phene relationship** : Several different gene can cause the same terata, though not necessarily by the same route. For example, there are more than twenty genes which affect eye color in *Drosophila melanogaster.* The mutants causing the same defect may be either recessive or dominant. For example, in fowl the trait of rumplessness i.e. absence of tail is controlled by either recessive or dominant gene.

In some cases the same mutation may behave as a recessive or a dominant depending on the genetic background. Thus, in mice the fused gene i.e. fusion or absence of ribs and/ or absence of tail is dominant in *Mus musculus musculus* but recessive in *Mus musculus bactrianus.*

The proportion of affected individuals in a population and degree of effect of mutant genes are dependent on both genetic and environmental factors. For example fowl carrying **rumpless gene** can be selectively bred to produce a 'normal' tail phenotype. Similarly in *Drosophila* carrying **Bar eye gene,** the size of the eye and the number of facets in the eye decreases by about 100 facets during development.

2. **Autophene, allophene and pleiotropy :** Not all genetic terata are the result of intrinsic action of genes in the affected tissues.

i) **Creeper mutation** (cp/cp) in fowl affects the limbs forming abnormally short limbs known as **Phocomelia** and the small eyes called **microphthalmia;** the embryo does not survive till hatching. Transplants of the cp/cp limb rudiments in the normal hosts produce phocomelia limbs. However transplants of cp/cp eye rudiments in the normal hosts produce normal eyes. Therefore the cp gene I intrinsically affects the eye development. This is known as **autophene** but only indirectly affects the eye development and that is known as **allophone.**

ii) Multiple effects of one gene are **pleiotropy.** Any given gene mutation essentially affects the production or structure of one transcribed RNA molecule. The translation product of this RNA (i.e. mRNA), the defective protein, may ultimately result in various defects due to correlation of various biochemical reactions in the body e.g. death in rat due to gray lethal mutant gene and sickle cell anemia in human beings.

5.5 Teratogenesis due to drug (Thalidomide)

Few reports on the drug

Thalidomide was a drug, which after years of extensive animal tests, was first marketed as an over-the-counter sedative: it came to be used by pregnant women in many countries during the late 1950s and early 1960s as a treatment for morning sickness. In fact, before being marketed, the danger signs had already appeared during the 1950s at the University Clinic at Bonn. Thalidomide had been tested on 140 children, seven of whom were less than a year old. Forty children, most of whom had brain damage, had been given the drug for up to nine weeks. The parents were not asked for their permission, nor were they informed that their children were being treated with an entirely new sedative. Doses used were 11 to 20 times higher than

the recommended dose for adults. Half the children were mentally disturbed or had brain damage.

Other children also received Thalidomide in the same high dosage. One child had a circulatory collapse, one died from a congenital heart defect, a three-month-old baby died from heart failure, a twentyone-month-old baby temporarily lost her vision. The doctor responsible stopped using the drug when he heard that his medical colleagues had similar experiences with Thalidomide (Although twelve years would elapse before Thalidomide was withdrawn from the market).

In 1955, one year before the commencement of the marketing of Thalidomide in its various formulae, three physicians, along with a Professor Kloos, took part in a symposium arranged by Chemie Grünenthal at which they reported to the company unsatisfactory experiences with Thalidomide. However, these were ignored. In 1956 the pharmaceutical companies (then) SmithKline and French (now SmithKline Beecham) revealed that even when used in very high doses Thalidomide could not induce sleep in mice. When administered at doses 50 times larger than that claimed by Chemie Grünenthal to be 'sleep inducing' this company could still not achieve the hypnotic effect in animals that it had on humans. Nor when given 650 times the dose effective in humans. This was substantiated and confirmed at the thalidomide trial by pharmaceutical Companies Richardson-Merrel and Ciba.

In November 1956 and October 1957, Thalidomide was marketed in Germany by Chemie Grünenthal. In 1957, after launching Contergan (Thalidomide) in West Germany, reports began to appear regarding peripheral neuritis which revealed thalidomide's toxic effects on the nervous system of the user.

Such a suspicion was suggestive enough to cause Dr. Frances Kelsey, the Medical Officer of the American Food and Drug Administration, to reject the pharmaceutical company's application to market Kevadon (Thalidomide) in the United States, because among other reasons, she was not satisfied that the drug would be safe to take during pregnancy. In pregnancy and during the lactation period the female organism is under great strain. Sleeplessness, unrest and tension are constant complaints. The administration of a sedative and a hypnotic that will hurt neither mother nor child is often necessary.

The potential danger to new drugs to the fetuses was exemplified by unrelated drug thalidomide. Thalidomide is a mild sedative (tranquilizer) that was prescribed for use in pregnancy in many European countries in the late 1950's. In 1961 the German scientist Lenz reported a possible connection between this drug and an increase frequency of a human congenital abnormality of the limbs known as Amelia, where there are no limbs and the closely related phocomelia where there is no development of long bones of limbs and flipper-like hands or feet attached directly

to the trunk. A daily intake of thalidomide for one week during early pregnancy was sufficient to induce limb effect. In 1959-1961 thousands of babies in West Germany and hundreds in other countries such as Japan, were born with partial or complete absence of limbs or limbs with defects (Fig. 5.1). This has led to extreme caution in the introduction of new drugs for commercial distribution, for the pregnant mothers. Since they may cause irreparable harm to human embryos.

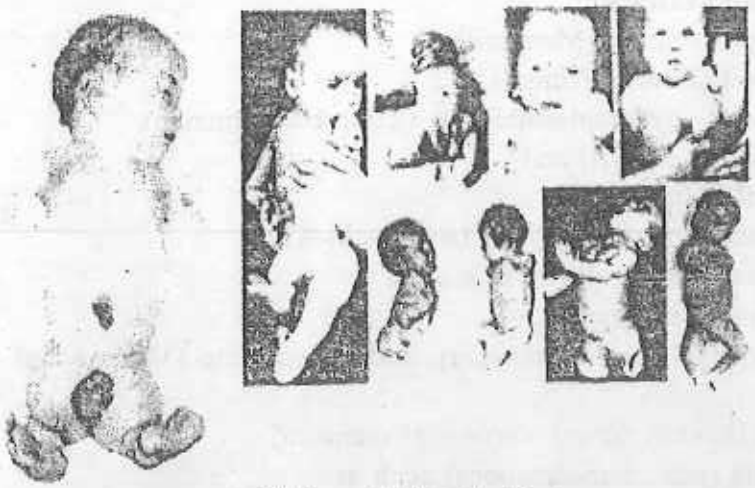


Fig 5.1 : Victims : Thalidomide infants

Teratogenicity tests have particular problems which make the results even more difficult to extrapolate to humans than other animal tests. In addition to the usual variation in metabolism, excretion, distribution and absorption which can exist between species, there are also differences in placental structure, function and biochemistry. Foetal and placental metabolism, and the handling of foreign compounds, are different in different species, and the use of several species does not necessarily overcome the problem. The difficulties are highlighted by aspirin, a proven teratogen in rats, mice, guinea-pigs, cats, dogs and monkeys, yet despite many years of extensive use by pregnant women, it has not been linked to any kind of characteristics malformation.

An unexpected finding was that the mouse and rat were resistant, the rabbit and hamster variably responded, and certain strains of primates were sensitive to thalidomide developmental toxicity. Different strains of the same species of animals were also found to have highly variable sensitivity to thalidomide.

Teratogenic drugs : A teratogen is an agent that can disturb the development of the embryo or fetus. Teratogens halt the pregnancy or produce a congenital malformation (a birth defect). Classes of teratogens include radiation, maternal infections, chemicals, and drugs.

Drugs that are capable of acting as teratogens include :

- ACE (angiotensin converting enzyme) inhibitors such as:
  - benazepril (Lotensin),

- captopril (Capoten),
- enalapril (Vasotec),
- fosinopril sodium (Monopril),
- lisinopril (Zestril, Prinivil),
- lisinopril + hydrochlorothiazide (Zestoretic, Prinzide),
- quinapril (Accupril) and
- ramipril (Altace).

- Acne medication isotretinoin (Accutane, Retin-A).
- Alcohol ingested chronically or in binges.
- Androgens (male hormones).
- Antibiotics tetracycline (Achromycin), and doxycycline (Vibramycin), and streptomycin.
- Anticoagulant (blood-thinner) warfarin (Coumadin).
- Anticonvulsants (seizure medications) such as:
  - phenytoin (Dilatin),
  - valproic acid (Depakene, Valprotate),
  - trimethadione (Tridione),
  - paramethadione (Paradione), and
  - carbamazepine (Tegretol).
- Anti-depressant drug lithium (Eskalith, Lithob).
- Antimetabolite/anticancer drugs methotrexate (Rheumatrex) and aminopterin.
- Antirheumatic agent and metal-binder (chelator) penicillamine (Ciprimene, Depen).
- Antithyroid drugs such as:
  - thiouracil/propylthiouracil and
  - carbimazole/methimazole.
- Cocaine..
- DES (diethylstilbestrol), a hormone.
- Thalidomide (Thalomid) which was approved by the FDA for the treatment of a complication of leprosy (erythema nodosum leprosum).

Antimitotic drugs :

It might be thought that antimitotic drugs used in cancer therapy would be especially harmful to the rapidly growing embryo.. However only aminopetrin (a folic acid antagonist) has proved to be teratogenic in man. This chemical has been used in order in man. This chemical has been used in order to bring about abortion. When it fails to induce abortion, the offspring is likely to show multiple malformations.

Other drugs :

1. Quinine ingested by a pregnant mother can cause deafness and alcohol cause physical and mental retardation in the infant.
2. Teratogenic effect of certain other drugs such as busulphan (for leukemia) and chlorambucil (for Hodgkin's disease) have also been reported.
3. Of the antibiotics, only tetracycline may give rise to an anomaly. When these drugs are administered during the period of enamel formation (for teeth), they may produce yellowing of deciduous teeth.
4. Epileptic mothers taking anticonvulsant drugs such as phenytoin and barbiturates are about three times as likely as normal mothers to give birth to malformed babies.
5. And in some cases such harmless drugs such as caffeine and aspirin have teratogenic effects at very low doses.
6. The teratogenic effect of lysergic acid diethylamide (LSD) is unproven. Some studies have implicated LSD in the appearance of defects in the hands and feet of offspring whose mother's have taken this drug either before or during pregnancy.

5.6 Hormones

None of the hrmones has been implicated in teratogenesis following their administration to the pregnant women. However in mice and rabbits , cortisone is known to induce cleft palate in the offspring. It also causes cleft palate in human fetuses but in less percentage.

Side-effects and drug interactions

Teratogenicity is thalidomide's most severe toxicity, and it is labeled as pregnancy category X. It is lipid soluble and readily crosses the placenta, so it should never be taken by pregnant women or those who could become pregnant. Even one dose of a 50 mg capsule can cause severe birth defects. The teratogenic risk is highest during the critical period, which is days 20-40 of gestation or days 35-50 after the last menstrual period.

The risk of additional, potentially severe birth defects outside the critical period is unknown but may be significant. Therefore, women should not use this drug any time during pregnancy. Phocomelia is a very common birth defect seen with thalidomide use. It is characterized by defective, shortened limbs resulting in flipper hands and feet (Fig. 5.1). In more severe cases, the complete absence of limbs can occur. Additionally, the fetus can develop external ear abnormalities, hypoplastic or

completely absent bones, facial palsy, eye abnormalities, and gastrointestinal and genitourinary tract malformations. Approximately 40 percent of exposed fetuses die at or shortly after birth, with bowel atresia being the most common cause of death.

5.7 Mechanism for teratogenicity

There are over 24 different proposed mechanisms for the teratogenicity. Currently, the most widely held theories involve either thalidomide's anti-angiogenic effect or its direct toxic effect on the segmental sensory neurons, with resulting limb deformities. The McCredie-McBride hypothesis states that neural tissue normally has an inductive effect on the development of the limb, and because neural tissue is damaged by thalidomide, the limb bud subsequently becomes malformed.

Teratogenic effects of thalidomide

- fetal limb growth retardation (arms,legs,hands,feet)
- ingrown genitalia
- absence of lung
- partial/total loss of hearing or sight
- malformed digestive tract, heart, kidney
- stillborn infant

Teratogenesis review

Birth defects are known to occur in 3-5% of all newborns. They are the leading cause of infant mortality in the United States, accounting for more than 20% of all infant deaths. Seven to ten percent of all children will require extensive medical care to diagnose or treat a birth defect. And although significant progress has been made in identifying etiologic causes of some birth defects, approximately 65% have no known or identifiable cause.

It was previously believed that the mammalian embryo developed in the impervious uterus of the mother, protected from all extrinsic factors. However, after the thalidomide disaster of the 1960s, it became apparent and more accepted that the developing embryo could be highly vulnerable to certain environmental agents that have negligible or non-toxic effects to adult individuals.

Wilson's 6 principles.

Along with this new awareness of the in utero vulnerability of the developing mammalian embryo came the development and refinement of *The Six Principles of Teratology* which are still applied today. These principles of teratology were put forth by Jim Wilson in 1959 and in his monograph *Environment and Birth Defects*. These principles guide the study and understanding of teratogenic agents and their effects on developing organisms :

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.
2. Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence. There are critical periods of susceptibility to agents and organ systems affected by these agents.
3. Teratogenic agents act in specific ways on developing cells and tissues to initiate sequences of abnormal developmental events.
4. The access of adverse influences to developing tissues depends on the nature of the influence. Several factors affect the ability of a teratogen to contact a developing conceptus, such as the nature of the agent itself, route and degree of maternal exposure, rate of placental transfer and systemic absorption, and composition of the maternal and embryonic/fetal genotypes.
5. There are four manifestations of deviant development (Death, Malformation, Growth Retardation and Functional Defect).
6. Manifestations of deviant development increase in frequency and degree as dosage increases from the No Observable Adverse Effect Level (NOAEL) to a dose producing 100% Lethality (LD100).

