MOLECULAR STUDIES OF SOMA-GERM CELL INTERACTIONS USING MUSSEL GONAD AS AN EXPERIMENTAL MODEL

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The development of male or female phenotype is the result of a combination of sex determination and subsequent sexual differentiation. The pivotal event in determining the sex of an individual is the differentiation and specialization of the gonads. Once formed, the gonads control the subsequent sexual phenotype.

First of all one has to know when and where inside the reproductive system certain soma-germ line interactions occurs before one can start the biochemical and molecular characterization of the factors and genes involved. In this regard, we predicted that the gonad of bivalve molluscs belonging to the genus *Mytilus* can provide favorable features for studies into cellular and molecular mechanisms of sexual differentiation. One of the great advantages of *Mytilus* gonad model for molecular analysis is the cyclic morphogenetic changes, due to which the soma-germ cell interactions can easily be followed from the adult animals during each year. In this contribution we will briefly review the general molecular features of the soma-germ cell interactions in the gonad of bivalve molluscs. We will also discuss the possible significance of our results in a developmental and evolutionary perspectives.

Our experiments were performed mostly on the gonad (mantle) of *Mytilus galloprovincialis* Lmk. of Galicia (NW Spain).

Mytilus gonad/mantle as a model for studying molecular mechanisms of morphogenesis and cell differentiation. In *M.galloprovincialis*, gonad is not a permanent differentiated organ, but consists of gonoducts that invade the mantle somatic tissue during each annual reproductive cycle. Thus, gonad and mantle tissues are morphologically fused in this species. We suggested that gonad development in the mantle tissue is an example of epithelial/mesenchymal interactions in the adult state. According to this concept, the aim of the study was to use biochemical and immunochemical methods for identifying and characterizing the mantle cell polypeptide markers whose expression is seasonally and morphogenetically regulated (Mikhailov et al., 1996a). We showed for the first time that *M. galloprovincialis* mantle, of both males and females, contains polypeptides (with an apparent MW of 45 to 53 kDa) specific for connective tissue ("mantle connective tissue polypeptides"; MCTPs). Electrophoretic, immunoblotting and immunofluorescent experiments demonstrated that MCTPs are primary localized in the adipogranular (ADG) cells, and their expression in the mantle/gonad is seasonally regulated. MCTP expression directly correlates with the volume of mantle connective tissue, but inversely correlates with gonad follicle concentration. MCTPs are overexpressed during a period of sexual rest, when the mantle consists of connective tissue mainly, whereas mature gonads contain only trace amounts of MCTPs (Fig. 1). Moreover, there is a temporal correlation between the onset and decrease of MCTP expression and the appearance and disappearance of the ADG cells in the mantle tissue. MCTP localization in the mantle tissue should not be associated with the ADG cells only, because positive immunofluorescence was also detected in the membrane of gonad follicles (but not in oocytes or spermatozoa) and superficial mantle epithelium. Three types of MCTP distribution in the mantle can be distinguished: (1) the cellular or cytoplasmic type; (2) the matrix- or membrane-associated type; and (3) the labeling associated with extracellular granules. It could be speculated that MCTPs are synthesized by ADG cells, excreted as protein-rich granules which could be used to support the process of gonad development and morphogenesis. At present, it is unclear whether MCTPs have functional significance, but our data suggest the possibility that these polypeptides are involved in soma/germ cell interactions occurring each year in *M. galloprovincialis* mantles. Identification of the factors responsible for the sequential development, maturation, and involution of the mantle/gonad tissues allow us to elucidate the mechanisms restricting the expression of MCTPs to certain cell types and periods of the annual cycle. Moreover, MCTPs make possible investigations on the temporal control of tissue-specific gene expression during the mantle/gonad morphogenesis. This is an important first step in understanding how various events of mantle connective tissue and gonad formation are coordinated.

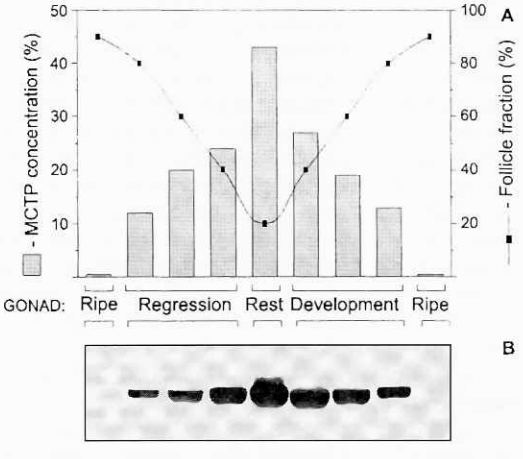

Figure 1. Patterns of MCTP expression in male mantle during annual cycle. **A** - results of densitometric analysis of mantle proteinograms; **B** - immunoblot analysis of the same mantle proteinograms. The results show that MCTP expression directly correlates with the volume of mantle connective tissue, but inversely correlates with follicle concentration.

