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9-3-2012

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EvoDevo

Sequencing and analysis of the gastrula transcriptome of the brittle star *Ophiocoma wendtii*

Roy Vaughn[1], Nancy Garnhart[2], James R Garey[1], W Kelley Thomas[2] and Brian T Livingston[3*]

Abstract

Background: The gastrula stage represents the point in development at which the three primary germ layers diverge. At this point the gene regulatory networks that specify the germ layers are established and the genes that define the differentiated states of the tissues have begun to be activated. These networks have been well-characterized in sea urchins, but not in other echinoderms. Embryos of the brittle star *Ophiocoma wendtii* share a number of developmental features with sea urchin embryos, including the ingression of mesenchyme cells that give rise to an embryonic skeleton. Notable differences are that no micromeres are formed during cleavage divisions and no pigment cells are formed during development to the pluteus larval stage. More subtle changes in timing of developmental events also occur. To explore the molecular basis for the similarities and differences between these two echinoderms, we have sequenced and characterized the gastrula transcriptome of *O. wendtii*.

Methods: Development of *Ophiocoma wendtii* embryos was characterized and RNA was isolated from the gastrula stage. A transcriptome data base was generated from this RNA and was analyzed using a variety of methods to identify transcripts expressed and to compare those transcripts to those expressed at the gastrula stage in other organisms.

Results: Using existing databases, we identified brittle star transcripts that correspond to 3,385 genes, including 1,863 genes shared with the sea urchin *Strongylocentrotus purpuratus* gastrula transcriptome. We characterized the functional classes of genes present in the transcriptome and compared them to those found in this sea urchin. We then examined those members of the germ-layer specific gene regulatory networks (GRNs) of *S. purpuratus* that are expressed in the *O. wendtii* gastrula. Our results indicate that there is a shared 'genetic toolkit' central to the echinoderm gastrula, a key stage in embryonic development, though there are also differences that reflect changes in developmental processes.

Conclusions: The brittle star expresses genes representing all functional classes at the gastrula stage. Brittle stars and sea urchins have comparable numbers of each class of genes and share many of the genes expressed at gastrulation. Examination of the brittle star genes in which sea urchin orthologs are utilized in germ layer specification reveals a relatively higher level of conservation of key regulatory components compared to the overall transcriptome. We also identify genes that were either lost or whose temporal expression has diverged from that of sea urchins.

Keywords: Brittle star, Gene regulatory networks, Evolution, Transcriptome

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Background

Sea urchins (Class Echinoidea) have been used as model organisms in developmental biology for more than a century. Over the last two decades intensive work has led to a fairly detailed understanding of the gene regulatory network (GRN) controlling the differentiation of the embryonic germ layers during development in the species *Strongylocentrotus purpuratus* [1-6]. An initial draft of the *S. purpuratus* genome was completed in 2006 [7] and is now in its third revision [6]. Several expression databases for various embryonic stages have also been constructed using expressed sequence tags (ESTs) [8-11], microarrays [12], and NanoString RNA counting [13]. Here we begin to examine the conservation and divergence in the gene regulatory networks expressed at the gastrula stage in a member of a different echinoderm class, the Ophiuroidea. Our results indicate that, although there are differences that reflect changes in the developmental processes, there is a shared 'genetic toolkit' central to the echinoderm gastrula, a key stage in embryonic development.

The echinoderms consist of five living classes: Asteroidea (starfish), Echinoidea (sea urchins and sand dollars), Ophiuroidea (brittle stars) Holothuroidea (sea cucumbers), and Crinoidea (sea lilies and feather stars). The crinoids appear first in the fossil record and are clearly the most basal anatomically. The other four classes appear to have all diverged within a very short geological period around 500 million years ago [14], and the exact phylogenetic relationship of the brittle stars to the other classes remains uncertain due to conflicts between molecular,
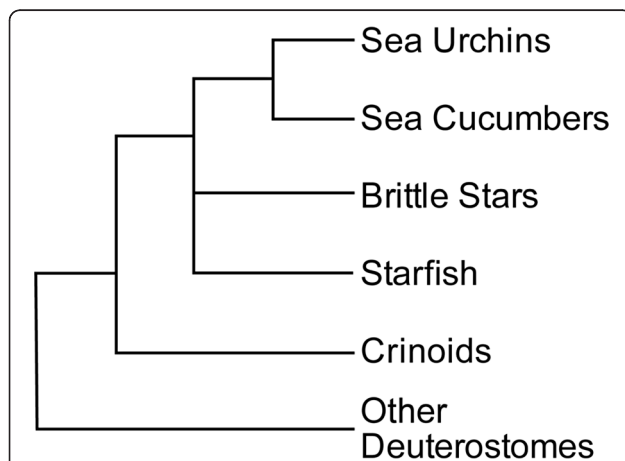
morphological, and embryological evidence [15,16] (Figure 1). The embryos of all echinoderm classes share some features, including holoblastic cleavage and similar cell movements during gastrulation. However, there are notable differences, such as the formation of micromeres in sea urchins but not brittle stars (Figure 2), the absence of pigment cells in brittle stars, and the formation of an embryonic skeleton in sea urchins and brittle star embryos, but not in the other groups. What is currently unclear is how these similarities and differences in development are reflected in the pattern of gene transcription. Davidson and Erwin [17] have suggested that key gene regulatory subcircuits central to the formation of major morphological features ('kernels') are very highly conserved by stabilizing natural selection, both because they are critical to the formation of a complete viable body and because their internal linkages and feedback loops make their component genes mutually dependent. A refinement of this idea is that some of the component transcription factors may be exchanged for others as long as the overall input/output logic and reliability of the circuit and its resulting function are maintained [18]. This suggests that many of the regulatory kernels shown to be important in sea urchin gastrulation would be conserved in the other echinoderm groups.

The set of genes that control skeleton formation in echinoderms may represent such a circuit under evolutionary constraints. All echinoderms form skeletons as adults; however, only sea urchins and brittle stars form extensive embryonic skeletal spicules. It has recently been shown that most of the same regulatory genes that underlie skeletogenesis in the sea urchin embryo are also expressed in the construction of the adult skeleton in both sea urchins and starfish [19]. The embryonic skeletons of sea urchins and brittle stars are thus thought to be derived characters resulting from early activation of an adult gene regulatory network in the embryo.

The process of embryonic skeletogenesis has been extensively studied in sea urchins [20]. Asymmetric fourth and fifth cleavages produce four small micromeres and four larger micromeres at the vegetal pole. Descendants of the larger micromeres ingress into the blastocoel just prior to gastrulation and become the primary mesenchyme (PMC), which soon produces the embryonic skeleton. Micromeres are a derived character unique to crown group sea urchins (euechinoids) [21]. Brittle stars and the more basal sea urchin groups form very similar embryonic skeletons from apparently homologous mesenchymal cells without prior unequal cleavages [22], Figure 2.

