Identification of Three Required Positive Cis-Regulated Inputs of the Sea Urchin Pigment Cell Gene Polyketide Synthase 1

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IDENTIFICATION OF THREE REQUIRED POSITIVE CIS-REGULATORY INPUTS OF THE SEA URCHIN PIGMENT CELL GENE POLYKETIDE SYNTHASE 1

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ABSTRACT

Sea urchin pigment cells are single cells of mesodermal origin embedded in the aboral ectoderm. Strongylocentrotus purpuratus polyketide synthase 1 (Sp-PKS1) is required for the biosynthesis of the echinochrome pigment. Evidence suggests that pigment cells are immune cells. In order to reconstruct the gene regulatory network of pigment cells a bottom-up approach combined with comparative genomics has been used in this study. We compared the cis-regulatory regions of five pigment cell genes, Sp-Pks1, flavin monooxygenase 1, 2, and 3 (Sp-Fmo) and sulfotransferase (Sp-Sult), across three different species, Strongylocentrotus purpuratus, Mesocentrotus franciscanus, and Strongylocentrotus fragilis. The computational tool used was multiple expectation maximization motif elicitation analysis. Thirty cis-regulatory motif candidates were identified, three of which were considered for further analysis. The functionality of these motifs was tested by injecting embryos with a -2KbPks-Gfp DNA construct having one of the three motifs mutagenized. All three motifs resulted to be functional cis-regulatory sequences. Specifically, they contained DNA-binding sites for transcriptional activators of Sp-Pks1.

Keywords: polyketide synthase, sea urchin, pigment cells, transcriptional cis-regulation, differentiation, gene regulatory networks

INTRODUCTION

The broad objectives of this study were to gain further knowledge on the gene regulatory networks (GRNs) that regulate cell-type specification and differentiation during embryo development. The development of sea urchin embryo pigment cells was used as the research model. To uncover the structure of the GRN regulating the
specification and differentiation processes of pigment cells a bottom-up approach was used, starting with the analysis of the cis-regulatory sequence of a differentiation gene (Calestani and Rogers 2010). Specifically, through the integration of computational and experimental approaches, we aimed to characterize the cis-regulatory architecture of Sp-Pks1, a gene required for the biosynthesis of the larval pigment echinochrome (Calestani et al. 2003). This work will help to uncover the gene regulatory connections between cell specification and the terminal process of cell differentiation, which are poorly understood.

The pigment cells of the sea urchin larvae are of mesodermal origin, specifically they develop from the non-skeletogenic mesoderm cells (NSM; Cameron et al. 1991; Ruffins and Ettensohn 1996). Pigment cells are the first out of the four NSM types to be specified. Pigment cell precursors migrate into the blastocoel during the early gastrula stage and embed in the ectoderm by the pluteus stage (Gustafson and Wolpert 1967; Gibson and Burke 1985; Kominami et al. 2001). The specification process of pigment cells is triggered by a Delta/Notch (D/N) signaling pathway (Sherwood and McClay 1999; Sweet et al. 1999, 2002; McClay et al. 2000; Oliveri et al. 2002; Croce and McClay 2010; Materna and Davidson 2012). Another factor required for the specification of pigment cells and other NSM cells is the membrane protein Numb, which acts synergistically with Notch (Range et al. 2008). At the 7th cleavage stage the D/N signaling directly activates the transcription factor gcm, which is expressed in a ring of Veg2 cells and is essential for pigment cell development (Ransick et al. 2002; Ransick and Davidson 2006). By the end of the mesenchyme blastula stage, pigment cell precursors are restricted to the aboral NSM by a regulatory process involving Nodal signaling from the oral ectoderm and repression of aboral NSM genes by oral NSM genes (Duboc et al. 2010; Materna et al. 2013). Vice versa, there is also a repression of oral NSM genes by aboral NSM genes (Solek et al. 2013; Materna et al. 2013). Studies have shown that other cell signaling pathways are involved in the NSM development; Hedgehog signaling from the endoderm is required to develop the normal number of pigment cells (Walton et al. 2009); later in development Eph/Ephrin signaling from the aboral and ciliary band ectoderm is required for the migration of pigment cells, insertion into the ectoderm, and acquisition of the stellar shape typical of pigment cells (Krupke et al. 2016). Moreover, a transcription factor belonging to the E-proteins family, SpE-Alt, is required for the correct timing of pigment cell precursor ingestion into the blastocoel (Schrankel et al. 2016). SpE-Alt does not seem to be a regulator of Sp-Pks1 since Sp-Pks1 is still expressed in SpE-Alt knock-down embryos (Schrankel et al. 2016). The regulatory relationships of SpE-Alt with the cell signaling described above have not been characterized.

