What We Know About Urodele (Especially Axolotl) Early Development: A Review of Gamete Formation and Fertilization

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INTRODUCTION

For over a century, the axolotl egg, larva, and adult have been used to unravel the mysteries of development, metamorphosis, regeneration, and ontogenetic evolutionary transformations. Embryologically, the axolotl, like other amphibians, is an anamniote (lacking an amnion) that develops outside the maternal body from a spherical egg two millimeters in diameter. The fertilized egg is shed into the water inside two protective membranes, the vitelline membrane, secreted by the follicle cells of the ovary, and the egg jelly layers, secreted by the oviduct. The egg first cleaves to form a multicellular blastula containing three adjacent prospective areas: ectoderm, mesoderm, and endoderm. During gastrulation, these areas are rearranged into three concentric germ layers by a series of morphogenetic movements, positioning the endoderm on the inside, the mesoderm in the middle, and the ectoderm on the outside. These layers then undergo regional morphogenesis and differentiation into the tissues and organs of the larva. The larva begins to feed within a week or so after hatching. Its form is typical of urodele larvae. A broad dorsal fin runs down the back to the tip of the tail. A narrower ventral fin begins just behind the cloaca and ends at the tip of the tail. The head is broad and squared off anteriorly.

There are many reasons why embryologists find axolotls so useful (Brunst, 1995a; Nieuwkoop, 1996). First, although *Xenopus* eggs have traditionally been used for injection studies (e.g. Gall et al., 1999), axolotl eggs and embryos are larger and more easily manipulated molecularly and surgically, including the injection into eggs or blastomeres of molecules such as RNA. For example, Andeol et al. (1998) have injected *Xenopus* proto-oncogene c-Myc RNA into fertilized axolotl eggs to investigate post-transcriptional control of cell cycle regulation, and Lefresne et al. (1998) have found the axolotl to be an excellent alternative model to *Xenopus* for studying the changes in cell cycling that occur at the midblastula transition (when cell cycles lengthen).

Second, many embryological experiments require the experimental substitution or rearrangement of parts by microsurgical techniques, and again, the large size of the axolotl embryo makes this easy to do. Microsurgical experiments on amphibian embryos are done in a balanced salt solution using glass or tungsten needles, iris scissors, and watchmakers' forceps, with the embryo lodged in a depression in a suitable substrate such as agar or wax. An excellent manual describing the instruments and techniques for embryonic microsurgery has been published by Hamburger (1960). Another advantage of axolotl embryos is the ability to create a heritable triploid cell marker. Triploid embryos can be made by heat or cold-shocking freshly spawned eggs to suppress second polar body formation (Fankhauser, 1955). Triploid cells can be recognized after transplantation by their larger nuclei and the presence of three or more nucleoli within the nuclei.

Third, embryonic amphibian tissues can be easily cultured *in vitro*, often using only a balanced salt solution. Both surgical and culture techniques are highly useful for assessing the developmental potential of embryonic cells and tissues, and for investigation of intercellular interactions.

Fourth, about three-dozen mutant genes are known in the axolotl that affect specific periods of development, as well as cell and tissue function (Briggs, 1973; Malacinski and Brothers, 1974).

Fifth, as pointed out by Nieuwkoop (1996), the single-layered nature of the urodele embryo facilitates observation of gastrulation and neurulation, which is more difficult in the double-layered *Xenopus*.

Comprehensive reviews on the subject of urodele developmental biology and regeneration were published in Volume 40, 1996, of the International Journal of Developmental Biology and on axolotl development by Armstrong and Malacinski (1989). We have recently reviewed urodele limb and spinal cord regeneration elsewhere (Nye et al, in press; Chernoff et al, in press). Here, we begin to review both older and recent studies describing earlier events happening in axolotl development. This is the first in a series of articles. First, we will describe gamete structure and function, events occurring during fertilization, and establishment of dorsoventral polarity in the urodele zygote. In future articles, we will summarize descriptions of the major features of axolotl cleavage, gastrulation, and organogenesis employing the Bordzilovskaya and Detlaff (1979) staging series. We will also describe studies on the developmental effects of several of the more well-studied axolotl mutant genes.

