Universidade do Minho
Escola de Ciências da Saúde

Sofia Rodrigues

Spatial-temporal control of the early stages of somitogenesis in the chick embryo

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Spatial-temporal control of the early stages of somitogenesis in the chick embryo

Tese de Doutoramento em Ciências da Saúde

Trabalho efectuado sob a orientação do
Professora Doutora Isabel Palmeirim

Maio de 2006

A minha primeira palavra vai para a Professora Isabel Palmeirim. Isabel, nunca esquecerei o dia que me acolheste, em Paris! Obrigada pela orientação, amizade e por teres aberto uma porta para mim, com acesso ao mundo da investigação e principalmente, da biologia do desenvolvimento.

À Escola de Ciências da Saúde na Universidade do Minho, e em particular à Professora Doutora Cecília Leão gostaria de agradecer o voto de confiança ao aceitar-me como estudante de doutoramento nesta escola. Cabe-me ainda frisar a excepcional qualidade dos cursos organizados pelos investigadores da Escola de Ciências da Saúde, os quais contribuiram grandemente para o meu crescimento científico.

Ao Instituto Gulbenkian de Ciência agradeço as excelentes condições de trabalho e o facto de me ter proporcionado o acesso a uma cultura cientifica e uma massa crítica excepcionais. A muitas pessoas no IGC que me aconselharam durante estes anos e me ajudaram no curso do meu trabalho, Moisés, Joaquín, Antonio, Maria, Ana Teresa, Cristina … E ainda, uma palavra especial ao professor António Coutinho, a quem agradeço os seus conselhos sempre sensatos que muito encorajaram as minhas opções de carreira científica.

To the “Institut d’Embryologie Cellulaire et Moléculaire” for hosting me in the first stages of my scientific life. In particular, to Marie Aimeé Teillet, thank you so much for having shared with me the beautiful world of quail-chick chimeras.

A todo o Grupo da Segmentação, de Lisboa a Braga, aos que estão e aos que estiveram, obrigada a todos pelo excelente ambiente de laboratório e pelas saudáveis discussões científicas, que sempre me proporcionaram. Catarina, a ti um obrigada especial por teres sido uma grande parceira desde sempre!

Ao Grupo das Integrinas que também desencadearam óptimas discussões cientificas, ao Gabriel, em particular, pela ajuda preciosa nas esperiências de imaging, e à Solveig, em especial, que esteve sempre presente para o bom e para o menos bom!

Ao Grupo da Simetria e Desenvolvimento por me manterem os dias agradáveis a escrever a minha tese e, claro, por gostarem (?) que eu esteja no vosso espaço!

À Leonor Saúde, de quem obtive o maior apoio moral, prático, profissional, pessoal, … enfim, durante os dias menos fáceis do meu doutoramento e que também se tornou uma grande mentora na ausência da Isabel.

À Dulce, à Leonor e à Susana – obrigada amigas, pela companhia e amizade nestes ultimos tempos da minha tese.

À Mae e ao Pedro, obrigada – sem vocês esta última parte teria sido muito mais difícil!

E por fim ao Joca e em especial à Beatriz, a quem dedico esta tese, e agradeço ter-me ‘tirado’ tanto tempo que afinal não era preciso para mais nada!

A segmentação é o processo que conduz à formação dos sómitos, estruturas embrionárias metaméricas que se formam na parte mais rostral da mesoderme presomítica (MPS) e que estão na base da segmentação do corpo dos vertebrados. Sabe-se actualmente que o processo de segmentação é controlado por um relógio molecular que opera ao nível das células da MPS e que se manifesta pela transcrição cíclica de vários genes, cujo tempo de ciclo corresponde precisamente ao tempo necessário para a formação de um sómito. O número de ciclos de expressão que ocorrem em cada célula pré-somítica confere-lhe uma informação temporal que se traduz numa informação posicional no eixo antero-posterior do embrião.

No curso desta tese de doutoramento, mostramos que ciclos de expressão dos genes do relógio estão já a decorrer nas células do território somítico prospectivo. Este estudo, realizado em embriões de galinha no estadio de 6 sómitos, também mostrou que as futuras células medianas (Futura Mediana (FM)-MPS) e futuras células laterais (Futura Lateral (FL)-MPS) dos sómitos se encontram em territórios distintos e organizados no eixo antero-posterior da linha primitiva. O facto das oscilações dos genes cíclicos atravessarem estes dois territórios ao longo deste eixo, implica que o relógio da segmentação está a proporcionar uma informação posicional bidimensional (no eixo antero-posterior e no eixo médio-lateral) às celulas da MPS. Notavelmente, bandas oblíquas de expressão dos genes cíclicos foram também observadas na MPS. Experiências de transplantação de tecidos efectuadas no nosso laboratório revelaram que apenas as células do território FM-MPS contêm a informação para segmentar, a qual possivelmente lhes é transmitida pela região imediatamente anterior, denominada fenda mediana (FM). Transplantes heterotópicos e interespecíficos da FM e células FM-MPS entre a galinha e a codorniz permitiram-nos verificar que, de facto, a FM é um importante centro sinalizador, responsável pela instrução das células vizinhas mais caudais no processo de segmentação.

Uma outra parte deste trabalho focou-se no estudo dos mecanismos moleculares responsáveis por activar o início do relógio da segmentação durante o desenvolvimento embrionário da galinha. Os nossos resultados mostraram que o relógio da segmentação

não é activado por nenhum sinal derivado dos primeiros passos da formação do embrião, e propuseram que o nó de Hensen poderia ser o responsável por iniciar o programa da segmentação. O resultado de uma série de transplantes heterotópicos e isocrónicos do nó de Hensen revelaram que este transmite um sinal que é capaz de não só induzir as oscilações dos genes cíclicos em células cujo destino não era a formação de sómitos, mas também de reiniciar o programa da segmentação nestas células.

A parte final desta tese consistiu em estudar as diferenças entre os sómitos mais anteriores e os sómitos mais posteriores do embrião. O facto dos sómitos mais rostrais não originarem estruturas segmentadas e de o fenótipo de várias mutações que afectam a somitogénese não apresentar defeitos ao nível destes sómitos, tem levado à ideia generalizada que os sómitos anteriores são diferentes dos mais posteriores. Uma análise detalhada da expressão de *notch1*, *delta1*, *hairy1*, *hairy2*, *lunatic fringe* and *hey2* ao nível dos primeiros 10 sómitos mostrou que a sua expressão não é a mesma ao longo do eixo antero-posterior, e que as diferenças observadas não são sempre ao mesmo nível somítico. Por outro lado, a monitorização da formação dos primeiros sómitos *in vivo*, permitiu-nos concluir que os primeiros 2 sómitos se formam mais rapidamente, e de forma quasi-concomitante, que os sómitos mais posteriores. No seu conjunto, os resultados deste trabalho apontam para a existência de diferenças relevantes entre os sómitos rostrais e caudais no que se refere às suas características moleculares e temporais no embrião de galinha.

Segmentation is built on repetitive structures, named somites, which are formed progressively from the most rostral part of presomitic mesoderm (PSM). This process is controlled by a molecular clock operating at the level of the PSM, being evident by the cyclic transcription of several genes. Remarkably, the transcription cycle of these genes is precisely the time required to form one somite.

In the course of this PhD thesis it was shown that the molecular clock underlying somite formation already operates in the prospective somitic territory at 6-somite stage embryos. Furthermore, this work demonstrated that at this stage the medial (PM-PSM) and lateral (PL-PSM) prospective PSM cells present clearly segregated territories in the primitive streak. Interestingly, the fact that the oscillations of the cycling genes cross the PL-PSM and PM-PSM territories in a posterior to anterior direction implies that the segmentation clock provides the PSM cells with bidimensional positional information. In aggrement, cross-stripes of the molecular clock genes expression were observed in the PSM. Grafting experiments performed in our laboratory further revealed that only the PM-PSM cells contain the information for segmentation, that is possibly transmitted by the median pit (MP), which is the morphological Hensen's node at the stage of 6-somites. To test this possibility, we performed heterochronic, heterotopic, interspecific quail-chick MP and PM-PSM grafts and checked for the capacity of the MP region to induce somite formation in an ectopic competent region of a younger host embryo. The analysis of these experiments revealed that in fact, the MP is able to induce the formation of ectopic somites as well as the expression of the cycling genes in induced PSM tissue. Hence, the MP seems to be an important signalling centre detaining the information for segmentation. These findings also propose that the MP is the functional organizer at the stage of 6-somites in chick embryos.

Another important subject in the line of this thesis was to uncover the molecular mechanisms underlying the onset of the segmentation clock during chick embryonic development. We showed that the segmentation clock was not activated at the very beginning of embryo formation, since both spontaneous and artificially generated double axis which derive from the same embryonic blastoderm, presented different phases of the

expression of the cycling genes in their PSMs. Conversely, the phase of the cycle was always the same when the caudal part of the embryos was joined together. These results led us to verify whether Hensen's node was responsible for triggering the initiation of the segmentation clock. So, a series of heterotopic interspecific quail-chick Hensen's node grafts were performed and our results showed that the node is able to induce ectopic PSM tissue and to reinitiate the segmentation clock. The same observation was made when chick node grafts were transplanted to the lateral blastoderm of the same embryo. Taken together these findings revealed that the node emits a signal(s) able to induce the oscillations of the cycling genes in cells that are not fated to become somites and that it also has the capacity to restart the segmentation programme in these cells.

In the final part of this thesis we focused on the study of possible differences between the formation of anterior and posterior somites, in the chick embryo. The fact that rostral somites do not give rise to segmented structures and are not disrupted in several somitogenesis-related mutants has led to the generally accepted idea that they are different from caudal somites. In addition, it was reported in zebrafish and mouse that the first somites appear to form faster than more posterior ones. We analysed the expression of *notch1*, *delta1*, *hairy1*, *hairy2*, *lunatic fringe* and *hey2* at the level of the first 10 somites, and we found that the somitic expression pattern profile is not the same along the anterior-posterior axis and that the differences are not observed at the same somite level. We have additionally designed a time-lapse experiment that allowed us to monitor somite formation *in vivo*. A detailed analysis of the monitored embryos indicates that the first 2 to 3 somites form faster, almost concomitantly, than the more posterior ones. Thus, we conclude from this work that there are relevant differences in anterior *versus* posterior somites in what concerns their molecular and temporal characteristics in the chick embryo.

The work developed during the course of the present PhD thesis was based on studies performed at the early steps of the chick embryonic development. The ultimate aim of this study was to investigate the cellular and molecular basis of the onset of the segmentation clock in the chick embryo. A general introduction to the themes of this thesis is presented in Chapter One.

First, we wanted to investigate whether the segmentation clock was operating at the level of the prospective somitic territory. To answer this question we have carried out a detailed analysis of the expression pattern of the cycling genes at this level, in 6-somite stage chick embryos. We established that medial (M) and lateral (L) presomitic cells arise from distinct prospective territories, and that these cells are differently committed in what concerns segmentation and somite formation. Furthermore, this work also revealed that the expression of the cycling genes at this level defines a 'wave' that spreads along the longitudinal axis of the PSM prospective territory, which implies that the segmentation clock is providing cellular positional information in at least two dimensions: not only along the AP but also along the ML presomitic axis. These results are presented in the form of an article, which has been published, followed by a set of results as supplemental material. These data are presented in Chapter Two.

The next step on this work was to understand how the segmentation programme was activated in prospective PSM cells, by investigating the mechanisms underlying the onset of the segmentation clock. So, this work began by analysing whether the oscillations of the cycling genes were an intrinsic property of the cells of the prospective somitic territory or, in contrary if they could be induced by a diffusible signal from the organizer. A series of detailed grafting experiments consisting of both *in ovo* and *in vitro* interspecific Hensen's node grafts were performed in order to test this possibility. The expression of the cycling genes in the PSM of induced secondary axes put forward the hypothesis that the node has a role in activating the segmentation clock in prospective

somitic cells, and also that the initiation of the segmentation programme can be a consequence of an inductive signal from the organizer. These results were organized in the form of an article and are presented in Chapter Three.

In the last set of results, we have studied possible differences between anterior and posterior somites. The fact that rostral somites do not give rise to segmented structures and are not disrupted in several somitogenesis-related mutants has led to the generally accepted idea that they are different from caudal somites. To test this hypothesis we designed a series of experiments aiming at understanding the molecular and temporal differences between the formation of anterior versus posterior somites in the chick embryo. These results are presented in the form of an article, which has been published, followed by a set of results as supplemental material. These data are presented in Chapter Four.

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A	Anterior
Aei	After eight
APH	Axial paraxial hinge
AVE	Anterior Visceral Endoderm
bHLH	Basic Helix-Loop-Helix
Bea	Beamter
Bmp	Bone morphogenetic protein
CNH	Chordal neural hinge
Des	Deadly seven
Dll1	Delta-like 1
Dll3	Delta-like 3
DV	Dorso-ventral
Ephr	Ephrin
Erk	Extracellular signal-regulated kinase
Fgf	Fibroblast growth factor
FgfR	Fibroblast growth factor receptor
Foxc	Fork-head winged helix
fss	Fused somites
Hes	Hairy/Enhancer-of-Split
HH	Hamburger and Hamilton
Hox	Homeobox
IgG	Immunoglobulin G
Itga5	Integrin $\alpha 5$
L	Lateral
L-PSM	Lateral presomitic mesoderm
Lfng	Lunatic fringe
M	Medial
MAPK	Mitogen- activated protein kinase
Mesp	Mesoderm posterior
Mib	Mind-bomb

MP	median pit
M-PSM	Medial presomitic mesoderm
mRNA	messenger Ribonucleic acid
N1	Notch1
N2	Notch2
NICD	Notch intracellular domain
P	Posterior
PM-PSM	Prospective medial presomitic mesoderm
PM-PSM	Prospective lateral presomitic mesoderm
PS1	Presenilin1
PS2	Presenilin2
PSM	Presomitic Mesoderm
QCPN	Quail cell perinuclear (antibody)
RA	Retinoic acid
raldh2	Retinaldehyde dehydrogenase 2
rbp-jk	Recombining binding protein- jk signal
RptpΨr	Receptor protein tyrosine phosphatase Ψr
S	Segment border
Shh	Sonic hedgehog
su(h)	Suppressor of hairless
vt	Vestigial tail
VAD	Vitamin A deficient
Wnt	Wingless Wnt

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Chapter 1

Introduction

1. 1. The segmented nature of the vertebrate body

The vertebrate column is a classical landmark among vertebrates that is composed by a series of similar metameres along the body axis, which are the vertebrae. The orchestration between the vertebrate column and its associated musculature provides all vertebrates with an efficient protection to the internal vital organs, while conferring a high degree of mobility to the adult body.

The segmentation of the internal body plan has its origin early during embryonic development, and it is firstly evidenced with the appearance of transient segmented structures called somites. Somites emerge from the presomitic mesoderm (PSM) as epithelial spheres in an anterior to posterior sequential manner, bilateral to the axial midline of the embryo (Fig.1).

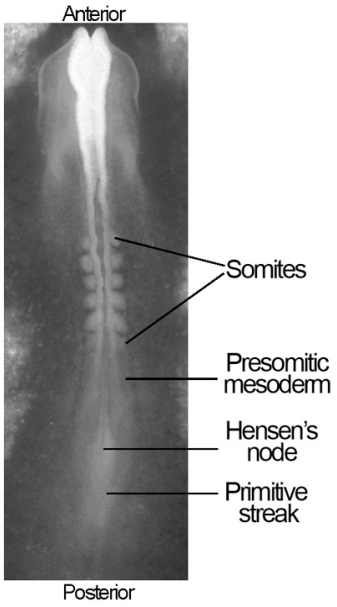


Figure 1. Somite formation in the chick embryo

Dorsal view of a 6-somite stage chick embryo. Epithelial somites arise from the rostral-most part of the presomitic mesoderm in an anterior to posterior direction. Concomitant with this process, the cells at the Hensen's node/primitive streak region are constantly renovated as a consequence of gastrulation.

The temporal periodicity of somite formation is remarkably precise. In the chick a pair of somites forms every 90 minutes, while in the mouse and zebrafish embryos it buds off every 2 hours and 30 minutes, respectively. Interestingly, in zebrafish, mouse and amphioxus embryos it was reported that the rostral-most somites form faster than more posterior ones, evidencing a relevant difference between anterior and posterior somites (Kimmel *et al*., 1995; Tam, 1981; Schubert *et al*., 2001).

Somites are epithelial transient structures that eventually differentiate into

several cell types. During somitic maturation, the cells facing the surface ectoderm differentiate into the dermomyotome. This somitic cellular subtype will in turn specifically originate the dermis of the back and the skeletal muscle precursors. Conversely, the ventral part of the somite de-epithelialises and gives rise to the sclerotome, which originates the skeletal elements of the vertebrate body. Somites are subdivided into anterior (A) and posterior (P) compartments that differ in their cell adhesive properties, essential for the maintenance of borders between segments (Stern and Keynes, 1987). Each vertebra originates from the posterior compartment of one sclerotome together with the anterior part of the following sclerotome, by a process called re-segmentation (Brand-Saberi and Christ, 2000) (Fig.2).

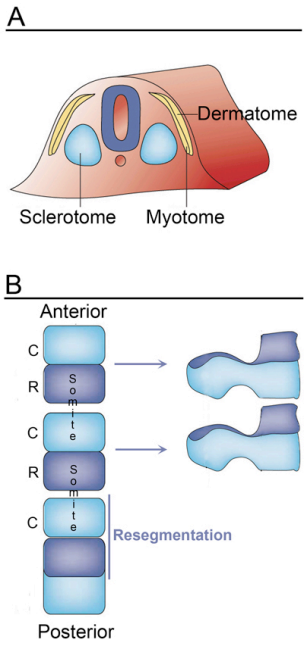


Figure 2. Resegmentation in Vertebrate Embryos

(A) Diagram representing somitic cell types during somite maturation.
(B) Schematic diagram of the resegmentation event that precedes vertebrae formation. During this process the rostral and caudal halves of somites segregate, and re-fuse with their neighboring halves to form vertebrae. C, caudal; R, rostral; *Adapted from Saga and Takeda, 2001.*

Neural crest cells and motor axons migrate exclusively through the anterior part of the somite, imposing a segmental organization to the precursors of the peripheral nervous system (Hirsinger *et al*., 2000).

In what concerns somitic derivatives, the first four somites differ from more posterior somites since they do not contribute to segmented structures. Instead, quail-chick grafts demonstrated that somites one and two contribute to the parasphenoid and to the basioccipital, exoccipital and supraoccipital, whereas somites three and four contribute to segments of the basioccipital and exoccipital

(Fig.3). These grafts also showed that somite five marks the border between head and neck, meaning that it corresponds to the first motion segment, therefore undergoing the process of re-segmentation (Couly *et al*., 1993; Huang *et al*., 2000).

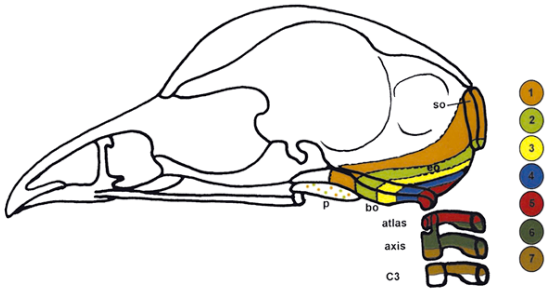


Figure 3. Anterior-most somites do not form segmented structures

Schematic illustration representing the derivatives of the anterior-most somites in the chick embryo. All somites are numbered from cranial to caudal, being each somite labelled by a different colour. This figure illustrates that somites 1 to 4 exclusively give rise to the basal neurocranium, whereas somite 5 already contributes to the first cervical vertebra.

bo basioccipital bone; *C3* third cervical vertebra; *eo* exoccipital bone; *p* parasphenoidal bone; *so* supraoccipital bone. *Adapted from Huang et al., 2000.*

1. 2. The beginning of a vertebrate embryo

Most of our understanding of the process of vertebrate segmentation largely relies on work performed on the chick embryo, especially due to its accessibility in the early stages. Once a chick egg is fertilized, it undergoes active cleavage becoming a blastula. When the egg is laid the chick embryo is called a blastoderm, where an *area pellucida* and a surrounding *area opaca* can be observed. At this stage the embryo consists of two layers, which are the epiblast and the hypoblast. The embryo proper arises from the epiblast whereas the hypoblast originates extraembryonic structures. At the time of laying, the anterior-posterior (AP) axis of the embryo is already determined, which is morphologically evident by a slight thickening in the posterior region, denominated Koller's sickle. At about 10 hours of incubation the primitive streak appears in the posterior pole of the *area pellucida*, from cells deriving from Koller's sickle. The formation of the primitive streak marks the beginning of the process of gastrulation. Gastrulation is the process by which the ectoderm, mesoderm and endoderm layers are formed. During streak elongation, epiblast cells move toward the midline of the extending primitive streak and ingress ventrally undergoing an epithelial to

mesenchymal transition. These cells then migrate to form extraembryonic and posterior tissues.

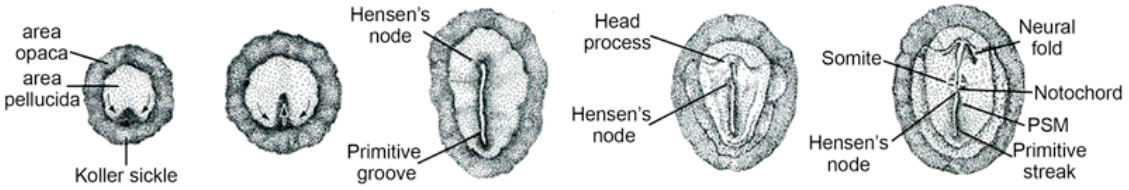


Figure 4. Early gastrulation stages in chick development

Schematic diagram representing the onset of gastrulation in the chick embryo. Gastrulation initiates with the appearance of the primitive streak, which arises from Koller's sickle in the posterior part of the embryo. As the embryo elongates its anterior part undergoes the process of organogenesis whereas at its posterior part gastrulation is still occurring. Rostral is to the top. *Adapted from Gilbert, 2000.*

Thus, this dynamic cellular movement implies that the cell populations in the primitive streak are in a constant flux throughout gastrulation (Cui *et al*., 2005). At the stage of full extension of the primitive streak, stage 4 HH (Hamburguer and Hamilton, 1951), Hensen's node becomes evident at its anterior end and a groove appears along the streak midline (Fig.4). Hensen's node is a bulb-like thickening lying at the cranial tip of the primitive streak. As gastrulation proceeds, the primitive streak gradually regresses toward the posterior margin, maintaining Hensen's node at its anterior end. As the streak and the node regress along the midline, a series of daughter cells from the node are laid down, giving rise to different axial structures in the embryo (Fig.4). The first cells that ingress through the node at stage 4 HH give rise to prospective notochord, prechordal mesendoderm, floor plate cells, prospective medial somites and endodermal precursors. Subsequently, the more posteriorly located cells that migrate through the primitive streak are mesodermal precursors, which will originate specific types of mesoderm according to their relative position in the axial midline. Hence, progressing along the primitive streak in a rostral to caudal direction, the most anterior cells to migrate will originate the lateral somitic precursors, the next are the intermediate mesoderm progenitors, then the lateral plate mesoderm precursors and finally the extraembryonic mesoderm (Fig.5) (reviewed by

Solnica-Krezel, 2005).

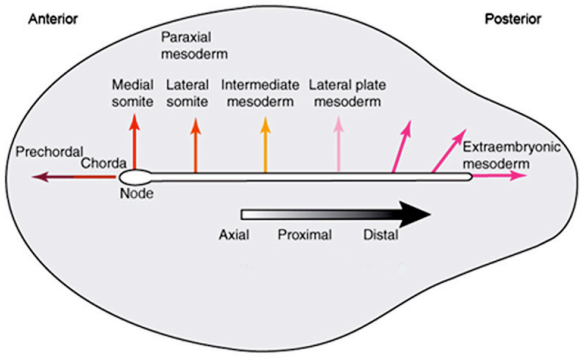


Figure 5. Mesodermal cell fates during vertebrate gastrulation.

Schematic diagram of a chick embryo at stage 4 HH, when the primitive streak is most elongated. The colored arrows point to the specific fate of the mesodermal cells that ingress at the corresponding axial level. *Adapted from Solnica-Krezel, 2005.*

As the avian embryo forms and takes shape a distinct anterior-to-posterior gradient of developmental maturity becomes obvious: while embryo organogenesis has started in the anterior part, in its posterior end gastrulation is still taking place. As mentioned before, Hensen's node is located at the tip of the primitive streak and it has a remarkable function during gastrulation involving the fate of its own cells and the tissues surrounding it. Outstandingly, the node has the capacity of inducing and patterning an ectopic secondary axis, which is the reason for being called the embryonic organizer.

1. 3. The organizer in vertebrate embryos

At the initial stages of embryonic development a small group of cells possesses unique morphogenetic and inductive properties that are crucial for the early establishment of the vertebrate body plan. The concept of organizer was first recognized with the classical grafting experiments of Spemann and Mangold in 1924 (Spemann and Mangold, 1924). These experiments reported the formation of a complete secondary axis when the dorsal blastopore lip of a pigmented amphibian embryo (*Triturus taeniatus*) was transplanted to the ventral side of a non-pigmented host embryo (*Triturus cristalus*) (Spemann and Mangold, 1924). Remarkably, the authors observed that this ectopic axis consisted of neural tube, somites, pronephros and gut that derived from the host. Conversely, notochord and floor plate were always derived from graft. The fact that the neural tube arose from prospective epidermis tissue and that somites

originated from prospective lateral plate tissue, led to the conclusion that induction by the organizer resulted in a change of fate of the host cells near the graft. Hence, the dorsal blastopore lip in amphibians was designated the embryonic organizer due to its ability to induce an axis of near normal proportions and arrangement, from a region fated to become non-neural ectoderm and non-axial mesoderm (reviewed by Gerhart, 2001). Taken together, the experiment of Spemann and Mangold on the organizer has established two important concepts. The first is the notion of induction that refers to the change of fate of a group of cells in response to signals from other cells. The second is the concept of competence that relates to the ability of the host cells to respond to the inductive signals. The impact of these findings on modern experimental embryology makes them one of the milestone experiments of the 20th century. Thereafter, several other works contributed to the identification of the organizer equivalent structure in other vertebrate embryos such as the chick Hensen's node (Waddington, 1932), the fish embryonic shield (Oppenheimer, 1936; Shih and Fraser, 1996), the frog blastopore lip (Gimlich and Cook, 1993; Smith and Slack, 1983) and the mouse node (Beddington, 1994).

Elegant embryonic manipulation experiments in chick embryos demonstrated that the cells that make up the node are constantly changing during gastrulation. This situation enables continuous molecular interactions between neighbouring cells that are crucial for embryonic patterning and cell fate determination (Joubin and Stern, 1999). Thus, this work shed new light into the definition of node, considering it as a population of cells that is not fixed but that is changing with time as development proceeds.

1. 4. Models for organizer activity in vertebrate AP patterning

1. 4. 1. Separable head and trunk organizers

Ever since the discovery of the organizer, a considerable effort has been made to explain how a single population of cells is able to induce a complete embryonic axis. One of the initially proposed explanations for the organizer

activity was the ‘separable head and trunk organizers’. From the time this hypothesis was proposed significant amount of data came up, providing either supporting or contesting evidence to this model.

The notion of separable head and trunk organizers appeared based on experiments performed by Spemann in amphibian embryos, which revealed that young organizer grafts induced complete secondary axes when transplanted to an ectopic site, while older organizer grafts only induced posterior axes (Spemann 1931 and 1938). These studies were subsequently supported by experiments in the chick embryo (Storey *et al*, 1992). In addition, this idea was further validated by the finding that neither early nor late stage mouse nodes seemed to be able to induce forebrain and midbrain structures, in contrast to other vertebrate organizers (Beddington, 1994; Tam *et al*., 1997; Tam and Steiner, 1999). It is now clear that the inability of the mouse node to induce anterior neural tissue was due to the absence of prechordal mesendoderm precursors from the graft, at the stages where the node grafting experiments were performed (Kinder *et al*., 2001). Consistent with this, studies in chick and zebrafish described that once prechordal mesendoderm precursors leave, the organizer is no longer able to induce anterior-most structures (Storey *et al*., 1992; Izpisua-Belmonte *et al*., 1993; Lemaire *et al*., 1997; Saude *et al*., 2000). So, these experiments supported the idea that the induction by the node of progressively different structures along the axis is due to the different molecular properties of the organizer as the embryo develops.

The fact that the node grafting experiments in the mouse revealed that anterior head structures were not induced was initially interpreted as evidence for the existence of an independent head organizer. In fact, this hypothesis was supported by the finding that the formation of anterior-most structures required the function of genes expressed in the anterior visceral endoderm (AVE), a group of cells with extraembryonic fate that is located anterior to the node region. This was demonstrated by the phenotype of mouse mutants for genes expressed in the AVE, such as *otx2* and *lim1*, that exhibited normal formation of trunk and tail but presented lack of head structures (Acampora *et al*., 1995; Matsuo *et al*.,

1995; Ang *et al*., 1996; Shawlot and Behringer, 1995). In addition, ablation experiments of the AVE showed that the resulting embryos did not develop anterior-most head structures (Thomas and Beddington, 1996). These results together with the evidence that mouse *hnf3β* mutants, characterised by the absence of the node and its derivatives, still developed a neural tube displaying a correctly patterned AP axis (Ang and Rossant, 1994; Weinstein *et al*., 1994), were another indication that anterior brain-inducing signals could be provided by a signal located outside the node.

Despite the undeniable evidence that the AVE plays an important role in the normal induction of anterior brain structures, ectopic grafts of the AVE do not induce anterior neural genes unless these grafts are combined with anterior and posterior epiblast fragments of the early gastrula mouse embryo (Tam and Steiner, 1999). Similarly, the chick hypoblast, which is the avian AVE equivalent, is also unable to induce neural tissue when grafted into competent epiblast (Foley et a., 2000). Hence, taken together these data do not support the existence of a head organizer independent from the node, located at the AVE.

However, work performed in the frog *Xenopus laevis* and zebrafish embryos revealed the existence of specific signals that induced either head or trunk/tail structures (see below) (Bouwmeester, *et al*., 1996; Glinka *et al*., 1997; Glinka *et al*., 1998; Agathon *et al*., 2003), providing additional proof of separable head and trunk/tail organizers. This idea was again contested by the fact that, in reality, no other structure apart from the organizer (as long as it contains prechordal mesendoderm precursors) is able to induce complete secondary axes. The alternative view is that the regions located outside the node that are important for head induction, most probably function in combination with cell populations that have previously received neural inductive signals (Stern, 2001). Altogether, the current available data does not support the 'separable head and trunk organizers'.

1. 4. 2. Activation-transformation model

An alternative to the head and trunk organizer model is the activation–

transformation model, proposed by Nieuwkoop in 1952 (Nieuwkoop, 1952). The experiments leading to the proposal of this model were performed in *Xenopus* and consisted in grafting longitudinal strips of double-layered competent gastrula ectoderm perpendicular to the presumptive neural plate of early gastrula embryos, at different AP levels. The author observed that implanting the grafts into the prospective forebrain region of the host gave rise to a graft whose proximal-distal axis exclusively developed forebrain structures. Conversely, implantation of the graft into prospective hindbrain region led to a graft that exhibited a hindbrain character in its proximal part (in contact with the host tissue) and a forebrain character in its most distal part. Consistent with these results, when a graft was implanted into a more caudal region, its proximal part always adopted the AP character of the host neural plate region with which it was in direct contact, while its distal part always developed into a tissue with anterior neural character. Based on these experiments Nieuwkoop postulated that the induction and patterning of the nervous system along the AP axis occurred in two steps. In the first step, an activator signal was responsible for inducing nervous tissue with 'anterior' (forebrain) character. In the second step, 'transformation' signals, originally proposed to derive from the organizer, progressively posteriorised nervous tissue into more caudal fates (Nieuwkoop, 1952; Nieuwkoop *et al*., 1999).

Genetic evidence supporting this model has been provided mainly by studies performed in amphibians. The finding that antagonists of the Bone Morphogenetic Protein 4 (BMP4) such as Chordin, Noggin and Follistatin induced the expression of anterior neural markers in *Xenopus* (Sasai *et al*., 1995; Lamb *et al*., 1993; Hemmati-Brivanlou, *et al*., 1994) indicated that these molecules could be responsible for the initial "activation" step of the above model. Furthermore, a study using explants and tissue recombinants of early neural plate in frog embryos reported that a combination of prospective forebrain and posterior axial tissues induced midbrain and hindbrain fates (Cox and Hemmati-Brivanlou, 1995). Consistent with the Nieuwkoop model, this work also showed that the Fibroblast Growth Factor (Fgf) signalling mimicked the

caudalising activity of posterior tissues, thus providing evidence that Fgf was functioning as a posteriorising signal in *Xenopus* embryos. The caudalising role of the Fgf signalling was subsequently confirmed by other works and found that it can also function by inducing the expression of downstream target genes (Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Pownall *et al*., 1996; Muhr *et al*., 1997; Hardcastle *et al*., 2000; Streit *et al*., 2000). In addition to Fgfs, many more studies have suggested that the Wingless-Wnt (Wnt) and Retinoic Acid (RA) signalling pathways also function as caudalising factors (Stern *et al*., 2006). Additional evidence has also proposed that the mesoderm layer is an important posteriorising signalling source that directly patterns the overlying ectoderm (Itasaki *et al*., 1996; Bang *et al*., 1997; Muhr *et al*., 1997; Poznanski and Keller, 1997; Woo and Fraser, 1997; Gould *et al*., 1998; Bang *et al*., 1999; Gaunt *et al*., 1999; Muhr *et al*., 1999; Chen *et al*., 2000; Wacker *et al*., 2004).

Embryological manipulations in the chick embryo demonstrated that the hypoblast, the avian equivalent of the AVE, induces the transient expression of early anterior neural markers, such as *sox3* and *otx2* when transplanted to the *area opaca* of a host embryo. Furthermore, the same experiments also showed that the hypoblast is not able to induce the development of anterior forebrain structures (Foley *et al*., 2000). These results led to the proposal that the activation step of the Nieuwkoop model was not sufficient to induce anterior neural character tissues, but rather that it induced a pre-neural state, which must be consolidated by stabilising/maintenance signals. Hence, a modified version of the "activation-transformation" model was proposed where a stabilising step followed the initial activation of the presumptive neurectoderm in order to fix and retain a neural character that could later be posteriorised during the transformation step of this model (Foley *et al*., 2000; Stern and Fraser, 2004).

1. 4. 3. The time-space translator model

Homeobox (Hox) genes are known for conferring positional identity along the AP axis in vertebrate embryos. These transcription factors are lined up into four clusters (from Hoxa to Hoxd) that are sequentially expressed along the axis.

Genes at the 3' end of the cluster are expressed anteriorly and earlier than the genes at the 5' end that are expressed later in progressively more posterior regions (Deschamps and Van Nes, 2005).

A recent work in *Xenopus* described the temporal collinear activation of a series of Hox genes in non-organizer mesoderm during the frog gastrulation and further reported that the expression of these genes is excluded from the organizer (Wacker *et al*., 2004). In addition, this study also analysed Hox gene expression in induced hyperventralised embryos (with blocked Wnt activity and organizer development) and dorsalised embryos (with activation Wnt activity that leads to hyper organizer development): The expression of Hox genes is downregulated in the ectoderm of ventralised embryos but remains normal at the mesoderm layer; the expression of these genes is totally abolished in the dorsalised frog embryos. Organizer grafts into ventralized embryos restored both the formation of well-developed embryos and the normal sequence of Hox gene expression zones, which led the authors to state that the normal sequence of Hox expression requires an interaction between the non-organizer mesoderm and the organizer (Wacker *et al*., 2004). This study further demonstrated that the early Hox expression is transient unless it is stabilized by signals from the organizer. These findings prompted the authors to propose the "time-space translator" model for AP patterning that is based on the timed interactions of the non-organizer mesoderm with the Spemman organizer. This model postulates that different portions of the non-organizer mesoderm interact with the organizer at different time points establishing positional values along the AP axis. This positional information is translated into a spatial pattern by the sequential activation of Hox gene expression along the axis as the embryo elongates (Wacker *et al*., 2004; Stern *et al*., 2006).

1. 5. The molecular players in axis induction

1. 5. 1. Neural induction

The discovery of the Spemann organizer raised the interest to understand the mechanisms underlying the induction of neural tissue. Experiments performed in amphibians demonstrated that dissociated animal caps differentiated into neural tissue, whereas intact animal caps differentiated into epidermis (Godsave and Slack, 1989; Gunz and Tacke, 1989; Sato and Sargent, 1989). These findings led to the proposal of the default model for neural induction, which states that that the default tendency of the embryonic ectoderm is to form neural tissue, unless it is prevented to do so. Consistent with this, it was found that BMP proteins are strong inducers of epidermis (Suzuki *et al*., 1997; Wilson and Hemmati-Brivanlou, 1995). Conversely, the organizer expresses several BMP antagonists whose misexpression leads to neural induction in animal cap assays in *Xenopus* (Lamb *et al*., 1993; Hemmati-Brivanlou *et al*., 1994; Sasai *et al*., 1995; Bouwmeester *et al*., 1996; Hansen *et al*., 1997). Accordingly, the normal expression pattern of these factors during development is consistent with their role in neural induction (Chang and Hemmati-Brivanlou, 1999; Hawley *et al*., 1995; Hemmati-Brivanlou and Thomsen, 1995).

However, subsequent studies in amniotes revealed an increasingly complexity in the regulation of neural fate induction as well as suggesting that the model for neural induction was perhaps too simplistic. The reasons for this are supported by studies in the chick where it was demonstrated that misexpression of BMP antagonists was not sufficient to induce neural tissue, in contrast to the results obtained in amphibians (Streit *et al*., 1998; Linker and Stern, 2004). Also, mouse mutants for BMP antagonists still develop a nervous system (McMahon *et al*., 1998; Bachiller *et al*., 2000), which is a strong indication that BMP inhibition may not be the only determinant for the process of neural induction. The fact that initiation of neural induction occurs before the onset of gastrulation, involving both the activity of Fgf and the inhibition of Wnt signalling pathways in the chick embryo, further supported this idea that BMP antagonists are not the only players

in the process of neural induction (Streit *et al*., 2000; Wilson 2000; Wilson *et al*., 2001).