Studies designed to test the teratogenic potential of environmental agents use animal model systems (e.g., rat, mouse, rabbit, dog, and monkey). Early teratologists exposed pregnant animals to environmental agents and observed the fetuses for gross visceral and skeletal abnormalities. While this is still part of the teratological evaluation procedures today, the field of Teratology is moving to a more molecular level, seeking the mechanism(s) of action by which these agents act. Genetically modified mice are commonly used for this purpose. In addition, pregnancy registries are large, prospective studies that monitor exposures women receive during their pregnancies and record the outcome of their births. These studies provide information about possible risks of medications or other exposures in human pregnancies.

Understanding how a **teratogen** causes its effect is not only important in preventing congenital abnormalities but also has the potential for developing new therapeutic drugs safe for use with pregnant women.

5.8 Suggested reading

1. Stirling DI. Thalidomide and its impact in dermatology. Semin in Cutan Med Surg. 1988;17(4):231-42.
2. Stephens TD. Proposed mechanisms of action in thalidomide embryopathy. Teratology. 1988;38:229-39.
3. McBride WG. Thalidomide embryopathy. Teratology. 1977;16(1):79-82.

Unit 6 ❐ Immunocontraception – an overview

Structure

6.1 Introduction

Although several different choices and approaches are available for contraception in women, the choices for men are currently limited to condoms and vasectomy. Male hormonal contraceptives developed over the past several years have now advanced to clinical trials, and the outcome of these studies may determine whether the suppression of sperm production through androgen regulation can become a realistic product. Immunocontraception, an alternative non-hormonal method, has been studied for many years, with the major emphasis on immunization of females to prevent pregnancy or fertilization.

6.2 Objectives

This specialized section of the unit of the module of Developmental Biology gives a clear picture of the idea of Immunocontraception. When the reader finishes this particular unit one will be able to understand

- What is contraception ?
- Basic differences between contraception and immunocontraception
- Different proteins involved in immunocontraception
- An overview of immunocontraception

6.3 What is contraception ?

Contraception is the prevention of fertilization without destroying fertility by natural,

mechanical or chemical means. In other words, a method or system which allows intercourse and yet prevents conception is called contraceptive method. This contraception may be temporary when the effect of preventing pregnancy lasts, but the fertility returns immediately or within a few months of its discontinuation.

6.4 What is immunocontraception ?

Immunity = Body defense mechanisms

Contraception = Protection against unplanned pregnancy

Immunocontraception= The use of body defense mechanisms to provide protection against an unplanned pregnancy.

6.5 Immunocontraception differs from contraception

Immunocontraception is a birth control method that uses the body's immune response to prevent pregnancy. It is used to control populations of wild animals (e.g. white-tailed deer) or feral animals (e.g. mustangs), because it is more humane than culling, and cheaper and less labor-intensive than spaying or castrating animals. It is not popular for domestic animals and is not used in humans.

One drug often used for immunocontraception is porcine zona pellucida or PZP. It is made from the zona pellucida of pigs. It is similar enough to that of other animals that a female animal vaccinated with PZP will produce antibodies against her own oocytes, which prevent fertilization.

6.6 Proteins in immunocontraception

Glycoproteins in ZP1, 2, and 3 are targets for immunocontraception.

In non-mammalian animals, the zona pellucida (called vitelline layer) plays an important role in preventing breeding of different species, especially in species that fertilize outside of the body (e.g. fish).

The zona pellucida is commonly used to control wildlife population problems by immunocontraception. When the zona pellucida of one animal species is injected into the bloodstream of another, it results in sterility of the second species due to immune response. This effect can be temporary or permanent, depending on the method used. In New Jersey, Porcine zona pellucida is used to keep deer populations low, and this process is commonly referred to as "spay-vac".

Overall picture :

Immunocontraception is a birth control method that uses the body's immune

response to prevent pregnancy. The Humane Society of the United States continues to lead development of this emerging technology, which offers a humane means of controlling animal populations in situations where it is necessary and appropriate to do so.

Immunocontraception is one of youngest branch of immunology. Investigations on immunocontraception field endure in last hundred years due to revolutionary advancement was made with apparition of genetic, molecular biology and reproductive immunology.

6.7 Advancement in immunocontraception

Antigenic basis of the first contraception vaccine was whole cells or tissue extracts, so that the most important antigen of the vaccines was not been precisely defined. In last twenty years, the concept of immunocontraception was established on the one-antigen or one-epitopes based vaccines. There are several advancement of immunocontraception relating classical approach in problems of contraception. The advancement refers to the comfort, prices, efficacy, complications, and possibility nonselective acting on animal populations. Classical contraception is inapplicable for treatment of animal population without engaged many of competent persons which can provide the procedure. To that effect, contraception vaccination is revolutionary procedure. This possibility comes as results of development in technology of recombinant DNA and creating a new microorganisms, which might express certain antigens. Live microorganisms like antigenic basis of contraception vaccine enable possibility for epidemic immunization whole population of animals. At the same time, this model of the immunization adapted for people, lead in epidemic model of the immunization with characteristics of biological weapon.

6.8 Examples of bio-control approach

- Interfering with fertilization
- Preventing development of embryo
- Preventing development of the reproductive system
- Interfering with lactation

Unit 7 ❐ Role of thyroxin in metamorphosis in amphibians

Structure

7.1 Introduction

Many animals complete their embryonic development without coming to resemble a young adult. Instead the embryo in these species forms a larva, a transition during which the animal is free-living but sexually immature. Many classes of the different invertebrate phyla, even some vertebrates, e.g. amphibians form larvae. The animals thus possess a free-living larval stage interposed between the stages of embryo and adult. The change from larva to adult can include a radical reorganization, of both body plan and physiology, called metamorphosis.

Amphibian metamorphosis is a complex process regulated by a number of external (environmental) and internal (hormonal) processes. The transformation from larval to adult form in amphibians provides excellent models for developmental biologists examining tissue and cell differentiation and morphogenesis. Metamorphosis is also an excellent model for endocrinologists because most of the changes during larval development and metamorphosis are under the direct influence of hormones. The metamorphic hormones (the thyroid hormones, and steroids) also function by altering gene expression; thus metamorphosis is an excellent tool for examining gene regulation and hormone-regulated gene expression. Because metamorphic rates are determined by various environmental changes that are translated into hormonal changes, with the hormones functioning at the molecular level, metamorphosis is also an excellent model for integrative studies.

7.2 Objectives

This particular unit of Developmental Biology gives a concise idea about the

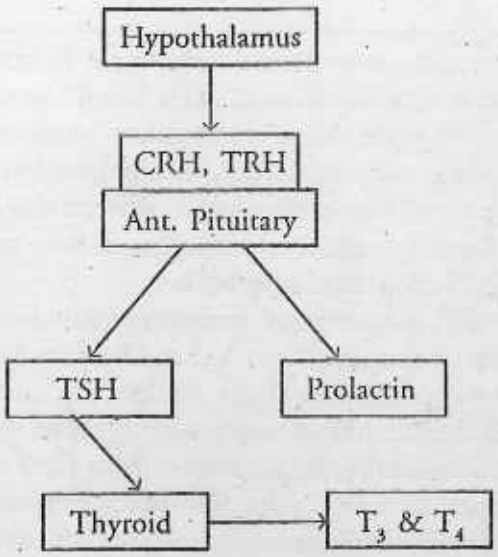
metamorphosis of first class of vertebrates to conquer the land, and most present-day amphibians still return to the water to reproduce. When the topic will be covered entirly, the reader will be able to understand

- metamorphic changes
- Hormonal action
- Control of metamorphosis by thyroid hormone

7.3 Definition of metamorphosis

Metamorphosis is a postembryonic extension of the developmental potential and involves a dramatic change in habit, habitat, morphology, physiology and behavior of larva so that it is transformed into the adult having entirely different habitat and structure.

Hormonal control of Amphibian metamorphosis :



7.4 Some experiments and their results

The control of metamorphosis by thyroid hormones were demonstrated first by Gudernatsch in 1912 who discovered that tadpoles metamorphosed prematurely when fed powdered horse thyroid gland . Allen (1916) found that when he removed or destroyed the thyroid rudiment of early tadpoles, the larvae never metamorphosed, but grew into giant tadpoles. Subsequent studies by Saxen et.al, 1957; Hanken & Hall 1988 showed that steps of anuran metamorphosis are regulated by increasing

amounts of thyroid hormone. Some events (development of limbs) occur early, while other events (regression of tail, remodeling of the intestine) occur later, after the thyroid hormones have reached higher concentrations. This experiment gave rise to a **threshold model.** Suggesting different events of metamorphosis are triggered by different concentration of thyroid hormones.

Metamorphic changes :

The metamorphic changes of amphibian development are brought about by

(1) the secretion of the hormone thyroxine (T_4) into the blood. By the thyroid gland.

(2) The conversion of T_4 into a more active hormone , tri-iodothyronine (T_3) by the target tissues and

(3) The degradation of T_3 in the target tissue.

T_3 binds to the nuclear thyroid hormone receptors (TRs) with much higher affinity than does T_4 and causes them to become transcriptional activators of gene expression. Thus the levels of T3 and TRs in the target tissues are essential for producing the metamorphic response in each tissue.

TRH in action :

The hypothalamic hormone that stimulates TSH release in mammals is thyrotropin releasing hormone (TRH). TRH is active in stimulating TSH release in frogs only when they are post metamorphic. In tadpoles effecting hormone driving TSH release is corticosterone releasing hormone (CRH), which also stimulates the release of adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal cortex to secrete corticosteroids. These adrenal steroids have been shown to regulate, at least in part, the production of enzymes that convert T_4 to T_3 in target tissues. Recent evidence indicates that thyroid hormones stimulate the pituitary to produce another hormone, prolactin that also plays a part in regulating some aspects of metamorphosis.

In short, during the stages leading up to metamorphic climax, there is an increase in TSHG, T_3 ,T_4 and prolactin, the very high levels of T_3 & T_4 then act on the hypothalamus as part of negative feedback loop to lower TSH and perhaps CRH production to levels appropriate for juveniles.

Prolactin antagonizes :

Prolactin has different effects in different vertebrates. In experiment when high levels of mammalian prolactin injected into tadpoles showed slower metamorphic changes. This means that prolactin in the frog antagonizes the actions of T_3 & T_4. Because it interferes with the formation of T_4 & T_3 receptors.

Thyroid hormone action is tissue specific :

A single hormone T_3 , initiates many changes. For example muscles in the developing limbs are stimulated to increase in size and to differentiate, while muscles in the tail are caused to wither and disappear. Some of the changes induced by T_3 are the following :

Organ/System	Changes from larva to adult
Movement	Tail fins to legs
Respiration	Gills and skin to lungs
Nutrition	Diet herbivore to carnivore
Gut	Lengthy to short
Skull and mouth	Extensive morphological changes
Nitrogen excretion	Ammonia to urea
Skin	Epidermis thin to stratified
Mucous glands	none to numerous

It is probably fair to say that every tissue organ system is affected by this hormone. Not only are there dramatic morphological changes during metamorphosis, some fundamental metabolic machinery also gets retooled.

7.5 Time of metamorphosis regulated by hormone levels

A principal factor is the concentration of thyroid hormones, whose action is modified in some target tissues by corticosteroids from the adrenal glands and by prolactin. Thyroid hormones concentrations increase during the progressive changes of metamorphosis, as do levels of corticosteroids and prolactin. Several experiments in which the levels of these hormones have been controlled to some extend clearly show that metamorphic events are induced by different levels of thyroid hormones. Shortening of the intestine and growth of the hind limbs occur at very low thyroxin levels, while tail regression occurs only at much higher levels.

These kinds of results support the idea that each of the different local responses to the hormones has a threshold concentration. Until the hormone reaches or surpasses its threshold, the local response will not occur. While this model seems reasonable, it does not tell us much about the mechanisms involved.

7.6 Regulation of molecular events by thyroid hormones

A) Control of protein synthesis

Liver cells undergo important metabolic changes during metamorphosis. For Ex.

The production of urea depends upon enzymes of the arginine – ornithine cycle and these are low or absent in young tadpole liver but are actively synthesized during metamorphosis.

B) Control of differentiation

i) Stem cells continuously generate the RBC of tadpole; in low levels of thyroxin. they express larval hemoglobin gene. As the thyroxin level in stem cell's proliferation is stimulated and new population of erythroblasts are diverted into pathways of adult hemoglobin synthesis. This shows that the action at the transcription level "selecting" a specific genetic programme by depressing adult hemoglobin gene.

ii) Like wise synthesis of enzymes needed for regression of tail, gut and gills during metamorphosis depends upon thyroxin stimulation. Histolysis of tail tissues is brought about by the action of variety of hydrolytic enzymes (e.g. cathepsin, collagenase and phosphatase) which are synthesized to 200 times their original levels before the tail is resorbed.

7.7 Regulation by hormones receptors

All hormones –including T_3, T_4 and prolactin – act by way of receptor proteins. The concentration and the properties of particular receptor types in a given tissue determine, at least in part, the response. The earliest response to thyroid hormones is an increase in transcription of thyroid hormone receptor genes (TR genes), of which there are at least two. Thus, T_3 induces greater levels of its receptor, a positive feedback loop that stimulates increased amounts of its receptor and thus the potential for an increased sensitivity to that hormone.

