GENETIC CONTROL OF EMBRYONIC DEVELOPMENT

ESTABLISHING CELL ASYMMETRIES – Lecture 1

1. Characteristics of Cell Differentiation

The differentiated state is stable (e.g., neurons vs. the lac operon in bacteria). Pattern formation: the difference between an arm and a leg is "not in the ingredients, it's in how they are mixed". The same genes that control development control body form and evolution. Cells are the true miracle of evolution. Once the basic building block, the eukaryotic cell, became available, the form of metazoans evolved by changing the arrangement of cells with respect to each other.

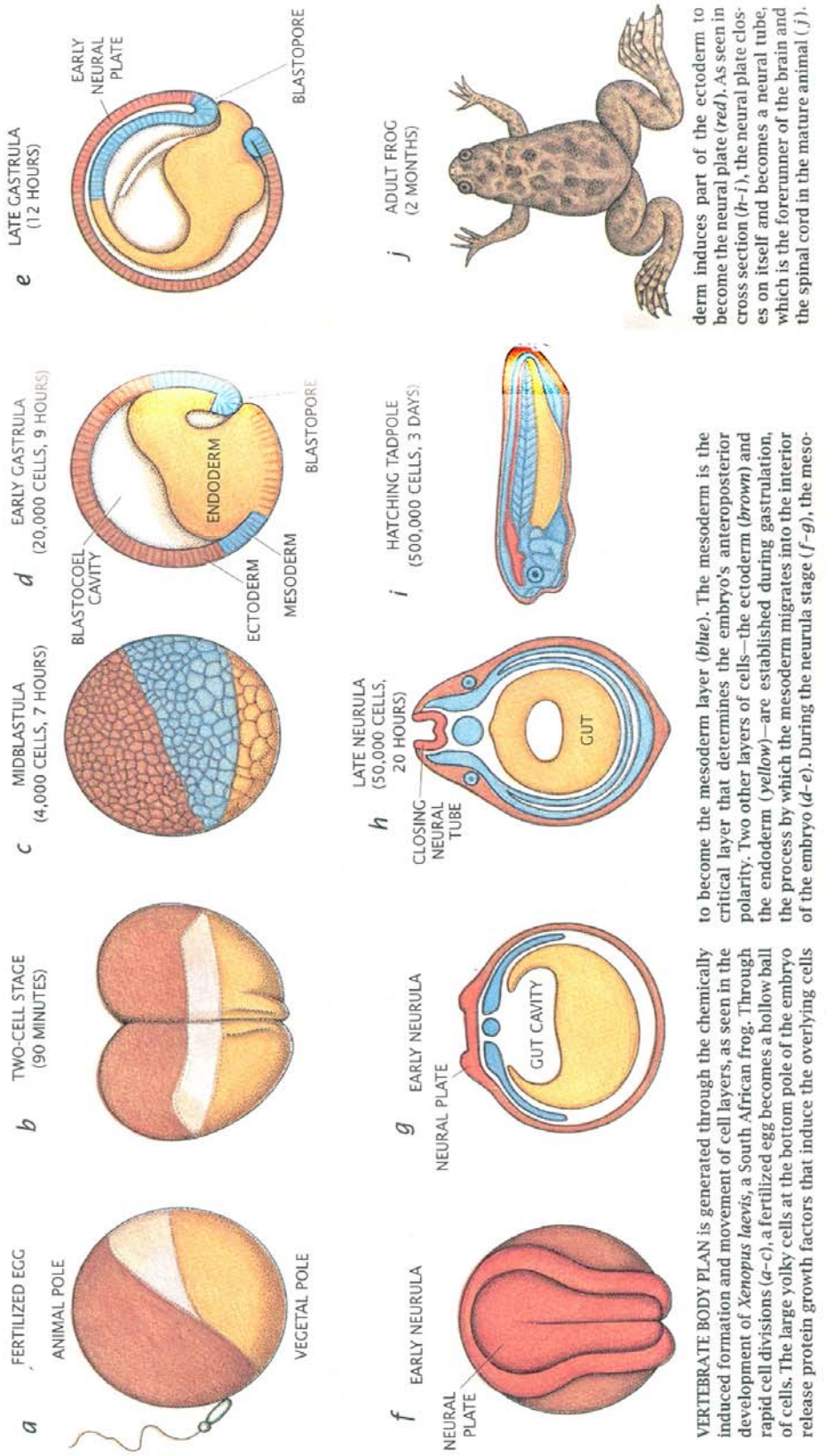
Differences between cells first arise as a result of two broad mechanisms: 1) **cytoplasmic determinants**, which are molecules asymmetrically localized in the cytoplasm of the egg (or of somatic cells, see below), which become unequally distributed among cells after cell division and then affect the activity of genes. 2) **cell-cell interactions**, in which cells induce new fates on their neighbors. Both mechanisms are used over and over in the course of development.

2. Early development is so rapid that many important molecules must be made during oogenesis and stored in the egg.

Eggs stockpile materials required for early development. In *Xenopus*, the rapid rate of division during early development allows little time for new synthesis. A female lays 1500 eggs and each is 1.2 mm in diameter. The first 12 cleavages take place synchronously every 30 minutes (compared to 1-2 days for eukaryotic cells and 20 min. for *E.coli*). This rapid pace of division is achieved through an increased number of replication origins and eliminating the G1 and G2 phases of the cell cycle. At the 4000 cell stage, or mid blastula transition (MBT), the cells start to divide slower and asynchronously, and start synthesizing RNA.

There is no RNA synthesis until MBT, but the initial differences between cells are already laid down by this stage. These decisions are made using maternal molecules (determinants) already present in the egg. The marginal zone (equatorial region) gives rise to mesoderm. The mesoderm involutes through the circular blastopore, starting on the dorsal side. The blastopore gives rise to the anus in deuterostomes (and to the mouth in protostomes). The involuted mesoderm (and possibly endoderm too), induces the ectoderm to form the central nervous system in the overlying ectoderm. By the end of these morphogenetic movements the body plan is outlined, and the places of future organs determined (e.g., muscle, kidney). The neural plate forms into a tube – Neurulation. Remarkably, the general outlines of a molecular

pathway that regulates dorsal development from fertilization to gastrulation are beginning to emerge.



VERTEBRATE BODY PLAN is generated through the chemically induced formation and movement of cell layers, as seen in the development of *Xenopus laevis*, a South African frog. Through rapid cell divisions (*a–c*), a fertilized egg becomes a hollow ball of cells. The large yolky cells at the bottom pole of the embryo release protein growth factors that induce the overlying cells to become the mesoderm layer (*blue*). The mesoderm is the critical layer that determines the embryo's anteroposterior polarity. Two other layers of cells—the ectoderm (*brown*) and the endoderm (*yellow*)—are established during gastrulation, the process by which the mesoderm migrates into the interior of the embryo (*d–e*). During the neurula stage (*f–g*), the mesoderm induces part of the ectoderm to become the neural plate (*red*). As seen in cross section (*h–i*), the neural plate closes on itself and becomes a neural tube, which is the forerunner of the brain and the spinal cord in the mature animal (*j*).