Interestingly, a recent work has challenged the initial experiments performed in *Xenopus* animal caps and revealed that BMP inhibition is not sufficient to induce specific neural markers (Linker and Stern, 2004). Furthermore, the results obtained in this study showed that neural induction in the chick only requires BMP inhibition as a late step and that this process involves signals other than Fgfs and Wnt antagonists. Taken together these findings led to the proposal that rather than being a default state, neural induction involves a succession of signalling events that still remain to be identified (Linker and Stern, 2004).

1. 5. 2. Anterior-posterior patterning

In parallel to the studies on vertebrate neural induction, experimental embryologists also became interested in the search for the molecular players involved in patterning the whole embryo. During vertebrate development, the differential inhibition by the organizer along the AP axis controls the formation of either the anterior or progressively more posterior parts of the embryo. Studies in the frog embryo have identified Cerberus as a potent head inducer that is a multifunctional antagonist of the BMP, Wnt and Nodal signalling pathways. This brought up the idea that inhibition of all three signalling pathways was required to form head structures (Piccolo *et al*., 1999; Silva *et al*., 2003). Consistent with this idea, loss of function experiments performed in mice, zebrafish and Xenopus embryos have provided broad evidence for the requirement of BMP, Wnt and Nodal signalling in head formation (reviewed in Niehrs *et al*., 2004).

The formation of more posterior structures was shown to be dependent on the inhibition of BMP by its antagonists *chordin*, *noggin* and *follistatin* (De Robertis *et al*., 2000). So, a common feature for inducing anterior and posterior structures seems to be the inhibition of BMP signalling. In contrast, studies performed mainly in mouse and frog embryos showed that both Wnt and Nodal signalling pathways are required for trunk formation (Conlon *et al*., 1994; Vincent

et al., 2003; Hoppler *et al*., 1996; Kiecker and Niehrs, 2001; Beck and Slack, 1999). Thus, the induction of trunk structures seems to require the activity of Wnt and Nodal together with the inhibition of BMP signalling.

Interestingly, work in zebrafish has reported the existence of a tail organizer independent of the classical shield. This study showed that overexpression of a combination of Wnt, Nodal and BMP signalling pathways leads to the formation of extra tails, providing evidence for the requirement of all these factors in the induction of tail structures (Agathon *et al*., 2003). Furthermore, a tail organizing activity was also described in frog and chick embryos whose tails transplanted to an ectopic region have the ability to organize a posterior axis (Gont *et al*., 1993; Knezevic *et al*., 1998).

The Fgf signalling pathway also has a prominent role in patterning the vertebrate AP axis. Loss of function studies performed in frog, mouse and zebrafish embryos where several components of the Fgf pathway were disrupted revealed a strong phenotype in axis development, specifically compromising the formation of posterior regions (Bottcher and Niehrs, 2005). Furthermore, Fgfs are important caudalising factors required to pattern the neurectoderm and mesoderm along the AP axis (Doniach, 1995; see section 1.4.2). The overexpression of Fgfs in chick, mouse and Xenopus embryos leads to the upregulation of posterior members of the Hox complex, indicating that Fgf regulates the AP axis by regulating the expression of the Hox genes (Pownall *et al*., 1996; Partanen *et al*., 1998; Bel-Vialar *et al*., 2002). Consistent with this, the overexpression of a dominant negative form of the Fgf receptor 1 (Fgfr1) represses the posterior expression of the *Hoxb-9* gene (Godsave and Durston, 1997; Pownall *et al*., 1996).

Retinoids are molecular derivatives of vitamin A and are strongly implicated in the process of AP patterning along the vertebrate axis. RA signalling regulates the cascade of genes that leads to neural differentiation in the anterior part of the embryo and its function is directly opposed to the activity of the Fgf signalling pathway in the posterior embryonic axis (Diez del Corral *et al*., 2003; Shiotsugu *et al*., 2004). In fact, this mutual opposition of Fgf and RA pathways controls not

only neural differentiation but also mesoderm segmentation (Diez del Corral *et al*., 2003; Sirbu *et al*., 2006; see section 1.9). Furthermore, the interplay between these pathways directly regulates Hox gene expression along the axis. Several studies have shown that exposure to RA prevents the expression of posterior Hox genes while exposure to Fgf signalling leads to their rostral expansion (Bel-Vialar *et al*., 2002; Liu *et al*., 2001; Maden, 2002; Omelchenko and Lance Jones, 2003). In a similar way to Fgf signalling, RA signalling also functions a caudalising factor to pattern the neurectoderm along the axis (Durston *et al.,* 1989; Ruiz i Altaba and Jessell, 1991; Kessel, 1992; Avantaggiato *et al.,* 1996; Blumberg *et al.,* 1997; Muhr *et al*., 1997; Grandel *et al.,* 2002; Kudoh *et al.,* 2002; Diez del Corral *et al.,* 2003; Oosterveen *et al.,* 2003; Sockanathan *et al.,* 2003; Molotkova *et al.,* 2005).

Taken together, these facts suggest that the orchestration of the axial patterning results from Bmp, Nodal, Fgf, Wnt, and RA signals that generate positional information and ultimately lead to the correct development of the embryo.

1. 6. The origin of somitic mesoderm

The chick embryo is a very useful model for performing cell-tracing techniques, which have been vastly used to outline cell migration paths and to accurately establish cellular prospective territories (reviewed by Hirsinger *et al*., 2000). Taking advantage of the use of such techniques several studies have provided the accurate localization of prospective somitic cells. In the chick, somitic precursor cells are already detected at pre-primitive streak stages before the onset of gastrulation. At this time in development these cells are located in the posterior epiblast and when primitive streak starts to appear they remain posteriorly located on both sides of the midline (Hatada and Stern, 1984; Lopez-Sanchez, 2001). At the stage of full extension of the streak, stage 4 HH, prospective somitic cells have moved to the anterior part of the primitive streak and are also located within Hensen's node. While the node regresses throughout

development these cells ingress laterally through the streak, giving rise to the unsegmented paraxial mesoderm, on each side of the neural tube (Stern *et al*, 1988; Selleck and Stern, 1991; Catala *et al*., 1996; Psychoyos and Stern, 1996). From the stage of 16 to 20 somites onwards, the remnants of Hensen's node and primitive streak become a mass of mesenchymal cells called the tail bud, which continues to undergo gastrulation and to provide new somitic cells until the total number of somites has been achieved (Catala *et al*., 1995; Schoenwolf *et al*., 1992).

Likewise, in the earliest stages of mouse development the somitic progenitor cells are initially located in the epiblast layer and then become situated in the anterior part of the primitive streak. These cells then ingress through the streak and will give rise to the two bands of PSM tissue on each side of the axial structures. The similarity in the morphogenetic movements and patterning of the somitic precursors in both chick and mouse embryos evidences a clear conservation in terms of topological fates among these vertebrates (Lawson *et al*., 1991; Smith *et al*., 1994; Parameswaran and Tam, 1995; Tam and Beddington, 1987).

1. 7. Somite formation in the light of classical models

The formation of somites from the PSM is the first conspicuous segmentation event occuring during vertebrate development. In fact, significant efforts are being made to understand the mechanisms underlying this process and there are three main classical models that explain the periodicity of somite formation: the Meinhardt's model, the cell cycle model and the clock-and-wavefront model.

Meinhardt proposed that prior to the formation of each somite, presomitic cells undergo several oscillations between two alternate states corresponding to the prospective A and P somitic compartments (Meinhardt, 1986). The author postulated that the confrontation between cells of incompatible A and P states would result in a physical boundary between consecutive somites, since there is

no intermingling between cells from different states. However, in this case the juxtaposition of these two segregated states would also lead to the formation of a physical barrier in the middle of a somite. To overcome this problem Meinhardt postulated a third oscillating state, called segment border (S), which corresponds to the somitic boundary. While distinct cell adhesive characteristics allowed the identification of A and P somitic compartments (Keynes and Stern, 1987), no S cells have been identified so far. In addition, a study using confocal time-lapse microscopy challenges the Meinhardt's model by showing that some degree of cell intermingling between A and P somitic compartments does occur during somite boundary formation (Kulesa and Fraser, 2002).

In 1988, Primmett and collaborators demonstrated that a single heat-shock applied to the chick embryo gives rise to several segmentation abnormalities that are repeated along the AP axis with a regular interval of 6 to 7 somites (Primmett *et al.*, 1988). The time required to form 6 somites in the chick is 9 hours, which corresponds to the time necessary for the completion of the cell cycle in the PSM (Primmett *et al.*, 1989). Consequently, it was postulated that the cell cycle would function as an internal cellular clock related to the process of segmentation: cells located in the rostral PSM share some degree of cell cycle synchrony, increase their adhesive properties and thus assemble and give rise to a somite. However, no correlation between the duration of the cell cycle in PSM and the rate of somitogenesis, which takes 90 minutes in the chick embryo, has been found so far.

The clock-and-wavefront model postulated the existence of two independent phenomena accounting for periodic somite formation (Cooke and Zeeman, 1976). On one hand, there is an intrinsic clock compelling presomitic cells to oscillate synchronously between a permissive and a non-permissive state. On the other hand and concomitantly, a wavefront travels along the embryonic axis establishing an AP gradient of differentiation. This model predicts that in order to form a somitic boundary, a group of PSM cells oscillating synchronously has to be reached by the wavefront of differentiation. Experimental data gathered so far seem to support both assumptions of the

clock-and-wavefront model.

Interestingly, several modified versions of this model have been proposed integrating recent available data and allowing experimental predictions concerning the process of somite periodicity (Kerszberg and Wolpert, 2000; Schnell and Maini, 2000; Dubrulle and Pourquie, 2002; Baker *et al*., 2006). The clock and wavefront model will be further discussed in the next sections.

1. 8. The segmentation clock underlying somite formation

In 1997, Palmeirim and collaborators provided the first molecular evidence for the existence of an intrinsic oscillator controlling the periodicity of somite formation in the presomitic cells of chick embryos. They observed that, within groups of embryos with the exact same number of somites, the basic Helix-Loop-Helix (bHLH) transcription repressor *hairy1* displayed remarkably different patterns of expression in the PSM. The authors have also demonstrated that this dynamic expression was reiterated every 90 minutes, corresponding exactly to the time required to form a pair of somites (Fig.6).

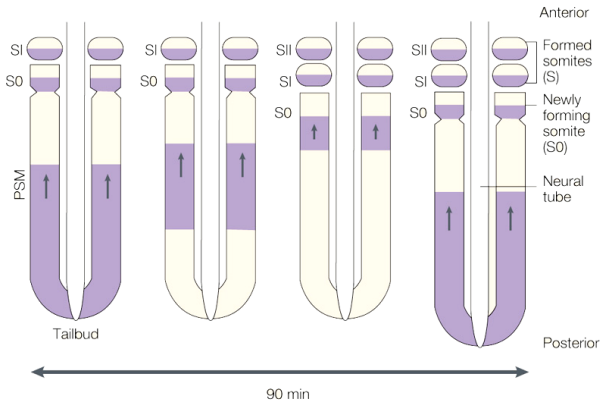


Figure 6. The segmentation clock in vertebrate embryos

Schematic diagram representing the oscillatory behaviour of hairy1 in the chick PSM. The *hairy1* mRNA cyclic expression appears as a caudal-to-rostral wave that crosses the PSM, every 90 minutes. This is precisely the time required to form one pair of somites. Once an epithelial somite forms, the *hairy1* expression becomes restricted to the posterior somitic compartment and a new cycle of expression begins in the caudal PSM. *Adapted from Saga and Takeda, 2001.*

Moreover, these oscillations of the *hairy1* mRNA were shown to be an

autonomous property of PSM cells and neither a consequence of cell migration nor dependent on a diffused signal travelling within the PSM. This pioneer study has demonstrated that presomitic cells undergo several periodic oscillations of the *hairy1* gene expression before they incorporate a somite, conceptually describing a caudal wave that progresses anteriorly and stabilizes in a narrow stripe in the rostral PSM (Palmeirim *et al.*, 1997). Interestingly, recent evidence has been provided showing that dissociating PSM cells *in vitro* become unable to maintain synchronous oscillations. These findings imply that cell communication or a community effect is essential for the synchronization of the normal cyclic gene expression in these cells (Maroto *et al*., 2005). Consistent with these results, Masamizu and collaborators also observed that dissociated PSM cells present individual oscillations of the Hairy/Enhancer-of-Split-1 (*Hes1*) gene with an unstable period and amplitude, which suggests that cell-cell communication is also important to stabilize the cellular oscillators (Masamizu *et al*., 2006).

After the discovery of the *hairy1* gene in the chick embryo, many other genes were reported to have a cyclic expression at the level of the PSM, suggesting that the segmentation clock involves a complex genetic network. It is now clear that the molecular mechanism underlying somitogenesis is highly conserved among vertebrates, since periodic gene transcription has also been described in mouse, frog, zebrafish and medaka. The majority of the cycling genes codes for Hes family targets of the Notch signalling pathway such as *hairy1* and *hairy2* in the chick (Palmeirim *et al.*, 1997 and Jouve *et al.*, 2000), *hey2* both in chick and mouse (Leimeister *et al.*, 2000), *hes1* and *hes7* in mouse (Jouve *et al.*, 2000; Bessho *et al.*, 2001b), *her1* and *her7* in zebrafish (Holley *et al.*, 2000; Oates and Ho, 2002) *esr9* in the frog (Li *et al.*, 2003) and *her7* in medaka (Elmasri *et al*., 2004). Other cycling genes encode a modulator of the Notch signalling pathway, *lunatic fringe* (*lfng*), in the chick and mouse (McGrew *et al.*, 1998; Aulehla and Johnson, 1999; Forsberg *et al.*, 1998), a Notch ligand, *deltaC*, in zebrafish (Jiang *et al.*, 2000) and the Notch intracellular domain (NICD) (Huppert *et al*., 2005; Morimoto *et al*., 2005). Furthermore, *axin2* and *nkd1* that function as repressors of the Wnt signalling pathway, were shown to exhibit an

oscillatory expression pattern in the mouse PSM (Aulehla *et al.*, 2003; Ishikawa *et al*., 2004). Taken together, these findings strongly suggest that Notch and Wnt signals are both implicated in the regulation of the segmentation clock.

Interestingly, a recent work has reported a new class of cycling genes, *snail1* and *snail2*, which present an oscillatory expression in the PSM of mouse and chick embryos, respectively (Dale *et al*., 2006). The relevance of this work is that the cyclic expression of the *snail* genes seems to be Notch independent but Wnt and Fgf signalling dependent. The authors suggest that these genes may be providing the link between the segmentation clock and the wavefront of differentiation (see section 1. 9) (Table 1).

TABLE 1. Cyclic genes reported in vertebrate species

Chick	Mouse	Zebrafish	Frog
hairy1 (Palmeirim *et al*., 1997) ***hairy2*** (Jouve *et al*., 2000) ***hey2*** (Leimeister *et al*., 2000) ***lunatic fringe*** (McGrew *et al*., 1998; Aulehla and Johnson, 1999) ***snail*** (Dale *et al*., 2006)	***hes1*** (Jouve *et al*., 2000) ***hes7*** (Bessho *et al*., 2001b) ***hey2*** (Leimeister *et al.*, 2000) ***lunatic fringe*** (Forsberg *et al*., 1998) ***axin2*** (Aulehla *et al*., 2003) ***Nkd1*** (Ishikawa *et al*., 2004) ***NICD*** (Huppert *et al*., 2005; Saga *et al*., 2005) ***snail*** (Dale *et al*., 2006)	***her1*** (Holley *et al*., 2000) ***her7*** (Oates *et al.*, 2002) ***deltaC*** (Jiang *et al*., 2000)	***esr9*** (Li *et al*., 2003)

1. 8. 1 The segmentation clock machinery

The mechanism that generates the cyclic expression pattern of the segmentation clock genes is still not completely understood and the most common explanation for this process is that it relies on feedback inhibition. The first direct evidence for the molecular mechanism that generates the oscillatory behaviour of the cyclic genes was presented in a study performed in cell culture (Hirata *et al.*, 2002). The authors demonstrated that not only *hes1* mRNA, but

also Hes1 protein, undergo oscillations of expression with the same periodicity as somite formation. These oscillations are produced by a negative feedback loop in which Hes1 protein periodically represses its own transcription, suggesting that a similar mechanism could be responsible for the transcriptional oscillations generated by the segmentation clock. In addition, it was demonstrated in this work that *hes1* mRNA oscillations are blocked in the absence of a functional Hes1 protein, suggesting that this protein might regulate its own promoter in the mouse embryo (Hirata *et al.*, 2002). Another study in mouse embryos revealed that both *hes7* mRNA and Hes7 protein oscillate in the PSM tissue (Bessho *et al.*, 2003). This work showed that at each time point the localisation of the Hes7 protein domains do not overlap with the regions where *hes7* mRNA is expressed. Also, the transcription of the *hes7* gene is constitutively activated in the absence of Hes7 protein and it is downregulated when the Hes7 protein is stabilized. Therefore, Hes7 oscillations in the PSM also rely on a negative autoregulatory loop (Bessho *et al.*, 2003), which is in accordance with the finding that Hes7 protein instability is crucial for cyclic gene expression (Hirata *et al*., 2004). In zebrafish, the cyclic genes *her1* and *her7* seem to negatively regulate their own expression, although there is no data regarding their protein expression in the PSM (Holley *et al.*, 2002; Oates and Ho, 2002). A recent study has further reported that Her13.2, which functions downstream of the Fgf signalling, augments the autorepression of *her1* by physically interacting with the Her1 protein (Sieger *et al*., 2006). This data strongly suggests that the function of *her13.2* is to mediate the regulation of the cycling genes by the Fgf signalling in the zebrafish embryo. In the mouse, the structural counterpart of the *her13.2* is the *hes6* gene that is not expressed in the PSM, implying different regulations of the clock machinery in these vertebrate species.

Another negative feedback loop involving the periodic production of Lfng protein was described in the chick PSM (Dale *et al.*, 2003). In addition to *lfng* mRNA, Lfng protein cycles with the same periodicity as somite formation. Furthermore, overexpression of *lfng* in the chick PSM impairs the cyclic expression of the Notch downstream targets, *hairy1*, *hairy2* and endogenous

lfng. Since Lfng is a modulator of Notch activity, it seems that the oscillations of segmentation genes are due to periodic inhibition of Notch activation (Dale *et al.*, 2003). In the mouse, *notch1* is expressed throughout the anterior PSM and it does not present a cyclic expression pattern. However, recent findings showed that unlike its RNA expression, a processed NICD antibody exhibits a dynamic pattern in the PSM, indicating that the activity of Notch1 oscillates in the posterior PSM of mouse embryos (Huppert *et al*., 2005; Morimoto *et al*., 2005). Also, it seems that no Notch1 activity is found in *delta-like1* (*dll1*) mutants while it stops oscillating in *lfng* mutant embryos. These results led to the conclusion that the Dll1 ligand is necessary for the activation of Notch1 while Lfng seems to act as a suppressor of Notch activity, thus generating the oscillatory activation of Notch1 (Morimoto *et al*., 2005). Altogether these data are in agreement with the previous findings in the chick (Dale *et al*., 2003), suggesting that in the mouse posterior PSM, *lfng* is activated by NICD and suppressed by Hes7, while in turn Lfng acts to inhibit the Notch activity (Morimoto *et al*., 2005) (Fig.7).

Studies performed in zebrafish revealed that the function of Lfng might be different from the one in the chick. In zebrafish, *lfng* mRNA does not oscillate in the PSM (Prince *et al.*, 2001; Leve *et al.*, 2001) and it seems that the periodic activation of Notch is undertaken by the cyclic gene *deltaC* (Jiang *et al.*, 2000). In contrast, *lfng* oscillates both in the chick and in the mouse PSM (McGrew *et al.*, 1998; Aulehla and Johnson, 1999; Forsberg *et al.*, 1998) although its constitutive expression does not abolish cyclic expression of endogenous *lfng* in the mouse embryos (Serth *et al.*, 2003). This implies that the activity of Notch alone cannot be the only determinant of cyclic gene expression in the mouse embryo, which is in agreement with recent findings on the role of presenilins in the control of the segmentation clock (Huppert *et al*., 2005) (see Section 1.10). Furthermore, the function attributed to the Wnt signalling pathway as a regulator of the clock by acting upstream of the Notch pathway is an alternative explanation for the above results in the mouse embryo (Aulehla *et al.*, 2003). Also, it was shown that both *axin2* and *nkd1*, which function as repressors of the Wnt pathway by binding to Dishevelled, are expressed in the PSM in a cyclic fashion. In the *vestigial tail* (*vt*)

mutant mouse, which is the hypomorphic mutant of *wnt3a,* there are caudal segmentation defects and, interestingly, *axin2* is not expressed and the expression of *nkd1* is greatly reduced (Aulehla *et al.*, 2003; Ishikawa *et al*., 2004). In addition, the *nkd1* expression is upregulated and arrests its oscillatory pattern in Hes7 mutants. These findings suggest that the transcription of *nkd1* and *axin2* depend on the Wnt signalling pathway, although the oscillatory behaviour of *nkd1* also relies on the function of Hes7 (Ishikawa *et al*., 2004). An interesting feature of the cyclic behaviour of these Wnt antagonists is that the *nkd1* expression oscillates in phase with the expression of *lfng* and *hes7*, whereas *axin2* exhibits a different expression phase of these genes. Taken together these data suggest that there is a complex regulatory feedback loop involving the Wnt and Notch pathways. Supporting this idea, Lef/TCF binding sites were described in the Dll1 promoter (Galceran *et al.*, 2004; Hofmann *et al*., 2004). Furthermore, the Wnt signalling pathway may be regulated by Notch signalling either via the transcriptional regulation of *nkd1* by Hes7 or by the NICD inhibition by Dishevelled (Ishikawa *et al*., 2004; Axelrod, *et al*., 1996). Conversely, since the expression of *axin2* oscillates alternately with the one of *nkd1*, it is also possible that the cyclic transcription of the *nkd1* gene originates a negative feedback loop directly regulating the *axin2* oscillations (Ishikawa *et al*., 2004). These findings establish an additional molecular link between the Wnt and the Notch pathways during somitogenesis. So far the involvement of Wnt signalling in the regulation of periodicity of somite formation has only been reported in mouse embryos.

Taken together these data indicate the existence of three types of negative feedback loops by incorporating data from zebrafish, chick and mouse, although the possible interactions between these loops are still not understood: 1) a direct feedback loop that generates the cyclic expression of Hairy/ Enhancer-of-Split family of bHLH repressors (Her1/Her13.2 and Her7 in zebrafish and Hes1 and Hes7 in the mouse); 2) an indirect feedback loop that establishes periodic activation of Notch signalling (DeltaC in zebrafish and Lfng in the chick and mouse); 3) another indirect feedback loop that promotes periodic activation of

Wnt signalling (Axin2 and Nkd1 in the mouse) (Fig. 7).

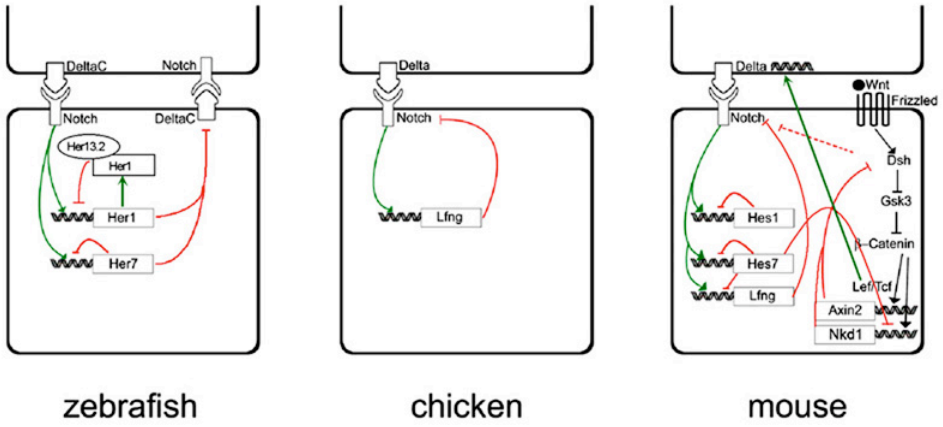


Fig. 7. Feedback loops underlying periodic oscillations of cycling genes. In zebrafish, the periodic oscillations of *her1* and *her7* mRNA are generated by autoregulatory feedback loops and Her13.2 directly interacts with Her1, augmenting its autorepression. The her genes may also be involved in *deltaC* periodic expression, which in turn would periodically modulate Notch signalling activity. In the chick, Lfng indirectly represses its own expression by periodic modulation of the Notch signalling pathway. In the mouse, cyclic transcription of hes7 is regulated by the periodic expression of its own protein that in turn regulates the periodic repression of *nkd1*. The same molecular feedback loop generates Hes1 oscillations in different cell types. Additionally, the cyclic expression of *axin2* and *nkd1*, direct targets of the Wnt pathway, regulate the Wnt signalling by a mechanism of feedback inhibition. The *lfng* gene is activated by Notch and suppressed by Hes7, while in turn Lfng acts periodically to inhibit Notch activity. Finally, Lef/TCF binding sites were described in the Dll1 promoter, suggesting a direct interaction between the Wnt and Notch signalling pathways in the regulation of the segmentation clock.

It has been suggested that a negative feedback loop in which the expression of a gene is repressed by its own protein product is insufficient to generate or maintain sustained oscillations (Hirata *et al.*, 2002). However, two mathematical models based on the experimental data from zebrafish and cell culture studies show that mRNA and protein oscillations can be produced if transcriptional and translational delays are taken into account (Lewis, 2003; Monk, 2003). Nevertheless, delayed feedback will only set up the pace of oscillations if the mRNA and protein half-lives are effectively small in relation to the delay. Surprisingly, these mathematical models reveal that delay-driven oscillations are very resistant to parameter changes (Lewis, 2003; Monk, 2003). A simulation where Notch signalling is impaired shows a progressive failure in the regularity of the oscillations which in agreement with the desynchronization theory, could contribute to explain the lack of defects in the first somites of zebrafish and mouse Notch mutants (Lewis, 2003; Jiang *et al.*, 2000).

1. 9. The wavefront: a partner of the clock

The clock-and-wavefront model proposes an explanation for the temporal and spatial regulation of somitogenesis. It predicts the existence of an intrinsic cellular oscillator operating in parallel with a wavefront of differentiation, whose progression rate determines the correct positioning of somitic boundaries, and consequently the somitic size (Cooke and Zeeman, 1976). As previously described, the molecular evidence for the existence of an oscillator was provided by the cyclic expression of a number of genes, whereas the wavefront position seems to be regulated by Fgf and Wnt signalling (Dubrulle *et al.*, 2001; Sawada *et al.*, 2001; Aulehla *et al.*, 2003), by RA signalling and possibly by an unknown pathway involving the T-box gene, *tbx24* (Diez del Corral *et al*., 2003; Nikaido *et al.*, 2002).

In the chick, *fgf8* defines a decreasing caudal to rostral gradient of expression in the two posterior thirds of the PSM. AP inversion experiments of PSM tissue demonstrated that AP somitic compartments are already determined in the anterior third of the PSM, in contrast to the caudal two thirds of this tissue where segment polarity is still undetermined (Dubrulle *et al.*, 2001). The transition between these two regions occurs at the level of the determination front that progressively regresses as a consequence of embryo elongation and seems to correspond to the anterior limit of the *fgf8* gradient of expression. The progressive posterior displacement of the determination front ensures that the somitic boundaries form at constant intervals, thus converting the periodic oscillations of the segmentation clock genes into a reiterated spatial pattern along the PSM (Pourquie, 2003). Either inhibiting or overexpressing Fgf8 at the level of the determination front alters the position of somitic boundaries, inducing the formation of larger or smaller somites, respectively. Hence, it seems that Fgf8 maintains posterior PSM cells in an immature state, thus negatively regulating the wavefront of differentiation in the chick embryo (Dubrulle *et al*., 2001). A recent work further reported that in the chick, the Fgf gradient is translated into a graded activation of the extracellular signal-regulated kinase (ERK) mitogen-

activated protein kinase (MAPK) pathway along the PSM (Delfini *et al*., 2005). The involvement of Fgf signalling in the control of the wavefront progression was also studied in zebrafish and mouse embryos where the ERK and Akt kinases, which mediate the intracellular response downstream of Fgf signalling, are also functioning in the posterior PSM (Sawada *et al.*, 2001; Dubrulle and Pourquie, 2004). Furthermore, experimental manipulations of MAPK levels in the PSM of zebrafish embryos leads to a variation in somitic size, strengthening the idea that Fgf signalling determines the position of segment border formation (Sawada *et al.*, 2001). The expression of the intronic *fgf8* probe was analysed in mouse and chick embryos and this allowed the detection of the transcription site of this gene (Dubrulle and Pourquie, 2004). These authors concluded that while the mature *fgf8* mRNA is present in a posterior-to-anterior gradient within the PSM, the *fgf8* precursor mRNA is confined to the tail bud. Hence, they proposed the "gradual mRNA decay" model which states that the Fgf8 gradient results from the fact that cells that actively transcribed the gene in the posterior region of the embryo, are gradually displaced away from the initial site of transcription concomitantly with the decay of the fgf mRNA (Dubrulle and Pourquie, 2004).

Previous studies have shown that presomitic tissue represses neuronal differentiation and that Fgf signalling can mimic this action (Diez del Corral *et al.,* 2002). On the other hand, the somitic mesoderm promotes maturation events, which are correlated with the activation of RA signalling in rostral PSM and somites, as indicated by the expression of the RA-synthesizing enzyme *raldh2* (Diez del Corral *et al.,* 2002 and 2003). Explant culture experiments using a RA agonist (TTNPB) and Vitamin A deficient (VAD) quail embryos, which lack biologically active RA, have additionally demonstrated that RA downregulates the expression of *fgf8* in the PSM (Maden *et al*., 1996; Diez del Corral *et al.,* 2003). Conversely, Fgf8 soaked beads placed in the chick PSM represses the expression of *raldh2* indicating that Fgf signalling regulates the onset of RA synthesis in presomitic tissue (Diez del Corral *et al.,* 2003). Consistent with these data, work performed in *Xenopus* embryos also showed that *raldh2* is expressed in the rostral PSM while the *cyp26A1*, an enzyme involved in RA catabolism, is

expressed in the most caudal part of the PSM tissue (Moreno and Kintner, 2004). These patterns of gene activity suggest that Fgf and RA signalling pathways are mutually inhibitory, acting in parallel to establish the position of the wavefront of differentiation along the AP axis of the vertebrate embryo.

In the mouse, *wnt3a* is strongly expressed in the tail bud and it was proposed to establish a decreasing caudal to rostral gradient of expression that regresses as the embryo elongates. Hence, in the mouse embryo, the *wnt3a* gene seems to play a similar role to the one attributed to Fgf signalling in both chick and zebrafish PSM (Aulehla *et al.*, 2003). Furthermore, *fgf8* is downregulated in the tail bud and PSM of *wnt3a* hypomorphic mutants, suggesting that *wnt3a* acts upstream of *fgf8* in the regulation of the wavefront position. Since there is evidence that Fgf signalling may enhance Wnt/ß-catenin signalling, Fgf8 might act as a relay enhancer of Wnt signalling in the PSM of mouse embryos (Aulehla *et al.*, 2003).

In the zebrafish, a mutation in the *tbx24* gene leads to embryos totally devoid of somitic boundaries, which corresponds to the phenotype of the *fused somites* (*fss*) mutants (Van Eeden *et al.*, 1996; Nikaido *et al.*, 2002). This suggests a role of the *tbx24* gene in the formation of somitic boundaries. Although *tbx24* is expressed in anterior and intermediate PSM, its function seems to be restricted to the rostral PSM. In fact, in *fss* mutants segmentation genes specifically expressed in the anterior PSM are downregulated and the anterior stripe of the *her1* cyclic gene is lost, whereas its expression appears to be normal in the posterior PSM. These data suggest that *tbx24* plays a role in the maturation process of anterior PSM cells that might be independent of the molecular clock. Since the formation of somitic boundaries is such a finely tuned process, it is conceivable that a signal in the anterior PSM controls the precise site of the determination front. Although *tbx24* has only been described in the zebrafish embryo, this gene is nevertheless a good candidate signal to work in combination with the RA signalling in the anterior PSM regulating the wavefront of differentiation. On the contrary, the Fgf and Wnt posterior gradients seem to be playing the opposite role positioning the determination front by maintaining

cells in an immature state (Fig.8). It would now be interesting to look for functional homologues in other vertebrate models and to understand how the T-box pathway interacts with Fgf and Wnt signalling pathways.

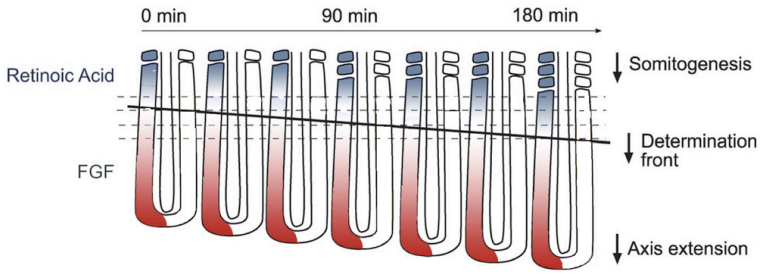


Figure 8. The wavefront of differentiation in the chick embryo

Diagram representing the opposing gradients of FGF signalling (in red) and of retinoic acid (in blue), which define the position of the determination front (thick black line). The gradual displacement of the determination front regulates the future position of somitic boundaries. *Adapted from Pourquie, 2004.*

1. 10. Somitogenesis related mutants

1. 10.1. Notch signalling mutants

As described above, most cycling genes code for components of the Notch signalling pathway, suggesting that it plays an important role in the regulation of the segmentation clock. Indeed, the analysis of Notch-related mutants in the mouse reveals that somites are always affected, namely in the *notch1* (Conlon *et al.*, 1995), *dll1*, *delta-like3* (*dll3*) (Hräbe de Angelis *et al.*, 1997; Kusumi *et al.*, 1998), *lfng* (Zhang and Gridley, 1998; Evrard *et al.*, 1998), *presenilin1* (Wong *et al.*, 1997), the γ-secretase components *nicastrin* (Li *et al*., 2003), *Pen-2* (Huppert *et al*., 2005) and *APH-1* (Ma *et al*., 2005; Serneels *et al*., 2005), *mind bomb1* (Koo *et al*., 2005), *rbp-jk* (Oka *et al.*, 1995), *pofut1* (Shi and Stanley, 2003) and *hes7* (Bessho *et al.*, 2001b) mutants. In general terms, these defects consist of disrupted AP polarity and somitic malformations in what concerns shape and alignment along the axis. In addition, the majority of these mutants exhibit a specific disruption of posterior somite formation whereas the most anterior somites are less affected. An exception to this is the double mutant for *presenilin1*; *presenilin2* (*PS1*;*PS2*), where the canonical Notch signalling is inactive, which does not form any somites (Donoviel *et al*., 1999). Surprisingly, it

was recently reported that the *notch1*;*notch2* (*N1*;*N2*) double mutant exhibits the formation of anterior somites (Huppert *et al*., 2005). The fact that *PS1*;*PS2* mutants lack both anterior and posterior somite formation and that the *N1*;*N2* mutants still form anterior somites suggests that the formation of rostral-most somites requires an activity of the Presenilin protein different from its role as a γ-secretase and independent from Notch signalling (Huppert *et al*., 2005). Remarkably, this work also proposes that tailbud somitogenesis requires more NICD protein than trunk somitogenesis, which is an interesting explanation for the sparing of the anterior somites in the Notch related mutants (Huppert *et al*., 2005).

TABLE 2. Notch signalling-related mouse mutants involved in somitogenesis

With a somitic phenotype	Without a somitic phenotype
notch1 (Conlon *et al*., 1995) ***notch1*;*notch2*** (Huppert *et al*., 2005) ***delta-like1*** (Hräbe de Angelis *et al*., 1997) ***delta-like3*** (Kusumi *et al*., 1998) ***mindbomb-1*** (Koo *et al*., 2005) ***lunatic fringe*** (Zhang and Gridley, 1998; Evrard *et al*., 1998) ***presenilin1*** (Wong *et al*., 1997) ***presenilin1*;*presenilin2*** (Donoviel *et al*., 1999) ***rbp-jk*** (Oka *et al*., 1995) ***hes7*** (Bessho *et al*., 2001b) ***pofut1*** (Shi and Stanley, 2003)	***hes1*** (Jouve *et al*., 2000) ***hes5*** (Ohtsuka *et al*., 1999)

Thus far, both *hes1* and *hes5* are the only Notch signalling mutants that do not exhibit a somitic phenotype (Jouve *et al*., 2000; Ohtsuka *et al*., 1999) (Table 1.2). These results reflect a possible compensation by other cyclic *hes* genes such as *hes7* (Bessho *et al*., 2001a). In mice, a mutation in the α5 integrin subunit (*itga5*) gene, which codes for a subunit of the fibronectin receptor,

exhibits a somitic phenotype that resembles the one from the Notch pathway mutants (Yang *et al*., 1993). Similarly, the fibronectin mutant also lacks somites but its phenotype is stronger than the one for *itga5* since no somites are observed along the whole axis.

In zebrafish, several somite mutants have been isolated from a large-scale screen and the detailed analysis of their phenotypes shows a striking resemblance to the Notch signalling mouse mutants (Jiang *et al.*, 1996; Van Eeden *et al.*, 1996). In fact, it is now known that the phenotypes of the zebrafish mutants *deadly seven* (*des*), *after eight* (*aei*), *beamter* (*bea*) and *mind bomb* (*mib*) are due to mutations in *notch1*, *deltaD*, *deltaC* and in an ubiquitin ligase that binds Delta, respectively (Holley *et al.*, 2000; Holley *et al.*, 2002; Itoh *et al.*, 2003; Julich *et al*., 2005b). Injection of morpholinos targeted for these genes recapitulates the phenotype of each of these zebrafish mutants (Holley *et al.*, 2002; Itoh *et al.*, 2003; Julich *et al*., 2005b). Morpholino knockdown experiments for the *her1*, *her7* and *suppressor of hairless* (*su* (*h*)) genes revealed that in the absence of the proteins coded by these genes, the embryos exhibit a somitic phenotype (Holley *et al.*, 2002; Henry *et al.*, 2002; Oates and Ho, 2002; Gajweski *et al.*, 2003; Sieger *et al.*, 2003) (Table 3).

