

Transfer of a large gene regulatory apparatus to a new developmental address in echinoid evolution

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Of the five echinoderm classes, only the modern sea urchins (euechinoids) generate a precociously specified embryonic micromere lineage that ingresses before gastrulation and then secretes the biomineral embryonic skeleton. The gene regulatory network (GRN) underlying the specification and differentiation of this lineage is now known. Many of the same differentiation genes as are used in the biomineralization of the embryo skeleton are also used to make the similar biomineral of the spines and test plates of the adult body. Here, we determine the components of the regulatory state upstream of these differentiation genes that are shared between embryonic and adult skeletogenesis. An abrupt “break point” in the micromere GRN is thus revealed, on one side of which most of the regulatory genes are used in both, and on the other side of which the regulatory apparatus is entirely micromere-specific. This reveals the specific linkages of the micromere GRN forged in the evolutionary process by which the skeletogenic gene batteries were caused to be activated in the embryonic micromere lineage. We also show, by comparison with adult skeletogenesis in the sea star, a distant echinoderm outgroup, that the regulatory apparatus responsible for driving the skeletogenic differentiation gene batteries is an ancient pleisiomorphic aspect of the echinoderm-specific regulatory heritage.

embryonic and adult skeletogenesis | gene network evolution | sea urchin | sea star

The geometrical, elegantly formed bilateral skeleton of the sea urchin embryo is one of its most prominent features. Its vertex underpins the aboral vertex of the embryo body wall, producing its triangular shape, and its rods extend four arms that project anteriorly of the mouth, mounting rows of cilia that assist in transfer of food microorganisms for ingestion. Its exact form is species-specific, but an embryonic skeleton is a canonical character of all indirectly developing modern sea urchins (i.e., the “thin spined” or euechinoid sea urchins). The skeleton is constructed during and immediately after the gastrula stage of development, by dedicated cells all belonging to a polyclonal lineage consisting of all descendants of four fifth-cleavage large micromeres (micromere lineage). These micromeres arise at the vegetal pole of the egg in a stereotypic series of unequal cleavages. The micromere lineage undergoes a set number of further cleavages during blastula stage while they reside in the center of the vegetal plate, and then, well before gastrular invagination of the archenteron, these cells acquire motility, and singly ingress into the blastocoel. In *Strongylocentrotus purpuratus*, the subject of most of the work in this article, there are 16 skeletogenic micromeres at time of ingress, and this number just doubles as skeletogenesis begins. The skeletal rods or spicules are deposited on the inner wall of the blastocoel within syncytial cables formed by the now mesenchymal descendants of the micromere lineage and are positioned according to signals produced by the ectoderm (ref. 1; for review of embryonic skeletogenesis, see ref. 2).

Even the nearest outgroup to the euechinoids, the “pencil urchins” or cidaroids, lack a precociously invaginating skeletogenic micromere lineage (3–5). Their skeletogenic cells ingress with other kinds of mesodermal cell types at gastrulation, and

they form the skeleton much later, at the very end of embryogenesis just before feeding begins. This distinction provides a time line for the evolutionary origin of the euechinoid type of skeletogenic micromere lineage, because the only sea urchin-like survivors of the Permian-Triassic extinction 250 mya were two cidaroid lineages (6); the first euechinoids appear only later, in the Triassic. No other class of echinoderms produces a skeletogenic lineage either [for phylogeny of the echinoderm classes, see [supporting information \(SI\) Fig. S1](#)]. Indirectly developing crinoid, sea star, and sea cucumber embryos have no skeleton at all, and, although ophiuroids do, they have no micromere lineage and build the skeleton differently (7, 8). The euechinoid skeletogenic micromere lineage and its embryonic skeletal structures thus may be considered a derived euechinoid character that appeared in the euechinoid common ancestor probably not long after 250 mya.

