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# SOX PROTEINS AND NEUROGENESIS

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# ABSTRACT

The primordium of the central nervous system is specified in a process called neural induction at which point the ectoderm is subdivided into the epidermal ectoderm and the neural plate. With time the neural plate will thicken, fold and form the neural tube. The progenitor cells within the neural tube will later give rise to all neurons and glial cells in the adult central nervous system. Soon after the formation of the neural tube neural progenitor cells stop proliferate and start to express neuronal characters. These events are tightly regulated by a number of different signaling pathways. In this thesis I have studied how SoxB proteins regulate neurogenesis and the maintenance of the neural progenitor pool.

In Paper I we examined the role of Sox21 in regulating neurogenesis. We show that Sox21 promotes neurogenesis, opposite to the activity of the SoxB1 factors (Sox1-3) that has been shown to repress neurogenesis. Sox21 and Sox1-3 bind a similar set of target genes and the effect of Sox21 is mediated through a direct counteraction of Sox1-3 activity. Therefore the intrinsic balance of Sox21 and Sox1-3 activities is important in deciding whether a cell should remain progenitor or commit to differentiation. The proneural proteins Ngn2 and Mash1 have been shown to shift this balance by upregulating Sox21 expression as a way to promote neurogenesis.

Since a balanced expression of Sox21 and Sox1-3 is important to maintaining the neural progenitor pool we wanted to find out more about how Sox21 transcription is regulated. In Paper II we identified a Sox21 enhancer that is active in neural progenitors. Sox1-3 and E proteins synergistically activate this Sox21 enhancer and upregulate *Sox21* transcription in vivo. Sox1-3/E protein mediated activation of the Sox21 enhancer is repressed by Sox21, suggesting a cross-regulatory interaction between Sox1-3 and Sox21. Interestingly, the proneural protein Ngn2 is interfering with this Sox1-3/E protein activity, suggesting a novel mechanism where Ngn2 is opposing Sox1-3 activation of neural progenitor gene expression.

Both Notch signaling and the Sox1-3 factors are functionally similar in suppressing neurogenesis but it is not clear to whether these signaling pathways are functionally interacting. In Paper III we have addressed this question. We show that both Notch and Sox1-3 repress proneural function, but they do so at distinct regulatory levels. Notch signaling is repressing transcription of proneural proteins and E proteins whereas Sox1-3 suppresses the proneural proteins function to promote neurogenesis. Overexpression of Sox3 maintain the progenitor pool even in the absence of Notch signaling, whereas Notch signaling requires Sox1-3 activity to maintain neural progenitors in an undifferentiated state.

# LIST OF PUBLICATIONS

I. **Magnus Sandberg**, Magdalena Källström and Jonas Muhr.
Sox21 promotes the progression of vertebrate neurogenesis.
*Nature Neuroscience* (2005) 8, 995-1001.

II. **Magnus Sandberg**, Maria Bergsland, Idha Kurtsdotter and Jonas Muhr.
Identification of Sox21 enhancer elements reveal cross-regulatory interactions between group SoxB proteins.
*Manuscript.*

III. Johan Holmberg, Emil Hansson, Michal Malewicz, **Magnus Sandberg**, Thomas Perlmann, Urban Lendahl and Jonas Muhr.
SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation.
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# LIST OF ABBREVIATIONS

| | |
|---|---|
| SRY | Sex Determining Region Y |
| CDK | Cycline Dependent Kinase |
| BMP | Bone Morphogenic Protein |
| FGF | Fibroblast Growth Factor |
| SOX | SRY Related HMG Box |
| CNS | Central Nervous System |
| Raldh2 | Retinaldehyd Dehydrogenase 2 |
| RA | Retinoic Acid |
| sHH | Sonic Hedgehog |
| NICD | Notch Intracellular Domain |
| bHLH | Basic Helix-Loop-Helix |
| HMG | High Mobility Group |

# 1 INTRODUCTION

Life is complex. It starts with the fusion of two gametes that form a zygote. The first cells of the embryo, the stem cells, have the capacity to give rise to all cells in the adult organism. How does one single cell produce a complex organism? Two fundamental requirements for this are specification and amplification. Gastrulation is the earliest specification event where cells in the embryo undergo a drastic reconstruction through extensive cell migration that will give rise to three germ layers, endoderm (the inner layer), mesoderm (the mid layer) and ectoderm (the outer layer). The endoderm will subsequently form the inner lining of the digestive- and respiratory tract whereas the mesoderm will gives rise to bone, muscle and circulatory system. The ectoderm is further sub-divided into the epidermal ectoderm that later will form the skin and the neural plate, the primordium of the nervous system.