We have sequenced and characterized the 40-h gastrula transcriptome of the brittle star *Ophiocoma wendtii*. The gastrula stage was chosen because it represents the point in development at which the three primary

Figure 1 Phylogeny of echinoderms. All evidence indicates that crinoids are the most basal. The other four groups all diverged within a very short geological timeframe around 500 million years ago. Urchins and sea cucumbers are generally considered to form a clade of the most derived. It remains unclear whether the brittle stars group more closely with this clade or with starfish, due to conflicts between molecular, morphological, and embryological evidence [14-16].
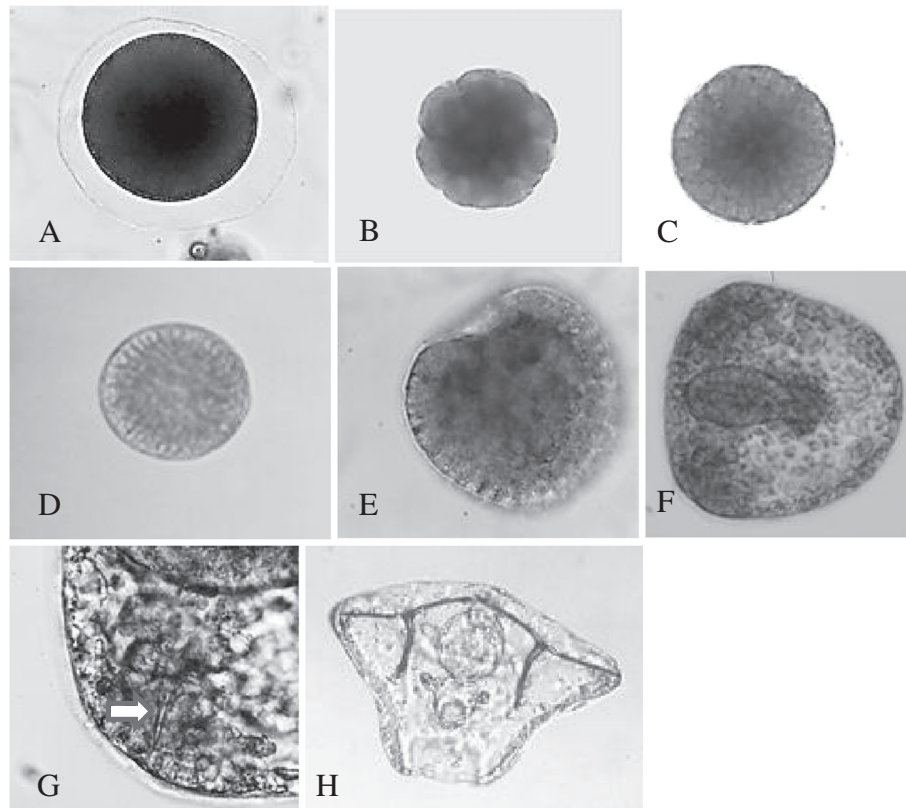
Figure 2 *Ophiocoma wendtii* embryonic development. Stages (**A**) egg, (**B**) 16 cell (5 h), (**C**) hatched blastula (16 to 18 h), (**D**) mesenchyme blastula (24 h to 26 h), (**E**) early gastrula (28 to 30 h), (**F**) gastrula (38 to 40 h), (**G**) ventrolateral cluster with skeletal spicule (arrow) at 40 h, (**H**) pluteus (80 h).

germ layers diverge, with ingression of mesenchymal cells and invagination of the gut. At this point in sea urchins the gene regulatory networks that specify the germ layers are established and the genes that define the differentiated states of the tissues have begun to be activated. The early gastrula therefore expresses the greatest number and diversity of developmentally important genes. We report here that the brittle star gastrula expresses genes of all functional classes and appears to share many key developmental regulatory components with other echinoderms. Some regulatory genes, as well as genes expressed in differentiated tissues in the sea urchin gastrula, were not expressed in the brittle star gastrula.

Methods
Animals and embryos
Brittle stars (*O. wendtii*) were collected from reefs and rubble piles in the shallow waters of Florida Bay near the Keys Marine Laboratory, Long Key, Florida, between April and October. Animals were sorted by sex, with gravid females identified by the presence of swollen purple gonads visible through the bursal slits. Sperm was obtained from two to three males by injection of 1 to 3 mL of 0.5 to 1.0 M KCl. Shedding of eggs from females was induced by a combination of heat and light shock. Animals were placed in containers in the dark with aeration at 30 to 32°C. Periodically the animals were exposed to bright light. Developing embryos were cultured at 25 to 27°C in filtered sea water. RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol.

Characterization of transcriptome sequences
Sequencing and assembly of contiguous sequences was carried out as described by Meyer *et. al.* [23]. The comparisons of gastrula transcriptomes were followed by an all-by-all BLAST [24] approach where each comparison was databased. These results were then queried for the identification of orthologous genes using a reciprocal best BLAST (RBB) strategy, and for the identification of gene families following the method of Lerat, *et al.* [25] as implemented previously [26,27]. Gene families and singletons were then annotated using Homology Inspector (HomIn) software, a Java program that stores and queries a set of gene families using the database tool

db4o for Java version 7.12 [28]. HomIn links gene families with annotation information including Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology Database (KO) categories [29], Clusters of Orthologous Groups of proteins (COGs) [30,31], Gene Ontology (GO) categories [32], or any other available annotation.

Search for GRN components
Glean3 predicted protein sequences for genes involved in the S. purpuratus developmental gene regulatory network were retrieved from SpBase [6] using the official gene name. These were used as queries to search the brittle star gastrula transcriptome sequences using TBLASTN at default settings. The best hit for each query was then used to search back against both sea urchin protein sequences and GenBank reference proteins using BLASTX. Sea urchin genes which had RBB hits to brittle star with e-values of 1e-9 or better in both directions were designated as present in the brittle star gastrula transcriptome. These sequences can be found in Genbank using accession numbers JX60067 to JX60106.

Database and analyses
Results from the automated BLAST searches were saved to a Microsoft Access (Microsoft Corporation, Redmond, WA, USA) database. This database and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) were used for the analyses involving presence/absence of expression, functional classes, and numbers of matches to other databases. Rarefaction curves were generated using EcoSim software [33]. For the functional class analysis, KEGG ortholog clusters were used if they included genes from at least one animal taxon. When a KEGG cluster participates in more than one pathway within a functional class, it was counted only once within the larger functional class. For example, K00128 aldehyde dehydrogenase (NAD+) is part of five different pathways within the class of carbohydrate metabolism and two pathways in lipid metabolism, among many others, but was counted only once within each class in Figure 3B, and once in the total number of distinct KEGG animal clusters in Figure 3A.

