Cellular aspects of somite formation in vertebrates

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The authors declare no competing interests

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Abstract: Vertebrate segmentation, the process that generates a regular arrangement of somites and thereby establishes the pattern of the adult body and of the musculoskeletal and peripheral nervous systems, was noticed many centuries ago. In the last few decades, there has been renewed interest in the process and especially in the molecular mechanisms that might account for its regularity and other spatial-temporal properties. Several models have been proposed but surprisingly, most of these do not provide clear links between the molecular mechanisms and the cell behaviours that generate the segmental pattern. Here we present a short survey of our current knowledge about the cellular aspects of vertebrate segmentation and the similarities and differences between different vertebrate classes in how they achieve their metameric pattern. Taking these variations into account should help to assess each of the models more appropriately.

Key words: somite, segmentation, metamerism, mesoderm, model, embryonic patterning

1 Introduction

The vertebrates are defined by having a vertebral column: a regular, periodic arrangement of bony elements forming the backbone, which is associated with corresponding periodic arrangements of other tissues including muscles (to bend the backbone), elements of the vasculature, and the peripheral nerves that supply them as well as conveying sensory information. This repeated arrangement of segments comprising derivatives of different germ layers in the embryo ("metamerism") is now more generally referred to as "segmental pattern". In the embryo, the vertebral bodies and associated processes, part of the intervertebral discs, the dermis, the skeletal muscles, and intersegmental vessels arise from repeated structures, called "somites"[1]. These were first described as long ago as the 17th Century by Malpighi, while observing chick embryos at different stages of development [2]. Over the last half-century there has been renewed interest in understanding the mechanisms that ensure that the correct number of somites form, and those that ensure that somites are scaled to the size of the whole embryo, so that smaller and larger embryos are correctly proportioned. In the last quarter century, since the discovery of genes whose expression oscillates in synchrony with somite formation, most of the interest has focused on molecular aspects of the process and in understanding the oscillators. However there has been comparatively little recent work in connecting these molecular events to the cellular processes that result in somites forming from the (pre-somitic) mesoderm laid down after gastrulation, including the cellular changes that precede and accompany somite formation. This review offers a short survey of these cellular events. We begin by describing our knowledge in the chick embryo, where perhaps most of this work has been done. We then compare the process in other groups of vertebrates, and give a short description of the models that have been proposed to explain the periodicity and scaling of somite formation. We emphasize that species differences need to be taken into account when assessing the degree to which each model can explain the process of segmentation.

2 Chick somitogenesis

2.1 Formation of mesodermal structures

During gastrulation three germ layers (endoderm, ectoderm and mesoderm) are formed and organized into the trilaminar embryo, by ingression of cells through the primitive streak (equivalent to the blastopore of other animals). Thereafter, as the primitive streak shortens and its tip (Hensen's node) regresses caudally, mesoderm cells derived from the epiblast move out of the node and anterior streak to deposit the notochord at the midline [3], flanked by the pre-somitic mesoderm (PSM) on either side. The PSM then forms somites, flanked at progressively more lateral positions by the intermediate mesoderm, the lateral plate and extraembryonic mesoderm (EM) (Fig 1 A) [3]. The more lateral the tissue, the more posterior the location at which its precursors ingress through the primitive streak: the midline notochord arises from the tip, the (paraxial) somites just behind, followed by the intermediate mesoderm, future heart, lateral plate and extraembryonic mesoderm through the most posterior streak. The final head-tail position of the cells in each tissue relates to the timing of ingression: later ingressing cells become located more caudally. Fate mapping using the lipophilic dyes DiI and DiO, as well as single cell labelling by injection of Lysinated-Rhodamine-Dextran were used to generate fate maps of the node and primitive streak cells contributing to the mesoderm [4,5]. The anterior portion of the node gives rise to the notochord, the lateral portion of the node contributes to the medial parts of somites, and the anterior portion of the primitive streak contributes to the lateral parts of somites (Fig 1 B) [4]. Cells contributing to the PSM and somites are found in the anterior 2/3 of the primitive streak [5]. Grafting experiments have shown that cells of the node that normally give rise to somites are not committed to their fate whereas notochord precursors are [6]. The fate of mesodermal precursors along the length of the streak corresponds roughly to the future medial (anterior streak) to lateral (posterior streak) axis of the future embryo, but there are no sharp boundaries between these prospective streak regions [5].

2.2 Are there stem cells in the node?

Several studies have suggested that the node contains a population of stem cells that self-renew and contribute to differentiated progeny in the somites and notochord. Labelling

a single node cell with Lysinated-Rhodamine-Dextran yielded progeny within the PSM organized into clusters, about 6-7 somite-lengths apart [4,7]. The clustering suggested that the PSM precursors resident in the node divide at time intervals and immediately release one daughter cell to the PSM, where they proliferate further to form a cluster. In mouse and chick, when a group of GFP-labelled cells of the chordoneural hinge (CNH), a late-stage equivalent of the node, was grafted to the node-streak border it contributed to axial and paraxial structures and to the tail bud. This grafting was repeated three times into successive host embryos; the pattern of cellular contribution was consistent with self-renewal, and suggested that cells are plastic, with the ability to reset their developmental stage [8], [9]. Retrospective clonal analysis performed in mouse embryos using the LaacZ reporter, which relies on a rare homologous recombination event to give single cell-labelling, produced clones in the neural tube, notochord and somite mesoderm as well as in the tail bud, suggesting that the founder cell has self-renewed as well as giving rise to descendants in two germ layers; these results led to the concept of neuromesodermal (NM) progenitors [10]. Based on developmental timing, cell cycle, and type of progeny it was inferred retrospectively that the labelling may have happened in the node [10].

In a recent study [11], anterior epiblast cells at the primitive streak stage (from a position that normally never contributes to the node) were grafted to a position adjacent to the node from where they could be carried into the node by gastrulation movements. Graft-derived cells contributed to the node, notochord and somites (with expression of appropriate markers), while some labelled cells remained in the node, suggesting that the node may act as a niche to capture some precursors that then become resident in it. This was tested further by serial single cell transplantations, which led to progeny appearing both in the node and in node-derived tissues, supporting the idea that the node is a niche specifying self-renewal of resident cells. Live cell tracking of node cells suggested that this niche is located in the posterior part of the node (adjacent to the anterior primitive streak). Single cell RNA sequencing revealed that cells that become resident in the posterior node have enriched expression of markers of the G2/M transition of the cell cycle [11].

2.3 Somite formation in the chick embryo

Somites are generated in head-to-tail order on both sides of the midline. As the unsegmented pre-somitic mesoderm (PSM) is laid down by the regressing node, somites bud

off from the anterior PSM (Fig 2). Consequently, PSM material is added posteriorly but 'removed' anteriorly. As a somite matures, 'younger' somites continue to be generated from the remaining PSM. Thus, anterior somites are developmentally older than posterior ones.

The PSM is organized as a loose mesenchyme (Fig 2 B, C) [12], whereas the newly formed somite comprises elongated, columnar epithelial cells radially aligned around a centre filled with irregularly organized, randomly oriented, mesenchymal cells, an arrangement described as a rosette with a somitocoele (Fig 2 B, D) [12,13]. The somitic epithelial cells are polarized like a classic columnar epithelium, being narrowest at their luminal (apical) end and widening basally, where the nucleus is predominantly located [12]. Most of the mitochondria are basal, Golgi bodies are apical to the nucleus, microtubules and microfilaments reside along the lateral cell walls, and coated vesicles concentrate apically with ribosome-rich protrusions [14]. Somite epithelial cells attach to one another apically by desmosomes and basally by tight junctions [12] (Fig 2 D'). Apically, there are numerous actin and α-actinin filaments [15,16]. The basal surface of the somite is covered with a lamina containing laminin, fibronectin, collagen, and cytotactin (Fig 2 D') [12,17–21].

Somites are transient structures that undergo subsequent changes. Each somite is subdivided into a rostral and a caudal half, and dorsoventrally into sclerotome and dermomyotome (which then generates myotome and the dermatome, future epaxial muscle and dermis precursors) as well as ventrolaterally migrating precursors of hypaxial, limb and body wall muscles (Fig 2 B, E, F). The anterior portion of the sclerotome allows migration of neural crest cells and dorsal root ganglia, which directs segmentation of the peripheral nervous system [18,22]. Later in development the somite undergoes resegmentation, whereby each vertebra arises from the caudal half of one somite and the rostral (anterior) half of the following one [23,24] and each somite develops region-specific characteristics according to its axial position. Thus, the process of segmentation, from somite formation to their differentiation, is fundamental for forming the final body plan.

