**INTRODUCTION**

Little is known about the morphogenetic complexity of the last common ancestor of modern multicellular animals, but it is generally thought to be an extremely simple organism without a body axis, multiple cell layers and tissues [reviewed in 1]. We can reconstruct this hypothetical animal—the Urmetazoa—by identifying common features in embryonic development of distantly related extant clades, specifically bilaterians, cnidarians, ctenophores and sponges. Among these groups, bilaterians are represented by long-favourite developmental model systems and several hypotheses have been proposed regarding morphogenetic complexity of their last common ancestor—the so-called Urbilateria or protostome-deuterostome ancestor [reviewed in 2]. Recent studies demonstrate surprising similarity between cnidarian and bilaterian gene content and development [3–7]. For example, the expression of Wnt genes is associated with blastopore and site of gastrulation in cnidarian and chordate embryos [3,5,8,9]. Even more surprisingly, TGF-β ligands that are involved in determination of the dorsal-ventral axis in bilaterians are also asymmetrically expressed during cnidarian development [6,7,10]. Without attempting to homologize the embryonic axes between cnidarians and bilaterians, the existence of two perpendicular embryonic axes, one directed by a Wnt gradient, and the other by a TGF-β gradient in the last common ancestor of living cnidarians and bilaterians appears plausible.

Until recently, developmental genetic data have not been available from sponges, whose adult body plan has not changed since before the Cambrian explosion [11,12]. Molecular phylogenies agree that the sponge lineage(s) diverged from the main (eu)metazoan lineage before all other major extant phyla [13–19]. Unlike the morphologically more complex eumetazans, sponges are considered to lack true tissue-level organization and metazoan-specific cell types such as neurons and muscles. Historically, these fundamental differences in the body plans have led to a prevailing view that sponges are living representatives of an evolutionary intermediary between unicellular choanoflagellate protists and the eumetazans [20]. Indeed, many adult sponges, such as the adult demosponge *Amphimedon queenslandica* (formerly known as *Rinorea* sp.; Fig. 1A), have highly plastic body shapes and lack an apparent anterior-
posterior (AP) axis of symmetry (Fig. 1A). However, most sponge embryos and larvae have an obvious AP axis with radial symmetry. This similarity to other metazoans is lost at metamorphosis when the growing sponge assumes its sessile body form (Fig 1B–F). Importantly, the formation of a patterned larva with a range of cell types distributed along the AP axis and allocated into different cell layers indicates that sponge embryos must have a requirement for localised signals [21,22].

The recent sequencing of the genome of the demosponge *Amphimedon queenslandica* by the Joint Genome Institute greatly facilitates reconstruction of the genetic repertoire that was present in the last common ancestor to all contemporary metazoans and reveals the innovations that lead to evolution of the first branches in the animal tree of life [23–28]. Amongst these innovations must have been a suite of signalling pathways that allow for communication in a range of multicellular contexts, including cell specification and patterning [22]. The highly conserved Wnt and TGF-β signalling pathways are fundamental to a plethora of developmental processes in bilaterian animals. In addition to specification of the first embryonic axes, these pathways interact to specify cells and to pattern tissues in many morphogenetic contexts, ranging from the formation of embryonic organizers [29–32], vertebrate skeleton [33] and the development of limbs in *Drosophila* and other bilaterians [34–36]. The primacy of Wnt and TGF-β pathways in intercellular communication and cell fate diversification suggests that their evolution may have been concomitant with the origin of multicellularity [22,37]. Here we address this issue by investigating the expression of Wnt and TGF-β genes during embryonic development in *Amphimedon queenslandica*. The asymmetrical expression of both genes in *Amphimedon* embryos indicates that sponges, and hence also the last common ancestor to living metazoans, utilized these two signalling pathways in embryonic patterning.

