Focus Article

The maternal muscle determinant in the ascidian egg

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Muscle formation in ascidian embryos has been investigated for more than a century as a representative example of cell fate specification by localized maternal factors within the egg cytoplasm. Observations of colored cytoplasm in combination with micromanipulation techniques have suggested the presence of a muscle-forming factor. The molecular basis has been elucidated with the discovery of macho-1. *macho-1* mRNA is already present in the unfertilized egg, and translocates to the posterior region of the egg during ooplasmic movements. It encodes a zinc-finger transcription factor that positively regulates the expression of target genes. macho-1-binding *cis*-elements have been identified in muscle-specific zygotic genes. Maternally localized *macho-1* appears to have originated in the ascidian lineage, but it activates a muscle-forming developmental program that is shared by the vertebrates. macho-1 is also involved in establishment of the anterior–posterior axis as a competence factor in mesenchyme induction in the posterior region. It is suggested that translation of the macho-1 protein is initiated at the eight-cell stage, and that the protein is inherited by all descendant blastomeres of the posterior-vegetal region. The macho-1 activities in nonmuscle descendants are suppressed or modified by cell interactions during the cleavage stages. In addition to the primary muscle specified by maternal macho-1, ascidian embryos develop secondary muscle, whose fate is determined by cell interactions. Dozens of maternal mRNAs show similar localization to *macho-1*, and these are known as postplasmic/PEM RNAs, being also involved in various posterior-specific developmental events. Evolutionary aspects relevant to macho-1 and tail muscle formation are also discussed in this article.

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INTRODUCTION

How do embryonic cells adopt specific fates during development? Maternal information stored in particular regions of the egg cytoplasm plays crucial roles in cell fate specification processes. This review focuses on the mechanisms that are responsible for muscle formation in the ascidian tadpole larva (Figure 1), which has long been employed as a classical model of localized determinants within the egg cytoplasm. mRNA for *macho-1* (an abbreviation derived from the Japanese term *maboya no choomoshiroi idenshi* 1, meaning "particularly interesting gene 1 in the ascidian") is the most upstream player in the muscle-forming cascade, being stored and localized in the egg cytoplasm as maternal mRNA, and encoding as a transcription factor.

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HISTORICAL BACKGROUND

Eggs of most animals are spherical, and no heterogeneity or specific localization of cytoplasm is recognizable at first sight in many cases. However, the results of various classical studies in experimental embryology involving micromanipulation techniques have suggested the presence of localized activities of tissue-forming factors along the animal-vegetal axis, as well as other axes, of eggs.[2] For a long time, it was hypothesized that certain factors that are localized within the ooplasm, become partitioned into specific blastomeres, and eventually specify cell fates. Molecular

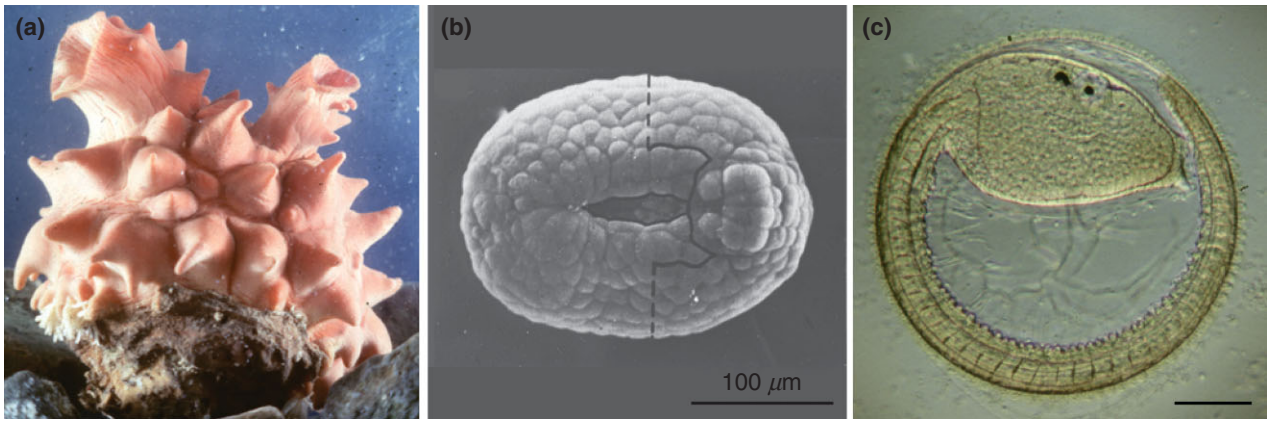

FIGURE 1 | The ascidian, *Halocynthia roretzi*. (a) Adult. (b) Neurula with closing neural tube on the dorsal side.[1] (c) Tadpole larva just before hatching at 35 h of development.

