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Short Communication

Developmental Expression Patterns of Alpha 2 Macroglobulin in Amphioxus: An Insight into Liver Evolution

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Abstract

It has been considered that hepatic caecum of amphioxus is precursor of vertebrate liver. Alpha 2 macroglobulin (A2m) ancient native immune factors are mainly synthesized in liver in vertebrate and is essential for liver formation in fish. In this study, the developmental expression patterns of A2m in amphioxus *Branchiostoma japonicum* were examined to seek some embryonic functionally evidence for the view about liver origin. It was demonstrated that A2m expression is mainly specific in primitive gut at early larvae stages, and then is in both whole gut and hepatic caecum in 25-day larvae and adult amphioxus. These suggested that the entoderm-derived gut and hepatic caecum in amphioxus are not fully functionally separated as liver and gut in vertebrate, and may represents an ancestral archetype of differentiation of liver and gut in vertebrate in evolution. In addition, the detection of abundant A2m transcript in amphioxus embryos at 2-4 cell stages revealed that A2m is a maternal molecule, which was further verified by Northern blot assay of A2m on unfertilized eggs.

INTRODUCTION

Amphioxus, a cephalochordate, has been regarded as an organism most closely related to the ancestor of ancient vertebrates [1-3]. Amphioxus has a hepatic caecum, the pouch that protrudes forward as an out pocketing of the digestive tube and extends along the right side of the posterior part of the pharynx, which has long been considered to be the precursor of vertebrate liver [3-6]. Recently it has been proved that liver-specific genes such as prealbumin, antithrombin, plasminogen, tachylectin, glutathione-S-transferase and alanine aminotransferase are specific expressed in hepatic caecum, which functionally support the homology of the hepatic caecum to vertebrate liver [7-13].

Alpha 2 macroglobulin (A2m) is an old native immune molecule [14], which presents widely among animalia including mammals [15], birds [16,17], reptiles [18], amphibians [19,20], fishes [21,22] and invertebrates such as shrimp [23,24], crab [25,26] and scallop [27]. Its basic function is as a broad-spectrum protein inhibitor through unique trap mechanism [28]. In vertebrates, the liver is the major production site of A2m [29,30]. In zebrafish it was found that A2m is essential for liver formation and was reasoned that A2m would provide a useful molecular marker to study development of the liver [31].

Recently we have cloned full length of A2m of amphioxus *Branchiostoma japonicum* and created a phylogenetic tree based on multiple alignment of the A2m sequence with other known A2m proteins. Furthermore, it has been shown that A2m was mainly expressed in the digestive canal including the hepatic caecum in a tissue-specific manner [32]. However, studies of expression of A2m and other liver-specific gene just focus on adult amphioxus. The aim of the study is to investigate the developmental expression patterns of A2m in amphioxus to further probe into the evolution of liver.

MATERIALS AND METHODS

Animals and embryos

Amphioxus *B. japonicum* were collected at the breeding season from the sandy bottom of the sea near Shazikou in the vicinity of Qingdao, transported to the laboratory and maintained at ambient photoperiod and temperature. The naturally fertilized eggs were pooled, and cultured at room temperature, and the developing embryos and larvae were collected at desired stages. For whole mount *in situ* hybridization, the embryos and larvae collected were fixed in 4% freshly prepared formaldehyde in 100 mM MOPS in PBS (pH 7.4) containing 500 mM NaCl at 4°C overnight and stored in 70% ethanol at−20°C until used.