How could this character have arisen, in terms of the heritable genomic regulatory program that controls development? More precisely, what regulatory innovations could have been responsible for inserting skeletogenesis into a micromere embryological address? A starting point is at the differentiation gene batteries that generate the biomineral matrix. Earlier evidence indicated that many specific protein components of the biomineral matrix are present in both demineralized embryonic skeletal spicules and demineralized adult endoskeleton, i.e., spines and test plates (9). Analysis of biomineralization genes in the *S. purpuratus* genome sequence (10) proves this is the case, although, for some families of these genes, certain paralogues are used in adult skeletons, some in embryonic and some in both. Thus, to begin, we may consider that our problem resolves at base into the regulatory changes that might have been required to cause mobilization of what were adult skeletogenic differentiation gene batteries in the blastula stage micromere lineage cells. This is when in development downstream skeletogenic differentiation genes begin to be expressed in the micromere lineage (for review, see ref. 11).

Recently the GRN was solved that embodies the program for development of the micromere lineage, all of the way from its initial specification, to expression of skeletogenic differentiation genes (12). Above the differentiation genes there are 23 genes in this network, all encoding transcription factors, except for some that encode signaling functions, another essential aspect of the developmental role of the micromere lineage. Interactions among these regulatory genes generate a progressive sequence of regulatory states, beginning with those that define the distinct functional identity of the micromeres, then enhancing and stabilizing the expression of the definitive set of regulators that

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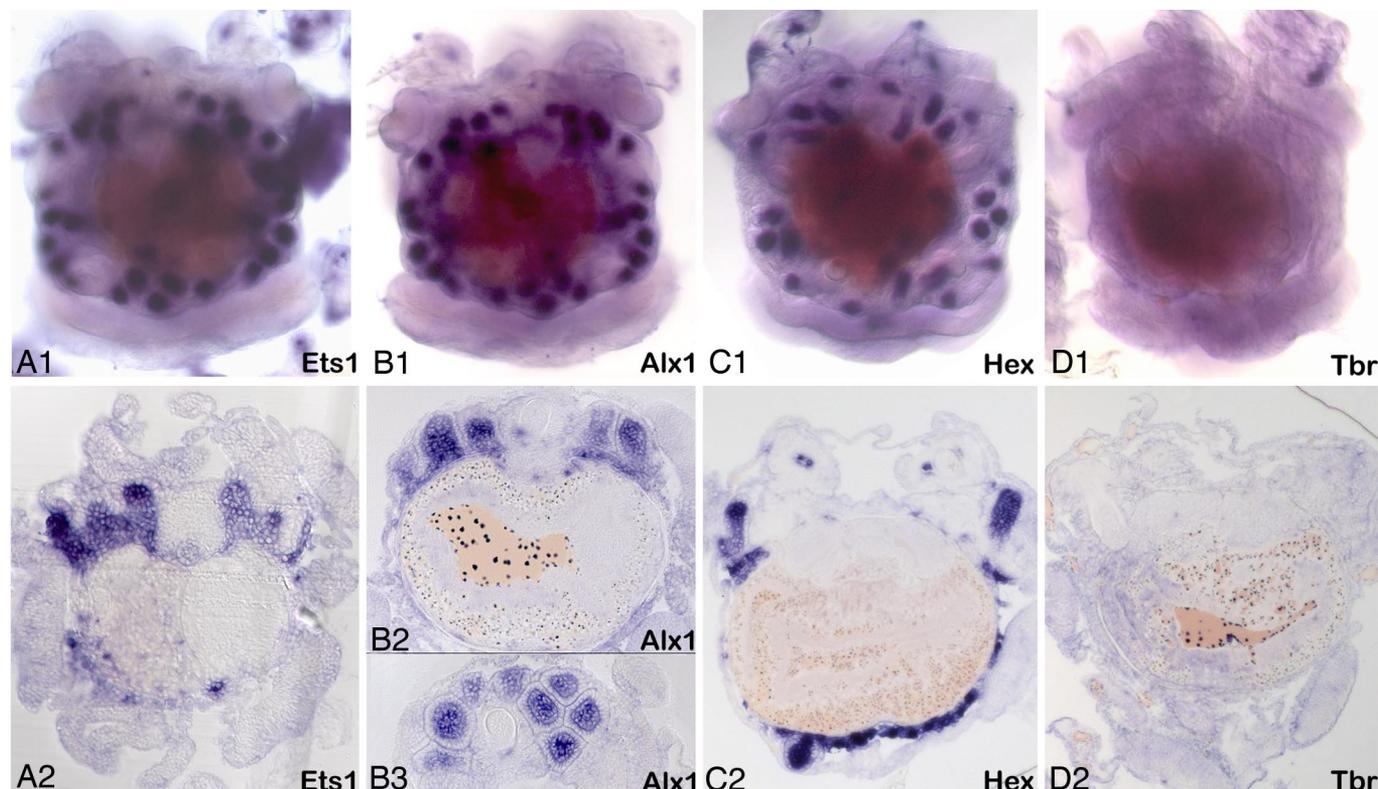