## 1.1 NEURAL INDUCTION

The event at which the ectoderm is subdivided into epidermal- and neural ectoderm is generally called neural induction. Hans Spemann and Hilde Mangold first described neural induction in 1924 by the discovery of the organizer. In this study the dorsal blastopore lip of a newt was shown to have the potential to exert an organizing effect on the surrounding cells. When transplanting the organizer from a donor embryo to a recipient it induced the formation of a second embryo. The role of the organizer was for a long time questioned and the supposed inductive signals produced by the organizer could not be identified. Not until fifty years later some of the factors produced by the organizer was cloned which would again re-vitalize the neural induction field. In a series of papers it was shown that the organizer produces the BMP inhibitors, Chordin, Noggin and Follistatin (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Piccolo et al., 1996; Zimmerman et al., 1996). The repression of BMP signaling has proven to be the most important function of the organizer when inducing the neural ectoderm. BMP repression and Fgf expression are the two major intercellular signaling pathways important for manifesting the neural ectoderm whereas BMP and Wnt signaling promote the non-neural lineage of the ectoderm (Levine and Brivanlou, 2007; Wilson et al., 2001).

## 1.2 NEURULATION

The vertebrate organizer will induce the neural ectoderm from the anterior to the posterior to establish the anterior posterior axis. As the organizer is migrating posteriorly a groove like structure along the midline of the embryo is formed. This structure is called the primitive streak and the neural ectoderm on each side of the primitive streak is called neural plate. Initially the neural plate is defined and distinguished from the epidermal ectoderm by the expression of factors such as Fgf, BMP inhibitors and SoxB proteins. Soon after the initial molecular separation the neural plate is starting to become morphologically different from the rest of the ectoderm. The neural progenitors change shape to become more columnar and elongated, causing a thickening of the neural plate. Eventually cells at the border between the neural- and non-neural ectoderm start to bend causing a ridge like structure called the neural fold. The neural folds rise causing the neural tissue to invaginate and fold up on itself to the point when the neural folds meet and fuse. Subsequently the neural ectoderm will pinch off and separate from the non-neural ectoderm. In this way a tube is formed in the dorsal part of the embryo extending the rostrocaudal axis. This process is called neurulation and the tubelike structure formed is called neural tube. The neural tube will give rise to the different components of the CNS (Smith and Schoenwolf, 1997).

Neurulation start at the anterior half of the embryo and progress toward the anterior following the caudal regression of the organizer. In this way the rostral parts of the embryo and the neural tube will start to develop and mature before the caudal parts. The anterior part of the neural tube is divided into the fore-, mid- and hindbrain regions that in the adult comprise cortex to brain stem. The part caudal to the hindbrain is called the neural tube and will eventually form the spinal cord (Altmann and Brivanlou, 2001).

The caudal most region of the neural plate surrounding the migrating primitive node is called the caudal stem region. Cells within this region is kept in a stem cell like state due to the presence of Fgf produced by the organizer and the underlying unsegemented paraxial mesoderm(Diez del Corral et al., 2002; Riese et al., 1995; Shamim and Mason, 1999). As the node migrates caudally the neural plate, rostral to the node, undergo neurulation and the paraxial mesoderm becomes segmented,

forming the somites. With the formation of the somites there is a switch of the morphogens expressed by the mesoderm. From having an unsegmented mesoderm producing Fgf the somites start to express the enzyme Retinaldehyde Dehydrogenase 2 (Raldh2) that will synthesizes Retinoic Acid (RA). RA is counteracting the effect of the Fgf pathway and the increasing RA concentrations will promote patterning and differentiation of the neuronal progenitors within the neural tube (Diez del Corral et al., 2002; Diez del Corral et al., 2003; Papalopulu and Kintner, 1996).

## 1.3 DORSOVENTRAL PATTERNING OF THE NEURAL TUBE

Ventral to the neural tube is a tubelike mesoderm structure that reaches form the hindbrain to the caudal most part of the neural tube. This structure is called notochord and is important in inducing the dorsoventral patterning of the neural tube. The notochord is formed by a set of mesoderm cells that, as soon as they have left the regressing node, aggregate. Immediately after the formation of the notochord it will start to produce the morphogen Sonic Hedgehog (sHH) a signal that will induce the floor plate in the ventral most part of the neural tube. Floorplate and notochord will create a ventral to dorsal sHH gradient, across the neural tube, essential in establishing the patterning of the neuronal progenitors within the ventral part of the neural tube(Diez del Corral et al., 2003; Ericson et al., 1995; Placzek et al., 1990; Yamada et al., 1991). The appearance of patterning genes within the neural tube coincides with the start of RA production in the somites. RA has been shown to induce some of the patterning genes independent of sHH signaling. It has also been suggested that Fgf is blocking sHH signaling. With the appearance of RA this block is lost allowing the initiation of patterning gene expression (Novitch et al., 2003; Pierani et al., 1999; Wilson et al., 2004).