2.4 Occipital somites

In the chick embryo, the first pair of somites to form is the second pair, followed by the first and third pairs almost simultaneously [25]. The first pair of somites does not have an identified anterior boundary and is therefore termed 'rudimentary' or 'incomplete' [26]. This first pair of somites does not have visible cranial and caudal halves [26,27] and disappears by

stage HH10 [25]. Charcoal labelling revealed that the first five pairs of somites generate occipital sclerotome, which contributes to the base of the skull [26,27]. Caudal to the occipital bone, the atlas (C1) and the axis (C2) vertebrae allow for extension and rotation of the head on the neck. DiI/DiO labelling revealed that the anterior portion of somite 5 contributes to the atlas, thus this is the only vertebra deriving from a single somite [24], whereas C2 is generated from three pairs of somites (5-7) because the odontoid process of C2 is derived from the body of C1 [24].

Staining for neural crest cells using antibody HNK-1 revealed that the cranial halves of the first five (occipital) sclerotomes are invaded by neural crest cells. However, no sensory ganglia develop in these somites. When occipital neural tube is grafted to the trunk region in a host embryo, donor-derived sensory ganglia develop at the trunk level, suggesting that the lack of sensory ganglia at occipital level is a property of the occipital somites themselves rather than due to intrinsic regional differences in the neural tube [27].

3 Mesenchyme to epithelial transition (MET)

3.1 PSM epithelialization

The PSM comprises both mesenchymal and epithelial cells. First, cells undergo MET as somites are formed [12], and later formed somites undergo EMT as ventromedial somite cells de-epithelialize to form the mesenchymal sclerotome [28]. *Paraxis*, first identified in mouse, is a basic helix-loop-helix transcription factor expressed in somites and anterior PSM [29]. Homozygous *paraxis* null mutant mice fail to generate epithelialized somites, but are able to segment correctly by appropriate numbers of cells grouping together, suggesting that *paraxis* regulates somite epithelialization but is not required for segmentation [30]. In *Xenopus laevis,* where no epithelial somites are formed (see below), both gain and loss of function of *paraxis* cause defects in cell elongation, rotation, alignment and expression of adhesion molecules, resulting in abnormally-shaped somites [31].

3.2 The core cells

The amniote somite comprises an epithelial rosette with its centre filled with mesenchymal core cells [12]. This centre is referred to as a ‘somitocoele’ [13], ‘myocoele’ [25] or ‘arthrotome’ [32,33]. The origin, fate, behaviour and developmental role of the core cells

are intriguing questions. They have been proposed to contribute to sclerotome [13], but possibly also to myotome [34]. When a single epithelial cell in an established somite was injected with lysinated fluorescein-dextran and followed for twenty-four hours, the cell, or its progeny, either stayed in the rosette or migrated to the core. However, when a single somitocoele cell was labelled, its progeny remained in the core [35]. In contrast, two-photon time-lapse imaging revealed that core cells continuously egress to the epithelial rosette during somite formation [36]. Quail-chick transplants of somitocoele cells demonstrated that this population contributes to the lateral sclerotome, with some migration towards the notochord, and then, after six days, contributed to the ribs and periphery of intervertebral discs [37,38]. Two days after microsurgical ablation of the somitocoele, embryos had fused articular processes and fused vertebral bodies with no intervertebral joints or discs, leading the authors to suggest that the neighbouring sclerotomal cells cannot adapt to form vertebral joints in the absence of the somitocoele population [33]. When GFP-labelled somitocoele is ectopically grafted into a somite rosette it contributes to structures according to its new position, suggesting that the fate of the somitocoele is not irreversibly determined [39]. This is in agreement with the observations of egression from the core to the rosette and vice-versa [36], suggesting that either the cell fate choices are flexible or that differently committed cells migrate to places appropriate to their identity [36].

3.3 Role of surrounding tissues in epithelialization

After separation of the PSM of chick [40] or snapping turtle [41] from the neural tube, notochord and node (but not from the ectoderm or endoderm), the PSM still forms somites. However, chick PSM explants isolated from all surrounding tissues never undergo segmentation, and PSM cells tend to disperse especially at the posterior end [40,42]. When mouse tail bud PSM was cultured in isolation from all other tissues, no signs of epithelialization were seen, one or two transitory boundaries were observed in a minority of the explants and expression of *paraxis* was diminished in the anterior two-thirds of the PSM. In contrast, when tailbud PSM was separated from all adjacent tissues but not from the surface ectoderm, epithelial somites expressing *paraxis* did form [43]. Moreover, wrapping PSM in an ectoderm sheath yielded similar results, suggesting that surface ectoderm plays a role in epithelialization, but not in segmentation of the PSM [43]. In chick, removal of ectoderm at PSM levels delays *paraxis* expression, suggesting that the ectoderm is involved

in the early phase of *paraxis* expression [44]. Isolation of PSM from the neural tube with metal foil does not initially disrupt *paraxis* expression, but after 24h the expression is dramatically diminished, suggesting that the neural tube plays a role in *paraxis* expression later in development [44]. *Paraxis* was not expressed when ectoderm removal was combined with neural tube separation, suggesting that PSM epithelialization and *paraxis* expression rely on both the adjacent ectoderm and the neural tube. However, when PSM is isolated from the ectoderm with metal foil there is no *paraxis* expression but somite morphology is normal up to 12 hours, whereas after 24 hours the resulting somites did not form dermomyotome and remained mesenchymal, with no *paraxis* expression, suggesting that PSM alone is capable of generating epithelial somites but ectoderm is required to maintain epithelial organization [45]. When paraxis is electroporated into newly formed chick somites, the dermomyotome cells remained epithelial 24h later, indicating that *paraxis* alone can maintain epithelial structure [45]. An independent study performed in chick PSM explants showed that epithelialized somites can be generated by PSM alone, provided its fibronectin matrix is not destroyed [46]. However, enzymatically isolated PSM, with or without its fibronectin matrix, expresses *paraxis* six hours after incubation, suggesting that neither ectoderm nor fibronectin matrix is necessary for *paraxis* expression [46]. Nonetheless, only those PSM explants that retained their fibronectin matrix formed epithelialized somites, indicating that paraxis expression is not sufficient to generate somites [46]. When the fibronectin matrix is enzymatically destroyed in the presence of ectoderm, no somites form [46]. *In situ* hybridization and RT-PCR revealed strong expression of fibronectin (*Fn1*) in the ectoderm and of its receptor *Itga5* in the PSM, suggesting that ectoderm is a source of fibronectin, which is essential for somite formation [46]. Consistent with this, 3D time-lapse imaging of anterior-PSM revealed that when fibronectin matrix assembly is inhibited, epithelialization slows down and somite formation halts [36].

3.4 Adhesion within the segmental plate mesoderm

PSM cells are less adhesive than somite cells, hence cell adhesion was proposed to have a role during somitogenesis [47]. When chick somites or PSM are dissociated in culture, somite cells tend to reassociate into somite sized-clumps, but PSM cells display only a limited tendency to adhere even after prolonged culture [48]. However, anterior-PSM cells also reassociate, suggesting that an increase in cell-cell adhesion precedes somite formation [48].

N-cadherin is a cell adhesion molecule expressed in PSM and somites of chick, mouse and zebrafish but not frogs [49–51]. In chick somites N-cadherin (but not NCAM or laminin) is expressed in the region of intercellular junctions [20]. PSM explants *in vitro* do not aggregate into somites in the presence of function-blocking antibodies against N-cadherin, whereas anti-NCAM antibodies have no effect [20]. Homozygous mouse mutants for N-cadherin have small irregular somites with a disturbed epithelial organization [52,53]. Blocking N-cadherin with antibodies *in vivo* also generates multiple rows of ectopic somites, extending more laterally than the normal ones [54]. In mouse N-cadherin mutants, somites fragment into rostral and caudal halves [53]. In N-cadherin/cad11 double mutants, each half somite further disintegrates into cell clusters, suggesting that PSM cells have the ability to aggregate independently into small epithelial clusters and the role of N-cadherin and cad11 is to integrate rostral and caudal somitic halves [53]. When chick somites are mechanically stretched they form anterior and posterior semi-somites; the authors concluded that mechanical strain can be morphologically instructive to generate anterior- and posterior-semi somites [55]. However, as von Ebner's fissure subsequently forms in the middle of the somite, this raises the possibility that applying a mechanical force may split the somite prematurely, rather than being instructive for border formation.

