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Somite formation in the chicken embryo

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ABSTRACT Somites are epithelial blocks of paraxial mesoderm that define the vertebrate embryonic segments. They are responsible for imposing the metameric pattern observed in many tissues of the adult such as the vertebrae, and they give rise to most of the axial skeleton and skeletal muscles of the trunk. Due to its easy accessibility in the egg, the chicken embryo has provided an ideal model to study somite development. Somites were first described in the chicken embryo by Malpighi in the 17th century, soon after the invention of the microscope. Most of the major concepts relating to somite segmentation and differentiation result from studies performed in the chicken embryo (Brand-Saberi and Christ, 2000). In this review, we will discuss how studies on somites in avian embryos have contributed to our understanding of key developmental processes such as segmentation, control of bilateral symmetry or axis regionalization.

KEY WORDS: *presomitic mesoderm, muscle, segmentation clock*

Somite segmentation

Somites form bilaterally on both sides of the neural tube and notochord from the presomitic mesoderm (PSM) which is produced by ingression of the epiblast during gastrulation in the primitive streak and tail bud. Together with the head mesoderm anteriorly, somites and PSM form the paraxial mesoderm. Somite formation proceeds until 52-55 somites are formed in the chicken embryo. This number is similar to humans but different from zebrafish which form 30 somites or from mice which form 65. Soon after their formation, somites subdivide into a ventral mesenchymal compartment, the sclerotome, fated to give rise to the axial skeleton and a dorsal epithelial compartment, the dermomyotome which will form the skeletal muscles of the body and the dermis of the back. Somitic derivatives subsequently acquire a regional identity under the influence of Hox genes, thus defining the characteristic anatomical domains of the body, such as the cervical, thoracic or lumbo-sacral regions. While the overall sequence of anatomical domains is conserved in evolution, the number of vertebrae contributing to these different regions is different from that seen in mammals and for example, chicken and quail embryos have 14 cervical vertebrae while most mammals including mice have only 7 (Burke *et al.*, 1995, Christ and Ordahl, 1995).

The study of somite patterning and differentiation has made tremendous progress since the 1960s. The development of the quail-chick chimera technique has allowed a precise mapping of the fate of the somitic derivatives, leading to the characterization of all the lineages derived from the somites (Le Douarin, 1969). The description of highly sensitive protocols for *in situ* hybridization has made possible very detailed characterization of gene expression during avian somitogenesis (Henrique *et al.*, 1995, Palmeirim *et al.*, 1997). Studies of somite patterning and differentiation have also been significantly enhanced by the introduction of the *in ovo* electroporation technique (Yasugi and Nakamura, 2000). This technique permits to overexpress plasmids driving expression of a construct of interest together with a fluorescent protein in embryonic tissues. Specific methods have been developed to target either the early paraxial mesoderm or the various somitic compartments (Dubrulle *et al.*, 2001, Gros *et al.*, 2004, Iimura and Pourquie, 2008). Using this technique, detailed molecular dissections of the various signaling pathways implicated in somite patterning have been performed. Compared to mouse embryos which form a cup shaped embryo during early stages of development, the chicken embryo is flat and thus much easier to observe during early somitogenesis stages. Moreover, avian embryos are much easier to culture than mammalian embryos as for instance, they do not require specific control of CO_2 or O_2 concentration (Chapman *et al.*, 2001, New, 1955). Combined to the recent development of transgenic chicken and quail reporter lines which express fluorescent proteins, this has enabled the development of sophisticated imaging protocols (McGrew *et al.*, 2008, Sato and Lansford, 2013).

Abbreviations used in this paper: AP, antero-posterior; PSM, presomitic mesoderm.

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Somite segmentation

Studies in the chicken embryo have contributed to major advances in the field of vertebrate segmentation. In the chicken embryo, pairs of somites are periodically formed at the tip of the PSM with a defined rhythm of 90 minutes per pair. This rhythm is characteristic of the species and while it is similar in quail, somite formation in mouse exhibits a 2 hour-period and in zebrafish a 30 minute-period. Microsurgical inversions of the PSM coupled to time-lapse microscopy demonstrated that the PSM kept its endogenous segmentation schedule, leading to an inverted series of segments (Christ *et al.*, 1974b, Menkes and Sandor, 1969, Palmeirim *et al.*, 1998). This argued that segmentation exhibits a high degree of autonomy at the tissue level. The observation of regular groups of more densely packed cells along the PSM using scanning electron microscopy led to propose the existence of prepatterned segments (somitomeres) along the PSM (Jacobson, 1988, Packard and Meier, 1983). However, such a prepattern could not be identified at the molecular level except in the anterior PSM, therefore arguing against the existence of the somitomeres.

