Functional specialization of cellulose synthase genes of prokaryotic origin in chordate larvaceans

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SUMMARY
Extracellular matrices play important, but poorly investigated, roles in morphogenesis. Extracellular cellulose is central to regulation of pattern formation in plants, but among metazoans only tunicates are capable of cellulose biosynthesis. Cellulose synthase (CesA) gene products are present in filter-feeding structures of all tunicates and also regulate metamorphosis in the ascidian Ciona. Ciona CesA is proposed to have been acquired by lateral gene transfer from a prokaryote. We identified two CesA genes in the sister-class larvacean Oikopleura dioica. Each has a mosaic structure of a glycosyltransferase 2 domain upstream of a glycosyl hydrolase family 6 cellulose-like domain, a signature thus far unique to tunicates. Spatial-temporal expression analysis revealed that Od-CesA1 produces long cellulose fibrils along the larval tail, whereas Od-CesA2 is responsible for the cellulose scaffold of the post-metamorphic filter-feeding house. Knockdown of Od-CesA1 inhibited cellulose production in the extracellular matrix of the larval tail. Notochord cells either failed to align or were misaligned, the tail did not elongate properly and tailbud embryos also exhibited a failure to hatch. Knockdown of Od-CesA2 did not elicit any of these phenotypes and instead caused a mild delay in pre-house formation. Phylogenetic analyses including Od-CesAs indicate that a single lateral gene transfer event from a prokaryote at the base of the lineage conferred biosynthetic capacity in all tunicates. Ascidians possess one CesA gene, whereas duplicated larvacean genes have evolved distinct temporal and functional specializations. Extracellular cellulose microfibers produced by the pre-metamorphic Od-CesA1 duplicate have a role in notochord and tail morphogenesis.

KEY WORDS: Extracellular matrix, Notochord, Tunicate, Lateral gene transfer, Appendicularian, Metamorphosis, Oikopleura

INTRODUCTION
Cellulose is the most abundant natural product in the biosphere with a variety of functional roles. Despite this abundance, the capacity to synthesize cellulose is restricted to relatively few phyla. Among prokaryotes, soil bacteria of the family Rhizobiaceae (Agrobacterium tumefaciens and Rhizobium spp) use cellulose in anchoring to host plant tissues during infection (Matthysse 1983; Smith et al., 1992). In Acetobacter xilinum, cellulose fibrils maintain bacterial cells in an aerobic environment in liquid and protect the cells from UV radiation (Williams and Cannon, 1989). Within the plant kingdom, cellulose plays a key role in structural support and the oriented deposition of cellulose microfibrils is crucial to patterning through anisotropic growth during development (Smith and Oppenheimer, 2005). The social amoeba, Dictyostelium, requires cellulose for stalk and spore formation (Blanton et al., 2000), and cellulose synthesis is also present in some fungi, although its function remains unclear (Stone, 2005). Among metazoans, cellulose biosynthesis is found only in the tunicate subphylum (Brown, 1999).

Cellulose is produced by multimeric cellulose synthase terminal complexes (TCs) inserted in the plasma membrane (Brown, 1996). In plants, the TCs are in the form of a rosette that moves through the cell membrane as cellulose fibrils are extruded (Paredez et al., 2006). In bacteria (Brown et al., 1976), various algae (Brown and Montezinos, 1976; Itoh, 1990) and tunicate ascidians (Kimura and Itoh, 1996), the TCs are disposed in a stationary, linear organization. The tunicates comprise larvaceans, ascidians and thaliaceans. Post-metamorphic stages of the latter two groups incorporate cellulose into a tough integument, the tunic, which surrounds the animal and forms in part the filter-feeding apparatus (Hirose et al., 1999; Kimura and Itoh, 2004). Pre-metamorphic, non-feeding, larval ascidians are also surrounded by a tunic composed in part of cellulose, and in addition to its protective function, cellulose has a role in the control of Ciona metamorphosis. Insertional mutagenesis in the promoter of the C. intestinalis cellulose synthase (CesA) gene caused a drastic reduction of cellulose in the larval tunic resulting in a swimming juvenile (sj) mutant, where the order of metamorphic events was disrupted (Sasakura et al., 2005). Sj larvae initiated metamorphosis in the trunk without prior tail resorption. Further analysis of metamorphic pathways in C. intestinalis (Nakayama-Ishimura et al., 2009) revealed that cellulose represses initiation of papillae retraction and body axis rotation until larval settlement has occurred.