**RESULTS**

*Amphimedon* embryogenesis

*Amphimedon queenslandica* embryos develop in brood chambers, with different developmental stages found together in one chamber (Fig. 1F). Early cleavage stages are milky-white and are found mainly at the edges of the brood chamber (Fig. 1F–G). At this time, cell divisions appear highly asymmetric and asynchronous, and the embryos are composed of irregularly shaped macromeres of various sizes with small micromeres interspersed between them (Fig. 1G). A solid blastula, with more uniformly sized cells is formed at the end of this process, and it does not display any morphological asymmetry (Fig. 1H). Different cell populations present in the blastula sort themselves into layers in a process that we consider to be gastrulation [21]. At the end of gastrulation, the outer layer is composed of smaller micromeres including pigment cells that give embryos a beige colour; bigger macromeres are present in the inner cell mass (Fig. 1I). While no asymmetry can be observed in live embryos, cleared beige coloured embryos reveal striking anterior-posterior asymmetry, with the outer layer significantly thicker at the posterior pole (Fig. 1I). Pigment cells initially distributed throughout the outer layer soon begin migration towards the posterior pole (Fig. 1J), where they coalesce into a spot, and then begin outwards migration resulting in formation of a narrow pigment ring (Fig. 1J–L). At the same time, multiple cell types migrate along the anterior-posterior axis to yield a highly patterned larva that consists of multiple cell layers, each of which contains a number of distinct cell types [21, Fig. 1L–M].

Wnt and TGF-β ligands are present in *Amphimedon*

We isolated Wnt and TGF-β genes from *Amphimedon* using a combination of EST and genome trace searches and RACE cloning. The deduced *Amphimedon* Wnt protein contains a signal peptide and 24 conserved cysteines characteristic for this family
Expression of Wnt and TGF-β genes during Amphimedon development

We studied the expression of the identified genes using whole mount in situ hybridization on Amphimedon embryos and larvae. Amphimedon Wnt gene is expressed from the early stages of development (Fig. 2). There is no evidence that Wnt transcripts are maternally deposited in oocytes or eggs. During cleavage, the Amphimedon embryo consists of large macromeres of varying size and shape surrounded by many tiny micromeres [Fig. 1G; 21]. Wnt transcripts first can be detected in very small micromeres that are uniformly distributed throughout the embryo and interspersed between the macromeres (Fig. 2A). At the next recognizable stage of development, the blastula stage, the embryo consists of more evenly sized cells [Fig. 1H; 21]. At this stage, Wnt-expressing cells are enriched in the inner part of the embryo (Fig 2B). Before any morphological asymmetry in the embryo can be detected by cytological indicators [21, 22, unpublished], Wnt-expressing cells become restricted to the inner cell mass on one side of the embryo (Fig. 2C, D). We have called this stage early gastrulation based on these localized Wnt expression patterns. As gastrulation progresses and separation of outer and inner layer becomes apparent, the Wnt-expressing cells become confined to the outer layer at the posterior pole (Fig 2E, F). The posterior pole is relative to larval swimming direction and where the pigment cells will eventually coalesce and form a ring [Fig. 1J–M, 21, 22]. The Wnt expression domain overlaps with the pigment spot and ring (Fig. 2G–I) and this expression continues within the pigment ring in the free swimming larva (Fig. 2J).