The existence of a somitomeric prepattern was further challenged by the identification of the periodic expression of the transcription factor *C-HAIRY1* in the chicken embryo PSM (Palmeirim *et al.*, 1997). This provided the first evidence for an oscillator associated to segmentation which was called "Segmentation clock" (Palmeirim *et al.*, 1997). While several different hypotheses aiming at explaining the sequential and rhythmic production of somites had been proposed (Bellairs, 1985, Keynes and Stern, 1988, Meinhardt, 1986), identification of the segmentation clock supported the "clock and wavefront" model (Cooke and Zeeman, 1976) which postulated the existence of an oscillator controlling the rhythmicity of segmentation. This work was quickly followed by the identification of the Notch target *Lunatic Fringe* (*LFNG*) as another cyclic gene oscillating in phase with *C-HAIRY1* in the chicken embryo PSM (Aulehla and Johnson, 1999, McGrew *et al.*, 1998). Following this pioneering work, cyclic genes showing periodic expression patterns were identified in other vertebrates such as mouse, fish as well as in invertebrates such as Tribolium, suggesting that the segmentation clock might represent a component of an ancestral segmentation system (Forsberg *et al.*, 1998, Holley *et al.*, 2000, Sarrazin *et al.*, 2012). Cyclic genes downstream of other pathways such as *SNAIL2* and *PAPC* or *AXIN2* which are downstream of FGF and Wnt signaling respectively were also identified in chicken embryos (Chal *et al.*, 2017, Dale *et al.*, 2006, Krol *et al.*, 2011).

First insights into the regulatory network underlying the segmentation clock oscillations indicated that periodic Notch signaling is involved in the regulation of hairy-like genes (*C-HAIRY1* and *2* in chicken and *Hes1* in mouse) a process involving Lunatic Fringe driving periodic Notch inhibition (Dale *et al.*, 2003, Morimoto *et al.*, 2005). Comparison of the whole set of cyclic genes identified using a microarray strategy between chicken, mouse and zebrafish demonstrated that the same signaling pathways but not the same genes are periodically regulated in these different species (Krol *et al.*, 2011). The onset of the segmentation clock has been first studied in the chicken embryo where the first two oscillations are observed during production of the head and prechordal mesoderm while the third oscillation marks the production of the first somite (Jouve *et al.*, 2002).

In the clock and wavefront model, the wavefront corresponds to a maturation wave that moves posteriorly as the embryo elongates and allows the periodic conversion of the response to the oscillator into a spatial series of segments (Cooke and Zeeman, 1976, Hubaud and Pourquie, 2014). Microsurgical inversions of somite-size fragments along the PSM identified a level, termed determination front, at which segmental identity becomes fixed (Dubrulle *et al.*, 2001). This level is slightly posterior to the level where the first stripes of *CMESO1/Mesp2* gene expression are observed. Posterior to the determination front, inversions lead to normal somitogenesis whereas anteriorly, inverted somites are formed. Further studies in the chicken embryo identified an FGF signaling gradient peaking in the tail bud which plays an important role in segmentation. The FGF gradient was proposed to define the position of the determination front as a threshold level at which cells become competent to respond to the periodic signal of the segmentation clock (Delfini *et al.*, 2005, Dubrulle *et al.*, 2001). Thus, the size of the future segment would be defined by the distance traveled by this threshold during one oscillation of the clock. These predictions are supported by experiments showing that the size of the segment can be predictably changed by interfering with FGF signaling. For instance, grafting FGF-producing beads, which is expected to delay the wavefront progression, leads to the formation of smaller somites while treatment with FGF inhibitors such as SU5402 results in the production of larger somites consistent with an acceleration of wavefront regression (Dubrulle *et al.*, 2001). A parallel gradient of Wnt signaling is established in mouse and chicken embryos and does also play a role in specifying the determination front position (Aulehla *et al.*, 2003, Aulehla *et al.*, 2008). Graft of Wnt3a-producing beads leads to the formation of smaller somites, suggesting that Wnt is also involved in positioning the determination front. Furthermore, overexpression of constructs activating FGF signaling in the PSM by electroporation maintains expression of posterior PSM markers such as Brachyury and prevents expression of the Mesp2-related genes eventually blocking segmentation (Delfini *et al.*, 2005, Dubrulle *et al.*, 2001). Remarkably, this FGF gradient is established via an unusual mechanism wherein transcription of the Fgf8 ligand mRNA stops as the descendents of the tail bud PSM precursors enter the posterior PSM (Dubrulle and Pourquie, 2004). Due to the elongation movements involved in PSM production, PSM cells become progressively located more anteriorly in the PSM as the amount of *FGF8* they contain gradually decays. This mechanism results in the establishment of a dynamic FGF signaling gradient that regresses together with the progressive elongation of the embryo.