Larvaceans do not live inside a rigid tunic, but instead repetitively secrete and discard a complex, gelatinous filter-feeding house. The house comprises cellulose (Kimura et al., 2001) and on the order of at least 30 proteins (Spada et al., 2001; Thompson et al., 2001), and is secreted by a polyploid oikoplastic epithelium (Ganot and Thompson, 2002). Of 11 characterized house proteins, none show significant similarity with proteins in the sequenced ascidian genomes of C. intestinalis or C. savignyi, or in a broader sense, with any proteins in public databases, suggesting that these innovations are specific to the larvacean

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lineage. Another important distinguishing feature of larvaceans is that they are the only tunicates to retain the chordate tail after metamorphosis. Traditionally, this has been considered neotenic, with both the common chordate and common tunicate ancestors viewed as having a free-swimming larval stage and sessile adult stage (Garstang, 1928; Nielsen, 1999; Lucalli, 2005; Stuch, 2008a). Sequence analysis of rRNA genes (Wada and Satoh, 1994; Wada, 1998) and more extensive molecular phylogenomic datasets (Delsuc et al., 2006; Delsuc et al., 2008) support an opposing view that the primitive life cycle in chordates was entirely free-living, as in extant larvaceans, and place the larvaceans basal to ascidians and thaliaceans in the tunicate lineage.

To date, tunicate CesAs have only been identified in two ascidians, *Ciona savignyi* and *C. intestinalis*, each with one CesA gene encoding a protein with mosaic structure comprising an N-terminal cellulose synthase core domain and C-terminal cellulase-like domain. This mosaic domain organization has not been found in plant or bacterial CesAs. Phylogenetic analyses of ascidian CesAs suggested that the genes might have been acquired from a prokaryote by horizontal gene transfer prior to the split of *C. savignyi* and *C. intestinalis* (Matthysse et al., 2004; Nakashima et al., 2004). Matthysse et al. concluded that information on larvacean cellulose synthases would be essential to resolving whether a single horizontal gene transfer event was responsible for acquisition of cellulose synthases would be essential to resolving whether a single horizontal gene transfer event was responsible for acquisition of cellulose synthase core domain and C-terminal cellulase-domain and C-terminal cellulase-domain support the hypothesis of a single lateral gene transfer event from a prokaryote at the base of the tunicate lineage. Spatial-temporal expression and knockdown experiments demonstrate that the two *O. dioica* CesA genes have distinct functional roles, one acting in the pre-metamorphic, and the second in the post-metamorphic, phase of the life cycle.

**MATERIALS AND METHODS**

**Animal collection and culture**

*Oikopleura dioica* were maintained in culture at 15°C (Bouquet et al., 2009). For in vitro fertilizations, females were collected in watch glasses, washed with artificial seawater (Red Sea, final salinity 30.4-30.5 g/l) and left to spawn. Sperm from 3-5 males was checked for viability and used for fertilization. Embryos were left to develop at room temperature.

**Cellulose synthase cloning**

Putative *O. dioica* cellulose synthase genes were identified using the amino acid sequence of the *C. intestinalis* cellulose synthase (BAD10864) as a query in Tblastn against the *O. dioica* genomic shotgun dataset. Total RNA was isolated from 4 hours post-fertilization (hpf) and (BAD10864) as a query in Tblastn against the *O. dioica* (10μl) and 4-hpf embryos for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds were conducted, with a final extension for 5 minutes at 72°C. RT-PCR amplification of each was run to 40 cycles of amplification. In all qRT-PCRs, 18S rRNA was used as a normalization control.

**Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNAs were isolated from each stage using RNeasy (Qiagen). For RT-PCR, 1 μg of total RNA was subjected to RT using M-MLV RT (Invitrogen). Real-time PCRs (DNA Engine Opticon 2; MJ Research Waltham, MA, USA) were run using cDNA templates synthesized from an equivalent of 10 ng total RNA, 10 μl of Quantitect qPCR 2X Master Mix (Qiagen), 0.2 μM primers (see Table S1 in the supplementary material) in a total volume of 20 μl. After initial denaturation for 15 minutes at 95°C, 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds were conducted, with a final extension for 5 minutes at 72°C. RT negative controls were run to 40 cycles of amplification. In all qRT-PCRs, 18S rRNA was used as a normalization control.

