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REVIEW

Growth Factors and Early Mesoderm Morphogenesis: Insights from the Sea Urchin Embryo

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Summary: The early morphogenesis of the mesoderm is critically important in establishing the body plan of the embryo. Recent research has led to a better understanding of the mechanisms that underlie this process, and growth factor signaling pathways have emerged as key regulators of the directional movements of mesoderm cells during gastrulation. In this review, we undertake a comparative analysis of the various essential functions of growth factor signaling pathways in regulating early mesoderm morphogenesis, with an emphasis on recent advances in the sea urchin embryo. We focus on the roles of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) pathways in the migration of primary mesenchyme cells and the formation of the embryonic endoskeleton. We compare the functions of VEGF and FGF in sea urchins with the roles that these and other growth factors play in regulating mesoderm migration during gastrulation in *Drosophila* and vertebrates. genesis 52:158–172. © 2014 Wiley Periodicals, Inc.

Key words: growth factors; mesoderm cell migration; mesoderm differentiation; gastrulation, sea urchin; skeletogenesis; primary mesenchyme cells; VEGF; FGF

INTRODUCTION

Coordinated cellular movements are a hallmark of gastrulation in metazoans (Stern, 2004). Mesoderm cells execute some of the most prominent of these movements and engage in striking, long-distance migrations following their transformation from an epithelial to a mesenchymal phenotype. The directional movements of mesoderm cells during gastrulation play a critically important role in establishing the body plan of the embryo and establish new tissue relationships that underlie subsequent inductive interactions.

Growth factors regulate many developmental processes, including early axis specification, germ layer formation, and organogenesis (Dorey and Amaya, 2010; Hogan, 1999; Wilson and Leptin, 2000; Wu and Hill, 2009). Recent studies have shown that growth factors, acting through their cognate cellular receptors, also regulate the motile behavior of mesoderm cells and function as directional cues that guide these migratory cells through embryonic tissues. In keeping with their diverse effects on cells, growth factors also regulate the differentiation of the early mesoderm in many organisms.

The sea urchin is a valuable experimental model for analyzing the role of growth factors in mesoderm morphogenesis. The optical transparency and morphological simplicity of the sea urchin embryo make it possible to analyze the migration of cells *in vivo* at high resolution, both in undisturbed embryos and in embryos that have been subjected to molecular manipulations. In addition, mesoderm cells can be purified in large

Abbreviations: EMT, epithelial-to-mesenchymal transition; FGF, fibroblast growth factor; HUVECs, Human umbilical vein endothelial cells; NSM, non-skeletogenic mesoderm; PDGF, platelet derived growth factor; PMCs, primary mesenchyme cells; RTKs, receptor tyrosine kinases; VEGF, vascular endothelial growth factor; VLC, ventro-lateral clusters.

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numbers and their motility studied in culture, under well-defined conditions. Newly developed chemical inhibitors of growth factor signaling pathways, well established gene knockdown approaches, and recent advances in echinoderm genomics, have all made it possible to systematically explore the developmental functions of growth factor pathways in early developmental processes in the sea urchin.

Here, we discuss recent advances in understanding the roles that growth factor signaling pathways play in regulating early mesoderm morphogenesis in sea urchins. We highlight the functions of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) pathways in regulating these processes, with an emphasis on the migration and differentiation of primary mesenchyme cells (PMCs) and the formation of the embryonic skeleton. We compare the functions of growth factors in early sea urchin development with the roles that these and other growth factors play in early mesoderm morphogenesis in *Drosophila* and vertebrates.

GROWTH FACTOR-MEDIATED MESODERM CELL MIGRATION AND SKELETOGENESIS IN SEA URCHINS

Primary Mesenchyme Cell Migration and the Formation of the Embryonic Skeleton

The sea urchin embryonic mesoderm consists of the PMCs, which secrete the calcified embryonic endoskeleton, and the non-skeletogenic mesoderm (NSM), which gives rise to pigment cells, muscle cells, coelomic pouch cells, blastocoelar cells, and possibly other cell types (Ruffins and Ettensohn, 1996; Shoguchi *et al*., 2012; Solek *et al*., 2013). Skeletal morphogenesis has been studied intensively, and several reviews discuss this process in detail (Ettensohn, 2013; Killian and Wilt, 2008; Lyons *et al*., 2012; Wilt and Ettensohn, 2007). In brief, the PMCs are progeny of the large micromeres, four blastomeres that arise at the vegetal pole as a consequence of the fourth and fifth cleavage divisions, which are unequal in the vegetal region of the embryo (Fig. 1a). In the pre-hatching blastula, the progeny of the large micromeres are integrated into the developing embryonic epithelium (Fig. 1b). As the embryo begins to gastrulate, PMCs undergo an epithelial-to-mesenchymal transition (EMT) and ingress into the blastocoel (Fig. 1c). After a brief period of quiescence, the cells begin to migrate away from the vegetal pole, adhering to the wall of the blastocoel and translocating along a thin basal lamina that covers the basal surfaces of the epithelial cells that form the outer wall of the embryo (Fig. 1d). The PMCs migrate by means of filopodia, which engage in continuous cycles of extension and retraction (Malinda *et al*., 1995; Miller *et al*., 1995). Some of these filopodia fuse with those of nearby PMCs, forming cable-like cytoplasmic strands that join the PMCs in a single, continuous syncytium (Hodor and Ettensohn, 1998, 2008). As the PMCs migrate and fuse during early gastrulation, they gradually position themselves in two clusters of cells (ventro-lateral clusters, or VLCs) at specific locations along the blastocoel wall (Fig. 1e). The VLCs are linked by two chains of PMCs, one located ventrally and the other dorsally. This syncytial arrangement of PMCs, which is well-formed by the mid-gastrula stage, is known as the sub-equatorial ring. Soon after the subequatorial ring forms, a single chain of PMCs migrates longitudinally from each VLC toward the animal pole of the embryo, with its advance led by numerous filopodia (Fig. 1f).

PMCs secrete the calcified embryonic endoskeleton, which forms from two triradiate spicule rudiments, one of which is deposited within each VLC late in gastrulation (Fig. 1f). These two rudiments elongate and branch in a characteristic manner, forming mirror-image spicules that together constitute the complete endoskeleton of the early (pre-feeding) larva (Fig. 1g,h) (Okazaki, 1975; Guss and Ettensohn, 1997). The spicules are deposited within the PMC filopodial cables; therefore, the spatial arrangement of the PMCs (and the resultant positioning of the cables) serves directly as a template for the embryonic endoskeleton. Both the branching pattern and fine structure of the endoskeleton are highly consistent within individuals of a species but vary among different species of sea urchin. Several additional skeletal elements arise during later larval development (Smith *et al*., 2008; Yajima and Kiyomoto, 2006). The skeleton serves as the primary determinant of the shape of the larva and plays an important role in its orientation, swimming, and feeding (see Ettensohn, 2013 and references therein).

In addition to the PMCs, some classes of NSM cells also migrate directionally during gastrulation. In particular, pigment cells and blastocoelar cells, two cell populations that function in immune surveillance (Li *et al*., 2013; Solek *et al*., 2013), undergo EMT during gastrulation and migrate to specific locations within the embryonic ectoderm and blastocoel, respectively (Gibson and Burke, 1987; Kominami *et al*., 2001; Tamboline and Burke, 1992). The guidance cues that direct these movements have not been identified. In contrast, considerable progress has been made in dissecting the mechanisms of PMC guidance, as discussed below.