One of the striking aspects of amniote segmentation is the tight coordination between somite production on the right and the left side. Retinoic acid (RA) plays an important role in the control of the bilateral symmetry of somitogenesis as inhibition of the RA biosynthetic enzyme, Raldh2 either by mutation in mouse or fish or by treating chicken embryos with a chemical inhibitor (Disulfiram), results in a lateralized desynchronization of somite formation (Kawakami *et al.*, 2005, Vermot *et al.*, 2005, Vermot and Pourquie, 2005). Situs reversal using grafts of Sonic Hedgehog beads in the chicken embryo leads to a concomitant reversal of the somitogenesis defects (Vermot and Pourquie, 2005). Thus RA acts to buffer somitogenesis against desynchronizing influences of the left-right machinery. Strikingly, the somitogenesis defects

are observed in opposite sides in mouse and chicken embryos, exhibiting a right delay of somite formation in mouse while in chicken it is observed on the left side (Vermot *et al.*, 2005, Vermot and Pourquie, 2005). This behavior is paralleled by the asymmetric expression of the nuclear receptor *NR2F2* (Coup-Tf2) which shows a stronger expression in the right mouse PSM while in chicken it is on the left (Vilhais-Neto *et al.*, 2010). Nr2f2 interacts with RA signaling in the PSM potentially explaining the reversed defect of somitogenesis in the two species. Since in both species, Nodal is located on the left side, this also argues that RA is not acting by buffering Nodal action. RA signaling acting via its co-activator Rere/Atrophin2 antagonizes FGF signaling and is required to maintain the bilateral symmetry of the FGF gradient (Vilhais-Neto *et al.*, 2010). Interestingly, Fgf8 which is a key element of the posterior gradient controlling segmentation was shown to act as determinant of the left identity in mouse while in the chicken it is a right determinant (Boettger *et al.*, 1999, Meyers and Martin, 1999). Together, these data argue that RA signaling acts to buffer the desynchronizing action of the left-right determinant FGF signaling to maintain the bilateral symmetry of somite formation.

Rostro-caudal patterning of somites and resegmentation

In the 19th century, based on histological observations of chicken embryo development, Remak concluded that vertebrae do not derive from a single pair of somites but rather from the fusion of two consecutive pair of half-somites, a process he called resegmentation (reviewed in Brand-Saberi and Christ, 2000). This implies that somites can be subdivided into an anterior and a posterior compartment. The first evidence for segmentation in the PSM corresponds to bilateral stripes of expression of the transcription factors of the Mesp2 family which encompass a domain of the size of a future segment (Buchberger *et al.*, 2002, Buchberger *et al.*, 1998). Immediately after their formation, these stripes resolve into a smaller domain which marks the future anterior domain of the forming somite, thus defining the rostro-caudal identity of the future somitic compartments. Several other genes, including *C-HAIRY1* and *UNCX4.1*, or *LFNG* and *TBX18* are expressed either in the anterior or the posterior compartment of the forming somite respectively (McGrew *et al.*, 1998, Palmeirim *et al.*, 1997, Schragle *et al.*, 2004, Tanaka and Tickle, 2004). This antero-posterior subdivision of the somites is materialized in the sclerotome by the fissure of Von Ebner which separates the two compartments (Von Ebner, 1888). While both sclerotome halves give rise to the vertebral body, the caudal half forms the vertebral pedicle and the rostral half produces the intervertebral disk (Goldstein and Kalcheim, 1992). Axons of the motoneurons and neural crest cells only migrate in the anterior compartment of the sclerotome (Bronner-Fraser, 1986, Keynes and Stern, 1984, Rickmann *et al.*, 1985). Inversion of portions of the anterior PSM along the AP axis results in an inversion of the AP polarity of the forming somites (Keynes and Stern, 1984, Palmeirim *et al.*, 1998). Strikingly, axons of the motor neurons still migrate through the anterior compartment of the inverted tissue (Keynes and Stern, 1984). Such microsurgical manipulations demonstrated that only the anterior portion of the sclerotome is permissive for the migration of neural crest cells or motoneurons axons. The posterior sclerotome is refractory to their migration. Replacement of host half-somites with either rostral or caudal donor half-somites show that only half somites with the same identity do mix (Stern and Keynes, 1987). Therefore, the antero-posterior compartmentalization of the somites is determined prior to somite formation in the anterior PSM and it plays a key role in peripheral nervous system segmentation (Krull, 2001).