identification of such factors had to wait until molecular approaches became available. Such approaches have now clarified that various macromolecules are indeed localized within the egg cytoplasm, or show a polarized distribution.[3,4]

The first remarkable example of visible localization was reported in ascidian eggs by Conklin in 1905.[5] Phylogenically, ascidians are the closest relative to vertebrates, and develop into tadpole larvae with a simplified chordate-type body plan (Figure 1). Conklin described ascidian embryogenesis in surprising detail. Regions in the egg cytoplasm of *Styela partita* showing various colors and textures allowed him to trace the cell lineages during embryogenesis. Yellow-colored cytoplasm that moved after fertilization, and was inherited by muscle-lineage blastomeres, was especially evident (Figure 2(a), published as a color print more than a century ago). Eventually, muscle cells in the tadpole larva acquired a yellow color. This observation led Conklin to propose that the yellow cytoplasm contains muscle-forming factors. This monumental observation has been cited in many developmental biology textbooks, the yellow cytoplasm in ascidian eggs being referred to as the yellow crescent or myoplasm. The myoplasm is rich in pigment granules in some ascidian species, and also rich in mitochondria that enable the actively contractile muscle cells of the tadpole larva to efficiently produce energy.[6] Conklin's observations clearly demonstrated that embryos are able to segregate certain localized substances into distinct cell lineages.

Blastomere isolation experiments have shown that ascidian embryos develop in a mosaic manner,[8,9] isolated blastomeres being able to assume their developmental fate in a cell-autonomous manner, as if still part of the whole embryo. Thus, ascidian eggs have been regarded as typical mosaic eggs. Similarly, when mitosis is permanently arrested at the cleavage stages, the arrested blastomeres in the embryos eventually express the specific differentiation markers for several tissues, including muscle, which would be expected for their cell lineages.[10] These findings highlight the importance of cytoplasm that is partitioned into each embryonic cell, and exclude involvement of cell interactions in fate specification at later stages of development. More direct evidence of localized factors in egg cytoplasm has been obtained by egg cytoplasmic transfer experiments. Fragments of various regions of eggs at various stages of ooplasmic movement have been prepared and fused with non-muscle blastomeres using cell fusion methods. The regions that correspond well with Conklin's yellow cytoplasm have activity to promote ectopic muscle formation (Figure 2(b)).[11] Similar experiments have mapped, within the egg cytoplasm, the distributions of cytoplasmic activities for formation of epidermis and endoderm, and those responsible for gastrulation and unequal cleavages. These factors are already present in unfertilized eggs, and shift during ooplasmic movements to become relocalized at sites corresponding to the appropriate region in the fate map.[12]

MOLECULAR IDENTIFICATION OF THE MUSCLE DETERMINANT

Advances in molecular biology techniques ultimately led to the identification of the localized maternal muscle determinant as *macho-1* maternal mRNA.[7] To isolate localized mRNAs that are present only in the vegetal hemisphere, polymerase chain reaction-based subtraction hybridization screening was carried out. Four hundred embryos of *Halocynthia roretzi*, which spawns relatively larger eggs, were bisected into animal and vegetal hemispheres at the eight-cell stage

