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DISSERTAÇÃO MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO 2015

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Acknowledgments

"Fifty percent of what we know is wrong. The problem is that we do not know which 50% it is."

- Timothy Noakes, PhD -

I would like to thank Dra. Leonor Saúde for giving me the opportunity to work in her lab, for the constant support during this past hard working year, and most importantly for believing in my work. I couldn´t have asked for a better supervisor.

To all members of LSaúde Lab for the help and advice they gave me throughout this year. I know that I'm not the easiest person to work with so I realy do appretiate the support given.

To Professor Solveig Thorsteinsdottir for being my internal supervisor.

To all of my friends, for making my life better by being there and making me laught when I was down. You are the best!

And last but not least, to my family for the unconditional love and support that they gave me all of these years. I love you guys!!!

Resumo:

Durante o desenvolvimento, diferentes eventos acontecem ao longo dos eixos de um embrião, para que um individuo adulto seja formado. Estes eventos podem dividir-se em dois tipos: (1) Eventos simétricos; (2) Eventos assimétricos.

Um dos eventos simétricos chave é a somitogénese. A somitogénese é o processo pelo qual temos iniciação da segmentação do sistema músculo-esquelético, através da formação dos sómitos, segmentos mesodérmicos transitórios. Este processo de segmentação ocorre progressivamente na mesoderme pré-somítica, numa direcção anterior para posterior. Os pares de sómitos formam-se ao mesmo tempo dos dois lados e sempre com a mesma periodicidade segundo o modelo, proposto em 1976 por Cooke and Zeeman, "Clock and Wavefront model". Este modelo supõe a existência de uma interacção de factores, cuja produção é cíclica, que marca o "tempo" que as células passam numa zona indeterminada da mesoderme paraxial. Associada a esta ciclicidade de factores encontra-se-ía ainda um gradiente de um outro factor, distinto dos primeiros, que seria responsável por determinar o espaço onde se dá a maturação das células. Quando as células atingem uma determinada região do gradiente, estas interpretariam a combinação de ambos os sinais como informação para se diferenciarem e aglomerarem, formando um novo sómito.

Um conjunto de eventos que se enquadra no ponto (2), é o da formação e organização dos órgãos internos. Um exemplo clássico é o da formação do coração, que está localizado no lado esquerdo do organismo (no direito no caso da galinha) e que sofre uma torção de maneira a que as aurículas fiquem em cima e os ventrículos em baixo. Outro exemplo é o da formação das vísceras que, de maneira a ficarem alojadas correctamente na cavidade abdominal, têm que sofrer uma série de rotações.

Todos estes processos assimétricos têm como base um conjunto de mecanismos que distribui, assimetricamente várias moléculas sinalizadoras, entre eles Shh, Wnts e FGFs, que vão controlar uma cadeia genética altamente conservada entre espécies. Hoje em dia sabe-se que este conjunto de mecanismos, iniciadores de assimetria, são variados e que apresentam algum grau de conservação entre espécies, actuando em diferentes tempos do desenvolvimento. Alguns destes mecanismos são: (1) A distribuição assimétrica de iões Ca^{2+}, antes da gastrulação; (2) Estabelecimento de um gradiente assimétrico de determinantes via cílios, presentes no organizador, durante a gastrulação; (3) Expressão diferencial de uma cadeia de genes durante a formação de sómitos. Esta cadeia é a cadeia de Nodal-Pitx2, que se sabe ser assimétrica, tendo expressão no lado esquerdo do embrião.

Sabe-se no entanto que os processos de simetria e assimetria, que formam um individuo adulto, ocorrem ao mesmo tempo, pelo que é necessário um, ou vários, mediadores para que a informação seja transmitida corretamente para as diferentes vias. Uma incorreta transmissão desta informação induz graves problemas no correcto posicionamento das diferentes estruturas que constituem um organismo, pelo que, uma boa compreensão dos eventos que coordenam as diferentes vias, é necessária.

Um dos mediadores é o ácido retinóico (RA), que é a versão oxidada e activa da vitamina A. Estudos revelaram que de facto o RA tem um papel importante na protecção dos sómitos contra sinais assimétricos, uma vez que a sua inibição leva a graves problemas na formação dos sómitos, observando-se uma distribuição assimétrica de expressão génica. Um destes genes é o lunatic fringe (*Lfng*), que faz parte do "relógio molecular" da somitogénese, e está envolvido na diferenciação dos sómitos.

Mais recentemente foi descoberto um novo efector nesta via de protecção. Este efector é o factor de transcrição *Dmrt2.* Este factor de transcrição, embora pertença a uma família maioritariamente associada à determinação sexual, não só é necessário para a formação simétrica dos sómitos, como é para o correcto estabelecimento da assimetria esquerda-direita. O seu papel exacto nas vias responsáveis por estes eventos, ainda é vastamente desconhecido, pelo que um estudo detalhado sobre este factor de transcrição é necessário.

O objectivo deste projeto seria aprofundar o nosso conhecimento relativamente ao papel do *Dmrt2* na formação de estruturas simétricas e assimétricas ao longo do desenvolvimento. Para que tal fosse possível iria ser utilizado o embrião de galinha como modelo, uma vez que este é o modelo em que menos se conhece o papel do *Dmrt2*, pelo que a importância de estudos neste organismo é maior.

Para que estas funções sejam desvendadas, iriam ser feitas experiências de ganho de função, através da electroporação do *Dmrt2*, clonado no vector de expressão pGAGGS, em embriões de galinha no estádio $HH3^{+}$ e em cultura New, para perceber qual o seu papel na padronização esquerda-direita. Tal não foi possível pois durante o processo de clonagem percebemos que a região a 5' do gene não estava bem anotada, pelo que o desenho de primers forward era impossível. Ainda foi tentada uma 5'-RACE (técnica de amplificação de extremidades de DNA), para obter a região 5', mas sem sucesso.

Para perceber quais os genes que estariam a regular a sua atividade, partimos para uma abordagem de "gene candidato". Para tal foram feitas manipulações na sinalização de sonic hedgehog (Shh), uma vez que este gene tem a mesma expressão que *Dmrt2* mas mais cedo no desenvolvimento. Estas manipulações foram realizadas pondo uma solução de ciclopamina, inibidor da via de sinalização de Shh, in ovo, em embriões por volta do estádio HH4.

Para ver se a expressão de *Dmrt2* era afectada, realizamos hibridações *in situ* para o *Dmrt2*, com uma sonda produzida por nós em laboratório, através de plasmídeos infectados com uma porção do *Dmrt2*, que já tinha sido isolada, no âmbito de um projecto anterior. Apesar de se terem utilizado concentrações diferentes de droga, não se notou diferenças significativas na expressão entre embriões manipulados *versus* controlo.

Tendo em conta estes resultados propomos que a abordagem futura a seguir, será a de optimizar os protocolos usados, nomeadamente o da 5'-RACE, e o da inibição de sinalização hedgehog, via ciclopamina. Deveram ainda ser feitas experiências adicionais, após realizar a experiência de ganho de função, tais como ver a expressão de genes candidatos, que estejam a ser regulados via Dmrt2, e o registo do fenótipo observado. Este registo pode ter como parâmetros a posição de órgãos com disposição assimétrica, como por exemplo o coração.

Palavras chave: Eixo esquerdo-direito; Assimetria; Dmrt2; Galinha

Abstract:

In order to form a well-developed body, different events need to happen. These events can be symmetric or asymmetric. An example of a symmetric event is the formation of the axial skeleton and it's associated muscles. An asymmetric event is for instance the establishment of organ morphology and *situs*. It is known that these events happen in the same time window and in embryonic territories closely located. A well-coordinated set of pathways is therefore needed, otherwise an incorrect transfer of information occurs, leading to serious problems in both somite and organ formation and positioning.

It was shown that in zebrafish, Dmrt2a, a transcription factor of the DM domain family, protects the somites from asymmetric signals and conveys asymmetric signals to place the heart. It is known that zebrafish *dmrt2a*/*terra* is expressed in the anterior region of the PSM and somites. In addition, our lab detected *dmrt2a/terra* transcripts in the zebrafish KV from the 3-somite stage until the 10-somite stage, showing that this transcription factor has a function in the transition of information from the KV to the LPM.

This transcription factor was also shown to have an asymmetric expression in the chicken's Hensen's node and a symmetric expression in the somites, although its function in this embryo has not been accessed..

In this study we tried to assess the role of the Dmrt2 in the establishment of left-right asymmetry in the chick embryo. We tried to clone the full length of *dmrt2*, by standard PCR and by 5'-RACE, in order to do experiments of gain-of-function, but without success. After this we tried to understand if Sonic hedgehog (Shh) is regulating the asymmetric expression of *Dmrt2* in the chicken node, by inhibiting Hedgehog signalling, with different concentrations of cyclopamine, a drug that

inhibits Patched. *In situ* hybridizations were done in order to understand if *Dmrt2* expression was downregulated when Hedgehog signaling was inhibited. No conclusive results were obtained.

The lack of results and the technical hurdles will be discussed and future experiments and expected results will be presented.

Keywords: Left-Right axis; asymmetry; Dmrt2; chick.

Table of Contents:

1. Introduction

In order to form a well-developed body, different events need to happen. These events can be symmetric or asymmetric. An example of a symmetric event is the formation of the bilaterally symmetric structures known as somites. An asymmetric event is for instance the positioning of the organs inside the body cavities.

It is known that these events happen in the same time window and in embryonic territories closely located. A well-coordinated set of pathways is needed, otherwise an incorrect transfer of information would occur.

When we have an interruption in one of the components of these pathways we have severe developmental abnormalities such has, *situs inversus,* atrophic musculature, and skeletal malformations. Due to these problems, studies in this area of investigation are crucial. To have a better comprehension of the events behind this we first have to look to each one separately.

1.1. Symmetry and its pathways

1.1.1. Somitogenesis

An example of a symmetric event is somitogenesis. Somitogenesis is a fundamental process that initiates segmentation in developing embryos, through the formation of somites. These transient metameric and epithelial structures are located symmetrically on either side of the axial structures (i.e. the notochord and the neural tube) and will give rise to our axial skeleton, skeletal musculature, dermis and the tendons. Temporal and spatial regulation is key to proper somite development.

The vertebrate segmentation occurs progressively in the presomitic mesoderm

(PSM) in a rostral-to-caudal direction [1,2].

As somites bud off from the anterior end of the PSM, the posterior end of the PSM, is constantly replenished by cells entering from the tail bud [3].

The time required to form a new pair of somites as well as the total number of somites formed is constant and species-specific depending on the temperature. In the chick embryo, at 37ºC, a somite pair is formed every 90 minutes, in a total of 50 somite pairs formed. In the human, a new pair of somites is formed every 4–5 hours, in the mouse every 120 minutes and in the zebrafish every 30 minutes [4]. This process of differentiation of the PSM to somites is done in a time and space dependent manner, which can be explained by the clock and wavefront model proposed by Cooke and Zeeman, in 1976. This model postulates that a biochemical oscillator – clock – is operating synchronously is PSM cells, while a gradient of maturation – wavefront – sweeps the embryos along the rostral-to-caudal axis (A-P axis), determing the size of each pair of somites (Figure 1.1). This model has been proposed to be translating temporal information into positional information in PSM cells [5].

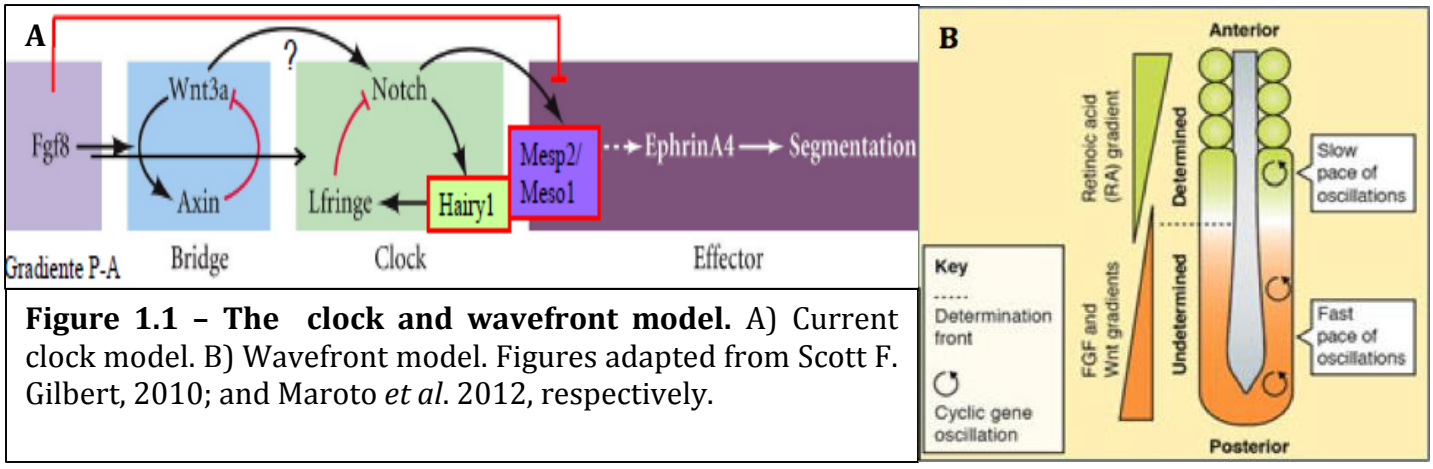


Figure 1.1 – The clock and wavefront model. A) Current clock model. B) Wavefront model. Figures adapted from Scott F. Gilbert, 2010; and Maroto *et al*. 2012, respectively.

1.1.1.1. The segmentation clock

The segmentation clock is responsible for the dynamic and periodic expression of mRNA of a number of "clock" genes across the PSM in a posterior to anterior fashion, with a periodicity that matches somite formation (Figure 1.1. A).

The molecular evidence for the existence of this segmentation clock came with the discovery of the first cyclic gene, the avian basic-helix-loop-helix (bHLH) transcription factor *hairy1.* The chick *hairy1* gene shows a dynamic and reiterated expression pattern in the PSM with the exact same periodicity of somite formation [6]. These *hairy1* messenger RNA (mRNA) oscillations occur autonomously in PSM cells and because they are synchronized with adjacent cells, describe a wave of expression starting at the posterior PSM and moving towards the anterior PSM, where it slows down and eventually stops, concomitant with somite formation (Figure 1.1. A). Therefore, PSM cells undergo several periodic oscillations of *hairy1* gene expression before they incorporate into the next somite [6].

Hairy1 is a bHLH transcription factor downstream of the Notch-Delta pathway. Other bHLH transcription factors with a cyclic behaviour were next found namely, Hairy2 in chick, Hairy and enhancer of split 1 (Hes1) and Hes7 in mouse, Hairy and enhancer of split-related 1 (Her1) and Her7 in zebrafish, just to name a few.

Indeed, the analysis of mouse and zebrafish mutants for several components of the Notch pathway revealed that cyclic gene expression and somite boundary formation were disrupted to varying degrees. Nevertheless, the anterior somites developed normally and only the posterior ones were affected in the Notch mutants [7][8][9]. These findings showed that Notch signalling is not entirely necessary for somite formation but instead suggested that its failure leads to a gradual perturbation in somite segmentation.

These oscillations in gene expression are generated by Notch activation that induces

both Lunatic fringe (Lfng) [10][11] and Hairy1 [6], in chicken, that exert inhibitory action upon their own promoters, thus leading to a negative feedback loop.

Lfng is activated, by Notch intracellular domain (NICD), acting post-translationally in the notch receptor, altering its sensibility to Delta, leading to a decrease in notch-delta signaling [5]. *Hairy1* expression, also activated by NICD, will then be responsible for the inhibition of Lfng and, when its protein expression levels get high enough, its own. When the concentration of Hairy 1 is low enough the notch-delta signaling becomes active again and a new cycle is started.

