Mesenchymal cells in ancestral spongiomorph urmetazoa could be the mesodermal precursor before gastrulation origin

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Abstract: This review proposes a possible scenario for the origin and early evolution of the gastrulation process based on sponge cell organization and developmental biology. We first assume that modern sponges are metazoans derived from a common urmetazoan ancestor which contained primitive multicellular organization and spongiomorph structure. Then, the concept of gastrulation is contrasted among sponge classes and other animals, probably indicating that developmental processes in sponges are mainly based on cellular terms and not in terms of germ layers and gastrulation process. Tissue organization, most likely a cnidarian’ innovation, could be the base for germ layer body plan organization, but is absent in sponges. A coherent alternative is proposed, in which their organization would be based on mesenchymal and mesothelial cell differentiation programs. Finally, sponge developmental genes of the homeobox family are reviewed and the NKL group is further discussed, since it is involved in patterning of mesenchymal cells derived from mesoderm in Drosophila, as well as in mesenchymal cell programs in mouse mesoderm. As a general conclusion, the proposed scenario would have primitive spongiomorph mesohyl (mesenchyma) as the precursor for all metazoan germ layers and the Tlx gene family (NKL) as a primary molecular control for mesenchymal lineage, regulating proliferation and differentiation. Gastrulation would thus be originated in the cnidarian lineage, generating the three germ layers, body plan patterning by Hox complex, true epithelium, and three stem cell systems keeping homeostasis, one for each germ layer.

Keywords: Gastrulation, homeobox, Porifera, urmetazoa, mesoderm

Introduction

Transition from unicellular to multicellular grade of organization was a major evolutionary step that occurred relatively late in evolution. While fungi and several plant groups have independently reached multicellularity, the question of monophyletic versus polyphyletic evolution of metazoans has raised extensive debate. Up to the middle of the 20th century, organisms without typical tissues, such as sponges, were separated in Parazoa, which were considered as a side offshoot of the major evolutionary tree, comprising all the other Eumetazoa (Hyman 1940). However, recent comparative studies on gene structure, protein primary sequences and ribosomal RNA have gathered a broad set of data, giving a strong support to the monophyly of Metazoa (reviewed in Müller 2001). Molecular studies strongly suggest that Porifera is the oldest metazoan group, and that choanoflagellates are the parent group among Protista, being thus close to the evolutionary root of multicellular animals (Brooke and Holland 2003, King et al. 2003).

Multicellular grade of organization, namely the programmed cell proliferation and differentiation, the spatial order and functional integration of differentiated cells, the maintenance of cell population homeostasis, ordered growth, as well as the response to injury by controlled regeneration and self-recognition, have all appeared during the evolution of spongiomorph urmetazoan, from which modern Porifera and other metazoans diverged. It is thus interesting to address the question of which mechanisms underlie the multicellular organization in sponges, and govern the cell differentiation and their spatial organization. Since this spatial order is established early in embryogenesis, during the separation and positioning of germ layers, sponge type of gastrulation is discussed at morphologic level and in the light of the recent molecular data, shedding perspectives on the evolution of tissues, germ layers and gastrulation.

Gastrulation is known to have responded to changes in the environment and egg architecture during evolution, indicating the plasticity of these developmental processes. Leptin (2005) was able to create a complete and up-to-date definition despite the wide diversity of gastrulation types, “the period during the early development of animals when major cell and tissue movements remodel an initially unstructured group of cells, requiring coordinated control of different types of cellular activities in different cell populations. A hierarchy of genetic control mechanisms, involving cell signaling and transcriptional regulation, sets up the embryonic axes and specify the territories of the future germ layers”. According to
this definition gastrulation would be more than integrated cell movements and generation of multilayered organisms. The intimate correlation among axes patterning and gastrulation was also clearly defined in Martindale (2005): “The developmental events that generate axial organization during the early cleavage stages lead to the site of gastrulation, and therefore determine the onset of differential gene activity that is responsible for the specification of distinct mesoderm and endodermal germ-layer fates”. Gastrulation in deuterostome includes a body-organizing center by which cells move and receive inductive cues in order to differentiate according to axes patterning (Willmer 1994, Holland 2000, Leptin 2005, Martindale 2005). Spiralians, which include the protostomes: molluscs, annelids, polyclad flatworms, sipunculids, echiurans, and nemerteans, display a common pattern of embryogenesis referred to as spiral cleavage, which specify cell fates (Willmer 1994, Martindale 2005). During spiralian gastrulation, the specified blastomeres are placed according to the body axes (Technau and Scholz 2003). Basal metazoans, such as cnidarians and ctenophores also have cell lineages allocated during gastrulation (Martindale 2005). As a conclusion, in both Protostome and Deuterostome the not terminally differentiated blastomeres are allocated according to their designated differentiation program and embryonic axes, thus forming multilayered animals with specific tissue organization, which will be the base for adult body plan (Holland 2000, Technau and Scholz 2003, Leptin 2005).

If gastrulation definition is based only on integrated cell movements and generation of axial patterned organisms we should be careful, and keep in mind that it is a very primitive feature also observed in colonial protists, such as Dictyostelium (amoeba). Gastrulation definition should not be based on primitive features also observed in non Metazoan clade. This unicellular amoeba does not gastrulate, but the colony has integrated cell movements that generate a patterned axis without cell layers. They experiment social life in stressed conditions, joining together to form moving streams of cells that converge at a central point where they culminate in a fruiting body that releases spores. The chemotaxic cAMP is secreted mostly in the aggregation central and works as a morphogen that attracts and patterns the axis of the fruit body (Ginsburg and Kimmel 1997). As discussed later, the sponge larvae have antero/posterior axis and probably a chemioattractant patterning it (Degnan et al. 2005, Leys and Ereskovsky 2006), but these primitive features should not be sufficient to assume gastrulation in Porifera. Nevertheless, a distinguishing feature is the cell layer organization in sponge larvae. It is acquired just after cell movements in the blastula and this cell layer organization is not observed in Dictyostelium. In conclusion, comparative analysis of deuterostome, protostome, sponges and amoeba development indicates that integrated cell movements in sponge larvae formation is slightly more complex than Dictyostelium multicellular organization.

Sponge embryology

Morphological and molecular data place the extant sponges in phylogenetically related groups. Here we follow the innovative division, and not completely consensual, of Porifera into four classes: Demospongiae, Homoscleromorpha, Hexactinellida and Calcispongia (Borchiellini et al. 2004, Ereskovsky 2004, Boury-Esnault 2006).

As expected for an old group with a long evolutionary history, different sponge taxa have a great variety of embryological processes, and this diversity generated old controversies in the literature (Ereskovsky 2004). As reviewed by Leys (2004), one group classifies sponges as Parazoa, because they postulate that sponges do not undergo gastrulation, arising from a separate unicellular ancestor (Rasmont 1979, Ereskovsky and Korotkova 1997). Another group believes that gastrulation occurs at metamorphosis, when intensive cell movements completely rearrange the cell layers and give rise to the definitive sponge tissues (Brien 1937, Lévi 1963, Tuzet 1963, Brien 1967, Fell 1974, Simpson 1984). A third group recognizes gastrulation cell movements of ingressions, epiboly, delamination and invagination just after blastula stage (Lévi 1956, Efremova 1997, Boury-Esnault et al. 1999). Here sponges are regarded as Metazoa, and personal views on the available data are proposed. Sponge embryogenesis and adult organization should be understood in cellular terms and not in terms of gastrulation and germ layers, stressing a complementary differentiation of flagellated and amoeboid cells, and their mutual interaction in three-dimensional structures.