TABLE 3. Notch signalling-related zebrafish mutants and morphants involved in somitogenesis

Zebrafish mutants	Zebrafish morphants ^{MO}
notch1 - deadly seven (des) (Holley *et al.*, 2002) ***deltaD - after eight (aei)*** (Holley *et al.*, 2000) ***deltaC – beamter (bea)*** (Julich *et al*., 2005) ***ubiquitin ligase - mind bomb (mib)*** (Itoh *et al*., 2003)	***her1*^{mo}** (Holley *et al.*, 2002; Henry *et al*., 2002; Oates and Ho, 2002; Gajewski *et al*., 2003) ***her7*^{mo}** (Oates and Ho, 2002; Henry *et al*., 2002; Gajewski *et al*., 2003) ***her1*^{mo}*+her7*^{mo}** (Oates and Ho, 2002; Henry *et al*., 2002) ***notch1*^{mo}** (Holley *et al*., 2002) ***suppressor of hairless*^{mo}** (Sieger *et al*., 2003) ***deltaC*^{mo}** (Holley *et al*., 2002) ***deltaD*^{mo}** (Holley *et al*., 2002)

Also in these zebrafish mutants the disruption of somite formation presents different anterior limits of defects, reinforcing the idea that there are relevant differences between rostral and caudal somites (Henry *et al*., 2002; Oates and Ho, 2002; Oates *et al*., 2005).

Despite the somitic defects observed in the mouse and zebrafish mutants, somitic derivatives like the sclerotome and dermomyotome present a more or less organised segmental pattern, indicating that a basic metameric pattern is nevertheless accomplished. It seems that the Notch signalling pathway is important to coordinate the periodicity of somite formation and to specify somitic AP polarity, although its downregulation is not sufficient to abolish overall segmentation. Consistent with this idea, Huppert and colleagues proposed a redundant function of the Notch, Wnt and Presenilin signalling pathways underlying the control of somitogenesis (Huppert *et al*., 2005).

Phenotypic analysis of Notch signalling mutants strongly suggested that the defects observed could be due to a disruption in molecular segmentation and, therefore, the expression pattern of the cycling genes was studied in these mutants. The expression of *lfng* is downregulated in *dll1*, *dll3*, *pofut1* and *rbp-jk* mutant mice but it is only slightly reduced in the *notch1* mutant (Barrantes *et al.* 1999; Shi and Stanley, 2003). On the contrary, the cyclic behaviour of *lfng* is not affected in *hes1* knockout mice (Jouve *et al.*, 2000). In *hes7* null mice *lfng* transcription is constitutively upregulated in all presomitic cells (Bessho *et al.*, 2001b). The *hes1* gene expression is severely downregulated in *dll1*, *dll3* and *hes7* homozygous null embryos (Jouve *et al.*, 2000; Dunwoodie *et al.*, 2002; Bessho *et al.*, 2001b). Moreover, the transcription of *hey2* is also downregulated in the PSM of *hes7* knockout mice (Bessho *et al.*, 2001b). Additionally, the transcription of *hes7* is constitutively upregulated in *hes7* mutants, as demonstrated by the expression of intronic probes in the PSM (Bessho *et al.*, 2003). The cyclic expression of the recently described *snail1* gene was also analysed in several Notch signalling mutants. The oscillatory behaviour of this gene was not altered in these mutants, indicating that Notch signalling is not required for *snail1* cyclic expression in the mouse embryo (Dale *et al*., 2006).

In zebrafish, the cyclic expression of *her1*, *her7* and *deltaC* is impaired in the *aei* (*deltaD*), *bea* (*deltaC*), *des* (*notch1*) and *mib* (*ubiquitin ligase*) mutants and in the *suppressor of hairless* (*su* (*h*)) morpholino-knockdown experiments (Holley *et al.*, 2000; Holley *et al.*, 2002; Oates and Ho, 2002; Jiang *et al.*, 2000; Sieger *et al.*, 2003; Julich *et al*., 2005b). Additionally, *her1* is impaired in *deltaC* morpholino injected embryos (Holley *et al.*, 2002). The inhibition of Her1 function in the PSM leads to a loss of the cyclic expression of both *her1* and *deltaC*. In this knockdown experiment *her7* expression is decreased but its cyclic behaviour is maintained (Holley *et al.*, 2002; Oates and Ho, 2002). Nevertheless, a decrease in Her7 function disrupts the dynamic expression of *deltaC*, *her1* and *her7* (Oates and Ho, 2002). Cyclic gene expression in the PSM is lost by reducing the receptor protein tyrosine phosphatase Ψr (RPTP Ψr) using morpholino antisense oligonucleotides, suggesting a requirement for RPTP Ψr in the control of the clock upstream of, or in parallel with, Delta/Notch signalling (Aerne and Ish-Horowicz, 2004). A recent work has further reported that *tortuga* mutants in zebrafish exhibit altered PSM expression of the *her1*, *her7* and *deltaC* genes, caused by a defect on the degradation of these cyclic genes mRNA at the post-transcriptional level (Dill and Amacher *et al*., 2005).

Overall, these studies show that the oscillations of the cycling genes are in fact disturbed in Notch signalling mutants, reaffirming the function of the Notch pathway in driving the segmentation clock and showing that this role is conserved among vertebrates. Jiang and colleagues (2000) observed that while in normal development PSM cells oscillate synchronously, in Notch signalling mutants they drift out of synchrony eventually leading to defective somitogenesis. The authors show that in zebrafish Notch signalling mutants, the expression pattern of *deltaC* is normal at first, but then it becomes desynchronized, which is another hypothesis that explains the sparing of the first somites in these mutants. This finding led to the proposition that the essential function of the Notch signalling pathway is to maintain the oscillations synchronized in adjacent PSM cells (Jiang *et al.*, 2000). In conformity with these results, Her6 was described to be an output of the Notch signalling pathway that, together with Her4, is required

for maintaining the synchronization of cyclic gene expression within the PSM in zebrafish embryos (Pasini *et al*., 2004).

As described above, most of the Notch signalling mutations specifically disrupt posterior somite formation. However, two recent studies have shown that mutations in the zebrafish *itga5* gene specifically disrupt anterior somite formation (Julich *et al*., 2005; Koshida *et al*., 2005). Julich and colaborators showed that these mutations give a somitic phenotype that is complementary to the posterior somite defects observed in the Notch pathway mutants (Julich *et al*., 2005), reinforcing the existence of differences in the regulation of the specification of anterior and posterior somites. Surprisingly, the phenotype of the *itga5* mutants in mouse embryos is different from the one in zebrafish. In mice, these mutants form the anterior-most seven to ten somites and their posterior axis becomes truncated (Yang *et al*., 1993; Goh *et al*., 1997). The comparison of this result with the one in zebrafish embryos suggests that the function of integrinα5 in what concerns the regulation of the anterior versus posterior somites is different in these vertebrate species.

1. 10. 2. Fgf, Wnt and T-box signalling mutants

An interesting feature of the phenotypes of the mutations caused by disruption of the Fgf, Wnt and T-box signalling pathways is the fact that the posterior PSM is not specified, while the anterior somites are apparently unaffected. The zebrafish mutations belonging to this group include the *no tail* and *pipetail* mutants, in which the mutated genes are the T-box gene *brachyury* and the *wnt5* gene, respectively. The phenotype of these mutants displays posterior truncations and more caudal somites do not form (Schulte-Merker *et al*., 1994; Hammerschmidt *et al*., 1996; Rauch *et al*., 1997). A complex interaction involving the *no tail*/*brachyury* gene and the Fgf signalling pathway was also described accounting for the specification of posterior mesodermal structures (Griffin *et al*., 1995, 1998). In contrast, a mutation in the *spadetail* gene that encodes a transcription factor of the T-box family that has a complementary

function to *no tail*, reveals a defective formation of anterior somites, whereas tail somites appear normal (Kimmel *et al*., 1989). As described before, an exception to the phenotype of the T-box group of mutations is the *fused somites*, which consists in the disruption of the *tbx24* gene. In these mutants the PSM is correctly specified but it fails to segment along the whole embryonic axis (Van Eeden *et al*., 1996; Nikaido *et al*., 2002). In mice, mutations that affect the *wnt3a* gene, the t-box genes *brachyury* and *tbx6*, the *Fgfr1*α and the double mutant for the *TCF1* and *lef1* genes result in a fate switch of the caudal-most PSM into neural tissue (Deng *et al*., 1994; Takada *et al*., 1994; Yamaguchi *et al*., 1994; Wilson *et al*., 1995; Yoshikawa *et al*., 1997; Chapman and Papaioannou 1998; Yamaguchi *et al*., 1999; Galceran *et al*., 1999). In these mutants the rostral-most somites form normally and the posterior part of the embryos exhibits the formation of three neural tubes. Conversely, mice carrying a mutant allele for *Lef1* exhibit a correct PSM specification and the formation of anterior somites (Galceran *et al*., 2004). Taken together the available experimental evidence suggests that a complex genetic network involving the Wnt, Fgf, and T-box signalling pathways is responsible for regulating the posterior PSM fate in the tail of mouse embryos (Arnold *et al*., 2000; Galceran *et al*., 1999; Xu *et al*., 1999; Yamagushi *et al*., 1999) (Table 4).

TABLE 4 . Fgf, Wnt and T-box related mutants involved in somitogenesis

	Wnt signaliing mutants	**T-box signalling mutants**	**Fgf signalling mutants**
Zebrafish	***pipetail - wnt5*** (Hammerschmidt *et al*., 1996; Rauch *et al*., 1997)	***no tail – brachyury*** (Schulte-Merker *et al*., 1994) ***spadetail*** (Kimmel *et al*., 1989) ***fused somites - tbx24*** (Van Eeden *et al*., 1996; Nikaido *et al*., 2002)	
Mouse	***wnt3a*** (Takada *et al*., 1994; Yoshikawa *et al*., 1997) ***tcf1/lef1*** (Galceran *et al*., 1999) ***lef1*** (Galceran *et al*., 2004)	***brachyury*** (Wilson *et al*., 1995) ***tbx6l*** (Chapman and Papaioannou, 1998)	***fgfr1*** (Deng *et al*., 1994; Yamagushi *et al*., 1994)

Mice mutants harbouring the Wnt3a hypomorphic allele *vestigial tail*, present a reduced Wnt3a activity and their somitic morphological defects become apparent later than in the null mutants for this gene. Hence these *vt/vt* embryos have become a useful tool for studying the expression behaviour of the cycling genes in a reduced Wnt activity environment. The analysis of the *axin2*, *lfng* and *snail1* cycling genes in the PSM of these hypomorphic mutants revealed that their cyclic expression is impaired (Aulehla *et al*., 2003; Dale *et al*., 2006). These results are consistent with an important role for the Wnt pathway in the regulation of the segmentation clock machinery.

1. 11. Molecular Events in the PSM leading to somite formation

The PSM can conceptually be organized in two distinct regions. In the first region, a pre-patterning of the PSM is established by the oscillations of the clock genes in the posterior PSM. In the second region, the expression of several segmentation genes is activated, prefiguring boundary formation and somitic AP polarity in the rostral-most third of the PSM. As described above, the transition between these two regions is regulated by a wavefront of differentiation that depends on the interaction between the Fgf and Wnt signalling pathways in the posterior PSM and the RA and possibly another pathway involving the T-box signalling in the more anterior part of the PSM. A recent study has further established that the *mesoderm posterior-2* (*mesp2*) gene is responsible for arresting the oscillation of Notch activity in the anterior PSM and to initiate the segmentation programme in this domain. Hence, this gene seems to have a crucial role in the switch between these two regions (Morimoto, 2005).

1. 11. 2 Maturation events in anterior PSM

Once presomitic cells cross the determination front, the segmental pre-patterning established by the activity of the cyclic genes in the posterior PSM is translated into a series of morphogenetic changes prior to epithelial somite formation. Concomitantly with the general process of PSM maturation there are

two main events that occur at the anterior part of this tissue. The first is the establishment of the somitic rostral-caudal polarization and the second is the process that leads to the actual boundary formation.

1. 11. 2. 1 Establishing AP polarity in anterior PSM

One of the mechanisms establishing the AP polarity within a presumptive somite involves the activity of the *mesp* genes and the Notch/Delta signalling pathway (reviewed by Saga and Takeda, 2001). The *mesp* genes were characterized in most vertebrate embryos (Buchberger *et al*., 1998; Saga *et al*., 1997; Sawada *et al*., 2000; Sparrow *et al*., 1998) and act downstream of the Notch signalling pathway. In the mouse, a complex genetic network is established involving the *dll1, dll3* and the *presenilin* genes and consequently their expression patterns become restricted either to the rostral or the caudal compartment of the future somite (reviewed by Saga and Takeda, 2001). A recent study further showed that *mesp2* acts as a restrictor of the *lfng* expression in the anterior PSM, suppressing the oscillatory Notch activity and hence, initiating the segmentation program in this region (Morimoto *et al*, 2005). These genetic interactions are important not only for correct boundary formation but also for subsequent somitic differentiation (Saga *et al*., 1997; Takahashi *et al*, 2000; Takahashi *et al*, 2003).

The *tbx18*, encoding a T-box transcription factor and the *uncx4.1* that encodes a paired-related homeobox transcription factor, are also important players in the pre-patterning of somitic AP polarity. In vertebrate embryos, both these genes are segmentally expressed in the anterior PSM region (Kraus *et al*., 2001; Haenig and Kispert, 2004; Begemann *et al*., 2002; Mansouri *et al*., 1997; Schragle *et al*., 2004). The analysis of homozygous mutants for the *tbx18* gene revealed that the anterior sclerotome acquires a caudalised character (Bussen *et al*., 2004), whereas mice mutants for *uncx4.1* present severe skeletal malformations lacking elements of the posterior sclerotome (Leitges *et al*., 2000; Mansouri *et al*., 2000). These findings confirm a role of the *tbx18* and *uncx4.1* genes in maintaining anterior and posterior somite characteristics, respectively.

The *ripply1* gene was recently identified in the zebrafish embryo as a nuclear protein associated with the transcriptional corepressor Groucho. This gene is expressed in the anterior PSM of both zebrafish and mouse embryos (Kawamura *et al*., 2005). In zebrafish *ripply1*-deficient embryos, somite boundaries do not form, the expression of several segmentation related genes is not properly terminated and the initially establishment of AP somitic polarity is not maintained (Kawamura *et al*., 2005). So, the maintenance of AP somitic compartments is one of the crucial roles this gene has during the process of PSM maturation.

The process of PSM maturation also relies on the activity of the fork-head/winged helix (Foxc) transcription factors, which are strongly expressed in the rostral PSM of mice (Kume *et al*., 2001) and zebrafish embryos (Topczewska *et al*., 2001a). Mutational analysis of *foxc1* and *foxc2* in the mouse has shown that no somites form along the whole AP axis and that the expression of some AP polarity genes in anterior PSM such as *mesp1* and *mesp2*, *ephrinb2*, *lfng* and *dll1* are either absent or disrupted (Kume *et al*., 2001). In the zebrafish embryo the phenotype of the *foxc1a* morphant resembles the one of *fss* mutants and it also disrupts the expression of some Notch-related rostrocaudal markers in anterior PSM (Nikaido, *et al*., 2002; Topczewska *et al*., 2001b). Altogether, these results indicate an important role of the *foxc* genes both in the establishment of AP polarity and in maturation events in anterior PSM.

1. 11. 2. 2. Forming somitic boundaries

The events preceding somitic epithelialization involve an increasing accumulation of cell-adhesion molecules in the anterior PSM region, that eventually leads to the compaction of the PSM cells into an epithelial segment (Duband *et al*., 1987). Several cell adhesion molecules are involved in this process, and interfering with their function causes severe somitic disruption (George *et al*., 1993; Kimura *et al*., 1995; Linask *et al*., 1998; Horikawa *et al*., 1999; Yang *et al*., 1999; Kim *et al*., 2000; Rhee *et al*., 2003). Furthermore, recent work in zebrafish embryos showed that Integrinα5 and its ligand Fibronectin have

a specific role in maintaining anterior somitic borders (Julich *et al*., 2005; Koshida *et al*., 2005). These studies also demonstrated that the α5 integrin subunit acts downstream of the *tbx24* pathway and in conjunction with the Notch signalling and the Eph/Ephrin pathways to regulate the cellular changes during epithelialization (reviewed by Chong and Jiang, 2005).

Just before a somitic boundary forms, members of the Eph/Ephrin pathway are important players in the specification of the mesenchymal to epithelial transition. These genes code for cell surface molecules that induce several cellular events from cell polarization to columnar shape acquisition, evidencing their function in the cellular change that is associated with somite epithelialization (Barrios *et al*., 2003). Furthermore, they exhibit a striped expression pattern in the rostral-most part of the PSM in zebrafish and chick, which is consistent with a role of this pathway in AP patterning (Durbin *et al*., 1998; Schmidt *et al*., 2001). In the mouse, no phenotype is observed in *epha4* or *ephrinb2* mutants leading to the hypothesis that there may be a redundant activity of this gene family in this species (Helmbacher *et al*., 2000; Wang *et al*., 1998). Conversely, in Notch signalling mutants *ephA4* stripes are absent or disrupted, suggesting that Eph signalling acts downstream of the Notch signalling pathway (Barrantes *et al*., 1999).

Prior to epithelial somite formation the Rac1 and Cdc42, which belong to the Rho family of GTPases, play important roles in the mesenchymal to epithelial transition during somitogenesis. In this work the authors verified that different levels of Cdc42 determine the binary decision between epithelial and mesenchymal states, whereas a correct level of Rac1 activity was also shown to be necessary for proper epithelialization (Nakaya *et al*., 2004). In addition, they demonstrate a functional interaction between Rac1 and Paraxis, that is a transcription factor known to be essential for somitic epithelialization (Burgess *et al*., 1996).

Grafting experiments in chick embryos have further shown that ectopic boundary formation can be achieved when a presumptive anterior somitic compartment is transplanted to a heterotopic region in the PSM (Sato *et al*.,

2002). In addition, the authors found that boundary induction can be mimicked by grafting cells that overexpress either Lfng or a constitutively active form of the Notch receptor. Hence, this work proposed that modulation of Notch activity *via* Lfng triggers cleft formation (Sato *et al*., 2002). More recently, these authors found a second inductive action along the dorso-ventral (DV) axis during fissure formation. In this work, they show that when relocated into a non-segmenting region of PSM the ventral-most cells taken from the presumptive boundary are sufficient to induce an ectopic fissure in host cells. These findings suggest that discrete unidirectional signals along both the AP and the DV axes act coordinately to achieve the formation of the intersomitic boundary (Sato and Takahashi, 2005).

Taken together, the analysis of the currently available data on somitogenesis suggests the requirement of a complex genetic coordination in order to correctly form and pattern somites in space and time.

Chapter 2

Evidence for medial/lateral specification and positional information within the presomitic mesoderm

Development 128, 5139-5147 (2001)

The present Chapter presents a detailed analysis of the expression pattern of the cycling genes at the level of the prospective somitic territory, in 6-somite stage chick embryos. We established that medial and lateral presomitic cells arise from distinct prospective territories, and that these cells are differently committed in what concerns segmentation and somite formation. Furthermore, this work also revealed that the expression of the cycling genes at this level defines a 'wave' that spreads along the longitudinal axis of the PSM prospective territory, providing cellular positional information in two dimensions: not only along the AP but also along the ML presomitic axis.

Development 128, 5139-5147 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2768

Evidence for medial/lateral specification and positional information within the presomitic mesoderm

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Accepted 14 September 2001

SUMMARY

In the vertebrate embryo, segmentation is built on repetitive structures, named somites, which are formed progressively from the most rostral part of presomitic mesoderm, every 90 minutes in the avian embryo. The discovery of the cyclic expression of several genes, occurring every 90 minutes in each presomitic cell, has shown that there is a molecular clock linked to somitogenesis. We demonstrate that a dynamic expression pattern of the cycling genes is already evident at the level of the prospective presomitic territory. The analysis of this expression pattern, correlated with a quail/chick fate-map, identifies a 'wave' of expression travelling along the future medial/lateral presomitic axis. Further analysis also reveals the existence of a medial/lateral asynchrony of expression at the level of presomitic mesoderm. This work suggests that the molecular clock is providing cellular positional information not only along the anterior/posterior but also along the medial/lateral presomitic axis. Finally, by using an in vitro culture system, we show that the information for morphological somite formation and molecular segmentation is segregated within the medial/lateral presomitic axis. Medial presomitic cells are able to form somites and express segmentation markers in the absence of lateral presomitic cells. By contrast, and surprisingly, lateral presomitic cells that are deprived of their medial counterparts are not able to organise themselves into somites and lose the expression of genes known to be important for vertebrate segmentation, such as *Delta-1*, *Notch-1*, *paraxis*, *hairy1*, *hairy2* and *lunatic fringe*.

Key words: Somite, Segmentation, Molecular clock, Primitive streak, Chick embryo, *hairy1*, *hairy2*, *lunatic fringe*

INTRODUCTION

In the vertebrate embryo, somites constitute the basis of the segmental pattern of the body. They not only give rise to segmented structures such as vertebrae, intervertebral disks, epaxial muscles and ribs, but they also impose a segmental pattern upon a variety of tissues, such as peripheral nerves, ganglia and vascular primordia (Keynes and Stern, 1984; Rickmann et al., 1985; Teillet et al., 1987; Wilting et al., 1997). Somites appear progressively, in a rostral-to-caudal order, as pairs of epithelial spheres that bud off at the cranial end of the presomitic mesoderm (PSM). Somites are formed in a remarkably coordinated fashion and the time required to form each pair of somites is fixed and characteristic of each species. In the chick embryo, this budding off occurs every 90 minutes at the thoracic level. Concomitant with epithelial somite formation at the anterior end of PSM, gastrulation continues in the most posterior part of the embryo, causing progressive PSM formation and embryo elongation (Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998). By using the quail/chick chimaera technique, a cell lineage study in six-somite stage embryos demonstrated that somitic cells originate from a region located in the posterior midline of the sinus rhomboidalis (posterior open neural plate), not adjacent to the median pit (a region containing the midline precursor cells) (Catala et al., 1996).

A previous study (Palmeirim et al., 1997) has presented evidence for the existence of a molecular clock that underlies the process of chick somitogenesis by showing that, in 15- to 20-somite stage embryos, PSM cells undergo several cycles of *hairy1* gene expression, with a 90-minute periodicity, corresponding to the time required to form one segment. These *hairy1* mRNA oscillations of expression occur in each PSM cell until it is incorporated into a somite. As previously described, the expression of *hairy1* mRNA appears as a caudal to rostral 'wave' that spans across the entire length of PSM once during the formation of each somite. This same type of behaviour has also been described for a gene coding for a closely related transcription factor, *hairy2* (Jouve et al., 2000), and for another gene encoding the secreted protein *lunatic fringe* (McGrew et al., 1998; Aulehla and Johnson, 1999). Cycling genes expressed during somitogenesis have also been recently described in mouse (lunatic fringe (Forsberg et al., 1998; Aulehla and Johnson, 1999); *Hes1* (Jouve et al., 2000); *Hey2* (Leimeister et al., 2000)) and in zebrafish embryos (*her1*) (Holley et al., 2000).

For many years, scientists have tried unsuccessfully to disturb the pattern of somite formation. Heterotopic grafts of both anterior to posterior and posterior to anterior part of the PSM of a chick embryo to another chick embryo of the same stage give rise to somite formation that respects the segmentation timing of the donor embryo (Packard, 1978). Similarly, the anterior/posterior inversion of a region of PSM tissue leads to a caudal to rostral somite formation in the grafted tissue (Menkes and Sandor, 1977; Palmeirim et al., 1998). In fact, PSM is able to segment even in complete isolation from environmental tissues (Packard and Jacobson, 1976; Sandor and Fazakas-Todea, 1980). This can be observed by the progressive appearance of *Delta-1* stripes in cultured explants of isolated PSM (Palmeirim et al., 1998). In these explants, despite disruption of morphological somite formation, genes known to be involved in the segmentation process are present, exhibiting a normal pattern of expression. Therefore, segmentation and somite formation are distinct processes: the former is independent of environmental tissues and the latter is dependent on the overlying ectoderm (Sosic et al., 1997; Palmeirim et al., 1998).

The aim of this work was to evaluate the behaviour of the molecular clock at the PSM prospective territory, during the formation of one somite. A detailed whole-mount and cross-section analysis of the expression patterns of cycling genes (*hairy1*, *hairy2* and *lunatic fringe*) show that concomitantly with an anterior/posterior PSM 'wave' of expression, a medial/lateral 'wave' can also be observed both in PSM and in its prospective territory. This result has driven us to evaluate further the heterogeneity within the medial/lateral PSM axis. We have found that the information for morphological somite formation and PSM genetic segmentation is present in medial but not in lateral PSM cells. We present data to support the idea that medial and lateral halves of PSM originate from distinct prospective territories and are also differently committed in what concerns segmentation and somite formation.

MATERIALS AND METHODS

Eggs and embryos

Fertilised chick (*Gallus gallus*) and quail (*Coturnix coturnix japonica*) eggs obtained from commercial sources were incubated at 37ºC in a 45% humidified atmosphere. The embryos were staged according to the number of formed somites (Hamburger and Hamilton, 1951). We consider a somite to be completely formed when we observe a definite cleft separating it from the PSM. We refer to the forming somite as somite 0 (caudal cleft not yet completely formed). The newly formed somite is referred to as somite I, as proposed by Christ and Ordahl (Christ and Ordahl, 1995).

RNA probes

The digoxigenin or fluorescein-labelled RNA probes were produced as described previously: *hairy1* (Palmeirim et al., 1997), *hairy2* (Jouve et al., 2000), *lunatic fringe* (Sakamoto et al., 1997), *Delta-1* and *Notch-1* (Henrique et al., 1995), *paraxis* (Barnes et al., 1997), *BMP-4* (Francis et al., 1994), *Ch-Tbx6L* (Knezevic et al., 1997) and *Noggin* (Connolly et al., 1997).

Whole-mount in situ hybridisation

Embryos and explants were fixed overnight at 4°C in 4% formaldehyde 2mM EGTA in phosphate-buffered saline (PBS), rinsed in PBT (PBS, 0.1% Tween 20), dehydrated through a methanol series and stored in 100% methanol at –20°C. Whole-mount in situ hybridisation was performed according to the procedure described by Henrique et al. (Henrique et al., 1995). For double in situ hybridisation, two RNA probes were hybridised simultaneously, one labelled with digoxigenin-UTP and the other with fluorescein-UTP. Both types of labelling were detected by alkaline-phosphatase-coupled antibodies (Roche). After the development of the first reaction, and in order to eliminate the alkaline-phosphatase extremities coupled to the first antibody, a 30 minute incubation at 70ºC with MABT was performed, preventing any cross-reaction. The RNA probes were detected either with NBT-BCIP (blue staining) or INT-BCIP (red staining).

Cross-sections

The *hairy1*, *hairy2* and *lunatic fringe*-labelled embryos were dehydrated in a series of ethanol, embedded in methacrylate (TECNOVIT 8100) and processed for sectioning at 20 μm using an ultramicrotome (LKB Ultrotome). The slides were mounted in Neomount (Merck) and photographed using a Leica DC 200 camera coupled to an Olympus BH-2 microscope.

Quail-chick grafts

Chicken and quail eggs were incubated for 30 to 36 hours in order to obtain six-somite stage embryos. A window was performed in the shell of the chicken eggs, Indian ink was injected into the sub-germinal cavity and the vitelline membrane was pulled apart using a tungsten microscalpel. Quail embryos were collected from the egg yolk into resin-coated petri dishes in PBS without Ca^{2+}/Mg^{2+}. At the caudal part of the embryo we identify the sinus rhomboidalis (open neural plate). At the centre of this structure, a pit can be observed. We consider the median pit to be the region comprising the pit itself and the slope surrounding it. Quail midline fragments, which extended caudally from the posterior limit of median pit over 100-150 μm, were grafted homotopically into stage-matched chick hosts (*n*=6) (see Fig. 4A). In order to avoid the grafting of prospective neural cells, the graft tissue only comprised the deeper layer under the surface epithelium. The chimaeras were incubated overnight, after which they were fixed in modified Carnoy solution (100% ethanol, 37% formaldehyde solution and glacial acetic acid (6:3:1, V:V:V)), embedded in paraffin, serially sectioned (5-7 μm) and processed overnight with the monoclonal antibody (mAB) QCPN (Developmental Studies Hybridoma Bank), followed by a HRP-conjugated anti-mouse IgG antibody (Southern Biotechnology) and DAB revelation to evidence quail nuclei, as described by Charrier et al. (Charrier et al., 1999).

Embryo culture experiment

Fifteen- to 20-somite stage embryos were collected from the egg yolk into resin-coated petri dishes in PBS without Ca^{2+}/Mg^{2+}. All the microsurgical procedures were performed in the left side of the embryo, taking the right-hand side PSM as a control. Different incisions were made, using a tungsten microscalpel, in order to create two types of ablations.

Lateral PSM ablation

A slit was performed in the ectoderm between the intermediate mesoderm and the entire length of PSM. After a brief treatment with 4× pancreatin (Gibco), a longitudinal incision was made in the PSM, dividing it into a medial (M-PSM) and a lateral (L-PSM) half. The L-PSM was removed by cutting transversally the anterior and posterior extremities of this piece of tissue.

Medial PSM ablation

A slit was made in the ectoderm between the neural tube and the PSM. The PSM was again longitudinally subdivided into two halves and the M-PSM was excised.

Foetal calf serum was then used to inactivate pancreatin and the operated embryo was cultured as described by Palmeirim et al.

(Palmeirim et al., 1997). After 6-9 hours of incubation (the time required to form four to six pairs of somites; note that under these conditions somitogenesis proceeds normally), the embryos were fixed in 4% formaldehyde-PBS without Ca^{2+}/Mg^{2+}, 2 mM EGTA pH8, overnight at 4°C. They were then washed in PBT, progressively dehydrated and kept in methanol at –20ºC. Finally, we assessed for morphological somite formation and for *Delta-1* and *Tbx6L* gene expression by performing whole-mount in situ hybridisation with the appropriate antisense RNA probes.

Explant culture experiment

Embryos with 15 to 20 somites were processed as described above in the embryo culture experiment. Subsequently, two types of explants were precisely delimited, excised and cultured. The entire left-hand side of the PSM was subdivided into a medial and a lateral half by performing a longitudinal incision throughout the three embryonic germ layers. Two transverse cuts were then made: one precisely beneath somite 1 (cells from somite 0 are included in both explants) and another in the most posterior part of PSM. Both explants were incubated separately for 6 hours using the in vitro tissue culture technique already described (Palmeirim et al., 1997). Explants were then fixed and hybridised with *hairy1*, *hairy2*, *lunatic fringe*, *paraxis*, *Notch-1*, *Delta-1*, *Tbx6L*, *BMP-4* and *Noggin* digoxigenin-labelled antisense RNA probes. All hybridised explants and embryos were photographed as whole-mounts in PBT/0.1% Azide, using a Leica DC 200 camera.

RESULTS

Variable expression patterns of *hairy1, hairy2* and *lunatic fringe* genes at the level of sinus rhomboidalis

Because in six-somite stage embryos the presumptive somitic territory has been located in the posterior midline region of the sinus rhomboidalis (Catala et al., 1996), we assessed for the pattern of expression of *hairy1* (*n*=37), *hairy2* (*n*=41) and *lunatic fringe* (*n*=24) in this domain. At the level of sinus rhomboidalis the embryos presented very different patterns of expression: some embryos present a strong staining in this region (Fig. 1A,E,H) while others are not stained at all (Fig. 1C,F,I). As expected, and already described for 15- to 20-somite stage embryos, very different expression patterns were observed, at the level of PSM, defining a PSM caudal-to-rostral wave of expression for each of these genes (Fig. 1A-I) (Palmeirim et al., 1997; McGrew et al., 1998; Aulehla and Johnson, 1999; Jouve et al., 2000).

In order to compare the expression patterns of *hairy1*, *hairy2* and *lunatic fringe* genes at the level of sinus rhomboidalis, we performed double whole-mount in situ hybridisation with the following combination of genes: *hairy1*/*hairy2* (*n*=14), *hairy1*/*lunatic fringe* (*n*=16) and *lunatic fringe*/*hairy2* (*n*=7), in six-somite stage embryos (Fig. 2A-I). The *hairy1* expression domain is located within the borders of the sinus rhomboidalis (Fig. 2A-F). The *lunatic fringe* gene presents a pattern of expression that is frequently coincident with the one of *hairy1* (Fig. 2E,F). Nevertheless, in some embryos, the region of expression of *lunatic fringe* extends slightly more posteriorly (Fig. 2D). By contrast, the domain of expression of *hairy2* is broader, extending beyond the limit established by the expression patterns of both *hairy1* and *lunatic fringe* (Fig. 2A-C,G-I). Analysis of these results allows us to conclude that in the posterior part of the sinus rhomboidalis, the expression patterns of these genes is variable and that this variation is not superimposed. (Fig. 1, Fig. 2).

A wave of expression is in progress at the level of sinus rhomboidalis

The whole-mount analysis did not permit a precise localisation of the cells undergoing *hairy1*, *hairy2* and *lunatic fringe* cycles of expression. We analysed a series of posterior-to-anterior cross-sections, which reveals the way cells are progressively

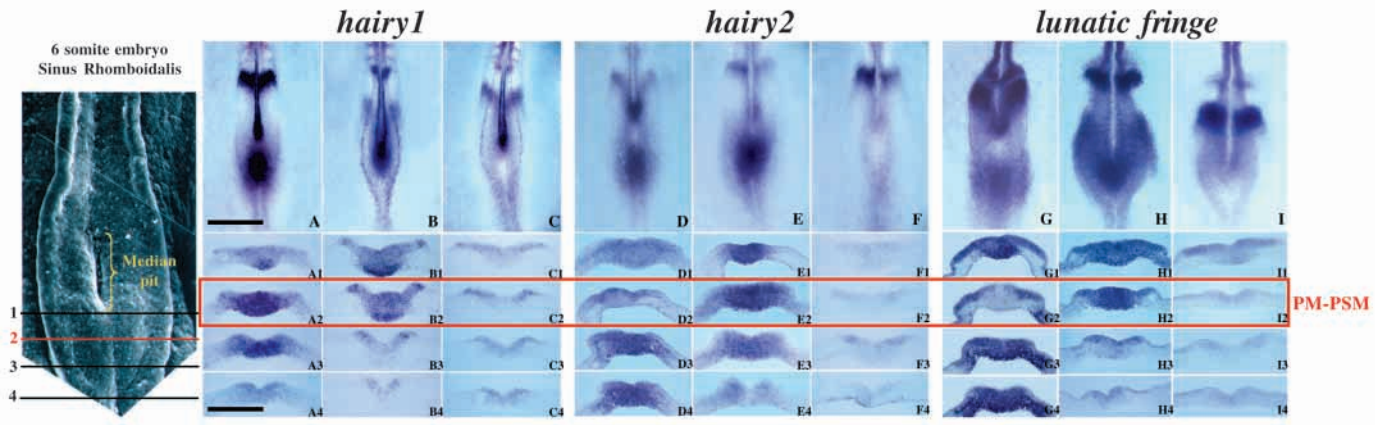


Fig. 1. Variability of *hairy1*, *hairy2* and *lunatic fringe* expression patterns is evident at the level of sinus rhomboidalis. In situ hybridisation with the *hairy1* (A-C), *hairy2* (D-F) and *lunatic fringe* (G-I) antisense mRNA probes, showing the variety of expression patterns observed at the level of PSM and sinus rhomboidalis, in six-somite stage embryos. (Left) Electron scanning micrograph of the sinus rhomboidalis of a six-somite stage embryo (dorsal view). The lines define the AP levels of the transverse sections. (A1-I1) Cross-sections at the level of the posterior limit of the median pit. (A2-I2) Cross sections 80-100 μm caudal to the posterior limit of the median pit. (A3-I3) Cross-sections 160-180 μm caudal to the posterior limit of the median pit. (A4-I4) Cross-sections 240-260 μm caudal to the posterior limit of the median pit. We consider the median pit to be the region that comprises the pit itself and the slope surrounding it. A transverse section at the same level, in two different embryos hybridised with the same probe, evidences distinct patterns of expression (e.g. A2-C2). Transverse sections at adjacent levels also evidence distinct patterns of expression, within the same embryo (e.g. G2-G3). PM-PSM, prospective medial–presomitic mesoderm. Rostral is towards the top. Scale bar: 250 μm in whole-mount embryos; 110 μm in cross sections.

