Distinct regulatory states control the elongation of individual skeletal rods in the sea urchin embryo

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Abstract

Background: Understanding how gene regulatory networks (GRNs) control developmental progression is a key to the mechanistic understanding of morphogenesis. The sea urchin larval skeletogenesis provides an excellent platform to tackle this question. In the early stages of sea urchin skeletogenesis, skeletogenic genes are uniformly expressed in the skeletogenic lineage. Yet, during skeletal elongation, skeletogenic genes are expressed in distinct spatial sub-domains. The regulation of differential gene expression during late skeletogenesis is not well understood.

Results: Here we reveal the dynamic expression of the skeletogenic regulatory genes that define a specific regulatory state for each pair of skeletal rods, in the sea urchin Paracentrotus lividus. The vascular endothelial growth factor (VEGF) signaling, essential for skeleton formation, specifically controls the migration of cells that form the postoral and distal anterolateral skeletogenic rods. VEGF signaling also controls the expression of regulatory genes in cells at the tips of the postoral rods, including the transcription factors Pitx1 and MyoD1. Pitx1 activity is required for normal skeletal elongation and for the expression of some of VEGF target genes.

Conclusions: Our study illuminates the fine-tuning of the regulatory system during the transition from early to late skeletogenesis that gives rise to rod-specific regulatory states.

KEYWORDS
biomineralization, gene regulatory networks, MyoD, Pitx, sea urchin, skeletogenesis, vascular endothelial growth factor

1 | INTRODUCTION

Transcription factors, signaling molecules, and regulatory RNAs form gene regulatory networks (GRNs) that drive cell fate specification during embryogenesis. The GRNs control the regulatory state of the cells, which is the specific combination of transcription factors within the cell nucleus. The regulatory states define which genes are activated and which genes are repressed and by that drive cell fate specification and morphogenesis. As development progresses, successive cascades of regulatory genes are activated: early regulatory genes activate...
the expression of late regulatory genes and enable the refinement of regulatory states, cell fate, and function. Understanding how early GRNs activate the late, elaborate GRNs and how these regulatory cascades encode developmental progression is a key to the understanding of the genetic regulation of morphogenesis.

Sea urchin larval skeletogenesis is a prominent model for dissecting the genetic regulation of developmental progression. The larval skeleton of the sea urchin is made of two calcite rods, the spicules, that are engulfed within a tubular spicule cavity, generated by the skeletogenic cells. During embryogenesis, the skeletogenic cells go through epithelial to mesenchymal transition and enter the blastocoel, fuse through their filopodia and form a pseudopodia cable that links them into a syncytium. The skeletogenic cells organize in a ring with two ventrolateral cell clusters in which the tri-radiate spicules form. The spicules elongate within the pseudopodia cable and generate the body, anterolateral and mid-ventral rods. A set of skeletogenic cells then migrate from the ventrolateral clusters and guide the elongation of the postoral rods; skeletogenic cells from the longitudinal chain migrate to form the distal part of the anterolateral rods. The growth rate differs between the rods, and particularly, the postoral rod is the last to grow but has the fastest elongation rate. The spicule rods grow rapidly at their tips and slower at their girth, suggesting that mineral deposition is enhanced at the growing spicule tips compared to the back. Relatedly, as the spicules elongate, the expression of some skeletogenic genes becomes restricted to the cells proximal to the growing tips, possibly to enhance spicule growth at these sites. As all the skeletogenic cells are connected through their cytoplasm, the regulation of localized gene expression within the skeletogenic-syncytium is quite intriguing.

A prominent regulator of skeletal growth and gene expression is the vascular endothelial growth factor (VEGF) signaling pathway. At the gastrula stage, the VEGF ligand is secreted from two lateral ectodermal domains while the VEGF receptor (VEGFR) is

**FIGURE 1** Sea urchin larval skeletogenesis and gene expression. (A) Sea urchin embryo at the gastrula stage. AL, anterolateral rods; MV, mid-ventral rods; BR, body rods; D, dorsal skeletogenic chain; L, longitudinal skeletogenic chain; V, ventral skeletogenic chain. The pseudopodia cable between the skeletogenic cells is marked in black lines. VEGFR expression is marked in red and VEGF is marked in blue, based on. (B) Sea urchin embryo at the pluteus stage. PO, postoral rods; p-AL, proximal anterolateral rods; d-AL, distal anterolateral rods. (C-H) Differential expression of SM50 and SM30 at 2 dpf and the effect of VEGFR continuous and late inhibition. (C-E) SM50 expression in control (C), late VEGFR inhibition (D), and continuous VEGFR inhibition (E). (F-H) SM50 expression in control (F), late VEGFR inhibition (G), and continuous VEGFR inhibition (H). Numbers at the bottom indicate the number of embryos that show this phenotype (left) out of all embryos scored (right), conducted in three independent biological replicates. (I-N) Embryo morphology and gene expression at the time of the addition of VEGFR inhibitor (25 hpf) in the late inhibition experiments. (I) Embryo morphology, arrowheads point to the tri-radiate spicules. Gene names are indicated at the bottom in (J-N).
expressed in the neighboring skeletogenic cells, where active spicule growth occurs (Figure 1A, N15,16). Inhibition of sea urchin VEGF signaling by genetic manipulation or using the VEGFR-specific inhibitor, axitinib, distorts skeletogenic cell migration and completely blocks spicule formation.7,16,21 At the pluteus stage, VEGF is expressed at the tips of the postoral and anterolateral rods, and VEGF is expressed at the ectodermal cells near these tips (Figure 1B15,16). A weak expression of VEGFR is also observed at the tips of the body rods, but VEGF is not expressed near these cells.15 Accordingly, late inhibition of VEGF signaling inhibits the elongation of the postoral and distal anterolateral rods and downregulates the expression of skeletogenic genes at the tips of these rods, but not at the body rods (Figure 1C-H21-23). Thus, VEGF signaling is one of the factors regulating localized gene expression and directing spicule growth, yet little is known about the regulatory states at the tips of the rods and the mechanisms that mediate the transcriptional response to VEGF signaling.