Regulation of PMC Migration by Ectodermal Cues

The complex and highly reproducible pattern of PMC migration during sea urchin gastrulation originally suggested that external cues from the ectoderm play an important role in directing PMC movements. Gustafson and Wolpert (1961), Okazaki *et al*. (1962) and Galileo

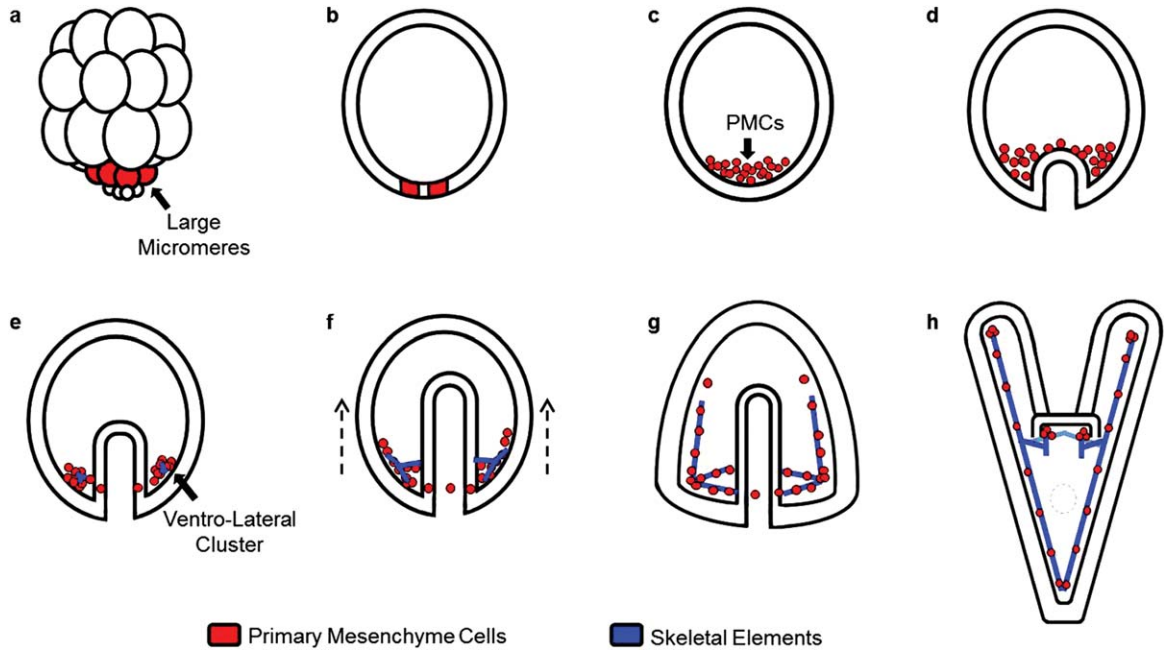


FIG. 1. Schematic diagram of sea urchin embryonic development and skeletogenesis. Figures show: (a) mid-cleavage stage, when the large micromeres form; (b) hatched blastula, when the presumptive PMCs (large micromere progeny) are embedded in the epithelial wall of the embryo near the vegetal pole; (c) mesenchyme blastula, when PMCs undergo EMT and ingress into the blastocoel; (d) early gastrula, when PMCs spread on the wall of the blastocoel and begin to migrate; (e) mid gastrula, when the ventro-lateral clusters (VLCs) are evident; (f) late gastrula, when the triradiate spicule rudiments (blue) form and PMCs begin to migrate from each cluster toward the animal pole (arrows); (g) prism, when the skeletal rods have elongated further and the migration of PMCs toward the animal pole is complete; and (h) pluteus, showing the final structure of the embryonic skeleton.

and Morrill (1985) observed that even before PMC migration begins, there is a distinctive, shingle-like arrangement of ectodermal cells at the sites where the future PMC ring will form. More significantly, perturbation of ectodermal patterning with $NiCl_2$ was shown to alter PMC migration and skeletogenesis (Armstrong *et al.*, 1993; Hardin *et al.*, 1992). A series of cell transplantation and cell marking studies showed that PMC guidance cues arise progressively during development and that PMCs are specifically competent to respond to these cues (Ettensohn and Malinda, 1993; Ettensohn and McClay, 1986; Malinda and Ettensohn, 1994; Peterson and McClay, 2003). Additional evidence that PMCs respond to signals from the ectoderm came from studies showing that localized photoablation of ectoderm cells blocked the deposition of skeletal material by neighboring PMCs (Ettensohn and Malinda, 1993) and from the finding that several mRNAs encoding biomineralization–related proteins are expressed selectively in the VLCs, where skeletal growth is initiated presumably in response to ectodermal cues (Harkey *et al.*, 1992; Guss and Ettensohn, 1997). Although these various studies clearly showed that local ectodermal cues regulate PMC migration, gene expression, and skeletogenesis, the molecular nature of the cues remained unknown until relatively recently.

Growth Factors and Primary Mesenchyme Morphogenesis

Growth factor signaling pathways have been linked to a wide variety of essential processes during embryonic development. Most growth factor receptors are receptor tyrosine kinases (RTKs), a family of transmembrane glycoproteins that function in transmitting extracellular signals into the cell (reviewed in Hubbard and Till, 2000). An RTK typically consists of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic domain with catalytic tyrosine kinase activity. Various RTKs differ mostly in the architecture of their extracellular domains, and in the absence of a ligand usually exist as monomers. The binding of a ligand to a specific receptor triggers the dimerization and autophosphorylation of the receptor, leading to the sequential phosphorylation and activation of downstream effectors of RTK signaling. RTKs exert their effects on cells through a variety of intracellular signaling pathways, including the RAS-MAPK, PI3K-AKT, and phospholipase C-Ca^{2+} pathways (Goetz and Mohammed, 2013; Koch and Claesson-Welsh, 2012). These intracellular pathways often culminate in the activation of specific transcription factors, which in turn regulate gene expression (Hubbard and Till, 2000; Lemmon and Schlessinger, 2010; Stuttfeld and Ballmer-Hofer, 2009).

VEGF Signaling Regulates PMC Migration

In a comprehensive survey of RTK signaling genes in the genome of the purple sea urchin, *Strongylocentrotus purpuratus*, Lapraz *et al*. (2006) identified two genes that encode VEGF receptors (*vegfr-Ig7 a*nd *vegfr-Ig10*) and three that encode VEGF ligands (*vegf1*, *vegf2*, and *vegf3)*. Of these, *vegf3* and *vegfr-10* are the most highly expressed VEGF ligand and receptor, respectively, during embryogenesis (Tu *et al*., 2012) and have been studied in the greatest detail. Orthologs of *vegfr-Ig10* are expressed selectively by PMCs in the sea urchin species *Paracentrotus lividus* (Duloquin *et al*., 2007), *S. purpuratus* (Rafiq *et al*., 2014), and *Lytechinus variegatus* (Fig. 2). The *vegfr-Ig10* gene is transcribed selectively in PMCs at least in part through essential positive inputs from a PMC-specific transcription factor, ALX1 (Rafiq *et al*., 2012). VEGFR-Ig10 is thought to be unique to sea urchins, as it has an unusual architecture of 10 extracellular immunoglobulin (Ig) domains (Lapraz *et al*., 2006) while most VEGF receptors have 7 Ig domains (Koch and Claesson-Welsh, 2012).

