Mechanisms of somite formation and subdivision

Ana Sofia Teixeira Franco Dias

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Department of Cell and Developmental Biology

University College London

London

I declare that the work presented in this thesis is of my own.

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Ana Sofia Teixeira Franco Dias

To J. D. and A. D.

Abstract

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Somitogenesis is the process by which the presomitic mesoderm is segmented along the vertebrate axis. A clock and wavefront system has been suggested to control multiple aspects of this process including somite size specification, timing of formation and rostral-caudal patterning of somites. This thesis investigates an *ex vivo* experimental model that generates multiple somites from non-somitic posterior primitive streak tissue through exposure to the BMP inhibitor Noggin. These somites from almost simultaneously in a nonlinear arrangement and acquire an axial identity of cervical somites, showing a normal size, morphology, expression of somitic markers and giving rise to late somite derivatives when transplanted into a secondary host, but form in the absence of the cyclic expression of key molecular clock genes. However, the experimental somites fail to become patterned into rostral and caudal halves, a feature necessary for the segmentation of the peripheral nervous system. The findings in this thesis raise the possibility that the main function of the clock operating in the presomitic mesoderm cells that

generate somites might be distinctive for the different aspects of somitogenesis and suggest that it could be mainly required for rostral-caudal patterning of the somites, coupling it with somite formation.

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List of abbreviations

A	Anterior
C	Caudal
DM	Dermomyotome
DML	Dorsal medial lip
EMT	Epithelial-to-mesenchymal transition
HH st	Hamburger and Hamilton stage of development
LFng	Lunatic Fringe
MET	Mesenchymal-to-epithelial transition
MRFs	Muscle regulatory factors
P	Posterior
PNS	Peripheral nervous system
PPS	Posterior primitive streak
PSM	Presomitic mesoderm
R	Rostral
RA	Retinoic acid
VLL	Ventral lateral lip

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Chapter One: Introduction

1.1 Somites and the vertebrate Bauplan

Embryogenesis is the process by which a unicellular zygote forms the sophisticated assembly of different tissues that elaborate the functional interacting organs of the developed adult body. Vertebrate embryogenesis differs extensively across the clade in the early stages of development but converges in the Pharyngula, which is the phylotypic stage that represents the general Bauplan of the vertebrate body, before diverging again at later stages to shape the anatomical diversity of the vertebrate subphylum (von Baer, 1828; Raff, 1996) [known as the hourglass model (Sander, 1983; Duboule, 1994; Raff, 1996)]. The vertebrate phylotypic stage is characterized by several features, among which is the segmentation of the paraxial or presomitic mesoderm (PSM) into segmental units known as somites. Somites are transient developmental features that appear as epithelial spheres along both sides of the notochord and neural tube. The term “somite” was first used by Balfour (Balfour, 1881) to describe the metameric units of the PSM in amphibians, but had been previously termed “urwirbel” or “protovertebra” by Remak (Remak, 1850), whereas the avian somite was first described by Williams (Williams, 1910). The somites determine the initial

segmental pattern along the body axis during embryogenesis and, later on in development, this segmental pattern is imposed upon other developing structures of the body (Christ and Wilting, 1992; Keynes and Stern, 1988, 1984). The segmentation of the vertebrate body into somites is a pre-requisite for movement, allowing for innervated muscle sets and joints to form and subsequently enabling movements in discrete regions to be integrated as general movements of the whole body. The competence of the adult body for movement involves effort, which is a capability of the muscles. The skeletal muscle cells of the body (axis, wall and limbs) originate from the somites (Christ et al., 1990; Wachtler and Christ, 1992). Movements of the body require support by elements of cartilage and bone in the vertebral column, which are also derived from the somites (Christ and Wilting, 1992), reflecting the initial segmental pattern laid down by them.

1.2 The chick embryo as a model to study somite formation

Given the fundamental significance of somites during development, understanding their role and development has been a subject of intense scientific investigation over the years and a great amount of information has been established by studies in the chick embryo (see

below). Our current knowledge of somite boundary formation (Kulesa and Fraser, 2002; Sato et al., 2002), somite differentiation (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000; Huang et al., 2000; Kato and Aoyama, 1998; Huang and Christ, 2000; Chevallier et al., 1977; Denetclaw and Ordahl, 2000; Cinnamon et al., 1999; Christ et al., 1983), regional patterning of the derivatives of the somites along the body axis (Kieny et al., 1972; Nowicki and Burke, 2000) as well as the understanding of the mechanisms regulating the segmental organization of the PSM (Christ et al., 1974; Menkes and Sandor, 1977; Packard and Jacobson, 1976; Packard and Meier, 1983; Bellairs and Veini, 1984; Primmett et al., 1988, 1989; Palmeirim et al., 1997; Jouve et al., 2002; Dubrulle et al., 2001) come largely from studies using this model system. Important findings using the chick embryo as an experimental model also include the discovery that the subdivision of the somites along the rostrocaudal axis guides emerging neural crest cells and motor nerves and imposes segmental organization in the peripheral nervous system (PNS) (Keynes and Stern, 1984, 1988; Kuan et al., 2004) and the discovery that periodic cycles of gene expression preempt somite formation (Palmeirim et al., 1997).

The chick embryo has long been an attractive model system used to investigate developmental processes since it is easy to access and observe as it develops almost entirely outside the mother. When the egg is laid it contains a pre-gastrula-stage embryo, the blastoderm, as cleavage occurs while the egg travels along the oviduct (Bellairs et al., 1978). Despite the difficulty in obtaining pre-laying embryos, the cleavage divisions have been studied (Bellairs et al., 1978) and a series of stages have been described for the chick embryo development before laying (Eyal-Giladi and Kochav, 1976). However, the majority of the experimental work is led on embryos at post-laying stages. It is very convenient to work with the latter because it is usually not necessary to house adult animals and fertilized eggs can be obtained from commercial sources (poultry farms), stored at 18^{o}C for up to two weeks or/and incubated at 38^{o}C for various periods of time to reach the stage of development needed for a particular experiment. The stages of chick embryo development from laying to hatching have been described by Hamburger and Hamilton (HH st.1 to st.46) based on structural developmental landmarks (Hamburger and Hamilton, 1951). The Hamburger-Hamilton (HH) staging system is independent of the incubation conditions and therefore represents a good reference standard for researchers worldwide (Hamburger and Hamilton, 1951). The fact that the early

chick embryos develop as flat discs and can be cultured *ex ovo* constitute an advantage for observation of normal development using live imaging. Another major advantage in using the chick embryo as an experimental model is the easiness with which they can be surgically manipulated and that can be done both *in ovo* (by cutting a window into the egg shell to reveal the embryo and subsequently re-sealing it for incubation), and *ex ovo* (by explanting the embryo and continue its development supported by suitable culture conditions) in which embryos can be cultured and manipulated in vitro to make observations on cell-type specification or on the behavior of cell populations, making chick embryos a versatile and unique animal model (New, 1955; Chapman et al., 2001; Nagai et al., 2014). Chick embryo culture methods include the culture technique of New (New, 1955) which consists of growing the blastoderm on a piece of vitelline membrane stretched out tightly over a glass ring; the filter paper frame (Chapman et al., 2001), which consists of growing the embryo in a dish containing semi-solid medium and using a filter paper frame as a carrier to transfer the embryo, and the modified Cornish pastry method (Nagai et al., 2014) which consists of growing the blastoderm folded in half and sealed around the edges, forming a pouch with the embryo growing on the top. Classical embryological manipulations including heterotypic grafting of tissues, tissue

ablations, and tissue grafts combined with the classical tissue transplantation technique used to make chick-quail chimaeras make a huge contribution to the study of the mechanisms of tissue interactions and of cell lineages due to the fact that donor quail tissue can be clearly distinguished from the host chick embryo (Le Douarin 1969, 1973) using a specific nuclear stain (LeDouarin, 1973) or a quail specific antibody (QCPN, developed by Dr B.M. Carlson). In addition, it is possible to do experiments using synthetic beads which can be loaded with inhibitors or enhancers of signaling cascades and applied locally close to target embryonic tissues (*in ovo* or *ex ovo*) that can be very useful to identify the signals involved in tissue patterning and cell determination. These classical methodologies have been complemented by other approaches to map the fate of cells such as cell labeling by injection of fluorescent dyes, and by technical advances of methods of transient transgenesis to misexpress genes in a time and space controlled manner by targeted microinjection and electroporation of expression vectors (Itasaki et al., 1999; Scaal et al., 2004; Voiculescu et al., 2008) that allow the study of gene function and promoter activity, making the chick model system even more powerful. Additionally, it is also possible to generate germline transgenic chickens, using a lentiviral vector to deliver genetic material. This method led to the establishment of a

transgenic chicken line carrying a transgene that includes the CAG enhancer/promoter driving ubiquitous expression of green fluorescent protein (GFP) (McGrew et al. 2004) which is extremely useful for transplantation experiments and studies of cell lineage analysis.

All of the technical advances mentioned above, together with the availability of the chicken genome sequence (Consortium, 2004; Wallis et al., 2004) and molecular resources including a large collection of expressed sequence tags (EST) (Hubbard et al., 2005) facilitate the generation of valuable molecular tools and make the chick embryo a very strong model in developmental biology as well as in comparative genomics and evolution studies.

1.3 The origin of the somites: somite precursor cells

To understand somite formation one must first understand where the cells that form the somites derive from. In amniote embryos, the mesoderm layer is initially formed during the process of gastrulation from cells of the Hensen's node and adjacent epiblast that ingress through the primitive streak (Psychoyos and Stern, 1996; Selleck and Stern, 1991). Prospective single-cell lineage analysis has shown that

the progenitors of the medial half of the somites are localized in the lateral quadrant of the node, and the progenitors of the lateral half of the somite are located in the primitive streak behind the node (Selleck and Stern, 1991; Stern et al., 1988). The progenitors of the lateral somites ingress through the streak from the region behind the node and do not behave as resident cells (Psychoyos and Stern, 1996; Selleck and Stern, 1991; Selleck and Stern, 1992). But the primitive streak does not only constitute a pathway for ingression and will also contain resident cells that are the precursors of the medial part of the paraxial mesoderm (or presomitic mesoderm), during its regression (Hatada and Stern, 1994; Psychoyos and Stern, 1996; Schoenwolf et al., 1992; Selleck and Stern, 1991). Detailed labeling studies in the chick have demonstrated the existence of this stem cell population in the streak, which subsequently has also been shown in the mouse (Nicolas et al., 1996; Tzouanacou et al., 2009). The primitive streak has also been proposed to prefigure some organization along the rostral-caudal axis (Selleck and Stern, 1991). The precursors of the axial and paraxial (presomitic) mesoderm are located close to the rostral end of the primitive streak whereas the precursors of the lateral mesoderm and extraembryonic tissues are located more caudally (Alev et al., 2010; Nicolet, 1965, 1967, 1971; Psychoyos and Stern, 1996; Rosenquist, 1966, 1967, 1970, 1971,

1972, 1983; Schoenwolf et al., 1992; Selleck and Stern, 1991; Spratt and Condon, 1947; Spratt, 1957; Tam and Tan, 1992). In the chick embryo at HH st.5 the paraxial mesoderm precursors reside in the most rostral third of the primitive streak (Schoenwolf et al., 1992; Selleck and Stern, 1991). Subsequent to ingression the mesodermal cells migrate rostrally and laterally and those that become located medially form the paraxial mesoderm and those located laterally form the intermediate/lateral plate mesoderm (Schoenwolf et al., 1992; Selleck and Stern, 1991). In the chicken embryo, the unsegmented paraxial mesoderm referred to as the presomitic mesoderm (PSM), that lies between the notochord and the intermediate mesoderm, separates at regular time intervals into individual somites in a rostral-caudal direction (Lash et al., 1984; Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998), a process that is termed somitogenesis.

1.4 Somitogenesis

During somitogenesis (or somite formation) in the chicken embryo, a new pair of somites forms at the rostral end of the presomitic mesoderm (PSM) on each side of the notochord by epithelialization of blocks of cells that undergo a mesenchymal-to-epithelial transition

(MET) (excepting for the cells inside the somite that remain mesenchymal, the somitocoele cells) at an approximate rate of one pair every ninety minutes and a total of fifty-two somites are formed (Lash et al., 1984; Christ and Ordahl, 1995; Keynes and Stern, 1988; Christ et al., 1972; Gossler and Hrabe de Angelis, 1998). Somite formation is an extremely robust process and an intrinsic property of the paraxial mesoderm (Packard, 1976; Christ et al., 1974; Menkes, 1977). Explanted fragments of the PSM continue to segment when cultured isolated from the embryo in the absence of the normal PSM surrounding tissues (Packard, 1976). It has been shown that just the fibronectin network of the ectoderm is required to form somites from isolated PSM (Rifes et al., 2007). In addition, somite formation is regulated at the level of the whole tissue. If fragments of the PSM are isolated and transplanted in an inverted orientation, somitogenesis proceeds in the original rostrocaudal orientation (Christ et al., 1974; Menkes, 1977), reiterating that somitogenesis is an autonomous process within the PSM.

At the rostral end of the PSM, the cells become organized in an arrangement reminiscent of an epithelium as the nuclei of the cells become aligned and regularly spaced in close contact (Beloussov and Naumidi, 1983; Marcelle et al., 2002; Revel et al., 1973). Each

somite initially forms as an epithelial sphere of columnar cells whose apices all face a central cavity or lumen, the somitocoele, containing mesenchymally arranged cells (Lash et al., 1984; Christ and Ordahl, 1995; Keynes and Stern, 1988; Christ et al., 1972; Gossler and Hrabe de Angelis, 1998). The process of somite formation is accompanied by increased cell adhesiveness (Bellairs et al., 1978; Cheney and Lash, 1984), correlating with an increase in cell adhesion molecules at the apical end of the epithelial somite cells (Duband et al., 1987). Various adhesion molecules are expressed in this luminal layer, including NCAM and N-Cadherin (Duband et al., 1987; Kimura et al., 1995). It has been shown that actin and α-actinin are also concentrated in the apical zone of epithelial somite cells (Lash et al., 1984; Ostrovsky et al., 1983). Each somite is surrounded by extracellular matrix that is condensed to form a basement membrane at the external surface of the somite (Solursh et al., 1979). The extracellular matrix connects the somite with adjacent structures: neural tube, notochord, ectoderm, endoderm, aorta and Wolffian duct. Thus, signals reaching the somites from the axial organs, endoderm or ectoderm must pass through an extracellular matrix compartment, which contains collagens, fibronectins, laminins, tenascins and other components (Bellairs, 1979; Crossin et al., 1986; Duband et al., 1987; Rickmann et al.,

1985; Thiery et al., 1982). In the rostral and caudal directions, each somite is also connected by extracellular matrix of the intersomitic cleft to its older and younger siblings. The epithelial cells of the somite are polarized, with the Golgi zone near the luminal cytoplasm and tight junctions close to the luminal cell surface (Lipton and Jacobson, 1974; Revel et al., 1973). During somite formation more cells are added both by mitosis within the plate or by recruitment of progenitor cells at the caudal end of the segmental plates (tail bud) as a consequence of continuing caudal gastrulation movements, maintaining the length of this tissue relatively constant during axis extension (Catala et al., 1995; Gont et al., 1993).

1.4.1 Somite epithelialization and intersomitic boundary formation

One of the most striking features of somitogenesis is the segmentation process itself that regularly generates individualized somites from the PSM continuous tissue (Lash et al., 1984; Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998). The mesenchymal cells at the rostral end of the PSM undergo the process of MET and change their shape to form a spherical somite composed of a single layer of epithelial cells and some mesenchymal cells in the lumen, the somitocoele cells (Keynes and Stern, 1988;

Christ et al., 1972; Gossler and Hrabe de Angelis, 1998). In the chicken embryo, the MET of the caudal margin cells within an epithelial somite precedes MET of the cells of the rostral side, which remain mesenchymal for longer (Duband et al., 1987; Takahashi and Sato, 2008). Therefore, the forming epithelial somite presents a horseshoe shape (Duband et al., 1987) (see Figure 1.1). The SNAIL superfamily of transcriptional repressors, including SNAIL2 in the chicken and SNAIL1 in the mouse, have been shown to repress genes associated with an epithelial state such as *E-cadherins* and are likely to maintain the mesenchymal state of the PSM (Batlle et al., 2000; Cano et al., 2000; Savagner et al., 1997). Ectopic activation of *Snail2* in the chicken PSM can block incorporation of PSM mesenchymal cells in the epithelial somites (Dale et al., 2006). The onset of the epithelialization process of the PSM correlates with the downregulation of *Snail1* and *Snail2* genes (Sefton et al., 1998). At this point, the rostral PSM morphological changes include an increase in cell number and density and cells become arranged as an epithelium at the peripheral sides of the PSM (Keynes and Stern, 1988; Tam and Trainor, 1994) (see Figure 1.1; in yellow). The expression of the bHLH transcription factor *Paraxis* in the PSM is associated with this change in cell organization and its expression is subsequently maintained in the epithelial somites (Burgess et al.,

1995). *Paraxis* is an essential regulator of the epithelial organization in the PSM (Burgess et al., 1995, 1996; Barnes et al., 1997) and it has been suggested that the overlying ectoderm is necessary to induce and maintain its expression via *Wnt6* that activates *Paraxis* via β-Catenin-dependent signaling (Correia and Conlon, 2000; Sosic et al., 1997; Linker et al., 2005) (see Figure 1.1: in green). Mice null mutants of the *Paraxis* gene fail to form epithelial somites (Burgess et al., 1996; Johnson et al., 2001). However, in these mice mutants, the PSM still segments in portions of normal somite size (Burgess et al., 1996). This normal segmental arrangement suggests that the segmental patterning of the PSM is independent of the epithelialization process and that the MET of cells within the somite is distinct from the process of segment border formation. Chicken embryos treated with *Paraxis* antisense oligonucleotides also fail to form epithelial somites (Barnes et al., 1997). In accordance with these results, experiments in which PSM explants were cultured *in vitro* resulted in a segmented pattern of gene expression in the explants but in the absence of epithelialization (Palmeirim et al., 1998). N-cadherin, a cell adhesion molecule, is also upregulated prior to somite epithelialization (Tam and Trainor, 1994) and targeted mutation of the gene results in segmentation defects, with smaller and irregular somites (Radice et al., 1997). The Rho family of small

GTPases, including Rac1 and Cdc42, has been implicated in cytoskeletal dynamic, mediating forces that change cell polarity and motility (Etienne-Manneville and Hall, 2000; Fukata et al., 2003; Ridley et al., 2003; Settleman, 2001; Van Aelst and Symons, 2002). Rac1 and Cdc42 have been shown to play a role in stabilizing epithelial structures through regulation of cell-cell adhesion mediated by cadherins (Braga, 2002; Fukata and Kaibuchi, 2001; Schock and Perrimon, 2002). They have also been identified as essential for development, as shown by the lethal effect in *Rac1* and *Cdc42* knockout mice (Chen et al., 2000; Snapper et al., 2001; Sugihara et al., 1998; Yan et al., 2003). The roles of Rac1 and Cdc42 have been studied in the context of the MET during somite formation in the chicken embryo (Nakaya et al., 2004). The maintenance of a correct level of Rac1 activity is necessary for somite epithelialization. Nakaya and colleagues (2004) have reported that cells with activated or inhibited Rac1 fail to undergo correct epithelialization and maintained *Paraxis* positive cells in a mesenchymal state. Although Rac1 does not affect transcription of *Paraxis*, Rac1 mediated cytoskeletal re-arrangement appears to be required for *Paraxis* to function as an epithelialization factor during somitogenesis (Nakaya et al., 2004). Cdc42 levels of activity appear critical for the defining decision between epithelial and mesenchymal states (Nakaya et al., 2004).

Nakaya and colleagues (2004) have also shown that "*Cdc42 activity needs to be low for cell epithelialization, whereas cells require high activity to maintain their mesenchymal state*" (Nakaya et al., 2004). It is possible that the Cdc42 activity is suppressed in the presomitic mesodermal cells undergoing MET by factors acting on the somitic cells emanating from the overlying ectoderm, which are known to be required for epithelialization (but not for intersomitic boundary formation), for which Wnt molecules are good candidates (Nakaya et al., 2004; Correia and Colon, 2000).

In the chicken embryo, somite epithelialization is preceded by the formation of an intersomitic boundary. *Mesp2* and *cMeso1* are homologous b-HLH transcription factors expressed in the rostral PSM just prior somite formation in the mouse and chicken, respectively, and appear to play an important role in the inductive events of intersomitic boundary formation (Saga and Takeda, 2001). Knockout mice for *Mesp2* show severe defects in boundary formation (Saga et al., 1997). Mesp2/cMeso1 transcription factors have been identified to regulate molecules able to act intercellularly between the rostral and caudal boundary border cells, such as *Ephs* (Nomura-Kitabayashi et al., 2002; Nakajima et al., 2006). The Eph family of receptor tyrosine kinases comprises membrane-spanning receptor

proteins involved in intercellular signaling (Holder and Klein, 1999; Palmer and Klein, 2003). Ephrin ligands for these receptors are classified into two groups: Ephrins A, which preferentially bind EphA receptors and Ephrins B, which preferentially bind EphB receptors. EphA4 exceptionally binds to both groups of ligands (Gale et al., 1996). Knockout mice for *EphA4* do not exhibit defects in boundary formation (Dottori et al., 1998) however, experiments done in the chicken embryo suggest that *EphA4* functions as a mediator for boundary formation (Watanabe et al., 2009). It has been shown that in the chicken, *cMeso1* upregulates *EphA4* in the cells located caudally to a forming boundary (Watanabe et al., 2009) which in turn activates *EphrinB2*-reverse signals (downstream signaling) in the immediate rostral border cells, where *EphrinB2* signal seems to be sufficient to produce a somitic boundary and it has been suggested that it promotes epithelialization (Watanabe et al., 2009). *EphrinB2* has been suggested to cooperate with low Cdc42 in the somitic cells and is implied in somitic boundary formation (Watanabe et al., 2009).

1.4.2 Rostrocaudal patterning of the somites

In the rostral-most third of the PSM, regional differences in gene expression define the rostral and the caudal patterning of the developing somites and such patterning arises before the formation

of morphological distinct somites (prior to epithelialization and prior to boundary formation) (Del Amo et al., 1992; Hrabe de Angelis et al., 1997; Jen et al., 1997; del Barco Barrantes et al., 1999; Saga and Takeda, 2001). In this region of the PSM, it has been identified that the initiation and maintenance of the rostral-caudal patterning is controlled by Delta-Notch signaling pathway interacting with the basic helix-loop-helix (b-HLH) transcription factor *Mesp2* in the mouse and *MESO1* in the chicken embryo (Del Amo et al., 1992; Hrabe de Angelis et al., 1997; Jen et al., 1997; del Barco Barrantes et al., 1999; Saga and Takeda, 2001). Notch ligand Delta-like 1 (*Dll1*) (Hrabe de Angelis et al., 1997) is expressed uniformly in the caudal-most two-thirds of the PSM but its expression becomes restricted to the future caudal half of the somites in the rostral-most third of the PSM and is maintained in the caudal half of the formed somites (Bettenhausen et al., 1995). Mutation of *Dll1* in the mouse causes a complete loss of rostrocaudal somite patterning and subsequent rostralization of the somite derivatives (Hrabe de Angelis, 1997). Hence, identifying how *Dll1* expression is regulated in the rostral PSM stands crucial to understand the establishment of the rostrocaudal patterning of the somites. It has been proposed that the expression of *Dll1* in the rostral PSM is dependent on two Notch signaling pathways because *Psen1* homozygous knockout mice lack

expression of *Dll1* in the rostral PSM (Takahashi et al., 2000). It has been suggested that Psen1-dependent Notch signaling pathway is involved in inducing *Dll1* expression in the prospective somite caudal half and Psen1-independent Notch signaling pathway is involved in inhibiting *Dll1* expression in the prospective somite rostral half (Takahashi et al., 2000). It has been identified that the expression of *Dll1* in the rostral PSM is further established by *Mesp2*/*MESO1* basic helix-loop-helix (bHLH) transcription factors mediated by the Notch pathway in the PSM, because expression of *Notch1* is reduced in *Mesp2* knockout embryos (Saga et al., 1997; Takahashi et al., 2000). *Mesp2* is initially expressed in the rostral-most third of the PSM (Saga et al., 1997) where it has been shown to downregulate *Dll1* (Saga et al., 1997; Takahashi et al., 2000). In *Mesp2* knockout mice, rostral *Dll1* fails to be suppressed (Saga et al., 1997; Takahashi et al., 2000) and rostral-caudal patterning of the somites is lost. *Mesp2* becomes expressed in the prospective rostral half of the somite, where it has been shown to be crucial to suppress *Dll1* and generate the stripped pattern of *Dll1* expression (Saga et al., 1997; Takahashi et al., 2000). The mechanism that restricts *Mesp2* expression to the prospective rostral half of the somite is not fully understood, but it seems to involve *Psen1*-dependent Notch signaling since *Mesp2* expression pattern is lost in *Psen1* knockout mice (Koizumi et al.,

2001).

1.5 Models of somite formation

The process of somitogenesis is an extremely complex multistep process and comprises a series of events that occur simultaneously as the embryo develops. A great amount of information has been gathered on the major features of cell behavior, establishment of the fate of the cells, regional properties during the development of individual somites and also on several possible mechanisms that generate and control the metameric pattern of somites, the precise timing of somite formation and the specification of axial identity. Once the paraxial mesoderm has been specified, the temporal order of formation of somites of regular size is constant. Over the past decades many theoretical models have been proposed to explain the complex process of somitogenesis and how the several aspects of somite formation can be integrated and to explain experimental data (Cooke and Zeeman, 1976; Stern et al., 1988; Primmett et al., 1989; Bard, 1990; Kerszberg and Wolpert, 2000; Schnell and Maini, 2000; Collier et al., 2000; Pourquie, 2003; Dequeant et al., 2006; Benazeraf and Pourquie, 2013; Aulehla et al., 2008; Santillan and Mackey, 2008; Morelli et al., 2009; Schroter and Oates, 2010; Murray et al.,

2011; Hester et al., 2011). Here, three of the most widely accepted models for somitogenesis will be considered.

1.5.1 Clock and wavefront model for somitogenesis

The clock and wavefront model, originally proposed on a theoretical basis by Cooke and Zeeman (1976), proposes the existence of a clock operating in the cells of the PSM and a wave to determine regional development (Figure 1.2). Cooke and Zeeman postulated the existence of a longitudinal “positional information” gradient along the rostral-caudal axis of the embryo, the wavefront, which determines the generation of segments by controlling the time at which a group of cell will segment together. They called this a *catastrophe,* a rapid change of state, which could correspond to an increase in cell adhesiveness of the cells to become a somite. Then, a *smooth cell oscillator*, the clock, periodically interacting with the wavefront allows groups of cells to segment together. Therefore, according to this model, the size of a unit (somite) is determined by the speed of the wavefront and the timing of somite formation is controlled by the frequency of the oscillator (Cooke and Zeeman, 1976).

1.5.1.1 Cycling genes

Experimental evidence that supports this model comes from the discovery of the periodic expression of genes in the PSM with the period equal to that of somite formation (Palmeirim et al., 1997). Several genes of the Notch signaling pathway have been identified to be expressed in the PSM and their cyclic expression is considered to arise as a result of a segmentation clock acting within the cells of the PSM (Palmeirim et al., 1997; Aulehla and Johnson, 1999; Forsberg et al., 1998; Jiang et al., 2000; McGrew et al., 1998; Bessho et al., 2001; Dequeant et al., 2006, Jouve et al., 2000; Leimeister et al., 1999; Leimeister et al., 2000; Holley et al., 2000; Jiang et al., 2000; Oates and Ho, 2002; Sawada et al., 2000; Elmasri et al., 2004; Li et al., 2003). The first oscillatory gene to be identified was *c-hairy1* (Palmeirim et al., 1997), which encodes a transcription factor homologous to Hairy/enhancer of split (*Hes*) in *Drosophila* and is also a downstream target of Notch signaling (Nusslein-Volhard and Wieschaus, 1980; Ish-Horowicz et al., 1985). Several genes, mainly from the Notch signaling pathway, have been subsequently identified to cycle their expression in the PSM. Lunatic Fringe (*Lfng*) was also described to have oscillatory expression in the PSM of the chick and mouse in the same fashion as *c-hairy1* (Aulehla and Johnson, 1999; Forsberg et al., 1998; Jiang et al., 2000; McGrew et al., 1998). In the

mouse, other cycling genes include *Hes1, Hes7, Hey1-3* and *Nrarp* (Bessho et al., 2001; Dequeant et al., 2006, Jouve et al., 2000; Leimeister et al., 1999; Leimeister et al., 2000). In the chicken PSM cycling genes also include *c-hairy-2* and *cHey2* (Jouve et al., 2000; Leimeister et al., 2000). In the zebrafish oscillating genes include *her1, her7* and *DeltaC* (Holley et al., 2000; Jiang et al., 2000; Oates and Ho, 2002; Sawada et al., 2000). In the medaka oscillating genes include *her7* and *hey1* (Elmasri et al., 2004) and in *Xenopus, esr9* and *esr10* (Li et al., 2003). Moreover, It has also been shown that Notch activity levels oscillate in the caudal PSM (Huppert et al., 2005; Morimoto et al., 2005) and, thus, PSM cells experience pulses of oscillatory *Notch* mRNA and Notch activity. It has been suggested that the mechanism that drives the oscillations of the cycling genes relies on feedback inhibition loops (Henry et al., 2002; Oates and Ho, 2002; Holley et al., 2002; Lewis, 2003; Jiang et al., 2000; Mara et al., 2007; McGrew et al., 1998; Forsberg et al., 1998; Aulehla and Johnson, 1999; Dale et al., 2003; Serth et al., 2003; Iso et al., 2003; Hirata et al., 2004; Bessho et al., 2003; Jensen et al., 2003; Monk, 2003; Barrio et al., 2006; Aulehla et al., 2003). The mechanisms that have been proposed to underlie the periodic oscillations of the cycling genes in zebrafish, chick and mouse are summarized in Figure 1.3. The above data suggests that Notch activity seems to

play a role in the segmentation clock. Surprisingly, the nature of the generator (or pacemaker) driving the oscillations of gene expression remains to be identified and is a subject of debate (Aulehla et al., 2008; Ozbudak and Pourquie, 2008; Giudicelli and Lewis, 2004). In addition to Notch, components of the Wnt signaling pathway have also been shown to be linked to the segmentation clock in the mouse (Aulehla et al., 2003; Ishikawa et al., 2004; Suriben et al., 2006; Dequeant et al., 2006). m*Axin2* (a negative regulator of the Wnt pathway) (Aulehla et al., 2003) and m*Dact1* (encoding a Dishevelled-binding regulator) (Suriben et al., 2006) mRNAs also show oscillatory expression in the PSM but with a reverse phase when compare with *Notch* genes (Aulehla et al., 2003). Also, *Nkd1* (an antagonist of Wnt) has been shown to oscillate in phase with the *Notch* genes and it is transcriptionally regulated by *Hes7* (a negative regulator of *Notch* signaling) and it has been suggested that it takes part at the communication level between the Wnt and Notch pathways (Ishikawa et al., 2004). A non-dynamic expression profile across the PSM has been identified for chick homologues of the mouse *Wnt* cyclic genes (Gibb, 2009), which suggests a species difference or that there might be other *Wnt* components oscillating in the PSM of the chicken embryo, non-homologous to the *Wnt* cyclic genes in the mouse.

1.5.1.2 Interactions between Wnt, Notch and Fgf pathways

Dequeant and colleagues (2006) proposed that the interaction between the Wnt and Notch and Fgf pathways may be an important mechanism contributing for the robustness of the segmentation clock and the cross talk between these pathways has been confirmed by analysis of mouse mutants and manipulation in the chicken embryo (Ferjentsik et al., 2009; Aulehla et al., 2003; Dequeant et al., 2006; Niwa et al., 2007; Gibb et al., 2009; Dale et al., 2006; Sewell et al., 2009; Wahl et al., 2007) (Figure 1.4). It has been shown that the oscillatory expression of the Notch-related genes *mLfng* and *mNrarp* is *Wnt3a*-dependent (Aulehla, 2003; Sewell, 2009) and also that *mLfng* transcription is ectopically activated as a result of misexpression of Wnt-regulated *mAxin2* (Aulehla, 2003). This suggests that Wnt signaling could act upstream of Notch signaling in the context of the segmentation clock. Additionally, Wnt signaling regulates *Dll1*, a ligand of Notch, expression in the mouse PSM (Hofmann, 2004; Galceran, 2004). However, downregulation of Notch signaling also results in downregulation of the Wnt target *mAxin2* in the mouse PSM (Aulehla, 2003). Hence, the interaction between Notch and Wnt is not clear and it is a possibility that both pathways reciprocally regulate each other (Gibb, 2009). Moreover, in the absence of Notch signaling, expression of Fgf target genes is

severely downregulated in the PSM (Ferjentsik, 2009) and mouse mutants for Fgf signaling lose dynamic expression of *mLfng* in the PSM (Wahl, 2007). Several studies indicate that Fgf signaling is involved in regulating Notch target gene expression in the PSM (Niwa, 2007; Kawamura, 2005; Ishimatsu, 2010). However, the interaction between Notch and Fgf signaling is also not completely clear, since in the absence of FGF signaling, Notch-related cyclic genes are not immediately halted and continue to be expressed in the PSM of both chick and mouse (Gibb, 2009; Delfini, 2005; Dubrulle, 2001) (see Figure 1.4).

1.5.1.3 The Wavefront

According to the clock and wavefront model, the segmentation clock is assumed to interact with a wavefront of positional information to specify the segments at the rostral PSM (Cooke and Zeeman, 1976) and to maintain the cells in the caudal PSM in an undifferentiated state (see Figure 1.2). This process is thought to be controlled by a combination of gradients present in the PSM, involving the FGF, the Wnt/β-catenin and the retinoic acid (RA) signaling pathways (Dubrulle et al., 2001; Dubrulle and Pouquie, 2004; Vermot and Pourquie, 2005; Moreno and Kintner, 2004; Diez del Corral et al., 2003; Aulehla et al., 2003; Aulehla et al., 2008; Sawada et al., 2001).