GAMETE STRUCTURE AND FUNCTION

Sperm

The testes of the male axolotl are bilaterally paired teardrop-shaped structures lying on the dorsal wall of the abdomen posterior to the liver, stomach, lungs and spleen. Sperm leave each testis via a series of fine vasa deferentia. The vasa deferentia empty into the two Wolffian ducts that run to the cloaca (Brunst, 1955b). At 18^{o} in the laboratory, spermatogenesis takes ~60 days. The mature sperm spend another 60 days in the testes before moving into the cloaca to be packaged into spermatophores. Unlike urodeles caught in the wild, there is no obvious seasonal variation in the initiation of spermatogenesis in laboratory-kept axolotls. As in other urodeles, however, spermatogenesis passes through the testis in an anterior to posterior wave, once initiated (Miltner and Armstrong, 1983).

The appearance of the axolotl sperm (see Chung and Malacinski, 1985) is typical of urodeles, as described in detail for the newt *Pleurodeles waltl* by Picheral (1979) (Fig. 1). The sperm is very slender. The head region consists of a narrow, sleeve-like acrosome bounded by an acrosomal membrane and

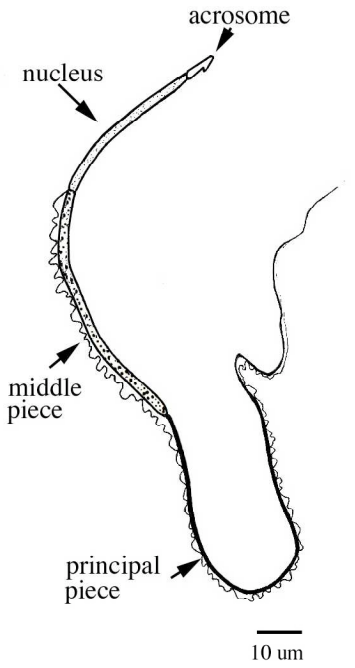

Fig. 1. Drawing of a urodele spermatozoon.

containing lytic enzymes that aid in digesting a path through the egg jelly and the vitelline membrane. It lies just under the plasma membrane and terminates in a knob and a curved barb. The space within the acrosomal sleeve is the subacrosomal space, into which fits the prefatorium, a sharp rod enveloped by a sheath. The posterior end of the rod fits into a deep depression in the anterior end of the nucleus, and the posterior end of the sheath fits over the anterior end of the nucleus. The nuclear chromatin is densely packed, and a non-chromatin structure, the nuclear ridge, lies on one side of the nucleus within the nuclear membrane. Posterior to the nucleus is a cylindrical neck, or connecting piece, that fits into a posterior depression in the nucleus. The neck has a high concentration of basic proteins, and two centrioles are located at its posterior end.

The tail of the urodele sperm consists of three main components: the axial fiber, the marginal filament, and the undulating membrane. The axial fiber extends straight from the neck and supports the tail. The marginal filament is organized from the more posterior of the two centrioles. It extends at an angle from the neck and courses along the edge of the undulating membrane. This wavy membrane is composed of two apposing layers of plasma membrane. The tail can be divided into a middle piece, in which mitochondria are arranged around the axial fiber, and the more posterior principal piece that contains no mitochondria.

Egg

The ovaries of the female axolotl are large, bilaterally paired organs lying in the abdominal cavity (Brunst, 1955b). If the body cavity is opened, oogonia and oocytes in various stages of development are visible, embedded in the thin walls of the ovaries. The oviducts are long, white, convoluted tubes running from the ovaries to the cloaca. For a detailed review of amphibian oogenesis and fertilization, see Malacinski (1985).

As in many vertebrates, axolotl primary oocytes undergo growth and differentiation while arrested in the diplotene stage of the first meiotic metaphase. Differentiation involves the production of messenger RNA, ribosomal and transfer RNA, proteins, carbohydrates, fats, yolk, and melanin granules. Ribosomal genes are amplified by replication of the nucleolar organizer region. Within the germinal vesicle (the nucleus), the replicates are packaged into hundreds of nucleolar bodies that synthesize rRNA precursors for 18, 28 and 5.8S rRNA. These rRNA's are used to make the millions of ribosomes that are concentrated in the oocyte cytoplasm (Davidson, 1986).

