Post-Graduate Degree Programme (CBCS)
in
ZOOLOGY
(M.Sc. Programme)

SEMESTER-III

Theory Paper-Minor Elective

Developmental Dynamics
ZDSE(MN)T 302

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
Kalyani, Nadia
West Bengal, India

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Director's Message

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Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2020 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Manas Kumar Sanyal, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and coordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

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Theory (Discipline Specific Elective - Minor) - [ZDSE(MN)T-302]

Module	Unit	Content	Credit	Page No.
ZDSE(MN)T 302 Developmental Dynamics	I	Common features of development: Genomic equivalence; cytoplasmic determinants;	**2**	
	II	Imprinting, Cloning of animals.		
	III	Techniques for the study of development: i) Cell labeling and tagging ii) Cell sorting.		
	IV	Model organism *Xenopus*/ Zebra fish: early embryonic development and major classes of molecules expressed, regional and genetic specification.		
	V	Model organism *C. elegans*: early embryonic development and major classes of molecules expressed, regional and genetic specification.		
	VI	Stem cells: Application of Adult Stem Cells, iPS Cells; Stem cell niches; Trans-differentiation.		
	Total counseling session 6hrs			

UNIT I

Common features of development: Genomic equivalence; cytoplasmic Determinants

Objective:

In this unit we will discuss about Common features of development: Genomic equivalence; cytoplasmic Determinants.

GENOMIC EQUIVALENCE

According to this theory, all cells of an organism contain an equivalent complement of genetic information but some exceptions occur in some animal cells where loss, gain or rearrangement of nuclear DNA has been observed. Mosaicism is another example of genomic non-equivalence in which cells within the same individuals have a different genetic makeup. Individuals showing mosaicism are referred to as mosaics. Mosaicism can be caused by DNA mutations, epigenetic alterations of DNA, chromosomal abnormalities (change in chromosome number and structure) and the spontaneous reversion of inherited mutations and somatic hypermutation. Mosaicism can be associated with changes in either nuclear DNA or mitochondrial DNA. It can exist in both somatic cells (somatic mosaicism) and germ line cells (germ line mosaicism). Another example of such exceptions are chromatin diminution(programmed DNA elimination) in somatic cells of some nematodes, selective amplification of r-RNA in Xenopus oocyte, DNA excision and rearrangement during mammalian lymphocyte maturation that result in formation of different types of antibody molecules by random rearrangement of series of genetic segments of immunoglobulin gene , phenomenon known as somatic hypermutation.

The existence of this genomic equivalence was not so much proved as assumed (because every cell is the mitotic descendant of the fertilized egg), so one of the first problems of developmental genetics was to determine whether every cell of an organism indeed had the same set of genes, or genome, as every other cell.

Metaplasia

The first evidence for genomic equivalence came from embryologists studying the regeneration of excised tissues. The study of salamander eye regeneration demonstrated that even adult differentiated cells can retain their potential to produce other cell types. Therefore, the genes for these other cell types' products must still be present in the cells, though not normally expressed. In the salamander eye, removal of the neural retina promotes its regeneration from the pigmented retina, and if the lens is removed, a new lens can be formed from the cells of the dorsal iris. The regeneration of lens tissue from the iris (called Wolffian regeneration after the person who first

observed it, in 1894) has been intensively studied. Yamada and his colleagues found that a series of events leads to the production of a new lens from the iris. The nuclei of the dorsal side of the iris begin to synthesize enormous numbers of ribosomes, their DNA replicates, and a series of mitotic divisions ensues. The pigmented iris cells then begin to dedifferentiate by throwing out their melanosomes (the pigmented granules that give the eye its color) These melanosomes are ingested by macrophages that enter the wound site. The dorsal iris cells continue to divide, forming a globe of dedifferentiated tissue in the region of the removed lens. These cells then start synthesizing the products of differentiated lens cells, the crystallin proteins. These crystallins are made in the same order as in normal lens development. Once a new lens has formed, the cells on the dorsal side of the iris cease mitosis

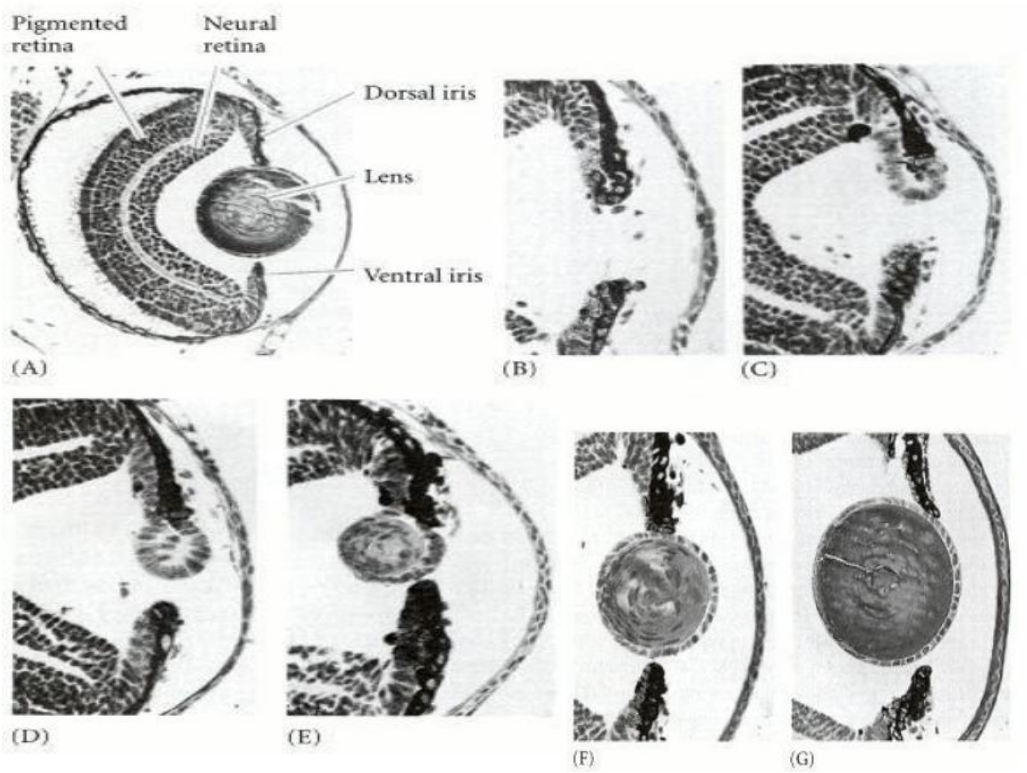


Figure: Wolffian regeneration of the salamander lens from the dorsal margin of the iris. (A) Normal eye of the larval-stage newt *Notophthalmus viridescens.* (B-G) Regeneration of the lens, seen on days 5, 7, 9, 16, 18, and 30, respectively. The new lens is complete at day 30. (From Reyer 1954; photographs courtesy of R. W. Reyer.)

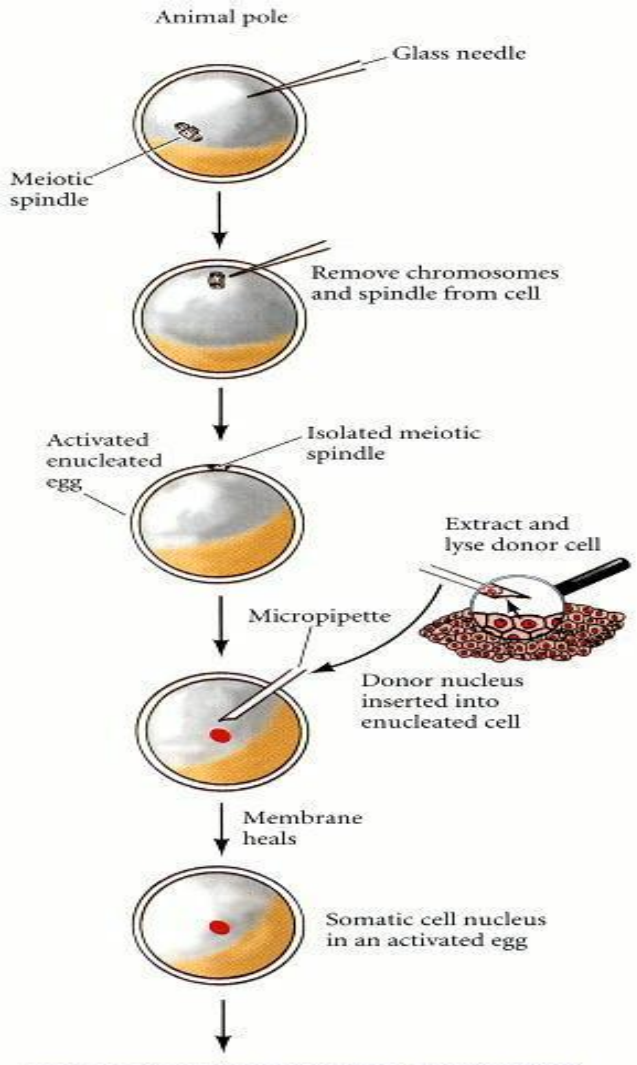
These events are not the normal route by which the vertebrate lens is formed. The lens normally develops from a layer of head epithelial cells induced by the underlying retinal precursor cells. The formation of the lens by the differentiated cells of the iris is an example of metaplasia(or transdifferentiation), the transformation of one differentiated cell type into another. The salamander iris, then, has not lost any of the genes that are used to differentiate the cells of the lens.

Animal cloning and genomic equivalence- Animal cloning is the process by which an entire organism is reproduced from a single cell taken from the parent organism and in a genetically identified manner.

The technique used to clone whole animals such as sheep, is referred to as reproductive cloning. In reproductive cloning, researchers remove a mature somatic cell, such as a skin cell or others. Then DNA of the donor animals somatic cell is transferred into an egg cell or oocyte by removing the own nucleus of oocyte . Removing of oocyte nucleus is done by micropipette. Then egg is allowed to develop into an early stage embryo in the test tube and then implanted into the womb of an adult female animal. Ultimately, the adult female gives birth to an animal that has the same genetic makeup as the animal that donated the somatic cell. This young animal is referred to as a clone. Reproductive cloning may require the use of a surrogate mother to allow development of the cloned embryo, as was the case for the most famous cloned organism- Dolly.

Nuclear transfer technology and animal cloning

Nuclear transfer can be used to produce genetically modified organisms. Nuclear transfer involves the replacement of an oocyte nucleus with the nucleus of a somatic cell, which is then reprogrammed by the oocyte and becomes able to recapitulate the whole of development despite the differentiated state of the donor cell. This technique has been used to generate cloned animals.



Nuclear transfer was first reported in Xenopus in 1952. Since then, it has been widely used in amphibians, in 1977, a mouse was generated by nuclear transfer technology by using a cell from a very early embryo. In 1980, cloned sheep and cattle were generated using early embryo cells as donors. The major breakthrough came in 1995, when Ian Wilmut and Keith Campbell produced two live lambs- Megan and Morag by nuclear transfer of embryonic cells grown in culture. Within a tear , a lamb had been produced from an adult cell, this lamb was called Dolly. Since then a number of lambs have been generated

using this technology, some of which are transgenic.

Embryo reconstruction by nuclear transfer involves the transfer of a single nucleus to an unfertilized oocyte or one cell zygote from which the genetic material has been removed. For livestock, the recipientcell of choice is usually an unfertilized oocyte wherein metaphase-II of meiotic-II is in arrested condition. The first step is to enucleate the recipient oocyte. Then genetic material from the donor cell is then introduced into the enucleated oocyte. The next phase involves the activation of the reconstructed embryo. In nature, this is achieved by fertilization and involves calcium mobilization. Activation can be mimicked by a variety of chemical agents such as phorbol esters. In practice, this step is often simplified, since, the electric pulse used to fuse the cells can also activate the embryo. Finally, the embryo is cultured to a stage at which it can be transferred to a synchronized recipient animal for development to term.

Amphibian cloning: The restriction of nuclear potency

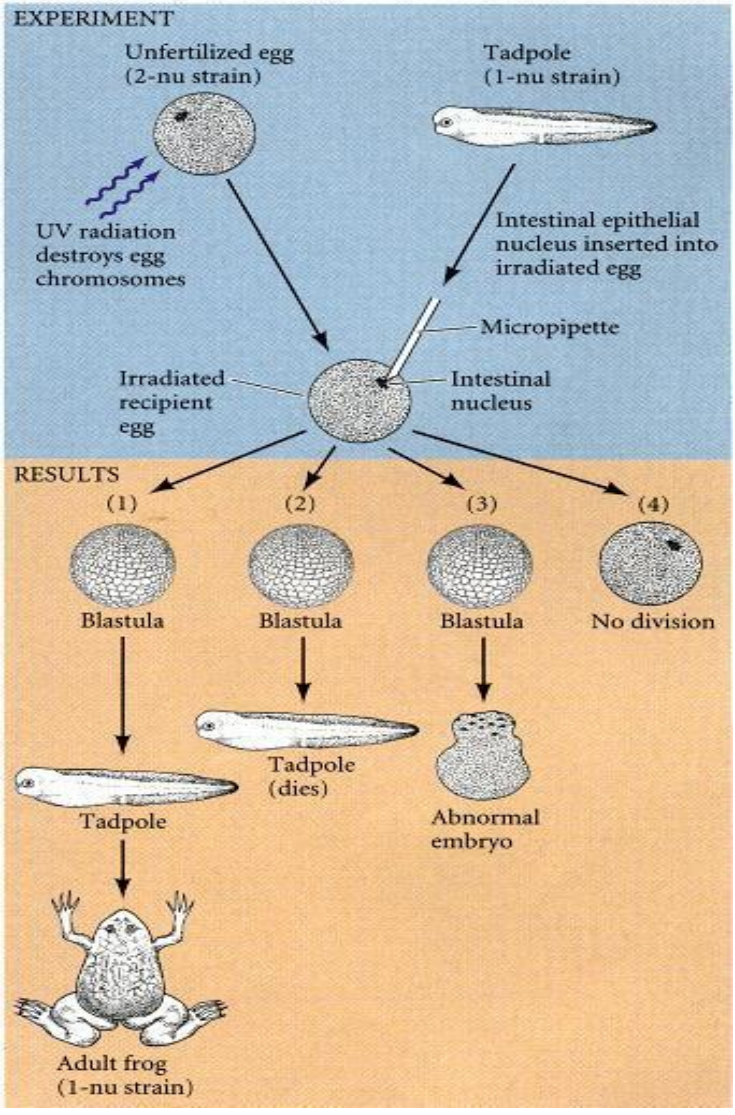
The ultimate test of whether the nucleus of a differentiated cell has undergone any irreversible functional restriction is to have that nucleus generate every other type of differentiated cell in the body. If each cell's nucleus is identical to the zygote nucleus, then each cell's nucleus should be **totipotent** (capable of directing the entire development of the organism) when transplanted into an activated enucleated egg. Before such an experiment could be done, however, three techniques for transplanting nuclei into eggs had to be perfected: (1) a method for enucleating host eggs without destroying them; (2) a method for isolating intact donor nuclei; and (3) a method for transferring such nuclei into the host egg without damaging either the nucleus or the oocyte.

All three techniques were developed in the 1950s by Robert Briggs and Thomas King. First, they combined the enucleation of the host egg with its activation. When an oocyte from the leopard frog (*Rana pipiens*) is pricked with a clean glass needle, the egg undergoes all the cytological and biochemical changes associated with fertilization. The internal cytoplasmic rearrangements of fertilization occur, and the completion of meiosis takes place near the animal pole of the cell. The meiotic spindle can easily be located as it pushes away the pigment granules at the animal pole, and puncturing the oocyte at this site causes the spindle and its chromosomes to flow outside the egg. The host egg is now considered both activated (the fertilization reactions necessary to initiate development have been completed) and enucleated. The transfer of a nucleus into the egg is accomplished by disrupting a donor cell and transferring the released nucleus into the oocyte through a micropipette. Some cytoplasm accompanies the nucleus to its new home, but the ratio of donor to recipient cytoplasm is only $1{:}10^5$, and the donor cytoplasm does not seem to affect the outcome of the experiments. In 1952, Briggs and King, using these techniques, demonstrated that blastula cell nuclei could direct the development of complete tadpoles when transferred into the oocyte cytoplasm.

What happens when nuclei from more advanced developmental stages are transferred into activated enucleated oocytes? King and Briggs found that whereas most blastula nuclei could produce entire tadpoles, there was a dramatic decrease in the ability of nuclei from later stages to direct development to the tadpole stage. When nuclei from the somatic cells of tailbud-stage tadpoles were used as donors, normal development did not occur. However, nuclei from the germ cells of tailbud-stage tadpoles (which could give rise to a complete organism after fertilization) were capable of directing normal development in 40% of the blastulae that developed. Thus, most somatic cells appeared to lose their ability to direct development as they became determined and differentiated.

Amphibian cloning: The pluripotency of somatic cells

Is it possible that some differentiatied cell nuclei differ from others in their ability to direct development? John Gurdon and his colleagues, using slightly different methods of nuclear transplantation on the frog *Xenopus,* obtained results suggesting that the nuclei of some differentiated cells can remain totipotent. Gurdon, too, found a progressive loss of potency with increasing developmental age, although *Xenopus* cells retained their potencies for a longer period than did the cells of *Rana*. The exceptions to this rule, however, proved very interesting. Gurdon transferred nuclei from the intestinal endoderm of feeding *Xenopus* tadpoles into activated enucleated eggs. These donor nuclei contained a genetic marker (one nucleolus per cell instead of the usual two) that distinguished them from host nuclei. Out of 726 nuclei transferred, only 10 (1.4%) promoted development to the feeding tadpole stage. Serial transplantation (transplanting an intestinal nucleus into an egg and, when the egg had become a blastula, transferring the nuclei of the blastula cells into several more eggs) increased the yield to 7% . In some instances, nuclei from tadpole intestinal cells



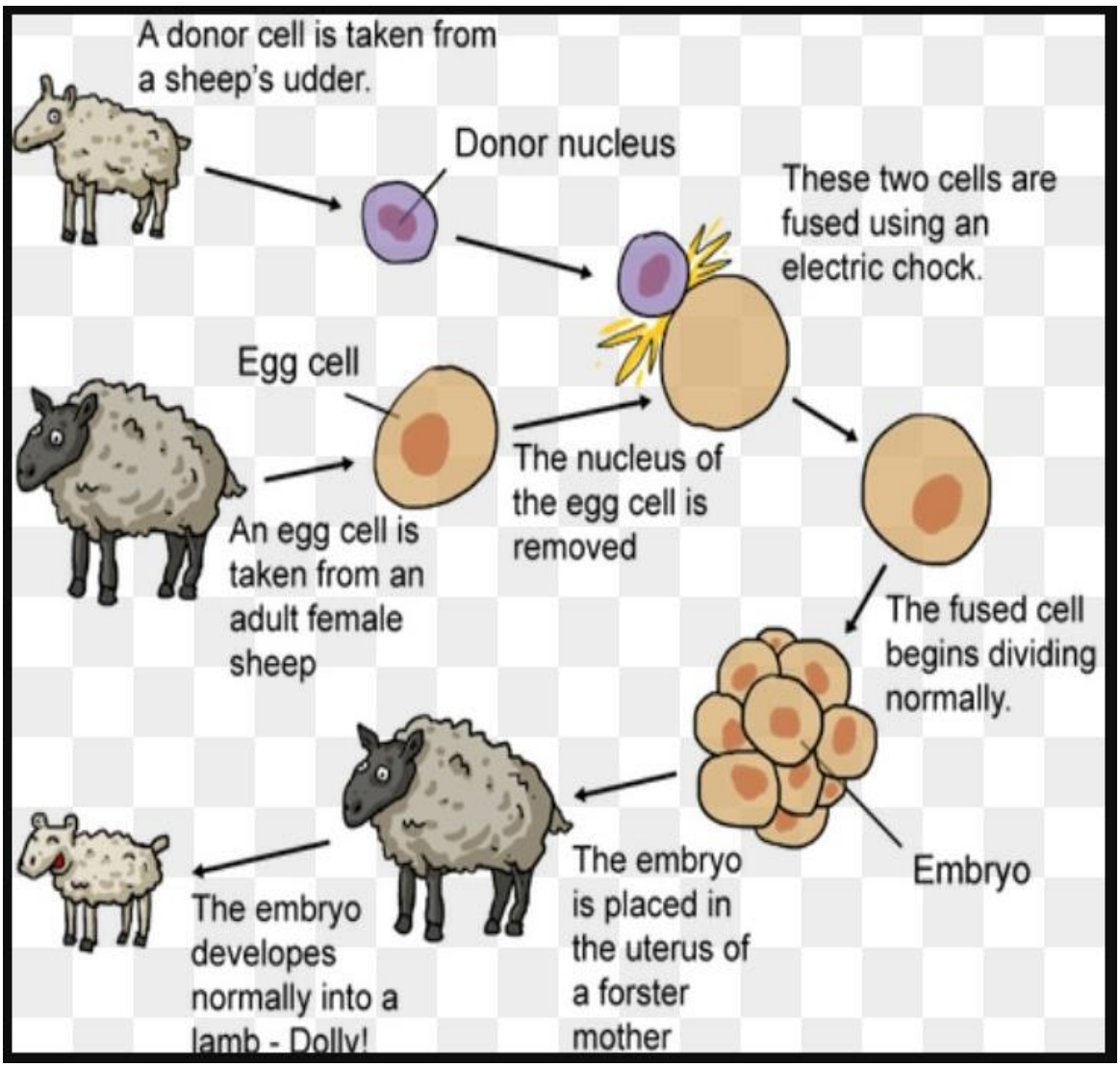
were capable of generating all the cell lineages—neurons, blood cells, nerves, and so forth—of a living tadpole. Moreover, seven of these tadpoles (from two original nuclei) metamorphosed into fertile adult frogs; these two nuclei were totipotent.

Procedure used to obtain mature frogs from the intestinal nuclei of *Xenopus* tadpoles. The wild-type egg (with two nucleoli per nucleus; 2-nu) is irradiated to destroy the maternal chromosomes, and an intestinal nucleus from a marked (1-nu) tadpole is inserted. In some cases, there is no cell division; in some cases, the embryo is arrested in development; but in other cases, an entire new frog, with a 1-nu genotype, is formed. (After Gurdon 1968,Gurdon 1977.)

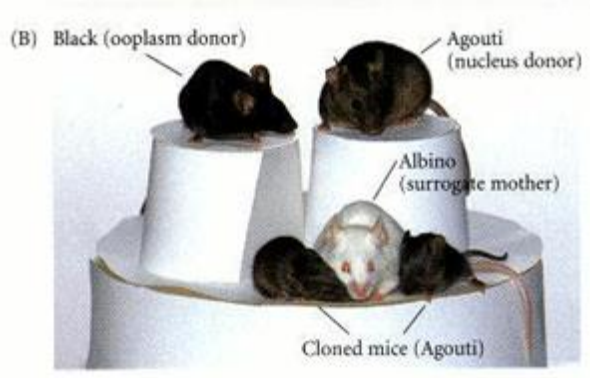
King and his colleagues criticized these experiments, pointing out that (1) not enough care was taken to make certain that primordial germ cells, which can migrate through to the gut, were not used as sources of nuclei, and (2) the intestinal epithelial cells of such a young tadpole may not qualify as a truly differentiated cell type because such cells of feeding tadpoles still contain yolk platelets. To answer these criticisms, Gurdon and his colleagues cultured epithelial cells from adult frog foot webbing. These cells were shown to be differentiated; each of them contained a specific keratin, the characteristic protein of adult skin cells. When nuclei from these cells were transferred into activated, enucleated *Xenopus* oocytes, none of the first-generation transfers progressed further than the formation of the neural tube shortly after gastrulation. By serial transplantation, however, numerous tadpoles were generated. Although these tadpoles all died prior to feeding, they showed that a single differentiated cell nucleus still retained incredible potencies.

Cloning of mammals

1997, Ian wilmut announced that a sheep had been cloned from a somatic cell nucleus from an adult female sheep. This was the first time that an adult vertebrate had been successfully cloned from another adult. He took cells from the mammary gland of an adult (6 year old) pregnant ewe and put them into the culture. Culture medium was formulated to keep the nuclei in these cells at the resting stage of the cell cycle (G_o). They then obtained oocytes (the maturing egg cell) from a different strain of sheep and removed their nuclei. The fusion of the donor cell and the enucleated oocyte was accomplished by bringing the two cells together and sending electrical pulses through them. The electric pulses destabilized the cell membrane, allowing the cells to fuse together. The resulting embryos were eventually transferred into the uteri of pregnant sheep. Of the 434 sheep oocytes originally used in this experiment, only one survived, named- Dolly. Thus, it appears that the nuclei of adult somatic cells can be totipotent. This result has been confirmed in cows and mice. In mice, somatic cell nuclei from the cumulus cells of the ovary were injected directly into enucleated oocytes. These re-nucleated oocytes were able to develop into mice at a frequency of 2.5%. Interestingly, nuclei from other somatic cells (such as neurons or Sertoli cells) that are similarly blocked at the G_0 stage did not generate any live mice. Cumulus cell nuclei from cows have also directed the complete development of oocytes into mature cows.



Cloned mammals, whose nuclei came from adult somatic cells. (A) Dolly, the adult sheep on the left, was derived by fusing a mammary gland cell nucleus with an enucleated oocyte, which was then implanted in a surrogate mother (of a different breed of sheep) who gave birth to Dolly. Dolly has since produced a lamb (Bonnie, at right) by normal reproduction.



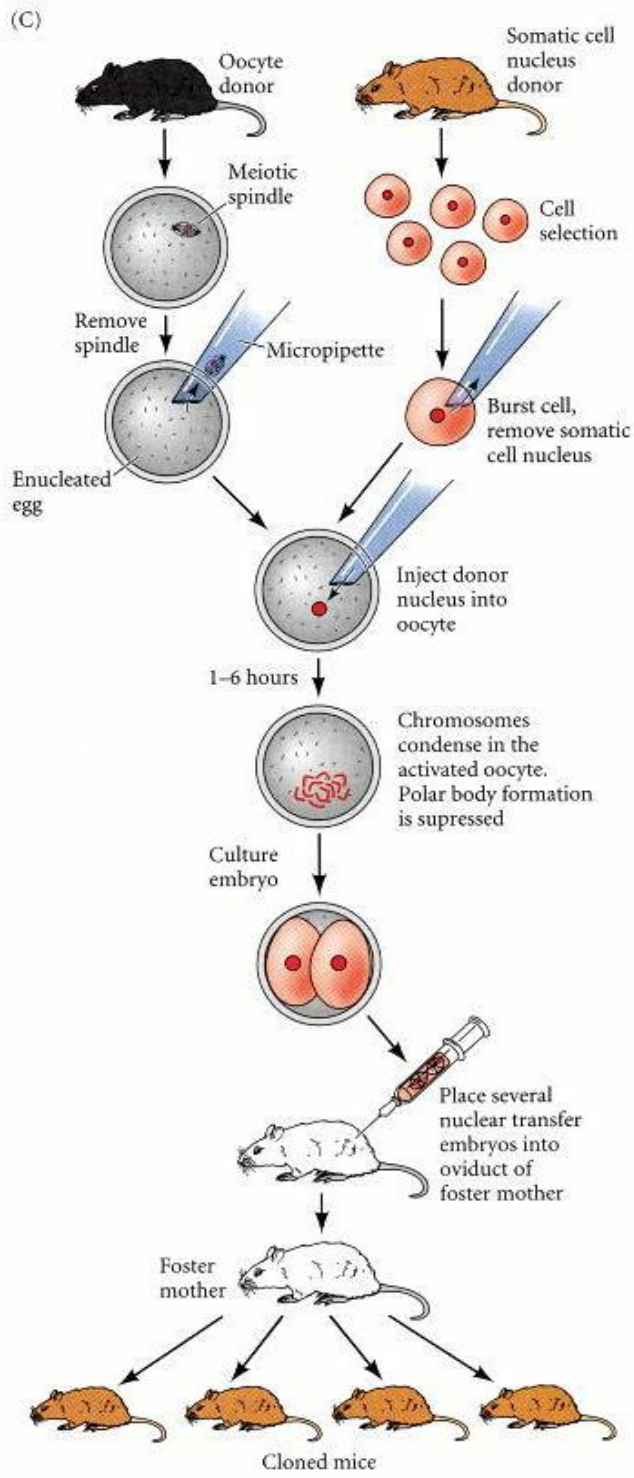


Figure
(B) Cloned mice and their "parents." The upper left black mouse is the oocyte donor, while the upper right brown (agouti) mouse is the nucleus donor. The white mouse in the lower row is the mouse into whose uterus the resulting embryos were implanted. The two agouti mice beside her are cloned mice, derived from the injection of the agouti nucleus into the oocyte of the black-furred parent (C) Procedure used for cloning mice. (A photograph by Roddy Field, © Roslin Institute; B courtesy of T. Wakayama and R. Yanagimachi.)