Scientific American July 1990 De Robertis, Oliver and Wright

3. Germ cell determinants

In 1875 Hertwig found that sperm contributed a nucleus. In 1833, Van Beneden and Boveri argued that each parent contribrutes a set of chromosomes. Ascaris was a very favorable material.

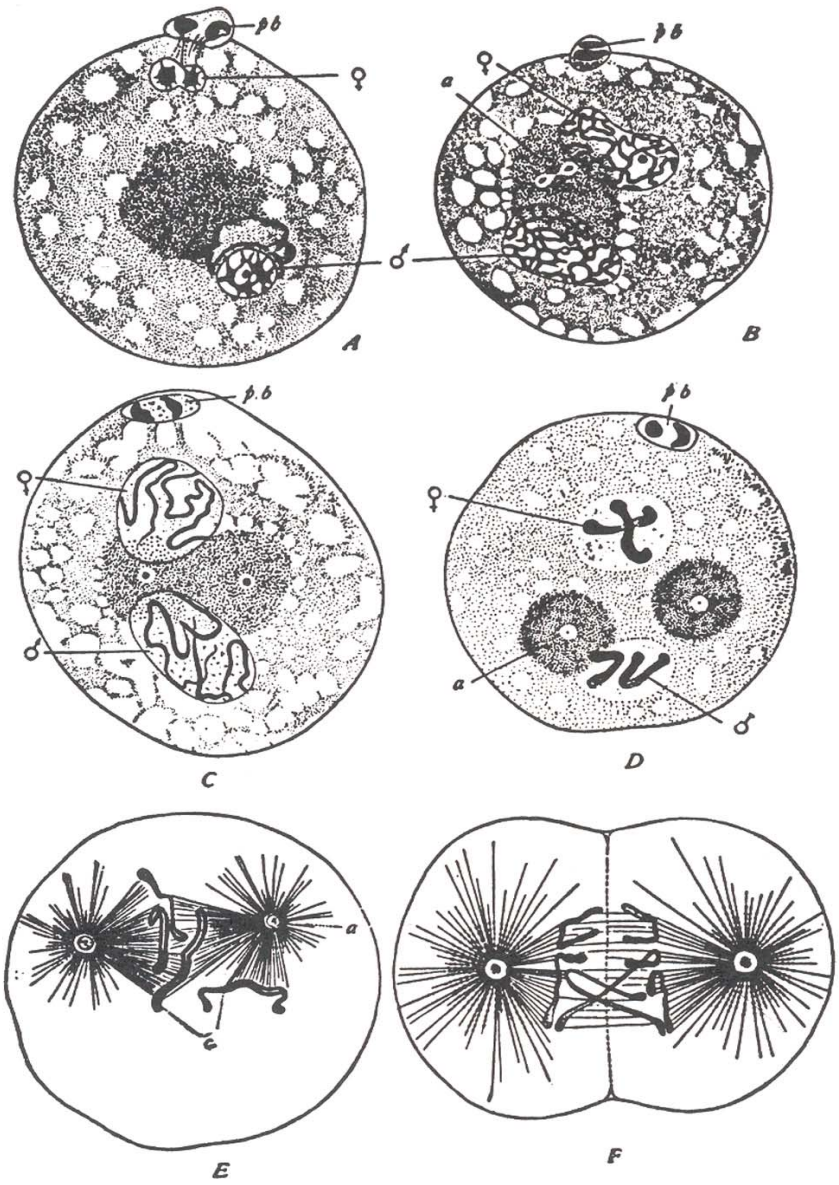


Fig. 23–7. Fertilization of the egg of *Ascaris megalocephala* var. *bivalens*, illustrating Van Beneden's demonstration that each parent contributes an equal number of chromosomes. *A*, the sperm has entered the egg and its nucleus has swollen (♂). The female nucleus is completing the second meiotic division and is eliminating the second polar body (pb). Each nucleus contains two chromosomes (the diploid number for this variety of *Ascaris* is four). *B*, both pronuclei (♀, ♂) have swollen; the centrosphere *(a)* contains the dividing centrioles, *C*, chromosomes start condensing. *D*, two chromosomes clearly visible in each nucleus. *E*, first division; the nuclear membranes dissolve, and the chromosomes align in a common metaphase plate. *F*, first cleavage anaphase (only three chromosomes shown on this section). In *Ascaris*, as in frogs, the pronuclei do not fuse before first cleavage. In other species, such as sea urchins, the membranes of the ♂ and ♀ pronuclei fuse before the first cleavage. (Drawing by T. Boveri, 1888.)

Boveri showed that Ascaris, which has only two chromosomes, has a process of chromosome fragmentation (called chromatin diminution) in all somatic cells except for the germ cell precursors. However, if the eggs are centrifuged, granules of the germ plasm are redistributed and cells do not fragment the DNA. This suggested that the type of cytoplasm inherited by cells is important. Weissman proposed an influential (at that time) theory of heredity stressing that the germ cells and the soma (body) are separate lineages. But his theory of development was incorrect.