We recently studied the molecular machinery activated by the VEGF pathway during sea urchin skeletogenesis and discovered hundreds of genes that respond to VEGFR inhibition, including regulatory and vascularization-related genes.7 Moreover, five upstream transcription factors and three signaling genes that drive skeletogenesis are homologous to vertebrate factors that control vascularization.6,7,24 The VEGF pathway participates in biomineralization in all studied echinoderms15,16,25-27 while in many other phyla it controls tubulogenesis and prominently, vascularization.24,28-31 This led us to propose that sea urchin skeletogenesis and vertebrate vascularization diverged from a common ancestral tubulogenesis program, uniquely co-opted for biomineralization in the echinoderm phylum.7,24

Two of the genes that respond most significantly to VEGF inhibition encode the transcription factors, MyoD1 and Pitx1 that could have a role in the regulation of skeletogenic gene expression.7 Sea urchin Pitx1 is a homolog of the vertebrates’ Pitx family of paired-related homeodomain transcription factors that regulate the formation of multiple organs, including biomineralization-related processes such as teeth and hindlimb development.32-41 MyoD1 is a sea urchin homolog of the vertebrates MyoD family of basic helix loop helix (bHLH) transcription factors that are typically involved in myogenic determination and differentiation.42 Interestingly, Pitx2 and genes from the MyoD family cooperate to regulate skeletal muscle differentiation in vertebrates.42 However, sea urchin Pitx1 and MyoD1 are not a part of the myogenesis regulatory network in the sea urchin embryo43 and are exclusively expressed in skeletogenic cells downstream of VEGF signaling.7 The expression and role of these genes in late sea urchin skeletogenesis were not studied before.

While the GRN that controls the early stages of skeletogenesis is known in great details,6,8,10-12,44 the regulatory states and mechanisms that control skeletal elongation and differential gene expression are not fully understood. Here we investigate the expression of key skeletal regulatory genes and their response to early and late perturbations of VEGF signaling during skeletal elongation. We study the dynamic expression pattern of Pitx1 and MyoD1 and the role of the transcription factor Pitx1 in skeletal elongation and regulation of skeletogenic gene expression. Our studies portray the transition between the initial regulation of spicule formation to the intricate regulation of the growth of individual skeletal rods.

2 | RESULTS

2.1 | Rod-specific regulatory states are formed during skeletal elongation and postoral gene expression depends on late VEGF signaling

To illuminate the regulatory states that form in the skeletogenic lineage during skeletal elongation, we studied the spatial expression of key skeletogenic regulatory genes at the prism and pluteus stages, in control embryos and under late and continuous VEGFR inhibition (Figures 2 and 3). In continuous VEGFR inhibition, the embryos are grown with VEGFR inhibitor, axitinib, from the time of fertilization and on, which causes complete loss of skeletal growth and a highly irregular positioning of the skeletogenic cells (Figures 2A,B and 3A,B), in agreement with previous studies.15,16 In late VEGFR inhibition, the inhibitor is added at 25 h post-fertilization (hpf), which is after the skeletogenic cells are arranged in a ring with two ventrolateral cells clusters and just after the spicule first forms (Figure 1I). Late VEGFR inhibition does not interfere with the growth of the body rods, but prevents cell migration required for the generation of the postoral rods and the elongation of the distal part of the anterolateral rods (Figures 2 and 3, panels A, B), in agreement with previous studies in other sea urchin species.16,21

We studied the expression of early and late skeletogenic transcription factors and signaling molecules responsible for different functions in the skeletogenic cells. The transcription factors Ets1/2, Alx1, Erg, and Hex drive early skeletogenic cell specification,6,10,44,45 are necessary for the skeletogenic epithelial to mesenchymal transition46 and represent the early upstream skeletogenic GRN. At
FIGURE 2  Legend on next page.
the time of the addition of VEGFR inhibitor, these genes are expressed in the skeletogenic cells and their expression is enriched at the skeletogenic lateral cell clusters (Figure 1J-M). The fibroblast growth factor (FGF) and its receptor, FGFR2, are expressed in the skeletogenic cells after they ingress into the blastocoel and are important for skeletal elongation.\textsuperscript{16,47} We also studied the transcription factors Pitx1 and MyoD1, the ligand, VEGF, and the receptor, VEGFR, mentioned above.