The observation of a caudal-rostral gradient of FGF (Dubrulle et al., 2001; Sawada et al., 2001; Dubrulle and Pourquie, 2004) and Wnt/β-catenin signaling (Aulehla et al., 2003; Aulehla et al., 2008) that accompanies the PSM as the embryonic axis grows caudally, is thought to be reminiscent of the wavefront postulated by the clock and wavefront model (Dubrulle et al., 2001). Additionally, a rostro-caudal gradient of RA signaling establishes an opposing gradient of activity to FGF/Wnt (Diez del Corral et al., 2003; Moreno and Kintner, 2004; Vermot and Pourquie, 2005). The high transcription levels of *fgf8* in the tailbud region is thought to generate the graded gradient of FGF8, with higher signaling levels in the caudal PSM and decreasing gradually towards the rostral end of the PSM (Dubrulle and Pourquie, 2004). As cells enter the PSM, leaving the tailbud region, *Fgf8* transcription ceases and consequently the concentration of *Fgf8* mRNA decays, resulting in the generation of a caudal-rostral *Fgf8* mRNA gradient, which is then translated into a gradient of Fgf8 protein (Dubrulle and Pourquie, 2004). Upon binding to Fgfr1 in the PSM, FGF signaling is mediated by several pathways: MAPK, ERK and PI3K (Bottcher and Niehrs, 2005). In the chicken embryo ERK is also found in the PSM with a graded caudal-rostral distribution (Sawada et al., 2001; Delfini et al., 2005), thought to result in the activation of the downstream targets *Dusp4* (Niwa et al., 2007),

Dusp6 (Dequeant et al., 2006; Li et al., 2007), *Snail1* (Dale et al., 2006) and *Pea3* (Roel and Nusslein-Volhard, 2001). The gradient of Wnt signaling in the PSM has been suggested to be established in the same way as the FGF8 gradient (Aulehla et al., 2003). Indirect evidence based on the expression patterns of downstream targets of *Wnt3a*, the ligand that activates the Wnt/β-catenin signaling in the PSM, suggest that *Wnt3a* could also be produced at the level of the tail bud and the gradient established by decrease of protein levels (Aulehla et al., 2003; Aulehla and Herrmann, 2004). On the other hand, RA signaling gradient is rostro-caudal, with highest levels of RA activity in the rostral PSM and the somites and absent activity in the caudal PSM/ tail bud (Rossant et al., 1991). The RA-synthesizing enzyme, Raldh2, is highly expressed in the rostral PSM and somites (Niederreither et al., 1997) whereas Cyp26A1, involved in RA degradation, is highly expressed in the tail bud (Fujii et al., 1997; Sakai et al., 2001).

During axis elongation, the wavefront moves in a caudal direction and the FGF8/Wnt signaling levels are maintained constant in relative positions along the PSM (Dubrulle and Pourquie, 2004). It has been suggested that the differentiation status of the PSM could be controlled by a system of FGF/Wnt/RA signaling gradients. Dubrulle and colleagues (2001, 2004) have observed that different

levels of FGF/Wnt activity along the PSM relate with regions of different structure along the PSM and also with regions of different states of segmental determination. In the caudal two-thirds of the PSM, the FGF/Wnt signaling is high, cells have a mesenchymal organization and are maintained in an immature state. In the rostral third of the PSM, the FGF/Wnt signaling is low and RA signaling is high and cells become more densely organized and the MET process initiates culminating with epithelial somite formation at the rostral end of the PSM (Dubrulle et al., 2001). The border that separates these two regions of FGF/Wnt activity has been termed "*determination front"* (Dubrulle et al., 2001) and correlates with the level at which a transition of differentiation status occurs: *"FGF8 is sufficient to maintain the caudal identity of presomitic mesoderm cells and (…) down-regulation of FGF8 signaling at the level of the determination front is required to enable cells to proceed further with the segmentation process"* (from Dubrulle et al., 2001). Several transcriptional changes occur once cells pass the level of the "determination front". Caudal PSM markers are downregulated, such as *Tbx6* and *Msgn1.* Additionally, *Mesp2*, a crucial factor in establishing the somite rostrocaudal polarity and somite boundaries, is also activated in the rostral PSM (Saga et al., 1997; Saga, 2007; Morimoto et al., 2005, 2006, 2007). Finally, transcriptional

oscillations of the segmentation clock genes are arrested once cells pass the PSM level of the so-called “determination front”. Experimental manipulations involving the inversion of small pieces of PSM (Dubrulle et al., 2001) were performed to help identify the specific position of the “determination front”: rotation of small pieces of the rostral-most one-third of the PSM resulted in the inversion of the pattern of the somites (somite determination), whereas inversion of small pieces in the caudal-most two-thirds of the PSM do not. The point of transition between these two states was interpreted as the position of the “determination front” (Dubrulle et al., 2001). Given the cell motility and the cell intermixing in the caudal PSM (Selleck and Stern, 1991; Benazeraf et al., 2010), it is difficult to understand whether the caudal PSM cells are indeed not yet determined to form a segment or, alternatively, whether the cells have not yet sorted to the correct allocated positions to form a segmental unit.

Since the discovery of the cycling genes, this model has been favored over the others and many authors have proposed newer versions the clock and wavefront model (Aulehla et al., 2008; Murray et al., 2011; Santillan and Mackey, 2008; Schroter and Oates, 2010; Hester et al., 2011; Dequeant et al., 2006; Pourquie, 2003). According to the clock and wavefront model, an increase in the size

of the somites could be caused by an acceleration of the wavefront progression, while keeping the period of the clock oscillations constant or by slowing down the oscillation of the clock, while the speed of the wave remains constant. This mechanism could account for the experimental observation that in manipulated *Xenopus* embryos where half of the cells of the embryo are ablated (Cooke, 1975), the number of somites remains constant but fewer cells are allocated to each somite.

1.5.2 Reaction-diffusion model for somitogenesis

Another theoretical model, the reaction-diffusion model, was proposed by Meinhardt (1982; 1986) to explain somite formation and somite regional specification (Meinhardt, 1982; 1986). This model postulated that somitic cells can adopt one of two possible states, *A* or *P*, respectively corresponding to the *anterior* (rostral) or *posterior* (caudal) halves of a somite (unit) and that the cells can transiently oscillate between these two states (see Figure 1.5; B, C and D). According to the model, the frequency of the oscillations is equivalent to the rate of unit formation, and the timing of unit formation and the number of oscillations required to form a unit are determined by a long-range gradient of a morphogen along the PSM. According to the model, the states *A* and *P* are mutually exclusive and somitic cells

can switch between one state and the other until they reach a stable state (Meinhardt, 1986). Consequently, a pattern of *APAPAP(*…) stripes forms in an rostral to caudal direction along the PSM. The transition between the two states could represent a change in the specification of a unit and could represent consecutive rostral and caudal sub-compartments of a somite (unit). Finally, sequential *A/P* pairs would become arranged along the PSM, resulting in the specification of somites with regional differences (i.e. rostral and caudal sub-compartments). Meinhardt further proposed that the mechanism by which the somitic cells could change between two states was the presence of a gradient of a morphogen along the PSM with increased levels at the caudal end of embryo (see Figure 1.5; A). Cells would oscillate between states under the influence of the concentration of the morphogen and would count the number of oscillations so that more oscillations would lead to a more caudal state. Meinhardt predicted that the model *"would obtain strong support if the oscillations in the mesoderm before somite formation could be detected"* (from Meinhardt, 1986). Experimental support towards these predictions comes from the discovery of genes with oscillatory cyclic expression in the PSM, such as *Hairy1* which has been identified to have a cyclic oscillatory expression in PSM of the chick embryo (Palmeirim et al., 1997). As somites progressively form

at the rostral end of the PSM, the expression of *Hairy1* is maintained only in the caudal half of the somites: caudal-half state specification (*Hairy1* positive) and rostral-half state specification (*Hairy1* negative) resemble the *P* and *A* states postulated by Meinhardt in the reaction-diffusion model. Meinhardt additionally postulated the existence of a long-range gradient of a morphogen in the PSM (Meinhardt, 1986). The subsequent observation of a caudal-rostral gradient of FGF in the PSM (Dubrulle et al., 2001; Sawada et al., 2001; Dubrulle and Pourquie, 2004) could constitute evidence for the gradient of a morphogen in the PSM. This gradient correlates with the postulated positional information gradient of a morphogen in the PSM that according to the reaction-diffusion model of Meinhadt is required by the pre-somitic cells to start oscillating between two states and start the formation of a segmented pattern (Meinhadt, 1986).

1.5.3 Cell cycle model for somitogenesis

The cell cycle model was proposed by Primmett (1988; 1989) and it links somite formation with the cell cycle phase of the cells in the PSM (Primmett et al., 1989; Primmett et al., 1988; Stern et al., 1988) (see Figure 1.6). This model was postulated based on the following premises: a) the time interval between the formation of two successive somites is ninety minutes to one-hundred minutes,

equivalent to one-seventh of the duration of the cell cycle (which corresponds to nine to ten hours); b) there is some degree of cell cycle synchrony in the cells of the PSM; and c) application of a single heat shock to chick embryos blocks the cell cycle. The results of the transient application of heat shock are several somitic anomalies separated by six to seven somites (Primmett et al., 1989; Primmett et al., 1988; Stern et al., 1988). The premise that there is *some* level of cell cycle synchrony along the PSM means that not all cells in the PSM are in the same phase of the cell cycle. This cell cycle synchrony of cohorts of PSM cells can be achieved due to the fact that the precursors cells destined to form somites leave the region of the node and enter the PSM in a rigorous temporal order by which they are derived from the node. In this manner, the more rostral cells of the PSM are older because they ingress first through the primitive streak to join the PSM and the more caudal cells of the PSM correspond to younger cells which join the PSM subsequently. This order is maintained in the PSM. Primmett and Stern postulated the existence of a time window during the cell cycle, between time points *P1* and *P2* (ninety minutes apart), within which cells become competent to form a somite (Primmett et al., 1989; Primmett et al., 1988; Stern et al., 1988) (see Figure 1.6). The synchrony of different cohorts of cells in the PSM accounts for the prediction that a group of

PSM cells can reach the *P2* point in cell cycle before the others (these cells are termed *pioneer cells*) and can produce and emit a segmentation signal to the other cells in the PSM. Consequently, any cell inside the *P1-P2* window would be responsive to this signal and as a response would increase adhesion to other cells so that this fraction of PSM cells could then segment together to form a somite unit (see Figure 1.6). Additionally, this model does not rely on the oscillatory expression of genes in the PSM, such as the Notch signaling pathway genes, for the specification of somite size and timing of somite formation and so far no direct link has been found between the cell cycle and the oscillatory expression of genes in the PSM. However, because it is yet not entirely understood for what aspect of somitogenesis the oscillatory expression of genes account for (somite size specification/ timing of somite formation/ rostral-caudal subdivision) it is possible that the cell cycle synchrony could constitute an extra level of regulation to maintain cells in synchrony to segment in the PSM. However, unlike the reaction-diffusion model of Meinhardt which accounts for somite polarity (1982, 1986), the cell cycle model does not predict how cells in the somite get allocated to a specific somite sub-compartment, therefore an additional level of complexity would be required to specify the polarity of the somites, as it has been proposed in the model by Meinhardt (1982, 1986).

1.5.4 Unresolved questions

Much debate remains over the mechanism that generates periodic formation of normal sized somites and although periodic Notch signaling is clearly important for somite development, some observations suggest that it may not be exclusive in determining the size and morphology of somites. Mutants of the Notch signaling pathway genes, such as *Notch1*, the Notch ligand *Delta-like 3,* the activated Notch protease presenilin and the Notch effector RBPJk, show disruptions in segmentation but the somites still form and only later fall apart (Conlon et al., 1995; Dunwoodie et al., 2002; Evrard et al., 1998; Oka et al., 1995; Shen et al., 1997; Takahashi et al., 2003; Wong et al., 1997; Zhang and Gridley, 1998; Zhang et al., 2002). Mutants of the *Mesp* genes, which interact with the Notch signaling pathway, show disrupted rostral-caudal subdivision of the somites but no effect on the initial formation of the somites (Sawada et al., 2000). Moreover, it has been observed that occipital somites form with a different periodicity compared with more caudal somites (Hamburger and Hamilton, 1951; Hamilton and Hinsch, 1956; Huang et al., 1997), in the absence of the oscillatory expression of genes of the segmentation clock (Rodrigues et al., 2006) and have different rostral-caudal subdivision character to the trunk somites (Lim et al.,

1987). These findings are not consistent with the commonly accepted Clock and Wavefront model (Cooke and Zeeman, 1976) and suggest that a distinctive mechanism might be responsible for allocating the cells to form each occipital somite. In addition, the occurrence of spatially periodic perturbations of somite formation after heat shock treatment in the chick embryo (Primmett et al., 1989) and zebrafish (Roy et al., 1999) have been shown to be linked to the cell cycle in the PSM (Primmett et al., 1989). The cell cycle model proposed by Primmett (Primmett et al., 1989) does not require oscillatory gene expression (like the genes of the Notch signaling pathway) but proposed that somitogenesis is controlled by the cell cycle, based on cell cycle synchrony in the PSM. The cell cycle model is distinctive in accounting for the periodicity of segmentation defects after heat shock (cells were suggested to be sensitive to heat shocks only at a particular phase of the cell cycle, which occur at regular spatial intervals in the PSM) (Primmett et al., 1989). Finally, it is also important to note that studies in transgenic mice expressing activated Notch (NICD) throughout the PSM, have shown that segmentation and rostral-caudal patterning of the somites can be uncoupled and provides evidence towards the assumption that cyclic expression of Notch is required for the correct rostral-caudal patterning of the somites (Feller et al., 2008). Additionally, studies in

mutant *mespb* knockin mice embryos have shown that epithelial somites can form without rostral-caudal polarity, suggesting that segment formation and establishment of rostral-caudal polarity are genetically separate events (Nomura-Kitabayashi et al., 2002). It is then possible that the role of the segmentation clock is mainly to regulate somite polarity, as postulated by the "clock and trail model for somite formation, specialization and polarization (Kerszberg and Wolpert, 2000). In addition, the discovery that ectopic somites can be generated in a non-sequential, non-linear arrangement, by treating posterior primitive streak grafts with the BMP4 antagonist Noggin, offers the experimental opportunity to separate the 'segmentation clock' from the timing of somite formation and explore directly the role of the 'segmentation clock' in somite formation or patterning (Streit and Stern, 1999; work presented in this thesis).

1.6 The formation of somite compartments and somite derivatives

1.6.1 Somite maturation

The course of somite maturation, in the same manner as the process of somite formation, progresses from rostral to caudal levels of the

embryonic axis (Williams, 1910; Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998). Accordingly, the sequence of the somites along the axis reveals a rostrocaudal gradient of developmental progress with the more rostral somites at later stages of maturation and the youngest somites gradually positioned more caudally (Williams, 1910; Christ and Ordahl, 1995; Christ et al., 1974). Given that the state of maturation of a given pair of somites is different along the rostrocaudal axis, a dynamic staging system has been established to characterize the somites according to their developmental status (Christ and Ordahl, 1995), so that the newly formed youngest somite is denominated somite I and the successive rostral somites, gradually maturing, are denominated sequential Roman numbers (Figure 1.7). In the avian embryo, somites I to IV are developmental compartments (Lawrence, 1990) arranged as spheres of epithelial cells (Williams, 1910; Christ and Ordahl, 1995; Bellairs, 1979; Meier, 1979; Keynes and Stern, 1988), composed of 2000-3000 cells (Herrmann et al., 1951; Ordahl, 1993; Venters et al., 2008), a single cell in radius, with a central somitic lumen containing the mesenchymal somitocoele cells (Huang et al., 1994, 1996) and an outer surface covered by a basement membrane which stabilizes their epithelial structure and connects them to adjacent structures via extracellular matrix (ECM) (Bellairs, 1979; Crossin et al., 1986;

Duband et al., 1987; Kimura et al., 1995; Rickmann et al., 1985; Solursh et al., 1979; Tam and Trainor, 1994; Tan et al., 1987). The epithelial somites possess a rostral-caudal polarity, which is thought to be initially specified in the cranial portion of PSM mediated by Delta-Notch signaling (Del Amo et al., 1992; Hrabe de Angelis et al., 1997; Jen et al., 1997; del Barco Barrantes et al. 1999; Saga and Takeda, 2001), that will later on become manifest morphologically in the rostral and caudal regions of the sclerotome (Keynes and Stern, 1984; Stern et al., 1986). Experiments in which the most rostral portion of the PSM or the epithelial somite are inverted along the rostro-caudal axis have established that this polarity is established prior to somite formation (Aoyama and Asamoto, 1988). On the other hand, the specification of the dorso-ventral and medial-lateral axes is controlled by signals emanating from adjacent structures (Christ and Ordahl, 1995; Kieny et al., 1972; Brent and Tabin, 2002). If an epithelial somite is rotated dorso-ventrally (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992; Aoyama, 1993) or medial-laterally (Ordahl and Le Douarin, 1992), it develops as the neighbouring non-rotated somites, showing that at these stages, the cells are multipotent and that the specification of the dorso-ventral and the medial-lateral compartments occurs under the influence of extrinsic signals. From somite V, eight to ten hours after formation of

the epithelial somites (Christ and Ordahl, 1995), the somites start to mature and molecular and morphological changes take place leading to a dorso-ventral polarization of the somites and, consequently, the formation of two main somitic compartments, the sclerotome and the dermomyotome. The ventral somite half undergo an epithelial-to-mesenchymal (EMT) transition. Disruption of the basement membrane in this region of the somite releases the cells of the ventral surface of the epithelium to form the mesenchymal sclerotome (Jacob et al., 1975; Christ et al., 2004) and the dorsal epithelium of the somite becomes more tightly organized, increases in height, forming the dermomyotome (Ordahl and Le Douarin, 1992; Christ et al., 2004). Given that during development the chick embryo folds laterally, the sclerotome comes to lie more medially and the dermomyotome more laterally. Hence, the somites subdivide into the sclerotome, ventromedially and the dermomyotome, dorsolaterally (Keynes, 1988). The dermomyotome later subdivides further: the cells situated immediately beneath the ectoderm eventually disaggregate and give rise to the dermis of the trunk and to some muscle cells (Christ et al., 1992); the cells that appear between the dermomyotome and the sclerotome remain closely packed as the myotome, forming the axial skeletal muscle (Christ *et al.,* 1978; 1992).

1.6.2 Somite compartments

During the process of somite maturation, the somites subdivide into compartments within which various cell types differentiate controlled by environmental signals, including myocytes, fibrocytes, adipocytes, chondrocytes, osteocytes, endothelial cells, pericytes and epineural and perineural cells (Christ and Ordahl, 1995; Scaal and Christ, 2004; Jacob et al., 1974; Christ et al., 1992; Ordahl and Le Douarin, 1992; Aoyama, 1993).

1.6.2.1 Sclerotome

The sclerotome is the ventromedial compartment of the somite that gives rise to the vertebral column, ribs, tendons and meninges of the spinal cord (Hall, 1977; Wallin et al., 1994; Christ et al., 1979, 1998, 2000; Kurz et al., 1996; Halata et al., 1990; Brent et al., 2003; Huang et al., 1994, 1996, 2000; Mitapalli et al., 2005). It is the source of fibrocytes of connective tissue and tendons and osteocytes precursors of the vertebral bodies, vertebral arches, intervertebral discs and proximal and distal parts of the ribs (Christ and Wilting, 1992; Brand-Saberi and Christ, 2000; Christ et al., 2004). The sclerotome compartment forms from a process of epithelio-mesenchymal transition (EMT) of the ventromedial cells of the

epithelial somite (Christ et al., 2004). It is therefore initially characterized by a mesenchymal arrangement of prospective sclerotome cells that first appear in the somite at stage IV (Christ and Ordahl, 1995) and these are subsequently joined by the mesenchymal cells of the somitocoele that eventually give rise to part of the ribs and the intervertebral discs (Huang et al., 1994, 1996). EMT in the sclerotome is also accompanied by a decrease in cell adhesion and down-regulation of N-cadherin and an increase in cell mobility (Hatta et al., 1987; Duband et al., 1987; Takeichi, 1988). Sclerotome mesenchymal cells appear to be polarized (Trelstad, 1977) and actively migrate towards the notochord to form the perinotochordal tissue (Chernoff and Lash, 1981; Williams, 1910; Jacob et al., 1975). It is not clear what mechanisms control the EMT in the ventromedial somite or whether it depends on signals emanating from the notochord or the ventral neural tube. Although notochord grafts exert ventralizing effect on the dorsal somite forcing it to undergo EMT (Brand-Saberi, 1993), it has been shown that ablation of the axial structures does not influence EMT in the ventromedial somite (Teillet and Le Douarin, 1983) and does not prevent ventro-medial cell dissociation and partial sclerotome formation (Christ et al., 1972; Veini and Bellairs, 1991; Hirano et al., 1995). Additionally, it has also been shown that Shh signaling from

the axial structures is not required for cell dissociation (Christ et al., 2004) and EMT is not required for sclerotome induction since the expression of the sclerotome markers, such as *Pax1*, precedes EMT (Brand-Saberi, 1993; Christ et al., 2004; Teillet and Le Douarin, 1983; Peters et al., 1999). It is possible that EMT in the ventromedial somite is initiated due to the lack of signals to maintain the epithelial state such as *Wnt6* that is expressed in the epidermal ectoderm and regulates epithelialization of the paraxial mesoderm (Schmidt et al., 2004). The sclerotome is also characterized by the specific expression of two genes of the Pax family of transcription factors, *Pax1* and *Pax9*. In the chick (and mouse) embryo, *Pax1* mRNA is first expressed in the ventral part of the early somite (future sclerotome compartment) and in the mesenchymal cells of the somitocoel (Deutsch et al., 1988; Ebensperger et al., 1995; Borycki et al., 1997) but subsequently during somite maturation *Pax1* mRNA becomes restricted to the ventral portion of the sclerotome (and down-regulated in the dorsal and lateral portions of the sclerotome) (Deutsch et al., 1988; Wallin et al., 1994; Peters et al., 1995; Muller et al., 1996). *Pax1* expression remains in the intervertebral discs at the time of chondrification of the vertebral bodies (Deutsch et al., 1988; Wallin et al., 1994; Peters et al., 1995). On the other hand, *Pax9* mRNA expression is first detected in the dorsolateral

sclerotome of somites IV-V (Müller et al., 1996), at HH st.12 (Hamburger and Hamilton, 1951) and subsequently its expression pattern partly overlaps with *Pax1* in the intervertebral discs (Peters et al., 1995). *Ondulated* mice mutants, containing a point mutation in the paired box of *Pax1* (Balling et al., 1988), lack vertebral bodies, intervertebral discs and proximal ribs but present neural arches, indicating a function of *Pax1* in ventral rather than dorsal sclerotome development (Chalepakis et al., 1991; Wallin et al., 1994; Dietrich and Gruss, 1995). *Pax1* expression is induced by signals emanating from the notochord and floor plate of the neural tube (Ebensperger et al., 1995; Furumoto et al., 1999). In vivo ablation of the notochord and floor plate of the neural tube in the chick embryo results in an absence of *Pax1* positive sclerotomal cells (Teillet et al., 1998; Teillet and Le Douarin, 1983; Goulding et al., 1993; Ebensperger et al., 1995; Brand-Saberi et al., 1993; Dietrich et al., 1993). Noggin (expressed in the notochord at the time of sclerotome formation) (Streit and Stern, 1999; Tonegawa and Takahashi, 1998; Hinsinger et al., 1997; Sela-Donenfeld and Kalcheim, 2002; McMahon et al., 1998) and Shh (secreted by the notochord and the floor plate of the neural tube) (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Chang et al., 1994; Marti et al., 1995) have been identified to act synergistically to initiate and maintain *Pax1* expression in the

sclerotome and are thought to play a role in early formation and maintenance of the sclerotome (Fan and Tessier-Lavigne, 1994; McMahon et al., 1998; Fan et al., 1995).

Distinct sclerotomal derivatives form from different subdomains of the maturing sclerotome. The central subdomain of the sclerotome close to the myotome compartment presents high cell density and develops under the influence of FGF8 produced by the myotomal cells (Stolte et al., 2002; Huang et al., 2003) giving rise to the pedicle part of the neural arches and the proximal ribs (Christ et al., 1979, 1992). The rostral and caudal borders of the central subdomain of the sclerotome constitute the syntetome subcompartment (Brent et al., 2003). These cell express *scleraxis,* a basic helix-loop-helix transcription factor (Cserjesi et al., 1995), induced by a still unknown signal activated by myotomal FGF8 and FREK (Brent et al., 2003). The syndetome contains the precursor cells of the tendons that connect the vertebrae with the epaxial muscles (Brent et al., 2003). The ventral subdomain of the sclerotome generates the vertebral bodies and the intervertebral discs (Hall, 1997; Wallin et al., 1994; Christ et al., 1979, 1998, 2000). The dorsal domain of the sclerotome generates the dorsal part of the neural arches and the spinous process (Ebensperger et al., 1995; Monsoro-Burq et al., 1996; Monsoro-Burq and Le Douarin, 2000). The lateral subdomain of the

sclerotome gives rise to the distal ribs (Huang et al., 1994, 2000, 2003; Olivera-Martinez et al., 2000) and the medial subdomain gives rise to blood vessels and meninges of the spinal cord (Halata et al., 1990; Kurz et al., 1996).

R/C sub-compartments of the sclerotome and the establishment of the metameric pattern of the vertebrate body

Although somites are transient embryonic structures, their initial segmental pattern is extremely important because it imposes the segmental organization of other structures in body such as the vertebral column, thoracic wall, skeletal muscle, smooth muscle, connective tissue, endothelial cells of the adult body, blood vessels and nerves (Christ and Jacob, 1980; Wachtler et al., 1981; Brand et al., 1985; Solursh et al., 1987; Schramm and Solursh, 1990; Lance-Jones, 1988; Noden, 1991). The subdivision of the somitic sclerotome into rostral and caudal halves is essential for imparting segmental organization to the peripheral nervous system (PNS) (Stern et al., 1986). In the PNS, motor nerves as well as neural crest cells can only migrate through the rostral half of the sclerotome and are inhibited from entering the caudal half. The ability of cranial half-somite cells, and extracellular matrix to support immigration of neural crest cells and nerves appears to be acquired prior to segmentation

and is not shared by cells of the caudal half-somite (Keynes and Stern, 1988; Ranscht and Bronner-Fraser, 1991). The subdivision of the sclerotome into rostral and caudal halves is also essential for the resegmentation event by which the caudal sclerotome of one somite and the rostral sclerotome of the next somite form a single vertebral body, allowing single muscle groups to interact with two vertebrae and allowing intervertebral movement (Huang et al., 1996; Huang et al., 2000; Keynes and Stern, 1984; Rickmann et al., 1985).

1.6.2.2 Dermomyotome and myotome

Subsequent to sclerotome formation, the remaining dorsolateral epithelium is referred to as the dermomyotome compartment of the somite that gives rise to the epaxial skeletal muscle of the back and hypaxial skeletal muscle of the ventro-lateral body wall, limb muscles, endothelial cells, dorsal dermis, smooth muscle cells and cartilage of the scapula blade (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000; Huang and Christ, 2000; Denetclaw et al., 1997; Chevallier et al., 1977; Denetclaw and Ordahl, 2000; Cinnamon et al., 1999; Christ et al., 1983). It is the source of the myogenic and dermogenic precursor cells (Gros et al., 2005; Kalcheim et al., 2006; Brand-Saberi and Christ, 2000). Dermomyotome formation and maintenance depend on dorsalizing

signals emanating from neighbouring structures, involving Wnt1 and Wnt3a secreted by the dorsal neural tube and contact-dependent Wnt4, Wnt6 and Wnt7a from the surface ectoderm (Spence et al., 1996; Dietrich et al., 1997; Fan et al., 1997; Marcelle et al., 1997; Capdevila et al., 1998; Ikeya and Takada, 1998; Wagner et al., 2000; Rodriguez-Niedenfuhr et al., 2003). Myotome formation depends on balanced signaling of Shh from the notochord/floor plate that induces expression of muscle regulatory factors (MRFs), *MyoD* followed by *Myf5* and also on Wnt signalling from the dorsal neural tube for maintenance of expression of the MRFs (Munsterberg et al., 1995; Munsterberg and Lassar, 1995; Christ et al., 1992; Rong et al., 1992; Borman and Yorde, 1994; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Cossu et al., 1996; Geetha-Loganathan et al., 2005). The dermomyotome comprises a central epithelial sheet adjacent to the ectoderm (DM) and medial and lateral curved borders or lips (DML: dorsal medial lip and VLL: ventral lateral lip, respectively) that bend inwards from where cells delaminate and migrate underneath to form the myotome compartment of the somite, which becomes located between the dermomyotome and the sclerotome (Christ et al., 1978; Kaehn et al., 1988; Denetclaw et al., 1997; Kahane et al., 1988). DM markers include *Pax3* and *Pax7* (Williams and Ordahl, 1994; Muller et al., 1996; Brand-Saberi et al.,

1993; Pourquie et al., 1993; Goulding et al., 1993, 1994). During somite maturation the DM undergoes EMT giving rise to myoblasts that generate skeletal muscle and dermoblasts that generate the subectodermal dorsal dermis, loosing Pax genes expression and start expressing dermal markers such as *dermo-1* (Li et al., 1995; Scaal et al., 2000). The process of DM cell mesenchymalization is accompanied by a change in the orientation of the mitotic spindles from a parallel to a perpendicular orientation of the daughter cells in relation to the dermomyotomal sheet (Ben-Yair and Kalcheim, 2005; Ben-Yair et al., 2011). The dissociating daughter cells relocated dorsally towards the ectoderm form the dermis and the daughter cells relocated ventrally contribute to the growth of the myotome. Only the myoblasts express N-*cadherin* whereas the dermoblasts do not (Ben-Yair and Kalcheim, 2005; Cinnamon et al., 2006). DML cells form the epaxial myotome and differentiate into the back muscles (Ordahl and Le Douarin, 1992) and the VLL cells form the limb girdle muscle and the hypaxial muscle (Ordahl and Le Douarin, 1992). There have been two main models proposed for the origin of the myotome (Denetclaw and Ordahl, 2000; Denetclaw et al., 2001; Kahane et al., 1998; Cinnamon et al., 1999; Kahane et al., 2002). According to the “incremental growth” model of myotome formation, there is a direct translocation of muscle precursor cells from the DML

and the VLL into the myotome, where they elongate rostrocaudally below the dermomyotome. Myotome expansion then proceeds medio-laterally as more myocytes are added and displace older myocytes laterally that become further apart from their progeny (Denetclaw and Ordahl, 2000; Denetclaw et al., 2001). According to the “intercalating growth” model (Cinnamon et al., 1999, 2001; Kahane et al., 2002; 1998), myotome formation involves two consecutive steps. The first muscle precursor cells originate from the dorsomedial epithelial somite (Kahane et al., 1998; Kahane and Kalcheim, 1998). After somite dissociation these “pioneer” cells migrate rostrally underneath the dermomyotome before tranlocating into the myotomme and later contribute to both epaxial and hypaxial muscles (Cinnamon et al., 1999; Kahane et al., 2002). Subsequently, DML and VLL cells are added to the rostral and caudal dermomyotome lips from where they translocate into the myotome where they elongate, intercalating with the pre-existing “pioneer” muscle fibers (Kahane et al., 1998; Cinnamon et al., 1999; Kahane et al., 2002; Cinnamon et al., 2001).

1.7 Somite axial identity

Although the somites of different axial levels initially resemble each

other, the derivatives of each one of these mesodermal segments generate regional differences that constitute the specific patterns of the different parts of the vertebral column (Kessel and Gruss, 1990). In the chicken embryo, somites differentiate into morphologically different vertebrae, dependent on their position in the rostrocaudal axis and this ultimately defines the axial formula: fourteen cervical vertebrae, seven thoracic vertebrae, twelve to thirteen lumbosacral vertebrae and five coccygeal vertebrae (Burke et al., 1995; Gadow, 1933; Nickel et al., 1977) (Figure 1.8). Genes that specifically affect the body segment pattern, *Hox* genes, were first identified in the *Drosophila* (Lewis, 1978) but have subsequently been identified in most metazoans (Duboule, 1998; Minelli and Perrufo, 1991; Akam, 1989). *Hox* genes are a family of homeobox containing transcription factors encoding transcription regulators active in pattern formation and expressed along the rostrocaudal axis in most metazoans (Affolter et al., 1990; Shashikant et al., 1991; McGinnis and Krumlauf, 1992), controlling the identity and morphology of individual segments (Kessel and Gruss, 1990). The positional identity of the somites along the axis of the embryo is specified by a unique code of combined expression of *Hox* genes (Kessel and Gruss, 1990; 1991). Therefore, regionalization can be characterized by specific patterns of *Hox* gene expression (Kessel and Gruss, 1991), which has been

termed the "*Hox* code". In vertebrates, there are four clusters of *Hox* genes (A, B, C and D) situated on four different chromosomes, comprising thirty-nine *Hox* genes. For each of the clusters, the *Hox* genes are arranged sequentially in the chromosome with an arrangement that prefigures their rostrocaudal order of expression along the embryonic axis during development, a phenomenon known as colinearity (Lewis, 1978; Duboule and Dolle, 1989; Gaunt, 1988; Graham et al., 1989) (Figure 1.8). Classical experiments have identified that axial identity determination takes place in the PSM cells before somite formation (Kieny et al., 1972; Jacob et al., 1975). Heterotopic transplantation of prospective-thoracic PSM cells into the cervical region of the host embryo results in the formation of ectopic ribs at the cervical region (Kieny et al., 1972; Jacob et al., 1975). In mice, loss- or gain-of-function mutations that affect the expression of *Hox* genes in the PSM result in an alteration of fates of the somite derivatives with altered morphology of the vertebrae (Crawford, 1995), which have been identified as homeotic transformations (Kessel and Gruss, 1991). Alteration of *Hox* gene expression in the PSM results in respective alteration of the axial identity of the vertebrae (Carapuco et al., 2005). Moreover, *Hox* gene expression boundaries along the rostrocaudal axis have been transposed according to morphology among vertebrates (Gaunt, 1994; Burke et

al., 1995). For instance, the same *Hox* genes are expressed in specific morphological transitions in the chick and mouse, as for example *Hox c-6,* which is expressed in the first thoracic vertebrae of chick (somite nineteen) and of mice (somite twelve) (Burke et al., 1995). During the chicken embryonic body axis extension, *Hox* genes are activated in a temporal collinear manner in the primitive streak (Forlani et al., 2003; Juan and Ruddle, 2003; Wacker et al., 2004; Iimura and Pourquie, 2006); as cells are added to the PSM, as a consequence of the process of gastrulation at the caudal end of the embryo, *Hox* genes are expressed in a collinear manner in the PSM: 3' *Hox* genes start their activity first followed later by 5' *Hox* genes as the axis of the embryo elongates (Gaunt, 1988; Dolle et al., 1989; Graham et al., 1989; Kessel and Gruss, 1991). The gradient of FGF, Wnt and RA signaling has been identified to be involved in the process of somite axial identity specification (Dubrulle et al., 2001). Perturbation of the FGF8 signaling gradient leads to the formation of smaller somites (Dubrulle et al., 2001), which is accompanied by a change in the expression of *Hox* genes (Dubrulle et al., 2001). Thus, *Hox* gene expression matched the respective (smaller) somite rather than the axial position (Dubrulle et al., 2001). Additionally, perturbation of the Wnt and RA signaling also causes a change in the *Hox* gene expression pattern and a change in the axial identity of

the vertebrae (homeotic transformation) in mouse embryos (Kessel and Gruss, 1991; Greco et al., 1996; Ikeya and Takada, 2001; Hogan et al., 1992; Duston et al., 1989; Conlon, 1995). Increasing RA signaling in mouse embryos, leads to homeotic transformations of the vertebrae to more rostral identities (Kessel and Gruss, 1991). It has been identified that there are binding sites for the morphogen RA in the regulatory regions of *Hox* genes and that RA can regulate the 3' expression of *Hox* genes (Hogan et al., 1992; Conlon, 1995), which can trigger the spatial collinear expression of the *Hox* family along the rostrocaudal axis. Homeotic transformations and change in *Hox* gene expression are also observed in *Wnt3a* hypomorphic embryos (Greco et al., 1996) and *Wnt3a* mutant embryos (Ikeya and Takada, 2001). In the PSM cells might start to sequentially express *Hox* genes correlating with the time they spent in the primitive streak (Gaunt, 1994; Gaunt and Strachan, 1994; 1996) or alternatively the exact time of ingression of the cells could be determined by the *Hox* genes expressed prior to it (Iimura et al., 2009; Iimura and Pourquie, 2006).