Demospongiae

The vast majority of sponges belongs to the Demospongiae. As reviewed by Ereskovsky (2004) and Leys and Ereskovsky (2006), there are three main types of development in this class. The most common generates the parenchymella larvae, another type generates disphaerula larvae, represented by Halisarcula, and the direct development is represented by Tetilla.

As reviewed by Borjevic (1970), total chaotic cleavage is followed by formation of a morula, which generates parenchymella larvae either by delamination or by micromeres appearing and centrifugal migrating to the periphery. In both cases micromeres and macromeres are segregated in external and internal layers of a solid swimming larva. Subsequently, micromeres differentiate into the flagellated larval cell layer that covers either the whole larva or only the anterior part. During metamorphosis, the flagellated epithelium disintegrates and micromeres migrate inward to become choanocytes or, sometimes, are partially or completely phagocyted during metamorphosis. In this case the adult is generated directly from a group of non-flagellate amoeboid cells. Nevertheless, the use of a tracer indicated that ciliated cells of the haptosclerid larva may form the future flagellated cells of the adult, the choanocytes (Leys and Degnan 2002). Maldonado (2004) recognized, in this case, gastrulation movements as “mixed delamination”. Such cell movement without a clear establishment of cell lineage according to the body plan is here assumed not sufficient to be defined as gastrulation. For instance, flagellated choanocyte can be generated either by amoeboid archaeocytes or flagellated micromeres. Moreover, all adult cells and structures can be generated from non-flagellate amoeboid cells without
the maintenance of an organization previously established during larva formation (Borojevic 1966a). Cells dissociated from a parenchymella larva re-aggregate directly to form juvenile sponge rather than a larva like organism (Borojevic and Lévi 1965). This property of generating all sponge cell types is unique to amoeboid mesenchymal stem cells only, whether derived from the larva or from the adult organism, since differentiated flagellated cells from the external layer of the parenchymella and from adult choanoderm cannot do so (Huxley 1911, Borojevic 1963, 1966a). Moreover, other studies with cell tracer would reveal the contribution of ciliated/flagellated cell lineage for the adult generation, as previously observed by Leys and Degnan (2002).

Extensive studies done by Degnan et al. (2005) in Haliclona development (as Reniera; Demosponge, parenchymella), suggested that gastrulation occurs after late blastula, followed by antero-posterior axis patterning. Sponge homologues of metazoan developmental transcription factors seems to be expressed in regions, layers and cell types of polarized Haliclona larva, as expected after gastrulation (Larroux et al. 2006). These authors observed “fertilization followed by a period of cell division yielding distinct cell populations which, through a gastrulation-like process, become allocated into different cell layers and patterned within these layers”. There are some divergences in Haliclona gastrulation and we suggest keeping the status of gastrulation-like. For instance, already determined cells such as sclerocytes (and probably pigmented cells too) sort out to external embryo layer, become fully differentiated during this process and come back to internal layer to be positioned posteriorly, a process of fully differentiated cell migration among germ layers not common in metazoan gastrulation. Moreover, all adult cells and structures can be generated from non-flagellate amoeboid cells without the maintenance of an organization previously established during larva formation (Borojevic 1966a), and sponge type of gastrulation seems to be unnecessary to specify the body plan of adult sponges.

As already suggested, the body structure of metazoans is more similar to larvae than adult Haliclona and it can be used as evidence for the hypothesis of larval neotenic evolution during the transition from the spongiomorph urmetazoan to multilayered animals with patterned axis (Larroux et al. 2006, Leys and Ereskovsky 2006). In this case, the sponge gastrulation-like process could be proposed as homologous to other metazoan gastrulation.

Larva must adhere on the substrate and undergo metamorphosis to form porocytes and channels, thus completing the development of the functional aquatic system (Borojevic 1971). This extrinsic induction to undergo metamorphosis and generate sponge body plan is quite different from the intrinsic signals of the gastrulation. This argument is valid only for the group that believes that gastrulation occurs during metamorphosis.

The simplest development, albeit not the most primitive one is that of Tetillidae (Spirophorida), in which there is no free larval stage. The substrate-adherent egg generates, by equal divisions, an amoeboid cell mass, which differentiates directly into an adult sponge (Watanabe 1978). This is an indication of the cellular organization of sponges, contrary to gastrulation positioning cell types in an ordered body plan. This is repeated in asexual reproduction from gemmules or involution bodies, as well as from re-aggregated cells in sponge regeneration from dissociated cells (Wilson 1907).

In Polymastia (Hadromerida), gastrulation is also missing. Eggs are retained at the substrate by mucus produced by the mother-sponge during egg-laying (Borojevic 1966b). After equal divisions, they give rise to a single-layered flat blastula containing only one cell type with short flagella, and only a virtual central lumen (Maldonado 2004). These creeping benthic larvae have an antero-posterior axis, since they are larger at the front (clavoblastula) and no dorso-ventral difference could be observed. After a long free life, they settle and convert directly to clumps of amoeboid cells, giving rise to the adult sponge in a process similar to the hatching of the gemmule (Borojevic 1966b).

The most striking example of Demospongiae cell movement similar to gastrulation is the larva formation in some species of the highly derived group Halisaricida (disphaerula larvae type), pointed by Maldonado (2004). As reviewed by Ereskovsky, (2004) and Leys and Ereskovsky (2006), Halisaricida does not follow parenchymella larva formation but the disphaerula type. Before larval differentiation, few cells migrate from the epithelium of the coeloblastula into the blastocoel and remain peripheral and close to the overlying epithelium without proliferating. The posterior-lateral ciliated blastoderm then invaginates and reorganizes into a monolayered internally ciliated tube suspended in a fluid-filled blastocoel. The lumen of the internal tube and the blastocoel are transitory cavities, since they are filled by proliferation of the internal cells as well as by late cell migration. Because the adult form does not preserve the primitive archenteron structure, and because other external cells migrate inward after gastrulation and generate internal structures, it is problematic to assume gastrulation strictu sensu during Halisaricida larval differentiation (Lévi 1956, Harrison and de Vos 1991, Ereskovsky and Gonobobleva 2000). We could not recognize here a process that should define the layers from which all adult structures are generated.

**Hexactinellida**

In the early development of Hexactinellida, the least studied class of sponges, a hollow blastula is formed, the coeloblastula (reviewed in Boury-Esnault et al. 1999, Leys 2003, Ereskovsky 2004, Leys and Ereskovsky 2006). Cleavage is total, equal and pseudospiral, and generates a hollow blastula. Subsequent delamination renders a two-layered stereoblastula. The external layer gives rise to ciliated micromeres, sclerocytes, choanoocytes (which later become collar buds) and spherulous cells. Internal macromeres envelop everything and gives rise to the internal, mostly syncytial, reticular tissue. Antero-posterior polarity of the larva (called trichimella) is evident already during early embryogenesis. The swimming trichimella is devoid of larval skeleton and has no functional filter feeding choanoocyte chambers. Such generalized, and not regionalized, delamination around Hexactinellida coeloblastula is not common in bilaterians. Since metamorphosis has not yet been described in Hexactinellida, it is difficult to affirm
that delamination in hexactinellids is devoid of a clear establishment of functional cell lineages, as happens in protostomes for instance. Moreover, it is not strange to assume that, as in demosponges, such morphogenesis based the origin of gastrulation.