**Whole mount in situ hybridization**

Fragments of 470, 466 and 861 bp for the *Od-CesA1*, *Od-CesA2* and *Od-Brachury* (AF204208) genes, respectively, were PCR-amplified using specific primers (see Table S1 in the supplementary material) and cDNA libraries generated from 4 hpf (*Od-CesA1* and *Od-Brachury*) and day 4 (*Od-CesA2*) animals. PCR products were cloned into pCRII-TOPO vector (Invitrogen). Sense and antisense RNA probes were synthesized by in vitro transcription of linearized plasmids with either T7 (Promega) or SP6 (Takara Bio) RNA polymerase in the presence of digoxigenin-labeled UTP (digoxigenin RNA Labeling Mix; Roche Molecular Biochemicals). Embryos at 5 hpf and day 4 animals were fixed in 4% paraformaldehyde, 0.1 M MOPS (pH 7.5) and 0.5 M NaCl at 4°C overnight, rinsed with 0.1 M MOPS (pH 7.5) and 0.5 M NaCl and then transferred to fresh 70% ethanol for storage at ~20°C. Prior to hybridization, embryos were rehydrated in 50 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100. Hybridization and detection of probes were performed as described by Seo et al. (Seo et al., 2004).

**Confocal analysis of cellulose microfibrils**

Embryos and animals were fixed in 4% paraformaldehyde, 0.1% saponin, 0.1 M MOPS (pH 7.5) and 0.5 M NaCl at 4°C overnight. Fixed animals were rinsed with PBS/0.1% saponin/0.1% Tween 20 (S/PBS-T) and then blocked with 3% BSA+S/PBS-T at 4°C overnight. Cellulose content was probed by incubation in 1% BSA+S/PBS-T containing rCBD-Protein L (10 μg/ml; Fluka) and mouse IgG (10 μg/ml; Sigma) at 4°C overnight, followed by incubation in Rhodamine-Red-X-conjugated goat anti-mouse IgG (1:200 in 1% BSA+S/PBS-T) at 4°C overnight. The rCBD-Protein L reagent can recognize other polysaccharides, notably, chitin. We therefore also performed specific staining for chitin using a chitin-binding probe (New England BioLabs that recognized other structures distinct from those that we determined as cellulose with the rCBD-Protein L reagent. Finally, digestion with cellulase specifically eliminated the cellulose staining detected by rCBD-Protein L. To visualize cell shapes, cortical F-actin was stained with Alexa Fluor 488 Phalloidin (10 units/ml; Molecular Probes). Nuclei were counterstained with 1 μM To-Pro-3 iodide (Molecular Probes). Specimens were mounted in Vectashield (Vector Laboratories) and analyzed at 20°C with a Leica TCS laser scanning confocal microscope (Plan Apo 40× oil immersion 1.25 NA objective) using Leica v2.5 and Zeiss LSM 5 software.

**Morpholino knockdown experiments**

Nucleotide sequences of the morpholino oligonucleotides (MOs) are given in Table S1 in the supplementary material. For *Od-CesA2* knockdown, a mixture of two MOs was used. The concentration of MO in the microinjection solution was 0.75 mM. MOs were injected into fertilized eggs before the first cleavage. In vitro fertilization of eggs and method of injection were as previously described (Clarke et al., 2007), except that sperm was obtained from pools of 10 males in 50 mm diameter petri dishes maintained on ice, and siliconized quartz capillaries (Sutter, QF100-70-10) pulled on a Sutter P2000 laser puller were used to prepare injection needles in place of aluminosilicate capillaries. The volume of injected solution was ~4 pl. To detect the splice modification of *Od-CesA1* and *Od-CesA2* genes, total RNA was extracted from the 1- and 4-hpf embryos for *Od-CesA1* and 10-hpf embryos for *Od-CesA2* by using Lysis II Buffer in the Cells-to-cDNA Kit (Ambion) according to...
manufacturer’s instructions. To generate cDNA, 10 μl of cell lysate was subjected to RT using M-MLV RT (Invitrogen). Nested PCR was performed using Dynazyme (Finzymes) and specific primers (see Table S1 in the supplementary material).