Dorsoventral patterning is depending on the dorsoventral concentration gradient of sHH across the neural tube. This concentration gradient is initiating transcription of different sets of transcription factors throughout the dorsoventral axis of the neural tube, similar to the patterning of the developing limb and the somites (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Niswander et al., 1994; Riddle et al., 1993). In the neural tube sHH will manage the expression of a group of transcription factors belonging to the homeobox transcription factors. This group is divided into

ClassI (Pax7, Irx3, Dbx1, Dbx2 and Pax6) and ClassII homeobox factors (Nkx2.2 and Nkx6). ClassII factors require sHH for their expression and the different members have a unique concentration threshold to be expressed. The lower sHH concentration needed the more dorsal the factor will be expressed. ClassI factors are also regulated by sHH in a concentration dependent manner but instead of being expressed in the presence of sHH they are repressed. The repression of ClassI factors imposed by sHH is mediated through the ClassII factors. In this way cross-repressive pairs of ClassI and ClassII homeodomain factors are responsible for establishing discrete domains in the spinal cord that later will generate specific types of neurons (Briscoe et al., 2000; Ericson et al., 1997; Sander et al., 2000).

Floor plate and notochord does not only produce sHH but also BMP repressors. The expression of these repressors is important in setting up the dorsoventral patterning in the neural tube (Liem et al., 2000; McMahon et al., 1998). In addition to the floor plate there is a second signaling centre in the dorsal part of the neural tube called the roof plate. The roof plate is induced by BMP expressing cells in the overlying ectoderm. Through the production of BMP4, -5 and –7, the roof plate induces dorsal patterning genes such as Pax3 and Msx. Ablation of the roof plate or deletion of BMP signaling causes a dorsal expansion of the ventral progenitor domains on behalf of the dorsal progenitor domains (Lee et al., 2000; Liem et al., 1997; Liem et al., 1995; Nguyen et al., 2000; Timmer et al., 2002; Wine-Lee et al., 2004).

In addition to producing BMP the roof plate is also producing Wnt. Wnt proteins are responsible for promoting proliferation in the neural tube but they are also involved in promoting the dorsal progenitor domains in the neural tube (Megason and McMahon, 2002; Muroyama et al., 2002). Sommer and colleagues have suggested a model where Wnt promote proliferation and BMP differentiation. This would create a balance between the two signaling pathways responsible for coordinating growth and patterning in the dorsal part of the neural tube (Ille et al., 2007).

## 1.4 NEUROGENESIS

The neural tube consists of bipolar cells spanning the width of the neural epithelium. Having one end in contact with the basal lamina of the exterior and the other end bordering the central lumen, or the ventricle. When the neural progenitors exit cell cycle they detach from the ventricle and migrate laterally to line the exterior margin of the neural tube. With time this will divide the neural tube into three regions, the ventricle-, intermediate- and marginal (or mantle zone) zone. Looking at a crossection of the neural tube the innermost region, closest to the ventricle, is called the ventricular zone. This region consists of dividing neural progenitors and is defined by the expression of for instance homeobox-, Notch- and SoxB proteins. As the neural progenitor exit cell cycle and detach from the ventricle it migrate laterally into the intermediate zone. This is a transition state that is characterized by the expression of the first post mitotic neuronal protein NeuroM. Finally the postmitotic neural progenitors settle in the lateral most domain of the neural tube, the marginal zone. Cells in this region express pan-neuronal markers such as Neuronal Nuclei (NeuN), Neuron-specific class III β-tubulin (Tuj1) and Neurofilamnet 1 (NF-1) (Diez del Corral and Storey, 2001; Hollyday, 2001).

The production of RA by the somites does not only coincide with the start of dorsoventral patterning of the neural tube but also with the appearance of post-mitotic neural progenitor markers like NeuroM. NeuroM is one member of a larger family of proneural basic-helix-loop-helix (bHLH) proteins and the onset of their expression seems to be dependent on the somites production of RA (Diez del Corral et al., 2002; Diez del Corral et al., 2003).

### 1.4.1 Proneural Proteins

The proneural proteins were discovered in the late 1970´s as a group of proteins involved in regulating early steps in neural development in *Drosophila*. Most of them are expressed in proliferating neural ectoderm progenitors, promote neurogenesis and share the basic Helix-Loop-Helix (bHLH) domain. In these early studies it was shown that the proneural proteins need dimerizing partner to bind the recognized consensus

sequence CANNTG (E box) to regulate transcription. To be able to bind DNA proneural proteins heterodimerize with a group of bHLH proteins called E-proteins that are related to the *Drosophila* gene *Daughterless*. Both dimerization and DNA binding is mediated through the bHLH domain, where the basic domain is responsible for binding to the DNA and the HLH domain for the dimerization. (Bertrand et al., 2002; Ellenberger et al., 1994; Ferre-D'Amare et al., 1993; Garcia-Bellido, 1979; Murre et al., 1989a; Murre et al., 1989b; Villares and Cabrera, 1987). A number of proneural proteins are expressed throughout the ventricular zone and their expression is patterned spatially and temporally promoting differentiation of the neural lines derived from the progenitor domain where they are expressed. (Guillemot et al., 1993; Lee et al., 1995; Lo et al., 2002; Ma et al., 1996; Mizuguchi et al., 2001; Parras et al., 2002; Pattyn et al., 2000).