The full-grown amphibian oocyte has a characteristic regional structure and polarity. The large germinal vesicle, still arrested in diplotene of the first meiotic division, is located in the endoplasm of the animal hemisphere. The melanin granules are concentrated in the cortical cytoplasm of the animal hemisphere, giving it a dark brown color. There is a gradient of yolk platelet size along the animal-vegetal axis, with the smallest platelets in the animal cytoplasm, and the largest ones in the vegetal cytoplasm, giving the vegetal hemisphere a creamy color. These asymmetries give the full-grown egg a clear animal-vegetal axis of polarity, around which its contents are radially symmetrical.

A full-grown oocyte cannot be fertilized until it has undergone cytoplasmic maturation. Maturation occurs during ovulation and results in the breaking of diplotene arrest, the continuation of meiosis to the second meiotic metaphase, and the acquisition of the capacity to fuse with sperm. Renewed meiosis is associated with the breakdown of the germinal vesicle membrane and mixing of the nucleoplasm with the cytoplasm. Germinal vesicle breakdown occurs 9-10 hours after the initiation of maturation and is manifested externally as a white spot near the animal pole, where the first polar body will be produced (Fig. 2).

Maturation is stimulated by progesterone, which is secreted by the follicle cells surrounding the oocytes. Studies on *Xenopus* and newt oocytes have shown that maturation requires an increase in protein synthesis and requires the production and phosphorylation of a protein originally called maturation promoting factor (MPF) (Wasserman, et al., 1985; Iwao et al., 1997; Sakamoto et al., 1998). MPF, also called mitosis promoting factor, functions in normal cell cycling during proliferation as well as in meiosis, and contains two subunits. The larger subunit is cyclin B, which accumulates during S and is degraded after M phase of the cell cycle. The small subunit is cyclin-dependent kinase, which initiates M by phosphorylating proteins that cause chromatin condensation, nuclear envelope degradation, and mitotic spindle organization. MPF is bound to centrosomes, and when added to cells in interphase, results in disassembly of long microtubules and the assembly of shorter, more dynamic microtubules that form the mitotic spindle (Gilbert, 2000). In all amphibians, progesterone secreted by follicle cells shortly before ovulation is thought to activate a protein called c-mos, which then activates a phosphorylation cascade that in turn activates MPF. In the newt *Cynops pyrrhogaster*, no MPF is present in oocytes prior to progesterone treatment, and removal of the germinal vesicle prevents the expression of MPF (Sakamoto et al., 1998). This indicates that the egg nucleus in newt eggs participates in the activation of MPF (Iwao et al., 1993). In the axolotl oocyte, progesterone treatment results in qualitative and quantitative changes in protein synthesis, phosphorylation of certain proteins and dephosphorylation of others, prior to the appearance of MPF (Gautier and Tencer, 1986). The pattern of protein synthesis is unaffected either by enucleation of the oocyte or treatment with α-amanitin (an inhibitor of transcription), suggesting that control

Fig. 2. Axolotl oocyte with first polar body.

of protein synthesis during maturation is at the level of translation.

It has also been observed that maturation of the axolotl oocyte is accompanied by a change in the permeability of the oocyte plasma membrane, allowing H+ to exit and Na+ to enter. This exchange depolarizes the membrane from the normal range of -40 to -90 (inside) to +30. The Na+ influx is associated with alkalinization of the cytoplasm, the significance of which is not yet clear (Baud and Barish, 1985).

FERTILIZATION AND ESTABLISHMENT OF POLARITY

Fertilization

The axolotl egg is fertilized internally. Spermatophores, filled with mature sperm, are shed by the male axolotl. These are picked up by the female, and inserted into her cloaca. The sperm are stored in spermathecal tubules within the cloaca. Spermathecal tubules are simple tubuloalveolar glands that secrete glycosaminoglycans to bathe stored sperm. The sperm are expelled by contraction of myoepithelial cells surrounding the spermathecal tubules (Sever and Kloepfer, 1993). The sperm fertilize the eggs as they move into the cloaca from the oviduct. Chung and Malacinski (1985) have studied sperm penetration in the axolotl egg by scanning electron microscopy. At the site of penetration, a depression, or sperm pit, is seen on the egg surface; it appears as a dark spot due to the concentration of pigment there. The sperm pit becomes free of the microvilli that normally cover the surface of the egg. The entire sperm enters the egg, after which the entry site becomes covered with long microvilli, perhaps a sign of wound-healing activity of the egg cortex. Pigment concentration and folding of the egg cortex have been observed in fertilized but uncleaved axolotl eggs after experimental wounding with platinum needles (Luckenbill, 1971). Ultrastructural studies of fertilization have not yet been conducted on axolotl eggs, but are available for *Pleurodeles* (Picheral 1977a, b). The descriptions given here are based on his work. To fuse with the egg, the sperm must first traverse the egg jelly and bind to and penetrate the vitelline membrane. This penetration is aided by the acrosomal reaction, in which the acrosome undergoes exocytosis of its lytic enzymes via calcium-mediated fusion of the acrosomal membrane with the overlying plasma membrane, exposing the perfatorium. The movements of the sperm tail drive the sharp-pointed perfatorium through the egg jelly and vitelline membrane, aided by the lytic action of the acrosomal enzymes.

