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A polymorphic, thrombospondin domain-containing lectin is an oocyte marker in *Hydractinia*: implications for germ cell specification and sex determination

BRAHIM MALI[1], R. CATHRIONA MILLANE[2], GÜNTER PLICKERT[3], MARCUS FROHME[1] and URI FRANK[*,2]

[1]*Molecular Biology and Functional Genomics, Technical University of Applied Sciences, Wildau, Germany,* [2]*School of Natural Sciences & Martin Ryan Marine Science Institute, National University of Ireland, Galway, Ireland and* [3]*Institute of Zoology, University of Cologne, Germany*

ABSTRACT We have identified a novel, multidomain, polymorphic lectin in the marine cnidarian *Hydractinia echinata*. The gene is expressed in oocytes and was therefore named *CEL* for *cnidarian egg lectin*. The predicted protein has an unusual domain architecture, consisting of variable numbers of thrombospondin type 1 domains, flanked by one N-terminal and two C-terminal galactose binding lectin domains. The diversity of the gene's transcripts results from allelic polymorphism as well as alternative splicing. *Hydractinia* is dioecious and its sex has been reported previously to be genetically determined. We found intersexual colonies that were functional males, but had immature *CEL*-positive oocytes alongside mature sperm in the same gonads. Intersexuality was observed to be common in one population but not found in others. Hermaphroditic, self-fertile colonies were found in one locality; however, in these cases gonads contained either male or female gametes without mixed ones. Intersexuality that was considered to be a very rare event is apparently a more common phenomenon, at least in some populations. True hermaphroditism also occurs in this species. *CEL* can be considered as a marker for early oocyte differentiation and may play a role in germ cell specification and sex determination in cnidarians.

KEY WORDS: *alternative splicing, hermaphroditism, Cnidaira, intersexuality*

Specification of germ cells is a major theme in developmental and evolutionary biology. Depending on the animal group, germ line specification may either be controlled by inherited maternal factors, or by inductive signals (Extavour and Akam, 2003).

In many animals, a boundary between somatic and germ-line cells is formed after germ-line specification has occurred during embryogenesis. Somatic mutations are prevented by this mechanism to be transferred to the next generation. A different mechanism is found in basal invertebrates where germ cells are continuously formed throughout adult life from pluripotent stem cells that also give rise to somatic cells (Frank *et al.*, 2009). The mechanisms for germ cell specification in these animals, and the anatomic location at which it occurs, are unknown.

An additional important question is sex specification. In mammals, this process occurs downstream of germ cell specification as a response to signals emitted by the gonadal somatic tissue (Kocer *et al.*, 2009). In contrast, sex determination in cnidarians, at least in certain species, occurs upstream of germ cell specification, and is controlled by the stem cells rather than by inductive signals from somatic tissues. This is evident from experiments showing that transplantation of adult male stem cells to adult females recipients causes sex reversals (Müller, 1964). Hence, the gonads probably emit a signal that induces pluripotent cells of a defined sex to become gametes. The situation may be different in other cnidarians, where sex reversals occur naturally.

Hydractinia is a colony forming, clonal cnidarian. Two North Atlantic sibling species, *H. echinata* and *H. symbiolongicarpus* have been studied since decades, mainly in the context of development and allorecognition (Frank *et al.*, 2001). *Hydractinia*'s stem cells, called interstitial cells or i-cells, are pluripotent and are

Abbreviations used in this paper: CEL, cnidarian egg lectin; ISH, In situ hybridization, SNP, single-nucleotide polymorphism; TSP, thrombospondin.