TR proteins belong to the same class as ecdysone receptors, e.g. the steroid hormone receptor super family. As with ecdysone, these receptors work as heterodimers, in which each TR protein is joined with a molecule from a class of retinoic acid receptors called RXR (retinoic acid –like receptor). There is some evidence that prolactin serves to decrease expression of TR genes, which may explain in part why prolactin counteracts some actions of thyroid hormones. It seems likely that local tissue-specific responses and the regulation of hormone sensitivity are mainly due to the levels of TR proteins, particular TR family members involved, level(s) and type(s) of RXR, and probably other accessory proteins that confer transcriptional specificity. We know that T_3 stimulates transcription of some genes, such as the gene for adult globin, while decreeing transcription of others. Similarly, we know that steroid hormone receptor members posses the kind of specificity capable of turning on some genes and turning off others.

Unit 8 ❐ Role of juvenile hormone & ecdysone in insect metamorphosis

Structure

8.1 Introduction

While the hormonal control of vertebrate development is of great interest for understanding our own sexuality, the molecular mechanisms of hormone action have been analyzed most successfully for the insect molting hormone ecdysone. Study of this hormone is advantageous because of i) it is steroid hormone. Its target action is straightforward, ii) effects of ecdysone can be seen directly on the puffing pattern of the polytene chromosome of Dipterans larvae and iii) Drosophila mutants facilitated the molecular analysis of ecdysone action.

Insect metamorphosis is a special form of molting. Usually molting comprises mainly the casting off of an old cuticle and the acquisition of a new one. This change of cuticle is necessary for growth and metamorphosis to occur because the fully sclerotized cuticle is rigid and does not expand.

8.2 Objectives

The unit of this module of Developmental Biology covers the basic concept of role of hormones in insect metamorphosis. This unit will assist you to develop an idea about the control of molting and metamorphosis of insect. When the topic will be covered finally, you will understand

- What metamorphosis is
- How metamorphosis is controlled
- Hormonal circuits during molting
- Molecular biology of 20 hydroxy ecdysone

8.3 Definition of metamorphosis

Metamorphosis is a biological process by which an animal physically develops after birth or hatching, involving a conspicuous and relatively abrupt change in the animal's form or structure through cell growth and differentiation. Some insects, amphibians, molluscs, crustaceans, cnidarians, echinoderms and tunicates undergo metamorphosis, which is usually (but not always) accompanied by a change of habitat or behaviour.

8.4 Molting—an essential part of insect development

In order for the insect to grow in size, the restrained of this exoskeleton must be removed. All insect larvae shed their cuticle, usually more than once, in process called **molting**. The stage of larval development between the molts is often called **instars**. Insect species vary in number of molts. The interval between the two molts is known as **stadium**.

Some insects, like grasshoppers produce an embryo that look approximately like their adult forms, smaller and not fully differentiated. This kind of larval development, called **hemimetabolus**, molting allows for an increase in size and further differentiation of the different tissues.

In many other insects, however, including *Drosophila*, the larvae appears to be quite different from the adult. After a number of larval molts, in which the larvae increases in size, the larva constructs an external cuticle called a puparium; once in its puparium, the larva is called a **pupa**. The adult insect emerges from puparium. This kind of development is called **holometabolus**.

8.5 Hormonal circuits in molting

The sequence of events of molting cycles and metamorphosis of insects has been found under precise hormonal control. Certain stimuli associated with the state of nourishment cause the neurosecretary cells of the brain to discharge the 'brain hormone', which is carried by neurosecretary cell axons to the corpora cardiaca (insect endocrine glands), from where it is released into the blood. This hormone then stimulates the thoracic glands to produce a hormone which causes the epithelial cells to begin the processes which lead to molting.

The metamorphosis of insects appears to be regulated by effector hormones controlled by neurosecretory peptide hormones in the brain. The molting process is initiated in the brain, where neurosecretory cells release prothoracicotropic hormone

(PTTH) in response to neural, hormonal, or environmental factors. PTTH is a family of peptide hormones with a molecular weight of approximately 40,000, and it stimulates the production of ecdysone by the prothoracic gland. Ecdysone, however, is not an active hormone, but a prohormone that must be converted into an active form. This conversion is accomplished by a heme-containing oxidase in the mitochondria and microsomes of peripheral tissues such as the fat body. Here the ecdysone is changed to the active hormone 20-hydroxyecdysone.

Each molt is occasioned by one or more pulses of 20-hydroxyecdysone. For a molt from a larva, the first pulse produces a small rise in the hydroxyecdysone concentration in the larval hemolymph (blood) and elicits a change in cellular commitment. The second, large pulse of hydroxyecdysone initiates the differentiation events associated with molting. The hydroxyecdysone produced by these pulses commits and stimulates the epidermal cells to synthesize enzymes that digest and recycle the components of the cuticle. In some cases, environmental conditions can control molting, as in the case of the silkworm moth *Hyalophora cecropia*. Here, PTTH secretion ceases after the pupa has formed. The pupa remains in this suspended state, called diapause, throughout the winter. If not exposed to cold weather, diapause lasts indefinitely. Once exposed to two weeks of cold, however, the pupa can molt when returned to a warmer temperature.

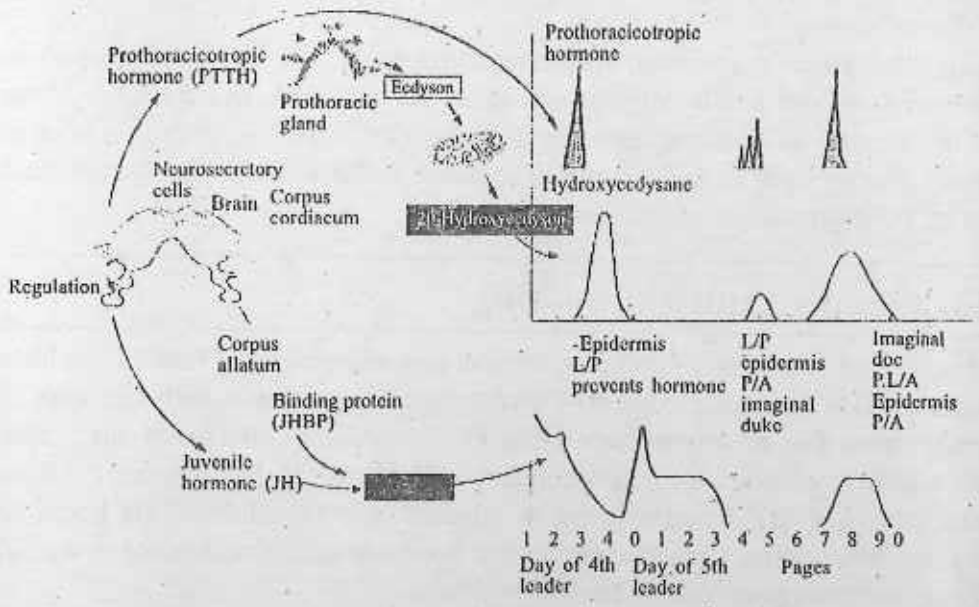


Fig. 8.1 Schematic diagram illustrating the control of molting and metamorphosis in the tobacco hornworm moth. There appear to be critical sensitive periods when the presence or absence of JH determines whether a tissue is retained at the same stage or changes to a more mature state. Different tissues have different sensitive periods. (After Nijhout 1994.)

The second major effector hormone in insect development is **juvenile hormone** (JH). JH is secreted by the corpora allata. The secretory cells of the corpora allata are active during larval molts but are inactive during the metamorphic molt. This hormone is responsible for preventing metamorphosis. As long as JH is present, the hydroxyecdysone-stimulated molts result in a new larval instar. In the last larval instar, the medial nerve from the brain to the corpora allata inhibits the gland from producing juvenile hormone, and there is a simultaneous increase in the body's ability to degrade existing JH. Both these mechanisms cause JH levels to drop below a critical threshold value. This triggers the release of PTTH from the brain. PTTH, in turn, stimulates the prothoracic glands to secrete a small amount of ecdysone. The resulting hydroxyecdysone, in the absence of JH, commits the cells to pupal development. Larval-specific mRNAs are not replaced, and new mRNAs are synthesized whose protein products inhibit the transcription of the larval messages. After the second ecysone pulse, new pupal-specific gene products are synthesized, and the subsequent molt shifts the organism from larva to pupa. It appears, then, that the first ecdysone pulse during the last larval instar triggers the processes that inactivate the larva-specific genes and prepare the pupa-specific genes to be transcribed. The second ecdysone pulse transcribes the pupa-specific genes and initiates the molt.

From the 1950s until recently, it had been thought that the type of molt was determined by the juvenile hormone titre at the time of the ecdysone pulses. High levels of JH induced larvae, intermediate levels of JH produced pupae, while low levels of JH produced adults (see Piepho 1951). However, when the titre of JH could actually be determined, it was found that it fluctuated during the final instar period, having specific peaks and troughs. Metamorphosis is not correlated with or caused by a progressive decline in JH activity. The control of metamorphosis appears more complex (Figure 8.1).

As shown in Figure 8.2, in the tobacco hornworm moth *Manduca sexta*, there are specific times when different cells are sensitive to juvenile hormone. As a general rule, if JH is present during a JH-sensitive period, the current developmental state is maintained, whereas if JH is absent during that period, this tissue will progress to a more mature developmental state. The onset and duration of the JH-sensitive period appears to be an autonomous state of the cell and is not controlled by hormones (Nijhout, 1994). (It has been hypothesized that this may be a time when JH receptors are available in these tissues). In each larval instars, there is a period where the presence of JH prevents the larval epidermis from transforming into pupal epidermis. If JH is present, the epidermis continues to be pupal, if JH is absent, it becomes pupal. During the penultimate instar larva, JH titers are able to retain the epidermis in its larval condition. During the last instars, there are two windows of JH sensitivity.

The first is for the epidermis. At this time, though, ecdysone levels have dropped significantly. Thus, the epidermis will be transformed from larval epidermis to pupal epidermis. The second JH sensitive period concerns the imaginal disc tissue. At this time, however, the JH titer has risen again, so that the imaginal discs are not instructed to evert and differentiate. The molt transforms the larva into a pupa (Nijhout and Wheeler, 1982). The next time the ecdysone pulses occur, no JH is seen during the critical periods. The epidermis transforms from pupal to adult, and the imaginal discs are allowed to evert and differentiate. Injection of JH into the pupa at this time can cause it to molt again into a second pupa (Williams, 1959).

8.6 Role of ecdysone : the molecular biology of 20-hydroxyecdysone activity

Ecdysone receptors : 20-hydroxyecdysone cannot bind to DNA by itself.20 E first binds to nuclear receptors. These proteins, called ecdysone receptors (EcRs), are almost identical structure to the thyroid hormone receptors of amphibians. An EcR protein forms an active molecule by pairing with an Ultraspiracle (Usp) protein. In the absence of the hormone-bound EcR, the Usp protein binds to the ecdysone-responsive genes and inhibits their transcription. This inhibition is converted into activation when the ecdysone receptor binds to the Usp.

Although there is only one gene or EcR, the EcR mRNA transcript can be spliced in at least three different ways to form three distinct proteins. All three EcR proteins have the same domains for 20 E and DNA binding, but they differ in their amino -terminal domains. The type of EcR in a cell may inform the cell how to act when it receives a hormonal signal. is therefore possible that the different receptors activate different sets of genes when they bind 20E.

Binding of 20-hydroxyecdysone to DNA: During molting and metamorphosis, certain regions of the polytene chromosomes of *Drosophila* puff out in the cells of certain organs at certain times. These chromosome puffs represent areas when DNA is being actively transcribed. Moreover these organ specific patterns of chromosome puffing can be reproduced by culturing larval tissue and adding hormones to the medium or by adding hydroxyecdysone to an earlier stage larva. When 20 E is added to larval salivary glands, certain puffs are produced and others regress. Puffing is mediated by the binding of hydroxyecdysone at specific places on the cromosomre.

8.7 Molts are driven by ecdysone production

Insect molting and metamorphosis are controlled by two effector hormones: the steroid 20 hydroxyecdysone (20E) and the lipid juvenile hormone (JH). 20

hydroxyecdysone initiates and coordinates each molt and regulates the changes in gene expression that occur during metamorphosis.

The molting process is initiated in the brain, where neurosecretary cells release prothoracotropic hormone (PTTH) in response to neural, hormonal or environmental signals. PTTH is a peptide hormone with a molecular weight of approx. 40,000 and it stimulates the production of ecdysone by the prothoracic gland. Ecdysone is modified in peripheral tissues to become the active molting hormone 20E. Each molt is initiated by one or more pulses of 20E. PTTH secretion is regulated by both environmental and autonomous signals, produce waves of ecdysone production. As the first wave ends, during the first instar, a small pike in ecdysone concentration occurs, soon followed by a more intense wave of ecdysone release. Stimulated by the hormone, the epithelial cells of the body surface withdraw from the cuticle and produce a molting fluid containing proenzymes that after activation will digest the old cuticle. The epithelium then generates a new cuticle. Because it is distensible, the new cuticle expands as the larva grows until this cuticle, too, becomes hard and inelastic, and the processes repeated again. During the latter portion of the third instar in *Drosophila,* a spike in ecdysone levels again begins the process of molting.