Reference:

Developmental Biology. 6th edition.

Gilbert SF. Sunderland (MA): Sinauer Associates; 2000.

CYTOPLASMIC DETERMINANTS

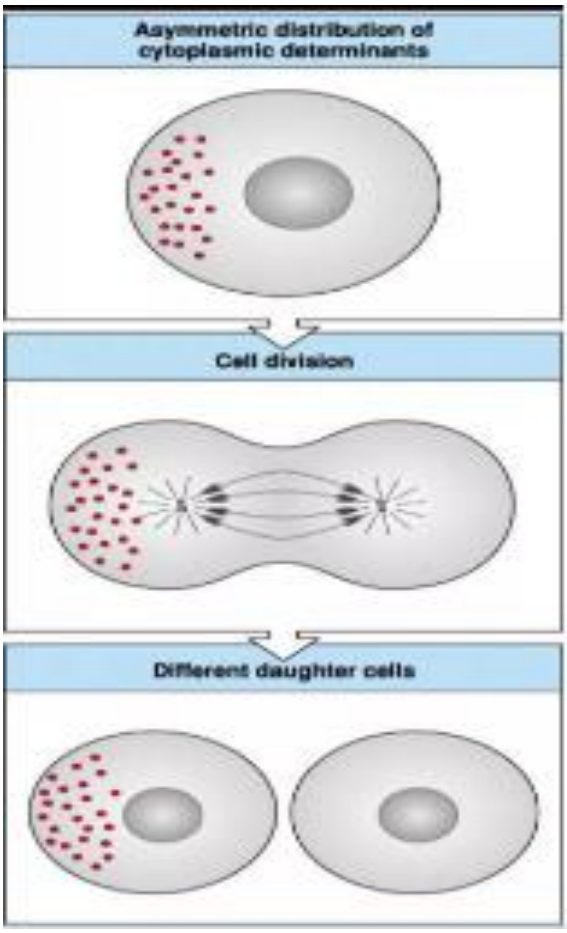
Cytoplasmic determinants are special molecules which play a very important role during oocyte maturation, in the female's ovary. During this period of time, some regions of the cytoplasm accumulate some of these cytoplasmic determinants, whose distribution is thus very heterogenic. They play a major role in the development of the embryo's organs. Each type of cell is determined by a particular determinant or group of determinants. Thus, all the organs of the future embryo are distributed and operating well thanks to the right position of the cytoplasmic determinants. The action of the determinants on the blastomeres is one of the most important ones. During the segmentation, cytoplasmic determinants are distributed among the blastomeres, at different times depending on the species and on the type of determinant. Therefore, the daughter cells resulting from the first divisions are totipotent : they can, independently, lead to a complete individual. That is not possible after the cytoplasmic determinants have been distributed in the differentiated blastomeres.

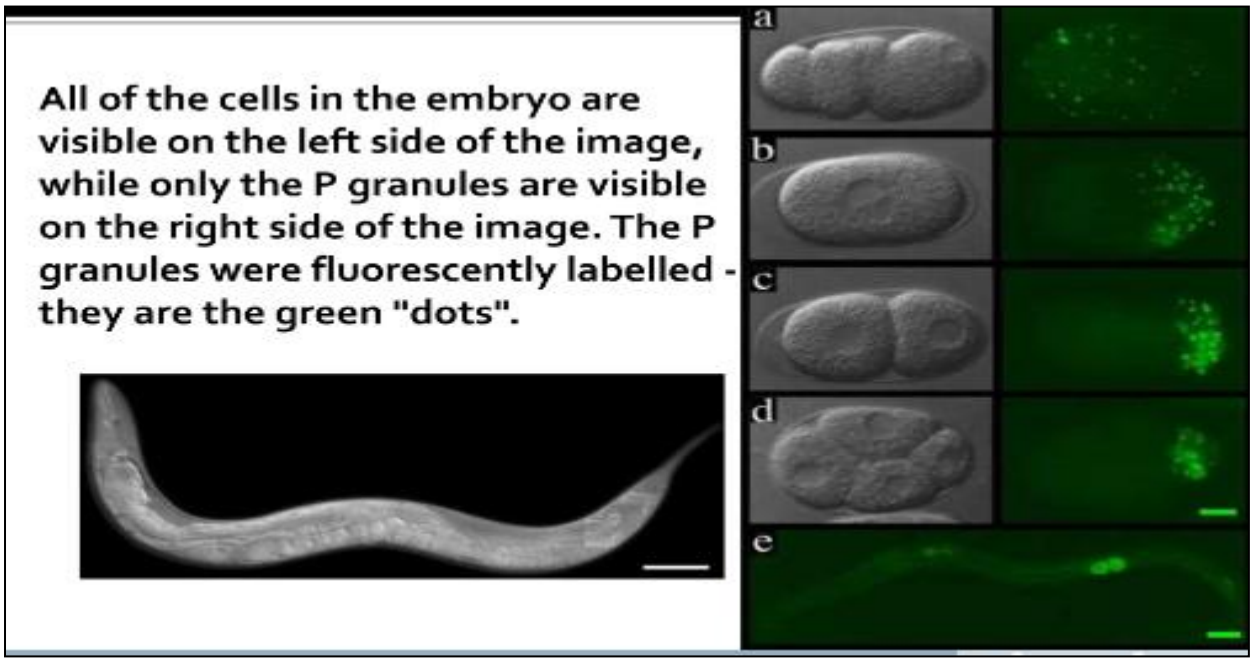
Major Steps:

i. The asymmetric segregation of cytoplasmic determinants is due to asymmetric localization of molecules (usually proteins or mRNA) within a cell before it divides. ii. During cell division one daughter cell receives most or all of the localized molecules, while other daughter cells receives less (or none) of these molecules.

iii. This results in two different daughter cells, which then takes on different cell fates based on differences in gene expression

iv. The localized cytoplasmic determinants are often RNAs encoding transcription factors, or the transcription factors themselves.





Early *Caenorhabditis elegans* embryo

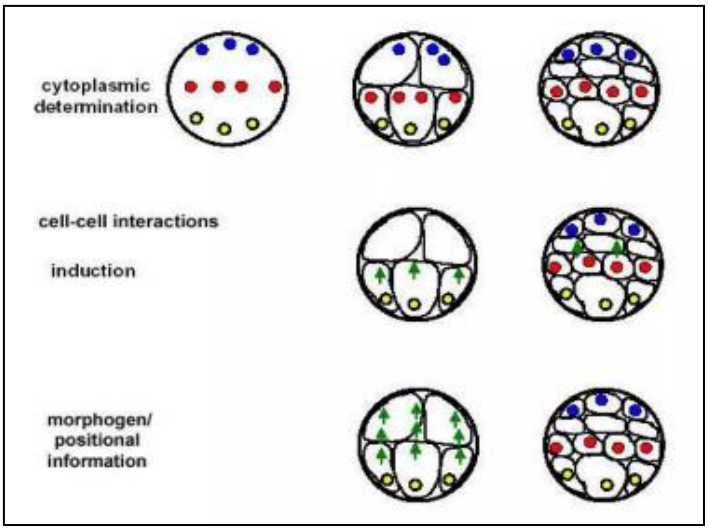
a. A newly fertilized embryo with dispersed **P granules**

b. P granules are localized in the posterior end of the embryo

c. After the first division the **P granules** are only present in the smaller posterior cell

d. Another unequal division give rise to single cell containing **P granules**

e. When the larvae hatches **P granules** are localized in the primordial germ cells



Mosaic development

During the mosaic development, the future embryo contains all the distinct cytoplasmic determinants that are distributed in distinct cells. Regions of the organism differentiate very quickly if each cell contains specific cytoplasmic determinants since the first divisions : then the cell divides to give all the other cell of its type, and the same process happens in all types of cells in the organism. As a result, in the case of the mosaic development, cell totipotence disappears very quickly during segmentation. Indeed, each new created cell determines a new region of the future organism, and it is independent from the other ones : t

hus development is independent from interaction between cells. It is most of all known in certain animals as nematodes C. elegans, or ascidians (marine animals).

Regulation development

Other animals show regulation development; their cells show totipotence for a longer time. In these animals, cytoplasmic determinants are more rapidly distributed between newly created cells. Mammals are an example of animals that undergo regulation development.

Probable questions:

1. Discuss the role of genes in development.
2. What is genetic equivalence in developmental biology?
3. What is Metaplasia? How it is related to genetic equivalence?
4. What are cytoplasmic determinants?
5. Discuss the Role of Cytoplasmic determinants in embryology.
6. Discuss the role of Cytoplasmic determinants in Cell divisions.

Suggested reading:

1. Gilbert S.F. 2010. Developmental Biology, IX Edition, Sinauer Associates, Inc., Publishers,
2. Das N. 2012. Fundamental Concept of Developmental Biology. New Central Book Agency

UNIT II

Imprinting and Cloning of animals

Objective:

In this unit we will learn about Imprinting and Cloning of animals.

- ## Genomic imprinting

Genomic imprinting, process wherein a gene is differentially expressed depending on whether it has been inherited from the mother or from the father. Such "parent-of-origin" effects are known to occur only in sexually reproducing placental mammals. Imprinting is one of a number of patterns of inheritance that do not obey the traditional Mendelian rules of inheritance, which assume indifference about the parental origin of an allele (an allele is any one of two or more genes that may occur alternatively at a given site on a chromosome). Traits are therefore able to be passed down maternal or paternal lines.

- ## Imprinting mechanisms

Imprinting can occur when one of the gene's parental alleles is silenced throughout the embryonic development of the individual by an alteration in parental DNA made during parental gametogenesis (the formation of gametes, or sperm in males and eggs in females). The other parental allele is therefore allowed expression during embryonic development. A mechanism by which this occurs is DNA methylation (the addition of a CH_3, or methyl, group to specific regions of DNA) at imprinting control regions (ICRs). Intracellular DNA-reading mechanisms exist after fertilization to check that the correct parental allele has been allowed differential expression.

- ## Imprinting and fetal development

Imprinting has been able to explain certain predicaments of life in utero. A number of imprinted genes are related to embryonic and fetal growth and thus the extraction of resources from the uterine environment for growth. Mother and father, however, have different interests in how resources are extracted, because of asymmetrical parental investment in each given child. This arises from the fact that mothers can only have one child every nine months for approximately 20 years, whereas a father could conceivably impregnate many different women from puberty until death.

Systematic knockout (inactivation) studies of key imprinted genes, especially as performed on mice, have provided support for the hypothesis that imprinted genes that allow expression of paternally inherited alleles tend to drive more extraction of nutrients from the mother during gestation and after birth to produce a larger child. In contrast, imprinted genes that allow expression of maternally inherited alleles will tend

to drive mechanisms to prevent the disproportionate utilization of resources by the fetus. A commonly cited example of this differential resource transfer in mice is the paternally expressed gene *Igf2* (insulin-like growth factor 2), which enhances fetal growth and placental nutrient transport capacity, and the maternally expressed *Igf2* receptor (*Igf2r*), which degrades excessive Igf2 protein.

Many of the effects of imprinted genes occur in the placenta, a crucial site for resource and nutrient transfer. For example, an overgrown placenta (hydatidiform mole) results when maternal imprints are missing. Additionally, in Silver-Russell syndrome (or Russell-Silver syndrome), a maternal uniparental disomy (both copies of a chromosome or partial chromosome are inherited from one parent), growth restriction is present. Similar effects are found in other cases of disordered imprinting. Preeclampsia, for example, in which disordered imprinting has been implicated, also demonstrates growth restriction in utero. Many of these diseases can be understood only within the context of imprinting as a common mechanism of parental conflict and manipulation of the phenotypic outcome of children.

- **Imprinting and cognition and behaviour after birth**

Although only approximately 100 human genes are known to be subject to parent-of-origin effects, these prove to have tremendous implications for the development and eventual adult attributes of people, including cognitive and behavioral attributes. Imprinting effects, similar to other genetic expression patterns, may manifest as earlier age of onset effects or changes in severity.

In Turner syndrome, which affects females and is characterized by the deletion of one X chromosome (females normally carry two X chromosomes), a parent-of-origin effect may influence social and intellectual functioning. The affected female could have received her only X chromosome from her father (Xp) or her mother (Xm). Studies have suggested that neurodevelopment in Xp Turner syndrome patients is similar to that of healthy females, whereas neurodevelopment in Xm patients is altered. Other studies, however, have yielded conflicting results.

Cloning of animals

- **What is Animal Cloning?**

Modern biotechnology revolutionized the world of science and medicine. One biotechnological technique in particular which remains a topic of controversy is **animal cloning**. Cloning refers to the process of creating an identical genetic copy of one organism. Cloning can be done on genes, single-celled organisms, and even entire animals. Clones also occur naturally, as is the case for identical twins; identical twins

originate from the same embryo and thus contain the same genes. Other instances of natural cloning include bacteria that reproduce by making exact copies of themselves. Scientists also clone single genes by inserting them into single-celled organisms such as bacteria and yeast. Each time these organisms reproduce, the gene is cloned and replicated.

The first recorded successful attempt at animal cloning gave birth to Dolly the Sheep in 1996. Dolly was cloned from another sheep and lived for six years. Dolly was genetically identical to its mother sheep, which would not have been the case if it was as a result of sexual reproduction. Sexual reproduction involves the fusion of haploid gametes from the father and the mother to result in a genetically different and unique offspring; on the other hand, cloning would result in an identical genetic copy of the parent.

- ## Animal Cloning:

Animal cloning is more difficult than plant cloning because animal cells lose their totipotency on reaching the gastrula stage of animal development. However, animal tissue cultures from tumours and embryonic tissue cells have been successful. Standard techniques are available for isolating animal cells and tissues from different systems. Some more important examples of animal cloning are tissue culture, somatic cell fusion, cell cloning and creating transgenics.

- ## Animal Cloning Process

The animal cloning process can occur via two separate methods: **artificial twinning** or **somatic cell nuclear transfer**. The process of artificial twinning is done in many labs in order to induce the birth of identical animal twins. The embryo to be transferred into the mother's womb is split into two, and each developing embryo is allowed to develop to form its own organisms. The resulting offspring would then form identical twins. Twinning can be used to increase livestock and produce more offspring than normal. The other process, somatic cell nuclear transfer, is more intricate and involves several steps that include:

- Enucleation: The host egg is enucleated. Its DNA is removed, and its genetic information is lost.
- Nucleus transfer: The nucleus of the animal to be cloned is removed.
- Insertion: The nucleus of the animal to be cloned is inserted into the enucleated host egg to produce a zygote.
- Stimulation: The zygote is stimulated to divide via electric current.
- Embryo transfer: The zygote is then transplanted into the surrogate mother's uterus.

- **Applications of Animal Cloning and Genetic Engineering:**

Examples of animal products (medicines) produced through genetic engineering are:

(i) Chick embryo fluid which produces vaccines for influenza, measles and mumps,

(ii) Duck embryo fluid which produces vaccines for rabies and rubella.

- **How Sheep 'Dolly' Cloned?**

Ian Wilmut and his associates at the Roslin Research Institute, Scotland, took cells from ewe (mother sheep's udder). An udder cell is different from a skin cell or a muscle cell or a nerve cell.

They managed to store these udder cells in nutrient deprived culture. This checked the starved cells from dividing, and switched off their active genes.

Now, one udder cell complete with its nucleus was selected, as this nucleus carries the mother's genetic information.

Meanwhile, unfertilized egg cell was taken from a different ewe (host mother sheep). Its nucleus was sucked out leaving an empty cell containing all the necessary components to produce an embryo. This cell was now ready to receive udder cell nucleus.

They now fused udder cell nucleus with the empty egg cell by electrical stimulation. Then this egg cell had the mother's nucleus.

When a normal or altered egg is implanted in a different female is termed 'surrogate mother'. This means, substitute mother.

Then the altered egg was cultured for six days. Out of many resulting embryos, one was implanted in the uterus of the surrogate mother, where it grew into a lamb.

Thus, Dolly was born genetically identical to mother sheep as her first cell nucleus came from mother's cell.

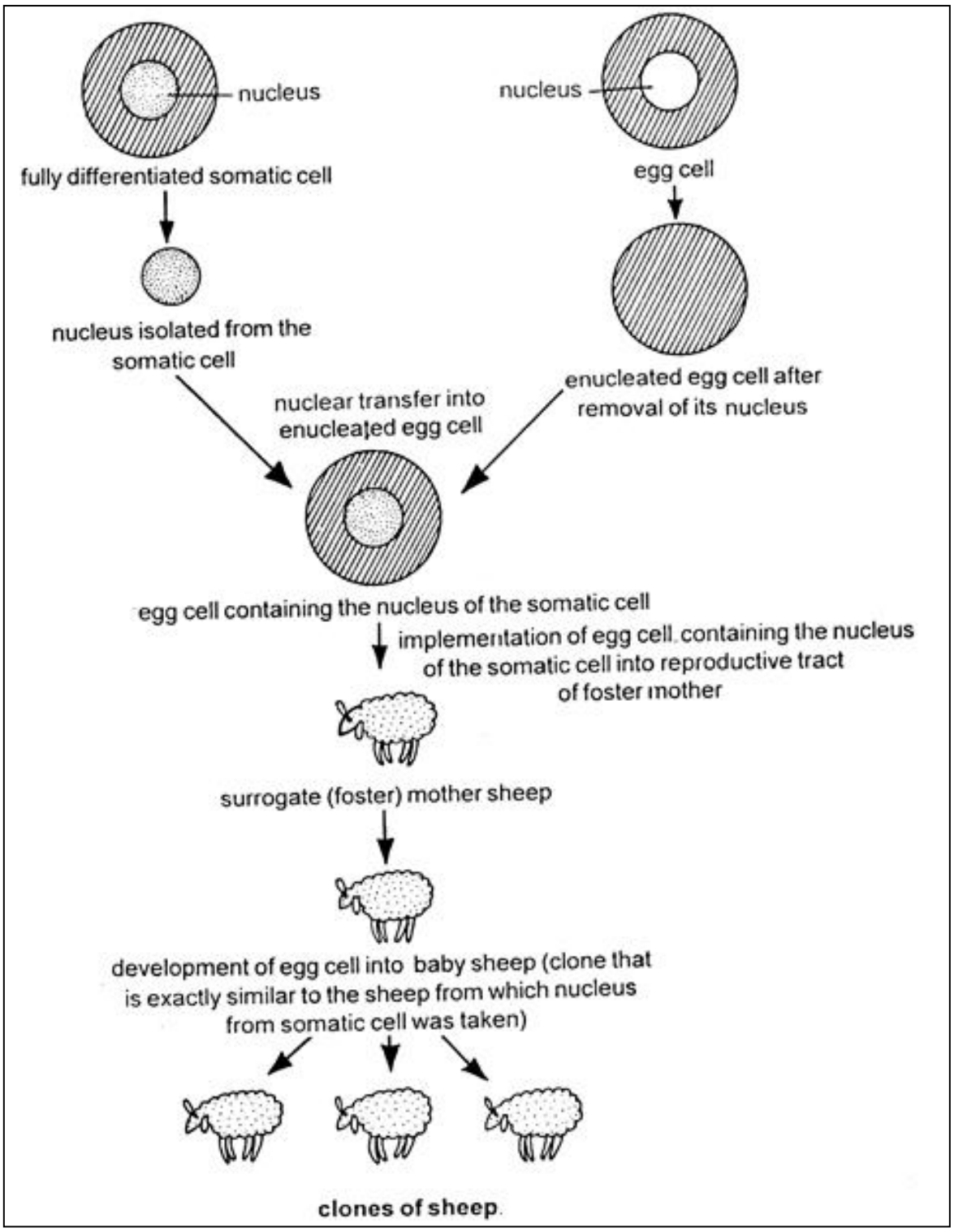


Fig: Steps involved in the cloning of the sheep Dolly

- **First Human Clone:**

The birth of the first human clone (December 26, 2002), a baby girl called Eve by scientists, was announced by Brigitte Boisselier, head of a company named Clonaid. According to Boisselier, the child is an exact genetic duplicate of her mother.

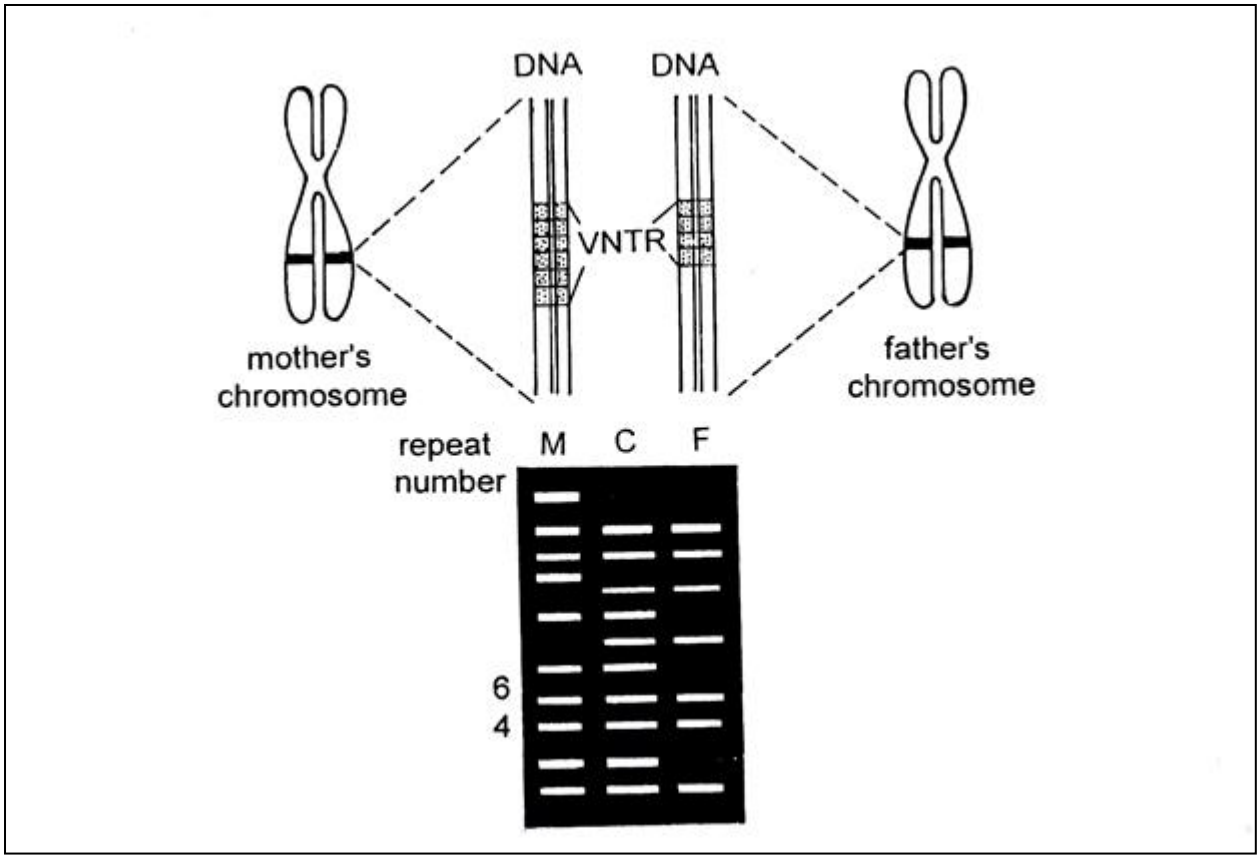


Fig: Variable Number Tandem Repeats (VNTRs). M= mother, C = Child, F = Father

To clone, scientists slip the nucleus of an adult cell, like a skin cell, into an unfertilized egg from which its own genetic material has been removed.

Then, if stimulated to act like a fertilized egg, the newly altered genetic material can then direct the egg to divide and grow into an embryo, then a foetus, then a newborn, if all goes well. Yet, as scientists have discovered, only rarely does all go well.

In animal work so far only about 1 to 5 per cent of cloning attempts succeed, said Randall Prather, a cloning expert. That is, for every 100 eggs, one to five clones are born.

- **Will clones look and behave exactly the same?**

Even if clones are genetically identical with one another, they will not be identical in physical or behavioral characteristics, because DNA is not the only determinant of these characteristics. A pair of clones will experience different environments and nutritional inputs while in the uterus, and they would be expected to be subject to different inputs

from their parents, society, and life experience as they grow up. If clones derived from identical nuclear donors and identical mitocondrial donors are born at different times, as is the case when an adult is the donor of the somatic cell nucleus, the environmental and nutritional differences would be expected to be more pronounced than for monozygotic (identical) twins. And even monozygotic twins are not fully identical genetically or epigenetically because mutations, stochastic developmental variations, and varied imprinting effects (parent-specific chemical marks on the DNA) make different contributions to each twin (SIMON and John, 1999)

Additional differences may occur in clones that do not have identical mitochondria. Such clones arise if one individual contributes the nucleus and another the egg—or if nuclei from a single individual are transferred to eggs from multiple donors. The differences might be expected to show up in parts of the body that have high demands for energy—such as muscle, heart, eye, and brain—or in body systems that use mitochondrial control over cell death to determine cell numbers (Finnila et al., 2001; Mccreath et al., 2000).

- **Animal Cloning Pros and Cons**

Just like any other scientific advancement, animal cloning has a list of pros and cons. While it certainly may help advance research and medicine, it is still a topic of controversy.

Benefits of Cloning Animals

The benefits of cloning animals can be seen in a variety of fields, such as medicine and agriculture. These benefits include:

1. **Cloning animals would allow us to balance environmental habitats.**

Our planet is flexible in its ability to sustain like, but we are learning that our ecosystems have less elasticity. If an animal becomes extinct or disappears because their habitat does not support life in needed ways, then the outcomes in the local region can be very dramatic. Yellowstone National Park saw a more significant erosion of their riverbeds when wolf populations started declining, and that is one example of many.

Cloning could encourage us to restore this equilibrium by replacing or even reintroducing cloned animals that are either extinct or endangered.

2. **Cloning animals would create more security in the global food supply.**

There will be somewhere between 9 billion to 10 billion people living on our planet by the year 2050 if current population trends continue. That figure could double in the century after. It is up to us to figure out how to solve that hunger crisis today so that tomorrow's generations can flourish. Cloning animals is a reasonable approach that could help to stabilize our supply of animal-based proteins.

Although this advantage won't solve hunger by itself, a higher level of food availability will reduce conflicts, encourage innovation, and push scientists toward the innovative results that are likely necessary for the future.

3. Cloning animals could advance scientific discoveries in other fields.

The scientific processes that allow us to clone animals could be useful in the duplication of specific cells found throughout the body. We could potentially take the techniques discovered in this field to produce new tissues or organs as needed. It wouldn't place the life of the animal in danger, and the information gained from these processes could create new breakthroughs in the area of human medical science. We could evaluate the cloned cells to determine the usefulness of each process to determine how progression can occur in these fields.

4. Cloning animals could help pet parents find greater comfort.

Losing a pet hurts as much as it does because these animals often become family members. The presence of cats, dogs, and others add structure to our daily routines. These companions help to keep us active, and they can even support us in our efforts to overcome the roadblocks that happen in life.

Pets give us purpose. The processes of cloning animals would allow people to protect their memories of a beloved companion with an identical animal created by scientists. This effort would still create a unique animal for the species, but it would also offer some continuity and support that could lessen emotional reactions.

5. Cloning animals does not create exact duplication.

When we clone animals, there is not an exact duplication that occurs. The genetic material gets put into an embryo that contains unique cells. These processes then make it possible for the clone to produce offspring later in life. The work that scientists performed with Dolly the Sheep created six lambs in total as she was bred with a Welsh Mountain ram. The first was named Bonnie, and she was born in 1998.

Then Dolly had twins that her caregivers called Rosie and Sally. In the fall of 2001, she would have triplets that were called Cotton, Darcy, and Lucy. Those offspring were not sterile as some researchers predicted, showing the full potential of what this scientific process could create.

6. Cloning animals allows us to preserve endangered species.

Researchers were able to save the Przewalski's horse only because 13 horses were captured from a wild herd and kept in a zoo in the 1940s. Even then, two of the horses were hybrids. Standard breeding practices helped to save the species, which now counts in the thousands of individuals.

We won't have the same kind of luck with the Northern White Rhino. The last known male of the species died in March 2018. With only two females of the same subspecies currently living, the only way to save them is through the cloning process. This work

gives us the ability to preserve endangered animals – and maybe even bring extinct ones back into our world.