The *P. lividus* and *L. variegatus* orthologs of *vegf3* are expressed in the ectoderm overlying the VLCs (Fig. 3), where the spicule rudiments first appear, and are expressed later in development in localized regions of the ectoderm that overlie sites of rapid skeletal growth, but only on the ventral side of the embryo (Adomako-Ankomah and Ettensohn, 2013; Duloquin *et al*., 2007). *Lv-vegf3* is initially expressed in a ring of prospective ectoderm in the vegetal region of the blastula and subsequently resolves to the ectoderm overlying the VLCs (Fig. 3), a phenomenon that might explain the gradual resolution of ingressed PMCs into two clusters during gastrulation.

The striking, complementary expression patterns of *vegf3* and *vegfr-Ig10* suggest a possible role for VEGF signaling in PMC morphogenesis, a hypothesis supported by gene knockdown studies in three different species of sea urchins (Fig. 4) (Duloquin et al., 2007; Adomako-Ankomah and Ettensohn, 2013). Morpholino knockdown of *vegf3* or *vegfr-Ig10* in *P. lividus* leads to a dramatic perturbation of PMC migration and a complete inhibition of skeletogenesis, though the specification and ingression of PMCs is unaffected (Fig. 4a–f, Duloquin *et al*., 2007). Overexpression of *vegf3* leads to an increased number of PMCs and the formation of supernumerary skeletal rods. Similarly, knockdown of *vegf3* in *L. variegatus* or *S. purpuratus* dramatically disrupts the directional migration of PMCs and blocks skeletogenesis (Fig. 4g–n, Adomako-Ankomah and Ettensohn, 2013). In these species, a potent and specific inhibitor of VEGF receptors, axitinib, which phenocopies *vegf3* knockdown, was used to show that VEGF signaling is required for both the initial migration of PMCs to form the sub-equatorial ring and the later phase of migration, during which a chain of PMCs migrates from each VLC toward the animal pole of the embryo (Adomako-Ankomah and Ettensohn, 2013).

PMC migration is dependent on the dynamic motility of filopodia (Malinda *et al*., 1995; Miller *et al*., 1995; Okazaki, 1965). Inhibition of VEGF signaling with a *vegf3* morpholino or axitinib reduces the length and number of filopodia extended by migrating PMCs *in*

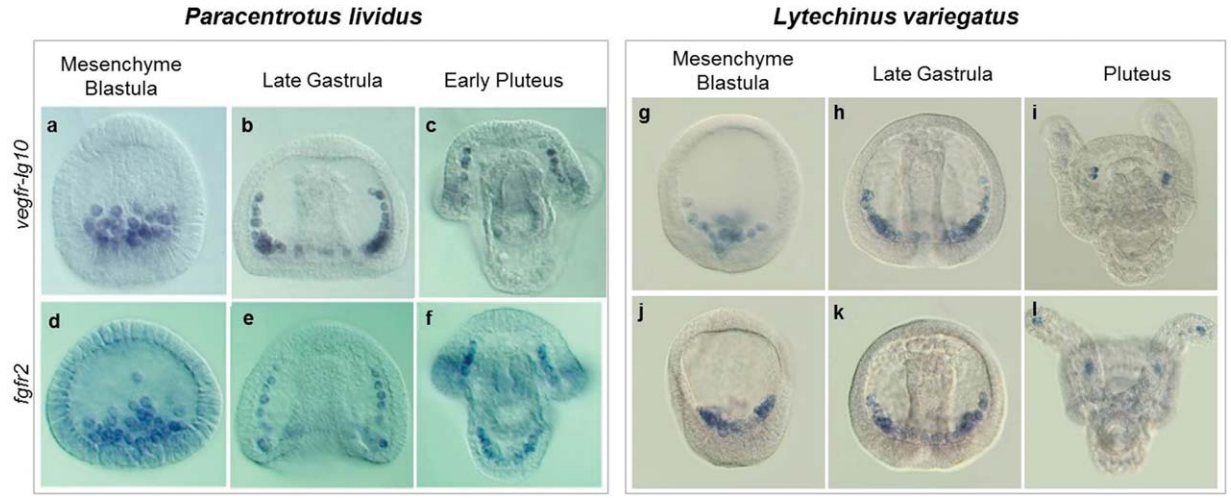


FIG. 2. VEGF and FGF receptors are expressed in the PMCs. Whole mount in situ hybridization analysis of the expression patterns of *vegfr-Ig10* (**a–c**, **g–i**) and *fgfr2* (**d–f**, **j**,**k**) during embryogenesis in *P. lividus* (a–f) and *L. variegatus* (g–l) shows that both genes are expressed in the migrating PMCs from the mesenchyme blastula stage to the pluteus stage. *vegfr-Ig10* and *fgfr2* are expressed uniformly in all PMCs at early stages (a, d, g, j), but at later stages both receptors are selectively expressed in PMCs at the VLCs (b, c, e, f) and at regions of rapid skeletal growth (h, i, k, l). *P. lividus* images are adapted and reproduced with permission from Duloquin *et al*., 2007 (a–c) and Röttinger *et al*., 2008 (d–f).