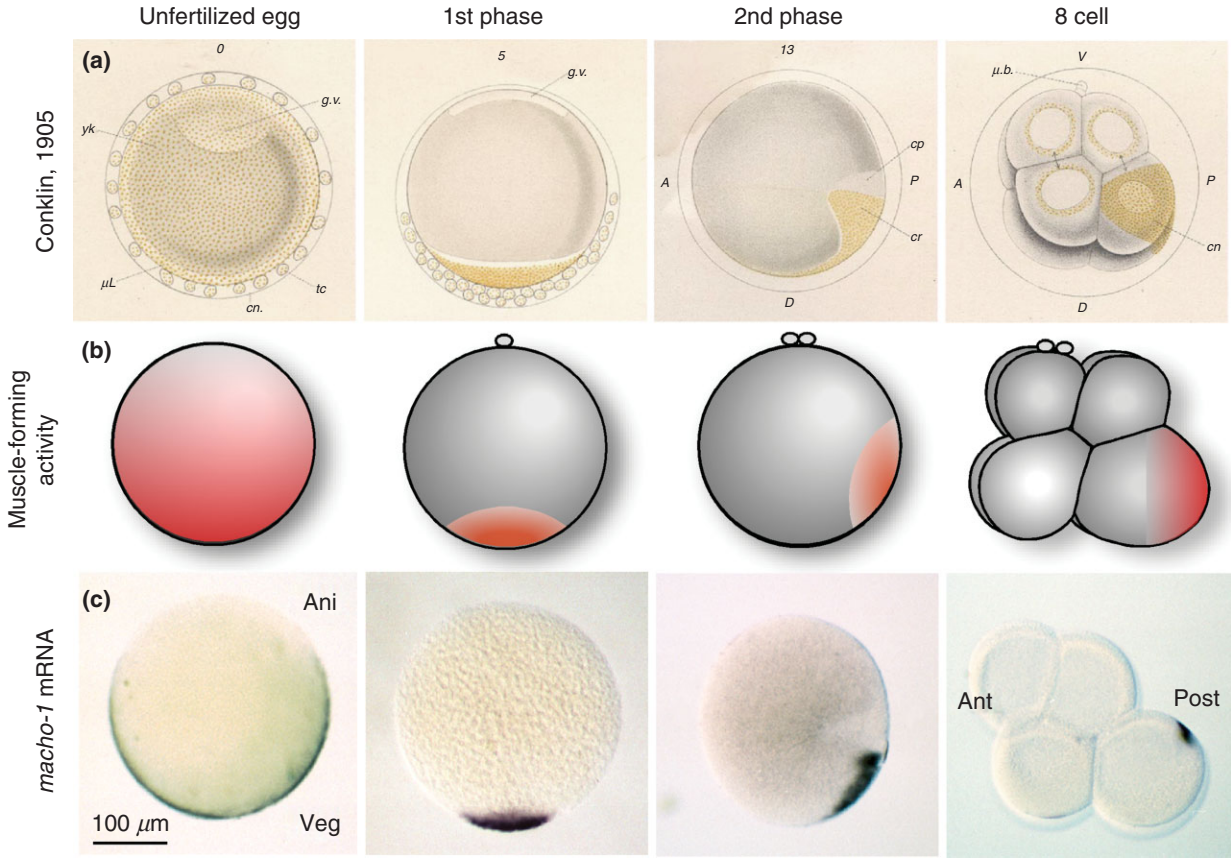

FIGURE 2 | Ooplasmic movements and localization of various factors. Cytoplasmic and cortical reorganization proceed in two major phases during the first cell cycle. (a) Yellow myoplasm in *Styela partita*. Copy of the original drawings by Conklin (1905).[5] Yellow cytoplasm is localized to the unfertilized egg cortex. It is concentrated at the vegetal pole just after fertilization, then gradually moves toward the future posterior pole before the first cleavage takes place. It is segregated into the posterior-vegetal muscle lineage blastomeres, and eventually into tail muscle cells of the tadpole larva. (b) Distribution of muscle-forming activity inferred from the results of cytoplasmic transfer experiments. (c) Localization of maternal mRNA of *macho-1*. Ani, animal pole; Veg, vegetal pole; Ant, anterior; Post, posterior. (Reprinted with permission from Ref 7. Copyright 2001 Nature Publishing Group)

with a glass needle. mRNA was purified from both hemispheres and converted to cDNA, and then animal cDNA was subtracted from vegetal cDNA. The distribution of mRNAs for 50 subtracted clones was examined by in situ hybridization, and three clones showed staining that corresponded well to the localization of the inferred muscle determinant (Figure 2(b) and (c)). Two of them included *macho-1* cDNAs, and the other had a cDNA of *PEM*, which is involved in generation of the posterior-specific cleavage pattern and unequal cell divisions.[13]

Loss-of-function-type experiments offer a straightforward interpretation of the functions of genes. *macho-1* mRNA was depleted by injection of antisense phosphorothioate oligodeoxynucleotides (S-oligo). Unfertilized eggs were injected, kept overnight, and fertilized the next day after a time sufficient for depletion of the targeted RNA. At hatching, the macho-1-depleted larvae had an apparently normal trunk region, but tail formation was severely perturbed (Figure 3(a) and (c)), the tail muscle cells being significantly reduced (Figure 3(b), (d), and (e)). Expression of the *muscle actin* gene is initiated during the cleavage stage in muscle-lineage cells (Figure 4), and this expression was also abrogated (Figure 3(f) and (g)). The defect in muscle formation was rectified by injection of *macho-1* mRNA, supporting the specificity of the S-oligo. In these cases, ectopic muscle formation was also observed. Removal of the coding region of the putative DNA-binding domain abolished the rescuing ability of the mRNA. Simple overexpression of macho-1 elicited by injection of wild-type mRNA resulted in aberrant embryos, in which most of the embryonic cells expressed the *muscle actin* gene at the cleavage stage, and much ectopic muscle was formed in larvae that lacked any morphological head and tail (Figure 3(h) and (i)). Recently, antisense morpholino oligodeoxynucleotides (M-oligo or MO) have