**RESULTS**

**Duplicated CesA genes in *O. dioica***

Two loci homologous to *C. intestinalis CesA* (Ci-CesA) were identified in the *O. dioica* genomic database. To clone both genes (Od-CesA1 and Od-CesA2), primers designed from these regions were used in a series of PCRs with cDNA from pools of 4-hpf embryos for Od-CesA1 and day-4 animals for Od-CesA2. As a result, a 9-exon Od-CesA1 gene and a 10-exon Od-CesA2 gene, coding 1143 and 1252 amino acid residues, respectively, were identified (Fig. 1). Both encoded proteins had a mosaic structure with a cytoplasmic cellulose synthase core region featuring a glycosyltransferase 2 (GT-2) domain and a C-terminal extracellular glycosyl hydrolase family 6 (GH-6) cellulose-like domain (Fig. 1C). This organization was similar to Ci-CesA and C. savignyi CesA (Cs-CesA), and the *O. dioica* sequences had 51-57% amino acid similarity to the *Ciona* enzymes. This mosaic structure is not found in any other CesAs, and at present is unique to tunicate CesAs. In bacterial and fungal GH-6 cellulases, two aspartic acid residues are implicated in catalytic function (Rouviere et al., 1999), with the most C-terminal residue demonstrated to be crucial (Koivula et al., 2002). Alignment of urochordate GH-6 domains with bacterial and fungal domains (see Fig. S1 in the supplementary material) reveals both of these residues to be modified in *Ciona* CesAs and Od-CesA1, whereas Od-CesA2 lacks the most C-terminal one. This raises questions as to the functional activity of the urochordate GH-6 domains. Cellulase activity is essential to cellulose biosynthesis in both prokaroyotes and eukaryotes, although its precise role is unclear (Delmer, 1999). BLAST searches of the *O. dioica* genomic database revealed several putative GHF-9 cellulases, a family known in plants, bacteria, fungi and animals, including *Ciona* (Davidson and Blaxter, 2005). Among these, Korrigan is essential for cell-wall biosynthesis in Arabidopsis (Nicol et al., 1998). It is probable that some urochordate GHF-9 cellulases are active in cellulose biosynthesis as opposed to only being involved in digestion of dietary cellulose.

Bayesian phylogenetic analysis revealed higher phylogenetic affinity of tunicate GT-2 domains with corresponding bacterial domains than those in plants (Fig. 2A). GH-6 family proteins are found only in bacteria and fungi and are absent in plants and all animals except tunicates. Tunicate CesA/GH-6 domains showed an affinity intermediate to bacterial and fungal cellulases (Fig. 2B). It has been proposed that ascidians acquired the CesA gene by horizontal transfer from bacteria (Matthysse et al., 2004; Nakashima et al., 2004; Sasakura et al., 2005). Based on phylogenetic analyses, placing larvae nearer the base of the urochordate lineage than ascidians and thaliaceans (Wada and Satoh, 1994; Delsuc et al., 2006), the findings here indicate that horizontal transfer of the CesA gene occurred in the urochordate ancestor prior to divergence of the sister classes.

We identified two CesA paralogs in *O. dioica*, whereas *C. intestinalis* and *C. savignyi* each possess only one CesA gene. There are no conserved splice sites in the *O. dioica* CesA paralogs. This contrasts with the *Ciona CesA* homologs that share 14 conserved splice sites, including one that is conserved with Od-CesA2. In Table 1, amino acid similarities among the tunicate CesA proteins are shown for the whole sequence and the GT-2 and GH-6 domains. Within the urochordate lineage, the GH-6 cellulase domains are evolving more rapidly than the GT-2 glycosyl transferase domains. Overall, the Od-CesA1 and Od-CesA2 proteins exhibit slightly higher similarity to each other than either does to the individual *Ciona* CesA proteins. However, Bayesian trees using the GT-2 domains (Fig. 2A) or the GH-6 domains (Fig. 2B), yield different topologies. The GT-2 domain analysis suggests that Od-CesA2 has greater affinity to the ascidian CesAs than does Od-CesA1. The GH-6 domain analysis suggests duplication of an ancestral CesA gene in the larvalplosis.

Table 1. Pairwise identities among tunicate CesAs

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<tr>
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<th>Od CesA1</th>
<th>Od CesA2</th>
<th>Ci CesA</th>
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<tr>
<td>Od CesA1</td>
<td>62.8</td>
<td>55.0</td>
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<td>Od CesA2</td>
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<td>56.7</td>
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<tr>
<td>Ci CesA</td>
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<td>Cs CesA</td>
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The pairwise identities (%) were calculated using ClustalW and whole CesA (A), GT-2 domain (below the table diagonal in B) and GH-6 domain (above the table diagonal in B) amino acid sequences. Od, *O. dioica*; Ci, *C. intestinalis*; Cs, *C. savignyi*. 