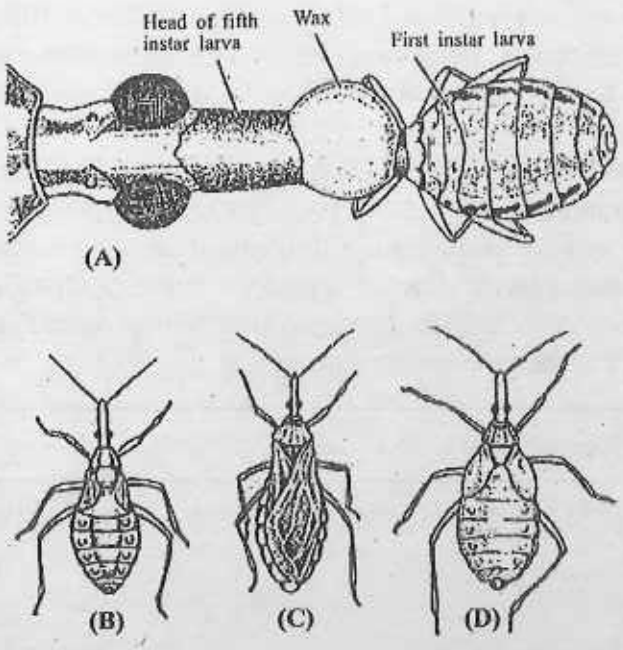


Figure 8.2 Demonstration of hormonal control of insect metamorphosis. (A) Technique of producing precocious "adult" from first instar larva of *Rhodnius* by fusing it to the head of a molting fifth instar larva. (B) Normal fifth instar larva of *Rhodnius.* (C) Normal adult *Rhodnius.* (D) "Sixth instar larva" produced when corpora allata from a fourth instar lava were implanted into the abdomen of a fifth instar larva. (After Wigglesworth 1939).

About JH :

It is the corpora allata, and the juvenile hormone produced by them that determine whether the result of a molt will simply be an increase in larval size, or pupation and metamorphosis. Removing the corpora allata surgically during the second instar will cause the next molt to undergo pupation, one fall instar early. On the other hand, implanting an actively secreting corpus allatum into a late third- instar larva may in the next molt result in a giant larva than a pupa. Puparium formation and pupation are initiated when an ecdysone wave occurs during very low levels of JH, or even in its absence. Recent measurements show that some JH is present in late third-insar larvae, and that the precise timing of JH release and the presence or absence of active JH receptors are also involved in regulating pupation.

8.8 Experiments of metamorphosis

Control of insect metamorphosis was shown by the dramatic experiments of Wigglesworth (1934), who studied *Rhodnius prolixus*, a blood-sucking bug that has five instars before undergoing a striking metamorphosis. When a first-instar larva of *Rhodnius* was decapitated and fused to a molting fifth-instar larva, the minute first instar developed the cuticle, body structure, and genitalia of the adult. This showed that blood-borne hormones are responsible for the induction of metamorphosis (Figure 8.2).

Wigglesworth also showed that the corpora allata, near the insect brain, produces a hormone that counteracts this tendency to undergo metamorphosis (Figure 1D). If the corpora allata was removed from a third-instar larva, the next molt turned the larva into a precocious adult. Conversely, if the corpora allata from fourth-instar larvae were implanted into fifth-instar larvae, these larvae would molt into extremely large "sixth-instar" larvae rather than into adults.

8.9 Suggested reading

Nijhout, H. F. 1994. *Insect Hormones*. Princeton University Press, Princeton

Unit 9 ❐ Significance of totipotency & pleuropotency of cells during animal development

Structure

9.1 Introduction

In higher animals, development involves the progressive restriction of cell fates. Every time a cell divides, its descendants may have to choose between alternative developmental pathways, and once that choice is made, the decision is usually irreversible. Differentiated animal cells placed in isolation therefore can not give rise to new individuals. There are examples where cells have a limited ability to dedifferentiate and recapitulate certain developmental processes, as seen in limb regeneration. However, in no case has it been possible to use a differentiated animal cell to recapitulate embryonic development.

Although differentiated animal cells cannot recapitulate the entire developmental programme, it is possible for the nuclei from those differentiated cells to do so. This type of experiment, where the nucleus of a differentiated cell is used to replace the nucleus of a fertilized egg, shows that all the information required to generate the animal is retained in the nuclei of differentiated cells.

9.2 Objectives

Through the study of this unit of the module, anybody can learn the basic impression of the potency of cells during development.

When the study will be finished, the person who reads shall be able to realize the

- Uniqueness of cell potency
- What those potency meant

- Basis of totipotency
- Nature of pluripotency
- Totipotency vs pluripotency action
- Significance of the potency

Potency - the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either totipotent or pluripotent - to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes referred to as stem cells.

Potency specifies the differentiation potential (the potential to differentiate into different cell types) of the stem cell.[4]

- **Totipotent** (a.k.a **omnipotent**) stem cells can differentiate into embryonic and extraembryonic cell types. Such cells can construct a complete, viable, organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent
- **Pluripotent** stem cells are the descendants of totipotent cells and can differentiate into nearly all cells, i.e. cells derived from any of the three germ layers.
- **Multipotent** stem cells can differentiate into a number of cells, but only those of a closely related family of cells.
- **Oligopotent** stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells.

Unipotent cells can produce only one cell type, their own, but have the property of self-renewal which distinguishes them from non-stem cells (e.g. muscle stem cells

Totipotency is the ability of a single cell to divide and produce all the differentiated cells in an organism, including extraembryonic tissues. Totipotent cells formed during sexual and asexual reproduction include spores and zygotes. Zygotes are the products of the fusion of two gametes (fertilization). In some organisms, cells can dedifferentiate and regain totipotency. For example, a plant cutting or callus can be used to grow an entire plant.

Human development begins when a sperm fertilizes an egg and creates a single totipotent cell (zygote). In the first hours after fertilization, this cell divides into identical totipotent cells. Approximately four days after fertilization and after several cycles of cell division, these totipotent cells begin to specialize.

Totipotent cells have total potential. They can specialize into pluripotent cells that can give rise to most, but not all, of the tissues necessary for fetal development. Pluripotent cells undergo further specialization into multipotent cells that are committed to give rise to cells that have a particular function. For example, multipotent blood stem cells give rise to the red cells, white cells and platelets in the blood.

Importantly, totipotent cells must be able to differentiate not only into any cell in the organism, but also into the extraembryonic tissue associated with that organism. For example, human stem cells are considered totipotent only if they can develop into any cell in the body, or into placental cells that do not become part of the developing fetus. This fact is an important aspect of the stem cell controversy because the human embryonic stem cells used for research purposes are pluripotent; they are collected from human embryos that have developed past the totipotent cell stage. All human embryos used in stem cell experimentation are destroyed in the process.

9.3 Basis of totipotency

The molecular mechanisms controlling totipotency are not well understood and are a subject for current research. In particular, a February 2006 report in Science suggests that in the model organism *Caenorhabditis elegans*, multiple mechanisms including RNA regulation maintain totipotency at different stages of development.

In cell biology, the definition of pluripotency has come to refer to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system). Pluripotent stem cells can give rise to any fetal or adult cell type. However, alone they cannot develop into a fetal or adult animal because they lack the potential to contribute to extraembryonic tissue, such as the placenta.

In contrast to pluripotent stemcells, many progenitor cells are multipotent, i.e. they are capable of differentiating into a limited number of tissue types.

9.4 Pluripotent (biological compounds)

Pluripotent, embryonic stem cells originate as inner mass cells within a blastocyst. The stem cells can become any tissue in the body, excluding a placenta. Only the morula's cells are totipotent, able to become all tissues and a placenta.

Pluripotency can also be used (albeit less commonly) to describe the ability of certain substances to produce several distinct biological responses.

For example, in immunology many cytokines are pluripotent, in that each of these compounds can activate specific behavior in some cell types and inhibit other behavior in other cell types. Interferon gamma represents an excellent example of pluripotency. In most somatic cells it inhibits growth and upregulates expression of Major Histocompatibility Complex (MHC) antigens in a general anti-viral response. In B

lymphocytes (B cells) it stimulates antibody class switching, and in Natural Killer (NK) cells this protein hormone stimulates maturation. In macrophages it activates intracellular killing.

Pluripotent cells have the ability to phagocytize bacterial cells and lyse red blood cells. Victims with the disease Typhoid Lymphoma have a defect in the beta nucleotide in the nucleus of the pluripotent cell. This causes the cell to lyse red blood cells, eventually leading to a death by suffocation due to the lack of oxygen in the body.

9.4 Experiments to understand totipotency

The experiments of this kind were performed on *Xeonopus*. Nuclei taken from tadpole cells or from different kinds of adult cell were injected into UV-irradiated eggs. In many cases, the eggs containing tadpole nuclei developed into normal swimming tadpoles, and in few cases these produced viable adults. Adult cell nuclei from many different cell types were also able to support full development but at a much lower efficiency. However, nuclei from some adult cell types consistently failed to allow development. The results from such experiments firstly confirm the totipotency of adult cell nuclei, showing there is no irreversible change to genetic information in the nucleus during development. However, it becomes more difficult to recapitulate the entire developmental programme as development proceeds, and indeed becomes impossible in certain cell types, such as neurons. Thus, although there is no change to the genetic information in most differentiated nuclei, the DNA does undergo some change that reduce the ability of the nucleus to be reprogrammed by the intracellular environment of the egg.

9.6 Totipotency vs. pluripotency

In animal development, cell fate *and potency* are progressively restricted. For example, at the 16 –cell stage, each blastomere of a mouse embryo is totipotent; i.e. it can potentially give rise to every cell type in the adult if transferred to another embryo. Later the morula differentiates to form the inner cell mass and the trophoectoderm. The inner cell mass gives rise to the embryo, and differentiate to form three germ layers as well as other extra-embryonic structures. At this point, individual cells are still pluripotent since they can generate several different cell types, but they are no longer totipotent, since certain fates are now unavailable. Cell fates are increasingly restricted until a cell is terminally differentiated (can form only a single type). Some cells, e.g. hepatocytes, continue to divide but only produce identical daughters. Other cells, e.g. neurons, become quiescent (i.e. they exit from

the cell cycle and do not divide further). Stem cells are exceptional because they are never terminally differentiated. Instead of dividing to produce two identical daughters, stem cells produce dissimilar daughter cells, only one of which undergoes terminal differentiation.

9.7 Few more examples

Stem cells are undifferentiated cells that have the ability to become any type of body tissue or specialized cell. Scientists believe that cures for many diseases could be found from research using stem cells. There are four basic kinds of human stem cells: embryonic stem cells (hES cells), which are obtained from 5-7 day old blastocysts; fetal stem cells, which are obtained from 4-6 week old fetuses that have been aborted either spontaneously or through procured abortions; placental/cord blood stem cells, which are obtained from the umbilical cord or placenta immediately after birth; and adult stem cells, which are obtained through a biopsy of mature tissues or from bone marrow of a post-natal human being (not necessarily the tissue or bone marrow of an adult). These stem cells can also be classified according to whether they are totipotent or pluripotent. Embryonic stem cells are virtually totipotent, meaning they can become *any* type of human cell, including at their earliest stage before any differentiation has occurred those cells that make-up the trophoblast (the outer-layer of the blastocyst, which eventually becomes the placenta). Fetal stem cells, placental/cord stem cells, and adult stem cells are all pluripotent, meaning they are not totipotent, but they can become some or many types of cells found in the human body. Currently it is thought that fetal stem cells are more pluripotent, i.e., they can become more types of cells, than placental/cord stem cells, while placental/cord stem cells are more pluripotent than adult stem cells.

9.8 Significance

The current thinking in the scientific community is that embryonic stem cells may be more useful than adult stem cells in creating treatments and possible cures for diseases such as Parkinson's, Alzheimer's and spinal cord injuries. From a scientific perspective, however, the downside of totipotent embryonic stem cells is that they are more difficult to control and manipulate in the lab than pluripotent stem cells. Some researchers are exploring ways to increase the pluripotency of non-embryonic stem cells. Some studies are beginning to support the theory that adult stem cells are much more pluripotent than originally thought, and are able to turn into many more types of cells and tissues than previously suspected.

Unit 10 ❐ Roles of maternal effect gene, segment polarity gene, zygotic gene and homeotic gene in development of *Drosophila*

Structure

10.1 Introduction

Development has been an important area of research for many years. Impounding informations and research findings have revolutionarised the idea or the pattern of animal development particularly the morphogenesis in different animals. Researches have been able to identify the similarities as well as differences in the basic plan of development from a fertilized egg to an adult in organisms ranging from the sea urchin to mammals.

In this chapter we will discuss the genetic control of animal development. The central idea of this chapter is to discuss effect of different genes in the various phases of development. However, the discussion will not be focused to comment on hundreds of genes that are required for a complete development, but on the primary genes that are required for the early development an animal.

Extensive studies and researches in the field of developmental biology and the availability of mutants called as *developmental mutants* are found to affect the body plan in *Drosophila*. Undoubtedly, the most extensive and spectacular examples of genes that control development have been identified in the fruit fly, *Drosophila melanogaster*. The Drosophila genome sequence have become available in late 1999 and it has been determined that it includes about 13,600 protein-coding genes and this advantage has facilitated to identify the role of each such gene in normal development, and the available mutant specifies the actual role it plays in the developmental event. In our discussion we will pay particular attention to those that affect the segmented body plan of the organism, both in the larva and in the adult.

10.2 Maternal effect genes organize the egg cytoplasm

Early *Drosphila* development occurs in following way—

a) The structure of the egg becomes organized as it develops in the ovary of the female.

b) Store of messenger RNA (mRNA) along with yolk protein and other cytoplasmic molecules are passed into the egg from the surrounding maternal cells.

c) Immediately after the fertilization, the Zygote nucleus in the egg divides, beginning a remarkable series of 13 mitotic divisions.

d) Each of these divisions takes 5 or 10 minutes, which means that the DNA in the nucleus is replicated constantly at a very rapid rate.