3.5 Differential cell adhesion and PSM

When PSM is mechanically dissociated with a glass needle the subsequent shaping of the somites is normal and somite formation occurs simultaneously with that on the unmanipulated side [56,57]. *In vitro* explants of chick PSM [48] or mouse tail bud PSM [58] also undergo sorting. When chick PSM was cut into anterior and posterior halves and cells of either half labelled, dissociated, and cultured together, anterior-PSM cells were found to aggregate in the centre whereas posterior-PSM cells remained dissociated and were displaced to the periphery [48]. However, when tail bud PSM is similarly treated, the posterior-PSM cells form a few small aggregates from which anterior-PSM cells are excluded [58].

Another example of cell sorting within PSM was provided by modulating levels of extracellular signalling kinase (ERK). Cells with elevated ERK have high motility and migrate out of the PSM to accumulate at the periphery. In contrast, cells with decreased ERK levels display lower motility and aggregate within the posterior-PSM forming clumps that segregate

from wild type cells, but are later incorporated into somites, suggesting that cells with similar levels of ERK cluster together and those with different levels repel each other [59].

Cell mixing was also observed within the PSM [4,59–62], raising interesting questions such as whether somites can form based on their intrinsic adhesion properties, when those adhesion properties are established, and whether PSM cells sort as they enter from the tail bud.

3.6 Cell motility within the PSM

PSM cells are motile [59,60,63]. Restricting cell motility within the PSM results in spherical cells, disrupted polarization, and abnormal somites [14,64]. A gradient of decreasing cell motility has been proposed from posterior-to-anterior along the PSM, which opposes the increasing cell density gradient [60]. When PSM cell movements were measured and subtracted from local ECM movements no local directional bias was found, and the distribution and angles of cellular protrusions were random, suggesting that PSM cells do not undergo chemotaxis [60]. FGF seems to be implicated in PSM motility: loss-of function reduced motility and gain-of-function increased motility in the anterior-PSM, suggesting that FGF is necessary for, and sufficient to induce, the graded, random movements of cells observed along the PSM [60].

3.7 Cell polarity within the PSM

RhoGTPases are involved in cell polarity in a variety of models [65]. When constitutively active *Cdc42* was electroporated into chick somite precursor cells, the daughter cells contributed to the mesenchymal somite compartment. In contrast, when *Cdc42* was inhibited, the precursor cells contributed to the epithelial somite compartment, suggesting that *Cdc42* acts as a binary switch, with high levels required to retain mesenchymal properties and low levels resulting in an epithelial arrangement [66]. Overexpression of *paraxis* in the PSM causes cells to contribute to the somite epithelium. Co-electroporation of *paraxis* with dominant-negative *Rac1* resulted in a mesenchymal phenotype, suggesting that the epithelialization effect of *paraxis* is suppressed by *Rac1* [66]. Conversely, in mouse *paraxis* mutants the expression levels of RhoGTPase family members are not changed [67]. These

results suggest that the interaction between *paraxis* and *Rac1* may happen post-translationally, for example by regulating cytoskeletal function [66].

4 Patterning of segmental mesoderm

4.1 Mediolateral axis

In chick embryos, the somite later rotates by 45°, so that the original medio-lateral (M-L) axis is re-aligned to a dorsoventral (D-V) orientation. After ventral body wall closure more lateral structures become located ventrally [68]. This process transforms the initial M-L patterning of the non-axial mesoderm to a D-V arrangement.

In chick, medial and lateral somite precursors originate from two different sources: medial-PSM derives from the lateral node whereas lateral-PSM is derived from the anterior primitive streak [4,61]. Switch-graft experiments, where the lateral node is grafted to the anterior streak or vice-versa, showed that the lateral node continues to contribute to notochord and medial somites according to its original fate, whereas the anterior streak switches fate and contributes to the notochord, demonstrating that lateral-PSM precursors in the streak are not yet committed to their fate [6]. The fates of medial- and lateral-PSM remain plastic even after somite formation: switching between newly formed medial and lateral-half somites had no developmental consequence, with cells contributing to structures according to their new positions [69]. Moreover, when PSM is grafted into the lateral plate mesoderm, it does not form somites, but changes fate to lateral plate mesoderm [70]. These findings suggest that there is an extrinsic mechanism acting in a position-dependent manner to regulate mesodermal fate.

The notochord is the source of noggin whereas the lateral plate mesoderm is a strong source of BMP4 [70,71], setting up a gradient of BMP activity [72]. When cells expressing BMP4 are grafted into the PSM, somite formation is inhibited, but when Noggin-soaked beads are grafted into lateral plate mesoderm ectopic somites form [71]. Moreover, exposure of posterior primitive streak to either Hensen's node or Noggin re-specifies prospective lateral plate mesoderm to a somite identity, generating "bunches of grapes" [71,73,74].

BMP induces expression of the lateral-somite marker *Sim1* [75], but suppresses expression of *Swip1*, a medial-somite marker (Fig 3. A) [76]. Both markers are expressed in formed somites but not in the PSM. At the same time Shh from the notochord induces medial

identity in the neighbouring PSM, which cooperates with noggin to antagonize BMP 'lateralising' signals [75,76].

In addition to regulation of M-L identity by the BMP4/noggin gradient, PSM cells appear to be able to sort into medial and lateral domains as revealed by DiI labelling of medial and lateral somite precursors, which are first dispersed throughout the posterior PSM, but later (anteriorly) sort to medial and lateral aspects of the PSM [4,61]. Interestingly, when medial-PSM is ablated the remaining lateral-PSM does not segment, whereas removal of the lateral-PSM does not affect medial PSM segmentation, which led the authors to conclude that the medial-PSM acts as a pacemaker for somite formation [77].

4.2 Dorsoventral axis

In chick, the dorsoventral (D-V) axis of somites is not determined until after somite formation. Rotation of newly formed somites by 180° about their D-V axis still results in the dermomyotome and the sclerotome forming in their normal positions [78,79]. These results suggest that the dorsoventral axis is not fixed until after somite formation. D-V patterning of the somites relies on external structures [80]. In chick, grafting the notochord, or neural tube floor plate, dorsal to the somites supresses development of dorsal somitic derivatives, demonstrating the ventralizing action of the notochord [81,82]. In mouse, grafting sonic hedgehog (shh)-expressing cells dorsal to the PSM mimics the notochord graft experiments, suggesting that shh from the notochord induces sclerotomal fate [83]. Removal of the notochord and floor plate also dorsalizes paraxial mesoderm, whereas removal of the dorsal neural tube and surface ectoderm generates mesenchymal somites expressing sclerotome markers, indicating that the somite has been ventralized [80].

Dorsoventral patterning has been studied on a molecular level (Fig 3 B). A source of noggin reduces or completely abolishes Wnt1 expression in the neural tube, whereas BMP4 promotes Wnt1 expression [79]. Neural tube-derived Wnt1 upregulates noggin and WNT11 at the dorsal somite [84], and WNT11 in turn is antagonized by shh from the notochord [79]. Loss of Wnt11 expression in dermomyotome correlates with absence of involution of myotome precursor cells and myotome disorganization, which led to the suggestion that Wnt11 may regulate myotome ingression from the dermomyotome [79].

4.3 Rostrocaudal identity within a somite

When “compound somites” are constructed *in ovo* by grafting either single or multiple rostral-halves or caudal-halves from a quail donor into rostral or caudal halves of a chick host in different combinations, the cells of “like” identity mix, destroying the segmental boundaries. Conversely, whenever “unlike” rostral and caudal cells are adjacent, a border is generated, suggesting that the alternation of rostral and caudal identity in the sclerotome serves to maintain the separation between these compartments [85]. Indeed, a border (‘von Ebner’s fissure’) is seen in the middle of each sclerotome, where rostral and caudal cells meet in the middle of the somite.

The subdivision of the sclerotome into rostral and caudal halves also serves to dictate the segmentation of the peripheral nervous system. Motor axons [22] and neural crest cells [18,86] both migrate exclusively through the rostral half of each sclerotome. To determine whether this is due to a property of the neural tube (from which both emanate) or to the subdivision of the somite, rotation experiments of both structures were performed [22]. Rotation of the neural tube did not change the pattern of peripheral nerve segmentation, whereas rotation of the somitic mesoderm led to inversion of the pattern so that the peripheral nervous system components formed in the now caudal (originally rostral) part of each somite. Therefore, the alternation of rostral and caudal somite halves determines segmentation of the peripheral nervous system. A key component responsible for the restriction of axonal outgrowth through rostral somites in chick was recently identified: protein disulphide isomerase (PDI) [87,88]. PDI is expressed in the caudal somite; silencing its expression *in ovo* by siRNA knockdown promotes sensory and motor axon outgrowth into the caudal half of somites, suggesting that PDI mediates contact repulsion of axons from the caudal somite [87,88]. In mouse and chick neural crest cells, the caudal somite migratory route depends on repulsive signalling by semaphorin and neuropilin and Ephrin/Eph in the caudal somite [86,89–92].