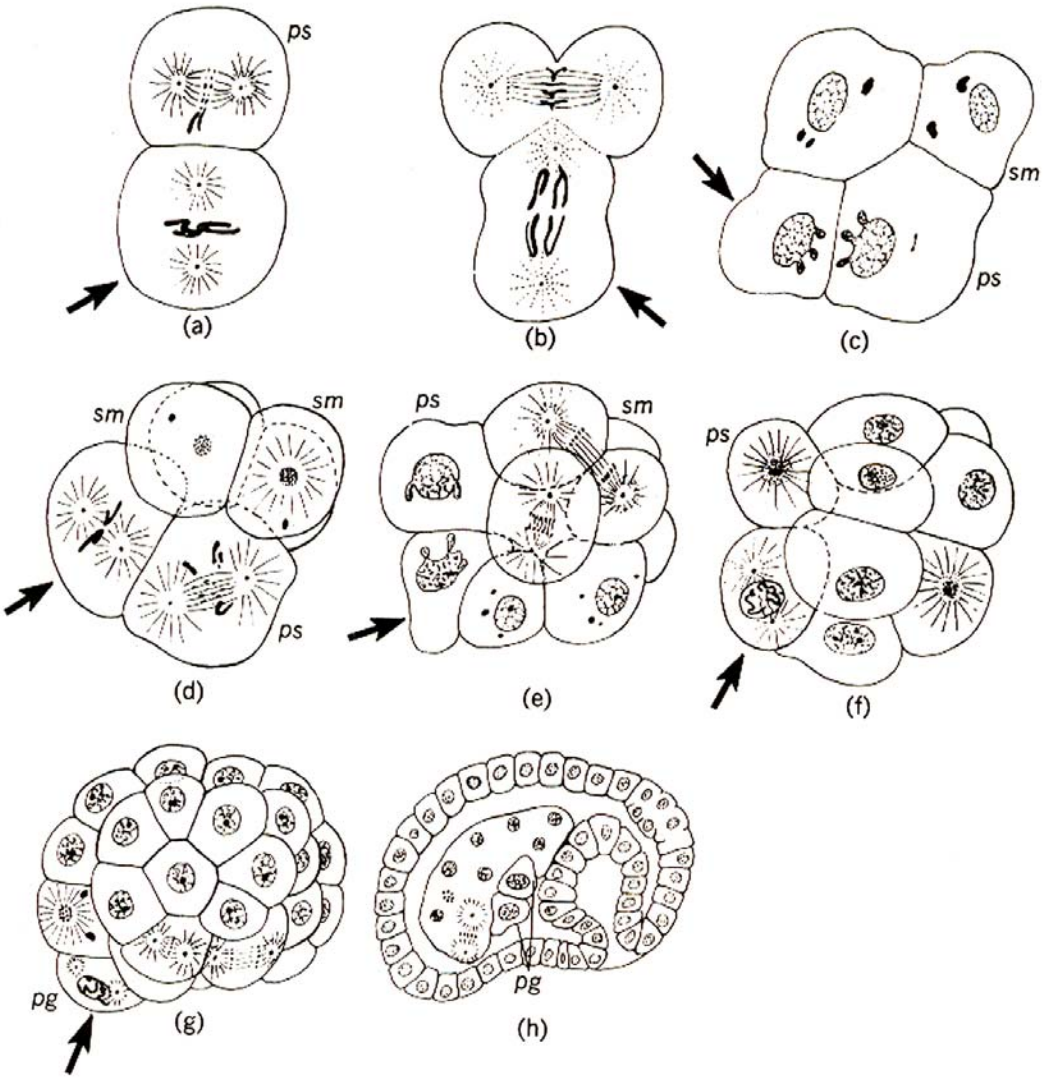


Fig. 23–13. Chromosomal diminution and determination of primordial germ cells in *Ascaris megalocephala*. *ps,* primordial somatic cell, yet to undergo diminution; *sm,* somatic cell which has undergone diminution. *arrow,* germ-line stem pg cell. *(a)* Second cleavage in progress. In the primordial somatic cell chromosome diminution is in progress. *(b)* Later stage, elimination-chromatin at equator of upper spindle. *(c)* 4-cell stage showing eliminated chromatin in the cytoplasm of the upper two cells. *(d)* Third cleavage in progress, second diminution at ps. *(e)* 10-cell embryo showing mitosis of somatic cells with diminished nuclei each containing many small chromosomes. *(f)* 12-cell embryo. *(g)* About 32 cells, fourth diminution in progress, leaving primordial germ cell (pg) (in prophase). *(h)* Gastrula completed with two primordial germ cells. (From the studies of T. Boveri, 1899.)

Boveri reasoned that there had to be a cytoplasmic substance being segregated into the germ cell that protects it from chromatin diminution. In 1910 he centrifuged Ascaris and showed that multiple germ cells were formed.

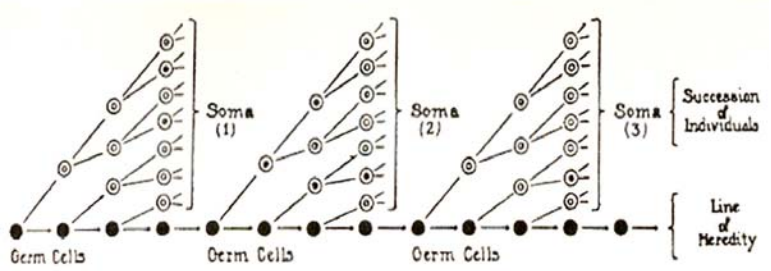


Fig. 23–8. The Weissman theory of heredity. In each generation the germ cell gives rise on the one hand to the body or soma and on the other to more germ cells, which carry the line of heredity. (From E.B. Wilson, 1897.)

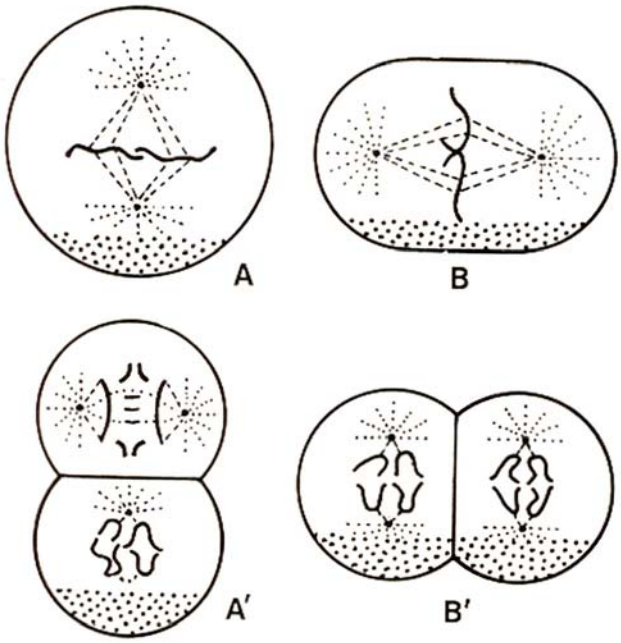


Fig. 23–14. Cytoplasmic control of chromosome diminution in *Ascaris*. *A*, *A′*, normal eggs; *B*, *B′*, centrifuged eggs. The shaded area indicates the distribution of a hypothetical cytoplasmic material. In blastomeres containing this material the two large chromosomes remain intact; in blastomeres lacking it the chromosomes undergo diminution (*A′*, top cell).

4. Nuclear transplantation and therapeutic cloning

Weissman's idea that DNA might be lost during differentiation of the soma was disposed of by ligature experiments and by nuclear transplantation in *Xenopus,* which showed that somatic cells can be totipotent.

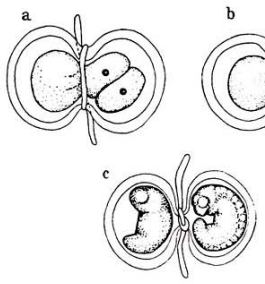


Fig. 23–9. Spemann's demonstration that embryonic nuclei are totipotent until the 16-cell stage.