Recent studies showed that components of the Wnt pathway have a cyclic expression in the same phase with Notch pathway components, and have a role in somitogenesis [12] [13]. In fact, mutants for Wnt3a showed a downregulation of the expression of Lfng and Dll1 in the tailbud [13]. This function of Wnt signalling is regulated by a negative feedback loop of Axin2 in the PSM [12] [14]. This Axin2 is a critical component of the Wnt signaling pathway that acts as a scaffold for the β-catenin destruction complex.

Although Wnt signalling is implicated in the somitogenesis process, components of the FGF pathway, like *snail1* and *snail2,* have also been showed to have cyclic expression. The determining agent in linking FGF signaling to Notch-related cyclic gene expression and, ultimately, proper somite segmentation in the zebrafish embryo is Hairy/Enhancer-of-Split molecule, Her13.2. Expression of *her13.2* is induced by FGF-soaked beads and decreased by an FGF signalling inhibitor. FGF-induced Her13.2 acts as a dimerization partner for Her1, and the formation of this heterodimer is required for transcriptional feedback repression on Her1 promoter, ensuring its cyclic expression, and for somitic border formation throughout the entire A-P axis [15] [16]. Another study described cyclic expression of *snail1* and *snail2*, in mouse and chick embryos, respectively, which required FGF and Wnt

pathways and was Notch-independent. This link of SNAIL proteins and the FGF pathway is observed when FGF signalling is inhibited. *snail1* expression is lost in the PSM of mouse mutants for FGFR1 [17]. When the activity of FGFR1 is blocked, *snail2* was found to be downregulated in the PSM, whereas it was upregulated in the neural tube and the lateral plate. Overexpression of FGF8 by electroporation blocked somite formation, but no ectopic expression of *snail2* was observed in the paraxial mesoderm of FGF8-overexpressing embryos. These experiments suggest that FGF signalling is necessary, but not sufficient, for *snail2* expression in the chick PSM. Nevertheless, *snail2* misexpression ceased *lfng* oscillations and impaired epithelial somite formation [18].

In summary, FGF and Notch clusters of cycling genes are activated in phase, while genes belonging to the Wnt cluster present an opposite cycling phase to the Notch/FGF cluster.

1.1.1.2. The wavefront

The wavefront component of the model states that between the determined and undetermined region of a developing embryo is a determination front, corresponding to the intersection of FGF8 and retinoic acid (RA) gradients, the first going from caudal to rostral, and the second going rostral to caudal (Figure 1.1. B) [19]. Once the PSM cells cross this front, the somitogenic program is activated and deposition of extracellular matrix (ECM) starts the individualization of somites [20].

The posterior-to-anterior gradient of *fgf8* mRNA in the PSM was the first one to be described in several vertebrate embryos (mouse, zebrafish and chick) [21][22][23]. In fact, displacing the position of the determination front by altering the extent of the Fgf8 gradient results in the shift of the somite boundary position [21]. This is because Fgf8 is responsible for maintaining the PSM cells in an undifferentiated

state.

What we see is a more intense RNA gradient, in the posterior part of the embryo, fading out as we reach the anterior part of the PSM. This more intense RNA gradient is because *fgf8* mRNA is transcribed in PSM progenitors but not in the PSM itself. This fading out of the gradient is established by the cells movements, which are occurring when cells leave the tailbud, and also due to the long half-life of *fgf8*. As the mRNA gets more mature the expression of FGF8 becomes more fade-out, because of the degradation of the more mature mRNA. This diminishing of protein expression leads to a more permissive environment for cells to differentiate. The precise regulation between undifferentiated and differentiated fronts is done by the counteracting gradient of RA. Once the cells contact with this determination front of low FGF8 and increasingly RA concentration they start the differentiation process, which culminates in a mesenchymal-to-epithelial transition (MET).

1.1.2. Formation of the axial skeleton and it's associated muscles

After the individualization of the somite, a maturation process begins. Two major compartments will form within the somite: the sclerotome and the dermamyotome (this will then divide to from the myotome and the dermatome) (Figure 1.3.) [22].

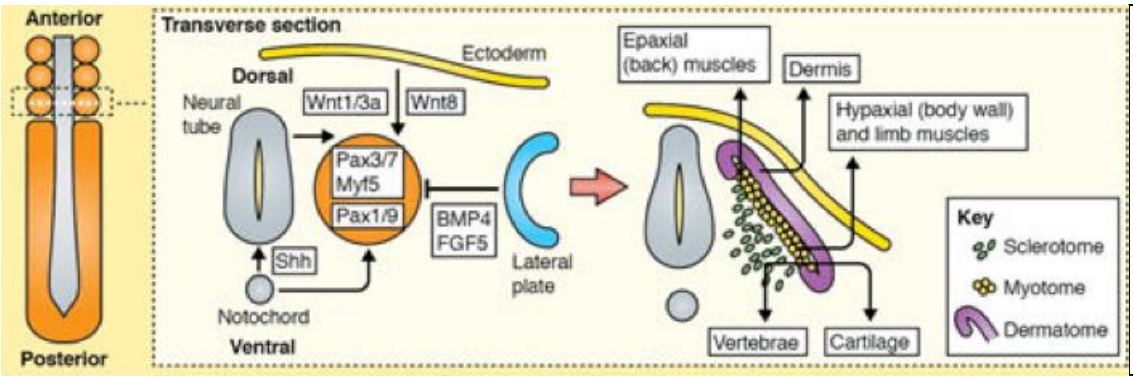


Figure 1.3 – Somite differentiation. Image adapted from Maroto *et al.* 2012

The sclerotome is formed when cells from the ventral part of the somite de-

epithelized and turn into mesenchymal cells. The ventromedial portion of the somite is induced to become the sclerotome by paracrine factors, especially Noggin and Sonic hedgehog, which are secreted from the notochord [23][24]. This paracrine factors will induce the expression of Pax1, which is required for their epithelial-to-mesenchymal transition and subsequent differentiation into cartilage [25]. The sclerotome will then give rise to the vertebrae and rib cartilage, and the syndetome (tendon precursor) in a process called somite resegmentation. In this resegmentation we will have the division of the sclerotome in a rostral and caudal segment (Figure 1.4.). Each caudal segment combines with the next anterior sclerotome, forming the vertebral rudiment. Meanwhile the motor neurons, that will innervate the muscles, migrate trough the anterior part of the sclerotome dividing it in half, helping in this way the resegmentation process.

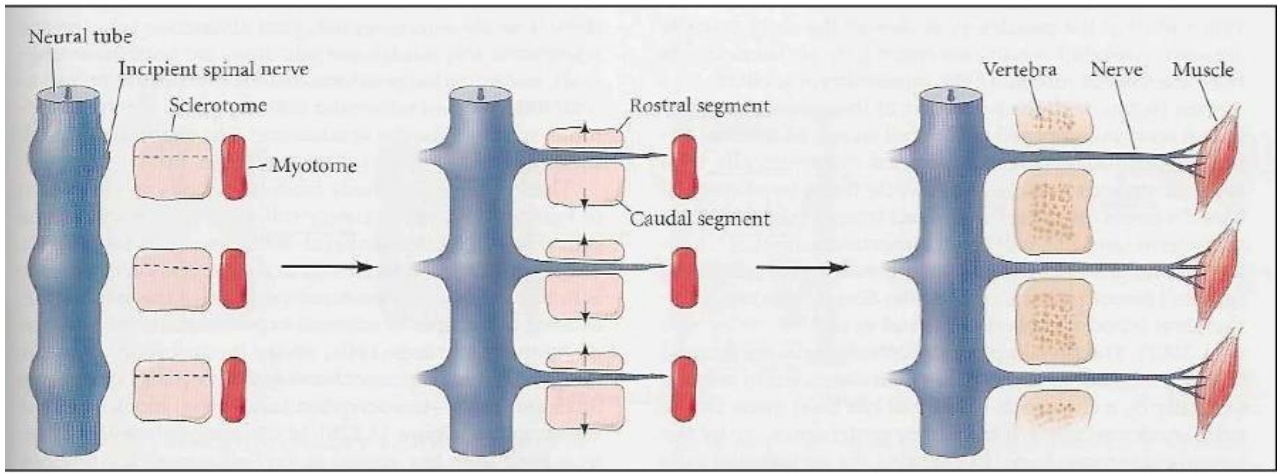


Figure 1.4 - Resegmentation of the sclerotome. Each caudal segment combines with the next anterior sclerotome, forming the vertebral rudiment. Meanwhile the motor neurons, that will enervate the muscles, migrate trough the anterior part of the sclerotome dividing it in half. Image adapted from Scott F. Gilbret, 2010.

The dorsal cells of the somite maintain the epithelial identity forming the dermamyotome (precursor of skeletal musculature and dermis of the dorsal skin). This dermamyotome will then be divided in myotome and dermatome.

Myotome formation involves two sequential steps: first, cells from the dorsomedial edge of the dermomyotome move underneath and form the epaxial myotome (musculature of the back and intercostals); in a second phase the central dermomyotome cells de-epithelialize and form the hypaxial musculature and the dorsal dermis of the trunk. This differentiation is due to Wnt signalling, namely Wnt1/3a from the neural tube, Wnt7a and Wnt8c from the ectoderm, that participate in the induction of the myogenic program in the epaxial and hypaxial lips respectively [26][27][1]. This myogenic program starts with the expression of the myogenic factors Myf5 (myogenic factor 5) and MyoD (myogenic differentiation 1), in Pax3/7 positive cells, eventually generating the epaxial and hypaxial muscles and also part of the dermis of the back.

The dermatome, the precursor of dermis tissue, is formed through signals via Neurotrophin 3 (NTF3) from the neural tube [27].

1.2. Asymmetry and its pathways

Although symmetric developmental processes are crucial, asymmetric developmental events are also needed in order to have a fully developed body.

One of the main mechanisms for the establishment of Left-Right asymmetry is the Nodal cascade, which takes place during somitogenesis, and is present in all vertebrate model systems and some invertebrates. Nodal is expressed on the left side of the node and the lateral plate mesoderm (LPM) [28][29], and it was shown that inhibition of Nodal will lead to right isomerism (formation of two right sides)

[30], making it a left-side determinant.

Other genes in this cascade are the *Lefty* gene family, composed by *lefty1* and *lefty2* [31], which are antagonists of Nodal signalling, thus creating a regulatory network between *Nodal* and the *Lefty* genes. So, when Nodal protein reaches a cell in the left side, it will activate *Nodal* in the left LPM (Lateral Plate Mesoderm), that when Nodal protein reaches a certain concentration, activates *Lefty1* and *2*. Lefty1 will be expressed in the midline inhibiting the expression of Nodal in the right side. Lefty2 will restrict the domain of Nodal on the left LPM [32][33].

This expression of *nodal* in the left LPM will lead also to the expression of the homeobox transcription factor *pitx2.* Pitx2 will promote the asymmetric establishment of the internal organs [33]. The mechanism by which Pitx2 contributes to this left-right asymmetry is not yet fully understood.

This conserved cascade can be activated differentially according to the organism. In the mouse and chick we have the activation of Nodal cascade, in the node, by Notch-Delta signalling [34][35]. Although Notch-Delta activates the Nodal cascade in the mouse and chick, in zebrafish we have the activation of Charon, an inhibitor of Nodal, wich is expressed in Kupffer's vesicle, the node homologous LR organ [36].

Another molecule that is responsible for the asymmetric expression of *nodal* is Fgf8. The gene *fgf8* is expressed in the chick, mouse and zebrafish, but has a different role in one of each organism. In mouse *fgf8* acts as a positive regulator of *nodal*, therefore acting as a left determinant [37]. In the chick *fgf8* acts has a repressor of *nodal*, having its expression in the right side of the Hensen's node, thus acting like a right determinant [38]. In zebrafish, the function of *fgf8* has not been determinated, although *fgf8* mutants have LR abnormalities [39].

One molecule that is also responsible for the expression of *nodal* in the left side of the perinodal region, is Shh (Sonic hedgehog) that has its expression restricted to

the left side around stage HH4+ in chick [28][29]. Studies showed that the asymmetrical *shh* expression at stage HH5 is considered to be itself upstream of asymmetric *nodal* expression, because right-sided implantation of Shh producing cells induces ectopic *nodal* expression in right LPM [28], whereas Shh antibody administration at stage HH5 effectively suppresses initiation of *nodal* expression in the left LPM [40].

One question that remained unanswered for a long time was how is this differential gene expression established. The ways by which these asymmetric processes are established are diverse, and can be through three distinguish mechanisms that act in different developmental times (Figure 1.8)[33].

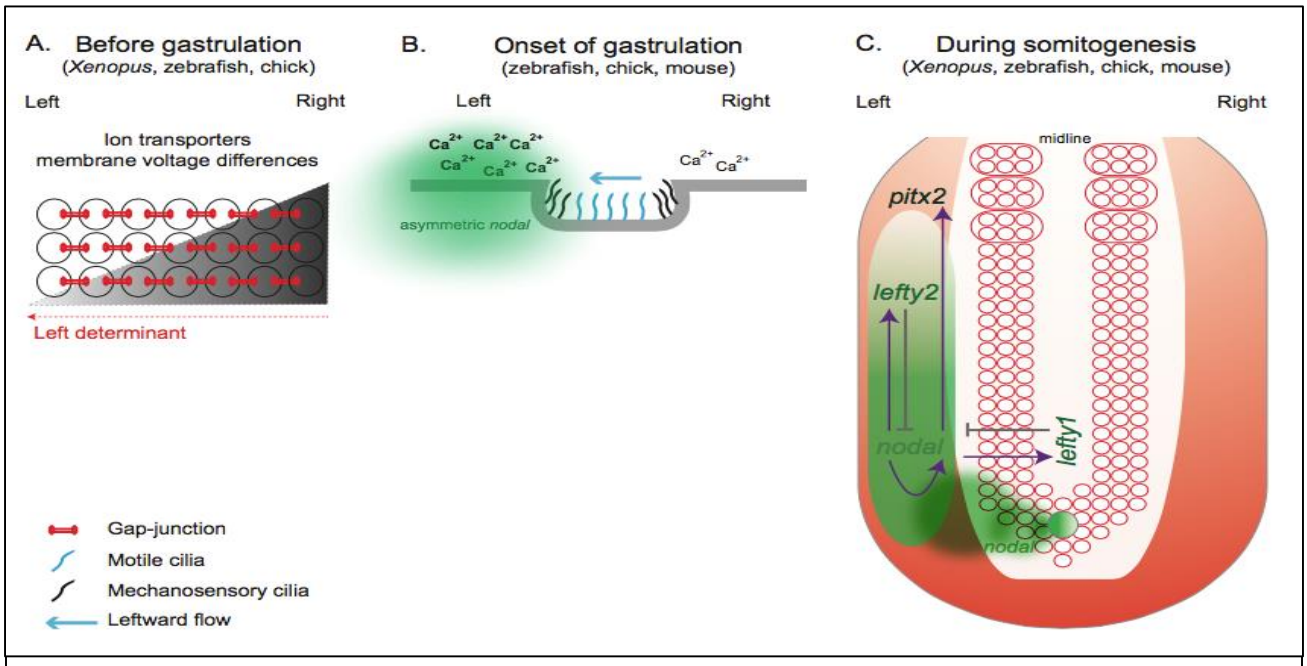


Figure 1.8 - Set of events that might culminate with the establishment of the left-right patterning in different vertebrates. A) Prior to gastrulation in *Xenopus*, zebrafish and chick, ion transporters asymmetrically distributed in the embryo generate differences in membrane voltage potential between the left and right side. B) In mouse, it is though that mechanosensory cilia present in the node epithelia sense the leftward fluid flow created by motile cilia and as a consequence trigger an asymmetric Ca^{2+} release which will induce an asymmetric *nodal* expression around the node. C) A conserved Nodal cascade is activated at the onset of gastrulation in *Xenopus*, zebrafish, chick and mouse. Nodal is asymmetrically transferred from the node to the left LPM. Figure adopted from Lourenço and Saude, 2010.