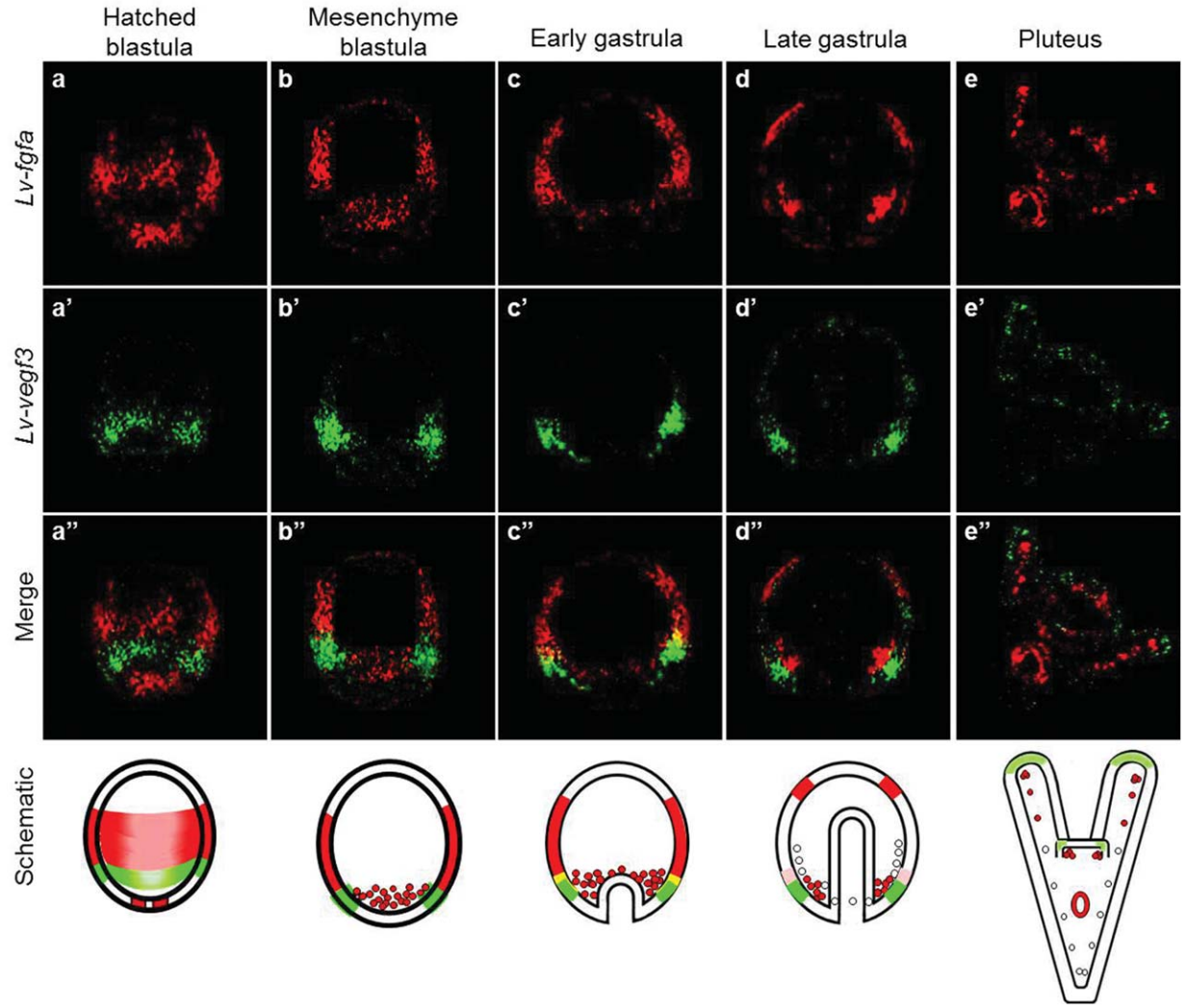


FIG. 3. *fgfa* and *vegf3* ligands are expressed in the ectoderm of the sea urchin embryo. Double fluorescent in situ hybridization analyses of *Lv-fgfa* (**a–e**) and *Lv-vegf3* (a'–e') expression show that their expression domains are entirely separate in the ectoderm at the hatched blastula (a–a") and mesenchyme blastula (b–b") stages. Their expression domains overlap at early gastrula (c–c") stage, and by the late gastrula (d–d") and pluteus (e–e") stages, *Lv-fgfa* and *Lv-vegf3* are once again expressed in independent domains. Schematic diagrams illustrate expression patterns. Adapted and reproduced with permission from Adomako-Ankomah and Ettensohn, 2013.

vivo (Fig. 5, Adomako-Ankomah and Ettensohn, 2013). These changes in filopodial morphology appear to be associated specifically with an impairment of PMC pathfinding, as PMC translocation *per se* is not dependent on VEGF. Thus, inhibition of VEGF signaling *in vivo* does not prevent PMCs from spreading on the basal lamina or moving away from the vegetal plate, and the velocity of PMC migration in an *in vitro* motilility assay is not affected by axitinib. In addition, and somewhat surprisingly, PMCs are still capable of engaging in cell–cell fusion when VEGF signaling is inhibited, despite the reduced number and length of filopodia (Adomako-Ankomah and Ettensohn, 2013).

In other model systems, VEGF functions as a chemoattractant and a regulator of filopodial dynamics (Gerhardt, 2008). For example, in quiescent endothelial vessels, VEGF-A induces the specification of tip cells and the formation of new filopodia (Hellstrom *et al.*, 2007). VEGF-A also regulates filopodia extension and directional cell migration during angiogenic sprouting in the developing retina (Gerhardt *et al.*, 2003). Human umbilical vein endothelial cells (HUVECs) and adult human dermal microvascular endothelial cells have been shown to migrate toward regions of higher VEGF concentrations in three-dimensional matrices *in vitro*, and these migrating cells exhibit an asymmetric distribution of filopodia, with protrusions localized predominantly along the leading edge of the cell (Shamloo *et al.*, 2008, 2012). Interestingly, application of exogenous VEGF gradients to HUVECs increases the average number of filopodia/cell, a finding reminisicent of the effects of VEGF on PMC filopodial activity. A variety of

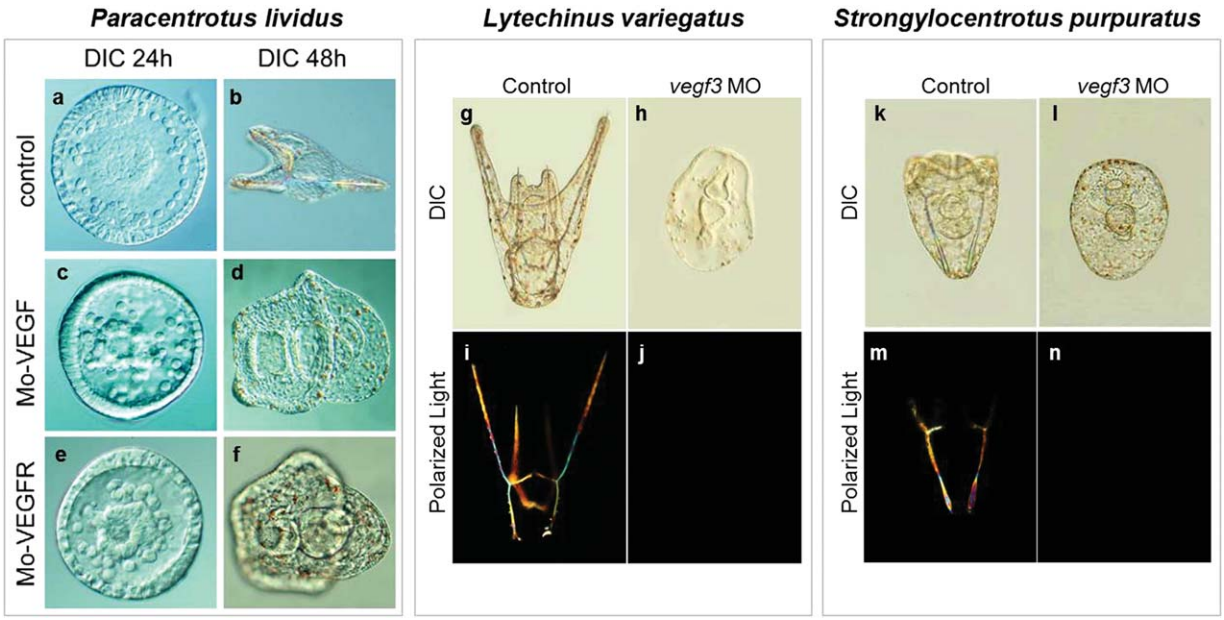
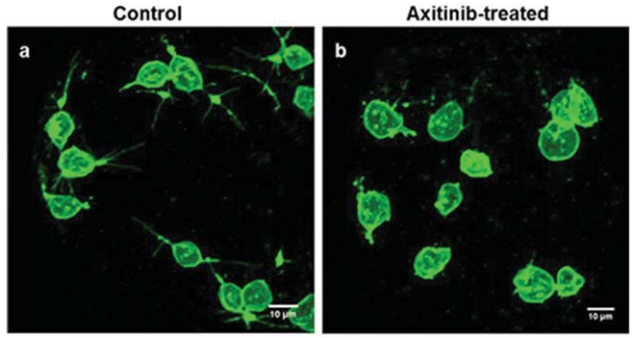


FIG. 4. Perturbation of VEGF signaling inhibits PMC migration and skeletogenesis in three species of sea urchin. DIC images of *P. lividus* control embryos (**a, b**), *vegf3* (**c, d**) and *vegfr-Ig10* (**e, f**) morphants at the late gastrula (a, c, e) and pluteus (b, d, f) stages show that PMC migration and skeletogenesis are blocked in *vegf3 and vegfr-Ig10* morphants. DIC (**g, h, k, l**) and polarized light (**i**, **j**, **m, n**) images of *L. variegatus* (g–j) and *S. purpuratus* (**k**–n) *vegf3* morphants and control embryos at the pluteus stage show that no skeletal elements form in *vegf3* morphants. Adapted and reproduced with permission from Duloquin *et al*., 2007 and Adomako-Ankomah and Ettensohn, 2013.