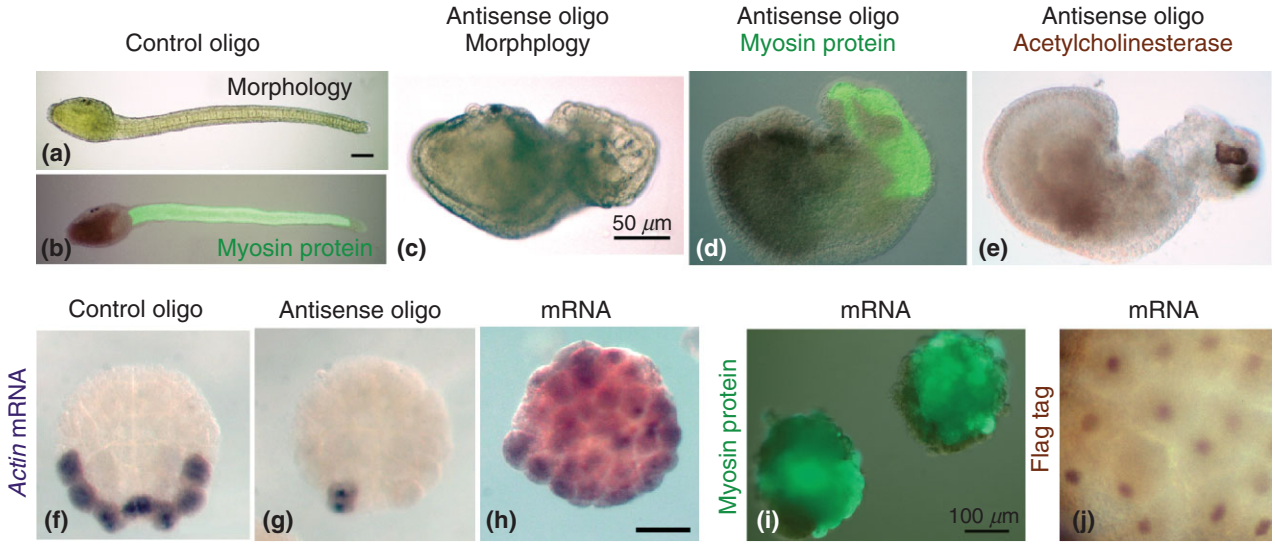

FIGURE 3 | macho-1 as a muscle determinant. (a),(b) Larval morphology and myosin expression in tail muscle cells in the control experiment. (c)–(e) Larvae in which antisense oligonucleotide against *macho-1* mRNA has been injected. (f)–(h) *Muscle actin* mRNA expression at the 110-cell stage. (i) Myosin expression in larvae into which *macho-1* mRNA has been injected. (j) Nuclear localization of flag-tagged macho-1 protein that was translated from injected mRNA, in every nucleus at the 110-cell stage. (Reprinted with permission from Ref 7. Copyright 2001 Nature Publishing Group)