4.4 When is the rostrocaudal identity of somite halves established?

Newly formed somites already express rostrocaudal markers; it was proposed that somite polarity is established at the anterior PSM, just prior to somite formation [85].

However, a subsequent study concluded that rostro-caudal polarity is not fully determined in the anterior-PSM [93]. Chick PSM cells from the prospective '-2' somite region were labelled with DiI and DiO (to mark anterior and posterior), and then grafted into the '-1.5' region where a prospective boundary would be expected to form. The anterior DiI labelling was found to be in the posterior part of the resulting somite and the posterior DiO labelling was found in the anterior part of the following somite. The posterior somite marker *Dll1* was expressed in the posterior part of newly formed somites, coinciding with anterior DiI labelling. The authors proposed that during normal somitogenesis PSM cells are biased as to their antero-posterior identities but not fully determined until after morphological segmentation [93].

In contrast, another study suggested that rostrocaudal somite polarity is established much earlier than the anterior-PSM: rotation of small fragments of chick PSM by 180° at different antero-posterior levels of the PSM results in reversal of somite polarity, as indicated by Uncx4.1 expression [94]. However, rotation of fragments in the posterior PSM level does not affect rostro-caudal somite polarity. This was interpreted to mean that rostro-caudal somite polarity is not specified in the posterior PSM but becomes fixed at a 'determination front' situated about half-way along the PSM. This 'determination front' was proposed to correspond to a threshold of FGF signalling [94].

However, rotation of the PSM also rotates the time-order of somite formation, so that a rotated piece behaves relatively autonomously [22]. It has also been shown that there is considerably more cell mixing at the posterior/caudal end of the PSM than more anteriorly, as cell movements gradually cease [4,60,62]. These results leave open all possibilities: cells entering the PSM from the primitive streak may already have their rostrocaudal identity imprinted on them, or they may acquire this at some time/position during their stay in the PSM.

4.5 Establishing somite boundaries

The separation of a newly generated somite from the PSM implies that a border has formed between it and the anterior-PSM. Based on sagittal PSM sections of chick embryos, it was proposed that border formation begins in a dorsal-to-ventral direction as cells of the core-PSM join an epithelialized sheet of dorsal-PSM, and that formation of the posterior somite wall precedes that of the anterior wall [14]. This proposal was supported by later

studies, again based on sagittal sections [66,95]. In contrast, time-lapse coronal imaging of chick embryos suggested that the anterior wall forms before the posterior wall, and that the border propagates in a medial-to-lateral direction [63]. However, using the same technique, another study concluded that the medial-PSM epithelializes first and 'recruits' adjacent cells to form somites, with the posterior wall forming before the anterior one, and the lateral wall forming last of all [36].

Grafting experiments suggested that the PSM contains border-inducing regions [93,95]. When cells are grafted from the presumptive '-1' border into a more anterior location, an ectopic fissure forms that generates two small somites instead of one as on the unoperated side, suggesting that border instructive activity resides just caudal to the -1 border [95]. Grafting ventral-most cells of the '-1' region produced the same result, but when these cells were removed from an embryo, no fissure formed, suggesting that the ventral-most cells of the '-1' region are sufficient to generate a fissure, which forms in a ventral-to-dorsal direction [93]. These findings contradict the proposal that border formation propagates from dorsal-to-ventral [15]. Blocking ectodermal signals with a piece of foil or by replacing the ectoderm with endoderm results in fissure formation, indicating that ectoderm does not influence border formation [93].

5 Non-amniote somitogenesis

Having discussed somitogenesis in amniotes, we now briefly describe these events in non-amniote groups (fish and amphibians) to highlight similarities and differences that may affect our understanding of the cellular and molecular processes governing different aspects of segmentation.

5.1 Segmentation in fish

The teleost fish somite is predominantly myotome, with very little sclerotome [96] (Fig 4 A, C). In zebrafish *(Danio rerio)*, each somite separates from the PSM as an envelope of cells embracing the rounded cells within it [96]. Time-lapse imaging of zebrafish PSM revealed that prior to separation of somites from each other, the initially intermingled prospective border cells undergo segregation, alignment along the medio-lateral axis, and anterior-posterior elongation; however, cells between the border cells do not undergo those changes. The

furrow between prospective somites forms when border cells selectively de-adhere [97]. When viewed from the lateral side, the cuboidal somites change shape into chevrons soon after separation from the PSM [98]. After this separation, the somite undergoes 90° rotation in an anterior-to-lateral direction, with individual cells rotating at different rates. This rotation immediately precedes myotome differentiation (Fig 4 A) [99]. Cell labelling performed in the zebrafish revealed that the medial PSM cells contribute to the medial somite, then elongate before migrating laterally to form the superficial, slow muscle fibres (Fig 4 C). A subset of PSM medial cells contribute to the muscle pioneer cells, whereas lateral PSM cells differentiate into the deeper, fast muscle fibres [100] (Fig 4 C). Other single cell lineage tracing studies in zebrafish demonstrated that lateral fast muscles derive from the anterior somite border cells, whereas the medial muscle fibres originate from posterior somite border cells [99,101].

In the zebrafish, the sclerotome is much smaller than its chick counterpart and forms ventromedially as an epithelial cell cluster after a somite separates from the PSM [96]. As in amniotes, somites are subdivided into rostral and caudal halves. Although motor axons and neural crest cells co-localize with the anterior sclerotome, sclerotome removal does not disrupt the segmental pattern of DRGs or peripheral nerves [96], suggesting that unlike amniotes, segmentation of the peripheral nervous system is a property of the neural tube rather than of the adjacent somites. Also, resegmentation differs from the amniotes. It has been described as 'leaky', as each somite half can contribute to more than two vertebrae regardless of their initial rostrocaudal position within the somite [102]. In contrast to amniotes, zebrafish vertebral bodies derive from the notochord rather than from somites, as removal of the notochord leads to an absence of vertebral centra [103]. Also, the vertebral centra form normally in the zebrafish mutant *fused somites* (which have no rostro-caudal somite polarity and irregular somite boundaries) [103,104].

It has been postulated that there is no dermomyotome in fish [98,105–107], but in the zebrafish, there is a thin layer of 'external cells' on the outer surface of the myotome, underlying the ectoderm [108]. These external cells were also reported in lamprey, lung fish, skate, sturgeon, herring, eel, pacu, sea bass, and sea bream [109–115]. Based on positional homology and expression of the dermomyotome markers *Pax3* and *Pax7*, the external cells could be equivalent to amniote dermomyotome [116]. In pearl fish and brown trout, the external cells express *Pax-7* [117,118]. In brown trout embryos the external cells de-epithelialize and migrate in a lateral-to-medial direction to contribute to lateral fast muscles,

consistent with the myotomal nature of the external cells [119]. In zebrafish, single cell tracking demonstrated that the cell population at this location contributes to muscle and dermis (external cells, hypaxial muscle precursors, muscle progenitors, dermal cells) [99].

5.2 Segmentation in urodele and anuran amphibians

In urodele amphibians (the Mexican axolotl and the Ribbed newt), PSM cells are organized into a double-layered palisade that is elongated in the dorsoventral direction, as viewed in SEM sagittal images (Fig 5 A) [120]. Later, the PSM cells re-shape and possibly turn by 90° to form a rosette-shaped somite with cells radiating from the centre but without a central somitocoele (Fig 5 A) [120,121]. Subsequent rearrangements involve apical retraction of dorsoventrally oriented somite cells, which reposition themselves into an antero-posterior orientation, and elongate to form myotome (Fig 5 A) [120]. Those reoriented cells form multinucleated myotome cells by extending cell processes, establishing end-to-end contact and merging together, probably involving cell fusion [120]. Both the rosette reorientation and myotome fusion proceed in medio-lateral direction in the somite and in head-to-tail order [120].

SEM studies of development in two anuran amphibians, the African clawed frog (*Xenopus*) and the Southern Leopard frog (*Rana*), described PSM cells to be organized into double-layered, polyhedral blocks (Fig 5 C), elongating in a mediolateral direction to form spindle-shaped cells that become organized at right angles to the head-tail axis (Fig 5 D) [120–122]. Next, the cells of the PSM bend individually and independently rotate, 90° in *Xenopus*, and 45° in *Rana*, with the medial edge moving forwards to isolate from the PSM (Fig 5 B) [120–122]. The cellular rotation starts from the middle of the somite and extends dorsally and ventrally [122,123]. There is no somitocoele in *Xenopus* somites but a “pre-somitocoele” can be found in the unsegmented PSM [121,122]. In *Rana*, but not in *Xenopus*, myotome cells undergo fusion [120–122]. As described above for urodeles, the fusion in *Rana* proceeds from medial to lateral in the six most-caudal somites, where tandemly aligned cells send their protrusions to fuse immediately after segmentation [120]. So, in *Xenopus* a single somite is one cell long whereas in *Rana* a somite is more than one cell in length [121,122].