Similar to Wnt expression, TGF-β expression is first detectable during cleavage stage in small micromeres, which do not appear cytologically different from the Wnt expressing cells (Fig. 3A). However, at the blastula stage TGF-β-expressing cells are more prominent in the outer region (Fig. 3B), while Wnt expressing cells are enriched in the inner region (Fig. 2A), indicating that these are largely different populations of cells. During gastrulation, TGF-β expression becomes restricted to the outer layer, with stronger domains of expression at anterior and posterior poles of the embryo (Fig 3C, D). Thus, at the gastrula stage, the posterior domain of TGF-β expression overlaps with Wnt-expression at the posterior pole. At the pigment spot stage (Fig. 1J, 3E–F), TGF-β expression is maintained in the centre of the spot and throughout the outer layer, except in pigment cells making the outer portion of the spot and the region immediately adjacent to the spot (Fig. 3E, F). The anterior pole domain of TGF-β expression is particularly prominent at the spot stage (Fig 3F, G). The anterior region of the larva consists of a different cell type than the majority of the outer layer [21]. As the pigment cells begin to move concentrically away from the posterior pole to form the pigment ring [Fig. 1K, 21, 22], TGF-β is expressed inside of the ring (Fig. 3H). As pigment ring formation progresses, TGF-β expression is not longer detected in the centre of the ring, but delineates the inner rim of the ring (Fig. 3I, J). During the ring formation stages, TGF-β expression continues throughout the outer layer except of the narrow band of cells just outside of the ring (Fig. 3I, J). TGF-β expression in the embryo gradually decreases, and the transcripts are not detected in the swimming larvae (not shown).

DISCUSSION

We have identified a Wnt and a TGF-β gene from the Amphimedon genome, extending the origin of these important gene families to before the divergence of sponge and eumetazoan lineages. While
outward migration, blastula stage, of it.

TGF-β ligands are expressed during Amphimedon embryogenesis in complex and localized patterns. The early expression of Wnt and TGF-β in Amphimedon embryos is compatible with a role in establishing axial polarity. The localization of Wnt-expressing cells to the future posterior pole is the earliest morphogenetic asymmetry detected in Amphimedon (Fig. 2C, D), occurring prior to the initial cell sorting event that creates the embryonic cell layers. We can not discern if the initial localized expression of Wnt is in the same cells that express Wnt later in development because of a lack of cell lineage data. Regardless, it is evident that Wnt-expressing cells are restricted early to the posterior pole and Wnt transcripts localize to this pole continuously through to the larval stage. The migration of pigment cells towards Wnt-expressing cells at the posterior pole is indicative of the existence of differential signals along the AP axis and is compatible with Wnt and/or TGF-β contributing to the establishment of this axis. Also migrating with the pigment cells along the outside of the embryo are sclerocytes-cells responsible for the synthesis of siliceous spicules [21,23]. Upon reaching the posterior end of the larva, the sclerocytes appear to ingress into the inner cell mass [21]. The movements of pigment cells and sclerocytes are consistent with a role for these metazoan-specific ligands interacting to establish axial polarity in sponge embryos in a manner akin to that observed in other metazoa [1,30,45,46]. For example, the vertebrate organizer is localized by interactions between Wnt and TGF-β signalling pathways [29–31], with TCF and SMAD, as respective effectors of these pathways, cooperating to regulate gene expression [31]. In both Amphimedon and the cnidianian Nematoctella, Wnt expression is restricted to the posterior ends of the larva [3,5], although mechanisms of gastrulation are different and there is limited evidence for these poles being homologous.

The intersecting expression of Wnt and TGF-β at the posterior end of the larva later in development also is compatible with these signalling pathways regulating the formation of the pigment ring [i.e. tissue morphogenesis, 22]. A zone of intersecting Wnt and TGF-β expression occurs anterior of the leading edge of the concentric front of migrating pigment cells (compare Fig. 2I and 3 H–J), and may be providing positional information [47] in manner similar to that observed during the formation of limbs in Drosophila [34] and the head organizer in cnidarians [48].