One of these mechanisms that are observed in chick, zebrafish and *Xenopus*, prior to gastrulation, is the establishment of a differential membrane potential due to an asymmetric distribution of ions done by channels present in the cell membrane. As a consequence, we will have an asymmetric distribution of extracellular Ca^{2+}, via gap-junction communication channels (GJC). If we have GJC inhibition, LR patterning problems will appear [41][42]. Also Ca^{2+} accumulation was shown to induce an asymmetric activation of Notch on the left side of the node that then translates this differential activity into asymmetric *nodal* expression. Perturbing this early asymmetric ion flux, will lead to randomized gene expression and organ heterotaxia [43].

Another mechanism, that happens during gastrulation, is the presence of motile and mechanosensory cilia [33][34]. One theory states that a morphogen is transported by the leftward flow created by the motile cilia, present in the node cells, thus breaking the symmetry. This leftward flow is possible because the cilia are posteriorly tilted [32]. The mechanism by which mechanosensory cilia break symmetry is due to an asymmetric intracellular Ca^{2+} flux, which will induce asymmetric nodal expression [45]. This asymmetric Ca^{2+} is generated by polycystin-2 (PKD) calcium activated channel [46]. This cilia mechanism of breaking symmetry can be found on the mouse node, zebrafish Kupffer's vesicle, and *Xenopus* gastrocoel roof plate [33].

There are no motile cilia in chicken embryos so the way of breaking symmetry in this organism is by leftward cell movements, downstream of the H^+/K^+-ATPase pump activity. This alternative strategy was seen in chick embryos at stage HH4 [47]. These cell movements stopped at stage HH5 by a N-cadherin dependent process, that is localized in the right side of the node, leading to asymmetric expression of *nodal* and *fgf8* [48].

1.3. Protection of symmetric events

As we saw, in order to have a well developed body a coordinated set of events, either symmetric or asymmetric, need to occur. Since these contradictory processes occur in the same timeline and in close territories the problem of how these events are protected from each other arises. Studies in the area showed that a set of conserved mediators are responsible for this protective barrier. Some of these mediators are RA, Snail and Dmrt2 (former Terra) (Figure 1.9).

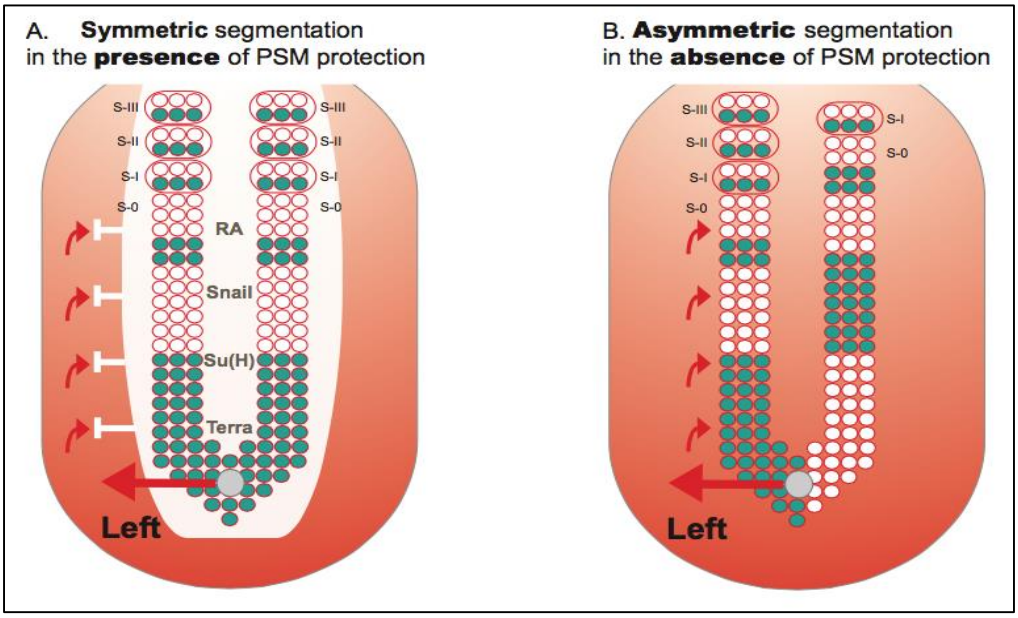


Figure 1.9 - Protection of PSM segmentation from LR asymmetric patterning cues. A) PSM is protected from LR signals that come from the node and are implicated in left-right patterning (red arrows). This protection consists of a "shield" (white) which so far has been shown to be composed by RA, Snail, Su(H) and Terra. In its presence, cyclic gene expression (blue) and somite formation are symmetric between the left and right sides. B) In the absence of this protection, cyclic gene expression becomes desynchronised between both sides. Consequently, somite formation proceeds in an asymmetric way, with the left side exhibiting more somites than the right (this biased asymmetry towards the right side is seen in mouse and fish embryos, while in chick asymmetries are biased to the left side). Figure adopted from Lourenço and Saude, 2010.

1.3.1. Retinoic acid

Retinoic acid (RA) is a morphogen derived from retinol (vitamin A) that plays important roles in cell growth, differentiation, and organogenesis [49]. It has been shown that RA acts has a buffer, during somitogenesis, preventing LR signals from disrupting symmetric signals that will form the somites [50]. When we have a blocking in the production of RA, in chicken, mouse and zebrafish embryos a desynchronization of somite formation between the two embryonic sides is observed [51][52]. This desynchronization is accompanied, in chicken embryos, by an expression domain of Lfng extend more anteriorly on the left than on the right embryonic side, in disulphiram-treated embryos (disulphiram inhibits *raldh2,* the enzyme responsible for converting retinol into RA). No asymmetric expression was seen for *fgf8* in the chicken embryos [52]. In mouse expression of the cyclic genes *hes7* and *lfng* are out of phase between the left and right sides. The same erratic expression pattern is observed in *deltaC*, *her1* and *her7* in zebrafish. Also consistent with the somite phenotype, is the anterior displacement of the wavefront seen by the anterior expansion of *fgf8* on the right side of the PSM. These observations showed that the primary target of the uncoordination process is the oscillations of the clock [52][53].

One question that arises is: what are the mechanisms by which RA is protecting the symmetric signals from the asymmetric ones? One of the molecules of this RA pathway is *Snail,* namely *snail1* that is transiently expressed in the right LPM, in both chick and mouse embryos, having a role in organ lateralization. It was seen that this transient expression of *snail1* corresponds to the period of RA expression in the PSM. If we have absence of RA signalling, during that period, *snail1* expression is affected leading to its expression on the right anterior PSM. This erratic expression of *snail1* leads to asymmetric expression of the cyclic genes *snail2* e *lfng* leading to

asynchronous somitogenesis [33].

1.3.2. Dmrt2

Recently the role of a new mediator in the crosstalk between symmetry and asymmetry was discovered. This mediator is *Dmrt2*, a transcription factor of the DM domain family, that is a family previously involved in sex determination [54]. Despite being mainly expressed in developing gonads and associated with sex differentiation, not all the vertebrate *dmrt* genes are associated exclusively with this function. So far, from the eight known *dmrt* genes, five of them have already been implicated in other developmental processes other than sex differentiation. *dmrt* genes have been detected in the central nervous system, nasal placodes and in the somites [55].

Dmrt2 in particular has a role in somitogenesis, more specifically in somite differentiation in mice and zebrafish, having its expression in the dermomyotome of developing vertebrate somites [56][57][58]. Mouse *dmrt2*$^{-/-}$ mutants die due to abnormal rib and sternal development, having also defects in the expression pattern of dermomyotomal and myotomal transcription factors [57].

In zebrafish, we have *Dmrt2a* and *Dmrt2b,* due to the genome duplication event, with different functions. Zebrafish injected with *Dmrt2a*-morpholino, displayed a randomization of clock-specific genes, such has *deltaC*, *her1*, *her7* and left dterminants like *pitx2* and *spaw*; a randomization of the heart position was also seen [59]. The *dmrt2b* gene is functionally divergent from *dmrt2a* regarding its role during somite formation. Instead of being necessary for symmetric somite formation, it is in fact involved in the regulation of somite differentiation at the level of slow muscle development through the regulation of the Hedgehog pathway [60]. *Dmrt2a* and *dmrt2b* share the same expression pattern in the anterior region of the PSM and somites expression pattern. Although these genes have similar expression

patterns, it was showed that *dmrt2a* is also expressed in the KV, in the 3-somite stage until the 10-somite stage, from where *dmrt2b* is in fact absent [33]. This observations lead to the suspicion that Dmrt2a is acting at the level of the KV to regulate both pathways, by doing the transition of information from the KV to the LPM, and that its expression in the anterior PSM region and somites is probably involved in somites differentiation. If this is true, it may help to understand why *dmrt2b* is not necessary for symmetry somite formation and only somite differentiation. A plausible explanation for the fact that *dmrt2b* is involved in the regulation of the LR asymmetry pathway, despite being absent from the KV, is that *dmrt2b* is regulating the Hedgehog signalling at the level of the midline, whose integrity is necessary for the correct establishment of the LR asymmetry pathway.

The role of the *dmrt2* in the chick embryo is still unknown; a role in left-right patterning, similar to *dmrt2a* in zebrafish, is a plausible hypothesis, due to the asymmetric expression in the left side of Hensen's node in HH4^{+} stage embryos [59], that is similar to the expression pattern of other genes that are known to have a role in LR patterning, like *shh* and *nodal* [28]. *Dmrt2* is also expressed in the somites [59]. Although the expression of *Dmrt2* in chick is known, its function is not. Revealing this function can lead to major breakthroughs in this area, thus giving a more complete vision of the processes behind LR patterning, and consequently to the problems that are behind LR defects, such as *situs inversus*.

1.4. Aim of this project

The aim of this project was to evaluate the function of Dmrt2 in LR patterning in the chicken embryo and uncover potential upstream regulators. In order to do so our initial idea was to do gain-of-function experiments and perform drug treatment experiments respectevly.

2. Materials and Methods

2.1. Eggs and embryos

Fertilized chicken (*Gallus gallus*) eggs were obtained from commercial sources (Sociedade Agrícola Quinta da Freiria, Portugal) and incubated at 37°C in a 17% humidified incubator. Embryos were staged according to the Hamburger and Hamilton development table (Figure 2.1.) [61].

2.2. Total RNA extraction from chicken embryos

Total RNA was isolated from entire embryos at stage HH10 and from the Hensen's node of stage HH4 embryos. To disrupt the cells and dissolve cellular components, maintaining the integrity of the RNA, 1 ml of Trizol reagent (Gibco-BRL) was added to the tubes per each 50-100 mg of tissue, which was thoroughly ressuspended. Next, the mixture was incubated for 5 minutes at 30°C, to allow the complete dissociation of protein complexes. This was followed by a dilution with 0,2 ml of Chloroform per each 1 ml of Trizol reagent initially used, a shaking for 30 seconds and an incubation for 3 minutes at 30°C. Then, the mixture was separated into three phases through a centrifugation at 4°C for 30 minutes at 6.000 g. This centrifugation places the red phenol-chloroform phase lower, followed by an interphase and finally a colourless upper aqueous phase where the RNA remains. The aqueous phase was transferred to another eppendorf tube. To precipitate the RNA, 0,5 ml of Isopropyl Alcohol were added per each 1 ml of Trizol reagent initially used, followed by an incubation of 10 minutes at 30°C. The RNA was then centrifuged at 4°C during 15 minutes at 10.000 g. The supernatant was discarded, the pellet washed once with 1 ml of 75% EtOH, per each 1 ml of Trizol, and centrifuged at 4°C during 15 minutes at 10.000 g. Finally, the RNA pellet was allowed to air dry, dissolved in 30 μl of RNase

free water and stored at -80°C.

2.3. Reverse transcription to obtain cDNA

The reverse transcription is a method that allows obtaining complementary DNA (cDNA), from a RNA sample. This reaction is done by an enzyme, which is found in viruses, called reverse transcriptase. This technique functions as a standard PCR, with the difference that the template is RNA, not DNA. With this technique we are cloning expressed genes by reverse transcribing the RNA of interest, not simply generating copies of a gene. For the general process see Figure 2.2.

For the reverse transcription we used the Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit #K1631, #K1632 (Thermo Scientific). For this reaction we used a random hexamer primer provided by the kit. The reason why we used random primers is because they initiate cDNA synthesis from the total RNA population (rRNA and mRNA), instead of the oligo(dT) that give us exclusively the mRNA population. Therefore, using random primers for first strand synthesis results in a greater complexity of the generated cDNA (since we started with a bigger pool of RNAs) compared with the oligo(dT) (exclusively the mRNA population).

2.4. Primer design

Sets of gene specific primers for *dmrt2* were design. For the sake of simplicity we have grouped the primers into 3 types. Type 1 (Table 2.1.) and Type 2 (Table 2.2.) primers were design using Primer BLAST from NCBI. Type 3 Primers (Table 2.3.) were designed using Primer3. The type 1 primers have restriction sites added to its 5' and 3' ends, for vector cloning. The type 2 primers differ from the type 1 simply because they are missing the restrictions sites. In the type 3 primers we designed

only reverse primers using specific characteristics such as, length (23-28 nt), melting temperature (T_m≥65°C), GC% (50-70%) and not complementary to the 3'-end of the Universal Primer Mix (For the specific numbers consult table 2.3.).

2.5. Cloning and sequencing

2.5.1. Standard PCR using chicken cDNA

For the standard PCR using type 1 primers, four master mixes of solutions were prepared. Mix 1 was done with forward and reverse primers with a Xho1 restriction site; Mix 2 was done with forward primer and reverse primers with a EcoR1 restriction site; Mix 3 was done with a forward primer with a Xho1 restriction site and a reverse primer with a EcoR1 restriction site; Mix4 was done with a forward primer with a EcoR1 restriction site and a reverse primer with a Xho1 restriction site. The PCR program was the one described in Table 5. The polymerase used for this PCR was Phusion High-Fidelity DNA polymerase (#F-530L, Thermo Scientific).

2.5.2. Optimization PCR using chicken cDNA

An optimization PCR protocol was done using the different combinations of Type 2 Primers (Table 2.2.), and different annealing temperatures (obtained by subtracting 5^0C of the Tm of primer), in a one-degree interval (one bellow and one higher of the calculated temperature). The polymerase used in these reactions was Taq DNA Polymerase (recombinant) (#EP0402, Thermo Scientific). The optimization protocol is described in Figure 2.3.

2.5.3. 5’-RACE using chicken cDNA

5’-RACE is a method for performing 5’-rapid amplification of cDNA ends (RACE). The way that this is done is by producing a cDNA sample that has an additional SMARTer sequence (3–5 modified bases that anneal to the extended cDNA tail). After this a two rounds PCR is done with different primers in order to have a Double-stranded 5'-RACE fragment (for the mechanism in detail consult Figure 2.4.) The 5’-RACE was done using the SMARTer RACE cDNA Amplification Kit (PT4096-1, Clontech), starting with 1 µg of chicken total RNA (for the RNA sampling see section 2.2.). For our gene-specific primers (GSP) we used the type 3 primers (Table 2.3.).

2.5.4. Gel electrophoresis and band extraction

2.5.4.1. Gel electrophoresis

The success of the amplification was determined by an agarose gel electrophoresis. The gel was prepared by dissolving agarose (Gibco-BRL) in 1x TAE buffer (40 mM Tris-acetate; 2,0 mM EDTA pH 8,5), to a final concentration of 2% (for Standard and Optimized PCR), and 1,2% (for 5’-RACE). To dissolve the agarose, the mixture was heated until a transparent solution was obtained and, when cooled, Red Safe 20 000X (Intron Biotechnology). This is a substance that intercalates with DNA bases, becoming fluorescent when exposed to ultraviolet (UV) light, allowing visualization of DNA molecules. The agarose mixture was then transferred to a gel mould with the appropriate comb in place. The gel was allowed to polymerize and was then covered with 1x TAE buffer. In eppendorf tubes, the following mixtures were made: 50 µl of PCR/5’-RACE product + 5 µl of 6x Loading Buffer (6 mM EDTA; 0,5% Bromophenol Blue; 40% Sucrose); 5 µl of Smart Ladder (5 µl/lane) (Eurogentec molecular weight

marker that allows the determination of the DNA fragment size). The samples were briefly centrifuged and loaded in the gel wells (25 µl of product/well). An electric field of 100V was applied until the appropriate resolution was achieved. Since the nucleic acids are negatively charged, the DNA runs from the negative pole (black) towards the positively charged pole (red) of the electrophoresis apparatus. In the end, the gel was placed on a UV light box and a photograph was taken using the ImageLab program.