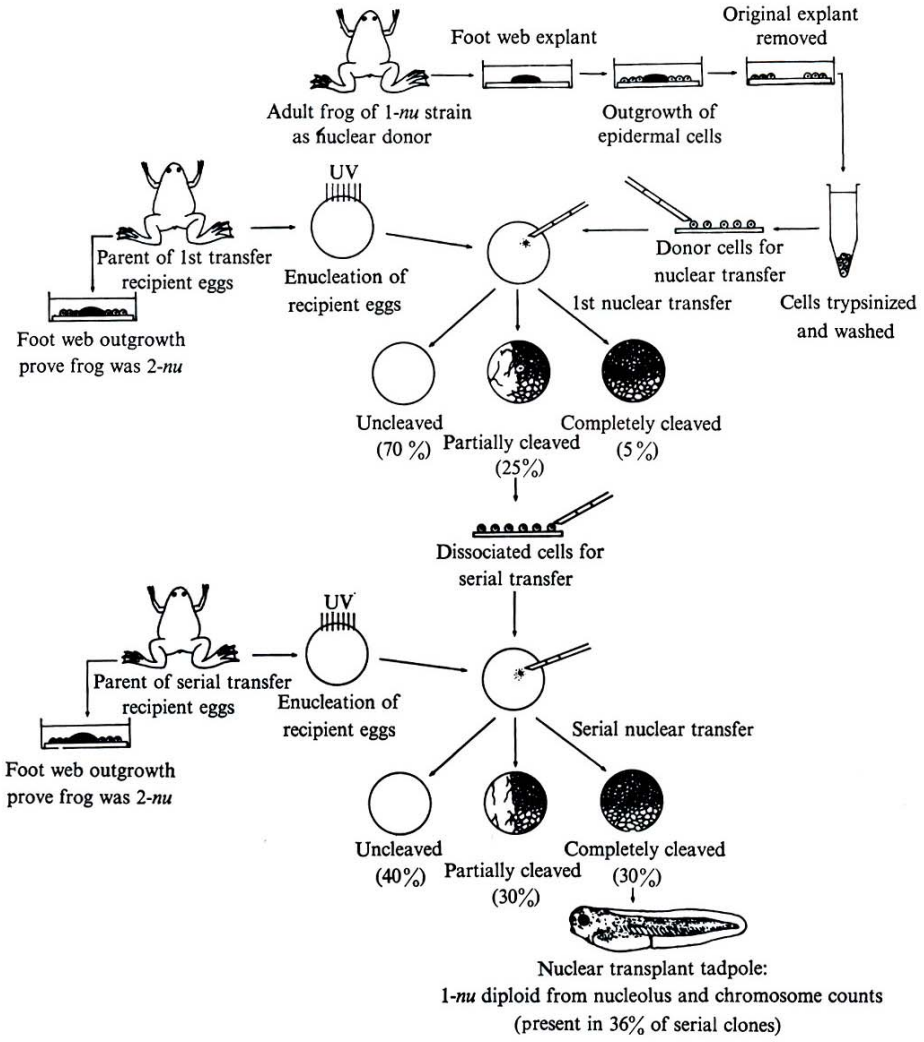


Fig. 23–10. Serial nuclear transplantation in *Xenopus*. Skin cells from the foot web of one-nucleolus frogs (the number of nucleoli provides a genetic marker) were cultured and shown by immunofluorescence to produce keratin, which is a characteristic of differentiated skin cells. Nuclei from these cells were transplanted into eggs whose own nucleus had been killed with ultraviolet light. Nuclei from partial blastulae (see text for details) were then transplanted into new recipient eggs, and swimming tadpoles were obtained. Thus adult skin cells contain all the genes necessary to build a tadpole of the stage indicated. (Courtesy of J.B. Gurdon.)

You must have heard about Dolly the sheep, who passed away in 2003. Alan Colman's company has now used cloning to inactivate the α1-3 galactosyl transferase gene in pigs for xenotransplantation. They circumvented the need for pig ES cells. Stock values went up 44% in one day (Jan. 2002).

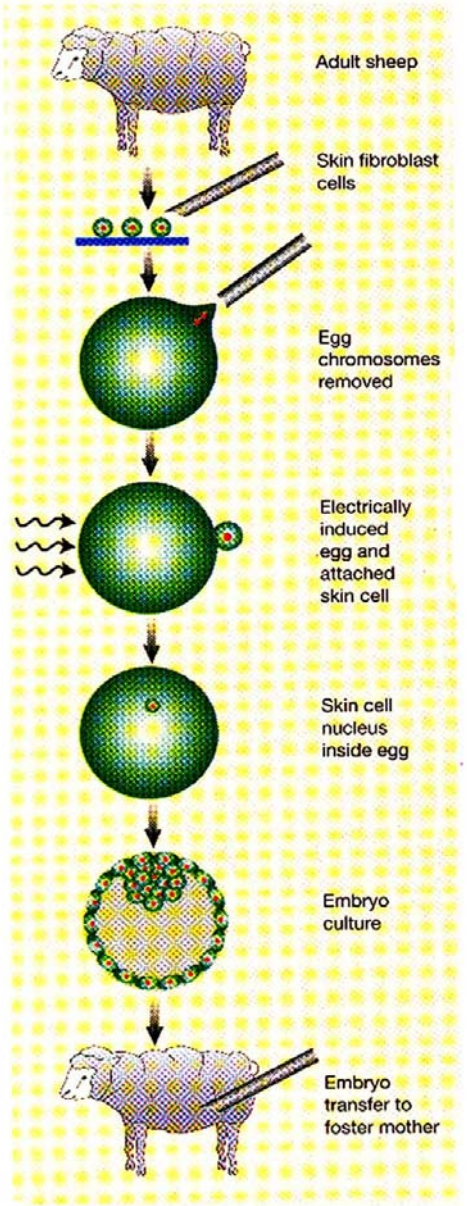


Figure 1 **Nuclear-transplantation techniques in mammals. The genetic material is removed from the recipient cell (an egg), then replaced by a nucleus from a donor cell. The resulting embryo is then transferred to a surrogate mother. The clones are genetically identical to the donor.**

From Gurdon and Colman Nature *402*, 743-746 (1999)

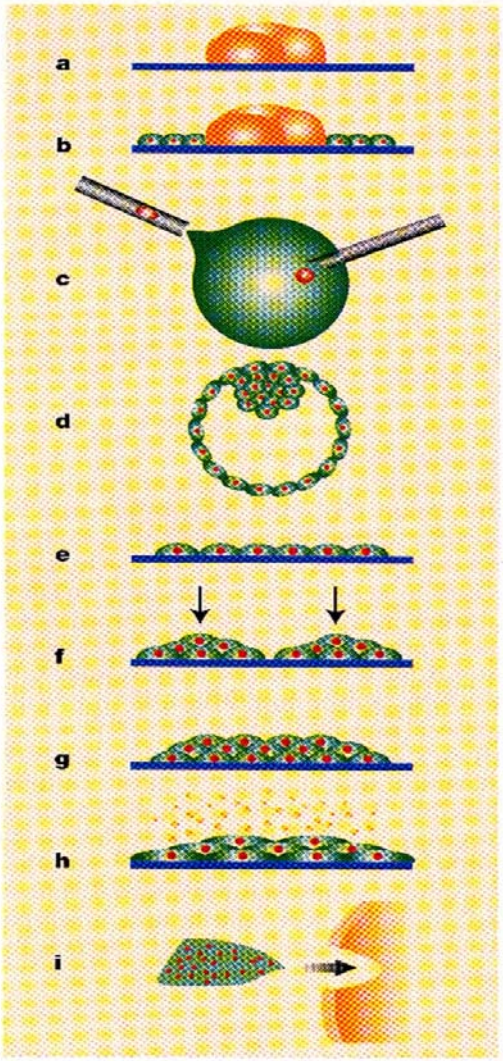


Figure 3 **Steps involved in therapeutic cloning. a, An explant is taken from an adult tissue donor. b, The tissue sample is grown up in culture (cell proliferation). c, Nuclei from the outgrowth cells are transplanted into enucleated eggs (eggs from which the genetic material has been removed). d, The nuclear-transplant eggs are cultured to the blastocyst stage. e, Embryonic stem-cell lines are derived from the inner cell mass. f, The cells are transfected with genes encoding proteins that will cause cell suicide if cells begin to proliferate out of control. g, Selected cells begin to proliferate. h, Differentiation factors are added and the cells begin to differentiate into specialized cell types. i, The replacement tissue is transplanted into the original donor.**

If all nuclei had identical information, then the initial differences must reside in the cytoplasm of the egg.