Experimental evidence suggests that pigment cells have a role in the immune system of sea urchin larvae (Service and Wardlaw 1984; Gibson and Burke 1987; Hibino et al. 2006; Castoe et al. 2007; Kiselev et al. 2013; Solek et al. 2013; Ho et al. 2016; Schrankel et al. 2016; Buckley et al. 2017). Pigment cells synthesize the echinochrome A pigment, which has been shown to have antimicrobial properties and produce hydrogen peroxide when oxidized (Perry and Epel 1981; Service and Wardlaw 1984; Brasseur et al. 2017). Pigment cells migrate to the gut when larvae are in seawater containing the bacterium Vibrio diazothrophicus (Ho et al. 2016). This immune response is mediated by members of the IL17 cytokine family (Buckley et al. 2017). Moreover, pigment cells interact with other immune cells of the larva such as globular, amoeboid, and filopodial
cells, especially in the proximity of bacteria and in response to wounding (Ho et al. 2016). Sp-Pks1 is required for echinochrome A biosynthesis and is exclusively expressed in pigment cells and their precursors starting between 15 and 18 h in S. purpuratus (Calestani et al. 2003). At the blastula stage, Sp-Pks1 is expressed in a ring of about 20 NSM precursors surrounding the skeletogenic mesoderm (SM) and by the end of the mesenchyme blastula stage is restricted to the aboral NSM (Calestani et al. 2003). At the gastrula stage, Sp-Pks1 expression is detected in cells migrating into the blastocoel (Calestani et al. 2003). Gene expression is maintained throughout the pluteus stage in cells embedded in the aboral ectoderm, coincident with the distribution of pigment cells (Calestani et al. 2003; Gibson and Burke 1985; Cameron et al. 1991; Ruffins and Ettensohn 1996).

With an integration of classical promoter deletions and comparative genomics approaches a previous study led to the identification of 500 bp (between -1.5 and -1Kb) that are required for the correct spatial and temporal expression of Sp-Pks1 (Calestani and Rogers 2010). Within this cis-regulatory region, our study determined that the transcription factors GATAE, GCM, and KRL-LIKE, known to be expressed in pigment cell precursors, are direct positive regulators of Sp-Pks1 (Calestani and Rogers 2010).

In this work, in order to predict DNA-binding sites for additional regulators of Sp-Pks1, we performed a comparative genomics analysis by using multiple expectation maximization motif elicitation (MEME; Bailey and Elkan 1994). Putative cis-regulatory sequences from three sea urchin species, Strongylocentrotus purpuratus (SP), Mesocentrotus franciscanus (MF), and Strongylocentrotus fragilis (SF) were used. To increase the predictive power of our comparative genomics analysis we included other pigment cell differentiation genes co-regulated by GCM and GATAE, hence they are likely to belong to the same differentiation gene battery, flavin monooxygenase (Sp-Fmo) 1, 2, and 3, and sulfotransferase (Sp-Sult; Calestani et al. 2003).

MATERIAL & METHODS

Embryo Culture
Gametes were obtained from adult S. purpuratus by injection with 0.5 M KCl. The eggs were fertilized in filtered seawater containing penicillin at 20 U/ml and streptomycin at 50 μg/ml, and then incubated at 15 °C for the duration of development.

MEME Computational Analysis
Cis-regulatory sequences of five coexpressed differentiation genes (Sp-Pks1, Sp-Fmo 1, 2, 3, and Sp-Sult) from three sea urchin species (S. purpuratus, M. franciscanus, and S. fragilis) were aligned using Genboree Bioinformatics (www.genboree.org). The aligned sequences corresponded to the first intron, which includes a Sp-GCM DNA-binding site (Calestani and Rogers 2010). The aligned sequences were compared using multiple expectation maximization motif elicitation (MEME) analysis (Bailey and Elkan 1994). The MEME motifs 2, 13, and 30 were mapped on the -2Kb region and considered for further analysis.