***Address correspondence to:** Uri Frank. University Road, Galway, Ireland. Fax: +353-9149-4526. e-mail: uri.frank@nuigalway.ie

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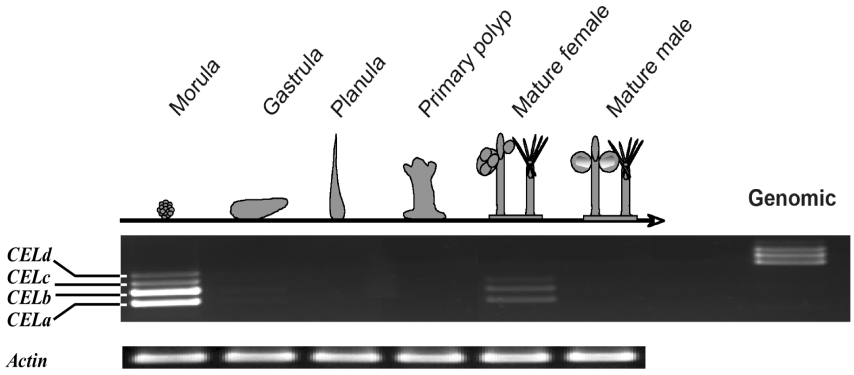

Fig. 1. Semi-quantitative RT-PCR on pooled total RNA and genomic DNA from animals sampled in the Sylt site. *RT-PCR results show mRNA expression levels of* CEL *isoforms and* Actin *in four stages of developmental as well as in mature male and female colonies.*

primarily located in the epithelial interstices of the gastrovascular system of the adults, called stolons (Müller *et al.*, 2004). I-cells can migrate into newly growing parts of the colony where they differentiate into somatic and germ cells. *Hydractinia* is dioecious and its sex has been reported to be genetically determined with a few observed mutants that display intersexual phenotypes (Hauenschild, 1954).

Here we report a novel gene encoding a multi-domain, polymorphic and alternatively spliced lectin that also includes thrombospondin repeats from *H. echinata*. The predicted protein has a structure hitherto undescribed. The gene is expressed during oocyte differentiation but not in sperm progenitors, and its transcripts are still detectable in early embryos. As a female germ cell marker it revealed surprising data on sex specification in *Hydractinia* and we propose it to be a useful tool also for studies on germ cell specification. We have named the gene *CEL* for *cnidarian egg lectin*.

Results

Identification and sequence analysis of the Hydractinia CEL cDNA

By analyzing an expressed sequence tags (EST) database from the hydroid *H. echinata* we identified a cDNA clone (GenBank accession number CO536184) encoding a putative protein that included lectin domains and thrombospondin type 1 (TSP) repeats. We named the gene *CEL* for *cnidarian egg lectin*, due to its expression pattern (see below). The complete cDNA sequence of the gene was 1341bp long. It contained an open reading frame encoding 427 amino acid residues. Protein database searches at (http://www.cbs.dtu.dk/services/SignalP/ and http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock) revealed that the predicted CEL had a signal peptide sequence at its N-terminus, 2 thrombospondin type 1 repeats in the middle part, flanked by one galactose-binding lectin domain at the N-terminus, and 2 other, tandem repeated lectin domains at the C-terminus.

The primary structure of the galactose binding lectin domains of CEL is similar to the rhamnose-binding lectin (RBL) family. RBLs have structural features composed of two or three tandemly repeated carbohydrate recognition domains (CRD) of about 95 amino acid residues including eight conserved cysteines (Hosono *et al.*, 1999). Initially discovered in sea urchin eggs, several forms of RBLs have been described in fish eggs (e.g. Hosono *et al.*, 1999). This family of lectins is abundantly expressed in the ovary and egg of the steelhead trout.

The thrombospondin (TSP)1 domain was first described in the thrombospondin protein where it is repeated three times. TSP1 repeats have later been identified in a wide set of secreted and membrane-bound proteins (for review see Adams and Tucker, 2000). The copy number of TSP1 in proteins varies from 1 to 18. TSP1 has been reported to be a multifunctional domain, regulating several cellular responses. A possible role of fish thrombospondin in oogenesis has been proposed recently, based on its expression pattern in ovaries (Wu *et al.*, 2009).