4. A cytoplasmic determinant in Ascidians; mosaic eggs

Some eggs have what is called mosaic development. When individual blastomeres are separated from each other, they will adopt fixed fates, such as muscle. In other words, mosaic eggs develop cell autonomously, with little influence from signls from their neighbors. Some ascidian eggs have regions of different pigmentation, and it is possible to visualize that after fertilization these regions undergo extensive movements and eventually become included in the cells that give rise to certain tissues. For example, in his classic 1905 paper, Edwin Conklin showed that the ascidian *Styela* has a region of yellow cytoplasm (rich in mitochondria), which eventually gives rise to mesoderm and muscle. If the embryos are compressed so that the yellow cytoplasm is distributed into more cells than usual, the cells that acquire it will give rise to muscle cells, suggesting that this yellow cytoplasm contains determinants for muscle tissue.

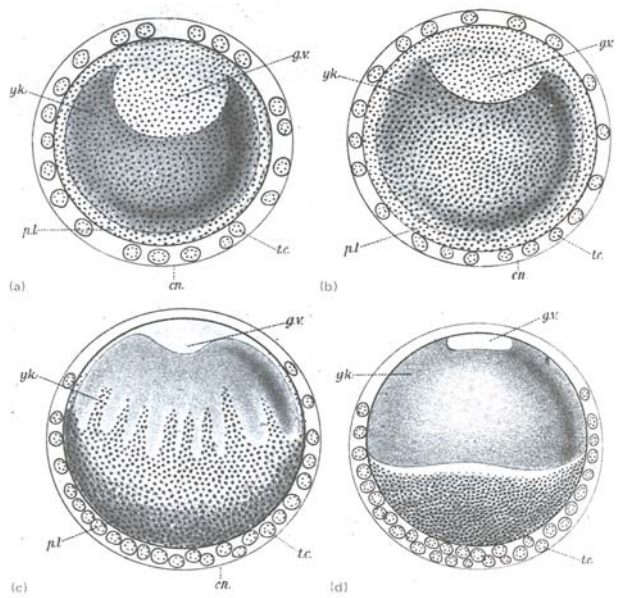


Fig. 7.2. Figures of the living eggs of *Cynthia* (*Styela*) *partita*; maturation and fertilization: (a) Unfertilized egg before the breakdown of the germinal vesicle (g.v.), showing central mass of gray yolk (yk.), peripheral layer (p.l.) of yellow cytoplasm, test cells (t.c.), and chorion (cn.). (b) Similar egg during the disappearance of the nuclear membrane, showing the spreading of the clear cytoplasm of the germinal vesicle at the animal pole. (c) Another egg about 5 minutes after fertilization, showing the streaming of the peripheral protoplasm to the lower pole where the spermatozoon enters, thus exposing the gray yolk (yk.) of the upper hemisphere; the test cells are also carried by this streaming to the lower hemisphere. (d) Later stage in the collection of the yellow cytoplasm. Clear cytoplasm lies beneath and extends a short distance beyond the edge of the yellow cap.

From E. G. Conklin (1905). *J. Acad. Nat. Sci. Philadelphia* **13**, 1.

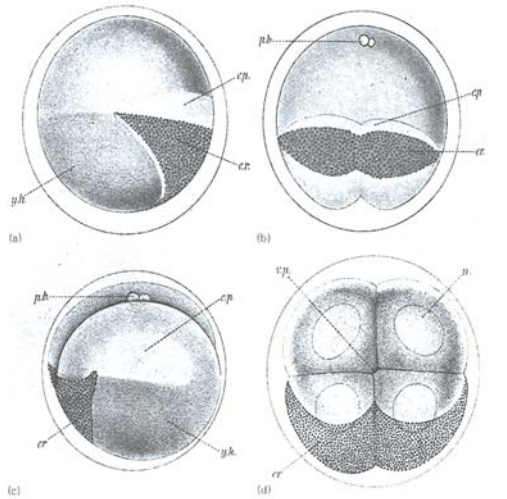


Fig. 7.1 (a)–(d). Living eggs of *Cynthia* (*Styela*) *partita*: (a) Right side view of fertilized egg showing the formation of the crescent (cr.) from the yellow hemisphere (y.h.); in (a)–(c) the future dorsal pole is below. The yellow crescent marks the posterior end. Above the yellow crescent is an area of clear protoplasm (c.p.). (b) First cleavage of an egg, viewed from the posterior region and showing the form taken by the yellow crescent during the division, and also, the enlargement of the area of clear protoplasm and its extension toward the polar bodies (p.b.). (c) Left side view of egg of same stage as (b) showing the lateral limits of the yellow crescent, the clear protoplasm in the upper (future ventral) hemisphere, and the yolk (yk.) in the lower. The anterior portion of the lower hemisphere is composed of light gray material; this is the gray crescent and gives rise to chorda and neural plate. (d) Four-cell stage seen from the vegetal pole (v.p.); the yellow crescent covers about half of the posterior blastomeres. (n), nucleus. From E. G. Conklin (1905). *J. Acad. Nat. Sci. Philadelphia* **13**, 1.

The co-segregation of an hypothetical muscle cell determinant with the yellow cytoplasm of *Styela* was revealed by another classic experiment by Whittaker in 1979. He used an experimental trick. The enzyme acetylcholinesterase is a good marker of muscle differentiation but does not normally appear until the embryo is 9 hours old and has several hundred cells. When developing embryos are placed in sea water containing cytochalasin B (an inhibitor of actin microfilaments), the cells no longer divide. The nuclei, however, continue to multiply, and the acetylcholinesterase activity appears at the normal time if these cleavage-arrested embryos are incubated for 9 hours.

He found that the potential to produce acetylcholineterase, which was present in the unfertilized egg became progressively segregated into subsets of cells during cleavage.

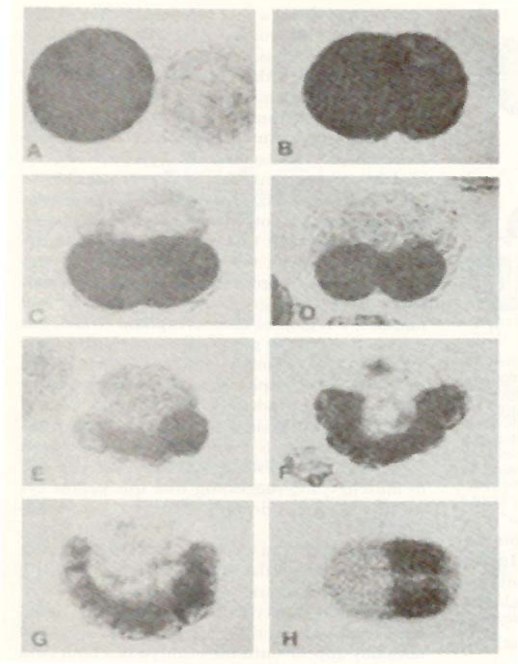


Fig. 23–12. Acetylcholinesterase (AchE) development in cleavage-arrested embryos. To stop cytokinesis embryos were placed in cytochalasin B at following stages: *A*, 1-cell; *B*, 2-cell; *C*, 4-cell; *D*, 8-cell; *F*, 16-cell; *F*, 32-cell; *G*, 64-cell. The embryos were kept for 15 to 16 hours after fertilization at 18°C, before being stained histochemically for the muscle enzyme AchE. *H*, AchE in a 9-hour, non-arrested control embryo. The enzyme stains the region of the tail muscles of the future swimming larva.

