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Effect of NEUROD4 and SCRATCH2 transcription factors on the control of proliferation, migration and transcription in embryonic neural progenitors

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Efeito dos fatores de transcrição NEUROD4 e SCRATCH2 no controle da proliferação, migração e transcrição em progenitores neurais embrionários

Dissertação apresentada ao Programa de Pós-graduação em Mestrado em Biologia de Sistemas do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do título de Mestre em Ciências.

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RESUMO

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SCRATCH2 e NEUROD4 são fatores de transcrição co-expressos na zona intermediária do tubo neural embrionário, onde progenitores neurais que emergiram recentemente do ciclo celular residem e irão migrar para as camadas mais externas do tubo neural. Tanto o NEUROD4 quanto o SCRATCH2 reconhecem a sequência E-box para a regulação da transcrição de genes alvo, sugerindo que eles podem atuar juntos na regulação gênica no início da diferenciação neural. Com isso, propomos a hipótese de que esses fatores de transcrição podem inibir a proliferação celular ou regular genes relevantes para os estágios iniciais de diferenciação. Para isso, superexpressamos NEUROD4 e SCRATCH2 no tubo neural por eletroporação in ovo. Em seguida, quantificamos o número de células mitóticas (ppH3-positivas). A superexpressão de NEUROD4 reduziu a porcentagem de células positivas para pHH3. No entanto, a superexpressão de SCRT2 não alterou esse índice mitótico. Finalmente, para identificar possíveis genes-alvo regulados por SCRT2, realizamos análises de bioinformática em dados de RNAseq e CUT & RUN realizados em tubos neurais superexpressando SCRT2. Nossa análise sugere que os genes LHX9 e BARHL1 são possíveis alvos indiretos do SCRT2, regulados pela ativação dos fatores de transcrição NEUROG1 e ASCL1. Em seguida, usamos os dados de scRNAseq do tubo neural de camundongo E11.5, E12.5 e E13.5 para correlacionar os níveis de expressão de SCRT2 e NEUROD4 com os genes-alvo potenciais em células individuais.

Nossos dados mostram que nas células neurais E11.5, aquelas que apresentam níveis elevados de SCRT2, o fator de transcrição ISLET-1 não é expresso. ISLET-1 é um marcador para o interneurônio dorsal 3 na medula espinhal. Por outro lado, ISLET-1 é expresso em células com altos níveis de NEUROD4. Em E12.5, SCRT2 é expresso em duas linhagens celulares que expressam os marcadores para o interneurônio dorsal 4 (DI4) e 5 (DI5). Os níveis de expressão

de NEUROD4 e ISLET-1 eram muito baixos neste estágio. Em 13,5 o SCRT2 permaneceu associado às subpopulações DI4 e DI5. Assim, propomos que SCRT2 reprime ISLET-1 em precursores pós-mitóticos iniciais e pode desempenhar um papel na restrição do domínio DI3. Além disso, em estágios posteriores, o SCRT2 pode contribuir com o estabelecimento ou manutenção dos compartimentos DI4 e DI5.

Palavras chave: NEUROD4. SCRATCH2. Neurogênese. Proliferação. Atividade transcricional.

ABSTRACT

DE LA CRUZ ANTICONA, S.M. Effect of NEUROD4 and SCRATCH2 transcription factors on the control of proliferation, migration and transcription in embryonic neural progenitors. 2021. (108). Dissertação Mestrado em Biologia de Sistemas – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2021.