Results and discussion
Embryonic development of O. wendtii
The key stages of O. wendtii development are shown in Figure 2. The egg is pigmented, and pigment granules are retained during cleavage stages but disappear in the blastula. Cleavage is radial and holoblastic and is equal throughout cleavage, such that the micromeres characteristic of the sea urchin fourth cleavage division are not produced. A hollow blastula is formed and cells ingress into the blastula to initiate gastrulation. The number of ingressing cells seems much larger than is typical in sea urchins, but we have not quantified the number or traced the lineage of individual cells. Archenteron formation occurs through invagination and convergent extension. A second group of mesenchyme cells forms at the tip of the archenteron and gives rise to the coelomic pouches, but no pigment cells appear. The skeletogenic mesenchyme cells gather in ventrolateral clusters as in sea urchins and begin to form the mineralized skeleton. The timing of development to hatching blastula is similar to sea urchins. However, following the invagination of endoderm, brittle star development proceeds at a slower rate relative to sea urchins. There is an initial invagination at 26 to 30 h post-fertilization, but this persists for several hours before overt endomesoderm development proceeds. Also, unlike that seen in sea urchins, the elongation of the skeletal rods is delayed relative to the extension of the archenteron, such that the archenteron has extended one-third to halfway across the blastocoel before skeletal elements appear. When the gut is fully formed the skeleton is still composed of relatively small tri-radiate spicules. These then elongate such that the pluteus larva is very similar to that of sea urchins. The stage at which we isolated RNA for sequencing analysis is similar to Figure 2F. We chose that point when skeletal elements were first visible.

Sequencing and assembly
Pyrosequencing was performed on mRNA from gastrula stage brittle star embryos. After cleaning and trimming, there were 354,586 sequencing reads with a total of 75,031,136 bp(Figure 4A). Sequencing read lengths ranged from 16 to 439 bp, with approximately three-fourths of the reads being between 200 and 300 bp. Less than one percent were longer than 300 bp. Reads of 15 bp or shorter after trimming were not used for contig assembly. A total of 14,261 contigs were assembled, with a combined length of 5,488,581 bp (Figure 4B). Median length increased by 23% over that of the unassembled reads (282 vs. 229), while average length increased by 81% (384 vs. 212). Roughly two-thirds had lengths between 100 and 400 bp. The average number of reads per contig was 16.3, with a median of 5, a mode of 2, and a maximum of 8,989. Coverage or depth ranged from 1x to 8549.4x, with an average of 7.1, median of 3.5, and standard deviation of 44.6 (Figure 4C).

Automated annotation
Reciprocal best BLAST (RBB) searches identified brittle star transcripts putatively corresponding to a total of 3,385 orthologous genes in other databases (Figure 5). The brittle star sequences were translated in all six reading frames, and blastp was used to query the SpBase sea urchin Glean3 protein models. There were 3,303
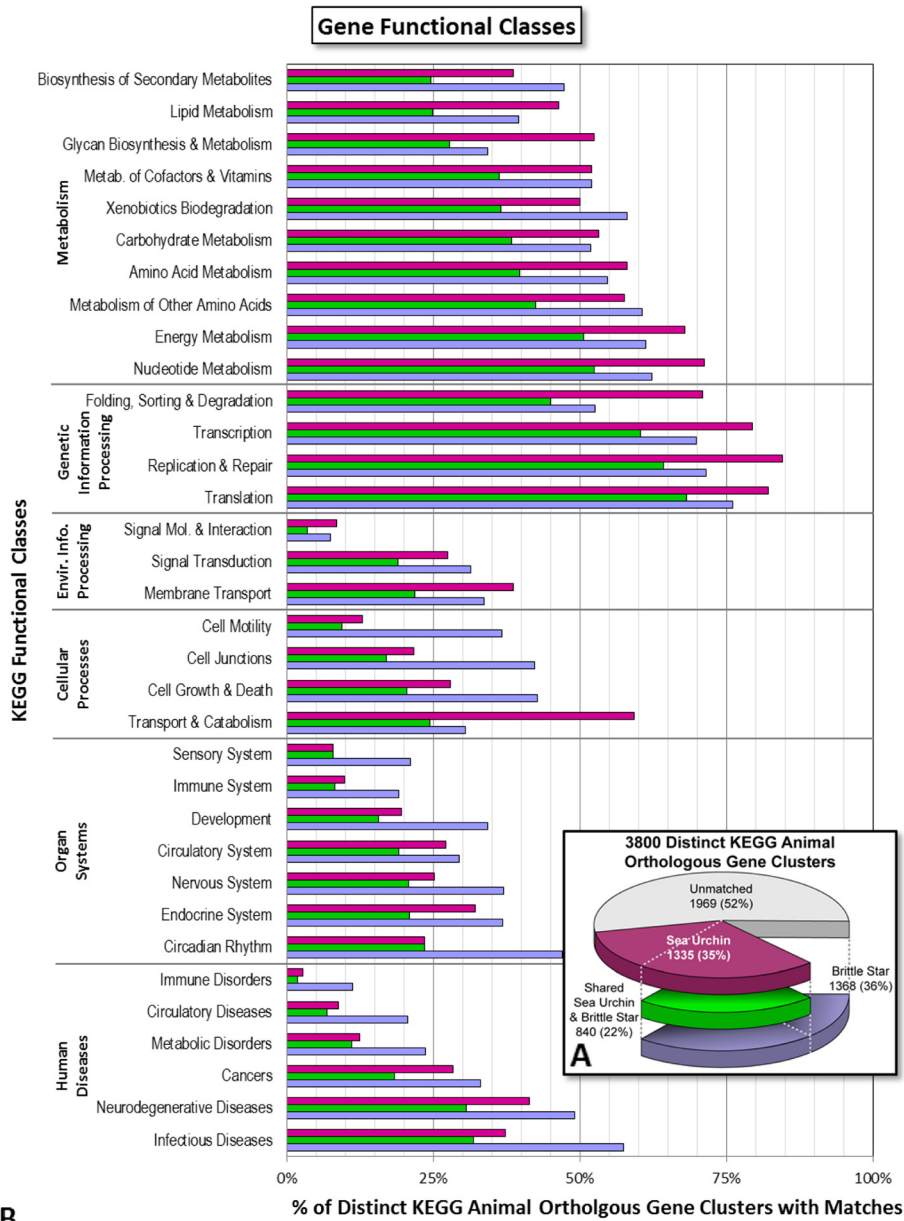
Figure 3 Gene functional classes found in brittle star gastrula transcriptome. (A) *Ophiocoma wendtii* sequences were compared to the KEGG Orthology database by reciprocal best BLAST. Of 3,800 distinct KEGG animal gene clusters, 36% had significant matches to brittle star (blue), and 35% had matches to sea urchin (purple). Green shows the overlap between these two sets, indicating the KEGG clusters that match to both organisms (22%). **(B)** When sorted into functional classes, an average of 43%, 39%, and 28% of the KEGG clusters within each class had matches to brittle star, to sea urchin, or to both, respectively, with a majority of classes having similar representation in both organisms.

matches between brittle star and the sea urchin genome [7]. Of these, 1,863 also matched to the sea urchin combined UniGene transcriptome libraries [34]. The KEGG Orthology database [35] produced 1,368 matches. More than two-thirds (2,309 or 68%) of the identified brittle star genes had matches to more than one dataset. Almost a quarter (840 or 24.8%) matched to all three.