Our results suggest that sponge embryos are patterned by signalling mechanisms strikingly similar to those controlling cell specification and patterning in bilaterians and cnidarians. These signalling systems evolved and interacted early in metazoan evolution prior to the first cladogenic events that predate the Cambrian explosion (Fig. 4). Wnt and TGF-β signalling pathways appear to have acted combinatorially to specify and pattern cells in the last common ancestor to all extant metazoa (Fig. 4). In addition, a hedgehog-like cell surface signal–Hedgling–is expressed in overlapping patterns with Wnt and TGF-β during ring formation [25]. The developmental expression of these signalling systems, along with that of many metazoan-specific transcription factor families [23], indicates that the last common ancestor to all living metazoa already possessed the regulatory capacity to form complex body plans, using the same molecular components as animals living over 550 million years later [49]. The evolution of this canonical zootypic network may have been the necessary precursor for the diversification of all contemporary metazoan body plans. The differential expansion and elaboration of this network in the eumetazoan lineage, compared to the sponge lineage, provided the foundation for the extensive body plan diversification seen in this clade, including possibly the origin of a second body axis. Along with the diversification signalling pathways in eumetazoans was the origination of Hox genes [24] and expansion of a range of developmental transcription factor gene families [23, 28, unpublished], which enabled further elaboration of ancestral gene regulatory networks.

Figure 3. Expression of TGF-β in Amphimedon embryos. (A) TGF-β expressing micromeres are distributed uniformly during cleavage. (B) At the blastula stage, TGF-β-positive cells are more prominent in the outer layer. (C, D) During gastrulation, TGF-β expression is restricted to the outer layer, with two stronger domains at the anterior and posterior poles. (E, F) As the pigment cells migrate to the posterior pole, TGF-β expression disappears from the posterior pole leaving the area just outside the pigment spot clearly devoid of TGF-β transcripts. A weak TGF-β expression domain persists in the very center of the spot. (F, G) The anterior pole expression remains strong at the spot and ring stages. (H) As the pigment cells begin their outward migration, TGF-β expression is strong inside of the forming ring and in the outer layer except of the pigment ring itself and area just outside of it. (I, J) In the later ring, TGF-β expression clears from the center of the ring, but persists in the inner rim of the ring. (J) The embryonic expression gradually fades at late ring stages, and expression in some cells of the follicle layer on the surface of the embryo becomes more apparent. Scale bar, 100 μm. doi:10.1371/journal.pone.0001031.g003
Whole mount in situ hybridization

Whole mount in situ hybridizations were performed as described in [36] using complete coding sequences cloned into pGEMT vector (Promega) as templates for probe synthesis. Embryos were photographed whole mount and then subsequently processed for sections. Samples were dehydrated in ethanol and infiltrated with Epon 812 resin in a BioWave microwave oven (Pelco) before polymerisation overnight at 60°C in a conventional oven. Sections were cut at 5 μm on an Ultracut T ultramicrotome (Leica) and mounted in Histomount.

Phylogenetic analyses

Phylogenetic analyses of Wnt and TGF-β sequences were performed for the purpose of assigning orthology. Detailed description of the methods used is included in Supplement S1.

SUPPLEMENTARY INFORMATION

Supporting Information

Supplement S1

Found at: doi:10.1371/journal.pone.0001031.s001 (0.04 MB DOC)

Figure S1

Found at: doi:10.1371/journal.pone.0001031.s002 (0.13 MB DOC)

Figure S2

Found at: doi:10.1371/journal.pone.0001031.s003 (0.04 MB DOC)

Figure S3

Found at: doi:10.1371/journal.pone.0001031.s004 (0.03 MB DOC)

Figure S4

Found at: doi:10.1371/journal.pone.0001031.s005 (0.04 MB DOC)

Figure S5

Found at: doi:10.1371/journal.pone.0001031.s006 (0.04 MB DOC)

ACKNOWLEDGMENTS

We gratefully acknowledge the significant contribution and support of The US Department of Energy Joint Genome Institute in the production of *Amphimedon reniera* genomic and EST sequences used in this study through the Community Sequencing Program (http://www.jgi.doe.gov/sequencing/vhw/CSP2005/reniera.html).

Author Contributions

Conceived and designed the experiments: BD MA. Performed the experiments: MA CL KG. Analyzed the data: BD MA MA CL SD. Wrote the paper: BD MA CL SD.