Production of GFP Reporter Constructs
The -2Kb-Gfp control construct was previously prepared (Calestani and Rogers 2010). The -2Kb with either the mutated motifs 2, 13, or 30 were produced by de novo double-stranded oligonucleotide synthesis (Gen-Script USA Inc.). The de novo double-
stranded oligonucleotide had SacI and MluI sites at the 5’ and 3’ ends, respectively. Motif 2 was mutated from ACCACGCACTA to GAAGTATGAC. Motif 13 was mutated from TGGCACTACA to CAATGTCGTG. Motif 30 was mutated from GGGATTTCC to ATAGCGCAA. The cis-regulatory DNA mutagenized fragments were cloned into the EpGFPII reporter vector between SacI and MluI (Arnone et al. 1997). Each construct sequence was verified by restriction digestion and sequencing. Reporter constructs were linearized by SacI digestion and purified (QIAquick PCR purification kit, Qiagen) prior to embryo injection.

**Embryo Injections**

Embryo injection was performed as previously described (McMahon et al. 1985; Arnone et al. 2004). Injection solutions were prepared at a concentration of 1000 molecules/pl of linearized plasmid in 0.12 M KCl with the addition of 5× molar excess of restriction digested sea urchin genomic DNA (carrier DNA). Approximately 2 pl of injection solution was delivered to each embryo. Embryos injected with each GFP reporter construct were observed at the pluteus stage using fluorescence microscopy (Olympus BX60).

**RESULTS**

In order to identify additional cis-regulatory sequences in the Sp-Pks1 promoter we used a comparative genomics approach. Specifically, we compared cis-regulatory regions of five coexpressed genes that are coregulated by GCM and GATAE, and hence are likely to belong to the same differentiation gene battery, Sp-Pks1, Sp-Fmo 1, 2, and 3, and Sp-Sult (Davidson 2006; Calestani and Rogers 2010). These five genes were compared in three different species of sea urchins, *S. purpuratus* (SP), *M. franciscanus* (MF), and *S. fragilis* (SF; Figure 1). The MEME computational analysis identified thirty conserved motifs (Table I) ranging from 11 to 14 bp.

We experimentally tested three motifs because they mapped close to the validated GCM, GATAE, and KRL-LIKE DNA-binding sites: motif 2, 13, and 30 (Table I; Figure 2). Motif 2 (*pksm2*) is localized at -1,227 bp of Sp-Pks1, 38 bp upstream of a GCM binding site, 153 bp downstream of a GATAE site, and 79 bp upstream of a KRL-LIKE site. Motif 13 (*pksm13*) is localized at -1,340 bp of Sp-Pks1, 151 bp upstream of a GCM binding site, 40 bp downstream of a GATAE site, and 192 bp upstream of a KRL-LIKE site. Motif 30 (*pksm30*) is localized at -561 bp of Sp-Pks1, 610 bp downstream of a GCM binding site, 819 bp downstream of a GATAE site, and 568 bp downstream of a KRL-LIKE site.

The function of each motif was tested by mutagenesis of the Sp-Pks1 -2Kb region fused to the reporter *gfp*. All three mutagenized constructs showed a drastically reduced expression of *gfp*: while 44.83% of the embryos injected with the control construct showed expression in pigment cells only, 0%, 1.56%, and 2.8% of the embryos injected respectively with the *pksm2*, *pksm30*, and *pksm13* mutagenized constructs showed *gfp* expression in pigment cells only (Table II; Figure 3). These results indicate that the predicted *pksm2*, *pksm13*, and *pksm30* motifs contain DNA-binding sites for positive transcriptional regulators of *Sp-Pks1*. 
Motif 2 in BLOCKS format