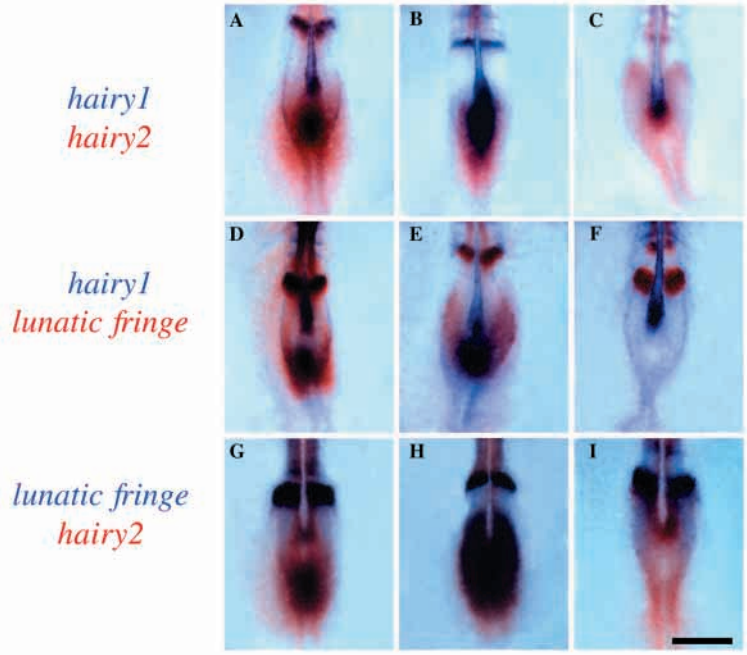


Fig. 2. Double whole-mount in situ hybridisation shows that at the level of sinus rhomboidalis the expression pattern of the cycling genes is not coincident. (A-C) Double in situ hybridisation in six-somite stage embryos with *hairy1* (blue staining) and *hairy2* (red staining) RNA probes. The expression domains of these genes are distinct, as evidenced by the caudal extension of the *hairy2* domain in relation to *hairy1*. (D-F) Double in situ hybridisation with *hairy1* (blue staining) and *lunatic fringe* (red staining) RNA probes. At the level of sinus rhomboidalis, we observe that, in some embryos, the expression pattern of *lunatic fringe* is different from *hairy1*, extending more posteriorly (see D). (G-I) Double in situ hybridisation with *lunatic fringe* (blue staining) and *hairy2* (red staining) RNA probes. The expression pattern of *hairy2* protrudes more caudally than that of *lunatic fringe* (see G-I). At the level of PSM, the expression domains of the three genes coincide at its anterior border, although at its caudal limit they are not completely coincident. Rostral is towards the top. Scale bar: 300 μm

organised in the sinus rhomboidalis. Starting at the posterior limit of the sinus rhomboidalis, a cross-section shows a superficial layer, with a slight groove, forming a continuous sheet of cells over a layer of mesenchymal tissue (Fig. 1, cross-sections at level 4). Rostral to this region, the superficial median groove disappears and the layer of mesenchymal cells is progressively enlarged (Fig. 1, cross-sections at levels 2 and 3). Progressing anteriorly, the median pit is reached. This region contains the prospective notochord and floor plate cells (Catala et al., 1996), as well as a region essential for the caudalward regression of Hensen's node (Charrier et al., 1999).

Highly variable levels of expression can be observed in the mesenchymal tissue underlying the prospective neural plate cells, from the posterior limit of the median pit (Fig. 1, cross-sections at level 1) to a region located slightly caudal to the sinus rhomboidalis (Fig. 1, cross-sections at level 4), defining a dynamic 'wave' of expression that moves in the longitudinal axis of this region. The analysis of these results suggests that simultaneous to the caudal-to-rostral 'wave' displacement that occurs at the level of PSM, another 'wave' of expression is in progress in the most posterior part of the embryo, at the level of sinus rhomboidalis (Fig. 1A-I).

This gradient of expression defines a 100-150 μm sub-region, located just behind the median pit (see Materials and Methods), that very often presents a clear asynchrony with the more posterior regions (compare Fig. 1 cross-sections at levels 2 and 3). This sub-region was not described by Catala et al. (Catala et al., 1996) as being part of the somitic prospective territory.

The medial part of PSM originates from deep cells located just behind the median pit

The somitic prospective territory described by Catala et al. (Catala et al., 1996), is located in the posterior part of sinus rhomboidalis but it is not adjacent to the median pit. Their work states that the more superficial layers of the region adjacent to the median pit give rise to the neural tube, but they did not assess for the fate of the deeper mesenchymal layers. We used the quail-chick chimaera technique to determine the fate of the cells located just behind the median pit, underneath the presumptive neuroectoderm. The latter was lifted and a 100-150 μm fragment of deep mesenchymal tissue was surgically removed and replaced by the equivalent tissue from a quail embryo of the same stage (Fig. 3A). One day after the graft, the chimaeras were analysed using the QCPN monoclonal antibody, which allows the recognition of quail cells in the chimaeras. The quail donor cells are exclusively located along the entire length of medial PSM (M-PSM) and in medial epithelial somites (Fig. 3B,C). Furthermore, no quail cells are observed in the lateral PSM (L-PSM) (Fig. 3B,C). These results show that medial PSM cells arise from a region located just behind the median pit, evidenced by quail cells only contributing to PSM and somites and being restricted to the most medial part of these tissues. These results

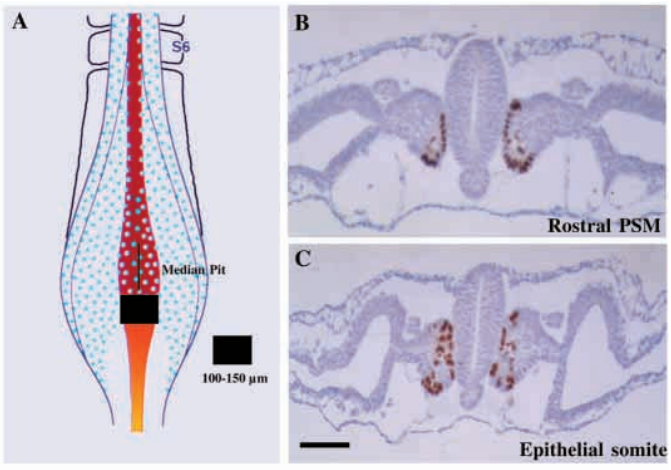


Fig. 3. Quail/chick chimaera fate map of deep mesenchymal tissue extending 100-150 μm posterior to the median pit. (A) Schematic diagram illustrating the localisation of transplanted quail tissue into the six-somite chick embryo. (B) Cross-sections at the level of rostral PSM and (C) epithelial somite, of chimaeras incubated for 24 hours, showing the localisation of the quail donor cells in the medial part of these tissues. Note that a cross section in the more rostral PSM evidences the Wolffian duct. Scale bar: 70 μm

extend the accepted idea that, at the primitive streak, more anterior cells will give rise to more medial mesodermal structures, while more posterior cells will give rise to more lateral mesodermal structures, to the cellular organisation within the PSM prospective territory.

Somites are not formed in the absence of medial PSM cells

As medial PSM cells originate from a different region of that of lateral PSM cells, we wondered whether they were equally committed to form somites. To answer this question, we performed ablations of the L-PSM territory corresponding to the length of approximately ten prospective somites on one side of 15 to 20-somite stage embryos (*n*=20) (Fig. 4A). These operated embryos were then cultured for 6-9 hours, which corresponds to the time required to form 4-6 pairs of somites. The analysis of the results showed that the same number of somites was formed in both operated and control PSM (Fig. 4B). Nevertheless, in the absence of L-PSM tissue, the first three to four developed somites were smaller than the control ones. By contrast, the most posterior formed somites are the expected size, showing the recovery capacity of PSM lateral tissue (Fig. 4B).

In another series of experiments we excised the M-PSM, keeping in place the L-PSM tissue (*n*=33) (Fig. 4E). After 6-9 hours of culture, the control PSM segmented and gave rise to four to six pairs of somites, as expected. Strikingly, no somite formation can be observed in the operated PSM, suggesting that in the absence of M-PSM cells, the lateral half of this tissue is not able to form somites (Fig. 4F). The results obtained suggest that M-PSM cells have the information for somite formation, in contrast to L-PSM cells.

We performed whole-mount in situ hybridisation in both medial and lateral PSM ablated embryos, using the *Delta-1* antisense RNA probe. The results obtained showed that somites formed in the absence of L-PSM cells express *Delta-1* in their posterior border, precisely in the same way as the non-operated PSM (Fig. 4C). By contrast, when the M-PSM domain is excised, not only does the L-PSM tissue not form morphological somites, but *Delta-1* expression pattern is perturbed: its anterior limit is shifted posteriorly and stripes of *Delta-1* expression cannot be observed in the operated PSM (Fig. 4G). Whole-mount in situ hybridisation was also performed using *Ch-Tbx6L*, a presomitic marker (Knezevic et al., 1997), which confirmed the presence of PSM tissue in both types of ablations (Fig. 4D,H).

The information for molecular segmentation is also segregated within PSM

It has already been established that the process of molecular

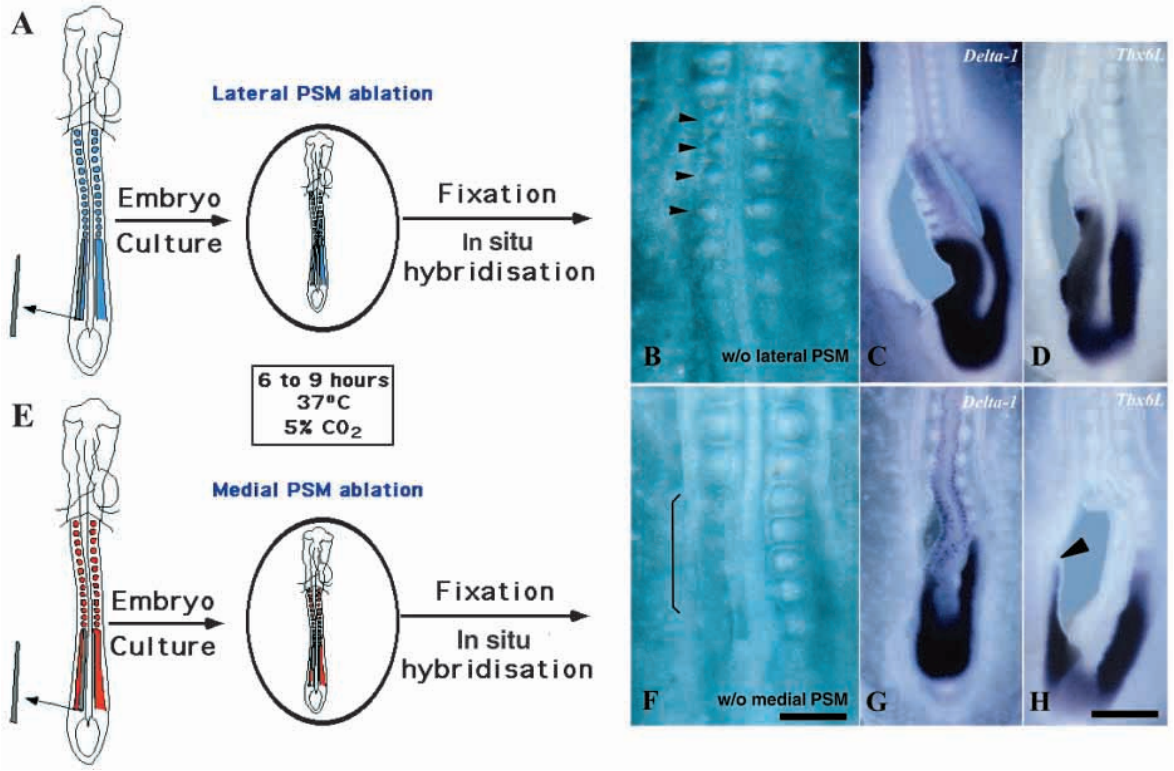


Fig. 4. Somites are not formed in the absence of medial PSM cells. (A,E) The type of PSM ablation performed on 15 to 20 somite stage embryos. (B) Dorsal view of an embryo cultured for 9 hours after lateral PSM ablation. The black arrowheads indicate somites formed during the in vitro culture period. The same number of somites is formed both in operated and control sides, although in the former the size of the somites appears smaller. (C) Lateral PSM ablated embryo hybridised with *Delta-1*. Somites formed in culture express *Delta-1* in its normal pattern of expression. (D) Lateral PSM ablated embryo hybridised with *Tbx6L* present a normal pattern of expression. (F) Dorsal view of an embryo cultured for 9 hours, whose medial PSM has been removed. The brackets indicate the operated side where no somites have been formed in the absence of medial PSM tissue. (G) Medial PSM ablated embryo hybridised with *Delta-1* reveals that the remaining lateral PSM does not express the *Delta-1* gene and that it is restricted to a more caudal domain, when compared with the control PSM. (H) Medial PSM ablated embryo hybridised with *Tbx6L* clearly shows that lateral PSM tissue remains in the operated side (arrowhead). Scale bars: 200 μm in B-F; 300 μm in C,D,G,H.

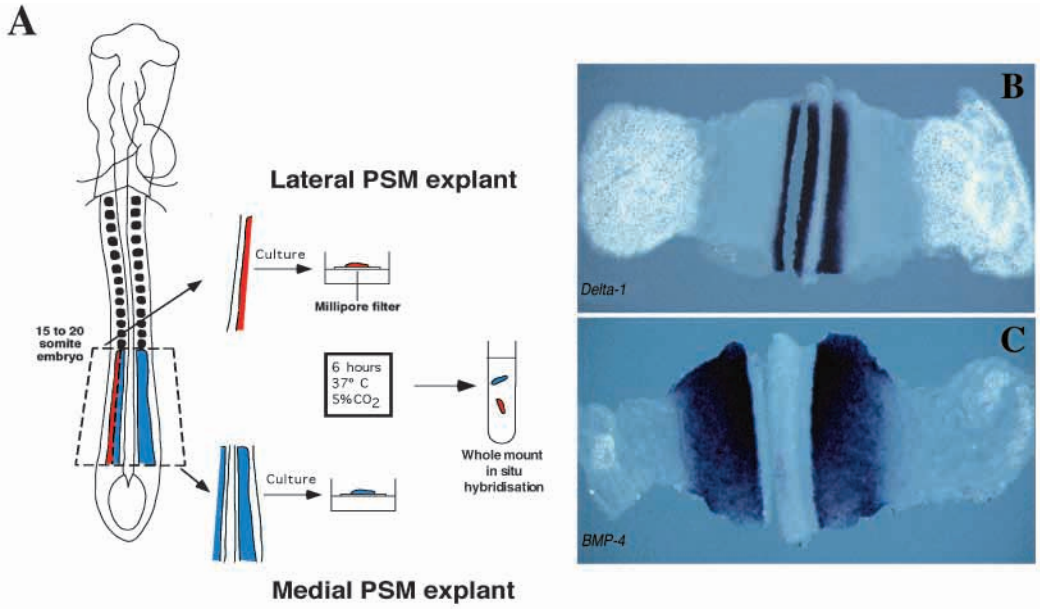


Fig. 5. Explant culture experiment: control experiment. (A) Two types of explants were generated, one comprising the lateral PSM (left explant) and the other containing the corresponding medial PSM, the axial structures and the control PSM (right explant). In order to ensure that the PSM tissue was being subdivided into equivalent medial and lateral halves, some explants were randomly taken, immediately fixed and hybridised either with probes for *Delta-1* (B) or with *BMP-4* (C). These control experiments show that the *Delta-1* domain of expression is longitudinally subdivided into two halves. In the *BMP-4* hybridised explant, a thin domain negative to this gene is present corresponding to the lateral PSM tissue. Scale bar: 250 μm.

segmentation is independent of the epithelialization phenomenon that leads to somite formation. We therefore assessed for the molecular segmentation of PSM in the absence of either medial or lateral PSM cells, by analysing the expression of the following molecular markers: *Delta-1*, *Notch-1*, *paraxis*, *hairy1*, *hairy2*, *lunatic fringe* and *Tbx6L*. We performed an in vitro culture of separated lateral and medial PSM explants devoid of the caudal part of the embryo (Fig. 5A), since it is likely to influence the results by constantly adding cells from the prospective PSM territory. As a control for the microsurgical incision, some explants were randomly chosen, and immediately fixed and hybridised with probes for *Delta-1* (*n*=5) and *BMP-4* (*n*=4) (Fig. 5). In these control explants, we can observe that the PSM *Delta-1* domain is divided in two halves (Fig. 5B) and that a *BMP-4*-negative domain, corresponding to the L-PSM tissue, remains axial to the *BMP-4* expression domain (Fig. 5C). The expression of *Delta-1*, *Notch-1*, *paraxis*, *hairy1*, *hairy2*, *lunatic fringe* and *Tbx6L* was assessed after 6 hours of culture. The analysis of the results obtained shows that the M-PSM domain, isolated from the neighbouring L-PSM domain, is not only able to form somites, as previously described, but also expresses all the tested molecular markers, in a similar pattern to the non-operated PSM (Fig. 6A-I). By contrast, L-PSM cells, isolated from more medial cells, lose the expression of *Delta-1* (*n*=16), *hairy1* (*n*=11), *hairy2* (*n*=17) and *lunatic fringe* (*n*=15) genes (Fig. 6A,G-I). *Notch-1* (*n*=9) and *paraxis* (*n*=10) are weakly expressed in cells located in the most anterior part of L-PSM (Fig. 6B,C), corresponding to the cells that were already expressing these genes at the moment of the operation. However, de novo expression is never detected in more posterior cells, in contrast to what happens in the control PSM (Fig. 6B,C). The expression domain of *BMP-4* and *Noggin* is conserved in both explants: the *BMP-4* domain is still delimiting the L-PSM half, the same way *Noggin* remains in the frontier between PSM and lateral plate tissue

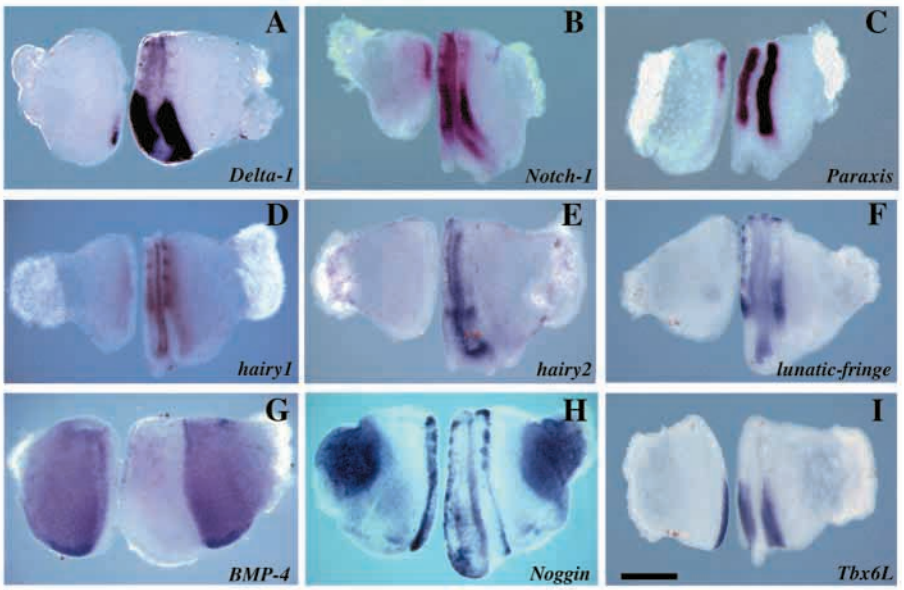


Fig. 6. Explant culture experiment: molecular segmentation markers are not expressed in the absence of medial PSM. Dorsal view of medial (right) and lateral (left) PSM explants, generated as described in Fig. 5 and subsequently hybridised with probes for *Delta-1* (A), *Notch-1* (B), *paraxis* (C), *hairy1* (D), *hairy2* (E), *lunatic fringe* (F), *BMP-4* (G), *Noggin* (H) and *Tbx6L* (I) antisense RNA probes. The expression of molecular segmentation markers (*Delta-1*, *Notch-1* and *paraxis*) as well as cycling genes (*hairy1*, *hairy2* and *lunatic fringe*) is affected in the isolated lateral PSM cells. Nevertheless, the usual expression patterns of *Tbx6L*, *BMP-4* and *Noggin* are maintained. Scale bar: 300 μm.

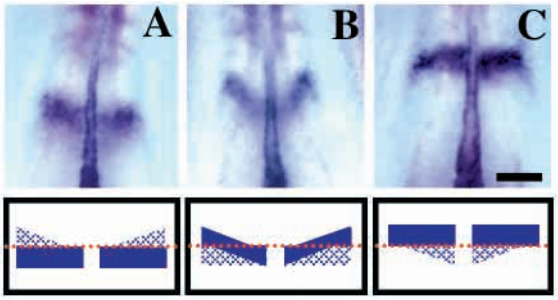


Fig. 7. A mediolateral asynchrony originates oblique stripes in the PSM. (A-C) Dorsal view of PSM/newly formed somites of six-somite stage embryos hybridised with *hairy1*. Rostral is towards the top. The schematics depict the PSM stripe anterior progression. The transition between two horizontal stripes originates an oblique stripe in the PSM: the transition between a posterior to an anterior stripe implies the upregulation of the gene in the lateral domain of the anterior stripe concomitant with the downregulation in the lateral part of the posterior stripe. In a second phase, cells in the medial domain follow the same progression in their expression. Scale bar: 100 μm.

(Fig. 6G,H). *Tbx6L* (*n*=19) remains expressed in the isolated L-PSM, confirming once again the presence of PSM tissue (Fig. 6I).

DISCUSSION

The molecular clock is already operating at the prospective PSM territory

Originally, it was demonstrated that presomitic cells begin expressing pulses of *hairy1* mRNA as they enter the paraxial mesoderm. The presence of cycling genes transcripts at the level of the tailbud has been mentioned (Aulehla and Johnson, 1999), although this region gives rise to several embryonic tissues that have not been fate-mapped. A cross-section analysis on the expression patterns of *hairy1*, *hairy2* and *lunatic fringe* genes, in 6-somite stage embryos, reveals an oscillation of expression of these genes from the posterior limit of the median pit to a region slightly posterior to the sinus rhomboidalis. The results from our quail/chick cell lineage study add new data to the previous fate-map performed by Catala et al. (Catala et al., 1996), allowing us to determine that this region, which exhibits a cyclic behaviour, corresponds exactly to the somitic prospective territory. In our study, we clearly show that the levels of *hairy1*, *hairy2* and *lunatic fringe* mRNA are already oscillating in prospective PSM cells well before they are incorporated into PSM, and that the cells presenting this cyclic behaviour are restricted to the prospective PSM territory (Fig. 1). It is also evident that within the prospective PSM territory we have a ‘wave’ of expression of cycling genes spreading in its longitudinal axis (Fig. 1).

The double whole-mount in situ hybridisation allowed us to perform a combinatory analysis of the expression patterns of the cycling genes. It is clear that cells located within the frontier defined by the sinus rhomboidalis, undergo cycles of expression of *hairy1*, *hairy2* and *lunatic fringe*. Nevertheless, the patterns of expression of these genes are very often not overlapped (Fig. 2). This can be explained either by an asynchrony in the transcription of the genes or as a consequence of different lifetimes of their mRNAs. Furthermore, posterior to the sinus rhomboidalis, mesenchymal cells undergo cycles of expression of *hairy2* and *lunatic fringe* but unexpectedly, they never seem to express the *hairy1* gene. In an even more posterior region, we can only observe transcription cycles of *hairy2*, while the expression of *hairy1* and *lunatic fringe* genes is never observed in this region.

The number of mRNA cyclic waves of expression exhibited by PSM cells depends on the time these cells spend until they incorporate a somite. Given that we have shown that prospective PSM cells are already undergoing cyclic waves of mRNA expression in their prospective territories, these cells must undergo more than the 12 cycles of expression they perform in the PSM (Palmeirim et al., 1997). However, as development proceeds, the PSM length is gradually reduced, suggesting that prospective somitic cells spend progressively less time in the PSM tissue. Moreover, the number of cycles undergone before somite formation could be maintained constant if, at later stages of development, prospective PSM cells spent more time in their prospective territory. Indeed, in our quail-chick chimaeras, quail donor cells can still be observed in this prospective PSM region after 24 hours of incubation. These remaining quail cells, or their progeny, are thus undergoing several cycles of expression without exiting the prospective PSM region.

The molecular clock is providing medial/lateral positional information

In 1996, Catala et al. determined the fate of several superficial regions of the sinus rhomboidalis by performing quail/chick orthotopic grafts at the six-somite stage, concluding that somite precursors are located in the posterior part of sinus rhomboidalis, not adjacent to the median pit (Catala et al., 1996). We performed grafts of the deep layer of the region caudal to the median pit (see Materials and Methods) and found that, 1 day after the operation, quail cells were situated in the M-PSM adjacent to notochord and neural tube. Thus, these cells located behind the median pit (which contains the precursors of more axial structures) and under the prospective neural plate will give rise to medial presomitic cells. Hence, we designate this region PM-PSM (presumptive medial – presomitic mesoderm; see Fig 1, Fig. 3). In agreement with our fate map, Charrier et al. (Charrier et al., 1999) showed that the paraxial mesodermal marker *Tbx6L* expression domain is juxtaposed to the *HNF3β* axial marker expression domain. Our result redefines, in the chicken embryo, the anterior limit of the prospective PSM territory, which is similar to the one described for the mouse embryo (Wilson and Beddington, 1996; Tam et al., 2000).

Our results show that within the prospective PSM territory, more anterior cells will be located in more medial positions. This suggests that more posterior cells will be located in more lateral positions. A DiI fate map created by Selleck and Stern (Selleck and Stern, 1991) at stage 4 (HH) has already revealed different origins for medial and lateral PSM cells. Therefore, the ‘wave’ of expression of the cycling genes spreading along the longitudinal axis of the PSM prospective territory corresponds to a ‘wave’ spreading along the future medial/lateral PSM axis. In agreement with this, if we re-analyse the stripes of expression of the cycling genes at the level of PSM, we can observe a medial/lateral asynchrony,

which is evidenced by the appearance of cross-stripes. In fact, an asynchrony in the medial/lateral expression could explain the appearance of a cross-stripe as a transition state between two horizontal stripes (Fig. 7). A very interesting conclusion is that the molecular clock is providing cellular positional information for both anteroposterior and mediolateral axes.

Medial and lateral PSM cells are differently committed to form somites

Medial and lateral PSM cells have different prospective territories, as reported in this study for six-somite stage embryos and for stage 4 embryos (HH) (Selleck and Stern, 1991). However, the fact that both medial and lateral parts of PSM have different origins does not imply that these two domains are differently committed as far as somite formation is concerned. We assessed for this issue, by performing both medial and lateral PSM ablations. Our results show that the M-PSM is able to form somites in the absence of the L-PSM, but surprisingly, no epithelial somite formation can be observed when the L-PSM is isolated from its medial counterpart. Selleck and Stern (Selleck and Stern, 1992) suggested that, at stage 4 (HH), cells from the lateral sector of the node (prospective medial PSM cells) are the ones that determine the spacing of the metameric pattern. Our experimental data clearly demonstrate that immediately before somites are formed, the information for morphological somite formation is only present in the medial PSM cells. Therefore, it is likely that medial PSM cells have to recruit lateral ones in order to form somites. Furthermore, PSM and epithelial somites can be subdivided into a medial and a lateral compartment according to their fate (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000). Hence, medial and lateral parts of PSM have different origins, are differently committed to somite formation and give rise to different embryonic structures.

Medial/lateral PSM dissociation disturbs the intrinsic program of PSM molecular segmentation

Several data from the literature support the idea that molecular segmentation occurs independently of epithelial somite formation (Burgess et al., 1996; Sosic et al., 1997; Palmeirim et al., 1998). In mice, targeted inactivation of several genes that are known to be important for vertebrate somitogenesis leads to a disruption of morphological somite formation (Conlon et al., 1995; Oka et al., 1995; Hrabe de Angelis et al., 1997; Kusumi et al., 1998), although somite derivatives such as muscles and skeleton retain a segmented pattern. In addition, morphological somite formation can be impaired by the ablation of the PSM upper-layer ectoderm (Sosic et al., 1997; Palmeirim et al., 1998), whereas genetic segmentation of PSM is not dependent on any signal coming from neighbouring tissues, making it an intrinsic property of this tissue (Palmeirim et al., 1998). For example, in cultured isolated PSM, epithelial somites do not form, although striped expression pattern of *Delta-1* is observed, corresponding to the *Delta-1* pattern of expression at the level of somites. The dynamic expression pattern of the cycling genes exhibits a remarkable degree of autonomy. The pattern of expression of these genes is completely independent of any signal coming either from the posterior or anterior part of the PSM, as cutting the PSM into as many as ten pieces, does not disturb the expression pattern of these genes (data not shown). In this work, we not only demonstrate that morphological somite formation is prevented in the absence of M-PSM, but also that the process of molecular segmentation is perturbed. The L-PSM isolated from its medial counterpart loses the expression of molecular segmentation markers such as *Notch-1*, *Delta-1*, *paraxis*, *hairy1*, *hairy2* and *lunatic fringe* (Fig. 6). For the first time we have disturbed the expression pattern of the cycling genes in L-PSM cells by isolating them from the medial ones.

In the chick embryo, the PSM is neighboured by the lateral mesoderm that expresses high levels of *BMP-4*. The PSM is protected from the action of *BMP-4* protein by the product of *Noggin* expressed in the border between PSM and lateral mesoderm (Tonegawa and Takahashi, 1998). In cultured explants, the domains of expression of these genes are maintained (Fig. 6G,H). These results suggest that, in the absence of M-PSM cells, its lateral counterpart is unlikely to be re-specified into lateral mesoderm.

It is now generally accepted that the Notch and Delta signalling pathway plays a role in the process of somitogenesis. The analysis of mouse mutants for the transmembrane receptor *Notch1* (Conlon et al., 1995), for its ligands *Delta1* and *Delta3* (Hrabe de Angelis et al., 1997; Kusumi et al., 1998) and for the transcription factor *RBPJ-kappa* (Oka et al., 1995) has demonstrated the importance of these proteins for establishing the somitic boundaries and defining anterior and posterior somitic identities. Furthermore, it has recently been observed that the *Delta1* mutant mice lack the dynamic expression of *Hes1* (the gene claimed to be the *hairy2* homologue in the mouse) (Jouve et al., 2000). Several lines of research are now trying to unveil the relationship between the Notch and Delta signalling pathway and the molecular clock that underlies somitogenesis (Aulehla and Johnson, 1999; Holley et al., 2000; Jiang et al., 2000). Taking this into account, our observation that *hairy1*, *hairy2* and *lunatic fringe* genes are not expressed in the isolated lateral PSM could be explained by the absence of an operating Notch and Delta signalling pathway in this tissue.

The absence of expression of the segmentation genes, including the cycling genes, in the L-PSM deprived from its medial counterpart, cannot be explained by the lack of a signal provided by axial structures, as PSM is able to undergo normal segmentation isolated from these structures (Palmeirim et al., 1997; Palmeirim et al., 1998; McGrew et al., 1998; Jouve et al., 2000). Therefore, it is more likely that a signal supplied by M-PSM would be responsible for the upregulation of these genes in the L-PSM. However, the nature of this signal remains to be identified.

In summary, we unveil that medial PSM cells are the ones possessing the information for molecular segmentation and somite formation. We propose that in order to control morphological somite formation and molecular segmentation, a signal travels along the mediolateral PSM axis. Furthermore, we demonstrate that the molecular clock that underlies somitogenesis is already operating in PSM precursor cells. At this level, a ‘wave’ of expression of *hairy1*, *hairy2* and *lunatic fringe* genes spreads along the longitudinal axis of the whole PSM prospective territory, which corresponds to the future mediolateral PSM axis. A very interesting conclusion of this work is that the molecular clock is providing cellular positional information in at least two dimensions: not only in the anteroposterior but also in the mediolateral PSM axis.

We thank São Alpiarça and Françoise Lapointe for their excellent histological work. We are very grateful to Sólveig Thorsteinsdóttir, Delphine Duprez, Chaya Kalcheim and Martin Catala for critically reading this manuscript. The QCPN monoclonal antibody developed by Bruce M. and Jean A. Carlson was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Financial support was provided by Instituto Gulbenkian de Ciência where I. P. is a Principal Investigator, by Fundação para a Ciência e Tecnologia project nº 34599, by the Centre National de la Recherche Scientifique, and by the Association Française pour la Recherche contre le Cancer (ARC) contract nº 5578. C. F. is a PhD student supported by the Praxis XXI/BD/21583/99 and S. R. is financed by Fundação para a Ciência e Tecnologia project nº 34599.

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Horloge moléculaire et segmentation des vertébrés : qui fait quoi ?

Catarina Freitas, Sofia Rodrigues, Jean-Baptiste Charrier, Marie-Aimée Teillet, Isabel Palmeirim

> Chez les vertébrés, les somites sont les structures responsables de la mise en place du squelette axial segmenté et de la segmentation du système nerveux périphérique. Les mécanismes moléculaires qui contrôlent la formation progressive des somites le long de l'axe rostro-caudal de l'embryon n'ont pas encore été complètement élucidés. L'analyse des profils d'expression de quelques gènes à expression cyclique dans le mésoderme présomitique et dans son territoire présomptif au niveau de la ligne primitive révèle que les cellules précurseurs des cellules somitiques possèdent dès leur origine une information positionnelle, non seulement selon l'axe rostro-caudal, mais aussi selon l'axe médio-latéral. De plus, ces cellules semblent présenter des propriétés différentes en ce qui concerne le processus de segmentation. <



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Chez les vertébrés adultes, il existe plusieurs types de structures ayant un caractère segmentaire comme les vertèbres et les côtes, les disques intervertébraux, les muscles et les vaisseaux qui leur sont associés, les nerfs et les ganglions spinaux, etc. La segmentation du corps des vertébrés se manifeste très tôt au cours du développement embryonnaire par l'apparition de structures métamériques transitoires, les somites (➔). La plupart de nos connaissances sur le développement et la différenciation des somites provient d'études réalisées chez l'embryon d'oiseau. Cependant, les grandes étapes du processus de formation des somites sont équivalentes chez les autres vertébrés, en particulier chez les mammifères.

(➔) m/s 1997, n°10, p. 1145

Chez l'embryon de poulet, la première paire de somites apparaît vers 24 heures d'incubation sous la forme de deux sphères constituées de cellules épithéliales, localisées de part et d'autre du tube neural et de la notochorde, en arrière du niveau des futures vésicules otiques *(Figure 1)*. Une fois la première paire de somites formée, la segmentation se poursuit le long de l'axe rostro-caudal de l'embryon au sein de deux bandelettes de tissu mésenchymateux, le mésoderme para-axial non segmenté appelé aussi mésoderme présomitique (MPS). De façon progressive et régulière, un groupe de cellules de la partie la plus rostrale du MPS acquiert une structure épithéliale et une séparation apparaît entre le nouveau somite formé et le MPS mésenchymateux. Néanmoins, la taille du MPS reste relativement constante. En effet, alors que de nouveaux somites se forment dans sa partie rostrale, les cellules du MPS se multiplient et d'autres cellules, issues de la ligne primitive par le processus continu de gastrulation, s'ajoutent à sa partie caudale. Des études expérimentales de ce processus coordonné ont montré, chez l'embryon d'oiseau, que les cellules appartenant au MPS sont en quelque sorte préprogrammées pour constituer un somite à un moment donné du développement et cela indépendamment de la présence des tissus

adjacents ou de l'orientation rostro-caudale du MPS dans l'embryon [1]. En effet, on peut inverser l'orientation d'un segment de MPS dans l'embryon sans changer le sens de son processus propre de segmentation.

Le nombre total de somites et le temps nécessaire à leur formation sont constants et caractéristiques d'une espèce donnée. Chez le poulet, les somites apparaissent à raison d'une paire toutes les 90 minutes en moyenne et, à la fin de la somitogenèse, après 4 jours d'incubation, 52 à 53 paires de somites auront été formées.

Au cours des étapes de la segmentation, on note, chez les embryons d'oiseaux et de mammifères, un gradient rostro-caudal de différenciation du mésoderme para-axial. En effet, alors que les cellules de la ligne primitive sont encore en cours de gastrulation, les cellules du MPS et des somites épithéliaux sont multipotentes et celles des somites les plus antérieurs ont engagé un processus de différenciation dans plusieurs lignages déterminés. Ces différents lignages constituent le sclérotome ventralement et le dermo-myotome dorsalement dans chaque somite. Finalement, les principaux tissus dérivés des somites sont le squelette axial du corps (les vertèbres et les disques intervertébraux ainsi que les côtes et une partie des ceintures scapulaire et pelvienne), le derme dorsal et les cellules musculaires squelettiques à l'exception de celles de la tête (pour revue, *voir* [2] et [3]).