	DMSO CONTROL (n=57)	AXITINIB-TREATED (n=63)
Average filopodia per cell	4.7	2.1
Average length of filopodia present	11.5	4.8

FIG. 5. VEGF signaling regulates the number and length of filopodia extended by PMCs. Fluorescence images of control embryo (**a**) and axitinib-treated embryo (**b**) 1.5 hours after PMCs were scattered at the mesenchyme blastula stage by microsurgical manipulation to reduce cell density within the blastocoel. PMCs in control embryos (a) extend more filopodia than PMCs in axitinib-treated embryos (b), and these filopodia are longer in control embryos. Table shows a comparison between the average number of filopodia per cell and the average length of filopodia extended by PMCs in control embryos and axitinib-treated embryos. Adapted and reproduced with permission from Adomako-Ankomah and Ettensohn, 2013.

studies suggest that VEGF also acts as chemoattractant for developing neurons (Chauvet *et al*., 2013). In sea urchins, the finding that knockdown of *vegfr-Ig10* results in mis-targeting of PMCs (Duloquin *et al*., 2007) strongly supports the view that VEGF acts directly as a PMC chemoattractant, although this has not been shown directly; that is, by demonstrating that isolated PMCs migrate toward a localized source of purified VEGF *in vitro*.

A homolog of *vegf3* has also been identified in the sea urchin *Hemicentrotus pulcherrimus*. Similar to previous observations, Hp*vegf* expression is restricted to two domains of ectoderm overlying the VLCs, and the overexpression of this ligand leads to the formation of supernumerary skeletal rods (Fujita *et al*., 2010). Interestingly, overexpression of the *H. pulcherrimus* homolog of heparan sulfate 6-O-endosulfatase, which regulates interactions between heparan sulfate and several growth factor ligand and receptor pairs, is sufficient to suppress the formation of these extra rods in embryos overexpressing *vegf3* (Fujita *et al*., 2010). These data point to a possible role for heparan sulfate proteoglycans in mediating VEGF signaling during skeletogenesis.

The expression pattern of *vegf3* has been examined in embryos of two other groups of echinoderms: sea stars (*Patiria pectinifera*) and brittle stars (*Amphipholis kochii*) (Morino *et al*., 2012). In brittle stars, which

form an embryonic endoskeleton, a *vegf3* ortholog is expressed during early embryogenesis in a ring-like pattern which then resolves into two domains of ectoderm, as seen in *L. variegatus*. In contrast, *vegf3* is not expressed in sea star embryos, which lack an endoskeleton, though this gene is expressed at late larval stages in epithelial cells that overlie the primordia of the adult skeleton (Morino *et al*., 2012). These comparative studies point to an important, conserved role of VEGF signaling in echinoderm skeletogenesis and suggest that evolutionary modifications in VEGF expression played a role in the appearance of new patterns of skeletogenesis within the phylum.

Recently, the pattern of expression of a second VEGF ligand, *vegf2*, was described in the sea urchin *Strongylocentrotus intermedius* (Kipryushina *et al*., 2013). Unlike *vegf3*, which is expressed specifically in the ectoderm, *vegf2* is ubiquitously expressed during early embryonic development but at later stages is expressed selectively by PMCs, particularly in the scheitel region at the posterior apex of the larva. The function of *vegf2* is unknown, though its expression pattern raises the possibility this ligand might also play a role in skeletogenesis.

VEGF Signaling Regulates PMC Gene Expression and Later Skeletal Morphogenesis

In addition to its essential role in PMC guidance, VEGF also regulates skeletal morphogenesis through its effects on PMC gene expression. Several studies have shown that the initial deployment of a cell-type specific transcriptional program in the micromere-PMC lineage occurs by a cell-autonomous mechanism (Ettensohn, 2009; Ettensohn, 2013; Oliveri *et al*., 2008; Rafiq *et al*., 2012). By the mid-gastrula stage, however, key elements of this transcriptional program come under the influence of extrinsic signals, a regulatory mechanism which ensures that skeletal growth is tightly coordinated with the development of the embryonic ectoderm. For example, ectodermal cues result in the localized upregulation of numerous biomineralization-related genes in regions of active skeletal growth within the PMC syncytium (Adomako-Ankomah and Ettensohn, 2011; Guss and Ettensohn, 1997; Harkey *et al*., 1992; Illies *et al*., 2002; Rafiq *et al*., 2012; 2014). VEGF signaling is one of several signaling pathways that control these regional patterns of gene expression and skeletal rod growth within the PMC network.

The role of VEGF as a direct "differentiation factor" was first suggested by the observation that forced expression of VEGF3 is sufficient to stimulate spicule growth by a partially enriched population of PMCs (Duloquin *et al*., 2007). It has long been known that the formation of spicules by isolated micromeres requires horse serum (McCarthy and Spiegel, 1983; Okazaki, 1975; Page and Benson, 1992), and VEGF may be the active factor in serum. Recent studies have shown that purified, recombinant VEGF3 stimulates biomineral formation by cultured micromeres and controls the pattern of skeletal growth in a concentration-dependent manner (Knapp *et al*., 2012). Relatively high levels of VEGF favor growth in the *a*-axis (i.e., the formation of triradiate spicule rudiments), while lower levels of VEGF favor growth in the *c*-axis (i.e., in a direction perpendicular to the plane of the triradiate spicule). These effects are consistent with patterns of skeletal growth *in vivo*; *vegf3* mRNA is expressed at high levels during gastrulation when the triradiate spicules are synthesized, and at lower levels at the prism and pluteus stages, when additional skeletal rods (e.g., the postoral and anterolateral rods) form by branching and extend in the direction of the *c*-axis.