BLAST analysis showed that CEL is similar to Rhamnospondin from the congeneric hydroid, *Hydractinia symbiolongicarpus* (Schwarz *et al.*, 2007), and to some predicted proteins from the sea anemone, *Nematostella vectensis*, and the freshwater polyp, *Hydra* spp. CEL also displayed similarity to numerous TSP-containing proteins, such as hemicentins, which are not lectins, and to some lectins that lack TSP domains, such as RBL (Hosono *et al.*, 1999). Rhamnospondin, in contrast, also contains the combination of TSP1 repeats and galactose binding lectin domains, but the overall structure of CEL is different. CEL, therefore, has a unique structure that has not been reported previously, but the combination of galectin binding domains with TSP1 repeats in a single protein may be cnidarian-specific.

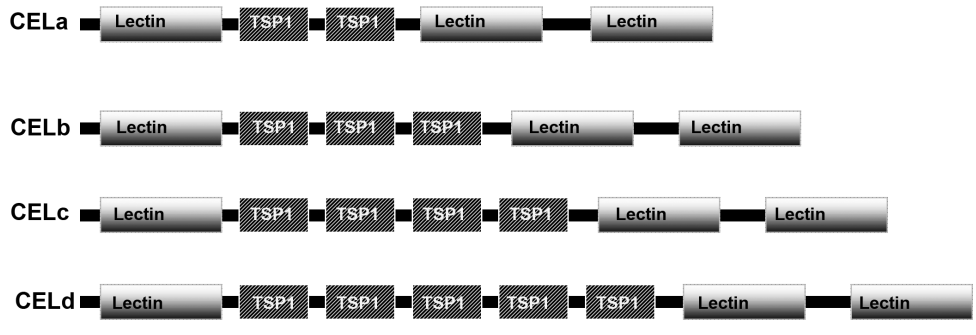

Fig. 2. Schematic representation of the domain structure of the various cnidarian egg lectin (CEL) isoforms. The isoforms differ in the number of TSP1 repeats.

Cloning and analysis of CEL isoforms by RT-PCR

We designed PCR primers spanning the entire cDNA of *CEL* and performed Semi-quantitative RT-PCR analysis of *CEL* expression in different developmental stages. We performed the experiments with cDNAs from animals originating from two localities, Sylt, Germany; and Galway Bay, Ireland. The analysis revealed

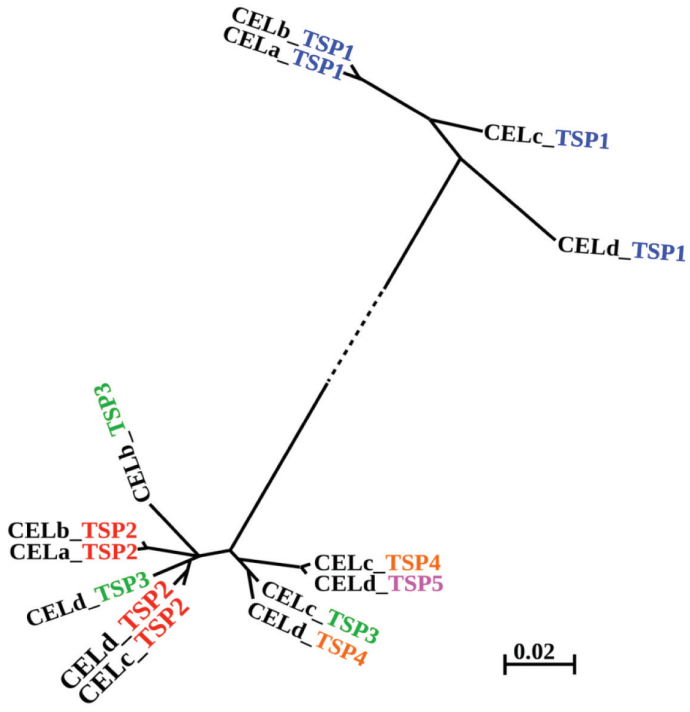

Fig. 3. Neighbor joining analysis of the thrombospondin1 (TSP1) domains, visualized as an unrooted tree. *Bar indicates distance in units of nucleotide substitutions per site. Long branch is plotted with dashed line and represents 0.18.*