Mytilus gonad/mantle as a model for studying molecular mechanisms of sexual differentiation. A very low incidence of hermaphroditism suggests that *M. galloprovincialis* is a strictly gonochoric species. In all the species of *Mytilus* that have been investigated, it has been difficult or impossible to sex the adult animals by external phenotypic characteristics. Moreover, there is no any accessory sex glands or structures so that males and females are indistinguishable without gonad/mantle biopsy examination. Our study focused on sexual differentiation of the reproductive system in bivalve molluscs led to identification of sex-associated protein expression in the gonad/mantle tissue of *M. galloprovincialis*. One of these proteins named "male-associated polypeptide" (MAP) was biochemically and immunochemically characterized (Mikhailov et al., 1995 a,b; 1996b). Using 2D-

electrophoresis followed by Western immuno-blot, MAP was identified as a single spot having an apparent MW value about of 40 kDa and p*I* of about 5.5 (Fig. 2). MAP concentration in male mature gonads achieved up to 10% of the soluble protein while in female ones only traces of this protein could be detected. Moreover, MAP revealed the positive immuno-blot reaction with antibodies against the protein of ejaculatory bulb (named esterase S; Korochkin, 1995) of *Drosophila virilis*, belonging to the carboxylesterase (EC 3.1.1.1) family (Mikhailov et al. 1995b). From the other hand, anti-MAP antibodies recognized esterase S from *D. virilis* ejaculatory bulb. Male-specific esterase S is overexpressed in ejaculatory bulb of *Drosophila* males and introduced into organs of female sex tract during mating (Korochkin, 1995). In *M. galloprovincialis*, the highest MAP concentrations occurred during the period of reproduction, and significantly declined at the stage of sexual rest. We suggested that *Mytilus* MAP and *Drosophila* esterase S may be characterized by not only immunochemical but also metabolic and functional similarity. Accordingly, we have addressed the question concerning the enzyme activity of MAP: it is similar to that of esterase S or differ from it? Using the optimized protocol, we could recover the carboxylesterase activity after SDS-PAGE of mussel male gonad extract. Substrate-stained bands were located in gels at the position typical for MAP fraction. Moreover, followed immuno-blot analysis of the same male gonad samples showed that these bands contain MAP (see Fig. 2). These results suggested that MAP is characterized by carboxylesterase activity. Additional support for such a conclusion came from the results of the electrophoretic separation of male gonad extract in non-dissociating buffer system followed by staining for carboxylesterase activity or immunoblotting with antibodies against MAP or esterase S. In male gonad, up to five bands hydrolysing α- and β-naphthyl acetates were detected. The antibody staining showed that only one of these bands characterized by slowest electrophoretic mobility and highest enzymatic activity revealed the positive immuno-blot reaction specific for MAP. Finally, adding anti-esterase S antibodies to male gonad extract of *M. galloprovincialis* decreased significantly esterase activity toward β-naphthyl acetate of both, gonad extract and MAP containing gonad fraction.

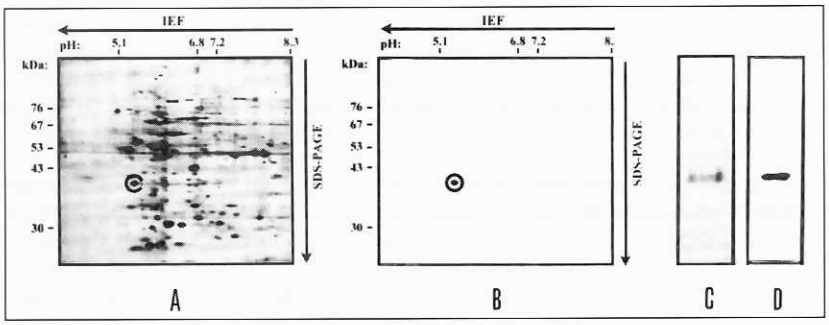

Figure 2. Electrophoretic analysis of MAP. **A** - 2D dimentional gel of male gonad proteins stained by polychromatic silver stain solution, and **B** - corresponding Western immunoblot; cycles indicate MAP spots. **C** - SDS-PAGE gel of male gonad proteins stained by β-naphthyl acetate, and **D** - corresponding Western immunoblot with anti-MAP antibodies.

Using immunofluorescent technique, we found MAP in the mantle connective tissue as well as in the membrane of gonad follicles and epithelium of gonad ducts but not in sperm cells. Nevertheless, the levels of MAP expression depend on presence or absence of mature spermatozoa in the gonad follicles. In ripe gonads before spawning, MAP is expressed at high levels around the gonad ducts. In spent gonads (after complete spawning), it is detected at much less concentrations. The mechanical elimination of spermatozoa from the gonad does not provoke any decreasing of MAP concentration. Interestingly, MAP was not found in spermatozoa obtained by biopsy of gonad follicles. In contrast, we showed that this polypeptide is present in spawned sperm cells (Mikhailov et al., 1996 a,b). Thus, it is possible that spawning may be required to establish the trafficking mechanisms that control whether MAP is retained or excreted by the gonad. In this regard, we suggest that MAP may be implicated in the processes of sperm release in *Mytilus*.

Summarizing, our data highlight MAP detected in male gonad of *M. galloprovincialis* as a new member of sex-specific (male-predominant) carboxylesterase family. Based on substrate and antigenic specificities, isoelectric point, and amino acid composition, MAP appears to be related not only to *Drosophila* esterase S but also to several mammalian carboxylesterases. Remarkably, rat testicular carboxylesterase is highly concentrated in interstitial Leydig cells, and it can bind to several organophosphorus compounds. The latter indicates that Leydig cells may be protected from the toxic effects by extremely high levels of testicular carboxylesterase (Yan et al., 1995). This suggest that a polypeptides with carboxylesterase activity, such as esterase S and MAP, may play a role in detoxifying processes in male reproductive tract/system of fruitflyes and mussels, correspondingly, although further studies are required to test this possibility.

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