In 2001, Nishida and Sawada (Nature *409*, 724-729) isolated a muscle cell determinant from the ascidian egg. They isolated an RNA enriched in the vegetal half of the fertilized egg. Although they work in Tokyo, they called the new gene *macho-1* (no reason given as to why). The gene encodes a transcription factor with five C2H2 zinc fingers. The localization of *macho-1* mRNA follows the movements through the embryo of the hypothetical muscle determinant proposed by Conklin and Whittaker.

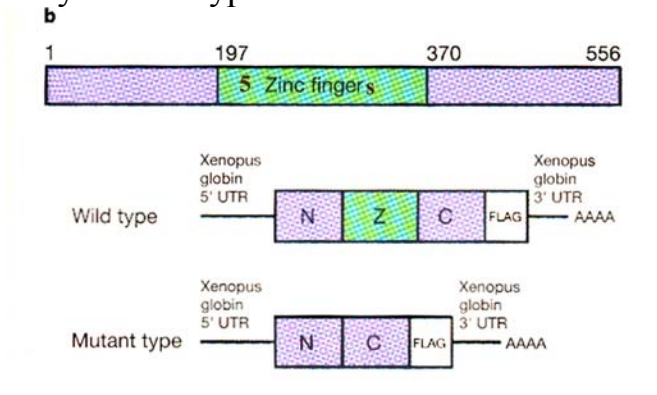
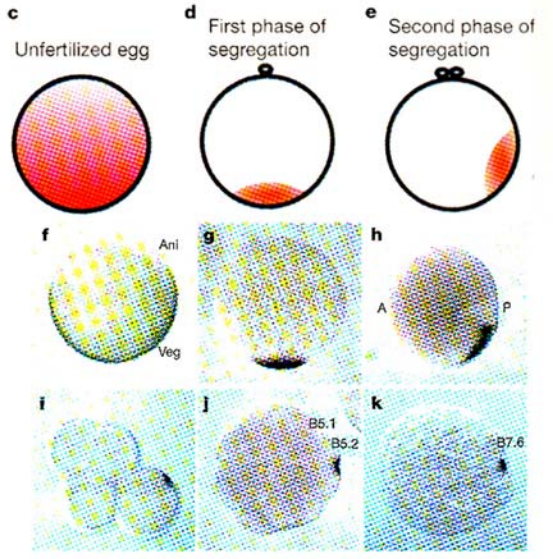


Figure 1 Structure and localization of macho-1 transcript. **b**, Structure of macho-1 protein and constructs for *in vitro* mRNA synthesis. Macho-1 has five zinc fingers in the central part. The cDNA clone is 2,210 bp long and encodes a 556-amino-acid ORF. The wild-type construct encodes the full length, but the mutant construct lacks the zinc-finger domain. **c–e**, Localization of muscle determinants in unfertilized egg (**c**), and eggs after the first (**d**) and second (**e**) phase of ooplasmic segregation[7]. Animal pole is top, and posterior is to the right. **f–h**, Distribution of maternal macho-1 mRNA shown by *in situ* hybridization in eggs at stages corresponding to **c–e**. A, anterior; P, posterior. **i–m**, Localization of macho-1 mRNA during embryogenesis. Anterior is to the left. **i**, Eight-cell stage, lateral view. *In situ* hybridization signal is localized to tiny spots in B4.1 blastomeres. Sixteen-cell stage (**j**) and 110-cell stage (**k**) embryos shown in vegetal view.



From Nishida and Sawada

NATURE | VOL 409 | 8 FEBRUARY 2001 | www.nature.com

Depletion of *macho-1* mRNA with antisense oligonucleotides leads to the lack of expression of muscle actin in primary muscle cells. Injection of myogenic cytoplasm of *macho-1* depleted eggs lost its ability to promote muscle formation. Injection of *macho-1* mRNA led to rrescue of the loss-of-function phenotypes and to ectopic muscle expression.

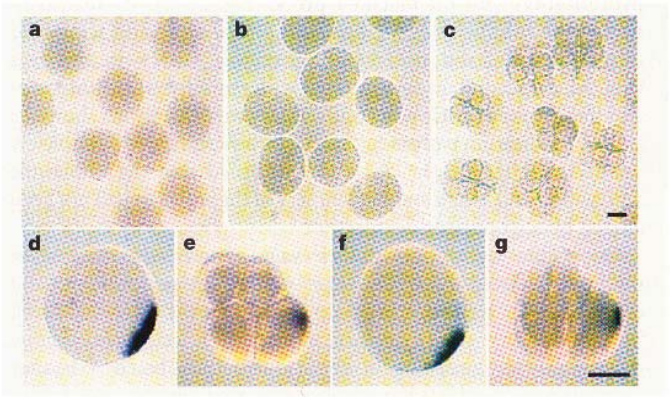


Figure 2 Depletion of maternal mRNA by antisense oligonucleotide. **a–c**, Antisense oligonucleotide-injected unfertilized eggs (**a**), eggs after the second phase of ooplasmic segregation (**b**) and eight-cell embryos (**c**) probed for macho-1 mRNA. **d**, **e**, Sense oligonucleotide-injected controls at the second phase (**d**) and the eight-cell stage (**e**). **f**, **g**, Antisense oligonucleotide complementary to macho-1 was injected, then the specimens were probed for *HrWnt-5*, whose mRNA shows a similar localization pattern as macho-1 mRNA. Scale bars, 100 μm.

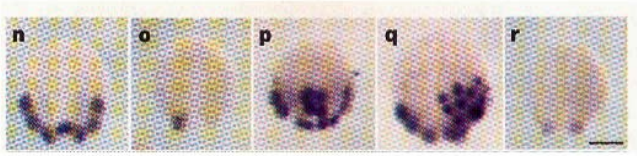


Figure 3 Depletion of macho-1 mRNA results in loss of primary muscle cells, and its overexpression causes ectopic muscle formation.

n–r, Embryos at the 110-cell stage fixed and in *in situ* hybridized with the muscle actin (*HrMA4*) probe. **n**, Uninjected control. Actin expression is observed in ten precursor blastomeres of primary muscle cells. **o**, macho-1-depleted embryos. **p**, **q**, Synthesized macho-1 mRNA was subsequently injected. Actin is ectopically expressed in endoderm (**p**) and epidermis (**q**) blastomeres. **r**, Subsequent injection of mutant mRNA has no effect.

From Nishida and Sawada
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Conclusion: almost 100 years after Conklin, we how have a molecule required and sufficient to act as a muscle cytoplasmic determinant.

5. Somatic cells have cytoplasmic determinants.

We are now learning that cells of adult tissues are very much like embryos. They have stem cells (e.g., intestinal villi, skin, blood) that divide asymmetrically; one of the daughters remains as a stem cell and the other one marches through a program of cell differentiation that ends in apoptosis, without leaving progeny. Mouse bone marrow stem cells can contribute to skeletal muscle. Adult Neural stem cells propagated from CNS (in the presence of high FGF) can give rise to hematopoietic cells. The principles we learn in embryos apply to the maintenance of adult tissues.