1.8 The role of BMP-4 signaling and Noggin in mesoderm patterning and somite specification

Bone morphogenetic proteins (BMPs) are secreted signaling molecules and members of the transforming growth factor-β (TGF-β) superfamily of proteins which includes TGF-βs, activins, inhibins, Growth Differentiation Factors (GDFs), Glial Derived Neurotrophic Factors (GDNFs), Nodal, Lefty and anti-Mullerian hormone (Kingsley, 1994; Derynck and Miyazono, 2008). The BMP subfamily can be further classified into further subgroups, according to their osteogenic activities: BMP-2/4 group, the BMP-5/6/7/8 group, the Growth and Differentiation Factor (GDF)-5/6/7 group and the BMP-9/10 group (Derynck and Miyazono, 2008; Kawabata et al., 1998; Cheng et al., 2003). BMP proteins (ligands) can signal via the canonical, Smad-dependent pathway or non-canonical pathways. In the canonical pathway, cell signaling transduction by BMPs follows binding to type II and type I transmembrane serine-theonine kinase receptors, forming of a heterotetrameric complex (Heldin et al., 1997). After formation of the heterotetrameric complex, the constitutively active type II receptor transphosphorylates the type I receptor which in turn phosphorylates the R-Smads (receptor regulated Smad 1/5/8). Then the phosphorylated Smad 1/5/8 associates with the co-Smad (co-mediator Smad4) and translocate to the nucleus where it

subsequently associates with coactivators/corepressors for gene expression regulation (Horbelt et al., 2012; Heldin and Moustakas, 2012). BMPs can bind to three of the seven type I receptors for the TGF- β family of ligands, type IA BMP receptor, type IB BMP receptor and type IA activin receptor and three of the four type II receptors, type II BMP receptor, type II activin receptor and type IIB activin receptor (Heldin et al., 1997; Holbert et al., 2012). The different receptors share structural properties comprising a short extracellular domain, a single transmembrane domain and an intracellular domain containing a serine-threonine kinase domain (Heldin et al., 1997). Various non-canonical, Smad-independent pathways, including the MAPK pathway, can also lead to gene expression regulation (Yamaguchi et al., 1995; Deynck and Zhang, 2003; Zhang, 2009). BMP signaling is additionally regulated by intracellular regulators (Yao et al., 2000), plasma membrane co-receptors which interact with type I and type II receptors, such as endoglin (Toporsian et al., 2010) and extracellular antagonists, such as Noggin, Chordin, Follistatin, Dan, Cerbrus, Gremlin (Glinka et al., 1998; Hsu et al., 1998, Joseph and Melton, 1997; Maniani and Harland, 1998; Nakamura et al., 1990; Pearce et al., 1999; Piccolo et al., 1996, 1999; Smith and Harland, 1992; Stanley et al., 1998; Zimmermann et al., 1996). Therefore, BMP activity in embryogenesis

is dependent on the integration of several signaling mechanisms and regulators (see above). BMPs were originally identified as factors that induce bone formation (Urist et al., 1979; Wozney et al., 1988) but subsequent studies further identified that these proteins regulate several developmental processes throughout embryogenesis: they have been implicated in the specification of the dorso-ventral axis in *Drosophila* (Wozney et al., 1988; Lyons et al., 1991), in mesoderm formation during gastrulation in mice (Winnier et al., 1995; Mishina et al., 1995), in the formation of the ventral mesoderm during gastrulation in *Xenopus* (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Harland et al., 1994; Schmidt et al., 1995; Dosch et al., 1997) and primitive streak formation, dorso-ventral patterning of the neural tube, mesoderm specification and somite patterning in the chicken embryo (Streit et al., 1998; Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995; Pourquie et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Tonegawa and Takahashi, 1997, 1998). In the chicken embryo, the mesoderm layer is initially formed during the dynamic process of gastrulation from cells of the node and adjacent epiblast that ingress through the primitive streak (Psychoyos and Stern 1996; M. A. Selleck and Stern 1991). The precursors of the axial and paraxial (pre-somitic) mesoderm are located close to the rostral end of the streak (whereas

the precursors of the lateral mesoderm and extraembryonic tissues are located caudally) (Nicolet 1965, 1967, 1971; Psychoyos and Stern 1996; Rosenquist 1966, 1970; Schoenwolf et al. 1992; M. A. Selleck and Stern 1991; Spratt and Condon 1947; Spratt 1957; Tam and Tan 1992). In the chick embryo at HH st.5, the paraxial mesoderm precursors reside in the most rostral third of the primitive streak. Subsequent to ingression the mesodermal cells migrate rostrally and laterally and those that are located medially form the paraxial mesoderm and those located laterally form the lateral plate (Schoenwolf et al. 1992; M. A. Selleck and Stern 1991). It has been identified that the level of BMP-4 activity imparts medial-lateral patterning to the mesoderm and to the somites (Pourquie et al., 1996; Hirsinger et al., 1997; Garcia-Martinez and Schoenwolf, 1992; Tonegawa et al., 1997). During early neurulation in the chicken embryo, BMP-4 mRNA is expressed in the developing neural plate and in a wide region caudal to Hensen's node including the ectoderm overlying the prospective lateral plate mesoderm and the caudal part of the primitive streak mesoderm (Watanabe and Le Douarin, 1996; Streit et al., 1998; Tonegawa and Takahashi, 1998; Pourquie et al. 1996). As development proceeds, BMP-4 mRNA is expressed in the presumptive lateral plate mesoderm at HH st.10, and at HHst-13 becomes strongly expressed in the lateral plate mesoderm along the

rostro-caudal axis next to the presomitic level, but not detected in the somite mesoderm (Watanabe and Le Douarin, 1996; Tonegawa et al. 1997; Roberts et al., 1995; Pourquie et al., 1996). Ectopic administration of BMP-4 on the presomitic mesoderm of the chicken embryo at HH st.14 results in a lateralization of the presomitic mesoderm in a concentration-dependent manner: at low concentration results in a change of somitic mesoderm to lateral somite characteristics and at high concentration results in complete respecification of somite mesoderm to lateral plate, providing evidence that BMP-4 acts as a lateralizer during mesoderm formation and is one of the signals involved in mesoderm specification (Tonegawa et al, 1997, Pourquie et al, 1996; Hirsinger et al., 1997). These observations are consistent with previous experiments done in younger chicken embryos at HH st.4 in which prospective somitic mesoderm from the primitive streak was grafted into the prospective lateral plate region of the primitive streak, resulting in contribution of the graft to the lateral plate, providing evidence that lateral plate provides a signal for lateralization (Garcia-Martinez and Schoenwolf, 1992). The BMP-4 antagonist Noggin, expressed in the Spemann's organizer and originally discovered as a dorsalizing factor in *Xenopus* (Smith and Harland, 1992), has been shown to counteract BMP-4 activity by binding to it and preventing an interaction with its

receptor (Piccolo et al., 1996; Zimmerman et al., 1996). Noggin binds to BMP2/4 and Gdf7 subclasses of BMPs with picomolar affinity and to other BMPs at lower affinities (Chang and Hemmati-Brivanlou, 1999; Re'em Kalma et al., 1995; Zimmerman et al., 1996). Noggin expression pattern during somitogenesis in the chicken embryo is also compatible with an antagonizing role to BMP-4 (Streit and Stern, 1999; Tonegawa and Takahashi, 1998; Hinsinger et al., 1997; Sela-Donenfeld and Kalcheim, 2002). At the beginning of neurulation (HH st.5) Noggin in expressed in the node and from HH-st.8 it becomes expressed in the notochord adjacent to the somite territory (up to somite X), in the mesodermal cells caudal to Hensen's node where it overlaps with the BMP-4 expression domain, in the caudal neural tube and also in the lateral border of the PSM (region that coincides with the intermediate mesoderm which expresses BMP-4) (Streit and Stern, 1999; Tonegawa and Takahashi, 1998; Hinsinger et al., 1997; Pourquie et al., 1996). At the level of the young epithelial somites, Noggin is expressed in the lateral portion of the epithelial somites I to III and becomes progressively more medial as somites start maturing (Tonegawa and Takahashi, 1998; Hinsinger et al., 1997; Sela-Donenfeld and Kalcheim, 2002). Since BMP-4 is expressed in the adjacent lateral plate mesoderm, it has been suggested that Noggin prevents lateralization of the somites by counteracting BMP-4 activity

(Capdevila and Johnson, 1998; Tonegawa and Takahashi, 1998; Hinsinger et al., 1997; Pourquie et al., 1996, Tonegawa et al., 1997). It has been shown that mesoderm growth and patterning of somites are severely affected in *Noggin* null mice embryos, which consequently die at birth with a severely malformed skeleton (somite derivative) (Brunet et al., 1998; McMahon et al., 1998). Bmp-4 haploinsufficiency suppresses this phenotype (Wijgerde et al., 2005). Therefore, Noggin-mediated regulation of BMP-4 activity is crucial for the specification of mesoderm and somites. The role of BMP-4 activity/Noggin in somite specification is further illustrated in several studies in the chicken embryo. Ectopic administration of BMP-4 on the paraxial mesoderm prevents pre-somitic cells from differentiating into somites (Tonegawa et al. 1997) and BMP-4 inhibition by ectopic Noggin is sufficient to convert prospective lateral plate/ extraembryonic mesoderm into somite fate (Streit and Stern 1999; Tonegawa and Takahashi 1998). It is possible to generate ectopic somites independently from the main axis by means of quail/chick transplantation experiments (Streit and Stern 1999): explants of the most posterior primitive streak (PPS) (prospective lateral plate/extraembyonic mesoderm) transplanted together with Noggin producing cells (CHOB3.A4 cells or Noggin-transfected COS cells)

into the extraembryonic area opaca of a chick host embryo give rise to ectopic somites (Streit and Stern, 1999).

1.9 Aims of the study

Somitogenesis is an intricate multistep process involving timing, somite size specification and patterning. Given that these processes are tightly integrated and happen in concert with maturation of the PSM and body axis extension in the developing embryo, it has been difficult to disentangle whether there is one prevailing mechanism controlling the specification of all aspects of somite formation or whether there are several mechanisms working in parallel to individually regulate each aspect. Because experimentally it has been difficult to separate all of these features of somite formation as they occur synchronously during somitogenesis in the normal embryo, prevailing models to explain somite formation have been postulated to encompass all of aspects, which have been thought to be regulated at the same time.

Preliminary experiments have provided the interesting observation that posterior primitive streak explants (prospective lateral plate) treated with the BMP4 antagonist, Noggin, generate experimental somites that show expression of the somite marker *Paraxis* but form

in groups resembling ‘bunches of grapes’, a different arrangement compared with how axial endogenous somites form in an orderly and periodic fashion (Streit and Stern, 1999). This means that it is possible to generate experimental somites *de novo* from non-somitogenic tissue and provides a unique experimental paradigm to study somitogenesis since experimental somites are not linearly arranged and are confronted with other somites in a three-dimensional arrangement.

To be able to use the experimental somites as a model to study aspects of somitogenesis it is important to first establish a reliable protocol to generate them in good numbers and to characterize them in terms of size, morphology, expression of somitic markers and potential to give rise to late somite derivatives if placed in the right environment. These are the aims of Chapters Three and Four.

Subsequently, the periodicity of experimental somite formation is addressed. To investigate whether the experimental somites form sequentially and periodically like the endogenous somites or synchronously, the formation of the experimental somites from a GFP-transgenic (CAG-GFP chick) donor posterior primitive streak explant treated with Noggin protein will be time-lapse imaged with an

inverted fluorescence microscope. This is the aim of the first part of Chapter Five.

The experimental somites will be used as an experimental paradigm to explore the potential roles of the molecular clock in the different events of somite formation because it allows to separate the aspects of somite size specification from the timing of somite formation, which in the normal embryo are coincident events. To assess the molecular clock in the posterior primitive streak explants treated with Noggin protein and investigate if the formation of experimental somites is prefigured, or not, by periodic expression of the “cycling genes”, a time-course experiment is performed, including multiple time-points separated by 45 minutes intervals, following treatment of the posterior primitive explants with Noggin protein and subsequent analysis by *in situ* hybridization for expression of key “cycling genes” *Hairy1*, *Hairy2* and *LFng*. Since the subdivision of the somites into rostral and caudal halves is thought to be a manifestation of the molecular clock (Takahashi et al., 2003), the rostrocaudal subdivision of the experimental somites will be investigated by examining the expression of known rostral half markers, *Hairy2* (Jouve et al., 2000) and *EphA4* (Schmidt et al., 2001) and caudal half markers, *Hairy 1* (Palmeirim et al., 1997*), Lunatic Fringe* (Aulehla and Johnson, 1999;

McGrew et al., 1998) *Uncx4.1 (*Schragle et al., 2004) and *Meso2* (Buchberger et al., 1998*),* by *in situ* hybridization. As an additional test of the somite patterning, the migration paths of neural crest cells and motor axons through somites (that will grafted in place of an endogenous somite in a secondary host) which are known to normally only migrate through the rostral half of the sclerotome (Keynes and Stern,1988) will be analyzed. These are the aims of the second part of Chapter Five.

Finally, given that occipital somites form almost simultaneously in the normal embryo and show molecular differences compared with the other more caudal somites (Alev et al., 2010; Lim et al., 1987; Rodrigues et al., 2006), one might reason that the experimental somites formed from posterior primitive streak explants treated with Noggin protein, could have an occipital somite identity. Therefore, to investigate the axial identity of the generated somites, the expression of known *Hox* genes (Burke et al., 1995; Gaunt, 1994) in the experimental somites will be analyzed by in situ hybridization. This is the aim of Chapter Six.

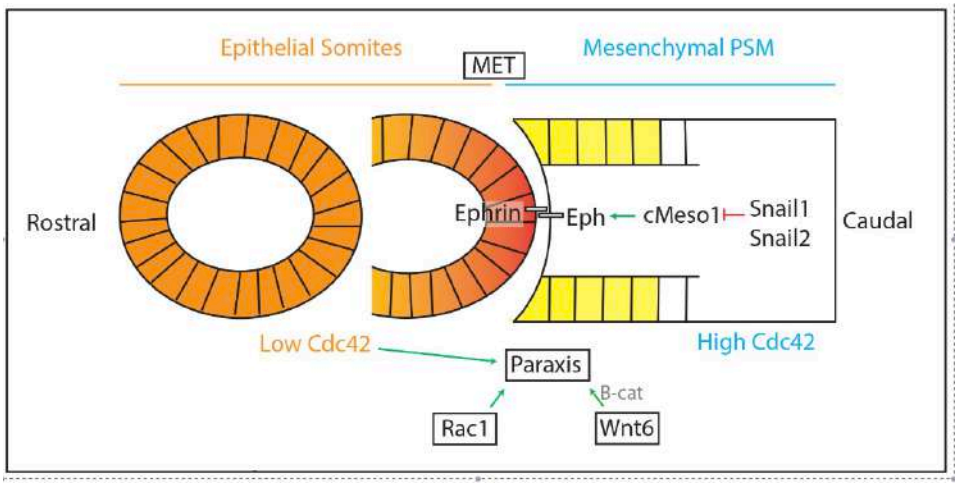

Figure 1.1. Summary diagram showing a two-dimensional representation of the forming epithelial somites and rostral PSM microenvironment and the roles of Cdc42, Paraxis and intercellular signaling in the MET process during somitogenesis in the chicken embryo.

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Newly formed epithelial somite (orange) is lined by a layer of epithelial cells and has a central cavity, the somitocoele (left); epithelialization of the caudal border of the epithelial somite precedes epithelialization of the rostral border, hence the forming somite presents a horseshoe shape (middle); in the rostral PSM cells become arranged as an epithelium at the peripheral sides of the PSM prior to somite formation (yellow). *Paraxis* starts to be expressed at the level of the rostral PSM, induced by ectodermal *Wnt6*. Low activity of *Cdc42* is necessary for somitic MET and high activity of *Cdc42* is required for mesenchymal state maintenance. *Rac1* activity is required for *Paraxis* function as an epithelialization factor. MET correlates with the downregulation of *Snail* genes. *cMeso1* upregulates *EphA4* in the cells located caudally to a forming border which in turn activates *Ephrin*-reverse signal in the rostral border cells, where it is sufficient to produce a somitic boundary and promotes epithelialization by repressing *Cdc42*. MET, mesenchymal-epithelial transition; PSM, presomitic mesoderm.

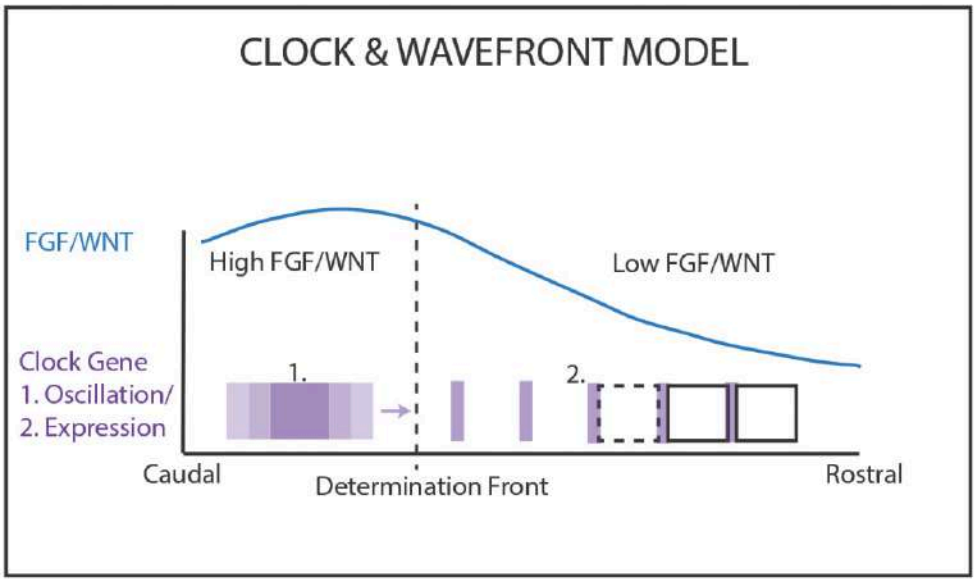


Figure 1.2. Summary diagram representing the Clock and Wavefront model.

Figure 1.2. Summary diagram representing the Clock and Wavefront model.

(**Top of graph**) The distribution of the strength of the FGF/WNT signaling (y axis) is illustrated as a blue line representing a decreasing gradient along the rostrocaudal axis of the PSM (x axis). The vertical dotted line illustrates the position of the determination front. (**Bottom of graph**) Representation of the rostrocaudal axis of the embryo with the somites (black squares, on the right), the clock gene oscillations (purple band, on the left) and the clock genes fixed expression (purple blocks, on the right). The dotted square represents the position of the next somite to form. Region (1) represents the travelling waves of oscillatory gene expression in the PSM. Region (2) represents the fixed stripes of gene expression after arrest of the oscillating gene expression. High FGF/WNT signaling maintains oscillations of gene expression in the PSM. The determination front fixes the position where the travelling waves of oscillatory gene expression are arrested and is proposed to define the caudal boundary of the prospective somite to be specified. PSM, presomitic mesoderm.

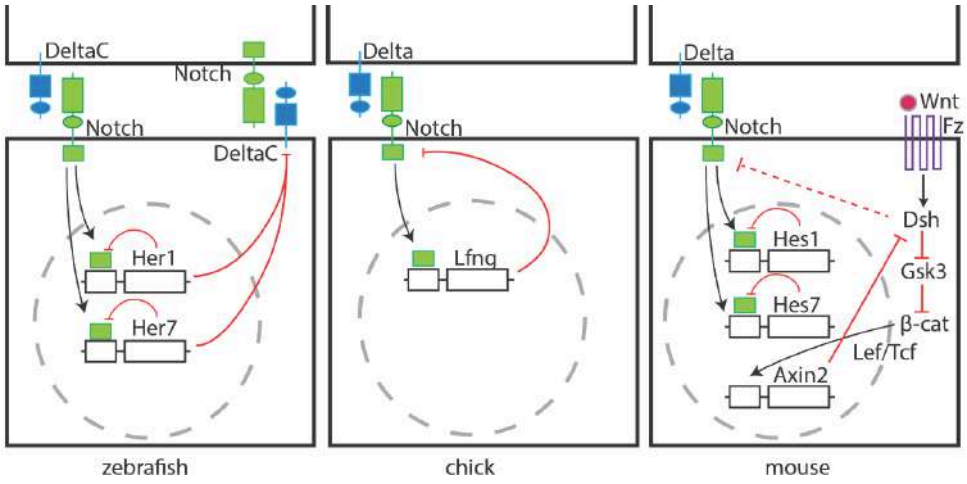


Figure 1.3. Summary diagram of the feedback loops that have been proposed to underlie the periodic oscillation of the cycling genes in zebrafish, chick and mouse.

Figure 1.3. Summary diagram of the feedback loops that have been proposed to underlie the periodic oscillation of the cycling genes in zebrafish, chick and mouse.

Zebrafish. *Her1* and *her7* show oscillatory expression in the zebrafish PSM cells, which has been proposed to be required for the oscillations of the cycling genes (Henry et al., 2002; Oates and Ho, 2002; Holley et al., 2002). Self-repression of *her* genes has been proposed as the basis for the oscillations of the zebrafish clock (Oates and Ho, 2002, Holley et al., 2002; Lewis, 2003) and are thought to be involved in periodic expression of *DeltaC* which in turn periodically activates Notch activity (Jiang et al., 2000). It has also been suggested that DeltaC oscillatory expression acts to synchronize adjacent cells (Jiang et al., 2000; Mara et al., 2007). **Chick.** *Lfng* shows oscillatory expression in the PSM cells, with the same periodicity as somite formation (McGrew et al., 1998; Forsberg et al., 1998; Aulehla and Johnson, 1999). It has been proposed that Lfng protein (a modulator of Notch activity) indirectly represses its own expression by periodic modulation of Notch signaling pathway (Dale et al., 2003). Overexpression of *Lfng* in the chick PSM impairs the cyclic expression of the Notch downstream targets *hairy1* and *hairy2* and endogenous *Lfng* (Dale et al., 2003; Serth et al., 2003). **Mouse.** *Hes* genes encode for transcriptional repressors of the bHLH family, downstream of Notch signaling (Iso et al., 2003). *Hes7/hes1* mRNA and Hes7/Hes1 protein show oscillatory expression in the mouse PSM cells (Bessho et al., 2003; Hirata et al., 2004). It has been proposed that *hes7*/1 oscillatory expression in the PSM relies on periodic delayed autorepression by its own protein (Hirata et al., 2004; Bessho et al., 2003; Jensen et al., 2003, Lewis, 2003; Monk, 2003; Barrio et al., 2006). Additionally, *axin2* (a target of the Wnt

pathway) is also expressed in a cyclic fashion in the mouse PSM cells (Aulehla et al., 2003). Wnt signaling-*Axin2* negative feedback loop has been proposed to drive the mouse clock (Aulehla et al., 2003). In the mouse PSM, Notch and Wnt act antagonistically through the binding of *Dsh* to the Notch intercellular domain (Aulehla et al., 2003).

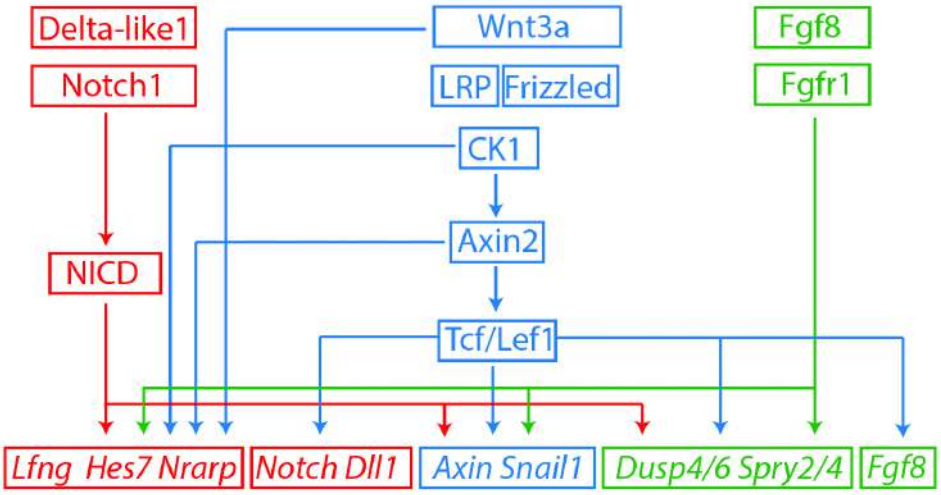


Figure 1.4. Interactions between Notch, Wnt and Ffg signaling pathways.

Figure 1.4. Interactions between Notch, Wnt and Ffg signaling pathways.

Crosstalk between the Notch (red), Wnt (blue) and Fgf (green) signaling pathways involved in the segmentation clock in the PSM (Ferjentsik et al., 2009; Aulehla et al., 2003; Dequeant et al., 2006; Niwa et al., 2007; Gibb et al., 2009; Dale et al., 2006; Sewell et al., 2009; Wahl et al., 2007). NICD, Notch intracellular domain; *Lfng*, *Lunatic fringe*; *Hes7*, hairy and enhancer of split related 7; Nrarp, Notch-regulated ankyrin repeat protein; LRP, low density lipoprotein receptor-related protein; CK1, casein kinase 1; Fgfr1, Fgf receptor 1; Dusp 4/6, Dual specificity phosphatase 4/6; *Dll1, Delta-like 1.* (Reproduced from Gibb et al., 2010).

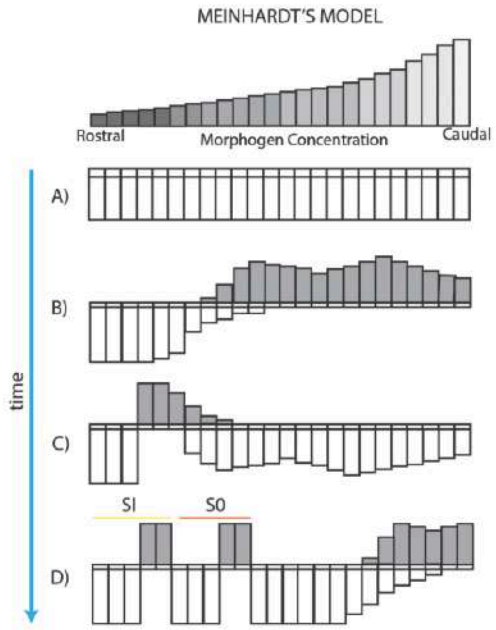


Figure 1.5. Summary diagram representing Meinhardt's model.

Figure 1.5. Summary diagram representing Meinhardt's model.

At the top of the figure, the rostrocaudal concentration of a morphogen represents the long-range gradient that provides positional information. **A-D:** Steps of gradual generation of a periodic and sequential pattern under the control of the long-range gradient of a morphogen. **A:** Initially, all the cells present a rostral state (white). **B:** A specific morphogen concentration defines a transition zone where the cells change their state to a caudal state (grey). **C:** At the level of the transition zone, somite formation is preceded by a series of oscillations between rostral (white) and caudal (grey) states, before they stabilize. This is proposed to be linked to the period of an oscillator operating in the PSM. Cells located too far from the transition, stabilizing zone revert to rostral state. **D:** According to this model, once the oscillations between states are arrested and the cells stabilize their states at the rostral end of the PSM, a somite border forms between the caudal cells of one somite and the rostral cells of the next somite to form. The process continues as the morphogen gradient moves caudally and defines a new transition zone, where the oscillations between states start again and new boundaries form. S0, next somite to form; S1, newly formed somite; PSM, presomitic mesoderm. (Reproduced with modifications from Meinhardt, 1986).

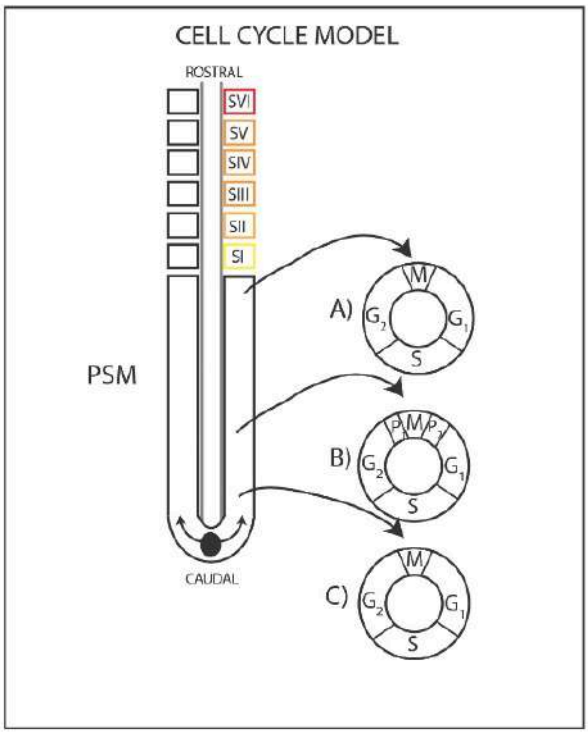


Figure 1.6. Summary diagram representing the Cell Cycle model.

Figure 1.6. Summary diagram representing the Cell Cycle model.

The model postulates a correlation between the process of somite formation and the synchrony of the cell division cycle of the cells in the PSM. Schematic representation of the caudal part of the chicken embryo (**left**); Schematic representation of three cell cycles (A, B, C) and associated levels of somitogenesis (**right**). **A:** At the lvel of somite formation in the rostral PSM, the cells are at the M phase of the cell cycle. **B:** Cells are allocated to a specific somite during cell cycle B, preceding somite individualization by one full cell cycle. According to the cell cycle model, the allocation of a group of PSM cells to form a somite is proposed to occur during a 90 min. window between the P1 and P2 points on either side of the M phase of cell cycle B. “Pioneer” cells that reach the P2 point are then able to signal only to the cells within the P1-P2 window, instructing them to group together to form a somite. **C:** Cells at the caudal end of the PSM are at the M phase of the cell cycle C and will still experience another full cell cycle prior to being allocated to a specific somite. PSM, presomitic mesoderm; S, somite. (Reproduced with modifications from Primmett et al., 1989).

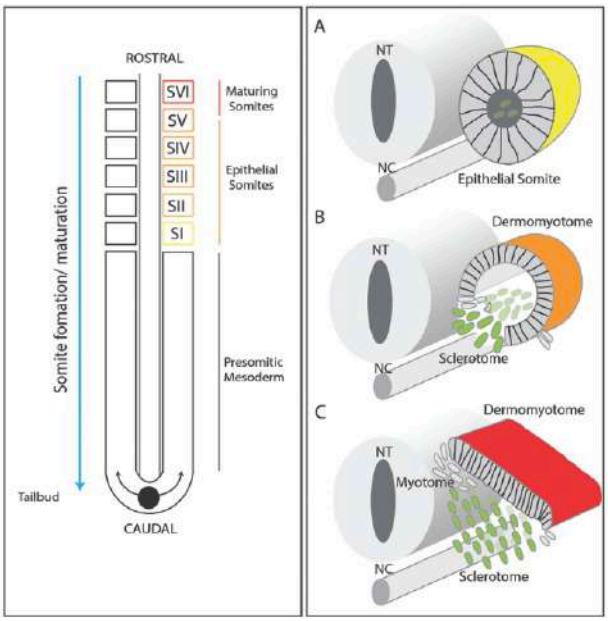


Figure 1.7. Schematic representation of the sequential formation and maturation of somites.

Figure 1.7. Schematic representation of the sequential formation and maturation of somites.

(right) Diagram representing the caudal part of a chick embryo. The PSM is produced caudally at the level of the primitive streak and the tail bud (arrows) and forms as two rods of mesenchymal cells on either side of the neural tube. Cells remain in the PSM until they segment into epithelial somites at the rostral end of the PSM, and somite formation progresses in a rostrocaudal sequence. One pair of somites forms every 90 min. The sequence of the somites along the axis reveals a rostrocaudal gradient of developmental progress with the more rostral somites at later stages of maturation and the youngest somites gradually positioned more caudally. Somite nomenclature, as defined by Christ and Ordahl (1995) is indicated inside the somites. The newly formed youngest somite is denominated SI and the successive more rostral somites are denominated sequential Roman numbers (SII, SIII, SIV, SV…).
(left) Schematic representation of the main morphological events during somite formation and maturation. **A:** Somites form as epithelial spheres, a single cell in radius, with a central lumen containing mesenchymal somitocoele cells. **B, C:** From SV, somites mature through compartmentalization and cell lineages specification guided by extrinsic cues, resulting in the formation of the ventromedial mesenchymal sclerotome (green), the dorsolateral epithelial dermomyotome (red) and the myotome (white). S, somite; NT, neural tube; NC, notochord; PSM, presomitic mesoderm.