The skeletogenic regulatory genes form distinct regulatory states at the tips of each pair of rods and the expression at the tips of the postoral rods strongly depends on late VEGF signaling. The most dorsal cells of the body rods express the genes encoding the transcription factors Ets1/2, Alx1, and Erg and the signaling receptor, FGFR2, at the prism and pluteus stages, independently of late VEGF signaling (Figures 2 and 3, panels C-E,J,M,N). The expression of Alx1 in skeletogenic cells at the tips of the body rods was also observed in Sun and Ettenshon\textsuperscript{21} and Chang and Su.\textsuperscript{23} A faint expression of VEGFR2 is detected in these cells at both time points and is reduced under VEGFR inhibition (Figures 2 and 3, panels K,M,N). The cells along the mid-ventral rods express the genes encoding the transcription factors Alx1 and Erg at the prism and pluteus stages, independently of late VEGF signaling (Figures 2 and 3, panels D,E,M,N). The cells at the tips of the anterolateral rods express the transcription factors Ets1/2 and the signaling molecular FGF, FGFR2, and VEGFR, at the prism and pluteus stages (Figures 2 and 3 panels C,J,K,M). Ets1/2 expression at the tips of the anterolateral rods was also observed in Chang and Su.\textsuperscript{23} The expression of VEGFR in this domain is reduced in late VEGFR inhibition while the expression of the other regulatory genes is unchanged (Figures 2 and 3 panels C,J,K,N). The expression of erg and FGFR2 is observed in cells along the anterolateral rods at the prism stage, in both control and late VEGFR inhibition, and the expression of alx1 is detected in this domain only in late VEGFR inhibition, suggesting an inhibitory role of VEGF signaling on alx1 (Figure 1D,E,J-K,M,N). The cells at the tips of the postoral rods express the genes encoding the transcription factors Ets1/2, Hex, MyoD1 and Pitx1 and the signaling molecules, FGF, FGFR2, and VEGFR2, and the expression of all these genes strongly depends on late VEGF signaling (Figures 2 and 3 panels C,F-K,M,N).

In contrast to the specific effect of late VEGFR inhibition, continuous inhibition of VEGFR eliminates the expression of most of the genes in prism and pluteus stages, except from ets1/2, alx1 and erg expressed in scattered skeletogenic cells in this condition (Figures 2 and 3, panels C-K). The expression of the ligand, VEGF, did not show a distinct change under VEGFR inhibition (Figures 2 and 3L). Thus, at the prism and pluteus stages, the expression of skeletogenic regulatory genes is localized at specific skeletogenic cells forming distinct regulatory states of which, the regulatory state at the tips of the postoral rods requires late VEGF signaling.

### 2.2 Late VEGF signaling mildly affects the expression of VEGF targets but is essential for pitx1 and myoD1 expression

Late VEGF signaling has a milder effect on the expression of the skeletogenic regulatory genes compared to continuous VEGF inhibition (Figures 2 and 3), hence we wanted to study the differences between these two treatments on the expression of VEGF target genes at the pluteus stage. We tested the effect of VEGFR inhibition on the expression levels of genes encoding the receptor, VEGFR, the transcription factors Pitx1 and MyoD1, the spicule matrix protein SM30, and the cytoskeleton remodeling protein Rhogap24f2. SM30 is one of the most abundant matrix proteins found occluded in the sea urchin spicules.\textsuperscript{48,49} Rhogap24f2 is a GTPase-activating protein that is expressed in the skeletogenic cells and its perturbation leads to ectopic spicule branching.\textsuperscript{7} All these genes were shown to depend on VEGF signaling at the gastrula stage\textsuperscript{7,15,16,23} and here we studied the effect of VEGFR inhibition on their expression levels at the pluteus stage, 2 and 3 days post-fertilization (dpf).
FIGURE 3  Legend on next page.
We found a distinct difference in the transcriptional response of VEGF targets between continuous and late inhibition of VEGF signaling at 2 dpf and 3 dpf (Figure 4A-D). Continuous VEGFR inhibition reduces the expression of all tested skeletogenic genes to less than 20% of their expression in control embryos (Figure 4A,C). This agrees with the strong effect of this perturbation on the spatial expression of the skeletogenic regulatory genes in the pluteus stage (Figure 3). However, late inhibition of VEGF signaling reduces the expression of the genes by less than 50% of their normal expression at 2 dpf and 3 dpf, except for the genes that encode the transcription factors Pitx1 and MyoD1, which show stronger reduction (Figure 4B,D). These results agree with the effect of late VEGFR inhibition on the expression of the skeletogenic regulatory genes that mostly affect the expression at the postoral rods, where pitx1 and myoD1 are exclusively expressed at the pluteus stage (Figures 3). To complete this set of experiments, we studied the spatial expression of these two genes and the effect of VEGFR inhibition at 3 dpf (Figure 4E-G). Skeletogenesis does not recover in continuous VEGFR inhibition at 3 dpf, but the only effect of late VEGFR inhibition is a delayed elongation of the postoral and the anterolateral rods (Figure 4E). At this time, myoD1 and pitx1 are expressed within a few skeletogenic cells at the tips of postoral rods and their expression is completely abolished in both, continuous and late inhibition of VEGF signaling (Figure 4F,G).
To investigate pitx1 and myoD1 spatio-temporal expression throughout skeletogenesis we studied their expression every few hours from gastrula to pluteus stage (Figure 5). At the time of skeletal initiation, both genes are expressed within the skeletogenic ventrolateral clusters as previously reported (24 hpf, Figure 5A,I). At 27-32 hpf, myoD1 remains localized at the ventrolateral cell clusters (Figure 5B-D) while the expression of pitx1 expands to neighboring cells, and is observed within the dorsal, longitudinal, and ventral skeletogenic chains (Figure 5J-L). From prism to pluteus stages (34-44 hpf), the expression of pitx1 becomes localized to cells at the tips of the growing postoral rods (Figure 5M-P), which is similar to the expression of myoD1 at the same time points (Figure 5E-H). Thus, after the initial activation of pitx1 and myoD1 in the lateral skeletogenic cells clusters, the expression pitx1 expression expands while myoD1 expression remains localized. Later the expression of both genes is localized in the migrating cells at the tips of the growing postoral rods.