REFERENCES

1. Martindale MQ (2005) The evolution of metazoan axial properties. Nat Rev Genet 6: 917–27.
2. Erwin DH, Davidson EH (2002) The last common bilaterian ancestor. Development 129: 3021–32.
3. Lee PN, Pang K, Matus DQ, Martindale MQ (2006) A WNT of things to come: evolution of Wnt signaling and polarity in cnidarians. Semin Cell Dev Biol 17: 536–9.
4. Miller DJ, Ball EE, Technau U (2005) Cnidarians and ancestral genetic complexity in the animal kingdom. Trends Genet 21: 536–9.
5. Hayward DC, Samuel G, Pontynen PC, Catmull J, Saint R, et al. (2002) Molecular evidence for deep evolutionary roots of bilaterality in animal development. Proc Natl Acad Sci U S A 103: 11195–200.
6. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, et al. (1999) Requirement for *Wnt*β in vertebrate axis formation. Nat Genet 22: 361–5.
10. Rentasch F, Anton R, Saina M, Hammerschmidt M, Holstein TW, et al. (2006) Asymmetric expression of the BMP antagonists chordin and gremlin in the sea anemone _Nematostella vectensis_: implications for the evolution of axial patterning. Dev Biol 296: 375–87.

11. Li CW, Chen JY, Hua TE (1998) Precambrian sponges with cellular structures. Science 279: 879–82.

12. Botting JP, Butterfield NJ (2005) Reconstructing early sponge relationships by using the Burgess Shale fossil _Edaphia globosa_, Walcott. Proc Natl Acad Sci U S A 102: 1534–9.

13. Cavalier-Smith T, Gao EE (2003) Phylogeny of choanosoa, apusozoa, and other protozoa and early eukaryote megalovae. J Mol Evol 56: 540–63.

14. Medina M, Collins AG, Silberman JD, Bogin ML (2001) Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc Natl Acad Sci U S A 98: 9707–12.

15. Peterson KJ, Butterfield NJ (2005) Origin of the Eumetazoa: testing ecological predictions of molecular clocks against the Proterozoic fossil record. Proc Natl Acad Sci U S A 102: 9347–52.

16. Borchiellini C, Manuel M, Alivon E, Bouny-Esaun N, Vacclet J, et al. (2001) Sponge parapophyly and the origin of Metazoa. J Evol Biol 14: 171–9.

17. Manuel M, Borchiellini C, Alivon E, Le Parco Y, Vacclet J, et al. (2003) Phylogeny and evolution of calcareous sponges: monophyly of calcinea and calcarea, high level of morphological homoplasies, and the primitive nature of axial symmetry. Syst Biol 52: 311–39.

18. Cavalier-Smith T, Allepuz MTF, Gao EE, Bourn-Esaun N, Vacclet J (1996) Sponge phylogeny, animal monophyly, and the origin of the nervous system. 18S rRNA evidence. Can J Zool 74: 2031–2045.

19. Halanych KM (2004) The new view of animal phylogeny. Ann Rev Ecol Evol Syst 35: 229–256.

20. Brusca RC, Brusca GJ (2003) The Invertebrates. Sinauer Associates, Sunderland.

21. Leys SP, Degnan BM (2002) Embryogenesis and metamorphosis in a haplo- sclerid demosponge: Gastrulation and transdifferentiation of larval ciliated cells to choanocytes. Invertebr Biol 121: 171–189.

22. Degnan BM, Leys SP, Larroux C (2005) Sponge development and antiquity of animal pattern formation. Integr Comp Biol 45: 331–341.

23. Larroux C, Fahey B, Lubiñich D, Himman VF, Gauthier M, et al. (2006) Developmental expression of transcription factor genes in a demosponge: insights into the origin of metazoan multicellularity. Evol Dev 8: 150–73.

24. Larroux C, Fahey B, Degnan SM, Adamski M, Rodhsar DS, et al. (2007) The NK homeobox gene cluster predates the origin of Hox genes. Curr Biol 17: 706–710.