Note that the *O. wendtii* data were compared against data in each of the other datasets in Figure 5 separately. Therefore, brittle star sequences with hits to multiple datasets do not necessarily represent RBB matches between every component in the annotation, but merely represent significant hits between brittle star and more than one of the other datasets independently.
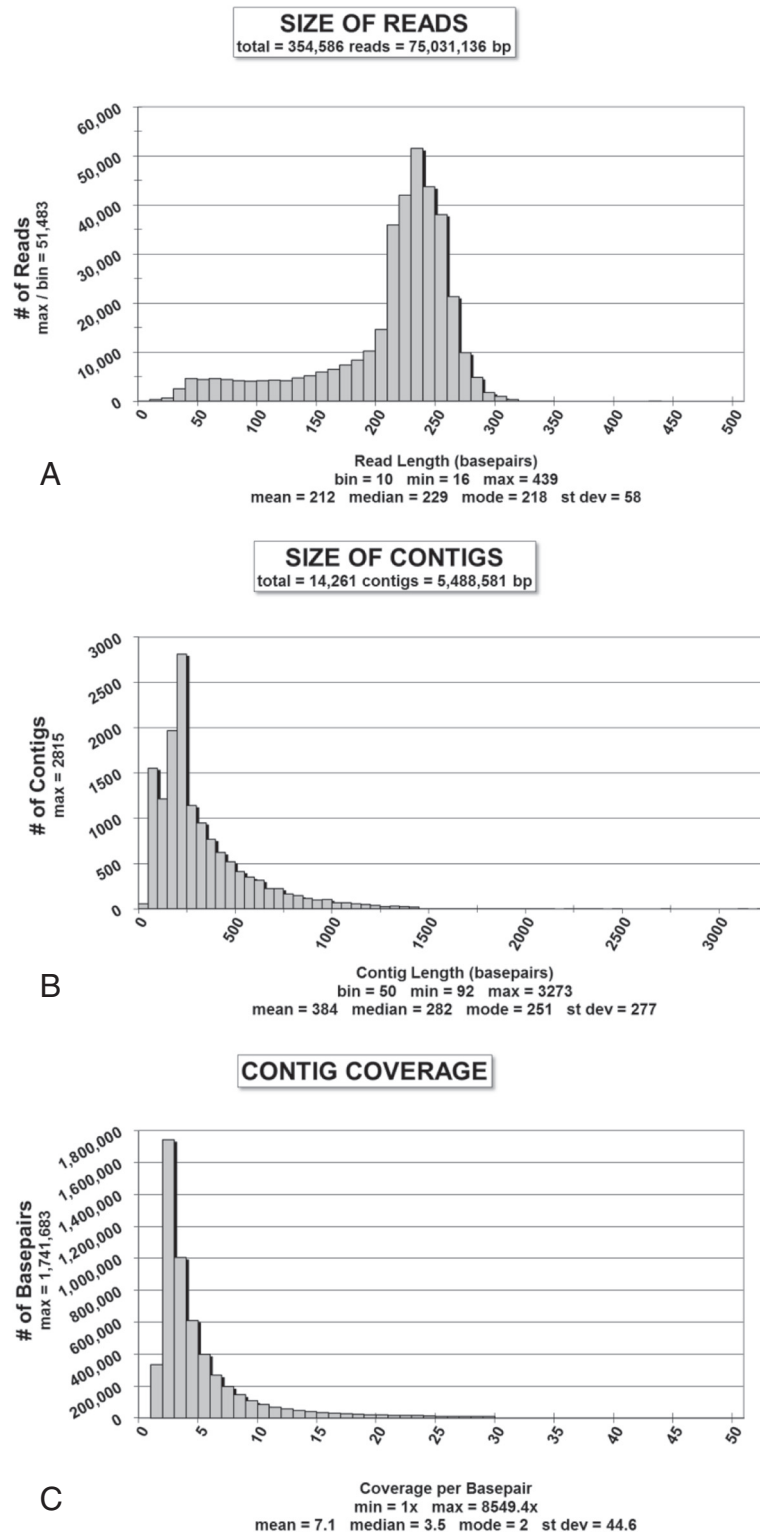
Figure 4 Pyrosequencing of brittle star transcriptome. (**A**) After cleaning and trimming, 354,586 reads totaled 75,031,136 bp. Approximately 3/4 had lengths between 200 and 300 bp. Less than one percent were longer than 300 bp. (**B**) A total of 14,261 contigs were assembled, with a combined length of 5,488,581 bp. Median length increased by 23% over that of the unassembled reads. Roughly two-thirds of the reads had lengths between 100 and 400 bp. Four percent were longer than 1,000 bp, creating a long right-hand tail to the distribution. (**C**) The number of times a given nucleotide position is present in the reads used to assemble the contigs ranged from 1x to 8549.4x. Eighty-one percent were represented one to five times, while less than one percent had more than 100× coverage.

Figure 5 BLAST identification of brittle star genes. Automated BLAST was used to align *Ophiocoma wendtii* cDNA sequences to both the genome and transcriptome of the sea urchin *Strongylocentrotus purpuratus*, as well as to the KEGG Orthology database. The areas of the smaller circles represent the number of significant reciprocal best BLAST hits to the indicated datasets. Overlaps indicate matches of the same brittle star sequences to more than one dataset, and in nearly all such cases the matches from the different datasets are mutually consistent. For reference, the large dashed border represents the size of the *S. purpuratus* genome (~23,300 genes).

Examination of the results reveals that the individual hits are mutually consistent in terms of genes identified. The brittle star data set has many times more sequences than the sea urchin gastrula UniGene set available on the NCBI UniGene database, and the average length of the brittle star sequences is shorter. To assess whether we could make meaningful comparisons between these different data sets, we plotted the data as rarefaction curves. In ecology, rarefaction uses repeated random resampling of a large pool of samples to estimate the species richness as a function of the number of individuals sampled. Here we used it to estimate how thoroughly each data set represents the full transcriptome. In Figure 6, the curve for sea urchin has a much steeper initial slope, and therefore matches to a significant number of KEGG clusters even with many fewer sequences. This likely occurs because the sea urchin sequences are longer on average. The brittle star curve rises more gradually, but plateaus near the end, indicating that the sequencing captured most of the genes present in the transcriptome. If we assume that the two organisms express roughly the same number of genes at equivalent developmental stages, then the rarefaction curves indicate that this is indeed a meaningful comparison.

Comparison of the brittle star gastrula transcriptome to the *S. purpuratus* genome as well as to the *S. purpuratus* gastrula transciptome and to the KEGG Orthology database, allowed us to identify 3,385 sequence matches.

Gene functional classes

O. wendtii sequences were compared to the KEGG Orthology database [24] by reciprocal best BLAST (Figure 3A). The KEGG Orthology database contains clusters of genes orthologous among a large number of organisms. Of the 3,800 clusters relevant to animals, 1,368 (36%) had significant matches to brittle star. Similarly, 1,335 KEGG clusters (35%) had matches to sea urchin gastrula. These numbers include 840 KEGG clusters (22%) with matches to both organisms.

