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Induced stem cell neoplasia in a cnidarian by ectopic expression of a POU domain transcription factor

R. Cathriona Millane[1], Justyna Kanska[1], David J. Duffy[1], Cathal Seoighe[2], Stephen Cunningham[3], Günter Plickert[4] and Uri Frank[1,*]

SUMMARY

The evolutionary origin of stem cell pluripotency is an unresolved question. In mammals, pluripotency is limited to early embryos and is induced and maintained by a small number of key transcription factors, of which the POU domain protein Oct4 is considered central. Clonal invertebrates, by contrast, possess pluripotent stem cells throughout their life, but the molecular mechanisms that control their pluripotency are poorly defined. To address this problem, we analyzed the expression pattern and function of *Polynem* (*Pln*), a POU domain gene from the marine cnidarian *Hydractinia echinata*. We show that *Pln* is expressed in the embryo and adult stem cells of the animal and that ectopic expression in epithelial cells induces stem cell neoplasms and loss of epithelial tissue. Neoplasm cells downregulated the transgene but expressed the endogenous *Pln* gene and also *Nanos*, *Vasa*, *Piwi* and *Myc*, which are all known cnidarian stem cell markers. Retinoic acid treatment caused downregulation of *Pln* and the differentiation of neoplasm cells to neurosensory and epithelial cells. *Pln* downregulation by RNAi led to differentiation. Collectively, our results suggest an ancient role of POU proteins as key regulators of animal stem cells.

KEY WORDS: iPS cells, Cnidaria, Interstitial cells, I-cells, Nematocyte, Differentiation

INTRODUCTION

A major focus in biomedical research is the role of stem cells in normal development, regeneration and disease, and the potential to use them in cell-based therapy. Understanding the basic biology of these cells and their evolutionary origin are therefore of particular interest.

Pluripotent stem cells are undifferentiated cells that are able to contribute to all adult somatic lineages and to the germ line. They have been best studied in mammals, in which they form the inner cell mass of the blastocyst. Following implantation and gastrulation, these cells lose pluripotency and become progressively committed to individual lineages. Various studies have revealed that the transcription factors Oct4 (also known as Pou5f1), Sox2 and Nanog are central in generating and maintaining pluripotency (Niwa et al., 2000; Boyer et al., 2005). *Oct4* and *Nanog* are silenced in adult somatic cells (Yamaguchi et al., 2005; Feldman et al., 2006), whereas *Sox2* continues to be expressed in adult neuronal stem cells. Forced expression of *Oct4*, *Sox2*, *c-Myc* and *Klf4* in mouse fibroblasts can reprogram them back to pluripotency (Takahashi and Yamanaka, 2006; Okita et al., 2007). Other gene combinations have also been reported to be capable of reprogramming somatic cells (Takahashi et al., 2007; Hanna et al., 2008; Kim et al., 2009), but only *Oct4*, a POU domain gene, is always indispensable.

Pluripotency outside the Mammalia is less well understood, but research in chick (Lavial et al., 2007) and frogs (Morrison and Brickman, 2006) has suggested that POU domain proteins fulfill a similar role at least in some non-mammalian vertebrates. An *Oct4*-like gene has also been identified in fish (Lunde et al., 2004), but its role in maintaining pluripotency is unclear. These reports, together with the absence of comparative studies on invertebrates, have led to the proposal that POU-mediated pluripotency is a vertebrate innovation (Frankenberg et al., 2010).

The reasons for the scarcity of information on invertebrate pluripotent cells are twofold. First, the most established invertebrate model organisms, flies and worms, have early committing embryonic cells and offer limited access to pluripotent cells. Indeed, most stem cell research on these animals has been conducted in the contexts of germ cells and tissue stem cells (Pearson et al., 2009; Joshi et al., 2010). Second, other invertebrates that do contain pluripotent stem cell populations, such as cnidarians and planarians (Müller et al., 2004; Reddien and Sanchez Alvarado, 2004), provided, until recently, only limited access to gene expression manipulation. Studies on cnidarians and planarians have revealed some conserved mechanisms in stem cell self-renewal and differentiation (Reddien et al., 2005b; Teo et al., 2006; Khalturin et al., 2007), but have not addressed the molecular control of pluripotency directly. Therefore, the present literature leaves the evolutionary history of pluripotency unclear.

We have been studying stem cells in the early diverging phylum Cnidaria, using the clonal marine hydrozoan *Hydractinia echinata* as a model. *Hydractinia* stem cells were first studied by August Weismann in the late 1800s (Frank et al., 2009), leading to his prominent germ plasm theory (Weismann, 1892). *Hydractinia* is a dioecious, colony-forming cnidarian. The sexually produced planula larva metamorphoses within 24 hours into a polyp, which reproduces clonally to form a colony of genetically identical individuals that share a gastrovascular system (Fig. 1). Specialized polyps develop gonads and release eggs or sperm daily in light-controlled cycles. To

[1]School of Natural Sciences and Ryan Institute, National University of Ireland, Galway, University Road, Galway, Ireland. [2]School of Mathematics, Statistics and Applied Mathematics, National University of Ireland, Galway, University Road, Galway, Ireland. [3]Glycoscience Group, National Centre for Biomedical Engineering Science, National University of Ireland, Galway, University Road, Galway, Ireland. [4]Biozentrum Köln, University of Cologne, Zülpicher Str. 47b, 50674 Köln, Germany.

*Author for correspondence (uri.frank@nuigalway.ie)

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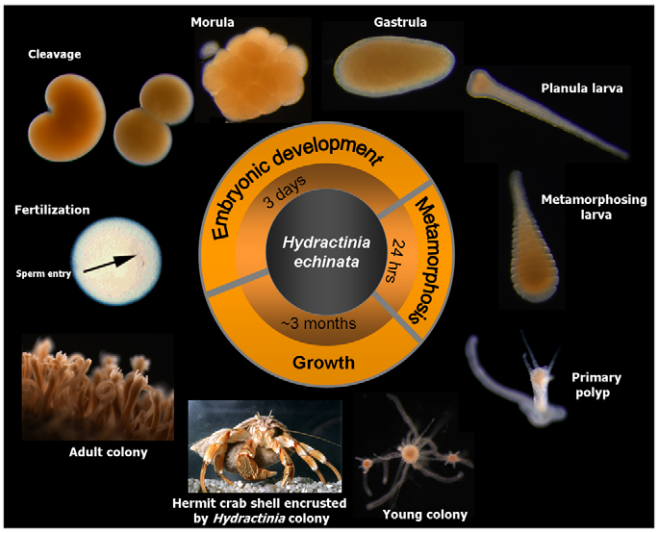

Fig. 1. The life cycle of *Hydractinia*.