7. Cloning animals gives us the opportunity to produce the most desirable traits.

When scientists work on animal cloning, they are doing what others have done through selective breeding for more than 1,000 years. The outcomes are similar to what we can achieve through natural reproductive processes that include human interference. This work is a chance to create precise, desired traits in animals.

We might use animal cloning to produce dairy cows that offer more milk. Researchers could look at the idea of cloning specific hens as a way to improve commercial egg production. Livestock animals could be bred through this process to produce more meat per carcass. The applications are almost limitless when we look at the full potential of this technology.

8. Cloning animals could help us to reduce human disease.

One of the most problematic illnesses that humans battle each season is influenza. Flu epidemics have killed millions of people in the past, especially when a new strain of the virus begins to circulate through the population. About 1 in 5 people will get sick with it each year, even with the availability of annual vaccines to prevent it.

The flu virus originates in birds, pigs, and other animal species. Our animal cloning processes could work to stop its development by creating more resiliency against its activity when it forms. It's an opportunity to stop the adverse impacts of disease before it even gets a chance to begin.

9. Cloning animals would not impact the quality of the food supply.

Rulings in 2008 from research at the time found that it is safe for people to consume animal products from cloned species. The FDA ruled that any livestock species can enter the commercial food chain. That means scientists can look for ways to improve the nutrient profile of the proteins we consume so that everyone has a chance to eat healthier without changing their habits. Think of this advantage as the beef version of working with enriched flour.

Disadvantages of Cloning Animals

Disadvantages of cloning are also a topic of discussion. Ethical and scientific concerns can be found when conducting animal cloning, such as:

1. Cloning animals is expensive.

If you have a prized bull that you want to clone, then the cost will be somewhere in the neighborhood of $20,000. That's the cheapest price you'll currently find from reputable providers of this service. You could create a duplicate of your cat for about $25,000, but replicating the beloved family dog is going to be double that rate.

Scientists have found ways to clone horses as well, but you'll pay just as much for a clone (if not more) than you would for a championship line Thoroughbred. Wealthy individuals and corporations might benefit from this science today, but it isn't in the price range for the average family.

2. Cloning animals reduces the genetic diversity of that species. When animals come from the same genetic profile, then a reduction in diversity occurs at the genetic level. This process may not impact the overall health of the species at first, but it can cause long-term problems if it were to continue. The likelihood of genetic disease and other health issues rises when parents with similar genetics have offspring.

When there isn't genetic variation, then a population loses the ability to respond to changing environmental variables. Even though cloning is meant to save a species, this disadvantage can lead them closer to extinction or population decline.

3. Cloning animals would eventually slow the rate of reproduction. Animals that have the highest levels of genetic similarity tend to have the lowest rate of reproduction. We already see this disadvantage taking place with cheetahs, who as a species shares 99% of their genome with other individuals. Male cats have low sperm production rates, and there is little genetic variety passed along when mating is successful. Severe congenital disabilities occur frequently with cubs.

Cloning animals would produce a similar outcome. Breeding programs with cheetahs are already unsuccessful, so it is not unreasonable to think a similar problem would occur with other species.

4. Cloning animals has a history of creating abnormal pregnancies. Almost half (45%) of the pregnancies that animal cloning techniques produce fail by the third trimester. When compared to the experiences from natural reproduction methods, the results are more significant and place the mother's life in more danger. Even when a pregnancy comes to term using a cloned embryo, it is more common for a C-section to be necessary. Dystocia and development abnormalities also appear more often, creating a higher risk of losing the mother during the birthing process.

5. Cloning animals can create long-term health issues to manage. Although advancements in animal cloning are reducing this risk, the older methods of reprogramming cells didn't always reset the biological clock. Dolly the Sheep had shorter telomeres than others of her species, and this genetic trait may have been a contributing factor to her living six years instead of the expected nine.

Cloned animals can experience life-threatening health issues at any time because the cell programming can alter their genetic profile. This process can reduce reproductive capabilities in some species, and some outcomes don't have a known cause.

6. Cloning animals could result in sterility issues. This disadvantage is another issue that is coming to a resolution in the near future, but it still impacts the current generation of animal clones. When scientists create a clone, then there is a higher risk that the animal will not have the capability of producing

offspring. If that impairment doesn't exist, the risk factors stay higher than they do with natural reproduction for the next generation.

In the cases when offspring can be born from reproductive efforts, the risks of congenital disability stay high until the second generation – what we would call "grandchildren." We also have no long-term data about how family lines could be impacted by cloning processes.

7. Cloning animals could result in unforeseen consequences. Restoring life to our planet seems like a worthy goal on paper, but it could produce more harm than good in some situations. Extinction from centuries ago altered the natural habitats so that the world could adjust to the change. Introducing wooly mammoths back into society could create massive problems with animal management in the wild. Even small introductions, like the dodo bird, could create problems with homeostasis.

We have no way to know how modern diseases would impact the ancient creatures we might try to revive through cloning. It could cause viruses to mutate, introduce new pathogens, or expose people to harmful bacteria.

8. Cloning animals could eventually lead to cloning humans. There is no supporting scientific evidence to suggest that a cloned human embryo exists, although genetic alteration reports have come from China in 2019. Cloning humans is more complicated than it is for other mammals because of the location of spindle proteins on the chromosomes.

Removing the nucleus removes these proteins. That process causes interference with cell division. As our technologies improve, the science that leads to better animal cloning could lead to improved human cloning techniques. Then we would need to start answering the deep philosophical questions that come when taking such an action.

9. Cloning animals could result in more cancer-related issues. The activity of stem cells is similar to the behaviors of many cancers. Both have the ability to continue dividing almost indefinitely. When we look at the idea of cloning, we know that there is a limit of about 60 division cycles before detrimental mutations can start forming. That means our ability to clone animals is restricted to a specific number before the odds start shrinking even more. If that information translates to humans, then there could be limitations to the number of feasible medical treatments using this technology.

10. Cloning animals leads to higher levels of embryo destruction. Scientists created Dolly the Sheep by implanting almost 300 cloned embryos over time. Despite all of that work, only 13 total pregnancies occurred. Less than 20% of nuclear transfers that involve somatic cells will develop into an embryo, and only half of them that reach this stage are of a high enough quality to qualify for implantation.

Even if animal cloning is only useful for therapeutic purposes, the destruction of the embryo would be necessary. That means we are destroying the potentiality of life in favor of what currently exists.

- **What are some of the ethical issues related to cloning?**

Gene cloning is a carefully regulated technique that is largely accepted today and used routinely in many labs worldwide. However, both reproductive and therapeutic cloning raise important ethical issues, especially as related to the potential use of these techniques in humans.

Reproductive cloning would present the potential of creating a human that is genetically identical to another person who has previously existed or who still exists. This may conflict with long-standing religious and societal values about human dignity, possibly infringing upon principles of individual freedom, identity and autonomy. However, some argue that reproductive cloning could help sterile couples fulfill their dream of parenthood. Others see human cloning as a way to avoid passing on a deleterious gene that runs in the family without having to undergo embryo screening or embryo selection.

Therapeutic cloning, while offering the potential for treating humans suffering from disease or injury, would require the destruction of human embryos in the test tube. Consequently, opponents argue that using this technique to collect embryonic stem cells is wrong, regardless of whether such cells are used to benefit sick or injured people.

Probable questions:

1. What is Genomic imprinting?
2. What is the relation between imprinting and fetal development
3. What is Animal Cloning?
4. Describe the process of animal cloning.
5. Describe the process how Sheep 'Dolly' was cloned?
6. What are the benefits of cloning animals?
7. Discuss about the disadvantages of animal cloning.
8. Discuss about the ethical issues related to cloning?

Suggested reading:

1. https://www.britannica.com/science/genomic-imprinting
2. HALL JG. Genomic imprinting: nature and clinical relevance**.** *Annu Rev Med* 1997, 48: 35-44.
3. SIMON DK, JOHNS DR. Mitochondrial disorders: clinical and genetic features**.** *Annu Rev Med* 1999, 50: 111-27.
4. FINNILA S, AUTERE J, LEHTOVIRTA M, HARTIKAINEN P, MANNERMAA A, SOININEN H, MAJAMAA K. Increased risk of sensorineural hearing loss and migraine in patients with a rare mitochondrial DNA variant 4336A>G in tRNAG ln . *J Med Genet* 2001 Jun, 38(6): 400-5.
5. MCCREATH KJ, HOWCROFT J, CAMPBELL KH, COLMAN A, SCHNIEKE AE, KIND AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells**.** *Nature* 2000 Jun 29, 405(6790): 1066-9.

UNIT III

Techniques for the study of development: i) Cell labeling and tagging ii) Cell sorting

Objective:

In this unit we will discuss about techniques for the study of development like i) Cell labeling and tagging ii) Cell sorting.

Cell labeling

The visualization of cells and cellular structures as well as the tracking and modulation of nucleic acid and proteins in living cells is mandatory to identify, map and analyze the underlying biochemical processes of cell signaling.

Fluorescent-labeled chemical reporters allow the direct detection of a specific target however, only a fraction of them is cell-permeable. The lack of cell-permeability can be overcome by protein and nucleic acid internalization kits that allow an efficient and low-toxic internalization of proteins/peptides and nucleotides, respectively.

Cell labeling and lineage tracing in zebra fish gastrula stage embryos have demonstrated that cells destined to form the pronephros arise from the ventral mesoderm, in a region partially overlapping with cells fated to form blood (Fig. 3A) (Kimmel *et al*., 1990). These cells emerge shortly after the completion of epiboly as a band of tissue, the intermediate mesoderm (IM), at the posterior lateral edge of the paraxial mesoderm (Fig. 3B and C). In zebra fish, unlike other non-teleost vertebrates, the IM gives rise to both kidney and blood cells. The size and positioning of the IM are significantly influenced by dorsoventral and anterior–posterior axis patterning molecules, such as the ventralizing factors bone morphogenetic proteins (BMPs) and their inhibitors, and the Cdx family of homeobox genes.

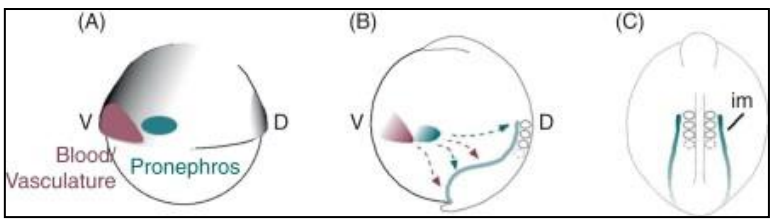


Fig. 3. Origins of the intermediate mesoderm. (A) Approximate positions of cells in a shield stage embryo destined to contribute to the blood/vasculature and pronephric lineages in the ventral (V) germ ring. (D; dorsal shield). (B) Migration of cells during gastrulation to populate the intermediate mesoderm (im) (C).

Estradiol Glow is the ovarian steroid hormone 17-β-Estradiol labeled with a novel low molecular weight orange/red fluorophore, thus retaining its chemical properties and its biological activity. This allows for a wide range of applications including

- analysis of *in vivo* and *in vitro* steroid uptake in real time
- monitoring of intracellular and subcellular steroid transport
- studies on steroid-binding proteins
- use as tracer for steroid immunoassays (to replace isotopes or enzymes)
- photodynamic elimination of estrogen sensitive tissues and cells in experimental settings with possible future clinical implications

Cell labeling can also occur indirectly through the fusion of fluorescent proteins (FPs) to the protein of interest. In this way, one can follow the destiny of the protein under study that is, intracellular trafficking, subcellular localization, co-localization with other proteins, and many other processes. When indeed the matter of study is not the lipid bilayer but the specific function exerted by a protein, it is advantageous to directly label the subject and thus directly follow its destiny. However, for the production of FP-tagged constructs cloning and plasmid design is required and biochemical/structural analysis is necessary to prove that no alterations in the functionality/structure have occurred. Constructs need then to be transfected into the cells for expression of the protein. Transfection can be carried out by many different methods, including electroporation and DNA complex formation with a lipid carrier (e.g., Lipofectamin™) or with polymers (e.g., polyethylenimine (PEI)). The efficiency of transfection depends strongly on the cell type, size of the plasmid, and cell state (e.g., cell confluency). Indeed, some cell lines do not tolerate very well the introduction of exogenous DNA, therefore transfection causes high mortality that can be due either to the overexpression of the FP or to the treatment with the transfection reagent. It has to be pointed out that, in general, transfection of tumor cell lines is more successful than transfection on primary cells. Although the use of FP tags does not require the addition of organic solvents or solubilizing agents in which the chemical dyes need to be resuspended and which may alter membrane properties, in any case also transfection agents may be cause of high toxicity.

Stem Cell Labeling and Implications

Stem cells contribute to the development of tissues and organs and are responsible for their functioning and maintenance throughout life. They reside in various tissues, and divide to either reproduce themselves or give rise to progeny that undergoes a limited number of divisions and ends up as a functional cell of that tissue.

The importance of stem cell labelling

To explore the prospects of healthy tissue rejuvenation or damaged tissue regeneration, it is necessary to uncover the cellular mechanisms supporting the life cycle of stem cells. This means tracking the propagation of stem cells in living tissues, tracing the fate of their progeny, and investigating cell distribution in organs.

Traditional experiments involved tagging the DNA in dividing cells with modified nucleotides. When a cell which has incorporated the tag into its genome divides, all its progenies will be tagged, too. However, so far it has been impossible to use more than two tags simultaneously.

Advances in stem cell labelling

Being able to mark dividing cells with different tags enables cellular processes to be explored in great detail. The problem was that previously, it was impossible to use more than two tags. We recently established a method for labeling three or four DNA tags at once. The use of three or four independent tags allows for marking several cohorts of cells born at distinct timepoints to observe the stem cell progeny passing through the different maturation stages.

Various combinations of pulse and cumulative marking highlights specific subpopulations among dividing stem cells. For example, injecting mice with three tags sequentially revealed the conveyor belt pattern of division and migration of cells in the intestine. Using this technique, we were able to analyze the patterns and parameters of neural stem cell division in the adult brain.

Determination of cycle re-entry, a critical parameter of the cell division kinetics, allowed for the conclusion that neural stem/progenitor cells in the hippocampus, a brain area with continuous neurogenesis, undergo approximately four cell divisions before exiting the cell cycle.

The same approach can be used for parallel analysis of various other dividing cells in an organism. We expect that various combinations of tags and labeling schemes will reveal new complex features of dividing stem cells, such as the distinct steps of their cell cycle entry or exit or the alternation between quiescent and proliferative states, under both normal and pathological conditions.

The future for dividing stem cell labeling

Identifying molecular mechanisms underlying the cell cycle entry or exit or the alternation between quiescent and proliferative states is currently being considered as a critical direction in the field of stem cell research. It is hoped that our technique will contribute to this direction by allowing for identification of stem cells which are undergoing or have recently undergone switch from one state to another.

When combined with the various approaches of genetic tracing, such as Cre-Lox recombination, our method will reveal the proliferative history of definite cell lineages that is critical not only for adult stem cell research, but also for tissue engineering and developmental biology.

Cell tagging

Generally, fluorescent tagging, or labeling, uses a reactive derivative of a fluorescent molecule known as a fluorophore. The fluorophore selectively binds to a specific region or functional group on the target molecule and can be attached chemically or biologically.

Introduction

Fluorescent proteins (FPs) have been used as protein tags since the mid-1990s mainly for cell biology and fluorescence microscopy. These tags have not only revolutionized cell biology by enabling the imaging of almost any protein, they are also used in biochemical applications. An important example is the immunoprecipitation and affinity purification of FP-tagged proteins, which was enabled by the development of affinity resins with high yield, purity, and affinity such as ChromoTek's Nano-Traps

The use of fluorescent tags is widespread in the studies of protein–nucleic acid interactions for imaging and structural/functional studies on both single-molecule and population levels. The sensitivity of the methods used usually means a low amount of required material. Proteins exhibit intrinsic fluorescent properties, changes in which can be observed during binding.

Types of fluorescent proteins

Most researchers use intrinsically fluorescent proteins GFP, mNeonGreen, TurboGFP, RFP, or mCherry. Alternatively, extrinsically fluorescent or self-labelling proteins have been introduced that require the covalent coupling of a fluorescent molecule to the non-fluorescent protein, e.g. the protein tags SNAP, CLIP, and Halo. These self-labelling fluorescent proteins have certain performance advantages over intrinsically FPs due to their fluorescent dyes' properties.

- **Green fluorescent proteins**

Jellyfish Green Fluorescent Protein (GFP) and its derivatives are still the most frequently used fluorescent proteins in biomedical research. Recently, additional green fluorescent proteins have been introduced that are derived from other organisms. This

FPs owns the same basic folds as GFP but diverge widely on sequence-level. Therefore, they require novel, dedicated research tools such as antibodies.

- **Red fluorescent proteins**

Red fluorescent proteins (RFPs) are FPs that emit red-orange fluorescence light. The first RFP that became commercially available was DsRed. It was derived from *Discosoma sp.* sea anemones in 1999.

DsRed has some immanent practical problems: (i) It has a maturation time of about 24 hours, which makes it unusable for short(er) time experiments. (ii) The tetrameric form of DsRed may compromise the function of proteins to which it is attached. (iii) Its photostability is rather low.

In consequence, DsRed was subjected to site-directed mutagenesis to become commonly applicable as a genetically encoded fusion tag. Eventually, monomeric RFP derivatives with better fluorescent performance (in terms of brightness and photostability) and higher maturation efficiency were created. In addition, derivatives were generated with orange, red, and far red fluorescence. These monomerized versions of RFP became the valuable research tools mCherry, mOrange, mRaspberry, mPlum (also known as the "mFruits"), mKO, mRFP (a.k.a. mRFP1), mRFPruby, mRuby, tagRFP, mKate2, and DsRed-Express etc.

Additional RFPs were identified in other anthozoans (i.e. anemones and corals), but these proteins were mostly tetramers, too. Thus, they have not been further optimized for use in research yet.

mRFP (also known as mRFP1)

The first monomeric variant of dsRed, genetically engineered in Roger Tsien's lab, was simply designated mRFP (or mRFP1), i.e. monomeric red fluorescent protein. Compared to dsRed, mRFP1 is characterized by slightly lower levels of absorption, quantum yield, and photostability. However, its maturation rate is about 10 times faster than that of DsRed, which results in a similar effective brightness when expressed in living cells.

mCherry

mCherry is probably the most commonly used RFP variant. It is a monomeric red fluorescent protein with broad applicability as a fusion protein in various cell types. Like other mFruit RFPs, mCherry is derived from the dsRed variant mRFP1 via directed evolution by Roger Tsien's lab. Compared to other mFruits, mCherry has the highest photostability, fastest maturation rate, and excellent pH resistance. It has, however, a lower quantum yield than mRFP1.

mPlum

Roger Tsien's lab has also generated a far-red monomeric derivative of mRFP1/dsRed, named mPlum. Far-red FPs are beneficial for whole-body imaging applications because

the main tissue absorbers such as water, lipids and hemoglobin are nearly transparent at the emission range of 650-900 nm. Like most red-shifted RFPs, mPlum features an extended Stokes shift.

- **Halo, SNAP, and CLIP**

The extrinsically fluorescent protein tags Halo, SNAP, and CLIP require the covalent capture of a small fluorescent ligand to turn into a fluorescent protein. To this end, these self-labelling protein tags are derived from enzymes that catalyze the formation of chemical bonds: The SNAP and CLIP tags are variants of O^6-alkylguanine-DNA alkyltransferase that react with benzylguanine and benzylcytosine derivatives, respectively. The HaloTag is derived from a haloalkane dehalogenase and reacts with alkylhalides (Figure 1).

In general, both intrinsically and extrinsically fluorescent proteins are fused to a protein of interest to enable its cellular imaging and detection. However, extrinsically fluorescent proteins require the addition of a reactive fluorophore, which has several advantages:

- Higher quantum yield and photostability
- Strong fluorescence in both live and fixed cells
- A broader selection of fluorescent dyes
- A single genetic construct allows the choice of distinct fluorophores for multicolor imaging/multiplexing
- Fluorescence only initiated upon addition of the label

The availability of three extrinsically fluorescent proteins with different substrate/ligand specificities enables their orthogonal use in multiplexed experiments, also in combination with intrinsically FPs.

Depending on the experimental needs, one may use cell permeant ligands based on tetramethylrhodamine (TMR), Oregon Green, diAcFAM, or Coumarin, which readily cross the cell membrane for labeling intracellular proteins. Alternatively, cell impermeant ligands based on impermeable fluorophores like Alexa Fluor® 488 and 660 may be applied for quick cell surface labeling.

HaloTag

The self-labelling HaloTag has been derived from the haloalkane dehalogenase enzyme DhaA from *Rhodococcus rhodochrous*. Its active site has been genetically modified to irreversibly bind chloroalkane linker substrates. Owing to this type of suicide inhibition, regeneration of its catalytic site for further dehalogenation is not possible anymore. Depending on the substrate chosen, the HaloTag converts into a fluorescent protein tag or may be immobilized on agarose beads, for example.

As haloalkane dehalogenases are absent in eukaryotic cells and most prokaryotes, including *E. coli,* there will be no background labelling.

SNAP-tag

The self-labeling protein tag SNAP-tag is derived from the human O^6-alkylguanine-DNA-alkyltransferase (hATG), which, as a wildtype protein, removes alkylation damage from DNA. The resulting hAGT variant used as SNAP-tag reacts covalently with O^6-benzylguanine derivatives (i.e. a fluorescent label conjugated to guanine or chloropyrimidine leaving groups via a benzyl linker) in an irreversible and highly specific manner. In the labeling reaction, the substituted benzyl group of the substrate is covalently attached to the SNAP-tag.

CLIP-tag

CLIP-tag is a modified version of SNAP-tag, engineered to react with benzylcytosine rather than benzylguanine derivatives. When used in conjunction with SNAP-tag, CLIP-tag enables the orthogonal and complementary labeling of two proteins simultaneously in the same cell.

Choice of fluorescent tag

The ideal fluorescent tag has a large quantum yield (brightness), has a good photostability, and is monomeric. The higher the quantum yield, the better the signal to noise ratio. Photostability is important since photobleaching will decrease fluorescence intensity, producing an artificially fast diffusion time as bleaching will mimic the disappearance of the fluorescence signal from the observation volume. GFP variants, such as eGFP and eYFP, and the newer variants are suitable for FCS. Others, such as CFP, mCherry, and dsRed, have low quantum yield and have the potential to form aggregates (dsRed).

Several fluorescent tags are commercially available for labeling oligonucleotides (e.g., TAMRA, the Cy dyes, Alexa dyes). When choosing which specific fluorophores to use, it is important to select dyes with high extinction coefficients and quantum yields so that they can be easily detected at the single-molecule level. Also, the dyes' excitation and emission spectra should be compatible with the optical setup such that independent excitation and emission detection is possible for each dye. Donor–acceptor dye pairs commonly used in our smFRET experiments include Alexa 555-Alexa 647, Alexa 555-Cy5, TAMRA-Cy5, and Cy3-Cy5.

There are several unknown parameters that can complicate dye selection. Notably, the structures of several commercial fluorophores are proprietary; thus, any perturbations they may introduce into the DNA remain unknowable. Also, fluorophores that serve as effective reporters in bulk experiments may be prone to blinking or bleaching in single-molecule experiments, making them poor choices for conformational dynamics studies using smFRET. Finally, some dyes are prone to interactions with the DNA (e.g., stacking

with the bases), which may change their fluorescence properties in ways that do not depend on DNA or MMR protein conformational changes.

Several chemical strategies exist to link fluorophores to DNA. Labeling the 5′- or 3′-ends of the DNA is one option; however, being limited to only the ends of the DNA may be problematic. While internally labeling DNA oligonucleotides allows flexibility in choosing labeling positions, there are fewer commercially available options for fluorophores. Two major types of internal labels are: (1) dyes attached covalently to thymine bases via a flexible linker extending from the major groove in the DNA, and (2) dyes (e.g., the Cy dyes) that can be incorporated directly into the DNA backbone. Note that these fluorophores are rigidly locked into a specific orientation relative to the DNA backbone as they are covalently attached at both ends, whereas fluorophores on a flexible linker have more conformational freedom. The Förster radius of a given FRET pair of fluorophores depends, in part, on the relative orientation of the transition dipoles of the two fluorophores (Lakowicz, 2006). Thus, limiting the conformational freedom of both fluorophores may lead to unpredictable changes in FRET due to changes in their relative orientations.

Probable questions:

1. What is cell labeling?
2. Discuss the utility of cell labeling.
3. What is the importance of stem cell labeling?
4. Discuss about the advances in stem cell labeling.
5. What are the criteria of choosing fluorescent tag?
6. What is fluroscent protein? How many types of fluorescent proteins are used generally?
7. Write short notes on Green fluorescent proteins.
8. Write short notes on Red fluorescent proteins.
9. What is Halo tag?
10. What is Clip tag?

Suggested reading:

1. https://www.ptglab.com/news/blog/fluorescent-protein-tags/
2. A guide to choosing fluorescent proteins; Shaner N.C., Steinbach P.A. and Tsien R.Y. (2005) Nature Methods 2(12), 905-909 doi: 10.1038/nmeth819.
3. https://www.fpbase.org/protein/mcherry/

UNIT IV

Model organism *Xenopus* / Zebrafish: early embryonic development and major classes of molecules expressed, regional and genetic specification

Objective:

In this unit we will discuss about Model organism *Xenopus* / Zebrafish: early embryonic development and major classes of molecules expressed, regional and genetic specification.

What is the early development of zebrafish?

Zebrafish develop rapidly from a one-cell zygote that sits on top of a large yolk cell. Gastrulation begins approximately 6 h post fertilization, hatching at 2 days as a free-swimming larva. Zebrafish reach sexual maturity around 3 months of age and can live for up to 5 years.

Why are zebrafish used to study embryonic development?

One reason that zebrafish are an important biomedical model is because zebrafish embryos are transparent and they develop outside of the uterus. This unique developmental process allows scientists to study the details of development starting from fertilization and continuing throughout development.

- ## Early Development in Fish

In recent years, the teleost fish *Danio rerio,* known as the zebrafish, has become a favorite organism of those who wish to study vertebrate development. Zebrafish have large broods, breed all year, are easily maintained, have transparent embryos that develop outside the mother (an important feature for microscopy), and can be raised so that mutants can be readily screened and propagated. In addition, they develop rapidly, so that at 24 hours after fertilization, the embryo has formed most of its tissues and organ primordia and display the characteristic tadpole-like form (see Granato and Nüsslein-Volhard 1996; Langeland and Kimmel 1997). Therefore, much of the description of fish development below is based on studies of this species

- ## Cleavage in Fish Eggs

In fish eggs, cleavage occurs only in the **blastodisc**, a thin region of yolk-free cytoplasm at the animal cap of the egg. Most of the egg cell is full of yolk. The cell divisions do not

completely divide the egg, so this type of cleavage is called **meroblastic** (Greek, *meros,* "part"). Since only the cytoplasm of the blastodisc becomes the embryo, this type of meroblastic cleavage is called **discoidal**. Scanning electron micrographs show beautifully the incomplete nature of discoidal meroblastic cleavage in fish eggs (Figure 11.1). The calcium waves initiated at fertilization stimulate the contraction of the actin cytoskeleton to squeeze non-yolky cytoplasm into the animal pole of the egg. This converts the spherical egg into a more pear-shaped structure, with an apical blastodisc (Leung et al. 1998). Early cleavage divisions follow a highly reproducible pattern of meridional and equatorial cleavages. These divisions are rapid, taking about 15 minutes each. The first 12 divisions occur synchronously, forming a mound of cells that sits at the animal pole of a large **yolk cell**. These cells constitute the **blastoderm**. Initially, all the cells maintain some open connection with one another and with the underlying yolk cell so that moderately sized (17-kDa) molecules can pass freely from one blastomere to the next (Kimmel and Law 1985).