Inhibition of VEGF signaling *in vivo* beginning at fertilization reduces the expression of many PMC-specific mRNAs that encode biomineralization proteins (Adomako-Ankomah and Ettensohn, 2013; Duloquin *et al*., 2007). It is unlikely that these changes are an indirect consequence of disrupting PMC migration; instead, they likely reflect a direct effect of VEGF signaling on PMC gene expression. In support of this view, it has been shown that blocking VEGF signaling with axitinib from the late gastrula stage, after PMC migration is largely complete, results in a selective inhibition of skeletal rod growth on the ventral side of the embryo (Adomako-Ankomah and Ettensohn, 2013). The local expression of several biomineralization genes by PMCs at the tips of the ventral rods is also dependent on VEGF signaling (Sun and Ettensohn, unpublished observations). These findings are consistent with the observed patterns of expression of *vegfr-10* and *vegf3* at late developmental stages; *vegfr-Ig10* is expressed selectively by PMCs at the tips of ventral skeletal rods, while *vegf3* is expressed in overlying patches of ectoderm (Adomako-Ankomah and Ettensohn, 2013; Duloquin *et al*., 2007). Because skeletal rods elongate by the addition of new biomineral at their tips (Decker and Lennarz, 1988; Ettensohn and Malinda, 1993), it seems likely that VEGF regulates skeletal growth by locally stimulating the expression of biomineralization proteins by PMCs at the tips of the rods. Though VEGF signaling plays an essential role in PMC gene expression and skeletal growth ventrally, other unidentified signaling pathways must operate on the dorsal side of the embryo.

FGF Signaling and Skeletal Morphogenesis in the Sea Urchin Embryo

FGFs are a conserved family of polypeptide growth factors ubiquitous among vertebrates and invertebrates (Ornitz and Itoh, 2001). FGFs are known to signal across many kinds of epithelial-mesenchymal boundaries, and their functions include the regulation of cell migration,

chemotaxis, cell adhesion, and cell differentiation (Böttcher and Niehrs, 2004; Dorey and Amaya, 2010).

FGF signaling has been shown to play a significant role in mesoderm formation in the sea urchin, as in other metazoans. The role of FGF has been studied in two sea urchin species, *P. lividus* and *L. variegatus*, with somewhat different results. Lapraz *et al*. (2006) identified two FGF receptors, *fgfr1* and *fgfr2*, and a single FGF ligand, *fgf 9/16/20*, in the *S. purpuratus* genome. The FGF ligand *fgfa* (which is the *L. variegatus* and *P. lividus* homolog of the *S. purpuratus fgf9/16/20* gene), like *vegf3*, is expressed in the ectoderm near the VLCs of PMCs during gastrulation in both species, though unlike *vegf3*, *fgfa* is also expressed in the migrating PMCs themselves (Fig. 3, Adomako-Ankomah and Ettensohn, 2013; Röttinger *et al*., 2008). In *L. variegatus*, *fgfa* is expressed in the PMCs prior to their ingression, a slightly earlier stage than in *P. lividus* (Adomako-Ankomah and Ettensohn, 2013; Röttinger *et al*., 2008). The *fgfa* and *vegf3* ligands are expressed in transiently overlapping, but mostly distinct domains in the ectoderm (Fig. 3). Throughout embryonic development, *Lv-fgfa* shows a highly dynamic pattern of expression, while *Lv-vegf3* expression is steadily maintained in the ectoderm overlying the VLCs and sites of rapid skeletal growth (Adomako-Ankomah and Ettensohn, 2013). In both species of sea urchin, *fgfr2*, which is an atypical, divergent FGF receptor (Lapraz *et al*., 2006), is expressed in the migrating PMCs (Fig. 2, Röttinger *et al*., 2008). The expression pattern of *fgfr1* has also been analyzed, and this gene is expressed in a very dynamic pattern in all three germ layers at different stages of development (Lapraz *et al*., 2006; McCoon *et al*., 1996).

Experiments blocking the FGF pathway have yielded different results in different species of sea urchins (Fig. 6). In *P. lividus*, knockdown of *fgfa* leads to multiple developmental defects: the directional migration of PMCs and skeletogenesis are perturbed, and the invagination and organization of the archenteron is inhibited (Röttinger *et al*., 2008). Knockdown of *fgfr1* leads to defects in the elongation of the archenteron and mild defects in skeletal rod elongation, while knockdown of *fgfr2* leads to developmental defects that are limited to the extension of skeletal rods. The double knockdown of *fgfr1* and *fgfr2* produces a phenotype that resembles an *fgfa* knockdown and leads to severe defects in gastrulation. In contrast, in *L. variegatus*, PMC migration is unaffected in *fgfa* morphants and these embryos form shortened but well-patterned skeletal elements, a much less severe phenotype than is observed in *P. lividus* (Adomako-Ankomah and Ettensohn, 2013). Controls using a splice-blocking morpholino indicated that the knockdown of *fgfa* in these studies was effective. Therefore, at least in *L. variegatus*, VEGF plays a much more prominent role than FGF in controlling PMC migration and skeletogenesis. It appears that the contribution of FGF signaling may vary among species, however, and studies on additional species are needed.

The VEGF and FGF pathways function synergistically in several systems. For instance, FGF and VEGF signaling are interdependent during embryonic coronary vasculogenesis and angiogenesis in quail explants and mouse embryonic hearts (Tomanek *et al*., 2010). In sea urchins, a connection between these pathways is indicated by the finding that signaling via VEGF3 is required for the expression of *fgfa* in PMCs (Adomako-Ankomah and Ettensohn, 2013). FGF signaling does not appear to

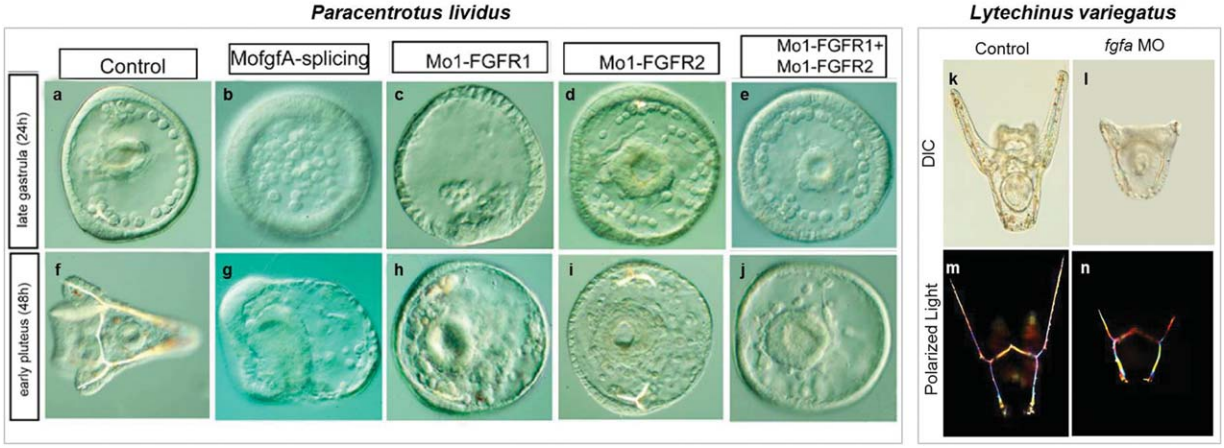


FIG. 6. Perturbation of FGF signaling inhibits PMC migration and skeletogenesis in *P. lividus*, but only partially inhibits skeletal rod elongation in *L. variegatus*. DIC images of *P. lividus* control embryos (**a, f**), *fgfa* (**b, g**), *fgfr1* (**c, h**), *fgfr2* (**d, i**) and *fgfr1+fgfr2* (**e, j**) morphants at the late gastrula (a–e) and pluteus (f–j) stages show that PMC migration and skeletogenesis are completely blocked in *fgfa and fgfr1+fgfr2* morphants, but not in *fgfr1* and *fgfr2* morphants. DIC (**k, l**) and polarized light (**m, n**) images of *L. variegatus* control embryos (k, m) and *fgfa* morphants (l, n) at the pluteus stage show that shortened skeletal rods form in *fgfa* morphants, and the migration of PMCs is unaffected in these embryos. Adapted and reproduced with permission from Röttinger *et al*., 2008 and Adomako-Ankomah and Ettensohn, 2013.

regulate *vegf3* expression, however. It has also been shown that the maintenance (but not the activation) of *vegfr-10* expression in PMCs is dependent on signaling through *vegf3*, revealing a positive feedback mechanism that operates during later development (Duloquin *et al*., 2007).