As in most vertebrates, the urodele sperm makes tangential contact with the egg membrane and fuses with it via the post-acrosomal plasma membrane. Picheral (1977a) has observed that the acrosome of the *Pleurodeles* sperm does not undergo any morphological changes until it has reached the junction between the middle and inner of the three jelly coats surrounding the egg. This suggests that penetration of the first two layers of egg jelly by the *Pleurodeles* sperm is accomplished strictly mechanically, and that the acrosome reaction might be triggered by the inner jelly layer. The fused membranes vesiculate, opening a channel through which the sperm components are drawn into the egg cytoplasm. The egg nucleus completes the second meiotic division, giving off the second polar body at the animal pole. The sperm pronucleus undergoes chromosome decondensation and migrates toward the middle of the animal hemisphere cytoplasm, where it combines with the egg pronucleus to restore diploidy (28 chromosomes in the axolotl).

The eggs of most species, including anuran amphibians, are monospermic; i.e. only one sperm penetrates the egg. In such eggs, polyspermy is avoided because sperm-egg fusion triggers first a fast, then a slow block that renders the egg incapable of fusing with additional sperm. The fast block is temporary and decays with time. It is electrical in nature and involves the depolarization of the egg membrane from a negative inside/positive outside potential to the reverse (Jaffe, 1976; Charbonneau et al., 1983; Jaffe and Creton, 1998). The slow block is permanent and results from the formation of a fertilization membrane from the vitelline membrane and a component contained in the thousands of membrane-bounded cortical granules that lie in the egg cortex (Glabe and Vacquier, 1978; Mozingo and Chandler, 1991). By contrast, fertilization in urodele (including the axolotl) eggs is naturally polyspermic (about 5.5 sperm/egg) (Fankhauser and Moore, 1941). Consistent with their polyspermy, urodele eggs exhibit no fast or slow block, nor do they contain cortical granules, although the perivitelline space expands and the vitelline membrane lifts off the egg (Charbonneau, et al., 1983).

All of the sperm pronuclei that penetrate the axolotl egg undergo chromosome decondensation and DNA synthesis (Wakimoto, 1979). In newts, only one sperm pronucleus moves toward the egg pronucleus, and this movement is inhibited if drugs that disrupt microtubules are administered to fertilized eggs (Iwao et al., 1997). The other (accessory) sperm nuclei conduct DNA synthesis more slowly than the zygote nucleus, have very much lower levels of MPF activity, and eventually degenerate. If the egg pro-

nucleus is damaged by ultraviolet radiation prior to fertilization, the entry of the zygote into cell cycling is delayed. Some accessory sperm pronuclei do not degenerate in this case. They form bipolar mitotic spindles, and cause multipolar cleavage. This suggests that the egg pronucleus controls the activation of MPF, and that accessory sperm pronuclei in normal newt eggs degenerate because they do not possess components essential for entry into M phase (Iwao et al., 1993).

Establishment of Dorsoventral Polarity and Bilateral Symmetry

In many species of amphibian eggs, the pigmented cortical cytoplasm rotates upward after fertilization. This results in the formation of a more lightly pigmented area of cytoplasm, the gray crescent, between the heavily pigmented animal cap and the vegetal hemisphere (Gerhart, et al., 1985). The crescent is more distinct in anurans than in urodeles, and often does not form in urodeles. When the gray crescent forms in the axolotl egg, it does so 4-6 hours after fertilization, giving the egg a visible dorsoventral polarity and bilateral symmetry. By marking the center of the crescent, Banki (1927a) found that the dorsal lip of the blastopore and hence the median plane of the embryo fell within 15^0 of the crescent center in 57% of the cases, and within 30^o of the center in 94% of the cases. In the monospermic anuran egg, the sperm can penetrate on any meridian of the animal, but not in the vegetal hemisphere. The sperm entry point (SEP) determines the meridional location of the gray crescent in these eggs; the crescent always forms on the side of the egg opposite the SEP. In the polyspermic urodele egg, sperm can penetrate at any site in the animal or vegetal hemisphere. It is not known whether any relationship exists between the meridian on which the gray crescent is centered and the entry point of any of the sperm, including the surviving one.