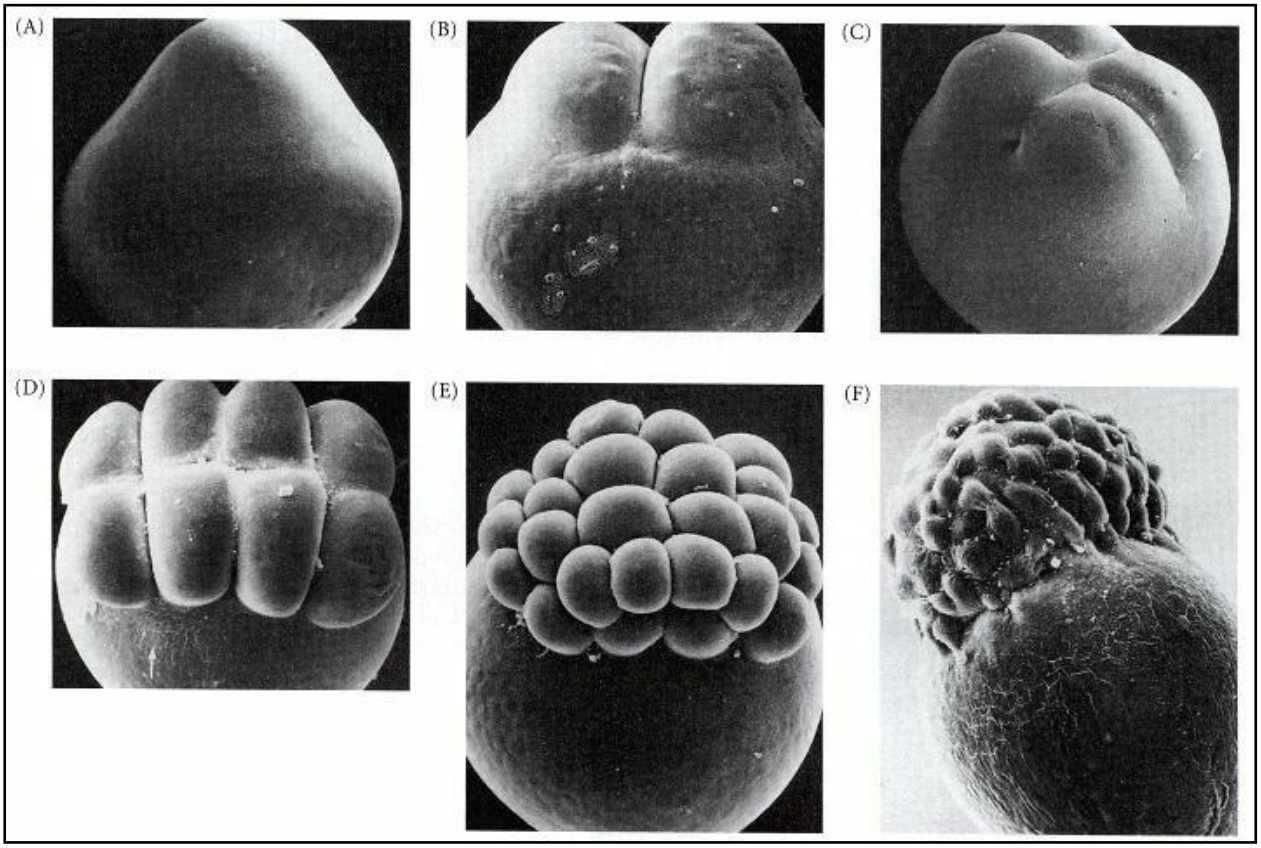


Figure 11.1

Discoidal cleavage in a zebrafish egg. (A) 1-cell embryo. The mound atop the cytoplasm is the blastodisc region. (B) 2-cell embryo. (C) 4-cell embryo. (D) 8-cell embryo, wherein two rows of four cells are formed. (E) 32-cell embryo. (F) 64-cell embryo, wherein the blastodisc can be seen atop the yolk cell. (From Beams and Kessel 1976; photographs courtesy of the authors.

Beginning at about the tenth cell division, the onset of the midblastula transition can be detected: zygotic gene transcription begins, cell divisions slow, and cell movement becomes evident (Kane and Kimmel 1993). At this time, three distinct cell populations can be distinguished. The first of these is the **yolk syncytial layer** (**YSL**). The YSL is formed at the ninth or tenth cell cycle, when the cells at the vegetal edge of the blastoderm fuse with the underlying yolk cell. This fusion produces a ring of nuclei within the part of the yolk cell cytoplasm that sits just beneath the blastoderm. Later, as the blastoderm expands vegetally to surround the yolk cell, some of the yolk syncytial nuclei will move under the blastoderm to form the **internal YSL**, and some of the nuclei will move vegetally, staying ahead of the blastoderm margin, to form **external YSL** (Figure 11.2A,B). The YSL will be important for directing some of the cell movements of gastrulation. The second cell population distinguished at the midblastula transition is the **enveloping layer** (**EVL**; Figure 11.2A). It is made up of the most superficial cells of the blastoderm, which form an epithelial sheet a single cell layer thick. The EVL eventually becomes the **periderm**, an extraembryonic protective covering that is sloughed off during later development.

Between the EVL and the YSL are the **deep cells**. These are the cells that give rise to the embryo proper. The fates of the early blastoderm cells are not determined, and cell lineage studies (in which a nondiffusible fluorescent dye is injected into one of the cells so that the descendants of that cell can be followed) show that there is much cell mixing during cleavage. Moreover, any one of these cells can give rise to an unpredictable variety of tissue descendants (Kimmel and Warga 1987; Helde et al. 1994). The fate of the blastoderm cells appears to be fixed shortly before gastrulation begins. At this time, cells in specific regions of the embryo give rise to certain tissues in a highly predictable manner, allowing a fate map to be made (Figure 11.2C; see also Figure 1.6; Kimmel et al 1990).

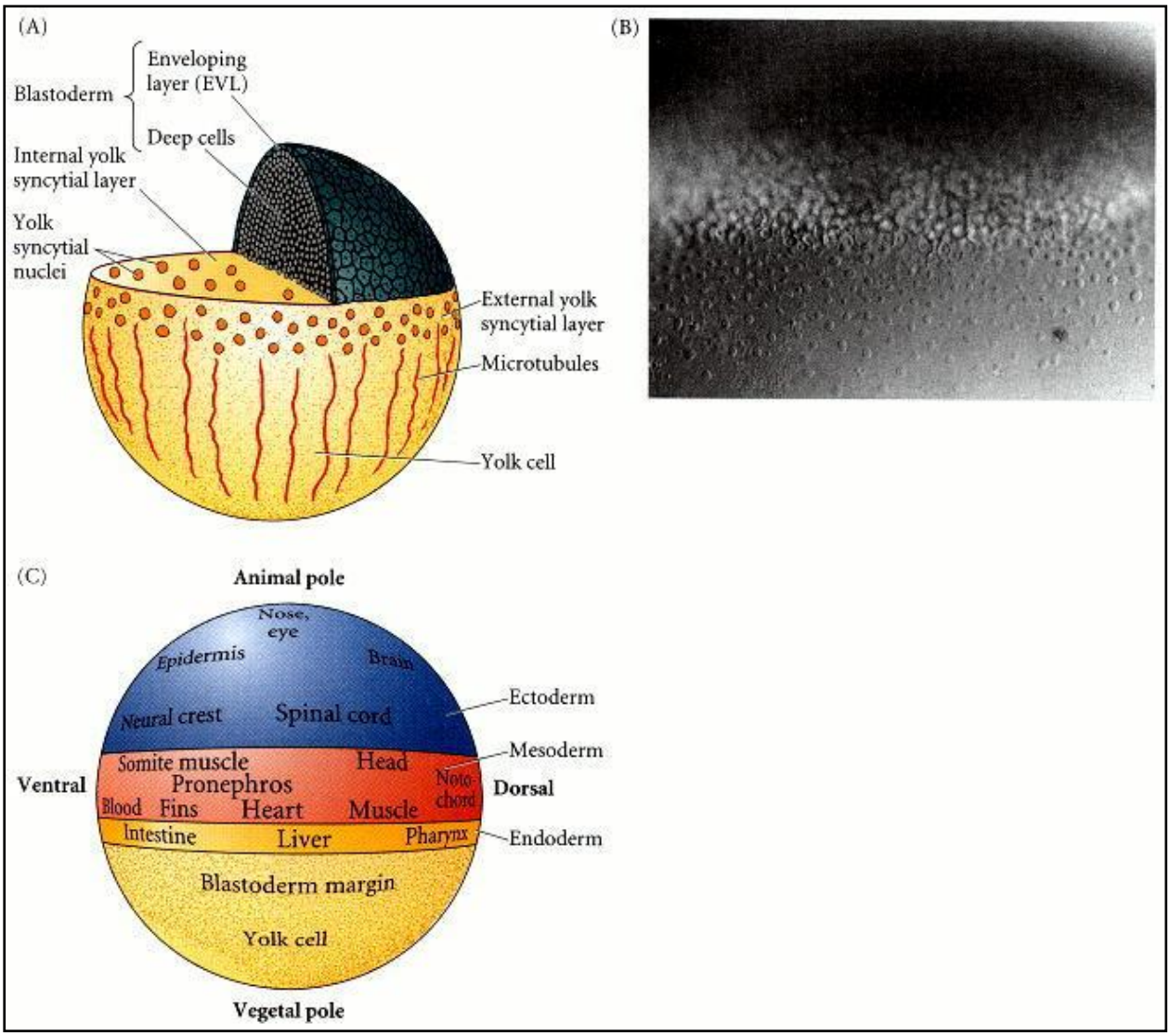


Figure 11.2

Fish blastula. (A) Prior to gastrulation, the deep cells are surrounded by the EVL. The animal surface of the yolk cell is flat and contains the nuclei of the YSL. Microtubules extend through the yolky cytoplasm and through the external region of the YSL. (B) Late-blastula stage embryo of the minnow Fundulus, showing the external YSL. The nuclei of these cells were derived from cells at the margin of the blastoderm, which released their nuclei into the yolky cytoplasm. (C) Fate map of the deep cells after cell mixing has stopped. The lateral view is shown, and not all organ fates are labeled (for the sake of clarity). (A and C after Langeland and Kimmel 1997; B from Trinkaus 1993, photograph courtesy of the author.)

- **Gastrulation in Fish Embryos**

The first cell movement of fish gastrulation is the epiboly of the blastoderm cells over the yolk. In the initial phase, the deep blastoderm cells move outwardly to intercalate with the more superficial cells (Warga and Kimmel 1990). Later, these cells move over the surface of the yolk to envelop it completely (Figure 11.3).

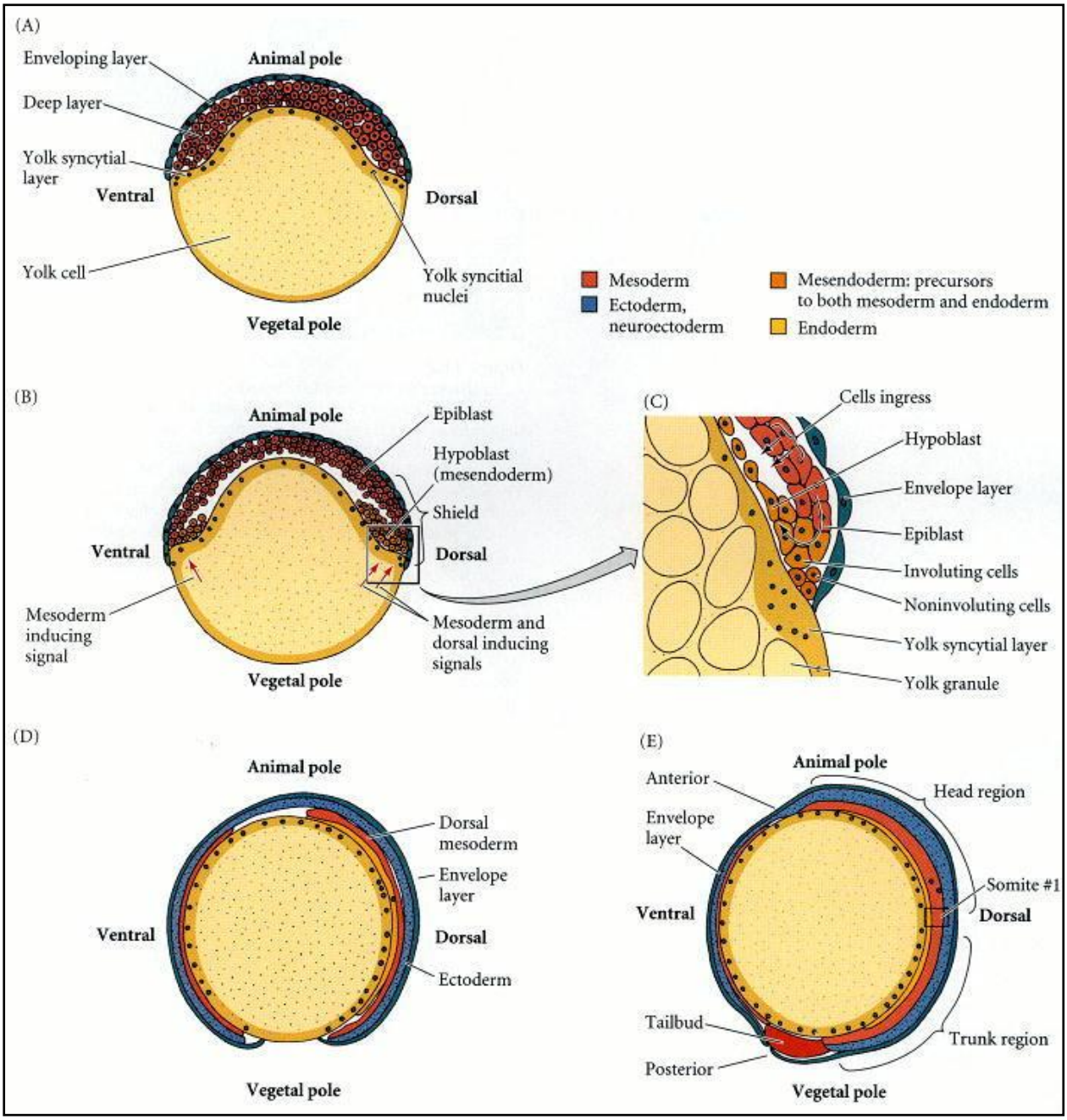


Figure 11.3

Cell movements during gastrulation of the zebrafish. (A) The blastoderm at 30% completion of epiboly (about 4.7 hours). (B) Formation of the hypoblast, either by involution of cells at the margin of the epibolizing blastoderm or by delamination of cells from the epiblast (6 hours). (C) Close-up of the marginal region. (D) At 90% epiboly (9

hours), mesoderm can be seen surrounding the yolk, between the endoderm and ectoderm. (E) Completion of gastrulation (10.3 hours). (After Driever 1995; Langeland and Kimmel 1997.)

This movement is not due to the active crawling of the blastomeres, however. Rather, the movement is provided by the autonomously expanding YSL "within" the animal pole yolk cytoplasm. The EVL is tightly joined to the YSL and is dragged along with it. The deep cells of the blastoderm then fill in the space between the YSL and the EVL as epiboly proceeds. This can be demonstrated by severing the attachments between the YSL and the EVL. When this is done, the EVL and deep cells spring back to the top of the yolk, while the YSL continues its expansion around the yolk cell (Trinkaus 1984, 1992). The expansion of the YSL depends on a network of microtubules in the YSL, and radiation or drugs that block the polymerization of tubulin inhibit epiboly (Solnica-Krezel and Driever 1994).

During migration, one side of the blastoderm becomes noticeably thicker than the other. Cell-labeling experiments indicate that the thicker side marks the site of the future dorsal surface of the embryo (Schmidt and Campos-Ortega 1995).

- ## The formation of germ layers

After the blastoderm cells have covered about half the zebrafish yolk cell (and earlier in fish eggs with larger yolks), a thickening occurs throughout the margin of the epibolizing blastoderm. This thickening is called the **germ ring**, and it is composed of a superficial layer, the **epiblast**, and an inner layer, the **hypoblast**. We do not understand how the hypoblast is made. Some research groups claim that the hypoblast is formed by the *involution* of superficial cells under the margin followed by their migration toward the animal pole (see Figure 11.3C). The involution begins at the future dorsal portion of the embryo, but occurs all around the margin. Other laboratories claim that these superficial cells *ingress* to form the hypoblast (see Trinkaus 1996). It is possible that both mechanisms are at work, with different modes of hypoblast formation predominating in different species. Once formed, however, the cells of both the epiblast and hypoblast intercalate on the future dorsal side of the embryo to form a localized thickening, the **embryonic shield** (Figure 11.4). As we will see, this shield is functionally equivalent to the dorsal blastopore lip of amphibians, since it can organize a secondary embryonic axis when transplanted to a host embryo. Thus, as the cells undergo epiboly around the yolk, they are also involuting at the margins and converging anteriorly and dorsally toward the embryonic shield (Trinkaus 1992). The hypoblast cells of the embryonic shield converge and extend anteriorly, eventually narrowing along the dorsal midline of the hypoblast. This movement forms the **chordamesoderm**, the precursor of the **notochord** (Figure 11.4B,C). The cells adjacent to the chordamesoderm, the **paraxial mesoderm** cells, are the precursors of the mesodermal

somites (Figure 11.4D, E). The concomitant convergence and extension in the epiblast brings the presumptive neural cells from all over the epiblast into the dorsal midline, where they form the **neural keel**. The rest of the epiblast becomes the skin of the fish. The zebrafish fate map, then, is not much different from that of the frog or other vertebrates (as we will soon see). If one conceptually opens a *Xenopus* blastula at the vegetal pole and stretches the opening into a marginal ring, the resulting fate map closely resembles that of the zebrafish embryo at the stage when half of the yolk has been covered by the blastoderm (see Figure 1.9; Langeland and Kimmel 1997).

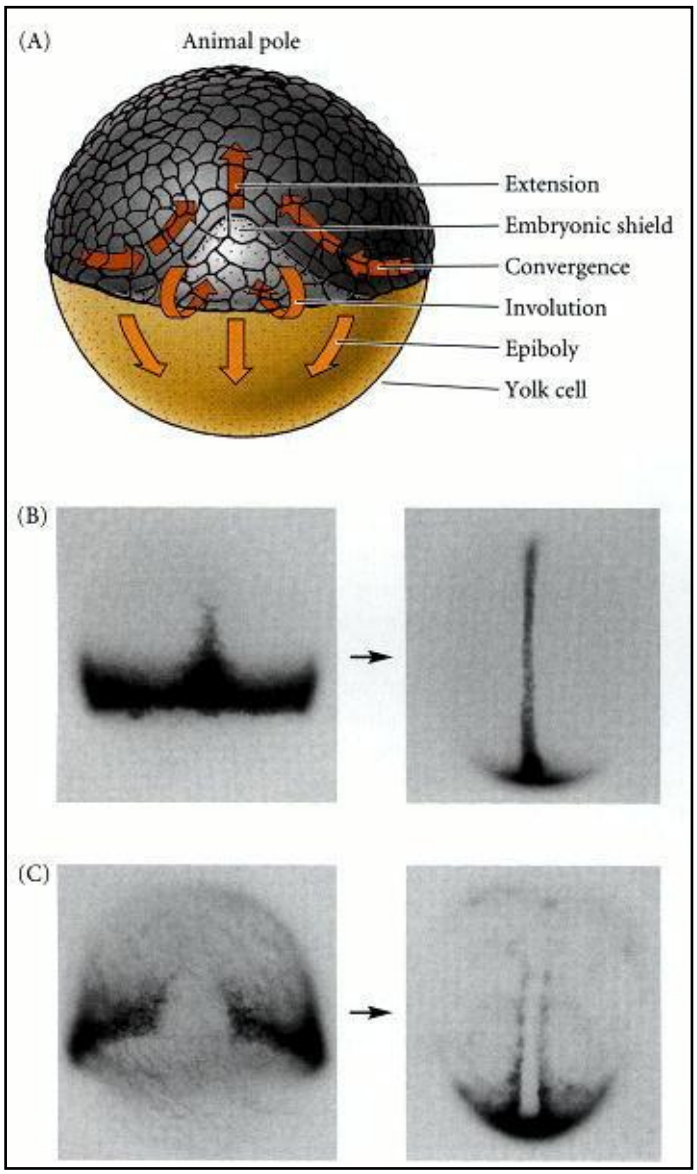


Figure 11.4

Convergence and extension in the zebrafish gastrula. (A) Dorsal view of the convergence and extension movements during zebrafish gastrulation. Epiboly spreads the blastoderm over the yolk; involution or ingression generates the hypoblast; convergence and extension bring the hypoblast and epiblast cells to the dorsal side to form the embryonic shield.

Within the shield, intercalation extends the chordamesoderm toward the animal pole. (B) Convergent extension of chordamesoderm is shown by those cells expressing the gene no tail, a gene that is expressed by notochord cells. (C) Convergent extension of paraxial mesodermal cells (marked by their expression of the snail gene) to flank the notochord. (From Langeland and Kimmel 1997; photographs courtesy of the authors.)

- **Axis Formation in Fish Embryos**

Dorsal-ventral axis formation: the embryonic shield

The embryonic shield is critical in establishing the dorsal-ventral axis in fishes. It can convert lateral and ventral mesoderm (blood and connective tissue precursors) into dorsal mesoderm (notochord and somites), and it can cause the ectoderm to become neural rather than epidermal. This was shown by transplantation experiments in which the embryonic shield of one early-gastrula embryo was transplanted to the ventral side of another (Figure 11.5; Koshida et al. 1998).

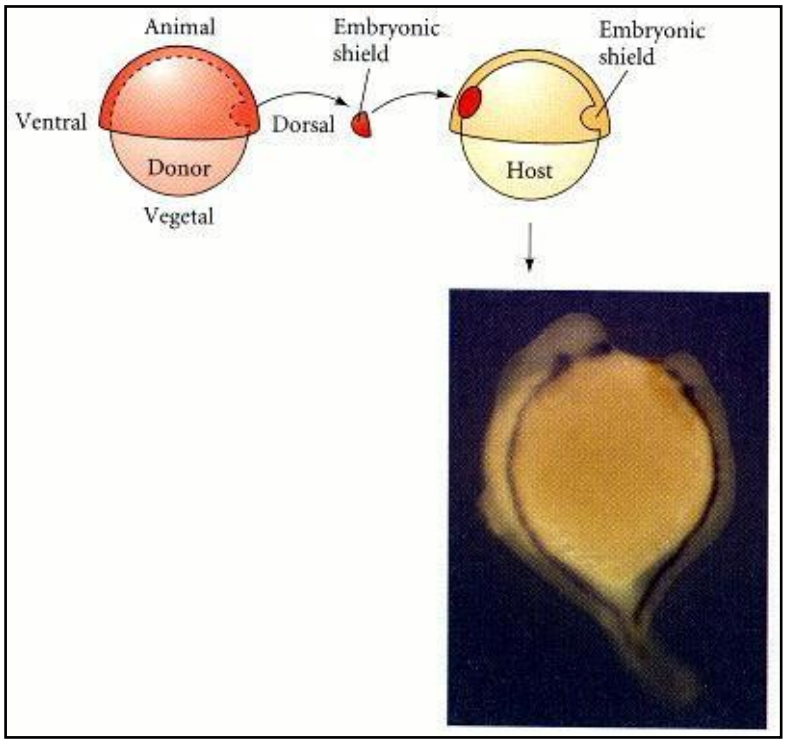


Figure 11.5

The embryonic shield as organizer in the fish embryo. A donor embryonic shield (about 100 cells from a stained embryo) is transplanted into a host embryo at the same early-gastrula stage. The result is two embryonic axes joined to the host's yolk cell. In the photograph, both axes have been stained for sonic hedgehog mRNA, which is expressed in the ventral midline. (The embryo to the right is the secondary axis.) (After Shinya et al. 1999; photograph courtesy of the authors.)

Two axes formed, sharing a common yolk cell. Although the prechordal plate and the notochord were derived from the donor embryonic shield, the other organs of the secondary axis came from host tissues that would have formed ventral structures. The new axis has been induced by the donor cells. In the embryo that had had its embryonic shield removed, no dorsal structures formed, and the embryo lacked a nervous system. These experiments are similar to those performed on amphibian gastrulae by Spemann and Mangold (1924; see Chapter 10), and they demonstrate that the embryonic shield is the homologue of the dorsal blastopore lip, the amphibian organizer.

Like the amphibian dorsal blastopore lip, the embryonic shield forms the prechordal plate and the notochord of the developing embryo. The precursors of these two regions are responsible for inducing the ectoderm to become neural ectoderm. Moreover, the presumptive notochord and prechordal plate appear to do this in a manner very much like that of their homologous structures in amphibians.* In both fishes and amphibians, BMP proteins made in the ventral and lateral regions of the embryo would normally cause the ectoderm to become epidermis. The notochord of both fishes and amphibians secretes factors that block this induction and thereby allow the ectoderm to become neural. In fishes, the BMP that ventralizes the embryo is **BMP2B**. The protein secreted by the chordamesoderm that binds with and inactivates BMP2B is a chordin-like paracrine factor called **Chordino** (Figure 11.6B; Kishimoto et al. 1997; Schulte-Merker et al. 1997).

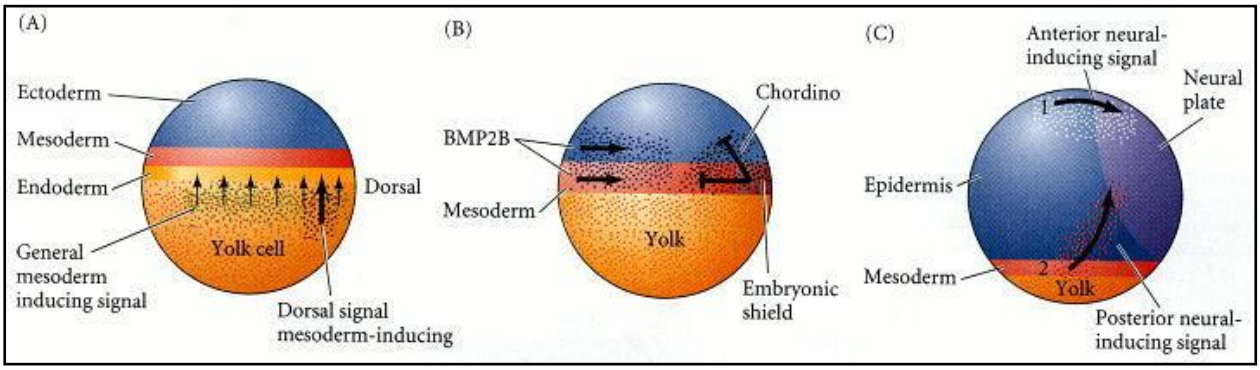


Figure 11.6

Axis formation in the zebrafish embryo. (A) Prior to gastrulation, the zebrafish blastoderm is arranged with the presumptive ectoderm near the animal pole, the presumptive mesoderm beneath it, and the presumptive endoderm sitting atop the yolk cell. The yolk syncitial layer (and possibly the endoderm) sends two signals to the presumptive mesoderm. One signal (lighter arrows) induces the mesoderm, while a second signal (heavy arrow) specifically induces an area of mesoderm to become the dorsal mesoderm (embryonic shield). (B) Formation of the dorsal-ventral axis. During gastrulation, the ventral mesoderm secretes BMP2B (arrows) to induce the ventral and lateral mesodermal and epidermal differentiation. The dorsal mesoderm secretes factors (such as Chordino) that block BMP2B and dorsalize the mesoderm and ectoderm (converting the latter into

neural tissue). (C) Recent studies have identified two signaling centers for anterior-posterior polarity, one (1) at the border of the neural and non-neural ectoderm, which induces anterior neural cell types, and the other (2) at the lateral margin, which generates a posteriorizing signal. (After Schier and Talbot 1998.)

If the *chordino* gene is mutated, the neural tube fails to form. It is hypothesized (Nguyen et al. 1998) that different concentrations of BMP2B pattern the ventral and lateral regions of the zebrafish ectoderm and mesoderm, and that the ratio between Chordino and BMP2B may specify the position along the dorsal-ventral axis. In fishes, however, the notochord may not be the only structure capable of producing the proteins that block BMP2B. If the notochord fails to form (as in the *floating head* or *no tail* mutations), the neural tube will still be produced. It is possible that the notochordal precursor cells (which are produced in these mutations) are still able to induce the neural tube, or that the dorsal portion of the somite precursors can compensate for the lack of a notochord (Halpern et al. 1993; 1995; Hammerschmidt et al. 1996).

The embryonic shield appears to acquire its organizing ability in much the same way as its amphibian counterparts. In amphibians, the endoderm cells beneath the dorsal blastopore lip (i.e., the Nieuwkoop center) accumulate β-catenin. This protein is critical in amphibians for the ability of the endoderm to induce the cells above them to become the dorsal lip (organizer) cells. In zebrafish, the nuclei in that part of the yolk syncytial layer that lies beneath the cells that will become the embryonic shield similarly accumulate β-catenin. This protein distinguishes the dorsal YSL from the lateral and ventral YSL regions (Figure 11.7; Schneider et al. 1996). Inducing β-catenin accumulation on the ventral side of the egg causes dorsalization and a second embryonic axis (Kelly et al. 1995). In addition, just prior to gastrulation, the cells of the dorsal blastopore margin synthesize and secrete Nodal-related proteins. These induce the precursors of the notochord and prechordal plate to activate *goosecoid* and other genes (Sampath et al. 1998; Gritsman et al. 2000.) Thus, the embryonic shield is considered equivalent to the amphibian organizer, and the dorsal part of the yolk cell can be thought of as the Nieuwkoop center of the fish embryo.