Si les aspects morphologiques et cellulaires de la somitogenèse ont été largement étudiés, le contrôle moléculaire du phénomène périodique et symétrique qui conduit à la formation des somites chez les vertébrés est demeuré pendant longtemps un des problèmes non résolus de la biologie du développement. Récemment, l'analyse, pendant le processus de formation somitique, de l'expression du gène *chicken-hairy1 (c-hairy1)*, homologue chez le poulet du gène de segmentation *hairy* de la drosophile, a montré que les cel-

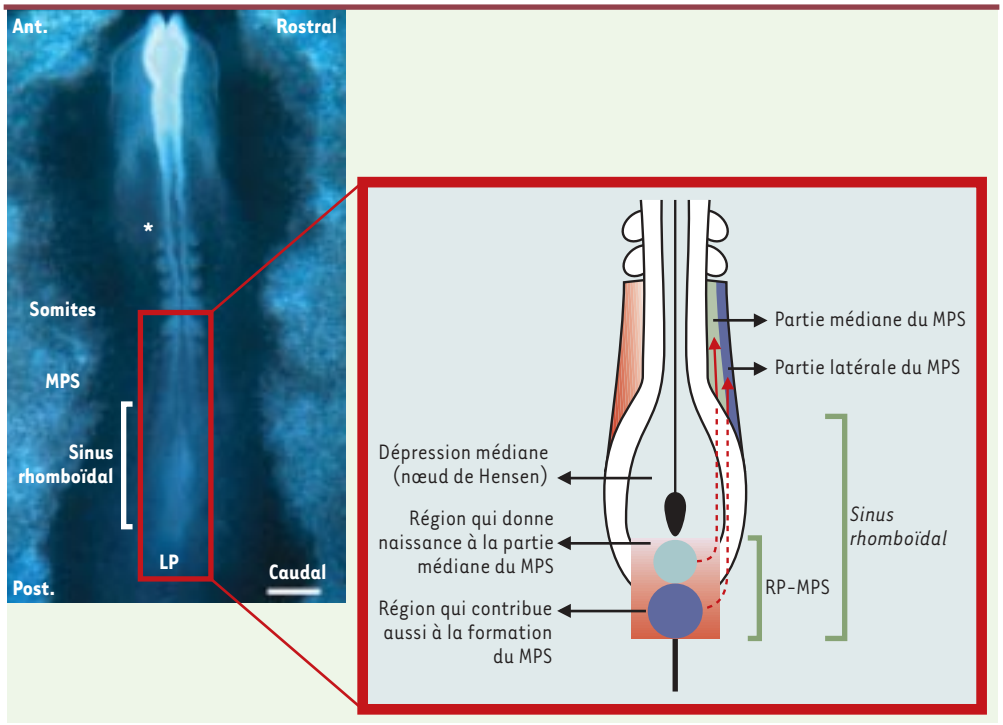


Figure 1. ***Embryon de poulet au stade six somites. À gauche.*** Vue dorsale d'un embryon de poulet de six somites. Le mésenchyme présomitique (MPS) s'étend jusque dans la partie caudale du sinus rhomboïdal où il prend naissance au niveau de la ligne primitive. ***À droite.*** Schéma représentant un grossissement de la partie caudale de cet embryon. La région du sinus rhomboïdal contient en son centre la dépression médiane où se localise l'organisateur de l'embryon (nœud de Hensen). Caudalement par rapport au nœud de Hensen, s'étendent, dans la ligne primitive, les territoires du futur MPS médian (petit cercle) et du futur MPS latéral (grand cercle). L'ensemble de ces deux territoires constitue la région présomptive des somites. Au cours de la gastrulation, le mouvement de régression du nœud de Hensen et de la ligne primitive s'accompagne de la mise en place des futures cellules somitiques représentées par les deux flèches rouges. La partie pointillée des flèches représente les cellules localisées sous la plaque neurale présomptive. L'expression des gènes de segmentation, *c-hairy1*, *c-hairy2* et *lunatic-fringe*, dans les cellules de la région présomptive des somites, oscille d'une manière coordonnée dans l'espace et dans le temps de sorte qu'on observe pour chaque gène une vague d'expression dans le sens longitudinal représentant la future disposition médio-latérale du MPS. Ces oscillations « médio-latérales », dans la région postérieure à l'organisateur, sont probablement maintenues jusqu'à l'allongement total de l'embryon. Le fait que la partie médiane du MPS continue à présenter des vagues d'expression des gènes de la segmentation et se segmente en l'absence de sa partie latérale, alors que la partie latérale du MPS montre des profils d'expression très perturbés et une absence de segmentation en l'absence de sa partie médiane, suggère que ces deux régions du MPS ne possèdent pas les mêmes propriétés en ce qui concerne le processus de la segmentation somitique. Les futures cellules médianes recevraient dès leur origine dans la ligne primitive l'information de segmentation alors que les cellules latérales recevraient cette information dans le MPS. Ant.: antérieur. Post.: postérieur. RP : région présomptive. LP : ligne primitive. Barre = 300 mm. Astérisque : localisation de la future vésicule otique.

lules du MPS subissent des oscillations d'expression de ce gène d'une durée équivalente au temps de formation d'un somite, c'est-à-dire de 90 minutes [4, 5]. En d'autres termes, toutes les 90 minutes, chaque cellule présomitique alterne une période de temps pendant laquelle des transcrits du gène *hairy1* sont détectés, et une autre période caractérisée par l'absence de ces mêmes transcrits. Ces oscillations se répètent jusqu'au moment où cette cellule est incorporée dans un somite épithélial nouvellement formé. Un léger décalage temporel, dans les oscillations d'expression de ce gène entre les différentes cellules présomitiques, entraîne, pour chaque somite qui se forme, l'apparition d'une vague d'expression de *c-hairy1* traversant le MPS dans toute sa longueur [4]. Ces oscillations du niveau d'expression de *c-hairy1*, réitérées toutes les 90 minutes, ont mis en évidence pour la première fois une horloge moléculaire liée à la segmentation somitique.

Plus récemment, deux autres gènes, *c-hairy2* et *lunatic fringe*, présentant également une expression en vague traversant le MPS, ont été identifiés [6-8]. Ces deux gènes sont à présent définis comme étant aussi liés à l'horloge moléculaire de la somitogenèse. Dans d'autres organismes comme la souris [6, 9] ou le poisson zèbre [10], des gènes d'expression cyclique ont également été identifiés dans le MPS.

Afin de mieux comprendre le rôle des gènes d'expression cyclique dans la segmentation des somites, il semblait nécessaire de déterminer si l'expression dynamique et rythmée de ces gènes, *c-hairy1*, *c-hairy2* et *lunatic fringe*, était déjà établie au niveau des cellules précurseurs du MPS, dans la ligne primitive.

Chez l'embryon de poulet, le nœud de Hensen (extrémité antérieure de la ligne primitive) est considéré comme l'organisateur de l'embryon. Au stade de 5-6 somites, le nœud de Hensen se localise au niveau d'une dépression, au centre du sinus rhomboïdal *(Figure 1)*. Le système de marquage cellulaire, par échanges de fragments d'ébauches embryonnaires équivalentes entre embryons de caille et de poulet, avait montré que certaines cellules du MPS ont pour origine la ligne primitive correspondant à la région postérieure du sinus rhomboïdal [11].

En étudiant attentivement, par hybridation *in situ in toto*, les profils d'expression des gènes d'expression cyclique au stade de six somites, nous avons noté que chacun de ces gènes présente un profil d'expression évolutif au niveau du sinus rhomboïdal. Cette observation suggère que les futures cellules du MPS subissent des cycles d'expression des gènes *c-hairy1*, *c-hairy2* et *lunatic fringe* avant même d'être incorporées dans le MPS. La localisation plus précise des cellules subissant des cycles d'expression de ces gènes a été obtenue à partir de coupes histologiques transversales de la région préalablement définie comme la région des somites présomptifs. L'analyse précise des profils d'expression génique montre qu'il existe à la

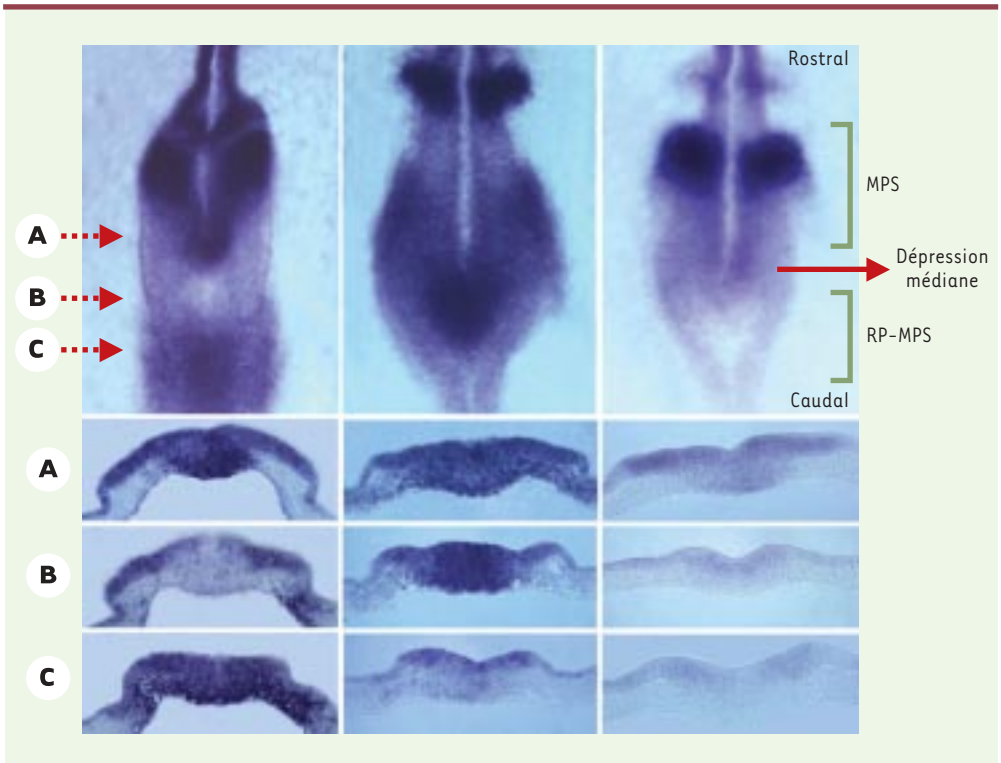


Figure 2. ***Expression dynamique du gène* lunatic fringe.** ***En haut.*** Vues dorsales de la partie caudale de trois embryons de poulet de 6 somites hybridés avec la sonde s'hybridant avec l'ARNm du gène *lunatic fringe*. On remarque que des embryons ayant le même nombre de somites présentent des profils d'expression très différents. Les mêmes types de profils sont retrouvés aux différents stades de la segmentation. ***En bas.*** Des coupes transversales à différents niveaux (A, B, C) du sinus rhomboïdal des embryons représentés ci-dessus montrent qu'il existe, d'une part, une vague d'expression caudo-rostrale au niveau du mésoderme présomitique (MPS) et, d'autre part, une vague distincte au niveau de la région présomptive du MPS (RP-MPS).

fois une vague d'expression s'étendant selon un axe caudo-rostral dans le MPS, et une vague d'expression progressant le long de la région d'origine des futures cellules somitiques, depuis le bord caudal de la dépression médiane (c'est-à-dire l'extrémité caudale du nœud de Hensen) jusqu'à la région caudale du sinus rhomboïdal *(Figure 2)*.

Afin de comprendre ce que représente la zone immédiatement postérieure au nœud de Hensen vis-à-vis des somites, nous avons donc remplacé cette région de l'embryon de poulet par la région équivalente provenant d'un embryon de caille au même stade, selon la technique éprouvée des chimères caille-poulet. Vingt-quatre heures après la greffe, des cellules de caille ont été repérées sur des coupes histologiques, à l'aide d'un anticorps spécifique (QCPN), dans la région médiane de la totalité du MPS et de quelques somites nouvellement formés [12]. Ce résultat montre que le territoire présomptif des somites s'étend depuis la limite caudale du nœud de Hensen jusqu'à la partie caudale du sinus rhomboïdal, région dans laquelle on observe les oscillations de plusieurs gènes de segmentation. De plus, la partie rostrale du territoire présomptif des somites donne naissance aux cellules les plus médianes du MPS et des somites. Cela montre que le futur axe médio-latéral du mésoderme para-axial est déjà présent selon l'axe rostro-caudal dans le territoire présomptif des somites, au niveau de la ligne primitive.

Quelle est la signification dans le processus de segmentation de l'existence de deux sous-populations de cellules présomitiques, médiane et latérale, déjà ségrégées au niveau de la ligne primitive ? Ces deux sous-populations cellulaires ont-elles un rôle différent en ce qui concerne ce processus ? Sont-elles engagées et déterminées de la même manière vers la formation des structures segmentées ?

Pour clarifier cette question, nous avons effectué, sur des embryons de poulet aux stades de 15 à 20 paires de somites, des ablations soit de la moitié médiane, soit de la moitié latérale du MPS d'un seul côté de l'embryon [12]. Les embryons opérés ont été cultivés *in vitro* pendant une durée correspondant à la formation de quatre à six paires de somites (soit six à neuf heures). Après excision du MPS latéral, on observe des somites en nombre égal du côté opéré et du côté témoin. La seule différence réside dans la taille des somites qui se révèlent plus petits du côté de l'ablation. En revanche, après ablation du MPS médian, nous constatons l'absence de structures segmentées du côté où a eu lieu l'ablation, bien que le MPS latéral soit présent comme en atteste l'expression d'un gène caractéristique du mésoderme présomitique, *cTbox6L*. Ces résultats ont été confirmés *in vitro* par des cultures d'explants, MPS médian ou MPS latéral. Le MPS latéral, en l'absence du MPS médian, perd l'expression des gènes critiques pour la segmentation (*C-Delta1*, *C-Notch-1*, *c-hairy1*, *c-hairy2* et *lunatic fringe*) et reste non segmenté, alors que le MPS médian peut effectuer, en l'absence du MPS latéral, une segmentation normale d'un point de vue moléculaire et morphologique [12].

Des expériences antérieures [13] avaient montré que des explants de MPS isolés des tissus adjacents (ectoderme, endoderme, tube neural et lames latérales) n'effectuent pas une segmentation morphologique bien que l'expression du gène *C-Delta1* suive un profil d'expression caractéristique fait de bandes qui marquent spécifiquement les segments. Ces résultats, associés à ceux que nous venons de présenter, montrent que le processus morphologique de formation des somites dépend de facteurs d'environnement et qu'il est distinct du processus de segmentation génétique, lequel requiert la présence du MPS médian, mais semble indépendant des autres structures.

En résumé, malgré son aspect homogène, le tissu présomitique est constitué de deux sous-populations cellulaires ayant une origine distincte et qui contiennent des informations différentes quant à la capacité de former des segments. Le MPS médian dérive de la région rostrale de la ligne primitive, en contact direct avec le nœud de Hensen *(Figure 1)*. Afin de former les segments successifs de l'embryon, les cellules issues de cette région s'associent vraisemblablement à des cellules provenant de régions plus caudales de la ligne primitive, correspondant à la région caudale du sinus rhomboïdal. Ces dernières constituent le MPS latéral. Les résultats de nos expériences suggèrent qu'un signal, de nature inconnue, en provenance du MPS médian, est responsable de la mise en place du programme moléculaire qui accompagne la segmentation dans le MPS latéral.

En 1998 [5] étaient décrites des oscillations d'expression de l'ARN messager de *c-hairy1* formant une suite de vagues qui balayent le MPS, de façon synchronisée avec la formation des somites. Les conclusions étaient que, pour chaque cellule présomitique, le nombre d'oscillations effectuées serait en rapport direct avec sa localisation rostro-caudale dans le mésoderme para-axial. Il apparaît maintenant qu'il existe un autre type d'oscillations définissant des vagues qui se déplacent dans le futur axe médio-latéral du territoire présomitique. Les données expérimentales que nous avons obtenues récemment suggèrent que l'horloge moléculaire, qui s'établit très tôt au cours du développement embryonnaire, selon un processus encore inconnu,

détermine l'information positionnelle des cellules présomitiques dans les deux dimensions, l'axe médio-latéral et l'axe rostro-caudal. ◊

REMERCIEMENTS

Ces recherches ont été financées par l'Instituto Gulbenkian de Ciência, par la Fundação para a Ciência e Tecnologia projet. n° 34599, par le Centre National de la Recherche Scientifique et par l'Association Française pour la Recherche contre le Cancer (ARC) contrat n° 5578. C.F. est boursière de Praxis XXI/BD/21583/99 et S.R. est financée par la Fundação para a Ciência e Tecnologia projet n° 34599.

SUMMARY

Molecuar clock and vertebrate segmentation: Who does what?

Somites are embryonic structures that constitute the framework on which the segmental pattern of the axial skeleton and peripheral nervous system of the vertebrate body is established. Somites appear in a sequential and periodic fashion along the rostro-caudal axis of the embryo, budding off from an unsegmented tissue named presomitic mesoderm (PSM). The oscillatory mRNA expression pattern of several segmentation genes at the level of PSM cells shows that the molecular clock underlying somitogenesis provides rostro-caudal positional information to these cells. In this paper, we show that medial and lateral presomitic cells arise from distinct prospective territories and are differently committed in what concerns segmentation and somite formation. Futhermore, our results strongly suggest that the molecular clock also supplies medio-lateral positional information to PSM cells. ◊

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TIRÉS À PART

I. Palmeirin

Supplemental Data

Unpublished results

2. 1. The median pit induces the formation of ectopic somites and PSM

The experiments described in the first part of this chapter, showed that ablation of the medial part of the anterior PSM results in the lack of somite formation in the lateral PSM tissue that is kept in place. Furthermore, we also demonstrated that the Prospective Medial PSM (PM-PSM) territory consists of a 100-150μm region located just posterior to the median pit (MP), in 6-somite stage chick embryos. Recent work from our group has further determined that cells belonging to the PM-PSM domain, in opposition to the lateral ones, are able to drive the segmentation process. In addition, it was established that at this particular level, such capacity is not fully determined, in contrast to the anterior PSM where the intrinsic segmentation capacity is restricted to the medial cells. The conclusion of this work is that these cells must have previously acquired such capacity, most likely through the influence of their environment (Freitas, 2004). Barrier insertion experiments unveiled that neither the caudal neural tube nor the open neural plate seem to be responsible for instructing the PM-PSM cells to form somites. The remaining structure in close proximity to this territory is the median pit, which is the region containing the prospective notochord and floor plate cells and conventionally considered to be the morphological Hensen's node at this stage (Catala *et al*., 1996; Freitas, 2004).

Following the analysis of the above data we reasoned that the MP could be instructing the PM-PSM cells with the information for segmentation. In the line of this hypothesis, we wondered whether PM-PSM cells that had previously been activated by the MP had the ability to induce the formation of somites. So, we started this study by performing interspecific quail-chick grafts of the PM-PSM to test if this region was able to induce ectopic somite formation. Heterochronic (different stages) grafts of the PM-PSM of 6-somite stage quail embryos were transplanted into competent lateral blastoderms of stage 4 HH chick embryos, and analysed one day after the grafting experiment (Fig.S1A-B). The morphological observation of these chimeras was not conclusive, and hence we

hybridised them with the *chordin* RNA probe followed by an immunohistochemistry with the QCPN (Quail Cell Perinuclear) antibody, which stains quail cells, in the chimeric embryos. The expression of these specific markers showed that the PM-PSM grafts self-differentiated into an unspecific tissue that totally derived from quail donor cells. These experiments showed that the PM-PSM region does not lead to axis formation or induction of somites (Fig. S1B').

Given that the PM-PSM cells that had previously been instructed to segment did not retain the capacity to induce ectopic somites, we sought to investigate whether the MP region could accomplish this task. In order to test this, we grafted the MP of 6-somite stage quail embryos into competent lateral blastoderms of stage 4 HH chick embryos, as previously described (Fig.S1C). The morphological analysis of these chimeras revealed that the grafted MP gives rise to secondary axial structures (n=11). However, the formation of somites in these ectopic axes was observed in few cases (3/11). We further analysed these chimeras by checking the expression of *tbox6l*, which is a specific marker for PSM tissue, and for the QCPN antibody. The analysis of the expression of these markers revealed that the ectopic axial structures were always of quail origin, whereas induced PSM (n=4/11) and somites (n=3/11) deriving from the host were observed in less cases (Fig.S1C'). Nevertheless, our results indicate that the median pit seems to contain the information for segmentation and that it is able to induce ectopic somites in a competent region, that was not fated to become PSM tissue.

The region of the MP grafts excised from the donor embryo in the above experiment comprised the pit itself and the slope surrounding it. The Axial Paraxial Hinge (APH) is a region located in the posterior slope of the MP. The relevance of the APH is that it contains bi-potent notochord and floor-plate precursor cells that are juxtaposed to a pool of PSM precursors. These fates are clearly evidenced by the confrontation of the expression of *hnf3*β and *tbx6l*, respectively. So, the APH is actually considered to be the chordal neural hinge at 6-somite stage, and this region was also proven to be crucial for embryo

elongation (Charrier *et al*., 1999). Given the importance of the role of the APH, we figured that this region alone could be detaining the information for segmentation and be instructing the PM-PSM cells to form somites. To test this possibility, we performed heterochronic grafts of the quail APH region into the lateral blastoderm of younger chick embryos, as previously described. However, the analysis of the resulting chimeras showed that the grafts do not develop at all, indicating that it is not the APH that is emitting a signal for the formation of somites (n=6) (Fig. S1D-D').

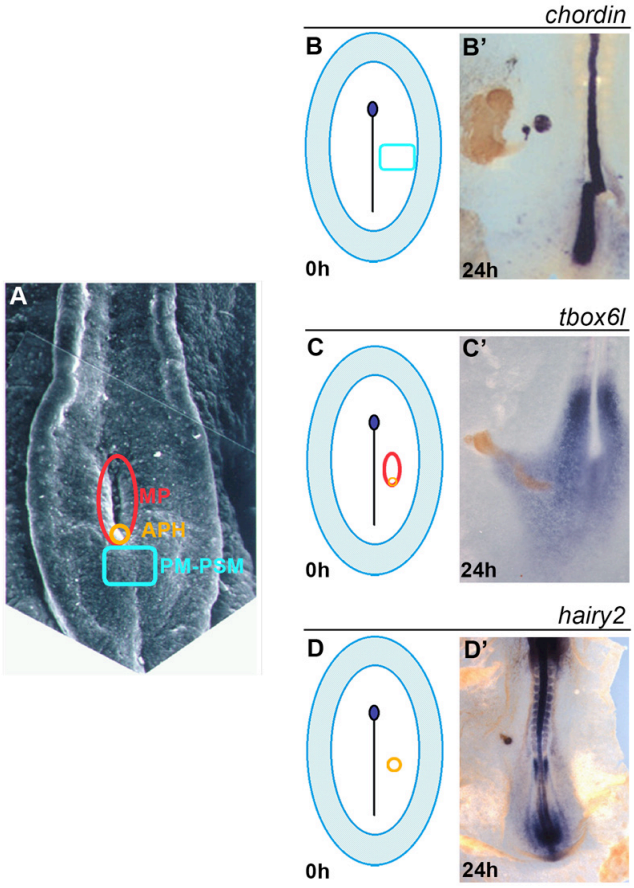


Fig. S1. Axis induction by PM-PSM, Median Pit and APH grafts

(A) Diagram representing the *sinus rhomboidalis* of a 6-somite stage donor embryo with the indication of the specific grafted regions. The MP, APH and PM-PSM regions are represented by red, orange and blue colour, respectively. (B-D) Left panels represent the grafts of the PM-PSM (B), MP region (C) and APH (D) into a stage 4 HH host embryo. (B') The PM-PSM graft developed into an undefined tissue of donor origin (QCPN brown staining). (C') The resulting secondary axis consists of axial structures derived from the graft (QCPN brown staining) and it exhibits the ectopic expression of the *tbx6l* gene, which is an indication of induced PSM tissue. (D') The graft did not develop, as shown by the QCPN staining on the left side of the host embryo. Rostral is towards the top. MP, median pit; APH, axial paraxial hinge; PM-PSM, prospective medial-presomitic mesoderm.

Taken together, our findings strongly suggest that the median pit is an important signalling centre that detains the information for inducing somite formation. Furthermore, these findings also show that at the 6-somite stage the median pit is not only the morphological node but it also retains organizing properties that are characteristic of a functional Hensen's node.

2. 2. The median pit induces the segmentation clock in ectopic PSM cells

As described above, the MP is an important signalling source able to induce somite formation in cells that were not committed to form somites. The next step on this study was to check whether the segmentation clock was properly operating in the induced PSM tissue that was derived form the median pit grafts (Fig.S2A-B).

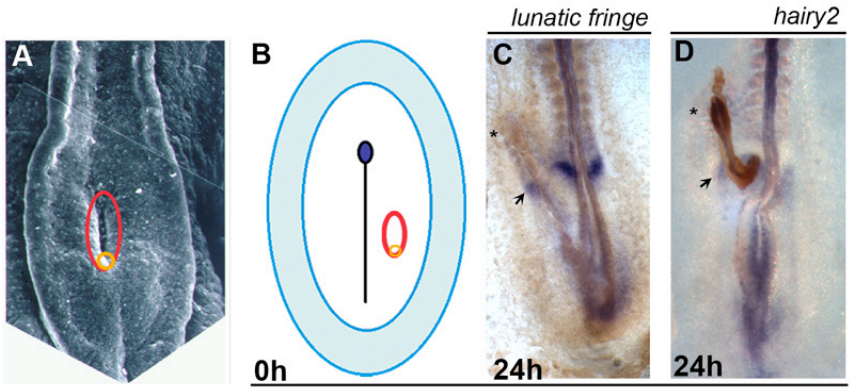


Fig. S2. Expression of the cycling genes in MP induced PSM tissue

(A) Diagram representing the *sinus rhomboidalis* of a 6-somite stage donor embryo with the indication of the specific grafted region. The MP and the APH region, contained within it, are represented by red and orange colours, respectively. (B) Panel representing the grafts of the MP region into a stage 4 HH host embryo. (C) The resulting secondary axis consists of axial structures, PSM and somites and it exhibits the ectopic expression of the *lunatic fringe* gene (D) A secondary axis was formed consisting of donor derived axial structures (QCPN brown cells) and induced PSM and somites. The ectopic PSM expresses the *hairy2* gene.
* indicates the presence of induced somites in the secondary axes.
Black arrow indicates the ectopic expression of the cycling genes in the induced PSM.

Thus, one day after the grafting experiments the chimeras were hybridised with either the *lunatic fringe* or the *hairy2* probes so that the expression of the cycling genes in both the host and induced PSM were compared. The analysis of the

chimeras revealed that in many cases the expression of the cycling genes is either absent or extremely difficult to analyse given the defectively patterned axis (n=43) (data not shown). Nevertheless, we obtained some cases (n=4) where the PSM expression of the cycling genes is clear in both induced and host axis (Fig.S2C-D). The fact that the induced PSM tissue is properly expressing the cycling genes evidences that the segmentation clock is also operating in the secondary axis. These results suggest that the MP has the ability to induce the complete segmentation programme in ectopic PSM cells.

2. 3 Materials and Methods

2. 3. 1 Median pit and PM-PSM grafts

Chick eggs were incubated for 18-20 hours to obtain stage 4 HH host embryos. The embryos were removed from the egg with their vitelline membranes and placed in a glass ring according to New (1955). Quail embryos were incubated for approximately 36 hours so that they developed until the stage of 5 to 6-somites and were collected from the egg yolk into resin-coated petri dishes in PBS without $Ca2^{+}$/$Mg2^{+}$. At the caudal part of the embryo we identify the *sinus rhomboidalis* (open neural plate). At the centre of this structure, a pit can be observed. We consider the median pit to be the region comprising the pit itself and the slope surrounding it. Either the MP or the Prospective Medial–Presomitic Mesoderm was surgically removed from the donor embryo and grafted to the posterior lateral blastoderm of the host. The chimeras were reincubated overnight in a humidified box at 37-38ºC and then harvested and fixed in 4% paraformaldehyde.

Chapter 3

The role of Hensen's node on the onset of the segmentation clock

Results organized in the form of an article

The work presented in this Chapter aimed at understanding what triggers the beginning of the molecular clock activity. The analysis of the expression of the cycling genes in the PSM of spontaneous and artificially generated double axes strongly suggests that Hensen's node could be the source of a signal responsible for activating the molecular clock. A series of heterotopic and isochronic node grafting experiments were performed, and we observed that the ectopic cyclic expression of the clock genes in induced PSM is different from the one of the host PSM. These results indicate that Hensen's node is in fact able to induce the triggering of the molecular clock and to restart the segmentation programme in induced PSM cells.

Introduction

The existence of a small group of cells in the vertebrate embryo that is able to generate a new embryonic axis when transplanted to a competent host region has been known for decades. This organizing centre, known as the organizer, has the capacity to induce head and trunk structures by recruiting competent host cells into a new embryonic fate. Ever since the description of the organizer in amphibian embryos (Spemann and Mangold, 1924) exhaustive studies have been performed to understand the mechanisms underlying neural induction (Stern, 2005). In contrary, fewer studies were made to specifically understand the induction of somitic mesoderm (Hornbruch *et al.*, 1979).

Somites are transient segmented structures that appear early during vertebrate embryogenesis. These structures form as epithelial spheres in an anterior to posterior sequential manner, from the unsegmented paraxial mesoderm, also called presomitic mesoderm (PSM), that is located bilaterally to the midline of the embryo. Their formation is controlled by a molecular clock operating at the level of somitic prospective cells. The segmentation clock is evidenced by the cyclic expression of several genes with a time period coincident with the formation of each somite (Freitas *et al.*, 2005), that can be represented in three successive phases. Phase I consists of a broad expression of the clock genes in the posterior PSM, whereas in phases 2 and 3 their expression becomes progressively narrower towards the anterior PSM, until it remains restricted to a stripe of expression coincident with either the caudal or the rostral part of the prospective somite (Palmeirim *et al.*, 1997).

In the chick embryo, the onset of cyclic gene expression occurs during the early gastrulation stages (Jouve *et al.*, 2002). Interestingly, the expression of the clock genes first appears in a region of the primitive streak that is fated to become extra-embryonic tissue in the chick embryo. However, dynamic gene expression is only observed when paraxial mesoderm precursor cells ingress through the primitive streak (Jouve *et al*., 2002). Despite the fact that the onset of cyclic gene expression was established, the mechanisms that underlie the

activation of the segmentation programme are poorly understood.

In the present work we investigated the early steps of the segmentation clock activity aiming at better understanding what triggers the initiation of the molecular clock. We start by analysing the PSM expression of the cycling genes in spontaneous and artificially generated double axes. These results showed that the segmentation clock was operating independently in these double embryonic axes, unless their posterior parts (comprising Hensen's node) were in close contact. In order to test whether Hensen's node could have a role in the activation of the segmentation clock we performed a series of chick intraspecific and quail-chick interspecific Hensen's node grafts into competent blastoderms of host embryos. Interestingly, the expression pattern of the cycling genes in the induced PSM tissues was different from the one of the host chick axis. These results proposed that the oscillations of the clock genes could be induced by a signal(s) from the organizer.

Taken together, the findings in the present study suggest that Hensen's node triggers the initial activation of the molecular clock and, furthermore, that it has the capacity to restart the segmentation programme in host recruited PSM cells that were originally not fated to become somitic mesoderm.

Results and Discussion

The segmentation clock is not induced by either fertilization or a downstream early event

First, we wanted to evaluate whether fertilization or a downstream early event could be responsible for inducing the segmentation clock. To test this possibility we analysed randomly harvested spontaneous double axes embryos and hybridised them with the *hairy1* probe in order to compare the expression pattern of this gene in the PSM of both axes. We figured that if the beginning of the clock was triggered by an event such as fertilization, spontaneous double axis embryos sharing the same blastoderm should present the same pattern of expression of the clock genes. The analysis of these twins revealed that the

hairy1 gene exhibits a different expression pattern in the PSM of both axes, when the embryos are physically separated, *i. e*., when their posterior trunk regions are apart one from each other (n=17) (Fig.1A). Conversely, the *hairy1* mRNA always presents the same phase of its cyclic expression in the PSM when the posterior trunk regions of the twin embryos are closely apposed (n=4) (Fig.B). Since the harvest of spontaneous double embryonic axes is a random event, the next step on this study was to artificially generate double embryonic axes, according to a previously described methodology (see materials and methods) (Wolff and Lutz 1947). This manipulation consisted of performing a fissure along the anterior-posterior axis of the blastoderm of non-incubated fertilized eggs, followed by their incubation. Two days after this operation we obtained the formation of physically separated double embryonic axes that exhibited the formation of approximately 15 somites (n=8).

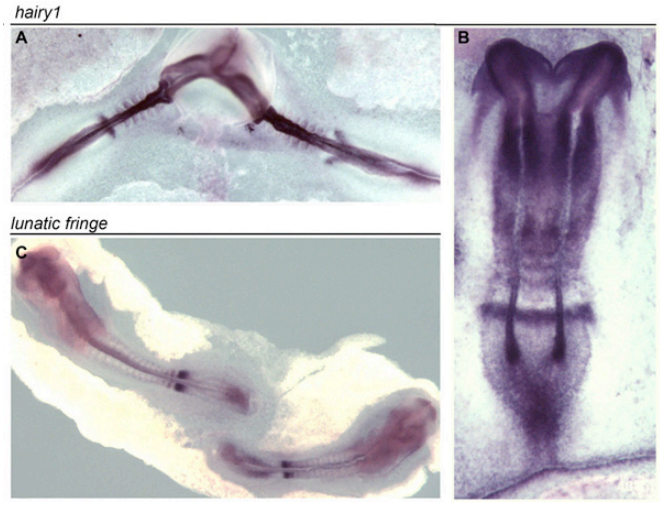


Fig.1. Expression of the cycling genes in double embryonic axis. (A-B) *hairy1* expression pattern in spontaneous double embryonic axes. (A) The cyclic PSM expression of *hairy1* is different in the twins that share anterior embryonic structures, but whose posterior regions are separated. (B) The cyclic expression pattern of *hairy1* is the same in the PSM of both axes when the trunk regions of the twins are closely apposed (C) The *lunatic fringe* expression pattern in the PSM of artificially generated double axis exhibits the same pattern in both axes.

The expression of *lunatic fringe* was analysed in the PSM of these double axes and, consistent with the above observations, the results obtained revealed that the *lunatic fringe* expression pattern is different in all artificially generated twins analysed (n=4) (Fig.1C). Taken together, the analysis of the cyclic

expression pattern of the *hairy1* and *lunatic fringe* genes on the PSM of double embryonic axes shows that an independent segmentation programme is operating on each twin embryo, unless the posterior part of these embryos is in close contact. Although both axes derive from the same blastoderm, our results suggest that these twin embryos have different clocks operating in their PSM tissues that were not activated by a signalling event during the very initial stages of chick development. Instead, these findings led us to the hypothesis that the primitive streak/ Hensen's node region could have a role in activating the segmentation clock.

Hensen's node induces the segmentation clock

In the line of our previous observations we aimed to further investigate the role of Hensen's node in the activation of the segmentation clock. The ability of Hensen's node to induce ectopic secondary axis specifically containing somitic mesoderm has been previously reported (Hornbruch et al., 1979). Thus, we performed heterotopic (different place) and isocronic (same age) interspecific stage 4 HH (Hamburger and Hamilton, 1951) quail-chick Hensen's node grafts into a competent region of a stage-match chick host. One day after the graft, the morphological observation of the resulting chimeras revealed the formation of a secondary axis that often exhibited the formation of somite-like structures (n=86). We subsequently performed *in situ* hybridisation for two of the clock genes, *lunatic fringe* and *hey2*, followed by an immunohistochemistry with the QCPN (Quail Cell Perinuclear) antibody, which allows the detection of quail cells among the chick host tissues. The analysis of these results revealed that the ectopically formed axial structures were invariably derived from the donor quail cells. Furthermore, the somites of these chimeras were either of mixed (quail donor/chick host) origin or totally formed by chick host cells (Fig 2A, B).

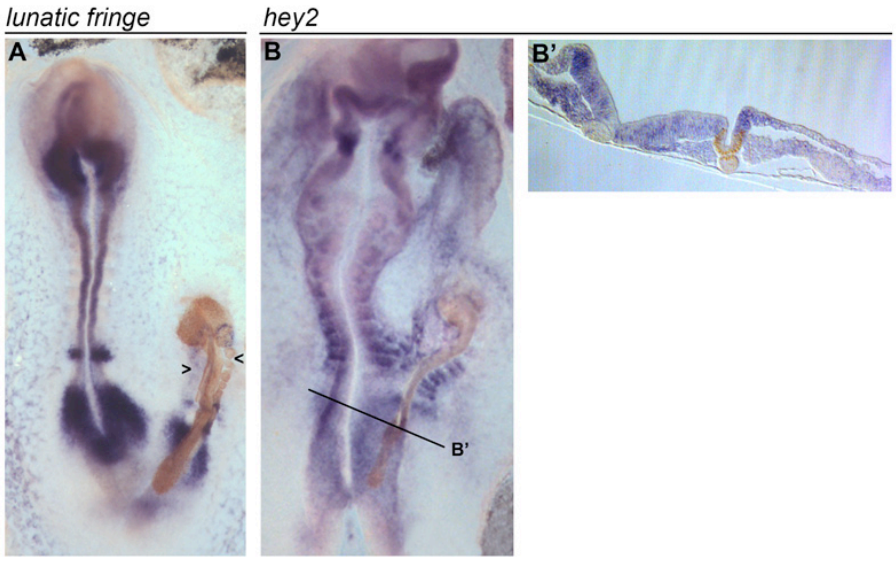


Fig.2. Cycling genes expression is observed in ectopically induced presomitic mesoderm (A) *lunatic fringe* expression in the PSM of both host and induced axis. The host axis is located on the left side and the induced secondary axis on the right. The PSM of the secondary axis is composed of chick cells. However, somites have double origin, being the ones on the right side derived from the donor (arrow head) and the ones closer to the main axis derived from the host (arrow head). The axial structures are totally derived from quail cells that are stained in brown. (B-B') *hey2* expression pattern is evident in the PSM and somites of both induced and host axis. Quail cells are stained in brown and are restricted to the axial structures of the induced secondary axes, as it can be observed by a cross section at the level of the PSM of both axis.

Conversely, in all the chimeras analysed the ectopic PSM tissue was composed of chick cells, evidencing the induced nature of this tissue (n=18). By *in situ* hybridisation, we observed that the *lunatic fringe and hey2* genes were expressed in these ectopically induced PSM tissues. Remarkably, the molecular clock in the induced PSM was in a different phase from that of the host PSM (n=8) (Fig. 2A-B'). This observation suggests that Hensen's node is capable of triggering a time count to host cells, which were not fated to become somites.

In order to further investigate this issue we performed two other types of node grafting experiments, that were carried out *in vitro* by using the New culture technique (see materials and methods) (New, 1955). The first experiment consisted of performing two heterotopic and isocronic stage 4HH quail-chick Hensen's node grafts into both sides of a competent chick donor blastoderm (n=45) (Fig.3A). The aim of this experiment was to induce the formation of two ectopic axes, one on each side of the host embryo, in order to subsequently

compare the expression of the cycling genes between the two ectopic PSM tissues and the one from the host.