**FIGURE 5** Spatiotemporal expression of myoD1 and pitx1 from gastrula to pluteus stage (24-44 hpf). (A-H) myoD1 spatiotemporal expression. During the gastrula, stage myoD1 is expressed at the skeletogenic ventrolateral clusters (A-D). At prism and pluteus stages, myoD1 is expressed at the cells at the tips of the growing postoral rods (E-H). (I-P) pitx1 spatiotemporal expression. At the early gastrula stage, pitx1 is expressed at the skeletogenic ventrolateral clusters (I). (J-L) During late gastrula stage, pitx1 is expressed within cells of dorsal (D), longitudinal (L), and ventral (V) chains. At prism stage (M, N) shrinks to the cells that form the postoral rods and is expressed at the tips of these rods in pluteus stage (O-P). Embryos at the gastrula stage are presented in lateral view except A and J where they are presented in ventral view (VV). At the prism and pluteus stages embryos are presented in the same orientation along the dorsal-ventral axis where the dorsal side is at the bottom of the image.
2.3 Downregulation of *pitx1* causes a significant reduction in elongation of the skeletal rods

The dynamic spatial expression of the *pitx1* gene and its strong dependence on VEGF signaling motivated us to study its role in sea urchin skeletogenesis. To do that we knocked-down the expression of *pitx1* by microinjection of two different morpholino antisense oligonucleotides (MOs) targeted to block the splicing of the gene at two distinct exons (Figure 6A). Random MO was injected as a control. To test the activity of the two MOs we designed polymerase chain reaction (PCR) primers that distinguish between the full transcripts and the truncated transcripts for each MO (Figure 6B,C). PCR on cDNA synthesized from uninjected embryos and from embryos injected with random MO produces one band that corresponds to the full transcript (Figure 6D,E, see Experimental Procedures for details). PCR of cDNA synthesized from embryos injected with sMO1 produces two bands that correspond to the truncated transcripts and the full transcript (Figure 6D). PCR on cDNA of embryos injected with sMO2 shows a single strong band of a truncated transcript (Figure 6E). This data confirms that both MOs block normal splicing as expected; however, sMO2 seems to be more efficient in inhibiting normal splicing of the *pitx1* transcript.

Downregulation of *pitx1* interferes with skeletal elongation and results in shorter skeletal rods at 3 dpf, as exemplified with representative embryos in Figure 7A-C. To quantify the effect, we measured the length of each rod in embryos injected with *pitx1* sMOs and in embryos injected with random MO as control (Figure 7D, see Experimental Procedures for details). The injections of both sMOs result in a significant decrease in the length of postoral, body, and distal anterolateral (d-AL) rods, with minor differences in the activity between the two sMOs (Figure 7E,F,H). The length of proximal anterolateral (p-AL) rods has been significantly decreased in sMO2 injected embryos but not in sMO1 (Figure 7G), possibly since sMO1 is less effective than sMO2 in blocking the splicing of *pitx1* (Figure 6E). Overall, our findings indicate that Pitx1 is important for the normal elongation of the body, AL, and postoral skeletal rods.

**FIGURE 6** *Pl-pitx1* MO design and test of splicing MOs. (A) Structure of the *Pl-pitx1* gene with target sites for the two splicing MOs and PCR primer locations identified. The coordinates correspond to the distance from the beginning of the first exon. The QPCR primers that were used to assess *pitx1* level in *pitx1* perturbation experiments are located at the fourth exon, a region that is unaffected by the splicing MO. (B, C) Primer design for testing the activity of sMO1 (B) and sMO2 (C) showing the expected PCR transcripts. (D, E) Gels testing sMO1 and sMO2, respectively, showing bands of a long (full) and short (truncated) transcripts. Un, uninjected embryos; raMO, embryos injected with random MO; sMO1, embryos injected with *Pl-pitx1* splicing MO1; sMO2, embryos injected with splicing MO2; L, long transcript; S, short transcript.
2.4 Pitx1 perturbation affects the level but not the spatial pattern of skeletogenic gene expression

The significant effect of the perturbation of pitx1 on the growth of all skeletal growth motivated us to study its effect on the expression of several VEGF targets. We studied the effect of pitx1 perturbation at the early phase of its expression when it is broadly expressed within the skeletogenic cells (late gastrula, /C24 30-32 hpf, Figure 5K,L) and during skeletal elongation, when pitx1 is localized to cell at the tips of the postoral rods (pluteus, 2 dpf, Figure 5P). For these experiments, we used sMO2 due to its improved efficiency in inhibiting the activity of pitx1 (Figure 6E). To quantify the effect of pitx1 downregulation on the level of pitx1 expression, we used quantitative polymerase chain reaction (QPCR) primers located at the fourth exon, which is unaffected by sMO2 (Figure 6A and Table S1).