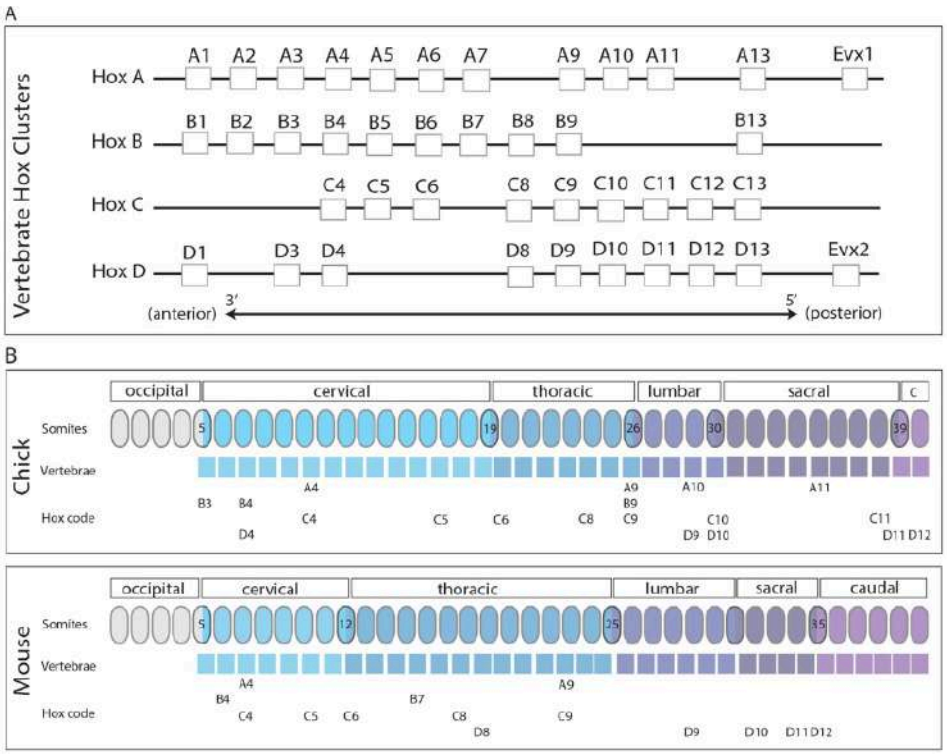


Figure 1.8. Hox code and the axial formula.

Figure 1.8. Hox code and the axial formula.

A: Schematic representation of the four vertebrate *Hox* genes clusters (A, B, C, D). *Hox* genes are shown as boxes in their order on the chromosome. **B:** Schematic representation of the axial formulae (vertebrae) and respective somite levels of the chick and the mouse. The *Hox* code is listed below each axial formulae and the rostral (anterior) boundary of *Hox* gene expression is indicated at the respective axial level.

Chapter Two: Materials and Methods

2.1 Avian embryos and somite nomenclature

Fertilized hens' (*Gallus gallus domesticus*) Brown Bovan Gold eggs and fertilized quail (*Coturnix coturnix japonica)* eggs were obtained from commercial sources (Henry Stewart & Co., UK and B C Potter Farm, UK, respectively). Transgenic hens' eggs (a line expressing Green Fluorescent Protein [GFP] in all cells) were obtained from the Roslin Institute, University of Edinburgh. Eggs were stored at 14°C until the beginning of each experiment after which they were incubated in a humidified atmosphere, at 38°C, to the desired stages. The embryos were staged according to the morphological criteria of Hamburger and Hamilton, henceforth referred to as ''HH-stage'' (Hamburger and Hamilton, 1951), further refined by noting the number of pairs of somites formed. Additionally, the shape of Hensen's node was carefully observed to stage embryos at around HH-stage 5 of development more precisely, according to Selleck and Stern (1991) (see Figure 2.1).

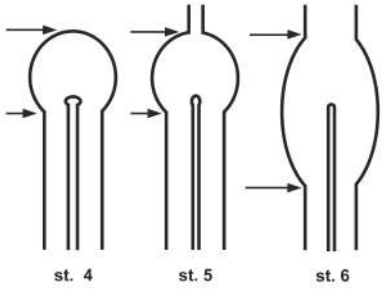


Figure 2.1. Diagram showing the changes in the shape of Hensen's node between HH-stage 4 and HH-stage 6. (adapted from Selleck and Stern, 1991).

The arrows indicate the rostral and caudal limits of Hensen's node. At HH-st.4, the node is a mass of cells at the rostral end of the primitive streak, with boundaries easily visible as indicated by arrows. At HH-st.5, the head process (rostral-most notochord) has emerged and is visible as a rod of condensed mesoderm extending forward from the rostral edge of Hensen's node. At HH-st.6 the caudal limit of the node becomes less distinct.

The classification of somite maturity was done according to the system defined by Christ and Ordahl (1995): the most recently formed somite is termed SI, anterior to SI the progressively older somites are denominated SII, SIII and so on (B. Christ and Ordahl 1995).

2.2 Microsurgical procedures

2.2.1 Modified New culture of young avian embryos

A modified culture method (Stern and Ireland, 1981) based on that first described by Dennis New in 1955 (New, 1955) was used for grafting experiments.

Fertilized chick eggs were incubated at 38°C for 19-22 hours to obtain HH-st.5 host embryos for transplantation experiments. The eggs were removed from the incubator and left to cool to room temperature. Incubated eggs were opened at the blunt end using coarse forceps. The thin albumen was poured out, collected in a small beaker and kept for culture. After removing the thick adhering albumen around the yolk, the latter was transferred into a large glass dish containing Pannett-Compton (PC) saline solution (Pannett, 1924). The yolk was carefully turned so that the embryo was facing up. Whilst submerged, the vitelline membrane enveloping the yolk was cut all the way around just below the equator and gently peeled from the yolk using semi-blunt forceps, taking the adhering embryo with it. The membrane containing the embryo was then transferred to a watch glass and positioned with the embryo facing up (ventral side up). A glass ring was placed on the membrane. The watch glass was then lifted out of the saline taking the ring with the membrane and

embryo with it. Then, the membrane was stretched around the ring and folded over it, positioning the embryo in the centre of the ring. The membrane and embryo were gently cleaned of any excess yolk with a stream of PC saline. At this point the experimental manipulation was performed (transplantation and bead grafts) after which the remaining saline was removed, inside and outside of the ring. Finally, the ring with the vitelline membrane was carefully transferred to a 35 mm plastic dish containing a pool of thin egg albumen (Stern and Ireland, 1981) and gently pressed down with two forceps allowing it to adhere to the bottom of the dish which was then sealed with a lid previously coated with albumen. The dishes were placed in a plastic box humidified using a piece of tissue paper soaked with distilled water and incubated for 3-24 hours, according to the experiment, in a 38°C incubator.

2.2.2 Somite transplantation experiment

Somite transplantation was performed *in ovo*. Fertilized hens' eggs were incubated resting on their sides for 40-48 hours to obtain HH-st.11-12 host embryos.

Preparation of the chick host embryo

The egg was removed from the incubator and placed on a plasticine

ring, keeping the orientation in which it had been incubated. Using a syringe with a 21G needle, the blunt end of the egg was pierced to withdraw 1 ml of egg albumen. A 1 cm x 1 cm square window was scored on the top of the shell using a scalpel and the piece of shell removed with forceps: the underlying shell membrane then moistened with Tyrode's saline solution and removed with forceps. The egg was filled with Tyrode's saline solution to float the embryo to the level of the window and 30 μl of Indian ink (Pelikan Fount India diluted 1:10 with Tyrode's saline) injected under the vitelline membrane below the embryo. A border of silicone grease was drawn around the edges of the window in the shell and filled with Tyrode's saline and fibre optic lights were placed tangentially to the surface of the egg to produce a "dark field" illumination (Hara, 1971).

Excision of a single somite

The vitelline menbrane was pierced above the last somite formed. Then the Tyrode's saline solution was replaced with Trypsin (Difco 1:250; 0.12% w/v in Tyrode's saline). Using a micro-knife a cut was made in the ectoderm next to the neural tube in the region of the last formed somite, lifted up and gently moved to the side. A second cut was made between the neural tube and the somite and gradually deepened without penetrating the endoderm and the same was done on the opposite side to free the somite from the intermediate

mesoderm. The somite was gently lifted up using the micro-knife and the neural tube as a lever and removed with a Gilson micropipette. Then, the Trypsine solution was removed and the embryo washed twice with fresh Tyrode's saline.

Grafting of the donor somite

An isolated ectopic somite was obtained from the donor embryo using light Trypsin (Difco 1:250; 0.12% w/v in Tyrode's saline) placed directly above the cultured embryo, inside the glass ring. The somite was picked up with a Gilson micropipette set to 2 μl and carefully placed into the Tyrode's bubble over the host embryo. It was manipulated with a micro-knife into the gap made by the excised host somite and covered with the layer of ectoderm. Then, the Tyrode's saline was carefully removed with a Pasteur pipette and 3 ml of thin egg albumen withdrawn from the egg through the original hole in the blunt part of the eggshell, lowering the host embryo back to its original position. Finally, the edges of the window on the shell were wiped with 70% alcohol and the egg sealed with PVC tape. The operated embryos were incubated on their sides (with the window upwards) for 2-4 days up to stage 22-25, in a 38°C humidified incubator.

2.3 Fixation of the embryos

After incubation in modified New culture (Stern and Ireland, 1981), embryos were submerged in Pannett-Compton saline solution and freed from the vitelline membrane using 21G hypodermic needles. The embryos were fixed overnight at 4°C (placed flat in a Petri dish) in a drop of fresh 4% paraformaldehyde in PBS, pH 7.0 and then rinsed in PBT (PBS with 0.1% Tween 20). For whole mount *in situ* hybridization the embryos were dehydrated through a methanol series and stored in absolute methanol at -20°C.

After incubation *in ovo,* the embryos were explanted and the extraembryonic membranes removed. Then the cavities were opened (brain vesicles and heart) to facilitate washing and the embryos fixed overnight at 4°C in fresh 4% paraformaldehyde in PBS, pH 7.0, then rinsed in PBT (PBS, 0.1% Tween 20), methanol cleared and stored as described above.

2.4 Synthesis of RNA antisense Digoxigenin-labelled probes for *in situ* hybridization

Digoxigenin-labelled RNA probes were *in vitro* transcribed from linearized plasmids according to standard procedure adapted from Sambrook et al. (Sambrook, 1989).The plasmids were cut at a 5'

restriction site with the appropriate restriction enzyme according to the manufacturer's instructions and the cut DNA was purified by phenol:chloroform (1:1) extraction, ethanol precipitated and resuspended in clean (commercial Ultrapure) RNAase-free water to a final concentration of 1μg/μl. *In vitro* transcription was carried out with the appropriate RNA polymerase (T3, T7 or SP6) at 37°C (40°C in the case of SP6) for 2 hours. The proportions of different reagents for the transcription reaction were the following**:** (for 3 μg of cut DNA): cut DNA (3 μl); DEPC water (22 μl); 5x transcription buffer (10 μl); digoxigenin-labelled nucleotide mix (5 μl); 10x DTT (5μl); RNAse inhibitor (1 μl); RNA polymerase (4 μl). After 2 hours at 37°C an aliquot of 1 μl was taken and ran in a 1%TAE agarose-gel to check transcription and 3 μl of DNAase (RNAse free. Promega RQ1 DNAase) was added to degrade the template DNA. The RNA was precipitated by adding 10 μl 4M LiCl and 250 μl of EtOH and incubated at -20°C, overnight. The pellet was washed with 300 μl of 70% ethanol and the probe was then resuspended in clean (commercial Ultrapure) RNAase-free water, at a concentration of approximately 1mg/ml and diluted in 10x the volume of hybridization buffer or to a final concentration of 100-500 ng/ml for use for *in situ* hybridization.

The digoxigenin-labelled antisense RNA probes used in the course

of this work were: *Noggin* (Connolly et al., 1997) *BMP4* (Liem et al., 1995), *Paraxis* (Sosic et al., 1997), *MyoD* (Pourquie et al., 1996), *Pax1* (a kind gift from A. Streit), *Hairy1* (Palmeirim et al., 1997), *Hairy2* (Jouve et al., 2000), *Lunatic fringe* (Sakamoto et al., 1997), *Meso2* (a kind gift from M. Maroto), *EphA4* (Schmidt et al., 2001*)*, *HoxB4*, *HoxB9* (kind gifts from Linda Ariza-McNaughton (Grapin-Botton et al., 1995) and *Uncx4.1* (Dale et al., 2003). Partial *Dapper1* and *Dapper2* cDNA probes were kindly provided by Dr. S. Dietrich (Alvares et al., 2009).

2.5 Whole mount *in-situ* hybridization

This experiment was performed according to standard protocols in the Stern lab (Stern, 1998; Streit and Stern, 2001), partly based on a method described by Domingos Henrique (Henrique et al., 1995). The stored embryos were re-hydrated by successive washes of 5 minutes in 75%, 50% and 25% methanol in PTW solution, followed by four washes of 10 minutes each in PTW. The embryos were then treated with proteinase K (10µg/ml) for 30 minutes (or 15 minutes for cultured embryos) at room temperature and re-fixed in 0.1% glutaraldehyde/4% paraformaldehyde in PTW for 20 minutes at room temperature. After two PTW washes of 10 minutes each, the

embryos were transferred to hybridization solution and pre-hybridized for 2 to 6 hours at 70°C. Hybridization was carried out overnight at 70°C in hybridization solution (Appendix 1) containing a digoxigenin-labelled RNA probe. The embryos were rinsed three times with a small volume of pre-warmed hybridization solution, then washed two times for 30 minutes each in pre-warmed hybridization solution followed by a wash in pre-warmed 1:1 hybridization solution: TBST (8 mg/ml NaCl, 0.2 mg/ml KCl, 2.5 mM Tris pH 7.6, 0.1% Tween-20). The embryos were then washed three times 30 minutes each in TBST and pre-blocked in 5% heat inactivated sheep serum in TBST for three hours followed by an incubation overnight at 4°C, in blocking solution containing alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim). After washing the embryos extensively in TBST for at least five hours, they were transferred to NTMT (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween-20). The alkaline phosphatase activity was visualized by incubating the embryos in NTMT containing 0.45 mg/mL nitro-blue-tetrazolium chloride (Boehringer-Mannheim), 0.35 mg/mL 5-bromo-4-chloro-3-indolyl phosphatase (Boehringer-Mannheim). After stopping the colour reaction, embryos were post-fixed in 0.1% glutaraldehyde/ 4% paraformaldehyde in PTW for 1 hour at room temperature and then stored in PTW.

2.6 Antibodies for whole mount immunocytochemistry

Whole-mount immunocytochemistry for GFP was performed using a monoclonal rabbit anti-GFP antibody (InVitrogen, 1:1000) and the secondary antibody Alexa Fluor 488-conjugated anti-rabbit IgG (InVitrogen, 1:1000) was used. A monoclonal antibody directed to neurofilament-associated protein (3A10, Developmental Studies Hybridoma Bank, 1:50) and a monoclonal antibody directed to the HNK-1 epitope (IC10, Developmental Studies Hybridoma Bank, 1:100) were also used. The secondary antibody used was HRP-conjugated anti-mouse IgG (1:1000).

2.7 Antibodies for whole mount double immunocytochemistry

Whole-mount double immunocytochemistry for Fibronectin and N-cadherin was performed using a monoclonal antibody directed to chick Fibronectin (VA1(3)-S Developmental Studies Hybridoma Bank, 1:100) and a polyclonal anti-N-cadherin antibody (ab12221 Abcam, 1:500). Secondary antibodies were Alexa Fluor 555- and 488- conjugated anti-mouse and anti-rabbit IgG (Molecular Probes, 1:200).

2.8 Whole mount immunocytochemistry

The manipulated embryos, previously fixed, were washed three times for 30 minutes in PBST (PBS with 1% Triton-X100) on a rocker at room temperature and then blocked overnight with gentle rocking at 4°C in blocking buffer (PBST with 1% BSA, 1% NGS). The embryos were incubated in primary antibody (or a mix of the two primary antibodies for double immunostaining) in blocking buffer, overnight at 4°C. The embryos were then washed in PBST, five times for 30 minutes, followed by incubation with blocking buffer, for 1 hour at room temperature. After this, embryos were incubated overnight in secondary antibody (or a mix of the two secondary antibodies for double immunostaining) in blocking buffer. Finally the embryos were washed three times for 10 minutes and three times for 30 minutes in PBST at room temperature and either:

(1) mounted in Vectashield (Vector Laboratories)

or

(2) washed in 100mM Tris-HCl pH7.4 for ten minutes and incubated in the developing medium (0.5mg DAB in 100ml TRIS pH 7.4 with 0.003% H2O2) for a few minutes until the brown peroxide reaction product had developed and finally washed briefly with water and fixed in 4% formaldehyde for storage at 4^{o}C.

2.9 Histological sections

Selected hybridized and post-fixed chick embryos were processed for histological sections (wax), progressively dehydrated/cleared as previously described (Izpisua-Belmonte et al., 1993): first, the embryos were washed in absolute methanol for 10 minutes, followed by a wash in propan-2-ol, 5 minutes. The embryos were then transferred to 1:1 tetrahydronaphthalene/wax, for 1 hour at 60^{o}C and finally immersed in wax, 3 times, for 30 minutes each, at 60^{o}C. Embryos were poured into molds, immersed in wax and allowed to set at room temperature. The blocks were wrapped in foil, stored at room temperature until sectioned. Sections (10 μm thick) were cut on a MICROM GmbH (Type HM315) microtome, and mounted onto SuperFrost®Plus microscope slides. Sections were dried overnight at 37^{o}C and the day after, rinsed in Histological-Clearing Agent, twice for 30 minutes each. Finally the sections were mounted in Canada balsam under a coverslip.

2.10 Photography of chick embryos

Embryos were photographed as whole mounts in PTW under microscope using a ZEISS AxioCam HRc camera and Zeiss Axiovision Application software. Either a zoom dissection microscope

with epifluorescence attachment (Olympus SZH10) or a compound (Olympus Vanox-T) microscope with bright field, epifluorescence and Differential Interface Contrast (Nomarski DIC) optics were used.

2.11 Time-lapse imaging of ectopic somite formation

Movies were made using an inverted microscope (Olympus IX71 with a Lumen-200 halogen fibre optic epifluorescence light source), acquiring images with a Hamamatsu C4742-96 monochrome camera at 1344 x 1024 pixels) using Simple-PCI software (Hamamatsu, supplied by Digital Pixel Inc.), which also controlled focus and a Prior filter wheel with Chroma fluorescence filters via a Prior Optiscan-2 interface. Frames were collected in bright field and fluorescence every 10 minutes.

2.12 Analysis of the molecular clock in the posterior primitive streak (PPS) explant treated with Noggin protein prior to ectopic somite formation – time-course experiment

To analyze if a molecular clock was present in the cells that form the ectopic somites, a time-course experiment was performed. The time-course included 6 time-points separated by 45 minutes intervals,

between 3 and 7.5 hours following treatment of the PPS explant with Noggin protein. The embryos were cultured for 3h, 4.5h, 5.15h, 6.45h or 7.5h and after each time point were fixed and analyzed by *in situ hybridization* for expression of *Hairy1*, *Hairy2* and *LFng.*

For each time point, 15-20 explants were analyzed.

2.13 Calculation of somite volumes

Somite volumes were calculated according to the equation of the volume of a sphere:

$V=4/3\ \pi\ r^3$

$r=1/2\ D$

V, volume; r, radius; D, diameter.

Measurements of the diameter were done in live HH st.12/13 host chick embryos for the epithelial endogenous somites SI, SII, SIII, SIV (8 embryos, n=24 epithelial endogenous somites) and on the experimental somites from the respective “bunches of grapes” (8 “bunches of grapes”, n=59 experimental somites), using a Widefield microscope with an Eyepiece Graticule.

Chapter Three: Optimization of an *ex vivo* method for Ectopic Somite Formation

3.1: Introduction

Two main reasons contribute for the attractiveness of somite formation as an experimental target: the first is the predictable regularity of their formation (timing) and the second is the relative consistency of size and cell number of each newly formed somite (the segmental pattern). In the normal embryo the timing of somite formation and the specification of somite size always go hand in hand, making it tempting to suggest that both aspects are controlled by the same mechanism. Expression of a number of genes (mainly related to the Notch signaling pathway) cycles at the same rhythm as somite formation in the pre-somitic mesoderm cells, and it is generally assumed that this "segmentation clock" regulates the timing of somite formation and the size of somites (Aulehla and Pourquie, 2008; Benazeraf and Pourquie, 2013; Cooke and Zeeman, 1976; Dequeant et al., 2006; Murray et al., 2011; Pourquie, 2003; Santillan and Mackey, 2008; Schroter and Oates, 2010). To test this assumption directly it is first important to establish an experimental model that might distinguish these two aspects of somite formation to

address the roles that the “segmentation clock” might have, and so far this has not been possible.

It has been reported that it is possible to obtain ectopic somites of fairly normal size, which express somite markers, if posterior primitive streak (which normally gives rise to lateral plate rather than somites) is treated with the BMP-inhibitor Noggin (Streit and Stern, 1999). Interestingly, these ectopic somites do not form linearly but rather in a three-dimensional arrangement resembling a “bunch of grapes” and appear not to arise by a sequential process (Streit and Stern, 1999). Therefore, this paradigm suggests that it might be possible to experimentally to separate the timing of somite formation from segmentation itself and presents an opportunity to investigate the role of the “segmentation clock” on somite patterning. In this study this approach is used as an experimental paradigm to study somite formation. This chapter describes the optimization of an experimental method to obtain ectopic somite formation reliably enough to study timing and other aspects of their formation. Before any important questions can be addressed the most efficient protocol for deriving ectopic somites has to be established, firstly, to validate the assay and secondly, to generate sufficient material to address these questions.

3.2: Methods

Host chick embryos

HH-st.5 host embryos were explanted and prepared for the transplant experiment in modified New culture (New, 1955; Stern and Ireland, 1981) (see Chapter Two: 2.2.1 Modified New culture of young avian embryos).

Posterior primitive streak (PPS) explants

Quail or GFP-transgenic chick HH-st.5 donor embryos were harvested through a window made with scissors in the blunt end of the egg. Four cuts were made in the vitelline membrane around the embryo and it was transferred using a spoon to a Petri dish containing Pannett-Compton saline. After removing excess yolk, the embryo was separated from the vitelline membrane using fine forceps and the region of the primitive streak corresponding to its most posterior 1/3 (prospective lateral/extraembryonic mesoderm, non-somitogenic tissue) was excised using fine (21G) hypodermic needles, picked up in a P20 Gilson pipette and the explant was placed in a drop of saline solution until transplantation. The selection of the region of the streak for transplant was made according to the fate map of Psychoyos and Stern (1996).

Original method for ectopic somite formation *de novo* from posterior primitive streak (non-somitogenic tissue) treated with Noggin producing cells (described by Streit and Stern (1999)

Noggin protein secreting cells and Noggin transfected cells

CHOB3.A4 cell culture

CHOB3.A4 is a subclone from a stable CHO cell line engineered to express *Xenopus* Noggin protein, described in Lamb et al., 1993 (kindly provided by Dr. Richard Harland, UC Berkley). Cells were grown in α-MEM medium without nucleotides (Gibco 320-2561AJ), 5% dialysed fetal bovine serum, 80 μM Methotrexate, 1% Sodium Pyruvate (1mM final) and 1% non-essential amino acid solution (0.1 mM final). CHOB3.A4 cells were usually passaged at 1:3 dilution (4 days to confluence). Culture supernatant from the CHOB3.A4 cells was prepared by 3 days culture of near confluent cells in low-serum medium, without the methotrexate which is normally included to maintain these cells. CHO cells do not spontaneously form aggregates in hanging drop culture. To produce aggregates suitable for grafting, the suspension of cells (approximately 3 x 10^6) was centrifuged in an Eppendorf tube (2 minutes, 1500 rpm, 10^{o}C). The medium was removed with a Pasteur pipette and the resulting pellet was re-suspended in 1 ml of Tyrode's solution (with Ca/ Mg). The

suspension of cells was centrifuged again (2 minutes, 1500 rpm at 10^{o}C). The Tyrode's solution was removed and the pellet was re-suspended in 1 ml of 2x Tyrode's solution and finally centrifuged for 10 minutes at 2100 rpm. The resulting pellet was loosened with a mounted insect steel needle run carefully around the edge of pellet and removed with a Gilson micropipette. It was then cut into suitably sized pieces for grafting using fine steel needles.

COS 1 cell transfection

COS 1 cells were grown in DMEM medium [containing 10% new calf serum (CS, Hyclone) and L- glutamine (Gibco-InVitrogen), dilution 1:100]. COS cells were usually passaged at a 1:10 dilution (3 days to confluency) and seeded when they were about 80-90% confluent. For transfection with Lipofectamine PlusTM the cells were seeded at 1.5 x 10^5 cells/ 35mm dish (8 cm^2), the day before transfection. The COS cells were then transfected with 1µg of pcDNA3-Noggin along with 4µl of LipofectamineTM reagent (InVitrogen) + 6µl of PlusTM Reagent (InVitrogen), for 4-6 hours, according to the manufacturer's instructions (see Table 3.1 for efficiency of transfections). The Lipofectamine suspension was then exchanged for DMEM + 10% CS and the COS cells were cultured for a further 24 hours. Cells were then harvested from culture, diluted at a concentration of 10^5 cells/ml

after which pellets containing 2000 cells were generated by setting up hanging drop cultures. The cell aggregates were used for transplantation into embryos 48h after transfection. In parallel with transfected COS cells, mock-transfected cells were used as a control.

Transplant and Noggin protein administration

The PPS explant (see above) was transplanted into a chick host embryo in modified *New* culture (see above), against the extraembryonic epiblast in the anterior-lateral area opaca, as described previously for neural induction assays (Storey et al., 1992; Streit and Stern, 1999). Pellets of Noggin-transfected COS cells/ control mock-transfected COS cells (2000 cells per pellet; see above), or concentrated cell aggregates of Noggin-secreting CHO cells (B3.A4)/ control CHO cells (see above), were then locally applied with a micropipette above the PPS explant. The excess saline was drained off and the transplanted host embryo was finally transferred onto a bed of thin egg albumin in a Petri dish (35 mm in diameter), covered with the lid and incubated at 38°C for different periods, ranging from 3 to 24h, according to the experiment.

Revised method for ectopic somite formation *de novo* from posterior primitive streak (non-somitogenic tissue) treated with Noggin protein

Posterior primitive streak (PPS) explants

To ensure that the experimentally produced somites arise from cells that would normally not contributed to this tissue, in all transplant experiments only the most posterior 1/4 portion of the streak from HH-st.5 donor embryos was used because according to the fate map of Psychoyos and Stern (1996) that region is fated to become extraembryonic mesoderm and does not contain prospective somite tissue.

Pre-incubation of PPS grafts in Noggin protein

An explant of the most posterior 1/4 of the primitive streak was excised from the donor embryo, picked up in a P20 Gilson pipette and immediately placed into a drop of Noggin protein solution (R&D Systems, catalog number: 6057-NG) at 1.5 µg/ml in PBS containing 0.1% BSA. It was incubated in a covered Petri-dish, on ice for 3 hours.

Noggin protein beads

Heparin-coated acrylic beads (selected to be approximately 75 μm in diameter; Sigma) were rinsed in PBS twice and loaded by incubation with Recombinant Human Noggin protein (R&D Systems, catalog number: 6057-NG) at a load concentration of 1.5 (or 2.5) μg/ml in PBS containing 0.1% BSA, for at least two hours on ice or overnight in the fridge. Once beads had been saturated with Noggin protein they were rinsed briefly in PBS prior implantation into the host chick embryo. Heparin-coated acrylic beads simply rinsed in PBS were used as controls.

Transplant and Noggin beads implantation

The PPS explant (see above) was transplanted into a chick host embryo in modified New culture, placed inside a small pocket made in the extraembryonic endoderm (germ wall) of the anterior-lateral area opaca. Then, 8-10 heparin-coated acrylic beads soaked in Noggin protein were locally applied with a micropipette positioned inside the pocket and surrounding the explant. This was to ensure that all the cells of the PPS explant were treated with Noggin protein as evenly as possible. The excess saline was carefully drained off to avoid disturbing the explant/ beads and the transplanted host embryo was finally transferred onto a bed of thin egg albumin in a Petri dish

(35 mm in diameter), covered with the lid and incubated at 38°C for the desired period of time, ranging from 3 to 24h, according to the experiment.

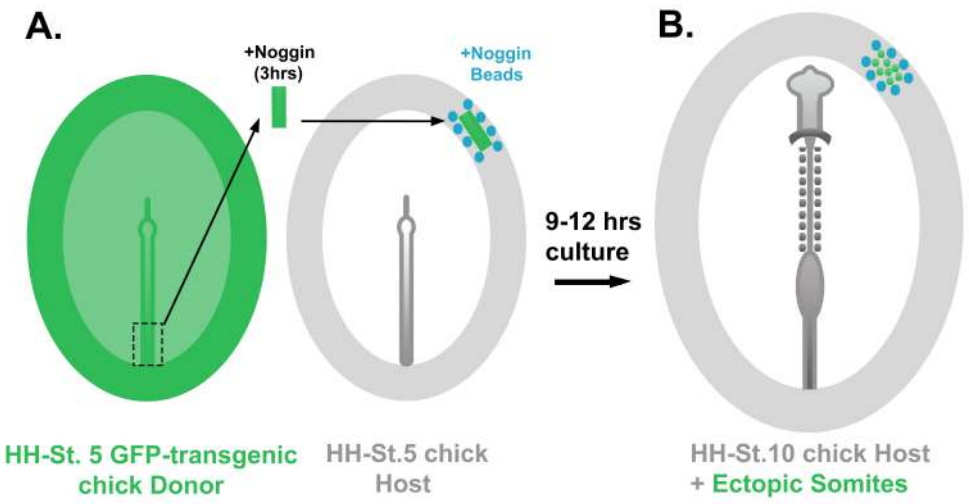


Figure 3.1. Experimental design for ectopic somite formation.

The diagram shows **(A)** the region of the donor GFP-transgenic chick primitive streak (dashed box) corresponding to its most posterior third selected to transplant. The posterior primitive streak explant (green) was incubated in Noggin solution (1.5 µg/ml) for 3 hours and then transplanted together with 8-10 Noggin beads (blue dots) into the extraembryonic area opaca of an HH-st.5 chick host embryo **(B)**. After 9-12 hours of incubation in modified New culture, the PPS explant gives rise to ectopic somites arranged as a “bunch of grapes”, independently from the main axis.
Abbreviations: PPS= Posterior primitive streak

3.3: Results

3.3.1: Original method for ectopic somite formation

As a starting point, the original method for ectopic somite formation was used. This method involved treating the posterior primitive streak (PPS) explant with Noggin-secreting CHOB3.A4 cells (Lamb et al., 1993) or Noggin-transfected COS-1 cells (Streit and Stern 1999). The percentage of host embryos forming ectopic somites was determined (see Table 3.2).

After numerous attempts (more than 150 transplants), the treatment of PPS explants with CHOB3.A4 Noggin-secreting cells never resulted in ectopic somite formation (see Table 3.2) perhaps due to the fact that the cell line used had been passaged many times and could have lost the ability to secrete Noggin (nevertheless, this method of Noggin administration is widely used in literature (Lamb et al., 1993) suggesting that in optimum conditions can be reliable). In contrast, treatment of PPS explants with Noggin-transfected COS-1 cells did result in ectopic somite formation as previously shown (Streit and Stern, 1999), but only 10% (20/200) of the cases generated ectopic somites. In an attempt to increase the percentage of embryos forming ectopic somites, the transfection method was

optimized (see Table 3.1) and the efficiency of COS-1 cells transfection was estimated for different concentrations of vector and duration of the Lipofectamin treatment (see Table 3.1). The maximum transfection efficiency obtained was 12%, which could explain why ectopic somites were generated in only 10% of the embryos.

Percentage of COS-1-transfected cells			
1 µg Vector			
4 hours in lipofectamin	5 hours in lipofectamin	6 hours in lipofectamin	4 hours + o/n inc
9.2%	12%	4.2%	9.2%
2 µg Vector			
4%	5%	2%	2%
3 µg Vector			
7%	10%	2%	2%

Table 3.1. Transfection efficiency of COS-1 cells.

COS-1 cells were transfected using 1 µg, 2 µg or 3 µg of pcDNA3-GFP expression vector and a 4:1, 4:2 or 4:3 ratio of Lipofectamine Plus reagent to DNA. The Lipofectamine mixture was applied to the seeded COS-1 cells in 35 mm dishes and left for 4h, 5h or 6h after which the Lipofectamine suspension was exchanged for DMEM+10%CS (or left overnight with DMEM+10%CS). Twenty-four hours post-transfection cells were examined using fluorescence microscopy to determine the transfection efficiency.

3.3.2: Optimized method for ectopic somite formation

To maximize the percentage of transplanted host embryos forming ectopic somites, a different experimental design was tested. This involved the pre-incubation of the posterior primitive streak (PPS) explant in Noggin protein solution (prior to transplantation) and further implantation of heparin-coated acrylic beads soaked in Noggin protein solution evenly around the explant on the host embryo (see Figure 3.1). This approach ensured a treatment of the PPS graft with an even and constant concentration of Noggin protein. The calibration of the Noggin concentration used for pre-treatments and for incubating the beads is presented on Table 3.3. PPS explants pre-incubated in 1.5 μg/ml Noggin protein solution and transplanted surrounded by 8-10 heparin acrylic beads soaked in the Noggin protein solution at the same concentration, resulted in ectopic somite formation in 66% of the cases, after 9 hours of incubation (Figure 3.2; see Table 3.2 for a comparison with the other methods).

Time Post Transplantation (hrs)	Noggin administration	Number of transplanted embryos	Number of embryos with ectopic somites	Success rate (%)
9	CHOB3.A4 Noggin-secreting cells	150	0	0
9	Noggin-transfected COS-1 cells	200	20	10
9	Noggin-beads (2.5 µg/ml)	90	60	66
9	Noggin-beads (1.5 µg/ml)	90	50	55
9	Noggin-beads (1.5µg/ml +PPS explant soaked in 1.5 µg/ml Noggin)	90	60	66

Table 3.2. Comparison of the percentage of transplanted host embryos forming ectopic somites after each different method of Noggin administration on PPS explants.

