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Sex differentiation of germ cells and their supporting cells in *Oryzias latipes*

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Introduction

Gonads in vertebrates consist of parenchymal tissue and interstitium. Female parenchyma is the ovarian follicle, and the male is the seminiferous tubule. The parenchymal tissue is made of germ cells and their supporting cells. Steroid producing cells, nervous systems and vascular systems reside in the interstitium. The supporting cells, granulosa cells in females and Sertoli cells in males, are considered to originate from celomic epithelial cells in fish, amphibians and most mammals. Oogonia and spermatogonia are differentiated from primordial germ cells. The spermatogonia always occur in seminiferous tubules, and granulosa cells always surround oocytes to constitute follicles (Fig. 1). Therefore, the sex differentiation of germ cells and supporting cells is supposed to proceed under the mutual interactions between these two types of cells. However, few experimental facts which directly indicate the presence of these interactions are available so far. In the present paper, we review the behavior of germ cells and supporting cells in *Oryzias latipes* during their sex-differentiation, and discuss on their possible interactions.

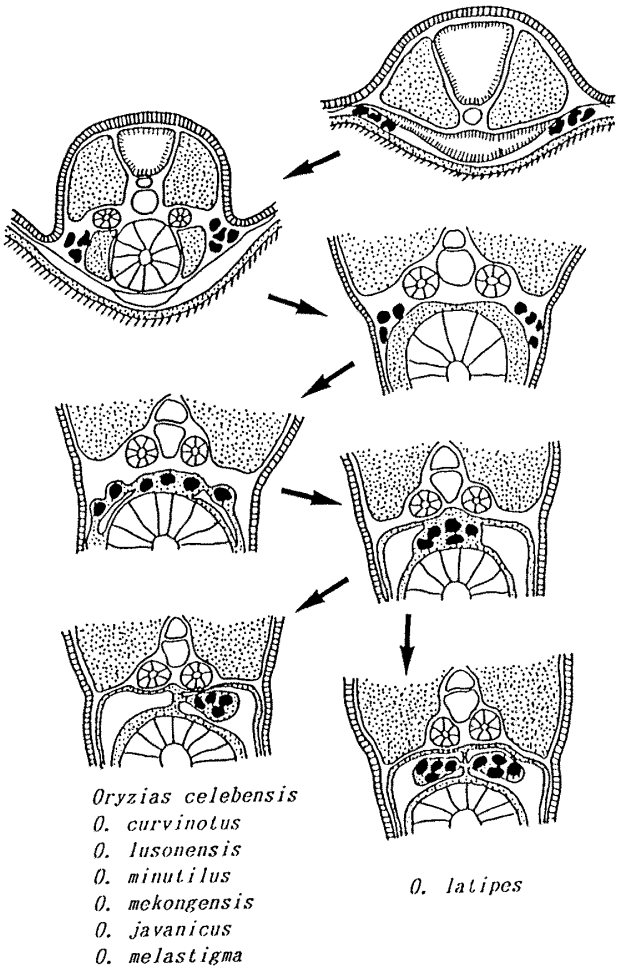

Fig. 2. The path of PGC migration in *Oryzias* fishes. Only in *O. latipes* gonads are formed on the both sides of mesentery.

Gonad development

The origin of primordial germ cells (PGCs) in fish can not be traced back earlier than late neurula (Johnston, 1951). In the neurula of *Oryzias latipes*, PGCs are identified in the peripheral endoderm (Gamo, 1961; Hamaguchi, 1982a). The subsequent path of PGC-migration is shown in figure 2. PGCs move to the cavity between lateral plate and ectoderm. Then, the somatomesodermal cells extend their thin cytoplasmic processes to cover the surface of PGCs. Consequently, PGCs are incorporated into the lateral plate, and completely surrounded by mesodermal cells. This is the first contact between