the presence of *CEL* mRNAs in early embryos (< 8 h post fertilization) and in mature female colonies. Levels of expression in mature male colonies, and in all other developmental stages of both males and females, were undetectable within 26 PCR cycles. Surprisingly, instead of one expected band at approximately 1.3 kb, the PCR reactions yielded three additional products running at approximately 1.5, 1.6 and 1.8 kb in the Sylt animals (Fig. 1), but only 2 bands in the Galway Bay animals, at 1.3 and 1.5 kb (not shown). The bands were excised from the gels, cloned and sequenced. Sequence analysis of the bands revealed different numbers of encoded TSP repeats between the clones. We have named the isoforms as *CELa* (i.e. the original EST clone with 2 TSP1 repeats), CELb (3 TSP1 repeats), *CELc* (4 TSP1 repeats) and *CELd* (5 TSP1 repeats). *CELd* was the longest cDNA sequence, comprising 1872 nucleotides including an open reading frame (ORF) encoding a 604-amino acid protein. The ORFs of *CELc* and *CELb* encoded 545 and 486 amino acid polypeptides, respectively (Fig. 2; Supplementary Fig. 1).

Alignment of the isoforms detected a total of 21 SNPs (single-nucleotide polymorphisms) that resulted in a change in the coded amino acid in 8 cases (Supplementary Fig. 1). Interestingly, in all SNP loci, *CELa* shared sequence with *CELb*, and *CELc* with *CELd* (Supplementary Fig. 1). This may indicate that *CELa* and *CELb* were alternatively spliced transcripts from one allele or gene, and *CELc* and *CELd* arose similarly from a different gene or allele (see below). To further examine the relationships among the *CEL* isoforms, their sequences were analyzed by neighbor joining and the analysis visualized by an unrooted tree (Fig. 3). The tree showed that the first TSP repeats of all *CEL* isoforms are more closely related to each other than to other repeats, whereas the other repeats did not show a clear relationship pattern, but were nevertheless highly similar (Supplementary Table 1).

Genomic analysis of CEL

To understand the occurrence of different *CEL* transcripts, we extracted genomic DNA from animals sampled at three localities: Sylt (Germany), Galway Bay (Ireland) and Roscoff (France). Primers spanning the start and stop codons of the *CEL* gene were used in PCR reactions. Similar to the results of the cDNAs, described above, genomic PCR experiments revealed differences between animals originating at the Sylt site in Germany and those that came from Roscoff and Galway Bay (not shown). Pooled DNA from Sylt-sampled *Hydractinia* yielded a total of 3 distinct *CEL* bands (Fig. 1). The Roscoff and Galway Bay populations, in contrast, had only a single *CEL* band, similar in size to the smallest band in the Sylt animals (not shown).

We have sequenced the entire genomic fragment of the Galway Bay animals from 800 bp upstream of the start codon until 18 bp downstream the stop codon. The structure of the *CEL* gene from the Galway Bay population consists of 7 exons and 6 introns (Fig. 4). The seven exons correspond to the predicted structural domains of *CELa* and *CELb*, which, also based on their common SNP pattern, are probably derived from a single gene and obtained by alternative splicing. Exon 1 encoded the N-terminal signal peptide. The individual lectin and TSP domains were each encoded by single exons. The nearly identical 5' and 3' UTR and introns 1-3 sequences from all localities, suggested that all the transcripts belong to one genetic locus. This locus is polymorphic

