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Signal-dependent regulation of the sea urchin skeletogenic gene regulatory network

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ABSTRACT

The endoskeleton of the sea urchin embryo is produced by primary mesenchyme cells (PMCs). Maternal inputs activate a complex gene regulatory network (GRN) in the PMC lineage in a cell-autonomous fashion during early development, initially creating a uniform population of prospective skeleton-forming cells. Previous studies showed that at post-blastula stages of development, several effector genes in the network exhibit non-uniform patterns of expression, suggesting that their regulation becomes subject to local, extrinsic cues. Other studies have identified the VEGF and MAPK pathways as regulators of PMC migration, gene expression, and biomineralization. In this study, we used whole mount *in situ* hybridization (WMISH) to examine the spatial expression patterns of 39 PMC-specific/enriched mRNAs in *Strongylocentrotus purpuratus* embryos at the late gastrula, early prism and pluteus stages. We found that all 39 mRNAs (including several regulatory genes) showed non-uniform patterns of expression within the PMC syncytium, revealing a global shift in the regulation of the skeletogenic GRN from a cell-autonomous to a signal-dependent mode. In general, localized regions of elevated gene expression corresponded to sites of rapid biomineral deposition. We used a VEGFR inhibitor (axitinib) and a MEK inhibitor (U0126) to show that VEGF signaling and the MAPK pathway are essential for maintaining high levels of gene expression in PMCs at the tips of rods that extend from the ventral region of the embryo. These inhibitors affected gene expression in the PMCs in similar ways, suggesting that VEGF acts via the MAPK pathway. In contrast, axitinib and U0126 did not affect the localized expression of genes in PMCs at the tips of the body rods, which form on the dorsal side of the embryo. Our results therefore indicate that multiple signaling pathways regulate the skeletogenic GRN during late stages of embryogenesis-VEGF/MAPK signaling on the ventral side and a separate, unidentified pathway on the dorsal side. These two signaling pathways appear to be activated sequentially (ventral followed by dorsal) and many effector genes are subject to regulation by both pathways.

The primary mesenchyme cells (PMCs) of the sea urchin embryo, which produce the embryonic and early larval skeleton, are a model system for studying specification, differentiation, and morphogenesis (Ettensohn, 2013; McIntyre et al., 2014; Wilt and Ettensohn, 2007). PMCs are the sole descendants of the four large micromeres, which arise at the vegetal pole of the embryo during cleavage. At the mesenchyme blastula stage, the large micromere progeny undergo an epithelial–mesenchymal transition (EMT) and ingress into the blastocoel, after which they are referred to as PMCs (Wilt and Ettensohn, 2007). After EMT, PMCs migrate along the blastocoel wall and arrange themselves in a characteristic, subequatorial ring pattern. The PMC ring consists of two clusters of cells (the ventrolateral clusters, or VLCs) that are connected by two cellular chains – a short ventral chain and a long dorsal chain. Late in gastrulation, a few PMCs from each VLC migrate toward the animal pole; these PMCs constitute the longitudinal chains (Fig. 1). As the PMCs migrate, their filopodia fuse, forming a cable-like structure that links the cells in a single, syncytial network (Hodor and Ettensohn, 1998). Despite the fact that the PMCs are joined in a syncytium, the exchange of gene products between cells is quite limited, and mRNAs and proteins are largely confined to the PMCs in which they are produced (Guss and Ettensohn, 1997; Harkey et al., 1992; Illies et al., 2002; Lapraz et al., 2009; Livingston et al., 2006; Urry et al., 2000; Wilt et al., 2008).

The arrangement of the PMCs during gastrulation prefigures the morphology of the skeleton. Skeletogenesis begins with the formation of one triradiate spicule rudiment in each VLC at the mid-gastrula stage. These two skeletal rudiments subsequently elongate and branch to form the two spicules of the skeleton, which exhibit mirror-image symmetry. The PMCs in the dorsal chain produce the body rods and those in the ventral chain form the ventral transverse rods. The PMCs in the longitudinal chains give rise to the

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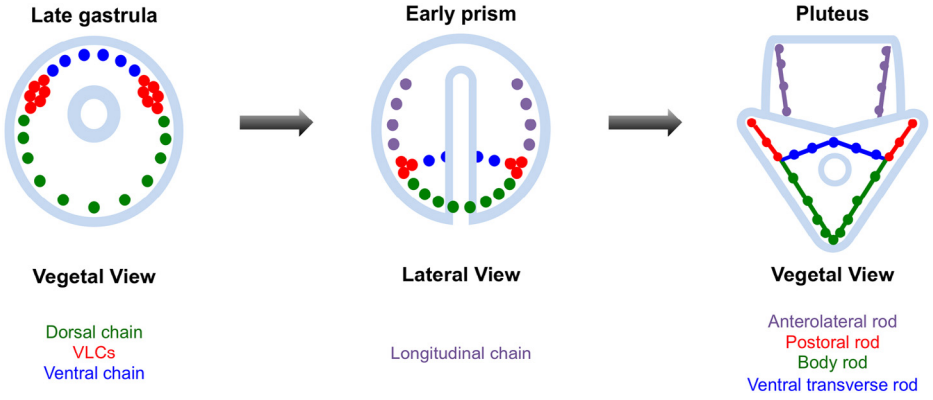

Fig. 1. Schematic diagrams of PMC patterning and skeletogenesis in *S. purpuratus* at the late gastrula, early prism and pluteus stage. PMCs are color-coded to indicate their position within the syncytial network and the specific skeletal rods that they produce. VLC = ventrolateral cluster.

dorsoventral connecting rods, which in *Strongylocentrotus purpuratus* curve ventrally, forming the anterolateral rods (Fig. 1). In many other species, the dorsoventral connecting rods branch to form the anterolateral and recurrent rods. During late stages of embryogenesis, the body rods, anterolateral rods, and postoral rods elongate rapidly through the addition of new biomineral at their tips, while the ventral transverse rods cease their growth (Ettensohn and Malinda, 1993; Guss and Ettensohn, 1997).