2.5.4.2. Band extraction

After photographed, the gel was placed under UV light and a band with approximately 1,4 Kb size (obtained from Standard and Optimized PCRs) and a band with 200/450 bp size (obtained from 5'-RACE), were excised with a clean sharp scalpel and placed in a eppendorf tube. The gel slice containing the DNA fragment was weighted and the DNA extraction from the agarose gel was carried out using the QIAQuick gel extraction kit. 3 volumes of Buffer QG (solubilization and binding buffer) were added for each volume of gel (100 mg ~ 100 µl), being the maximum amount of gel slice per QIAQuick column 400 mg (for gel slices > 400 mg more than one QIAQuick column was used). The tube was then incubated at 50°C for 10 minutes, until the gel slice was completely dissolved (to help dissolving the gel, the tube was vortexed every 2 minutes during the incubation). After that, one gel volume of Isopropanol was added to the tube. To collect the DNA, the content of the tube was transferred into a QIAQuick spin column (which was inserted in a 2 ml collection tube) and centrifuged for 1 minute at RT (the maximum volume of the column was 800 µl). The flow through was discarded, the column placed back in the same collection tube and 0,5 ml of Buffer QG were added, followed by a centrifugation of 1 minute at RT. To wash, 0,75 ml of Buffer PE were added to the

column, which was left stand 2-5 minutes before centrifugation of 1 minute at RT. The flow through was once again discarded and the column centrifuged for an additional minute at RT. The column was then placed into a sterile 1,5 ml eppendorf tube and the DNA eluted with 30μl of RNase free water, being centrifuged for 1 minute at maximum speed and RT. The DNA was then stored at -20°C. Quantification of purified DNA was done using a NanoDrop 2000**,** a full-spectrum (220-275 nm) spectrophotometer that measures 1 μl of samples with high accuracy.

2.5.5. Cloning

As our cloning vector, we used pGEM-T and pGEM-T Easy Vector Systems (Promega). The reason for using pGEM-T, was because this vector can be sequenced using universal primers making the process of sequencing much less laborious. Since the pGEM-T vector has a polyT tail, the transformation is also easier, since we only need to add a polyA to our sequence in order to successfully clone our vector.

The best-fit ratio for the pGEM-T transformation was calculated by using the ligation calculator of NEBioCalculator. The produced plasmids were introduced into Subcloning Efficiency™ DH5α™ Competent Cells (18265-017, Thermo Fisher Scientific) and the transformed bacteria were cultured in LB medium with 100μg/ml of Ampicillin (A9518-SG, Sigma). A background control, with the plasmid alone, was done, in order to evaluate the success of the tranformation. In order to identify the bacteria colonies that are effenciatly transformed we performed a blue-white selection test using XGal-4 99,5% 1g (Bio-37035, BIOLINE), at 20mg/ml in DMF.

2.5.6. Plasmid DNA isolation and purification

2.5.6.1. Lysis of bacterial cells

Purification of plasmid DNA was done using the Promega's Wizard® *Plus* SV Minipreps DNA Purification System. A 4 ml bacterial culture was pelleted by centrifugation at room temperature (RT) for 5 minutes, at 16.000 g (centrifugal force). The supernatant was discarded and the tube was inverted on a paper towel in order to remove the excess media. Next, the bacterial pellet was thoroughly resuspended in 250 µl of Cell Resuspension Solution (50 mM Tris-HCl pH 7,5; 10 mM EDTA; 100 µl/ml RNase A), followed by the addition of 250 µl of cell lysis solution (0,2 M NaOH; 1% SDS). The tube was inverted 4 times and incubated at RT for 5 minutes, until the cell suspension cleared. Then, in order to inactivate endonucleases and other proteins released during the lysis of bacterial cells (that can affect the quality of the isolated DNA), 10 µl of Alkaline Protease Solution (approximately 250 µg per sample) were added. The tube was inverted 4 times and incubated at RT for 5 minutes. Finally, 350 µl of Neutralization Solution (4,09 M guanidine hydrochloride; 0,759 M potassium acetate; 2,12 M glacial acetic acid) were added and the tube inverted 4 times, followed by a centrifugation of the bacterial lysate at RT for 10 minutes, at 16.000 g.

2.5.6.2. Purification of Plasmid DNA

The supernatant obtained in 2.5.6. was transferred to a spin column (inserted in a 2 ml collection tube), avoiding the transfer of any of the white precipitate, and centrifuged at RT for 1 minute at 16.000 g. The spin column was then removed from

the collection tube and the flow through was discarded. This was followed by the reinsertion of the spin column into the collection tube and the addition of 750 µl of column Wash Solution (162,8 mM potassium acetate; 22,6 mM Tris-HCl pH 7,5; 0,109 mM EDTA pH 8,0). The centrifugation occurred at RT for 1 minute, at 16.000 g. The spin column was once more removed from the collection tube and the flow through discarded. The column was reinserted into the collection tube, and the wash procedure repeated using 250 µl of column Wash Solution. This was centrifuged at RT for 2 minutes, at 16.000 g. The spin column was transferred to a new sterile 1,5 ml microcentrifuge tube and the plasmid DNA eluted in 30 µl of nuclease-free water, followed by a centrifugation at RT for 1 minute at 16.000 g. After eluting the DNA, the spin column was discarded. Before storing the DNA at -20°C (where it remains stable), 1 µl of the sample was collected in order to quantify the DNA. The concentration of the purified DNA was determined spectrophotometricaly using the Nanodrop 2000.

2.5.6.3. STETS (Dirty-Preps) protocol (only used for 5'-RACE plasmids)

After growing our colonies, 12 to 24 were inoculated into a 15 ml falcon with 5 ml of LB medium with Ampicillin (A9518-SG, Sigma). Colonies were lefted to grow O/N with agitation at 37°C. Next day the falcons were centrifuged for 30 seconds to 1 minute at 13000 rpm. After centrifuged the flowthrough was discarded. Next the pellet was ressuspended in 750 µl of STET (5% Triton, 5% Sucralose, 50 mM EDTA, 50 mM TrisHCl pH 8), plus Lysozime. The solution was vortexed and boild for 2 minutes at 100°C in a dry bath. Then we centrifuged at 13000 rpm for 10 minutes, and removed de pellet with a sterile toothpick. After removing the pellet, 750 µl of Isopropanol was added to the flowthrough followed by brief vortexing. Next the

solution was centrifuge for 10 minutes at 13000 rpm and washed with 500 µl of ethanol at 70%, and the falcons were centrifuged for an additional 2 minutes. After centrifuged the flowthrough was discarted and the pellet was left to dry. After all the ethanol evaporated the pellet was ressuspended in 30 µl of Diethylpyrocarbonate (DEPC, alkylating agent that destroys the enzymatic activity of RNase) treated water.

2.5.7. DNA sequencing

The DNA minipreps we sent to StabVida Company for sequencing. The sequences were analysed using Nucleotide BLAST from NCBI to confirm the degree of similiatity with the gene *dmrt2*.

2.6. Whole Mount *In situ* Hybridization

In situ hybridization is a technique that allows the identification and localization of a particular nucleic acid sequence in preserved embryonic tissues or sections. It consists in the annealing of a labelled probe to complementary sequences (in this case, the annealing between an antisense mRNA probe and its complementary sense mRNA) in fixed tissues, followed by its detection with a specific antibody conjugated with an enzyme that catalysis a colorimetric reaction.

2.6.1. Plasmid DNA linearization

The different plasmids, pGEM-T; TERRA 1, 2, 3 and 4 (plasmids with the chicken EST 465h15, already available in our lab as a glicerol stock at -80^{0}C) used in this work were linearized with the appropriate restriction enzyme (see Table 2.4.). We have chosen a restriction site located beyond the 3' prime end of the sense strand to

allow the RNA polymerase to transcribe the entire gene, without transcribing plasmid sequences. The plasmid linearization was done in a 20 µl reaction mix containing the following components: 5 µg of plasmid DNA; 1x Restriction Enzyme Buffer, specific for each enzyme; 10 units of the appropriate Restriction Enzyme (see Table 2.4.). The tube was briefly centrifuged, followed by an incubation at 37°C for 2,5 hours. After that time, it was briefly centrifuged and kept on ice.

2.6.2. Anti-sense RNA probe preparation for *in situ* hybridization

2.6.2.1. RNA transcription

Single-stranded mRNA probes were *in vitro* synthesised in RNase free conditions. The 20 µl reaction mixture contained: 1µg of linearized DNA template; 1x Transcription Buffer, specific for each RNA polymerase; 1x Digoxigenin (DIG)-RNA Labelling Mix; 40 units of Ribonucleases Inhibitor; 20 units of the appropriate RNA Polymerase (see Table 2.4.), which synthesizes RNA complementary to a DNA template. The mixture was then vortexed and incubated at 37°C for 4 hours. In the commercially available DIG-RNA labelling mix approximately 1/3 of the uridine oligonucleotides are labelled with DIG. DIG is a steroid only found in *Digitalis* plants, to which a specific antibody has been raised. In this way, it is ensured that the anti-DIG antibody does not bind to other biological material. Using this specific antibody is possible to detect the incorporated DIG labelled nucleotides and precisely detect the RNA probe. After the 4 hours incubation, 2 units of DNAse (RNAse free) were added to the tube, which was incubated at 37°C for 30 minutes. The tube was then briefly centrifuged and kept on ice, followed by visualization of the RNA by an agarose gel electrophoresis (see section 2.5.4.1. Material and Methods).

2.6.2.2. RNA precipitation

To precipitate the RNA, after transcription, the following solutions were added to the tube: 2,5 µl of 4 M LiCl; 1µl of 0,5 M EDTA; 75 µl of cold 100% EtOH. The RNA was left to precipitate at –80°C for 40 minutes or at –20°C O/N. Finally, to obtain the RNA probe, the tube was centrifuged at 4°C during 30 minutes, at 16.000 g. The supernatant was removed, the pellet washed with 500 µl of cold 70% EtOH and centrifuged at 4°C during 10 minutes, at 16.000 g. The supernatant was discarded, the pellet allowed to air dry and ressuspended in 20 µl of DEPC treated water. An agarose gel electrophoresis was used (see section 2.5.4.1. Material and Methods) to check the integrity of the RNA probe, which was then stored at -20°C.

2.6.2.3. RNA probe hidrolysis

After RNA precipitation, 5 µl of sodium carbonate 0,6 M and 5 µl of sodium bicarbonate 0,4 M were added. The solution was heated at 60^0C during 34 minutes. After this step, 200 µl of DEPC H_2O, 25 µl sodium acetate 3 M and 600 µl of ethanol 100% at -20^0C were added to the solution. The solution was placed at -20^0C O/N to percipitate the RNA. The next day the solution was centrifuged at 4^0C during 30 minutes, at 12.000 g. The supernatant was removed, the pellet washed with 1 ml of cold 70% EtOH and centrifuged at 4^0C during 15 minutes, at 12.000 g. The supernatant was discarded, the pellet allowed to air dry and ressuspended in 30 µl of DEPC treated water.

2.6.3. Whole-mount *in situ* hybridization protocol for chicken embryos

The embryos were fixed O/N in PFA 4% fixative. After O/N fixation, embryos were dehydrated in methanol (50% first, then 2x times 100% washes, 5 minutes each) and left at -20°C, at least O/N (and no more than 3 months).

On day 1 of the *in situ* hybridization protocol embryos were rehydrated in a 75%-50%-25% methanol series. Embryos were then permeabilized with Proteinase K (Roche #3115879001) at 10µg/mL. Exposure times to Proteinase K depended on embryo stage (one minute per stage, i.e HH1=1 minute, HH10=10 minutes and so on). After that the embryos were post-fixed in 4% formaldehyde and 0.1% glutaraldehyde (Sigma #G5882) in PBT, to inactivate the Proteinase K and prevent deterioration of the tissues. Finally, embryos were passed through Hybmix by rinsing in a 1:1 PBT:Hybmix solution, and then left to incubate in Hybmix at 65°C/70°C for at least an hour. Instead of the incubation time, some embryos were put in Hybmix and stored at -20°C for future hybridization. After the 1h incubation they were submerged in 500µL of probe (diluted in Hybmix in a concentration of 1 µg/mL). The RNA probes used were as followed: *nodal* (positive control) and *dmrt2*.

On day 2, the probe was recollected from the embryos and stored at -20°C to be reused. After that embryos were washed, for 30 minutes, twice in Hybridization Mix at 65°C/70°C, and then transitioned to TBST 1X by washing in a 1:1 TBST: Hybmix solution for 10 minutes at 65°C/70°C, and then twice in TBST 1X. The embryos were then washed for one hour in in 2% Blocking, 20% Sheep Serum solution in TBST 1X, at room temperature (RT) and with agitation, in 2% Blocking, 20% Sheep Serum solution in TBST 1X. Finally, the embryos were incubated O/N, at 4°C, with Anti-Digoxigenin-AP Fab fragments (11093274910, Roche), diluted 1:2000 in 2% Blocking, 20% Sheep Serum solution in TBST 1X.

On day 3, depending on how clean (less background) the probe's development is, embryos were either washed for ≈3h (often more) in TBST 1X, or washed all day, several times, in MABT. In the last case, they were then stored O/N, at 4°C, in TBST 1X, and the rest of the protocol happened on day 4. Embryos were then washed twice, for 10 minutes, at RT, in Alkaline Phosphatase (AP) Buffer (NTMT, 100mM $MgCl_2$, 100mM Tris-HCl pH9,5, 100mM NaCl and 1% Tween, in H_2O), and then developed in a 0.5ml BM Purple (11442074001, Roche) solution per well.

To photograph, the AP reaction was either temporarily paused in TBST 1X, or completely stopped in PBT. If paused in TBST 1X, the reaction was continued incubating again in NTMT followed by BM Purple. After stopping the reaction in PBT and photographing the embryos, they were placed in 1% Sodium Azide in PBT at stored at 4°C.

2.6.4. Image recording and analysis

Photographs of the embryos, in a Petri dish with agar 1% (1g Bactoagar : 100ml dH_2O), were taken using a LEICA DFC320 colour camera coupled to a LEICA M2 16 FA stereomicroscope and to a computer. Images were treated using the Photoshop programme and ImageJ 1.48u4.

2.7. New culture

The New culture is a technique for *in vitro* culture of early avian embryos. Eggs were incubated until stage HH3$^+$ of developmental was reached. The egg was opened and the albumin was removed using a plastic Pasteur pipette and kept aside. The yolk was transferred into a bowl containing 1X PBS (1X Phosphate Buffer Saline : 1x PBS: 136 mM NaCl; 2,7 mM KCl; 8,0 mM $Na_2HPO_4.H_2O$; 1,5 mM KH_2PO_4 pH 7,4-7,6) with 1% penicillin/streptomycin and the vitelline membrane was cut below the equator

of the yolk. Since early stage embryos are weakly attached to the vitelline membrane, this was peeled very slowly using a pair of forceps. The vitelline membrane, with the embryo still attached and with its ventral side up, was transferred with a spoon to a Petri dish and washed with PBS with 1% penicillin/streptomycin (15140122/15140163, Gibco). The vitelline membrane was then stretched around a 26 mm diameter glass ring, making sure that the embryo stays in the centre of the ring. When the periphery of the *area opaca* reaches the glass ring the development of the embryo slows down, so the use of larger rings recommended. The ring with the vitelline membrane and embryo attached was then lifted and transferred into a small plastic Petri dish, filled with egg albumin which is good bacteriostatic culture medium. (the strong bacteriostatic properties of the albumin made it possible to dispense strict sterile techniques).