As in fish, most of the anuran somite is myotome, with very little dermomyotome and sclerotome. The dermomyotome cells form a sheath over the myotome cells [120]. The

dermomyotome forms together with the myotome as somites bud off from the PSM [122,124], an observation that conflicts with earlier reports that the dermomyotome remains as an unsegmented curtain [121]. It is not clear whether the dermatome undergoes rotation. In *Xenopus*, lineage tracing and ablations of a cell population located in the lateral PSM, also referred to as the "lateral somitic frontier" (LSF), revealed that the sclerotome and dermomyotome derive from the LSF [125]. Thus, in *Xenopus*, unlike the chick, somite compartmentalization into sclerotome, dermomyotome and myotome appears to precede segmentation [125].

In urodeles, the myotome becomes subdivided into rostral and caudal halves and the somite undergoes resegmentation, but neither is the case in anurans [126,127]. In axolotl and the Japanese Fire-bellied newt, the myotome transiently expresses actin and myosin in rostral and medial myotome halves, whereas in the Oriental Fire-bellied toad and *Xenopus laevis* the expression encompasses the entire myotome, suggesting that rostro-caudal and medio-lateral somite polarity is urodele specific [126]. In axolotl, upon 180° reversal of unsegmented PSM, the rostro-caudal somite polarity is also reversed, leading the authors to conclude that the polarity is autonomous [126]. Homotopic GFP-somite grafts performed in axolotl revealed that a single vertebra is formed from two adjacent somites, consistent with resegmentation [127]. Resegmentation has also been described in the third group of living amphibians, the caecilians [128], suggesting that the anuran condition may be specialized.

6 Models of somite formation

Several models have been proposed to explain how the embryo regulates somite size and number and the rhythm of their formation during development, so as to ensure that the pattern is the same between individuals of the same species irrespective of the size of the individual. Historically, each model is mainly based on observations on one or another group of vertebrates. Here we will briefly present each model and how it may relate to current knowledge of cellular properties during somitogenesis and also to differences between vertebrate groups, as described above.

6.1 Somitomeres and pre-patterning

One proposal was that the PSM is already pre-patterned. Consistent with this, after 180° rotation of a PSM fragment along the antero-posterior axis, the rotated PSM continues

to segment autonomously (in the opposite direction to the remaining host PSM) [22,56,129]. Altering PSM continuity by UV irradiation, a simple cut, or the introduction of a PSM fragment from a donor neither affected segmentation [130,131], nor influenced neighbouring structures. To date, molecular evidence for an existing pre-pattern along the entire PSM is lacking [132], but morphological data were considered to support the hypothesis of pre-patterning. Based on SEM images of chick embryos, Meier (1979) claimed to see *'pairs of circulate parcels accumulate in tandem across the embryonic axis'* and that '*they form the basis for metameric pattern'.* He referred to these structures as somitomeres [133].

Later SEM studies described somitomeres in chick and quail [134], snapping turtle [135], medaka fish [136], newt [137], and mouse [138], with each model organism having a different number and size of somitomeres (reviewed in [139]). However, other SEM studies found no evidence of somitomere-like structures in the PSM of the amphibians *Rana, Ambystoma* and *Pleurodeles* [120]. As no somitomeres were seen by high resolution time-lapse microscopy in the teleost fish *Barbus conchonius,* another study concluded that somitomeres do not exist, at least in teleosts, but are an artefact of SEM preparation [140]. Keynes and Stern argued that in order to maintain a fixed arrangement of somitomeres, there should be little or no cell movement within the PSM, or, at least, that any movement should be restricted to a single somitomere [141], which is not the case, as described above [60,62]. Even Meier reported that somitomeres were not visible under the light microscope [133], as did subsequent studies using light microscopy or by mapping the packing density of the PSM [142]. The failure of methods other than SEM to reveal somitomere structures sheds doubt on whether they are mere artefacts [143].

6.2 Clock and wavefront model

In *Xenopus* embryos, reduction of the prospective PSM generates proportionally smaller somites, with fewer cells, but their pattern and timing of formation were normal. Conversely, in haploid embryos generated by treatment with heavy water (D_2O), which have more but smaller cells than normal embryos, somite size was normal but the cell number per somite was larger [144]. Together with mathematician Christopher Zeeman, a proponent of Thom's 'catastrophe theory' [145], Cooke developed a model to account for the regularity and constancy of segmentation: the 'clock and wavefront' model, to explain how the size of individual somites is determined by the length of tissue available [146]. The model proposes

a ‘wavefront’ (defined as a *‘front of rapid cell change’)* interacting with a ‘clock’ (*‘a smooth cellular oscillator’)* ensuring that a group of cells undergo the change at the same time as they experience the wave and consequently become committed to form a somite [146]. In the tissue reduction experiments, the front has a shorter distance to travel and therefore a group of cells enters the rapid change sooner, hence fewer cells pinch off to form each somite.

When an embryo is transected, the sequential progression of segmentation proceeds as normal despite the physical gap [147]. To account for this, the speed of the wavefront needs to be controlled by a mechanism that can sense the length of the entire embryo, such as a head-tail gradient. Slack therefore proposed replacement of the ‘wavefront’ with either a ‘chemical’ or ‘developmental’ gradient, thus the ‘clock and wavefront’ model should more appropriately be viewed as a ‘clock and gradient’ model [147].

6.3 Cell cycle model

Cooke and colleagues had also performed experiments to establish when, along the length of the *Xenopus* PSM, groups of cells become committed to segment together [148,149]. They used a single, discrete heat shock, reasoning that only PSM cells that were currently responding to the wavefront would be sensitive to the heat shock, thus the difference between the time of the shock and the time of appearance of the first affected (malformed) somite could indicate how long a period of time intervenes between the decision to segment and the act of segmentation itself. The experiments found that a single heat shock causes segmentation anomalies in somites that formed about 4-7 somites after the shock. This result was interpreted as suggesting that the cohort of cells that will segment together is established (specified) as about 4-7 somite lengths before they overtly undergo segmentation. The authors argued that the observed anomalies could not be clones of one initially perturbed cell, as cell division in gastrula mesoderm is too slow to allow the anomalies of the observed size to form in the time available [148].

Similar heat shock experiments in chick embryos produced as many as four repeated disturbances, at regular intervals of 6-7 somites, corresponding to a period of about 10 hours [150]. This is incompatible with the idea that the heat shock reveals when the PSM cells respond to the wavefront and commit to form a somite. Rather it suggests a repeated event, with a period of about 10 hours. Labelling a single cell within the PSM generates discrete clones in the somites to which they contribute, and cells labelled two thirds of the way

towards the posterior-PSM divide every 10 hours [62]. Inhibition of the cell cycle with chemical agents mimics periodic disturbances along the axis caused by the heat shock experiments [151]. The duration of the cell cycle within the PSM was measured as 9-10 hours, corresponding to the periodicity of the heat shock disturbances [151]. These results suggest that there is some cell cycle synchrony between the cells that will segment together, which led to the 'cell cycle model' (Fig 6) [62,151,152].

The model proposes that cells enter the PSM from the node/primitive streak in age order and at the same stage in the cell cycle, immediately after a precursor in the streak undergoes mitosis. This accommodates the existence of resident stem cells in the node whereby the stem cell remains in the node as its daughter enters the PSM [4,7]. This causes cells in the PSM to be arranged roughly in order of their cell cycle history, where phases of the cell cycle succeed each other in space as well as in time. The model explains the results of the heat shock and inhibitor treatments as they block the cell cycle, consequently altering the number of cells that adhere together to form a somite as well as the observed periodicity of the anomalies after a single heat shock [151].

In zebrafish, a single heat shock gave rise to as many as three periodic disturbances at five somite intervals, which in zebrafish corresponds to a time period of 2.5 hours [153]. This could challenge the cell-cycle model as the average cell cycle length within zebrafish PSM is four hours [154], but it is also possible that different species use different developmental mechanisms for this process.