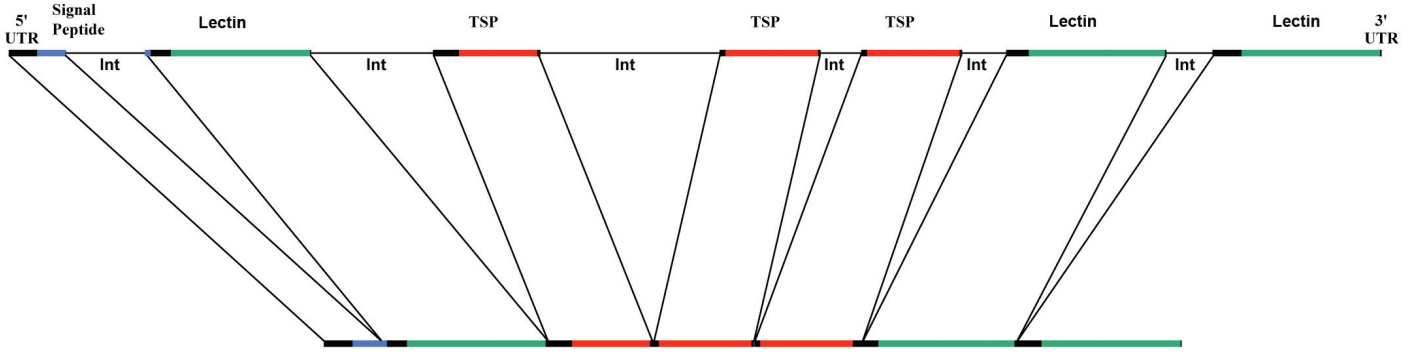

Fig. 4. Genomic structure of the *Cnidarian egg lectin (CEL)* gene from Galway Bay (Ireland). *Green and red lines represent exons encoding lectins and thrombospondins (TSPs), respectively; Int, introns; UTR, untranslated region. Lower part represents the spliced* CELb *transcript.*

in the Sylt population (Fig. 1) but not in the sampled Galway Bay or Roscoff ones. The various cDNAs in Sylt population could therefore be the result of alternative splicing, allelic polymorphism, or a combination of both. Fig. 1 shows higher expression level of *CELa* and *CELb*, compared with the other two isoforms, which could be explained by variable allelic expression in the Sylt population, or preferential alternative splicing.

The differences in allele polymorphism between the sampling localities might have resulted from differences in the sampling methods. Animals from Sylt were sampled by dredging over a large area, while in Galway Bay and Roscoff, animals were sampled using SCUBA and skin diving from areas that were at least one order of magnitude smaller.