2.8. Electroporation of chicken embryos

New culture embryos (Section 2.7 of Materials and Methods) were transferred to a silicon pool with a 2 mm^2 cathode (CUY701P2E electrode; Nepa Gene). The embryos were covered with Hank's balanced salt solution (GIBCO) and injected with the DNA solution (1 ml DNA; 0.1% Fast Green) in the prospective node territory. pCAGGS-green fluorescent protein (GFP) sample was used at a concentration of 1 mg/ml. Electroporation was performed with five pulses of 5 V for 50 ms at 500 ms intervals using an Electro Square Porator ECM830 (BTX) (Figure 2.5.).

2.9. Drug treatment

A hole was made on the round end of the chicken eggshell of embryos at the appropriate developmental stage, arround $HH3^+$ and HH4. After opening the shell, 20 µl of a cyclopamine (C4116-1MG, Sigma) solution at two different concentrations

(5 μM or 10 μM) was administrated. As a control we used DMSO (D2650, Sigma) dilluted in 1X PBS in the same concentrations has the cyclopamine solutions. After drug and DMSO administration, the embrios were sealed and re-incubated for a period of approximately 6 h. When embryos reached stage HH5 they were collected from the egg and transferred to a Petri dish containing 1X PBS and then any adherent yolk or vitelline membrane was removed. The embryo was transferred with a spoon to a Petri dish, which had been previously coated with charcoal-containing resin (rhodorsyl, Rhône-Poulenc, France). This dark surface allows a better visualisation of the embryo, since it contrasts with the transparent embryo. Embryos were examined for any phenotype abnormalites, and were then fixed O/N in 4% PFA in PBS and dehydrated the next day, for in situ hybridization (see section 2.6.2. of Materials and Methods chapter).

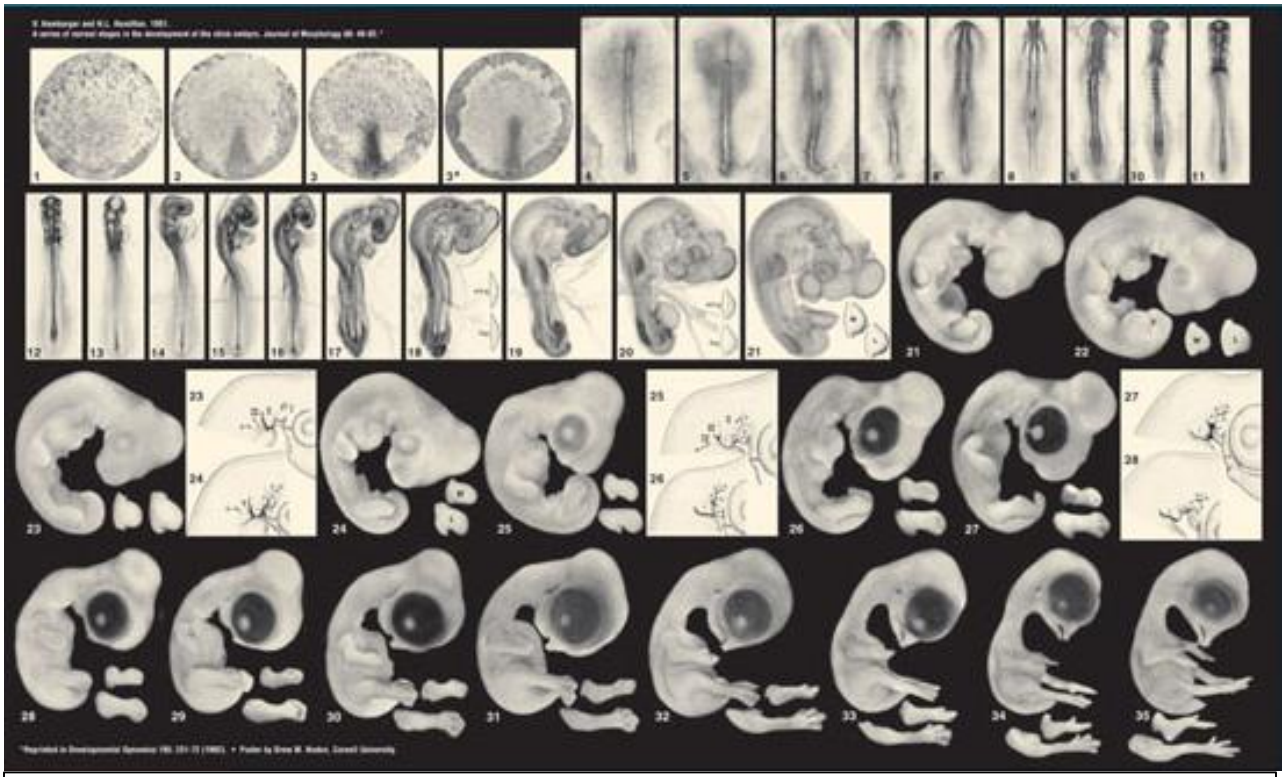


Figure 2.1. - Hamburger and Hamilton development table. Image adapted from Hamburger and Hamilton, 1951.

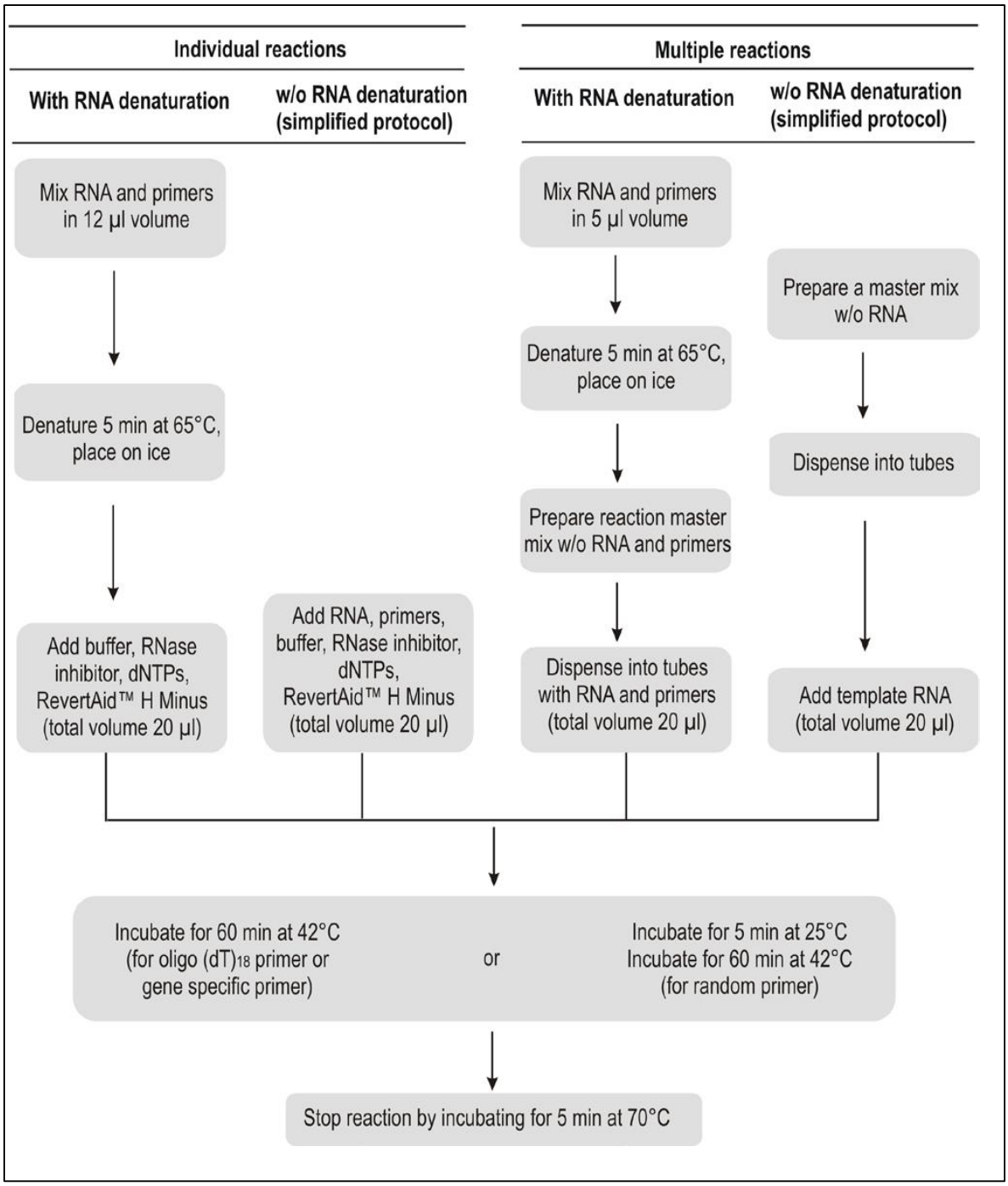


Figure 2.2. – Overview of cDNA synthesis procedures in individual and multiple reactions. Image adopted from Thermo scientific.

	Sequence (5'->3')	Length	Tm	GC%	Self 3' complementarity
pF1Dmrt2XhoI	CTCGAGGGCCACCATGGCCGAAGGACGCGCCGCCGGCGAGTGGGAAAT	48	77.8	70.8	-
pR1Dmrt2EcoRI	GAATTCTCATTTAAGGATGGATTCAACTGAAAACGACAGTG	41	61.4	36.6	-
pR1Dmrt2XhoI	CTCGAGTCATTTAAGGATGGATTCAACTGAAAACGACAGTG	41	63	41.5	-
pF2Dmrt2XhoI	CTCGAGGGCCACCATGGCCGAAGGACGCGCCGCCGG	36	77.6	77.8	-
pR2Dmrt2EcoRI	GAATTCTCATTTAAGGATGGATTCAACTG	29	54.6	34.5	-
pR2Dmrt2XhoI	CTCGAGTCATTTAAGGATGGATTCAACTG	29	57.3	41.4	-

Table 2.1. - Type 1 Primers. Used in standard PCR with Phusion polymerase, with the restriction sites for XhoI and EcoRI. (Primers designed by Rita Pinto, PhD student in our lab)

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
pF1 chick	ATGGCCGAAGGACGCGCCGCCGGCGAGTGGGAAAT	35	81.85	68.57	11.00	2.00
pR2 chick	TCATTTAAGGATGGATTCAACTG	23	54.68	34.78	5.00	2.00
pF1 chick	ATGGCCGAAGGACGCGCCGCCGGCGAGTGGGAAAT	35	81.85	68.57	11.00	2.00
pR3 chick	TCATTTAAGGATGGATTCAACTGAAAACGA	30	61.95	33.33	6.00	6.00
pF2 chick	ATGGCCGAAGGACGCGCCGCCGG	23	76.02	78.26	6.00	6.00
pR2 chick	TCATTTAAGGATGGATTCAACTG	23	54.68	34.78	5.00	2.00
pF2 chick	ATGGCCGAAGGACGCGCCGCCGG	23	76.02	78.26	6.00	6.00
pR3 chick	TCATTTAAGGATGGATTCAACTGAAAACGA	30	61.95	33.33	6.00	6.00
pF3 chick	ATGGCCGAAGGACGCGCCGC	20	70.87	75.00	5.00	5.00
pR2 chick	TCATTTAAGGATGGATTCAACTG	23	54.68	34.78	5.00	2.00
pF3 chick	ATGGCCGAAGGACGCGCCGC	20	70.87	75.00	5.00	5.00
pR3 chick	TCATTTAAGGATGGATTCAACTGAAAACGA	30	61.95	33.33	6.00	6.00
pF4 chick	ATGGCCGAAGGACGCGCCGCCGGCGAGT	28	80.39	75.00	11.00	11.00
pR2 chick	TCATTTAAGGATGGATTCAACTG	23	54.68	34.78	5.00	2.00
pF4 chick	ATGGCCGAAGGACGCGCCGCCGGCGAGT	28	80.39	75.00	11.00	11.00
pR3 chick	TCATTTAAGGATGGATTCAACTGAAAACGA	30	61.95	33.33	6.00	6.00

Table 2.2. - Type 2 Primers. Used in the optimization protocol for standard PCR with Taq polymerase. Primers rearranged in the different combinations used for forward (pF) and reversed (pR) primers.

	Sequence (5'->3')	Template strand	Length	Tm	GC%	Self 3' complementarity
pRACE1	GCACCCGATTTGTCTGGAAATG	Minus	22	60.42	50.00	1.00
pRACE2	TGTCTGGAAATGTCTGCGGG	Minus	20	60.32	55.00	0.00
pRACE3	TGAAAACGACAGTGGCTCAGGTG	Minus	23	63.39	52.17	2.00
pRACE4	GGTTCTCCTTAATGCTCAACTCCC	Minus	24	61.16	50.00	0.00
pRACE5	CTTCTTGTCCTCGGTGGCCTGCT	Minus	23	70	60.9	0.00

Table 2.3. - Type 3 Primers. Used in the 5'-RACE

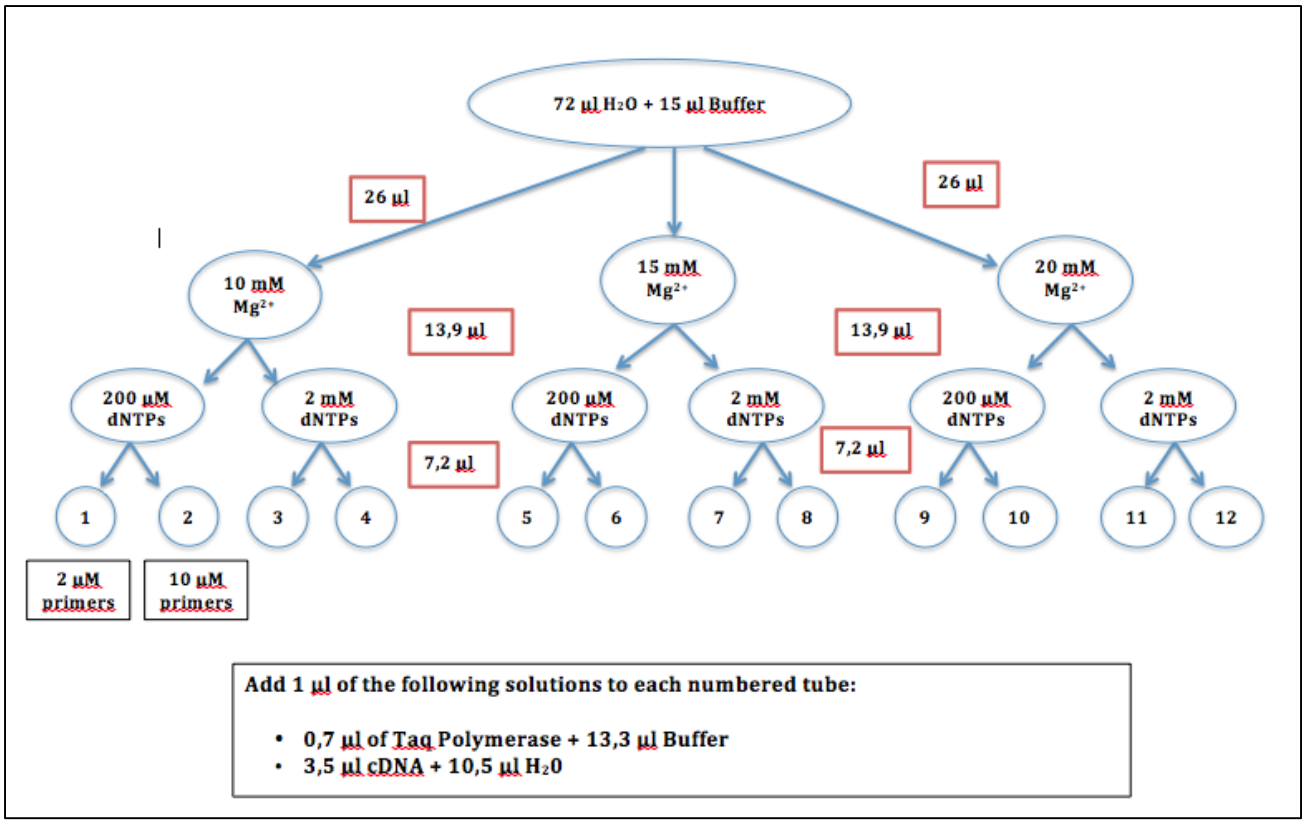


Figure 2.3.- Optimization protocol for PCR. Red boxes are the volume to add from each solution to the next tube.