<table>
<thead>
<tr>
<th>Motif No.</th>
<th>Number of Aligned Sequences</th>
<th>Consensus Sequence (IUPAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>HYCCCCSWSCCCCC</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>GSYGKASYMRGGGC</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>CCMMKTTCGCCGGTC</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>CDACHYTTSCCTCC</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>AYCCGTGTGCTCGG</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>TRWWTSYACTACA</td>
</tr>
<tr>
<td>13</td>
<td>15</td>
<td>TSYDSGTACCG</td>
</tr>
<tr>
<td>15</td>
<td>14</td>
<td>GMYRSRACSAACGGA</td>
</tr>
<tr>
<td>16</td>
<td>9</td>
<td>TSGMTGACGTGTTA</td>
</tr>
<tr>
<td>17</td>
<td>12</td>
<td>AKRTRRARAGA</td>
</tr>
<tr>
<td>19</td>
<td>6</td>
<td>TBKADGYGATATT</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>GYKSKRTGCTCGGT</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>GRKCTWCGCACCA</td>
</tr>
<tr>
<td>23</td>
<td>5</td>
<td>CATTCASGAGGAA</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>GTTYYCACCACCC</td>
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<td>18</td>
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<td>26</td>
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<td>28</td>
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<tr>
<td>29</td>
<td>6</td>
<td>GKCSSGCGGCC</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>CGCTGGGGCGATGCC</td>
</tr>
</tbody>
</table>

Motif 13 in BLOCKS format

Motif 30 in BLOCKS format

**Table 1.** Comparative genomics analysis to identify conserved cis-regulatory motifs. List of the consensus motifs identified by the MEME analysis. The motifs that were tested in vivo by mutagenesis are highlighted in bold.

**Figure 1.** MEME putative cis-regulatory sequence alignment of five coexpressed differentiation genes (Sp-Pks1, Sp-Fmo 1, 2, 3, and Sp-Sult) from three sea urchin species (SP, MF, and SF). Data shown are for the three motifs tested by mutagenesis.
**Figure 2.** Mutagenesis of putative cis-regulatory motifs within the -2Kb of Sp-Pks1. Single motifs were mutagenized (indicated with *) and DNA constructs were injected into fertilized eggs. GFP expression was observed at the pluteus stage. GFP expression present in pigment cells is indicated by a green box. The GFP white box indicates no expression of the construct. Color-coded are the cis-regulatory motifs considered in this study, *pksm2* in black, *pksm13* in brown, and *pksm30* in pink. Previously functionally validated DNA-binding sites for GCM (red), KRL-LIKE (green), and GATAE (blue) are also indicated (Calestani and Rogers 2010).

**Table II.** Cis-regulatory activity of the Sp-Pks1-Gfp reporter constructs. Data reported were obtained from pluteus stage embryos. Each replicate experiment was performed on embryos produced from a different set of parents. Embryos were considered GFP positive if they had more than two fluorescent cells; GFP expression in only one or two cells was considered background due to the position of integration in the genome. The total number of scored embryos was obtained by combining all the replicate experiments.

<table>
<thead>
<tr>
<th>Construct</th>
<th>% GFP Positive</th>
<th>% GFP Positive</th>
<th>% GFP Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(# of Replicates)</td>
<td>(# of Scored Embryos)</td>
<td>Pigment Cells Only</td>
</tr>
<tr>
<td>Control -2Kb (3)</td>
<td>48.28 (145)</td>
<td>44.83 (145)</td>
<td>3.45 (145)</td>
</tr>
<tr>
<td>pksm2 mutagenized (3)</td>
<td>0.70 (145)</td>
<td>0.00 (145)</td>
<td>0.70 (145)</td>
</tr>
<tr>
<td>pksm13 mutagenized (3)</td>
<td>7.70 (143)</td>
<td>2.80 (143)</td>
<td>4.9 (143)</td>
</tr>
<tr>
<td>pksm30 mutagenized (3)</td>
<td>2.34 (128)</td>
<td>1.56 (128)</td>
<td>0.78 (128)</td>
</tr>
</tbody>
</table>
Figure 3. Mutagenesis of putative cis-regulatory motifs 2, 13, and 30. (A-D) Overlay of DIC and fluorescence images of embryos injected with the -2Kb-Gfp control construct (A), the pksm2-Gfp mutagenized construct (B), the pksm30-Gfp mutagenized construct (C), and the pksm13-Gfp mutagenized construct (D). GFP expression is observed in the control but not in the mutagenized construct injected embryos.