2.5 µg/ml Noggin Treatment					
Hours post transplant	Total n	Number of embryos with ectopic somites	Total	Bunch of grapes	Average number of ectopic somites (St Dev)
5	30	0	0	0	0
9	90	60	67%	17%	7.6 (1.5)
12	75	33	44%	39%	7.5 (1.6)
15	68	38	56%	53%	8.2 (1.9)
1.5 µg/ml Noggin Treatment + PPS explant soaked in Noggin solution for 3 hrs prior to transplant					
Hours post transplant	Total n	Number of embryos with ectopic somites	Total	Bunch of grapes	Average number of ectopic somites (St Dev)
5	10	0	0	0	0
9	30	20	66%	72%	8.5 (2.3)
12	25	12	50%	75%	8.5 (2.2)
15	16	10	63%	81%	9 (2.3)

Table 3.3. Calibration of Noggin concentration.

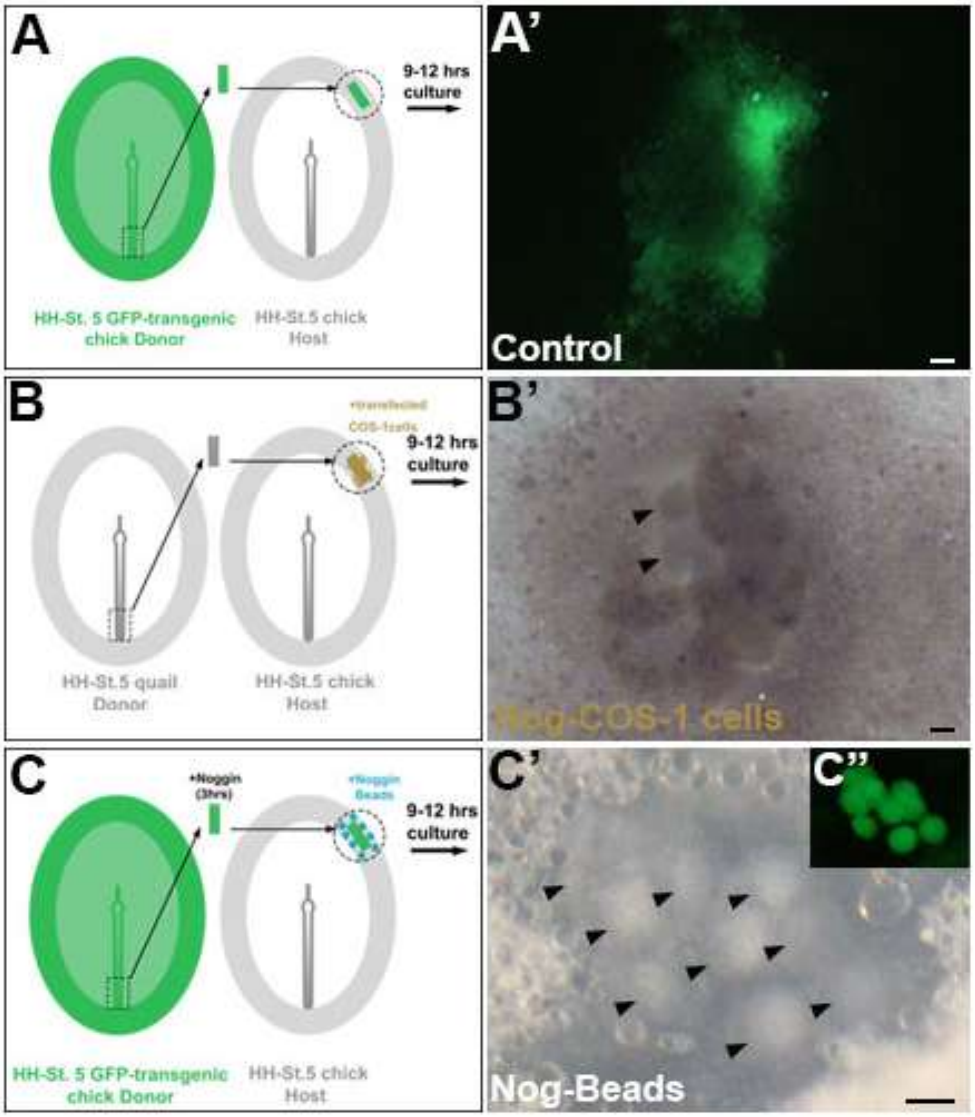


Figure 3.2. Posterior primitive streak explants (non-somite tissue) treated with Noggin protein and cultured for 9-12h

Figure 3.2. Posterior primitive streak explants (non-somite tissue) treated with Noggin protein and cultured for 9-12h.

A. Control explant.
The region corresponding to the most posterior third of the primitive streak (PPS) was explanted from a GFP transgenic chick HH-st.5 donor embryo then cultured in the extraembryonic area opaca of a chick host embryo for 9 hours. (**A')** Anti-GFP antibody staining reveals that in the absence of any treatment the GFP-positive explant cells spread as a sheet on the area opaca of the host chick embryo.
B. PPS explant cultured with Noggin-transfected COS-1 cells.
PPS explant cultured into the extraembryonic area opaca of a chick host embryo together with a pellet of Noggin-transfected COS-1 cells for 9 hours. **(B')** The explant gives rise to ectopic somites (arrows) (partially covered by the pellet of COS-1 cells).
C. PPS explant cultured with Noggin-beads.
PPS explant from a GFP-transgenic chick donor embryo cultured together with Noggin-beads (1.5 µg/ml). The region corresponding to the most posterior third of the primitive streak was explanted from a GFP-transgenic chick HH-st.5 donor embryo then cultured for 12 hours, after which a "bunch of grapes" of ectopic somites formed. The ectopic somites are entirely derived from the GFP positive explant cells, as shown by anti-GFP staining (**C"**). Black arrowheads point to each one of the experimental somites of the "bunch of grapes" (**C'**). Scale bar: 80 µm.

3.4: Discussion

The finding that posterior primitive streak (PPS) explants (whose fate is to contribute to the lateral plate in embryonic and extraembryonic regions) transplanted together with Noggin producing cells generate somites independently from the main axis (Streit and Stern, 1999), is consistent with the known effect of BMP inhibition by Noggin, which is sufficient to convert lateral/ extraembryonic mesoderm into somite fate (Streit and Stern, 1999; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998). What is striking about this finding is that the ectopic somites that form after this manipulation are not linearly arranged, do not form in a pair-wise orderly sequence as do endogenous somites. Providing that they have characteristics of real somites, this experimental model offers the unique chance to separate experimentally the timing of somite formation from the segmentation itself and can allow to further understanding the role of the “segmentation clock” genes during somitogenesis.

Firstly, I repeated the original method used to generate ectopic somites (Streit and Stern, 1999) and confirmed that it is possible to generate somites from explants of posterior primitive streak. However, the percentage of explants forming ectopic somites was

low, just 10% of cases. The low transfection efficiency of COS-1 cells (12%) suggests that this method of Noggin administration did not ensure a uniform and constant Noggin treatment of the PPS explant, accounting for why the majority of the explants did not generate ectopic somites. Therefore, this method was not reliable for the purpose of the current extensive study because it would require the manipulation of hundreds of chick host embryos to obtain just a few cases. Moreover, the large clumps of secreting cells required impedes observation of the somites that form beneath.

Then, an optimized method was developed for generating ectopic somites. GFP-transgenic chick donor embryos were used to obtain PPS explants, facilitating the observation of the ectopic somites generated, which were GFP-positive (Figure 3.2, C, C'). In an attempt to establish an experimental method that allowed for a high percentage of explants to form somites required for the current study, it was first important to set up a method by which the PPS explants could be treated by a constant source of high concentration of Noggin protein. Since Noggin is a heparin-binding protein that is highly conserved among vertebrates (Groppe et al., 2002; Paine-Saunders et al., 2002), recombinant human Noggin (R&D Systems) could be used to load heparin-coated acrylic beads (Sigma) at known

concentrations and these could be used to apply the protein along with the explant. Using this approach, beads were soaked in Noggin protein at a concentration of 1.5 μg/ml and implanted evenly around the PPS explant (which was also pre-incubated in the same Noggin solution prior to transplantation). There are several advantages for this method of Noggin administration using beads: the concentration of Noggin protein applied on the explant is known; the application of Noggin protein is simultaneous and even on all the cells of the explant; the visualization of the ectopic somites formed is not impaired because the beads are placed around the explant (rather than on top of the explant) and it is technically simpler because it does not involve cell culture or cell transfection methods. This method of Noggin administration was far more efficient, 66% of the hosts formed ectopic somites. The remaining host chick embryos that did not form ectopic somites appear to do so due to the culture conditions and due to technical difficulties during incubation in New culture. During the 9-12 hours culture period of the PPS explant and Noggin-beads on the area opaca of the host chick embryo, the normal development of the host involves a great extension of the embryonic rostro-caudal axis, and the movements associated with the morphogenesis of the head folds make the embryo expand medio-laterally (HH st.5 – HH st.12/14). Therefore, in some cases,

these movements interfere with the position of the PPS explant and the Noggin-beads in the area opaca and make it difficult for these to stay in their relative positions. This resulted in a shifting of the beads away from the PPS explant, thereby removing the PPS explants from an even exposure to Noggin protein, which is required for the formation of the “bunches of grapes”. It can be noted that pre-treatment of the PPS explant in Noggin protein solution increases the efficiency of ectopic somite formation but it is not absolutely required for their formation. Exposure of the PPS explants to Noggin-beads only or Noggin-transfected COS cells is sufficient to generate ectopic somites (Table 3.3, 2.5 µg/ml Noggin-beads – 53% of PPS explants form ectopic somites; Streit and Stern, 1999). Therefore, what appears to be critical is to get a high enough concentration of Noggin to inhibit BMP in the PPS explant, which at the time of explantation is expressed in the PPS (Watanabe and Le Douarin, 1996; Streit et al., 1998; Tonegawa and Takahashi, 1998; Pourquie et al. 1996). Thus, pre-treating the PPS explant in a direct source of Noggin increases the efficiency of this inhibition and enables the explants to change the fate of the cells towards a somitic fate.

In conclusion, this optimized method allows for a high percentage of host embryos forming ectopic somites and therefore is more

adequate for an extensive study of ectopic somite formation (for a summary of the experimental design used for generation of ectopic somites in the course of this work, see Figure 3.1).

Chapter Four: Characterization of experimentally generated somites

4.1: Introduction

To establish whether ectopic somites arising from posterior primitive streak (PPS) explants treated with Noggin protein, can be used to investigate aspects of somite formation and subdivision, one crucial question to address is whether these experimental somites are morphologically and functionally equivalent to normal somites.

In the normal embryo, somites bud off as epithelial spheres through a mesenchymal-to-epithelial transition (MET) of the cells at the anterior-most region of the pre-somitic mesoderm (PSM). At the anterior tip of the PSM, the cells become organized as an epithelium as the nuclei of the cells become aligned and regularly spaced in close contact (Beloussov and Naumidi ,1983; Marcelle et al., 2002; Revel et al., 1973; Trelstad et al., 1967), there are apical tight junctions between the cells close to the lumen (Bellairs et al., 1978; Bellairs and Veini, 1980; Cheney and Lash, 1984; Lipton and Jacobson, 1974; Revel et al., 1973) as well as each somite being surrounded by a basal lamina containing fibronectin and laminin

(Bellairs, 1979; Crossin et al., 1986; Duband et al., 1987; Kimura et al., 1995; Rickmann et al., 1985; Solursh et al., 1979; Tam and Trainor, 1994; Tan et al., 1987; Thiery et al., 1982). The process of epithelial somite formation is accompanied by increased cell adhesiveness (Bellairs et al., 1978; Cheney and Lash, 1984; Duband et al., 1987). Various adhesion molecules are expressed apically in the cells, including N-CAM and N-cadherin (Duband et al., 1987; Kimura et al., 1995; Tam and Trainor, 1994). Targeted mutations of the N-cadherin gene cause defects in somite formation with smaller irregular somites formed (Radice et al., 1997).

During normal development, each somite forms as an epithelial sphere of columnar cells whose apices all face a central cavity, the somitocoel, containing a loose aggregate of mesenchymal cells (Huang et al., 1996; Lash et al., 1984; Stern and Bellairs, 1984). In the chick embryo, an epithelial somite is approximately 100 μm in diameter and contains an estimated 1000-2000 cells (Ordahl and Le Douarin, 1992). Each somite is surrounded by extracellular matrix that is condensed to form a basement membrane at the external surface of the somite (Solursh et al., 1979). The extracellular matrix which connects the somite with adjacent structures contains multiple components including collagens, fibronectins, laminins and tenascins

(Bellairs, 1979; Crossin et al., 1986; Duband et al., 1987; Rickmann et al., 1985; Thiery et al., 1982). Each somite is also connected by extracellular matrix of the intersomitic cleft to its older (next more anterior) and younger (next more posterior) siblings. The bHLH-transcription factor *Paraxis* starts to be expressed in the anterior tip of the PSM and subsequently all the somites formed maintain expression of *Paraxis,* which has been identified as a key regulator of the epithelial organization in the PSM (Burgess et al., 1995; Burgess et al., 1996). In support of this, mouse mutants for *Paraxis* also fail to form epithelial somites (Burgess et al., 1996).

After their initial formation, the epithelial somites mature and several characteristic molecular and morphological changes take place. The ventral somite half undergoes epithelial-to-mesenchymal transition (EMT). Disruption of the basement membrane during EMT in this region of the somite releases the cells of the ventromedial surface of the epithelium to form the mesenchymal sclerotome (Jacob et al., 1975), to which the cells of the somitocoel also contribute (Huang et al., 1994; Huang et al., 1996). At this time, the dorsal epithelium of the somite becomes more tightly organized, increases in height and rotates laterally (Ordahl and Le Douarin, 1992). As a result, the somites become subdivided into the sclerotome (precursor to the

vertebrae) ventromedially and the dermomyotome (precursors of skeletal muscle and dermis) dorsolaterally (Keynes and Stern, 1988). Specification of these somite compartments can be assessed by expression of specific markers, such as the sclerotome/vertebral marker *Pax1* (Barnes et al., 1996) and the myotome/muscle marker *MyoD* (Cossu et al., 1996; Emerson, 1993). To determine if experimentally produced somites share the characteristics of endogenous somites, the expression of the somite marker *Paraxis* was analyzed by whole mount *in situ* hybridization, and the expression of N-cadherin and fibronectin by double immunocytochemistry. An analysis of the size and morphology of these somites was also carried out and compared to endogenous epithelial somites somites. Finally, the ability of experimental somites to generate normal somite derivatives was tested by grafting these in the place of endogenous somites, and subsequently analyzing their ability to incorporate into the axis and differentiate into dermomyotome and sclerotome, through expression of *Pax1*, and *MyoD.*

4.2: Results

4.2.1 Noggin treatment of posterior primitive streak explants generates somite-like structures of normal size and structure

After nine to twelve hours of culture of HH-st.5 posterior primitive streak explants with Noggin-beads (1.5 µg/ml), a 3-dimensional arrangement of ectopic somites emerges (with a structure reminiscent of a 'bunch of grapes') (Figure 4.1, A, D-F and H). These ectopic somites are entirely derived from the graft (GFP-positive, posterior primitive streak) as shown by anti-GFP staining (Figure 4.1, E") (n=25) and express the somite marker *Paraxis* (25/25) (Figure 4.1, E, E', G), presenting a morphology similar to that of endogenous early somites (Figure D-H). Also like normal somites, the cells making up these experimental somites are polarized with a basal outer surface surrounded by a fibronectin rich matrix (Figure 4.1, H in red) and an apical region expressing N-cadherin (5/5) (Figure 4.1, H, in green). Furthermore, the ectopic somites are of normal size compared with endogenous epithelial somites (Appendix 3, Table A3.1 and Table A3.2). Volumes were calculated based on measurements of endogenous and ectopic somite diameters from living embryos: 538323.29±209331.01 μm^3 for ectopic somites and 516783.49±221438.37 μm^3 for endogenous epithelial somites SI, SII,

SIII, SIV (t-test P=0.844 indicative of no significant difference) (Appendix 3, Graph A3.1).

4.2.2 Ectopic somites incorporate well into the axis and express sclerotome and dermomyotome markers

Single GFP-positive ectopic somites were isolated and grafted into the cervical region of the axis of normal HH-st.12 embryos in place of an endogenous somite (Figure 4.2, A). After 2-3 days of incubation *in ovo* they had incorporated into the axis (Figure 4.2, B-C) (n=26, reaching HH-st.25/27, all showing somite maturation) as shown by anti-GFP staining to reveal the cells derived from the grafted somite (Figure 4.2, B, B', B", C, C', C"). To verify the ability of the experimental somite to mature into normal somite derivatives, the expression of the sclerotome marker *Pax1* and the myotome marker *MyoD* (early myogenic marker) were assessed by in situ hybridization. The grafted ectopic somites expressed *Pax1* (Figure 4.2, E, E', E") and *MyoD* (Figure 4.2, D, D', D") similarly to the rest of the axis (8/8). Transverse sections through the grafted somite stained with anti-GFP showed overlap of the expression patterns of *Pax1* and *MyoD* with the GFP-positive cells derived from the grafted somite (Figure 4.2, D", E"). Anti-GFP staining also revealed the formation of some blood vessels (Figure 4.2, E") derived from the

grafted tissue. Although somite precursor cells do contribute to endothelium especially of the aorta as well as to intersegmental blood vessels (Beddington and Martin, 1989; Stern et al., 1988), vascular tissue is also a normal fate of the lateral plate, a normal derivative of the posterior primitive streak. Thus, ectopic somites are able to mature into characteristic derivatives of a somite, as shown by their morphology and expression of markers, but it is possible that some non-somite tissue also forms.

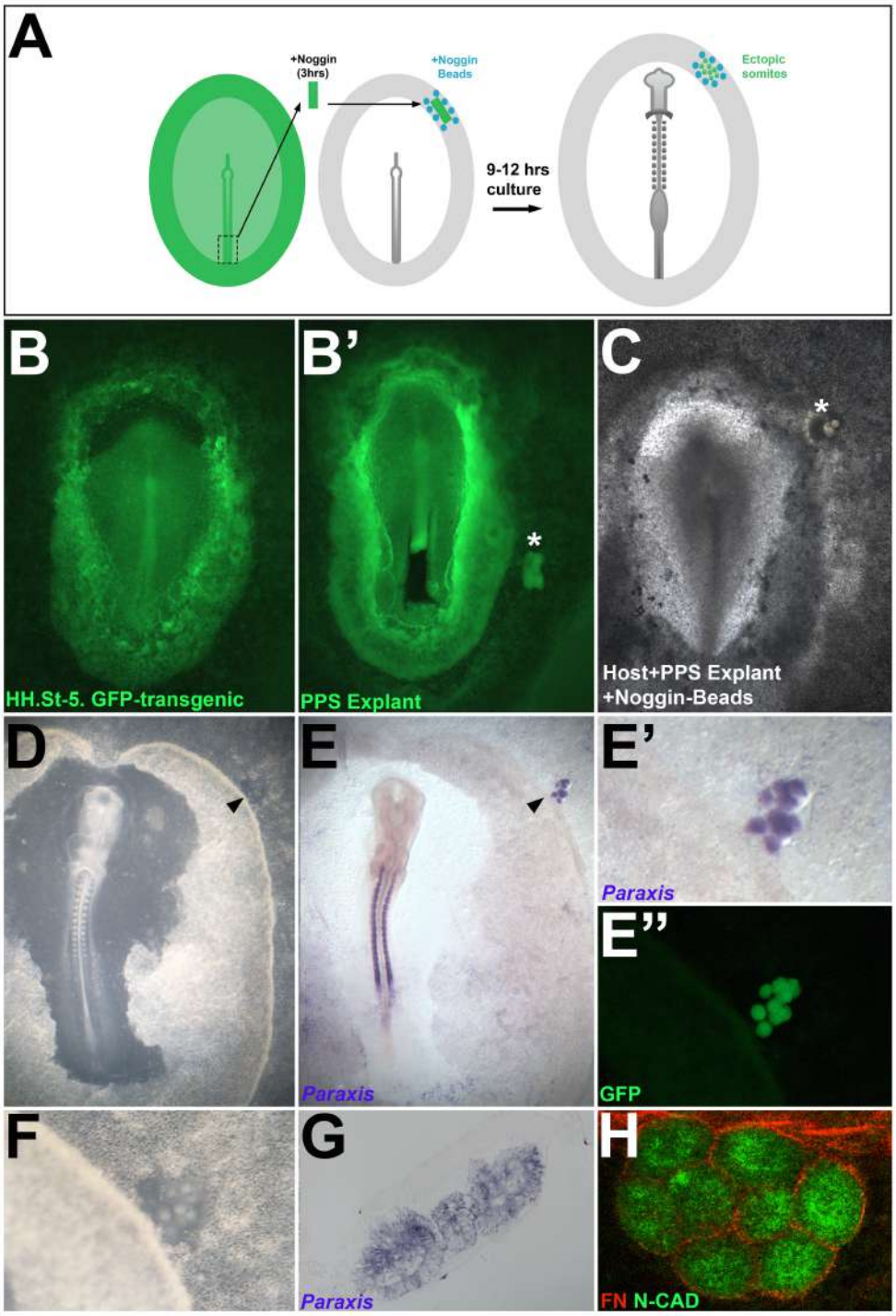


Figure 4.1. Somites formed from non-somite mesoderm treated with Noggin express *Paraxis* and N-cadherin and are surrounded by a Fibronectin matrix.

Figure 4.1. Somites formed from non-somite mesoderm treated with Noggin express *Paraxis* and N-cadherin and are surrounded by a Fibronectin matrix.

A-C. Experimental design. The posterior primitive streak explant (corresponding in size to the posterior third of the primitive streak) was excised from the HH-st.5 donor GFP-transgenic chick embryo, soaked in Noggin protein solution for 3h and transplanted into the extraembryonic area opaca of a HH-st.5 chick host embryo, surrounded evenly by 8-10 beads soaked in Noggin protein. After a further 9h-12h of incubation an arrangement of ectopic somites reminiscent of a "bunch of grapes" forms. **B.** GFP-transgenic chick donor embryo. **B'.** Posterior primitive streak explant excised to transplant (*). **C.** Transplanted HH-st.5 chick host embryo with a PPS explant surrounded by 10 Noggin-beads (*). **D, F.** After 12h incubation, the PPS explant gives rise to a "bunch of grapes" of ectopic somites which are entirely derived from the explant as shown by anti-GFP staining (n=25) **(E")**. **E, E'.** The ectopic somites express the somite marker *Paraxis* (25/25). **H.** The ectopic somites express *N-cadherin* and are surrounded by a matrix expressing Fibronectin (5/5). **G.** In histological sections, the ectopic somites have epithelial morphology.

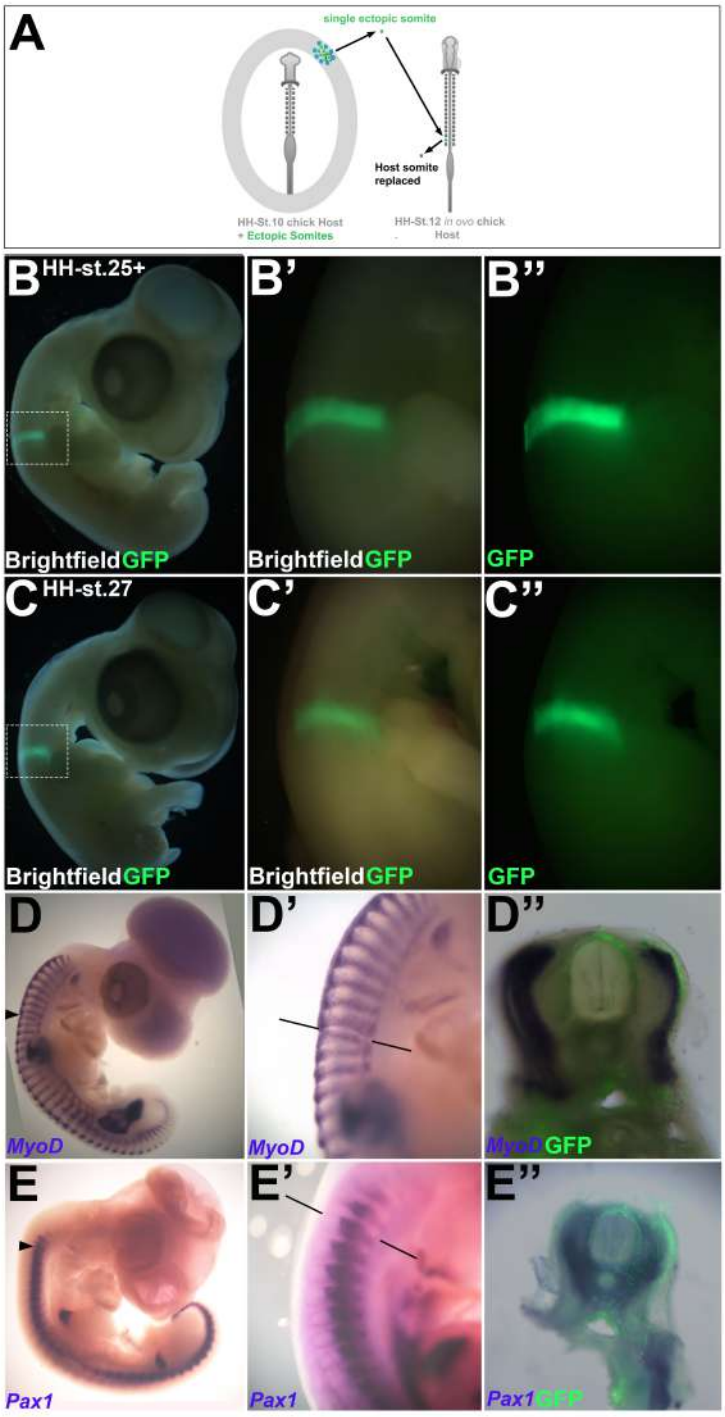


Figure 4.2. Experimentally generated somites grafted into the place of an endogenous somite express *MyoD* and *Pax1.*

Figure 4.2. Experimentally generated somites grafted into the place of an endogenous somite express *MyoD* and *Pax1* (A).

Experimental design. A GFP-positive ectopic somite was grafted in place of an endogenous epithelial somite into an HH-st.12 chick host, *in ovo.* (**B and C).** After 2 days **(B)** or 3 days **(C)** of incubation, the grafted ectopic somite incorporated well in the axis of the host chick embryo and was aligned with the endogenous somites, as shown by anti-GFP staining (n=26). (**B'** and **B")** are magnifications of the dashed white box in **B**. (**C'** and **C")** are magnifications of the dashed white box in **C**. (**D, D' and E, E').** The grafted ectopic somite matures normally and shows expression of the dermomyotome marker *MyoD* and the sclerotomal marker *Pax1*, respectively (8/8). (**D" and E")** Transverse sections cut at the level of the grafted somite. Anti-GFP staining shows that the *Pax1* and *MyoD* expression patterns are coincident with the incorporated GFP-positive ectopic somites.

4.3: Discussion

The above results suggest that non-somite mesoderm treated with Noggin generates somites that are equivalent to normal somites based on their size and morphology, expression of specific somite markers and ability to give rise to somite derivatives when placed in the right environment into the axis of a host embryo.

Six to fourteen experimental somites arranged as “a bunch of grapes” form nine to twelve hours after transplantation, entirely derived from the PPS explant treated with Noggin and appear similar to those described by Streit and Stern (1999). This analysis shows that experimental somites present a true somite morphology comprising cells forming a fully three-dimensional epithelial segment that has a basal surface on the outside and a lumen in the centre: they express N-cadherin and a fibronectin basal lamina. The measurements of somite diameters and, accordingly, the somite volumes calculated from living embryos show no significant difference in size between ectopic and endogenous somites (Appendix 3, Graph A3.1). In agreement with their morphology, ectopic somites also express the somite marker *Paraxis.*

In addition, the experimentally generated somites can give rise to normal musculoskeletal derivatives: when an individual ectopic somite is transplanted in place of an endogenous somite, it incorporates into the axis of the host and expresses the sclerotome marker *Pax1* (Barnes et al., 1996) and the dermomyotome marker *MyoD (*Cossu et al,. 1996; Emerson, 1993*)* in appropriate locations. In some cases, in addition to the late somite derivatives (sclerotome and dermomyotome), the grafted ectopic somite also contributed to the formation of some blood vessels (derived from the explant as shown by anti-GFP staining), which correspond to normal somite derivatives (Beddington and Martin, 1989; Stern et al., 1988). Alternatively, these blood vessels could also derive from some cells of the original PPS explant that failed to incorporate in the ectopic somite and, grafted together with the latter in the host, retain their original lateral plate mesoderm fate.

Taken together, these results indicate that the somite-like structures that form from PPS explant treatment with Noggin are bona fide somites. These findings support the idea that BMP inhibition by Noggin is sufficient to change the fate of prospective lateral mesoderm into somites (Streit and Stern, 1999; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998) and importantly, the resulting

ectopic somites are of normal size, morphology and able to generate normal somitic descendants.

Chapter Five: Somite formation without a clock?

5.1: Introduction

Timing of somite formation

The timing of somite formation is species specific and in the chick embryo a new pair of somites forms at an approximate rate of one pair every ninety minutes (Packard, 1976). To explicate the periodicity of somite formation, numerous theoretical models have been proposed (Aulehla and Pourquie, 2008; Bard, 1990; Benazeraf and Pourquie, 2013; Collier et al., 2000; Cooke and Zeeman, 1976; Dequeant et al., 2006; Hester et al., 2011; Kerszberg and Wolpert, 2000; Morelli et al., 2009; Murray et al., 2011; Pourquie, 2003; Primmett et al., 1989; Santillan and Mackey, 2008; Schnell and Maini, 2000; Schroter and Oates, 2010; Stern et al., 1988) from which the Clock and Wavefront model by Jonathan Cooke in collaboration with the mathematician Christopher Zeeman (Cooke and Zeeman, 1976) has received a lot of attention in the last fifty years. The Clock and Wavefront model postulated the existence of a molecular clock in the PSM and a wavefront of maturation of cells which dictates somites of correct size to form from the rostral end of the PSM (Cooke and Zeeman, 1976). This model was proposed

based on studies of the patterning process of somitogenesis in amphibian embryos (*Xenopus*) of normal and half size (embryos in which half of the cells had been removed) (Cooke, 1975) and accounted for the fact that the final number of somites formed from segmentation of the PSM was always the same irrespective of embryo size (Cooke, 1975; Cooke and Zeeman, 1976). According to this model, the size of the somites is the result of a close interaction between the cell-intrinsic 'clock' and the caudal travelling 'wave' along the axis to gate groups of cells to segment together. Somite size regulation in embryos of different sizes would be achieved by adjusting the speed of the wave (Cooke and Zeeman, 1976).

Molecular clock

The Clock and Wavefront model by Cooke and Zeeman (1976) has received further attention because of the identification of genes that are cyclically expressed in the PSM with a periodicity that generally coincides with the time necessary for one pair of somites to form, the so-called "cycling genes", mainly components of the Notch signaling pathway (General Introduction, 1.5.1.1 Cycling genes; Aulehla and Johnson, 1999; Bessho et al., 2001a; Forsberg et al., 1998; Holley et al., 2000; Jiang et al., 2000; Jouve et al., 2000; McGrew et al., 1998; Oates and Ho, 2002; Palmeirim et al., 1997; Sawada et al., 2000).

The first gene of the molecular clock to be described was the basic helix-loop-helix (bHLH) transcriptional repressor *c-hairy1* in the chick (Palmeirim et al., 1997). The periodicity of its expression coincides with the ninety minutes required for formation of one somite pair in the chick (Palmeirim et al., 1997). Its expression is highly dynamic and can be subdivided into three phases: first it is expressed in a broad domain in the caudal part of the PSM and the tail bud, next the wave of expression shifts rostrally and ceases in the caudal PSM (second phase) and finally ends in a band of expression that corresponds to the caudal half of S-1 (third phase). Every ninety minutes this expression cycle is reiterated and re-initiates from the tail bud (Palmeirim et al., 1997). Another cycling gene, *Lunatic fringe* (*Lfng*), a modulator of Notch activity and a target of Notch (Aulehla and Johnson, 1999; McGrew et al., 1998), has also been shown to have a similar cyclic expression with a ninety minutes period in the PSM, as well as c-*hairy2* (a downstream target of Notch) (Jouve et al., 2000). These cycling genes, that cycle in phase with Notch in the PSM are considered to be part of the "segmentation clock" and are thought to be components of the "clock' postulated in the "clock and wavefront" model.

Despite the identification of multiple cyclic genes and numerous

studies on the Clock and Wavefront model, since the discovery of the first gene identified in 1997 (Palmeirim et al., 1997), it is still unknown what accounts for the generation of these cell-intrinsic oscillations.

Rostrocaudal subdivision of somites

The oscillatory expression of the molecular clock genes, synchronized into travelling waves in the PSM results in the sequential production of regular stripes of gene expression along the rostrocaudal axis (McGrew et al., 1998; Palmeirim et al., 1997). The caudal level of each stripe correlates with the site of somite boundary formation (Dubrulle et al., 2001; Palmeirim et al., 1997). This molecular patterning of the PSM is thought to be the result of the interaction between the highly synchronous oscillations of genes and the rostral to caudal sequential arrest of the oscillations by a wavefront of cell maturation (Dubrulle et al., 2001; McGrew et al., 1998; Palmeirim et al., 1997). It is also thought that when cells stop oscillating they become committed to the rostral or caudal half of the somite and axial identity becomes determined (Dubrulle et al., 2001). Therefore, the segmentation clock is thought to control the timing of somite formation, the molecular patterning of the somites, as well as axial identity of the somites. The rostral-caudal polarity is an important characteristic of the newly formed epithelial somites that

will later become manifested morphologically in the rostral and caudal regions of the sclerotome (Keynes and Stern, 1984; Stern et al., 1986). The subdivision of the somite into rostral and caudal halves is also essential for imparting segmental organization to the peripheral nervous system (PNS) (Keynes and Stern, 1984; Stern et al., 1986). In the PNS, motor axons (Keynes and Stern, 1984; Rickmann et al., 1985; Stern et al., 1986) as well as neural crest cells (Bronner-Fraser et al., 1991; Rickmann et al., 1985) can only migrate through the rostral half of the sclerotome and are inhibited from entering the caudal half. The ability of rostral half-somite cells and extracellular matrix to support immigration of neural crest cells and nerves appears to be acquired prior to segmentation and is not shared by cells of the caudal half-somite (Alev et al., 2010; Keynes and Stern, 1984, 1988; Krull et al., 1997; Ranscht and Bronner-Fraser, 1991; Stern et al., 1986; Evrard et al., 1998; Wang and Anderson, 1997).