6.4 Reaction diffusion models

Alan Turing proposed a reaction-diffusion model to explain pattern formation without a pre-formed pattern: a chemical, the 'morphogen', provides instructions that are perceived and interpreted by cells to undergo changes [155]. The morphogen diffuses away from a local source through intercellular space forming a gradient that is interpreted differently by cells experiencing different concentrations [155]. Based on this, Meinhardt proposed a mechanism by which reaction-diffusion could explain somite formation [156]. It contains two components: a gradient and an oscillator, therefore it shares some properties with the variation of the 'clock and wavefront' model proposed by Slack [147].

Meinhardt's model proposes that a morphogen is secreted by the posterior end of the embryo, from where it diffuses anteriorly, forming a concentration gradient along the

PSM. The shorter the PSM the steeper its slope and *vice-versa*, allowing for size regulation [156]. The model also proposes that there are two cellular states within the PSM: anterior (A) and posterior (P). Cells oscillate between these two states, where any given cell promotes the opposing state in the neighbouring cell, giving rise to a regular A-P-A-P pattern within the PSM. On its own, the oscillator would form an irregular pattern, and the gradient provides regularity. Therefore the two elements of the reaction diffusion model are interdependent [156]. The gradient is smooth but is translated into steep thresholds by the cells to form a somite boundary.

This model is challenged by the observation that upon rotation of the chick PSM by 180° about the A-P axis, the resulting somites retain their original directionality [56,129]. If the model is correct, the directionality of the host gradient should reset the tightly linked oscillator of the rotated graft, and consequently the A-P-A-P cellular states should be reversed leading to a change in the direction of somite formation. Recently, a variation of the reaction-diffusion model, the 'progressive oscillatory reaction-diffusion' (PORD) model, was proposed (Fig 7) [157]. Like the 'cell cycle' model, but unlike both the 'reaction-diffusion' and 'clock and wavefront' models, the PORD model invokes only local cell interactions as generative of the somite pattern (Fig 7). Cotterell and colleagues performed a number of experiments in chick embryos to compare the performance of the PORD model to other models and found that it can explain many observations better than the clock-and-wavefront and other models involving global patterning events [157].

6.5 Wave and cell polarization model

The 'wave and polarization' model involves propagation of a kinematic wave of somitogenic cell determination interacting with the cell cycle and the mechanism of contact cell polarization [158]. The contact cell polarization idea stems from the observation of Beloussov and Naumidi (1983) of increased cell polarization in the chick anterior PSM, and cells arranging into epithelialized 'fans' prior to separating from the PSM [14]. Polezhaev proposed that non-homogeneous spatial distribution of polarized cells within the PSM occurs by the appearance of so-called 'primary polarized cells' [158]. The primary polarized cells are generated by the passage of the wave of somitogenic cell determination, but only PSM cells at a certain phase of the cell cycle can respond to the wave by changing their state of

polarization. The primary polarized cells serve as 'the centres of polarization' from which the polarized state spreads. The centres of polarization also secrete an inhibitor that prevents other mesenchymal cells from polarising. Polarization is possible when a mesenchymal cell is at a certain cell cycle phase and when the inhibitor levels do not exceed some critical value. The cells respond to changes in inhibitor concentration with some time delay. The model does not need synchronization of the cell cycle within the PSM. Polezhaev (1992) claims that the results of heat shock experiments can easily be explained by the wave and cell polarization model, but it is difficult to see how the repetitive perturbances could be achieved if there is no cell cycle synchrony. Rather, the model predicts irregular defects.

6.6 Clock and trail model

Kerszberg and Wolpert (2000) proposed the existence of an oscillator or 'the clock' in a proliferative region called the 'progress zone', which generates cells. As the proliferating cells contribute to the PSM at different phases of the clock, a wave is generated. In this system, the oscillatory status is permanently stamped upon a cell at the moment of leaving the 'progress zone' (entering the PSM) – therefore the clock is relatively cell-autonomous. In this respect, the model resembles the cell cycle model (see 1.7.3). A 'trail' is formed behind the 'progress zone'. Consequently, as PSM is laid down along the axis it is comprised of cells with cell autonomous behaviours that cannot be changed [159]. In this system the size of the somite is regulated by the speed of the 'clock', the faster the period, the smaller the somite formed. However, this model does not easily explain why a single 'heat-shock' generates multiple periodic perturbations in somite patterning.

7 Conclusion

This short review surveyed aspects of cell morphology and cell behaviour that change along the progression of cells from their ingression into the paraxial mesoderm, to their incorporation into a regular pattern of somites that will eventually dictate the overall body architecture of the adult and the organization of the musculoskeletal system. Some of these cellular changes differ greatly between different groups of vertebrates. We feel that these differences have barely been considered alongside the various mechanistic models proposed to explain the regularity and timing of somite formation, so the connections between molecular events of segmentation and cell behaviours are not yet sufficiently understood.

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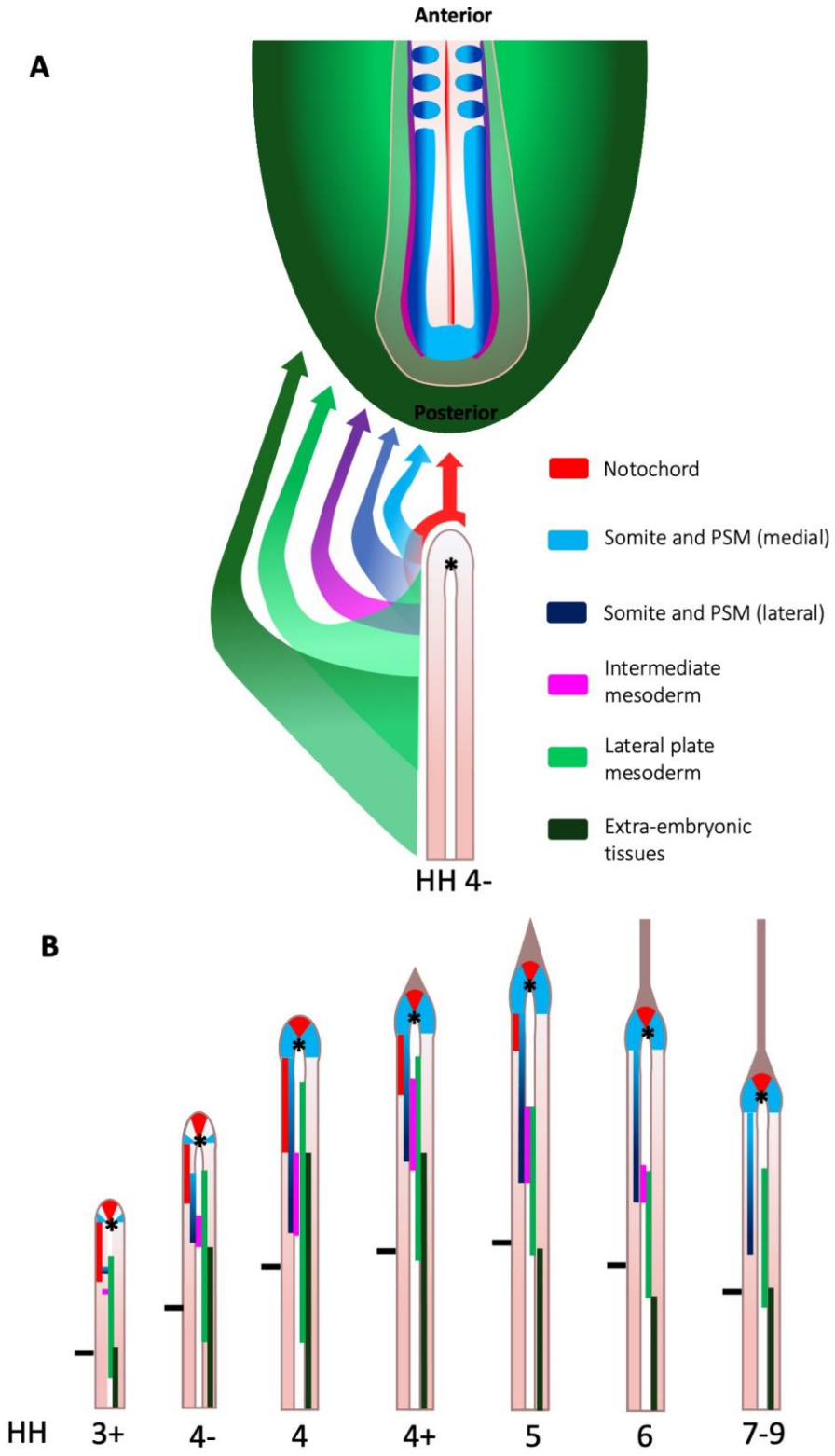

Fig 1. Fate map of the primitive streak and the node

A) The mesodermal cell progenitors which ingress further from the node will give rise to more lateral structures. B) Different stages of the node and the streak during their elongation and regression change the proportion of cells contributing to the mesodermal structures.