When sorted into functional classes (Figure 3B), an average of 43%, 39%, and 28% of the distinct KEGG clusters within each class had matches to brittle star, to sea urchin, and to both, respectively, with a range between 2% and 85%. Each KEGG functional class consists of a number of biochemical pathways. On average, 44%, 43%, and 30% of the KEGG clusters within each pathway had matches to brittle star, to sea urchin, and to both, respectively. Note that there is extensive overlap between the various KEGG functional classes and pathways, with many clusters falling into several different ones.

Overall, genes involved in metabolism and genetic information processing were the most highly conserved, as would be expected. The number of these 'housekeeping' genes found in sea urchins and brittle stars are similar, and the relationship between the number of genes observed in each group and the number shared between them is very consistent. There are fewer orthologs detected in the other KEGG orthology groups. Many pathways under 'Organ Systems' and 'Human Diseases' are vertebrate-specific and/or relate to functions which do not operate extensively until later stages of development or after metamorphosis and would not be expected to be expressed at the gastrula stage. This is true for both organisms. There is also more variation in the number of gene matches to sea urchins and brittle stars in these functional classes.

Genes involved with the cytoskeleton and cell junctions had considerably more matches to brittle star. Cell-adhesion genes are often large, with many exons, and with domains often repeated and shared between multiple genes [36]. These characteristics, along with the short lengths of the brittle star sequences, have the potential to produce an artificially high number of BLAST hits. However, this pattern was the exception, not the rule, across the other functional classes.

The sea urchin had a far greater number of matches to genes involved in endocytosis, lysosome and RNA degradation. Many of these genes again overlap with several
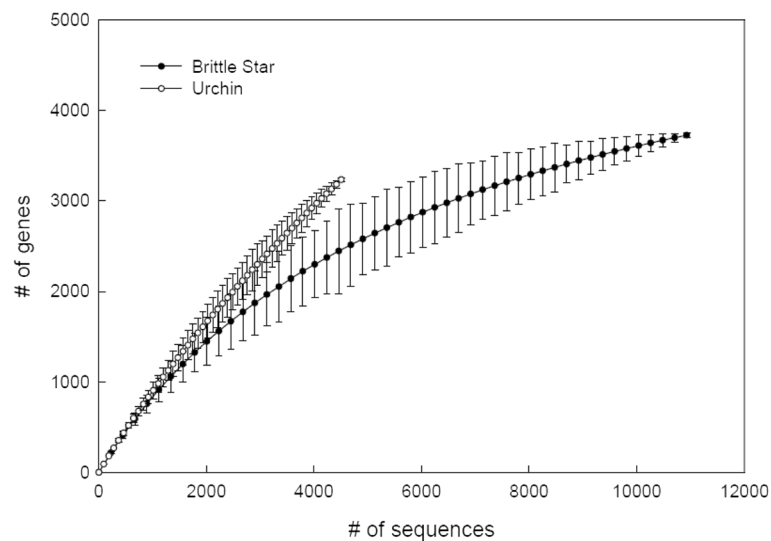
Figure 6 Rarefaction curves for sea urchin and brittle star. The steeper initial slope for the sea urchin curve indicates matches to a significant number of KEGG clusters even with many fewer sequences. The brittle star curve rises more gradually, but becomes asymptotic at the right, indicating that the sequencing captured most of the genes present in the gastrula transcriptome. If the two organisms express roughly the same number of genes at equivalent developmental stages, then the rarefaction curves indicate that comparison of these two data sets is indeed meaningful.

other pathways, but there is no clear pattern to account for the disparity.

Comparison to sea urchin developmental gene regulatory network

The gene regulatory networks that underlie the differentiation of the basic tissue types in sea urchin embryos have been fairly well-characterized. The temporal and spatial expression of these genes has been determined and many of the regulatory interactions between the various genes have been determined, either directly or indirectly by interference with gene expression. The majority of these genes are expressed concurrently at the gastrula stage, which makes this stage an excellent point to identify a global set of genes important to the process of early cell differentiation. Here we use the sea urchin *S. purpuratus* gastrula GRNs at 21 to 30 h of development [3-6] as a reference to look for conservation of genes expressed in the brittle star gastrula at 40 h of development, which is equivalent morphologically. At this point the skeletal spicules have just begun forming, the archenteron is one third to halfway across the blastocoel cavity and the equivalent of secondary mesenchyme has formed. The gut is not yet partitioned and no mouth has formed. The presence of the same genes expressed at the same stage in these two organisms would suggest a conservation of GRNs and a shared gastrula 'toolkit' of proteins. The absence of genes expressed in either organism would indicate that there is either a temporal

change in expression or that the gene is not expressed at all in the embryo of one group. Either is an indication of a change in a GRN. It is possible we could fail to detect some of those transcripts that are expressed at very low levels and recalcitrant to this RNA profiling. However, reciprocal BLAST searches using the brittle star gastrula transcriptome data and the *S. purpuratus* genome found homologs for a majority of genes involved in the sea urchin developmental gene regulatory network, including transcripts expressed at very low levels in *S. purpuratus*.

In sea urchins, a gradient of β-catenin initiated at the vegetal pole of the egg sets up and is soon reinforced by a circuit in the early embryo involving β–catenin/*lef1*, *wnt8*, *blimp1*, and *otx* in an intricate shifting relationship, creating a ring of gene expression which moves outward from the vegetal pole to specify endomesoderm [37]. *Hox11/13b* is also soon involved in this circuit [5,38]. Comparisons between sea urchins and starfish have revealed that just downstream from these early endomesoderm genes in the endoderm lies an extremely well-conserved kernel involving *blimp1/krox, otx, gatae, foxa*, and *brachyury* [39]. In starfish, *tbr (t-brain)* is also part of this kernel, a role which is likely deeply ancestral, as it is also expressed in vegetal pole endoderm precursors in both sea cucumbers and hemichordates [40,41]. However, in sea urchins *tbr* has lost this role in endoderm and has instead been co-opted into skeletogenesis [42]. In sand dollars it appears to play both these roles [43].

In Table 1 we compare some key endomesoderm and endoderm specific genes in the sea urchin to the

Table 1 Comparison of *Ophiocoma wendtii* gastrula transcripts to the *Strongylocentrotus purpuratus* endodermal and endomesodermal gene regulatory networks