At the late gastrula stage, the skeletal morphology of pitx1 morphants looks very similar to that of the control embryos injected with Random MO, (Figure 8A,B), and the expression level of some of VEGF targets is mildly altered (Figure 8C). Specifically, the expression of SM30 and VEGF is reduced while the expression of pitx1 itself is increased; yet these changes are minor. At the pluteus stage, embryos injected with pitx1 sMO2 show distinct delay in skeletal elongation compared to control embryos injected with Random MO (Figure 8J,K). The expression level of VEGF targets, SM30, SM50, and MyoD1 is significantly reduced by 2-fold or more, and the expression of pitx1 is upregulated (Figure 8L). The increase in the level of pitx1 under pitx1 downregulation at both time points suggests that Pitx1 is an auto-repressor of its own gene. That is, pitx1 knockdown results in downregulation of the expression levels of SM30, SM50, and MyoD1, of a similar order as VEGFR late inhibition (Figure 4B), and a delay in the elongation of all skeletal rods at 2 dpf.

We tested the effect of pitx1 downregulation on the spatial expression of VEGF targets at the same time points where we studied gene expression levels. We focused on the spicule matrix proteins, SM30 and SM50, and on pitx1 itself. At the gastrula stage, in control embryos injected with Random MO, SM30 is expressed in the skeletogenic cell clusters, in the longitudinal chain, and in dorsal skeletogenic cells next to the skeletogenic cell clusters (Figure 8D). In control embryos, SM50 is expressed in the same cells as SM30, but it is also expressed in the ventral skeletogenic chain (Figure 8F) and pitx1 is expressed in the skeletogenic cell clusters and in the cells near them (Figure 8H). The downregulation of pitx1 did not change the spatial expression of VEGF targets at the gastrula stage.
of these genes at this time point (Figure 8E,G,I). At the pluteus stage, in control embryos injected with Random MO (A) and pitx1 sMO2 (B) at 30 hpf, (C) Relative change in gene expression level in pitx1 sMO2 compared to Random MO, measured by QPCR. Bars show averages and markers indicate individual measurements. Ratio of 1 (dashed line) indicates that the expression of the gene is unaffected by pitx1 downregulation. Error bars indicate SD. One-tailed z-test statistical significance is represented by *P < .01; **P < .001. (D-I) Spatial gene expression in control embryos vs embryos injected with pitx1 sMO2, gene names are indicated at the bottom. (J-R) Similar to (A-I), tested at 2 dpf. QPCR experiments were performed in 3 biological replicates and spatial expression experiments were performed in 2 to 4 biological replicates. Exact numbers of studied embryos are provided in Table S2. (T-S) Pitx1 putative binding sites in the promoter regions of SpSM30a (S) and SpSM50 (T), that drive expression in the skeletogenic cells. Borders of active regions are marked magenta; first exon is marked in blue letters and the start of translation is highlighted in yellow. Pitx1 putative binding sites are highlighted in green, two overlapping sites in SpSM30a are marked in bold.
postoral rods (Figure 8Q). The downregulation of pitx1 results in smaller embryos due to the skeletal delay, but did not change the spatial expression of these genes at this time (Figure 8N,P,R).

The reduction in the expression levels of SM30 and SM50 at the pluteus stage suggests that Pitx1 could be a direct activator of these genes. The cis-regulatory regions controlling the expression of SM30 and SM50 were characterized in the sea urchin species, Strongylocentrotus purpuratus, which shows high similarity in its skeletogenic GRN and gene expression patterns to P. lividus. We used human and mouse position weight matrices of Pitx1/2/3 in the Jaspar database, to identify putative binding sites in the promoter regions of SpSM30a and SpSM50 that drive skeletogenic gene expression when fused to a reporter gene. We identified four putative Pitx binding sites in the promoter regions of SpSm30a and two putative Pitx binding sites in the promoter region of SpSm50 (Figure 8S,T). The function of these binding sites should be tested experimentally to confirm the direct regulation of SM30 and SM50 by Pitx1. Overall, our studies imply that Pitx1 acts as a transcriptional activator of the skeletogenic genes, SM30, SM50, MyoD1, and possibly, as a transcriptional repressor of its own gene (Figure 8).

3 | DISCUSSION

As embryo development progresses, the complexity of organs and embryonic morphology increases, and the complexity of regulatory states and GRNs that control these processes increases accordingly. Here we studied the regulatory changes that underlie the transition between the early stage of spicule formation to the late stages of skeletal elongation in the sea urchin larva, our findings are summarized in Figure 9. We detected specific regulatory states in each pair of skeletal rods, of which, gene expression at the tips of the postoral rods requires late VEGF signaling (Figures 2-4). The expression of these genes is first localized to the ventral-lateral skeletogenic cell clusters and later observed in cells at the tips of the growing postoral rods, and depends on VEGF signaling throughout skeletogenesis (Figures 4 and 5). Pitx1 knock-down reduces the elongation of all skeletal rods and downregulates the expression of some of VEGF targets, suggesting that this transcription factor mediates part of the transcriptional response to VEGF.
signaling (Figures 6-8). Below we discuss our findings and their implications on the regulation of the transition between spicule initiation and spicule elongation.