**Calcispongia**

The embryogenesis and larval morphology of the two subgroups of Calcispongia, Calcinea and Calcaronaea are so different that, considering differences in anatomy, cytology and spicule morphology, it has been questioned whether they belong to the same evolutionary lineage (Borojevic 1970). These are also quite distinct from sponges with siliceous or organic skeletons. Molecular analyses have settled this issue, and it is now considered that they do represent a well defined and monophyletic group (Manuel 2006). Molecular studies indicated that they may be closer to other Metazoa (Ctenophora, Cnidaria) than to other sponges (Cavalier-Smith et al. 1996, Collins 1998, Kruse et al. 1998, Zrzavy et al. 1998, Schutze et al. 1999, Borchiellini et al. 2001, Medina et al. 2001), although this proposal requires further analyses (Manuel et al. 2003, Manuel 2006).

Following an extensive study of calcareous sponges, Haeckel (1872) proposed that the simple tubular ascons are the most primitive sponges. The main argument was the structure of tubular calcareous sponges (ascons), which is constituted essentially by two layers (diploblast): the internal flagellated choanoderm and the external pinacoderm, corresponding to endoderm and ectoderm respectively. This larva was described as a blastula, and the following metamorphosis was assumed to be a gastrulation process. At this time an invagination of the non-flagellate cells in one pole forms a “gastraea”, a two-layered ovoid larva provided with a single “mouth”, whose illustration was provided in the monograph “Die Kalkschwämme” (Haeckel 1872). After settlement, this larva would give rise directly to a two-layered ascon with a single apical opening, and this theory of the origin of multicellularity was named the “Gastraea theory” (Haeckel 1874). This formation of the second germ layer by invagination of the hollow blastula is still frequently considered to be the primitive form of gastrulation (Wolpert 1992). Only Hammer (1908) and recently Leys and Eerkes-Medrano (2005) were able to capture a stage that represented Haeckel’s gastrula, a larva invaginating to form a ciliated gut, but not Metschnikoff (1879) and Borojevic (1968). Leys and Eerkes-Medrano (2005) observed such epithelial invagination occurring prior to and during attachment. The authors suggested that this event is difficult to capture because metamorphosis in calcaronaean sponges takes place very rapidly. An alternative interpretation for such difficulty to observe is a rare and aberrant development related with substrate adhesion failure (R. Borojevic, personal communication).

The idea of gastrulation during metamorphosis in calcaronaean sponges is not well accepted by modern spongologists and the main argument is that the hole is engulfed by the newly forming basal epithelium, and the ciliated cells dedifferentiate to form an inner cell mass. Thus the transient cavity that is formed by invagination is not the future gut.

**Calcinea**

As reviewed by Ereskovsky (2004) and Leys and Ereskovsky (2006), Calcinea has a typical simple coeloblastula, sometimes called the calciblastula. Such larvae are found in the simplest asconoid Calcinea, which explain why it was considered to be the simplest sponges. At the end of total and equal cleavages, blastomeres become flagellated, but a few large ones may remain without flagella and are located at the posterior pole. Antero-posterior polarity becomes expressed towards the end of larva formation. These non-flagellated cells have been interpreted as founders of the “endoderm” after their polar ingestion into the blastocoel, although there is no clear morphological evidence. Such process has generated many speculations on the evolutionary significance of these cells. Moreover, not all larvae of Calcinea have these non-flagellate cells; some species have a coeloblastula with only flagellated cells. The cell ingestion site is not a *conditio sine qua non* to generate Calcinea *bauplan*, contrasting with gastrulation in bilaterians. The equatorial section of the larva gives anterior and posterior halves that are equally able to generate a new sponge, regardless of the presence of the posterior non-flagellate blastomeres in only one of them. Also, while the inner cell mass is indeed preferentially formed at the posterior pole of the larva by polar ingestion, all the flagellate cells eventually lose their flagella during larval maturation. At the end of the free life, the whole larva thus collapses in a solid cell mass that settles to the bottom, and is converted into a small mass of amoeboid cells that generate a new sponge (Borojevic 1968).

Further sponge development, settlement and metamorphosis are dependent upon conversion of larval flagellate cells into amoeboid cells that can proliferate and generate the necessary cell mass. The larval flagellate layer is thus a temporary embryonic structure that has to disintegrate before the progression to larval metamorphosis. We find again here the general pattern of cell differentiation and morphogenesis already discussed for Demospongiae, where a clump of substrate-adherent amoeboid cells is necessary and sufficient to generate a new sponge. Hence, the progressive filling of the blastocoel does not correspond to the generation of a second embryological cell layer, but a conversion from flagellate cell types responsible for movement into amoeboid cells that can proliferate.

**Calcaronaea**

This group has a divergent and most complex development compared to other sponges and its earlier stages were recently reviewed (Eerkes-Medrano and Leys 2006). It is somewhat similar to the development of *Volvox*. In these viviparous sponges a single cell layer hollow blastula, the stomoblastula, is formed after total and unequal cleavage. It already has the two major cell lineages arranged in opposite poles, amoeboid and flagellated, corresponding to macromeres and micromeres respectively (Amano and Hori 1993, Ereskovsky 2004, Leys and Ereskovsky 2006). Micromeres can have inwardly directed flagella forming a sheet that turns inside
out (excurvation) through the opening between macromeres, the “mouth” of the stomoblastula, and generates an amphiblastula with outwardly directed flagellated cells at the anterior pole. During the long free life of the larva, posterior pole macromeres continue dividing slowly until larval settlement and metamorphosis. At this stage non-flagellated cells cover the flagellated external layer by a process similar to epiboly, thus generating the pinacoderm. Meanwhile, flagellated cells regress their flagellum, submerge inside and generate the other sponge cell types. It could be regarded as the establishment of cell lineages but these lineages are not respected during adult life. The adult mesenchymal stem cell, the archeocyte, generates all cell types, including pinacocytes. The conversion from flagellated cells responsible for larval movement to the proliferating totipotent ameboeid cells is similar to what is observed in choanoflagellates, as discussed latter. The functional organization of sponge larvae by cell sheet inversion and flagellar-mesenchymal conversion during metamorphosis are both cell movements commonly found in colonial protozoans or algae and probably they are not enough to define gastrulation.