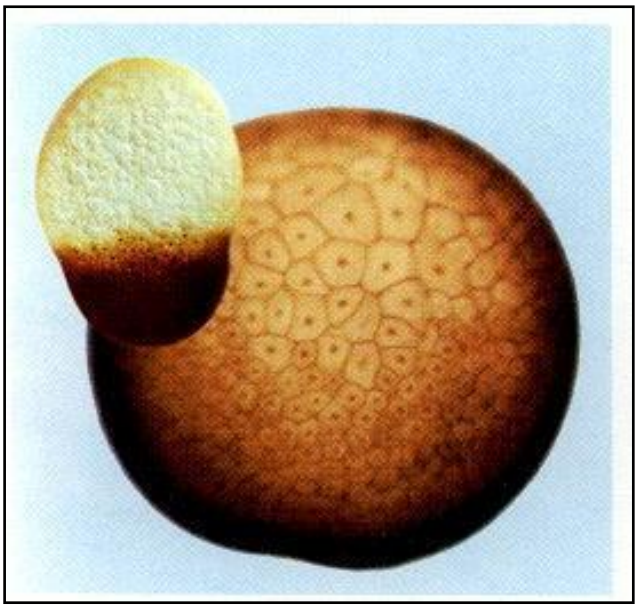


Figure 11.7

Nuclear localization of β-catenin marks the dorsal side of the Xenopus blastula (the larger one) and helps form its Nieuwkoop center beneath the organizer. In the zebrafish late blastula, nuclear localization of β-catenin is seen in the yolk syncytial layer nuclei beneath the future embryonic shield. (Photograph courtesy of S. Schneider.)

- **Anterior-posterior axis formation: two signaling centers**

As is evident from Figure 11.5, when a second dorsal-ventral axis is experimentally induced in zebrafish eggs, both the regular and the induced axes have the same anterior-posterior polarity. Both heads are at the former animal cap, and both tails are located vegetally. Indeed, the anterior-posterior axis is specified during oogenesis, and the animal cap marks the anterior of the embryo. This axis becomes stabilized during gastrulation through two distinct signaling centers. First, a small group of anterior neural cells at the border between the neural and surface ectoderm (a region that become the pituitary gland, nasal placode, and anterior forebrain) secrete compounds that cause anterior development. If these anterior neural cells are experimentally placed more posteriorly in the embryo, they will cause the neural cells near them to assume the characteristics of forebrain neurons. The second signaling center, in the posterior of the embryo, consists of lateral mesendoderm precursors at the margin of the gastrulating blastoderm. These cells produce caudalizing compounds, most likely Nodal-related proteins and activin (Figure 11.6C; Woo and Fraser 1997; Thisse et al. 2000). If transplanted adjacent to anterior neural ectoderm, this tissue will transform the presumptive forebrain tissue into hindbrain-like structures.

- **Left-right axis formation**

Little is known about left-right axis formation in fishes. In zebrafish, a gene called ***nodal-related-2*** is expressed solely in the left side of the lateral plate mesoderm[±] (Rebagliati et al. 1998). Nodal-related-2 is a paracrine factor of the TGF-β family. As we will see later in this chapter, proteins of the TGF-β family are critical in the establishment of the left-right axis throughout the vertebrate classes.

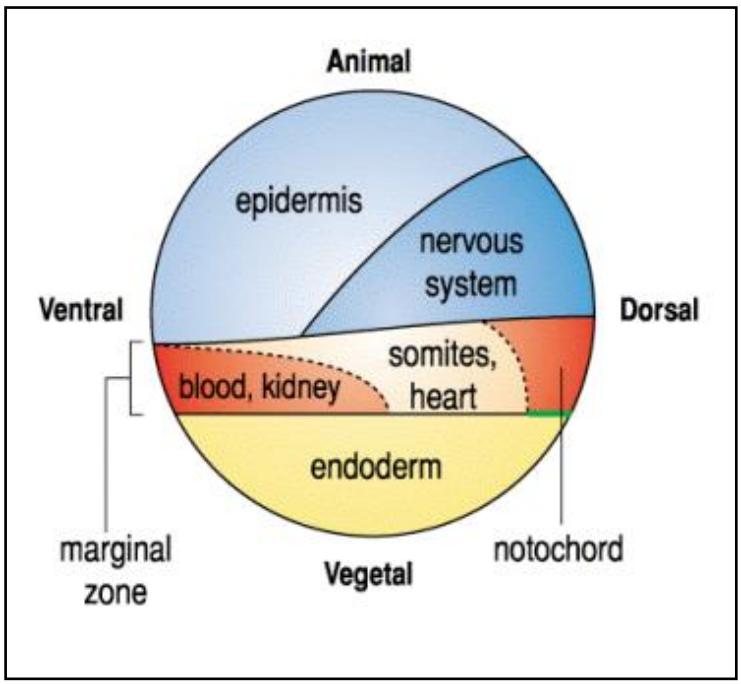
Xenopus Development

1.1, Introduction

The *Xenopus* egg is clearly polarized into a darkly pigmented animal hemisphere and a lightly pigmented vegetal hemisphere. After fertilisation the egg undergoes a series of very rapid, and synchronous, cleavage divisions (90 minutes for the first, then every 25-30 minutes – at 21˚C) that over a period of 7 hours will generate the 5,000 cells of the mid-blastula stage embryo. During this period there is no recognisable G1 or G2 phase in the cell cycle and most of the embryonic genome is silent. Development is dependent upon maternal molecules (e.g. mRNAs and proteins) synthesized during oogenesis. After 12 cell divisions the cell-cycle slows considerably becoming 50 minutes for cycle 13, 90 minutes for cycle 14 and 240 minutes for cycle 15. Cell division becomes asynchronous and both G1 and G2 phases are introduced, allowing the embryonic genome to be activated. This is known as the mid-blastula transition (MBT). Timing of the MBT appears to be dependent on the titration of a cytoplasmic factor against increasing DNA content, since the MBT is delayed by one cell cycle in haploid eggs and can be advanced experimentally by injecting DNA.

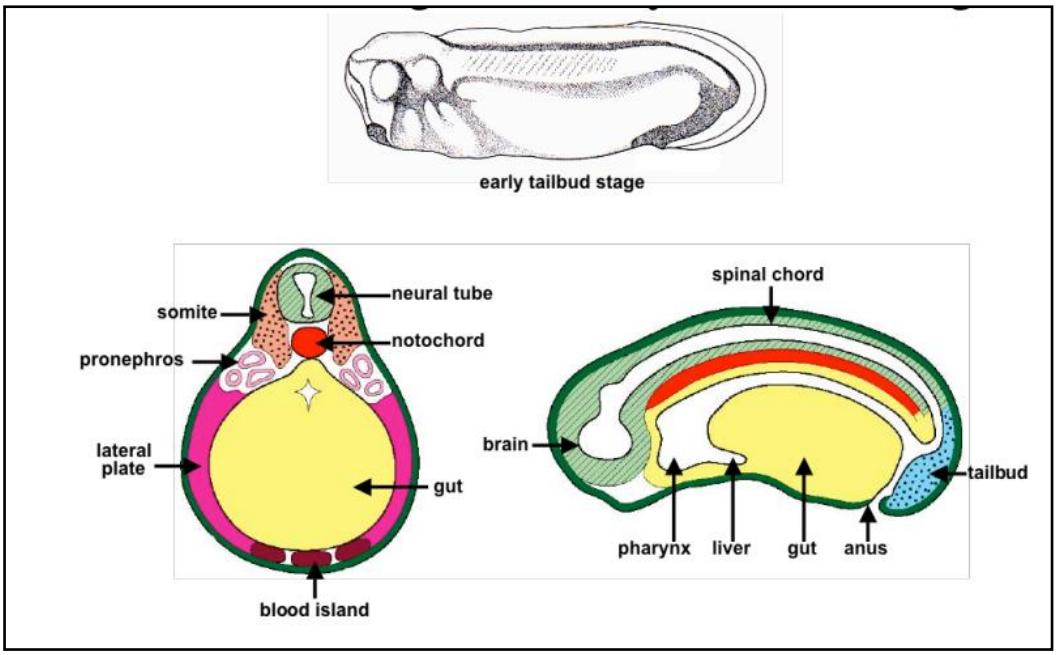
The blastula stage embryo is a ball of cells surrounding a fluid filled cavity, the blastocoel, which is displaced to the animal hemisphere by the large, yolk rich, vegetal blastomeres. Fate maps have shown that the animal hemisphere will form the ectoderm, while the vegetal hemisphere will form the endoderm. The equatorial region, which is known as the marginal zone, will form the mesoderm. After 10 hours the embryo is composed of 10,000 cells and undergoes gastrulation, when the germ layers are rearranged such that the endoderm forms the innermost layer, the ectoderm the outermost layer, and the mesoderm the middle layer. Ectoderm will form the skin and nervous system, mesoderm will form the notochord, muscle, cartilage and bone, the urogenital system, connective tissues, and blood, while endoderm will form the digestive system (the lining, not the surrounding smooth muscle and connective tissue), the respiratory system, liver, and pancreas. Gastrulation takes about six hours and is followed by neurulation (15-22 hours after fertilization), when the dorsal ectoderm folds to form the neural tube, the precursor of the central nervous system, and the

lateral edges of the epidermis fuse. Over the next three days the embryo undergoes organogenesis, when all the major tissues are formed and differentiation occurs. After 2-3 months the tadpole will metamorphose into the adult frog, which will take another year to become sexually mature



1.2, The vertebrate body plan

Following the completion of neurulation the embryo has acquired a body plan that is characteristic of all vertebrate embryos. The brain is already divided into forebrain, midbrain and hindbrain regions. the eyes are forming and an adhesive organ, the cement gland, forms in the “chin” region of the ectoderm. The branchial arches form posterior to the cement gland, in association with the pharynx, and will form the jaws and gills. A cross section through the anterior trunk shows that the mesoderm lies between the epidermis and the endoderm. The neural tube lies along the dorsal midline and beneath this is a mesodermal structure called the notochord, which is composed of large vacuolated cells that add rigidity to the tadpole. The notochord is also the source of signals that are responsible for patterning adjacent structures such as the neural tube and somites.

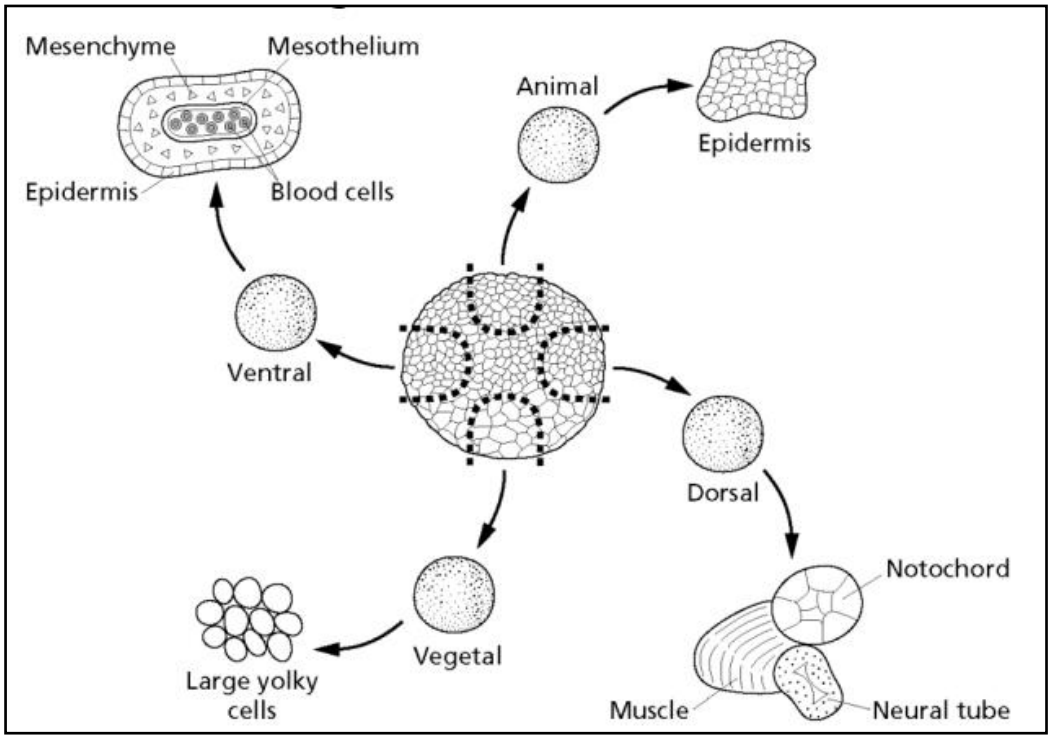


Flanking the notochord are the paired somites, which ultimately differentiate the dermis of the back (dermatome), skeletal muscle (myotome), and the cartilage and bone of the vertebrae (sclerotome). In amphibians, the myotome is easily the largest region of the somite, reflecting the requirement for powerful skeletal muscles for swimming. Somites form in an anterior to posterior sequence, until a total of about 40 are present. Beneath the somites are the pronephros and its associated tubules (a primitive but functional kidney), and the lateral plate mesoderm, which forms the smooth muscle of the digestive tract, the linings of the body cavities, and the dermis of the belly. On the ventral (belly) side of the embryo are the blood islands that differentiate the first population of blood cells. The mesoderm surrounds the yolk rich endoderm that differentiates the epithelial lining of the digestive tract and organs such as the liver and pancreas. Yolk is a source of food for the embryo, sustaining development until the fully differentiated tadpole can feed for itself. At the posterior tip of the embryo is the tailbud, which will grow to form the neural tube, notochord, and somites of the posterior 2/3rds of the tail.

1.3, Animal-vegetal polarity is maternally specified

The *Xenopus egg* possesses a distinct polarity even before it is fertilized, with a darkly pigmented animal hemisphere and a lightly pigmented vegetal hemisphere. Within the egg the yolk is mainly confined to the vegetal hemisphere, while the nucleus is located close to the animal pole. Several mRNAs have been identified that are localized to either the animal or vegetal hemisphere and some of these are involved in early patterning events. One such mRNA encodes the signalling molecule **Vg1**, while a second encodes the T-box transcription factor **VegT**. Both mRNAs are localized to the vegetal cortex during oogenesis but diffuse throughout the vegetal hemisphere prior to fertilization. Translation of vg1 mRNA begins during oogenesis, while translation of vegT mRNA is not initiated until after fertilization. Animal and vegetal explants can be isolated from

early blastulae and cultured in a simple salt solution for several days, where they will differentiate into recognisable tissues. Animal explants, called animal caps, will only form epidermis, even though they will also form neural tissue if left in the embryo. Vegetal explants don't differentiate but will express genes that are specific to the endoderm, but not genes that are specific to the ectoderm or mesoderm. (Differentiation of the endoderm requires it to interact with mesoderm, which is absent from vegetal explants). This demonstrates that ectoderm and endoderm are specified by maternal components inherited from the egg, but that neural tissue requires continued interactions with the embryo. Explants from the dorsal marginal zone differentiate dorsal mesoderm (e.g. notochord), while ventral and lateral explants differentiate ventral mesoderm. However, it is impossible to isolate pure mesoderm and marginal zone explants always include ectoderm and endoderm. Therefore, a role for inductive signalling in mesoderm formation, between endoderm and ectoderm, cannot be excluded (see section 2.1). It is notable that lateral marginal zone fragments form blood and very little, if any, muscle, yet their normal fate is to form lots of muscle and very little blood (see section 1.1).

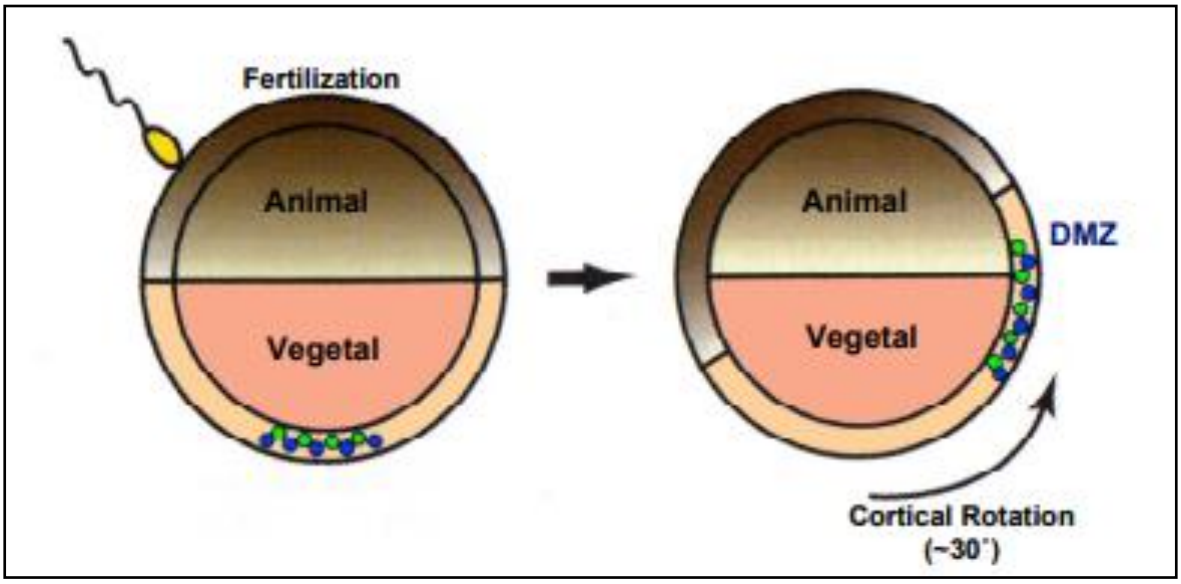


This suggests that the normal fate of marginal zone fragments is not specified by maternal localized determinants, but requires continued interactions with other regions of the embryo (see section 2.1). The identity of the maternal components that specify ectoderm is still unknown, but the determinant responsible for endoderm formation is believed to be VegT. This transcription factor induces the expression of endoderm specific genes (e.g. bix1-4, mix1-2, sox17) when injected into the animal cap hemisphere, showing that it is sufficient for endoderm formation. Moreover, injection of

antisense oligonucleotides for vegT into Xenopus oocytes results in degradation of maternal vegT mRNA (before it is translated) and the resulting embryos fail to express endoderm-specific genes. Thus VegT is also necessary for endoderm formation. VegT depleted embryos also form little or no mesoderm, yet maternal VegT is not inherited by this germ layer! The explanation is that VegT activates the expression of vegetal signals (e.g. Xnr1, derriere) that are required for mesoderm formation (section 2.3). Ectoderm is expanded in VegT depleted embryos, demonstrating that VegT represses ectodermal fates. Indeed, it is possible that ectoderm is a "default state" that forms in the absence of maternal VegT.

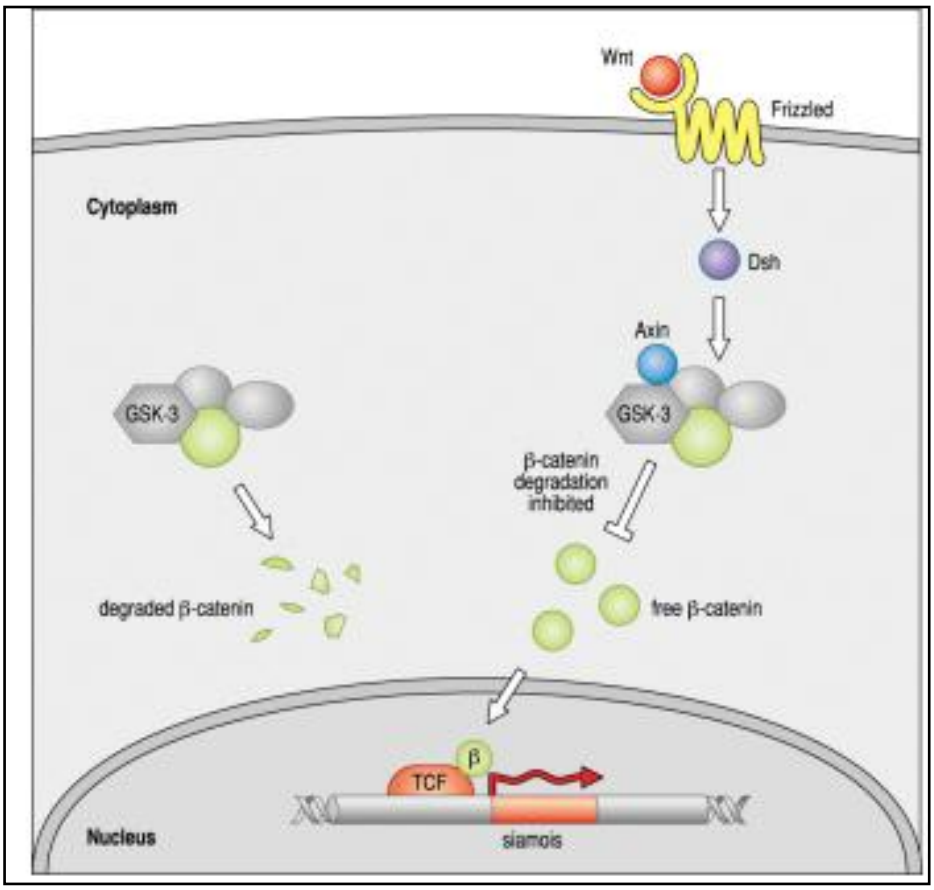
1.4, Cortical rotation

The unfertilized Xenopus egg is radially symmetric about the animal-vegetal axis and this symmetry is broken by a series of events set in motion by fertilization. Soon after fertiliozation (~30 minutes), microtubules have formed between the cortex and inner cytoplasm at the vegetal pole and the egg cortex rotates, by approximately 30°, relative to the inner cytoplasm. At the vegetal pole this movement is away from the sperm entry point and towards the future dorsal side. Cortical rotation causes the vegetal microtubules to form parallel arrays, in a ventral to dorsal direction. Small particles and membrane bound organelles, have been observed moving along these microtubules, from the vegetal pole towards the dorsal marginal zone.



Movement of these particles is blocked by treatments that Normal Maternal vegT vegT depleted disrupt the formation of microtubules, including UV-irradiation of the vegetal pole. Development of dorsal structures is severely disrupted by UV-irradiation and in extreme cases the embryos are completely ventralized (they lack notochord, muscle, pronephros, and neural tube, but lots of blood). In contrast, D_2O randomizes the orientation of the vegetal microtubules and treated embryos are hyperdorsalized (they

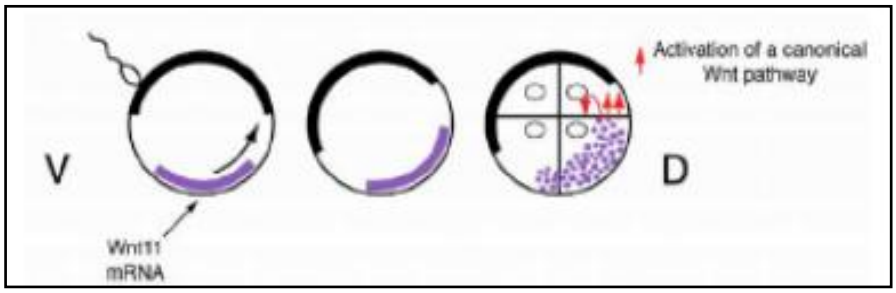
have an enlarged neural tube, notochord, and muscle, but less pronephros, lateral plate mesoderm, and blood). In the unfertilized egg, the vegetal pole contains factors that induce a second dorsal axis when injected into ventral blastomeres (vegetal pole cytoplasm is injected), but this activity moves towards the equator, on the future dorsal side, following cortical rotation. Movement is blocked by UV-irradiation of the vegetal pole, suggesting that a dorsal determinant is moved along the vegetal array of microtubules. Good candidates for this dorsal determinant are proteins called **dishevelled (Dsh)** and **GSK3 binding protein (GBP)**. Both proteins associate with the vegetal array of microtubules and become enriched on the dorsal side of the embryo following cortical rotation.



Dsh and **GBP** are components of the canonical **Wnt signalling pathway** that regulates gene expression by inducing nuclear localisation of the protein ß-catenin. Wnts activate cell surface receptors belonging to the frizzled family, which transduce the signal across the plasma membrane to Dsh. Activated Dsh, and GBP, interact with a protein complex that includes the enzyme glycogen synthase kinase 3 (GSK3), which phosphorylates ßcatenin and targets it for degradation. Dsh and GBP inactivate GSK3, allowing ßcatenin to accumulate. As expected from this model, ß-catenin is enriched on the dorsal side of the embryo, from early cleavage through to late blastula stages. Injection of ß-catenin mRNA into ventral blastomeres induces a second dorsal axis on the ventral side of the embryo. As a consequence conjoined twins are formed. Depletion of maternal ß-catenin mRNA, by injection of antisense oligonucleotides, blocks the formation of the primary

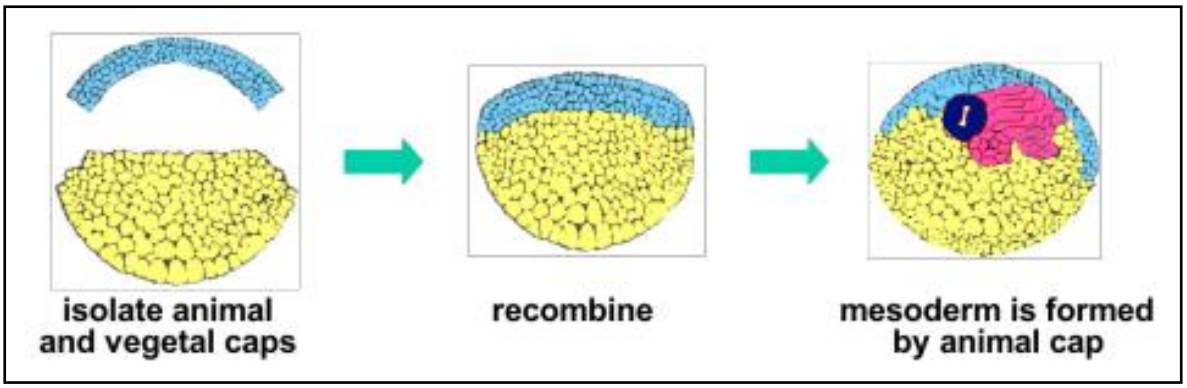
dorsal axis, demonstrating that ß-catenin is both necessary and sufficient for dorsal development. ßcatenin accumulates in nuclei on the dorsal side of the embryo at blastula stages, forming complexes with the transcription factor XTcf3, and activates transcription of dorsally expressed genes such as siamois, twin, and xnr3. Siamois and Twin are two closely related homeodomain transcription factors that induce secondary dorsal axes when misexpressed in ventral blastomeres (hence the names). Inhibiting Siamois blocks formation of the primary dorsal axis indicating that it is essential for dorsal development. Siamois and twin are normally expressed in dorsal-vegetal blastomeres, a region of the embryo known as the Nieuwkoop centre (see section 2.1).

Initially, it was thought that Dsh and GBP might be activated on the dorsal side of the embryo independently of Wnt signalling, but recent evidence suggests that this is not correct. Both maternal Wnt11 and a frizzled 7 are necessary for dorsal specification in *Xenopus* embryos, since deleting maternal mRNA for either gene, by injecting antisense oligonucleotides, causes a loss of dorsal tissues. In both cases, ß-catenin fails to accumulate opposite the site of sperm entry. The mRNA for Wnt11 is localized to the vegetal cortex of Xenopus oocytes and moves to the dorsal side of the embryo following cortical rotation. It is not known if this requires the parallel array of microtubules. Thus we have a picture in which cortical rotation shifts several components of the Wnt signalling pathway to the dorsal side of the embryo, where they prevent degradation of ß-catenin and stimulate transcription of dorsal specific genes.