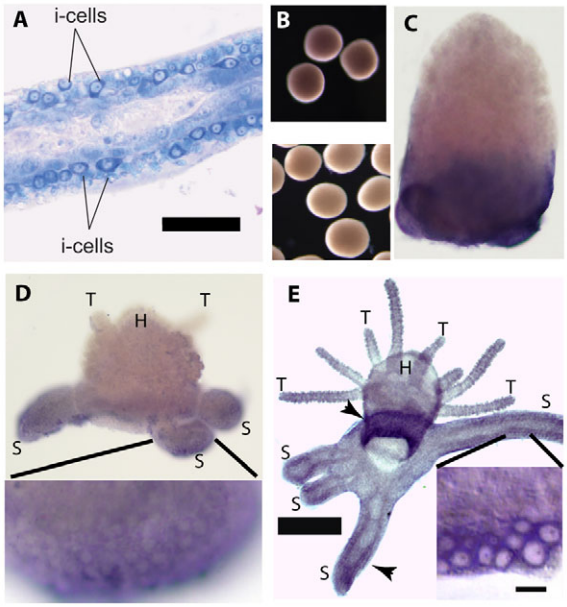

Fig. 2. i-cells in *Hydractinia* and expression of *Pln* mRNA. (**A**) May-Grünwald Giemsa staining of a *Hydractinia* stolon showing i-cells. (**B-E**) In situ hybridization of *Pln*. (B) A blastula showing ubiquitous staining. Antisense (top) and sense (bottom) probe results are shown. (C) Ten-hour metamorphosing polyp. (D) Eighteen-hour metamorphosing polyp. Inset shows an outgrowing stolon tip and individual i-cells at higher magnification. (E) Two-day post-metamorphosis animal. Staining (arrowheads) is visible in a ring-like structure at the lower part of the polyp and in the epidermal layer of the stolons. Inset shows individual i-cells at a higher magnification. H, head; S, stolon; T, tentacle. Scale bars: 50 μm in A; 200 μm in E (10 μm in inset).

enable a continuous production of germ cells, clonal growth and virtually unlimited regenerative ability, *Hydractinia* possesses migratory stem cells, called interstitial cells (i-cells). Their name originated from their location in the interstitial spaces of epithelial cells. The i-cells of *Hydractinia* are small (~7-10 μm), rounded or slightly spindle shaped, and have a large nucleus and basophilic cytoplasm (Fig. 2A). The i-cell population maintains pluripotency throughout the life cycle (Müller et al., 2004).

To address the unresolved issue of the evolution of pluripotency, we have studied the specific roles of a putative homolog of the key mammalian pluripotency gene *Oct4* in *Hydractinia* i-cells.

MATERIALS AND METHODS

Animal culture

Hydractinia echinata colonies growing on hermit crab shells were sampled in Galway Bay, Ireland, and cultured in natural seawater at 17-18°C under a 14/10 hour light-dark regime. They were fed daily with brine shrimp nauplii and once a week with ground fish. Embryos were collected ~1 hour after the onset of light and allowed to develop in small Petri dishes. Metamorphosis of mature larvae was induced by a 3-hour pulse treatment with 100 mM CsCl in seawater. Animals were then positioned on glass slides to complete metamorphosis (Frank et al., 2001). As the colonies grew, small pieces were removed and glued to a new glass slide to produce physiologically independent clonal colonies.

Cloning of a POU domain gene from *Hydractinia*

Degenerate primers directed against the POU and homeodomains of human *POU5F1* (*OCT4*) (for primers see Table S1 in the supplementary material) were used in PCR reactions and amplified a 390 bp fragment of a *Hydractinia* POU gene. The full-length coding sequence was obtained by RACE-PCR according to the SMART RACE protocol (Clontech). Nucleotide sequences are available at GenBank under accession numbers JF820067 (Pln); JF820068 (Myc); JG772275 (Piwi); JG772276 (Cmd); JG772277 (Nanos); JG772278 (Tac).

Pln RNAi

A 300 bp DNA fragment was amplified by PCR from colony cDNA. T7 or SP6 recognition sequences were added to the 5′ ends of the primers. Sense and antisense RNAs were synthesized by T7 and SP6 polymerases and annealed to generate double-stranded (ds) RNA. dsRNA was applied by soaking the animals in seawater containing 100 ng/μl RNA for 24 or 48 hours (Duffy et al., 2010). As control, we used non-coding dsRNA corresponding to a fragment of the pGEM-T plasmid.

Retinoic acid treatment

All-trans retinoic acid (RA) (Sigma; 50 mg/ml stock in DMSO) was added to seawater to a final concentration of 100 μM. Controls were treated with DMSO alone at the same concentrations.

In situ hybridization

In situ hybridization was performed as described (Gajewski et al., 1996; Teo et al., 2006). The full-length coding sequences were cloned into pGEM-T and pBluescript vectors, extracted and cleaned up using the standard alkaline lysis method. DIG-labeled RNA probes (Roche) were synthesized using SP6, T7 and T3 RNA polymerases according to the manufacturer's protocol (Fermentas). In situ hybridization was performed at 50°C.

Immunohistochemistry

We used rabbit polyclonal anti-Oct4 antibody (ab19857, Abcam). Animals were fixed in 4% paraformaldehyde in PBS for 20 minutes and then dehydrated in ethanol through four steps (25, 50, 75 and 100%). Samples were rehydrated and blocked for 30 minutes in 2% BSA in PBS (BSA/PBS), then blocked for 30 minutes in 5% goat serum in BSA/PBS (GS/BSA/PBS). The antibody was diluted 1:100 in GS/BSA/PBS and incubated for 1 hour at room temperature, followed by three washes with BSA/PBS and re-blocking as above. Pre-adsorbed secondary antibodies (Alexa Fluor 594 and 635 goat anti-rabbit IgG, A-11012 and A-31577, Invitrogen) were diluted 1:500 in GS/BSA/PBS and incubated for 1 hour at room temperature. Animals were mounted in Prolong Gold Antifade (P7481, Invitrogen). The nematocyst-specific antibody NCol-1 (Adamczyk et al., 2010) was a kind gift from Dr Suat Özbek (University of Heidelberg, Germany).

Microinjection

Plankton netting (100 μm) was glued to the bottom of a Petri dish. One- to two-cell stage embryos were pipetted into the dish and immobilized in the mesh holes. About 100 pl 1-2 ng/nl vector solution was microinjected into each embryo as previously described (Künzel et al., 2010).

Quantitative real-time PCR (qPCR)

Total RNA was isolated by the acid guanidinium thiocyanate-phenol:chloroform method and DNase digested. First-strand cDNA synthesis was performed using the Omniscript RT Kit (Qiagen). qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Green or TaqMan chemistries. Gene expression was normalized to that of *Gapdh*. For primers and TaqMan probes, see Table S1 in the supplementary material.