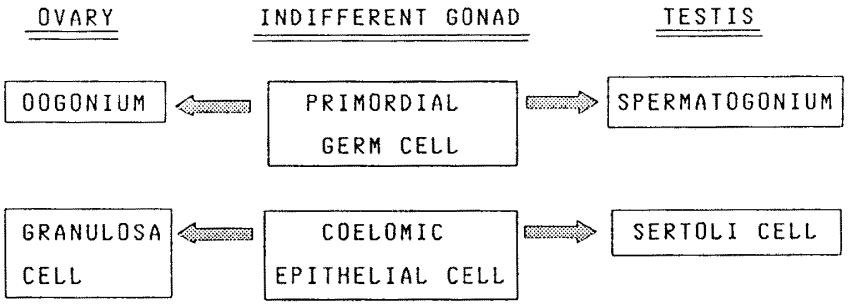

Fig. 1. The sex differentiation of germ cell and their supporting cells.

germ cells and their supporting cells which constitute the parenchymal tissue of gonads. From the free surface of PGCs before incorporation into lateral plate, thin filopodia-like protrusions can be observed, however, the PGCs in lateral plate mesoderm have a smooth contour and are in close contact with surrounding mesodermal cells. Nuage, a germ cell specific organelle, in PGCs begins to change morphologically from a nuage of a strand-like structure to an amorphous fibrous body according to the translocation of PGCs from endoderm to mesoderm, a suggestion that PGCs pass through first step of differentiation by the contact with mesodermal cells (Hamaguchi, 1985).

The gonadal anlage is established during the morphogenetic movement of the body-cavity formation which results from the separation of somatopleura from splanchnopleura. PGCs appear to move centrally toward the dorsal mesentery, but there is no evidence of active movement of PGCs. PGCs, completely surrounded by mesodermal cells, seem to be transferred passively with the extension of somatic mesoderm which becomes mesothelium (Hamaguchi, 1982a). Consequently, the gonadal anlage consists only of celomic epithelium which embraces PGCs. From the overview of the processes of gonad formation of this fish, it seems improbable that other type of cells than celomic epithelial cells take part in the germ-cell supporting cells in the gonad.

In *Oryzias latipes*, a pair of gonads protrude into celomic cavity in the both sides of dorsal mesentery, while in other species of *Oryzias*, the gonads are formed only on the right side (Hamaguchi, 1983, unpublished data).

Sex differentiation

In mammals, the differentiation of germ-cell supporting cells into Sertoli cells is the first indication of sex differentiation of gonads (Byskov, 1981; Magre & Jost, 1983). Sertoli cells are organized into a tubular form to constitute the seminiferous tubules. Whereas, in *O. latipes* germ cells precede the somatic cells in the sex differentiation.

(1) Proliferative activity in germ cells

The first symptom of the sex differentiation of this fish is the difference in the proliferative activity of germ cells between males and females (Fig. 3). (Tuzuki *et al.*, 1966; Satoh & Egami, 1972; Hamaguchi, 1979, 1982a). About 40 PGCs occur in endoderm and they hardly proliferate during the formation of gonads. There is no sexual differences in the number of PGCs in the gonads newly formed (Quirk & Hamilton, 1973). The PGCs resume their mitotic activity immediately after the completion of gonad formation, when female germ cells proliferate more actively than the males. Consequently, presumptive ovaries in the fry just after hatching contain about 200 germ cells, while presumptive testes about 80. Female germ cells (oogonia) continue to proliferate after hatching, but the proliferation of male germ cells (prespermatogonia) is arrested after hatching. After 10–15 days of a mitotically dormant stage, prespermatogonia resume their mitotic activity and begin to increase in number.

The second indication of sex differentiation can be noted in the timing of the initiation of meiosis (Tuzuki *et al.*, 1966; Satoh & Egami, 1972). Some of female germ cells begin to enter meiosis to