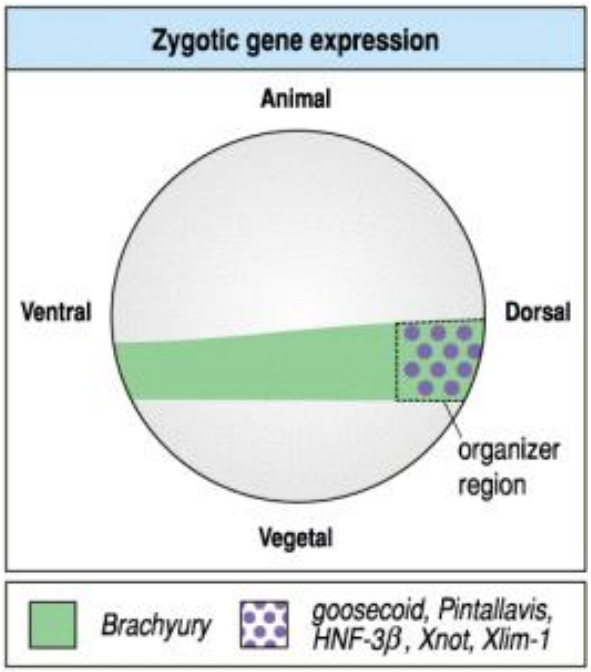


2.1, Mesoderm-induction

Mesoderm is formed in the marginal zone of Xenopus blastulae as a result of inductive signals released by vegetal blastomeres, a process known as mesoderm-induction. This was first shown by Peter Nieuwkoop (1969), who isolated animal and vegetal explants from axolotl mid-blastulae and found that they formed ectoderm and endoderm, respectively, when incubated alone (see section 1.3). However, they also formed mesoderm (notochord, muscle, pronephros) when combined. Since all the mesoderm was derived from the animal cap, Nieuwkoop concluded that it was induced by the vegetal hemisphere.



Mesoderm was not induced when explants were isolated from early gastrulae, indicating that mesoderm-induction occurs during blastula stages. Nieuwkoop's experiments were subsequently repeated on Xenopus and identical results obtained. Not all vegetal cells are able to induce dorsal mesoderm, indeed most will only induce ventral mesoderm. This was demonstrated by dividing the vegetal pole into small fragments along the dorsal-ventral axis and combining them with animal caps. Only the dorsal most fragment induced notochord and muscle, while lateral and ventral fragments induced blood.



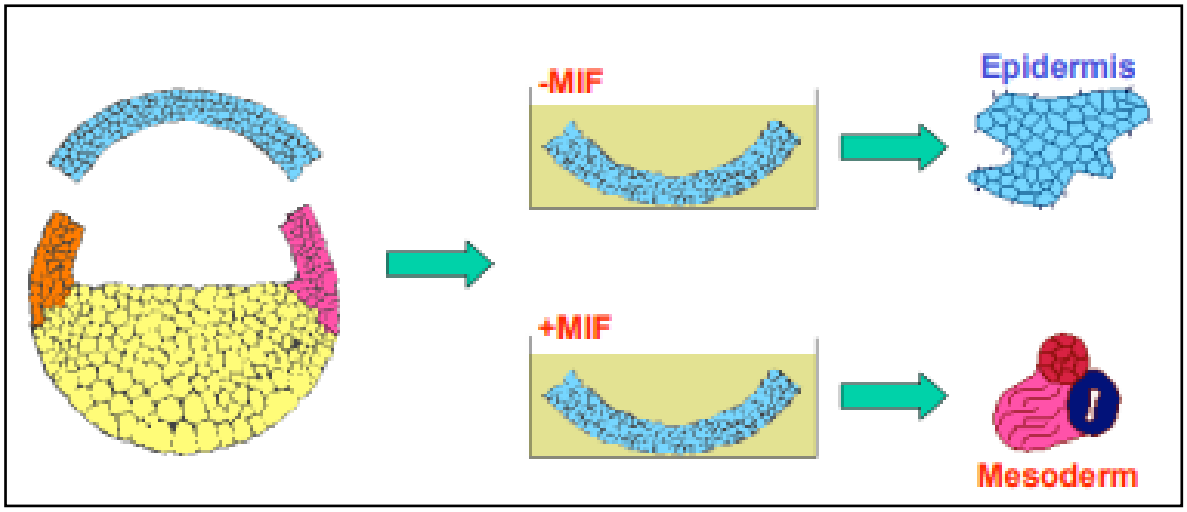
The small dorsal fragment will also induce a secondary dorsal axis when transplanted to the ventral side of a host blastula, without making any contribution to the mesoderm. Hence it releases a signal that induces dorsal mesoderm in the adjacent ventral marginal zone. This dorsal-vegetal fragment is the Nieuwkoop centre, the location of siamois and twin expression in late-blastulae (section 1.4), These results suggest that the vegetal hemisphere releases two inducing signals; a dorsal signal released by the Nieuwkoop centre and a ventral signal released by lateral and ventral blastomeres. Thus only two types of mesoderm should be initially specified in the marginal zone and

evidence in favour of this was obtained by taking explants from the marginal zone of early gastrulae. Only explants of the dorsal marginal zone differentiated notochord and muscle, while ventral and lateral explants differentiated blood, mesothelium and mesenchyme (see section 1.3).

Additional evidence is provided by the initial expression patterns of genes that are directly activated by mesoderm-inducing signals. These genes are first detected in late blastulae and while some (e.g. brachyury) are expressed throughout the marginal zone, others (e.g. goosecoid, pintallavis) are localized to the dorsal sector.

2.2, Mesoderm-Inducing-Factors

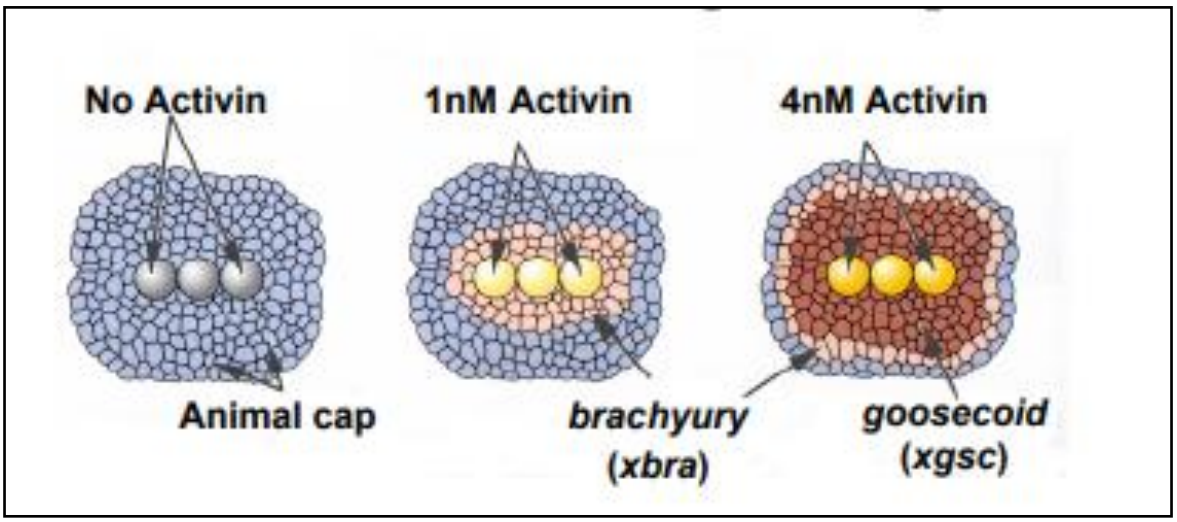
The blastula stage animal cap has been used as an assay for identifying molecules with mesoderm inducing activity, by incubating them in a simple salt solution containing the molecule to be tested. Isolated animal caps normally form round balls that differentiate as epidermis, but if a mesoderm inducing factor is present then the animal cap changes its morphology and differentiates mesoderm.



The changed morphology is particularly evident if dorsal mesoderm is induced, since the cap undergoes considerable elongation (this is because dorsal mesoderm undergoes convergent-extension, the main mechanism responsible for elongating the anterior-posterior axis during gastrulation and neurulation). Using this assay, a small number of proteins have been identified that have mesoderm inducing activity and most of them are extracellular signalling molecules belonging to either the **Fibroblast Growth Factor (FGF)** or **Transforming Growth Factor-ß (TGF-ß)** families. Members of the FGF family usually induce mesenchyme and mesothelium (ventral-type tissue) at low concentrations and muscle at high concentrations, but never notochord. The first member of the TGF-ß family to be identified as a mesoderm inducing factor was Activin, which induces endoderm at high concentrations, notochord and muscle at slightly lower concentrations, and mesenchyme and mesothelium (ventral-type tissues) at the lowest

inducing concentrations. Subsequently, Vg1, Derriere, and several Nodal-related (XNr) proteins were shown to have similar inducing activities, while Bone Morphogenetic Proteins (e.g. BMP4) were shown to induce blood, mesenchyme and mesothelium at all concentrations.

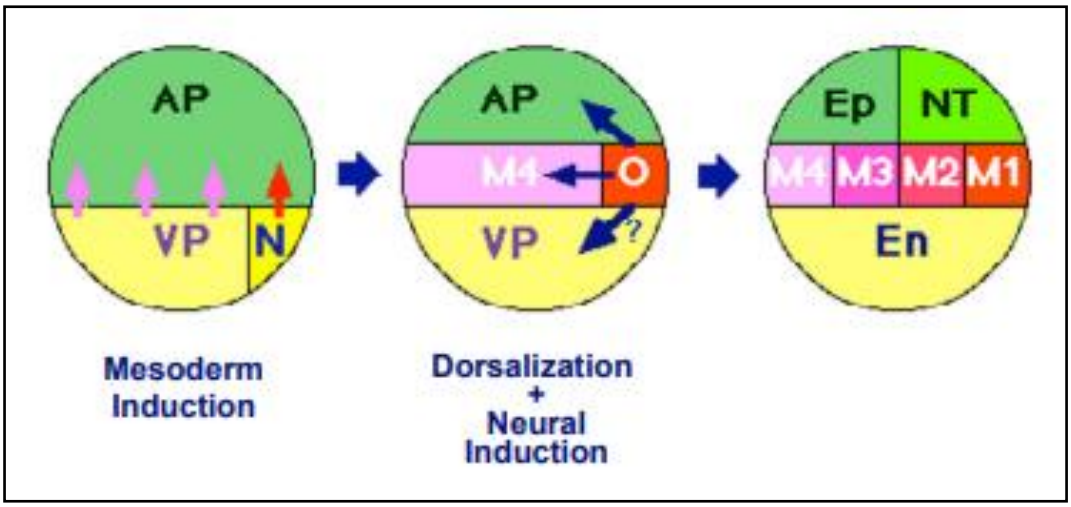
The concentration dependent effects of Activin were elegantly demonstrated by John Gurdon and his colleagues, who sandwiched beads soaked in Activin between two blastula stage animal caps. A few hours later they used in situ hybridisation to look at the expression pattern of *goosecoid (xgsc)* and *brachyury (xbra*) (section 2.1).



Xgsc was not expressed when low concentrations (1 nM) of Activin were used and xbra was localized to cells close to the beads. These cells expressed *xgsc* when high concentrations (4 nM) of Activin were used, while *xbra* was localized to cells further away. Radiolabelled Activin showed that this signalling molecule diffused away from the beads, forming a gradient with the highest concentration adjacent to the beads. The simplest interpretation is that low concentrations of Activin are sufficient to induce *xbra* expression and that higher concentrations are required to induce *goosecoid* expression. This suggests that the same molecule could be responsible for inducing both dorsal and ventral-type mesoderm: high concentrations secreted by the Nieuwkoop centre inducing dorsal-type mesoderm and low concentrations secreted by the remaining sectors inducing ventral-type mesoderm.

3.1, The Spemann Organizer

As described above (section 2.1), mesoderm inducing signals appear to establish just two territories within the marginal zone. A relatively small dorsal sector (90˚ of arc) that differentiates dorsal-type mesoderm (notochord) when isolated and a much larger ventrallateral sector that differentiates ventral-type mesoderm (blood) when isolated. The remaining mesodermal tissues - muscle, pronephros, and lateral plate - are specified by signals that are released from the dorsal mesoderm, a process known as dorsalization. The dorsal mesoderm also releases signals that induce the overlying ectoderm to form neural tissue, a process known as neural-induction.



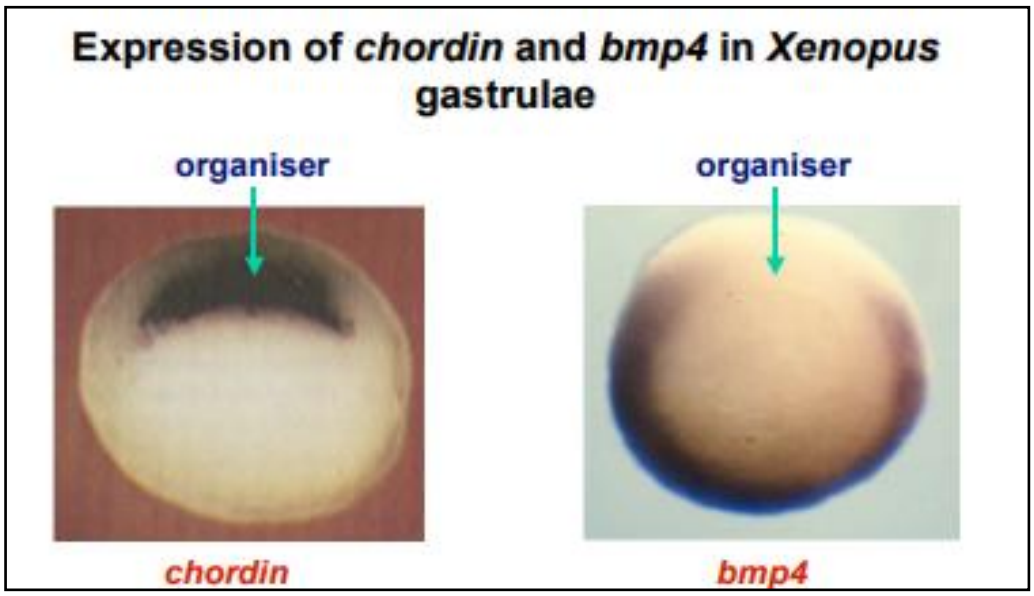
Dorsalization and neural-induction were first demonstrated in the experiments of Hans Spemann and Hilde Mangold (1924), who took the dorsal blastopore lip from an early newt gastrula and implanted it into the ventral side of a second, differently pigmented, host gastrula. The host developed a second dorsal axis, complete with neural tube, notochord, and somites. Within this axis the notochord and a few somite cells were derived from the donor, while the neural tube, and most of the somites, were derived from the host. Identical results have been obtained in Xenopus and the dorsal marginal zone ofthe amphibian gastrula is now known as the Spemann organizer. Similar axis inducing organizers have been identified in gastrulae from zebrafish (the shield), chicks (Hensen's node), and mice (the node) – like the amphibian organizer they all give rise to the notochord. Complete secondary axes, including heads, are only induced if the organizer is grafted at the beginning of gastrulation, at later stages secondary axes lack a head. This has lead to the suggestion that there are two organizers; a head organizer and a trunk organizer. As the mesoderm involutes, the head organizer moves away from the dorsal blastopore lip to be replaced by the trunk organizer.

3.2, Organizer signals

Following the discovery of the Spemann organizer, amphibian embryologists spent many years trying to identify the molecules involved. However, the technologies available at the time (1930-1960) were inadequate for the task and no progress was made. The first organizer signal was not identified until 1992, using molecular techniques that were only developed in the previous 10 years. Smith and Harland cloned organizer specific mRNAs and injected them into UV-irradiated Xenopus embryos. As described above (section 1.3), UV-irradiation of the vegetal pole soon after fertilization blocks cortical rotation and the formation of the Nieuwkoop centre, the resulting embryos lack all dorsal mesoderm. An mRNA that rescued dorsal development was identified and found to be localized to the Spemann organizer. It encodes a novel protein that Smith and Harland called Noggin. Injection of *noggin* mRNA into ventral

blastomeres induced a partial secondary axis that lack head structures, indicating that it mimicked the trunk organizer. Noggin protein dorsalized isolated ventral mesoderm and induced neural tissue in animal caps. Unfortunately, the amino acid sequence of Noggin provided few clues as to how it might work.

Two additional proteins, Chordin and Follistatin, with similar properties to Noggin were subsequently identified and their mRNAs were also found to be localized to the Spemann organizer.



Chordin induces a secondary dorsal axis (lacking head structures), while both Chordin and Follistatin neuralize animal caps. Inhibition studies (injecting antisense morpholino oligonucleotides that block translation of target mRNAs) have shown that all three proteins are necessary for the normal function of the organizer. Chordin and then Noggin are probably the most important, since they give the strongest phenotypes when depleted. However, depletion of all three proteins is necessary for full ventralization of the embryo. Follistatin was of special interest because its mode of action was known from mammalian physiology, where it binds to extracellular Activin. Follistatin bound Activin cannot activate its receptors. Subsequent studies showed that it also bound and inhibited BMP7, suggesting that a member of the TGF-ß family promoted ventral development in amphibian embryos and that dorsal development required inhibition of this signal by the organizer. It also suggested that Chordin and Noggin might act in a similar manner and biochemical studies demonstrated that they do indeed inhibit members of the TGFß family, in both cases BMP2 and BMP4 (but not Activin). Transcripts for bmp4 are uniformly expressed in Xenopus blastulae but are lost from the Spemann organizer and neural plate during gastrulation. Injection of bmp4 mRNA into the organizer causes a catastrophic loss dorsal structure, the resulting embryos only differentiating ventral mesoderm (blood) and ectoderm (epidermis). Moreover, a dominant negative BMP receptor (dnBMPR1), which specifically inhibits BMPs, dorsalizes *Xenopus* embryos. It induces a secondary dorsal axis, lacking head structures,

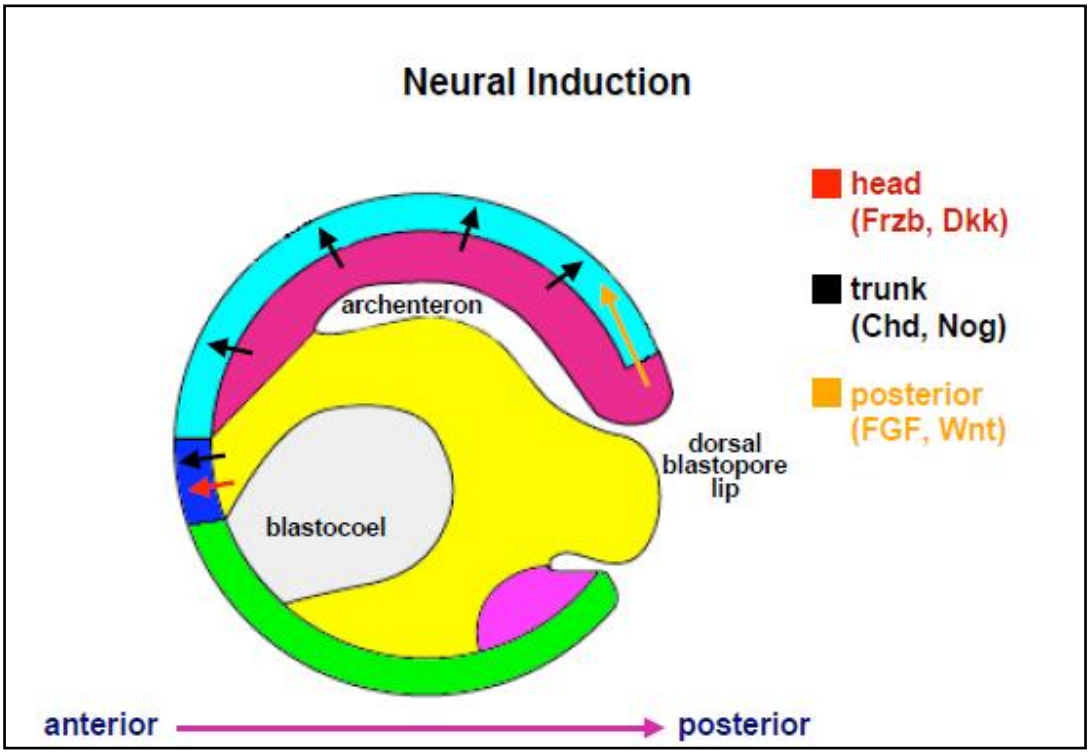
when expressed in ventral blastomeres and neural tissue when expressed in animal caps. BMP4, but not Activin, can also restore epidermal differentiation to dissociated animal caps, which would otherwise differentiate as neural tissue (In contrast to whole animal caps, which form epidermis, dissociated animal caps form neural tissue, presumably because an epidermalizing factor (BMP4) is diluted by dissociation). Taken together, the data suggested that BMP4 specifies ventral fates in both the ectoderm and mesoderm (and probably the endoderm as well) and that dorsal fates are activated in the absence of this signal. (In the nervous system this is known as the neural default model). Genetic studies in zebrafish support this conclusion. Mutations that disturb dorsal-ventral patterning during gastrulation always affect the BMP signalling pathway and either dorsalize (e.g. mutations in *bmp2* and *bmp7*) or ventralize (e.g. mutations in chordin) embryos.

3.3, Head-induction

Although BMP antagonists induce a secondary dorsal axis when expressed in ventral blastomeres, the axis always lacks the anterior regions of the head (although they frequently contain a hindbrain and otic vesicles). BMP antagonists therefore mimic the trunk organizer. How then does an embryo get its full head? Clearly the organizer must release additional signals. The hunt for genes specifically expressed in the Organizer identified ***frzb*, *cereberus*,** and ***dickkopf1* (*dkk1*)**, all of which appear to be required for head development. Transcripts for *cerberus* are localized to the anterior endoderm, while transcripts for *frzb* and *dkk1* are localized to the prechordal plate (mesoderm anterior to the notochord). Thus all three genes are specifically expressed in tissues that contribute to the head. They have only weak dorsalizing activity but they neuralize animal caps, cooperating with BMP inhibitors to promote strong neuronal differentiation. Coinjecting ***chordin*** and ***frzb*** mRNA gives secondary axes that are much more complete that than injecting *chordin* alone, with eyes usually being formed. However, the eyes are always cyclopic (fused across the midline), indicating that the most anterior regions (the forebrain) are still missing. Almost identical results are obtained

after coinjecting *chordin* and *dkk1* mRNAs, although the heads are often fully formed. Moreover, inhibition of Dkk1 function results in embryos that lack anterior head structures. Cerberus, on the other hand, will induce a fully formed head when injected alone. As described in section 2.3, Cerberus is a protein that binds, and inhibits, members of the Wnt, BMP, and Nodal families of extracellular signaling molecules, indicating that inhibition of Wnt and/or Nodal signaling, as well as BMP signaling, may be necessary for head induction. FrzB has a very similar amino acid sequence to the N-terminal domain of Frizzled receptors, which is known to be responsible for binding Wnt signaling molecules. This suggested that FrzB might be an inhibitor of Wnt signaling, binding and preventing Wnts from activating their receptors. Biochemical experiments proved that this was indeed the case and subsequent studies showed that Dkk1 also inhibits Wnt signalling. Thus inhibition of Wnt signallingappears to be the key event in head induction. Inhibition of Nodal signalling may also be required for full head

development, as suggested by experiments with the Nodal-binding domain of Cerberus (Cer-S). Embryos injected with Chordin, FrzB, and Cer-S have fully formed heads. The ability to inhibit BMP, Wnt, and Nodal signaling may explain the formation of a fully formed second head when *Cerberus* mRNA is injected into ventral blastomeres. These experiments suggest that inhibition of BMP, Nodal and Wnt signaling pathways are required for head induction in amphibians.



Although inhibiting BMPs induces only trunk tissues in axis duplication assays, it induces anterior (head) neural tissue in isolated animal caps. This paradox can be explained if the embryo produces posteriorizing signals that inhibit the development of anterior structures, transforming anterior into posterior fates. One candidate for a posteriorizing signal is Wnt3a, which blocks head development when over-expressed in *Xenopus* embryos. Wnt3a will also transform anterior neural tissue, induced in animal caps by Chordin, into posterior neural tissue. The role of Wnt inhibitors in head induction may be to prevent this transformation, allowing anterior neural tissue to differentiate. Other candidate molecules for posteriorizing signals include FGF4 and Retinoic Acid (RA). Both molecules transform anterior neural tissue into posterior neural tissue in animal caps lacking BMP signalling. *Fgf4* expression is localized to posterior and tail bud mesoderm, where it activates expression of the T-box transcription factor *xbra*. This gene has an essential role in the development of posterior structures, as evident from the "no tail" phenotype of mouse and zebrafish embryos mutant for the *brachyury* gene. A similar phenotype is seen in *Xenopus* embryos expressing a dominant-negative mutation for *xbra*. An almost identical phenotype is obtained with the dominant-negative FGF receptor, which blocks FGF4 signalling and

induction of *xbra* expression. (*xbra* is a direct target of FGF signalling in *Xenopus* blastulae and early gastrulae) FGF also activates expression of *Xenopus* caudal genes that in turn activate expression of posterior *hox* genes, such as *hoxb-9*. As a consequence, overexpression of FGF4 leads to more posterior specification of the nervous system and a reduction of anterior fates. A similar phenotype is also observed following addition of RA to embryo culture media during gastrulation. A posterior (high) to anterior (low) gradient of RA has been detected in the dorsal mesoderm of *Xenopus* neurulae, and high concentrations of RA have been shown to activate the expression of posterior *hox* genes.

Probable questions:

1. What is the early development of zebrafish?
2. Why are zebrafish used to study embryonic development?
3. Describe the early development in fish.
4. Describe the cleavage in fish eggs.
5. State the gastrulation in fish embryos.
6. Discuss about the formation of germ layers in fish embryos.
7. Discuss about the axis formation in fish embryos.
8. Describe the gastrulation in *C. elegans*.
9. 'Animal-vegetal polarity is maternally specified' in *Xenopus* –Explain.
10. What is cortical rotation?
11. State the importance of cortical rotation.
12. Write down the full form of Dsh and GBP.
13. Mention the role of Dsh protein in cortical roration.
14. Discuss various Mesoderm-Inducing-Factors and their role in *Xenopus* embryo.
15. What is Spemann Organizer?

Suggested reading:

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UNIT V

Model organism *C. elegans*: early embryonic development and major classes of molecules expressed, regional and genetic specification

Objective:

In this unit we will discuss about Model organism *C. elegans*: early embryonic development and major classes of molecules expressed, regional and genetic specification

Introduction

- ✓ The nematode worm *Caenorhabditis elegans* is a small (1 mm long), unsegmented, vermiform, free-living soil nematode.
- ✓ It is a relatively simple, and precisely structured organism, extensively used as a model organism for molecular and developmental biology.
- ✓ The body of an adult *C. elegans* hermaphrodite contains exactly 959 somatic cells, whose entire lineage has been traced through its transparent cuticle.
- ✓ Its genome has also been entirely sequenced, the first-ever for a multicellular organism.

Reasons for Selection *C. elegans* as Model Organism

- It has a rapid period of embryogenesis (about 16 hours), which it can accomplish in a petri dish.
- It has relatively few cell types.
- The predominant adult form is hermaphroditic, with each individual producing both eggs and sperm.
- Roundworms can reproduce either by self-fertilization or by cross-fertilization with the infrequently occurring males.
- Unlike vertebrate cell lineages, the cell lineage of C. *elegans* is almost entirely invariant from one individual to the next.
- *C. elegans* also has a small number of genes for a multicellular organism about 19,000.

- **What are the early stages of embryonic development?**

The early stages of embryonic development, such as fertilization, cleavage, blastula formation, gastrulation, and neurulation, are crucial for ensuring the fitness of the organism. Fertilization is the process in which gametes (an egg and sperm) fuse to form a zygote.

- **Cleavage and Axis Formation in *C. elegans***

Rotational cleavage of the *C. elegans* egg

The *C. elegans* zygote exhibits **rotational holoblastic cleavage** (Figure 8.42C).