Phylogenetic analysis

POU domain protein sequences from various animals, representing all POU families, were downloaded from GenBank and are listed in Table 1. The domain boundaries were determined by alignment to PFAM domains (http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock). The POU-specific domain, the POU homeodomain, and the linker between them were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) and adjusted manually. Phylogenetic trees were inferred using Bayesian and maximum likelihood methods. Bayesian phylogenetic inference was carried out using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001), allowing jumps between alternative amino acid models in order to select the most appropriate model as well as estimating model parameters. The Jones model of amino acid replacement (Jones et al., 1992) was selected with a posterior probability of 1. Variation in the rate of evolution across sites was modeled by allowing a proportion of invariant sites, and rate variation among remaining sites was approximated by a discretized gamma distribution with four rate categories (both the proportion of invariant sites and the shape parameter of the gamma distribution were treated as free parameters). The phylogeny was reconstructed from samples obtained with four chains and convergence was achieved after 1.1×10^6 generations. Trees and parameters were sampled every 100 generations with a burn-in of 10,000 generations to obtain a consensus tree and estimates of clade posterior probabilities. A bootstrapped (100 replicates) maximum likelihood phylogenetic tree was inferred using PhyML3.0 (Guindon and Gascuel, 2003), with the Whelan and Goldman (Whelan and Goldman, 2001) amino acid replacement matrix and rate variation modeled as described above. Trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree).

RESULTS

Cloning of a POU gene from *Hydractinia*

Using degenerate primers against the POU and homeodomains of mammalian Oct4 proteins, we amplified a POU domain gene fragment from *Hydractinia* cDNA. The full-length coding sequence of the gene was obtained by RACE-PCR. We have named the gene *Polynem* (*Pln*) owing to its knockdown phenotype (see below). BLAST analysis of the predicted Polynem protein on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) gave the highest hits to various class 3 POU proteins. Our phylogenetic analysis using both Bayesian and maximum likelihood methods, however (Fig. 3), clustered the gene within POU class 5, but the posterior probability and bootstrap support for this placement were poor. It is therefore not possible to definitively assign the gene to a particular POU class. Class 5 is known only in vertebrates, includes Oct4, and is closely related to class 3, which includes vertebrate and invertebrate sequences. The predicted amino acid sequence of Pln is shown in Fig. S1 in the supplementary material, aligned to human OCT4. The full alignment of POU genes used to generate the phylogeny has been deposited in TreeBASE under accession number S11321.

An additional *Hydractinia* POU gene fragment was identified in an unpublished EST database. Analysis of this sequence revealed that it is closely related to POU class 6. The gene has not been analyzed further owing to the incompleteness of its known coding sequence.

Expression pattern of *Pln*

To study the spatial expression of *Pln*, we performed whole-mount in situ hybridization. These experiments showed that early embryos express *Pln* ubiquitously (Fig. 2B). During and following

Table 1. Proteins used for the phylogenetic analysis of POU proteins

Protein	Accession	Organism	POU class
Pituitary-specific positive transcription factor 1 isoform alpha	NP_000297	*Homo sapiens*	1
POU domain, class 4, transcription factor 1	NP_006228	*Homo sapiens*	4
POU domain, class 2, transcription factor 3	NP_035269.2	*Mus musculus*	2
PIT-1-beta	AAA41852.1	*Rattus rattus*	1
Brain-specific homeobox/POU domain protein 3	CAA63049	*Gallus gallus*	4
Pou-domain protein	CAA41342	*Drosophila melanogaster*	4
Pit1	CAJ38811	*Platynereis dumerilii*	1
POU2F3	NP_055167	*Homo sapiens*	2
POU domain, class 2, transcription factor 1, isoform CRA_b	NP_002688	*Homo sapiens*	2
OCT1	AAM77920.1	*Homo sapiens*	2
POU domain, class 2, transcription factor 2	NP_002689.1	*Homo sapiens*	2
Nubbin	P31368	*Drosophila melanogaster*	2
Pou12	CAA69213	*Danio rerio*	3
Ventral veins lacking	NP_523948	*Drosophila melanogaster*	3
OCT6	CAA79158.1	*Homo sapiens*	3
	NP_002690	*Homo sapiens*	3
POU50	AAH43847	*Xenopus laevis*	3
POU3	ABD97868.1	*Acropora millepora*	3
	AAW23073	*Oikopleura dioica*	3
Polynem	JF820067	*Hydractinia echinata*	3/5
Oct4	AAH68268	*Mus musculus*	5
Pou2	AAH49295	*Danio rerio*	5
Xoct60	M60075.1	*Xenopus laevis*	5
Xoct25	NP_001079832	*Xenopus laevis*	5
POU6F1	NP_002693	*Homo sapiens*	6
POU6F1	AAH85139	*Mus musculus*	6
POU domain, class 6, transcription factor 1	CAA48481	*Danio rerio*	6
Pou domain motif 3, isoform A	NP_610377	*Drosophila melanogaster*	6