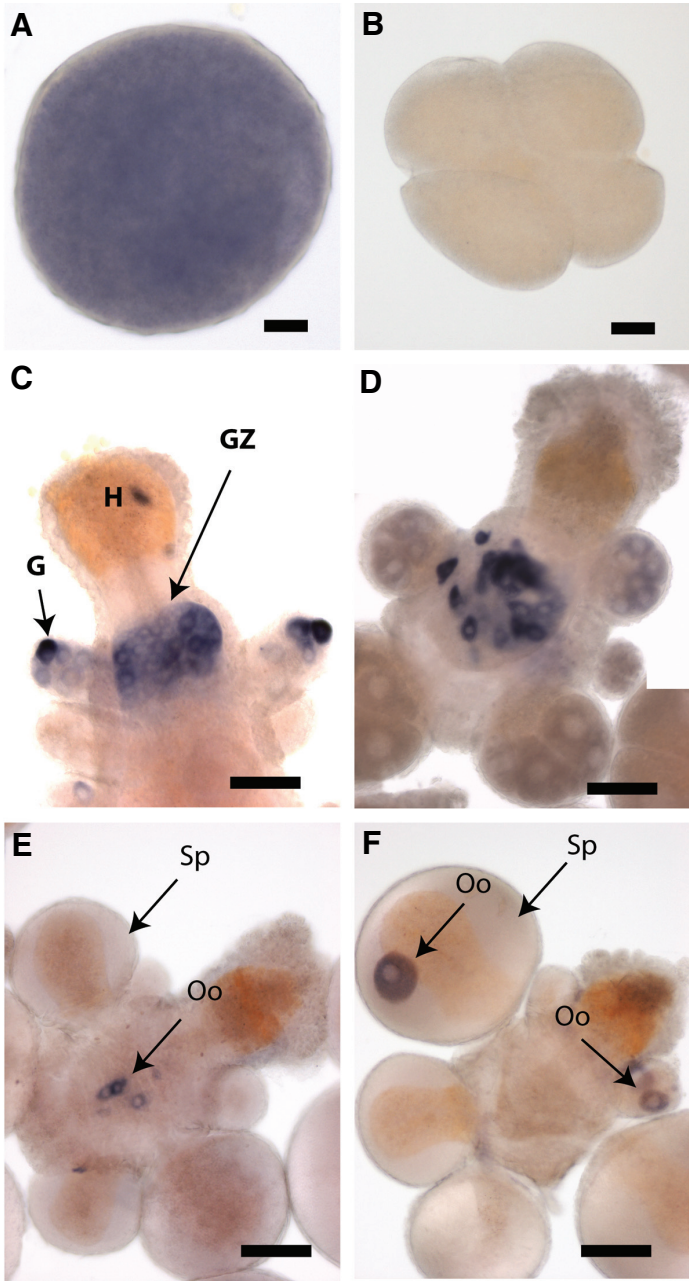

Fig. 5. ***In situ*** **hybridization of** ***Cnidarian egg lectin (CEL)*** **in** ***Hydractinia*** **from Sylt. (A)** *Fertilized egg.* **(B)** *Four-cell stage, sense probe.* **(C,D)** *Female sexual polyps containing gonads with* CEL *expressing oocytes at different stages.* **(E,F)** *Male colonies showing some* CEL*-positive oocytes. H, head; G, gonad; GZ, germinal zone; Sp, sperm; Oo, oocyte. Scale bars 20 μm in (A,B); 50 μm in (C-F).*

Analysis of CEL spatial expression by in situ *hybridization*

To determine also the spatial distribution of the *CEL* transcripts, whole mount *in situ* hybridization (ISH) analysis was performed at 7 different stages of *Hydractinia* development, including unfertilized egg, 3 h embryo, 24 h embryo, planula larva, 15 h post metamorphosis induction, primary polyp and male and female mature colonies. Sense and antisense RNA probes were generated from a cloned *CELa* cDNA fragment. The structure of the four-isoform cDNAs, having various numbers of overall similar TSP repeats (Supplementary Fig. 1; Supplementary Table 1), prevented a successful design of cRNA probes, which would selectively hybridize to only one of the four isoforms. Hence, a possible isoform-specific expression pattern could not be studied.

ISH revealed that in eggs and zygotes *CEL* mRNA was distributed uniformly (Fig. 5A). During the first cleavages, the ISH staining was still detectable, but the signal progressively faded, becoming undetectable after the late morula stage and in all other stages until the sexually mature colony. In mature colonies, *CEL* expression resumed but was restricted to only few cells. A *Hydractinia* colony is mainly composed of two polyp types, feeding polyps (gastrozooids) and sexual polyps (gonozooids), both emerging from the gastrovascular network, the stolons (Frank *et al.,* 2001). Sexual polyps do not feed but contain the gonads. Stem cells (i-cells) migrate from the stolons into gonozooids where they become committed to germ cells and enter the gonads where differentiation into gametes is completed. ISH on mature female colonies stained early oocytes in the germinal zone of sexual polyps and in gonads (Fig. 5 C,D). Onset of expression appeared to be depending on location, as *CEL* positive cells were only observed close to the gonads and rarely in the head region, but never at the base of the polyp, or in stolons. It was possible to observe *CEL*-expressing, migratory oocytes as they move into the gonad (Fig. 5 C,D). The *in situ* signal gradually faded as oocytes matured. This suggests that *CEL* expression commences just after oogenesis has started in germ cells before entering the gonads. It is the same region of the sexual polyp where i-cells stop expressing *Vasa*, a stem cell marker in *Hydractinia*, before they enter the gonad and complete oogenesis (Rebscher *et al.*, 2008). Possibly, this is also the stage where i-cells lose pluripotency to become committed to gamete differentiation. Despite the RT-PCR experiments on male colonies, revealing *CEL* expression below detection levels of 26 PCR cycles, we also performed ISH on 16 males, originating from Sylt, and 4 males, originating from Galway Bay. Surprisingly, in all 16 Sylt colonies, but not in the Galway Bay ones, there was at least one male sexual polyp containing a few *CEL* positive i-cells (Fig. 5 E,F). In some cases the ISH staining highlighted developing oocytes in the male gonads alongside mature sperm in a single gonad (Fig. 5F). Mature eggs, however, have never been observed in any of these male colonies, possibly due to inhibition of oocyte maturation by sperm-committed cells as suggested to be the case in *Hydra* (Bosch and David, 1986). No other *CEL* positive cells have been observed in any part of either male or female colonies, or indeed