The anterior PSM progressively becomes epithelial and somite formation mostly consists in creating a fissure separating the forming somite from the PSM (Nakaya *et al.*, 2004). Thus, formation of a posterior boundary is a key event in somite formation as it leads to the separation of an epithelial sphere surrounding a mesenchymal core (the somitocoele) from the PSM. The position of the future posterior boundary is marked by the interface between the *Mesp2 (MESO1*)-positive (Notch activated) and *Mesp2*-negative territories (Notch inactive) which arises as a new stripe of Mesp2 expression forms as a result of the segmentation clock oscillations (Oginuma *et al.*, 2008, Watanabe *et al.*, 2009). *MESO1* triggers expression of *EPHA4* which in turn regulates Cdc42 leading to fissure formation (Nakaya *et al.*, 2004, Sato *et al.*, 2002, Watanabe *et al.*, 2009). This process also involves the paraxial protocadherin PAPC acting downstream of *MESO1* to control endocytosis of CDH2 and promote fissure formation (Chal *et al.*, 2017). Grafting a fragment of the posterior primitive streak with Noggin beads in the area opaca of a chicken host results in the production of rosettes of paraxial mesoderm resembling somites but lacking the characteristic alignment of somites and their rostro-caudal identity (Dias *et al.*, 2014). Thus while this suggests that somitic boundaries tend to spontaneously form, in the embryo, the timing of their formation appears to be tightly regulated by the segmentation clock.

Mapping the fate of somitic cells

By microsurgery, newly formed somites can be easily removed from a chicken host embryo and replaced by a donor somite of a quail embryo from the same level (Ordahl and Christ, 1997). Using this technique, vertebrae, ribs, tendons, meninges of the spinal cord, dorsal dermis, some blood vessels and all skeletal muscles were shown to derive from somites (Brent *et al.*, 2003, Chevallier, 1975, Christ *et al.*, 2007, Olivera-Martinez *et al.*, 2000). The chicken embryo produces 55 somites, which for the 7 most anterior ones contribute to the occipital bones, while the rest forms the vertebral column (Christ and Ordahl, 1995, Couly *et al.*, 1993a, Huang *et al.*, 1997, Huang *et al.*, 2000a). A somitic contribution to the scapula was also identified (Chevallier, 1975, Huang *et al.*, 2000b, Shearman *et al.*, 2011). Detailed mappings of the contribution of individual somites to specific muscles and dermis regions along the AP axis have been performed, demonstrating significant migration of these derivatives along the AP axis (Beresford, 1983, Beresford *et al.*, 1978, Chevallier *et al.*, 1977, Christ *et al.*, 1974a, Christ *et al.*, 1976, Christ *et al.*, 1977, Christ *et al.*, 1983, Couly *et al.*, 1993b, Jacob *et al.*, 1979, Lance-Jones, 1988, Noden, 1983). Importantly, such studies showed that limb muscle precursors originate from the somite and migrate from the lateral dermomyotome into the lateral plate (Chevallier *et al.*, 1977, Christ *et al.*, 1974a). Using DiI labeling and quail-chick chimeras, somites were shown to be subdivided into a medial compartment that gives rise to sclerotome, paraxial muscles and dermis, while the lateral compartment produces the muscles of the limbs and

girdles (Ordahl and Le Douarin, 1992, Selleck and Stern, 1991). Remarkably, microsurgical separation of the medial and lateral PSM shows that only the medial cells can segment and maintain cyclic gene oscillations, suggesting that communication between the medial and lateral compartments is required for somite segmentation (Freitas *et al.*, 2001).