5.1.1: Investigating the timing of experimental somite formation

Since normal somites form from posterior primitive streak (PPS) explants treated with Noggin protein (Chapter Four), this raises the important question of whether they also form in sequence like

endogenous somites or with a different periodicity or simultaneously. If the periodic timing of a clock is required for the formation of somites of correct size, one would expect experimental somites to form in sequence and periodically like the endogenous somites. To investigate whether the experimental somites form sequentially and periodically or synchronously, the formation of the experimental somites generated from a GFP-transgenic (CAG-GFP chick) donor PPS explant was time-lapse imaged with an inverted fluorescence microscope.

5.1.2: Investigating the molecular clock in the posterior primitive streak (PPS) explants treated with Noggin protein, prior to somite formation

To originally reveal the molecular clock the two halves of multiple embryos containing fifteen (HH-st.12^{-}) to twenty (HH-st13^{+}) somites were analyzed by *in situ* hybridization for expression of c-*hairy1* after different incubation periods of thirty minutes to three hours (Palmeirim et al., 1997). Whereas c-*hairy1* expression was detected in the caudal half of the somites at all stages analyzed, in contrast a variety of expression patterns was observed in the PSM of the embryos with the same number of somites, revealing a highly

dynamic pattern of expression that repeated itself every ninety minutes, correlating with the formation of each new somite (Palmeirim et al., 1997). The PSM cells experience twelve cycles of expression before forming a somite (Palmeirim et al., 1997).

Therefore, to assess the molecular clock in the posterior primitive streak (PPS) explants treated with Noggin protein and investigate if the formation of somites is prefigured, or not, by periodic expression of the “cycling genes”, a time-course experiment was performed. This time-course experiment included multiple time-points separated by fourty-five minutes intervals (which corresponds to half of the wavelength of the originally described cyclic waves of expression), following treatment of the PPS explant with Noggin protein (Materials and Methods, 2.12 Analysis of the molecular clock in the posterior primitive streak (PPS) explant treated with Noggin protein prior to ectopic somite formation – time-course experiment). Embryos at these different time points prior to somite formation were analyzed by *in situ* hybridization for expression of the key “cycling genes” *Hairy1*, *Hairy2* and *LFng*. If cyclic expression of key Notch genes is present one would expect to observe strong variations of their expression patterns in PPS explants (strong expression vs. no expression) at the different time-points and between several PPS explants at the same time-point.

5.1.3 Investigating the rostrocaudal polarity of the experimental somites

Since the subdivision of the somites into rostral and caudal halves is thought to be a manifestation of the molecular clock (Takahashi et al., 2003), we investigated the rostrocaudal subdivision of the experimental somites by examining the expression of known rostral and caudal markers by *in situ* hybridization. We analyzed the expression of the caudal half markers *Hairy1* (Palmeirim et al., 1997)*, Lunatic Fringe* (Aulehla and Johnson, 1999; McGrew et al., 1998) *Uncx4.1 (*Schragle et al., 2004) and *Meso2 (*Buchberger et al., 1998*)* and the rostral half markers, *Hairy2* (Jouve et al., 2000) and *EphA4* (Schmidt et al., 2001*).* As an additional test of the somite patterning, we further analyzed the migration paths of neural crest cells and motor axons through somites (that had been grafted in place of an endogenous epithelial somite in a secondary host), which are known to normally only migrate through the rostral half of the sclerotome (Keynes and Stern, 1988).

5.2: Results

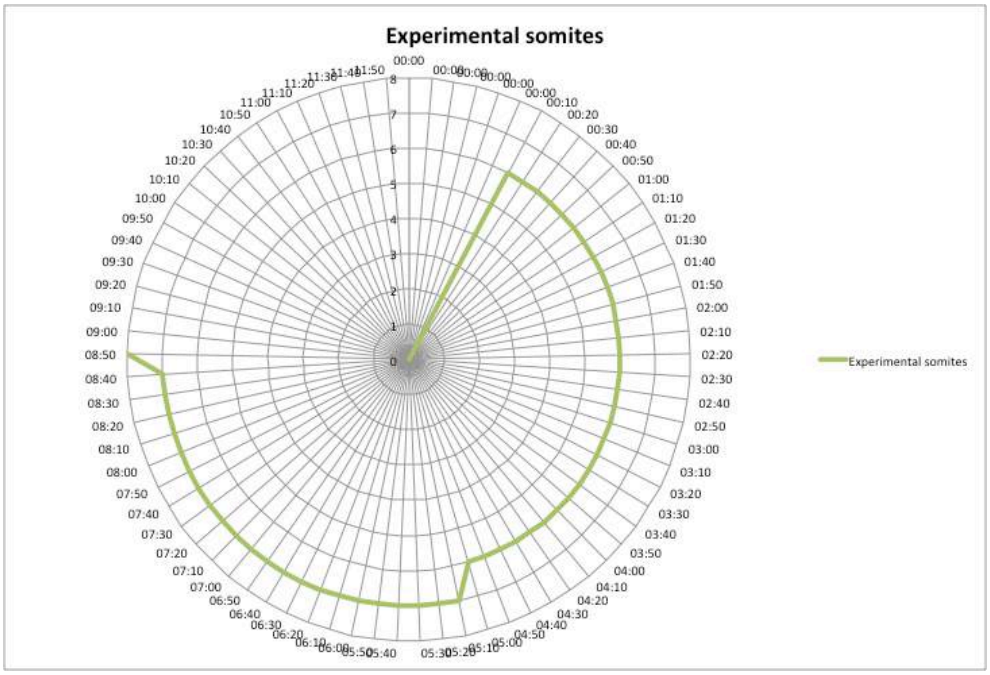
5.2.1 Timing of somite formation

Explants of posterior primitive streak (PPS) of HH-st.5 GFP-transgenic donor chick embryos soaked in Noggin protein solution for three hours and transplanted together with ten Noggin-beads, at a load concentration of 1.5 µg/ml, when cultured for nine to twelve hours generated “bunches of grapes” of 6-14 experimental somites. By filming these PPS explants (which are GFP-positive) using time-lapse fluorescence microscopy, the formation of experimental somites could be visualized and the timing of somite formation was addressed. Representative images of screenshots of a time-lapse movie capturing this process are shown in Figure 5.1. In the case presented, after ten hours of culture, seven somites formed simultaneously from the explant (Figure 5.1, D). A total number of eight somites formed from the explant within a period of two hours (Figure 5.1, D-E), between ten and twelve hours after transplant of the PPS explant with the Noggin-beads.

Additionally, to directly compare the timing of experimental and endogenous somite formation, the number of somites formed was counted at ten minutes intervals in the axis of a control chick embryo

and from a PPS explant using time-lapse imaging and the numbers plotted together in Graph 5.1. The embryos were imaged for twelve hours subsequently to the formation of the first somite, which is taken as t=0.00. The timing of experimental and endogenous somite formation is clearly different (Chart 5.1, A and B; Graph 5.1, green and grey, respectively). While the endogenous control somites form from the PSM with a sequential periodicity of approximately ninety minutes (Chart 5.1, B; Graph 5.1, grey), the experimental somite formation does not correlate with this periodicity (Chart 5.1, A; Graph 5.1, green). Experimental somites do not form sequentially and periodically but rather almost synchronously, six somites formed simultaneously, in the case presented in here (Chart 5.1, A; Graph 5.1, green).

A.


Experimental somites
Experimental somites

B.

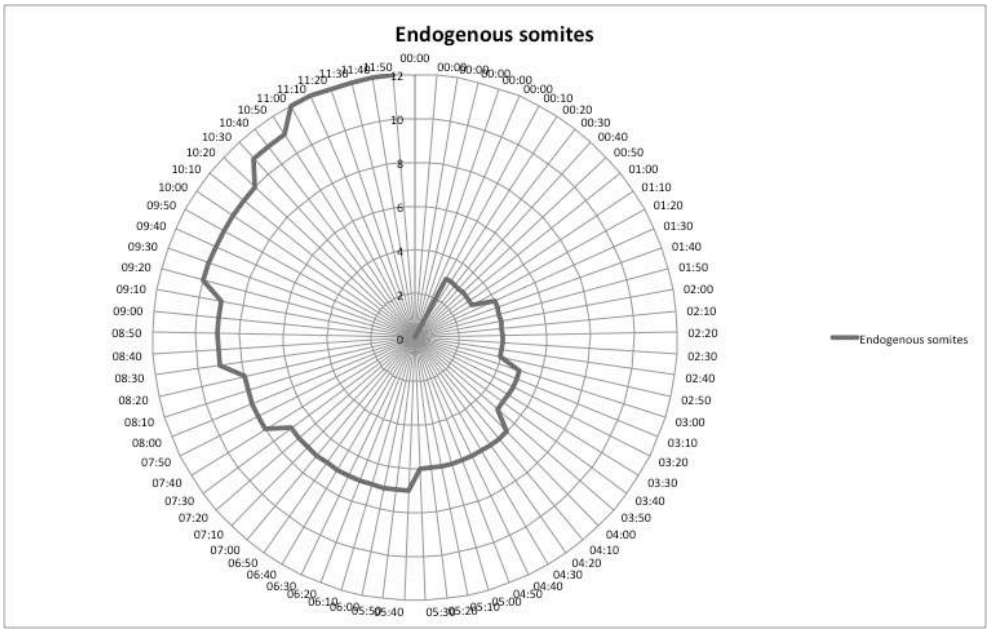
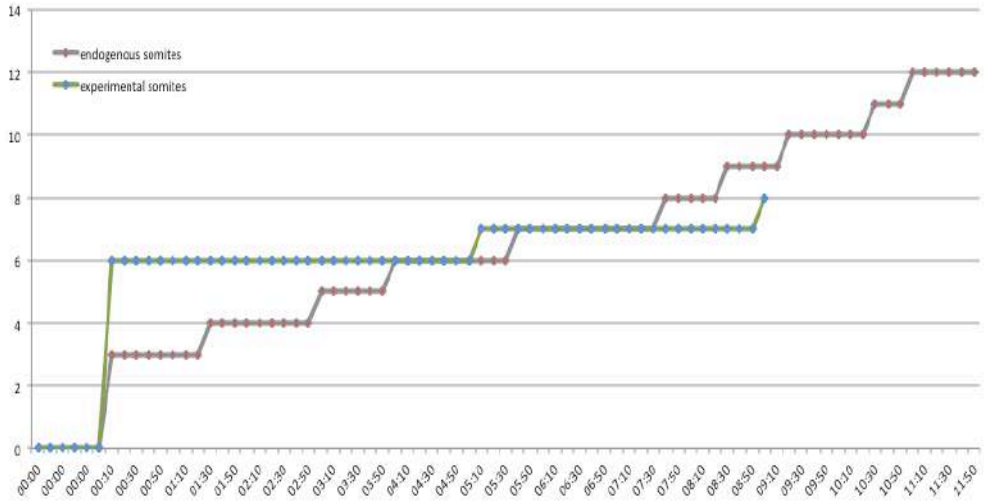

Endogenous somites
Endogenous somites

Chart 5.1.

Chart 5.1. Chart representation of the length of time required to form a new somite based on analysis of time-lapse imaging of somite formation. A. Experimental somite formation. B. Endogenous somite formation.

Time is read clockwise from t=0.00 to t=8:00 (**A**) or t=0.00 to t=12:00 (**B**), at 10 minutes intervals. Concentrical circles represent the number of somites formed ('0' in the middle). **A.** t=0.00: 6 somites form simultaneously. **B.** At t=0:00: the control embryos have 3 pairs of somites. Between t=01:10 and t=12:00 a total of 9 pairs of somites form in approximately 11 hours. The length of time required for a new somite to form is approx. 90 minutes.



Graph 5.1. Experimental vs. endogenous somite formation.

Graph 5.1. Experimental vs. endogenous somite formation.

This graph compares the number of somites formed and the timing of their formation in a control embryo (grey) and from a PPS explant (green). The somite counts were done at 10 minutes intervals, measured from time-lapse imaging of somite formation in wild-type chick embryos (grey) and PPS explants (green). T=0:00: first somites to appear. 6 somites form simultaneously from the PPS explant treated with Noggin solution for 3 hours and cultured for 9 hours.

5.2.2 Molecular clock in the experimental somites

Given that somites form almost synchronously, it suggests that experimental somites could form independently from the periodic and cyclic expression of genes of the molecular clock. To further investigate the molecular clock, the expression of the key Notch genes *Hairy1* (Palmeirim et al., 1997)*, Hairy2* (Jouve et al., 2000) and *Lunatic Fringe* (Aulehla and Johnson, 1999) was analyzed in the PPS explants prior to somite formation in a time-course, 3h (n=20), 4.15h (n=20), 5.15h (n=20), *6h (n=18), 6.15h (n=20) and 7.5h (n=20) following Noggin treatment. In this experiment, gene expression was analyzed by *in situ* hybridization and the expression of the genes in the endogenous PSM of the chick host embryo was used as a positive control for the expression in the PPS explant, although the *in situ's* were developed optimally to reveal the expression of molecular clock genes in the cells of the explant.

(*the 6h time point was done by co-workers and can be found *in* Dias et al., 2014).

In the chick host embryos there were visible variations in the pattern of gene expression in the PSM for the three markers analyzed [Figure 5.3; *Hairy1* (A-E), *Hairy2* (F-J) and *Lunatic Fringe* (K-O)]. These strong variants in gene expression correspond to characteristic phases of the molecular clock (Palmeirim et al. 1997).

Comparatively, in the PPS explants, such variations were not observed between time-points [Figure 5.3, insets in A-E (*Hairy1)*, F-J *(Hairy2)* and K-O (*Lunatic Fringe*)]. Additionally, when various embryos were analyzed at a same time-point for the expression of a single marker [Appendix 4, Figure A4.1 (3h), A4.2 (4.5h), A4.3 (5.15h), A4.4 (6.45h), A4.5 (7.5h)], the hosts accordingly showed strong variations in gene expression in the PSM between embryos but comparatively the PPS explants do not show significant differences in expression between them.

Altogether, these results suggest that somites can form non-periodically and in the absence of a ninety minutes cyclic expression of the molecular clock genes. However, the possibility that a molecular clock with a wavelength shorter than forty-five minutes could be operating in the PPS explants treated with Noggin protein prior to experimental somite formation cannot be completely excluded. Nonetheless, this possibility seems unlikely given that strong variations (strong expression vs. no expression) of the expression patterns for the different cycling genes markers in PPS explants were never observed at the different time-points and between several PPS explants at the same time point.

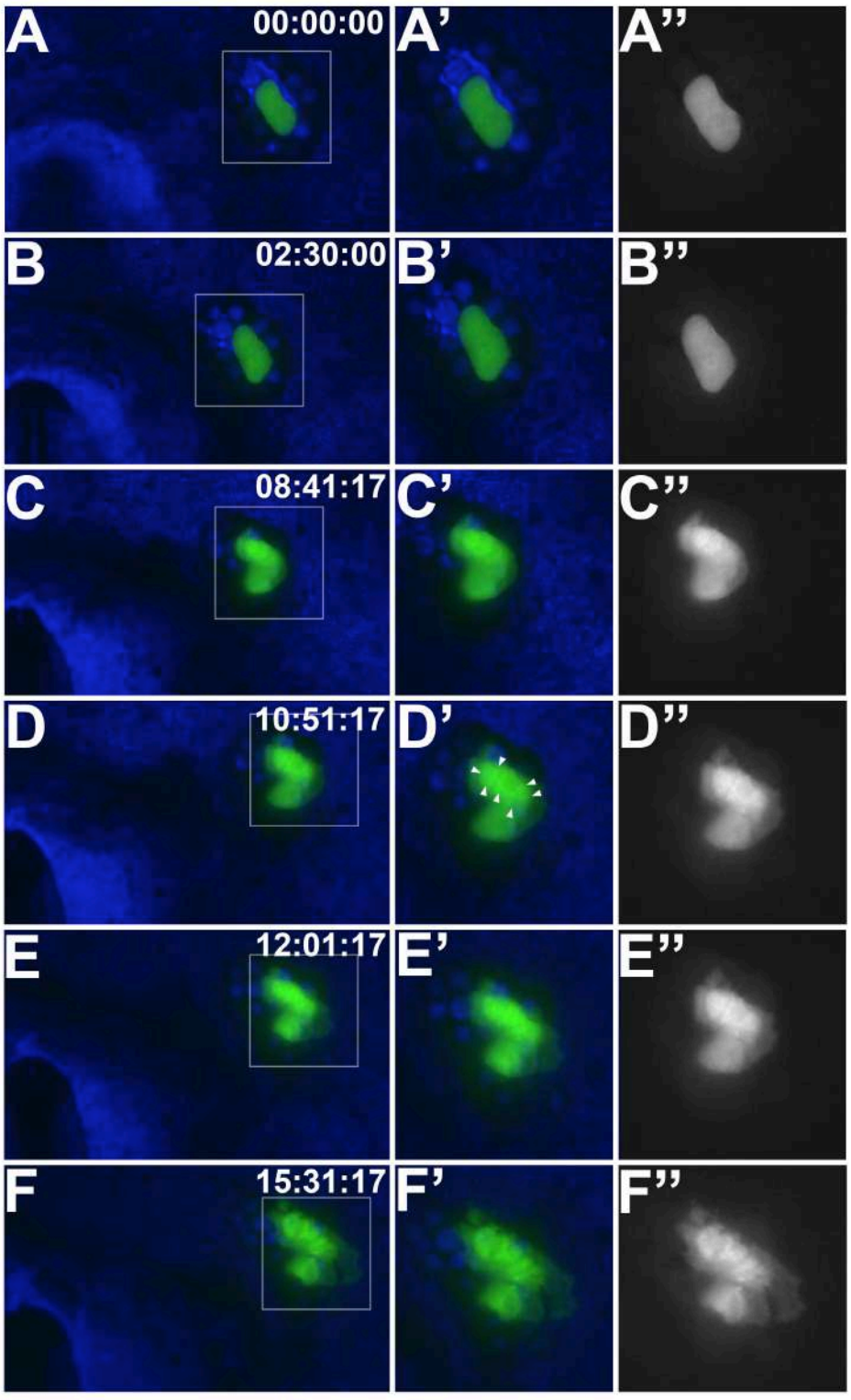


Figure 5.1. Time-lapse imaging of a PPS explant during experimental somite formation.

Figure 5.1. Time-lapse imaging of a PPS explant during experimental somite formation

(A to F) Representative images of a time-lapse movie of experimental somite formation from a GFP-positive PPS explant. A total of 7 somites form almost simultaneously between 10 and 11 hours after Noggin treatment. (**A-A")** The posterior primitive streak (PPS) explant from a HH-st.5 GFP-transgenic chick donor embryo (green), previously soaked in Noggin solution for 3 hours and transplanted into a host embryo surrounded by 10 Noggin-beads (for a detailed description of the experimental design see Chapter Three, Figure 3.1). (**B-B')** PPS explant 02:30 hours after the beginning of imaging. **(C-C')** PPS explant 08:40 hours after the beginning of imaging. No somites are observed at this point. **(D-D")** 10:51 hours after the beginning of imaging. 7 somites form simultaneously. White arrowheads indicate the position of each somite. **(E-E")** 12 hours after the beginning of imaging. 7 somites are observed. **(F-F")** 15 hours after the beginning of imaging, 8 somites are observed.

A'-F'- magnification of the white box in A-F.

A"-F"- bright field images correspondent to A'-F'.

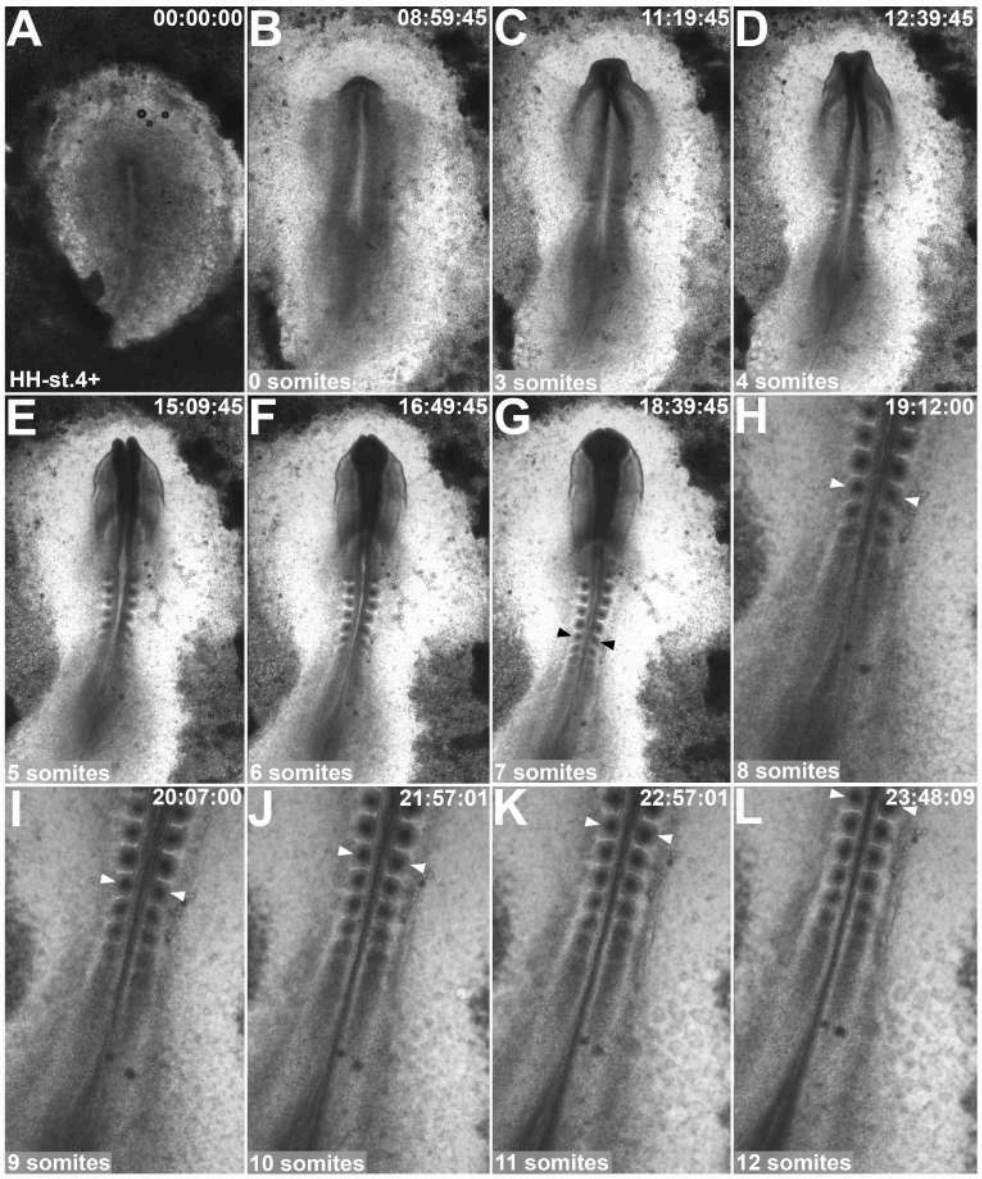


Figure 5.2. Time-lapse imaging of endogenous somite formation.

Figure 5.2. Time-lapse imaging of endogenous somite formation.

(A to L) Representative images of a time-lapse movie of endogenous somite formation. The endogenous somites form sequentially at an approximate rate of one pair every 90 minutes. **A.** The wild-type embryo at HH-st.4, at the beginning of image acquisition. **B.** The wild-type embryo at HH-st.6^{+}. No somites. Unsegmented paraxial mesoderm visible as two rods on each side of the neural plate. **C.** The first 3 pairs of somites form almost simultaneously. **D-L.** Each frame represents the formation of an extra pair of somites.

H-L. For reference, arrowheads always point to the position of the 6th somite formed.

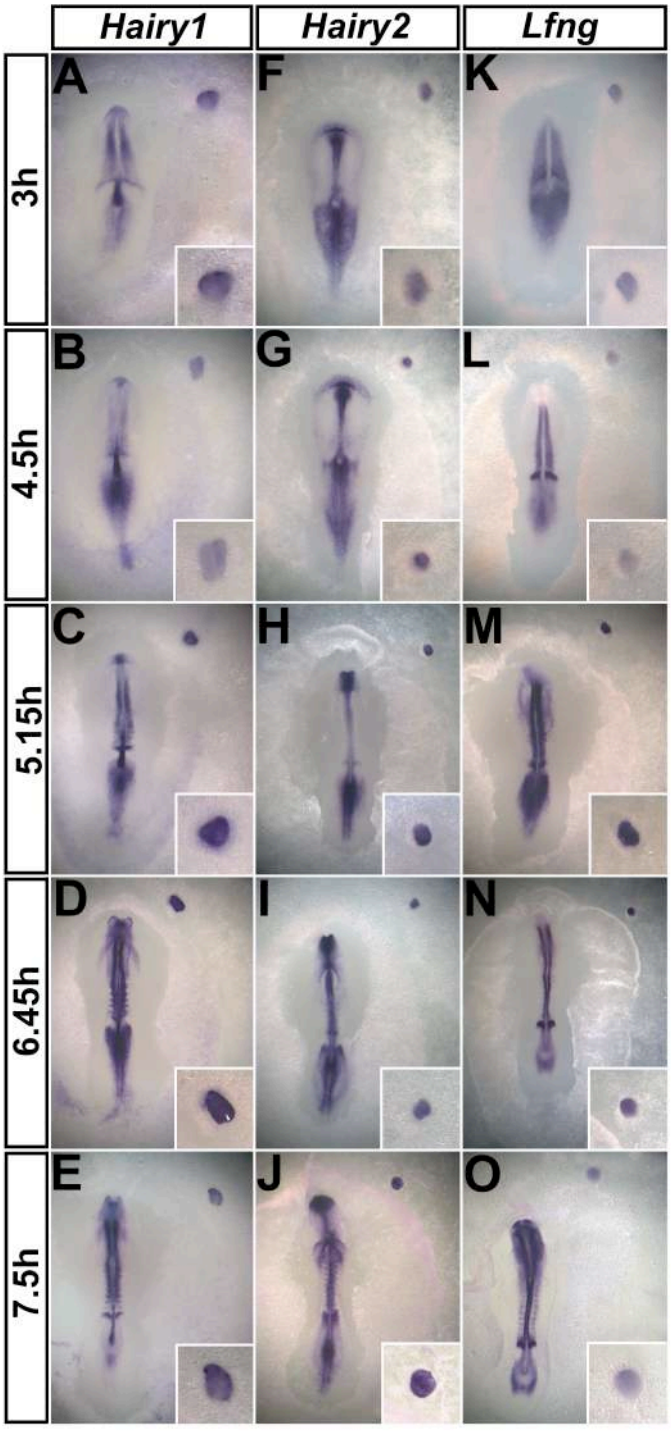


Figure 5.3. Experimental somite formation is not accompanied by cyclic expression of molecular clock key genes *Hairy1*, *Hairy2* and *Lunatic Fringe.*

Figure 5.3. Experimental somite formation is not accompanied by cyclic expression of molecular clock key genes *Hairy1*, *Hairy2* and *Lunatic Fringe.*

Experimental design: The posterior primitive streak explant was transplanted together with 8-10 Noggin-beads into the area opaca of an HH-st.5 chick host embryo and incubated for 3h, 4.5h, 5.15h, 6.45h or 7.5h. Embryos were fixed after each time point and the expression of *Hairy1* **(B-E)**, *Hairy2* **(F-I)** and *LFng* **(J-M)** was assessed by *in situ* hybridization. Patterns of expression of *Hairy1* **(B-E)**, *Hairy2* **(F-I)** and *Lfng* **(J-M)** are dynamic in the PSM region of the host chick embryo. The expression pattern of the genes is not significantly different in the PPS explant (insets).

5.2.3 Experimental somites lack coherent rostrocaudal subdivision

Newly formed somites in the axis are subdivided into molecularly distinctive rostral and caudal halves, a characteristic for which the molecular clock is thought to be required (Takahashi et al., 2003). Since experimental somites form in the absence of the cyclic expression of genes of the segmentation clock, we investigated if the somites were subdivided into normal rostral and caudal halves. The expression of known caudal half markers *Hairy1* (Palmeirim et al., 1997), *Meso2* (Buchberger et al., 1998), *LFng* (Aulehla and Johnson, 1999; McGrew et al., 1998), *Uncx4.1* (Schragle et al., 2004), and rostral half markers *Hairy2* (Jouve et al., 2000) and *EphA4* (Schmidt et al., 2001) was analyzed by *in situ* hybridization. None of these markers presented a compartmentalized rostral or caudal-restricted expression pattern: *Hairy1* (0/22), *Meso2* (0/22) and *EphA4* (0/19) were not expressed (Figure 5.4, A, B, C), *LFng* (22/24) and *Hairy2* (8/8) were weakly expressed throughout the entire somite (Figure 5.4, D and E) and *Uncx4.1* (13/19) was randomly expressed in the experimental somites (Figure 5.4, F). These expression patterns suggest that that the experimental somites are not correctly patterned into rostral and caudal halves.

The subdivision into rostral and caudal compartments of the newly formed somites, which subsequently give rise to sclerotome subdivision, is essential for segmentation of components of the peripheral nervous system (Bronner-Fraser et al., 1991; Keynes and Stern, 1984; Rickmann et al., 1985). Neural crest cells that emerge from the dorsal neural tube migrate through the rostral half of the sclerotome and not the caudal half (Bronner-Fraser et al., 1991; Rickmann et al., 1985). The neural crest derivatives, dorsal root ganglia and peripheral projections are restricted to the rostral half (Keynes and Stern 1984). Equally, motor axons enter the sclerotome only in the rostral half but avoid the caudal half (Keynes and Stern, 1984; Rickmann et al., 1985; Stern et al., 1986). We further tested the rostrocaudal subdivision of experimental somites by analyzing the migration of neural crest cells and motor axons through the somites: single GFP-positive experimental somites were isolated and grafted into the cervical region of the axis of normal HH-st.12 embryos in place of an endogenous somite and incubated for two to three days *in ovo* (Figure 5.5, A, C). At HH-st.22-25, the patterns of migration of neural crest cells and motor axons were analyzed in the experimental somites that incorporated into the axis by immunohistochemistry using antibodies directed to HNK-1 epitope (IC10) and to neurofilament-associated protein (3A10), respectively

and anti-GFP staining. The arrangement of motor nerves (Figure 5.5, C-C"; D-D") and neural crest cell migration (Figure 5.5, A-A"; B-B") were both disturbed. The disturbances included: extra motor roots within the whole grafted somite (Figure 5.5, C', C", arrowheads), the formation of multiple dorsal roots and duplication of ventral roots (Figure 5.5, D, arrowheads). Also, several smaller dorsal root ganglia formed within the whole grafted somite (Figure 5.5, A', A", arrowheads) and the ventral roots were duplicated (Figure 5.5, B, arrowheads). The anomalies suggest that the experimental somites are as a whole permissive for neural crest cells and motor nerve migration and provide additional evidence that the experimental somites are not rostro-caudally subdivided. Altogether, these results suggest that experimental somites lack coherent subdivision into rostral and caudal halves in the absence of the molecular clock, and therefore consistent with the proposal that the molecular clock is necessary for this aspect of segmental pattern (Takahashi et al., 2003).

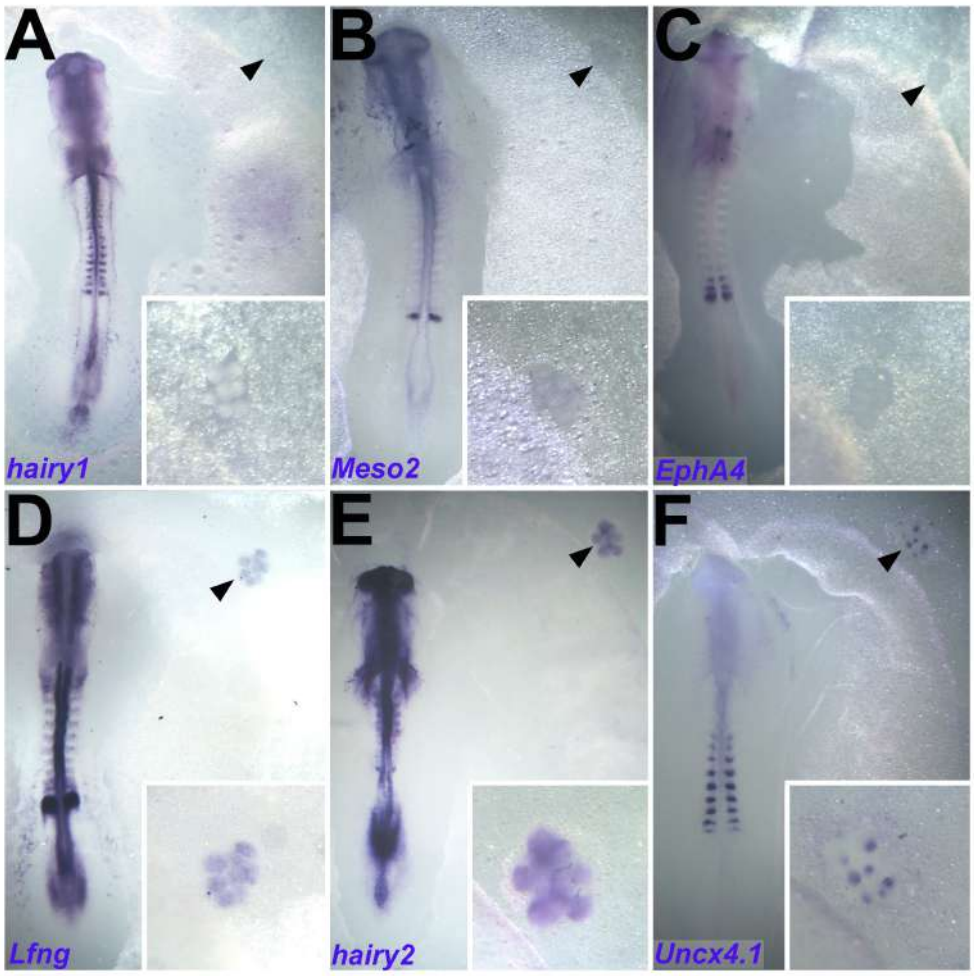


Figure 5.4. Experimental somites are not subdivided into rostral and caudal halves.

Figure 5.4. Experimental somites are not subdivided into rostral and caudal halves.