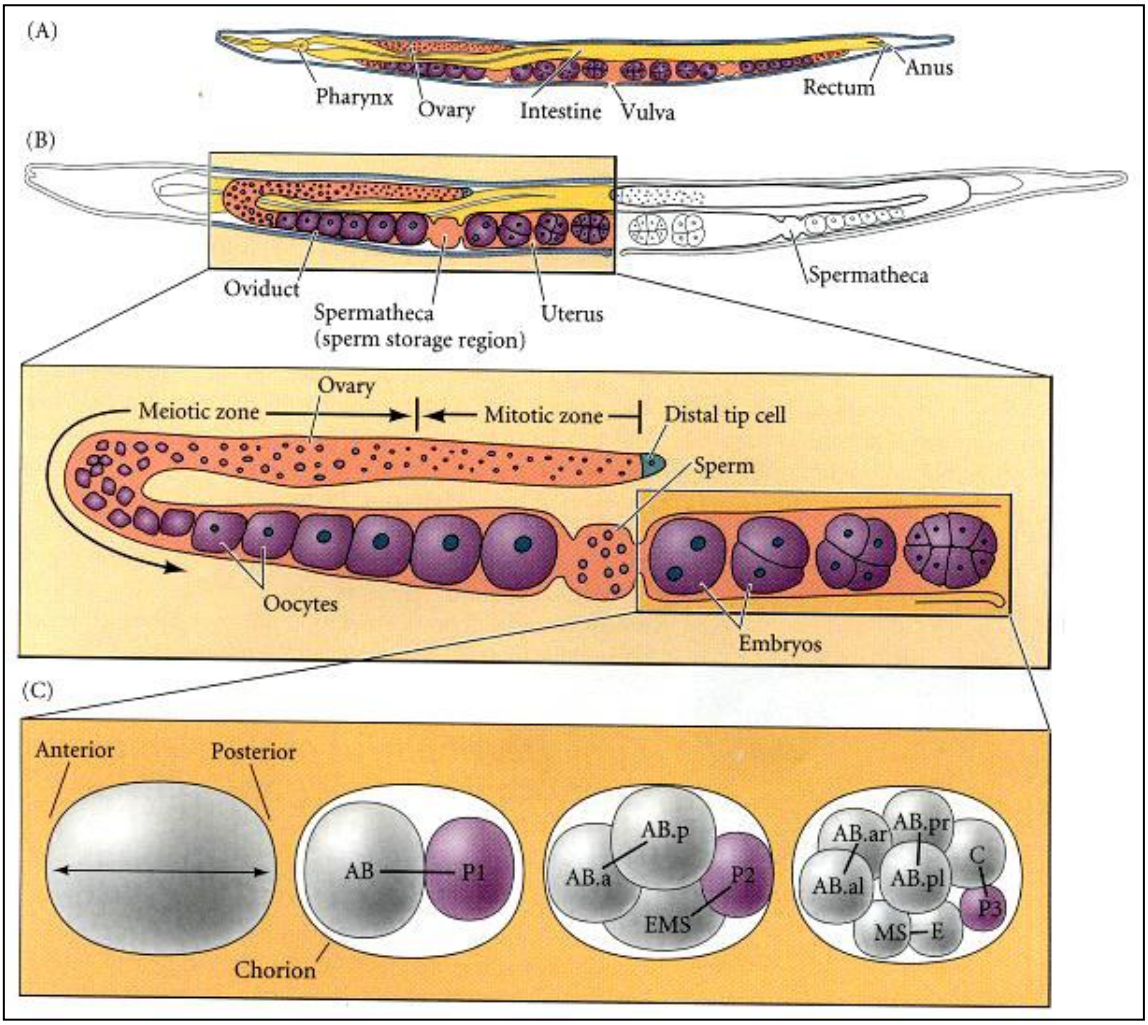

Figure 8.42

C. elegans. (A) Side view of adult hermaphrodite. Sperm are stored so that a mature egg must pass through the sperm on its way to the vulva. (B) The gonads. Near the distal end, the germ cells undergo mitosis. As they get further from the distal tip, they enter meiosis. Early meiosis forms sperm that are stored in the spermatheca. Later meioses form eggs that become fertilized as they roll through the spermatheca. (C) Early development, as the

egg is fertilized and moves toward the vulva. The p-lineage is stem cells that will eventually form the germ cells.

During early cleavage, each asymmetrical division produces one **founder cell** (denoted AB, MS, E, C, and D), which produces differentiated descendants, and one **stem cell** (the P1-P4 lineage). In the first cell division, the cleavage furrow is located asymmetrically along the anterior-posterior axis of the egg, closer to what will be the posterior pole. It forms a founder cell (AB) and a stem cell (P1). During the second division, the anterior founder cell (AB) divides equatorially (longitudinally; 90° to the anterior-posterior axis), while the P1 cell divides meridionally (transversely) to produce another founder cell (EMS) and a posterior stem cell (P2). The stem cell lineage always undergoes meridional division to produce (1) an anterior founder cell and (2) a posterior cell that will continue the stem cell lineage.

The descendants of each founder cell divide at specific times in ways that are nearly identical from individual to individual. In this way, the exactly 558 cells of the newly hatched larva are generated. The descendants of the founder cells can be observed through the transparent cuticle and are named according to their positions relative to their sister cells. For instance, ABal is the "left-hand" daughter cell of the Aba cell, and ABa is the "anterior" daughter cell of the AB cell.

Anterior-posterior axis formation

The elongated axis of the *C. elegans* egg defines the future anterior-posterior axis of the nematode's body. The decision as to which end will become the anterior and which the posterior seems to reside with the position of sperm pronucleus. When it enters the oocyte cytoplasm, the centriole associated with the sperm pronucleus initiates cytoplasmic movements that push the male pronucleus to the nearest end of the oblong oocyte. This end becomes the posterior pole (Goldstein and Hird 1996).

A second anterior-posterior asymmetry seen shortly after fertilization is the migration of the **P-granules**. P-granules are ribonucleoprotein complexes that probably function in specifying the germ cells. Using fluorescent antibodies to a component of the P-granules, Strome and Wood (1983) discovered that shortly after fertilization, the randomly scattered P-granules move toward the posterior end of the zygote, so that they enter only the blastomere (P1) formed from the posterior cytoplasm (Figure 8.43). The P-granules of the P1 cell remain in the posterior of the P1 cell and are thereby passed to the P2 cell when P1 divides. During the division of P2 and P3, however, the P-granules become associated with the nucleus that enters the P3 cytoplasm. Eventually, the P-granules will reside in the P4 cell, whose progeny become the sperm and eggs of the adult. The localization of the P-granules requires microfilaments, but can occur in the absence of microtubules. Treating the zygote with cytochalasin D (a microfilament inhibitor) prevents the segregation of these granules to the posterior of the cell,

whereas demecolcine (a colchicine-like microtubule inhibitor) fails to stop this movement (Strome and Wood 1983).

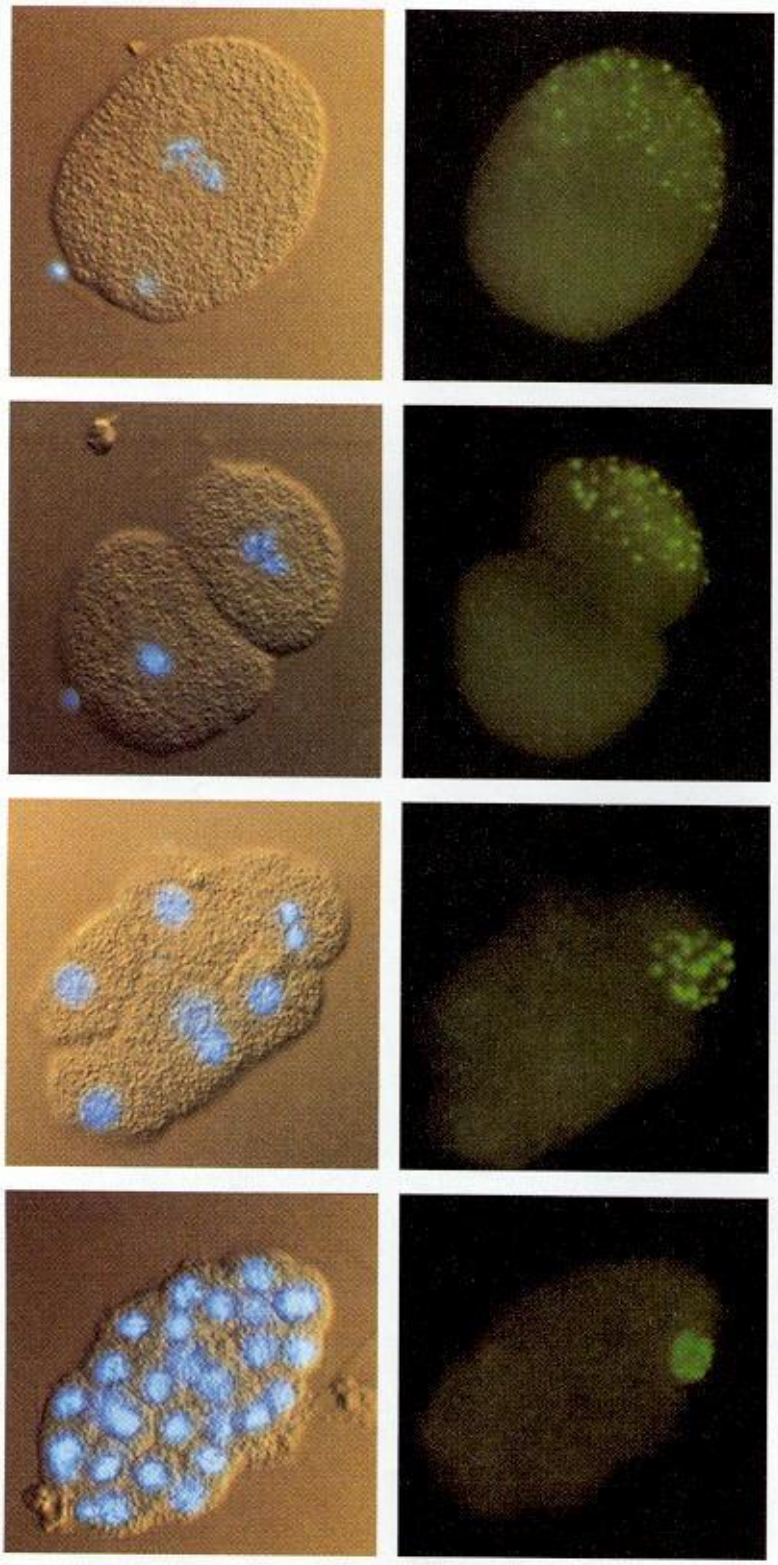

Figure 8.43

Segregation of the P-granules into the germ line lineage of the C. elegans embryo. The left column shows the cell nuclei (the DNA is stained blue by Hoescht dye), while the right

column shows the same embryos stained for P-granules. At each successive division, the P-granules enter into the P-lineage blastomere, the one that will form the germ cells. (*Photographs courtesy of S. Strome.)*

The partitioning of the P-granules and the orientation of the mitotic spindles are both deficient in those embryos whose mothers were deficient in any of the *par* (partition-defective) genes. The proteins encoded by these genes are found in the cortex of the embryo and appear to interact with the actin cytoskeleton (Bowerman 1999).

Formation of the dorsal-ventral and right-left axes

The dorsal-ventral axis of the nematode is seen in the division of the AB cell. As the AB cell divides, it becomes longer than the eggshell is wide. This causes the cells to slide, resulting in one AB daughter cell being anterior and one being posterior (hence their names, ABa and ABp, respectively). This squeezing also causes the ABp cell to take a position above the EMS cell that results from the division of the P1 blastomere. The ABp cell defines the future dorsal side of the embryo, while the EMS cell, the precursor of the muscle and gut cells, marks to future ventral surface of the embryo. The left-right axis is specified later, at the 12-cell stage, when the MS blastomere (from the division of the EMS cell) contacts half the "granddaughters" of the ABa cell, distinguishing the right side of the body from the left side (Evans et al. 1994).

Control of blastomere identity

C. elegans demonstrates both the conditional and autonomous modes of cell specification. Both modes can be seen if the first two blastomeres are experimentally separated (Priess and Thomson 1987). The P1 cell develops autonomously without the presence of AB. It makes all the cells that it would normally make, and the result is the posterior half of an embryo. However, the AB cell, in isolation, makes only a fraction of the cell types that it would normally make. For instance, the resulting ABa blastomere fails to make the anterior pharyngeal muscles that it would have made in an intact embryo. Therefore, the specification of the AB blastomere is conditional, and it needs the descendants of the P1 cell to interact with it.

Autonomous specification

The determination of the P1 lineages appears to be autonomous, with the cell fates determined by internal cytoplasmic factors rather than by interactions with neighboring cells. It is thought that protein factors might determine cell fate by entering the nuclei of the appropriate blastomeres and activating or repressing specific fate-determining genes. Have any transcription factors been found in autonomously determined cell lineages? While the P-granules of *C. elegans* are localized in a way

consistent with a role as a morphogenetic determinant, they do not enter the nucleus, and their role in development is still unknown. However, the SKN-1, PAL-1, and PIE-1 proteins are thought to encode transcription factors that act intrinsically to determine the fates of cells derived from the four P1-derived somatic founder cells, MS, E, C, and D.

The **SKN-1** protein is a maternally expressed polypeptide that may control the fate of the EMS blastomere, the cell that generates the posterior pharynx. After first cleavage, only the posterior blastomere, P1, has the ability to autonomously produce pharyngeal cells when isolated. After P1 divides, only EMS is able to generate pharyngeal muscle cells in isolation (Priess and Thomson 1987). Similarly, when the EMS cell divides, only one of its progeny, MS, has the intrinsic ability to generate pharyngeal tissue. These findings suggest that pharyngeal cell fate may be determined autonomously by maternal factors residing in the cytoplasm that is parceled out to these particular cells. Maternal effect mutants lacking pharyngeal cells, and isolated a mutation in the *skn-1* gene. Embryos from homozygous *skn-1*-deficient mothers lack both pharyngeal mesoderm and endoderm derivatives of EMS (Figure 8.44). Instead of making the normal intestinal and pharyngeal structures, these embryos seem to make extra hypodermal (skin) and body wall tissue where their intestine and pharynx should be. In other words, EMS appears to be respecified as C. Only those cells that are destined to form pharynx or intestine are affected by this mutation. Moreover, the protein encoded by the *skn-1* gene has a DNA-binding site motif similar to that seen in the bZip family of transcription factors (Blackwell et al. 1994).

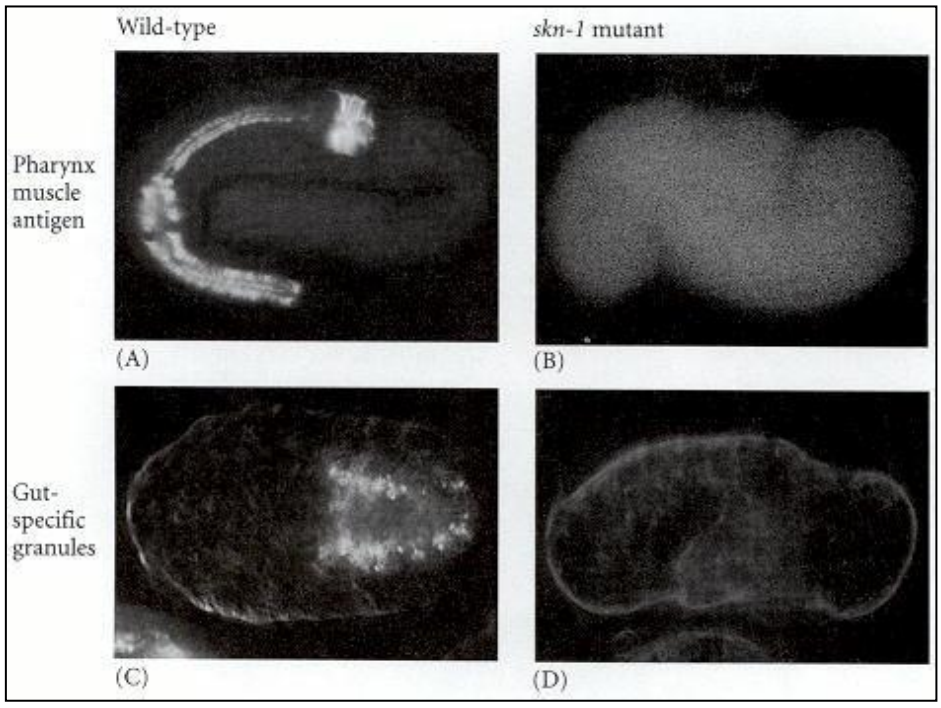

Figure 8.44

Deficiencies of intestine and pharynx in skn-1 mutants of C. elegans. Embryos derived from wild-type females (A, C) and females homozygous for mutant skn-1 (B, D) were tested for the presence of pharyngeal muscles (A, B) and gut-specific granules (C, D). A pharyngeal

muscle-specific antibody labels the pharynx musculature of those embryos derived from wild-type females (A), but does not bind to any structure in the embryos from skn-1 mutant females (B). Similarly, the birefringent gut granules characteristic of embryonic intestines (C) are absent from embryos derived from the skn-1 mutant females (D). (From Bowerman et al. 1992a; photographs courtesy of B. Bowerman.)

A second possible transcription factor, **PAL-1**, is also required for the differentiation of the P1 lineage. PAL-1 activity is needed for the normal development of the somatic descendants of the P2 blastomere. Thus, embryos lacking PAL-1 have no somatic cell types derived from the C and D stem cells (Hunter and Kenyon 1996). PAL-1 is regulated by the MEX-3 protein, an RNA-binding protein that appears to inhibit the translation of the *pal-1* mRNA. Wherever MEX-3 is expressed, PAL-1 is absent. Thus, in *mex-3*-deficient mutants, PAL-1 is seen in every blastomere. SKN-1 also inhibits PAL-1 (thereby preventing it from becoming active in the EMS cell).

A third putative transcription factor, **PIE-1**, is necessary for germ line fate. PIE-1 appears to inhibit both SKN-1 and PAL-1 function in the P2 and subsequent germ line cells. Mutations of the maternal *pie-1* gene result in germ line blastomeres adopting somatic fates, with the P2 cell behaving similarly to a wild-type EMS blastomere. The localization and the genetic properties of PIE-1 suggest that it represses the establishment of somatic cell fate and preserves the totipotency of the germ cell lineage (Mello et al. 1996)

Conditional specification

As we saw above, the *C. elegans* embryo uses both autonomous and conditional modes of specification. Conditional specification can be seen in the development of the endoderm cell lineage. At the 4-cell stage, the EMS cell requires a signal from its neighbor (and sister), the P2 blastomere. Usually, the EMS cell divides into an MS cell (which produces mesodermal muscles) and an E cell (which produces the intestinal endoderm). If the P2 cell is removed at the early 4-cell stage, the EMS cell will divide into two MS cells, and endoderm will not be produced. If the EMS cell is recombined with the P2 blastomere, however, it will form endoderm; it will not do so, however, when combined with ABa, ABp, or both AB derivatives (Figure 8.45)

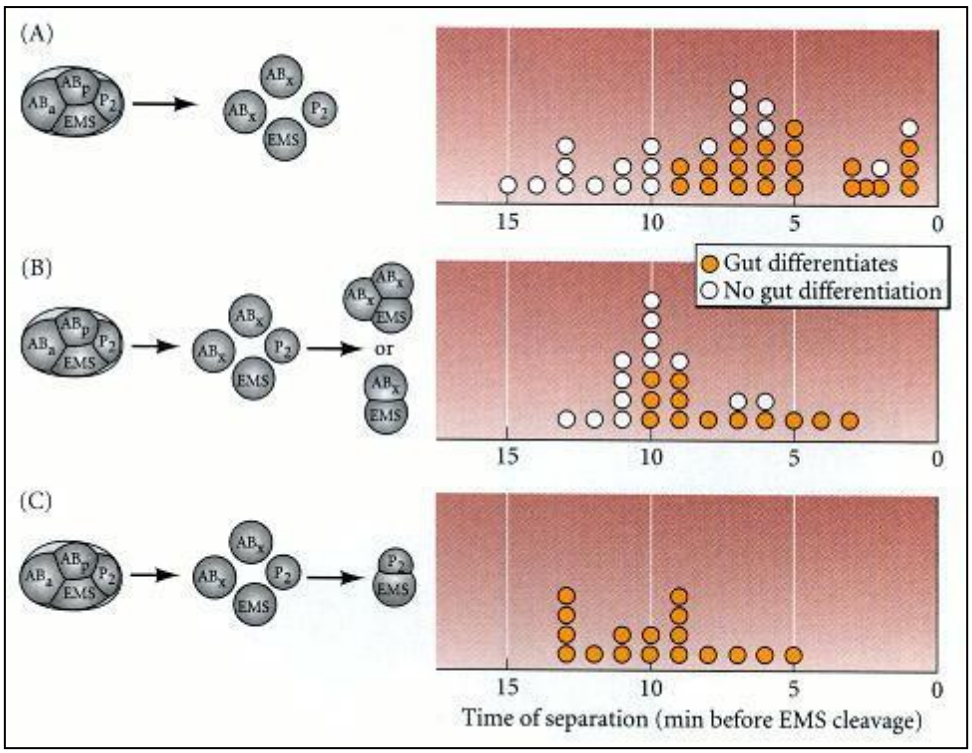

Figure 8.45

Results of isolation and recombination experiments, showing that cellular interactions are required for the EMS cell to form intestinal lineage determinants. (A) When isolated shortly after its formation, the EMS blastomere cannot produce gut-specific granules. If left in place for longer periods, it can. (B) If the EMS cell is recombined with either or both derivatives of the AB blastomere, it will not form gut-specific granules. (C) If recombined with the P2 blastomere, the EMS cell will give rise to gut-specific structures. (After Goldstein 1992.)

The P2 cell produces a signal that interacts with the EMS cell and instructs the EMS daughter that is next to it to become the E cell. This message is transmitted through the Wnt signaling cascade (Figure 8.46; Rocheleau et al. 1997). The P2 cell produces the *C. elegans* homologue of a Wnt protein, the MOM-2 peptide. The MOM-2 peptide is received in the EMS cell by the MOM-5 protein, the *C. elegans* version of the Wnt receptor protein, Frizzled. The result of this signaling cascade is to down-regulate the expression of the *pop-1* gene in the EMS daughter destined to become the E cell. In *pop-1*-deficient embryos, both EMS daughter cells become E cells (Lin et al. 1995).

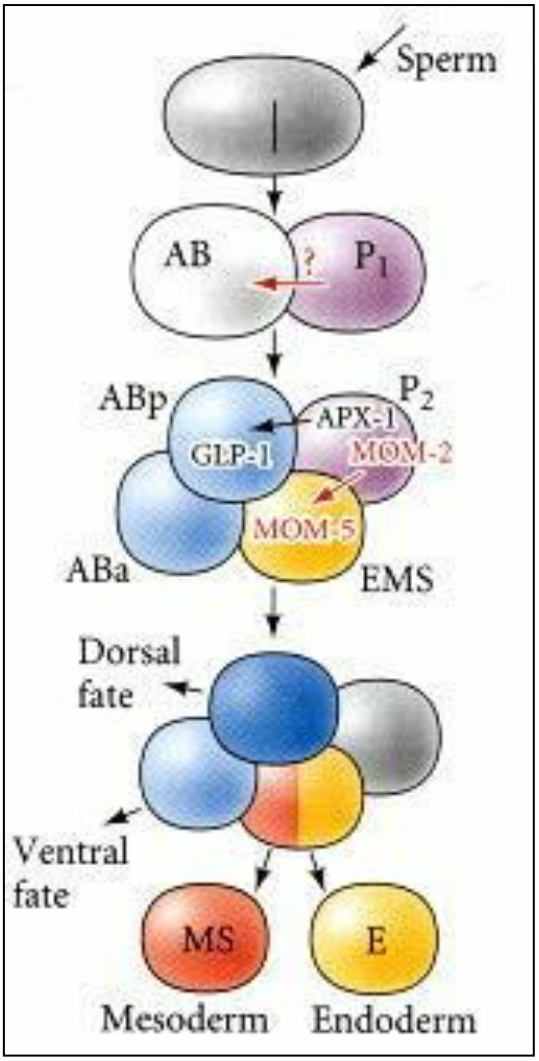

Figure 8.46

Cell-cell signaling in the 4-cell embryo of C. elegans. The P2 cell produces two signals: (1) the juxtacrine protein APX-1 (Delta), which is bound by GLP-1 (Notch) on the ABp cell, and (2) the paracrine protein MOM-2 (Wnt), which is bound by the MOM-5 (Frizzled) protein on the EMS cell. (After Han 1998.)

The P2 cell is also critical in giving the signal that distinguishes ABp from its sister, ABa. ABa gives rise to neurons, hypodermis, and the anterior pharynx cells, while ABp makes only neurons and hypodermal cells. However, if one experimentally reverses their positions, their fates are similarly reversed, and a normal embryo is formed. In other words, ABa and ABp are equivalent cells whose fate is determined by their positions within the embryo (Priess and Thomson 1987). Transplantation and genetic studies have shown that ABp becomes different from ABa through its interaction with the P2 cell. In an unperturbed embryo, both ABa and ABp contact the EMS blastomere, but only ABp contacts the P2 cell. If the P2 cell is killed at the early 4-cell stage, the ABp cell does not generate its normal complement of cells (Bowerman et al. 1992a). Contact between

ABp and P2 is essential for the specification of ABp cell fates, and the ABa cell can be made into an ABp-type cell if it is forced into contact with P2.

Moreover, these studies show that this interaction is mediated by the GLP-1 protein on the ABp cell and the APX-1 (anterior pharynx excess) protein on the P2 blastomere. In embryos whose mothers have mutant *glp-1,* ABp is transformed into an ABa cell (Mello et al. 1994). The GLP-1 protein is a member of a widely conserved family called the Notch proteins, which serve as cell membrane receptors in many cell-cell interactions, and it is seen on both the ABa and ABp cells. As mentioned in Chapter 5, one of the most important ligands for Notch proteins such as GLP-1 is another cell surface protein called Delta. In *C. elegans,* the Delta-like protein is APX-1, and it is found on the P2 cell (Mello et al. 1994). This APX-1 signal breaks the symmetry between ABa and ABp, since it stimulates the GLP-1 protein solely on the AB descendant that it touches, namely, the ABp blastomere. In doing this, the P2 cell initiates the dorsal-ventral axis of *C. elegans,* and it confers on the ABp blastomere a fate different from that of its sister cell.

- **Gastrulation in *C. elegans***

Gastrulation in *C. elegans* starts extremely early, just after the generation of the P4 cell in the 24-cell embryo (Skiba and Schierenberg 1992). At this time, the two daughters of the E cell (Ea and Ep) migrate from the ventral side into the center of the embryo. There, they will divide to form a gut consisting of 20 cells. There is a very small and transient blastocoel prior to the movement of the Ea and Ep cells, and their inward migration creates a tiny blastopore. The next cell to migrate through this blastopore is the P4 cell, the precursor of the germ cells. It migrates to a position beneath the gut primordium. The mesodermal cells move in next: the descendants of the MS cell migrate inward from the anterior side of the blastopore, and the C- and D-derived muscle precursors enter from the posterior side. These cells flank the gut tube on the left and right sides (Figure 8.47). Finally, about 6 hours after fertilization, the AB-derived cells that contribute to the pharynx are brought inside, while the **hypoblast** (hypodermal precursor) cells move ventrally by epiboly, eventually closing the blastopore. During the next 6 hours, the cells move and organize into organs, and the ball-shaped embryo stretches out to become a worm (see Priess and Hirsch 1986). This hermaphroditic worm will have 558 somatic cells. An additional 115 cells will have formed, but undergone apoptosis (see Chapter 6). After four molts, this worm will be a sexually mature adult, containing 959 somatic cells, as well as numerous sperm and eggs.

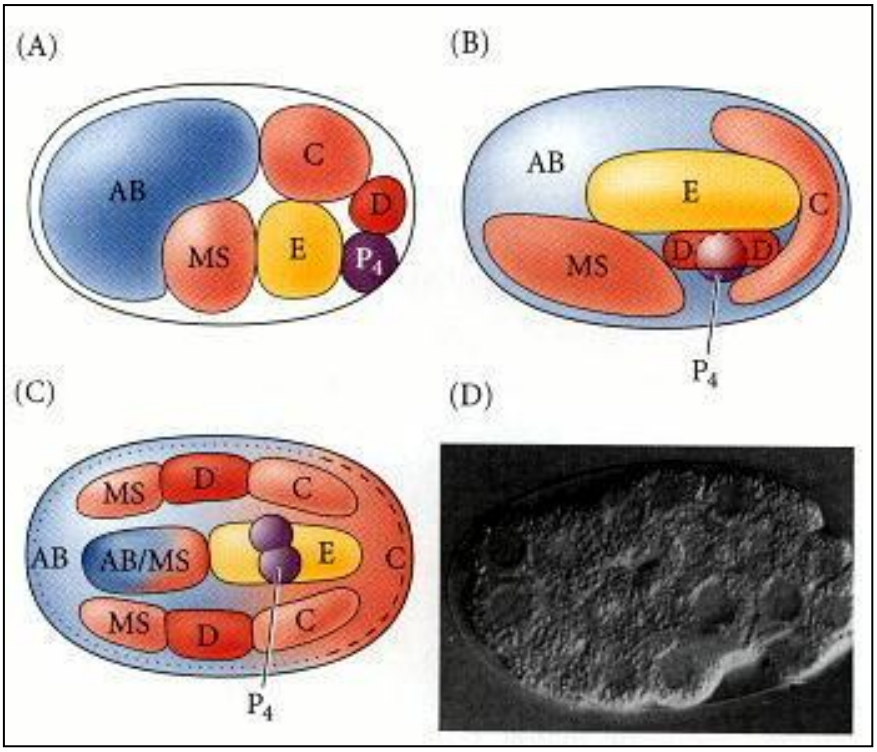

Figure 8.47

Gastrulation in C. elegans. (A) Positions of founder cells and their descendants at the 26-cell stage, just prior to gastrulation. (B) 102-cell stage, after the migration of the E, P4, and D descendants. (C) Positions of the cells near the end of gastrulation. The dotted and dashed lines represent regions of the hypodermis contributed by AB and C, respectively. (D) Early gastrulation, as the two E cells start moving inward. (After Schierenberg 1998; photograph courtesy of E. Schierenberg.)