**Homoscleromorpha: primitive gastrulation with primitive epithelium**

Homoscleromorpha have mostly free swimming, hollow flagellated blastulae, originated from combined process of total, equal and chaotic cleavage, histolysis and outward migration of micromeres (multipolar egression) (Boury-Esnault et al. 2003, Ereskovsky 2004, Leys and Ereskovsky 2006). This unusual outward migration of micromeres is only comparable with the centrifugal cell movements generating parenchymella larvae in true Demosponges. There is no such cell movement during gastrulation of other metazoans. This larva is called the cinctoblastula, and contains one-layered flagellated epithelium, basal lamina and belt desmosomes (Boury-Esnault et al. 2003). After settlement, cells of the anterior pole give rise to the large choanocytes typical of this group, and the posterior ones to pinacocytes, which are also flagellated, with the exception of those belonging to the basopinacoderm. During larval metamorphosis, the structure of the flagellated layer of the anterior pole is preserved, folding and invaginating by quite complex cell-sheet movements. After fragmentation it gives rise directly to several choanocyte chambers of the rhagon, a small leuconoid sponge with a simple aqueous system containing a group of choanocyte chambers arranged around the central exhalant aqueous lacuna and the osculum (N. Boury-Esnault, personal communication). Homoscleromorpha have a basal lamina under both larval and adult cell layers, with its typical components such as the collagen IV (Boute et al. 1996, Boury-Esnault et al. 2003). We believe that it may stabilize flat cellular sheets, equivalent to epithelia in other metazoans. We discuss further the similarities and differences on epithelia from Homoscleromorpha, others sponges, Dictiostelium and clades with true epithelium. Now it is just assumed that the existence of basal lamina and desmosome contribute significantly to the cell-sheet movements, permanence and stability of cell lineages and the larval and adult epithelial layers. Since gastrulation places cells in their definitive germ layer, we believe that true epithelium is important for ectoderm and endoderm origin, as discussed further on. If is assumed that metamorphosis is part of the gastrulation, thus Homoscleromorpha has gastrulation aspects more similar to other metazoans, as preservation of the epithelium. Ancestral homoscleromorpha would have aspects in its metamorphosis, such as invaginating epithelium, that could be evolutionary committed to originate gastrulation later on.

**Germ layers in sponges: Enantiozoa**

The assumption of germ layers in sponges is problematic, since the contribution of endoderm and ectoderm to adult sponge tissues is quite different from that in other basal metazoans, and it is commonly adopted the concept of gastrodermis and epidermis (Hyman 1940) to avoid embryological implications, i.e., the gastrodermis need not necessarily be endoderm throughout. These issues have direct implications in defining the nature of sponge gastrulation as discussed here.

The anterior flagellated pole of swimming sponge larvae (amphiblastula, cinctoblastula and parenchymella), which putatively corresponds to the animal pole of other larvae, gives origin to the internal choanoderm, while ameboeid cells at the posterior (vegetative) pole generate the outer layer of the sponge body (Borojevic 1970). Delage (1892) has proposed classifying sponges as Enantiozoa, the “inverted animals”, with an internal ectoderm (choanoderm) and external endoderm (pinacoderm). The intense phagocytic activity of the internal and external pinacoderm layer was associated with food ingestion, a feature of the endoderm in other metazoans (Willenz and van de Vyver 1982), and this would be an additional argument in support of the Enantiozoa concept. An alternative approach to this issue was proposed by Willmer (1970) and Lévi (1970) who considered that sponge embryogenesis and adult organization should be understood in cellular terms and not in terms of germ layers, stressing the complementary differentiation of flagellated and ameboeid cells, and their mutual interaction in three-dimensional structures. This is similar to the proposal of Morris (1993), who considered that the common feature of metazoan development and probably sponge development is the mechanism controlling the multicellular grade of organization, involving complex interactions among motile mesenchymal cells, epithelial cells and extracellular matrix.

**Germ layers in sponges: mesoderm, ectoderm or endoderm derived epithelium?**

As in any textbook, mesenchyme is here defined as being formed by elongated cells with abundant cytoplasmic extensions, immersed in an abundant, viscous extracellular matrix with few fibers. Mesenchymal cell morphology is commonly present in mesoderm (embryonic or extra-embryonic) and in the evolutionarily recent neural crest. Sponges have a similar connective tissue layer between covering cell layers, which was named mesohyl (Borojevic et al. 1967). The authors concluded that only morphological data was not sufficient to associate the sponge mesenchyma with mesoderm.
Even a simple cover layer of cells can be considered an epithelium (Allaby 1985). Nevertheless, there is a clear distinction between simple cell layer and true epithelial tissue composed by polyhedral and juxtaposed cells with only a small amount of extracellular substance, firmly attached by intercellular junctions, and both morphologically and functionally polarized by attachment to the basal lamina. These features are the basis for true functional epithelial covering layers on all external and internal surfaces of metazoans (excluding articular cartilage). The three germ layers may form epithelium. The mesoderm-derived epithelium is called mesothelium when covering the peritoneum, pleura and pericardium, and endothelium when covering blood and lymphatic vessels. Junctional complexes are less well developed in epithelia derived from mesoderm (except the renal duct with complete junctional structures) than in epithelia derived from ectoderm and endoderm. For instance, tight junctions, adherens junctions and gap junctions are less ordered in endothelia than in the epithelia derived from ectoderm and endoderm (Imhof and Aurrand-Lions 2004). It thus causes endothelial cells more dynamics and mobile. Despite the fact that sponges (at least the most simple asconoid ones) are considered classical diploblasts, i.e. composed of ectoderm and endoderm only, the working hypothesis here is that no sponge adult tissue has a typical epithelial structure, with the possible exception of the Homoscleromorpha. Basal lamina has not been observed in sponges (excluding Homoscleromorpha), in spite of the presence of a dermal membrane in a number of adult sponges and larvae (reviewed in Maldonado 2004). Sponges possess a flagellated component secreted by pinacocytes, consisting of collagen fibrils scattered between pinacocytes, which resembles the lamina reticularis zone of the vertebrate epithelial basal lamina (reviewed in Harrison and de Vos 1991). The classical desmosome and macula adherens have not been described in sponges (excluding Homoscleromorpha), but localized electron-dense deposits exhibiting tonofibril insertions have been reported in two distinct Demospongiae, suggesting the presence of specializations in pinacocyte membranes comparable to the macula adherens and desmosome (reviewed in Harrison and de Vos 1991). Pinacocytes associate through interdigitating membranes (Adell et al. 2004), but opposed membrane junctions are often bordered by gaps of 100-300 nm between cells. This is not so different from what is observed in colonial amoeba Dictyostelium, which does not form epithelium, but has adherens junctions connecting cells through their actin cytoskeleton (Grimson et al. 2000). These details are morphological evidences supporting that the simple epithelial structures of sponges are not sufficient to characterize them as true epithelium, but were committed to originate it later on along evolution. Nevertheless, some authors recognize true tissues in non Homoscleromorpha Demosponges (reviewed in Harrison and de Vos 1991).

Pinacocytes of some Demospongiae and Calcispongia have a large part of the cell body embedded in the mesohyl, and readily migrate into it where they convert to typical coelocytes with fibroblastoid morphology and collagen secretor activity (Fauré-Frémyet 1932, Harrison 1972). It was suggested that the ability to become motile is related to the general absence of specialized desmosomal junctions in sponge epithelia (reviewed in Harrison and de Vos 1991). The absence of solid adhesion and the ability to become mobile would attest against gastrulation in sponges because there is no conservation of cells into a specific germ layer, as introduced previously.

Loose attachment and the mesenchymal nature of pinacocytes are not characteristics of epithelia derived from ectoderm and endoderm, and it is here hypothesized having mesodermal like nature, as previously suggested by Bagby (1970). Assuming early spongimorphs at the base of metazoan origins, early pinacoderm would be ancestral mesothelium-like/endothelium-like tissue. Afterwards, when Porifera phylum branched off from the main metazoans lineage, the common ancestry of Cnidarians and Bilateria is here hypothesized to originate ectoderm and endoderm derived epithelia. This hypothesis is different from the classical view of ectoderm and endoderm being more ancient than mesoderm. Moreover, increasing number of authors argument in favor of mesoderm features among cnidarians, and this is in agreement with the hypothesis of ancient origin of mesoderm fulfilling internal structures and making volume for 3D structure, as discussed later on (Spring et al. 2000, 2002, Scholz and Technau 2003, Hayward et al. 2004, Martindale et al. 2004, Galle et al. 2005, Seipel and Schmid 2005).