Whole mount *in situ* hybridization

A2m gene expression was studied by whole mount *in situ*

hybridization on amphioxus embryos at developmental stages according to the method of Holland et al. (1992) [33] with small modifications. The vital line membranes of embryos at pre-hatching stages were carefully removed manually by watch forceps under stereomicroscope. Proteinase K (New England BioLabs, Ipswich, MA) treatment was performed at μg/ml for 5 min at 37°C. Prehybridization was performed at 60°C for 8hr. Probes were prepared from T-vector (Promega) containing the 867 bp A2m fragment (3'UTR region). Anti-sense probe was *in vitro* transcribed with Sp6 RNA polymerase and digoxigenin RNA labeling mix (Roche Applied Science). Sense probe was transcribed with T7 RNA polymerase and digoxigenin RNA labeling mix (Roche Applied Science) after Linearization with XhoI. Hybridization was performed at 60°C for 24 hr. Alkaline-Phosphatase (AP) conjugated anti-digoxigenin antibody was obtained from Roche Applied Science. The chromogenic reaction was performed with alkaline phosphatase buffer containing 4.5 μl /ml NBT and 3.5 μl/ml BCIP.

Northern blot

An aliquot of 3μg RNAs prepared from the unfertilized egg was electrophoresed and blotted onto Nylon membrane (Roche). The digoxigenin (Dig)-labeled Bjα2m riboprobes of about 867 bp were synthesized *in vitro* from the linearized plasmid DNA following the DIG-UTP supplier's instructions (Roche). The blots were hybridized as described by Fan et al. (2007) [11].