Somites are patterned by signals from surrounding tissues

Graft of a quail somite in an inverted dorso-ventral orientation in a chicken host leads to normal differentiation of the somitic derivatives (Aoyama and Asamoto, 1988). This indicated that somitic cells are equally plastic at the time of their formation, arguing for a role of the surrounding tissues in the specification of the various somitic derivatives. Ectopic grafts or ablations of the surrounding structures such as the notochord, the neural tube, the ectoderm or the lateral plate have established their role in the patterning of the different somitic regions. For instance, such manipulations have demonstrated the role of the notochord and floor plate in sclerotome induction (Pourquie *et al.*, 1993, Strudel, 1955), while the ectoderm and neural tube promote dermomyotome formation (Mauger, 1972, Stern and Hauschka, 1995). These experiments have led to the identification of the molecular signals implicated in the induction of the various lineages. Such signals include Sonic Hedgehog produced by the Notochord and floor plate for sclerotome induction (Fan and Tessier-Lavigne, 1994, Johnson *et al.*, 1994), Wnts produced by the ectoderm and neural tube for the dermomyotome (Hirsinger *et al.*, 1997, Marcelle *et al.*, 1997) and BMP4 produced by the lateral plate for the lateral somite fate (Pourquie *et al.*, 1996). The electroporation technique has also been very effectively used to perform high resolution imaging studies which have clarified the early stages of myogenesis from the myotome (Gros *et al.*, 2004). Early studies based on quail-chick chimeras argued that the medial lip of the dermomyotome was the major contributor to the myotome (Kaehn *et al.*, 1988, Ordahl *et al.*, 2001). High resolution live imaging following the fate of the derivative of the different dermomyotome compartments labeled with fluorescent proteins by electroporation demonstrated that all four lips of the dermomyotome contribute to the myotome in a specific order(Gros *et al.*, 2004).

Regional patterning of somitic derivatives

Soon after their formation, somitic derivatives become patterned according to their anatomical region. Substitution of the PSM from the future thoracic region by PSM of the future cervical region results in the formation of ribs in the neck region of transplanted animals (Kieny *et al.*, 1972). Such experiments demonstrated that regional identity of somitic derivatives is established early in the PSM. This regional identity of paraxial mesoderm derivatives is largely imparted by the Hox genes which code for a family of 39 transcription factors arranged in collinear order in four clusters in the chicken embryo chromosomes (Wellik, 2007). Hox genes are expressed in the paraxial mesoderm in a temporal sequence which reflects the position of the genes on the chromosomes (temporal collinearity)(Dolle *et al.*, 1989, Iimura and Pourquie, 2007). This sequential expression of the genes ultimately results in the formation of collinear domains along the body axis, with the genes located more 3' (anterior genes) in the cluster showing an expression boundary located more anteriorly than the 5' genes (posterior genes). Electroporation of Hox gene constructs in the chicken embryo epiblast shows that genes are able to control the timing of ingression of cells fated to form the PSM (Iimura and Pourquie, 2006). Strikingly, the more posterior the gene is expressed, the more it delays cell ingression suggesting that the collinear activation of genes in the epiblast controls the ingression schedule of paraxial mesoderm derivatives. Combined to the progressive elongation of the body axis, these properties are expected to result in the collinear distribution of Hox gene expression domains in the paraxial mesoderm potentially explaining how the temporal collinearity is translated into spatial collinearity. How Hox genes subsequently direct the regional morphology of vertebrae and other somitic derivatives remains poorly understood.

The more posterior Hox genes were also shown to control posterior elongation of the chicken embryo via a collinear repression of Wnt signaling which controls paraxial mesoderm production in the tail bud (Denans *et al.*, 2015). By slowing down axis elongation while somitogenesis progression remains constant, posterior Hox gene expression is expected to lead to a shrinking of the PSM ultimately leading to termination of axis extension. This mechanism was proposed to play a role in the control of segment number in the chicken embryo.

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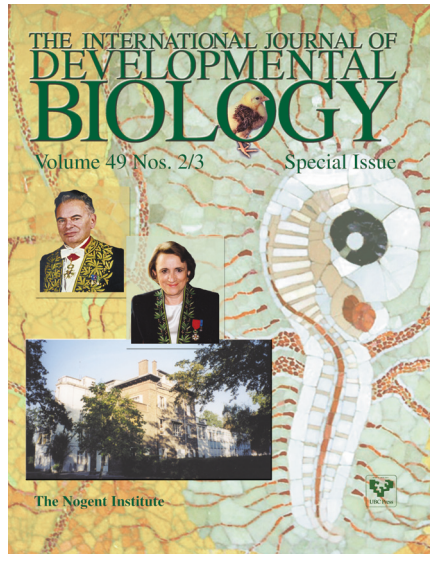

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