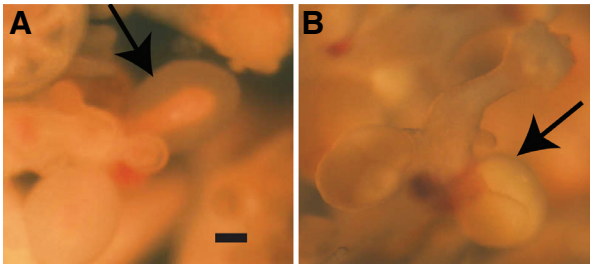

Fig. 6. Hermaphroditic colony. (A) *Male.* **(B)** *Female. Arrows point to maturing gonads. Scale bar 100 μm*

in any other developmental stage except of very early embryogenesis.

The apparently higher expression level in eggs and early embryos, as revealed by RT-PCR (Fig. 1), is an artifact as follows: RNA was extracted from whole mature colonies, in which the few *CEL*-positive oocytes were outnumbered by many *CEL*-negative somatic cells, as opposed to whole embryos where all cells were expressing *CEL* at low levels.

True hermaphroditic colonies

We have also identified 4 animals, sampled in Galway Bay, which were predominantly females, but contained also fully developed male sexual polyps (Fig. 6). The gonozooids were either male or female and no mixed sex polyps, similar to some Sylt males, were observed. Self-fertilization within these hermaphroditic colonies resulted in embryos that developed normally until 2 days post fertilization and then died, possibly due to inbreeding.

Discussion

Intersexuality, expressed as single oocytes in functional male gonads, has been reported to be a rare genetic mutation in this species (Hauenschild, 1954), or be the result of allogeneic stem cell invasion of the opposite sex following natural or experimental grafting of male and female histocompatible colonies (Müller, 1967; Müller, 1964; Müller *et al.,* 2004). Our results, using laboratory reared offspring from field-sampled animals and therefore non-chimeric, show that intersexuality is actually quite common in males, at least in the Sylt population. It may be rare or absent in other populations. Intersexuality was not evident in functional female colonies, but in the absence of an appropriate marker for early sperm differentiation, one cannot exclude single sperm-committed i-cells in normal female colonies. Self-fertilizing hermaphrodites were found in Galway Bay only; however, in these cases, male and female gametes were not observed in one gonad, or even one sexual polyp, but were restricted to individual polyps, either male or female. These field-collected animals could be chimeric, resulting from co-settlement of two, histocompatible larvae on the same shell. True hermaphroditism with both mature and functional sperm and oocytes has not been described in *Hydractinia* previously, but is known from other cnidarians, such as acroporid corals. Temperature-dependent sex was reported in the hydrzoan, *Clytia* (Carré and Carré, 2000), and fungiid corals undergo sex reversals naturally (Loya and Sakai, 2008). In the freshwater polyp *Hydra*, sex is determined by the stem cells. Female stem cells can only produce oocytes, while male stem cells can produce both. Male stem cells in *Hydra* repress oocyte differentiation (Bosch and David, 1986). Hence, cnidarian sex determining mechanisms seem to be diverse, but the data presented here provide further evidence that sex in *Hydractinia* is controlled by the germ cells rather than by the somatic tissues of the gonads, as oocytes were developing alongside sperm in the same microenvironment.

CEL is a marker for the oocyte differentiation pathway and as such may also be used for further studies on the transition from pluripotent cells to germ cells, a process that occurs continuously in hydroids. The function of the gene product, which is predicted to encode a secreted thrombospondin domain-containing lectin, and the nature of the carbohydrates it binds remain elusive. CEL could be used by the released egg as sperm-recognizing and binding factor in analogy to the egg receptor for sperm binding in sea urchins (Zigler, 2008) or the ZP glycoproteins in the zona pellucida of the mammalian egg (Chiu *et al.*, 2008). CEL may also have a role in the differentiation process from stem cell to oocyte. Alternatively, CEL might be an immune-related protein. The diversity of its transcripts, resulting from genomic polymorphism and/or alternative splicing, would be consistent with such a function.