Neural progenitors have the ability to form three main groups of cell types in the CNS, neurons, oligodendrocytes and astrocytes. The processes through which oligodendrocytes and astrocytes are formed are commonly called gliogenesis and start after the onset of neurogenesis. One consequence of deleting proneural proteins, apart from reduced neurogenesis, is premature gliogenesis. This temporal shift has been explained by the ability of proneural proteins to block gliogenesis during CNS development (Nieto et al., 2001; Sun et al., 2001; Tomita et al., 2000).

Induction of the neural fate is tightly linked to cell cycle arrest. Besides promoting neurogenesis, vertebrate proneural proteins also induce cell cycle exit through promoting the expression of Cycline Dependent Kinase (CDK) Inhibitors such as p21 and p27. CDK are important checkpoint proteins regulating the transition of dividing cells between the different stages of the cell cycle. The CDK inhibitors are repressing these transitions inducing cell cycle arrest (Farah et al., 2000; Mizuguchi et al., 2001; Mutoh et al., 1998; Novitch et al., 2001).

### 1.4.2 Notch signaling

Whereas proneural proteins promote neurogenesis there are a number of proteins and signaling pathways that oppose this activity. One important signaling pathway that is deeply involved in repressing the expression and activities of proneural proteins is the

Notch signaling pathway. The Notch proteins are membrane receptors that are expressed on the surface of progenitor cells throughout the ventricular zone. Signaling through the Notch receptors will keep the receiving cell in a progenitor state. The canonical Notch signaling involves receptor binding to the ligands Delta and Jagged (Serrate in *Drosophila*). Ligand binding promotes proteolytic cleavages of the Notch receptor resulting in the release of the Notch intracellular domain (NICD). The NICD is translocated to the nucleus where it co-operates with the transcription factor CSL to promote transcription of the target genes of the Hes/Her/Esr family. These proteins repress the expression of proneural genes (Fortini and Artavanis-Tsakonas, 1994; Schroeter et al., 1998; Struhl and Adachi, 1998). The interaction between Notch signaling and the proneural proteins go further beyond the repression of the proneural genes by Hes/Her/Esr. Proneural proteins up regulate the expression of the Notch ligands that will increase the canonical Notch signaling in the neighboring cells. In this way increased proneural protein expression in one cell will decrease the proneural protein expression in its neighboring cells through increased canonical Notch signaling. This will lead to differentiation of the cell expressing proneural proteins and ensure that the progenitor pool is maintained by inducing Notch expression in the neighboring cells. This intercellular communication between proneural proteins and canonical Notch signaling is called lateral inhibition (Artavanis-Tsakonas et al., 1999; Castro et al., 2006; Chitnis and Kintner, 1996; Coffman et al., 1993; Louvi and Artavanis-Tsakonas, 2006).

## 1.5 SOX TRANSCRIPTION FACTORS

In 1973 Goodwin, Sanders and Johns defined a group of non-histone nucleosome proteins called the ”High Mobility group” (HMG), a name they were given according to their mobility on an SDS-PAGE gel(Goodwin et al., 1973). This diverse group of protein contain a family of proteins called the HMG domain proteins that is characterized by having a conserved DNA binding domain, the so called HMG domain or HMG box. The members of this group can be divided into two subfamilies based on how many HMG boxes they have. The first subfamily has multiple HMG boxes and bind to DNA with low sequence specificity whereas the second subfamily has one single HMG box and bind to DNA with some sequence specificity. Sox proteins are members of the latter subfamily having one HMG box. The Sox group

was founded by the discovery of the *Sry* gene (Sex determining *R*egion Y), the gene on the Y chromosome responsible for male sex differentiation (Berta et al., 1990; Gubbay et al., 1990; Sinclair et al., 1990). Today the Sox group (Sry related HMG box) has grown to a total of around 30 genes. Based on structure and function the Sox group has been divided into a total of 10 subgroups named SoxA-SoxJ. They are expressed in most organisms and are involved in regulating development of a wide variety of cell lineages. Depending on the context they have been shown to act as both activators and repressors of gene transcription (Bernard and Harley; Bowles et al., 2000; Murakami et al., 2001).

The Sox proteins have several functional domains. The HMG box alone harbor a number of important functions, it bend and bind DNA, interacting with partner proteins and contain signals for nuclear import and export (Bernard and Harley). The HMG box is a region of approximately 80 amino acids comprising three α-helixes that together form a L-shaped structure. When binding to DNA, the HMG box causes a bend in the DNA backbone(Paull et al., 1993; Pil et al., 1993; Weir et al., 1993; Werner et al., 1995). It has been suggested that this bend will cause a conformational change of the nucleosome, increasing the accessibility to binding sites that earlier was hidden within the nucleosome (Ferrari et al., 1992; Grosschedl, 1995; Kornberg and Lorch, 1995). This function has been shown for the HMG protein Lef-1but there is today no direct evidence for Sox proteins having this function(Giese et al., 1995). There are studies supporting the importance of the DNA bending ability among the Sox proteins. In a Sox2 mutant that is not able to bend DNA, but still able to bind DNA, the protein activity was reduced causing mutant phenotypes *in vivo* (Pontiggia et al., 1994; Scaffidi and Bianchi, 2001).