* Hensen's node. (HH) Hamburger and Hamilton stages.

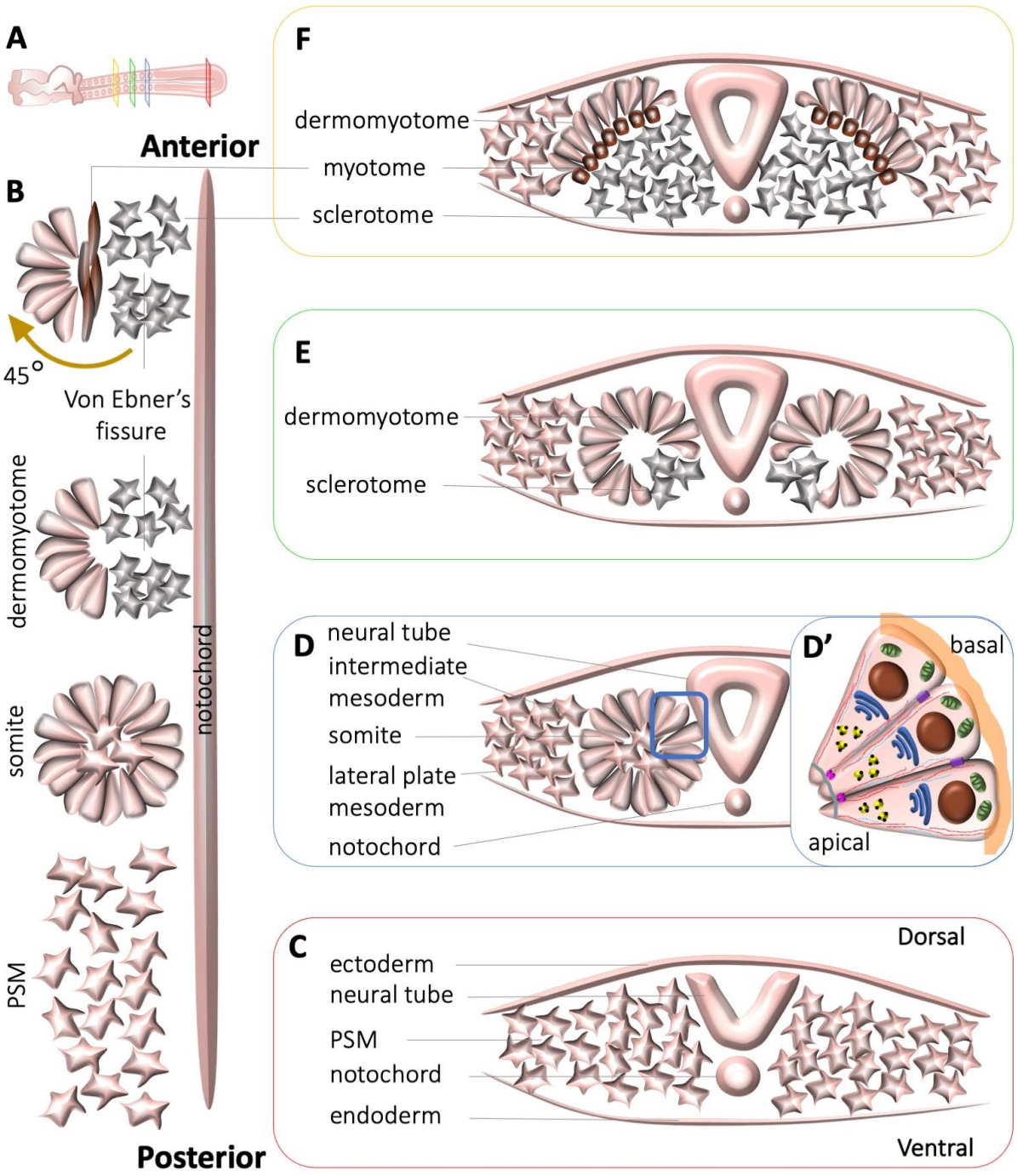

Fig 2. Somitogenesis in the avian embryo

A) Diagram of a chick embryo at stage HH 11. Coloured boxes correspond to C-F. B) Sagittal view of segmental mesoderm. C-F) Transverse views of segmental mesoderm at different axial levels in A. D' demonstrates epithelial organization: mitochondria (green), nucleus (brown), Golgi ribbon (dark blue), coated vesicles (yellow) with ribosomes (black), tight junctions (purple), desmosomes (pink), microtubules (light blue, microfilaments (red), basal lamina (orange), actin (grey).

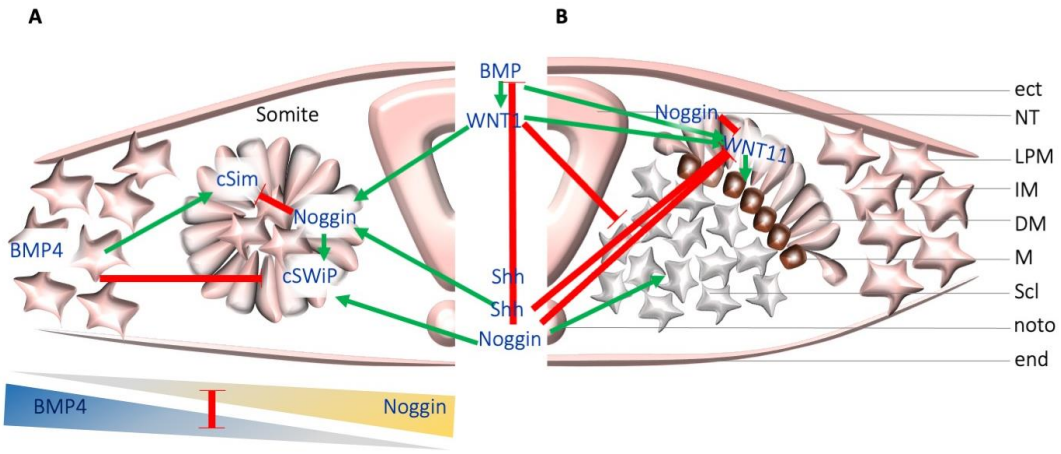

Fig 3. Patterning of the paraxial mesoderm

A) Medio-lateral somite patterning. B) Dorso-ventral patterning of paraxial mesoderm. Ect-ectoderm, NT- neural tube, LPM- lateral plate mesoderm, IM- intermediate mesoderm, DM- dermomyotome, M- myotome, Scl- Sclerotome, noto- notochord, end- endoderm.

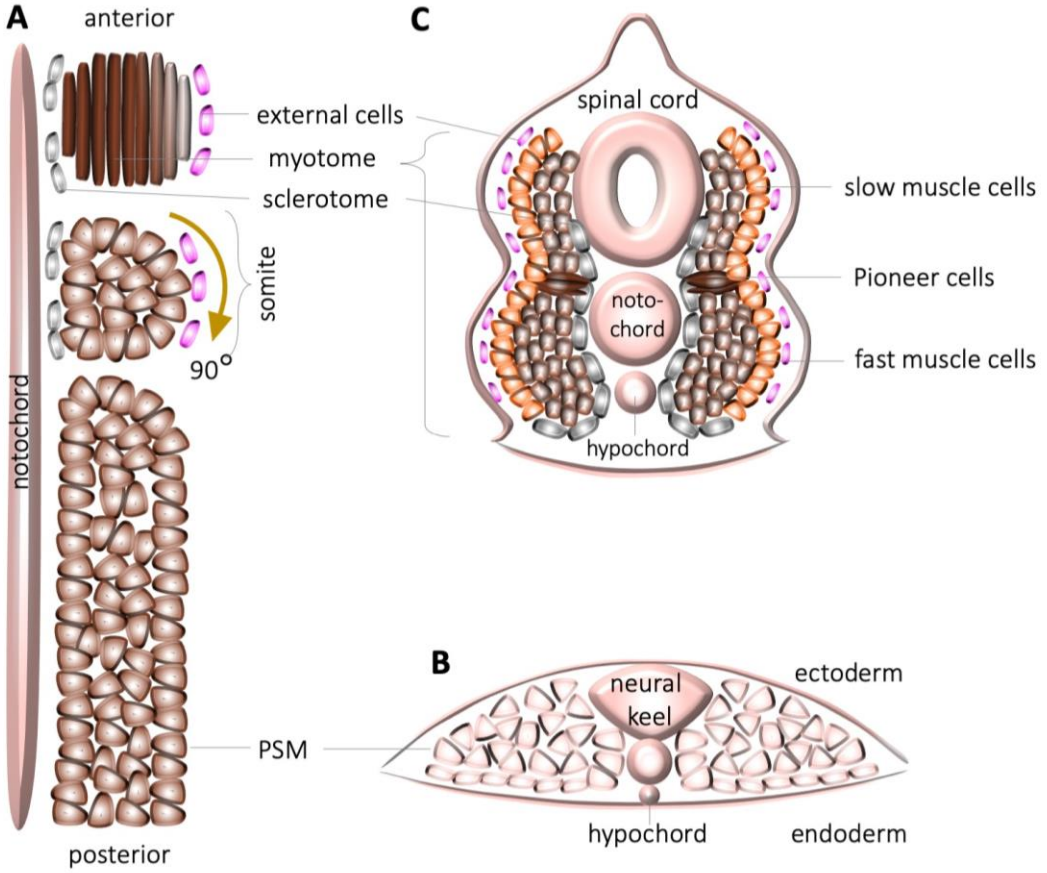

Fig 4. *Danio rerio* somitogenesis

A) Coronal longitudinal sections of segmental mesoderm. B) Transverse sections of PSM.