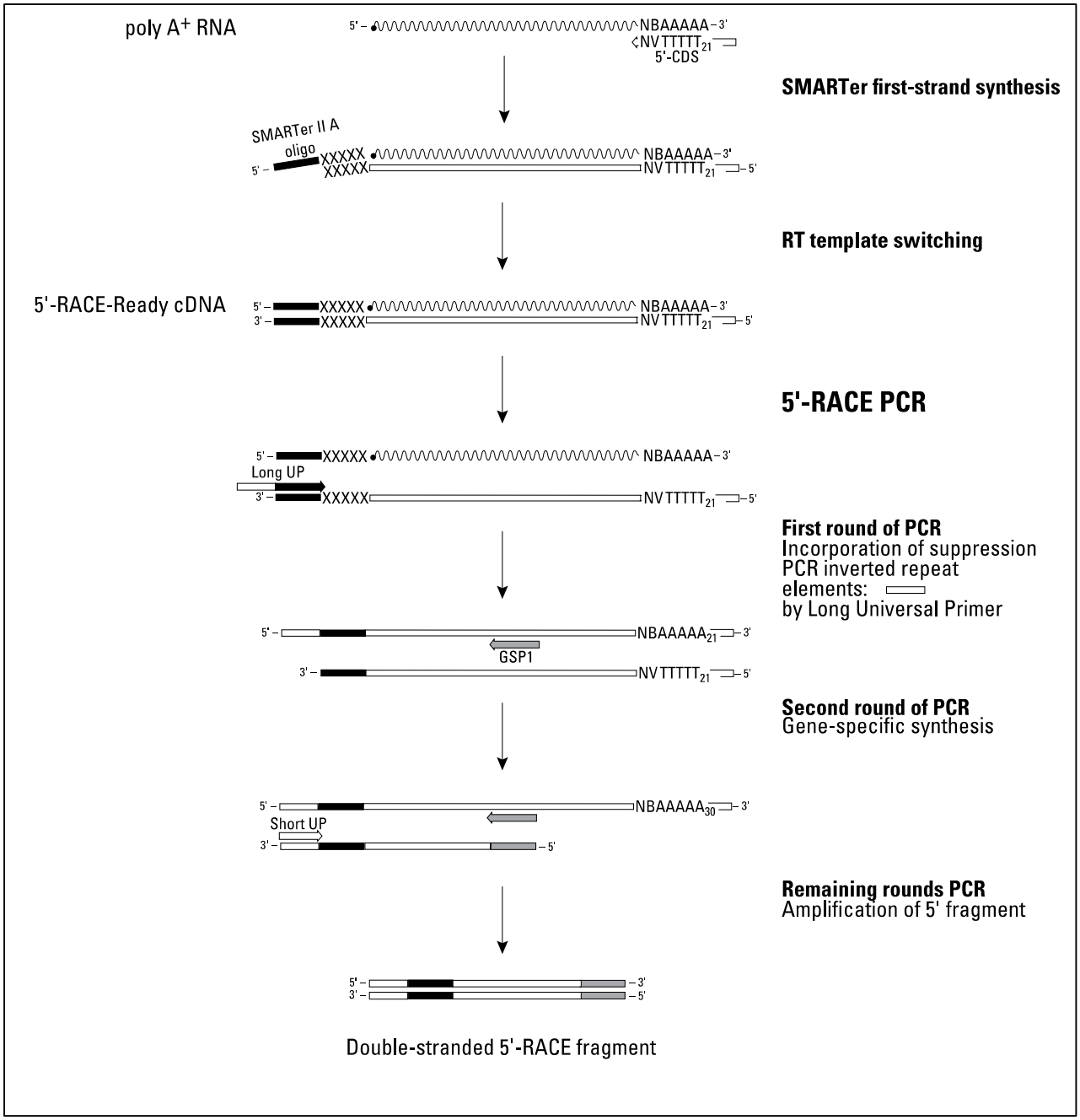


Figure 2.4. - Detailed mechanism of the 5'-RACE reactions. Image adopted from Clontech.

Plasmid	Linearization site	RNA Polymerase
Terra1,2,3,4 (Chicken)	SacI-HF	T3
pGEM-T	NcoI/SpeI	-

Table 2.4.- Appropriate restriction enzyme and RNA polymerase for each probe

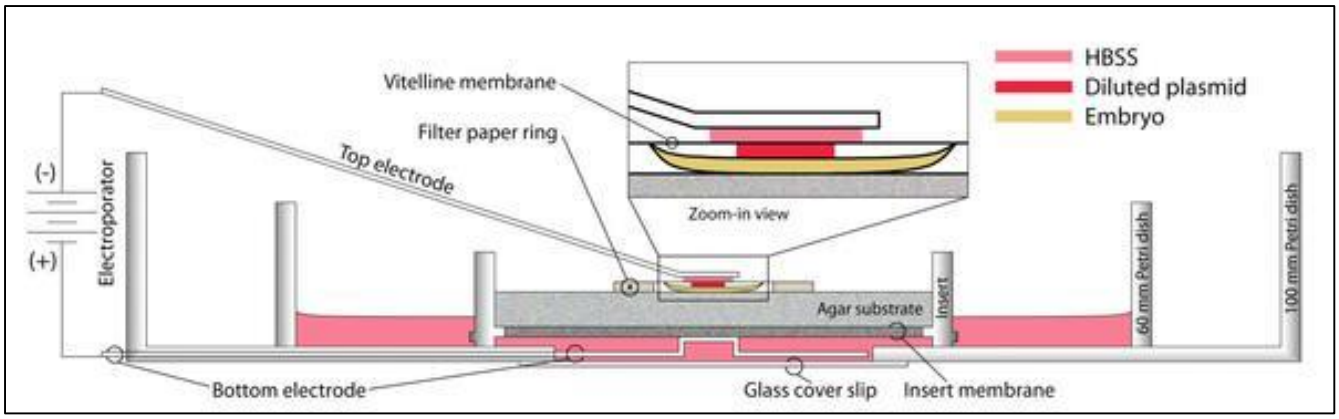


Figure 2.5. - Detailed set-up of the Electroporation. Image adopted from Cui *et al.* 2007.

3. Results

3.1. Attempts to clone the chicken *dmrt2* full-length gene

In the absence of Dmrt2a function, zebrafish embryos display a bilateral asymmetrical expression of clock-specific genes, such has *deltaC*, *her1* and *her7*, leading to asymmetric somite formation. In addition, these embryos show a left-right randomization of *pitx2* and *spaw* expression leading to an incorrect location of the heart [58].

The *dmrt2b* gene is functionally divergent from *dmrt2a.* Instead of being necessary for symmetric somite formation, it is in fact involved in the regulation of somite differentiation at the level of slow muscle development through the regulation of the Hedgehog pathway [59].

Dmrt2a in particular is expressed in the KV, leading to the suspicion that Dmrt2a is acting at the level of the KV to regulate both pathways, symmetric and asymmetric [32].

Mouse *dmrt2*$^{-/-}$ mutants die due to abnormal rib and sternal development, having also defects in the expression pattern of dermomyotomal and myotomal transcription factors [56]. In contrast to what happens in zebrafish, *dmrt2* is not expressed in the mouse node, which is in agreement with the absence of a LR phenotype in *dmrt2* mutant mice.

Although the role of the *dmrt2* in the chicken embryo is still unknown, a role in left-right patterning, similar to what was seen in zebrafish, is a plausible hypothesis, due to the asymmetric expression in the left side of Hensen's node, in HH4^{+} stage embryos, and in the somites, during somitogenesis [58]. Another interesting observation is that *dmrt2* expression pattern is similar to the expression pattern of other genes that are known to have a role in LR patterning, like *shh* and *nodal* [28].

In order to assess the function of Dmrt2 in left-right patterning our initial idea was to perform gain-of-function experiments. For these purpose, we would electroporate chicken embryos with Dmrt2 in the right side of the Hensen´s node, which is the opposite side of its normal expression, followed by phenotype analyses by accessing the position of the heart. To evaluate if *dmrt2* was regulating the Nodal-Pitx2 cascade, and consequently regulating the left-right patterning of the embryo, expression of *nodal* would also be accessed.

In order to do so, our lab purchased one chicken *dmrt2* EST (ChEST465h15) from Source BioScience. (Ref: WTSIp6101H15465Q). The sequence analysis revealed that it was missing the initial 200 bp of the first exon (Figure 3.1) and therefore would not be useful to perform the gain of function experiments. To resolve the problem full-length cloning of *dmrt2* was attempted.

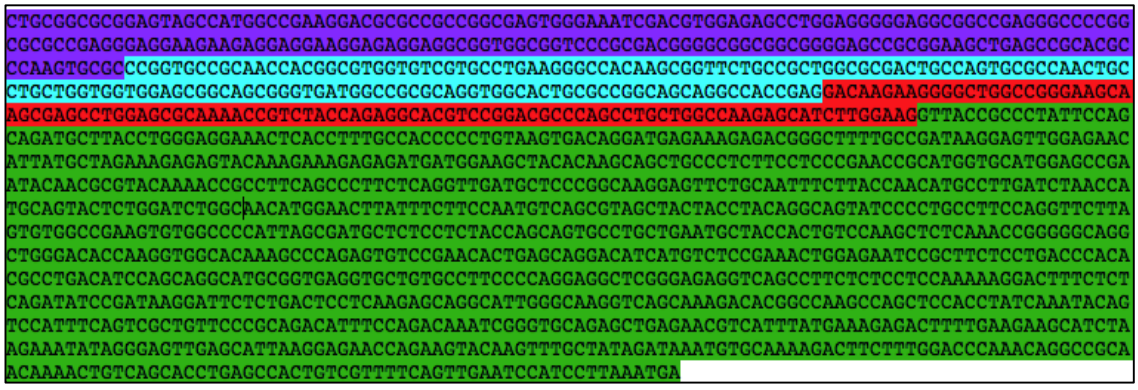


Figure 3.1 – Chicken *dmrt2* sequence present in Ensembl. In purple is the missing portion of *dmrt2's* first exon. In blue is the first exon, in red the second exon and in green is the third exon.

3.1.1. Standard PCR approach to clone the full-length *dmrt2*

Since the chicken *dmrt2* EST would not be useful to perform the gain of function experiments, full-length cloning of *dmrt2* was attempted using a standard PCR technique.

For this PCR, total RNA was extracted from HH10 embryos and from HH4 Hensen's nodes (where we know that *dmrt2* is expressed) [61]. The extracted RNAs were then reverse transcribed to cDNA for cloning (processes described in section 2.2 and 2.3 of the Materials and Methods chapter)**.**

Four gene specific primers with added restriction sites (two forward and two reverse) using primer BLAST from NCBI and having the *dmrt2* sequence present in Ensembl, as our BLAST sequence, were designed. The restriction sites used were XhoI and EcoRI, because our initial idea was to clone *dmrt2* in pGAAGS, a chicken expression vector. Since pGAAGs doesn't have a PolyT tail, restriction sites need to be added to 5' and 3' ends of our primers in order to successfully insert our gene in to the vector. To this set of primers we gave the denomination of type 1 primers (see **table 1** of Materials and Methods). The PCR reaction was done according to section 2.5.1 of the materials and methods chapter.

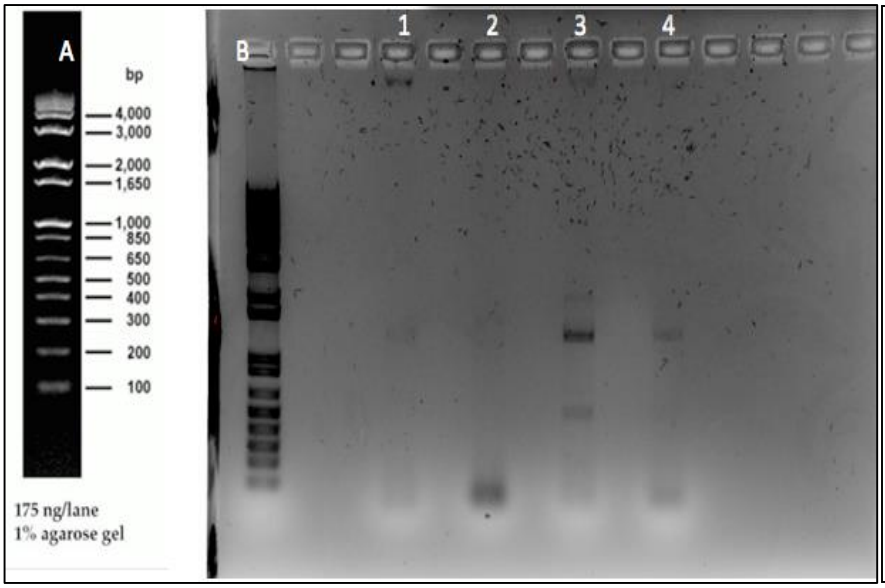


Figure 3.2 – Attempt to amplify the chicken *dmrt2* using Type 1 Primers. A) 1Kb Ladder Plus. B) Inverted color image 2% Agarose gel of PCR product obtained from Chiken total RNA. 1 - PCR product using Primer Mix 1. 2 - PCR product using Primer Mix 2. 3 - PCR product using Primer Mix 3. 4 - PCR product using Primer Mix 4. For more details see Materials & Methods Chapter.

From these reactions a 1400 bp band (same size as the sequence present in ESEMBL for the chicken *dmrt2)* was amplified, using mix 3 and 4 (see section 2.5.1 of Materials and Methods)(Figure 3.2). We isolated the bands from the agarose gel,

which correspond to the product of our interest (Figure 3.3), and proceeded to TA cloning using pGEM-T vector, and not pGAAGS, and then sent for sequencing. The reason for using pGEM-T, was because this vector can be sequenced using universal primers, unlike pGAAGs that needs specific primers for sequencing, making the process of sequencing much more laborious. Unfortunately the results for the sequences came back negative for *dmrt2* (results not showed).

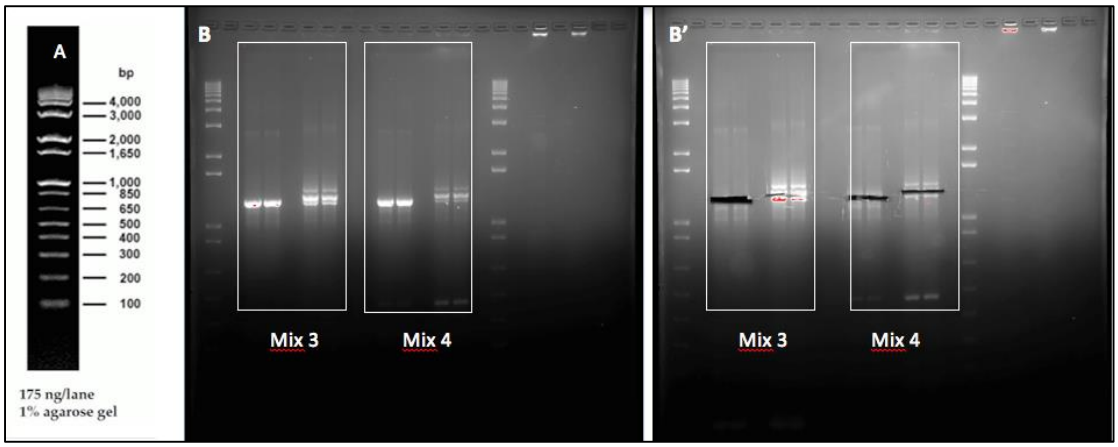


Figure 3.3 – 2% Agarose gel of PCR products from the isolated bands in figure 3.2. A) 1Kb Ladder Plus. B) PCR products from Primer Mix 3 and Primer Mix 4. B') Same gel after band extration.