The choanoderm is a flagellated flat cell layer, facing the choanoecel of the aquiferous system. Such simple filtering devices with cells containing an apical flagellum surrounded by a collar of microvilli are observed in protonephridia (flame cells), and were suggested to have existed in the ancestor of Bilateria (Dewel 2000). Protonephridia has a terminal flame cell, or crytocyte, and a proximal tubule. It occurs in most of the lower Metazoaa, and has long been regarded as the primitive excretory and osmoregulatory apparatus in animals. Despite the traditional view deriving protonephridia from ectoderm (Willmer 1994), Dewel (2000) further suggested that all Bilaterian nephridial systems have been derived ultimately from the mesothelium of coelomic cavities, which is derived from mesoderm. The hypothesis of mesoderm derived protonephridia was also suggested by Younossi-Hartenstein and Hartenstein (2000), who reported the development of these cells in polyclad flatworms. There is a morphological analogy between choanocytes and flame cells, and they also osmoregulate the multi-cellular structure. Only after molecular studies address these analogies it will be possible to distinguish if there is some degree of homology among them. Actually, we conclude that it is possible to think that sponges may have a mesenchymal/mesenthielal-like nature, a possible precursor for mesoderm evolutionary origin.

The structural similarity between choanoflagellates and sponge choanocyte does not necessarily mean that choanoflagellate-like organisms were ancestors of the extant sponges and the remaining metazoans. An alternative hypothesis regards choanoflagellates as derived from evolutionary simplification of sponges. Choanocyte would be an end branch of the animal evolution with no homolog flagellated collar cell identified in metazoans (Maldonado 2004). Thus, choanocytes-lacking larvae would be
architecturally closer to the remaining metazoans than adult sponges. Reviewing histological organization of adult sponge and larva, Maldonado (2004) identified in larva, but not in adult sponges, several evidences of more complex junction structures and external cell layer with lamina reticularis zone. These data were considered as being consistent with the hypothesis of neotenic evolution of metazoans from primitive spongiomorph larva. Nevertheless, evolutionary simplification is much more common in parasitic organisms than in free life style ones and Maldonado hypothesis needs more evidences to be credited.

**Germ layers and stem cell systems**

Differentiation of all cell lineages from a single progenitor with unlimited proliferation capacity and plasticity could be a hallmark of cell differentiation programs in primitive metazoans (Harrison and de Vos 1991). Sponges have a single totipotent stem-cell system (Borojevic 1966a, Borojevic 1970, Funayama et al. 2005) and it is based on archaeocytes. Its capacity for long-term proliferation is probably explained by keeping high telomerase activity, which prevents cell senescence (Koziol et al. 1998). Archeocytes have mesenchymal morphology and are also responsible for phagocytosis, including self-recognition in a primitive immune system (Willmer 1970).

In contrast to sponges, *Hydra* has three stem-cell systems, two of which produce cells for maintenance and growth of ectodermal and endodermal derived tissues. The third one is responsible for the mesenchymal interstitial cell lineages, which forms a thin mesoglea between epithelia (Bode 1996). In contrast to the autonomy of sponge mesenchymal amoeboid cells, *Hydra* only succeeds to regenerate from a small clump of cells if there is simultaneous presence of at least some tissues of the two epithelia in the regenerative blastema (Bekkum 2004). A different result was observed in marine hydroid *Hydractinia* (Müller et al. 2004). Despite the presence of three stem-cell types in normal condition (for ectodermal, endodermal and interstitial lineages respectively), interstitial stem cells can give rise to all cell types during stressful conditions. Moreover, the epithelial microenvironment had to be preserved in order to generate epithelial cell types from interstitial stem cell system.

Despite the lack of evidence for a third interstitial mesenchymal stem-cell system in Cnidaria other than hydroids, it is possible to recognize a broad gap spearing the stem-cell systems of Porifera and hydroids, potentially representing one of the major steps in the evolution of multicellularity. The emergence of a true epithelium in the common ancestor of Cnidaria/Bilateria, with a basal lamina and its own stem cell system, had pivotal importance. Epithelial cells do not usually cross the basal lamina to colonize tissues derived from other germ layers, and thus a stem-cell system is required for maintenance of each germ layer-derived tissue. It is possible that gastrulation coevolved with the basal lamina and stem-cell systems, since gastrulation would place cell layers and their microenvironment in a defined axis, as in Cnidaria and Bilateria. The totipotency of *Hydractinia* interstitial stem cells is observed only under stressful condition and is dependent of the epithelial microenvironment, which was already positioned by gastrulation. It could be viewed as an intermediary condition from one totipotent mesenchymal stem cell system in Porifera, to isolated germ layers with their own stem cell system in most Bilateria. This is not a rule, but a tendency, and exceptions are expected. For instance, ectoderm may switch for mesoderm lineage during axolotl tail regeneration (Echeverri and Tanaka 2002). Planarian have only one totipotent stem cell system, the neoblasts, despite the presence of three germ layers (Reddien and Alvarado 2004). This divergent stem cell plasticity would be responsible for the uncommon capacity for body regeneration. Even presenting spectacular capacity for regeneration, a preexisting ordered body is needed for perfect regeneration, and this order was built during planarian or axolotl gastrulation. This is different from sponges, which regenerate from masses of mesenchymal cells without any influence of preexisting gastrulation order and no true epithelium separating germ layers’ derivatives.

**Gastrulation: amoeboid (mesenchymal) and flagellated cells?**

Since sponges cells have sufficient autonomy to generate a functional body from a clump of amoeboid (mesenchymal) cells adherent to the substrate, the major requirement for their multicellular integration is the control of cell proliferation versus cell differentiation. This has already been proposed to be the major requirement for formation of multicellular bodies from flagellated Protozoa (Buss 1983).

Mitosis requires the use of the microtubule organizing center and interruption of flagellar activity, and it hampers the movement of the swimming flagellated colony. Cell divisions have to be restricted to amoeboid cells. They have to follow a temporal program in which the functional flagellated state is interspersed with dividing cells in the amoeboid state. For swimming organisms it may be advantageous to segregate amoeboid cells inside, as in swimming sponge larvae or colonial choanoflagellates (King 2004). In contrast to Buss (1983), we do not consider that this segregation should be considered as true gastrulation, since it may be caused simply by a proliferating state, as in colonial choanoflagellates. The essence of being a sponge is thus the coordinated and cooperative opposition of the amoeboid and flagellated state of their cells, as in a choanoflagellate colony (Lévi 1970). In this context, the genetic control of the self-renewal of dividing amoeboid cells versus differentiation of flagellated cells is the first requirement towards integration of the multicellular body, independently of gastrulation.