Downstream Effectors of Growth Factor Signaling in the PMCs

In the sea urchin embryo, the downstream effectors of VEGF and FGF signaling pathways that regulate mesoderm morphogenesis have not been identified. In other cell types, RTKs often signal via the MAPK and PI3K pathways (Koch and Claesson-Welsh, 2012; Perona, 2006; Schlessinger, 2000; Tulin and Stathopoulos, 2010), both of which are essential for skeletogenesis in the sea urchin embryo. Inhibition of PI3K during sea urchin development blocks the elongation of skeletal rods but not PMC specification, migration, the extension of filopodia, fusion or skeletal initiation (Bradham *et al*., 2004). The MAPK pathway, on the other hand, regulates PMC specification, ingression, migration and differentiation (Fernandez-Serra *et al*., 2004; Röttinger *et al*. 2004). Therefore, the perturbation of neither of these pathways results in phenotypes identical to VEGF or FGF knockdown. However, wash-in experiments blocking the MAPK pathway using the inhibitor U0126 post-PMC specification (i.e., after the hatched blastula stage) show that these embryos form truncated skeletal elements in a manner similar to results obtained upon the inhibition of VEGF signaling at later stages of embryogenesis (Adomako-Ankomah and Ettensohn, 2013; Adomako-Ankomah and Ettensohn, unpublished observations; Fernandez-Serra *et al*., 2004; Röttinger *et al*., 2004). Many of the biomineralization genes that are sensitive to VEGF signaling receive positive transcriptional inputs from ETS1, a known target of the MAPK cascade (Fernandez-Serra *et al*., 2004; Rafiq *et al*., 2012, 2014; Röttinger *et al*. 2004). In addition, the genes *pea3*, *pax2/5/8*, and *sprouty*, which are targets of the MAPK pathway, are also downstream of FGF signaling (Röttinger *et al*., 2008). The MAPK pathway is therefore a likely candidate for a signal transduction cascade that acts downstream of VEGF and FGF signaling, and its role in PMC specification (which is independent of VEGF and FGF signaling) may be as a result of other inputs into this versatile pathway during early embryonic development.

As noted above, inhibition of VEGF signaling leads to a reduction in the number and length of filopodia extended by migrating PMCs (Adomako-Ankomah and Ettensohn, 2013). Filopodial extension is regulated by cytoskeletal dynamics (Le Clainche and Carlier, 2008; Mattila and Lappalainen, 2008; Yang and Svitkina, 2011), and the small GTP-ases, Ras, Rho, and Cdc42 are usually associated with the regulation of actin polymerization during growth factor-mediated cell migration (Lauffenburger *et al*., 1996; Montell, 1999). These proteins often function by activating the Arp2/3 complex through proteins of the WASP family (Kurosaka and Kashina, 2008; Le Clainche and Carlier, 2008; Yang and Svitkina, 2011). *cdc42, arp3*, and *wasp* transcripts are enriched in the PMCs (Rafiq *et al*., 2012), and it is likely that, as in other systems, the VEGF signaling cascade regulates the activity of these proteins by post-translational modifications.

GROWTH FACTOR-MEDIATED MESODERM CELL MIGRATION IN OTHER METAZOANS: PARALLELS AND CONTRASTS

VEGF/PDGF Signaling and Mesoderm Migration in Vertebrates

In vertebrates, VEGFs are best known for their role in regulating the proliferation, sprouting, migration, and tubulogenesis of endothelial cells during angiogenesis and have been studied intensively in the context of tumorigenesis (Lamalice *et al*., 2007; Matsumoto and Claesson-Welsh, 2001; Perona, 2006; Tammela *et al*., 2008). The genomes of mammals commonly encode three VEGF receptors, VEGFR1, VEGFR2, and VEGFR3; and five VEGF ligands, VEGFA, VEGFB, VEGFC, VEGFD, and PlGF (placental growth factor) (Koch *et al*., 2011). Homologs of VEGF receptors are also present in several non-mammalian species; work from several groups has identified one PDGFR/VEGFR homolog in *Drosophila*, one VEGFR homolog in *Xenopus*, and four VEGFR homologs in zebrafish, though the functions of these various VEGF signaling components have not been fully explored (reviewed in Stuttfeld and Ballmer-Hofer, 2009).

Aside from its important role in angiogenesis, VEGF regulates other motile cell behaviors during development. For example, in the chick, VEGF signaling mediates the migration of cranial neural crest cells (McLennan *et al*., 2010) and cells in the posterior streak (Chuai and Weijer, 2009) during early embryogenesis. The VEGF pathway also regulates blood cell migration in the *Drosophila* embryo (Cho *et al*., 2002; Stuttfeld and Ballmer-Hofer, 2009). VEGF signaling is nonetheless not widely recognized as a regulator of mesoderm cell migration during gastrulation, and, at least at present, its central role in sea urchin gastrulation is distinctive. VEGF receptors are very similar in architecture, however, to platelet-derived growth factor (PDGF) receptors, which have been shown to play a critically important role in directed mesoderm cell migration and differentiation in the early vertebrate embryo. During *Xenopus* gastrulation, PDGF-A, which is secreted by ectoderm cells and binds to fibronectin-rich fibrils that

coat the blastocoel roof, acts as a guidance cue that regulates the orientation, radial intercalation and migration of dorsal, anterior mesoderm cells, which express PDGFR-α (Ataliotis *et al*., 1995; Damm and Winklbauer, 2011; Nagel *et al*., 2004; Nakatsuji and Johnson, 1983; Smith *et al*., 2009; Winklbauer and Muller, 2011). The role of PDGF in mesodermal guidance is less well characterized in other vertebrates; however, it has been shown that migration of mesoderm cells away from the primitive streak in the gastrulating chick embryo is regulated by PDGF signaling (Chuai and Weijer, 2009; Yang *et al*., 2008). PDGF also regulates both the polarization and protrusive activity, but apparently not the directional migration, of mesendoderm cells during zebrafish gastrulation (Montero *et al*., 2003).