Experimental design: PPS explants were incubated in Noggin protein solution for 3h and transplanted into the area opaca of a HH-st.5 chick embryo surrounded by 10 Noggin-beads. The embryos were cultured for 9 hours, which corresponds to 12 hours of Noggin treatment. **(A-F)** Expression of caudal half markers, *Hairy1*, *Meso2*, *LFng* and *Uncx4.1*, and rostral half markers *Hairy2 EphA4*, was assessed by *in situ* hybridization. *Hairy1* (**A**), *Meso2* (**B**) and *EphA4* (**C**) are not expressed in the experimental somites. LFng (**D**) and *Hairy2* (**E**) are uniformly and weakly expressed and *Uncx4.1* (**F**) is expressed randomly.

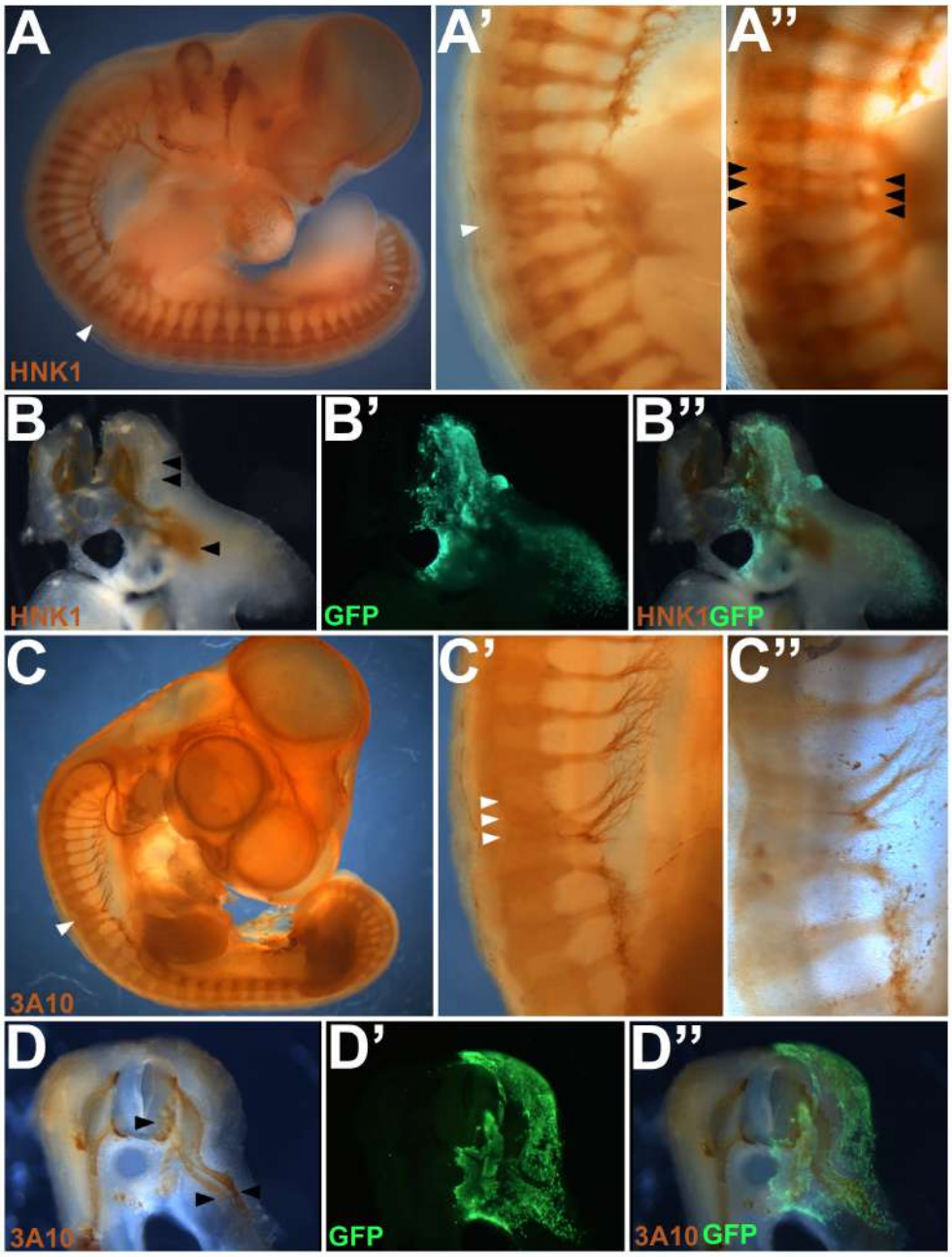


Figure 5.5. Migration paths of neural crest cells and motor axons is abnormal in experimental somites.

Figure 5.5. Migration paths of neural crest cells and motor axons is abnormal in experimental somites.

To analyze the rostrocaudal patterning of the experimental somites the migration paths of neural crest cells and motor nerves was analyzed in GFP-positive experimental somites grafted in the place of an endogenous somite and incubated for 2-3 days. The embryos were immunostained with IC10 (for HNK-1 epitope of neural crest cells), 3A10 (for neurofilament-associated protein) and anti-GFP. Several smaller dorsal root ganglia formed and the ventral roots were duplicated at the level of the grafted somite (**A-A”**, **B”**). Extra motor roots formed, multiple dorsal roots and duplication of ventral roots, at the level of the grafted somite (**C-C”**, **D”**).
Sections B and D are transverse at the level of the grafted somite.

5.3: Discussion

The formation of somites generated from HH-st.5 posterior primitive streak explants treated with Noggin occurs non-periodically and therefore, the periodicity of experimental somite formation is different from the periodicity of the endogenous somites formation (Chart 5.1, A and B; Graph 5.1, grey and green, respectively). Endogenous somites, caudal to the occipital somites, form with a periodicity of approximately one pair every ninety minutes (Figure 5.2; Chart 5.1, B; Graph 5.1, grey). Given that many experimental somites can form simultaneously (Figure 5.1; Chart 5.1, A; Graph 5.1, green), it suggests that the somites of correct size can form in the absence of the oscillatory expression of genes of the molecular clock and because somites form from PPS explant cells in the extraembryonic area opaca (independently from the PSM) they are also not exposed to a wavefront of maturation, raising the possibility that there could be an additional unknown mechanism controlling initial somite formation. To investigate the molecular clock in the PPS explant cells that generate the experimental somites, we analyzed the expression of the key molecular clock genes *Hairy1* (Palmeirim et al., 1997), *Hairy2* (Jouve et al., 2000) and *Lfng* (Aulehla and Johnson, 1999) in a time course experiment at several time-points at forty-five minutes

intervals, prior to somite formation and following treatment with Noggin protein. While the host exhibited different characteristic expression patterns representative of different phases of the molecular clock (Figure 5.3, A-O), by comparison the PPS explants presented only subtle variations in expression (Figure 5.3, insets in A-O) and not compatible (do not correlate) with the arrangement of experimental somites that later are generated from the explant. Furthermore and importantly, when several embryos were analyzed at the same time-point for the expression of any of the markers *Hairy1, Hairy2* and *LFng* (Appendix 4; Figures A4.1- 3h, A4.2- 4.5h, A4.3- 5.15h, A4.4- 6.45h, A4.5- 7.5h) the variations in expression patterns of the molecular genes were apparent in the host embryos but in the PPS explants the expression of the markers was comparatively uniform. Given that the PPS explant is first soaked in Noggin protein for two hours prior to transplant, we can not tell whether there is cyclic expression of genes during those first two hours of Noggin treatment prior to transplant into the host chick area opaca. However, the cells could only experience a maximum of two expression cycles prior to the beginning of our analysis at three hours after first exposure to Noggin, rather than the twelve cycles of cyclic expression that the cells experience in the PSM of the normal embryo before forming an endogenous somite (Palmeirim et al.,

1997). In addition, further experimental evidence provided by analysis of *Dapper1* and *Dapper2* expression (Dias et al., 2014), which are markers for PSM and formed somites, respectively (Alvares et al. 2009), are consistent with the results presented in this thesis and suggest that the formation of experimental somites is not prefigured by a "presomitic mesoderm-like" state. *Dapper1* is not expressed prior to (or after) somite formation and *Dapper2* is only expressed when the somites form (Dias et al., 2014). These results suggest that the experimental somites of normal size and morphology that form simultaneously, form independently of the "segmentation clock".

A feature of somite formation that is thought to be controlled by the segmentation clock is the patterning of the somites into rostral and caudal halves (Stern and Keynes, 1987). Since the ninety minutes molecular clock does not operate in the PPS explant cells that generate the experimental somites we investigated whether the somites presented a normal rostrocaudal patterning in the absence of such clock. We analyzed the expression of known caudal half markers and rostral half markers and as a further test for patterning we analyzed the migrations paths of neural crest cells and motor axons, which are known to migrate only through the rostral half of the

somites, in experimental somites that had been grafted in to a secondary host in place of an endogenous somite. All the rostral and caudal markers show different patterns of expression after formation of the experimental somites, *Hairy1*, *Meso2* and *EphA4* are not expressed, *Uncx4.1* is expressed randomly and *LFng* and *Hairy2* are expressed uniformly (Figures 5.4, A-F). Therefore, there is no indication of somite rotrocaudal polarity because there is no part of the somites that presents a pattern of expression coherent with the identity of a rostral or caudal half. Moreover, the boundaries between experimental somites are random since the somites form in a three-dimensional arrangement unlike in the normal embryo where boundary formation correlates with the apposition of rostral and caudal half identities (Stern and Keynes, 1987). In the experimental somites boundaries between adjacent somites are never accompanied by opposite R/C gene expression patterns. Also, there is no clear migration of neural crest cells and motor axons through a preferred half of an experimental somite grafted into a secondary host, a result which is further evidence of the lack of rostral-caudal polarity of the somites.

In summary, the synchronous formation of normal somites from PPS explants treated with Noggin in the absence of a molecular clock is

strong evidence that the initial formation of somites and size specification occurs independently of the segmentation clock. The experimental somites allow us to effectively separate the aspects of size specification from the timing of somite formation, which in the normal embryo are coincident events. Although somites of correct size form in the absence of the molecular clock, the experimental somites form simultaneously and without a normal rostrocaudal subdivision, which suggests that one of the functions of the molecular clock in the normal embryo might be to control these two aspects of somite formation, alongside the initial formation of epithelial somites. Therefore, these observations raise the possibility that in the normal embryo the principal function of the segmentation clock could be to impart rostral-caudal patterning and regulate the timing of, a currently not fully understood, self organizing program of somite formation in the PSM cells.

Chapter Six: Do ectopic somites have an axial identity?

6.1: Introduction

In the embryo the formation of the occipital somites, the most rostral four-five somites, does not follow a strict order and occurs almost simultaneously (Chapter Five, Figure 5.2; Hamburger and Hamilton, 1951; Hamilton and Hinsch, 1956; Huang et al., 1997). The first somite that forms is actually the second somite of the series, and it has clear rostral and caudal boundaries and subsequently, rostral to it, the most rostral somite of the series forms, smaller and with no defined rostral boundary, shortly followed by the formation of the third and fourth somite (Hamburger and Hamilton, 1951). The first (most rostral) somite forms caudally to the otic placode and is connected with the paraxial head mesoderm (Hamilton and Hinsch, 1956; Huang et al., 1997). Studies of fate map of avian somites have shown that the most rostral four somites and the rostral half of the fifth somite also differ from most caudal somites because they do not give rise to vertebrae but rather, contribute to the occipital bone (exo-occipital and basi-occipital) of the base of the skull and contribute to the otic (Lim et al., 1987) capsule (Couly et al., 1993). It has also

been shown that sensory ganglia do not develop in these somites (Le Douarin et al., 1992; Lim et al., 1987; Teillet et al., 1987) instead, the XII cranial nerve and Froriep's ganglion develop in these somites (Lim et al., 1987). In addition, occipital somites lack expression of some rostral/caudal markers: the first three somites never show expression of *Hairy1*, *Hairy2* or *LFng,* which are key molecular clock genes (Rodrigues et al., 2006).

Given that occipital somites form almost simultaneously in the normal embryo and show molecular differences compared with the other more caudal somites (Alev et al., 2010; Lim et al., 1987; Rodrigues et al., 2006), one might reason that the experimental somites formed from PPS explants treated with Noggin (that also form simultaneously), could have an occipital somite identity. Therefore, to investigate the axial identity of the generated somites, we examined the expression of *Hox* genes in the experimental somites (Burke et al., 1995; Gaunt, 1994). In the embryo, although the somites of different axial levels in general resemble one another, there are regional differences that constitute the specific rostral-caudal regional patterns of the vertebral column (cervical, thoracic, lumbar, sacral and caudal). These regionalizations can also be visualized by specific patterns of *Hox* gene expression (Kessel and Gruss, 1991),

which have been termed the "*Hox* code". For instance, *Hoxb-3* is expressed caudal to the caudal half of somite five with an increased level of expression between somite five and seven (Gaunt, 1994) and *Hoxb-4* is expressed caudally to somite seven (Burke et al., 1995), making these good markers of the rostral cervical somites. *Hoxb-6* is expressed caudally to somite fifteen (Gaunt and Strachan, 1996) and *Hoxb-9* is expressed caudally to somite twenty-six, at the level of the rostral lumbar region (Burke et al., 1995), therefore these genes can be used as markers of the thoracic and lumbar somites, respectively.

6.2: Results

To ask whether experimental somites, that form from PPStreated with Noggin, have an axial identity and to address the hypothesis that they could be occipital somites, we examined expression of the *Hox* genes *Hoxb-3*, *Hoxb-4*, *Hoxb-6* and *Hoxb-9* (Figure 6.1, D; H; L; P). PPS explants excised from an HH-st.5 chick donor embryo were incubated in Noggin solution (1.5 µg/ml) for three hours and then transplanted together with eight to ten Noggin beads into the extraembryonic area opaca of an HH-st.5 chick host embryo. After twelve hours of incubation in modified New culture (New, 1955; Stern

and Ireland, 1981), the PPS explant generated experimental somites arranged as a “bunch of grapes”, independently from the main axis and the expression of the *Hox* genes (above) was analyzed by whole mount *in situ* hybridization (Experimental design, Figure 6.1, A) with the expression pattern of the *Hox* genes in the host chick embryos acting as positive control for the expression in the experimental somites (Figure 6.1, D; H; L; P; insets).

Hoxb-3 and *Hoxb-4* were both expressed in the experimental somites (Figure 6.1, D; H, insets) suggesting that the experimental somites do not have an occipital identity. *Hoxb-6* and *Hoxb-9* were not expressed in the generated somites (Figure 6.1, L; P, insets), but in the embryo these are normally expressed in thoracic and lumbar somites, respectively. These results suggest that the experimental somites have an axial identity of cervical somites (somites eight to fourteen).

Experimental somites are not like occipital somites and their identity corresponds to the *Hox* genes expressed in the posterior primitive streak (PPS) at the time of transplant.

The region of PPS of HH-st5 chick donor embryos which is used to explant, also initially expresses the same complement of *Hox* genes

as observed in the experimental somites generated from it; *Hoxb-3* and *Hoxb-4* are expressed (Figure 6.1; C and G, respectively) while *Hoxb-6* and *Hoxb-9* are not expressed (Figure 6.1; K and O, respectively). We observed that the expression pattern of the *Hox* genes in the PPS of the chick embryo at the later developmental stage of HH-st.8 changes: *Hoxb-6* and *Hoxb-9* are now expressed unlike at HH-st.5 (Figure 6.1, M and Q, respectively) while *Hoxb-3* and *Hoxb-4* continue to be expressed in HH-st.8 PPS (Figure 6.1, E and I, respectively). Thus, we investigated whether experimental somites generated from PPS explants from an older donor chick embryo, at HH-st.8, would also express similar *Hox* genes to the streak – does the axial identity of the experimental somites depend on the *Hox* expression of the source donor tissue?

PPS explants excised from a HH-st.8 chick donor embryo were incubated in Noggin solution (1.5 µg/ml) for three hours and then transplanted together with eight to ten Noggin beads into the extraembryonic area opaca of an HH-st.5 chick host embryo. After twelve hours of incubation in modified New culture, the PPS explant generated experimental somites arranged as a “bunch of grapes”, independently from the main axis and the expression of the *Hox* genes was analyzed by whole mount *in situ* hybridization

(Experimental design, Figure 6.1, B). In contrast to the experimental somites generated from HH-st.5 donor tissue, the somites generated from HH-st.8 expressed *Hoxb-3*, *Hoxb-4,* as well as *Hoxb-6* and *Hoxb-9* (Figure 6.1, F; J; N; R, insets), correlating with the expression of these markers in the HH-st.8 donor streak tissue. This suggests that the axial identity of the somites that form from the posterior primitive streak is specified according to the *Hox* genes that are expressed in the streak at the time of transplant.

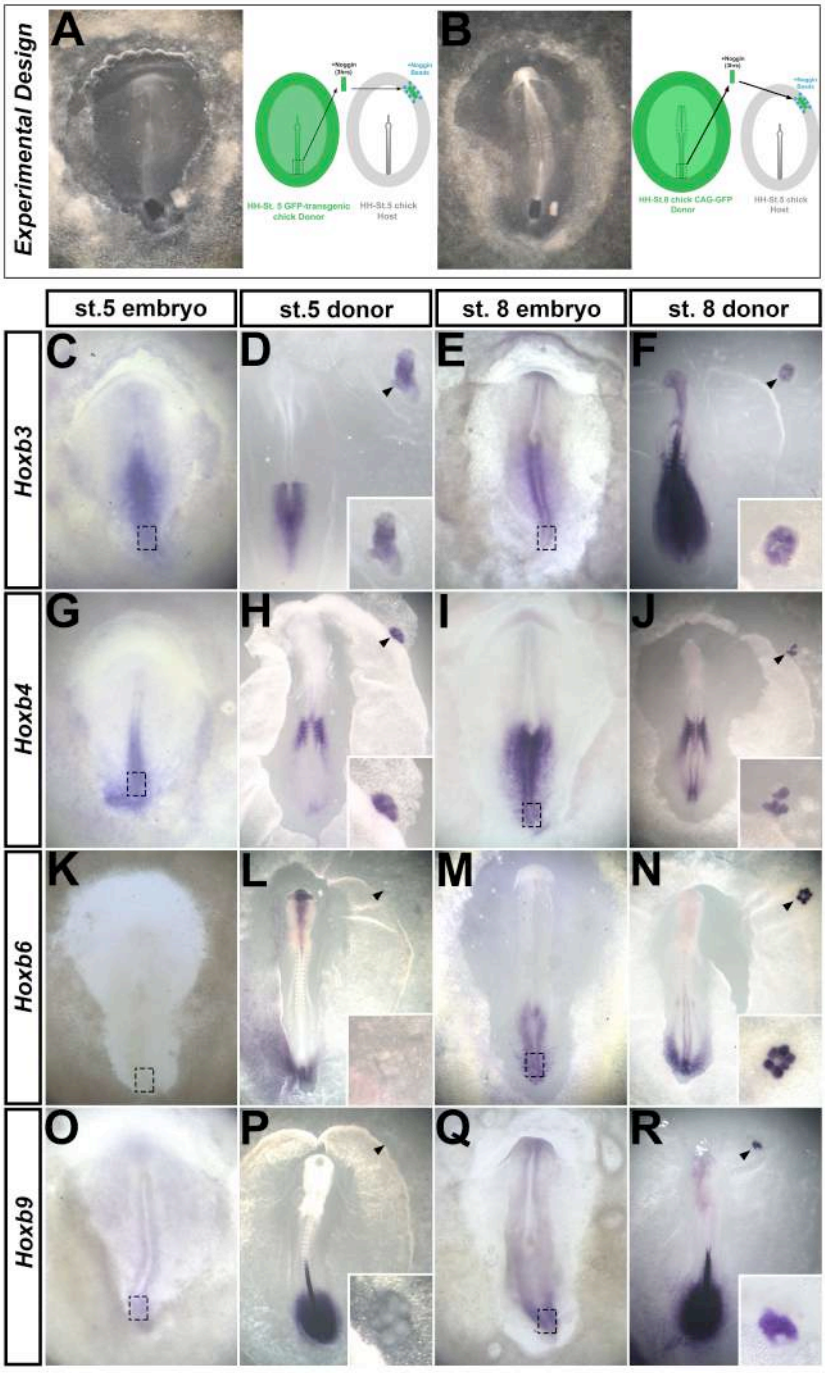


Figure 6.1. Ectopic somites have axial identity of cervical somites and express *Hox* genes as the streak at the time of transplant.

Figure 6.1. Ectopic somites have axial identity of cervical somites and express *Hox* genes as the streak at the time of transplant.

A. Experimental design. PPS explants excised from a HH-st.5 chick donor embryo were incubated in Noggin solution (1.5 µg/ml) for 3 hours, then transplanted together with 8-10 Noggin beads into the extraembryonic area opaca of an HH-st.5 chick host embryo and further incubated for 12 hours in modified New culture. **B.** Experimental design. PPS explants excised from a HH-st.8 chick donor embryo were incubated in Noggin solution (1.5 µg/ml) for 3 hours, then transplanted together with 8-10 Noggin beads into the extraembryonic area opaca of an HH-st.5 chick host embryo and further incubated for 12 hours in modified New culture. **C; G; K; O.** Expression of *Hoxb-3, Hoxb-4, Hoxb-6* and *Hoxb-9,* respectively, in the primitive streak of HH-st.5 chick donor embryo. **E; I; M; Q.** Expression of *Hoxb-3, Hoxb-4, Hox-b6* and *Hoxb-9,* respectively, in the primitive streak of HH-st.8 chick donor embryo. **D and H.** *Hoxb-3* and *Hoxb-4,* respectively, are expressed in the experimental somites (insets) generated from PPS explants from an HH-st.5 donor chick embryo (10/10). **L and P.** *Hoxb-6* and *Hoxb-9,* respectively, are not expressed in the experimental somites generated from PPS explants from an HH-st.5 donor chick embryo (8/8). **E and I.** *Hoxb-3* and *Hoxb-4,* respectively, are expressed in the experimental somites (insets) generated from PPS explants from an HH-st.8 donor chick embryo (8/8). **N and R.** *Hoxb-6* and *Hoxb-9,* respectively, are expressed in the experimental somites generated from PPS explants from an HH-st.8 donor chick embryo (4/4).

6.3: Discussion

Somites that form from PPS explants treated with Noggin protein are normal somites based on expression of marker genes, morphology and fate (Chapters Four, Five). Given that these somites also form in the absence of oscillatory expression of molecular clock genes and temporal sequence this posed the question of whether the experimental somites, which share some characteristics with the most anterior somites, could have an axial identity of occipital somites?

Occipital somites do not form in the same orderly fashion as the most posterior trunk somites (Chapter Five, Figure 5.2; Hamburger and Hamilton, 1951; Hamilton and Hinsch, 1956; Huang et al., 1997). Hence, at least for the first four to five somites in the normal embryo, somitogenesis does not obey a strict temporal order. In addition, cyclic gene expression of the molecular clock genes *Hairy1* (Palmeirim et al., 1997)*, Hairy2* (Jouve et al., 2000*)* and *LFng* (Aulehla and Johnson, 1999; McGrew et al., 1998) is not observed at the level of the occipital somites (Rodrigues et al., 2006). Therefore, these somites do not show somitic rostral/caudal polarized gene expression of these molecular clock genes and the boundaries

between them to not correspond to confrontations between rostral and caudal halves states of expression of these genes, which are not expressed at all (Rodrigues et al., 2006). Moreover, mutants of components of the Notch signaling pathway in mice (Barrantes et al., 1999; de la Pompa et al., 1997; Feller et al., 2008; Ferjentsik et al., 2009; Oka et al., 1995) and zebrafish (Henry et al., 2002; Oates and Ho, 2002; Oates et al., 2005) present different somitic phenotypes but the most rostral somites appear to be less affected. Although the mechanism of occipital somite formation is not subject of the research presented in this thesis, the formation of these somites is interesting evidence supporting the existence of an alternative mechanism to the “clock and wavefront” operating in the rostral most cells that generate the occipital somites.

However, despite the fact that experimental somites form almost simultaneously like occipital somites, an analysis of the expression of *Hox* genes (Figure 6.1, D, H, L, P, insets) suggests that these experimental somites are not occipital, and in fact have a cervical somite identity. Since these somites acquire an axial identity and form in the absence of a segmentation clock it supports the view that: firstly, normal (non-occipital, trunk) somites can form simultaneously in the absence of the segmentation clock, with correct size,

morphology and ability to generate normal somite derivatives; and secondly, axial identity could also be specified independently of the segmentation clock (Schroter and Oates, 2010). The identity of the experimental somites corresponds to the *Hox* genes that are expressed in the streak at the time of excision of the PPS explant (Figure 6.1) as *Hoxb3* and *Hoxb4* are expressed in the posterior primitive streak at both stages (Figure 6.1, C, G, E, I) while *Hoxb6* and *Hoxb9* are expressed only at HH-st.8 (Figure 6.1, M, Q) with the experimental somites generated from both stages following suit (Figure 6.1, D, H, F, J, insets).

These results provide further evidence that in the normal embryo that the *Hox* code present in the streak prior to somite formation is what controls their ultimate axial identity, as has previously been suggested by Iimura and Pourquie (2006), who suggest that the *Hox* code is fixed in cells prior to ingression in the streak (Iimura and Pourquie, 2006). Thus, it is the patterning of the streak that could ultimately control somite axial identity. However it must be noted there is also a population of self-renewing somite progenitors in the lateral quarter of the node that can progressively give rise to daughter cells that leave the node and give rise to the medial half of somites (Selleck and Stern, 1991). The axial identity of these cells

has been suggested to be controlled by the age of the cells, or length of time spent in the primitive streak or the length time in the PSM (Gaunt, 1994; Gaunt and Strachan, 1996).

In summary, the fact that experimental somites form simultaneously cannot be explained by the fact that they are occipital-like, rather they have a trunk axial identity, which is matched by the *Hox* code expressed in the primitive streak at the time of transplant. These results support the conclusion that true somites (that have been suggested to require the segmentation clock to control size, pattern and identity) can form correctly and acquire an axial identity in the absence of the segmentation clock (Schroter and Oates, 2010) and secondly that axial identity of somites in the axis may be controlled during patterning of the presomitic mesodermal progenitors in the primitive streak.

Chapter Seven: General Discussion

Somitogenesis is the process of periodic formation of sequential pairs of somites along the rostral-caudal axis of the vertebrate embryo and is an evident example of the process of segmental patterning during embryogenesis. Somite formation is an intricate process which encompasses PSM cell rearrangements, cell epithelialization, fissure formation, segment boundary formation and somite sub-compartment specification (Lash et al., 1984; Christ and Ordahl, 1995; Keynes and Stern, 1988; Christ et al., 1972; Gossler and Hrabe de Angelis, 1998; Packard, 1976; Christ et al., 1974; Menkes, 1977; Beloussov and Naumidi, 1983; Marcelle et al., 2002; Revel et al., 1973; Duband et al., 1987; Takahashi and Sato, 2008). Overall, the process of somitogenesis encompasses several aspects such as a specific timing (the rate of somite formation), somite size specification (the number of cells that segment together) and patterning (the rostrocaudal subdivision of somites) (Lash et al., 1984; Christ and Ordahl, 1995; Keynes and Stern, 1988; Christ et al., 1972; Gossler and Hrabe de Angelis, 1998; Del Amo et al., 1992; Hrabe de Angelis et al., 1997; Jen et al., 1997; del Barco Barrantes et al., 1999; Saga and Takeda, 2001). It has been challenging to experimentally separate the different aspects of somite formation

since these are tightly integrated and also happen in concert with maturation of the PSM and with rostral-caudal body axis extension in the developing embryo, which has made it difficult to determine whether there is one prevailing mechanism governing the specification of all aspects of somite formation, or whether there are several mechanisms working in parallel to individually regulate each aspect.

7.1 Prevailing model of somitogenesis and incongruent data

The model that has been favoured for explaining somitogenesis is the Clock and Wavefront model (Cooke and Zeeman, 1976). This model proposes the existence a *cellular oscillator* or "clock" operating in the cells of the PSM, which would periodically interact with a "wavefront" dependent on a gradient of *positional information* along the rostral-caudal axis of the embryo (Cooke and Zeeman, 1976; Slack, 1991). This interaction would set the time at which groups of cells in the PSM segment together to form somites, dictating the periodic pattern of formation of somites and boundary positions at which point cells become committed to a rostral or caudal compartment of the somite (Introduction, Figure 1.2). According to this model, the number of cells that segment together to form a somite and the frequency of segmentation would depend on the

frequency of the segmentation “clock” and on the velocity of the “wavefront” progression along the PSM (Cooke and Zeeman, 1976). The finding of genes which are expressed in a periodic fashion in the PSM, with a frequency that generally coincides with the rate of somite formation, provides evidence for the existence a “clock” acting within the cells of the PSM (Palmeirim et al., 1997; Forsberg et al., 1998; McGrew et al., 1998; Leimeister et al., 2000; Sawada et al., 2000; Holley et al., 2000; Jouve et al., 2000). Expression of these genes starts in a broad area at the caudal region of the PSM, moving rostrally until it narrows into a stationary band in the rostral or caudal half of the newly forming somite (Introduction, Figure 1.2). This periodic expression is not due to cell movements and is considered to be the result of a cell-intrinsic “segmentation clock” operating in the PSM cells (Palmeirim et al., 1997; Dale and Pourquie, 2000). This is the case for the oscillatory expression of c-*hairy-1* (Palmeirim et al., 1997) and other subsequently described Notch signaling related genes (Aulehla and Johnson, 1999; Forsberg et al., 1998; Jiang et al., 2000; McGrew et al., 1998; Bessho et al., 2001; Dequeant et al., 2006, Jouve et al., 2000; Leimeister et al., 1999; Leimeister et al., 2000; Holley et al., 2000; Jiang et al., 2000; Oates and Ho, 2002; Sawada et al., 2000; Elmasri et al., 2004; Li et al., 2003). Additionally, Dubrulle and co-workers described a gradient of

FGF8 in the caudal part of the embryo as a result of translation of a gradient of *fgf8* mRNA (*fgf8* mRNA transcription is limited to the caudal end of the embryo and it is gradually degraded in the forming tissues generating the gradient of *fgf8* mRNA), high in the caudal region and low in the rostral region of the PSM where segmentation takes place (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004), and separated by a border subsequently defined as a *determination front* (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004). It was proposed that this gradient of FGF8 signalling and related threshold of FGF8 at the level of the determination front could provide a physical basis for the "wavefront" of the Clock and Wavefront model (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004). Experimental correspondence for "clock" dependent genes and a rostral-caudal gradient of FGF8 able to correspond to a moving 'wavefront' of determination operating within the PSM, have led the field to generally favour the Clock and Wavefront model for explaining somite formation. However, the Clock and Wavefront model does not account for several important observations and experimental data (Hamburger and Hamilton, 1951; Hamilton and Hinsch, 1956; Lim et al., 1987; Oka et al., 1995; Conlon et al., 1995; Huang et al., 1997; Shen et al., 1997; Wong et al., 1997; Evrard et al., 1998; Zhang and Gridley, 1998; Sawada et al., 2000; Dunwoodie et al., 2002; Zhang et al.,

2002; Nomura-Kitabayashi et al., 2002; Takahashi et al., 2003; Rodrigues et al., 2006; Feller et al., 2008; Dias et al., 2014). Firstly, in mutants of the Notch signaling pathway genes *Notch1*, the Notch ligand *Delta-like 3,* the activated Notch protease presenilin and the Notch effector RBPJk, despite showing disruptions in segmentation and in rostral-caudal patterning of the somites, these somites form first, and only subsequently degenerate (Oka et al., 1995; Conlon et al., 1995; Shen et al., 1997; Wong et al., 1997; Evrard et al., 1998; Zhang and Gridley, 1998; Dunwoodie et al., 2002; Zhang et al., 2002; Takahashi et al., 2003). Moreover, zebrafish mutants of the *Mesp* genes, which interact with the Notch signaling pathway, show disrupted rostral-caudal subdivision of the somites but, again, no effect on the initial formation of the somites (Sawada et al., 2000). Additionally, it has been described that the occipital somites (five-most rostral somites) form with a different periodicity compared with more caudal somites (Hamburger and Hamilton, 1951; Hamilton and Hinsch, 1956; Huang et al., 1997; Dias et al., 2014), form in the absence of the oscillatory expression of genes of the segmentation clock (Rodrigues et al., 2006) and have different rostral-caudal patterning compared to more caudal somites (Lim et al., 1987). These observations are not consistent with the Clock and Wavefront model for somite formation and suggest that a distinct mechanism

must be accountable for allocating the PSM cells to form each occipital somite. Furthermore, it is also important to note that studies in transgenic mice expressing activated Notch (NICD) throughout the PSM in which up to eighteen unpatterned somites form, have shown that somite (segment) formation and somite rostral-caudal patterning can be uncoupled, and that cyclic expression of Notch might be mainly required for the correct rostral-caudal patterning of the somites rather than required for segment border formation (Feller et al., 2008). Similarly, studies in mutant *mespb* (zebrafish functional homologue of mouse *Mesp2*) knockin mice embryos have shown that epithelial somites can form without rostral-caudal polarity, suggesting that segment formation and establishment of rostral-caudal polarity may also be genetically separate events (Nomura-Kitabayashi et al., 2002). In the light of the above data, it is possible that a segmentation clock operating in the PSM could be dispensable for the initial allocation of cells to individual somites and rather required for coordination of other aspects of somitogenesis, as is somite patterning into correct rostral and caudal compartments. Furthermore, contrasting with the Clock and Wavefront model (Cooke and Zeeman, 1976), alternative models for somite formation have been proposed, namely the Cell Cycle model (Primmett et al., 1989; Stern et al., 1988; Collier et al., 2000) which postulates that

somitogenesis and the initial allocation of PSM cells into discrete somites could be coordinated in a cell-autonomous manner by the cell cycle, based on the discovery of cell cycle synchrony of the PSM cells (Primmett et al., 1989; Stern et al., 1988; Collier et al., 2000). In brief, this model proposes the existence of a time window in the cell cycle of the PSM cells during which cells of similar maturity turn on cell adhesion genes which would, one cycle later, trigger the adhesiveness of sub-sets of PSM cells and segregate to form a segment (somite) (Primmett et al., 1989; Stern et al., 1988; Collier et al., 2000; Introduction, 1.5.3 Cell cycle model for somitogenesis).