Gene	Found in *O.w.* gastrula	RBB to *S.p.* Genome [SpBase:]	RBB to NCBI RefSeq Proteins [NCBI:]	Role in *S.p.*
β-Catenin	Y	β-Catenin [SPU_004319]	*S.p.* β-Catenin [XP_786059.2]	Endoderm
Otx	Y	Otx [SPU_010424]	*S.p.* Otx [NP_999753.2]	Endoderm
Wnt	Y	Wnt5 [SPU_026277]	*S.k.* Wnt2 [NP_001158455.1]	Endoderm
Blimp1	Y	Blimp1/Krox [SPU_027235]	*B.f.* Zn-finger [XP_002587482.1]	Endoderm
Hox11/13b	Y	Hox11/13b [SPU_002631]	*S.p.* Hox11/13b [NP_999774.1]	Endoderm
Bra	N			Endoderm
Krl	N			Endoderm
Myc	Y	Myc [SPU_003166]	*S.p.* Myc [NP_999744.1]	Endoderm
SoxB1	Y	SoxB1 [SPU_022820]	*S.p.* SoxB1 [NP_999639.1]	Endoderm
Brn1-2-4	Y	Brn1-2-4 [SPU_016443]	*S.p.* Brn1-2-4 [XP_782909.2]	Endoderm
Tgif	Y	Tgif [SPU_018126]	*I.s.* Tgif [XP_002433653.1]	Endoderm
Hnf1	N			Endoderm
Eve	N			Endoderm
Hh	N			Endoderm
VEGF	Y	VEGF [SPU_030148]	*H.p.* VEGF BAI67115.1]	Endoderm
Dac	Y	Dac [SPU_028061]	*I.s.* Dachsund [XP_002407755.1]	Endoderm
Endo16	N			Endoderm
FoxA	Y	FoxA [SPU_006676]	*S.p.* FoxA [NP_001073010.1]	Endo + SMC
GataE	N			Endo + SMC
Kakapo	Y	Syne1 [SPU_013237]	*S.p.* Similar to CG33715-PD [XP_784190.2]	Endo + SMC
Apobec	Y	Hnrpr [SPU_019557]	*S.p.* Hnrpr [XP_793277.1]	Endo + SMC
Gelsolin	Y	Gelsolin [SPU_003985]	*S.p.* Gelsolin [XP_788777.1]	Endo + SMC

B.f. = Branchiostoma floridae, H.p. = Heliocentrotus pulcherrimus.
I.s. = Ixodes scapularis, O.w. = Ophiocoma wendtii.
S.k. = Saccoglossus kowalevskii, S.p. = Strongylocentrotus purpuratus.

transcripts present in the brittle star gastrula. Brittle stars express *β–catenin, lef1, otx, blimp1, wnt, hox/11/13b, and foxa* genes, suggesting that components of the endomesoderm and endoderm GRNs expressed early in development are conserved. *Gatae*, however, is not expressed. Many animal phyla employ *gata* genes in gut formation [44]. *Gatae* is a key component of the endoderm GRN in sea urchins and forms a feedback loop

that maintains expression of these genes in the endoderm [45]. *Otx* and *blimp1* constitute another portion of that feedback loop [5], and this could be sufficient for endoderm differentiation in brittle stars. Two genes that are activated by *gatae* in *S. purpuratus*, *brachyury (bra)* and *krüppel (krl)*, are not expressed at the gastrula stage in brittle stars. *Krüppel* expression in sea urchins is highest in the early blastula, and is mostly gone by the time of gastrulation in *S. purpuratus* [46]. Its absence from the brittle star data may therefore reflect a small shift in timing and/or low transcript abundance at the onset of gastrulation. *T-brain* is not expressed in the brittle star gastrula. This would seem to indicate that *tbr* expression is not required for skeletogenesis in brittle star embryos as it is in sea urchins, or for endoderm formation as in starfish.

The endoderm in *S. purpuratus* is derived from two tiers of blastomeres formed during cleavage from the macromeres: Veg2, closest to the vegetal pole, and Veg1 above that. The Veg2 derived endoderm in *S. purpuratus* expresses *myc, brn1/2/4, tgif* and *dac* genes at the gastrula stage [47]. All of these are expressed in the brittle star gastrula (Table 1). In contrast, the Veg1 genes *eve* and *hnf1* are not expressed in brittle stars. Together this suggests that a central early kernel of the endoderm GRN is conserved, although the expression of *gatae* and some genes it regulates are not. The expression of genes found in Veg2 endoderm is also largely conserved. The most likely explanation of our results is that the equivalent of Veg1 endoderm has not formed in the brittle star gastrula at the stage we examined. This suggests a heterochronic shift in the formation of the second tier of endoderm. This could also explain the absence of *brachyury*. It is a key player in gut formation in both protostomes and deuterostomes, though the details differ between taxa [48-51]. A shift in the timing of Veg1 endoderm formation could delay expression of *brachyury* in the brittle star. A less likely explanation is that a loss of this layer of endoderm has occurred in brittle stars, and that the gut is formed entirely by the equivalent of Veg2 endoderm. *Endo16*, one of the major differentiation gene products in endoderm is not expressed in brittle star gastrula.

Following endomesoderm specification, mesenchyme precursors all express *ets1/2, erg,* and *hex* in *S. purpuratus.* All three of these genes are expressed in the brittle star gastrula (Table 2). The sea urchin skeletogenic primary mesenchyme derived from the micromeres, the homologous vegetal plate mesoderm in starfish, and the larval structures that produce the adult skeletons in both animals all express many of the same genes as sea urchin micromeres [19,52,53], and a majority of these genes were found in the brittle star gastrula transcriptome as well (Table 2). In all cases of echinoderm skeleton formation studied, including brittle star embryos, *alx1* is

expressed. This is consistent with reports that ectopic expression of *alx1* in sea urchin non-skeletogenic mesenchyme (NSM) induces skeleton formation [52]. *Ets1* is expressed both maternally and zygotically, and is involved in all the above cases, activating a great number of downstream genes. *Ets1* and *alx1* were both found in the brittle star gastrula transcriptome. Just downstream from these in both sea urchin micromere and starfish vegetal plate mesoderm are a group of three genes, *erg, hex,* and *tgif,* which form a "lockdown" mechanism, stabilizing the specification state by feeding back to each other and to *tbr* and *ets1,* and feeding forward into tissue-specific differentiation genes [53]. All three were present in brittle star, as was *deadringer (dri),* which appears to play a similar role in all the skeletogenic cases. *Tbr* has not been found in brittle stars. In starfish, *tbr* is seen in both endoderm (discussed above) and mesoderm [53]. It does not appear to be involved in adult skeletogenesis in either starfish or sea urchins. Its absence in brittle stars reinforces the idea that it was not part of the ancestral skeletal GRN and that its role in sea urchin embryonic skeleton formation is derived.

The downstream differentiation genes found in *S. purpuratus* skeletogenic cells at the gastrula stage are also found in the brittle star gastrula (Table 2). The spicule matrix proteins of the sea urchin endoskeleton contain a single C-type lectin domain and repetitious stretches rich in proline and glycine [54-56]. The apparently loose constraints on primary structure in these proteins, and the resulting low sequence conservation make identification of brittle star homologs difficult. However, the brittle star gastrula transcriptome contains several transcripts encoding C-type lectin domains and repetitive regions. Several other proteins, including Cyclophilin and Ficolin, are all expressed in sea urchin PMC cells and associated with the skeleton, though their exact functions remain unclear. The brittle star gastrula transcriptome contains matches for *Cyclophilin* and F*icolin,* but not for *MSP130,* a major cell surface protein in sea urchin PMCs. Overall there is a remarkable conservation of the GRN leading to formation of mineralized tissue in the embryos of sea urchins and brittle stars.