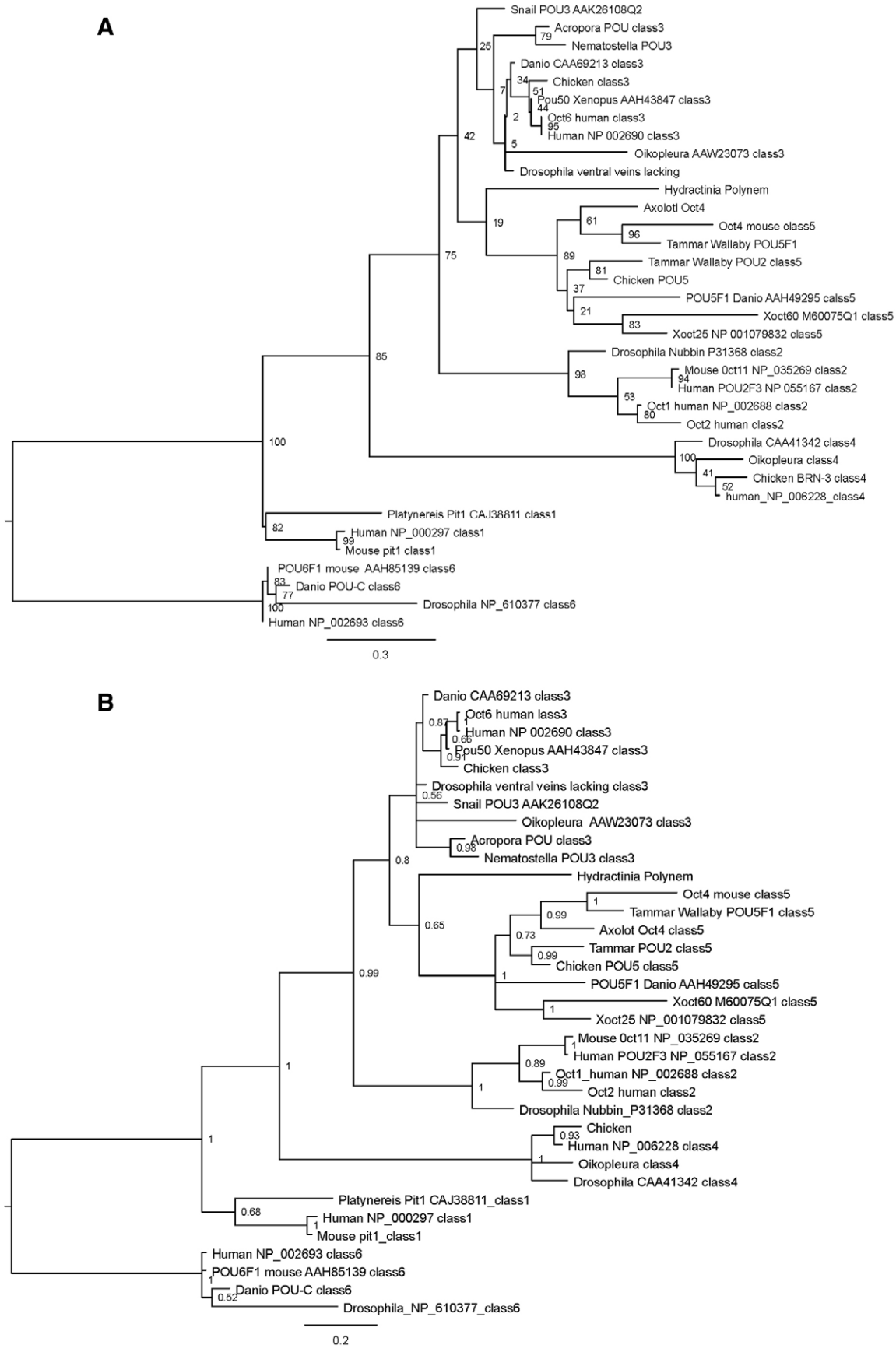

Fig. 3. Phylogenetic analysis of POU proteins. (**A**) Maximum likelihood. Numbers indicate bootstrap values. (**B**) Bayesian analysis. Numbers indicate posterior probabilities. The position of the root in both trees is arbitrary.

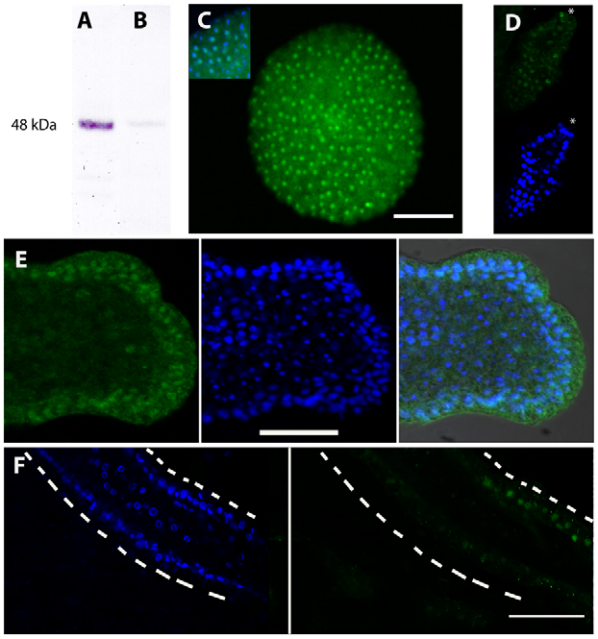

Fig. 4. Anti-human OCT4 antibody detects Pln. (**A**) Western blot of *Hydractinia* protein extract detects a protein at the expected size for Pln. (**B**) Blocking of antibody binding with a synthetic Pln peptide. (**C-F**) OCT4 immunoreactivity (green) and DAPI staining (blue). (C) Confocal image of an OCT4-stained morula. Inset, DAPI merge. (D) Budding polyp. Above, anti-OCT4; below, DAPI. Asterisks indicate oral pole. (E) Tip of a growing stolon. Left, anti-OCT4; middle, DAPI; right, merge. (F) Tentacle (outlined by dashed line) of a wild-type polyp. Left, DAPI; right, anti-OCT4. Scale bars: 100 μm in C; 50 μm in E,F.

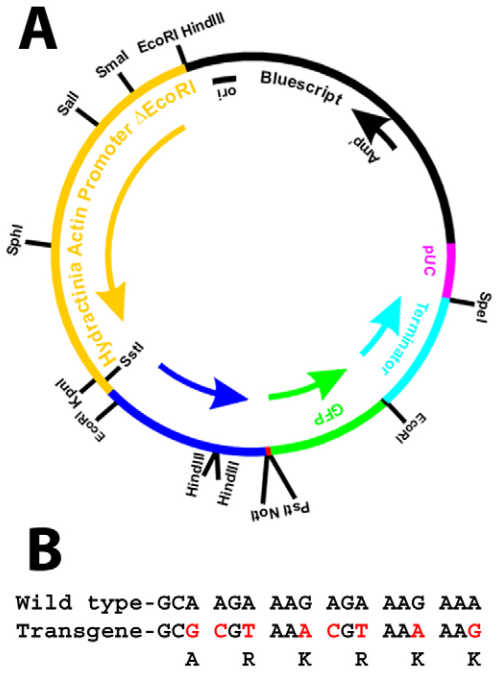

Fig. 5. Structure of the *Pln* expression vector. (**A**) Vector map. (**B**) The silent mutations (red) introduced into the *Pln* transgene, aligned with wild-type sequence.

metamorphosis, however, *Pln* transcripts were visible in i-cells (Fig. 2C-E). i-cells were identified by their morphology and anatomical location (Müller et al., 2004). The identification of i-cells and derivatives by morphology has been common practice in cnidarians (e.g. Fedders et al., 2004; Lindgens et al., 2004; Khalturin et al., 2007; Hartl et al., 2010) and is facilitated by their characteristic size and shape and by the relatively low number of cell types in these animals. Morphological identification, however, cannot distinguish between putative i-cell subpopulations, although some committed derivatives, such as nematoblasts, neuroblasts and gametes, can also be identified morphologically (e.g. developing nematocyst capsules) and by behavior (e.g. by forming characteristic clusters). Studies performed on *Hydra* have shown that large i-cells are the basic stem cells, whereas small i-cells represent the committed populations (for a review, see Bode, 1996). A number of i-cell markers have been identified in cnidarians, but their definition as such is also based on the morphology of the cells in which they are expressed, rather than on functional studies (Siebert et al., 2008; Hartl et al., 2010).