25. Adamski M, Matus DQ, Adamski M, Green K, Rokhsar DS, et al. (2007) The evolutionary origin of hedgehog proteins. Curr Biol in press.

26. Jackson DJ, Macis L, Reitner J, Degnan BM, Warhede G (2007) Sponge paleogenomics reveals an ancient role for carbonic anhydrase in skeletogenesis. Science 316: 1893–5.

27. Sakarya O, Armstrong KA, Adamski M, Adamski M, Wang IF, et al. (2007) A post-synaptic scaffold at the origin of the animal kingdom. PLoS ONE 2: e2506.

28. Simionato E, Ledent V, Richards G, Thomas-Chollier M, Kerner P, et al. (2007) Origin and diversification of the basic helix-loop-helix gene family in metazoans: insights from comparative genomics. BMC Evol Biol 7: 33.

29. De Robertis EM, Larrain J, Oegeschlager M, Wessely O (2000) The establishment of Spemann’s organizer and patterning of the vertebrate embryo. Nat Rev Genet 1: 171–81.

30. Green J (2002) Morphogen gradients, positional information, and _Xenopus_: interplay of theory and experiment. Dev Dyn 225: 392–408.

31. Nishita M, Hashimoto MK, Ogata S, Laurent MN, Urno N, et al. (2000) Interaction between Wnt and TGF-β signaling pathways during formation of Spemann’s organizer. Nature 403: 781–5.

32. Watabe T, Kim S, Candia A, Rothbacher U, Hashimoto C, et al. (1995) Molecular mechanisms of Spemann’s organizer formation: conserved growth factor synergy between _Xenopus_ and mouse. Genes Dev 9: 3038–50.

33. Zhou S, Eid K, Glawacki J (2004) Cooperation between TGF-β and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. J Bone Miner Res 19: 463–70.

34. Cohen B, Simcox AA, Cohen SM (1993) Allocation of the thoracic imaginal primordia in the _Drosophila_ embryo. Development 117: 597–608.

35. Maves L, Schubiger G (1998) A molecular basis for transdifferentiation in _Drosophila_ imaginal discs: interactions between wingless and decapentaplegic signaling. Development 125: 115–24.

36. Oskaynak E, Schneegergen PN, Jin DF, Clifford GM, Warren FD, et al. (1992) Organogenetic role-2. A new member of the transforming growth factor-β superfamily expressed early in embryogenesis. _J Biol Chem_ 267: 25220–7.

37. Suga H, Ono K, Miyata T (1999) Multiple TGF-β receptor related genes in sponge and ancient gene duplications before the parazoan-eumetazoon split. FEBS Lett 453: 346–50.

38. Adell T, Nefken I, Muller WE (2003) Polarization factor ‘Frazzled’ in the demosponge _Selenia domuncula_ identification, expression and localization of the receptor in the epithelium/pinacoderm. FEBS Lett 554: 363-8.

39. Adell T, Thakar AN, Muller WE (2007) Isolation and characterization of Wnt-pathway-related genes from _Polychaeta_. Cell Biol Int 31: 939–49.

40. Reinhardt B, Broun M, Blitz IL, Bode HR (2004) HhBMP-8b, a BMP-8 orthologue, acts during axial patterning and tentacle formation in hydra. Dev Biol 267: 43–59.

41. Flowers VL, Courteau GR, Foutka AJ, Weng W, Venuti JM (2004) Nodal/activin signalling establishes oral-aboral polarity in the early sea urchin embryo. Dev Dyn 231: 727–40.

42. Wolpert L (1996) One hundred years of positional information. Trends Genet 12: 359–64.

43. Broun M, Gee L, Reinhardt B, Bode HR (2005) Formation of the head organizer in hydra involves the canonical Wnt pathway. Development 132: 2907–16.

44. Davidson EH (2006) The Regulatory Genome: Gene Regulatory Networks In Development And Evolution. Academic Press.