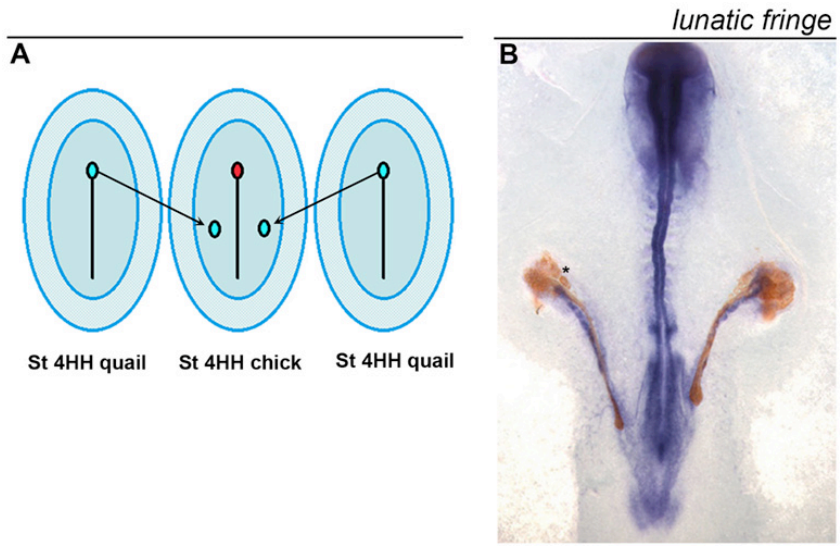


Fig.3. *hairy2* expression in ectopic secondary axis derived from a 2-node grafting experiment.
(A) Schematic diagram representing a 2-node grafting experiment. The quail nodes are coloured in blue and the chick node is coloured in red. (B) Two ectopic axes were formed on each side of the chick host. Although the ectopic axis on the right side presents the formation of somites, no PSM expression of the *hairy2* gene is observed in the ectopic embryos. In contrary, the PSM of the host shows a strong expression of this gene. (*) indicates quail-derived somites. Rostral is towards the top.

One day after the grafting experiment we observed the formation of two ectopic axes, one on each side of the host embryo that appeared to have somite-like structures (n=27). These chimeras were subsequently hybridised with the *hairy2* cycling gene followed by an immuno staining with the QCPN antibody. We reasoned that if the node were in fact imposing a new time count to newly recruited PSM host cells, the analysis of the molecular clock would reveal a different phase of the clock in the three axes. However, the analysis of these double stained chimeras showed that quail derived somites were often observed in the induced axes, but no clear PSM expression of the *hairy2* gene was ever observed in the ectopic axes. Conversely, the host embryo always presented a normal cyclic *hairy2* expression at the level of the PSM. Hence, the comparison of the phase of the clock between the three embryonic axes was not possible and these experiments came out to be inconclusive essentially due to technical difficulties.

The second experiment that we performed in the context of the previous results consisted in subdividing a stage 4HH quail Hensen's node into two halves and grafting each half into competent blastoderm on each side of a stage-match chick host embryo (n=33) (Fig.4A). The aim of this experiment was to induce the formation of two ectopic axes deriving from each node-half, and subsequently compare the expression of the cycling genes between the two ectopic and the host PSM tissues, as in the previous experiment.

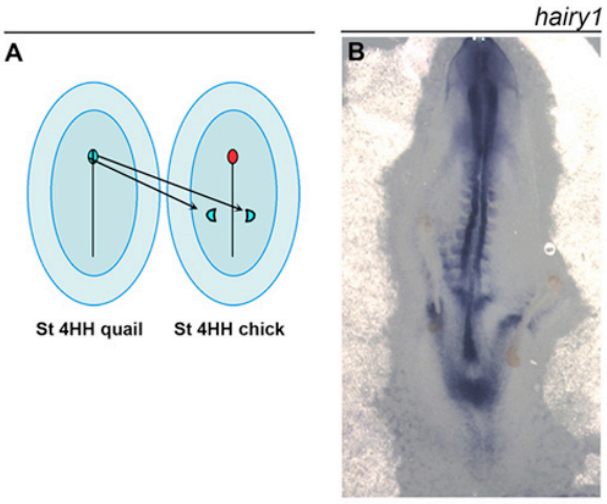


Fig.4. *hairy1* expression in induced secondary axis derived from a half-node grafting experiment
(A) Schematic diagram representing a half-node grafting experiment. The quail node is coloured in blue and the chick node is coloured in red. (B) Two ectopic axes were formed on each side of the chick host. The *hairy1* gene presents a different PSM expression pattern in all embryonic axes. Rostral is towards the top.

The morphological analysis of these chimeras one day after the experiment revealed the formation of two ectopic axes, one on each side of the host embryo, which often exhibited the formation of somite-like structures (n=19). Like in the previous examples, the chimeras were subsequently hybridised with the *hairy1* probe and then immuno stained with the QCPN antibody. Unfortunately, the analysis of these results showed that although ectopic somites were observed in the secondary axes, in most cases these axes presented no clear expression of the *hairy1* gene at the level of the PSM. This result is in contrast to the host PSM that always exhibited a normal cyclic *hairy1* pattern. Nevertheless, we obtained a single case where the *hairy1* expression could be observed in the ectopic PSM

tissue. The analysis of the *hairy1* expression in this case revealed that the phase of the clock appears to be different in the three embryonic axes (n=1)(Fig.4B). An interesting conclusion that comes out from this experiment is that the two node-halves, which derive from a common Hensen's node, were able to impose two different time counts to the newly induced PSM tissues located one on each side of the main embryonic axis. Thus, these data show that both halves of the node have a similar capacity to induce an ectopic axis. The fact that there are no differences related to the side of transplantation was also previously reported (Inagaki and Schoenwolf, 1993).

Taken together, the analysis of the node grafting experiments proposes that the segmentation clock is not an intrinsic feature of the PSM prospective cells but rather that it can be induced by the organizer activity during the process of axis formation. Hence, our results put forward the idea that the node has a role in triggering the onset of the segmentation clock.

The node is able to restart the segmentation programme

The experiments described above have established that Hensen's node is able to impose a time count to ectopically induced PSM cells that have been recruited from the host lateral blastoderm. This newly induced clock activity is evidenced by the fact that different phases of cyclic gene expression are observed in the host and in these induced PSM cells. We then wanted to assess whether the node imposes its own time count, or if, in contrary, it provokes the restart of this counting in the induced PSM cells. To address this question we performed intraspecific chick Hensen's node grafts by transplanting a stage 4HH node into the lateral blastoderm of the same embryo (Fig.5A). One day after the grafting experiment, the morphological analysis of these operated embryos showed that the main axis totally recovered from the node ablation, since it presented a normal development. In addition, we also observed that in most cases, a secondary ectopic axis formed adjacent to the main axis (n=35), but that the formation of somites was seldom apparent (n=3).

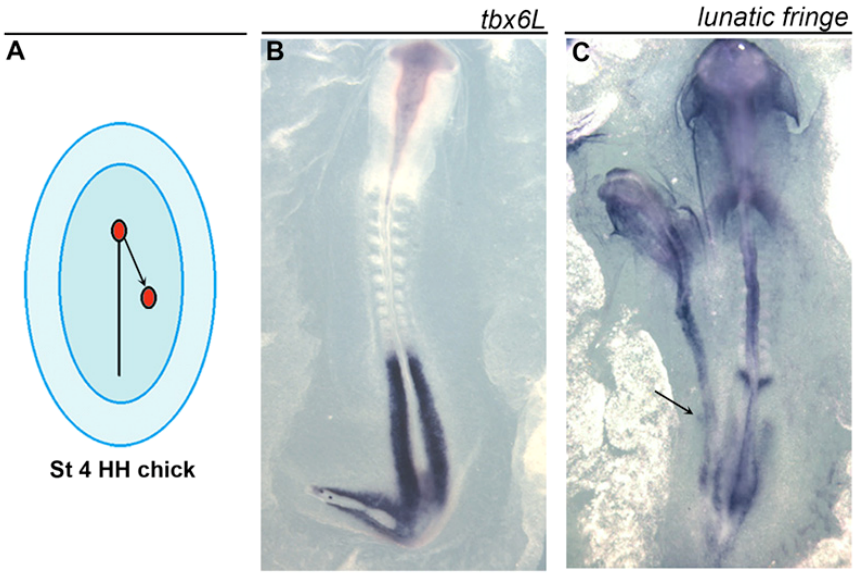


Fig.5. Expression of PSM markers in secondary axis derived from chick intraspecific node grafts.
(A) Schematic diagram representing the intra-specific node grafting experiment in the chick embryo. The chick node is coloured in red. (B) *tbx6l* is strongly expressed in the PSM of both the host and secondary axis. (C) The expression of *lunatic fringe* is evident at the level of the host's PSM. However, the induced axis also shows a faint expression of this gene at the level of the anterior PSM (arrow). Rostral is towards the top.

Given this result we hybridised a few embryos with the *tbx6l* gene, a PSM tissue marker, in order to check whether ectopic PSM had been formed. The analysis of these results showed that the *tbx6l* gene was ectopically expressed in the embryos we tested (n=2) (Fig.5B), which implied the existence of ectopic PSM tissue in the secondary axis derived from the node graft. So, the remaining cases were then hybridised with the *lunatic fringe* gene, so that its cyclic PSM expression pattern could be compared in the host and secondary axis. Surprisingly, a faint *lunatic fringe* expression was only observed in one embryo, which contrasted with the PSM expression of the main axis (Fig.5C). The analysis of clock gene expression in this single case suggests that the two axes present different phases of the molecular clock. Although very preliminary, this result suggests that Hensen's node is able to induce the restart of a new segmentation programme in the PSM cells of newly induced axis, rather than imposing its own timing.

The next step on this work was to investigate in how much time Hensen's node was able to induce the expression of the cycling genes. Hence, we grafted stage 4 HH quail nodes into competent lateral blastoderms of stage-match chick hosts and incubated the embryos for periods ranging from 1 to 10 hours. These embryos were subsequently hybridised with the *hairy2* gene and then immuno stained with the QCPN antibody. From the analysis of these embryos it becomes clear the site where the grafted node was located, since it is stained with the QCPN antibody. Furthermore, the expression of *hairy2* revealed that no ectopic staining of this gene is present after 5 hours of incubation (n=8) (Fig.6B-C'). In contrast, after a 6-hour incubation time a faint ectopic expression of *hairy2* appeared next to the node graft (n=1) (Fig.6E-E'). Consistent with these results, ectopic expression of this gene is also observed after 8 hours of incubation (n=1) (Fig.6F-F').

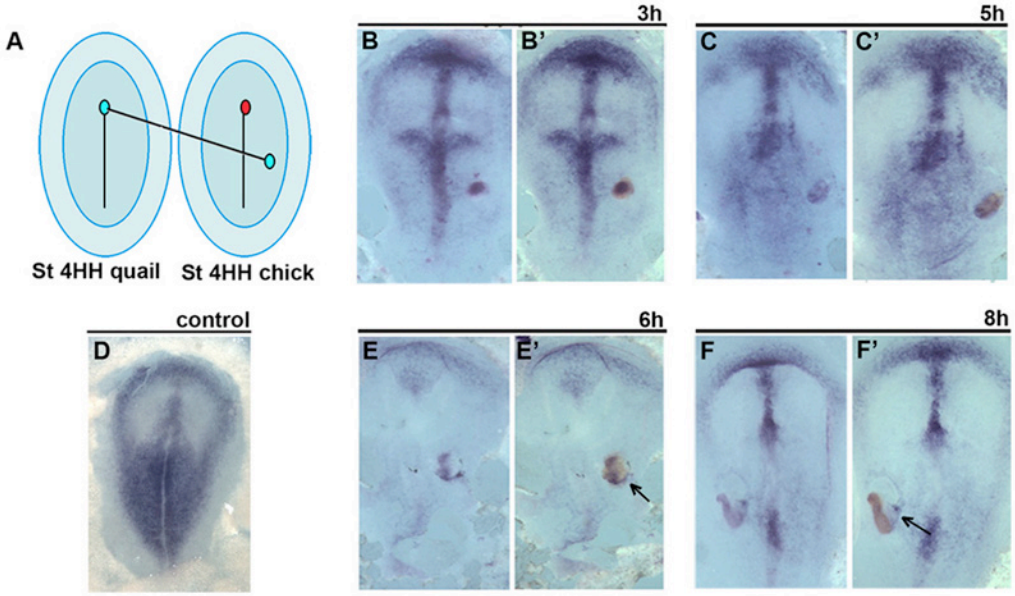


Fig.6. Expression of the *hairy2* gene in various time periods following a quail graft.
(A) Diagram representing an interspecific quail-chick node grafting experiment. The quail node is coloured in blue and the chick node is coloured in red. (B-C) *hairy2* expression is present in the host axis and within the quail node after 3 and 5 hours, respectively. (B'-C') These figures represent embryos (B-C) after DAB brown staining for the QCPN antibody. (D) Stage 4^{+} HH chick embryo hybridised with the hairy2 probe. (E-F) *hairy2* expression is present in the host axis. (E'-F) An ectopic expression of this gene becomes obvious adjacent to the quail node at 6 and 8 hours, respectively (arrows). Rostral is towards the top.

So, the conclusion of this work is that Hensen's node appears to induce the expression of *hairy2* after 6 hours in contact with the lateral blastoderm of the

chick host embryo. However, we found that the ectopic expression of the *hairy2* gene overlapped with its endogenous expression around the region of the node graft, which became a major practical difficulty of this work. In fact, the analysis of the expression of the *hairy2* gene in control embryos at stage 4 and stage 5 HH indicated that the expression of this gene is cycling at the site where Hensen's node was grafted throughout these experiments (n=38) (Fig.6D). Although the analysis of the ectopic induction of the *hairy2* gene does not provide direct evidence for the ectopic activation of the segmentation clock, this study contributes with important information for the future identification of the molecular mechanisms involved in this inductive event.

Taken together, the present work suggests that Hensen's node has a prominent role in activating the segmentation clock in prospective somitic cells, and that the initiation of the segmentation programme can be a consequence of an inductive signal from the organizer.

Materials and Methods

Induced double axis

A small window was opened in the shell of non-incubated fertilized chick and quail embryos and the anterior-posterior (AP) axis of the embryo was identified (a slight thickening of the blastoderm indicates the posterior part of the embryo) (Wolff and Lutz, 1947). A very thin glass micropipette was inserted under the blastoderm and a fissure in the AP direction was performed, dividing the whole embryo in two parts. The eggs were then sealed with tape and incubated at 37ºC for 48h.

Spontaneous double axis

Spontaneous double axis were harvested, fixed overnight in paraformaldehyde 4% and finally dehydrated in a methanol series before storing at –20ºC for posterior *in situ* hybridisation.

Hensen's node grafts

Chick and quail eggs were incubated at 37-38ºC in a humidified atmosphere for approximately 18-20h in order to obtain 4HH (Hamburguer and Hamilton, 1951) stage embryos. A window was performed in the shell of the chicken eggs. Indian ink was injected into the subgerminal cavity and the vitelline membrane was pulled apart using a tungsten microscalpel. The quail embryos were collected from the egg yolk into resin-coated petri dishes in PBS without Ca2+/Mg2+. Hensen's node was surgically removed from the donor embryo and grafted to the posterior lateral blastoderm of the host by using a glass micropipette. The eggs were sealed with tape and were reincubated for about 24h, after which the embryos were harvested and fixed overnight in 4% paraformaldehyde. Finally, the chimeras were washed in PBT, progressively dehydrated and kept in methanol at –20ºC.

Double Hensen's node grafts

Chick and quail embryos were incubated for approximately 18-20h in order to reach stage 4HH. The chick host embryos were removed from the egg with their vitelline membranes, which were stretched and draped around a glass ring according to New (1955). The embryos were then cultured in a 35mm petri dish on an albumen substrate obtained from fresh fertilized eggs. The quail embryos were harvested as described above and 2 Hensen's nodes were grafted to the posterior lateral blastoderm of the chick host: one on the right side and another on the left side. The chimeras were left to develop overnight in a humidified box at 37-38ºC and processed as described above.

Half Hensen's node grafts

The procedure and staging used to perform these grafts is similar to the double Hensen's node grafts described above. The only difference is that instead of grafting 2 nodes, a single node from quail embryos was used and subdivided in to halves that were grafted one on each side of the chick host.

Temporal sequence of the activation of the *hairy2* gene expression

Quail and chick embryos were processed as described above for the double Hensen's node grafts. In this experiment only one quail node was grafted to the posterior lateral blastoderm of the chick host and the chimeras were left to incubate for several time periods ranging from 1 to 10 hours. These embryos were then fixed in paraformaldehyde, washed in PBT, progressively dehydrated and kept in methanol at –20ºC for posterior *in situ* hybridisation and immunohystochemistry.

***In situ* hybridisation and immunohystochemistry**

All chimeras were first hybridised with a probe of interest according to the *in situ* hybridisation protocol of (Henrique *et al.*, 1995). Once the staining of the probe was completed, the embryos were left 1h in 1X PBS, 0.5% Triton, 0.5% Hydrogen peroxide (H2O2, Sigma) in order to block endogenous peroxydases, which was followed by a 3h pre-incubation in 1X PBS, 0.5% Triton, 5% goat serum (GS), 0.2% bovine serum albumin (BSA). The embryos were then incubated with the monoclonal antibody, the QCPN (Developmental Studies Hybridoma Bank) supernatant, diluted 1/5 in 1X PBS, 1% GS, 0.1% BSA, overnight at 4°C. After extensive washing, the embryos were pre-incubated for 3h with 1X PBS, 0.5% Triton, 5% GS, 0.2% BSA and left overnight at 4°C with the secondary antibody, goat anti-mouse IgG1 HRP conjugated (Southern Biotechnology Associates, Inc.), diluted 1/100 in 1X PBS, 1% GS, 0.1% BSA. Finally, detection of the quail nuclei was carried out using diaminobenzidine tetrahydrochloride (DAB) (Sigma).

RNA probes

The digoxigenin-labelled RNA probes were produced as previously described: *hairy1* (Palmeirim et al., 1997), *hairy2* (Jouve *et al.*, 2000), *lunatic fringe* (Sakamoto *et al.*, 1997), *hey2* (Leimeister *et al.*, 2000), *tbx6L* (Knezevic *et al.*, 1997).

Histology

Selected hybridised embryos were progressively dehydrated with ethanol, embedded in methacrylate (Tecnovit 8100) and processed for sectioning at 20 μm thickness using an ultramicrotome (LKB Ultrotome). The slides were mounted in Neomount (Merck) and photographed using a Leica DC 200 camera coupled to an Olympus IMT-2 DIC Inverted.

Acknowledgements

S.R. was supported by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/11839/2003). The authors are members of the FP6 European Network of Excellence 'Cells into Organs'.

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Chapter 4

Molecular characterization of the rostral-most somites in early somitic stages of the chick embryo

Gene Expression Patterns (2006), in press

The results presented in this Chapter aimed at studying possible differences between anterior and posterior somites. The fact that rostral somites do not give rise to segmented structures and are not disrupted in several somitogenesis-related mutants has led to the generally accepted idea that they are different from caudal somites. To test this hypothesis we designed a series of experiments to understand the molecular and temporal differences between the formation of anterior versus posterior somites in the chick embryo. We conclude from this work that there are relevant differences in anterior versus posterior somites in what concerns their molecular and temporal characteristics in the chick embryo.

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Gene Expression Patterns xxx (2006) xxx–xxx

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Molecular characterization of the rostral-most somites in early somitic stages of the chick embryo

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Received 18 October 2005; received in revised form 28 December 2005; accepted 7 January 2006

Abstract

Segmentation consists on the progressive formation of repetitive embryonic structures, named somites, which are formed from the most rostral part of the presomitic mesoderm. Somites are subdivided into anterior and posterior compartments and several genes are differentially expressed in either compartment. This has provided evidence for the importance of establishing the anterior–posterior polarity within each somite, which is critical for the correct segmented pattern of the adult vertebrate body. Although all somites appear morphologically similar, fate map studies have shown that the first 4 somites do not give rise to segmented structures, in contrast to more posterior ones. Moreover, in several somitogenesis-related mutants the anterior somites are not affected while posterior somites present clear defects or do not form at all. Altogether these data suggest relevant differences between rostral and caudal somites. In order to check for molecular differences between anterior and posterior somites, we have performed a detailed expression pattern analysis of several Notch signalling related genes. For the first time, we show that the somitic expression pattern profile is not the same along the anterior–posterior axis and that the differences are not observed always at the same somite level.
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Keywords: Chick embryo; Somite; Notch signalling; Somitogenesis mutants; Rostral somites; Anterior–posterior somitic polarity; *notch1*; *delta1*; *hairy1*; *hairy2*; *lunatic fringe*; *hey2*

1. Results and discussion

Somites are segmented epithelial structures generated early during vertebrate embryonic development that form in an anterior-to-posterior order from the rostral end of the presomitic mesoderm (PSM). In the chick embryo, each pair of somites forms with a strict 90-min periodicity and this correlates with the reiterated transcription of several genes in the PSM cells (Freitas et al., 2005). Once they are formed, somites are subdivided into an anterior and a posterior compartment that differ in their cell adhesive properties, essential for the maintenance of borders between segments (Stern and Keynes, 1987). Moreover, several genes are differentially expressed in either the rostral or caudal somitic compartment evidencing an anterior–posterior (AP) polarity within the somite that is critical for their subsequent differentiation into the vertebral column, ribs, skeletal muscles of the trunk and limbs and the dermis of the back (Saga and Takeda, 2001). In addition to giving rise to all segmented structures of the body, somites also impose a segmental organization to the precursors of the peripheral nervous system. Thus, AP polarity within the somite is an essential feature for establishing a correct segmented pattern in the adult vertebrate body (Hirsinger et al., 2000).

Fate map studies have demonstrated that the first 4 somites do not give rise to vertebrae but, instead, they contribute to the formation of both the occipital and the sphenoid

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doi:10.1016/j.modgep.2006.01.004

bone residing on the basis of the skull. Therefore, the border between head and neck is in the centre of somite 5, which corresponds to the first motion segment (Couly et al., 1993; Huang et al., 2000). Although somites appear morphologically identical along the whole AP axis, in fact the most rostral somites contribute to non-segmented structures in contrast to the posterior ones. Several mutations have been described in zebrafish and mice that affect somitogenesis. Most of these mutations are in components of the Notch signalling pathway and cause a progressive disruption of somitogenesis along the AP axis: in the posterior part of the embryo, somites are irregular in size and shape and lose their AP polarity (Pourquié, 2001). Interestingly, in these mutants the disruption of somite formation presents different anterior limits of defects, suggesting that there are relevant differences between rostral and caudal somites (Oates and Ho, 2002; Henry et al., 2002; Freitas et al., 2005; Oates et al., 2005). Recently, two studies have also shown that mutations in the zebrafish integrinα5 specifically disrupt anterior somite formation (Julich et al., 2005; Koshida et al., 2005). In addition, Julich et al. (2005) showed that these mutations give a somitic phenotype that is complementary to the posterior somite defects observed in the notch pathway mutants. The Notch signalling components such as *notch1*, *delta1*, *hairy1*, *hairy2*, *lunatic fringe* and *hey2* are known to be directly responsible for the accurate process of somitogenesis (Freitas et al., 2005). We have analysed the expression pattern of these genes at the level of the first 10 somites in order to check for molecular differences between rostral and caudal somites.

1.1. notch1 and delta1 expression pattern in anterior somites

In the chick, the expression of the *notch1* and *delta1* has been described in the paraxial mesoderm of 15–20 somites chick embryos. At the level of the PSM, *delta1* is present throughout this tissue and *notch1* is more restricted to its anterior part where it partially overlaps with *delta1*. In newly formed somites, these genes are expressed in a polarised fashion being restricted to the caudal compartment. As somites mature, the expression of *notch1* is downregulated (Palmeirim et al., 1998).

We have performed whole-mount in situ hybridisation in chick embryos ranging from 1 to 10 somites ($n = 133$). Our results show that at the level of the PSM, the expression pattern of *delta1* and *notch1* does not differ from what has been previously described in older embryos (Palmeirim et al., 1998). On the other hand, at the level of the somites a clear difference is promptly observed: neither *delta1* nor *notch1* present a polarised expression in the first 3 somites (Figs. 1A, B and D). Moreover, these somites do not exhibit any *delta1* expression. The salt and pepper expression that is detected at this level is exclusively neural tissue staining (Fig. 1B). The *delta1* and *notch1* polarised expression is first detected in the caudal compartment of the fourth somite as soon as it forms. During somitic maturation *delta1* expression is maintained in all somites while *notch1* remains expressed only in the most recently formed somites, SI and SII (see Section 2 for nomenclature) (Figs. 1D and E). These data show that the expression pattern of both *delta1* and *notch1* is different in the first 3 somites when compared to more posterior ones.

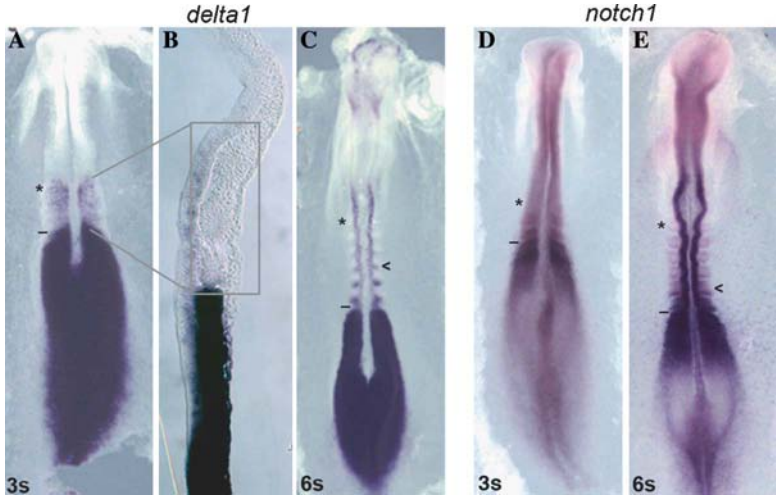


Fig. 1. Expression of *notch1* and *delta1* in early somitic stages. Embryos ranging from 1 to 10 somites were analysed by whole-mount in situ hybridisation for the expression of *delta1* (A–C) and *notch1* (D and E) genes. (A–E) At the level of the PSM, the expression of *notch1* is restricted to its anterior part where it partially overlaps with the expression of *delta1* that is present throughout the entire length of the PSM tissue. (A and B) *delta1* is never expressed in the first 3 somites. The salt and pepper expression observed at the level of the somites is exclusively neural tissue staining. (C) A polarised somitic staining becomes evident in the caudal half of the 4th somite and it is maintained in all subsequent somites. (D) *notch1* does not present a polarised expression in the first 3-formed somites. (E) At the stage of 6 somites a polarisation of *notch1* expression is observed in the caudal halves of SI and SII while the most anterior somites do not express *notch1*. Embryos are shown in a dorsal view with rostral to the top. * indicates the first formed somite; black line shows the transition between PSM and somite; < points to the somite where polarised expression is firstly observed.

1.2. Expression pattern of hairy1, hairy2, lunatic fringe and hey2 in the anterior-most somites

Having observed that the expression of *notch1* and *delta1* is different in rostral *versus* caudal somites, we have also performed an expression pattern analysis of other genes that are involved in the Notch signalling pathway, namely *hairy1* ($n = 155$), *hairy2* ($n = 84$), *lunatic fringe* ($n = 55$) and *hey2* ($n = 160$).

The expression pattern of these genes has already been described in 15- to 20-somite chick embryos. At the level of the PSM, these genes present a cyclic expression pattern that is reiterated every 90 min, corresponding to the time required to form a somite (Palmeirim et al., 1997; Aulehla and Johnson, 1999; Jouve et al., 2000; Leimeister et al., 2000). Once somites form, the expression of *hairy1* and *hey2* becomes restricted to the caudal compartment of the somite while *hairy2* and *lunatic fringe* become expressed in their rostral half.

From the analysis of our results, it is evident that *hairy1*, *hairy2*, *lunatic fringe* and *hey2* are cyclically expressed at the level of the PSM during the early stages of somitogen-

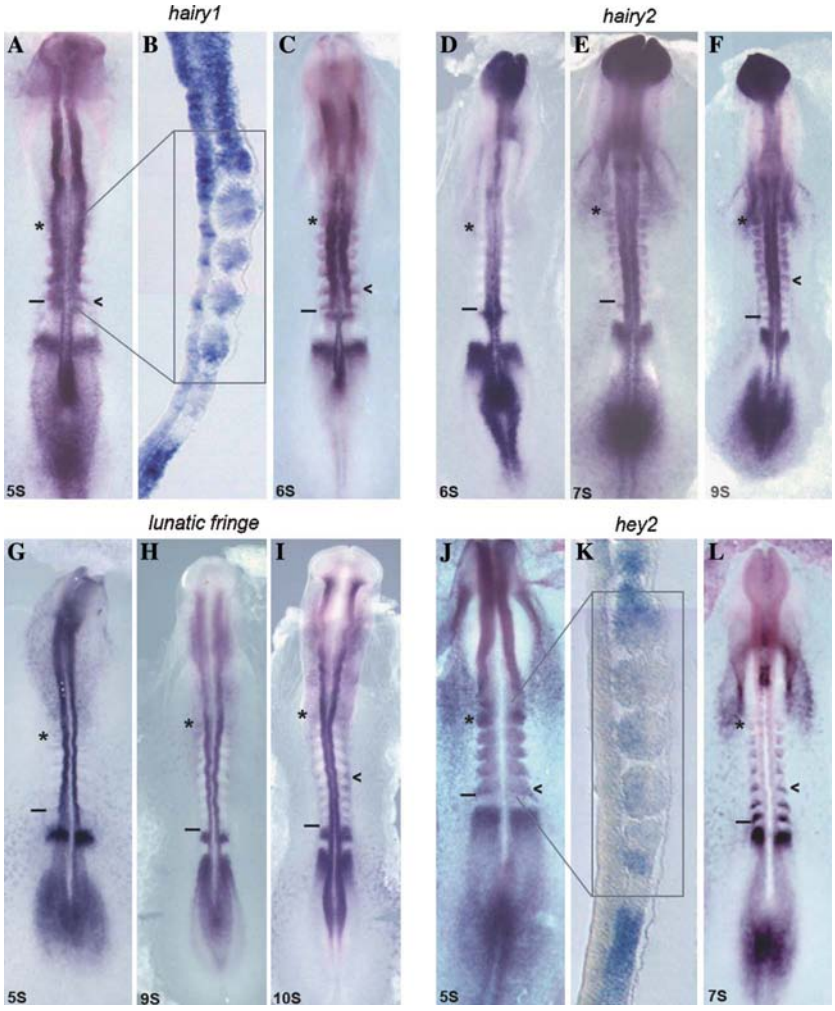


Fig. 2. Expression of *hairy1*, *hairy2*, *lunatic fringe* and *hey2* in the first formed somites. Embryos ranging from 1 to 10 somites were hybridised with *hairy1* (A–C), *hairy2* (D–F), *lunatic fringe* (G–I) and *hey2* (J–L) antisense riboprobes. At the level of the PSM, all these genes present a cyclic expression pattern. (A and B) *hairy1* is randomly expressed in the first 4 somites as it is confirmed by a sagittal section at this level. From the 5th somite onwards *hairy1* expression becomes restricted to the posterior somitic compartment. (C) In a 6-somite stage embryo, the 4 most rostral somites do not evidence any polarised expression of this gene while the 2 most recently formed somites (SII and SI) clearly show *hairy1* staining restricted to the posterior somitic compartment. (D) *hairy2* mRNA is not detected in the somites until the 6-somite stage. (E) At the stage of 7 somites, *hairy2* expression is present in the lateral part of the 4–5 most anterior somites. (F) At the stage of 9 somites, *hairy2* staining is evident in the anterior somitic compartment of the 6th somite (SV) and subsequently formed somites. Rostral to somite 6 no AP polarised expression of this gene is observed. (G and H) No somitic expression of *lunatic fringe* is observed before the stage of 9 somites. (I) At the stage of 10 somites, a polarisation of *lunatic fringe* expression appears in the anterior compartment of the 6th somite (SV) and in all other somites posterior to somite 6. In contrary, the 5 most rostral somites do not present any AP polarisation of the expression of this gene. (J and K) At the stage of 5 somites *hey2* is ubiquitously expressed in the 1st somite and it presents a non-polarised diffuse staining in SIII, SII and SI. The 5th somite already presents a polarised expression in the posterior somitic compartment. (L) In a 7-somite stage embryo, the first 4 most rostral somites do not present AP polarisation of the *hey2* expression while it can be clearly observed a polarised *hey2* staining in subsequently more posterior somites (SIII, SII and SI). All embryos are shown in a dorsal view with rostral to the top. * Indicates the first formed somite; black line shows the transition between PSM and somite; < points to the somite where polarised expression is firstly observed.

esis. In contrast, the expression of the cycling genes at the level of the somites is not as expected.

In the first 4 somites, the *hairy1* gene shows a random pattern of expression in the whole somite (Figs. 2A and B). Only from the 5th somite onwards does *hairy1* expression become restricted to the posterior half of the somite, being this pattern maintained in subsequently caudal somites (Fig. 2C).

hairy2 transcripts are not detected in the somites up to the 6-somite stage (Fig. 2D). At the stage of 7 somites, *hairy2* expression is upregulated in the lateral part of the first 4 or 5 somites although this does not correspond to an AP polarisation of the *hairy2* expression (Fig. 2E). A polarised expression of *hairy2* in the anterior somitic compartment only becomes evident from the 8th somite onwards. Interestingly somites 6 and 7, which do not present any *hairy2* expression at the time they bud off from the PSM, upregulate an anterior polarised expression in later stages (Fig. 2F). In agreement it has been described in the zebrafish that the anterior 5–6 somites simultaneously activate many markers, in contrast to the sequential activation of the same markers in more posterior somites (Weinberg et al., 1996).

lunatic fringe transcripts are never detected in the somites before the stage of 9 somites (Figs. 2G and H). A clear polarised expression of *lunatic fringe* in the anterior somitic compartment is only observed from the 10th somite onwards. As in the case of *hairy2*, a polarised expression of *lunatic fringe* is simultaneously activated in somites 6–10 in later stages (Fig. 2I).

Until the stage of 5 somites, *hey2* transcripts are strongly detected in the most rostral somite while somites SIII, SII and SI (see Section 2 for nomenclature) present a diffuse staining. None of these somites exhibit any AP somitic polarisation. From this stage onwards, the 4 most rostral somites never evidence an AP polarised expression of *hey2* while all the subsequently formed somites show a staining in the posterior somitic compartment (Fig. 2L).

For the first time, this work shows that the anterior-most 10 somites are molecularly different from more posterior ones. The first 3 somites never evidence a polarised expression of any of the genes analysed. Interestingly, the onset of the polarised expression of these genes does not emerge always at the same somitic level. Instead, the AP expression of each gene is switched on one at the time, originating an increasingly complex pattern that starts with the formation of somite 4 and culminates with the formation of somite 10 (Fig. 3).

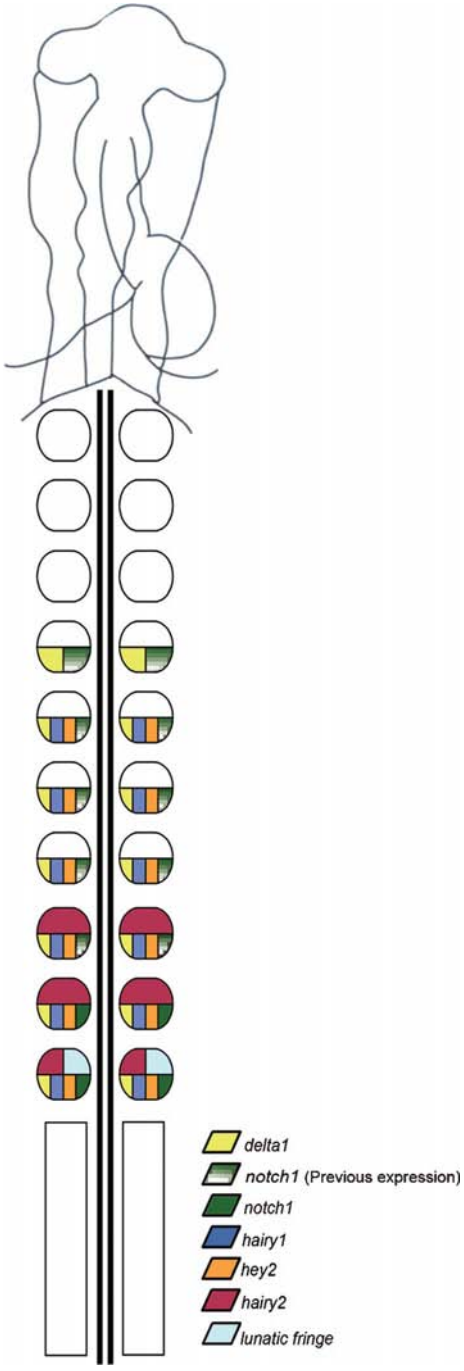


Fig. 3. Emergence of somitic AP polarised gene expression during the formation of the first 10 somites. Schematic diagram of a 10-somite chick embryo representing the gradual appearance of the somitic AP polarised expression of the genes analysed. Each gene has a colour code that is represented in the diagram. The coloured boxes exclusively denote the beginning of the AP polarised expression of these genes, not being meant to represent medial lateral differences in gene expression. This figure shows that the first 3 somites do not exhibit an AP polarised expression of any of the genes analysed. Also, this model represents the gradual appearance of the AP polarised expression of the studied genes, which reaches a maximum complexity at the level of somite 10. Note that although the *notch1* expression pattern starts to be polarised in somite 4 (see Section 1) its expression is lost as somites mature, remaining restricted to somites SI and SII (see Section 2 for nomenclature).

2. Experimental procedures

2.1. Eggs, embryos and somite nomenclature

Fertilized chick (*Gallus gallus domesticus*) eggs were obtained from commercial sources (Avipronto – Benavente, Portugal) and stored at 15 °C until the beginning of each experiment after which they were incubated for periods ranging from 24 to 48 h in a humidified atmosphere at

37 °C. The embryos were staged by the total number of somites formed, according to the Hamburger and Hamilton (1951) system of classification. 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 (Christ and Ordahl, 1995).

2.2. RNA probes

The digoxigenin-labelled RNA probes were produced as previously described: *hairy1* (Palmeirim et al., 1997), *hairy2* (Jouve et al., 2000), *lunatic fringe* (Sakamoto et al., 1997), *hey2* (Leimeister et al., 2000) and *delta-1* and *notch-1* (Henrique et al., 1995).

2.3. Whole-mount in situ hybridisation

Embryos were harvested and fixed overnight at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed in PBT (PBS, 0.1% Tween 20), dehydrated through a methanol series, and stored in 100% methanol at −20 °C. Whole-mount in situ hybridisation was performed according to the procedure described by Henrique et al. (1995). The RNA probes were detected with NBT-BCIP. Embryos were photographed as whole mounts in PBT using a Leica D200 camera.