Protein distribution of Pln

To study the distribution of Pln protein, we used a polyclonal anti-human OCT4 antibody in western blots and whole-mount immunohistochemistry. According to its manufacturer (Abcam), this antibody was raised against an undisclosed peptide within the 60 C-terminal amino acids of human OCT4 (POU5F1). In our hands, the antibody detected a single band of approximately the expected size of Pln in *Hydractinia* protein extract (Fig. 4A). Pre-incubating the antibody in a solution of a synthetic peptide corresponding to amino acids 385-414 of Pln effectively blocked antibody binding to the band on the blot (Fig. 4B), suggesting that the OCT4-specific antibody cross-reacted with Pln. Pln immunohistochemistry, using the anti-human OCT4 antibody, stained nuclei ubiquitously in early embryos (Fig. 4C). At later stages, it stained the nuclei of cells in regions where i-cells normally reside (Fig. 4D,E) and showed little staining in regions poor in i-cells (Fig. 4F). Interestingly, Oct4 immunoreactivity has also been reported in urochordates in a germ cell context (Rosner et al., 2009), suggesting sharing of epitopes between mammalian and invertebrate stem cell-specific POU proteins, which are expressed in germ cells as well.

Ectopic expression of *Pln*

In order to study the function of *Pln* in vivo, we generated transgenic animals that ectopically express the gene. We designed an expression construct that included the full coding region of *Pln* fused to the enhanced green fluorescent protein (eGFP) coding sequence and driven by the *Hydractinia Actin1* promoter (Fig. 5). The *Hydractinia* genome encodes at least three Actin genes. The promoter of *Actin1* is epithelial specific in post-metamorphic animals and is not active in i-cells (Künzel et al., 2010). We used this promoter to force the expression of *Pln* in epithelial cells, where it is not expressed naturally. Silent mutations were introduced into the coding region of the *Pln* transgene (Fig. 5) that enabled us to design a TaqMan probe specific for the endogenous gene. qPCR with this TaqMan probe did not amplify any product when the expression construct was used as template, but effectively amplified a product from cloned wild-type *Pln* sequence (data not shown). Control transgenic animals were generated by injecting embryos with a construct lacking the *Pln* coding sequences, thereby consisting of GFP coding sequence alone downstream of the *Actin1* promoter.

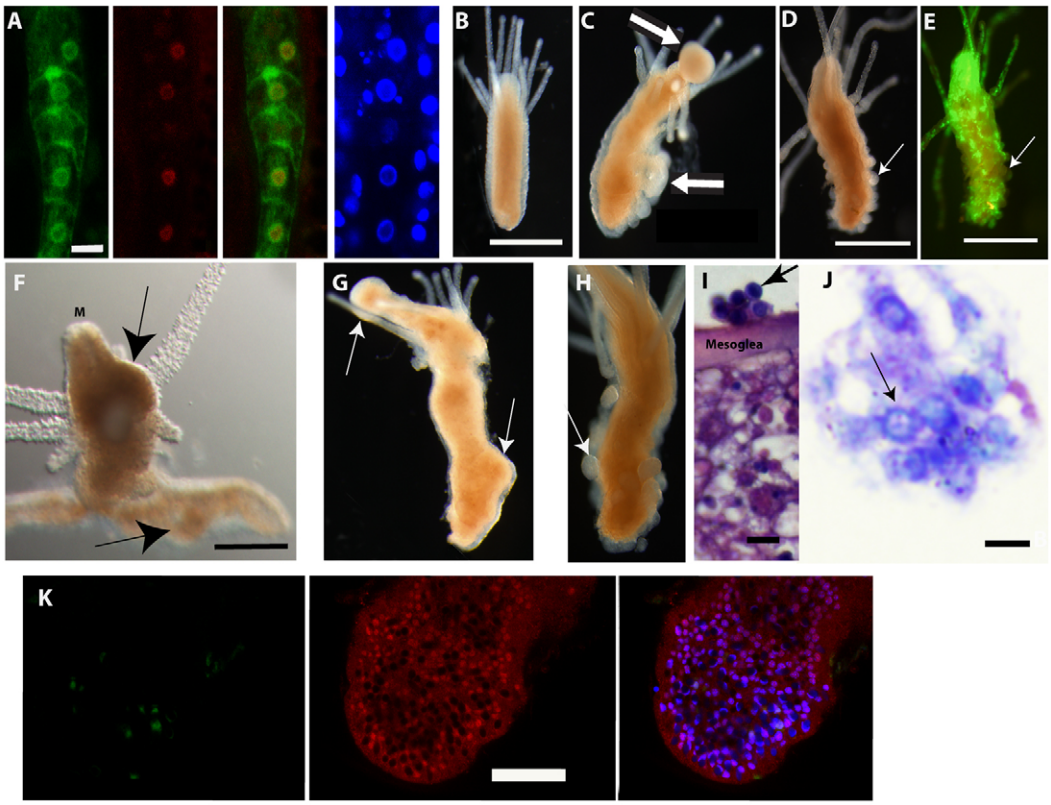

Fig. 6. Transgenic *Hydractinia*. (**A**) Confocal image of a *Pln* transgenic tentacle. From the left: GFP; anti-OCT4 (red); merge of GFP and anti OCT4; DAPI. (**B**) Control transgenic polyp expressing only GFP under the *Actin1* promoter. (**C-H**) *Pln* transgenic polyps with neoplasms (arrows). D and E are the same polyp under bright and blue light, respectively, showing non-fluorescent neoplasms. (F) An F1 animal resulting from crossing a transgenic female with a wild-type male. M, mouth. (**I**) Transverse section (5 μm) of polyp body wall showing small i-cell neoplasm (arrow) and lack of epithelial tissue on the mesoglea. (**J**) Transverse section (5 μm) through a neoplasm filled with i-cells (arrow), stained with May-Grünwald Giemsa. (**K**) Confocal image of a neoplasm stained with ani-OCT4 antibody. From left: GFP; anti-OCT4; merge of anti-OCT4 and DAPI. Scale bars: 10 μm in A; 500 μm in B-F; 10 μm in I,J; 50 μm in K.

One- or two-cell stage embryos were microinjected with the control or experimental constructs as described previously (Künzel et al., 2010). Stable GFP expression was evident within 48 hours. Confocal laser microscopy of *Pln* transgenic animals stained with the anti-OCT4 antibody showed that Pln protein was present in the nuclei of epithelial cells, co-localized with GFP, as expected (Fig. 6A). Epithelial cells in wild-type animals did not express *Pln* (Fig. 4F), further demonstrating the specificity of the antibody. *Pln* transgenic animals developed to planula larvae and were induced to metamorphose 4 days post-fertilization. Metamorphosis in *Pln* transgenic animals commenced normally. However, ~24 hours later, and during the next few days, all animals developed neoplasms (Fig. 6B-H). The neoplasms appeared as bumps all over the polyp body column, head and tentacles, as well as in the developing stolons, in some cases directly interfering with normal functions such as feeding. The neoplasm phenotype developed consistently in all transgenic animals, but some individuals seemed to be more severely affected, and most of these animals died within days or weeks post-metamorphosis. The differences in phenotype severity might have resulted from different proportions of transgenic to wild-type cells in the mosaic animals. Neoplasia has never been observed before in *Hydractinia* during decades of laboratory culture.