During that time the nucleus do not synthesis RNA. Cytokinesis does not take place and the nucleus produced by the first seven divisions remain in the centre of the embryo until the eighth division occurs.

e) After this event, i.e. during the eighth division, most of the nuclei start to migrate out from the centre and becomes localized at the periphery of the embryo. This is known as the syncytial blastoderm stage because the nuclei are not surrounded by individual plasma membranes. Subsequently, cell membranes do form and the embryo becomes known as cellular blastoderm (Fig. 10.1).

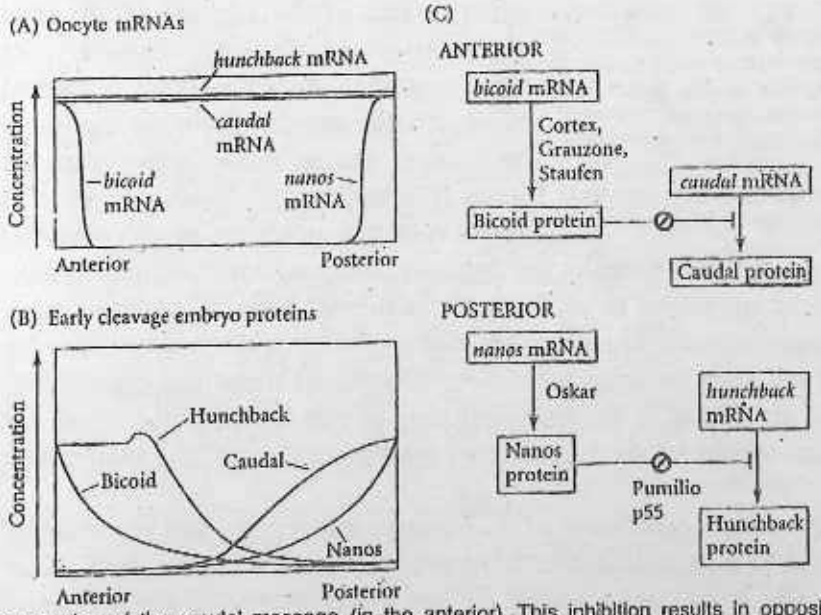


Fig. 10.1 : A model of anterior posterior pattern generation by the *Drosophila* maternal effectgenes. (A) The *bicoid, nanos, hunchback, and caudal* messanger RNA's are placed in the oocyte by the ovarian nurse cells. The *bicoid* message is sequestered anteriorly; the nanos message is sent to the posterior pole. (B) Upon translation, the Bicoid proteingradient extends from anteriorto posterior, while the Nanos protein gradient extends from posterior to anterior. Nanos inhibits the translation of the hunchback message (in the posterior), while Bicoid prevents the transaction of the caudal message (in the anterior). This inhibition results in opposing Caudal and Hunchback gradients. The Hunchback gradient is secondarily strengthened by the transcription of the hunchback gene in the anterior nuclei (since Bicoid acts as a transcription factor to activate hunchback transcription). (C) Parallel interactions whereby translational gene regulation establishes the anterior-posterior patterning of the Drosophila embryo. (C after macdonald and Smibert 1996.)

The genes that act to organize the structure of the egg cell are referred to as *maternal effect genes*. There are genes in the surrounding maternal tissues that are transcribed to produce mRNA molecules to be transported into the developing egg. It has been found that mutants defective in these genes failed to develop the polarity of the eggs. Therefore, the products of these genes (normal) are necessary in establishing the polarity of the embryo by designating which parts of the egg are dorsal or ventral and which are anterior or posterior. Thus they are known as *egg polarity genes*.

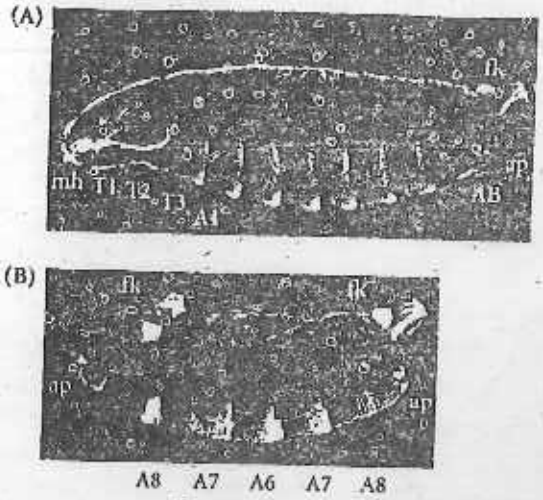


Fig. 10.2 : Phenotype of a strongly affected embryo from a female fly deficient in the *bicoid* gene. (A) Wild-type cuticle pattern. (B) *bicoid* mutant. The head and throax have been replaced by a second set of posterior telson structures. Abbreviations : fk, filzkorper neurons; ap, anal plates (both telson structures); T1-T3, thoracic segments ; A1, A8, the two terminal abdominal segments ; mh, head structures. (From Driever et al. 1990; photograph courtesy of W. Driever)

A concentration gradient of the products of the maternal effect genes is found. At about 128 nuclei stage (i.e., about 1.25 hrs after fertilization) between the seventh and eighth nuclear divisions the nuclei start to migrate to the periphery of the egg. The products of several mutant genes are localized in different regions of the egg. For example, the product of the maternal gene which defines the anterior end of the egg are localized at the anterior end of the egg. Similarly, the other maternal genes which not concerned with anterior end development are found to localize behind the anterior maternal gene (marked by *). However, prior to that event, the products of the maternal genes were found to evenly distributed in the egg and from this point a concentration gradient of the products are found to establish from the anterior to posterior axis.

At 1500 nucleic stage (about 2 hrs after fertilization) most of the nuclei reach the perimeter of the egg and start to make their own mRNA. At this stage the product of the maternal genes are found to be restricted only at the anterior end. Soon the maternal genes product would be over shadowed by the product of the own genes (Fig. 10.2).

The product (mRNA transcripts) of some of the maternal effect genes can be identified by their ability to hybridize with radioactive DNA probes obtained from cloned genes ; alternatively, their products can be identified by *antibodies that specifically bind to them*. The protein produced by translation of mRNA appear to be a part of system of determinants that organizes the early pattern of development in the embryo. A

combination of these protein gradients may provide positional information that specifies the fate of each nucleus or cell within the embryo. That information may then be

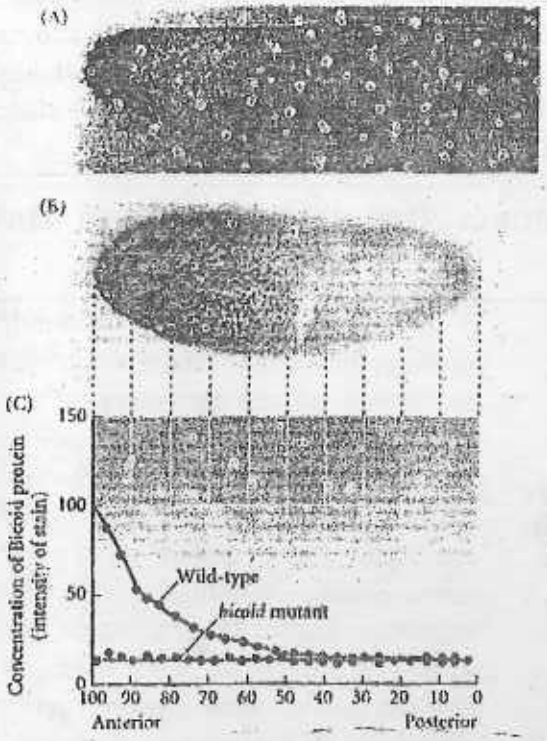


Fig. 10.3 : Gradient of Bicoid protein in the early *Drosophila* embryo. (A) Localization of *bicoid* mRNA to the anterior tip of the embryo. (B) Bicoid protein gradient shortly after fertilization. Note that the concentration is greatest anteriorly and trails off posteriorly. Notice also that Bicoid is concentrated in the nuclei. (C) Densitometric scan of the Bicoid protein gradient. The upper curve represents the Bicoid gradient in wild-type embryos. The lower curve represents Bicoid in embryo s of *bicoid* mutant mothers. (A from Kaufman et al. 1990 ; B and C from Driever and Nusslein-Volhard 1988b; photographs courtesy of the authors.)

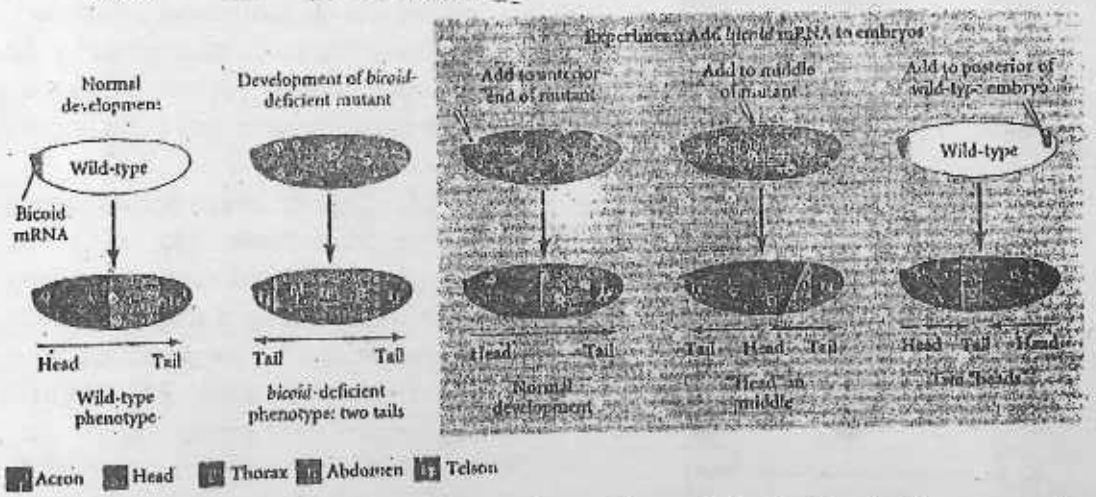


Fig. 10.4 : Schematic representation of the experiments demonstrating that the *bicoid* gene encodes the morphogen responsible for head structures in *Drosophila*. The phenotypes of *bicoid*-deficient and wild-type embryos are shown at the left. When *bicoid*-deficient embryos are injected with bicoid mRNA, the point of injection forms the head structures. When the posterior pole of an early-cleavage wild-type embryo is injected with *bicoid* mRNA, head structures form at both poles. (After Driever et al. 1990.)

interpreted by a cell as signals specifying the developmental path it should follow. For example, owing to the absence of specific signals in the egg, maternal effect mutation can produce an embryo with two heads or two posterior ends (Fig. 10.3 and 10.4).

In many cases, the phenotype associated with a maternal effect mutation can be reversed by injecting normal maternal mRNA into the mutant embryo. When this is done, the fly develop normaly, indicating that gene product is needed only for a short time at the earlier stages of development.

10.3 Zygotic segmentation genes favour and extend the developmental programme

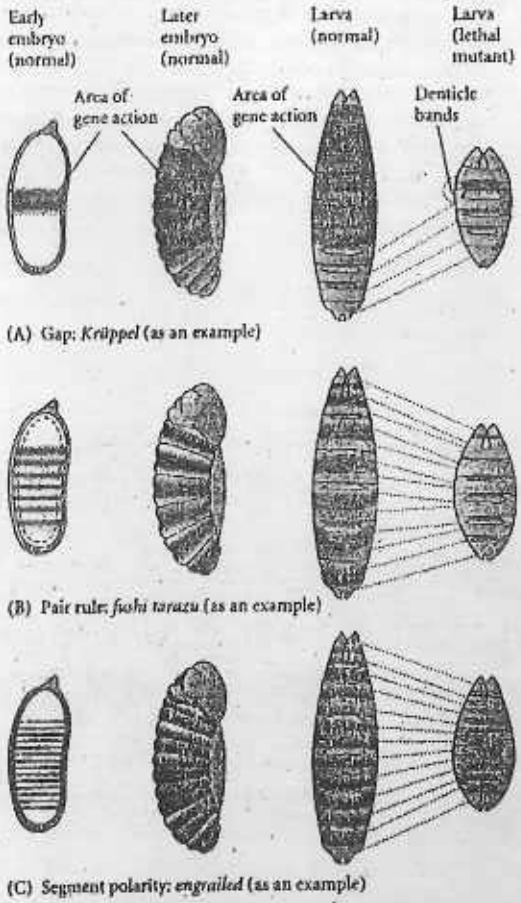


Fig. 10.5 : Three types of segmentation gene mutations. The left panel shows the early-cleavage embryo, with the region where the particular gene is normally transcribed in wild-type embryos shown in color. These are deleted as the mutants develop.

In *Drosophila*, at the eighth mitotic division the nuclei of the embryo start to migrate to the periphery of the embryo. The migration of the nucleus to the periphery under the influence of the expression of the products of the some genes called as *Zygotic genes*. Though, the stage at which the expression begins is not a zygote, but it is customary to refer them as Zygotic genes. Such Zygotic genes extend the developmenal programme beyond the pattern established by the metarnal genome include the zygotic segmentation genes and the homeotic genes.

The Zygotic segmentation genes fall into there classes 'like' gap genes, pair-rule gene, and segment polarity genes representing a rough hierarchy of gene action. So far geneticists have identified at least 24 Zygotic segmentation genes that are responsible for generating a repeating pattern of body segments within the embryo (Fig. 10.5).

