Temporal control of muscle gene expression in an ascidian embryo

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Background

In the larval tail of an ascidian, *Ciona intestinalis*, anterior 28 cells of 36 mononuclear striated muscle cells, which are called the primary lineage muscle cells, are differentiated autonomously. The primary lineage consists of three sub-lineages, B7.4, B7.8 and B7.5.

A maternal factor, Macho-1, has been identified as a muscle determinant localized in the posterior pole of the embryo. Under the control of Macho-1, three transcription factors, Tbx6-r.b, Zic-r.b and Mrf, activate muscle structural genes. However, the regulatory interaction among these three key regulatory factors is not fully understood.

Tbx6-r.b and Mrf directly bind to upstream regions of muscle structural genes. Indeed, two important *cis*-elements for specific expression of a muscle actin gene in embryos of another ascidian *Halocynthia roretzi*, contain an E-box and sequences similar to the Tbx6-r.b binding site motif. However, it has not been demonstrated that these elements bind Tbx6-r.b and Mrf. In addition, it has not been examined whether these two elements have distinct functions or whether they cooperatively activate their targets.

In *Halocynthia* embryos, the expression of the muscle actin gene begins at the 32-cell stage, while expression of other muscle structural genes begins later. It has not been elucidated how expression timings of various muscle structural genes are determined. In addition, it has not been examined when transcription of muscle structural genes ceases. If such transcription continues in late stage embryos or larvae, how is the transcription maintained?

Results

The present study determined precise expression profiles of genes for Tbx6-r.b, Zic-r.b and Mrf by fluorescence *in situ* hybridization, and examined regulatory mechanisms to activate and maintain the expression of these genes. *Tbx6-r.b* expression disappeared in the B7.4 and B7.8 lineages immediately after the initial expression at the 16- and 32-cell stages, respectively, and that *Tbx6-r.b* was re-activated in these lineages after one cell division. While it has been shown that the first wave of the expression is driven by a maternal factor, Macho-1, the present study revealed that the second wave of the expression was activated by Zic-r.b and Mrf. Expression of *Mrf* was controlled both by *Tbx6-r.b* and *Zic-r.b*.

Knock-down assays for *Tbx6-r.b* and *Mrf* revealed that Tbx6-r.b and Mrf were required for expression of a gene encoding myosin regulatory light chain (*Mrlc*) at the tailbud stage and that *Tbx6-r.b*, but not *Mrf*, was required for *Mrlc* expression at the 64-cell stage. A reporter assay identified two critical *cis*-regulatory elements in the upstream region of *Mrlc3*, which is one of three copies of *Mrlc* genes. The upstream element was important for the expression at the 64-cell stage, and the downstream one was important for the expression in late stage embryos. Both elements contained putative Tbx6-r.b binding sites, and the downstream element additionally contained an E-box. These putative Tbx6-r.b binding sites indeed bound Tbx6-r.b protein and the E-box bound Mrf protein *in vitro*, which was confirmed by an electrophoretic mobility shift assay. An analysis using reporter constructs revealed that the functional difference between these two *cis*-elements was not due to the slight difference between nucleotide sequences of these Tbx6-r.b binding sites, but due to the E-box, which was located only in the downstream element.

An analysis of gene expression profiles of five muscle structural genes, *Tnt*, *Mrlc*, *Tni*, *Mlc* and *Tnc,* revealed that these genes began to be expressed at various stages. Chromatin-immunoprecipitation followed by deep-sequencing (ChIP-seq) was employed to determine the occupancy of Tbx6-r.b in the upstream regions of these genes. This analysis showed that genes with higher degrees of Tbx6-r.b occupancy were expressed earlier and stronger. This finding was further confirmed by an analysis of expression profiles of seven muscle genes, and also by reporter constructs with synthetic upstream regulatory regions.

Fluorescence *in situ* hybridization showed that *Mrlc3* transcription was maintained at the middle tailbud stage. Although the Tbx6-r.b binding site in the downstream *cis*-element of *Mrlc3* was responsible for the expression in late stage embryos, *Tbx6-r.b* expression terminated during gastrulation. Because *Tbx15/18/22* was expressed under the control of *Mrf* just after the termination of *Tbx6-r.b* expression, and because Tbx6-r.b and Tbx15/18/22 recognize very similar sequences, *Tbx15/18/22* might regulate *Mrlc3* expression in late stage embryos.

Discussion

The present study revealed a regulatory circuit in which three key regulatory genes, *Tbx6-r.b*, *Zic-r.b*, and *Mrf*, regulated one another. This cross-regulatory circuit maintained the expression of *Tbx6-r.b* and *Mrf* in the muscle lineages during gastrulation. Under this cross-regulatory circuit, muscle structural genes were directly activated. In addition, it has been shown that *Tbx6-r.b* and *Zic-r.b* are initially activated by maternal factors, Macho-1, β-catenin/Tcf7, and Gata.a. Thus, the genetic pathway for muscle cell differentiation in *Ciona* larvae is now understood from the maternal factors to the expression of muscle structural genes.

Expression of muscle structural genes in early stage embryos was controlled by Tbx6-r.b in a quantitative manner. Regulatory elements that bind Tbx6-r.b more strongly induced gene expression earlier and more strongly. On the other hand, in late stage embryos, it was controlled combinatorially by Tbx6-r.b and Mrf. Thus, muscle structural genes are activated and maintained through two different mechanisms.