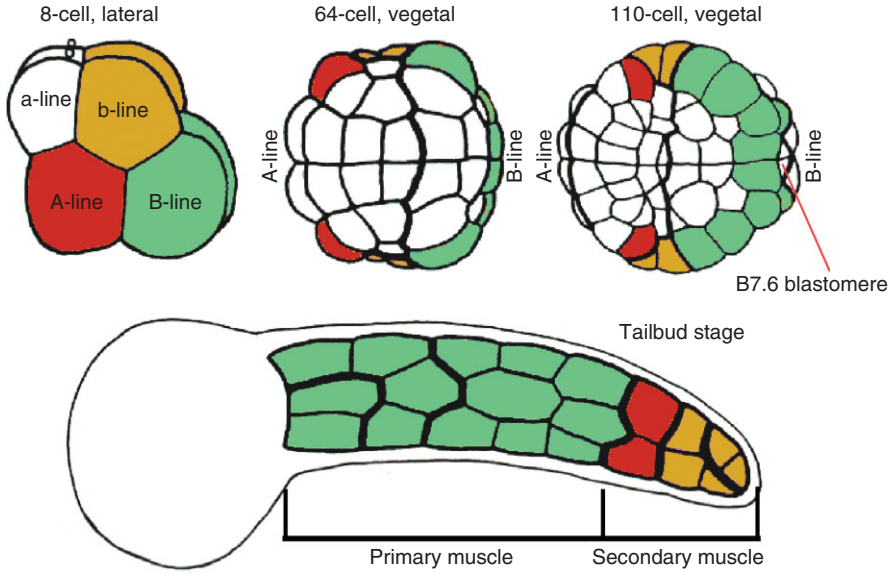

FIGURE 4 | The primary (green) and secondary (orange and red) muscle lineages.[33] Primary muscle cells are derived from the B-line (posterior-vegetal) blastomeres. Secondary muscle cells originate from the A-line (anterior-vegetal) and b-line (posterior-animal) blastomeres. macho-1 is involved in muscle fate determination in the primary lineage. Anterior is to the left. Note that the anterior-posterior order of muscle progenitor cells is reversed during the gastrula and neurula stages.

been commonly used to study gene functions, and M-oligo also effectively knocks down the functions of *macho-1*.[14] It is possible to deplete mRNA using S-oligo with the aid of endogenous RNase H activity, while M-oligo inhibits translation of the protein. Injection of M-oligo after fertilization also results in loss of muscle, indicating that translation of *macho-1* is initiated after the start of embryogenesis.

Thus, *macho-1* mRNA is both required and sufficient for specification of muscle fate during embryogenesis. However, this criterion is not enough to confirm conclusively that macho-1 is, in fact, the localized muscle determinant, i.e. whether it lies at the start of the cascade of muscle fate determination. For example, in frogs, maternally supplied β-catenin is required and sufficient for promoting the development of dorsal

structures. However, when the dorsal cytoplasm of β-catenin-depleted eggs is transferred to the ventral side of an intact egg, it still induces a secondary dorsal axis.[15] This indicates that the dorsal determinant is still present in embryos lacking β-catenin, and that β-catenin functions as a component of the machinery transducing the dorsal determinant, but is not the dorsal determinant itself. macho-1, however, is a true muscle determinant. A similar experiment to test macho-1 has been performed.[7] The posterior-vegetal cytoplasm of fertilized eggs has the ability to promote muscle formation when transferred into blastomeres fated to give rise to epidermis. By contrast, the posterior-vegetal cytoplasm of macho-1-depleted eggs does not have this ability. Therefore, macho-1 is clearly the most upstream player in the muscle-forming cascade.

macho-1 AS A TRANSCRIPTION FACTOR

The macho-1 protein has five zinc finger repeats showing highest similarity to that of vertebrate Zic family proteins, and an odd-paired protein of *Drosophila*. Zic are transcription factors that function mainly in the development of neural tissues, the neural crest and dorsal somites in vertebrates.[16] Ascidians also have a Zic ortholog. To determine whether macho-1 acts as a transcription factor that promotes or represses transcription, the effects of transcription-activation (VP-16) and transcription-repression (En^R) domains conjugated with macho-1 were examined. VP-16 fusion has the same effect as intact macho-1, while En^R fusion suppresses zygotic muscle gene expression.[14] Thus, macho-1 functions as a transcriptional activator. Consistent with its function as a transcription factor, flag-tagged macho-1 protein is transported into the nucleus (Figure 3(j)). A macho-1-binding consensus nucleotide sequence has been identified with SELEX, and by an electron mobility shift assay with mutated probes.[14,17] The sequence is t/cgGGg/tGg/tt/c for *Halocynthia* and CagcGGGgggC for *Ciona*. Mandatory nucleotides are indicated by capital letters. The two consensus sequences do not match perfectly, but it is evident that macho-1 binds to guanine-rich sequences. The genome sequence of *Ciona* is available, and several candidate-binding sites have been found in the 5' upstream regions of macho-1 downstream genes.[18]

Maternal macho-1 activates zygotic expression of various muscle-specific genes during cleavage stages, and these include the *muscle actin, muscle myosin*, myogenic factor *MyoD homolog*, *Tbx6*, and *snail* genes in both *Halocynthia* and *Ciona*.[14,17] Among these, MyoD and Tbx6 are also essential and sufficient to some extent for muscle formation in ascidians as transcription factors.[19,20] As MyoD and Tbx6 are also involved in muscle formation in various other organisms,[21,22] macho-1 activates a well-conserved developmental program of muscle formation, although macho-1 is probably unique to ascidians.