**Od-CesA1 and Od-CesA2 form different extracellular structures**

To analyze the temporal expression patterns of Od-CesA1 and Od-CesA2, qRT-PCR was performed using cDNAs at twelve different development stages from oocyte to day 6. Expression of Od-CesA1 was restricted to embryonic stages from 1 hpf to the hatching stage, whereas Od-CesA2 was expressed at later stages from hatching to day 6 (Fig. 3A). Respective spatial expression patterns of these genes were identified by in situ hybridization (Fig. 3B). Od-CesA1 was expressed at the lateral sides of the tail in tailbud embryos, whereas Od-CesA2 was expressed in the oikoplastic epithelium, responsible for secretion of the filter-feeding hood.

Cellulose microfibrils were first observed in pre-hatching tailbud embryos at 3 hpf (Fig. 3C). After hatching (4 hpf), the fibrils were seen to emerge laterally from the tail epidermis and aligned in an anterior-to-posterior orientation towards the tail tip. At 8 hpf, disintegration of the cellulose fibrils commenced in the anterior-most region of the tail and proceeded towards posterior regions of the tail over the next 2 hours. In parallel with the disappearance of cellulose fibrils, fin-like structures delimited by actin staining appeared along the tail margins. Cellulose staining in the oikoplastic epithelium initiated in local patches at 8 hpf and had spread over the entire trunk surface by 10 hpf. The appearance and disappearance of the cellulose fibrils corresponded very well to the spatial-temporal

We further compared the cellulose structures in *O. dioica* with those in *C. intestinalis* (Fig. 3D). In *C. intestinalis* tadpoles, the entire animal was surrounded by cellulose. Fibers aligned in an anterior-to-posterior orientation as in *O. dioica*, were not observed on the lateral side of the tail and were only present at the tail tip. Similar to *O. dioica* tadpoles, actin staining delimited the fin-like structure along the tail margins.

**Od-CesA1 is required for embryo hatching, notochord alignment and tail elongation**

We designed MOs to block either *Od-CesA1* protein translation (*cesa1start*) or mRNA splicing (*cesa1e2i2*) (Fig. 4A). To assess whether MOs targeting the splice junctions could interfere with endogenous *Od-CesA1* transcripts in vivo, we injected *cesa1e2i2* MO or a 5-mismatched control MO into one-cell stage embryos, allowed them to develop until 1 hpf or 4 hpf, and then performed RT-PCR using primers located in exon2 and exon4 of the *Od-CesA1* gene. Control MO-injected and uninjected embryos yielded expected wild-type products of 413 bp, whereas embryos injected with *cesa1e2i2* MO yielded a product of 318 bp (Fig. 4B). The nucleotide sequence of the shorter product extracted from *cesa1e2i2* MO-injected embryos revealed an excision of 95 bp from the 3′/H11032-end of exon2 due to activation of a cryptic splice donor site (Fig. 4C). This modification resulted in a frame shift downstream, creating a premature stop codon in exon3. This causes deletion of the last of the seven transmembrane helices and the entire GH-6 domain in the translation product of the incorrectly spliced mRNA (Fig. 4D). The ratio of the modified 318-bp product to the native 413-bp product was highest in 1-hpf embryos and decreased in 4-hpf embryos (Fig. 4B), suggesting that the efficiency of splice blocking decreased over this time interval.

Both translation blocking and splice-blocking MOs targeting *Od-CesA1* caused embryonic phenotypes (Fig. 5). In *cesa1start* MO-injected embryos, the predominant phenotype was a failure to elongate the tail and, additionally, an increase in failure of embryo hatching was observed. In *cesa1e2i2* MO-injected embryos there was an extensive failure of embryo hatching. To further assess the specificity of the MO effects we generated capped mRNA (cmRNA) from a rescue cDNA construct in which we had mutated three
nucleotides in the *cesa1e2i2* MO target sequence. Injection of the cmRNA alone into 330 embryos resulted in 74% developing normally, 5% exhibiting improper tail elongation and 21% failing to hatch, results similar to the injection of mismatch MOs and consistent with effects related to the mechanical perturbations of injection. When the cmRNA was co-injected with the *cesa1e2i2* MO, a rescue of hatching success was observed and this was dose-dependent (Fig. 5A).