Fig. 2. Representative WISH of probes for genes expressed in the micromere lineage to juvenile skeletonization centers of sea urchin larvae. (*Upper*) Larval WISH viewed from the left sides of the larvae, displaying labeling in ventral and dorsal spines. (*Lower*) Sections made after WISH, cut along the left-right axis, except B3, which is cut along the oral-aboral axis. Identity of probes is indicated in the figures. (A2, B2, and C2) Labeling in forming spines and plates, and, in C2, the tube foot discs as well. (D) The *tbr* gene is not expressed in the juvenile skeletogenic centers.

advanced sea star larvae for expression of eight key skeletogenic regulatory genes and one downstream gene (Table S1) and found results identical to those seen in sea urchin larvae. Representative examples are shown in Fig. 4, and the remainder are shown in Fig. S3. The skeletogenic regulatory apparatus may thus have existed as a modular GRN unit since early in echinoderm history, and it was this same unit that was placed into the embryological control system defining the micromere lineage regulatory state. There is presumptive evidence for another highly conserved GRN kernel, such as that responsible for endoderm specification discovered in a comparison of endomesoderm GRNs of the sea star and the sea urchin (16). But this proposition could only be substantiated only by determining whether the actual *cis*-regulatory linkages are as pleiomorphic as is the skeletogenic gene expression complex.

Discussion

We show here the added value for evolutionary analysis of a solved developmental GRN. The GRN provides a map for orientation of expression data. In Fig. 3, we show a clear boundary on the GRN map between the micromere regulatory apparatus that is shared with the juvenile skeletogenic centers and the apparatus that is particular to this embryonic lineage, and, from this, some conclusions can be drawn. Although we cannot be certain that the genes in the shared region of the map are indeed linked similarly in the two developmental contexts, we can be fairly certain of the parts that are micromere-specific.

The Micromere “Address”: Past and Present. In terms of embryological function the micromeres have first to interpret the anisotropic inputs sequestered uniquely within them and transform them into a zygotic regulatory state and then to

perform two general classes of function. These are to express essential intercellular signals and to develop skeletogenic potential. As we discuss in ref. 12, the micromere lineage GRN provides a complete causal explanation of these functions. The first two of these tasks are executed respectively by the apparatus in the violet and green portions of the comparison map in Fig. 3, and the third by that in the red is the shared portion.

One interpretation is that an earlier evolutionary state in the echinoid line was the presence of micromeres that operated the violet and green portions of the current GRN; i.e., that established their initial unique regulatory state to serve as an embryonic signaling center. There are two essential micromere signals. The first is the “Early Signal” (ES in the GRN), still not defined biochemically, which in echinoids executes a derepression function needed to permit the endomesodermal development of the adjacent cells. The second is the Delta signal, used in echinoids for specification of mesoderm, particularly pigment cells. Significantly, both are under control of the *pmar1-hesC* regulatory system, as can be seen in Fig. 3. Both of these genes encode repressors and this system functions as a double-negative gate (17–19). This gate performs the function of transducing the initial anisotropies sequestered in the micromeres into a micromere-specific regulatory state, because the *pmar1* gene is activated in response to these localized inputs. Thus, once presented in the micromeres, the Pmar1 repressor in turn represses the *hesC* gene, the source of an otherwise global zygotic transcriptional repressor that keeps the ES and Delta genes off outside the micromeres. Its target genes, here the *es* and *delta* genes, are thereby activated specifically in the micromeres. The micromere double-