As important as bending the DNA is the Sox proteins ability to bind DNA. Unlike most transcription factors the Sox proteins bind to the minor grove instead of the major grove(Harley et al., 1992; Love et al., 1995). *In vitro* Sox proteins recognize the consensus site 5´-WWCAAW-3´ (W being A or T). All Sox proteins bind this consensus with individual preferences for the nucleotides flanking the core sequence(Harley et al., 1994; Lefebvre et al., 2007; Mertin et al., 1999). It is important to note that these results come from *in vitro* experiments. When looking at the identified Sox target genes and the Sox sites in their regulatory regions

(enhancers), all of them do not fit to the consensus site(Lefebvre et al., 1997; Lefebvre et al., 1998).

All Sox proteins are able to bind the consensus site *in vitro* but they do not indiscriminately bind and regulate the expression of all known target genes *in vivo*. Sox proteins bind to DNA with low affinity and need co-factors to stabilize their binding. This suggests that the gene regulatory specificity of Sox proteins is decided partly by the interaction with different co-factors. Many of the identified co-factors are other transcription factors dependent on DNA binding. Looking at the Sox sites in the enhancers of their target genes they are often flanked by their co-factors target sites with only a handful of bases separating them(Yuan et al., 1995). This close proximity seem to be necessary to form a protein/DNA complex stable enough to regulate transcription. Mutating one of the binding sites, or changing the distance between them, is often enough to disrupt the stability of the active protein/DNA complex (Ambrosetti et al., 1997; Kamachi et al., 2001). Interaction of Sox proteins with their co-factors can to some extent explain how specificity is achieved regarding target site selection *in vivo*. It can also explain how the same Sox proteins can regulate different genes, in different tissues, at different time points during development (Kamachi et al., 2000; Lefebvre et al., 2007).

There are a number of examples where the interaction with co-factors has been mapped to the C-terminal end of the Sox proteins(Marshall and Harley, 2001; Poulat et al., 1997) but a surprisingly large amount of the interactions have been mapped to the conserved HMG domain (De Santa Barbara et al., 1998; Di Rocco et al., 2001; Hosking et al., 2001; Wilson and Koopman, 2002). Here are a handful of examples to illustrate the complexity of the interaction between Sox factors and their partner proteins.

For instance on the δ-crystallin minimal enhancer DC5 it was shown that the C–terminal of Sox2 is important for the activation of the enhancer whereas the HMG domain seemed replaceable. The DC5 enhancer is activated by Sox2 and Pax6 but could not be activated by Sox9 and Pax6. Making a chimeric protein fusing the HMG domain of Sox9 to the C-terminal domain of Sox2 rendered in a functional protein, highlight the importance of the C-terminal of Sox2 when binding the co-factor Pax6.

In the same study it was also shown that Sox9 depend on both the HMG domain and the C-terminal domain to activate its target gene *Col2a1* (Kamachi et al., 1999).

A domain in the C-terminal tail region behind the third α-helix of the HMG domain in SoxE factors has been shown to be essential to recruit and interact with a wide range of protein. These interactions were shown in a yeast two hybrid screen *in vitro*, therefore the biological relevance of these interaction has to be confirmed *in vivo*(Wissmuller et al., 2006). Moreover, members of the SoxD and SoxE groups are forming homo- and hetero dimers an interaction that is mediated through the HMG domain (Bernard et al., 2003; Lefebvre et al., 1998; Stolt et al., 2006).

Most of the Sox proteins are transcriptional activators, but there are some Sox proteins that are repressors. In the SoxB group the SoxB1subgroup (Sox1-3) consist of transcriptional activators whereas the members of the SoxB2 group (Sox14 and Sox21) are transcriptional repressors. The two subgroups show a high sequence similarity overall but they differ in the C-terminal region where the SoxB1 group have an activator domain whereas the SoxB2 group have a repressor domain. Looking at the whole Sox protein family this seems to be the general trend having the transactivator domain in the C-terminal region of the protein but there are exceptions (Chew and Gallo, 2009; Kamachi et al., 2000; Uchikawa et al., 1999). Sox5 and Sox6, members of the SoxD family, do not have a C-terminal transactivation domain but they are still able regulate transcription, both as activators and repressors. They rely on their partner proteins to activate or repress transcription. In this way they can both activate and repress transcription depending on the partner factors present(Hattori et al., 2008; Murakami et al., 2001; Stolt et al., 2008).