Given the multimeric structure of cellulose synthase complexes, the different degree of severity of phenotypes is perhaps not surprising. The splice-blocking MO created a prematurely truncated form of Od-CesA1 in which almost the entire GT-2 domain was still present but the last transmembrane domain and the cellulase domain were deleted. This might have created a dominant-negative form that could have efficiently poisoned multimeric complexes that also...
contained unmutated Od-CesA1 subunits. Conversely, the translation-blocking MO would reduce the quantity but not the quality of Od-CesA1 subunits produced. To test this idea, we created a truncated cmRNA to mimic the RNA species produced by the cesa1e2i2 MO (Fig. 4). Injection of this trCesA1 cmRNA did result in an increased ratio of hatching failure to improper tail elongation and did so in a dose-dependent manner (Fig. 5A), consistent with poisoning of multimeric complexes by dominant-negative subunits.

Production of cellulose fibrils under the different experimental conditions was assessed with cellulose staining. In cesa1start MO-injected embryos, cellulose production was restricted to the

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**Fig. 3. Cellulose synthase expression and patterning in Oikopleura dioica and Ciona intestinalis.** (A) Developmental expression profiles of CesA1 (white bars) and CesA2 (black bars) determined by qRT-PCR. oo, oocytes; 2-8, 2- to 8-cell embryos; 1h, 1 hour post-fertilization (hpf) embryos; TB, tail bud; H, hatching tadpole; ET, early tadpole; TS, tail shift; D2-D6, day 2 to day 6 animals. (B) Wholemount in situ hybridization patterns for CesA1 and CesA2 in 3.5-hpf embryos and day 4 animals. Ta, tail; Tr, trunk; A-P, anteroposterior axis; D-V, dorsoventral axis. (C) Confocal image stacks of cellulose staining (green) in O. dioica embryos showing actin (red) and DNA (blue). (D) Confocal image stacks of cellulose staining in C. intestinalis embryos. Actin and DNA staining as in C. Scale bars: 50 μm in C, D.

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**Fig. 4. Knockdown of the Oikopleura dioica CesA1 gene.** (A) Schema showing the target locations for the translation-blocking morpholino (MO; cesa1start) and splice-blocking MO (cesa1e2i2). Nested primers used for RT-PCR are indicated by arrowheads. (B) RT-PCR of cesa1e2i2 MO-injected embryos. The mRNA population isolated from MO-injected embryos yielded a smaller 318-bp band in addition to the wild-type 413-bp band at both 1 and 4 hpf. Ui, uninjected embryos; e2i2, cesa1e2i2 MO-injected embryos; Ct, 5-mismatch cesa1e2i2 MO-injected embryos. (C) Nucleotide sequences around the exon2 to exon3 junction in cDNAs generated from wild-type (wt) 4-hpf embryos and cesa1e2i2 MO-injected 4-hpf embryos. A 95-bp sequence (underlined) was deleted from exon2 in MO-injected embryos through use of a cryptic splice donor site upstream of the MO-targeted splice donor. A truncated cDNA (trCesA1) was created by introducing a C-to-T point mutation (black dot) in order to produce truncated capped mRNAs to test whether this construct mimicked the effect of the cesa1e2i2 MO. (D) Predicted (http://www.cbs.dtu.dk/services/TMHMM/) transmembrane domains (TM, red bars) in wild-type and MO-disrupted CesA1. The vertical axes indicate average values of the posterior probabilities of inside, outside and transmembrane helix. MO injection results in a truncated protein lacking the seventh transmembrane helix and the entire GH-6 domain of CesA1. The black arrowhead in the wt representation indicates the position of the introduced premature stop codon in the trCesA1 construct.
posterior portion of the tail, whereas in *cesa*2e2i2 MO- or tr*CesA1* mRNA-injected embryos no cellulose production was observed (Fig. 5C). In 5-mismatched MO-injected embryos, actin staining revealed a single linear row of notochord cells in the tail. In *cesa*1start MO-injected embryos, the alignment of notochord cells was perturbed, with some cells forming a ball-like agglomeration, and the shape of the cells was non-uniform. The point of notochord cell misalignment corresponded with the position of the anterior-most emergence of cellulose fibrils from the tail epidermis. Conversely, in *cesa*1e2i2 MO- or tr*CesA1* mRNA-injected embryos, where no cellulose fibril production was detected, Phalloidin staining revealed no typical linear arrangement of notochord cells. Co-injection of the rescue cmRNA with the *cesa*1e2i2 MO recovered tail cellulose expression domains and the correct linear alignment of notochord cells. None of the constructs used in this study caused a failure of embryos to produce cells expressing the notochord differentiation-specific marker brachyury (Fig. 5D), but the ability to correctly align these notochord cells was clearly impaired by reduced or failed extracellular cellulose production.