(a) Gap gene : The gap gene are apparently the first set Zygotic

segmentation genes to act. These genes seem to interprete the maternal anterior-posterior information in the egg and begin organization of the body segments. A mutation in one of the gap gones usually causes the absence of one or more body segments in an embryo.

(b) Pair-rule genes and Segment polarity genes— The other two classes of segmentation genes do not act on small groups of body segments but rather affect all segments. For example mutations in pair-rule genes every other segment, whereas mutation in segment polarity genes produce segments in which one part is missing and the remain part is duplicated as a mirror image. (Table-1)

Table-1 : Classes of genes involved in pattern formation of embryonic segments in *Drosphila*

Type of gene	Site of gene activity	Effect of mutant alleles and proposed function(s) of genes
Maternal effect genes	Maternal tissues (ovary)	Many maternal effect mutation alter the polarity of the embryo, initiate pattern formation by activating zygotic genes in nuclei in certain location in embryo.
Gap genes	Embryo	Mutant cause one or more segments to be missing ; Some may influence activity of pair rule genes, segment polarity genes and homeotic genes.
Pair-rule genes	Embryo	When mutated, cause alternate segments to be missing. Some may influence activity of polarity genes and homeotic genes.
Segment polarity genes	Embryo	Mutant alleles delete part of every segment and replace it with mirror images of remaining structures and may influence homeotic genes.
Homeotic gene	Embryo	Homeotic mutations cause parts of fly to form structures normally formed in other segments. Control the identities of the segments.

10.4 Homeotic genes specify the identity of each segment

One function of the zygotic segmentation genes is to regulate the expression of a separate set of gene that actually designate the final adult structure formed by each of the *imaginal discs*. It is to note that during the very early embryogenesis in developing

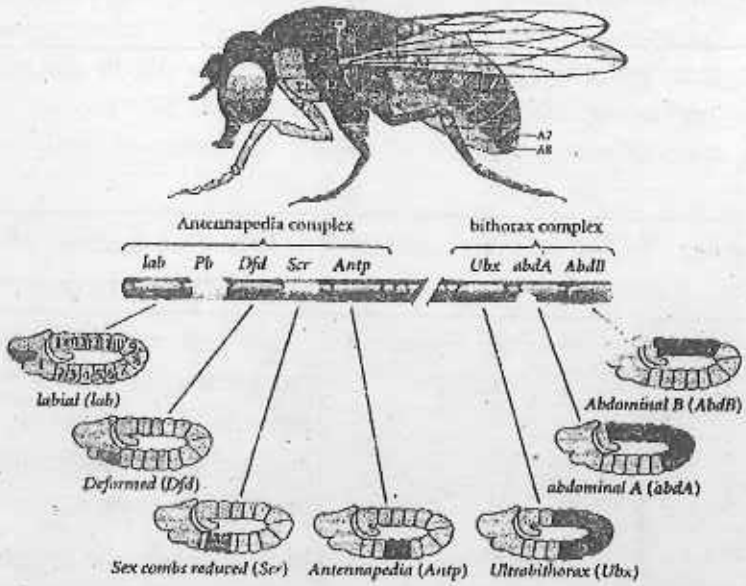


Fig. 10.6 : Homeotic gene expression in *Drosophila*. In the center are the genes of the Antennapedia and bithorax complexes and their functional domains. Below and above the gene map, the regions of homeotic gene expression (both mRNA and protein) in the blastoderm of the *Drosophila* embryo and the regions that form from them in the adult fly are shown. Darker shaded areas represent those segments or parasegments with the most product. (After Dessain et al. 1992 and Kaufman et al. 1990.)

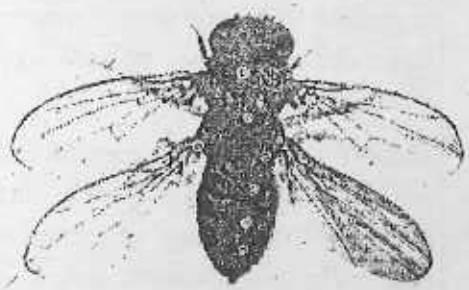


Fig. 10.7 : A four winged fruit fly constructed by putting together three mutations in *cis* regulators of the *Ultrabithorax gene*. These mutations effectively transform the third thoracic segment into another second thoracic segment (i.e., halteres into wings). (Photograph courtesy of E.B. Lewis)

larvae, precursor cells of many of the adult structures are organized as relatively undifferentiated paired structures called imaginal discs. This term comes from image, the name given to the adult form of the insect. Each imaginal disc occupies a definite position in the larva and will form a specific structure, such as a wing or a leg, in the adult body (Fig. 10.6 & 10.7).

Homeotic genes are involved to provide the segment identity and as such mutations in homeotic genes

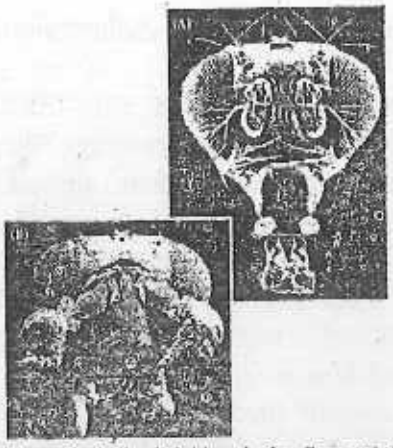


Fig. 10.8 : (A) Head of a wild-type fruit fly. (B) Head of a fly containing the *Antennapedia* mutation that converts antennae into legs. (From Kaufmann et al. 1990; photographs courtesy of T.C. Kaufmann).

cause one body part to be substituted by another and therefore produce some peculiar change in the adult. The effect of homeotic gene mutation can be best illustrated by the case of Antennapedio mutant, which have legs that grow from the head at a position where the *antennae* would normally be found (Fig 10.8).

10.5 Mechanism of action of genes which control embryonic development in *Drosophila*

Many development mutants of Drosophila are now been identified. Their effect on development in various combination have been examined and studied extensively at the molecular level. In our discussion, we will briefly comment on the moleculer mechanism of some of these genes briefly.

The earliest developmental program to operate in the egg is established by *maternal effect genes*. Such genes are active prior to fertilization and such genes are transcribed in the surrounding maternal tissues to produce mRNA molecules and such molecules are transported into the developing eggs. Using various molecular techniques, it is now possible to identify the proteins that such mRNAs are produced in the eggs. The proteins produced by translation of the mRNA appears to be part of a system of determinants that organize the early pattern of development in the embryo. A combination of these protein gradient may provide positional information that specifies the fate of each nucleus or cell in the embryo. The proof of such action can be observed when mutation in the maternal effect genes are studied. It has been observed that mutation of maternal effect genes can produce an embryo with two heads or two posterior ends. The observations strongly suggest that maternal gene products (proteins) act as signals specifying the developmental path that a cell should follow. In case of mutations in absence of such specific signals abnormal embryos are produced.

Hundreds of maternal effect genes and their products are identified of which some are named here.

The Bicoid protein, a product of bicoid gene, arise from the maternal nurse cell and injected into the anterior end of the unfertilized egg. The protein diffused through the syncytium, setting up a concentration gradient, highest at the anterior end and lowest the posterior end of the embryo. The other maternal effect gene products are also involved in setting up the anteiror posterior gradient. These are the Hunchback, Nanos and Caudal proteins. All are injected as mRNAs in to anterior region of the unfertilized eggs. The nanos mRNA is transported to the posterior part of the egg and attached to the cytoskeleton while it awaits translation. The hunchback and caudal mRNA become distributed evenly through the cytoplasm, but their proteins subsequently form gradient through the action of Bicoid and Nanos.

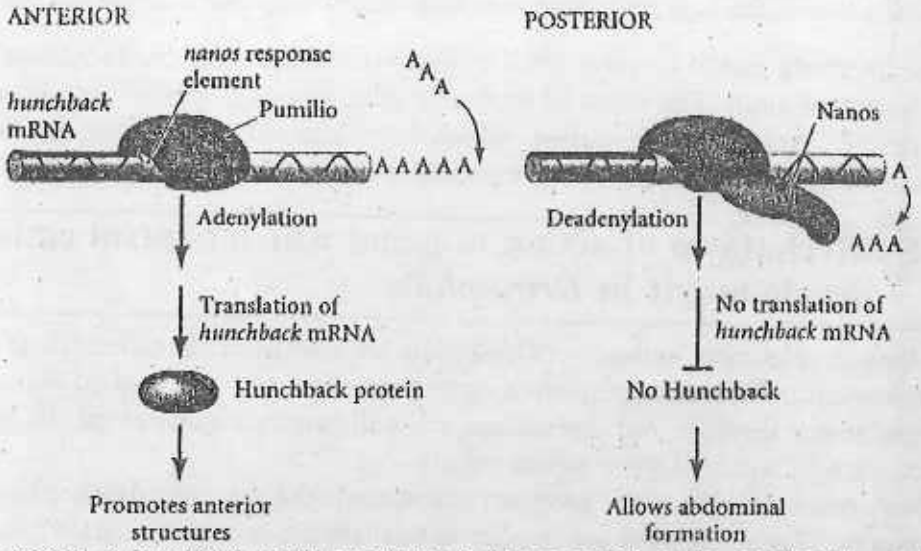


Fig. 10.9 : Control of *hunchback* mRNA translation by Nanos protein. In the anteriorof the embryo, Pumilio protein. In the anterior of the embryo, Pumilio protein binds to the Nanos response element (NRE) in the 3' UTR of the *hunchback* message, and the message is polyadenylated normally. This polyadenylated message can be translated into Hunchback protein. In the posterior of the embryo, where Nanos protein is found, Nanos binds to Pumilio to cause the dead-emylation of the *hunchback* mesage, thus preventing its translation (After Wreden et al. 1997).

Bicoid activates the hunchback gene in the embryonic nuclei, supplementing the hunchback mRNA in the anterior region and represses translation of maternal caudal mRNA. The result is an increase in the concentration of the Hunchback protein in the anterior region and a decrease in that caudal (Fig. 10.9).

Nanos represses translation of hunchback mRNA, contributing further to anterior-posterior gradient of the Hanchback protein.

The net result is a gradient of Bicoid and Hunchback in the anterior end while Nanos and Caudal operates in the posterior end. The gradient is supplimented with

Torso proteins (a maternal effect gene product) which accumulate at the extreme anterior and posterior ends. Similarly Dorsal protein gradient is formd dorsal to ventral axis.

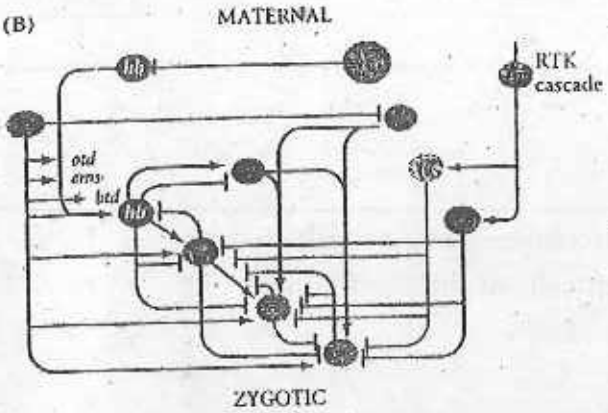


Fig.10.10 : Conversion of maternal protein gradients inot zygotic gap gene expression. (A) Gap gene expression patterns. (B) The gradients of maternal transcription factors Bicoid, Caudal, and Hunchback regulate the transcription ofthe gap genes. Hunchback and Caudal proteins come from both maternal messages and new zygotic transcription. These gap gene-encoded proteins diffuse, and the interactions between them are critical in activating the transcription of the pair rule genes. At the two tremini ofthe embryo, the interaction between Torso and Torso-like activates the *tailless* and *huckebein* gap genes. (B after Rivera-Pomar and Jackel 1996).

Zygotic segmentation genes do not become active until much later, when the embryo is no longer a zygote. They continue and extend the developmental program initiated by the maternal-effect genes. The Zygotic segmentation genes and their products interact with each other and the products of the maternal effect genes according to a hierarcheal pattern. The gap genes acting first, then the pair-rule genes and finally the segment polarity genes (Fig. 10.10).

Homeotic genes in *Drosophila* were originally identified by the altered phenotypes produced by mutant alleles. When genticits analysed the DNA sequences of several homeotic genes, they discovered a short DNA sequence of approximately 180 base pairs is the charteristic of homeotic gene, and the sequence is called the *homeobox*. Each homeobox codes for a protein functional region called *homeodomain*, Constituting of 60 amino acids that form four α-helices. Such protein acts as transcription factor and affect transcription.

Unit 11 ❐ Elementary idea of stem cell and its importance

Structure

11.1 Introduction

Stem cells are cells that has the capacity to divide indefinitely & which can give rise to more specialised cells. When they divide, stem cells produce a more specialized type of cells and more stem cells.

Some single stem cells in the early embryo are capable of generating all the structures of the embryo. These cells are known as *pleuripotent stem cells* and are capable of generating ectoderm, endoderm, mesoderm and germ cells.

These stem cells generated more pluroipotent stem cells as well as *committed stem cells*. CSCs can give rise to a smaller population of cells. For instance, one type of CSC is hemangioblast that gives rise to all the blood vessels, blood cells & lymphocytes.

CSCs can give rise to more CSCs or *progenitor cells* or *precursor*. These PCs are no longer the stem cells as they cannot produce more PCs, rather, they divide to form one or few related cell types.