The animals showed loss of epithelial tissues, resulting, in some cases, in small neoplasms positioned on the extracellular matrix (mesoglea), which was free of any epithelial cells (Fig. 6I). Histological cross-sections through the neoplasms revealed that they contained numerous rounded small cells (7-10 μm) with large nuclei, strongly resembling i-cells in morphology and staining properties (Fig. 6I,J). Anti-OCT4 antibody immunohistochemistry showed Pln protein in their nuclei (Fig. 6K). The neoplasm cells were also positive for *Vasa*, *Nanos*, *Piwi* and *Myc*, as revealed by in situ hybridization (Fig. 7). These genes are known stem cell and germ cell progenitor markers in cnidarians (Mochizuki et al., 2000; Seipel et al., 2004; Torras et al., 2004; Extavour et al., 2005; Rebscher et al., 2008; Hartl et al., 2010). Interestingly, *Piwi* was expressed in markedly fewer cells than the other stem cell markers (Fig. 7).

qPCR using TaqMan probes specific for the endogenous *Pln* gene showed a significant increase (up to 250-fold) in the amount of endogenous *Pln* transcripts in *Pln* transgenic animals, as compared with tissues from animals that expressed only GFP under the same promoter (Fig. 7A). Neoplasms, however, were not fluorescent (Fig. 6E,K), indicating that their cells expressed only endogenous *Pln* but not the transgene. This is consistent with the neoplasm cells being predominantly i-cells because the transgene was under the control of the *Actin1* promoter, which is quiescent in i-cells. Hence, even transgenic i-cells were not expected to express the transgene but were expected to, and did, express the endogenous *Pln* gene, in addition to other stem cell markers as noted above.

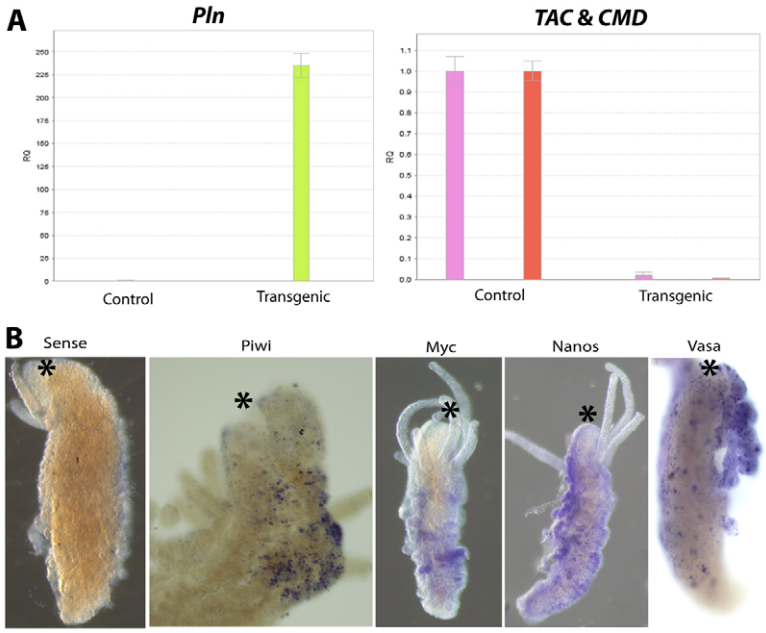

Fig. 7. Gene expression analysis in transgenic *Hydractinia*. (**A**) qPCR of control and transgenic animals showing *Pln* and epithelial marker [*TAC* (pink) and *CMD* (red)] expression. Expression is normalized to *Gapdh*. Error bars indicate s.d. (**B**) In situ hybridization of polyps showing high expression of stem cell markers in neoplasm tissues. Asterisks indicate the oral pole.

Metamorphosed transgenic animals displayed irregular cycles of increased neoplasia, with intermediate phases where neoplasms were less widespread. During the severe phases, whole polyps transformed into neoplasms and were no longer recognizable as polyps. This resulted in regression of the colony, being unable to feed, which then developed new polyps, initially without neoplasms. The colonies grew during these phases, until neoplasms spread again. The animals were generally in poor condition and their tissues were mechanically very labile, showing loss of epithelial tissues, as mentioned above (Fig. 6G,I). This was also evident by downregulation of the epithelial markers *Cytosolic malate dehydrogenase* (*CMD*) and *Tubulin alpha chain* (*TAC*), as assessed by qPCR (Fig. 7). These genes have been shown to be epithelial cell markers in *Hydra* (Hwang et al., 2007) and we confirmed them as having a similar expression pattern in *Hydractinia* (see Fig. S2 in the supplementary material). The loss of epithelial tissue often resulted in fragmentation, in which neoplasms or whole polyps fell off the colony. A short pronase digestion completely dissociated the animals into single cells within 2 hours, as compared with incomplete digestion after 4 hours of normal animals (not shown), which is also consistent with loss of epithelial cells. For a list of the *Pln* phenotypes see Table 2.

Of more than 80 transgenic animals, only three survived for more than 3 months. All the others died earlier when most of their epithelial tissues regressed and were replaced by stem cell neoplasms (Fig. 6I). The three surviving colonies were subcloned by cutting off small pieces and attaching them to glass slides. This resulted in 20 independent colonies for each of the three clones. These animals reached sexual maturity only very late, after more than a year of growth. Normally, sexual maturity is reached within 2-3 months in the laboratory. Sexual polyps bearing immature gonads did appear prior to the delayed sexual maturity; however, they tended to transform into neoplasms before maturing. One male and one female *Pln* transgenic clone (i.e. 40 colonies each) eventually released transgenic gametes. Attempts to fertilize transgenic eggs with transgenic sperm were unsuccessful. We therefore crossed transgenic eggs with wild-type sperm, resulting in 70 F1 transgenic animals at very low fertilization rates (<1%). These animals developed to planula

Table 2. Phenotypes of ectopic *Pln*-expressing animals

Phenotype	Frequency	Remarks
Nuclear GFP	All transgenic cells	Epithelial cells only
Nuclear OCT4 immunoreactivity	All transgenic epithelial cells and all i-cells	Using anti-human OCT4 antibody
Neoplasms	Variable in time	All animals had unstable neoplasia
Loss of epithelial cells and fragile tissue	All cases	Complete dissociation with short pronase treatment; spontaneous disintegration
Fertility	Always low	Only two animals out of 80 reached sexual maturity; fertilization rates of transgenic gametes was low
Death	Over 90%	Within 3 months post-metamorphosis
Differentiation following *Pln* downregulation	All cases in metamorphosed animals	Occurred after *Pln* RNAi or retinoic acid treatment
Stem cell gene expression	In all neoplasm tissues	*Pln*, *Myc*, *Nanos*, *Vasa*, *Piwi*; *Piwi* expression in fewer cells
Epithelial marker gene expression	Downregulated in transgenic animals	Upregulated following *Pln* downregulation