## 1.6 SOXB TRANCRIPTION FACTORS IN CNS DEVELOPMENT

### 1.6.1 The SoxB1 Group

Preceding neural induction the developing embryo is expressing a number of Sox proteins belonging to the SoxB1 group. In mouse, Sox2 is present as maternal transcript and start to be expressed by the embryo at the morula stage. At late blastocyst stages it is expressed throughout the whole hypoblast, the presumptive

embryo (Avilion et al., 2003; Wood and Episkopou, 1999). Sox2 is not the only SoxB1 protein expressed at these earl developmental stages. Sox3 is expressed in the epiblast of the gastrula and Sox1 is starting to be expressed in the neural ectoderm during neural induction(Wood and Episkopou, 1999). All of the studies presented in this thesis are made in chicken where the expression and function of the SoxB1 factors are similar, with one deviation from the pattern described. In chicken development Sox3 is expressed earlier then Sox2. Sox3 is expressed at Hamburger Hamilton stage 1-3 (H.H. 1-3; 12-13 hours incubation) throughout the epiblast, before the appearance of the primitive streak. Sox2 on the other hand is starting to be expressed at H.H. 4 (18-19 hours incubation) within the neuronal ectoderm at the time of neural induction(Rex et al., 1997a).

Despite differences in the onset of their expression, SoxB1 factors are much alike. At the time of neural induction their expression is restricted to the neural ectoderm where they seem to be redundant in function and in their role as promoters of the neural stem cell state (Bylund et al., 2003; Collignon et al., 1996; Graham et al., 2003; Li et al., 1998; Pevny et al., 1998).

The expression of the factors Sox2, Nanog, Oct3/4, myc and Klf4 is characteristic for the pluripotent stem cell state and involved in keeping embryonic stem cells in an undifferentiated state. This set of transcription factors is not only able to maintain the stem cell state. They can also induce embryonic stem cell gene expression in somatic cells, reprogramming them into a pluripotent progenitor state(Nakagawa et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

To find out more about stem cell maintenance during embryonic development and in the adult organism a lot of attention has been put into finding out more about how Sox2 expression is regulated. A large number of enhancer regions connected to the Sox2 gene have been identified. They are active at different time points and in different tissues during development forming a spatiotemporal patchwork of enhancers regulating Sox2 expression during development(Miyagi et al., 2004; Uchikawa et al., 2003; Zappone et al., 2000). One of the regulatory regions (SRR2) is expressed in embryonic stem cell and is bound and activated by both Sox2-Oct6 and Sox2-Oct3/4 complexes (Tomioka et al., 2002). The Sox2-Oct3/4 complex is also responsible for driving the transcription of a number of proteins expressed in the stem

cell and neuronal progenitor niche such as, Fgf4, UTF1, Nestin and Nanog (Ambrosetti et al., 1997; Kuroda et al., 2005; Nishimoto et al., 1999; Tanaka et al., 2004; Yuan et al., 1995).

With the onset of neurogenesis the SoxB1 factors are restricted to the ventricular zone. Their expression is quickly down regulated as cells stop proliferating and migrate laterally into the intermediate zone and is completely gone as the cells settle in the marginal zone. Over expression studies in chicken neural tube indicate that the SoxB1 factors are blocking neurogenesis downstream of proneural protein activity. In addition it seem as the proneural proteins activity to drive neural progenitor cells toward differentiation is dependents on their ability to down regulate the SoxB1 factors expression. How proneural proteins exert this down regulation is not known (Bylund et al., 2003; Graham et al., 2003).

### 1.6.2 The SoxB2 group

There is also a second SoxB sub-group called SoxB2. In most vertebrates this groups consist of two proteins namely Sox21 and Sox14. Sox14 is expressed during a brief period in post mitotic V2 interneuron and is not dealt with in this thesis. Sox21 is starting to be expressed at H.H.3 in chicken around the time when Sox2 and Sox3 are starting to be expressed. Similar to the expression of the SoxB1 proteins, Sox21 expression is restricted to the neural ectoderm during neural induction. Unlike the SoxB1 proteins that are evenly expressed in all neuronal precursors, the Sox21 expression is more patterned forming dorsoventral stripes along the entire rostrocaudal axis(Cunningham et al., 2008; De Martino et al., 1999; Hargrave et al., 2000; Rex et al., 1997b; Rimini et al., 1999).