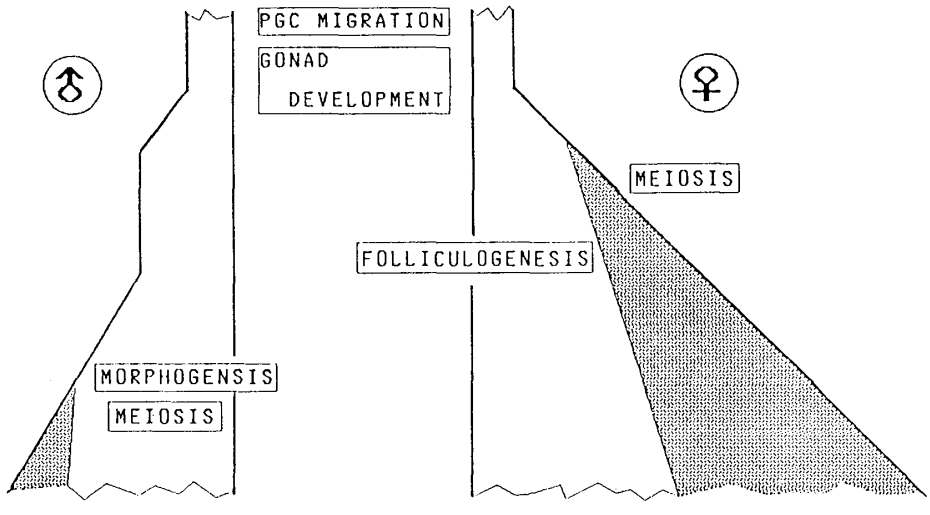

Fig. 3. The schematic illustration of the proliferative activity of germ cells during sex differentiation in medaka. The width indicates the population size of germ cells, and shadows designate the germ cells in meiosis.

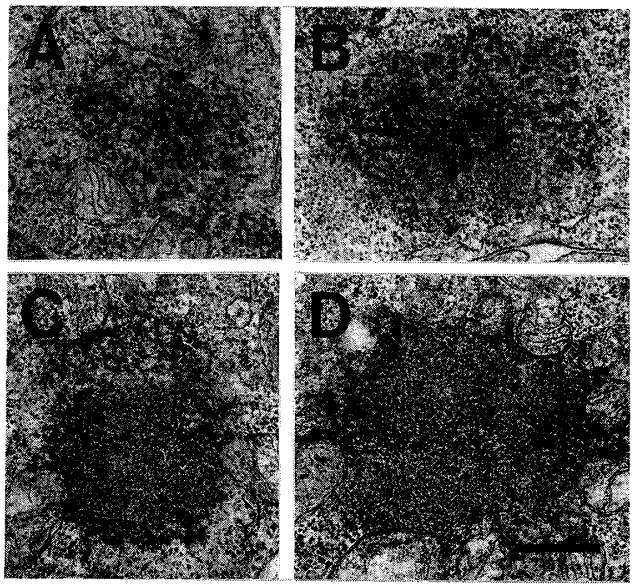

Fig. 4. Four types of nuages. A: a nuage of strand-like structure. B: a nuage of an intermediate structure in which strand-like structure is dominant. C: a nuage of an intermediate structure in which the portion of the amorphous mat is dominant. D: an amorphous body. Bottom bar: 0.5 μm.

differentiate into oocytes just after hatching. Oocytes pass through stages in meiotic prophase to reach diplotene stage, and form a follicle structure with supporting cells. On the other hand, the entry into meiosis in male germ cells is delayed for 40–50 days. Prespermatogonia are surrounded by supporting cells forming acinous structure, which develops into seminiferous tubule in this fish. The first cyst of spermatocytes occurs in these seminiferous tubules (Kanamori *et al.*, 1985; Hamaguchi, 1987).

(2) Morphology of nuages

The morphology of nuages is also sexually differentiated (Hamaguchi, 1982b). Nuages in *O. latipes* are polymorphic, and classified into four types (Fig. 4): nuages with a loosely woven strand-like structure (type A), amorphous fibrous bodies (type D), and two types of nuages of intermediate morphology (type B and C). These four types of nuages coexist in germ cells and their incidence varies according to the progress of the developmental stages (Fig. 5) (Hamaguchi, 1985, 1987). As mentioned above, most nuages in PGCs in endoderm are type A. According to the translocation of PGCs from endoderm to the mesoderm, nuages of amorphous types emerge and relatively increase. At the hatching, when the sex of the fry becomes manifest from the existence of early oocyte in females, the sex difference in the morphology of nuages becomes obvious. In prespermatogonia and spermatogonia, nuages of strand types (type A or B) are significantly increased. Whereas, the oogonia contain more nuages of types C and D. The incidences of type C and D nuages are more prominent in oocytes. There is no significant difference between PGCs in indifferent gonads and oogonia. These observations on the morphology of nuages suggest that germ cells in the male fry just after hatching have changed from primordial germ cells.