We hypothesized that the fact of our primers have added nucleotides to 5' and 3' ends, made them incapable of binding properly to our target gene, thus leading to the negative results that we obtained

3.1.2. Optimizing the PCR protocol

Since our results came negative for *dmrt2*, we decided to optimize the PCR protocol in order to successfully clone its full-length.

For this we redesign the primers, using the same method, but this time no restriction sites were added to the 5' and 3' ends of our primers. The concentrations

of the reagents, annealing temperatures, buffers (both KCl and $(NH_4)_2SO_4$ buffers, provided by the kit, were used) as well as the samples of cDNA were altered, resulting in different PCR reactions (Figure 2.3.). A variety of combinations of forward and reverse primers were done, since all the primers have different melting temperatures (Tm), leading to different affinities. For these primers we gave the denomination of type 2 primers (see Table 2 of Materials and Methods). All of these reactions had a control were no cDNA was added. The total volume of each reaction was 10 µl.

What we saw was that, the reactions with higher annealing temperatures, had more bands then the reactions with lower annealing temperatures. This was not expected since reactions with lower annealing temperatures are less specific, than reactions with higher annealing temperatures, thus generating more bands. Although we had production of bands, only two combinations of primers had bands of the desired size of 1200/1400 bp. The combinations were pF2+pR3 and pF3+pR3 (see Table 2 of Materials and Methods). Also reactions using the KCl buffer were more efficient then the reactions using the buffer $(NH_4)_2SO_4$. No significant differences were seen using different cDNA samples.

After having amplified a product of interest, we decided to repeat the reactions, but this time with a volume of 50 µl, in order to have enough volume of product to send for sequencing. Unfortunately only the pF3+pR3 reaction was able to amplify a 1400bp product. The bands were isolated and quantified. The concentrations were too low to send to sequencing, so we decided to increase, in increments of 0,5 µl, the volume of cDNA in the reaction (we begin with 1,5 µl and finished with 2,5 µl of cDNA). After doing the increments of 0,5 µl, we still were not able to produce enough product to send to sequencing.

3.1.3. Attempt to amplify the 5' end of *dmrt2* using 5'-RACE technique

Since our previous attempts to amplify the full-length of *dmrt2* by PCR failed, we hypothesized that the annotation for the *dmrt2* was not properly done. To test our hypothesis an analysis of the sequence present in Ensembl was done.

For this analysis, we started by looking for Expressed Sequences Tags (ESTs), for the *dmrt2.* These ESTs result from the sequencing of one of the extremities of cDNA library clones and are used as a support to our sequence since they are expressed portions of our genes. To do so the sequence, present in the Esembl, was run in the BBSRC ChickEST Database to see, if we have any ESTs for the missing portion of *dmrt2.* After running the sequence in the BBSRC ChickEST Database, only three ESTs were found, but none of them was for the the first 200 bp at 5' of our gene. This lead us to conclude that the 5'-UTR doesn't have any supporting evidences.

Since no EST for the 5' region was found, a 5'-RACE was done in order to amplify the first 200 bp of the gene. For this, new samples of RNA were extracted (see section 2.2. of Materials and Methods). A new set of gene specific primers with specific characteristics, for which we give the denomination of Type 3 primers (see table 2.3.), was design (see section 2.4. of Materials and Methods).

The first set of RACEs (with pRACE 1,2,3,4) did not amplifiy any product, so a positive control with mouse heart total RNA was done in order to see if the lack of product was due to our primers, and not because of some deficient component of the kit. A band of 1200 bp (expected result) was successfully amplified; leading us to conclude that the lack of product was indeed due to the designed primers.

In order to eliminate this problem and obtain a successful product a new primer, pRACE 5, was designed. This time we decided to design the primer with a shorter predicted product size (beginning the transcription in the end of the first exon and

not in the third exon as the previous ones), in order to have only the 5'-region of interest amplified. After running half of the 5'-RACE product (the other half was stored at -20°C, for cloning), bands of 450 bp and 200 bp were successfully amplified (Figure 3.4. B).

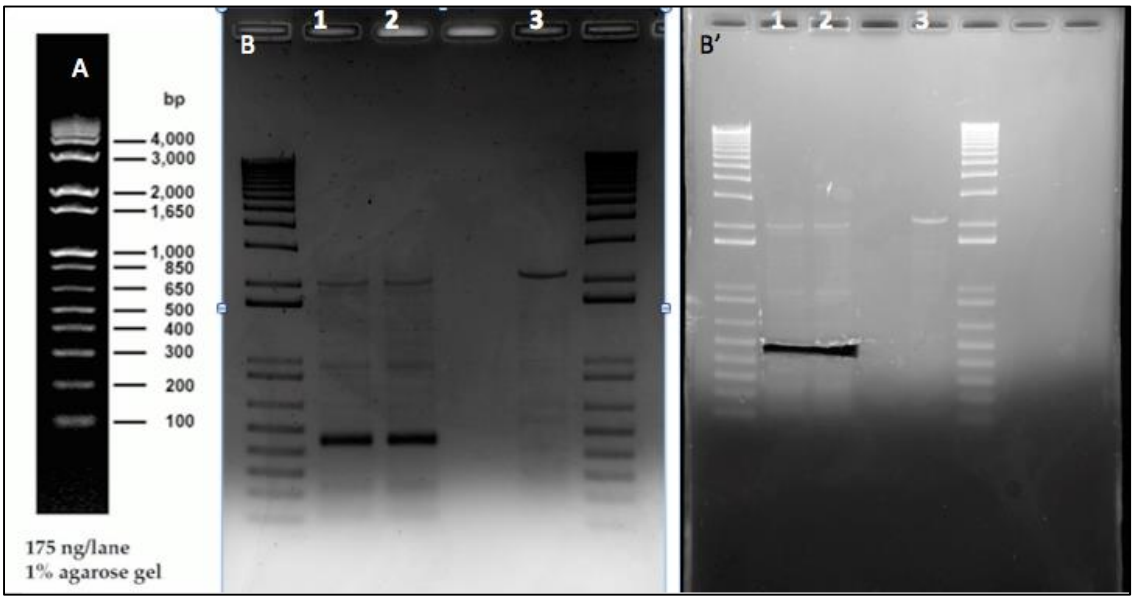


Figure 3.4 – 5'-RACE Type 3 primers were able to produce a 200bp and a 450bp bands. 1,2% Agarose gel of 5'-RACE product from HH10 chicken total RNA. A) 1Kb Ladder Plus. B) 5'-RACE products from Chicken total RNA (1) and (2), Control Mouse Heart Total RNA (3) (inverted color image). B') Extracted bands of 5'-RACE product.

Since the size of the 5'-region is unknown, we decided to isolate both bands (Figure 3.4. B') and clone them, along with the whole PCR product, that was stored, into the pGEM-T vector (see section 2.5.5. of Materials and Methods). A total of 29 inoculated colonies were purified using the Dity-Prep method (see section 2.5.6.3. of Materials and Methods), followed by double digestion of the plasmids to access the size of the inserts present in each isolated colony (see section 2.6.1. of Materials and Methods)(Figure 3.5.). After the double digestion of the plasmids, the 13 colonies with the strongest bands were purified using the mini-prep method (see sections

2.5.6.1. and 2.5.6.2. of Materials and Methods), and sent to sequencing. The results came negative for *dmrt2.* Since the portion 5' of our sequence of interest is unknown, this results were already expected.

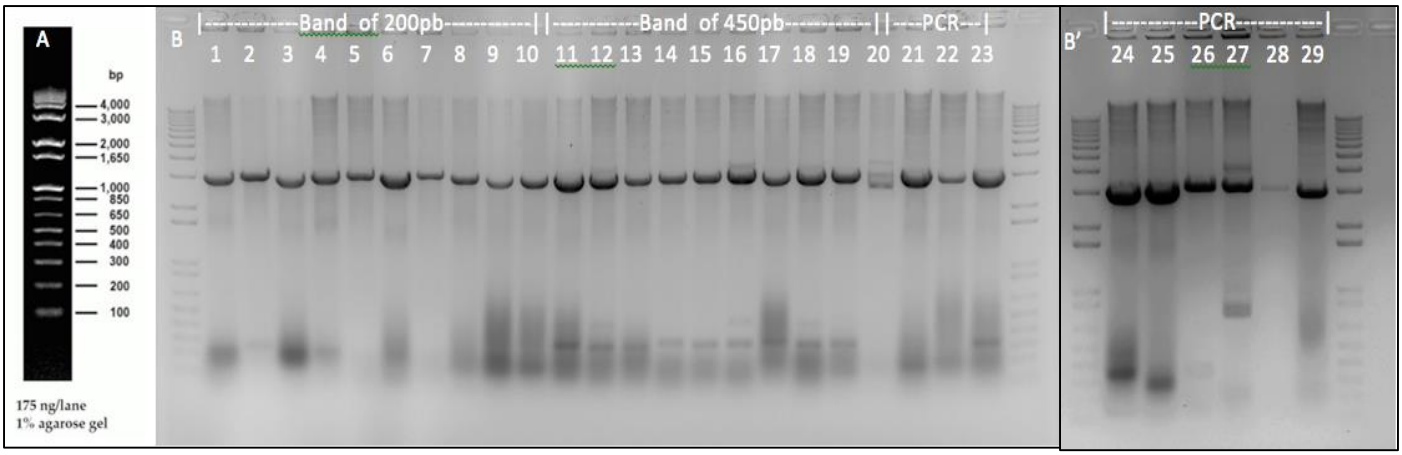
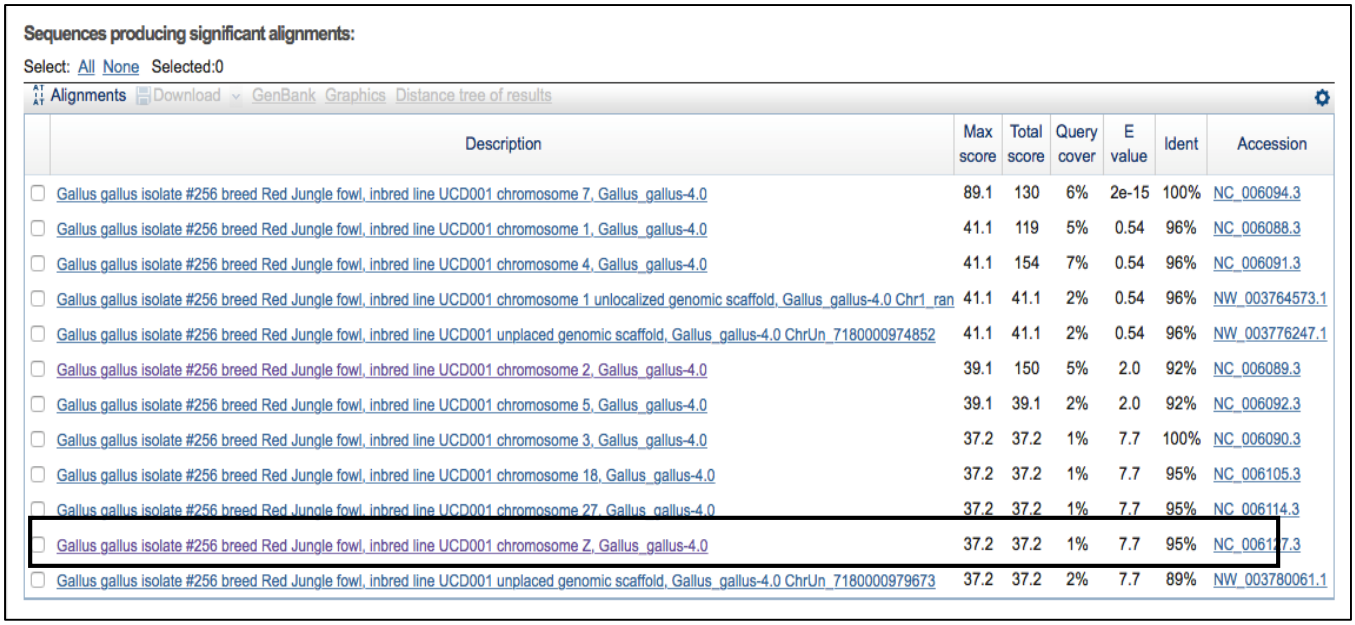


Figure 3.5 – Cloning of the 5'-RACE inserts. A) 1 Kb Ladder Plus. B) Inverted color images of 1% Agarose gels of the linearized plasmids containing 5'-RACE products. B') Second lane of the gel presented in B. Wells 1-10 were loaded with linearized plasmids containing a 5'-RACE insert of 200 bp. Wells 11-19 were loaded with linearized plasmids containing a 5'-RACE insert of 450 bp. Wells 20-29 were loaded with linearized plasmids containing the whole 5'-RACE product. All plasmids were digested using NcoI and SpeI.

Since our sequences were unknown a detailed analysis of the sequence was performed, in order to see if the isolated 5'-product was indeed the missing portion of *dmrt2*. For this analysis we started to clean the parts of the sequence that had sequencing errors (uncertain base matches). After cleaning the sequence of mismatched nucleotides, the sequence was then aligned with the sequence from pGEM-T in order to eliminate the portion of our sequence that belongs to our vector. After having the clean sequence a BLAST using the NCBI ref-seq genomic database (database of genomic sequences) was performed. For the positive matches for chromosome Z sequences a zoom-out of the alignment was done to see if our sequence was near of the 5'-end of the known sequence of *dmrt2*. None of the

sequences were near *dmrt2*, and had also a low score (meaning that only a small portion is from chromosome Z) (Figure 3.6.).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 7, Gallus_gallus-4.0	89.1	130	6%	2e-15	100%	NC_006094.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 1, Gallus_gallus-4.0	41.1	119	5%	0.54	96%	NC_006088.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 4, Gallus_gallus-4.0	41.1	154	7%	0.54	96%	NC_006091.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 1 unlocalized genomic scaffold, Gallus_gallus-4.0 Chr1_ran	41.1	41.1	2%	0.54	96%	NW_003764573.1
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 unplaced genomic scaffold, Gallus_gallus-4.0 ChrUn_7180000974852	41.1	41.1	2%	0.54	96%	NW_003776247.1
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 2, Gallus_gallus-4.0	39.1	150	5%	2.0	92%	NC_006089.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 5, Gallus_gallus-4.0	39.1	39.1	2%	2.0	92%	NC_006092.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 3, Gallus_gallus-4.0	37.2	37.2	1%	7.7	100%	NC_006090.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 18, Gallus_gallus-4.0	37.2	37.2	1%	7.7	95%	NC_006105.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome 27, Gallus_gallus-4.0	37.2	37.2	1%	7.7	95%	NC_006114.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 chromosome Z, Gallus_gallus-4.0	37.2	37.2	1%	7.7	95%	NC_006127.3
Gallus gallus isolate #256 breed Red Jungle fowl, inbred line UCD001 unplaced genomic scaffold, Gallus_gallus-4.0 ChrUn_7180000979673	37.2	37.2	2%	7.7	89%	NW_003780061.1

Figure 3.6 – 5'-RACE failed to amplify the 5' region of chicken *dmrt2*. BLAST nucleotide alignments results for the obtained 5'-RACE sequences. Black box indicate positive match for chromosome Z.