Experiments in the 1950's had indicated that the type of cytoplasm of neuroblasts in insects (grasshoppers) determines cell fate, for one could rotate the chromosomes 180° with a needle without affecting the outcome cell differentiation.

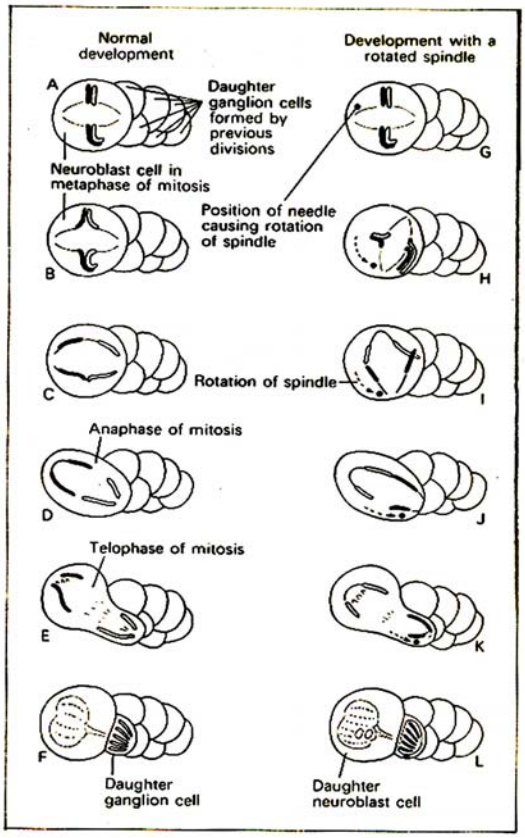
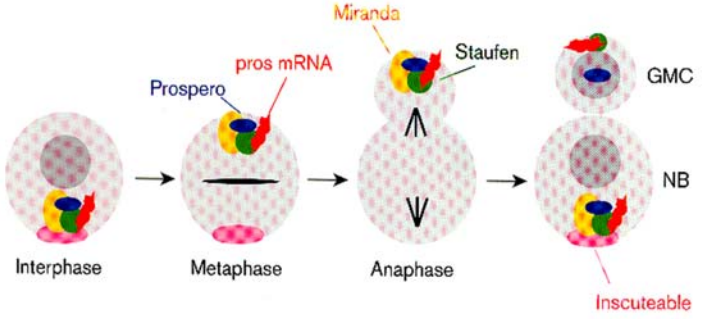


FIG. 25. The cytoplasmic determination of ganglion cell differentiation in grasshopper neuroblasts. In normal development of the grasshopper, *Chortophaga viridifasciata*, embryonic neuroblasts undergo a series of mitotic divisions in which one daughter cell becomes a differentiated ganglion cell and divides no further, while the other (larger) daughter cell remains an unspecialized neuroblast and divides further. A to F show normal development with the daughter chromosomes (only 2 pairs are illustrated) which are destined to enter the future neuroblast cell (in color). G to L show comparable stages of mitosis of neuroblast cells maintained in a hanging drop culture. A needle is pushed against the outside of the dividing neuroblast so as to cause a 180° rotation of the metaphase spindle. As a result the colored chromosomes enter the daughter ganglion cell. The divergent differentiation of the two daughter cells is therefore determined, not by properties of the chromosomes or spindle, but by the asymmetrical arrangement of some cytoplasmic components of the parent cell. [After Carlson, J. G. (1952). *Chromosoma (Berl.)* 5, 199-220.]

In *Drosophila*, RNA-binding proteins such as Staufen have been shown to migrate to the daughter cell at metaphase. Staufen binds prospero mRNA (a homeobox gene) that determines neuron (ganglion cell) fate. In mammals homologues of Staufen and of the other proteins have been found as well.



Miranda coordinates the asymmetric segregation of Prospero protein and its mRNA in neural precursor cells (NB). Miranda binds directly to both Prospero and Staufen which, in turn, binds *prospero* mRNA. The complex moves from the apical (bottom) to the basal (top) side of the cell, and is segregated to the daughter cell (GMC) at cytokinesis.

Conclusion: the asymmetric distribution of RNA is important in later development, in addition to eggs. The cleavage plane can determine cell fate:

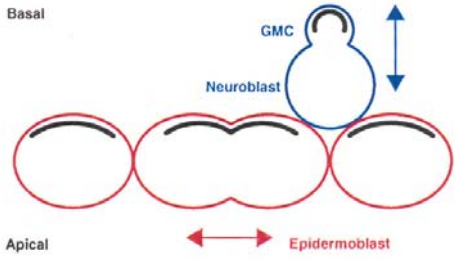
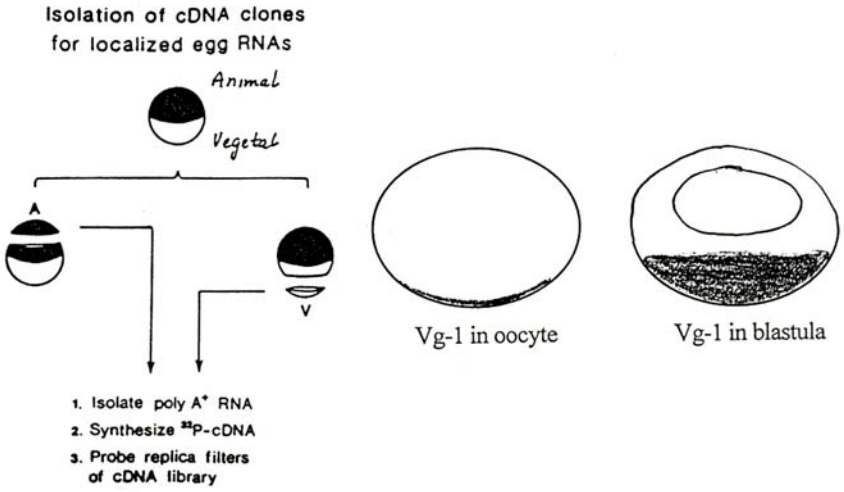


Figure 1 ***Drosophila*** **neuroblasts divide in an axis perpendicular to that of epidermoblast division.**

6. Cytoplasmic determinants in the *Xenopus* egg.

Vg-1 was isolated by D. Melton in 1987 as an RNA localized in the vegetal pole of the egg. In the oocyte it is tightly localized to the vegetal cortex; in the egg and blastula it is distributed more uniformly in vegetal cells.



Vg-1 encodes a growth factor of the TGFβ superfamily. Microinjection of synthetic mRNA (made for example by using SP6 polymerase) encoding a processable form of Vg1 causes endoderm and mesoderm differentiation. (Other TGFβs of the Nodal and Activin subfamilies have similar activities). Because Vg-1 is a maternal mRNA asymmetrically localized in the egg with biological activity, it is a very good candidate for an egg **cytoplasmic determinant.**

VegT was isolated more recently as a cytoskeleton-associated egg mRNA, and found to have a distribution almost identical to Vg1 in the oocyte and early cleavage. VegT encodes a T-box family transcription factor (the T stands for the short-tail mouse mutant Brachyury a.k.a. T). VegT has the same early distribution as Vg-1. VegT is a cytoplasmic determinant required for endoderm and mesoderm formation. The third important *Xenopus* egg determinant is β-*catenin*, which is stabilized in the dorsal side of the embryo..