**How old is mesoderm?**

The formation of mesoderm in jellyfish has already been suggested (Spring et al. 2002, Müller et al. 2003, Seipel and Schmid 2005) and is in accordance with the hypothesis of a spongiomorph mesenchymal structure originating mesoderm in the common ancestor of Ubilateria and Cnidaria. Actually, during medusa bud development, cells detach from the epithelial outer layer and ingress to form the entocodon, a proliferating cell mass separated from the ectoderm and
endoderm by an extracellular matrix (Boelsterli 1977). Cells from the entocodon proliferate and migrate to give rise to new tissues, such as the striated (including nonmyoepithelial cell layers) and smooth muscle tissues of the developing mesoderm (Spring et al. 2002, Müller et al. 2003, Seipel and Schmid 2005). The authors suggested the existence of a motile tri-layered cnidarians ancestor and a monophyletic descent of striated muscle in Cnidaria and Bilateria. The expression of mesoderm and myogenic cell line-specifying genes was assessed to substantiate that the entocodon in cnidarians is homologous to the mesoderm of bilaterians. These data come from JellyD1, related to an ancestral MyoD gene (Müller et al. 2003) brachyury, Mef2, Snail (Spring et al. 2002) and Twist (Spring et al. 2000). Based on mesoderm markers, gene expression, and morphological features, the authors suggested that gastrulation would be initiated at the blastula-larva transition, interrupted at the larva and polyp stages, and continued during entocodon formation. Following the authors, diploblasty evolved secondarily in Cnidarian larvae and polyps (Seipel and Schmid 2005). The attached polyps do not feed and would be an evolutionary simplification of a more complex form. The mesodermic form of life is faced with locomotory and other complex behaviors, and these would be the evolutionary pressure to retain genetic programs of other cell types.

These data are in agreement with the early origin of mesoderm features in spongiosquam urmetazoans (maybe not a true mesoderm), before the origin of true gastrulation processes. In the next section this hypothesis will be confronted with new genetic evidences.

**Genetic evidences**

Among all cloned sponge genes, the most significant to the discussion on sponge gastrulation and germ layers is the brachyury family of T-domain containing transcription factor (Manuel et al. 2004, Adell and Müller 2005). A consensual expression for brachyury in the blastopore and subsets of mesodermal cells in most metazoans has now emerged (Technau 2001). In embryos of the sea anemone Nemastostella, a basal Cnidaria, brachyury is expressed around the blastopore and its derivatives, the endodermal mesenteries (Scholz and Technau 2003). In Hydra and jellyfish polyps, expression is found in the endoderm of the mouth anlage (Technau and Bode 1999, Spring et al. 2002). The blastopore has a key function in the gastrulation movements of all metazoans, but the cell types it gives rise to, in a brachyury-dependent manner, vary (Marcellini et al. 2003). It specifies the cells in which it is expressed. Marcellini et al. (2003) observed after heterologous experimentations that brachyury orthologs from different phyla, including Cnidaria, were able to induce the specification of mesoderm and/or endoderm lineages in competent Xenopus tissue, and distinguished both ancestral and derived functions of brachyury proteins. Therefore, their observation could be directly linked to an ancestral function of brachyury in mesoderm specification and blastopore formation. A derived function is proposed for insects and tunicates, with no circumblastoporal expression, lost of the N-terminal peptide and inductive activity, in heterologous assay, for both endoderm and mesoderm.

Protein localization of Sd-Bra (sponge brachyury homologous) showed a granular pattern in the cytoplasm of some cells dispersed in the sponge tissue. Only in a few cells was the signal seen both in the cytoplasm and the nucleus (Adell and Müller 2005). Sd-Bra is also located in the cytoplasm of cultured sponge cells. Low amounts of Sd-Bra were found in almost all the cells that adhered to the plastic after 24 hours of culture; later the expression is restricted to a few cells of the already formed primmorphs, the sponge cells aggregate.

Brachyury expression in sponges is not restricted to embryo or larval development, but includes adult mesenchymal cells undergoing cell movements and specification. It is possible that the original feature of brachyury in urmetazoan spongiiomorphs was to regulate genes involved in morphogenetic movements and differentiation of mesenchymal cells, and was afterwards committed to gastrulation cell movements in other metazoans. Again, gastrulation in sponges seems to be very primitive but further data on sponge brachyury expression and function would shed light on this question.

**Hox complex: genetic programs for antero-posterior patterning, downstream gastrulation**

The processes of gastrulation are very flexible and appear to have changed rapidly during evolution in response to environmental changes and architecture of the egg. Nevertheless, it is possible to recognize homology among protostome and deuterostome gastrulation (Holland 2000). Gastrulation is based on common genetic interactions and Antp-Hox genes are part of these conserved gene interactions (Martindale 2005). Their expression is set up during gastrulation and they function as transcriptional regulators arranged in clusters along the chromosome (Hom complex in invertebrates and Hox complex in vertebrates), whose genomic organization reflects their central roles in patterning along the anterior/posterior (A/P) axis (Duboule and Dolle 1989, Graham et al. 1989, Megennis and Krumlauf 1992, Duboule 1994, Ferrier and Minguillon 2003).

If Cnidaria has true gastrulation with tissue movements remodeling an initially unstructured group of cells, with a hierarchy of genetic control mechanisms that sets up the embryonic axes and specifies the territories of the future germ layers, one should expect a Cnidaria Hox system associated with gastrulation. In a recent publication Kamm et al. (2006) concluded that axial patterning and diversification in the Cnidaria predate the Hox system, since there is no equivalent Hox cluster in Cnidaria. According to the authors, the cnidarian Hox genes are expressed in patterns that are inconsistent with the Hox paradigm. Nevertheless, another recent publication has divergent conclusions. Chourrat et al. (2006) characterized the full Hox/ParaHox gene complements and genomic organization in two cnidarian species, and suggested an ancestral ProtoHox cluster consisted of only two anterior genes. Non-anterior genes could have appeared independently in the Hox cluster after the separation of bilaterians and cnidarians. These conclusions were partially supported by the gene linkages of five genes (HoxC/HoxDa/HoxDb/Evy/HoxA) in a tandem...
array over about 50 kilobases (kb) and two other homeobox genes (Mnx and Rough) about 200 kb downstream. Mnx is also present in the neighborhood of chordate Hox and the Drosophila Rough is on the same chromosome arm, 3R, as the ANT-C and BX-C Hom complexes. These new data on gene structure, genomic organization and Cnox2-Pc (antterior Hox gene) expression during the establishment of an anterior-posterior axis (Masuda-Nakagawa et al. 2000) support true gastrulation in Cnidaria.

Identification of Hom gene clusters in sponges would be surprising. It would be a strong evidence supporting homology among bilaterians and sponges axial patterning and probably its regulation during gastrulation. After Seimiya et al. (1994), Coutinho et al. (1994) and Kruse et al. (1994), several other authors have tried to identify and clone sponge Antp-Hox genes. The chosen methodology was the use of heterologous PCR primers designed for conserved regions of homeobox genes of the Antp-Hox family (Seimiya et al. 1994, 1997, Coutinho et al. 1994, Kruse et al. 1994, Degnan et al. 1995, Hoshiyama et al. 1998, Manuel and Le Parco 2000, Wiens et al. 2003, Perovic et al. 2003, Hill et al. 2004, Larroux et al. 2006). None of these authors succeeded, and the “sponge” Antp-Hox genes reported by Degnan et al. (1995) (SpoxH1 and SpoxH2) were isolated from an ascidian contaminant DNA, as already indicated by Manuel and Le Parco (2000). We can not draw any conclusion until the sequencing of the sponge genome has been completed, but all the failures to identify a sponge Antp-Hox gene, using a precise methodology, could be an evidence that the sponge type of gastrulation, or its multicellular grade of organization, precedes the origin of Antp-Hox gene interactions and Bilateria type of antero-posterior axis patterning. If gastrulation does occur in sponges, it would be very simple and primitive when compared with that in other metazoans that already contain these genetic toolkits.