The intricate and reproducible pattern of the embryonic skeleton is regulated by interactions between PMCs and neighboring cells. Isolated micromeres cultured in plain seawater fail to produce spicules unless horse serum is added (Okazaki, 1975). Hardin et al. (1992) and Armstrong et al. (1993) showed that the induction of supernumerary triradiate spicule rudiments by $NiCl_2$ is a consequence of a ventralization of the ectoderm. Furthermore, Ettensohn and Malinda (1993) showed that photoablation of a patch of ectodermal cells at the tip of the postoral arm inhibits the elongation of the underlying postoral rod, suggesting that short-range, ectoderm-derived cues are required for rod elongation. The stereotypical pattern of elongation rates of the various skeletal rods observed *in vivo* also supports the view that local cues regulate skeletal growth (Guss and Ettensohn, 1997).

Vascular endothelial growth factor-3 (VEGF3) has recently been shown to play an important role in PMC migration, gene expression and biomineralization (Adomako-Ankomah and Ettensohn, 2013; Duloquin et al., 2007; Knapp et al., 2012). Whole mount *in situ* hybridization (WMISH) reveals that *vegf3* is expressed selectively by ectoderm cells that overlie sites of rapid skeletal growth (Adomako-Ankomah and Ettensohn, 2013; Duloquin et al., 2007). The localized pattern of *vegf3* expression is controlled by a complex cascade of signaling molecules and gene regulatory interactions that pattern the ectoderm (Li et al., 2013, 2014; McIntyre et al., 2013; Molina et al., 2013). Treatment of embryos with U0126, a MEK inhibitor, or axitinib, a VEGFR inhibitor, at late developmental stages results in an inhibition of biomineral deposition and the formation of truncated skeletal rods (Adomako-Ankomah and Ettensohn, 2013; Sun and Ettensohn, unpublished observations). Because receptor tyrosine kinases often signal via the MAPK cascade (Schlessinger, 2000), these observations suggest that VEGF3 regulates skeletal growth through this pathway.

A complex gene regulatory network (GRN) is deployed in the large micromere-PMC lineage (Oliveri et al., 2008; Rafiq et al., 2012, 2014). The PMC GRN is activated by polarized, maternal inputs and requires the unequal cleavage of vegetal blastomeres (Sharma and Ettensohn, 2010). These inputs entrain the cell-autonomous deployment of many downstream genes in the network, including many terminal effector genes (Ettensohn, 2013; Oliveri et al., 2008; Rafiq et al., 2012, 2014). The MAPK pathway is selectively activated in the large micromere-PMC lineage during early development, probably by cell-autonomous mechanisms, and this pathway plays an essential role in the deployment of the network (Fernandez-Serra et al., 2004; Rafiq et al., 2014; Röttinger et al., 2004).

The developmental consequences of the cell-autonomous phase of PMC differentiation are evident from the development of micromeres that have been cultured in seawater without serum or other supplements. Under such conditions, micromeres divide and their descendants undergo striking changes in behavior, becoming migratory and fusogenic (Hodor and Ettensohn, 1998; McCarthy and Spiegel, 1983; Okazaki, 1975), but they do not form spicules. It seems likely that this early, cell autonomous phase of GRN deployment is responsible for the activation of many effector genes that show maximal levels of expression at the blastula stage, prior to PMC ingression (Rafiq et al., 2014). At least two downstream effectors, *sm50* and *msp130*, are expressed at similar levels in the presence or absence of serum (Page and Benson, 1992). In addition, *sm50* is expressed at high levels even when early embryos are dissociated and the cells cultured under conditions that minimize cell–cell contacts (Stephens et al., 1989). Based on qualitative WMISH studies, the initial, cell-autonomous phase of GRN deployment appears to produce a relatively homogeneous population of cells; i.e., effector genes are expressed relatively uniformly among PMCs at the late blastula stage (Rafiq et al., 2012, 2014). Later in development, however, several effector mRNAs show non-uniform distributions within the PMC syncytium, suggesting that local signals regulate the expression of the cognate genes (Adomako-Ankomah and Ettensohn, 2011; Cheers and Ettensohn, 2005; Guss and Ettensohn, 1997; Harkey et al., 1992; Illies et al., 2002; Livingston et al., 2006).

To gain a better understanding of the regulation of skeletal morphogenesis by ectodermal cues, we analyzed and classified the spatial expression patterns of 39 PMC-enriched transcripts in *S. purpuratus* embryos at three late (post-blastula) stages of embryogenesis. We report that: 1) many genes in the skeletogenic GRN, including both regulatory genes and effector genes, show non-uniform patterns of expression within the PMC syncytium at late developmental stages, reflecting the influence of local, ectoderm-derived cues; 2) the effect of these local cues is to *maintain* the expression of effector genes that are initially activated by cell-autonomous mechanisms but decline in expression except in those regions where appropriate signals are provided; 3) the PMC syncytium consists of distinct subpopulations of PMCs with different molecular properties; 4) regions of elevated gene expression generally correspond to sites of skeletal rod growth; 5) multiple ectodermal signals regulate gene expression and skeletal growth. One of these cues, VEGF3, regulates gene expression and skeletal rod selectively on the ventral side