Similar to the SoxB1 factors the expression of Sox21 is restricted to the ventricular zone as neurogenesis is initiated, except for the post mitotic, $Sox14^{+}$, V2 interneurons. Sox21 expression is patterned along the dorsal ventral axis in the ventricular zone with higher expression in three domains (dorsal, medial and ventral) extending the rostrocaudal axis of the neural tube (Rex et al., 1997b; Rimini et al., 1999). All SoxB factors have been shown to bind and regulate similar target genes in vitro but Sox21 differ from the SoxB1 factors in being a transcriptional repressor

(Rimini et al., 1999; Uchikawa et al., 1999). In contrast to this Sox21 was shown to mediate transcriptional activation of the μ Opioid receptor distal promoter in vitro (Hwang et al., 2003). Over expression of the Sox21b gene during early zebrafish development caused various degrees of dorsalization. At early stages of gastrulation Chording expression was expanded and the later phenotypes ranged from a moderate tail shortening to anterior duplication of the body axis. In line with these results depleting Sox21a caused a ventralization of the embryo(Argenton et al., 2004). In another study Sox21 was shown to repress neurogenesis in a similar way as the SoxB1 group. This study was performed employing the *in vitro* model where Nerve Growth Factor (NGF) induces neurogenesis in PC12 cells. Over expressing Sox21 while inducing neurogenesis with NGF significantly reduced neurite outgrowth, a repression that was alleviated by the presence of the Sox21 cofactor YB-1(Ohba et al., 2004).

# 2 AIM

## 2.1 PAPER I

In this study we wanted to investigate the role of Sox21 in regulating neurogenesis. We did this by modulating the expression levels of Sox21 in the developing chicken neural tube using *in ovo* electroporation.

## 2.2 PAPER II

In this study we wanted to gain further insight into how the transcription of Sox21 is regulated. We did this by identifying and analyzing Sox21 enhancers in the chicken neural tube.

## 2.3 PAPER III

In this study we wanted to investigate the functional relationship between the Notch signaling and SoxB1 proteins. Both signaling pathways have been described key roles in establishing and maintaining the neural precursor pool but it is unsettled whether they use similar or distinct mechanisms to control progenitor maintenance.

# 3 RESULTS

## 3.1 PAPER I

In this study we show that Sox21 is expressed within the ventricular zone and that the expression is down regulated as the cells exit cell cycle and migrate out into the intermediate zone. Sox21 promote neurogenesis by counteracting the activity of SoxB1 proteins. The balance of SoxB1 and Sox21 activities decide whether the neural progenitor should remain as a progenitor or commit to differentiation. We also suggest that the proneural proteins Mash1 and Ngn2 promote differentiation by upregulating Sox21 expression.

## 3.2 PAPER II

In this study we present a Sox21 enhancer (Sox21D) that is active in Sox21 positive neural progenitors that is involved in the transcriptional regulation of *Sox21*. Sox1-3 are activating the Sox21D enhancer *in vitro* as well as promoting endogenous *Sox21* transcription when overexpressed in the chicken neural tube. SoxB1 proteins are activating Sox21D in synergy with E proteins, a synergy that is dependent on one E box within the Sox21D enhancer. By mutating this E box the enhancer activity is greatly reduced. Proneural proteins, like Ngn2, are not activating the enhancer but rather interfere with the transcriptional activation promoted by Sox1-3. The ability of Ngn2 to block this activation of the Sox21D enhancer is increased in the presence of E proteins. These results suggest that Sox1-3/E proteins are responsible for regulating the balance between Sox21 and Sox1-3 activities in neural progenitors. This balance is important to decide whether neural progenitors should remain undifferentiated or commit to differentiation. The results further imply that Ngn2 could interfere with the Sox1-3/E protein activity blocking the expression of their downstream targets.

### 3.3 PAPER III

In this study we show that Notch signaling block the expression of proneural proteins and E proteins. The repression of proneural proteins is mediated through the activation of Hes whereas the repression of E protein expression is Hes independent. The ability of Notch signaling to maintain the neural progenitor pool is dependent of Sox1-3 activities. Consequently, active Notch signaling is unable to repress neurogenesis in the absence of Sox1-3 function. Both Sox1-3 and Notch block the function of proneural proteins. However, in contrast to Notch, Sox1-3 do not repress proneural gene expression but alter their activity at a post transcriptional level. Altogether these data show that Notch and Sox1-3 use different mechanisms to oppose proneural proteins to preserve the undifferentiated neural precursor pool.

# 4 DISCUSSION

From Paper I we showed that neurogenesis is promoted by increasing Sox21 expression levels. This finding is contradicted by the results presented by Ohba *et al*. 2004. In this study Sox21 is repressing Nerve Growth Factor (NGF) induced neurogenesis in PC12 cells, a repressive activity that is counteracted by the addition of the Sox21 co-factor, YB-1. The different conclusions might have to do with how neurogenesis is scored in the two different studies. Sox21 is down regulated when neural progenitors become post mitotic and over expression of Sox21 does not induce a full range of neuronal phenotypes (Paper I), suggesting that the main role of Sox21 is to suppress the progenitor characters rather then to promote the expression of neuronal properties. The results presented by Ohba *et al*. suggest that Sox21 has to be down regulated or inactivated by cofactors such as YB-1 to allow the expression of the neuronal characters associated with NGF induced neurite outgrowth.