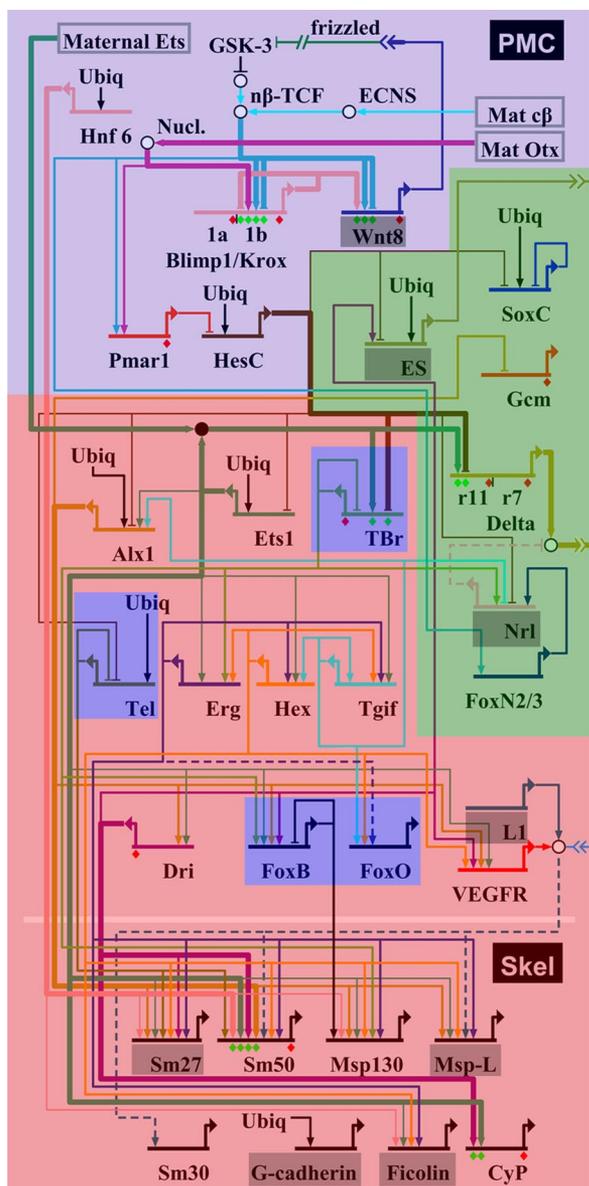


Fig. 3. Map of gene expression data on micromere GRN. Genes expressed in juvenile skeletogenic centers and micromere lineage are shown over the red background. Genes used only in micromere lineage (<24–30 h) are shown over violet and green backgrounds. Green denotes genes used in micromere-specific signaling functions. The violet territory at the top denotes the portion of the GRN that executes the initial micromere specification function (see text); the four additional blue genes are individual cooptations, as discussed. Several genes not studied here are denoted over gray backgrounds.

negative gate may have evolved initially as a controller of the micromere signaling apparatus.

The double-negative gate is also the precise junction between the rest of the micromere apparatus (blue in Fig. 3) and the part shared with the juvenile skeletogenic centers (red in Fig. 3). To effect the incorporation of the latter into the micromere GRN, two genes of the adult skeletogenic pathway need have been placed additionally under control of the double-negative gate, namely, *alx1* and *ets1*. This requires providing them with *cis*-regulatory systems that respond to ubiquitously active inputs and *hesC* repression (ref. 19; genes in this subnetwork are all ubiquitously expressed whether *pmar1* is overexpressed or *hesC* is prevented from being expressed).