**The cellulose-based filter-feeding house in post-metamorphic *O. dioica***

After tail elongation, metamorphosis occurs, with the tail switching from a posterior orientation to a final arrangement where the tail is orthogonal to the trunk and retains the notochord as its axial structure. Then the first filter-feeding house is inflated. The filter-feeding house is initially secreted as a compact rudiment by a specialized endodermal epithelium and several rudiment layers are often observed stacked above the trunk (Fig. 6A, upper panel). Upon escape of the animal from an inflated house, the outermost rudiment swells and is subsequently expanded by specific movements of the trunk and tail until the entire animal is contained within the mature structure (Fig. 6A, lower panel). Cellulose staining revealed the skeletal structure of the house rudiments and the food-concentrating filter and inlet filter are readily identified in pre-house rudiments (Fig. 6B). The inlet filter exhibited a meshwork composed of a single-warp and double-wef thread (Fig. 6C). The term of each cellulose bundle branched into smaller fibrils.

We also designed MOs to block *Od*-CesA2 mRNA splicing (*cesa*2e2i3 and *cesa*2e3i3) and injected a mixture of these MOs into one-cell embryos. RT-PCR using primers localized in exon2 and exon4 of the *Od*-CesA2 gene (see Fig. S2A in the supplementary material) on cDNAs isolated from 10-hpf embryos revealed successful targeting of the *Od*-CesA2 mRNA, with deletion of the entire GT-2 domain (see Fig. S2B in the supplementary material). Cellulose production in the *cesa*2e2i3/*cesa*2e3i3 MO-injected embryos was analyzed by cellulose staining and compared with that in 5-mismatched control MO-injected embryos. Injection of these MOs had no effect on hatching, notochord formation, tail elongation or the production of cellulose fibrils along the tail in early embryos as observed when *Od*-CesA1 was targeted. Instead, a minor phenotype was noted, where delayed cellulose
production on the epithelium retarded pre-house formation (see Fig. S2C in the supplementary material). This suggests that MO injection into one-cell zygotes exhibited relatively limited penetrance on the Od-CesA2 gene, which is expressed at high levels at later developmental stages than Od-CesA1.

DISCUSSION

We identified two larvacean CesA genes that show very distinct functional specializations. Od-CesA1 has a pre-metamorphic function to produce long cellulose fibrils along the larval tail, whereas Od-CesA2 is responsible for primarily post-metamorphic production of the cellulose scaffold that forms, in part, the complex filter-feeding house. Knockdown of Od-CesA1 using a splice-blocking MO resulted in a failure to produce cellulose fibrils along the tail and yielded a penetrant phenotype in which most embryos failed to hatch. Targeting of the same mRNA with a translation-blocking MO resulted in reduced production of cellulose fibrils and an elevated proportion of embryos that failed to hatch, but the major effect was failure to properly elongate the tail post-hatching.

Disruption of cellulose production in Ciona sj mutants did not impair embryo hatching. In this regard, it is notable that there is a large space between the Ciona chorion and embryo, whereas the Oikopleura embryo is tightly juxtaposed to the chorion. Thus, mechanical forces generated by the embryo might play a more significant role in hatching in Oikopleura. Cellulose fibrils might be implicated in these forces through facilitating sliding of trunk and tailbud cells against one another or through involvement in correct formation and ensuring sufficient rigidity of the tail. Relevant to this idea is that both MOs targeting Od-CesA1 exhibited clear effects on disrupting correct notochord formation. In splice-blocking MO-injected embryos, we failed to observe a typical linear arrangement of any notochord cells, whereas in translation-blocking MO-injected embryos, alignment of notochord cells was disrupted. The shape of the notochord cells was non-uniform and some cells formed a ball-like agglomeration corresponding positionally with emergence of the anterior-most cellulose fibrils from the tail epidermis.