Probable questions:

1. State the reasons for selection *C. elegans* as model organism
2. Describe the Cleavage in *C. elegans*.
3. Describe the Anterior-posterior axis formation in *C. elegans*.
4. Describe the dorsal-ventral and right-left axes formation in *C. elegans*.
5. Mention the role of SKN-1 protein in Autonomous specification in *C. elegans*.
6. Describe the role of different transcription factors in Autonomous specification in *C. elegans*.
7. Describe the events of Conditional specification in *C. elegans*.
8. Describe the gastrulation in *C. elegans*.

Suggested reading:

1. Bowerman B, Eaton B A, Priess J R. *Skn-1,* a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. Cell. 1992a; 68:1061–1075.
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5. Goldstein B, Hird S N. Specification of the anterioposterior axis in *Caenorhabditis elegans*. Development. 1996; 122:1467–1474.
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7. Han M. Gut reaction to Wnt signaling in worms. Cell. 1998; 90:581–584.
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10. Schierenberg, E. 1998. Nematodes, the roundworms. *In* S. F. Gilbert and A. M. Raunio (eds.), *Embryology: Constructing the Organism.* Sinauer Associates, Sunderland, MA, pp. 131–148.
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UNIT VI

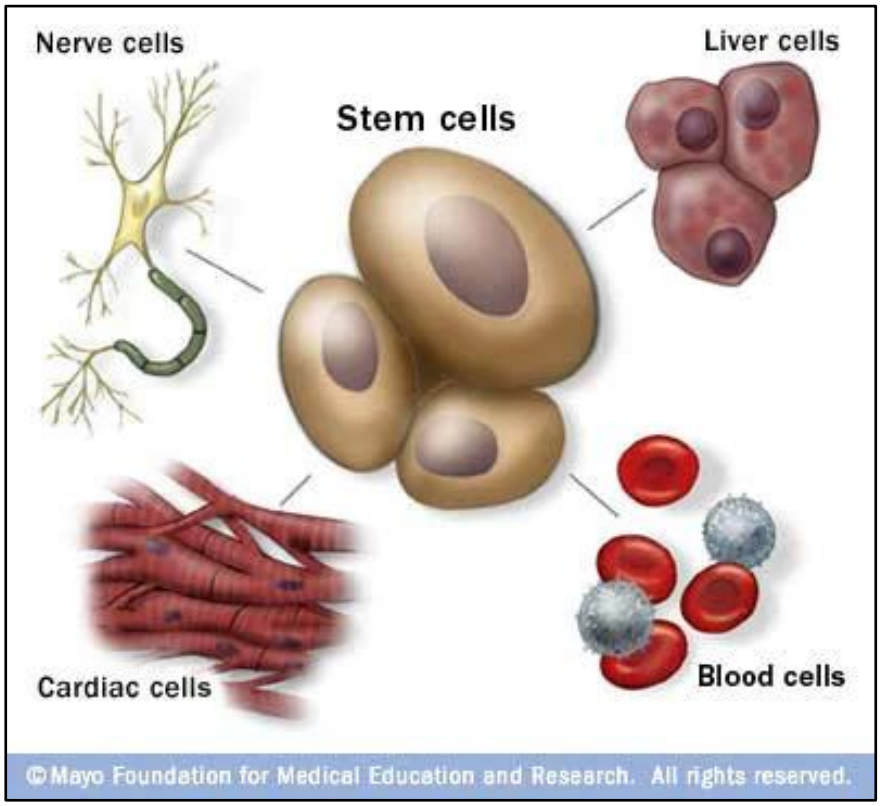
Stem cell: Application of adult stem cells, iPS cells, Stem cell niches, trans-differentiation

Objective:

In this unit we will discuss about Stem cell and application of adult stem cells, iPS cells, Stem cell niches, trans-differentiation.

Application of adult stem cells

Stem cells are the body's raw materials — cells from which all other cells with specialized functions are generated. Under the right conditions in the body or a laboratory, stem cells divide to form more cells called daughter cells.These daughter cells become either new stem cells or specialized cells (differentiation) with a more specific function, such as blood cells, brain cells, heart muscle cells or bone cells. No other cell in the body has the natural ability to generate new cell types.



Stem cell studies can help to:

- Increase understanding of how diseases occur. By watching stem cells mature into cells in bones, heart muscle, nerves, and other organs and tissue, researchers may better understand how diseases and conditions develop.
- Generate healthy cells to replace cells affected by disease (regenerative medicine). Stem cells can be guided into becoming specific cells that can be used in people to regenerate and repair tissues that have been damaged or affected by disease.
- People who might benefit from stem cell therapies include those with spinal cord injuries, type 1 diabetes, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, heart disease, stroke, burns, cancer and osteoarthritis.
- Stem cells may have the potential to be grown to become new tissue for use in transplant and regenerative medicine. Researchers continue to advance the knowledge on stem cells and their applications in transplant and regenerative medicine.
- Test new drugs for safety and effectiveness. Before using investigational drugs in people, researchers can use some types of stem cells to test the drugs for safety and quality. This type of testing will most likely first have a direct impact on drug development for cardiac toxicity testing.
- New areas of study include the effectiveness of using human stem cells that have been programmed into tissue-specific cells to test new drugs. For the testing of new drugs to be accurate, the cells must be programmed to acquire properties of the type of cells targeted by the drug. Techniques to program cells into specific cells are under study. For instance, nerve cells could be generated to test a new drug for a nerve disease. Tests could show whether the new drug had any effect on the cells and whether the cells were harmed.

Applications of stem cells: For any disease condition, there are two possible approaches to treatments. Treatment by drugs with or without surgical intervention. The second type of treatmentapproach is through regenerative remedies. This second option is rare, applicable to fewof the diseases where drugs cannot mend the situation andare not able to restore or regenerate the normal functionof the damaged tissue or organ. Regenerative remedies arecommonly applied through stem cell therapy and to someextent by organ transplantation. The stem cells dependingupon their nature of origin can differentiate into many orspecific kind of mature cell type to rebuild the tissue. Adult stem cells have the most potential and are thesafest for therapeutic utilization. Stem cell therapy is extensively used in clinic for curing hematological malignancies.However, for many other dreadful life threatening diseases,stem cell therapy is still in experimental phase and requiresmajor effort to bring them into clinical practice.

Cartilage defects and/or degeneration resulting frominjury, aging, and osteoarthritis are a major cause of jointpain and disability that seriously affect quality of life. Current surgical or pharmacological treatments canonly help in temporary relief and delay in disease progression. In recent years, new strategies have been devised to repair thedamaged cartilage using adult stem cells. *In vivo* studies on adipose derived mesenchymal stemcells for the treatment of articular cartilage defect has been reported. However, few researchers have reviewed the currentstatus and future developments of adult stem cell therapytowards cartilage tissue engineering. Both these techniqueshave elucidated the promises and limitations of stem celltransplantation for cartilage defect.

Intracerebral hemorrhage (ICH) caused by suddenincrease in blood pressure is not as common as ischemicstroke but is more serious and quickly causes brain andnerve damage. Depending on the location of hemorrhage andamount of injury long term treatments including physical, speech, and occupational therapy are applied but majority survive with some kind of permanent disability. In recent years, stem cell transplantation as well as cell engineering has shown promising results for various neurological diseases and regeneration of injured nerves. Application of suchtherapies assessed the safetyprofile, feasibility, and effectiveness of surgery combinedwith autologous bone marrow stromal cells (BMSC) transplantation for treating ICH.This kind of study indisputably showed encouragingclinical outcome. Application of stem cell in treating spinalcord injury has also been found to be very encouraging.

The most demanding area for stem cell application is the incurable neurodegenerative disorders suchas Alzheimer's disease, Parkinson disease, and Huntingtondisease. Though etiology and symptoms of these diseasesare well characterized, the underlying mechanism is yet tobe understood. Therefore, current treatments majorly aimtowards delaying progression of the disease. Lack of appropriate animal models simulating the human pathogenesis alsodemands development of human disease-specific models toidentify new drugs. Induced pluripotent stem cells or iPSCshave revolutionized the field of regenerative medicine and arenow being utilized as models for neurodegenerative disordersto understand the biology of pathogenesis and screen noveltherapeutics.

A major thrust in stem cell research is to experimentnew approaches for efficient and lineage specific differentiation of stem cells. Researchers have demonstrated that priming of mesenchymal stem cells (MSCs) with endothelial growth medium improvestherapeutic efficacy in the treatment of systemic arterialhypertension in a rat model. Enhanced differentiation ofneuronal stem cells by Oleanolic Acid (OA) were evaluated where it was found that Nkx-2.5 transcription factorpartially regulates this differentiation process. It was even studied that*in vitro* Strontium treatment of hADSCs enhances cell proliferation and osteogenic differentiation through expression of early and late osteoblasticbiomarkers such as ALP and HA, respectively. This studyclearly supports the use of Strontium in *in vitro* induction ofbone regeneration.The placenta, amniotic fluid, and umbilical cord areknown to be rich source for neonatal MSCs.

Cryopreservation of umbilical cord immediately after birth has alreadybeen commercial practice to tackle future life-threateningdiseases. However, such practice must be dealt with careand properly designed experiments.

There are several sources of stem cells:

1. Embryonic stem cells. These stem cells come from embryos that are 3 to 5 days old. At this stage, an embryo is called a blastocyst and has about 150 cells.These are pluripotent (ploo-RIP-uh-tunt) stem cells, meaning they can divide into more stem cells or can become any type of cell in the body. This versatility allows embryonic stem cells to be used to regenerate or repair diseased tissue and organs.

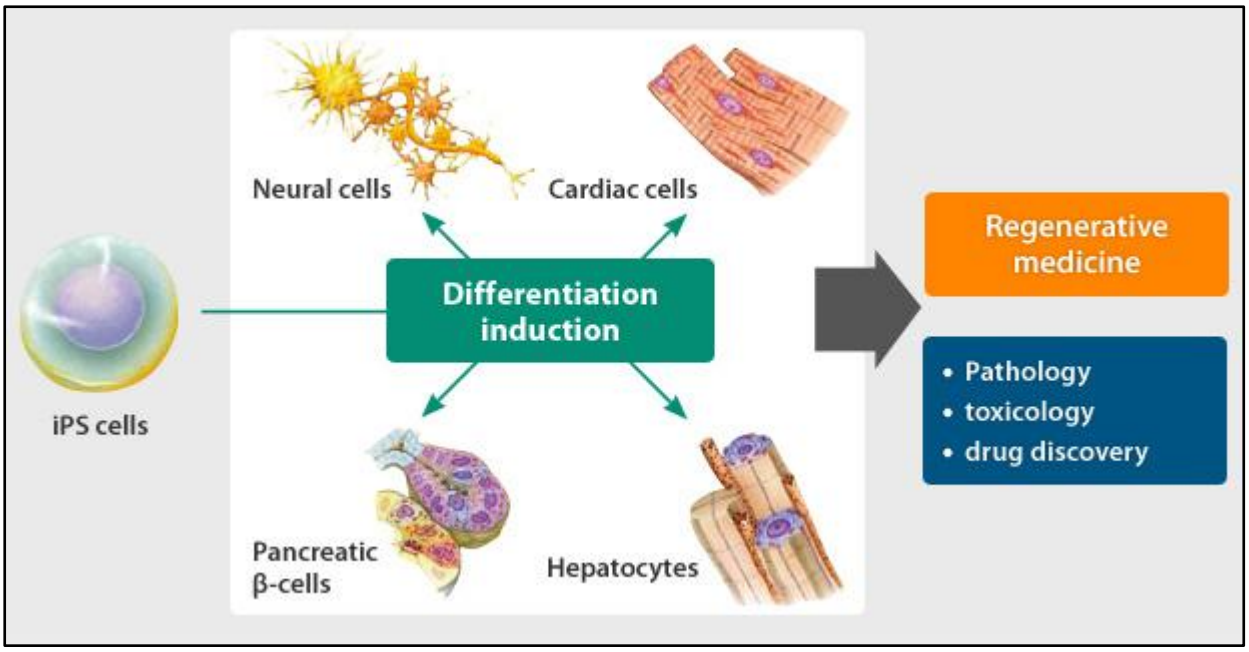
2. Adult stem cells. These stem cells are found in small numbers in most adult tissues, such as bone marrow or fat. Compared with embryonic stem cells, adult stem cells have a more limited ability to give rise to various cells of the body.Until recently, researchers thought adult stem cells could create only similar types of cells. For instance, researchers thought that stem cells residing in the bone marrow could give rise only to blood cells. However, emerging evidence suggests that adult stem cells may be able to create various types of cells. For instance, bone marrow stem cells may be able to create bone or heart muscle cells. This research has led to early-stage clinical trials to test usefulness and safety in people. For example, adult stem cells are currently being tested in people with neurological or heart disease.Adult cells altered to have properties of embryonic stem cells. Scientists have successfully transformed regular adult cells into stem cells using genetic reprogramming. By altering the genes in the adult cells, researchers can reprogram the cells to act similarly to embryonic stem cells.This new technique may allow use of reprogrammed cells instead of embryonic stem cells and prevent immune system rejection of the new stem cells. However, scientists don't yet know whether using altered adult cells will cause adverse effects in humans.Researchers have been able to take regular connective tissue cells and reprogram them to become functional heart cells. In studies, animals with heart failure that were injected with new heart cells experienced improved heart function and survival time.Although research into adult stem cells is promising and adult stem cells are more adaptable, adult stem cells may not be as versatile and durable as are embryonic stem cells. Adult stem cells may not be able to be manipulated to produce all cell types, which limits how adult stem cells can be used to treat diseases. Adult stem cells are also more likely to contain abnormalities due to environmental hazards, such as toxins, or from errors acquired by the cells during replication.

3. Perinatal stem cells. Researchers have discovered stem cells in amniotic fluid as well as umbilical cord blood. These stem cells have the ability to change into specialized cells.Amniotic fluid fills the sac that surrounds and protects a developing fetus in the uterus. Researchers have identified stem cells in samples of amniotic fluid drawn from pregnant women for testing or treatment — a procedure called amniocentesis.

- **iPS cells**

Induced pluripotent stem (iPS) cells, are a type of pluripotent stem cell derived from child or adult somatic cells(usually skin or blood). They have been reprogrammed and genetically modified through inducing genes and factors to be pluripotent. The basic paradigm in the use of iPS cells for cell therapypurposes is that they are first differentiated into the desired cell types of interest, and the resulting specializedtissue-specific cells are then transplanted as cell suspensions or more complex tissue constructs into patients. Mouse iPS cells from mouse fibroblasts were first reported in 2006 by Yamanaka at Kyoto University. Human iPS cells were first independently produced by Yamanaka and Thomson groups from human fibroblasts in late 2007. iPS cells are similar to embryonic stem (ES) cells in many aspects, including the expression of ES cell markers, chromatin methylation patterns, embryoid body formation, teratoma formation, viable chimera formation, pluripotency and the ability to contribute to many different tissues *in vitro*.iPS cells generated from patient cells can be used to create cells that replicate the tissue affected by disease. This technique offers the prospect of using iPS cells to test drug efficacy, side effects and toxicity and to develop new drugs and therapies.As the name implies, these cells are pluripotent, which means that they have the ability to form all adult cell types.Hence, iPSCs do not exist naturally and are instead generated ("induced" or "reprogrammed") in culture from somatic cells through ectopic co-expression of defined pluripotency factors.The most important disadvantage of iPS cell is the use of retroviruses to generate iPSCs as they are associated with cancer. More specifically, retroviruses can insert their DNA anywhere in the genome and subsequently trigger cancer-causing gene expression. A better understanding of epigenetic alterations and transcriptional activity associated with the induction of pluripotency and following differentiation is required for efficient generation of therapeutic cells. Furthermore, a long-term safety data must be obtained to use human iPScell based cell therapy for treatment of disease.

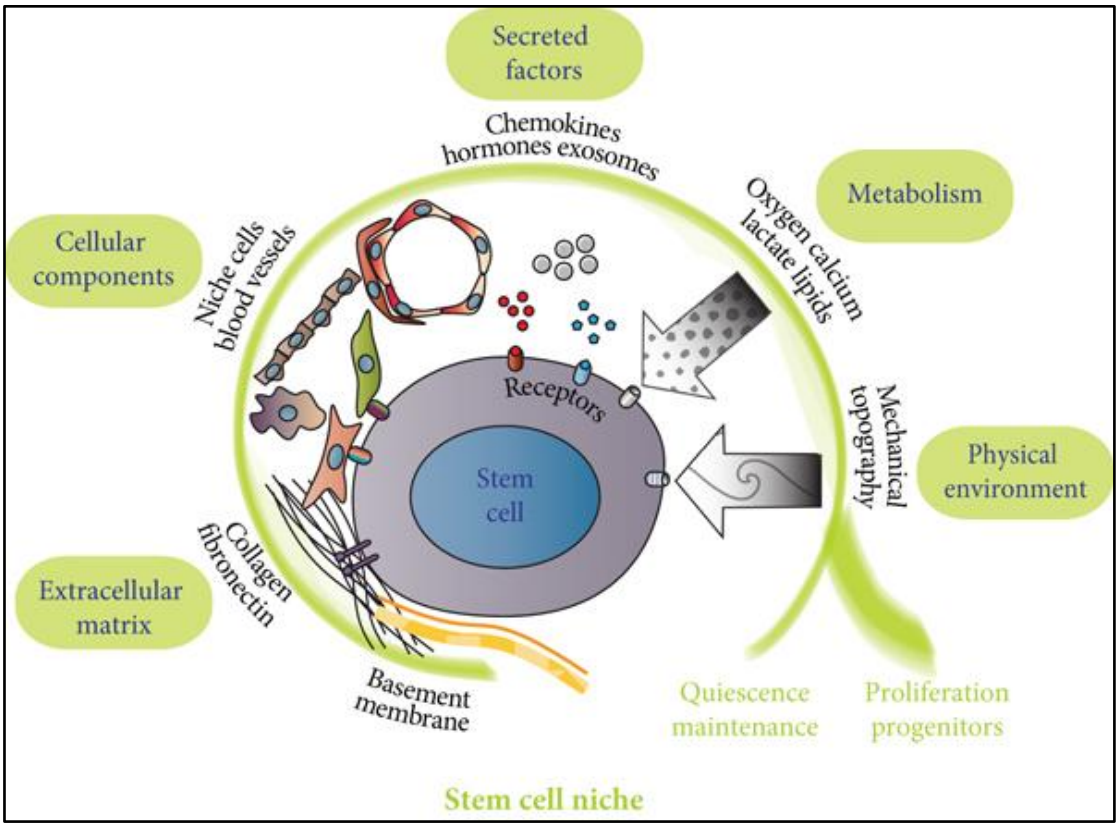
Ethical, legal, and social issues in using iPSCs fortherapy: Many guidelines for human experimentations such asthe Nuremberg Code (1947) and the related Declarationof Helsinki as well as the Belmont Report (1978) havebeen put forward to restrict unethical researchwith human subjects. Notably, the lattertwo guidelines are widely regarded as the cornerstonedocuments on human research ethics today. Althoughthese guidelines still apply today, things have changedand new possibilities have emerged.



(Courtesy: www.cira.kyoto-u.ac.jp)

A major challenge is/willbe, however, that such ethical rules must be acknowledged and abided by on global terms and not only in individual countries that all make up their own rules andethics. Therefore, with the advancement of technologyin the field of biomedicine and the emergence of newfields such as stem cell research and genome editing,these new technologies require a set of new specific rulesto be included in the regulations to enable their application in these broad fields, and specifically in the area ofregenerative medicine.Given that in cell therapy, the cells are injected intopatients as a live component with complex features andfunctions, applying the same regulations of drug therapyto this topic is not feasible. Therefore, a separate set ofrules and conditions is required for using cells, particularly stem cells, in cell-replacement therapies. There arecurrently several guidelines for the use of cell, tissue,and stem-cell products in treating patients, most notablythe US Food and Drug Administration (FDA) andthe European Medicines Agency (EMA) guidelines(https://www.ema.europa.eu/documents/scientific-guideline/guideline-human-cell-basedmedicinal-products_en.pdf). Specialist associations such as the International Society for Stem Cell Research (ISSCR) have also separatelydeveloped or updated specific guidelines for the use ofstem cells in cell therapy by the help of experts from allaround the world. These guidelines share many important outlines and differ from each other mostly inminor issues. According to them, the most importanttopics related to ethical, legal, and social considerationsof cell therapy include (i) manufacturing conditions and characterization of clinical-grade cells, (ii) genetic material and confidential personal information, (iii) informedconsent, (iv) genetic manipulation of the cells, and (v) intellectual property and patents, along with some other important issues.

- **Stem cell niches**



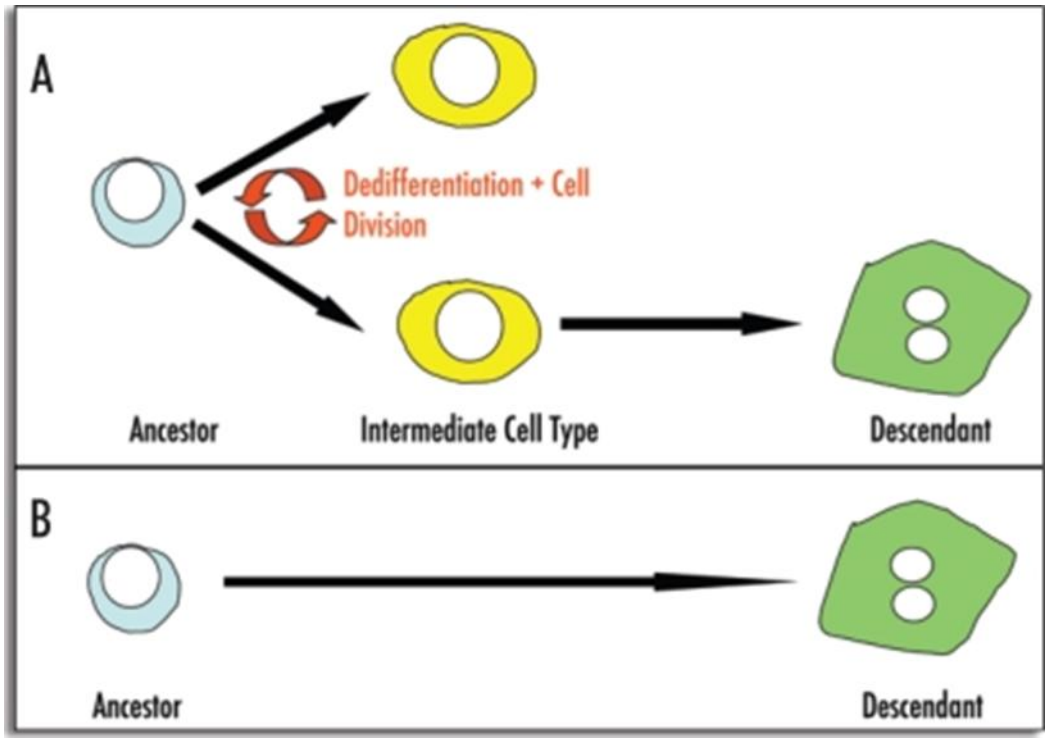
Stem-cell niche refers to a microenvironment, within the specific anatomic location where stem cells are found, which interacts with stem cells to regulate cell fate. The word 'niche' can be in reference to the *in vivo* or *in vitro* stem-cell microenvironment. During embryonic development, various niche factors act on embryonic stem cells to alter gene expression, and induce their proliferation or differentiation for the development of the fetus. Within the human body, stem-cell niches maintain adult stem cells in a quiescent state, but after tissue injury, the surrounding micro-environment actively signals to stem cells to promote either self-renewal or differentiation to form new tissues. Several factors are important to regulate stem-cell characteristics within the niche: cell–cell interactions between stem cells, as well as interactions between stem cells and neighboring differentiated cells, interactions between stem cells and adhesion molecules, extracellular matrix components, the oxygen tension, growth factors, cytokines, and the physicochemical nature of the environment including the pH, ionic strength (e.g. Ca2+ concentration) and metabolites, like ATP, are also important. The stem cells and niche may induce each other during development and reciprocally signal to maintain each other during adulthood.Scientists are studying the various components of the niche and trying to replicate the *in vivo* niche conditions *in vitro*. This is because for regenerative therapies, cell proliferation and differentiation must be controlled in flasks or plates, so that sufficient quantity of the proper cell type are

produced prior to being introduced back into the patient for therapy.Human embryonic stem cells are often grown in fibroblastic growth factor-2 containing, fetal bovine serum supplemented media. They are grown on a feeder layer of cells, which is believed to be supportive in maintaining the pluripotent characteristics of embryonic stem cells. However, even these conditions may not truly mimic *in vivo* niche conditions.

Adult stem cells remain in an undifferentiated state throughout adult life. However, when they are cultured *in vitro*, they often undergo an 'aging' process in which their morphology is changed and their proliferative capacity is decreased. It is believed that correct culturing conditions of adult stem cells needs to be improved so that adult stem cells can maintain their stemness over time.

- **Trans-differentiation**

Transdifferentiation is defined as the irreversible conversion of one cell type to another. It belongsto a wider class of cell type transformations called metaplasias which also includes casesin which stem cells of one tissue type switch to a completely different stem cell.



Transdifferentiation is associated with a discrete change in the programme of gene expression and there is a direct ancestor-descendant relationship between the two cell

types. At the molecular level, the cause of transdifferentiation is presumably a change in the expression of a master switch gene (selector or homeotic gene), whose normal function is to distinguish the two cell types in normal development. The term 'transdifferentiation' was first introduced by Selman and Kafatos to describe the transformation of the cuticle-producing cells to salt-secreting cells in the silk moth during metamorphosis from the larval to the adult moth. Numerous examples of transdifferentiation exist within the literature. For example, isolated striated muscle of the invertebrate jellyfish (Anthomedusae) has enormous transdifferentiation potential and even functional organs (e.g., tentacles and the feeding organ (manubrium)) can be generated in vitro. In contrast, the potential for transdifferentiation in vertebrates is much reduced, at least under normal (nonpathological) conditions. But despite these limitations, there are some well-documented cases of transdifferentiation occurring invertebrates. For example, in the newt, the lens of the eye can be formed from the epithelial cells of the iris. Other examples of transdifferentiation include the appearance ofhepatic foci in the pancreas, the development of intestinal tissue at the lower end of theoesophagus and the formation of muscle, chondrocytes and neurons from neural precursorcells. Although controversial, recent results also suggest the ability of adult stem cells fromdifferent embryological germlayers to produce differentiated cells e.g., mesodermal stem cells forming ecto- or endodermally-derived cell types. This phenomenon may constitutean example of metaplasia.

- **Clinical significance of transdifferentiation**

Understanding the individual molecular and cellularsteps leading to the identification of early markers suitable for diagnostic purposes or even in the production of therapeutic strategies for curing the disease. One such clinical application is for oesophageal adenocarcinoma, a condition that has been rising rapidly in recent years.

Cell-based therapies- Cellular therapies are now part of the newly emergingscience of regenerative medicine. The term 'regenerative medicine'refers to the stimulation of regeneration of damaged or defectivetissues. Regeneration of diseased or damaged organs constitutes oneof the fundamental challenges to tissue engineers. The reasons forwishing to produce tissues are simple enough: due to the shortage fortransplantation alternative strategies have to be found to replacediseased or damaged organs. There is currently intense interest in thefield of regenerative medicine because research findings from thisarea may turn out to be the panacea for a spectrum of degenerativedisorders including Parkinson's disease, diabetes and heart disease.

Probable questions:

1. What is stem cell?
2. Mention different sources from where stem cells can be achieved?
3. Mention the applications of Stem cell.
4. What is iPS cell?
5. What do you mean by stem cell niche?
6. What is trans-differentiation?
7. State the clinical significance of transdifferentiation.

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