Superimposed Cooptions. The skeletogenic regulatory apparatus can be defined as GRN circuitry that provides the immediate or penultimate inputs into the differentiation genes. The skeletogenic regulatory apparatus of the micromeres differs from that of the juvenile skeletogenesis centers by at least four genes that are used in the micromeres but not in the juvenile centers (of course, there could be others used in the juvenile and not in the micromeres). These are *foxB*, *foxO*, *tel*, and *tbrain* (*tbr*) (blue regions in Fig. 3). The two *fox* genes are activated very late and may not be intrinsic components of the skeletogenesis system per se, i.e., they may control cell biology functions, as suggested for instance by the *tel* target gene *msp130* (12), which encodes a cell surface glycoprotein. But *tel* and *tbr* are in the upstream region of the GRN and are under control of the double-negative gate. We know nothing of evolutionary significance for this story about *tel*, but *tbr* provides an explicit case of cooption, because this gene is expressed in endomesoderm in other echinoderms, including sea stars (20, 21), where it is obligatory for endomesoderm specification (21); in echinoids, *tbr* is expressed only in the skeletogenic micromere lineage. We may conclude that, perhaps typically, following the linkage of the pleisiomorphic skeletogenic regulatory apparatus into the micromere GRN, additional regulatory gene cooptions took place in the echinoid lineage. It is interesting that the route of these cooptions was the same: linkage into the double-negative gate (Fig. 3).

Skeletogenic Paleogenomics. Finally, we may inquire about the evolutionary origin of the pleisiomorphic skeletogenic regulatory apparatus of echinoderms. Although it lies beyond the scope of this article, an essential point is that many of the genes of this GRN circuitry are also expressed in nonskeletogenic mesoderm. Cooption of some additional regulatory genes, erection of stabilizing intergenic circuitry, and formulation of biomineralization gene batteries by linking them into the modified mesodermal regulatory circuitry must have taken place in the remote preskeletogenic stem group echinoderm lineage. A similar sequence of events engaging other kinds of mesodermal structural genes, superimposed on a broadly distributed mesodermal regulatory state, must underlie the evolutionary diversification of mesoderm in Bilateria.

Heterochrony. The precocious expression of skeletogenic functions in the micromere lineage is a classic case of evolutionary heterochrony in which a complex function pleisiomorphically larval and adult and, in some primitive echinoids, late embryonic has been moved forward in the life cycle to the early embryonic period (4, 13). Here, we can glimpse the mechanism of this heterochrony: Essentially, an entire adult tissue GRN subnetwork has been transferred to an embryonic developmental regulatory address. We show how this could have occurred by means of a few changes in the *cis*-regulatory linkages of a few genes at the top of the subnetwork hierarchy, plus a few cooptions. Of course, the subnetwork was inserted not into a vacuum but rather into an also-pleisiomorphic regulatory apparatus operating to define the vegetal endomesoderm of the egg. Unfortunately, we have little comparative evidence on the structure of the underlying vegetal specification GRN. But this could be a phylogenetically widespread system judging from clues such as the near universal eumetazoan nuclearization of β -catenin in embryonic cells at the vegetal poles of eggs in deuterostomes, lophotrochozoans, and cnidarians (22–25).

Concluding Remark. Whether or not the evolutionary pathway sketched out here is an accurate reconstruction of events, it is a plausible explanation, couched in terms of what would be at root genomic *cis*-regulatory changes that affect GRN architecture. It is these kinds of change that must be responsible for