In sea urchins, Delta-Notch signaling from the micromeres activates *gcm* in the adjacent NSM to form pigment cells [57,58]. Brittle star embryos do not form embryonic pigment cells. Neither do starfish, but they express *gcm* in ectoderm rather than mesoderm, and it does not depend on Delta signaling [18]. Neither *notch* nor *delta* is expressed in the brittle star gastrula (Table 2). *Gcm* is expressed in brittle star gastrula, but *gatac, gatae. six1/2,* and *scl* are not. This suggests that the GRN leading to pigment cells, not surprisingly, is not conserved in brittle stars. Likewise, most of the genes that are expressed in the *S. purpuratus* small micromeres (i.e. *soxe, foxy*), which are

Table 2 Comparison of *Ophiocoma wendtii* gastrula transcripts to the *Strongylocentrotus purpuratus* mesenchymal gene regulatory networks

Gene	Found in *O.w.* gastrula	RBB to *S.p.* Genome [SpBase:]	RBB to NCBI RefSeq Proteins [NCBI:]	Role in *S.p.*
HesC	Y	HesC [SPU_021608]	*S.p.* HesC [XP_796692.1]	Mesenchyme
Erg	Y	Erg [SPU_018483]	*S.p.* Erg [NP_999833.1]	Mesenchyme
Hex	Y	Hex [SPU_027215]	*S.p.* Hex [XP_001197103.1]	Mesenchyme
Ets1/2	Y	Ets1/2 [SPU_002874]	*S.p.* Ets1/2 [NP_999698.1]	Mesenchyme
Alx1	Y	Alx1 [SPU_025302]	*S.p.* Alx1 [NP_999809.1]	PMC
Tbr	N			PMC
Tgif	Y	Tgif [SPU_18126]	*l.s.* Tgif [XP_002433653.1]	PMC
FoxN2/3	N			PMC
Dri	Y	Dri [SPU_017106]	*S.p.* Dri [NP_999799.1]	PMC
FoxB	Y	FoxB [SPU_004551]	*S.p.* FoxB [NP_999797.1]	PMC
FoxO	Y	FoxO [SPU_009178]	*S.p.* FoxO [XP_001183650.1]	PMC
VEGFR	N			PMC
Delta	N			PMC
Spicule matrix genes	Possible	C-lectin [SPU_007882]	*S.p.* C-lectin [NP_999805.1]	Skeletal differentiation
MSP130	N			Skeletal differentiation
G-Cadherin	Y	G-Cadherin [SPU_015960]	*S.k.* G-Cadherin [XP_002741140.1]	Skeletal differentiation
Ficolin	Y	Fic [SPU_023548]	*B.f.* Ficolin [XP_002594892.1]	Skeletal differentiation
Cyclophilin	Y	CypL7 [SPU_008305]	*D.m.* Cyclophilin 1 [NP_523366.2]	Skeletal differentiation
Gcm	Y	Gcm [SPU_006462]	*S.k.* Gcm [XP_002733441.1]	SMC
Notch	N			SMC
Six1/2	N			SMC
Hnf6	Y	Hnf6 [SPU_016449]	*S.p.* Hnf6 [NP_999824.1]	SMC
GataC	N			SMC
Scl	N			SMC
Pks	Y	Pks [SPU_028395]	*S.p.* Pks [NP_001239013.1]	SMC

Table 2 Comparison of *Ophiocoma wendtii* gastrula transcripts to the *Strongylocentrotus purpuratus* mesenchymal gene regulatory networks *(Continued)*

FoxF	Y	FoxF	*S.p.* FoxF	Small micromeres
		[SPU_000975]	[XP_794135.1]	
SoxE	N			Small micromeres
FoxY	N			Small micromeres

B.f. = Branchiostoma floridae, D.m. = Drosophila melanogaster, I.s. = Ixodes scapularis.
O.w. = Ophiocoma wendtii, S.k. = Saccoglossus kowalevskii.
S.p. = Strongylocentrotus purpuratus.

not formed outside of euechinoids, are not expressed in the brittle star gastrula (Table 2).

In sea urchin ectoderm, Nodal patterns both the ventro-dorsal (oral-aboral) and left-right axes [59], but was not found to be transcribed in brittle star gastrula (Table 3); nor was its antagonist Lefty, which limits Nodal to the ventral side during sea urchin development [60]. On the other hand, a number of genes downstream from Nodal and key to specification of different ectodermal regions [61] were found in brittle star (Table 3). Most of the genes expressed in the *S. purpuratus* oral ectoderm are found in the brittle star gastrula transcriptome, including *chordin* and *BMP2/4*. Sea urchin BMP2/4 is expressed in the oral ectoderm, then diffuses to and specifies the aboral ectoderm by inhibiting Nodal [62], while Chordin helps pattern neural tissue in the ciliary band at the oral/aboral border by excluding BMP2/4 activity from the oral side [63]. Genes that, in the sea urchin, are activated by Nodal-independent early oral ectoderm input are found to be expressed in brittle star gastrula. These include *otxb1/2* and *hnf6.* Of the sea urchin genes that are activated at the boundary of ectoderm and endoderm, *foxj* is expressed in brittle star gastrula, but *lim1* and *nk1* are not.

Genes that are expressed in the sea urchin aboral ectoderm are not as uniformly expressed in brittle star gastrula. Genes expressed by 12 h of sea urchin development such as *sim* and *nk2.2* are expressed in brittle star gastrula, but not genes expressed later in sea urchin aboral ectoderm such as *hox7* and *msx*. The differentiation genes *spec1* and *spec2a* are also not found to be expressed in brittle star gastrula at the time examined. *Tbx2/3* is expressed in brittle star gastrula, but not *irxa* and *dlx*, which are activated by Tbx2/3 in sea urchins. Taken together this would suggest two heterochronic shifts in ectoderm determination between sea urchins and brittle stars. In sea urchins, all of the genes examined are expressed at the gastrula stage. In brittle stars, patterning by Nodal and Lefty is apparently complete by gastrulation and these genes are no longer expressed. Oral ectoderm is determined and specification of the aboral ectoderm is underway, but it appears that this process is not complete in the 40 h brittle star gastrula.

Conclusions

The brittle star *O. wendtii* exhibits radial holoblastic cleavages that are equal throughout, giving rise to uniform-sized blastomeres without the formation of the micromeres characteristic to sea urchins. Despite this, mesenchymal cells ingress and give rise to an embryonic skeleton, a developmental structure unique to echinoids and ophiuroids among the echinoderms. Mesenchymal cells also give rise to the coelomic pouches, but no pigment cells are formed in the embryo. Archenteron formation occurs much the same as in sea urchins, although there is a delay in gut elongation following invagination as well as in growth of the skeletal spicules initiated in the ventrolateral clusters. The resulting pluteus larva closely resembles that of sea urchins, albeit without pigment cells. The *O. wendtii* gastrula expresses genes from all functional classes at the gastrula stage. Brittle stars and sea urchins have comparable numbers of genes in most functional classes expressed at the gastrula stage.