Our studies and previous works demonstrate the specific regulatory states and GRNs that operate at the tips of the growing skeletal rods, possibly to support the distinct growth dynamics and function of these rods in the sea urchin larva (Figures 2, 3, and 9A). It was previously shown that the growth rate is rod-specific and that differential gene expression is observed within the skeletogenic cell lineage after the gastrula stage. Furthermore, the postoral and anterolateral rods form the feeding structure of the sea urchin larva that its growth and size depends on food availability and could require specific environmental-dependent regulation. Here we revealed the distinct regulatory states formed in each pair of skeletal rods that apparently control rod-specific gene expression (Figure 9A). Interestingly, the cells at the tips of the postoral and anterolateral rods express the highest number of signaling molecules, FGF, FGFR2, and VEGFR2. Furthermore, the ligand VEGF is secreted from the ectodermal cells located next to the tips of these rods. Possibly the VEGF and FGF pathways drive and guide the cell migration required for the growth of postoral and anterolateral skeletal rods. Thus, the rod-specific GRNs are probably controlling the timing and growth rates of each pair of rods according to distinct developmental and environmental cues.

Our findings indicate that the role of VEGF signaling in sea urchin skeletogenesis has two distinct phases, in agreement with previous studies. Early VEGF signaling is a key to the spatial organization of the skeletogenic cells, to the initial formation of the spicules, and to the expression of regulatory and differentiation genes at the ventrolateral cell clusters. Yet, after the skeletogenic cells form the dorsal, ventral, and longitudinal chains and generate the tri-radiate spicules, the elongation of body, the mid-ventral, and the proximal part of the anterolateral rods, do not require VEGF signaling (Figures 2 and 3). After spicule formation, VEGF signaling is required for the migration of the cells that form the postoral rods and the distal part of the anterolateral rods and for the expression of regulatory genes at the tips of the postoral rods, including the transcription factors, myoD1 and pitx1 (Figure 9B).

The sharp transition between the initiation and the elongation stage could suggest that early VEGF signaling controls cellular organizations essential for the formation of the spicule cavity. As mentioned above, the cells that build the body, proximal anterolateral and mid-ventral rods are positioned in their proper location and are connected through the pseudopodia cable at the time of the addition of VEGFR inhibitor in the late inhibition (25 hpf, Figure 1A). The distal anterolateral rods and postoral rods are the only rods that grow without a pre-existing pseudopodia cable, which could suggest that VEGF activity is required to grow new spicule cavity. Thus, early VEGF signaling is required for the initiation of the spicule cavity in the lateral skeletogenic cell clusters, and late VEGF signaling is essential for the generation of the postoral and distal anterolateral rods, possibly due to VEGF unique role in cell migration and cavity formation (Figure 9B).

The expression of the genes encoding the transcription factors, MyoD1 and Pitx1 is dependent on VEGF signaling throughout skeletogenesis and they are a part of the postoral specific regulatory state (Figure 9A). The expression of myoD1 and pitx1 initiates at the skeletogenic cell clusters downstream of VEGF signaling at the time of spicule formation. Late inhibition of VEGFR activity completely abolishes the expression of myoD1 and pitx1 at all tested time points (Figures 3-5). Thus, VEGF signaling is essential for the dynamic expression of myoD1 and pitx1 throughout skeletogenesis, first in the ventrolateral clusters and later at the tips of the growing postoral rods.

The knockdown of Pitx1 results in a significant delay in the growth of all skeletal rods (Figure 7), and down-regulates the expression of some of VEGF targets (Figure 8 and 9B). The reduction of the expression level of Pitx1 targets in pitx1 morphants is more significant at 2 dpf than at 30 hpf (Figure 8C, L). At both time points the spatial expression of pitx1 is more localized than the broad expression of its target genes, SM30 and SM50, and the spatial expression of these genes is unchanged under Pitx1 knockdown (Figure 8D-I, M-R). As the skeletogenic cells are connected in a syncytium it is possible that Pitx1 protein migrates to neighboring cells and activates genes in the vicinity of pitx1 expressing cells. However, it is unlikely that at 2 dpf, when pitx1 is localized to the tips of the postoral rods, it regulates the broad expression of SM30 or the expression of SM50 at the tips of the anterolateral and body rods (Figure 9). Probably, the observed reduction of SM30 and SM50 levels and the size reduction in all skeletal rods are due to the role of Pitx1 in regulating gene expression at the ventrolateral cell clusters during the late gastrula stage, and not because of its late localized expression in the pluteus. To study the late role of Pitx1 in regulating gene expression at the tips of the postoral rods, a late perturbation technique is required, which is beyond the scope of this work.