C) Transverse section post segmentation.

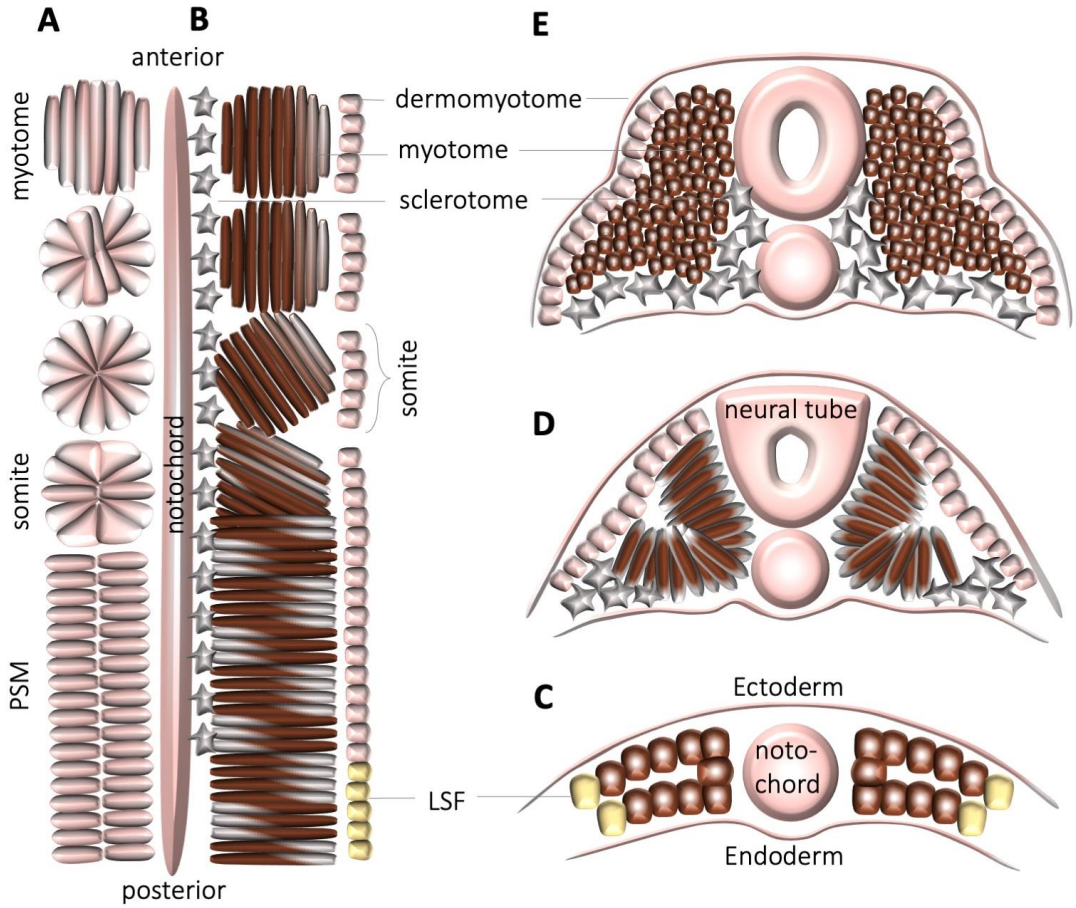

Fig 5. Amphibian somitogenesis

A) Coronal longitudinal sections of segmental mesoderm in the Mexican axolotl *Ambystoma mexicanum* and Clawed toad B) *Xenopus laevis*; C-E) *Xenopus laevis* transverse sections at different developmental stages. LSF=lateral somatic frontier.

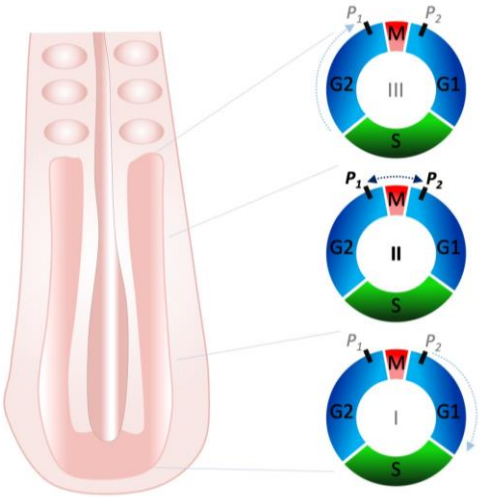

Fig 6. The cell cycle model

The cell cycle model proposes that 'gating' of cell groups into future somites could be accounted for by the existence of two unique points (P_1 and P_2) in the cell cycle, 90 minutes apart, defining a 'window' for local signalling. This event would happen roughly in the middle of the PSM, about two cell divisions prior to somite formation. As a cell reaches point P_2 in this position, it emits a signal, to which nearby cells between P_1 and P_2 is competent to respond. About one cell cycle later, the signalling event would trigger expression of a cell adhesion molecule, causing the synchronised cells to aggregate in clusters corresponding to each somite [151,152].

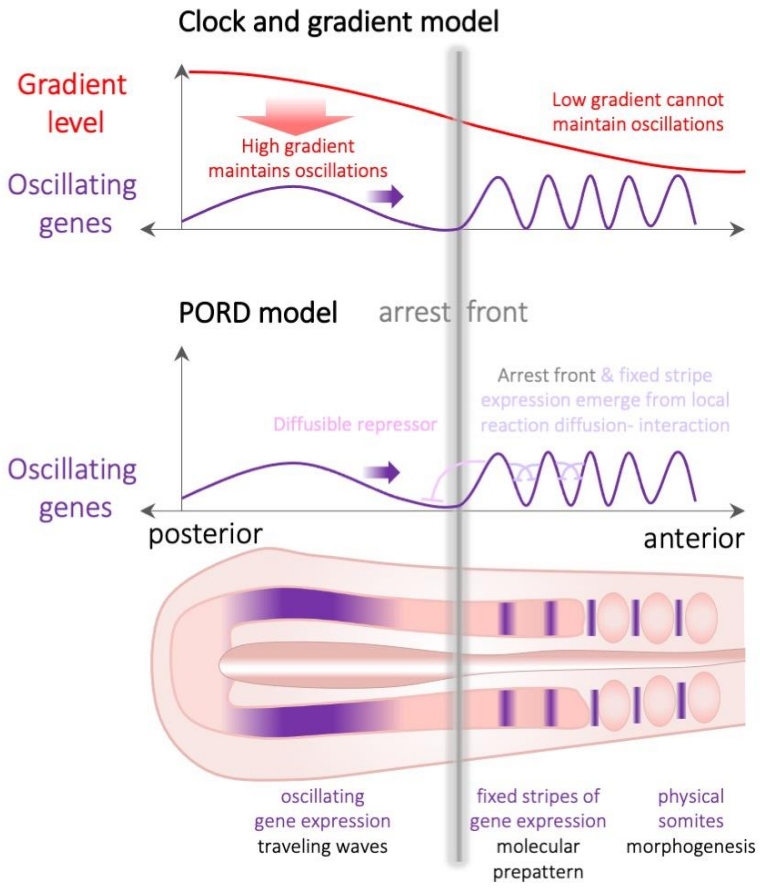

Fig 7. The 'clock and gradient' and 'progressive oscillatory reaction-diffusion' models

The PORD model proposes that a 'front' moves posteriorly as a result of local cell interactions, and cells progressively induce each other to change their state. In contrast to Slack's 'clock and gradient' model there is no need for gradient in PORD model.