2.4. Histology

Selected hybridised embryos were progressively dehydrated with ethanol, embedded in methacrylate (Tecnovit 8100) and processed for sectioning at 20 μm thickness using an ultramicrotome (LKB Ultrotome). The slides were mounted in Neomount (Merck) and photographed using a Leica DC 200 camera coupled to an Olympus IMT-2 DIC Inverted.

Acknowledgements

We thank Leonor Saúde and Raquel Andrade for critically reading the manuscript. S.R. was supported by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/11839/2003). The authors are members of the FP6 European Network of Excellence 'Cells into Organs'.

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Supplemental Data

Unpublished results

3. 1. Monitoring anterior somite formation time

The above experiments showed a detailed analysis of the *notch1*, *delta1* and the cycling genes *hairy1*, *hairy2*, *lunatic fringe* and *hey2* at the level of the first 10 somites, revealing clearly different expression patterns in the anterior somites *versus* more posterior ones. Consistent with these findings and with the idea that the rostral-most somites are different from more caudal ones, zebrafish, mice and amphioxus exhibit different rates of somite formation during development. It appears that in these embryos, the rostral-most somites form more rapidly than more posterior ones (Kimmel et al., 1995; Tam, 1981; Schubert et al., 2001). In the chick embryo, no experimental study has accurately been performed to establish the rate of somite formation during development. So, in this study we sought to investigate whether the rate of formation of the first somites in the chick was also faster than more posterior ones. To do this we designed a time-lapse monitoring experiment, which provided an accurate observation of somite formation *in vivo*.

Stage 6 HH chick embryos were cultured according to the New technique (New, 1955) and placed in a culture chamber apparatus where the temperature and humidity were carefully controlled (see materials and methods). At this stage the embryos had not formed any somites. A selected embryo was then monitored, by using an inverted microscope coupled to a camera, which was set up to take a photograph of the embryo every 5 minutes. The development of the embryo was followed for approximately 12 hours, after which the embryos had formed 8-10 somites (n=12) (Movie S1, supplemental material) (Fig.S1). The first conclusion we can withdraw from this experiment is that the formation of the first 3 somites is difficult to observe with this imaging setup. An explanation for this is the fact that the camera is photographing the embryo from its dorsal side, which makes the image slightly opaque due to the open neural plate layer standing precisely above the forming somites. In addition, we observed that cleft formation has a ball-and-socket arrangement (Kulesa and Fraser, 2002), reinforcing the difficulty to determine exactly when the somitic boundaries formed. Consequently we

often noted that when a somitic boundary was visible, the following somite had also already been formed (Fig.S.1J-K).

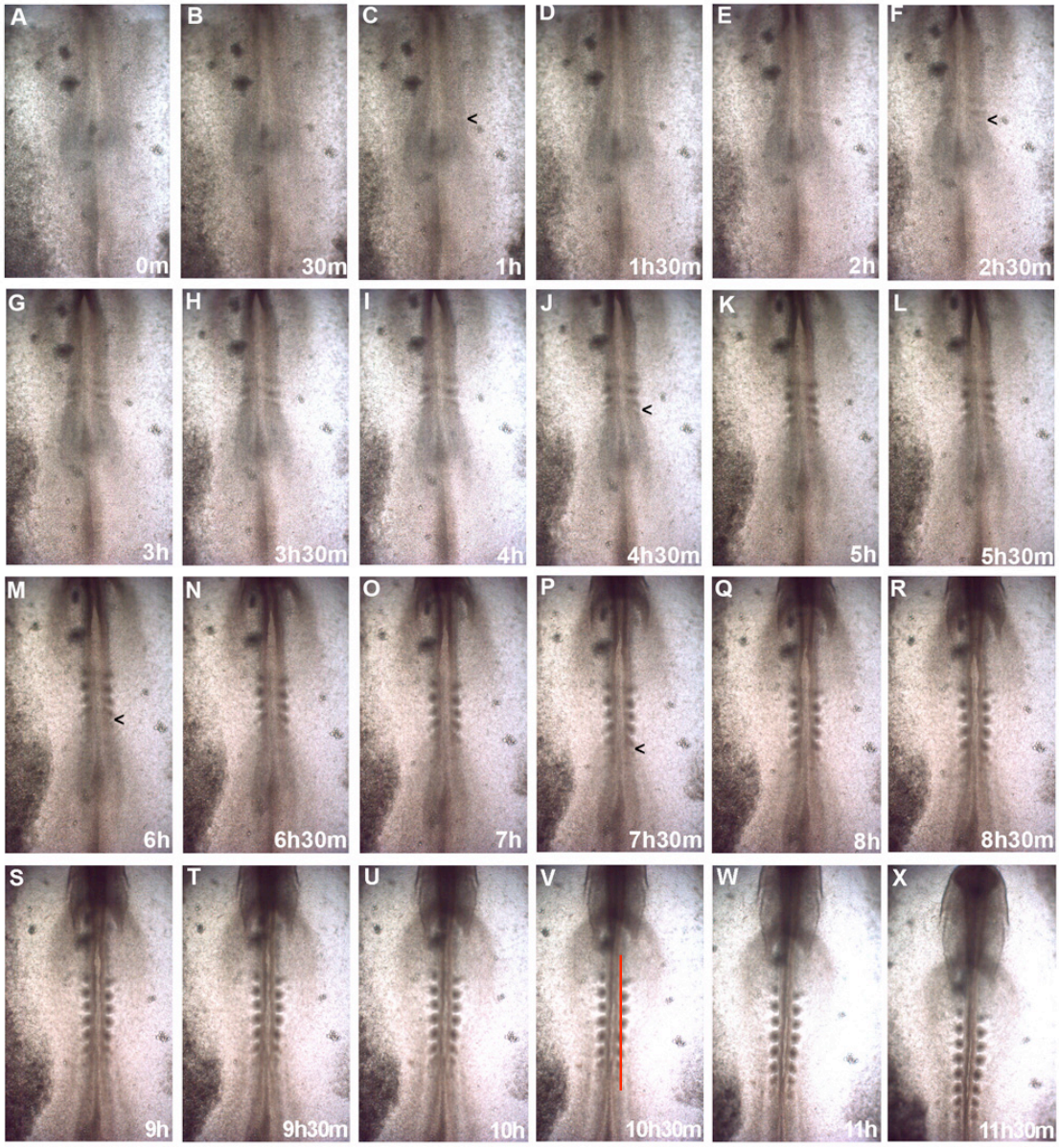


Fig.S1. Time-lapse microscopy for monitoring anterior somite formation in the chick embryo

(A-R) Photo frames from Movie S1 that present a time interval of 30 minutes between each other. These pictures represent anterior somite formation in the chick along an 11h30m time period. Arrowheads indicate the localization of what we consider clearly visible boundaries. Red line indicates a hypothetical line that represents the region from where the 3D kymograph was constructed (see text for details). Rostral is towards the top.

For this reason the exact counting of somites at this stage was uncertain, though it became progressively clearer as the embryo grew and elongated. Furthermore, this study also allowed us to conclude that the anterior-most somite does not present a definite anterior boundary and somite 2 is the first

to exhibit clear anterior and posterior boundaries (Fig.S.1E). This result is consistent with previous observations (Hamburguer and Hamilton, 1951).

In order to better visualise the timing of the formation of the anterior-most somitic clefts we stacked all time-points and sampled a hypothetic line along the AP axis passing through the somites (as in the example represented by the red line in Fig S1V). Thus, we obtained a kymograph, which is a 3D representation, in which the sampled lines are stacked in the X-axis and the time scale is represented in the Y-axis (Fig.S2). This procedure provided us with a more precise temporal visualization of somitic boundary formation along the time we monitored the embryo. A surprising observation that came up from the analysis of the kymograph was that the first 2 somites seem to have formed simultaneously. Conversely, the formation of somites 3 to 7 seemed to be sequential. Moreover, the kymograph indicates that somite 4 formed shortly after somite 3, and that from somite 5 onwards the time of boundary formation was maintained at a constant rate (see black lines in Fig. S2).

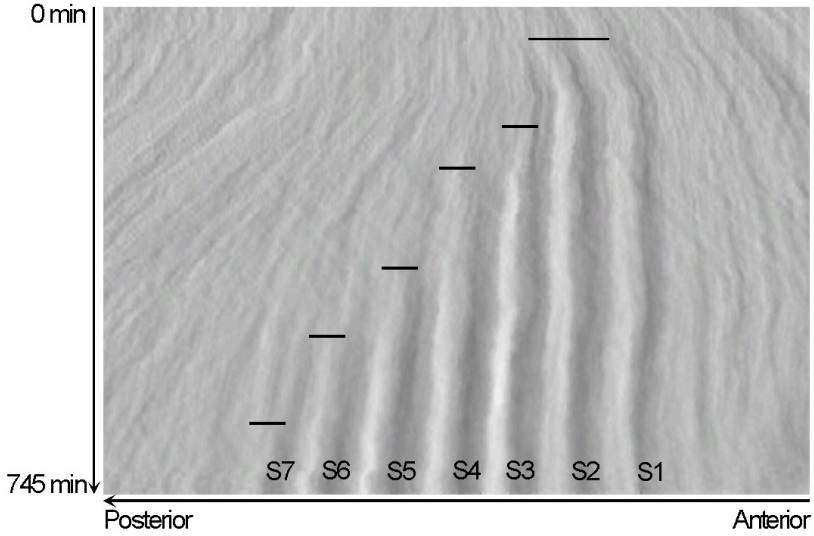


Fig. S2. Kymograph representing the formation of anterior-most somites along a time scale

The white stripes represent boundaries between somites whereas the dark stripes indicate the epithelial somites. The y-axis corresponds to a time scale where time 0 is at the top. The x-axis represents the AP direction of embryonic growth, where anterior is towards the right. Starting in an anterior to posterior direction, the first 2 white stripes are at the same level on the time axis (black bar), meaning that the boundaries of these somites (S1 and S2) formed at the same time. In contrast, the following white stripes, corresponding to somites 3 to 7 (S3 to S7), exhibit a rostral limit that is not coincident in time (black bars), meaning that these somites formed sequentially.

Taken together, these results indicate that, in fact, the anterior-most somites form faster than the more posterior ones. However, an improved visualization of the first somitic boundaries is currently being performed in our lab by using the confocal microscope to monitor *in vivo* the time of anterior somite formation.

3. 2. The time of the segmentation clock is altered in the PSM of the anterior-most somites

In parallel to the time-lapse somite-monitoring essay we performed explant culture experiments in order to check whether the time of the molecular clock differed in anterior versus posterior somites. We started this study by using 1 and 2-somite stage embryos in which one half of the embryo was cultured immediately and the other was incubated for 90 minutes (Palmeirim et al, 1997), which is the time conventionally required to form a pair of somites in the chick embryo. We then hybridised both halves with the *hairy1* RNA probe. The analysis of these results revealed that the expression pattern of these genes is not coincident in both explant halves of the embryos analysed (n=18) (Fig. S3A-B).

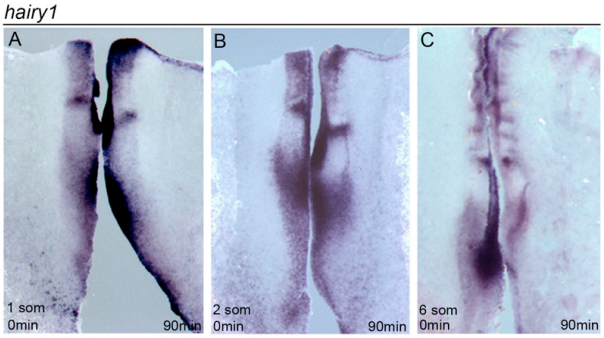


Fig. S2. The molecular clock works differently in anterior versus posterior somites. (A-C) Expression of the *hairy1* gene in explant halves incubated for 90 minutes (right side) and controls (left side). (B) The experimental halves of embryos with 1 and 2 somites present different expression patterns of this gene in the two explants. (C) Conversely, 6-somite stage embryos always exhibit the same expression pattern in the two explant halves.

In contrast, the analysis of the expression of *hairy1* in explants of 6-somite stage embryos presented the same expression pattern in both halves (n=5). These results strongly indicate that the anterior-most somites do not form in

90 minutes, which is in agreement with our previous data concerning the time of anterior somite formation. We also performed explant cultures of 1-somite stage embryos where the experimental halves were incubated for 60 minutes. In this experiment, the expression pattern of the cycling genes was different in both halves of all explants analysed (data not shown). These results imply that the cycling time underlying anterior somite formation is not 60 minutes, which supports our previous finding that the 2 anterior-most somites form simultaneously. Thus, these experiments strongly suggest that the molecular clock is operating faster in the PSM from where the anterior somites will form, although its precise timing underlying the formation of somites 3 and 4 was not disclosed.

3. 4. Materials and Methods

3. 4. 1. *In vivo* monitoring of time of somite formation

Chicken eggs were incubated for 24h in order to obtain embryos ranging from stage 4 to 6 (HH). A selected embryo was cultured *in vitro* by using the New culture technique (New, 1955) and placed in a sterile petri-dish containing a thin (about 1ml) layer of albumin. The dish was placed inside a chamber (Tempcontrol 37-2, Pecon, Germany), where it was maintained at 37 Cº and in a humidified atmosphere. Finally, the whole apparatus was placed under an Olympus IMT-2 DIC Inverted microscope and the embryo was visualised in a dorsal view. The time-lapse analysis was performed by taking a photograph of the embryo every 5 minutes, for 10-12 hours and by using a *Pixel link* camera coupled to the inverted microscope.

3. 4. 2. *In vitro* culture of chick explants

Chick embryos ranging from 1 to 7 somites were surgically removed from the yolk into resin-coated petri dishes with phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}. A transverse section was performed just anterior to the last formed somite and the caudal part of these embryos, including ectoderm and endoderm, was bisected along the midline. One half was immediately fixed in 4% paraformaldehyde in PBS, and the other half was

cultured for 60 or 90 minutes prior to fixation. Explants were cultured in petri dishes on polycarbonate filters (0.8 μm pore size; Millipore) floating on top of culture medium, Medium 199 (Sigma), supplemented with 5% heat inactivated fetal calf serum, 10% heat inactivated chicken serum, 1% L-glutamine (200 mM) and 1% penicillin-streptomycin (5000 IU/ml-5000 IU/ml) as described in Palmeirim et al. (1997).

3. 5. Legend of supplemental material

Movie S1. Time lapse monitoring of anterior somite formation.

Movie S1 shows the development of a chick embryo from stage 6+ HH to approximately stage 9 HH. A frame was taken every 5 minutes. The rostral part of the embryo is towards the right and thus the embryo grows from the right to the left side.

Chapter 5

General Discussion

5. 1. The segmentation clock is operating at the level of the prospective somitic territory

Originally, the segmentation clock was reported to operate at the level of the presomitic mesoderm and the authors considered it to be activated when prospective PSM cells entered the presomitic tissue, as a consequence of gastrulation (Palmeirim *et al*., 1997). Hence, it remained to be unveiled whether the molecular clock was already operating in gastrulating PSM prospective cells. To test this possibility we performed a detailed analysis of the expression patterns of *hairy1*, *hairy2* and *lunatic fringe* genes at the level of the primitive streak of 6-somite stage embryos. This work revealed that the expression of these genes oscillates in this territory, from the posterior limit of the median pit to a region slightly posterior to the *sinus rhomboidalis*. In addition, a quail/chick cell lineage study performed in our laboratory, together with a fate-map previously described by Catala and colaborators (Catala *et al*., 1996), allowed us to determine that this region corresponded precisely to the prospective somitic territory at the stage of 6-somites. So, our work clearly demonstrated that the levels of *hairy1*, *hairy2* and *lunatic fringe* mRNA are already oscillating in prospective PSM cells well before they incorporated the PSM tissue, and that the cells presenting this cyclic behaviour are restricted to the prospective PSM territory. Consistent with our data, it was also reported that the molecular clock is already functioning in prospective somitic cells at the time they ingress through the primitive streak, approximately at stage 4 HH (Jouve *et al*., 2002).

When the molecular clock was first described, the authors suggested that the number of mRNA cyclic waves of expression exhibited by PSM cells depended on the time these cells spent until they incorporated a somite (Palmeirim *et al*., 1997). Since the chick PSM tissue contains 12 prospective somites, these were the correspondent number of cycles that the PSM cells had to perform before incorporating a somite. Since our data shows that prospective PSM cells are already undergoing cyclic waves of clock gene expression in their prospective territories, we can then infer that these cells accomplish a higher

number of cycles than the ones at the level of the PSM, before they become epithelialised into a somite. In agreement with this, Jouve and collaborators proposed a counting mechanism based on the assumption that all somitic cells are generated from a common resident pool of stem cells located in the prospective somitic territory at the level of the primitive streak (Jouve *et al*., 2002). According to this model, the number of cycles performed by a somitic stem cell is directly correlated with the AP position of the somite they will eventually incorporate. These results are consistent with our quail-chick chimeras that show that quail donor cells are still observed in the prospective PSM region after 24 hours of incubation. These remaining quail cells, or their progeny, are thus undergoing several cycles of expression without exiting the prospective PSM region.

5. 2. The segmentation clock provides positional information in two dimensions

As described above, the segmentation clock is already operating at the level of the prospective somitic territory that, at the stage of 6-somites, is located from the posterior limit of the median pit to a region slightly posterior to the *sinus rhomboidalis*. Notably, precise isotopic (same place) quail-chick grafts of a 150μm region posteriorly adjacent to the MP revealed that the cells located in this territory exclusively give rise to medial presomitic cells. In agreement with our fate-map, it was shown that the expression domain of the *tbx6l* gene, which is a specific PSM marker, is juxtaposed to the MP expression domain of the *HNF3β* axial marker (Charrier *et al*., 1999). Additional grafting experiments in our laboratory have also determined the precise site of the prospective lateral somitic territory, which is located posteriorly to the medial prospective territory, at the basal part of the *sinus rhomboidalis* (Freitas *et al*., 2004). So, our results show that within the prospective PSM territory, more anterior cells will be located in more medial PSM positions and more posterior cells will be located in more PSM lateral positions. Interestingly, since the ‘wave’ of expression of the cycling genes

spreads along the longitudinal axis of the PSM prospective territory, this implies that this 'wave' of gene expression also spreads along the future medial/lateral PSM axis. In agreement with this, the stripes of expression of the cycling genes at the level of PSM also exhibit a medial-lateral asynchrony, which is manifested by the appearance of cross-stripes. In fact, an asynchrony in the medial-lateral expression could explain the appearance of a cross-stripe as a transition state between two horizontal stripes. Recently, a remarkable work has monitored the cyclic waves of expression of the *hes1* gene, *in vivo*, in the PSM of the mouse embryo at tailbud stages (Masamizu *et al*., 2006). Interestingly, the analysis of the waves of *hes1* expresssion does not evidence the existence of such oblique stripes at the level of the PSM. However, since it has previously been described that the mouse embryo also exhibits segregated medial and lateral prospective somitic territories at the level of the primitive streak (Wilson and Beddington, 1996; Tam *et al*., 2000; Eloy-Trinquet and Nicolas, 2002), we figure it is highly probable that a medial-lateral asynchrony is also occurring at the level of the PSM. Hence, the fact that no cross stripes are observed at this level in the mouse PSM is probably due to the short length of the PSM tissue of the mouse at the stage analysed, that does not allow such a precise observation. Similar real-time imaging of the molecular clock in the chick PSM would certainly be very informative. So, a very interesting conclusion that comes out of our work is that the molecular clock is providing cellular positional information in two dimensions, both in the anterior-posterior and in the medial-lateral axis.

5. 3. Medial and lateral PSM cells are differently committed to form somites

As reported previously in this study medial and lateral PSM cells have different prospective territories, which led us to wonder whether these two domains were also differently committed to form somites. We assessed this issue, by performing both medial (M-PSM) and lateral (L-PSM) PSM ablations. Our results show that the rostral-most M-PSM is able to form somites in the

absence of the L-PSM, but surprisingly, no epithelial somite formation can be observed when the L-PSM is isolated from its medial counterpart. Therefore, we find it likely that medial PSM cells have to recruit lateral ones in order to form somites. Furthermore, PSM and epithelial somites can be subdivided into a medial and a lateral compartment according to their fate (Ordahl and Le Douarin, 1992; Olivera-Martinez *et al*., 2000). Hence, medial and lateral PSM compartments have different origins, are differently committed to somite formation and give rise to different embryonic structures.

Conversely, grafting experiments, performed in our laboratory, at the level of the prospective somitic territory demonstrated that if the PM-PSM territory is replaced by the PL-PSM territory, these cells will give rise to the medial part of the PSM and somites (Freitas, 2004). So, this work led to the conclusion that in opposition to the M-PSM cells at the rostral part of the PSM, the PM-PSM region is still not determined to form somites. Furthermore, it seemed likely that such capacity to segment had to be acquired from the time the PM-PSM cells left their territory until they reached the rostral-most part of the PSM. In fact, several heterotopic grafting experiments performed in our laboratory have further suggested that a signal from the environment is actually instructing the PM-PSM cells with the capacity to form somites (Freitas, 2004).

5. 4. The median pit induces the formation of a secondary axis

As described above, the PM-PSM cells are not fully committed to form somites and such capacity is most likely obtained by a signal from the environment. Barrier insertion experiments conducted in our laboratory have additionally demonstrated that the most probable source of such a signal is the median pit region (Freitas, 2004). To test this possibility we performed heterotopic and heterochronic grafting experiments of the MP region and observed that the MP alone can induce the ectopic formation of somites and PSM. In addition our results also demonstrate that the molecular clock is operating in the ectopically induced PSM tissues. Thus, these results are

consistent with a role of the MP as a signalling source responsible for activating the segmentation programme in the PM-PSM region. An interesting conclusion from these grafting experiments is that the MP is able to induce the formation of ectopic secondary axis consisting of host-derived PSM and somites. This finding is in contrast to previous data that have established that the node at somitic stages is not able to induce an extra axis (Inagaki and Schoenwolf, 1993; Charrier *et al*., 2005). So, the notion that the MP is the organizer at mid-gastrulation stages is based on specific characteristics of Hensen's node, other than its inductive capacity.

In fact, several common features have been ascribed to Hensen's node at specific stages during development. In what concerns the node morphology and cell derivatives, Hensen's node first appears at stage 4 HH as a bulk of cells at the tip of the primitive streak, and it originates the prechordal plate, notochord and floorplate. The medial part of the somites was also reported to derive from the node at this stage (Selleck and Stern, 1991). Interestingly, in no other embryonic stage during development the somitic precursor cells originate from the node (see Chapter 2) (Knezevic *et al*., 1997; Charrier *et al*., 2005). At the stage of 6-somites the MP has been characterised into three distinct regions, *a*, *b* and *c*, and the morphological bulk of the node corresponds to region *b* (Charrier *et al*., 1999), which gives rise to notochord and floorplate structures (Catala *et al*., 1996). Despite this evidence, the precise localization of Hensen's node at 6-somite stage embryos has been debatable, since the anterior and posterior regions adjacent to zone *b* were also proposed to be part of the node (Charrier *et al*., 1999). A fate-map study of the tail bud region in the chick has also demonstrated that the chordal neural hinge (CNH), that results from the merge of the notochord and the caudal neural tissue, corresponds to the remnants of Hensen's node, whereas other tissues within the tail bud consist of primitive streak and posterior neural plate derivatives (Catala *et al*., 1995). In addition, the authors of this latter study observed that the cell movements within the tailbud are very similar to the earlier gastrulation movements, indicating that these cellular movements appear to be maintained during development. Another

common feature attributed to the node throughout all developmental stages is the expression of *hnf3β*, *shh* and *chordin* that is maintained from stage 4 HH to tailbud stages (Charrier *et al*., 1999; Lawson *et al*., 2001; Charrier *et al*., 2005). In addition, the expression domain of *tbx6l* is always observed at the level of the rostral primitive streak until the formation of the tail bud, and then it becomes restricted to the primitive streak remnants within the tail bud itself (Knezevic *et al*., 1997, 1998; Charrier *et al*., 2005). In summary, the analysis of the available data on the morphology, localization of the axial progenitors, cellular movements and gene expression patterns of the node indicates a clear segregation between the node region and the rostral part of the primitive streak along the chick development.

Our results based on the ability of the MP to induce a secondary axis, support the idea that, at the stage of 6-somites, Hensen's node is the morphological bulk of cells that corresponds to zone *b*. We reached this conclusion due to the fact that this specific region alone was able to induce the formation an ectopic axis when transplanted to competent blastoderm of a younger host, in contrast to the adjacent zone *c* (that consists of the axial paraxial hinge) or the PM-PSM, that were not able to induce ectopic axes (see Chapter 2, sections 2.1 and 2.2). At odds with our findings and with the fact that the node at stage 4 HH is the signalling center that drives the induction of ectopic axis formation, the CNH (corresponding to the remnants of Hensen's node within the tailbud) is not able to induce an axis. Instead, it was shown that the ventral mesenchyme is the only region within the tail bud that has the ability to induce the ectopic formation of PSM tissue (Knezevic *et al*., 1998).

5. 5. The specification of PSM precursor cells precedes the onset of segmentation clock

The mechanisms underlying the onset of the segmentation clock are poorly understood. During the course of this work we sought to investigate the very early steps of avian development aiming at better understanding what

controls the beginning of the clock activity. We started by analysing the oscillations of the cycling genes in both spontaneous and artificially generated double embryonic axes. These type of twin embryos derive from a common blastoderm and thus the comparison of the molecular clock gene expression on the PSM of both embryos allowed us to verify whether a signalling event at the very earliest stages of chick development, such as fertilization, could be activating the segmentation clock. We reasoned that if the molecular clock was operating in the same phase in both twin embryos, then the clock must have been activated prior to their separation, perhaps by fertilization or a downstream event. In contrast, if the molecular clock was operating in two different phases, then its activation most probably occurred after the twin axes were fully determined. In fact, the analysis of our data showed that when the double axes were divergent, the cycling genes always exhibited different phases of expression at the level of the PSM. This suggests that different molecular clocks were operating in the PSM of these twin embryos and that they were not activated by a signalling event in the early hours of chick development.

Consistent with this idea, the analysis of the *tbx6l* gene expression pattern during the early stages of chick development revealed that the onset of its expression precedes the observation of cyclic gene expression in PSM precursors cells (Knezevic *et al*., 1997; Jouve *et al*., 2002). While the expression of the *tbx6l* gene coincides with the position of the prospective somitic cells at stage X-XI (stages according to Eyal-Giladi and Kochav, 1976) (Hatada and Stern, 1994; Knezevic *et al*., 1997) the cyclic expression of the *hairy2* and *lunatic fringe* genes starts to be observed only between stage 3 + and 4 + HH, when the primitive streak has almost fully elongated (Jouve *et al*., 2002). In agreement with our results, the interpretation of these data strongly suggests that the appearance of PSM precursor cells and the activity of the segmentation clock are events that follow each other during development.

5. 6. Hensen's node triggers the onset of the segmentation clock

The observation that the molecular clock was operating in the same phase on the PSM of twin embryos whose axis were convergent, or in close contact, led us to put forward the hypothesis that perhaps the node had a role in triggering the segmentation clock activity. We tested this possibility by performing heterotopic interspecific grafting experiments that consisted in transplanting a quail Hensen's node into the competent blastoderm of a chick host. These experiments demonstrated that ectopic PSM tissue was induced (identified by the absence of QCPN labelling) and that the phase of the expression of the cycling genes at this level was different from the one observed in the PSM of the host embryo. So, this result implied that the node recruited lateral blastoderm host cells to a somitic fate, and also that it was capable of inducing a time count to these cells.

Grafting experiments at stage X-XI have reported that the posterior marginal zone is able to induce an ectopic organizer and a primitive streak when transplanted to the anterior pole of the embryo (Bachvarova *et al*., 1998). Furthermore, a 'node-inducing center' was identified in the middle third of the primitive streak at stage 4 HH, which induces ectopic expression of organizer markers when transplanted to the lateral blastoderm of stage-match chick embryos (Joubin and Stern, 1999). The interpretation of these results makes it tempting to postulate that perhaps the 'node-inducing center' and the posterior marginal zone also have the molecular information necessary to activate the segmentation programme.

5. 7. The segmentation clock is progressively activated during chick embryonic development

As previously described, the cyclic gene expression first appears as a chevron in the region of the middle primitive streak (Jouve *et al*., 2002), consistent with the idea that the node-inducing center, located within this region,

already contains the information for activating the molecular clock. Conversely, the proposal that the marginal zone cells at the stage of X-XI, also has this information, may apparently be difficult to explain. However, we find it possible that the information for the activity of the segmentation clock is already contained within the posterior marginal zone cells, meaning that these cells would be able to induce the onset of the segmentation clock when transplanted to an ectopic site. The fact that no cyclic gene expression is observed at this level suggests that the somitic prospective cells perhaps only start the actual counting of time when they enter the primitive streak to form PSM tissue. In agreement with this hypothesis, the posterior primitive streak in the mouse embryo was shown to contain the necessary information for activating Hox gene expression much earlier than overt Hox expression, even though Hox genes are not expressed in that region (Forlani *et al*., 2003).

Interestingly, the analysis of the initial expression of several other clock-related genes such as *delta1*, *notch1* and *wnt3a* reveals that their expression is not concomitantly activated at a specific stage in the chick embryo. Instead, the expression of *delta1* appears at stage XII, *notch1* becomes evident at stage 2 HH and the expression of the *wnt3a* gene has not been detected at any stage before the onset of primitive streak development (Skromne and Stern, 2001; Caprioli *et al*., 2002). Taken together, these data suggests that the main players involved in the segmentation clock machinery seem to be progressively activated during the early developmental stages, as if they were establishing a temporal network that will trigger the onset of a time-counting activity. This onset of the clock activity is then subsequently manifested by the cyclic gene expression of the molecular clock genes, at the time when the prospective somitic cells ingress the primitive streak to become PSM tissue.

5. 8. What is the molecular nature of the signals that activate the molecular clock genes during development?

Another important question that came up during the course of this work

was whether the time count induced by Hensen's node corresponded to an intrinsic timing of the organizer itself or if, in contrary, the counting of time was induced *de novo* in the newly recruited PSM cells. To distinguish between these two situations we made heterotopic intraspecific chick node grafts and observed that the expression pattern of the *lunatic fringe* gene was different in the ectopically formed PSM and the one of the main axis. So, we concluded that the organizer is able to reset the molecular clock in the ectopic PSM cells and provide them with a new segmentation programme.

The fact that *cvg1* and *cwnt-8c* are both expressed in the marginal zone and in the middle region of the primitive streak of the chick embryo (Seleiro *et al*., 1996; Hume and Dodd, 1993; Joubin and Stern, 1999), has shed new light into the search for the molecular players regulating the process of organizer induction. Actually, the overexpression of a combined source of these signals induces the expression of organizer markers when they are grafted to an ectopic site, in the chick (Joubin and Stern, 1999). Therefore, these results strongly suggest that the Vg1/activin and Wnt signalling pathways have a prominent role in embryonic axis induction. An attractive hypothesis that comes out of the analysis of these data is the possibility that these genes may be important players in the genetic cascade leading to the induction of the molecular clock activity. In the present study we observed that the expression of the *hairy2* gene seems to be activated 6 hours following the heterotopic graft of Hensen's node into the lateral blastoderm of a host embryo. This finding, together with the fact that the node is able to induce the restart of the segmentation clock in cells not fated to become somites, provides an important step to understand the molecular network underlying the activation of the clock mechanism.

It has been known for almost a decade that the implantation of Noggin-producing cells, in the presumptive lateral plate mesoderm territory of stage 8 HH chick embryos, leads to ectopic somite formation (Tonegawa and Takahashi, 1998). This result puts forward the hypothesis that the inhibition of BMP signalling could be responsible for the induction of somite formation. In fact, our findings that the organizer is able to restart the molecular clock are consistent

with this hypothesis, given the fact that the node is a strong source of BMP antagonists (see Chapter 1, section 1.5). Hence, an attractive possibility is that the Noggin-derived PSM could exhibit the expression of the molecular clock genes. In the future, it would be interesting to test whether the time taken by the implantation of the Noggin cells to induce somites, could somehow relate to our preliminary data that established the time of induction of the *hairy2* gene. This approach would certainly contribute to initiate the study of the genetic interactions leading to the activation of the molecular clock.

5. 9. Somitic AP polarity is progressively established during development

The analysis of the expression pattern of *hairy1*, *hairy2*, *lunatic fringe* and *hey2* at the level of the PSM of early somite-stage chick embryos showed that these genes present a consistent cyclic expression throughout all somitic stages (see Chapter 4). These findings indicate that the molecular clock mechanism controlling the periodicity of the first somites at the level of the PSM is the same as the one controlling the periodicity of more posterior somites. In addition, the fact that these genes and the *notch1* and *delta1* genes always exhibit a striped expression at the level of the rostral PSM suggests that AP polarity specification is occurring throughout all somitic stages in the chick embryo.

However, at the somitic level, these genes display distinct expression patterns in more rostral somites when compared to those described for 15 to 20-somite stages. Surprisingly, our results showed that the stabilization of the expression of the cycling genes in either the anterior or posterior somitic compartment is not observed in the most anterior somites, whereas more posterior somites always maintain a rostral or caudal expression of the *notch1*, *delta1*, *hairy1*, *hairy2*, *lunatic fringe* and *hey2* genes. Furthermore, we also found that the onset of the polarised expression of these genes occurs at different AP somitic levels and it is never observed before the formation of the 4^{th} somite. These results suggest the existence of a specific programme that progressively

switches on the *notch1* and *delta1* expression, followed by *hairy1* and *hey2* and finally by the activation of *lunatic fringe* and *hairy2* expression patterns. As previously discussed, the onset of the expression of *delta1*, *notch1*, *wnt3a* and the cycling genes also appears at progressively different times during the very early stages of chick development (see section 5.7), which is strikingly similar to this event of progressive activation of polarised gene expression at the level of the rostral-most somites. An attractive possibility is that the somitic AP positional information is sequentially generated during gastrulation (possibly by the progressive appearance of the *Hox* genes and of several clock-related genes) and only later in development, stabilized by a specific combination of molecular factors. In agreement with this idea, a study in *Xenopus* has proposed that successive transient Hox codes are provided to the gastrulating embryo during development, although each Hox code only becomes stabilized when mesodermal cells leave the node as it regresses along the AP axis (Wacker *et al*., 2004). Hence, it is tempting to propose that a similar mechanism could be operating to stabilize the progressively polarised expression of the cycling genes in somites, which would account for the observed molecular differences between most rostral and caudal somites.

An alternative possibility that could explain the observed molecular differences between anterior and posterior somites is that progressively different combinations of Hox genes along the AP axis, could lead to the progressive switch of the polarised expression of the Notch-related genes at the somitic level. In support of this hypothesis a recent study reported that the *Hoxa10* gene, which exhibits an anterior expression boundary at the level of the thoracic-lumbar transition, leads to a lumbar phenotype when it is ectopically expressed at the level of the PSM (Carapuço *et al*., 2005). Conversely, this result is not obtained when *Hoxa10* is overexpressed at the level of the somites. Hence, the conclusion of this work is that the patterning activity of *Hoxa10* is different in the PSM and in the somites. Notably, the authors propose that the PSM cells are already committed to a later specific function, that is imposed to these cells by a specific Hox gene patterning programme operating at this level (Carapuço *et al*., 2005).

Thus it would be interesting to test whether the ectopic expression of a specific combination of Hox genes at the level of the PSM that leads to the formation of the anterior-most somites, would lead to ectopic induction of the polarised expression of the Notch-related genes.

5. 10. Anterior-most somites are not AP polarised

As previously described, our data shows that the stabilization of the expression of the *notch1*, *delta1*, *hairy1*, *hairy2*, *lunatic fringe* and *hey2* genes, in either the rostral or caudal somitic compartment, is never observed in the anterior-most 4 somites. These results are consistent with previous studies that have demonstrated that the first 4 somites do not give rise to segmented structures (Couly *et al*., 1993; Huang *et al*., 2000). In addition, grafting experiments concerning the migration of the dorsal root ganglia (DRG), which derive from the neural crest cells, have demonstrated that these ganglia migrate through the anterior half of somites 2 to 6, but subsequently degenerate and do not originate motor neurons, in contrast with DRG at more posterior levels (Lim *et al*., 1987; Kant and Goldstein, 1999; Ferguson and Graham, 2004). In addition, it was shown that the microenvironment within the 5 rostral-most somites is responsible for the degeneration these ganglia (Kant and Goldstein, 1999).

In opposition to the results reported above, the expression of *uncx4.1* is observed in the caudal compartment of anterior somites as well as in more posterior ones (Schragle *et al*., 2004). Given the fact that our results show that the anterior-most somites do not express AP polarity markers and that no other molecular marker other than the *uncx4.1* gene was reported to be expressed at the level of anterior-most somites, we find it attractive to speculate that perhaps these somites are not AP polarised. Consistently, the null mutant for the *uncx4.1* gene in the mouse presents no phenotype at the level of the skull (Leitges *et al*., 2000; Mansouri *et al*., 2000), which is a strong indication that AP polarised expression of the *uncx4.1* gene activity at the level anterior somites, is not absolutely essential for the formation of the basal structures of the skull.