DISCUSSION

This study brings new insights into the GRN architecture required for NSM development and adds to the general knowledge of the structure and function of GRNs during the differentiation process. The use of comparative genomics involving the comparison of putative cis-regulatory sequences of five coexpressed and coregulated genes in three different sea urchin species was effective. Thirty conserved DNA motifs were identified (Table I). Three DNA motifs were tested for functionality by mutagenesis and all three include DNA-binding sites for positive regulators of Sp-Pks1 (Table II; Figure 3). The pksm2 and pksm13 motifs mapped very close to previously validated DNA-binding sites for GCM, GATAE, and KRL-LIKE, in the range of 38–192 bp, which suggests that they belong to the same cis-regulatory module (Table I; Calestani and Rogers 2010; Davidson 2006). The pksm13 motif is relatively close to the three previously validated DNA-binding sites but it might belong to an adjacent cis-regulatory module.
Previous studies demonstrated that the GRN for pigment cell development downstream of the D/N signaling is relatively shallow (Figure 4; Ransick et al. 2002; Calestani and Rogers 2010; Materna et al. 2013). The D/N signaling directly activates Sp-Gcm, which directly activates Sp-Pks1. This study, in combination with the study of Calestani and Rogers (2010), suggests that the expression of the Sp-Pks1 differentiation gene is locked-down by multiple positive transcriptional regulators. Two of these positive cis-regulatory inputs are acting downstream of the 7th-9th cleavage D/N signaling through GCM and GATAE and one is D/N independent acting through KRL-LIKE (Ransick et al. 2002; Ransick and Davidson 2006; Calestani and Rogers 2010). It is not known if the rest of the direct regulators identified are D/N dependent or independent. It is possible that at least some are parallel positive regulatory inputs into differentiation genes as was observed for the SM development (Amore and Davidson 2006; Oliveri et al. 2008; Sun and Ettensohn 2014). Each positive transcriptional regulator is not redundant but is required for Sp-Pks1 expression, resulting in an “all or nothing” transcriptional output. The “all or nothing” transcriptional output could be a mechanism that serves as a developmental check-point. Specifically, the cis-regulatory region of a differentiation gene, such as Sp-Pks1, might integrate the inputs of multiple genetic pathways that regulate earlier specification processes, and possibly coordinate pigment cell differentiation with the development of other embryonic territories.

Moreover, pigment cell specification and differentiation become independent from the D/N signaling through at least two positive feedback loops: one is produced by the activation of Sp-Gcm by itself, a second one is a triple positive feedback loop involving Sp-Gcm, Sp-GataE, and Sp-Six1/2 (Figure 4; Ransick and Davidson 2006, 2012). Mathematical models of GRNs suggest that the presence of multiple interconnected positive feedback loops is a more effective mechanism for locking down the regulatory state of cells as opposed to single positive feedback loops (Hornung and Barkai 2008; Ben-Tabou de-Leon 2010; Ben-Tabou de-Leon 2016). Further robustness of the pigment cell GRN is provided by a feed forward loop involving Sp-Gcm, Sp-GataE, and Sp-Pks1 (Figure 4; Ransick and Davidson 2006; Calestani and Rogers 2010; Materna et al. 2013).

Interestingly, as previously observed (Calestani and Rogers 2010), none of the deletion constructs produced a significant amount of ectopic expression of GFP. This suggests that direct repressors might not be required to restrict Sp-Pks1 expression to pigment cells. Instead, previous studies have shown that the earlier process of specification sets the boundaries of the differentiation gene batteries’ domain of expression (Levine and Davidson 2005; Oliveri et al. 2008; Solek et al. 2013; Materna et al. 2013). For example, alx1 represses gcm in the SM at the blastula stage (Oliveri et al. 2008), gcm represses the blastocoelar cell fate in pigment cell precursors, and not represses gcm in the oral NSM (Solek et al. 2013; Materna et al. 2013). Indeed, gcm seems to be a key regulator of pigment cell development. In fact, its ectopic expression in SM cells is sufficient to develop a pigment cell’s fate (Damle and Davidson 2012).

In conclusion, the transcriptional regulation of the pigment cell differentiation gene Sp-Pks1 involves at least six positive cis-regulatory inputs. The GRN architecture upstream of Sp-Pks1 is shallow and it includes multiple interconnected positive feedback loops, which contribute to lock-down its expression.
Figure 4. Graphic representation of the *S. purpuratus* pigment cell GRN upstream of *Sp-Pks1*. Arrows indicate positive regulatory inputs. Different embryonic territories are indicated and color-coded (SM and aboral NSM). The three positive regulatory inputs discovered in this study are indicated by red arrows, while the ones reported by previous studies are indicated in black (Ransick et al. 2002; Ransick and Davidson 2006; Calestani and Rogers 2010; Ransick and Davidson 2012; Materna et al. 2013).

REFERENCES