SECONDARY MUSCLE FORMATION

The muscle cell lineages of ascidians are shown in Figure 4. There are two types of muscle cells in the larval tail: primary muscle (28 out of the total of 42 muscle cells) in the anterior and middle regions of the tail, and secondary muscle (14 cells in *Halocynthia* and 8 cells in *Ciona*) in the posterior region.[23] The primary muscle cells are derived from B-line (posterior-vegetal, shown in green) blastomeres, while the secondary muscle cells originate from A-line (anterior-vegetal, red) and b-line (posterior-animal, orange) blastomeres. macho-1 is involved only in fate specification of the primary muscle cells, as the mRNA is partitioned into the B-line blastomeres, but not into the A- and b-lines. Although muscle is reduced in macho-1-depleted larvae, some muscle cells are always present and are often located at the tip of the tail (Figure 3(d) and (e)). The results of blastomere isolation experiments at the eight-cell stage suggest that the muscle cells formed in macho-1-depleted larvae are those of the secondary lineage.

The cell fate of secondary muscle lineage cells is specified during late cleavage stages, and is dependent on cell interactions and embryonic induction.[24] The mechanisms vary among ascidian species: Wnt signaling is involved in *Halocynthia*, while nodal and Notch signaling occurs in *Ciona*. The muscle-forming program can be activated by a cell-autonomous mechanism involving macho-1 and inductive cell interactions. Therefore, ascidian embryos produce the same type of cells using distinct mechanisms. In vertebrates, muscle cell formation is dependent on mesoderm induction, which is mediated by nodal signaling.[25] The secondary muscle cells of ascidians and vertebrates muscle cells may share a common origin. If this is the case, then the secondary muscle cells would be ancestral in ascidian evolution. Another possibility is that ascidians might have added secondary muscle cells to the tip of the tail during their evolution after the development of macho-1-mediated cell autonomous mechanisms. So far, we have been unable to conclude which of these possibilities is most likely, as all ascidian species examined to date have both primary and secondary muscle cells.

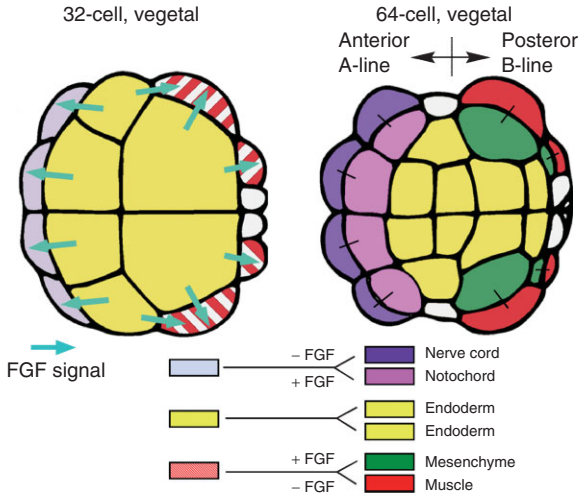

FIGURE 5 | Fate specification in the vegetal hemisphere at the 32-cell and 64-cell stages.[34] Anterior is to the left. An FGF signal (arrows) secreted from the vegetal endoderm blastomeres promotes asymmetric cell divisions in the marginal zone. The signal induces notochord and mesenchyme fates. The default fates are nerve cord and muscle, respectively. Sister blastomeres are connected with bars.

macho-1 AS A POSTERIOR DETERMINANT

Although macho-1 was first identified as a muscle determinant, it was later found to be also required for mesenchyme formation. Mesenchyme cells are not fully differentiated in the swimming tadpole, and give rise to cells in the tunic, which is a sheath surrounding the whole body of the adult ascidian.[26] The mechanism of fate specification in the vegetal hemisphere at the 32- and 64-cell stages is shown in Figure 5. An FGF signal (light blue arrows) secreted from the vegetal endoderm blastomeres (shown in yellow) promotes asymmetric cell divisions in the marginal zone, producing two daughters with distinct fates: an induced fate and a default fate. The FGF signal induces notochord fates (pink) in the anterior region and mesenchyme fates (green) in the posterior region. The default fates are nerve cord (purple) and muscle (red), respectively.[12,27] The mother cells receive the fibroblast growth factor (FGF) signal from endoderm located at one side of the cells, and are polarized by the directional signal to undergo asymmetric cell division. If mother cells receive the FGF signal at the opposite side, the positions of muscle and mesenchyme cells become reversed.[28]