There is some evidence that the position of the gray crescent in the axolotl egg may be specified prior to fertilization. Banki (1927b) observed that many unfertilized axolotl eggs exhibit a slight elevation of the pigment border on one side. Vital staining of this region showed that it coincided with the site of gray crescent formation. Further evidence that the structural pattern underlying gray crescent formation is present prior to fertilization is the fact that a crescent can be induced to form precociously in heat shocked, unfertilized eggs (Benford and Namenwirth, 1974). If these eggs are later fertilized, the dorsal side of most embryos formed where the gray crescent had been located (Beetschen, 1993). However, it is not known whether this precocious crescent coincides with the region of the raised pigment border. The determination of the position at which the gray crescent appears after fertilization is not irreversible. If the animal-vegetal axis of the fertilized egg is rotated 90^0 for 120 minutes, the gray crescent always forms on the side that opposes gravity in the rotated position, indicating that reorientation of cytoplasmic components under the influence of gravity can establish a new pattern of dorsoventral polarity and bilateral symmetry (Malacinski and Chung, 1981).

Experiments on axolotl oocytes maturing *in vitro* have shown that precocious gray crescent formation can also be induced by inhibitors of protein synthesis. If the oocyte has been enucleated, however, precocious crescent formation cannot be induced by inhibition of protein synthesis (Grinfeld and Beetschen, 1982; Gautier and Beetschen, 1983a, b). These results suggest first, that the maturing oocyte synthesizes a crescent-inhibiting protein that is removed at fertilization; and second, that after germinal vesicle breakdown and removal of the inhibitor protein, a nuclear factor interacts with the cytoplasm to establish conditions that permit formation of the gray crescent (Gautier and Beetschen, 1985).

PERSPECTIVES

The axolotl egg and early embryo will continue to be useful for developmental studies during oogenesis and fertilization. What is the significance of changes in permeability of oocyte membrane during oocyte maturation? Is the resulting membrane depolarization and alkalinization essential for enablement of sperm penetration? Of particular interest is solving the mystery of how only one sperm pronucleus survives to fuse with the egg pronucleus among the typical five sperm that enter the axolotl egg during the initial phase of fertilization. How does the egg nucleus activate MPF? Axolotl eggs have been used for study of post-transcriptional control of cell cycle regulation and cell cycle changes during the midblastua transition; they could be more widely used in cell cycling experiments. The mechanism of dorsoventral polarity establishment in axolotls may be different in that the gray crescent, which is typically associated with cortical rotation in amphibians, may not form after fertilization. When it is present, it is possible that this region of cytoplasm may have been specified specified prior to fertilization. Heat shocking and inhibition of protein synthesis both induce precocious grey crescent formation. What protein appears after germinal vesicle breakdown that permits formation of the gray crescent? How does it do this?

Many questions concerning urodele early development remain to be investigated. New uses for the early axolotl are being developed. Traditionally, embryotoxicity studies have been performed using *Xenopus* (Davies and Freeman, 1991). Recently, axolotl embryos were exposed to retinoids to study teratogenesis. Dose-dependent effects seen in embryonic tissues included neural tube defects, growth retardation and craniofacial and cardiovascular defects (Kratke et al., 2000). Chimeric mutant axolotls have been produced to study early embryonic heart development (Lemanski et al., 2001). DiI labeling has been used to study migratory pathways and cell fates in normal and transplanted neural crest cells (Epperlein et al., 2000). Attempts to make transgenic axolotls, which would be useful for a wide variety of cell marking and genetic gain-of-function studies, are being pursued (Malacinski, personal communication). Despite these innovations, more is still unknown than known about axolotl (and vertebrate) embryonic development. With this series of articles, we hope to stimulate interest in extending past and current research on development, using the axolotl as a model system.

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