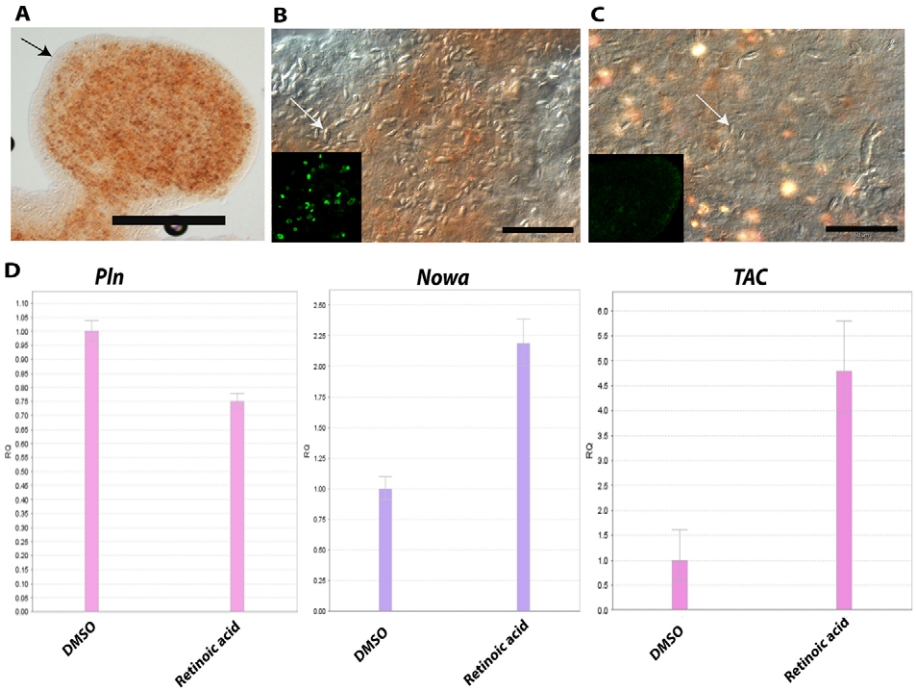

Fig. 8. Retinoic acid treatment of a transgenic *Hydractinia* polyp. (**A**) Bright-field image of a neoplasm treated with retinoic acid (RA). Arrow indicates thickened ectoderm. (**B**) DIC image of an RA-treated neoplasm. Numerous nematocytes (arrow) are visible. (**C**) DIC image of a control neoplasm treated with DMSO alone. Only a few nematocytes are visible (arrow). Insets in B and C show NCol-1 antibody staining of nematocytes. (**D**) qRT-PCR of RA-treated neoplasm showing downregulation of *Pln* (left) and upregulation of nematocyte (*Nowa*) and epithelial (*TAC*) markers. Expression is normalized to *Gapdh*. Error bars indicate s.d. Scale bars: 100 μm in A; 50 μm in B,C.

larvae, but died shortly after metamorphosis with neoplasms spreading in their tissues (Fig. 6F), showing that the phenotype is fully heritable.

Retinoic acid treatment of neoplasm-containing animals

Retinoic acid (RA) is known to cause differentiation of mammalian embryonic stem cells and to downregulate *Oct4* (Gu et al., 2005; Stavridis et al., 2010). Furthermore, RA has been found to promote neural differentiation in primary cell cultures of the sea pansy *Renilla koellikeri* (Estephane and Anctil, 2010). With this background in mind, we treated adult *Pln* transgenic polyps containing neoplasms with RA. The treatments resulted in downregulation of *Pln* expression and upregulation of *Nowa*, a nematocyte early differentiation marker (Engel et al., 2002) (Fig. 8). Nematocytes are cnidarian-specific stinging cells that belong to the neurosensory lineage. The numbers of nematocytes increased significantly in the neoplasms within a few days of RA treatment (Mann-Whitney *U*-test: $P<0.001$). Nematocytes were identified by morphology and by the nematocyst-specific antibody NCol-1. Counting was performed by randomly selecting ten RA-treated and ten untreated neoplasms and counting all NCol-1-positive nematocytes in a complete confocal *z*-stack. Treatment of wild-type embryos at 10 hours post-fertilization with RA was lethal.

The epithelial marker *TAC* was also upregulated following RA treatment. Consistent with this upregulation, neoplasms acquired thickened epithelia several days following the treatment (Fig. 8A). There was also an increase in gland cell numbers compared with untreated neoplasm tissue (not shown), but this has not been assessed quantitatively. These results, and recently published work by others (Estephane and Anctil, 2010), suggest that the function of RA in stem cell differentiation is conserved between cnidarians and vertebrates, at least in some aspects. Our data demonstrate the broad developmental potential of neoplasm cells, which are at least multipotent.

RNAi downregulation of *Pln*

Since RA may act through various mechanisms, we aimed to specifically downregulate Pln by RNAi. RNAi knockdown of Pln in neoplasm-containing transgenic animals for 24 hours resulted in similar effects to RA treatment (see Fig. S3 in the supplementary material). Treating 10-hour-old wild-type embryos with Pln dsRNA for 24 hours was lethal within the treatment time (Fig. 9A,B), suggesting that the gene is also required for normal development. RNAi downregulation of Pln in wild-type metamorphosed animals (3 days post-metamorphosis, 48 hours treatment) resulted in a significant increase in nematocytes, as assessed as for RA treatment above (Mann-Whitney *U*-test: P=0.001). This is why we named this Hydractinia POU protein Polynem – for 'many (POLY) NEMatocytes'. Control experiments using non-coding dsRNA had no effect on the animals (Fig. 9A). Collectively, these results suggest that Pln is required for maintaining stemness in i-cells, and that loss of Pln leads to the differentiation of stem cells.