(3) Lectins

The information on the sex differentiation of supporting cells is less available. In mammals, sex differentiation of supporting cells becomes manifest from their morphogenetic movement to construct a tubular structure (Byskov, 1981; Magre & Jost, 1983). In addition to the morphological character, the production of Müllerian inhibiting substance is also used as an indication of the functional differentiation of Sertoli cells (Taketo *et al.*, 1991). In *O. latipes*, follicle structure begins to be formed around oocytes at diplotene stage about 10 days after hatching (Satoh, 1974), indicating that female supporting cells begin to differentiate into granulosa cells. While, the differentiation of male supporting cells occurs at later stage of development at about 40–50 days after hatching (Kanamori *et al.*, 1985; Hamaguchi, 1987).

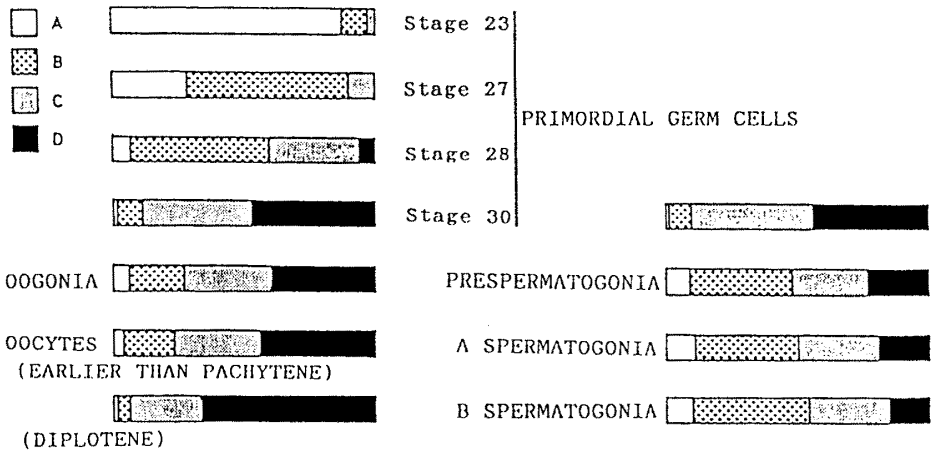

Fig. 5. The incidence of nuages of various types in germ cells at various stages of sex differentiation.

The lectins, which recognize the sugar residues, are recently used as an indicator of cytodifferentiation. We stained the histological sections of testes and ovaries with 13 lectins labeled with peroxidase, and found that the carbohydrate composition on the surface of germ cells as well as of supporting cells changes according to their developmental stages (Shibata, 1989). There were sex-related differences in the binding patterns of BPA, PNA and MPA. MPA and PNA bound to granulosa cells, but did not to Sertoli cells. On the other hand, male supporting cells were stained with BPA, but female ones were not. Primary gonial cells of both sexes, type A spermatogonia and oogonia, were stained with SBA, but secondary (type B) spermatogonia were not. From the investigation using the developing gonads, female germ cells in the fry just after hatching could be stained with SBA, whereas male germ cells could not be stained until the stage when the testicular tissue architecture began to be constructed. Male supporting cells in the fry at early stages also could not be stained with BPA (Table 1) (Matsunuma, Shibata & Hamaguchi, unpublished data). These observations coincide with the conclusion from morphological studies, that is, in *O. latipes* the ovarian development begins earlier than the development of the testes.

Interactions between germ cells and supporting cells

In mammals, the differentiation of male germ cells is believed to occur under the influence of the supporting cells (see review by McLaren, 1991). The first incidence in the sex differentiation of gonad is the differentiation of Sertoli cells, which inhibits the entry into meiosis of germ cells and induces PGCs to differentiate into prespermatogonia. Prespermatogonia differentiate into stem type of spermatogonia under the milieu in the seminiferous tubules. On the other hand, germ cells without inhibition by supporting cells enter meiosis to differentiate into oocytes which in turn affect the supporting cells to differentiate into granulosa cells (Fig. 6).

The situation in *O. latipes* is different from that in mammals. As mentioned above, the ovarian development occurs much earlier than the development of the testes. At the stage when the ovarian development is initiated in the female, male germ cells as well as supporting cells lie quite dormant. There is no symptom of the differentiation in supporting cells. Therefore, in *O. latipes* it is not plausible that "male" supporting cells inhibit the male germ cells from entering meiosis. That is, the interaction between supporting cells and germ cells in the initiation of testicular development is not evident in fish (Fig. 6).