Materials and Methods

Animals

Hydractinia echinata colonies, growing on gastropod shells that are inhabited by hermit crabs were collected from 3 different localities: One was on the Island of Sylt, Germany, the second site was Roscoff, France, and the third was Galway Bay, Ireland. The hermit crabs were removed and the hydroids cultured in artificial or natural seawater at 18°C under a 14:10 light:dark regime. Animals were fed brine shrimps nauplii 3-6 times a week. Under these conditions male and female colonies spawn nearly daily about an hour after the onset of light. Embryos were allowed to development for 3 days until the planula larva was competent to undergo metamorphosis. The larvae were induced to metamorphose into a primary polyp using a 3 h pulse treatment with 116 mM CsCl in seawater. Following the CsCl treatment, animals were positioned on glass cover slips on which they completed metamorphosis within 24 h.

Molecular biology

All molecular biology work was done using standard protocols unless otherwise stated. Total RNA was isolated from different developmental stages by acid guanidium and phenol/chloroform extraction and reverse transcribed. The *CEL* fragments from Sylt were amplified from cDNA of early embryos (~3hours post fertilization) using primers starting at the methionine and the stop-codon, respectively, introducing restriction sites on both ends for cloning
F/ATG*Nco*I: 5'CATGCCATGGATGGTGAATTTGCGATTA3';
R/TAA*Apa*I: 5'GATATGGGCCCTTATTTCACAATGCATCG3'. The fragments were cloned into the pBlueScript-vector and sequenced. *Hydractinia actin* was used as reference gene for semi-quantitative RT-PCR by using actin forward primer, 5'AAACCCTTTTCCAACCATCCTT3', and actin reverse primer, 5'TGGG CCAGATTCATCGTATTCT3'.

For the isolation of genomic DNA fertilized eggs were allowed to develop into primary polyps in the presence of 10 μg/ml tertacycline, replacing the water containing fresh antibiotic twice daily. Genomic DNA was isolated by SDS/ProteinaseK treatement and phenol/chloroform extraction. Nucleic acids were treated with a mixtured of 20 μg/ml RnaseA and 100 units/ml RnaseT1 and the DNA was purified by two consecutive ethanol-precipitations in the presence of 1M ammonium acetate.

Genomic *CEL* from Galway Bay was amplified from genomic DNA using primers from the 5' and 3' untranslated region:
CELfwd: 5'GGTTACTTGTTCTTCCTGCAGTTTTG3';
CELrev: 5'ATCCACATGTTATTTGCAAGTATACGAC3'). The PCR-product was gel-purified and sequenced directly by primer-walking using primers located inside the introns. The 5' upstream region of the *CEL* gene was isolated by a modified version of the splinkerette protocol. The PCR product was also gel purified and sequenced directly. Amount and quality of extracted nucleic acids was assessed spectrophotometrically and by gel electrophoresis, respectively.

RNA* in situ *hybridization

For *in situ* hybridization, fragments of the coding sequence (300-400 bp) were cloned into pGEM-T or BlueScript vectors and sequenced. Next, the vector was linearized by restriction digestion and used as template for sense and anti-sense, DIG-labeled RNA probe synthesis. *In situ* hybridization and probe detection was performed according to standard protocols.

Sequence analysis

For functional annotation of the *CEL* transcripts, the program BLASTX was used to compare nucleotide sequences to the non-redundant protein database of the National Center for Biotechnology Information (NCBI). Conserved protein domains were searched in the Pfam and SMART databases. To identify a possible signal peptide, the CEL protein sequences were submitted to the SignalP server. Multiple alignment was performed using CLUSTALW and CLUSTALX with default settings. Neighbor-joining analysis of the 4 CEL isoform sequences was performed with the programs NJPLOT (Perriére and Gouy, 1996).

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