There are a number of striking similarities between the roles of PDGF and VEGF in gastrulation in amphibians and sea urchins, respectively. In both groups of organisms, signals from the vegetal region of the early embryo pattern the presumptive ectoderm, thereby regulating the developmental expression of the guidance cues that will later direct mesoderm migration (Ettensohn *et al*., 2000; Nagel and Winklbauer, 1999). Another common feature is that PDGF signaling in *Xenopus*, like VEGF signaling in sea urchins, is not strictly required for the protrusive activity or motility of mesoderm cells, though it is essential for cell guidance (Adomako-Ankomah and Ettensohn, 2013; Nagel *et al*., 2004). These findings strongly suggest that VEGF and PDGF play similar roles in mesodermal cell migration in sea urchins (which lack PDGF and PDGF receptor genes) and amphibians, respectively. Yet there appear to be differences in the cellular functions and downstream effectors of these signaling pathways in the two taxa. Wortmannin, an inhibitor of the PI3K pathway, disrupts mesoderm guidance in *Xenopus* (Nagel *et al*., 2004) but not in sea urchins (Bradham *et al*., 2004). In addition, while PDGF signaling is required for the multilayered mass of amphibian mesoderm cells to undergo radial intercalation and spread on the overlying ectoderm (Damm and Winklbauer, 2011), PMCs do not require VEGF signaling to spread on the blastocoel wall or to organize themselves as a monolayer following ingression (Adomako-Ankomah and Ettensohn, 2013).

FGF Signaling and Mesoderm Migration

Twenty-four FGF ligands and four FGF receptors have been identified in vertebrates, though the complement of genes in these families varies among species (Tulin and Stathopoulos, 2010). FGF signaling has been shown to play an important role in mesoderm cell migration and differentiation in several taxa. In *Drosophila* embryos, mesoderm formation is primarily regulated by the FGF pathway (Bae *et al*., 2012). FGF signaling via the ligands *pyramus* and *thisbe*, which are expressed in the ectoderm, regulates the intercalation and spreading of mesoderm cells, which express the FGF receptor *heartless*. This role for FGF in mesoderm intercalation and spreading is comparable to that of PDGF in *Xenopus* gastrulation (Winklbauer and Muller, 2011), but clearly FGF does not have a similar role in PMC movements during sea urchin gastrulation (Adomako-Ankomah and Ettensohn, 2013; Röttinger *et al*., 2008). In *Drosophila*, FGF signaling later regulates the directional migration of mesoderm cells (Kadam *et al*., 2009; McMahon *et al*., 2010; Reim *et al*., 2012; Winklbauer and Muller, 2011). The FGF pathway also plays a role in *Xenopus* gastrulation by regulating the migration and differentiation of the dorsal mesoderm (Amaya *et al*., 1993; Isaacs *et al*., 1994). In the gastrulating chick embryo, the FGF4 and FGF8 ligands are thought to function as chemoattractants and chemorepellants, respectively (Chuai *et al*., 2012; Chuai and Weijer, 2009; Lunn *et al*., 2007; Yang *et al*., 2002). FGF signaling also regulates the EMT, migration, and specification of mesoderm cells at the primitive streak of the mouse (Boulet and Capecchi, 2012; Ciruna and Rossant, 2001; Sun *et al*., 1999). In vertebrates, the effects of FGFs on mesoderm migration during gastrulation have been studied primarily at the level of cell populations, and individual cell behaviors are less well understood.

CONCLUSIONS AND FUTURE DIRECTIONS

The directional movements of mesoderm cells are among the most prominent features of gastrulation. An exciting finding from recent studies is that, in organisms as diverse as fruit flies, sea urchins, and vertebrates, growth factors play an essential role in these movements. Growth factors do not seem to be strictly required for mesoderm motility; rather, their principal function is to orient the movements of cells within a complex extracellular matrix (Adomako-Ankomah and Ettensohn, 2013; Nagel *et al*., 2004). Surprisingly, the principal ligands (and their cognate receptors) that regulate early mesoderm morphogenesis vary among organisms, revealing considerable evolutionary flexibility in this developmental program.

In sea urchins, recent studies have shown that VEGF3 and an atypical VEGF receptor, VEGF-Ig10, play key roles in PMC migration, a directional cell movement that underlies the patterning of the embryonic skeleton (Duloquin *et al*., 2007). VEGF signaling also locally regulates PMC gene expression and biomineral deposition, effects that are independent of the function of this signaling pathway in cell guidance (Adomako-Ankomah and Ettensohn, 2013). Studies on the role of FGF signaling in PMC migration and skeletal morphogenesis in sea urchins have yielded different results in two species, pointing to possible interspecies variation in the role of this pathway (Adomako-Ankomah and Ettensohn, 2013;

Röttinger *et al*., 2008). Clearly, further analysis of FGF signaling in other sea urchins is needed. In addition, to date, studies on the role of growth factors in early mesoderm morphogenesis have focused on PMC migration, and little is known concerning their possible role in the directional movements of other classes of mesoderm cells.

Growth factor signaling may not be the only mechanism by which the ectoderm regulates PMC migration and differentiation. For instance, the homeobox gene *orthopedia (otp)*, which is expressed in the oral ectoderm, influences PMC migration and biomineralization (Di Bernardo *et al*., 1999, Cavalieri *et al*. 2003). Likewise, the tripartite motif-containing gene *strim1* and paired box transcription factor *pax2/5/8* are expressed in the ectoderm overlying the VLCs. A knockdown of *pax2/5/8* leads to a significant delay in skeletogenesis and the synthesis of shortened skeletal rods, while a knockdown of *strim1* inhibits PMC positioning and skeletogenesis (Cavalieri *et al*., 2011). At present, it is not known whether these molecules act by regulating VEGF expression and/or function, or by completely independent mechanisms. Clearly, there is a possibility for multifaceted interactions between various pathways that regulate mesodermal cell migration and skeletogenesis in the sea urchin embryo.

Growth factors have diverse effects on cells, and one general challenge is to distinguish the effects of signaling pathways on cell movements from their effects on cell differentiation, to the extent that these are separable. For example, blocking growth factor-mediated signaling *in vivo* might affect gene expression and cell differentiation indirectly, by perturbing cell movements that are required for subsequent tissue interactions. Conversely, effects on cell migration might be relatively *Indirect* consequences of perturbing cell specification. *in vitro* systems using purified cells can be used to tease apart some of these effects; for example, chemotaxis chambers can be used to analyze growth factor-mediated cell guidance under well-defined conditions (e.g., Shamloo *et al*., 2012). Studies using explants or whole embryos provide a different kind of information, as they reflect the role of growth factor signaling in complex, intact tissues, but specific cellular behaviors are more difficult to analyze and indirect effects are likely. Other experimental challenges are presented by the multiplicity of potential ligands and receptors (which are typically members of small gene families) and the possibility of crosstalk among them, factors that complicate the design and interpretation of gene knockdown and chemical inhibitor studies.

To deepen our understanding of the function of growth factor signaling pathways in mesoderm morphogenesis, it will be important to elucidate the downstream effectors of these pathways. As discussed above, at least with respect to the effects of VEGF on the expression of biomineralization genes, several lines of evidence suggests that the MAPK pathway may be responsible. The VEGF effectors that control directional migration may, however, operate entirely by post-transcriptional mechanisms. As noted above, pharmacological studies have implicated the PI3K pathway in growth factor-mediated guidance of mesoderm cells in vertebrates, but this pathway does not appear to be involved in sea urchins. The regulation of cancer cell motility by growth factor signaling pathways has been studied intensively (Balkwill, 2012; Fuxe and Karlsson, 2012; Hoshino *et al*., 2013), as have the intracellular pathways that underlie the chemotactic behaviors of *Dictyostelium* amoebae and mammalian leukocytes (Bagorda and Parent, 2008; Insall, 2013; Jin, 2013; Weiger and Parent, 2012). The extent to which these same signaling pathways operate in early embryonic cells that are responding to growth factors is an intriguing and open question.

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