On the other hand, the significance of the oocytes in the differentiation of granulosa cells is also the case in this fish. The oogenesis in *O. latipes* does not proceed synchronously among oocytes in an ovary, so oocytes at various stages of meiosis coexist (Satoh & Egami, 1972). Folliculogenesis occurs only around the oocytes at the diplotene stage. This fact indirectly suggests that the differentiation of granulosa cells is induced by the influence from oocytes.

Table 1. The change in the profile of binding of lectins (SBA and BPA) to germ cells (GERM) and their supporting cells (SUPP) during the gonad development.

DAYS AFTER HATCHING		FEMALE				MALE			
		BPA		SBA		BPA		SBA	
		GERM	SUPP	GERM	SUPP	GERM	SUPP	GERM	SUPP
TOTAL	0	–	–	–/+	–	–	–	–	–
LENGTH	15	–	–	–/+	–	–	–	–	–
5–6 mm	30	–	–	–/++	–	–	–	–/+	–
7–8		–	–	–/++	–	–/+	++	–/++	–
9–10		–	–	–/++	–	+	++	–/++	–
11–12		–	–	–/++	–	++	+++	–/++	–
ADULT FISH		–	–	–/++	–	++	+++	–/++	–

Staining: – Negative, + Weak, ++ Moderate, +++ Strong, –/+ Both negative and positive cells exist.

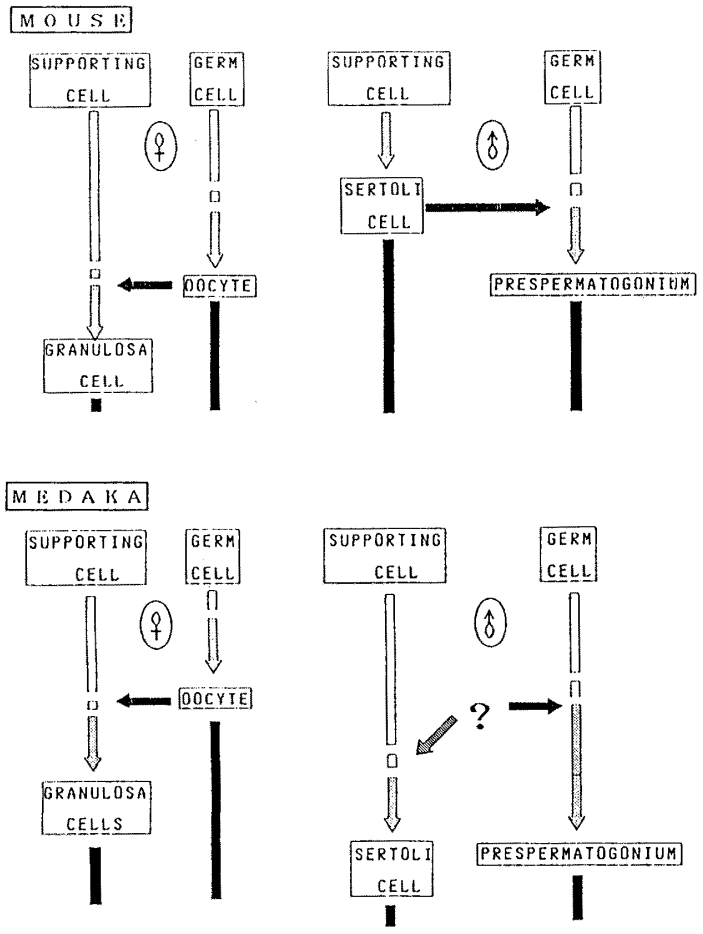

Fig. 6. The processes in sex differentiation of germ cells and supporting cells in mouse and medaka. See the text.