In Paper I we conclude that the balance between Sox1-3 and Sox21 activities are important for maintaining the progenitor state. We also showed that the proneural proteins are able to shift this balance by up regulating Sox21 expression in favor of neurogenesis. We know that Ngn2 do not activate Sox21 transcription through the Sox21D enhancer leaving us with two alternatives. Either Sox21 transcription is upregulated indirectly or through an unidentified Sox21 enhancer.

In Paper II we provide further evidence for how the balanced expression of Sox1-3 and Sox21 is maintained within the neural progenitors. Looking at the expression patterns, of Sox21 and Sox1-3, they are almost completely overlapping (Paper I). In line with this expression pattern we found that Sox1-3 are responsible for activating Sox21 expression via the Sox21D enhancer. We also show that Sox21 is able to repress the Sox1-3 mediated activation of its own enhancer, strengthening the notion that Sox1-3 and Sox21 regulate the expression of a similar set of target genes. Taking all these results together it is tempting to suggest a model where Sox1-3 activate Sox21 expression plus their own expression. In addition to this, Sox21 bind and repress the same regulatory regions to balance the Sox1-3 activity. This would create a regulatory loop between Sox1-3 and its antagonist Sox21. There are no identified

Sox1-3 enhancers, active in the neural tube, that are activated in this feedback fashion but there is one Sox2 enhancer active during earlier stages of development that is activated by Sox2 in synergy with Oct3/4(Tomioka et al., 2002). This hypothetical model could explain the balanced expression of Sox1-3 and Sox21 in neural progenitors.

Sox1-3 acts in synergy with E proteins to activate Sox21 transcription, an activation that is repressed by the proneural protein Ngn2. The exact mechanism by which this repression is mediated it unclear but it seems to be independent of binding to DNA via E-boxes. This result suggests a novel role in which Ngn2 interfere with Sox1-3 transcriptional activation as a step in promoting neurogenesis. Together with earlier results this suggest a posttranslational crossrepressive interaction between Sox1-3 and Ngn2(Bylund et al., 2003). The details surrounding this interaction is not clear but the key to this unresolved issue might be involving their common co-factors, the E proteins. Indeed it has previously been shown that Sox1-3 block the ability of Ngn2 to promote neurogenesis. Thus, it is possible that the balance of Sox1-3 and Ngn2 activities determines whether cells should remain progenitors or commit to differentiation.

Lets recapitulate. In Paper I we conclude that Ngn2 upregulate Sox21 expression to repress progenitor features. Later in Paper II we suggest a mechanism where Ngn2 downregulate the expression of progenitor characters by interfering with Sox1-3 activities. These results suggest two separate mechanisms in which Ngn2 would downregulate progenitor features. There are still some unresolved questions regarding the compatibility of the two mechanisms. The main question is the contradictory effect mediated by Ngn2 on Sox21 transcription. Forced expression of Ngn2 upregulate Sox21 transcription *in ovo* whereas Ngn2 block the SoxB1 mediated activation of the Sox21D enhancer. By identifying additional Sox21- and progenitor gene enhancers we could gain further understanding of how Ngn2 regulate Sox21 expression and neurogenesis.

In Paper III we conclude that Notch signaling and Sox1-3 are maintaining the progenitor properties through different mechanisms. This study supports the notion that Sox1-3 are able to repress proneural protein activities posttranslationally, an activity that is independent of Notch signaling. One the contrary the ability of Notch

signaling to promote progenitor cell maintenance depends on Sox1-3 activities. In the presence of a dominant negative Sox3, Notch signaling is unable to maintain cells in a progenitor state. E proteins could be a key factor in how Notch signaling is maintaining neural progenitors. Notch signaling is blocking the expression of E proteins. It is well established that proneural proteins need E proteins for their activity and reducing the amount of E proteins would diminish the activity of the proneural proteins. In addition Notch signaling is reducing the expression of Sox21. By decreasing the E protein expression levels, Notch will reduce Sox21 expression in keeping with the results presented in Paper II. These results again suggest that E proteins might be common denominators between the Notch pathway and Sox signaling.

The Sox21 knockout mouse exhibit epidermal hyperplasia indicating that Sox21 is important in regulating proliferation and differentiation of epidermal keratinocytes (Kiso et al., 2009). Apart from cyclic hair loss no phenotype was described in the CNS. From our results presented in Paper I it would be expected that the rate of differentiation and the number of neurons would be affected. The lack of phenotype in the CNS could be explained by an increased expression of redundant Sox proteins. Sox14, Sox5 and Sox6 are three possible candidates that might be responsible for this redundancy. It still has to be confirmed whether this is the case by analyzing the expression of these genes in the Sox21 knockout. One possible reason for the lack of CNS phenotype in the knockout and the decreased neurogenesis seen in our RNAi electroporation experiment could be due to the acute effect mediated through the RNAi. At the time points when we are decreasing the Sox21 levels, in chicken, neurons are born in large numbers. Before any redundant signaling system has been initiated, the acute reduction of Sox21 has already caused a significant decrease in the number of differentiating neurons.

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Enjoy!

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