Since none of the sequences were near *dmrt2,* and four exons for *dmrt2* in zebrafish, mouse and human were reported, we put the hypothesis that the chicken *dmrt2* has also four exons and not the three described in Ensembl.

In order to see if an extra exon was present in the chicken's *dmrt2*, and if our product was part of this missing exon, we align our sequencing results with a section of approximately 5 Kb at the 5'-end of *dmrt2*. None of the sequences aligned with the 5'-section meaning that our 5'-RACE product isn't the missing portion of *dmrt2.* Another conclution that we were able to take, was that the probability of the chicken *dmrt2* having an extra exon is extremely low, although it is not zero.

3.2. Shh signalling, a possible upstream regulator of *dmrt2* expression

It is known that Shh is one of the key molecules in left-right patterning and that is expression is asymmetric on the left side of Hensen's node at stage HH4 [28]. Since *dmrt2* has the same expression pattern as *shh* but at a later developmental stage [58], we hypothesised that Shh signalling could be an upstream regulator of *dmrt2* expression.

In order to determine the expression pattern of *dmrt2,* an anti-sense RNA probe was done using plasmidic DNA with the chicken EST 465h15 (expressed portion of *dmrt2*), that was already available in our lab (see sections 2.6.1 and 2.6.2. of Materials and Methods)(Figure 3.7.). After probe production, an *in situ* hybridizations of HH10 embryos were done in order to assess the quality of our probe (see section 2.6.2. of Materials and Methods). The control for the *in situ* hybridization was done using HH7 embryos and a *nodal* probe already available and tested in our lab. The *dmrt2* probe was functional showing expression at the level of the somites and anterior PSM (n=4) (as showed by our group in previous studies)(Saude et al., 2005) (Figure 3.8. B). Nodal showed expression in the perinodal and LPM domain, meaning that the process was done properly and that our expression is not an artefact (Figure 3.8. A).

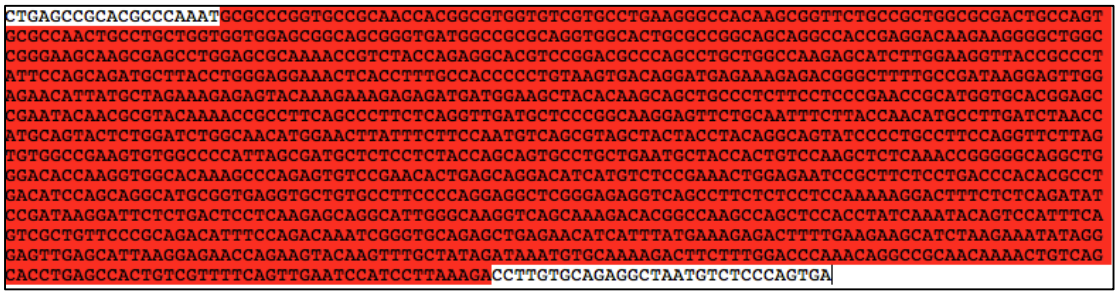


Figure 3.7 – Isolated chicken EST 465h15. Represented in red is the sequence of the chicken EST available in our lab.

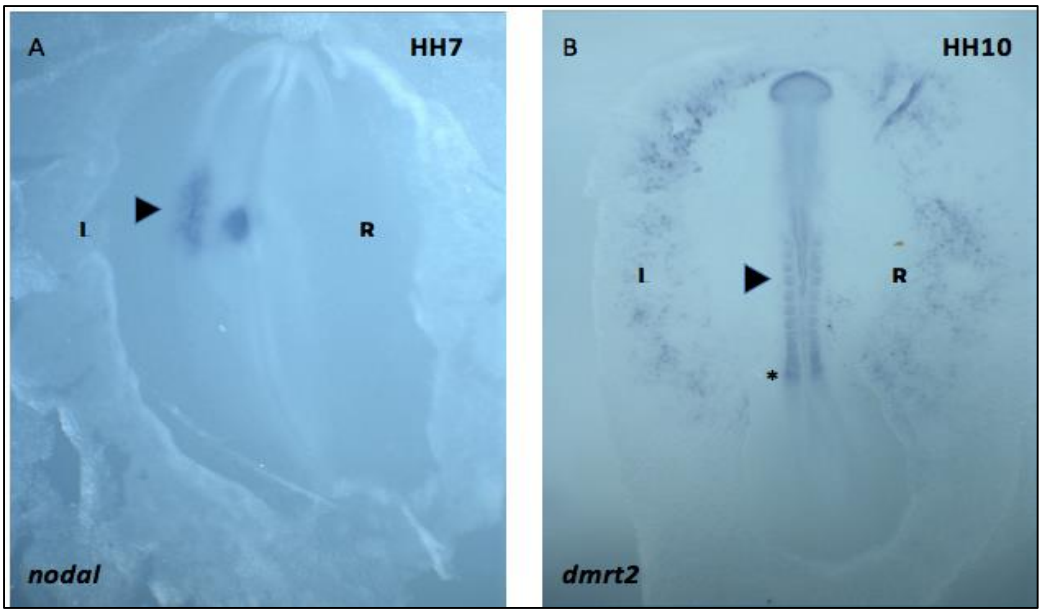


Figure 3.8 – Validation of the RNA probes. A) WISH using the *nodal* mRNA probe, showing expression in the perinodal and LPM (arrow head), in a HH7 embryo (n=3). B) Expression of WISH using the *dmrt2* mRNA probe, showing expression in the somites (arrow head) and anterior PSM (*) of stage HH10 embryo (n=4). L, left; R, right

To test our hypothesis, chicken embryos at stage HH4 were treated with a solution of Cyclopamine (a known Hedgehog signalling inhibitor) at 5 µM (n=17). As control, we treated embryos at the same stage with DMSO (n=16) (see section 2.9. of Materials and Methods). For this a hole was made on the round end of the chicken eggshell of embryos at the appropriate developmental stage, around HH3^{+} and HH4. After opening the shell, 20 µl of a 5 µM cyclopamine solution was dropped on top of the embryo. As a control we used DMSO diluted in 1X PBS in the same concentrations has the cyclopamine solutions. After drug and DMSO administration, the embryos were sealed and re-incubated for a period of approximately 6 h. When embryos reached stage HH5 they were collected. The embryos were then checked for any phenotype abnormalities, and then were fixed O/N in PFA 4% fixative and

dehydrated the next day, for in situ hybridization (see section 2.6.2. of Materials and Methods chapter).

No differences in the expression pattern, of *dmrt2*, between control and treated embryos were observed (Figure 3.9. A-B). In order to see if the reason for the unaltered expression was the amount of drug administrated, a new set of embryos was treated with a solution of cyclopamine at 10 µM (n=3). What we observed was that the expression of *dmrt2* was abolished in all the treated embryos, but not in the control ones (Figure 3.9. C). This result indicates that Shh signalling might be controlling the expression of dmrt2 in the chicken embryo although this need further confirmation due to the small sample size (n=3).

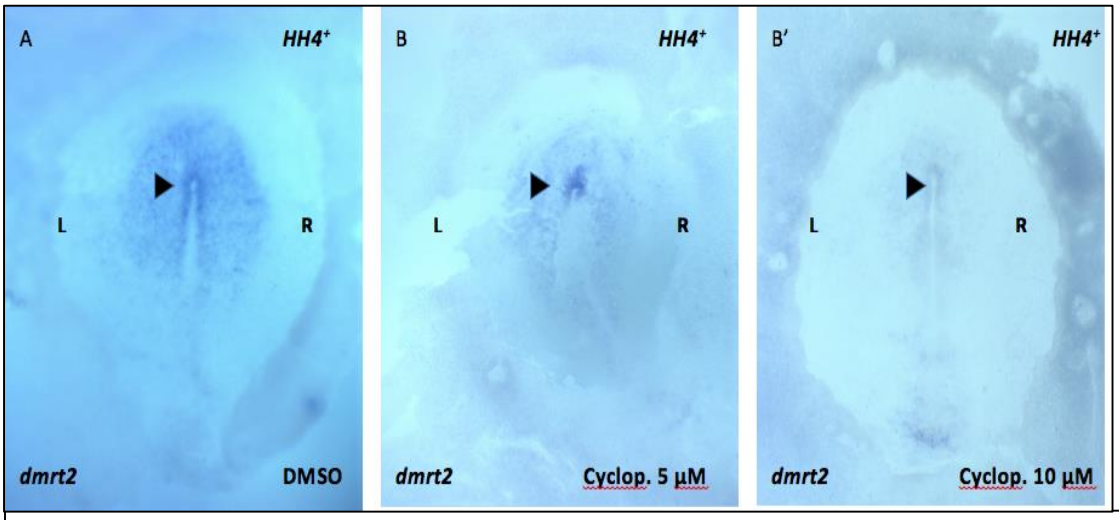


Figure 3.9 – Shh as a possible candidate gene for *dmrt2* regulation. WISH for *Dmrt2* in HH4 embryos treated with cyclopamine. A) Expression of *Dmrt2* in the Hensen's node (arrow head), of an embryo treated with a DMSO solution (control) (n=16). B) Expression of *Dmrt2* in the Hensen's node (arrow head), of an embryo treated with a 5 μM cyclopamine solution (n=17). B') Absence of expression of *Dmrt2* in the Hensen's node (arrow head), of an embryo treated with a 10 μM cyclopamine solution (n=3). L, left; R, right

4. Discussion

For a long time the DMRT family was linked to sex determination [60]. Although after a series of in situs and qPCRs, investigators started to see that members of the DMRT family were expressed in places not related to sex determination [54]. Dmrt2, in particular, was seen to have its expression in the anterior PSM and somites in different organisms [55][58][56].

In an initial report, it was shown that Dmrt2a in zebrafish had a role in somite differentiation [55]. Our lab later showed that Dmrt2a protects the somites from asymmetric signals, ensuring the symmetric formation of the somites, and at the same time has a second function conveying asymmetric signals for the correct placement of the internal organs [58].

Due to the genome duplication in zebrafish, an extra *dmrt2* does exist. This extra *dmrt2* is *dmrt2b* and although it has the same expression pattern in the anterior region of the PSM and somites as *dmrt2a*, it is functionally divergent. Dmrt2b is necessary for the establishment of left-right asymmetries in the LPM but it does not play a role in the protection of somites from asymmetric signals. It is however necessary for later somite differentiation at the level of slow muscle development through the regulation of the Hedgehog pathway [59].

In the mouse, Dmrt2 is absolutely necessary for somite differentiation [56] but seems to be dispensable for the establishment of asymmetries within the LR axis and it also seems to be irrelevant to protect the somites from asymmetric signals [58]. The non-conserved function of Dmrt2 between zebrafish and mouse is probably due to the differential expression of *dmrt2* in the laterality organ between zebrafish and mouse. Mouse *dmrt2* is not expressed in the node, while *dmrt2a* is expressed in the Kupfer's vesicle [33].

In the chicken embryo, it is clear that *dmrt2* is expressed, on the left side of Hensen's node during developmental stages when the information for the symmetric displacement of the somites as well the information for the asymmetric events are taking place [58]. These observations lead to the hypothesis that Dmrt2 is playing the role of coordinator, along the left-right axis in the avian embryo. Since *dmrt2* is also expressed in the anterior PSM and somites, a function in somite differentiation is also plausible. However, its exact function of Dmrt2 in the chicken embryo was never studied.

With this work our initial idea was to access the function of Dmrt2 in left-right patterning, in the chicken embryo. Although the best way to study the function of a gene is by combining loss-of-function (LOF) and gain-of-function (GOF) experiments, we decided to perform exclusively a gain-of-function (GOF) approach. The reason we chose a GOF approach was due to major technical problems involving the methodologies used for LOF experiments in the chicken embryo.

Some of these problems come from the fact that the genome of the chicken is not well annotated leading to problems in the specificity of methods like iRNA or morfolino (MO). Also the usage of RNAi and MOs in the avian model is still controversial and only a few studies in this model used these types of methods. Methods like dominant negative molecules would be difficult to design since Dmrt2 is a transcription factor and not a transmembrane receptor.

In order to perform GOF experiments, the full-length protein of Dmrt2 is needed. So we started by attempting to clone the entire coding sequence of the *dmrt2* chicken gene. To successfully clone the full-length, gene-specific primers were design according to the sequence deposited in the Ensembl database. After running our initial PCR we were unable to amplify *dmrt2*. To overcome this negative result a series of optimizations to the initial protocol were done. Unfortunately the cloning

of *dmrt2* was never achieved.

A hypothesis that we put forward was that our PCR was not working probably because the sequence deposited in the Ensembl database was not correctly annotated. And in fact a closer analysis of the deposited sequences led us to conclude that the supporting evidence for the 5' 200bp region of *dmrt2* were weak. This major technical difficulty makes the design of forward primers impossible giving us a major setback. Cloning using standard PCR techniques was no longer possible.

To bypass this problem, we decided to perform 5'-RACE to amplify the missing unknown 5'-region. Although our primers were designed based on the sequence of a EST, this approach also failed. One reason could be related to non-optimal protocol conditions for our set of primers.

It is important to emphasize that although our sequencing results, for both PCR and 5'-RACE, came negative for *dmrt2*, amplification of PCR products occurred using all set of primers. This led us to conclude that the process is working, but the specificity of our primers, regarding the conditions used in the protocol, is probably low. Taking this into account, the next step would be to design new primers, with more suitable parameters, and repeat the 5'-RACE since the 5'-end of *dmrt2* does not have any supporting evidence.

In the event of a successful amplification of the 5'-region of the *dmrt2* gene, we would proceed to vector cloning by adding the entire coding sequence to a chicken expression vector, like pGAAGs. This vector would have a fluorescent tag, like EGFP or mCherry, in order to see if our expression vector is being correctly expressed in space and time once electroporated. After cloning we would proceed to the electroporation of the *dmrt2-EGFP* construct in the right side of the prospective Hensen´s node. The electroporated embryos would be allowed to develop until

relevant stages and would be analysed by accessing the position of the heart, somite displacement and morphology.

The expected result would be a randomization of the heart postion, thus confirming a function of Dmrt2 in the left-right patterning in the chicken embryo. At a molecular level it would be interesting to see expression of *nodal* and *pitx2* in the right side, giving us proof that *dmrt2* is regulating the left-right patterning of the embryo, via Nodal-Pitx2 cascade. Another interesting observation whould be a disruption in the cyclic genes of the segmentation clock, showing a dual function for Dmrt2.

In this project, we also set up to find what are the molecular cues responsible for the asymmetric activation of *dmrt2* expression on the left side of the Hensen's node at stage HH5. The secreted factor s*hh* is expressed on the left side of the Hensen´s node at an earlier stage and than *dmrt2* expression in that same location. This led us to put forward the hypothesis that *shh* is an upregulator of *dmrt2* asymmetric expression.

To test this hypothesis, we performed drug treatment experiments with cyclopamine, an inhibitor of Hedgehog signalling, in order to see if the expression of *dmrt2* would be downregulated in this experimental situation. What we saw was that only the embryos treated with higher concentrations of cyclopamine (10 μM) had a knockdown in *dmrt2* expression. Although the number of embryos analyzed (n=3) was low, all of them presented a downregulated *dmrt2* expression pattern, meaning that the hypothesis of the *shh* signaling pathway as an upstream regulator of *dmrt2*, is a plausible and valid one. It is really important to emphasize the fact that the samples analyzed were smaller than the ideal due to: errors in the initial manipulation; to problems in the first days of the *in situ* hybridization protocol; and to the necessity to discard embryos that were not at the desired developmental

stage (HH4^{+} or HH5) by the time of analysis. The presence of different developmental stages in different embryos that were incubated under the same conditions can probably be due to the influence that certain environmental factors exert over development, particularly temperature. The fact is that sometimes the incubator was opened consecutively, which resulted in variations of more than 1°C, which can influence embryo growth.

Regarding future perspectives, we would suggest an identical assay contemplating a bigger sample and including all points criticized above, in order to obtain more conclusive results than the ones we are presenting here.

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