macho-1 acts as a competence factor in the induction of mesenchyme by the FGF signal. In the posterior marginal zone, macho-1-inheriting cells respond to the FGF signal by forming mesenchyme cells. In macho-1-knockdown embryos, the posterior cells respond to the FGF signal by forming notochord cells, as if acting as the anterior cells.[29] Thus, macho-1 plays a role in anterior-posterior discrimination. When macho-1 is overexpressed, most cells assume the muscle fate, as mentioned before. However, injection of a moderate amount of synthetic *macho-1* mRNA results in muscle development in cells that do not receive the FGF signal, and in mesenchyme formation in the position of notochord precursors in the anterior region. In addition, ectoderm precursor cells in the animal hemisphere that are injected with *macho-1* mRNA and treated with FGF protein assume the mesenchyme fate. Therefore, macho-1 as an intrinsic competence factor, in addition to the FGF signal as an external signal molecule, is sufficient for mesenchyme induction.

macho-1 mRNA is concentrated in a tiny region at the posterior pole at the eight-cell stage (Figure 2(c)). The blastomere that inherits the mRNA is a B-line primary muscle lineage cell, the B4.1 blastomere (Figure 4). Its localization differs from that of Conklin's yellow myoplasm at the eight-cell stage. Eventually, *macho-1* mRNA is inherited only by the B7.6 blastomeres at the posterior pole at the 110-cell stage (Figure 4). B7.6 blastomeres are no longer muscle precursors, but rather germ cell progenitors.[30] How is macho-1 protein preferentially partitioned to the blastomeres of muscle and mesenchyme precursors within the posterior-vegetal quarter after the eight-cell stage (Figure 5)? There is evidence that macho-1 translation is initiated at the eight-cell stage, and that the protein is distributed to every descendant of the B4.1 blastomere. This is because when the B4.1 blastomere is isolated and its descendant cells are continuously dissociated during subsequent cleavages, every cell develops into muscle, indicating that all of them inherit the macho-1 protein.[31] Formation of mesenchyme and endoderm is abrogated. As mentioned above, the FGF intercellular signal is required for mesenchyme development; otherwise the cells assume a default muscle fate. Similarly, specification of an endoderm fate requires either the FGF or bone morphogenetic protein (BMP) signal to totally suppress the function of macho-1. In the anterior quarter, formation of A-line endoderm (Figure 5, yellow) does not require cell interaction because macho-1 protein is not present there. When macho-1 is depleted, formation of the B-line endoderm also becomes cell-autonomous. Therefore, signaling plays a role in preventing macho-1 from functioning in endoderm cells. There is a clear difference in the response to the FGF signal between vegetal endoderm blastomeres and marginal mesenchyme blastomeres. FGF suppresses macho-1 function in endoderm, whereas the same

signal modifies macho-1 function in mesenchyme. The difference is likely due to Not and Zic transcription factors.[32] Thus, embryos do not have a mechanism that precisely segregates macho-1 protein into muscle and mesenchyme precursors; rather, macho-1 is distributed to every descendant after the eight-cell stage, and then its function is modulated by cell interactions.

macho-1 mRNA AS A MEMBER OF THE *postplasmic/PEM* RNAs

macho-1 mRNA does not diffuse within the cytoplasm, and it has been shown to colocalize with, and probably be anchored to, a single-layered network of cortical endoplasmic reticulum (cER) that is tethered to the plasma membrane.[35] The deep cytoplasm contains most ER, but no *macho-1* mRNA is present there. Therefore, the cER has a special property that allows anchoring of *macho-1* mRNA, probably attributable to a still unknown mRNA-binding protein. *macho-1* mRNA translocates during ooplasmic movement and becomes concentrated within a tiny region at the eight-cell stage, together with the cER.