Phylogenetic relationship among sponges homeobox genes

Many homeobox genes have been identified in sponges, as reviewed by Manuel and Le Parco (2000), Gauchat et al. (2000), Wiens et al. (2003) and Larroux et al. (2006). Unlike these authors, we considered the Antp-nonHox NK3/Bap gene family as a separate sub-group of the NKL family, as do Pollard and Holland (2000) and Jagla et al. (2001). Contrasting to the analysis done by Gauchat and co-workers, who did not included NK3/Bap genes, it is generally assumed that Seimiya et al. (1994) was the first to identify sponge Antp-nonHox genes of the NK-3/Bapx (prox1) and Msx (prox3) families. Another Antp-nonHox gene (BarBsh-Hb and RenBsh) was later added to this list, and they were classified into the Bsh/Bar homeobox gene family (Hill et al. 2004, Larroux et al. 2006). The nonAntp homeobox gene families, such as Iroquois (SUBDOIRX-a), POU (spou-l and spou-2) and PAX (sPax2/5/8) were further identified (Seimiya et al. 1997, Hoshiyama et al. 1998, Perovic et al. 2003, Larroux et al. 2006). Manuel and Le Parco (2000) could not classify several other closely related sponge genes into an obvious orthologous group of homeobox genes. This related group contained EfH-1 (Coutinho et al. 1994), prox2 (Seimiya et al. 1994), SpoxTA1 (Degnan et al. 1995) and EmH-3 (Richelle-Maurer et al. 1998). Coutinho et al. (2003) published a possible scheme for classifying these related and problematic sponge homeobox genes into the Lbx/Tlx gene family. This result was also confirmed by Gauchat et al. (2000), Wiens et al. (2003), Hill et al. (2004) and Larroux et al. (2006). Actually, Tlx and Lbx are closely related homeobox gene families (Pollard and Holland 2000, Jagla et al. 2001, Coutinho et al. 2003), suggesting a common evolutionary origin.

Ancestral NKL-like genes co-originating with spongiomorphs: genetic programs for mesenchymal cells?

The close homology among Tlx and Lbx gene family extends to human, mouse, amphibious, Anopheles and Drosophila genomic organization, raising the possibility of gene duplication followed by evolutionarily conserved co-regulation. Tlx and Lbx are linked in the vertebrate and Drosophila genomes, and they are believed to have evolved from an ancient gene cluster common in ancestral Bilateria (NKL-like), which has been secondarily split in the chordate ancestry (Pollard and Holland 2000, Jagla et al. 2001, Luke et al. 2003). From the ancestral NK gene cluster, only the Tlx–Lbx and NK3–NK4 linkages have been retained in chordates. No split has occurred in the 93DE (NKL) gene cluster of Drosophila or Anopheles, which retained the ancestral physical linkage of slouch, bap, tin, 93Bal/C15/311 (Tlx), ldl and lbe (Jagla et al. 2001). This is the opposite of the splitting of the Hox cluster in Drosophila and its conservation in chordates.

The Drosophila 93DE homebox gene cluster (NKL) appears to participate in a network of gene interactions that governs progressive cell fate decisions during Drosophila mesoderm patterning, just downstream from gastrulation (Jagla et al. 2001). Since it is now established that sponges have homeobox genes classified in the Nk3 and Tlx/Lbx families, that form the 93DE gene complex in Drosophila, it is reasonable to suppose that these are ancient homeobox gene families from which the 93DE/NKL homeobox gene complex evolved, but which was further split in the chordate NKL. Because homebox gene families Nk3 and Tlx/Lbx have only been found in animal lineages, as far as we know, we assume that these genes appeared in the early stages of metazoan evolution. Alternatively, but less probably, these gene families could have existed in other phyla (prokaryotes, fungi and plants), but were subsequently lost. The grouping of Ctenophora, Porifera and Nematoda Tlx genes in a phylogenetic tree is in agreement with the early origin of Tlx gene family in basal metazoans (Martinelli and Spring 2005).

We do not know the role and genetic map of Nk3 and Tlx/Lbx in sponges. Further investigations in these directions are key points to determine if the ancestral NKL-like genes co-originated with ancestral spongiomorphs. It will also address its probable co-evolution with mesoderm development, controlling proliferation and differentiation of mesenchymal cells. Recently, the NKL gene complex was linked to mesoderm origin and evolution (Garcia-Fernandez 2005).
The *Drosophila* 93DE homeobox gene complex is under the control of BMP2/4-dpp signaling during gastrulation. Acting as morphogen, the concentration of dpp (in a functional state) along the dorso-ventral axis of the *Drosophila* mesoderm determines the 93DE gene that will be expressed. Since NKL was split in the chordate lineage, we do not expect extended evolutionary conservation during mesoderm patterning in protostomes and deuterostomes, but analyzing each gene individually it is possible to recognize, in some aspects, similar functions inducing cell proliferation and differentiation control of mesodermally derived cells (Jagla et al. 1995, Newman et al. 1997, Park et al. 1998, Rovescalli et al. 2000, Lettice et al. 2001, Holland 2003). Genes of the NKL/93DE complex are not mesodermal markers, and they participate in other developmental processes, commonly controlling cell proliferation and differentiation. Since Nk3 (Bap) and Tlx gene families were described in sponges, further functional analysis of these sponge homeobox genes would distinguish deep homology among the Nk3 (Bap), Tlx and by consequence, the NKL complex. These functional analyses would thus address the hypothesis wether sponges are organized by mesenchymal cell programs which originated mesoderm germ layer.

Are Tlx genes deeply homologous?

The mouse Tlx-2/Hox11L1 promoter is an established model to test the presence of BMP2/4, as Tlx-2 is a direct target for the BMP2/4 signaling pathway (Guo et al. 2001). Tlx-2/Hox11L1 is expressed at the highest concentration of BMP in extraembryonic mesoderm of the ventral yolk sac (extraembriionary hematopoietic tissue) of the mouse embryo (Tang et al. 1998). This is similar to the expression of the homologous *C15* gene in the homologous BMP rich dorsal amnioserosa of the *Drosophila* gastrula (Jagla et al. 2001). This comparative expression analysis indicated evidences for homologously conserved gene interaction during dorsoventral patterning, since the BMP rich ventral region of arthropods is homologous to the BMP rich dorsal region in vertebrates. The comparison analysis can be extended to the functional level and new evidences of deep homology among Tlx genes were discussed. Results from hox11L1/tlx-2 knockout mice from diverse groups are different. First, postnatal lethality was associated with neurological problems (Hatano et al. 1997, Shirasawa et al. 1997). Later, Tang et al. (1998) observed embryonic lethality caused by proliferation failure in embryonic and extra-embryonic (BMP rich primitive hematopoiesis in yolk sac) mesoderm during gastrulation. This result is in agreement with Tlx expression in the BMP rich region, a hematopoietic region in *Drosophila* and vertebrates. Further analyses of functional and expression data suggest the involvement of the Tlx/Hox11 gene family in hematopoiesis control. The human Hox11/tc13/Ttx1 (homeobox gene/T-cell leukemia) is a proto-oncogene first described in leukemic T cell patients with accumulation of blast cells that fail to differentiate normally (Dube et al. 1991, Hatano et al. 1991, Kennedy et al. 1991). Its oncogenic activity has been confirmed in myeloid lineages by bone marrow transfection with retroviruses containing Hox11 (Hawley et al. 1994). After comparative expression analysis, clinical cases, knock-out and enforced expression, there is enough data suggesting that Tlx expression could be associated with proliferation of immature cells, mainly the BMP rich hematopoietic region, and with the delay or abrogation of their terminal differentiation. Additional data is in agreement with this hypothesis, as seen in the following sentences. Erythroblasts already committed to the differentiation program lose all differentiation markers and become mesenchymal adherent cells after enforced expression of Hox11/Ttx1 (Greene et al. 2002). Moretti et al. (1994) detected by RT-PCR the expression of the Hox11/Ttx1 gene in very uncommitted human bone marrow CD34+ cells, which are target cells for BMP signaling (Tang et al. 1998). Retrovirus-transfected ES cells expressing the Hox11 gene became representative of early stages of primitive yolk sac hematopoiesis, and contain primitive erythroblast and monocyte cell lineages (Keller et al. 1998). The cell lineage K562, representing yolk sac primitive hematopoiesis, expresses a Tlx gene, but only during the proliferative immature state (Coutinho et al. 2003). Hox11 enforced expression successfully immortalized cell lineages from yolk sac, which rendered 26 lineages characterized as primitive hematopoietic and eight as hemangioblasts (Yu et al. 2002). These data link Tlx gene family with induction of cell proliferation and abrogation of differentiation progress, or even programming cells to become primitive-hematopoietic cells of the yolk sac extra-embryonic mesoderm. If the role of Tlx gene family is deeply conserved, from sponges to vertebrates, then the hypothesis of an early origin of mesoderm will be supported.