RESULTS AND DISCUSSION

A2m mRNAs were examined in the developing embryos and

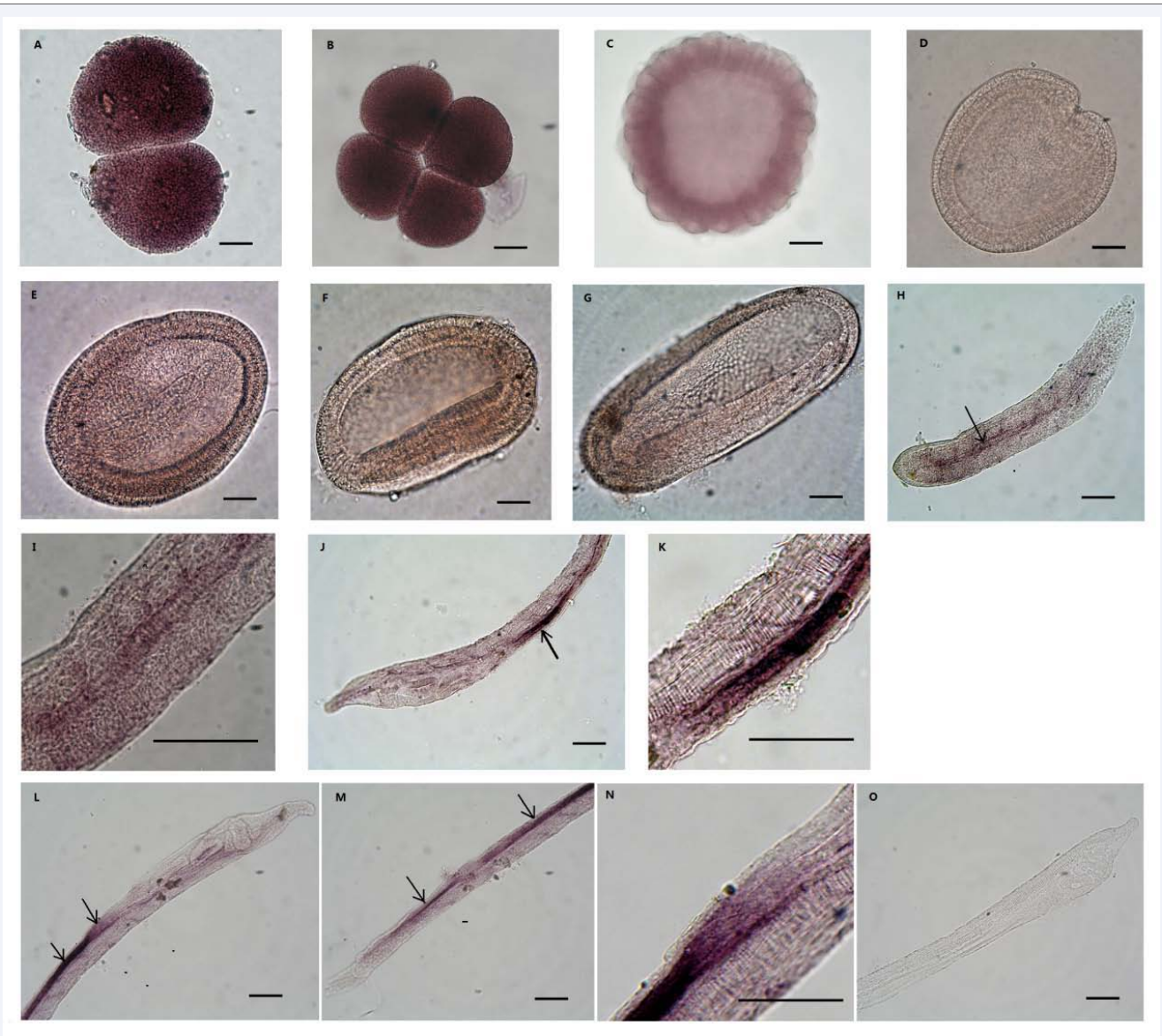


Figure 1 Expression of A2m mRNA in amphioxus embryos and larvae as analyzed by in situ hybridization. (A) 2-cell stage; (B) 4-cell stage; (C) blastula; (D) gastrula of 7 h; (E) neurula of 9 h; (F) neurula of 12 h; (G) neurula of 16 h; (H) 1-day larva, positive signals in primitive gut (arrow); (I) magnification of (H); (J) 3-day larva, positive signals in primitive gut (arrow); (K) magnification of J; (L) and (M) 25-day larva, positive signals in hepatic caecum primordium and whole gut (arrows); (N) magnification of (L); (O) 25-day larva used us control. Bars: 50μm.

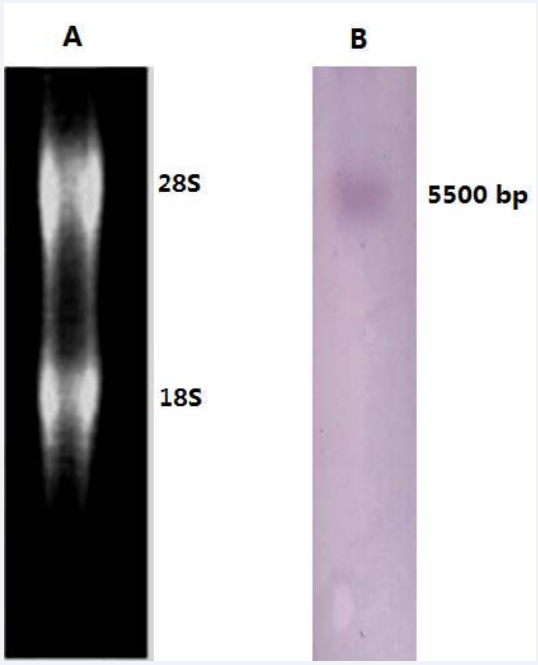


Figure 2 Northern blot analysis for A2m mRNA in unfertilized eggs. A total of 3 μg RNA was separated by electrophoresis on 1.2% agarose formaldehyde-denaturing gel (A) and a band of approximately 5500 bp transcript was hybridized (B).