5. 11. The molecular clock operates faster in the anterior-most somites

Several studies have reported that the anterior-most somites form faster in several vertebrate species (Kimmel *et al*., 1995; Tam, 1981; Schubert *et al*., 2001). These findings, together with our own observations of early somitic stage chick embryos, led us to suspect that anterior somites in the chick, could also be forming faster than the 90 minutes conventionally established. In fact, our time-lapse analysis that monitored the formation of the first somites *in vivo* revealed that these somites do not form in 90 minutes. Instead the first 2 somites seem to form concomitantly and somites 3 and 4 form very shortly after one another. The regularity of somite formation seems to be accomplished by somite 5 (see Chapter 4). These results led us to propose the hypothesis that perhaps the molecular clock, that regulates periodicity of somite formation at the level of the PSM, could also be accelerated in the PSM of these early somitic stages. To check this possibility we performed explant culture experiments and we verified that the cycling time underlying anterior somite formation was less than 90 minutes, in agreement with our previous time-lapse experiment

According to the 'clock and wavefront' model, periodicity and size of somite formation depends on an intrinsic cellular oscillator operating in parallel to a maturation wavefront at the level of the PSM (Cook and Zeeman, 1976) (see Chapter 1, section 1.7). A recent work has mathematically formulated the clock and wavefront model allowing the authors to make experimental predictions (Baker *et al*., 2006). Interestingly, it was mathematically predicted in this study that if the period of the segmentation clock were increased (i.e., operating slower) while keeping the regression of the wavefront constant, it would result in increased somitic size. In contrast, the authors show that decreasing the rate of axis elongation (i.e., decreasing the rate of wavefront progression) while keeping the segmentation clock constant, it would lead to the formation of larger somites. According to our results, the period of the segmentation clock is diminished in the PSM of the anterior somites, which could account for the fact that it is generally

observed that the size of these somites is smaller than more posterior ones (see Chapter 4) (Linask *et al*., 1998). The fact that anterior somites appear smaller than more posterior somites has also been reported in the mouse embryo (Tam, 1981). Therefore, we find it tempting to infer that the formation of the rostral-most somites, that exhibit a smaller size than more posterior ones, involves a decrease of the period of segmentation clock. In order to better understand the mechanism by which the interaction between the segmentation clock and the progression of the wavefront works along the embryonic axis, it would also be interesting to evaluate whether the periodicity of the posterior-most somites (generally larger than rostral somites) is slower than the 90 minutes conventionally established.

AppendiX

Running after the clock

Revision article published during the course of this thesis

Int. J. Dev. Biol. 49: 317-324 (2005)
doi: 10.1387/ijdb.041955cf

Running after the clock

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ABSTRACT The way we currently understand vertebrate development is undoubtedly associated with the research undertaken at the "Institut d'Embryologie Cellulaire et Moléculaire" at Nogent-sur-Marne during the last decades. Working in this Institute has been a privilege for many junior and senior researchers. Eight years ago, in this stimulating environment, an exciting observation followed by a series of revealing experiments gave rise to a novel field of research. This study provided evidence for the existence of a molecular clock underlying chick somite formation. In this review, we focus on the cascade of studies that have followed this discovery. Thus far, it has been demonstrated that the molecular clock is operating in several vertebrate models namely chick, mouse, zebrafish, frog and medaka, probably functioning to provide cells with multidimensional positional information. Loss and gain of function experiments and detailed gene promoter analyses have proved very useful in understanding how the clock machinery works. Recent data has also led to the fascinating hypothesis that the clock might not be an exclusive property of somitic cells, but rather a mechanism used by a wide range of embryonic tissues. Meanwhile, the clock "keeps ticking" and many questions are still waiting for an answer.

KEY WORDS: *molecular clock, cycling gene, segmentation, somitogenesis, vertebrate embryo*

Segmentation is an evolutionary successful feature that starts early during embryonic development with the formation of transient metameric structures called somites. Somites will give rise to the segmented structures in the vertebrate embryo such as vertebrae, intervertebral disks, ribs and skeletal muscles. These structures provide an efficient protection to the internal vital organs, while conferring a high degree of mobility to the adult body. Somites form as epithelial spheres in an anterior (A) to posterior (P) sequential manner, bilateral to the axial midline of the embryo. Each somite buds off periodically from the most anterior tip of the unsegmented mesenchymal paraxial mesoderm or presomitic mesoderm (PSM) (reviewed by Gossler and Hräbe de Angelis, 1998). For a given species, temporal periodicity of somite formation is so remarkably precise that it has retained the attention of embryologists for many decades. Several theoretical models have tried to explain the precision of somitogenesis. Many aspects of the cellular and molecular mechanisms underlying this process have been unveiled in the last years although many fundamental questions remain to be addressed.

Somite formation in the light of classical models

Three main classical models have been proposed to explain the periodicity of somite formation: the Meinhardt's model, the cell cycle model and the clock-and-wavefront model.

Meinhardt proposed that prior to the formation of each somite, presomitic cells undergo several oscillations between two alternate states corresponding to the prospective A and P somitic compartments (Meinhardt, 1986). As postulated, the confrontation between cells of incompatible A and P states would result in a physical boundary between consecutive somites, since there is no intermingling between cells from different states. Juxtaposition of these two segregated states would also lead to the formation of a physical barrier in the middle of a somite. To overcome this problem Meinhardt postulated a third oscillating state, called segment border (S), which corresponds to the somitic boundary. However, while distinct cell adhesive characteristics allowed the identification of A and P somitic compartments (Keynes and Stern, 1984), no S cells have been identified so far. In addition, a study using confocal time-lapse microscopy challenges the Meinhardt's model by showing that some degree of cell intermingling between A and P somitic compartments does occur during somite boundary formation (Kulesa and Fraser, 2002).

In 1988, Primmett and collaborators demonstrated that a single heat-shock applied to the chick embryo gives rise to several segmentation abnormalities that are repeated along the AP axis

Abbreviations used in this paper: EJM, ejemplo poner aquí ; QTR, quitar si no hay escribir desde aquí hacia arriba.

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0214-6282/2005/$25.00
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Printed in Spain
www.intjdevbiol.com

with a regular interval of six to seven somites (Primmett *et al.*, 1988). The time required to form six to seven somites in the chick is approximately ten hours, which corresponds to the time necessary for the completion of the cell cycle in the PSM (Primmett *et al.*, 1989). Consequently, it was postulated that the cell cycle would function as an internal cellular clock related to the process of segmentation: cells located in the rostral PSM share some degree of cell cycle synchrony, increase their adhesive properties and thus assemble and give rise to a somite. However, no correlation between the duration of the cell cycle in PSM and the rate of somitogenesis, which takes 90 minutes in the chick embryo, has been found so far.

The clock-and-wavefront model postulated the existence of two independent phenomena accounting for the periodic somite formation (Cooke and Zeeman, 1976). On one hand, there is an intrinsic clock compelling presomitic cells to oscillate synchronously between a permissive and a non-permissive state. On the other hand and concomitantly, a wavefront travels along the embryonic axis establishing an AP gradient of differentiation. This model predicts that in order to form a somitic boundary, a group of PSM cells oscillating synchronously has to be reached by the wavefront of differentiation. Experimental data gathered so far seem to support both assumptions of the clock-and-wavefront model.

The segmentation clock: the beginning of times

In 1997, Palmeirim and collaborators provided the first molecular evidence for the existence of an intrinsic oscillator operating in the presomitic cells of chick embryos. They observed that, within groups of embryos with the exact same number of somites, the basic Helix-Loop-Helix (bHLH) transcription repressor *hairy1* displayed remarkably different patterns of expression in the PSM. The authors have also demonstrated that this dynamic expression is reiterated every 90 minutes, corresponding exactly to the time required to form a pair of somites. Moreover, these mRNA oscillations were shown to be an autonomous property of PSM cells and neither a consequence of cell migration nor dependent on a diffused signal within the PSM. This pioneer study has demonstrated that presomitic cells undergo several periodic oscillations of the *hairy1* gene expression before they incorporate a somite, conceptually describing a caudal wave that progresses anteriorly and stabilises in a narrow stripe in the rostral PSM (Palmeirim *et al.*, 1997).

After the discovery of the *hairy1* gene in the chick embryo, many other genes have also been reported to have a cyclic expression at the level of the PSM, suggesting that the segmentation clock involves a complex genetic network. It is now clear that the molecular mechanism underlying somitogenesis is highly conserved among vertebrates, since periodic gene transcription has also been described in mouse, zebrafish, frog and medaka. The majority of the cycling genes code for Hairy/Enhancer-of-Split (Hes) family targets of the Notch signalling pathway such as *hairy1* and *hairy2* in the chick (Palmeirim *et al.*, 1997 and Jouve *et al.*, 2000), *hey2* both in chick and mouse (Leimeister *et al.*, 2000), *hes1* and *hes7* in mouse (Jouve *et al.*, 2000; Bessho *et al.*, 2001b), *her1* and *her7* in zebrafish (Holley *et al.*, 2000; Oates and Ho, 2002;) *esr9* in the frog (Li *et al.*, 2003) and *her7* in medaka (Elmasri *et al.*, 2004). Other cycling genes encode a modulator of the Notch signalling pathway, *lunatic fringe* (*lfng*), in the chick and mouse, (McGrew *et al.*, 1998; Aulehla and Johnson, 1999; Forsberg *et al.*, 1998) and a Notch ligand, *deltaC*, in zebrafish (Jiang *et al.*, 2000). Furthermore, work by Aulehla and colleagues (2003) has shown that a repressor of the Wnt signalling pathway, *axin2*, is also cycling in the mouse PSM (Aulehla *et al.*, 2003) (Table 1). More recently, nkd1, a wnt antagonist, has also been shown to exhibit an oscillatory expression pattern in the mouse PSM, suggesting a reciprocal interaction of Notch and Wnt signals in the regulation of the segmentation clock (Ishikawa *et al.*, 2004).

Looking at the mutants

The fact that the majority of the cycling genes code for components of the Notch signalling pathway suggests that it plays a role in the segmentation clock. Indeed, the analysis of *notch1* (Conlon *et al.*, 1995), *delta-like1* (*dll1*), *delta-like3* (*dll3*) (Hräbe de Angelis *et al.*, 1997; Kusumi *et al.*, 1998), *lunatic fringe* (*lfng*) (Zhang and Gridley, 1998; Evrard *et al.*, 1998), *presenilin1* (Wong *et al.*, 1997), *rbp-jk* (Oka *et al.*, 1995), *pofut1* (Shi and Stanley, 2003) and *hes7* (Bessho *et al.*, 2001b) mutants reveals a somitic phenotype. In general these defects consist of disrupted AP segment polarity, misaligned and misshapen caudal somites, while more rostral somites seem to be less affected. Although most of these mutations are lethal, the analysis of the embryos at early stages reveals defects in the organisation of the sclerotome and dermomyotome, eventually leading to severe perturbations in the axial skeleton. Thus far, both *hes1* and *hes5* are the only Notch signalling mutants without a somitic phenotype (Jouve *et al.*, 2000; Ohtsuka *et al.*, 1999). These results reflect a possible compensation by other cyclic *hes* genes such as *hes7* (Bessho *et al.*, 2001a). In zebrafish, several somite mutants have been isolated from a large-scale screening and the detailed analysis of their phenotypes shows a striking resemblance to the Notch signalling mouse mutants (Jiang *et al.*, 1996; Van Eeden *et al.*, 1996). In fact, it is now known that the phenotypes of the zebrafish mutants *deadly seven* (*des*), *after*

TABLE 1

CYCLIC GENES REPORTED IN VERTEBRATE SPECIES

Chick	Mouse	Zebrafish	Frog	Medaka
hairy1 (Palmeirim *et al.*, 1997)	*hes1* (Jouve *et al.*, 2000)	*her1* (Holley *et al.*, 2000)	*esr9* (Li *et al.*, 2003)	*her7* (Elmasri *et al.*, 2004)
hairy2 (Jouve *et al.*, 2000)	*hes7* (Bessho *et al.*, 2001b)	*her7* (Oates *et al.*, 2002)		
hey2 (Leimeister *et al.*, 2000)	*hey2* (Leimeister *et al.*, 2000)	*deltaC* (Jiang *et al.*, 2000)		
lunatic fringe	*lunatic fringe*			
(McGrew *et al.*, 1998;	(Forsberg *et al.*, 1998)			
Aulehla and Johnson, 1999)	*axin2* (Aulehla *et al.*, 2003)			
	ndk1 (Ishikawa *et al.,* 2004)			

eight (*aei*) and *mind bomb* (*mib*) are due to null mutations in *notch1*, *deltaD* and in an ubiquitin ligase that binds Delta, respectively (Holley *et al.*, 2002; Holley *et al.*, 2000; Itoh *et al.*, 2003). Injection of morpholinos targeted for these genes recapitulates the phenotype of each of these zebrafish mutants (Holley *et al.*, 2002; Itoh *et al.*, 2003). Although there are no zebrafish mutants for the *deltaC*, *her1*, *her7* and *suppressor of hairless* (*su* (*h*)) genes, morpholino knockdown experiments have revealed that, in the absence of the proteins coded by these genes, the embryos exhibit a somitic phenotype (Holley *et al.*, 2002; Henry *et al.*, 2002; Oates and Ho, 2002; Gajweski *et al.*, 2003; Sieger *et al.*, 2003). More recently, it has also been described that the zebrafish Her6 is an output of the Notch signalling pathway that, together with Her4, is required for maintaining the synchronization of cyclic gene expression within PSM (Pasini *et al.*, 2004). Similarly, cyclic gene expression in the PSM is lost by reducing the receptor protein tyrosine phosphstase ψ (RPTP ψ) using morpholino antisense oligonucleotides, suggesting a requirement for RPTP ψ in the control of the clock upstream of, or in parallel with, Delta/Notch signalling (Aerne and Ish-Horowicz, 2004) (Table 2).

Despite the somitic defects observed in the mouse and zebrafish mutants, the sclerotome and dermomyotome present a more or less organised segmental pattern, indicating that a basic metameric pattern is accomplished in the somitic derivatives. It seems that the Notch signalling pathway is important to coordinate the periodicity of somite formation and to specify somitic AP polarity, although its downregulation is not sufficient to abolish overall segmentation. This could be due to the fact that Notch activation is not the only determinant responsible for the formation of segments or because there is a certain degree of redundancy between Notch signalling components.

Phenotypic analysis of Notch signalling mutants strongly suggested that the defects observed could be due to a disruption in molecular segmentation and, therefore, the expression pattern of the cycling genes was studied in these mutants.

The expression of *lfng* is downregulated in *dll1*, *dll3*, *pofut1* and *rbp-jk* mutant mice but it is only slightly reduced in the *notch1* mutant (Barrantes *et al.* 1999; Shi and Stanley, 2003). On the contrary, the cyclic behaviour of *lfng* is not affected in *hes1* knockout mice (Jouve *et al.*, 2000). In *hes7* null mice *lfng* transcription is constitutively upregulated in all presomitic cells (Bessho *et al.*, 2001b). The *hes1* gene expression is severely downregulated in *dll1*, *dll3* and *hes7* homozygous null embryos (Jouve *et al.*, 2000; Dunwoodie *et al.*, 2002; Bessho *et al.*, 2001b). Moreover, the transcription of *hey2* is also downregulated in the PSM of *hes7* knockout mice (Bessho *et al.*, 2001b). Additionally, the transcription of *hes7* is constitutively upregulated in *hes7* mutants, as demonstrated by the expression of intronic probes in the PSM (Bessho *et al.*, 2003).

In zebrafish, the cyclic expression of *her1*, *her7* and *deltaC* is impaired in the *aei* (DeltaD), *des* (Notch1) and *mib* (Ubiquitin ligase) mutants and in the *su* (*h*) morpholino-knockdown experiments (Holley *et al.*, 2000; Holley *et al.*, 2002; Oates and Ho, 2002; Jiang *et al.*, 2000; Sieger *et al.*, 2003). Additionally, *her1* is impaired in *deltaC* morpholino injected embryos (Holley *et al.*, 2002). The inhibition of Her1 function in the PSM leads a loss of the cyclic expression of both *her1* and *deltaC*. In this knockdown experiment *her7* expression is decreased but its cyclic behaviour is maintained (Holley *et al.*, 2002; Oates and Ho, 2002). A decrease in Her7 function disrupts the dynamic expression of *deltaC*, *her1* and *her7* (Oates and Ho, 2002).

Overall, these studies show that the oscillations of the cycling genes are in fact disturbed in Notch signalling mutants, reaffirming the function of the Notch pathway in driving the segmentation clock and showing that this role is conserved among vertebrates. Jiang and colleagues (2000) observed that while in normal development PSM cells oscillate synchronously, in Notch signalling mutants they drift out of synchrony eventually leading to defective somitogenesis. The authors show that in Notch signalling zebrafish mutants, the expression pattern of *deltaC* is normal at first, but becomes desynchronised, which could account for the sparing of the first somites in these mutants. Thus, it was proposed that the essential function of the Notch signalling pathway is to maintain the oscillations synchronised in adjacent PSM cells (Jiang *et al.*, 2000).

Dissecting clock promoters

As discussed above there are several PSM genes that show a cyclic expression pattern, however the mechanism that generates this pattern is not completely understood. In the mouse, it was shown that the cyclic expression of *lfng* in the PSM is controlled at the level of transcription by periodic activation of its promoter (Morales *et al.*, 2002). Analysis of the *lfng* promoter by successive deletions of the 5'UTR sequence led to the identification of a *cis* -regulatory region that is able to recapitulate the cyclic expression of this gene. A human equivalent region also drives the cyclic expression of a reporter gene in the mouse PSM (Cole *et al.*, 2002; Morales *et al.*, 2002). Comparison of both human and mouse *cis* -regulatory regions disclosed an evolutionary conserved 110 kilobase fragment that is a strong candidate to regulate the periodic gene

TABLE 2

LOSS OF FUNCTION OF NOTCH SIGNALLING PATHWAY COMPONENTS INVOLVED IN SOMITOGENESIS

Mouse mutants	Zebrafish mutants
With a somitic phenotype	*notch1 - deadly seven (des)* (Holley *et al.*, 2002)
hes7 (Bessho *et al.*, 2001b)	*deltaD - after eight (aei)* (Holley *et al.*, 2000)
notch1 (Conlon *et al.*, 1995)	*ubiquitin ligase - mind bomb (mib)* (Itoh *et al.*, 2003)
rbp-jk (Oka *et al.*, 1995)	
presenilin1 (Wong *et al.*, 1997)	**Zebrafish morphants**
delta-like1 (Hräbe de Angelis *et al.*, 1997;	*her1*^{mo} (Holley *et al.*, 2002; Henry *et al.*, 2002;
Cordes *et al.*, 2004)	Oates and Ho, 2002; Gajewski *et al.*, 2003)
delta-like3 (Kusumi *et al.*, 1998)	*her7*^{mo} (Oates and Ho, 2002; Henry *et al.*, 2002;
lunatic fringe (Zhang and Gridley, 1998;	Gajewski *et al.*, 2003)
Evrard *et al.*, 1998)	*her1*^{mo}*+her7*^{mo} (Oates and Ho, 2002; Henry *et al.*, 2002)
pofut1 (Shi and Stanley, 2003)	*notch1*^{mo} (Holley *et al.*, 2002)
	suppressor of hairless^{mo} (Sieger–*et al.*, 2003)
Without a somitic phenotype	*deltaC*^{mo} (Holley *et al.*, 2002)
hes1 (Jouve *et al.*, 2000)	*deltaD*^{mo} (Holley"*et al.*, 2002)
hes5 (Ohtsuka *et al.*, 1999)	*her4*^{mo} (Pasini et al., 2004)
	her6^{mo} (Pasini et al., 2004)
	her6^{mo} *+ her4*^{mo} (Pasini et al., 2004)
	RPTP ^{mo} (Aerne and Ish-Horowicz, 2004)

^{mo} - morpholino

oscillations. Further dissection of this fragment has shown that it contains two E-boxes, which are binding sites for bHLH proteins, as well as a binding site for the CBF1 (Rbp-Jk; Su(H)) transcription factor (Cole *et al.*, 2002; Morales *et al.*, 2002). So it seems that cyclic bHLH Notch targets may regulate the segmentation clock acting as transcriptional repressors that bind to the E-boxes in the *cis* -regulatory region. Indeed, Hes7 binds E-boxes and thus it was proposed to mediate the cyclic repression of *lfng* (Bessho *et al.*, 2003). Mutations in the CBF1 binding site lead to a decrease in *lfng* expression in the mouse PSM (Cole *et al.*, 2002; Morales *et al.*, 2002), which indicates that this region functions as an activator element within the promoter. In addition, these experiments also demonstrate that the expression of *lfng* is distinctly regulated in the anterior and the posterior part of the PSM, which is in agreement with previous work performed in the frog and the fish (Jen *et al.*, 1999; Holley *et al.*, 2002). Analysis of the *her1* promoter in the zebrafish embryo has also demonstrated that a 5'UTR fragment is able to drive its dynamic expression in the PSM. This study also pointed to the existence of regulatory elements that distinctly control *her1* expression in either the anterior or the posterior PSM (Gajewski *et al.*, 2003). A complex interaction between repressor and activator clock elements in the promoter seems to be a general requirement for the oscillatory behaviour of segmentation genes.

Unveiling the clock mechanism

A negative feedback loop is a mechanism by which the expression of a gene is repressed by its own protein product. It has been suggested by several authors that the mechanism that drives the oscillations of the segmentation genes relies indeed on feedback inhibition.

The first direct evidence for the molecular mechanism that generates the oscillatory behaviour of the cyclic genes was presented in a study performed in cell culture (Hirata *et al.*, 2002). The authors demonstrated that not only *hes1* mRNA, but also Hes1 protein, undergo oscillations of expression with the same periodicity as somite formation. These oscillations are produced by a negative feedback loop in which Hes1 protein periodically represses its own transcription. This study suggested that a similar mechanism could be responsible for the transcriptional oscillations generated by the segmentation clock (Hirata *et al.*, 2002).

Recently, it was shown that in the mouse embryo both *hes7* mRNA and Hes7 protein oscillate in the PSM (Bessho *et al.*, 2003). Hes7 protein localisation domains do not overlap with the regions where *hes7* mRNA is expressed. Moreover, transcription of *hes7* is constitutively activated in the absence of Hes7 protein and downregulated following stabilization of Hes7 protein. Therefore, Hes7 oscillations in the PSM rely on a negative autoregulatory loop (Bessho *et al.*, 2003). Accordingly, Hes7 protein instability was shown to be crucial for cyclic gene expression (Hirata *et al.*, 2004). Also in the mouse embryo, it was shown that *hes1* mRNA oscillations are blocked in the absence of a functional Hes1 protein, suggesting that this protein might regulate its own promoter (Hirata *et al.*, 2002). In zebrafish, the cyclic genes *her1* and *her7* seem to negatively regulate their own expression, although there is no data regarding their protein expression in the PSM (Holley *et al.*, 2002; Oates and Ho, 2002).

Another negative feedback loop involving the periodic production of Lfng protein was described in the chick PSM (Dale *et al.*, 2003). In addition to *lfng* mRNA, Lfng protein cycles with the same periodicity as somite formation. Furthermore, overexpression of *lfng* in the chick PSM impairs the cyclic expression of the Notch downstream targets, *hairy1*, *hairy2* and endogenous *lfng*. Since Lfng is a modulator of Notch activity, it seems that the oscillations of segmentation genes are due to periodic inhibition of Notch activation (Dale *et al.*, 2003). Studies performed in zebrafish and mouse revealed that the function of Lfng might be different from the one in the chick. In zebrafish, *lfng* mRNA does not oscillate in the PSM (Prince *et al.*, 2001; Leve *et al.*, 2001) and it seems that the periodic activation of Notch is undertaken by the cyclic gene *deltaC* (Jiang *et al.*, 2000). In contrast, *lfng* oscillates in the mouse PSM (Forsberg *et al.*, 1998), although its constitutive expression does not abolish cyclic expression of endogenous *lfng* (Serth *et al.*, 2003). This implies that the activity of Notch alone cannot be the only determinant of cyclic gene expression in the mouse embryo.

An interesting finding that might explain the Lfng results in the mouse is the role recently attributed to the Wnt signalling pathway as a regulator of the clock by acting upstream of the Notch pathway (Aulehla *et al.*, 2003). It was shown that *axin2*, a negative regulator of the Wnt pathway, is expressed in the PSM in a cyclic fashion. In the *vestigial tail* mutant mouse, which is a hypomorphic mutant of *wnt3a,* there are caudal segmentation defects and, interestingly, *axin2* is not expressed. Thus, a negative feedback loop was proposed in which Wnt3a induces *axin2* expression and then Axin2 negatively modulates Wnt signalling. Since *axin2* mRNA oscillates alternately with *lfng* mRNA, it was suggested that Wnt and Notch pathways interact antagonistically through the binding of *dishevelled* to the Notch intercellular domain (Aulehla *et al.*, 2003). Recently, it was shown that the Wnt-responsive transcription factor Lef1 binds to several sites in the *dll1* promoter and enhancer regions and regulates its activity in the mouse PSM. This finding establishes a molecular link between the Wnt and the Notch pathways during somitogenesis (Galceran *et al.*, 2004). So far, the involvement of Wnt signalling in the regulation of periodicity of somite formation has not been reported in other vertebrate embryos.

It is possible to consider the existence of three types of negative feedback loops by incorporating data from zebrafish, chick and mouse, although the possible interactions between these loops are still not understood: 1) a direct feedback loop that generates the cyclic expression of Hairy/ Enhancer-of-Split family of bHLH repressors (Her1 and Her7 in zebrafish and Hes1 and Hes7 in the mouse); 2) an indirect feedback loop that establishes periodic activation of Notch signalling (DeltaC in zebrafish and Lfng in the chick); 3) another indirect feedback loop that promotes periodic activation of Wnt signalling (Axin2 in the mouse) (Fig. 1).

It has been suggested that a negative feedback loop in which the expression of a gene is repressed by its own protein product would be insufficient to generate/maintain sustained oscillations (Hirata *et al.*, 2002). However, two mathematical models based on the experimental data from zebrafish and cell culture studies show that mRNA and protein oscillations can be produced if transcriptional and translational delays are taken into account (Lewis, 2003; Monk, 2003). Nevertheless, delayed feedback will only set up the pace of oscillations if the mRNA and protein half-

lives are effectively small in relation to the delay. Surprisingly, these mathematical models reveal that delay-driven oscillations are very resistant to parameter changes (Lewis, 2003; Monk, 2003). Simulating a reduction in the rate of protein synthesis revealed no effect on the oscillation period (Lewis, 2003), which could explain the occurrence of *hairy1* oscillations in the chick PSM after cycloheximide treatment (Palmeirim *et al.*, 1997). Moreover, a simulation where Notch signalling is impaired shows a progressive failure in the regularity of the oscillations which, in agreement with the desynchronization theory, could explain the mild defects in the first somites of zebrafish and mouse Notch mutants (Lewis, 2003; Jiang *et al.*, 2000).

The wavefront: a partner of the clock

The clock-and-wavefront model proposes an explanation for the temporal and spatial regulation of somitogenesis. It predicts the existence of an intrinsic oscillator operating in parallel with a wavefront of differentiation, whose progression rate determines the correct positioning of somitic boundaries (Cooke and Zeeman, 1976). As previously discussed, the molecular evidence for the oscillator was provided by the cyclic expression of a number of genes, whereas the wavefront position seems to be regulated by Fgf and Wnt signalling (Dubrulle *et al.*, 2001; Sawada *et al.*, 2001; Aulehla *et al.*, 2003), by retinoic acid (RA) signalling and possibly by an unknown pathway involving the T-box gene, *tbx24* (Diez del Corral *et al.*, 2003; Nikaido *et al.*, 2002).

In the chick, *fgf8* defines a decreasing caudal to rostral gradient of expression in the two posterior thirds of the PSM (Dubrulle *et al.*, 2001). AP inversion experiments of PSM tissue demonstrated that AP somitic compartments are already determined in the anterior third of the PSM, in contrast to the caudal two thirds of this tissue where segment polarity is still undetermined (Dubrulle *et al.*, 2001). The transition between these two regions occurs at the level of the so-called determination front that progressively regresses as a consequence of embryo elongation and seems to correspond to the anterior limit of the *fgf8* gradient of expression. Either inhibiting or overexpressing Fgf8 at the level of the determination front alters the position of somitic boundaries, inducing the formation of larger or smaller somites, respectively. It seems that Fgf8 maintains posterior PSM cells in an immature state, thus negatively regulating the wavefront of differentiation in the chick embryo. Caudal to the determination front, the axial identity of PSM cells is also undetermined, since Fgf8 overexpression can induce an anterior shift of *hoxB9* and *hoxA10* expression domains (Dubrulle *et al.*, 2001). Additionally it was proposed that an interaction between the segmentation clock and the *hox* genes would establish the correct coordination between sequential segment formation and AP specification (Zakany *et al.*, 2001). Reinforcing this idea, transgenic mice expressing a dominant negative version of *Delta1* in the PSM showed subtle changes of *Hox* gene expression and alterations of vertebral identity resembling homeotic transformations (Cordes *et al.*, 2004).

The involvement of Fgf signalling in the control of the wavefront progression was also studied in zebrafish embryos (Sawada *et al.*, 2001). Fgf/mitogen-activated protein kinase (MAPK) signalling is functioning in the posterior PSM and it maintains these cells in an immature state. Experimental manipulations of MAPK levels in the PSM also lead to a variation in somitic size, strengthening the idea that Fgf signalling determines the position of segment border formation (Sawada *et al.*, 2001).

In the mouse, *wnt3a* seems to play a similar role to the one attributed to Fgf signalling in both chick and zebrafish PSM (Aulehla *et al.*, 2003). *wnt3a* is strongly expressed in the tail bud and it was proposed to establish a decreasing caudal to rostral gradient of expression that regresses as the embryo elongates. Furthermore, *fgf8* is downregulated in the tail bud and PSM of *wnt3a* hypomorphic mutants, suggesting that *wnt3a* acts upstream of *fgf8* in the regulation of the wavefront position. Since there is evidence that Fgf signalling may enhance Wnt/β-catenin signalling, Fgf8 might act as a relay enhancer of Wnt signalling in the PSM of mouse embryos (Aulehla *et al.*, 2003).

Previous studies have shown that presomitic tissue represses neuronal differentiation and that Fgf signalling can mimic this action (Diez del Corral *et al.,* 2002). On the other hand, the somitic mesoderm promotes maturation events, which are correlated with the activation of RA signalling in rostral PSM and somites, as indicated by the expression of the RA-synthesizing enzyme *raldh2* (Diez del Corral *et al.,* 2002 and 2003). Furthermore, explant culture experiments using a RA agonist (TTNPB) and Vitamin A deficient (VAD) quail embryos, which lack biologically

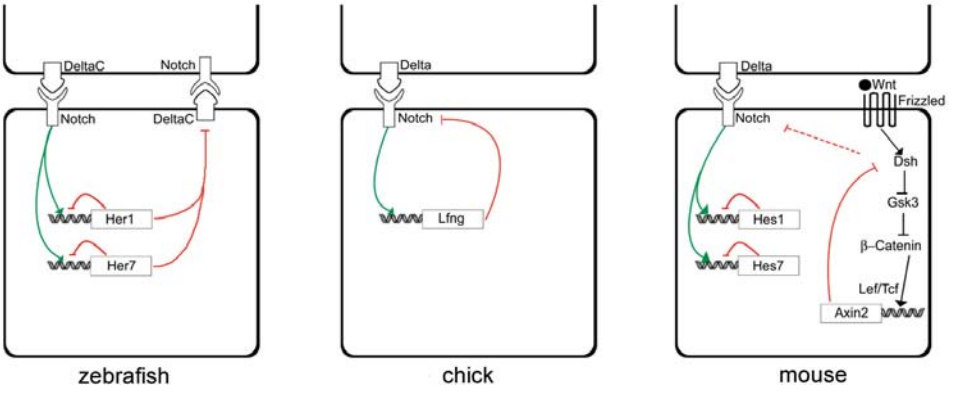


Fig. 1. Feedback loops underlying periodic oscillations of cycling genes. *In zebrafish, the periodic oscillations of* her1 *and* her7 *mRNA are generated by autoregulatory feedback loops and may be involved in* deltaC *periodic expression DeltaC in turn would periodically modulate Notch signalling activity. In the chick, Lfng protein indirectly represses its own expression by periodic modulation of the Notch signalling pathway. In the mouse, cyclic transcription of* hes7 *is regulated by the periodic expression of its own protein. The same molecular feedback loop generates Hes1 oscillations in different cell types. Additionally, the cyclic expression of* axin2*, a direct target of the Wnt pathway, regulates the Wnt signalling by a mechanism of feedback inhibition. It has been proposed that Wnt and Notch signalling pathways antagonistically interact since transcription of* axin2 *and* lfng *oscillates out of phase in the mouse PSM.*

active RA, have demonstrated that RA downregulates the expression of *fgf8* in the PSM (Diez del Corral *et al.,* 2003; Maden *et al.*, 1996). Conversely, Fgf8 soaked beads placed in the chick PSM represses the expression of *raldh2*, which indicates that Fgf signalling regulates the onset of RA synthesis in presomitic tissue (Diez del Corral *et al.,* 2003). These results show that Fgf and RA signalling pathways are mutually inhibitory and point to an important role of RA in inducing PSM cells maturation, in opposition to Fgf8. Future research will be important to reveal a possible interaction between the cycling genes (segmentation clock) and RA (wavefront) and their role in the control of somitogenesis.

The *fused somites* (*fss*) zebrafish mutant lacks all somitic boundaries and this phenotype is the result of a mutation in the *tbx24* gene (Van Eeden *et al.*, 1996; Nikaido *et al.*, 2002). This suggests the involvement of *tbx24* in the formation of somitic boundaries. Although *tbx24* is expressed in anterior and intermediate PSM, its function seems to be restricted to the rostral PSM. In fact, in *fss* mutants segmentation genes specifically expressed in the anterior PSM are downregulated and the anterior stripe of the *her1* cyclic gene is lost, whereas its expression appears to be normal in the posterior PSM. These data suggest that *tbx24* plays a role in the maturation process of anterior PSM cells and that it might be independent of the molecular clock.

Fgf and Wnt signalling pathways seem to control the positioning of the wavefront of differentiation (Dubrulle *et al.*, 2001; Sawada *et al.*, 2001; Aulehla *et al.*, 2003). Since the formation of somitic boundaries is such a finely tuned process it is conceivable that a signal in the anterior PSM controls the precise site of the determination front. This signal could work in combination with the Fgf and Wnt posterior gradients that maintain cells in an immature state. As discussed above, both RA and *tbx24* are good candidates for this signal. Since *tbx24* has only been described in zebrafish, it would be interesting to look for functional homologues in other vertebrate models and to understand how the T-box pathway interacts with Fgf and Wnt signalling pathways.

The clock in two dimensions

It is now well established that an intrinsic oscillator operates in PSM cells as it is revealed by the periodic expression of the cyclic genes. It seems that presomitic cells perceive the number of oscillations they undergo before incorporating a somite. This suggests that the segmentation clock constitutes a mechanism that provides AP positional information to these cells, determining their spatial organisation within the PSM (Palmeirim *et al.*, 1997). In the chick, a detailed analysis performed from stage 4 to 7HH (Hamburguer and Hamilton, 1951) showed that the cycling genes are expressed in the prospective somitic territory (Jouve *et al.*, 2002). This implies that presomitic cells are provided with their future AP positional information well before the first somite is formed.

In six somite stage chick embryos (stage 9- HH) a dynamic pattern of the cycling genes is also evident at the level of the presumptive presomitic territory, defining in this region an AP gradient of expression. A detailed quail-chick chimera fate map has revealed an anterior region located within this prospective territory that specifically gives rise to the medial part of the PSM and somites (Freitas *et al.*, 2001). Therefore, the AP gradient of expression in the prospective PSM territory describes a wave spreading throughout the future medial/lateral (ML) presomitic axis. Accordingly, a more careful analysis of expression pattern of the cycling genes at the level of the PSM unveils a ML asynchrony that is evidenced by oblique stripes of gene expression corresponding to a transition between two horizontal stripes (see supplemental data at: http://www.ijdb.ehu.es/ijdb200549023/49023esm317.mov). Thus, the segmentation clock is providing cellular positional information in at least two dimensions: not only along the AP but also along the ML presomitic axis (Freitas *et al.*, 2001).

Final remarks

The development of a living organism is highly regulated in space and time. The only biological clock known to operate during embryonic development is the segmentation clock underlying the highly coordinated process of somite formation in vertebrates. However, the nature of the signal that triggers the molecular clock, early in development, remains to be determined. The segmentation clock is currently perceived as a cross talk between Notch and Wnt signalling cascades, consisting of complex regulatory feedback loops that ultimately generate periodic gene oscillations in presomitic cells. The cyclic transcription will only be translated into visible periodic gene expression patterns if both 1) the half-life of the mRNA is shorter than its transcription cycle and 2) a group of neighbouring cells is synchronous. It is possible that other genes undergoing cyclic transcription are not detected and so, their existence cannot be ruled out.

Recently, it was shown that the involvement of the Notch signalling pathway in the process of segmentation is not exclusive to vertebrates since it has been shown to be essential for the formation of segments in spiders (Stollewerk *et al.*, 2003). Such a finding suggests that a common ancestor of both vertebrates and arthropods might have used the same molecular mechanism for segmentation. Therefore, the fact that *Drosophila* does not use the Notch signalling pathway to make segments suggests that arthropods that generate segments simultaneously might have lost this ancestral segmentation strategy during evolution. It remains to be clarified whether the Notch signalling pathway is used by all sequentially segmented arthropods as well as whether a clock-like mechanism drives the cyclic transcription of Notch targets in these organisms.

It is now evident that a conserved clock mechanism dictates the timing of segment formation. At this point, an interesting question that arises is whether this clock operates in tissues other than presomitic mesoderm. As a matter of fact, an *in vitro* study has shown that several mouse cell lines express *hes1* mRNA and protein in a cyclic fashion. This, points to the possibility that different types of cells might measure time using a similar clock mechanism (Hirata *et al.*, 2002). Work from our group has provided evidence for cyclic *hairy2* expression in the chick limb bud, indicating that the clock mechanism is also controlling limb outgrowth (Pascoal *et al.*, in preparation). It is therefore tempting to postulate that the rearrangement of cells into tissues and organs requires different coordinated events controlled by a common mechanism that counts time.

Acknowledgements

We thank Sólveig Thorsteinsdóttir, Raquel Andrade, Ana Tavares, Will Wood and Joaquín Rodríguez-León for helpful comments on the

manuscript. Animation (supplemental data) was produced by Spirituc (www.spirituc.com). Financial support was provided by Instituto Gulbenkian de Ciência and by Fundação para a Ciência e a Tecnologia (F.C.T.) project no. 34599. C.F. is supported by F.C.T. PRAXIS/XXI/BD/21583/99; S.R. is supported by F.C.T. project no. 34599; L.S. is supported by F.C.T. SFRH/BPD/6755/2001.

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