A majority of the genes involved in the sea urchin gene regulatory network were also found in the brittle star gastrula transcriptome (Table 4). The brittle star pyrosequencing data are completely consistent with our earlier results using a PCR-based candidate gene approach (not shown). For example, transcripts of *alx1, dri, gabp, ets1,* and *erg* were found by both methods, whereas *tbr, gatac,* and *gatae* were not. While this does not completely rule out these genes being expressed but undetected by our methods, their absence is striking given the overall conservation of expression between the two groups. The percentage of genes involved in gene regulatory networks expressed in *S. purpuratus* gastrula that are also expressed in *O. wendtii* gastrula exceeds the percentage of transcripts conserved overall (Table 4). However, this conservation is not uniform across the different tissue types found in echinoderm gastrulae. Some of these differences can be explained by heterochronic shifts in gene expression, although loss of gene expression is also a possibility. Some of the endomesoderm genes that are expressed in sea urchin gastrula at declining levels could be undetectable by the brittle star gastrula stage. Examination of the aboral ectoderm genes

Table 3 Comparison of *Ophiocoma wendtii* gastrula transcripts to the *Strongylocentrotus purpuratus* ectodermal gene regulatory network

Gene	Found in *O.w.* gastrula	RBB to *S.p.* Genome [SpBase:]	RBB to NCBI RefSeq Proteins [NCBI:]	Role in *S.p.*
Nodal	N			Oral ectoderm
Lefty	N			Oral ectoderm
Chordin	Y	Chordin [SPU_004983]	*S.k.* Chordin [NP_001158390.1]	Oral ectoderm
Sip1	N			Oral ectoderm
FoxG	N			Oral ectoderm
BMP2/4	Y	BMP2/4 [SPU_000669]	*S.p.* BMP2/4 [NP_001116977.1]	Oral ectoderm
FoxA	Y	FoxA [SPU_006676]	*S.p.* FoxA [NP_001073010.1]	Oral ectoderm
Bra	N			Oral ectoderm
Dri	Y	Dri [SPU_017106]	*S.p.* Dri [NP_999799.1]	Oral ectoderm
Hes	Y	Hes [SPU_006814]	*S.k.* Hes1 [NP_001158466.1]	Oral ectoderm
Hnf6	Y	Hnf6 [SPU_016449]	*S.p.* Hnf6 [NP_999824.1]	Oral ectoderm
FoxJ1	Y	FoxJ1 [SPU_027969]	*S.p.* FoxJ1 [NP_001073013.1]	Ecto/Endo border
Nk1	N			Ecto/Endo border
Lim1	N			Ecto/Endo border
Tbx2/3	Y	Tbx2/3 [SPU_023386]	*S.p.* Tbx2/3 [NP_001123280.1]	Aboral ectoderm
Lhx2 (Lim2)	Y	Lhx2 [SPU_021313]	*M.m.* Lhx2 [NP_034840.1]	Aboral ectoderm
Dlx	N			Aboral ectoderm
Nk2.2	Y	Nk2.2 [SPU_000756]	*S.p.* Nk2.2 [NP_001123283.1]	Aboral ectoderm
Hox7	N			Aboral ectoderm
Msx	N			Aboral ectoderm
Klf7	Y	Klf2/4 [SPU_020311]	*S.k.* Klf2 [NP_001161575.1]	Aboral ectoderm
IrxA	N			Aboral ectoderm
Hmx	N			Aboral ectoderm

M.m. = Mus musculus, O.w. = Ophiocoma wendtii, S.k. = Saccoglossus kowalevskii, S.p. = Strongylocentrotus purpuratus.

expressed in *O. wendtii* relative to *S. purpuratus* indicates that specification of aboral ectoderm has begun but is delayed in the brittle star. The same could be true for the Veg1 endoderm. Other differences in gene expression correlate with differences in embryonic development. Brittle star embryos do not possess micromeres or pigment cells. The second lowest percentage of GRN genes conserved (33%) is seen in the genes expressed in

S. purpuratus small micromeres and pigment cells (Table 4).

The highest percentage of GRN conservation is seen in the skeletogenic mesenchyme cells (PMCs in sea urchins). This is not surprising, since all adult echinoderms form mineralized structures. The GRN and differentiation genes that lead to mineralized structures must be conserved in order for the adult skeleton to form. In sea urchins this

Table 4 Conservation of genes between
Strongylocentrotus purpuratus* and *Ophiocoma wendtii

		% conserved in O. wendtii
S. purpuratus transcriptome		55
Early gastrula GRN		65
Endoderm		53
	Veg2 endoderm	70
	Veg1 endoderm	20
Primary mesenchyme		86
Non-skeletogenic mesenchyme		57
	Secondary mesenchyme	64
	Small micromeres	33
Oral ectoderm		67
Aboral ectoderm		58

GRN is activated in the embryo largely intact. The conservation of these genes in the *O. wendtii* gastrula suggests that is the case in brittle stars as well. An extensive analysis of spatial expression of the genes involved in these GRNs is the next step in confirmation of homology.

In sea urchins, Hesc is a transcriptional repressor ubiquitously expressed in the embryo, where its role is to repress the skeleton program. In the sea urchin micromeres, *hesc* is itself repressed by Pmar1 in response to nuclearized β-catenin, thereby de-repressing the skeleton circuits [64]. This double-negative *pmar1/hesc* gate appears unique to sea urchins as the mechanism that coupled the pre-existing programs of skeletogenesis and maternal β-catenin-mediated vegetal specification to produce the novelty of the embryonic skeleton, as it is not involved in adult sea urchin skeletogenesis [19,53]. Recent evidence suggests that other, as yet unknown, mechanisms related to the unequal cleavage that produces the micromeres are also involved [65]. Starfish, which do not build an embryonic skeleton, also express *hesc* throughout most of the embryo, but this expression appears to have no effect on mesodermal genes shared with sea urchin skeletogenesis, and *pmar1* has never been found in starfish [54].

Sea urchins express *pmar1* from fourth cleavage through mid-blastula, so we would not expect to see it expressed in the *O. wendtii* gastrula transcriptome. Using PCR, we have searched for, but never found, *pmar1* transcripts from any stage of brittle star development. We have, however, successfully amplified the *pmar1* homolog from brittle star genomic DNA, identified as such by the presence of a conserved intron [unpublished]. This suggests that activation of the adult skeletal GRN in embryos occurred differently in brittle stars than in sea urchins. Overall, our data suggest that embryonic skeleton formation in sea urchins and brittle stars represents convergent evolution by independent co-optation of a shared pathway utilized in adult skeleton formation.

Abbreviations

BLAST: Basic Local Alignment Search Tool; bp: Base pairs; EST: Expressed sequence tag; GRN: Gene regulatory network; NSM: Non-skeletogenic mesenchyme; PMC: Primary mesenchyme; RBB: Reciprocal best BLAST; SMC: Secondary mesenchyme.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BTL conceived of and oversaw the project, collected animals, cultured embryos, extracted RNA, and conducted BLAST comparisons against the sea urchin GRN. NG performed, and WKT oversaw and advised on, initial processing of the data and automated BLAST searches. RV assembled the annotation data, carried out the analyses and interpretation, and wrote most of the manuscript. JRG oversaw and advised on the analyses. All authors contributed to the final version of the manuscript.

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