Totipotency or totipotent stem cells : The very early mammalian cells that can form both the entire embryo and the fetal placenta (trophoblast) around it.

Pleuripotencey or pleuripotent stem cells : They are the Inner Cell Mass (ICM) of mammals that can form the embryo but not its surrounding tissues.

The restriction on the potency of stem cells is gradual and the potencies of these cells are determined by their surroundings. Once committed, however, they usually do not switch commitment. Once placed in a new environment, they will not change the type of cells which they can generate. Stem cells are critical for maintenance of

cell population that last for long periods of time and must be removed. Thus, in human stem cells are important for continual production of blood, hair, epidermis and intestinal epithelial cells (Fig. 11.1).

11.2 Stem cell niche

Many organs have stem cells that undergo continuous renewal. These tissues include the mammalian epidermis, hair follicle, intestinal villi, blood cells & sperm cells as well as *Drosophila* sperm and egg.

The ability of a cell to become an adult stem cell is determined by where it resides. The continuously proliferating stem cells are housed in compartments called *Stem cell niches* (regulatory microenvironment). There are particular places in the embryo that become stem cell niches.

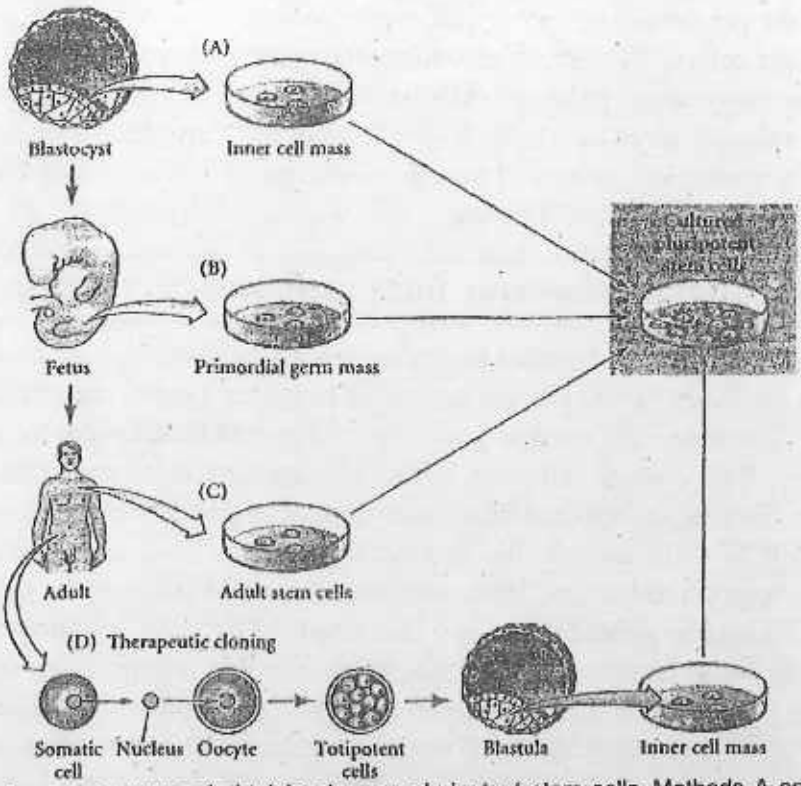


Fig. 11.1 : Four major ways of obtaining human pluripotent stem cells. Methods A and B have been documented to work; methods C and D remain experimental. (A) Cells from the inner cell mass of a blostocyst are cultured and become pluripotent embryonic stem cells. (B) The primordial germ cells a fetus are harvested and become pluripotent embryonic stem cells. (C) Adult stem cells are obtained and grown in a manner that allows them to become pluripotential. (D) "Therapeutic cloning," wherein the nucleaus of a somatic cell is transferred into an enucleated oocyte. The oocyte is activated and gives rise to a blastocytst, whose inner cel mass in harvested and clutrued to become pluripotent embryonic stem cells. (After NIH 2000.)

Stem cell niches regulate their continuous production of stem cells and their more differentiated progeny. Usually by paracrine (and sometimes by juxtacrine) factors they are produced in the niche cells. These paracrine factors retain the cells in an uncommited state. Once the cells leave the niche, the paracrine factor cannot search them and the cells begin the process differentiation.

For instance, sperms are continuously produced in the *Drosophila* testis. The stem cells for sperm resides in a regulatory microenvironment named *the hub*. The hub consists of about a dozen somatic testes cells and is surrounded by 5-9 germ stem cells. These germ stem cells that remain attached to the somatic cells remain as germ stem cell. However, their division is assymmetirc. Those remaining attached to the hub remain the stem cell population, while those daughter cells that divide in such a way that they are not touching the hub become the gonial blast cells that will divide to become the precursors of sperm cell.

The somatic cells of the hub are able to regulate stem cell proliferation by secreting the paracrine factor unpaired on the cells attached to them by activating the Jak STAT pathway in adjacent germ stem cells to specify their self renewal. Those cells that are distant form paracrine factor cannot receive this signal, so they begin their differentiation into sperm cell lineage.

11.3 Moleculer mechanism for pluripotency or totipotency

In mouse the earliest blastomeres can form both trophoblast cells and the embryonic precursor. These very early cells are said to be totipotent (Latin, capable of forming everything). The inner cell mass is said to be pluripolent (Latin, capable of forming many things). That is each cell of ICM can generate any cell type in the body but because the distinction between ICM and trophoblast has been established, it is thought that ICM cells are not able to form trophoblast.

Once the decision to become either torphoblast or ICM is done, the cells of these two regions express different genes. The trophoblast cells synthesized T box transcription factor *eomesodermin* and homeodomain containing, caudal like transcription factor Cdx2. Eomesodermin-activates those proteins characteristic of trophoblast layer [Russ et al 2000 ; Hanna. at 2002]. Cdx2 is responsible for down regulating Oct4 and Nanog, two transcription factors that along STAT 3 characterises ICM [Strumph et al 2005]. At eight cell stage cdx2, eomesodrmin and Oct4 are synthesized in all cells. But in blastocyst cdx2 and eomesodermin remains in trophoblast, whereas Oct 4 is maintained in ICM [Niwa et al 2005].

The expression of the 3 transcription factors characteristic of ICM— Oct 4, STAT 3 & Nanog— is critical for formation of the embryo and for maintenance of

pluripotency of ICM. Oct 4 is expressed first, and it is expressed in the morula as well as in the inner cell mass and early epiblast. Oct4 blocks cells to take on trophoblast fate. Later, Nanog prevents the ICM blastomeres from becoming hypoblast cells and stimulate blastomere for self renewal in epiblast.

The activated (phosphorylated) form of STAT 3 and also stimulate self renewal of ICM blastomere [Pesce and Scholar et al 2001 ; Chabers et al 2003 ; Mitri et al 2003]. If ICM blastomeres are removed in a manner-that let them retain their expression of Nanog, Oct4 and phosphorylated STAT 3 protein these cells divide and become embryonic stem cells. Pluripotency of these stem cells depend on their retaining in the expression of these 3 transcription factor.

11.3 Types of stem cells / stem cells of difrerent regions

1. Neural Stem Cells : Until recently, it was generally believed that once the nervous system is mature, no new neuron is born. However recent studies show adult mammalian brain is capable of producing new neurons and environmental stimulation can increase the number of these new neurons.

The existence of neural stem cells is now well established for the olfactory epithelium and the hippocampus [Kempermann et al. 1997a, b; Prag et al. 1999 ; Kornea & Rakie 1999 ; Kalo et al 2001]. These cells respond to sonic hedgehog and can proliferate to become multiple cell types for at least first year of a mouse's life [Alm & Joyner 2005]. It appears that the stem cells producing these neurons are located in the ependyma. Though these cells represnt only about 0.3 percent of ventricle wall cell population, they can be distinguished from other cells by their particular cell surface protein (Rietze et al 2001).

Mechanism by which neural stemeells are kept in a ready state of quiscence is not well known. Before they become neurons, neural stem cells are characterized by NRSE translational *inhibitor that prevents* neuronal differenciation by binding to a silencer region of DNA. However, when neuronal stem cells begin to differentiate, they synthesize a small, double stranded RNA that has same sequence as the silencer and which might bind NRSE and thereby permit neuronal differentiation [Kuwabara et al 2004].

2. Melanocyte stem cells : During the migration of neural crest cells, the cells that took the dorsal route become commiteed to form melanocytes. As they travel through dermis and epidermis, they eventually enter the developing hair follicles take up position at the base of the follicle bulge.

Here, they become melanocyte stem cells [Mayer-1973 ; Nishimura et al 2002]. A portion of these cells migrate outside the bulge at the beginning of each hair

development cycle to diferentiate into mature melanocyte and provide pigmentation to the hair shaft.

Nishinura et al. in (2005) have documented that the reason behind greying of hair and age in human and mice is that melanocyte stem cells become depleted from the bulge.

When the melanocytes are in stem cell niche, melanocyte stem cells are inhibited from differentiating because of the regulation of Mitf transcription factor.

Mitf activates the genes of the melanin pathway. The Mitf gene is itself activated by the Sox10 and Pax3 protein. However, on some of the genes activated by Mitf, Pax3 bind to same place on the enhancer as the Mitf, thus competing for the site [Lang et al. 2005]. So, even though Pax3 stimulates melanocyte differentiation by *activating Mitf, it also prevent* Mitf from functioning. Once outside the bulge, the Wnt signalling generated β-Catenin, which bind to a Lef/Tef transcription factor and displaces Pax3 from their sites. Thus allows Mitf to be expressed and activate the melanin producing genes.

3. Muscle progenitor cell : Adult muscles can regenerate following injury. The new myofibrils come from sets of stem cells or progenitor cells that resides along adult muscle fibre.

There may be more than one type of muscle stem cells and their function may overlap. [Polesskaya *et al.* 2003]. Lineage tracing using Chimeras indicate that these muscle progenitor cells are somatically derived myoblasts that have not fused and remain potentially available throughout adult life.

One type of putative stem cells, *the satellite cell,* is found within the basal lamina of mature myofibers. Satellite cells respond to injury or exercise by proliferating into myogenic cells that fuse and form new muscle fiber. These cells may be stem cells with the capacity to generate daughter cells for renewal or differentiation. In inactive form, these cells show Pax-7 that inhibit MYOD expression and muscle differentiation in these cells (Olguin & Olwin, 2004).

Another type of muscle stem cell [may be derived from somatic cells that migrate to form dorsal aorta] is activated by Wnt signalling from injured muscle tissue. Wnt signal appear to activate Pax 7 and it. Pax 7 protein activates the myoD family gene, thus, promoting muscle differentiation.

Comment : Most muscle progenitor cells express both Pax 7 (sattelite specific) gene and Pax 3. Recent studies also indicated that at least some satellite cells come from the central portion of the dermamyotome [Gros et al. 2005 ; Relaix et. al. 2005].

4. Development of blood cell : The bone marrow HSC is a remarkable cell in that it is the common precursor of all blood cells and lymphocytes. It is estimated that only about 1 HSC is present in every 10,000 blood cells.

The HSCs formed in the embryo are those that populate the bone marrow (in some instance, in spleen) of adult mammals. The adult HSCs riches in bone & spleen make chemo attractant proteins that attract the circulating stem cells into them (Christensen et al. 2004 ; Gotherd et al. 2005).

The HSC (or, CFU-M, L) appears to be dependent on the transcription factor SCL. Mice lacking SCL die from the absence of all blood & lymph lineages.

HSC is also dependent on endosteal osteoblasts that line the bone marrow and are responsible for providing the niche that attracts HSCs and keep them in a state of plasticity. These osteoblasts bind HSCs and provide several other signal.

a) one signal is provided by *jagged protein* which activate Notch protein on HSC surface.

b) A second signal comes from *Angioprotein-1* on osteoblast, which activates receptor tyrosine kinase Tie and on the surface of HSC (Arai et al. 2004).

c) A third signal is from Wnt pathway, localizing *β-catenin* into the nucleus. This pathway seems to be critical for self renewal of HSCs (Reya et. al 2003).

The HSC can give rise to blood cell precursor (common myeloid precursor cells" CMP or CFU-S) or to lymphocyte stem cells (CLP). The CMP produce *megakaryocyte /Erythroid Precursor Cells (MEP)l* which can generate either the red blood cell lineage or the platelets lineage.

CMP also produce *Granulocyte/Monocyte precursor cell (GMP)* which generate beasophil, eosinoplul, neutrophil and monocyte.

Eventually these cells produce progenitor cells that can divide but produce only one kind of cell in addition to renewing itself. eg. *Erythroid progenitor cell* [BFU-E] is a committed stem cell that can form only RBCs. Its immediete progeny is capable of responding the hormone *erythropoitin* to produce from recognizable differentiated member of erythrocyte lineage, *proerythroblast,* this cell begins to produce globin and cell is matured gradually to produce *erythroblast.* Eventually mammalian erythroblast expels the nucleus to produce *reticulocyte.* The final stage of differentiation form the erythrocyte (Fig. 11.2).

11.5 Stem cells and therapeutic cloning

Characters :

1] They are unspecialised cells.

2] They can divide until they are committed.

3] Pluripotency / totipotency.

4] They have to contain a niche.

5] They always transform into progenitor cells prior to formation of specialized cells.