DISCUSSION

Pln is similar to POU class 3 and class 5 proteins. In both the Bayesian and the maximum likelihood phylogenies, Pln was in a clade consisting entirely of class 5 proteins (Fig. 3), but the statistical support for the trees was poor. This probably resulted from the high similarity of the POU and homeo domains in classes 3 and 5. The branch separating the two classes was also poorly supported (Fig. 3). In addition to poor statistical support the inferred phylogenetic trees were found to be sensitive to modeling assumptions, resulting, in one case, in paraphyly of class 3 (data not shown). Hence, the position of Pln within the POU protein family could not be definitively resolved. It has to be noted that POU classification predates the massive sequencing projects of the 2000s (Verrijzer and Van der Vliet, 1993), resulting in many invertebrate POU genes not being considered. Upon discovery, these genes might have been

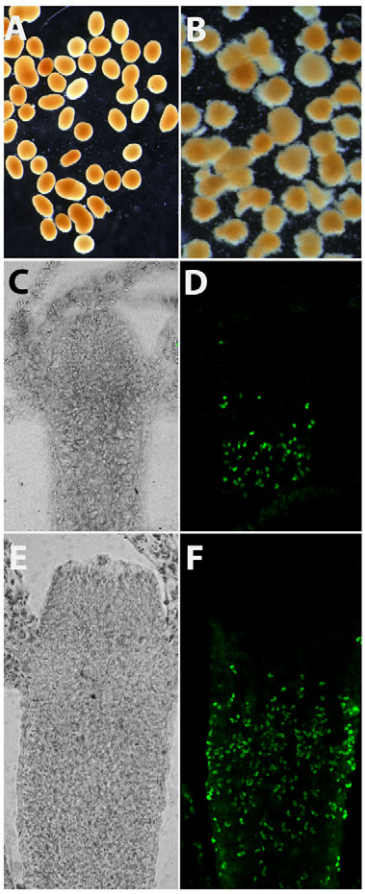

Fig. 9. *Pln* downregulation in wild-type animals by RNAi. (**A**,**B**) Early *Hydractinia* embryos, 24 hours into the experiment. (A) Control non-coding dsRNA. (B) *Pln* dsRNA. (**C-F**) Metamorphosed polyps stained with NCol-1 antibody to detect nematocytes. (C,D) Control, non-coding dsRNA. (E,F) *Pln* dsRNA. (A,B,C,E) Bright-field images; (D,F) blue light to detect nematocytes stained by NCol-1 antibody. The same animals are shown in C,D and in E,F.

annotated as class 3 because class 5 contained only very few known sequences and was less diverse than class 3. A new classification of POU proteins might be due.

Pln was expressed ubiquitously in early embryos, but later in development and in post-metamorphic stages it was expressed in i-cells based on their morphology and anatomic location (Frank et al., 2009). The typical morphology of i-cells and the relatively low number of cnidarian cell types have made their identification by morphology common practice in the cnidarian research community (e.g. Hartl et al., 2010). Cnidarian stem cell marker genes have thus far been defined by their expression pattern in morphologically identified i-cells, and *Pln* is the first i-cell gene to be studied at the functional level, using both ectopic expression and knockdown approaches.

Anti-human OCT4 antibodies cross-reacted with Pln. This was demonstrated by western blotting, which showed a single band of the expected size for Pln in *Hydractinia* protein extract (Fig. 4A), and by successfully blocking the antibody binding with a synthetic Pln peptide (Fig. 4B). The nuclear staining of the antibody is consistent with the expected subcellular localization of Pln, a putative transcription factor. Anti-Oct4 antibodies have also been used to stain stem cells and germ cells in the colonial tunicate *Botryllus schlosseri* (Rosner et al., 2009), further supporting an ancient role for POU3/5 proteins in animal stem cells.

The ectopic expression of *Pln* induced neoplasms, which have never been observed in *Hydractinia* before. The phenotype was consistent in all transgenic animals (n>80) and was also fully heritable (n>70) (Fig. 6F). The neoplasms were composed of cells that not only resembled i-cells in morphology and staining pattern, but also expressed the known cnidarian stem cell genes *Vasa*, *Nanos*, *Myc* and *Piwi* (Fig. 7). Interestingly, *Piwi* was expressed in only a subset of neoplasm cells and could mark a specific but as yet uncharacterized subpopulation. We also detected a substantial increase in *Pln* expression in transgenic animals. The silent mutations that we introduced into the transgene rendered it undetectable by the qPCR TaqMan probe employed, which was specific for the endogenous gene. Therefore, the recorded increase in expression of *Pln* is likely to have been a result of increased numbers of i-cells expressing the endogenous gene, following ectopic expression of *Pln* in epithelial cells.

A central question arising from our data is the origin of the neoplasm i-cells in this unprecedented high-impact phenotype, which was caused by ectopic expression of a single gene in epithelial cells. Two possible mechanisms could account for this observation: (1) the expansion of resident i-cells; (2) the reprogramming or dedifferentiation of epithelial cells following forced *Pln* expression. We think that the former mechanism is unlikely and favor the latter for the following reasons. First, the transgene was epithelial cell-specific and quiescent in i-cells and could therefore not have affected resident i-cells directly, even if they were transgenic. Second, we also observed neoplasms in areas normally poor in i-cells, such as in heads and tentacles of mature polyps (Fig. 6). In a previous study (Teo et al., 2006), we showed that global activation of the canonical Wnt pathway causes a proliferative burst of i-cells in stolons (where i-cells normally reside), but not in heads or tentacles, as occurred in the present study. Finally, transgenic animals lost epithelial tissues (Fig. 6I) and had reduced expression of epithelial markers, while upregulating endogenous *Pln*, *Nanos*, *Myc*, *Vasa* and *Piwi* (Fig. 7). This is consistent with dedifferentiation of transgenic epithelial cells into i-cells, which, upon dedifferentiation, lost the epithelial-specific transgene expression and reactivated their endogenous i-cell-specific *Pln* gene and other stem cell genes. We cannot rule out the possibility of expansion of resident i-cells, however.

RA treatment downregulated *Pln*, upregulated nematocyte and epithelial markers and induced the differentiation of neoplasm i-cells into nematocytes, epithelial cells and presumably other cell types such as gland cells. It has been shown previously that exogenous RA also affects axis formation in this species (Müller, 1984), further supporting an ancient role for RA in animal development. dsRNA-mediated knockdown of *Pln* caused an increase in nematocytes in wild-type animals and was lethal in embryos (Fig. 9). Based on these results and the forced *Pln* expression in epithelial cells, which induced i-cell neoplasms, we suggest that Pln is essential to maintain, and sufficient to induce, stemness in *Hydractinia* cells.

Evidence for the involvement of POU genes in invertebrate stem cells is scarce (e.g. Bhat and Apsel, 2004; Reddien et al., 2005a). Our results point to an ancient role of POU proteins in stem cells, but this function might have been lost in some lineages. The pluripotent cell type could have evolved in early metazoans by maintaining the expression of germ cell

transcription factors, forming a stable network based around class 3/5 POU genes. This network sustains an open chromatin structure (Niwa, 2007) that keeps these cells undifferentiated. Pluripotency is lost in somatic cells during gastrulation in many animals, including mammals, but their germ lines continue to express pluripotency genes that induce pluripotency in embryos of the next generation (Seydoux and Braun, 2006). Other animals, such as *Hydractinia*, retain POU3/5-expressing stem cells that give rise to somatic and germ cells throughout life. Our data suggest that the core pluripotency transcriptional network might be more highly conserved in animals than previously thought. Future studies will reveal the degree of conservation as well as lineage-specific adaptations.

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Competing interests statement
The authors declare no competing financial interests.

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