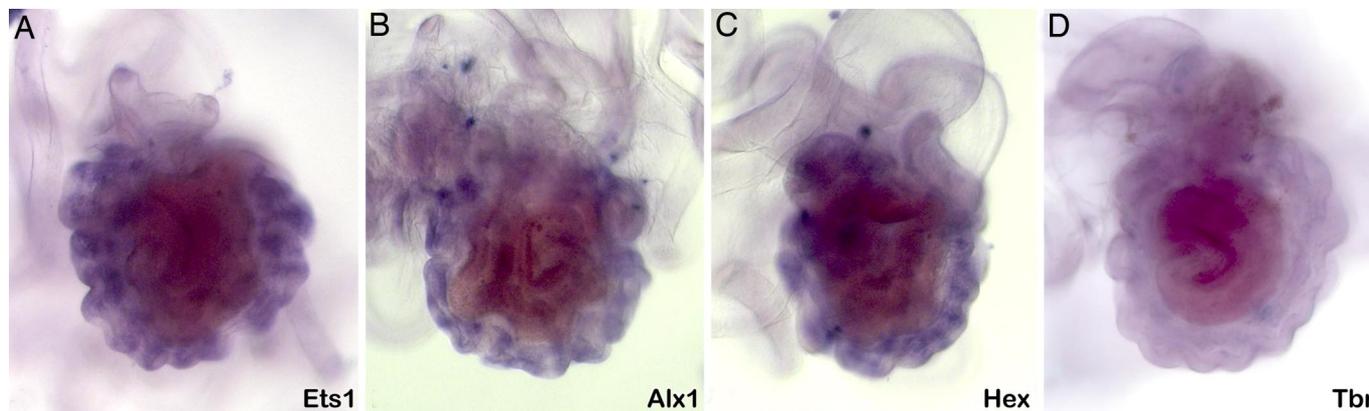


Fig. 4. Representative WMISH of probes for genes expressed in the sea urchin micromere lineage to juvenile skeletization centers of sea star larvae. Views are from the left side of the whole larvae. As in the sea urchin, *ets1* (A), *alx1* (B), and *hex* (C) are expressed in juvenile spines, whereas *tbr* (D) is not expressed.

features of morphology and function that arise physically during the life cycle through the operation of evolutionarily derived developmental pathways (26). Some specific and testable correlative predictions follow from the proposed pathway. For example, it might be predicted that, in cidaroids, the micromeres have the signaling capacities of euechinoid micromeres and that these could be under the control of the same double-negative regulatory gate as in the euechinoid, whereas the skeletogenic regulatory genes are not. A set of additional predictions that could be tested by reintroduction into sea urchin eggs, together with computational genomics, concerns the cis-regulatory modules of the sea star skeletogenic regulatory genes. If there is indeed a pan-echinoderm skeletogenic kernel, then the sea star regulatory modules thereof should display most of the same functional linkages and require most of the same inputs when introduced in reporter constructs into sea urchin eggs as are portrayed in the sea urchin GRN in Fig. 3.

Developmental GRNs provide a potent approach to definition of the mechanisms of evolutionary change in the body plans of embryos and adults. Only in the terms of GRN architecture can the actual nature of such changes be proposed and tested.

Materials and Methods

WMISH. Sea urchin larvae were grown as described in refs. 27 and 28, and similar methods were used to culture sea star (*A. miniata*) larvae. The larvae

were fed on *Rhodomonas lens* and/or *R. salina*. Adult skeleton was first visible from 2 and 4 weeks after fertilization in *S. purpuratus* and *A. miniata*, respectively. *S. purpuratus* and *A. miniata* larvae required, respectively, 6 and 9 weeks after fertilization to attain the stage competent for the metamorphosis.

Fixation and RNA extraction were done every week from the embryo through the late larval stages. Larvae were fixed in 4% paraformaldehyde as described in ref. 29. Twenty-four skeletogenic micromere genes from the sea urchin GRN and nine sea star orthologs isolated by library screening and degenerate PCR were studied by WMISH. Digoxigenin was used to label riboprobes of ≈ 1 kb. For genes with larger transcripts, several probes were made from different regions, and a mix of probes was used. Hybridization was for 1 week (29).

Viewing, Embedding, Sectioning, and Photography. After the staining reaction, the specimens were observed in an inverted microscope to determine the profile of their expression at all stages. For expression patterns that were not clear in whole mount display, larvae were embedded and sectioned. Staining patterns were examined in serial 8- μ m cryosections or in 4- μ m Durcupan sections.

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