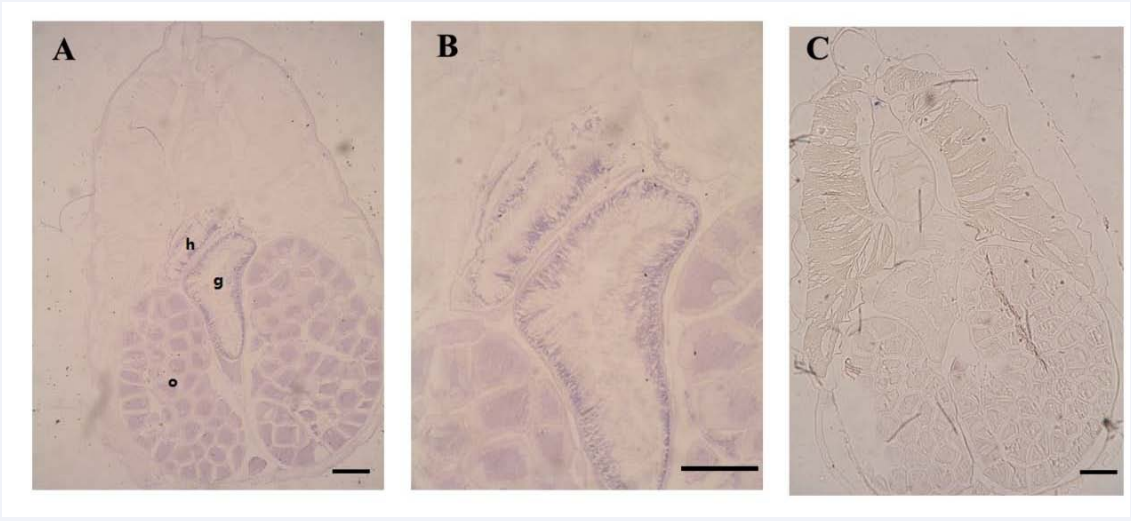


Figure 3 Localization of A2m mRNA in different tissues of by *in situ* hybridization. (A) Staining in hepatic caecum, foregut and and ovary; (B) magnification of (A); (C) the absence of A2m transcript in the control section. H: Hepatic caecum; F: Foregut; O: Ovary. Bars: 100 μm.

larvae by whole mount *in situ* hybridization. Abundant A2m transcript was detected at early cleavage stages (2-4cell) (Figure1A and 1B). The presence of abundant A2m mRNA in 2-4 cell stages suggested that A2m is maternally derived, due to lack of embryonic transcription at early cleavage stages. Further the maternal existence of A2m in eggs was reconfirmed by Northern blot analysis of A2m expression in unfertilized eggs. It was showed there was a band of approximately 5500 bp transcript in eggs, matching the expected size of A2m cDNA in *B. japonicum* (Figure 2). Similarly, existence of maternal A2m mRNA in early embryos was also reported in fish, which was proposed to play a protection role for early embryos development since A2m is an old and native immune molecule [34]. Whereas, due to the depletion of maternal transferred product, no obvious expression of A2m was detectable in subsequent stages from gastrula to late neurula (Figure 1D-G). This expression pattern was also reconfirmed by Quantitative PCR (not published).

Then A2m expression were specifically re-detectable in the primitive gut formation site in 1-day larvae (Figure1H,I) and much stronger expression was specifically detected in primitive gut in 3-day larvae (Figure1J,K). Whereas in 25-day larvae, A2m expression distributed the whole gut and hepatic caecum primordium (Figure 1L-N). In adult amphioxus, previously showed that location of A2m

expression are also in hepatic caecum and hind gut [32], but it is lack of information in foregut. As a complement, *in situ* hybridization was re-performed on the tissue section containing foregut and hepatic caecum. As Figure 3A and B revealed A2m expression were specifically located in foregut and hepatic caecum and ovary. Interestingly, the A2m expression in ascidian *C. intestinalis* was also detected in gut [35]. In vertebrate liver is the major synthesis site of A2m and A2m is essential for fish liver formation. The previous study on several other liver marker genes indicate that amphioxus hepatic caecum is homologous to vertebrate liver [7-11], which well matches the conclusion drawn from morphological studies [3-6]. However, the A2m expression patterns in amphioxus may hints that the entoderm-derived gut and hepatic caecum in amphioxus are not fully functionally separated as liver and gut in vertebrate, and may represents an ancestral archetype of differentiation of liver and gut in vertebrate in evolution.

CONCLUSION

In summary, this study provides an insight into the evolution of vertebrate liver, evidenced by the developmental expression patterns of A2m in the basal chordate amphioxus. Combined the previous study, it suggest that the entoderm-derived gut and hepatic caecum in amphioxus are not fully functionally separated as liver and gut in vertebrate and vertebrate liver may be evolved cooperatively from hepatic caecum and gut of amphioxus.

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