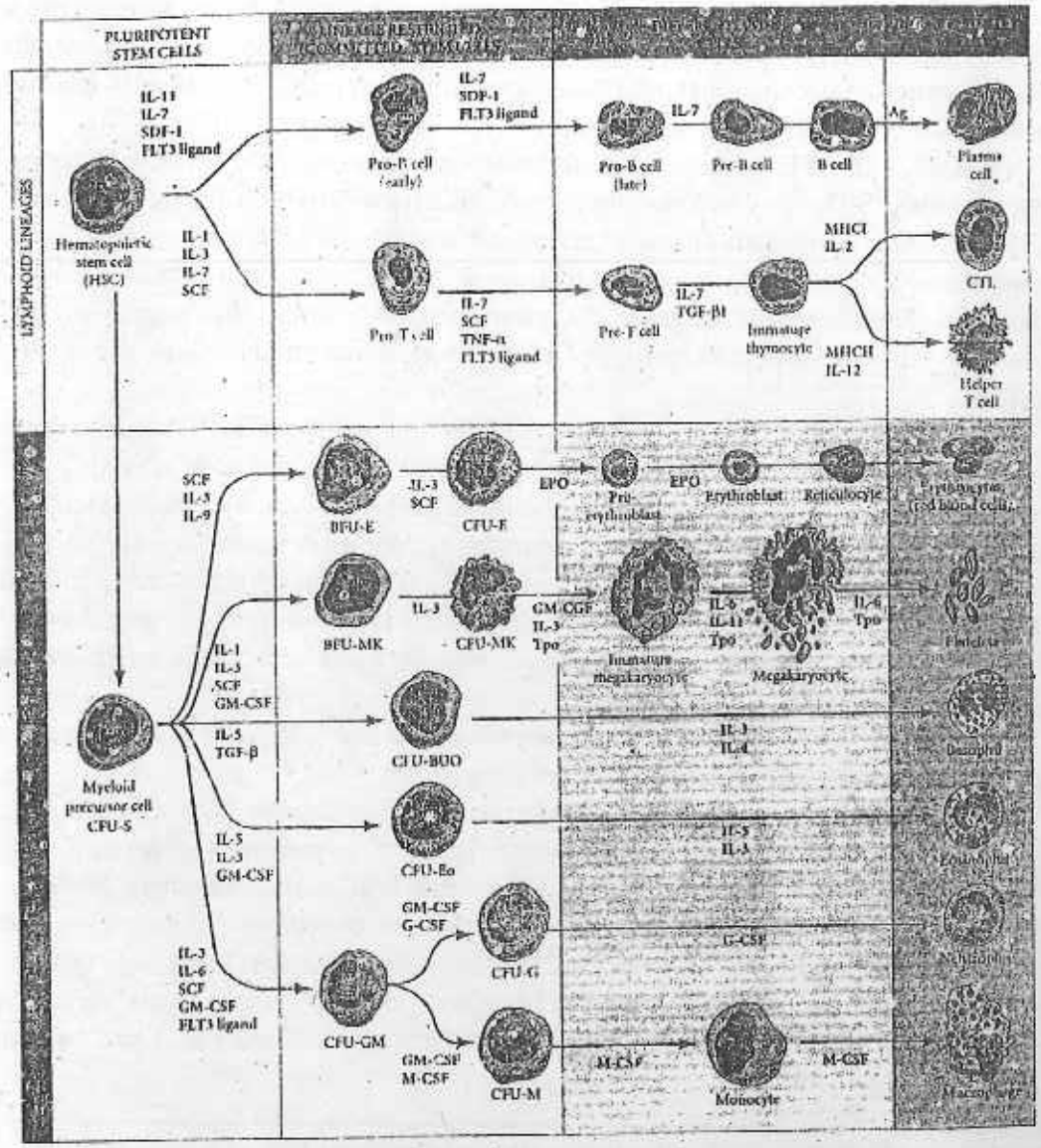


Fig. 11.2 : A model forthe origin of mammalian blood and lymphoid cells. (Other models are consistent with the data, and this one summarizes features from several models.) Factors affecting each step of differentiation are shown in red. Ag. antigen ; EPO. crythropoietin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor ; IL, interleukin ; LIF, leukemia inhibiting factor ; M-CSF, macrophage colony-stimulating factor ; MHCI, major histocampatability complex type I protein ; MHCII, major histocompatability complex type II protein ; SCF, stem cell factor ; SDF-1, stromal-derived factor-1; TNF, tumor necrosis factor ; Tpo, thrombopoietin. (After Nakauchi and Gachelin 1993 ; R&D Systems 1997.)

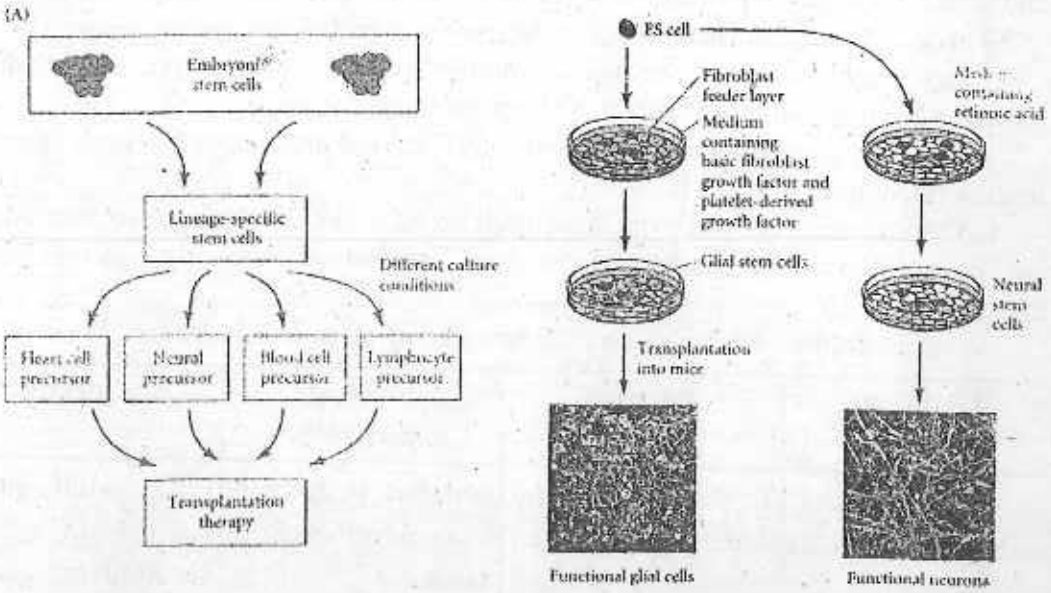
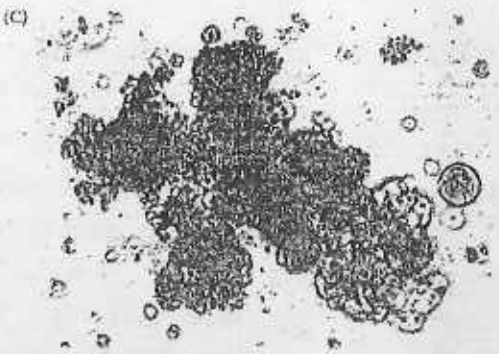


Figure 11.3 : Embryonic stem cell therapeutics, (A) Human embryonic stem cells (ES cells) can differentiate into lineage-specific stem cells, which can then be transplanted into a host. (B) The differentiation of mouse ES cells into lineage-restricted (neuronal and glial) stem cells can be accomplished by altering the media in which the ES cells grow. (C) Blood cells developing from human embryonic stem cells cultured on mouse bone marrow. (A after Gearhart 1998 ; B, photographs from Brustle et al. 1999 and Wickelgren 1999, courtsey of O. Brustle and J.W. McDonald ; C. photograph courtsey of University of Wisconsin.)



6] They are controlled by individual molecular cascade.

7] They follow contact inhibition and not form lump as in cancer.

8] There is no immuno rejection.

A) A recent review article that summarises the development of "genetic medicines".

B) Softer, D; 2006 ; from teratocarcinomas to embryonic stem cells and beyond : a history of embryonic stem cell research. Nat rev Gene 7 : 319-27.

11.6 Stem cell therapy

Embryonic Stem Cells and therapeutic cloning

ES cells are pluripotent, can be cultured indifinitely in an undifferentiated state

and retain their developmental potential after prolonged culture (Fig. 11.3).

Source : At present, human ES cells are obtained by two major sources.

a) they can be derived from ICM blastomere of human blastocyst, such as those left over from in vitro fertilization [Thomson *et al.* 1998].

b) they can be generated from germ cells derived from spontaneously aborted fetuses [Gearhart, 1998].

N.B. Some experimental evidence (Strelchenko *et. al.* 2004] suggests that it may also be possible to derive embryonic SC from late morulae before they go on to form blastocyst.

Table : Material and techniques of stem cell research

Technique	Purpose	Starting material	End product
1) Adult (or fetal) stem cell research.	To obtain undifferentiated stem cells for research and therapy.	Isolated stem cells from adult or fetal tissue.	Cells produced in culture to repair diseased or injured tissue.
2) ESC research	To obtain undifferentiated stem cells for research & therapy.	Stem cells from a blastocyst stage ambryo.	—Do—
3) Therapeutic cloning (nuclear transplantation)	To obtain undifferentiatied stem cells that are genetically matched to the recipient for therapy and tissue regeneration.	Stem cells from a blastocyst stage embryo produced from an enucleated egg supplied with nuclear material from recipient's own somatic cells.	—Do—
4) Reproductive Cloning.	To produce an embryo for implantation in womb, leading to birth of a child.	Enucleated egg supplied with material from a donor somatic cell.	Embryo (and eventual off spring) genetically identical to donor of nuclear material.

Aspects of ESC : It is thought that they can help in

— production of new neurons (in AD, PD).
— spinal cord injuries.
— produce new pancreas for diabetic.
— produce new blood cells for anaemics.
— produce new heart tissue for patients & deteriorating hearts.
— replenish new immune system.

Different experiment :

A) Kaufman & Colleagues (2001) direct human ES cells to become blood forming stem cells by placing them on mouse bone marrow or endothelial cells. These ES derived hematopoitic stem cells could further differentiate into numerous types of blood cells.

B) Human ESC. derived from germ cells were able to cure virus induced paraplegia in rats. These stem cells appear to do this by both differentiating into new neurons and by producing paracrine factors (BDNF and TGF-α) that prevent death of existing neurons [Kern et al. 2003].

C) ESC from monkey blastocyst have been able to cure a Parkinson like disease in adult monkeys whose dopaminergic neurons had been destroyed [Takagi *et al* 2005].

11.7 A potencial technique : therapeutic cloning

As human ESC develop, they express significant amount of MHC that can cause immune rejection [Drukker *et al* 2002]. To solve this problem, human ESC could be modified, or somatic cell nuclear transfer could be used to ensure that the stem cells are geneticaly identical to the person who would be receiving their progeny. To solve the problem therapeutic cloning is being introduced.

Process : In this technique, a nucleus from patient is inserted into an enucleated oocyte.

The resulting embryois grown in vitro until it has developed an ICM.

Cells from the ICM are then cultured to general stem cells that are gentically identical to the patient

Application : In "Parkinsonian" Mice

1) Barberi *et al* [2003] performed somatic cell nuclear transfer such that the nucleus of one type of mice was transferred to oocyte of another strain of mice.

2) These cells divided and became a blastocyst, and ESC were derived from blastocyst.

3) Then, the embryonic SC induced to become neural stem cells by growing on mesoderm and providing Fgf2 in medium.

4) The neural stem cells were then induced to become ventral neural stem cells by adding sonic hedge hog, to the medium.

5) Fgf2 & Fgf8 are then added to these cells, followed by exposure to brain derived neurotrophic factor (BDNF) and these produce cells that had characteristic of dopaminergic neurons.

Comment : When these cells are injected to affected mice, they restore the dopaminergic neurons and the mice become normal.

N.B. An interesting modification of this system is designed by Cowan & Collegauge (2005). They fused the somatic cell and an already existing ESC.

In many instances, the cells not only fuse, but so did their nuclei, to make a tetraploid nucleus. These hybrid cells kept the stem cell phenotype and could differentiate into 3 major germ layers.

This finding opens the possibility that transplantation of somatic nuclei into enucleated ESC may allow patient specific stem cells to be made without having to use early embryonic stages. This could circumvent many of the religious objections to using human ESC.

11.8 Multipotent adult stem cells

Numerous organs have committed multipotent stem cells, even in the adult. They are not as easy to use as Pluripotent embryonic SC, they are difficult to isolate and are often fewer than one out of every thousand cells in an organ.

Bone marrow transplantation has been used to transfer haematopioetic stem cells from one person to another in over 40,000 operations each year. This pluripotent haematopioetic stem cells are rare (1 in every 15,000 bone marrow cells) but such transplantation works well for people who are suffering from RBC. deficiencies or leukemias.

In mice, very few (perhaps even one) blood stem cells will reconstitute the muscles blood and immune systems (Asawa *et al* 1996) and a single mammary stem cell will generate an entire mammary gland in mics (Shackleton *et al* 2006).

When researchers have been able to isolate and culture such cells, they have proved to be very useful. Carvey & Colleagues (2001) have shown that when neural stem cells from midbrain of adult rats are cultured in a medium with a mixture of paracrine factors, they too, will diffentiate into dopaminergic neurons that can cure rat version of PD.

NOTE

মানুষের জ্ঞান ও ভাবকে বইয়ের মধ্যে সঞ্চিত করিবার যে একটা মহৎ সুবিধা আছে, সে কথা কেহই অস্বীকার করিতে পারে না। কিন্তু সেই সুবিধার দ্বারা মনের স্বাভাবিক শক্তিকে একেবারে আচ্ছন্ন করিয়া ফেলিলে বুদ্ধিকে বাবু করিয়া তোলা হয়।

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