The common participation of sea urchin Pl-Pitx1 and vertebrates’ Pitx2 in biomineralization, makes it interesting to compare these two developmental programs. Pitx2 regulates vertebrate teeth development from bud
formation up until generation of enamel.\textsuperscript{57,58} To the best of our knowledge, Pitx2 is not regulated by VEGF signaling during vertebrates’ tooth development. Vertebrates’ Pitx2 is one of the earliest genes specifically expressed in the oral ectoderm, where it is activated by FGF signaling and repressed by BMP signaling.\textsuperscript{34} Sea urchin BMP signaling is important for skeletal patterning as it represses VEGF and VEGFR expression.\textsuperscript{59} Other transcription factors that regulate tooth organogenesis such as, Dlx, Msx, Irx, and FoxJ\textsuperscript{35,57,58,60} are not involved in the regulation of skeletogenesis in the sea urchin embryo and are a part of the GRNs that control dorsal ectoderm specification.\textsuperscript{61,62} Overall, there are more differences than similarities between the mesodermal GRN that regulates sea urchin skeletogenesis and the ectodermal GRN that drives vertebrate enamel formation, implying that these two GRNs do not have a common evolutionary origin.

Interestingly, the transcription factors, Pitx2 and MyoD, are a part of the GRN that controls skeletal muscle differentiation in vertebrates.\textsuperscript{42} Specifically, Pitx2 activates the expression of \textit{myoD} in limb muscle precursors through direct binding to MyoD core enhancer.\textsuperscript{63} Moreover, Pitx2 and MyoD co-regulate the differentiation of all skeletal muscles, including limb, myotome, extraxicular and pharyngeal arch muscles.\textsuperscript{63} As discussed above, in the sea urchin embryo, \textit{pitx1} and \textit{myoD1} are co-expressed in the skeletogenic cells at the tips of the postoral rod (Figure 4) and Pitx1 activates \textit{myoD1} expression (Figure 9B). Other sea urchin orthologs of Pitx and MyoD, \textit{pitx2} and \textit{myoD2}, are expressed in the non-skeletogenic mesodermal cells, and MyoD2 is a part of the myogenesis GRN.\textsuperscript{64} Within the intense rewiring of the mesodermal derived GRNs, the apparent conservation of the Pitx-MyoD connection is quite remarkable. This could be an example of the “plug-in” concept, where a successful regulatory sub-circuit is inserted into various positions in the GRN hierarchy to drive diverse outcomes.\textsuperscript{65}

3.1 | Experimental procedures

3.1.1 | Animal and embryos

Adult \textit{Paracentrotus lividus} were obtained from the Institute of Oceanographic and Limnological Research (IOLR) in Eilat, Israel. The animals were kept in aquaria in a dedicated room, in artificial seawater (ASW) with a salinity of 39 ppt. Eggs and sperm were obtained by injection of 0.5 M KCl solution into the adult sea urchin species. Embryos were cultured at 18°C in 0.2 μ filtered ASW.

3.2 | Imaging

All embryos presented in this work were imaged by Zeiss Axioimager 2 and aligned in Photoshop CS6. Figures were made in Adobe Illustrator CS6.

3.3 | Axitinib (AG013736) treatment

A 5-mM stock solution of Axitinib (AG013736, Selleckchem, Houston, TX), was prepared by reconstituting the chemical in dimethylsulfoxide (DMSO). The embryos were treated with Axitinib at a final concentration of 150 nM. Control embryos were cultured in 150 nM solution of DMSO at no more than 0.1% (v/v).

3.4 | Whole mount in situ hybridization probe preparation

Total RNA of 30 hpf \textit{P. lividus} embryos was used to generate cDNA using the High capacity cDNA RT Super-script II Invitrogen 18 064-024. cDNAs of the target genes were PCR amplified, ligated, and inserted into pGemT (Promega A3600) or pJet plasmids (Thermofisher Scientific K1231). Primer list is provided in Table S1. RNA DIG probes were generated using Roche DIG labeling kit (Catalogue Number 1277073910) and SP6 polymerase 10810274001 or T7 polymerase 10881767001 (Sigma-Aldrich).

3.5 | WMISH procedure

WMISH was performed as described in Reference 7. Briefly, embryos were fixed by 1-hour incubation in 2% paraformaldehyde (PFA) in 4°C, followed by overnight incubation at 4% PFA at 4°C. Fixed embryos were washed three times in TBST buffer, once with hybridization buffer at room temperature, followed by prehybridization at 65°C for 1 hour. Probes were added to the hybridization buffer (0.3-1 ng/mL) and incubated overnight at 65°C. Probe was washed by hybridization buffer at 65°C twice, followed by addition of a 1:1 mix of the hybridization buffer and 2XSSCT. Embryos were then washed with 2XSSCT and 0.2XSSCT. Antibody staining was done similarly to,\textsuperscript{7} that is, washe with MABT, followed by first blocking solution with 10 mg/mL BSA and second blocking solution with 1 mg/mL BSA and 10% sheep serum. Then the embryos were incubated with anti-DIG antibody solution buffer at 4°C overnight. The embryos were washed with MABT, followed by two washes with alkaline phosphate buffer. The embryos...
were stained with staining Solution (10% dimethylformamide, 0.1 M Tris pH 9.5, 50 mM MgCl2, 0.1 M NaCl, 1 mM levamisole, 337 mg/mL NBT, 175 mg/mL BCIP) in room temperature until the color was visible. Staining was stopped with 50 mM EDTA in MABT buffer. The probes were stored in 50% MABT and 50% glycerol at 4°C.