More direct evidence is presented from the observation on the folliculogenesis around the testis-ova which appear in the testes of adult fish. By the administration of steroids, X-irradiation, starvation or exposure to high temperature, testis-ova (oocyte-like cells in testes) can be induced (Egami, 1955a, 1955b, 1955c, 1955d, 1956). The counting of the number of testis-ova in a cyst suggested that they deviate from normal course of spermatogenesis at early type B spermatogonial stage, an indication that spermatogonia in adult testes remain to be sexually bipotential (Shibata & Hamaguchi, 1988). Electron microscopic observation revealed that follicle structures are formed around the testis-ova, and that the granulosa cells of these follicles originate from Sertoli cells which constitute the cyst wall around a group of testis-ova at earlier stages of meiosis than the diplotene stage (Shibata, 1989). From the profile of the binding of lectins, the granulosa cells of the follicles around testis-ova can not be distinguished from those in the normal ovaries (Shibata & Hamaguchi, unpublished data). These facts indicate that Sertoli cells in *O. latipes* maintain their sexual bipotentiality, and that the differentiation of granulosa cells depends upon the existence of diplotene oocytes.

Perspective

Recently, a testis determining gene on Y chromosome (Tdy) in man and mouse was identified, and named *SRY* (*Sry*) (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). Though the functions of *SRY* has not been clarified in detail, the gene product is supposed to bind to DNA to elicit the expression of gene cascade toward the determination of testis development (Nasrin *et al.*, 1991). Eicher and Washburn (1986) proposed two gonad-determination pathways; the testis-determination (Td) pathway and the ovarian-determination (Od) pathway (Fig. 7). Each pathway is composed of a series of genes that function in a coordinated manner. From the sequence of the event during the sex differentiation of the gonad, they supposed that Tdy, the first gene in the Td pathway, functions before the first gene in the Od pathway. They also inferred that Tdy may inactivate the first gene of Od pathway to further guarantee testis development in the XY individual. Genes in Td and Od pathways have not been clarified. Eicher (1988) reported the existence of genes on autosomes (*Tda-1, Tda-2, Tas*) which are involved in the sex determination, and discussed about the possibility that the *Tda-1, Tda-2*, and *Tas* loci are some of the genes that comprise the either Td or Od pathway.

In *O. latipes*, the genetic mechanism of sex determination remains to be an open question. From the genetic analysis of the sex-limited characteristics (Aida, 1921), as well as from the sex ratio of the offspring of sex-reversed fish (Yamamoto, 1969), the male is regarded as heterozygous (XY). However, two alternative possibilities remain to be elucidated; first, the testis-determining gene on Y chromosome determines the sex of the gonad as is the case in mammals, second, the gene dosage on X chromosome determines the sex as in *Drosophila*.

The sequence of the events during the sex differentiation of the gonad suggests that the Td and Od pathways in *O. latipes* begin to express in reverse order comparing to mammals; the expression of Od pathway precedes the expression of Td pathway (Fig. 7). In addition, some mechanism by which the expression of Od pathway in germ cells is inhibited in XY individuals must be exerted.

Recently, the genetic analysis of the sex reversal in interspecific hybrid fish between *O. latipes* and *O. curvinotus* indicates that more than two genes on autosomes are involved in the sex determination (Hamaguchi & Sakaizumi, unpublished data). It is of interest which pathway these autosomal genes are concerned with.

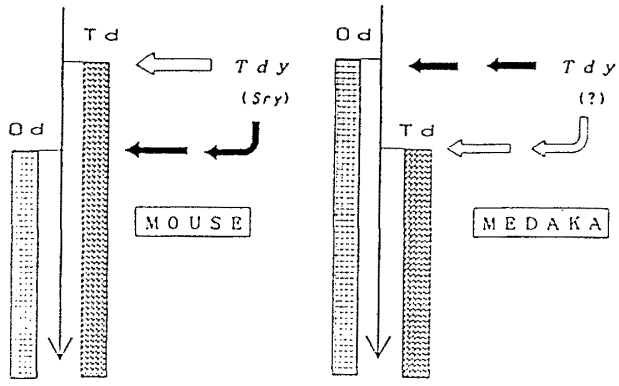

Fig. 7. The hypothetical mechanisms of genetic control on sex differentiation of cells in gonads in mouse (after Eicher & Washburn, 1986) and medaka. Testis determining gene on Y chromosome (*Sry*) triggers off (◁) the testis determination pathway (Td), and inhibits (◀) the expression of the ovary determination pathway (Od). If testis determining gene exists on Y chromosome of medaka, it must be also bifunctional, and the sequence of the expression of the functions may be reversed between mouse and medaka.

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