Interestingly, macho-1 is a member of the *postplasmic/PEM* RNAs that show an identical localization pattern during embryogenesis, as shown in Figure 2(c). So far, approximately 50 maternal mRNAs have been identified as *postplasmic/PEM* RNAs using various methods.[36] They encode a variety of proteins such as transcription factors, secreted signal molecules, kinases, and RNA-binding proteins. Some of them have been shown to play crucial roles in developmental events specific to the posterior region of the embryo, for example, the primary muscle formation mediated by macho-1, germ cell formation, and generation of the posterior-specific cleavage pattern. It has also been revealed that some postplasmic/PEM RNAs have localization elements (or zip codes) in their 3'UTR.[37,38] It appears that a number of maternal RNAs show the localization pattern similar to that of *macho-1*, and that this type of localization is remarkable and probably sole localization pattern in maternal mRNAs of ascidian eggs.[39]

EVOLUTIONARY CONSIDERATIONS

The macho-1 protein is a transcription factor with a zinc finger DNA-binding domain that shows highest similarity to that of vertebrate Zic family proteins. Ascidians also have an ortholog of the Zic gene.[40] The expression of ascidian *Zic* is temporally and spatially comparable to that of vertebrate Zic. Therefore, macho-1 and Zic of ascidians and vertebrate Zic may have originated from the same ancestral gene, although the function of macho-1 has greatly diverged since gene duplication of Zic and macho-1 in the ascidian lineage. Specification of a muscle cell fate by a localized maternal determinant appears to be invented in ascidians. macho-1 and Zic transcription factors activate the expression of distinct gene subsets.[17,41] The binding consensuses of these two are very similar, but the mandatory nucleotides (capital letters) are different in *Ciona*: CagcGGGgggC for macho-1 and CAGCggg for Zic, although recent results of ChIP assay indicated that Zic binds to AGTGTGCGCA.[42] Similarly, *Halocynthia* macho-1 is able to bind a vertebrate Zic-binding nucleotide sequence.[14] macho-1 and Zic diverged to activate distinct target genes, but how these two genes with similar DNA-binding domains discriminate the target nucleotides remains unclear.

Ascidians (Class Ascidiacea) are taxonomically divided into two orders: Enterogona and Pleurogona. The cleavage pattern and muscle lineages (Figure 4) are common in these two orders, at least in species that have a solitary lifestyle. However, both of these orders also include various species of colonial ascidians that form colonies of small individuals by asexual reproduction, budding, or blastogenesis. Many species of colonial ascidians produce eggs as large as 1 mm in diameter. These eggs develop slowly into giant larvae with a large number of muscle cells in the tail, for example, *Ecteinascidia tubinata* has more than a thousand muscle cells. Our knowledge of the development of these ascidians is still limited, and which of the primary and secondary muscle lineages proliferate is not known. It will be interesting to investigate the cell lineages and macho-1 homologs in these species. Another example of a derived alternative mode of tail muscle formation is found in direct developers, which skip the larval stages and develop an adult morphology directly; larval tail and muscle cells are not formed.[43] The cleavage pattern is conserved, and muscle and notochord lineage cells are also present, but do not undergo morphogenesis leading to tail formation and do not differentiate. Interestingly, in the eggs of an extreme direct developer, *Molgula tectiformis*, the *macho-1* gene is present and its mRNA is still localized.[44] Zygotic expression of the *Tbx6* gene is also observed in muscle-lineage cells. However, expression of muscle structural genes is suppressed. These observations suggest that, in this tailless ascidian, early events in the specification of a muscle cell fate are still maintained, but that later processes of muscle cell differentiation are abrogated. We have begun to clarify the evolutionary processes responsible for these alternative modes of ascidian development, although much still remains to be understood.

CONCLUSION

Muscle formation in ascidian embryos is an excellent example of cell fate specification by localized maternal factors within the egg cytoplasm. Cell fate specification and, more generally, establishment of embryonic axes by localized maternal factors is a common strategy employed as an initial step of embryogenesis in most animals, with the obvious exception of mammals. The molecular identities of such localized factors have been revealed mainly in model organisms, but the molecular natures of localized factors in other organisms remain unclear. With regard to ascidian eggs, the molecular identities of localized factors that govern the establishment of the primary axis, the animal-vegetal axis, are still unknown. Also, much remains to be clarified about how macho-1 evolved in the ascidian lineage. However, it is anticipated that further investigation of the *macho-1* gene and the muscle-forming mechanisms operating in ascidians with various modes of muscle development, and in closely related taxa such as the Appendicularia (larvaceans) and amphioxus (lancelets), will provide valuable clues.

ACKNOWLEDGMENTS

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FURTHER READING

Ascidian development site in BioMarCell. Available at:http://biodev.obs-vlfr.fr/recherche/biomarcell/ascidies/ascidiemenu.htm.

Movie collections of embryogenesis including ascidian development. Available at: http://www.celldynamics.org/celldynamics/gallery/timelapse.html.

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