Phylogenetic analyses of the Od-CesA genes show affinity with bacterial GT-2 domains and an intermediate affinity with bacterial and fungal GH-6 domains. These data support the hypothesis (Matthysse et al., 2004; Nakashima et al., 2004) of lateral gene transfer from a prokaryote to the chordate ancestor of the tunicate lineage (Fig. 7). However, the two domains give alternative topologies with respect to the evolution of CesA genes within tunicates. Analysis using the GT-2 domain suggests that gene duplication occurred in the common tunicate ancestor. At this point, two scenarios are possible. The horizontally transferred gene underwent gene duplication at the base of the tunicate lineage and was retained in larvaceans (Appendicularians), while being lost in ascidians. Alternatively, gene duplication occurred specifically in the larvacean lineage, with ascidians retaining the ancestral single-copy state. The gene(s) responsible for cellulose production in thaliaceans have not yet been isolated. Further details are discussed in the text.
the ascidians would have lost the homolog of Od-CesA1, whereas larvaceans retained it. Trees based on the more rapidly evolving GH-6 domain suggest that the gene duplication event occurred in the larvacean lineage after their split from ascidians. Molecular phylogeny of the GT-2 domain indicates that Od-CesA2 has more affinity to the Ci-CesAs than Od-CesA1 and this is corroborated by a respective degree of retention of intron positions and function, with Od-CesA2 being required for adult house formation in larvaceans and Ci-CesA necessary for adult tunic formation in ascidians. Characterization of the CesA complement in thaliaceans should help to resolve these alternative gain/loss scenarios.

Interestingly, in O. dioica, we found that cellulose is progressively degraded and lost along the larval tail (Fig. 3) and this precedes metamorphosis. This is at least superficially reminiscent of the loss of tail cellulose in ascidians (Nakayama-Ishimura et al., 2009) required for correct ordering of metamorphic events. In larvaceans, metamorphosis involves much less extensive morphological change than in ascidians. The longitudinal axis of the larval tail is aligned with the anteroposterior axis of the trunk in both groups. Whereas in ascidians the larval tail is resorbed and lost during metamorphosis, in larvaceans it merely undergoes migration to the ventral side of the trunk such that its longitudinal axis becomes orthogonal to the trunk. It remains a point of debate as to whether tail loss or retention is more representative of the ancestral tunicate. The morphological data suggests larvaceans are neotenic (Stach, 2008a), whereas molecular phylogenetic data (Delsuc et al., 2006; Delsuc et al., 2008) and a filter-feeding hypothesis on urochordate evolution (Satoh, 2009) places them basal to ascidians. Whereas repression of metamorphic initiation by Ci-CesA and/or cellulose in ascidians is alleviated through tail loss, in larvaceans the tail must be retained in juveniles and adults as an integral part of the feeding mechanism. Instead, tail cellulose is lost through developmental regulation of the Od-CesA1 paralog. It is possible that cellulose fibrils emerging from the larval tail of larvaceans would simply impair the supple sinuosoidal movement of the juvenile and adult tail required to regulate the flow of water through the filter-feeding house and to inflate new houses, rendering the timing of cellulose loss merely coincidental with the initiation of metamorphosis in this lineage. Experiments to prolong the expression of Od-CesA1 could be informative as to whether this would delay the metamorphic tailshift, suggesting a conserved role for cellulose in regulating timing of tunicate metamorphosis, or only impair post-metamorphic tail function.

The horizontal transfer of a prokaryotic gene giving rise to the extent tunicate CesAs is more than a mere curiosity. It has been speculated that the ability to secrete a protective covering could have significantly impacted life history strategies by prohibiting larval feeding and increasing evolutionary pressure on speed of development (Stach, 2008b). Thus, relative to other chordates, the notable acceleration of tunicate development, greatly accentuated in the fully planktonic larvaceans, might have been triggered by the ability to secrete a tunic after the lateral gene transfer event. In a larger sense, tunicates, which are uniformly filter-feeders, have combined the ability to synthesize cellulose with cellular mechanisms enabling the elaboration of complex extracellular structures, some of which are invariably associated with the filter-feeding mechanism. The sister vertebrates, lacking cellulose synthetic capability, exhibit a variety of more active feeding mechanisms, including filter-feeding, and have undergone considerable elaboration of skeletal, sensory and nervous systems compared with tunicates and the common chordate ancestor. Arguably therefore, the lateral gene transfer event has had a profound influence on the tunicate lineage, which has undergone secondary morphological simplification and is evolving at faster evolutionary rates than their vertebrate cousins (Delsuc et al., 2006). It will be of considerable interest to investigate how tunicate CesAs have been integrated into metazoan cell machinery in order to scaffold complex extracellular structures and to further explore the roles of CesA and cellulose in tunicate notochord formation and metamorphosis.

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