VegT is a transcription factor expressed in the vegetal part of the embryo. Its activity was depleted by DNA oligonucleotide depletion. Oocytes are removed surgically from the abdomen of a frog and injected with specific DNA oligonucleotides for the targeted maternal mRNAs. Endogenous RNAse H digests the RNA-DNA hybrids and the mRNA is degraded. The injected oocytes are placed in solutions containing vital dyes (such as Nile Blue Sulphate and phenol red), matured with progesterone for 12 hours (this induces meiotic division I) and transferred to the peritoneum of a host female that is laying eggs. The egg acquires a cover of jelly and becomes fertilizable by sperm. In the case of VegT this has been very successful. When VegT is depleted in this way, and embryos dissected

into animal, equatorial (middle) and vegetal (base) thirds, markers of epidermis and mesoderm are now found in the base fragment, that normally only contains endodermal markers. This indicates that the VegT transcription factor is required for endoderm formation.

Subsequent experiments using higher doses of oligonucleotides showed that neither mesoderm nor endoderm are formed. VegT depleted base (vegetal) fragments are unable to induce animal caps to form mesoderm in Nieuwkoop recombinants. Thus, VegT is required for the transcription of zygotic signals that induce mesoderm.

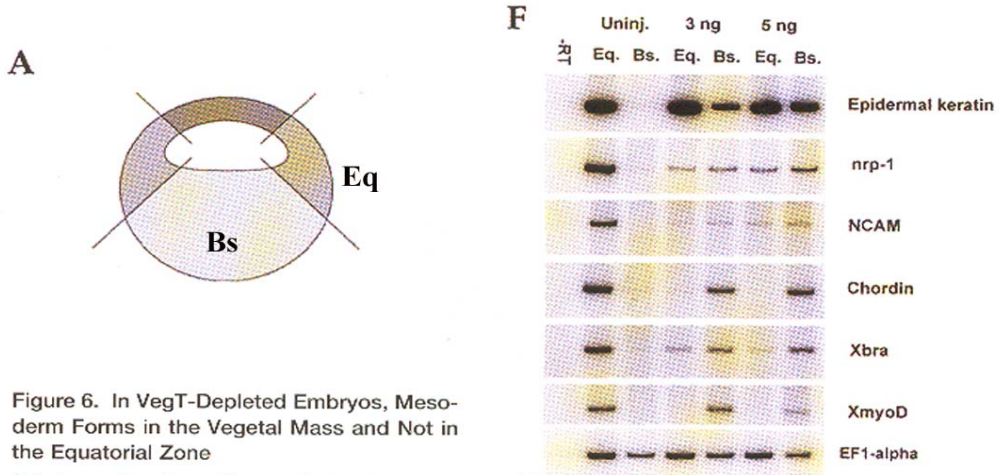
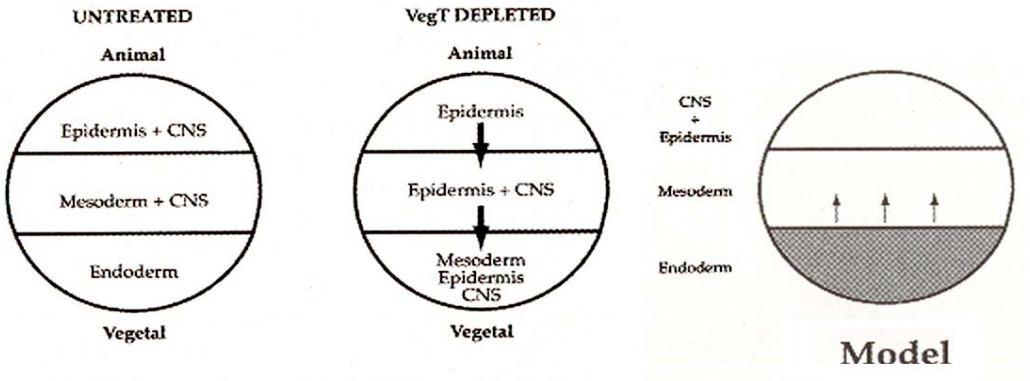


Figure 6. In VegT-Depleted Embryos, Mesoderm Forms in the Vegetal Mass and Not in the Equatorial Zone

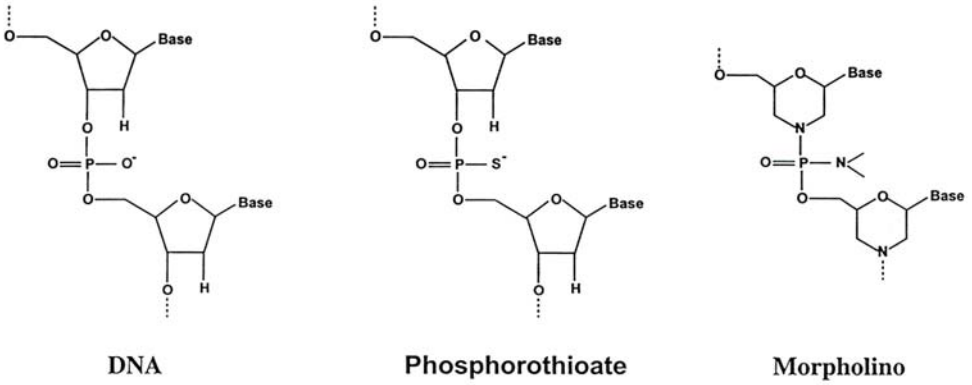
(A) shows the dissection carried out on control, uninjected and VegT-depleted midblastulae to explant the three regions, the animal caps, equatorial zones, and vegetal masses.

(F) Oocytes were uninjected or injected with 3 or 5 ng oligo, and equatorial zones (Eq.) and vegetal masses (Bs.) were dissected at the midblastula stage. The explants were cultured until the early tailbud stage and then analyzed by RT-PCR for ectodermal and mesodermal markers.



From Zhang et al., Cell *94*, 515-524 (2000)

Other methods to achieve loss of function in embryos in which targeted gene knockouts are not available will be mentioned. Phosphorothioate oligos are more stable. Morpholino antisense oligonucleotides (instead of the normal phosphodiester bonds) are very stable, and provide a way of blocking translation:



Additional tools are provided by dominant-negative protein constructs and by secreted inhibitors of growth factors, that function by binding and sequestering them in the extracellular space (noggin, chordin, cerberus), or by binding to but not activating the receptors (Antivin or Lefty). These secreted inhibitors can be very effective in blocking cell signaling, as will be seen below.

References Lecture 1

Gilbert, S.F. (2000). Developmental Biology. Six edition, Sinauer Associates.

De Robertis, E.M., Larraín, J., Oelgeschläger, M. and Wessely, O. (2000). The establishment of Spemann's Organizer and patterning of the vertebrate embryo. Nature Reviews Genetics *1*, 171-181. This paper is found in the course webpage and in http://www.hhmi.ucla.edu/derobertis/index.html (go to teaching), and is required reading.

Nishida, H. and Sawada, K. (2001). macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis. Nature *409*, 724-729.

Heasman, J. (1997). Patterning the *Xenopus* blastula. Development *124*, 4179-4191.

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