Knowledge of genetic controls in sponge stem cell biology is scarce. One of the most studied transcription factors known to be specifically expressed by sponge mesenchymal stem cells, the archaeocytes, is EmH-3 (Tlx homeobox gene family) (Richelle-Maurer et al. 1998, Richelle-Maurer and van de Vyver 1999). RT-PCR analysis of EmH-3 expression indicated that it is correlated with archaeocyte proliferation after gemmule hatching. It is down regulated during differentiation of a functional aquiferous system, which is the major morphogenetic phenomenon during the development of a functional sponge. The temporal expression of an EmH-3 homologous (*Renprox2*) in *Haliclona* demosponge development was recently published by Larroux et al. (2006). The RT-PCR results showed that this Tlx gene family is weakly expressed during early stages of development, when cleavage is the main way for cell proliferation. The mRNA level increases significantly during metamorphosis and juvenile form of adult sponge, a period of much higher mitosis activity. These expression data seem to be similar to what is observed in Bilateralen Tlx genes. Actually, increased Tlx/EmH3 expression is associated with mitoses of immature cells, and with delay or abrogation of their terminal differentiation.

Regulatory similarities between sponge *EmH*-3 and human Tlx promoters were proposed (Coutinho et al. 1998, 2003). Using a reporter-gene strategy, the EmH-3 promoter was shown to be operational in mouse 3T3 cell lineage and in the self-renewal and differentiation of the human cell line K562, a representative of human yolk sac hematopoiesis. The latter cells express the endogenous Tlx gene, but down regulates it when induced to differentiate with sodium butyrate. The
EmH-3 promoter was active in K562 undifferentiated cells and down regulated during their differentiation. This is in agreement with the expression pattern of the endogenous Tlx gene of K562 cells, and with the high expression of the Emh-3 gene in the sponge archaeocytes followed by its down regulation after differentiation (Richelle-Maurer and van de Vyver 1999). This heterologous model provided evolutionary evidence of a deep conservation of Tlx expression, associating Tlx/EmH3 expression with proliferation and delay or abrogation of terminal differentiation of immature sponge and yolk sac metazoan precursor cells (K562).

Genes originally performing a defined function during development can be co-opted during evolution to play secondary roles in different embryonic processes. It has happened with the Tlx gene family, which is also expressed in certain regions of the developing central nervous system, the mesenchymal cranial neural crest, and the proliferative fibroblastoid cell mass that gives rise to the spleen (Roberts et al. 1994, Logan et al. 1998). These are apparent secondary functions, since C13 (Drosophyla) is not expressed in neurons. Considering the control of proliferation and differentiation of extra embryonic mesoderm as the primary function of Tlx gene family in mouse and Tlx deep conservation, at least in expression regulation, the most plausible scenario for metazoan origin is a urmetazoan spongiomorph with mesenchymal stem cell lineages under the control of primitive NKL homeobox genes (Tlx for instance) for proliferation initiation and abrogation of terminal differentiating program. Following the discussion on sponge development presented before it would be the base for the later origin of mesoderm. Since the sponge genome contains TGF (BMP) receptors (Suga et al. 1999), a further speculative scenario would hypothesize that BMP signaling and Tlx genes formed a tool kit that originated in spongiomorph urmetazoan and was further evolutionarily conserved in archaeocytes, the sponge stem cell, and extra-embryonic mesoderm in vertebrates. These are very speculative thoughts, and additional functional studies with sponge and vertebrate homeobox genes would bring new evidence which would corroborate, or not, these hypotheses.

Conclusion

It was concluded from the analysis of sponge embryology that the developmental processes in these organisms are mainly based on cellular terms and not in terms of germ layers and gastrulation process. Sponge type of primitive gastrulation was here considered to be an individual sequence of complementary cell differentiation events converting flagellated cells into amoeboid morphology, as happened, in a simpler way, in choanosflagellate colonies. Based on functional and morphological data, amoeboid and flagellated sponge cells apparently belong to the mesenchymal and mesenchymal type rather than to the true epithelial type derived from ectoderm and endoderm. It is thus suggested that sponge would have precursors for mesoderm origin. The appearance of a “true” epithelium with a basal lamina would contribute to the major difference between the multicellular grade of organization of Porifera and Cnidaria, with the appearance of an ordered placement of tissues as a result of gastrulation, the preservation of cells in their adult tissue, and new epithelial stem-cell systems for endoderm and ectoderm, in addition to the earliest mesenchymal stem-cell system.

The apparent absence of Hox genes in sponges and the absence of this gene interaction system for antero-posterior axis patterning were here interpreted as evidence against the concept of a sponge gastrulation strictu senso. The presence of NKL genes in sponges would suggest ancestral NKL-like genes co-originating with ancestral spongiomorphs. Moreover, Nk3 (Bap) and Tlx would be the precursor of the Nkl complex. If functional conservation is expected among sponges and bilaterian NKL genes, the primary function in the ancestral spongiomorph would be associated with individual programs for mesenchelial and mesenchymal cell types, and would later contribute to the origin of mesoderm genetic programs. Expression and heterologous data corroborated the hypothesis of deep homology among Tlx genes. However, more data at the functional level could bring more solid evidences for it. If so, this genetic interaction would be one of the bases for the origin of mesoderm in triploblasts.

According to von Baer’s laws, “the general feature of a large group of animals appears earlier in development than do the specialized features of a smaller group” and “less general characters develop from the more general until the most specialized appear”. Bilaterians do not cross a diploblast phase during their development, but they cross a period with mesenchymal cells in BMP rich vegetal pole, which is homologous to vertebrate yolk sac extraembryonic mesoderm. Considering sponges at the base of monophyletic evolution of animals, and if our hypotheses are further confirmed by additional studies, a general and earlier feature among animal development is the mesenchymal cell mass which evolved to BMP rich mesoderm in invertebrates and to BMP rich yolk sac extraembryonic mesoderm in vertebrates.

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