3.6 cDNA preparation for QPCR experiments

For VEGFR inhibition experiments, total RNA was extracted from >1000 sea urchin embryos in each condition using RNeasy Mini Kit (50) from QIAGEN (#74104). For injected embryos, total RNA was extracted from 150 to 200 injected sea urchin embryos in each condition using RNeasy Micro Kit (50) from QIAGEN (#74004). Both kits were used according to the kits’ protocol. DNase treatment on the column was done using RNase-Free DNase Set-Qiagen (50) (#79254). RNA was reverse-transcribed using the High Capacity cDNA RT kit, AB-4368814 (Applied Biosystems) following the manufacturer’s instructions.

3.7 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (QPCR) was carried out in triplicates using a 384CFX-real-time thermal cycler and SYBR Green FastMix (Quantabio 95 072-012, Beverly, MA). Reaction conditions were as follows: 95°C for 3 min (one cycle), followed by 95°C for 10 s, 55°C for 30 s (40 cycles). Dissociation analysis was performed at the end of each reaction to confirm the amplification specificity. Primer set for all tested genes were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). A complete list of primer sequences used for the experiments is provided in Table S1.

3.8 QPCR expression levels quantification and differential expression analysis

Changes in gene expression were measured under different conditions and at the same developmental stage. Relative expression of genes was calculated by 1.9^-ΔΔCT method using Ubiquitin as an internal reference gene for normalization. 7,53

3.9 Pitx1 MO microinjection

Egg jelly coat was removed by changing the pH of the ASW to 5.3 by gradual addition of 1 M HCl. The eggs were incubated at pH 5.3 for 1 min. The pH was then risen to 8.0 by the addition of 1 mL of 1 M Tris-HCl solution-pH 8. The eggs were rolled onto the injection plates treated with 0.25% protamine sulfate solution and injected with an injection solution containing 0.12 M KCl, 0.5 μg/μl Rhodamine Dextran, and 800 μM morpholino antisense oligonucleotide (MO). Down-regulation of pitx1 was done by injecting two specific MOs targeting the splicing sites of the pre-mRNA. MOs were designed and synthesized by Gene Tools, Philomath, OR, according to Pl-Pitx1 sequence. Splicing MO 1 (sMO1) was designed to remove the third exon, whereas sMO2 was designed to remove the second exon (Figure 6A). The sequences are as follows: sMO1: 5’-CACATAAATACCTACTCTAACTCGT-3’ and sMO2: 5’GTACCTGAGATCGACAAGAATTATTA-3’.

Embryos injected with the same concentration of Random MO were used as control. Injected embryos were cultured at 18°C. At least three biological replicates, with different pairs of parents, were studied and imaged for each MO (see Table S2 for an exact number of embryos scored in each experiment).

3.10 Quantification of skeletal length and statistical analysis

The lengths of skeletal rods were manually measured at 72 hpf using a built-in straight ruler tool in Zen software (Figure 7D). Embryos were flattened under the coverslip and the length of the rods was measured as shown in Figure 7D. The measurements were repeated for three biological repeats with a total of 109 embryos measured for sMO1, 99 embryos for sMO2, 115 embryos for Random MO in sMO1 experiment, and 99 embryos for Random MO in the sMO2 experiment. The data were analyzed in Excel and the statistical analysis was performed using Student’s t-test in Excel and SPSS Statistics 21.

3.11 Verification of the activity of pitx1 sMOs

The activity of the splicing MOs was checked by PCR reaction of cDNA prepared as described above from injected embryos collected at 44 hpf. All primers used for the splicing check can be found in Table S1. For sMO1,
the primer pair F1 + R1 was used (Figure 6A,B). The expected length of the PCR amplicon of the primers pair F1 + R1 in uninjected eggs (Un) and random MO (raMO) was approximately 480 bp, and the truncated amplicon in the Pl-Pitx1 splicing sMO1 was 265 bp (excluding the third exon, Figure 6B). sMO2 was tested with a primer pair F2 + R2 (Figure 6A,C). The size of the expected amplicon for uninjected embryos and embryos injected with random MO was 819 bp, whereas the Pl-Pitx1 sMO2 was expected to produce a truncated amplicon of 290 bp due to the deletion of the second exon (Figure 6C).

3.12 Identification of putative Pitx1 bindings sites

We studied the promoter regions of SpSM30a and SpSM50 that were shown to drive expression in the skeletogenic cells.50,51,52 (Figure 8S,T). Putative binding sites were identified using the human and mouse Pitx1/2/3 position weight matrices (PWM) from Jasper, with a relative profile score of >80%. https://jaspar.genereg.net/analysis. Four putative Pitx binding sites are detected in the promoter region of SpSM30a and two putative Pitx binding sites are detected in the promoter region of SpSM50 (Figure 8S,T).

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AUTHOR CONTRIBUTIONS

Kristina Tarsis: Conceptualization (equal); formal analysis (equal); investigation (lead); methodology (lead); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). Tsvia Gildor: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (lead); supervision (equal); validation (lead); writing – review and editing (equal). Miri Morgulis: Formal analysis (supporting); investigation (supporting); methodology (supporting); visualization (supporting); writing – review and editing (equal).

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