7.2 The aims of this study

The study presented in this thesis took advantage of an experimental paradigm that allowed us to separate the aspect of somite size specification from the timing of somite formation, which in the embryo are coincident events. Evenly exposing posterior primitive streak (PPS) explants (prospective lateral plate mesoderm) with the BMP4 antagonist, Noggin, generates experimental somites equivalent to axial endogenous somites in terms of size, morphology, function, fate and expression of somitic markers, that form synchronously in the absence of a clock but lack patterning into rostral and caudal halves. This suggests that the main function of the molecular clock operating

in the PSM cells that generate somites might be distinctive for the different aspects of somitogenesis and suggests that it could be mainly required for patterning of the somites coupling it with the other aspects of somitogenesis.

The main aims of this study were to

- Optimize an *ex vivo* method to generate experimental somites.
- Characterize the experimentally generated somites in terms of size, morphology, expression of somitic markers and potential to give rise to derivatives if placed in the right environment.
- Investigate the timing of experimental somite formation and whether their formation is prefigured by periodic expression of the “cycling genes”.
- Analyze the rostrocaudal patterning of the experimental somites (by examining the expression of known rostral- and caudal- half markers and the migration paths of neural crest cells and motor axons).
- Investigate the axial identity of the generated somites.

Firstly, the experimental somites were morphologically and functionally characterized and compared with axial endogenous somites. Thus, the properties of size, morphology, the expression of

somite-specific genes and the ability to generate normal somite derivatives if put in the place of an endogenous somite were analyzed. The experimental somites present a normal size compared with endogenous epithelial somites (Appendix 3, Table A3.1, Table A3.2 and Graft A3.1). Somite volumes were calculated based on measurements of endogenous and ectopic somite diameters from living embryos: 538323.29±209331.01 μm^3 for experimental somites and 516783.49±221438.37 μm^3 for endogenous epithelial somites SI, SII, SIII, SIV (t-test P=0.844 indicative of no significant difference) (Appendix 3, Table A3.1, Table A3.2 and Graft A3.1). The experimental somites express the somite marker *Paraxis* (Figure 4.1, E, E') and also present a normal morphology when compared with endogenous epithelial somites, comprising cells forming a three dimensional epithelial segment that has a basal surface on the outside and a lumen in the centre: they express N-cadherin and a Fibronectin-rich basal lamina (Figure 4.1, G, H). Subsequent analysis using multi-photon confocal imaging of the experimentally generated somites done by co-workers (Dias et al., 2014) has now also clearly shown the expression of N-cadherin and Fibronectin in the experimental somites (Dias et al., 2014). Furthermore, the experimental somites can also give rise to late somite derivatives: when an individual ectopic somite was transplanted in the place of an

axial endogenous somite (Figure 4.2, A), it incorporates well in the axis of the host (Figure 4.2, B, B', B", C, C', C") and expresses the sclerotome marker *Pax1* (Figure 4.2, E, E') and the dermomyotome marker *MyoD* (Figure 4.2, D, D').

Secondly, the formation of the somites derived from PPS explants treated with Noggin does not occur at regular time periods as shown by time-lapse imaging of the formation of the experimental somites using time-lapse fluorescence microscopy (Chart 5.1, B; Graph 5.1, green; Figure 5.1) and their formation is not accompanied by the cyclic expression of the 'segmentation clock' genes in the PPS explant cells (Appendix 4, Figure A4.1-A4.5). The key components of the clock *Hairy1, Hairy2* and *Lunatic Fringe* show approximately the same pattern of expression for all the times points analyzed prior to ectopic somite formation (Figure 5.3; Appendix 4, Figure A4.1-A4.5). However, in the absence of the cyclic expression of key segmentation clock genes, the experimental somites fail to become normally patterned into rostral and caudal halves, suggesting that the molecular clock is required for normal rostral/caudal patterning, which has previously been suggested in other studies (Takahashi et al., 2003). The markers of the somite caudal-half *Hairy1* and *Meso2* are not expressed in the experimental somites (Figure 5.4, A, B),

Lunatic Fringe is expressed weakly and uniformly (Figure 5.4, D) and *Uncx4.1* is expressed in a random fashion (Figure 5.4, F). The somite rostral-half marker *EphA4* is not expressed in the experimental somites (Figure 5.4, C) and *Hairy2* is expressed uniformly (Figure 5.4, E). Correspondingly, the migration of neural crest cells and motor axons through the experimental somites was observed to be affected (Figure 5.5, A, A' and C, C"). Normally these populations of cells migrate through the rostral half of the somite and are inhibited to migrate through the caudal half (Bronner-Fraser et al., 1991; Keynes and Stern, 1984; Rickmann et al., 1985; Stern et al., 1986). After transplanting an experimental somite into the axis of a host embryo in the place of an epithelial endogenous somite and let it incorporate, the migration of neural crest cells and motor axons was analyzed and both these cells populations do not migrate through a preferred route within the experimental somite which is indicative of disrupted rostral/caudal correct patterning (Figure 5.5, A', A" and C', C").

It was already known that during somitogenesis in normal embryonic development, the occipital somites (five-most rostral somites) form with a different periodicity compared with more caudal somites (Chapter Five, Figure 5.2; Hamburger and Hamilton, 1951; Hamilton

and Hinsch, 1956; Huang et al., 1997; Dias et al., 2014), form in the absence of the oscillatory expression of genes of the segmentation clock (Rodrigues et al., 2006) and have different rostral-caudal patterning compared to more caudal somites, lacking expression of some of the rostral/caudal markers (Lim et al., 1987; Rodrigues et al., 2006). Consequently, in this study it was therefore investigated whether the experimental somites acquire an axial identity of occipital somites. An analysis of *Hox* gene markers suggested that, despite the fact that experimental somites form almost simultaneously like occipital somites, the experimental somites are in fact not occipital, but rather have the axial identity of cervical somites (Figure 6.1, D, H, L, P, insets). Given that the experimental somites acquire an axial identity and form in the absence of a segmentation clock it supports the view that: firstly, normal (non-occipital) somites can form simultaneously in the absence of the segmentation clock, with correct size, morphology and ability to generate somite late derivatives; and secondly, axial identity could also be specified independently of the segmentation clock (Schroter and Oates, 2010). Moreover, the PPS explant from an HHst.5 embryo used as donor tissue expressed the same set of genes as the experimental somites: *Hoxb-3* and Hox*b-4* but not Hox*b-6* or Hox*b-9* (Figure 6.1, C, G, K, O). *Hox-b6* and *Hoxb-9* only start to be expressed in the streak later

at HHst.8 (Figure 6.1, M, Q). Likewise, experimental somites generated from a PPS explant from an HH-st.8 embryo, not only express *Hoxb-3* and Hox*b-4* but also Hox*b-6* and Hox*b-9* (Figure 6.1, F, J, N, R, insets). In summary, the axial identity of the experimental somites corresponds to the *Hox* genes that are expressed in the streak at the time of excision of the PPS explant. These results provide further evidence that, in the normal embryo, the *Hox* code present in the streak prior to somite formation is what controls their ultimate axial identity, as has previously been suggested by Iimura and Pourquie (2006), who suggest that the *Hox* code is fixed in cells prior to ingression in the streak (Iimura and Pourquie, 2006). Thus, it is the patterning of the streak that could ultimately control somite axial identity.

7.3 Final remarks and future directions

The results of this study suggest that bona-fide somites of normal size, morphology and fate can form in the absence of a 'segmentation clock'. One aspect lacking in these experimental somites is their correct rostral-caudal subdivision, raising the possibility that the main requirement of the 'segmentation clock' could be to couple the specification of correct somite patterning with somite formation (by a still unknown mechanism). Additionally, the

results of this study suggest that somite formation can be uncoupled from long-range molecular gradients operating in the rostral-caudal axis, since the three-dimensional arrangement and non-periodic formation of the experimental somites is not compatible with it. A recently developed model to explain somite patterning, the Progressive Oscillatory Reaction-Diffusion model (PORD) (Cotterell, 2015) further corroborates this idea since this tested model proposes that somite formation and size regulation, unlike in the Clock and Wavefront model for somitogenesis, can be driven by short-range interactions rather than global positional information (Cotterell, 2015). Furthermore, it has also previously been suggested that the control of how many cells segment together to form a somite could also involve local cell-cell interactions (Stern and Bellairs, 1984). As the cells undergo MET, intrinsic properties of the cells as are cell-cell adhesion, the limits of cell size and the limitations on cell packing (mechanical forces and requirements for correct cell-cell tight-junction formation) during their formation into epithelial spheres with a central lumen, could explain how the experimentally generated somites form with the correct number of cells in the absence of the normal environmental cues. Indeed, there is additional experimental evidence that suggests that somites are self-organizing structures (Stern and Bellairs, 1984) controlled by cell interactions of adhesion

and “packing constraints” of cells undergoing MET (Nakaya et al., 2004). What exactly controls this intrinsic self-organizing mechanism is still not clear, and how it may be coordinated with the ‘segmentation clock’ to control the patterning of the somites is also unknown. Further detailed molecular analysis of experimentally generated somites could be a useful system for investigating the series of events that govern somite formation. Finally, the results of the present study raise the possibility that the inhibition of BMP by Noggin could mark the set off for epithelialization of groups of cells in the rostral PSM to form a somite. This possibility is consistent with previous findings showing that Noggin-null mice lack normal somites (McMahon et al., 1998). Furthermore, this work also suggests that BMP inhibition may serve to fix the axial identity of the cells in the PSM, prior to somite formation. One plausible possibility could be that inhibition of BMP by Noggin, or the cells leaving the primitive streak, could arrest their molecular clock thereby controlling the expression of *Hox* genes in the somites, which may happen in the embryo when the presomitic cells leave the primitive streak and become positioned next to the notochord where BMP is inhibited by secreted Noggin.

In conclusion, the work herein suggests that it is possible to separate the aspect of somite size specification from the timing of somite formation and from the segmentation clock. While the function of the clock might be to coordinate rostral-caudal patterning of somite progressively along the axis during development, somite size could be controlled by an alternative mechanism, for example involving local cell-cell interactions.

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Appendix 1: Publication

Somites Without a Clock
Ana S. Dias, Irene de Almeida, Julio M. Belmonte, James A. Glazier and Claudio D. Stern (January 9, 2014)
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Editor's Summary

Cell-Cell Interactions in Development

In vertebrate embryos, the number, size, and positional identity of mesodermal segments (somites) located bilaterally along the anteriorposterior axis is widely believed to be controlled by a molecular clock of oscillating gene expression interacting with a traveling wave of signals to determine how many cells make up a somite. **Dias *et al.*** (p. 791, published online 9 January; see the Perspective by **Kondo**) reveal that it is possible to generate somites of normal size, shape, and identity without either a clock or a wavefront. Instead, the findings suggest that somite size and shape are regulated by local cell-cell interactions.

Appendix 2

Reagents setup

Saline solution used for modified New (1955) technique of culture of chicken embryos: Pannett-Compton saline (Pannett and Compton, 1924)

Solution A

121 g NaCl

15.5 g KCl

10.42 g $CaCl_2.2H_2O$

12.7 g $MgCl_2.6H_2O$

H_2O to 1 litre

Solution B

2.365 g $Na_2HPO_4.2H_2O$

0.188 g $NaH_2PO_4.2H_2O$

H_2O to 1 litre

Both stock solutions were autoclaved for storage at room temperature, and once opened kept at 4°C. Before use, mixed in order 120 ml of solution A, 2700 ml H_2O, and 180 ml of solution B. To prevent infections 1:1000 dilution antibiotic/antimycotic (GIBCO) solution was added.

Saline solution used for dissection of grafts: Tyrode's saline.

10x concentrated stock

80 g NaCl

2 g KCl

2.71 g $CaCl_2.2H_2O$

0.5 g NaH_2PO_4

2 g $MgCl_2.6H_2O$

10 g glucose

H_2O to 1 litre

Stock solution was autoclaved for storage at room temperature. Before use, diluted 1:10 with distilled water.

Solutions for whole mount *in situ* hybridization

Hybridization buffer (HB)

50% Formamide

1.3x SSC (pH 5.3)

5 mM EGTA

50 μg/ml yeast RNA

0.2% Tween-20

0.005% CHAPS

100 μg/ml Heparin

Phosphate Buffered Saline (PBS)

8.76 g NaCl

2.14 g $N_2HPO_4.7H_2O$

2.3 g $Na_2H_2PO_4$

H_2O to 1 litre

pH 7.0

PTW

Phosphate Buffered Saline; 0.1% Tween-20 solution

Tris-Buffered Saline Tween-20 (TBST)

1.4 M NaCl

27 mM KCl

0.25 M Tris-HCl pH7.5

1% Tween-20

NTMT

0.1M NaCl

0.1M Tris-HCl pH 9.5

0.005M $MgCl_2$

0.1% Tween-20

Developing solution

NTMT with 4.5 μl BCIP (5-bromo-4chloro-3-indolyl phosphate: 7.5 mg/ml in 70% dimethylformamide) and 3.5 μl NBT (Nitro-blue teratzolium: 50 mg/ml in 100% dimethylformamide) per ml.

Solutions for imunohistochemistry

Blocking solution

PBS + 1% Bovine serum + 0.1% Triton X100

Appendix 3

Endogenous Epithelial Somites			
Axial Level	**Somite Stage**	**Diameter (μm)**	**Volume (μm^3)**
Embryo 1			
17	I	120	9.05×10^5
16	II	115	7.96×10^5
15	III	100	5.24×10^5
14	IV	100	5.24×10^5
Embryo 2			
16	I	70	1.8×10^5
15	II	80	2.68×10^5
14	III	70	1.8×10^5
13	IV	85	3.22×10^5
Embryo 3			
16	I	80	2.68×10^5
15	II	80	2.68×10^5
14	III	95	4.49×10^5
13	IV	100	5.24×10^5
Embryo 4			
17	I	80	2.68×10^5
16	II	90	3.82×10^5
15	III	100	5.24×10^5
14	IV	100	5.24×10^5
Embryo 5			
17	I	110	6.97×10^5
16	II	100	5.24×10^5
15	III	100	5.24×10^5
14	IV	95	4.49×10^5
Embryo 6			
17	I	95	4.49×10^5
16	II	90	3.82×10^5
15	III	90	3.82×10^5
14	IV	75	2.29×10^5
Embryo 7			
18	I	120	9.05×10^5
17	II	95	4.49×10^5
16	III	95	4.49×10^5
15	IV	95	4.49×10^5
Embryo 8			
19	I	125	1.02×10^5
18	II	125	1.02×10^5
17	III	125	1.02×10^5
16	IV	110	6.97×10^5

Table A3.1. Endogenous epithelial somite measurements.

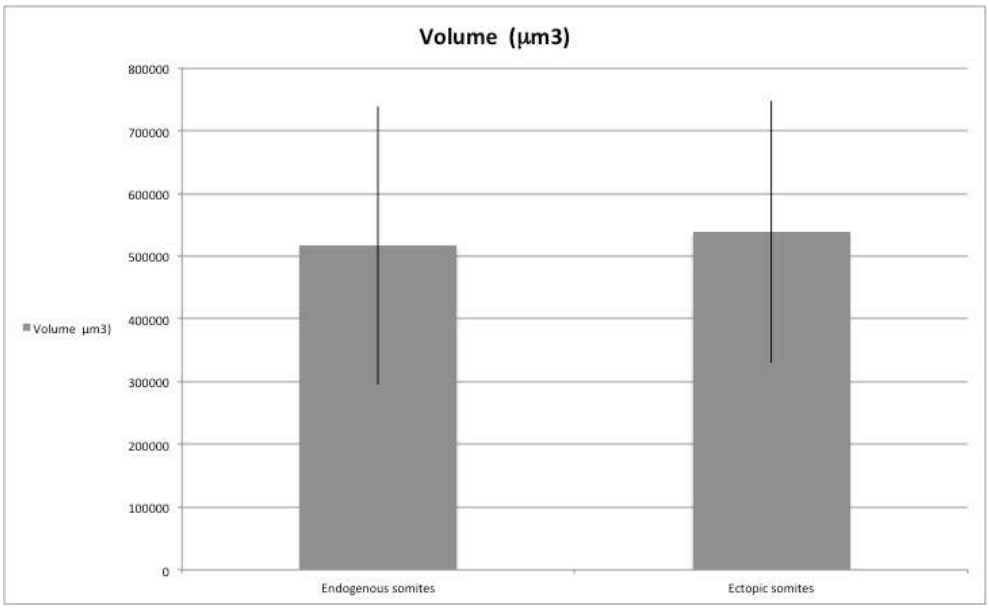
Diameter column shows the measurement of the diameter of the endogenous epithelial somites from live embryos (8 embryos, n=24 endogenous epithelial somites measured). Somite axial level is provided in Arabic numerals. Somite stages (Christ and Ordahl, 1995) are provided in Roman numerals and correspond to the

youngest epithelial endogenous somites (SI, SII, SIII, SIV) for each embryo.

"Bunches of grapes"- Experimental Somites					
Experimental Somite	Diameter (μm)	Volume (μm^3)	Experimental Somite	Diameter (μm)	Volume (μm^3)
Host-Embryo 1			*Host-Embryo 6*		
1	120	9.05×10^5	1	100	5.24×10^5
2	105	6.06×10^5	2	90	3.82×10^5
3	100	5.24×10^5	3	100	5.24×10^5
4	150	1.77×10^6	4	90	3.82×10^5
5	90	3.82×10^5	5	90	3.82×10^5
6	80	2.68×10^5	6	90	3.82×10^5
7	90	3.82×10^5	7	100	5.24×10^5
8	100	5.24×10^5	8	100	5.24×10^5
9	80	2.68×10^5	*Host-Embryo 7*		
Host-Embryo 2			1	100	5.24×10^5
1	75	2.21×10^5	2	110	6.97×10^5
2	75	2.21×10^5	3	110	6.97×10^5
3	100	5.24×10^5	4	80	2.68×10^5
4	120	1.05×10^6	5	80	2.68×10^5
5	120	1.05×10^6	6	100	5.24×10^5
6	110	6.97×10^5	7	110	6.97×10^5
Host-Embryo 3			8	125	1.02×10^6
1	100	5.24×10^5	9	125	1.02×10^6
2	85	3.22×10^5	*Host-Embryo 8*		
3	150	1.77×10^6	1	100	5.24×10^5
4	100	5.24×10^5	2	95	1.29×10^6
5	150	1.77×10^6	3	60	1.13×10^5
6	100	5.24×10^5	4	60	1.13×10^5
Host-Embryo 4			5	60	1.13×10^5
1	80	2.68×10^5	6	65	1.44×10^5
2	75	2.21×10^5	7	60	1.13×10^5
3	80	2.68×10^5			
4	85	3.22×10^5			
5	100	5.24×10^5			
Host-Embryo 5					
1	70	1.8×10^5			
2	90	3.82×10^5			
3	90	3.82×10^5			
4	100	5.24×10^5			
5	105	6.06×10^5			
6	110	6.97×10^5			
7	125	1.02×10^5			
8	100	5.24×10^5			
9	115	7.96×10^5			

Table A3.2. Experimental Somites measurements.

Table shows the measurements of the diameter and calculated volumes of experimental somites from “bunches of grapes” (“bunches of grapes” from 8 embryos, n=59 experimental somites measured). (Note that these experimental somites were formed in host chicken embryos from which the endogenous epithelial somite measurements were taken for size comparison in Table A3.1).



Graph A3.1. The experimental somites and the endogenous somites do not present a significant difference in their volumes.

Appendix 4

Figure A4.1-A4.5. Time-course of the expression of the molecular clock markers *Hairy1*-, *Hairy2*, and *Lunatic fringe.*

No evidence of oscillatory expression of the molecular clock genes, *Hairy1*, *Hairy2* or *LFng* in PPS explants, at different time-points of the time-course. A4.1 – 3 hours, A4.2 – 4.5 hours, A4.3 -5.15 hours, A4.4 – 6.5 hours and A4.5 – 7.5 hours after Noggin protein treatment of the HH-St.5 PPS explant. Different examples of the expression of the markers are shown for a single time-point. The host embryos show typical variations of expression patterns; by comparison the explants show comparatively invariance of expression patterns (insets).

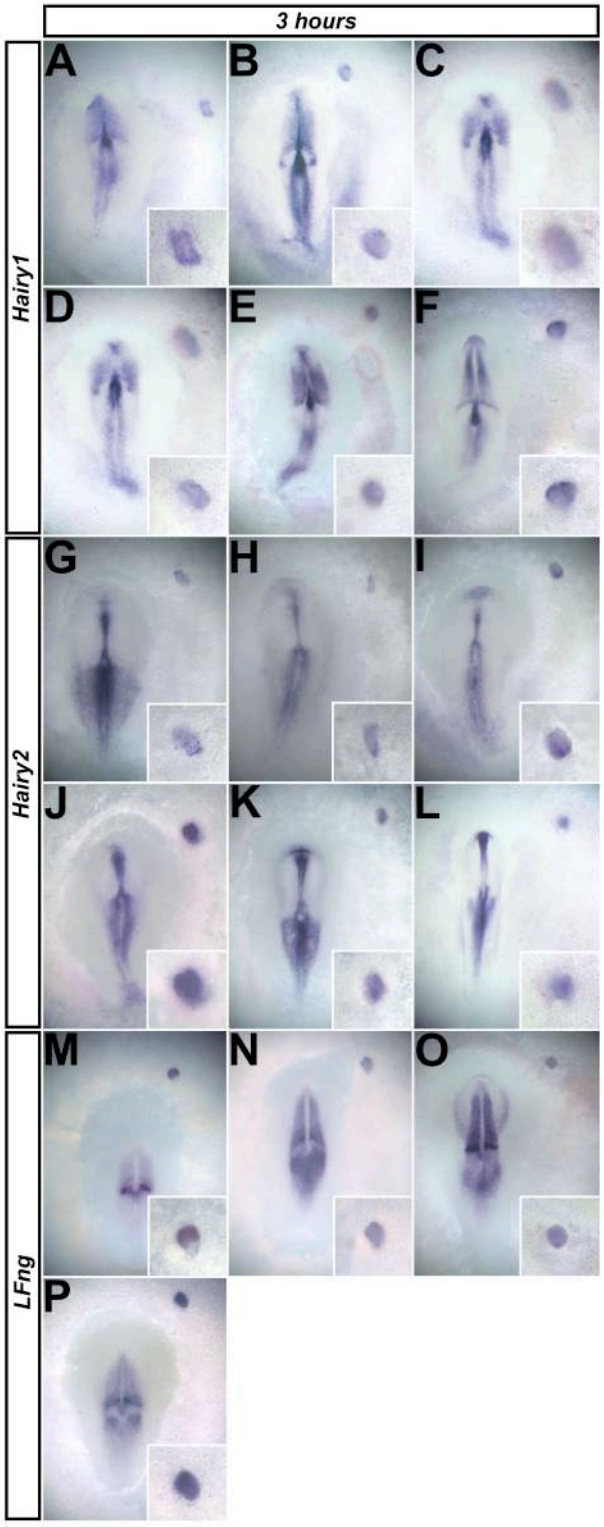


Figure A4.1

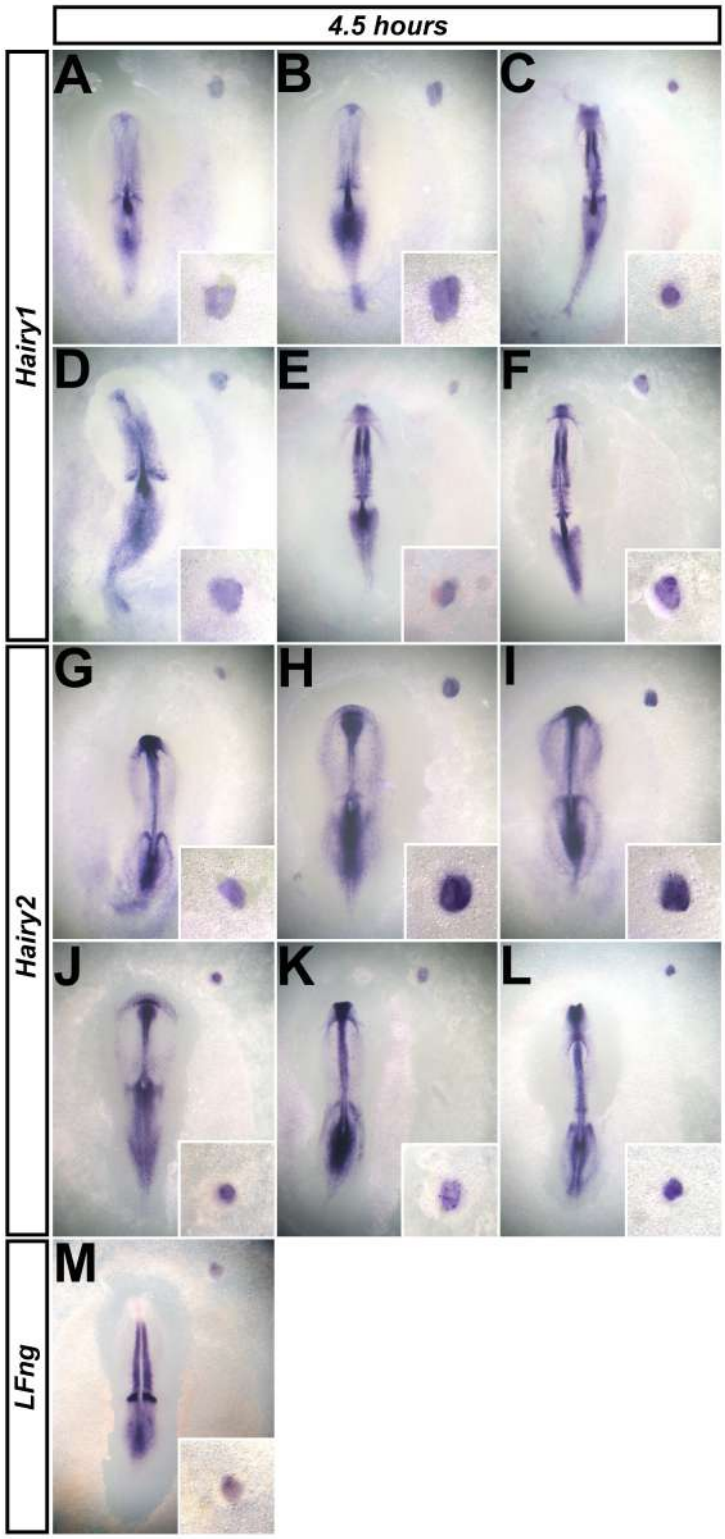


Figure A4.2

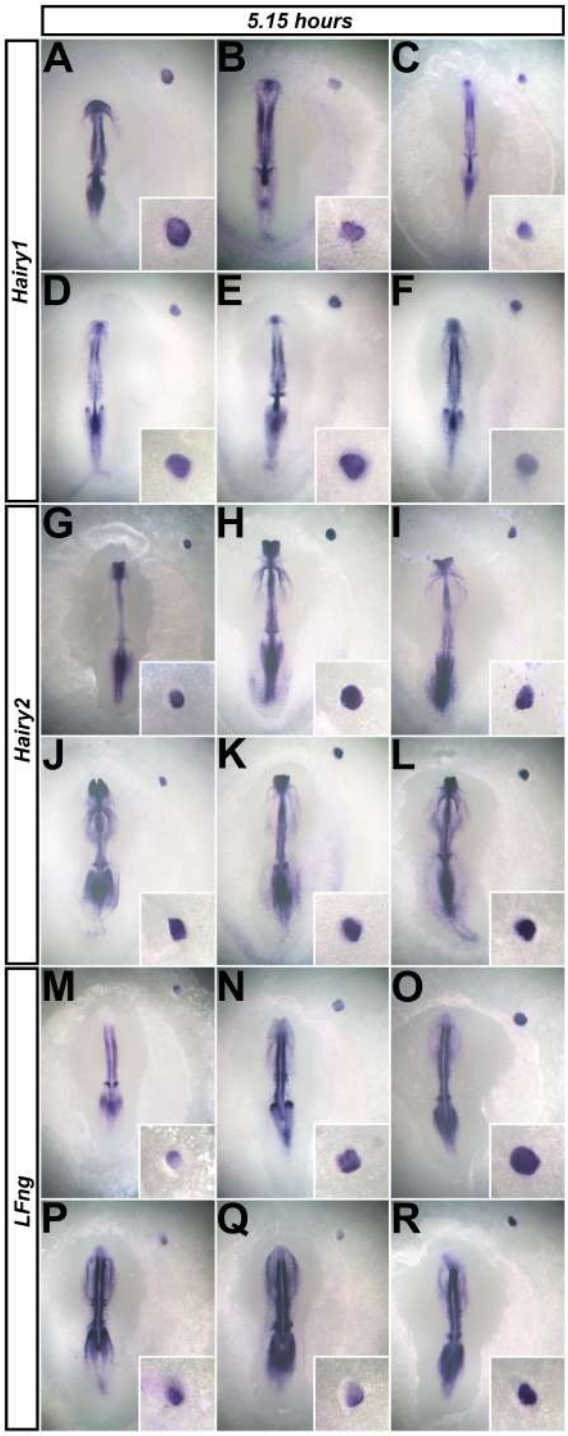


Figure A4.3

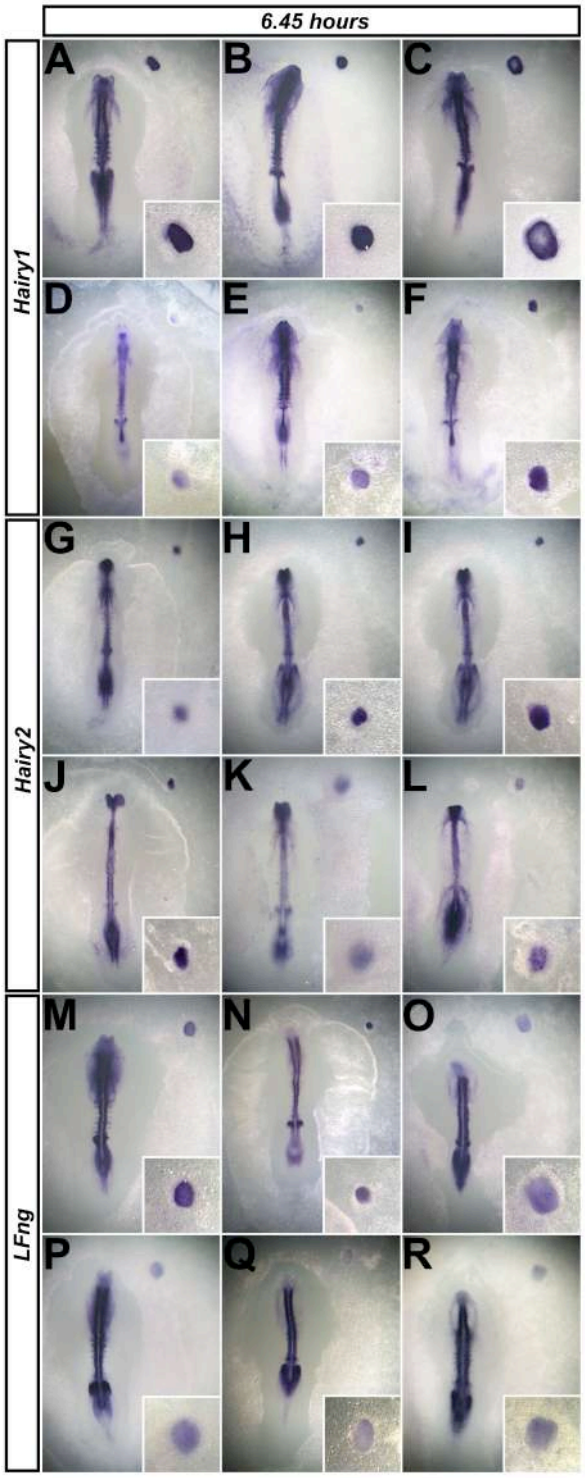


Figure A4.4

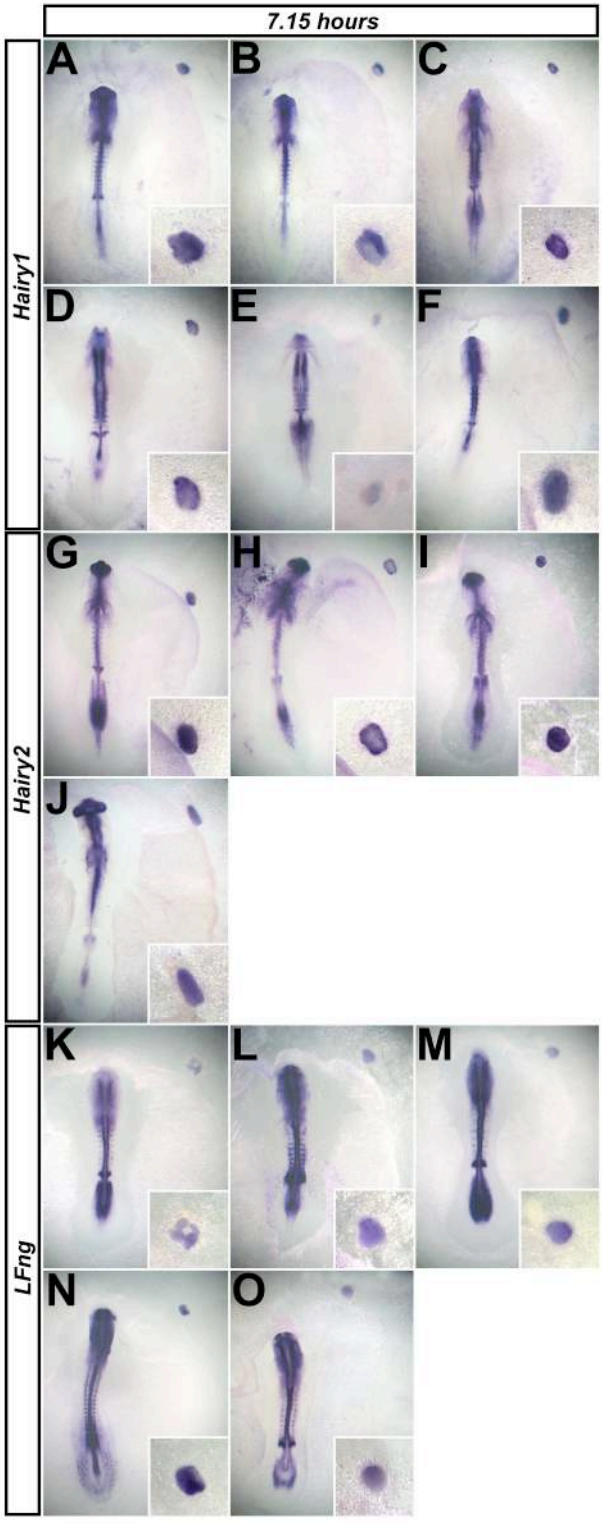


Figure A4.5