SCRATCH2 and NEUROD4 are transcription factors co-expressed in the intermediate zone of the embryonic neural tube, where neural progenitors that have recently emerged from the cell cycle, reside and will migrate to the outermost layers of the neural tube. Both NEUROD4 and SCRATCH2 recognize the E-box sequence for transcriptional regulation of target genes suggesting that they can act together in gene regulation in the beginning of neural differentiation. With this, we propose the hypothesis that these transcription factors can inhibit cell proliferation or regulate genes relevant to the initial stages of differentiation. For this, we overexpressed NEUROD4 and SCRATCH2 in the neural tube by *in ovo* electroporation. We then quantified the number of mitotic cells (ppH3-positive). Overexpression of NEUROD4 reduced the percentage of pHH3 positive cells. However, overexpression of SCRT2 did not alter this mitotic index. Finally, to identify possible target genes regulated by SCRT2, we performed bioinformatics analysis on RNAseq and CUT&RUN data performed neural tubes overexpressing SCRT2. Our analysis suggests that the LHX9 and BARHL1 genes are possible indirect targets of SCRT2, regulated through the activation of the transcription factors NEUROG1 and ASCL1. We then used scRNAseq data from E11.5, E12.5 and E13.5 mouse neural tube to correlate SCRT2 and NEUROD4 expression levels with the potential target genes in individual cells.

Our data show that in E11.5 neural cells those that have high levels of SCRT2, the transcription factor ISLET-1 is not expressed. ISLET-1 is marker for dorsal interneuron 3 in the spinal cord. Conversely, ISLET-1 is expressed in cells with high levels of NEUROD4. In E12.5, SCRT2 is expressed in two cell lineages that express the markers for dorsal interneuron 4 (DI4) and 5 (DI5). Both NEUROD4 and ISLET-1 expression levels were very low at this stage. In 13.5 SCRT2 remained associated with DI4 and DI5 subpopulations. Thus, we propose that SCRT2 represses ISLET-1 in early postmitotic precursors and might play a

role in restricting the DI3 domain. Also, in later stages, SCRT2 might contribute with the establishment or maintenance of DI4 and DI5 compartments.

Key words: NEUROD4, SCRATCH2, neurogenesis, proliferation, transcriptional activity.

INTRODUCTION

NEURULATION

Morphology

The central nervous system is one of the first systems to form during embryo development, involving different morphological and molecular processes that are essential to determine the correct anatomy of this system (SQUIRE et al., 2012). The Central Nervous System - composed by the brain and spinal cord - derives from the neural tube. The process by which the neural tube is formed is called neurulation and it encompasses a sequence of morphological changes of the cells that participate on it. Once the neural tube is formed, it will give rise to the future central nervous system after a complex progression of differentiation.

The onset of neurulation occurs after gastrulation, when the three embryonic sheets are already formed: ectoderm, mesoderm and endoderm. The neural plate is induced in the ectoderm. The induction process divides the ectoderm into non-neural ectoderm and neuroectoderm. The molecular signals that differentiate the ectoderm into neural and non-neural tissues is primarily dependent on inhibition of the BMP pathway, such as noggin or chordin (REICHERT; RANDALL; HILL, 2013; PATTHEY; GUNHAGA, 2014). The presence of these inhibitors initiates a series of morphological and molecular changes in the ectoderm. We will describe in detail the former here, and address the molecular changes in the next sections.

The first morphological phenotype generated by the BMP inhibitors is an apicobasal growth of the ectodermal cells and expression of early markers of the neural fate (SMITH; SCHOENWOLF, 1997; JIDIGAM et al., 2015). The result of the apicobasal growth is the thickening of the neuroectoderm, forming the neural plate.

The central region of the neuroectoderm will originate the Central Nervous System (CNS) and its associated structures such as the retina; the borders of the neuroectoderm will form the neural crest, which will establish the Peripheral Nervous System (PNS). Once the neural plate is formed, neural folds are arise from its border and it closes upon itself to establish a neural tube with a neural groove at its ventral-most point. In the cephalic region, three hinge points help rotate the tissue. Next, the neural folds meet in the center and merge, thus closing

the neural tube (YAMAGUCHI; MIURA, 2013). Once the folds merge, the neural tube separates from the non-neural ectoderm. As these folds fuse completely, the cells of the neural crest that are at the dorsal edge of the neural tube migrate laterally and ventrally to various specific target tissues (Figure 1) (GILBERT, 2010).

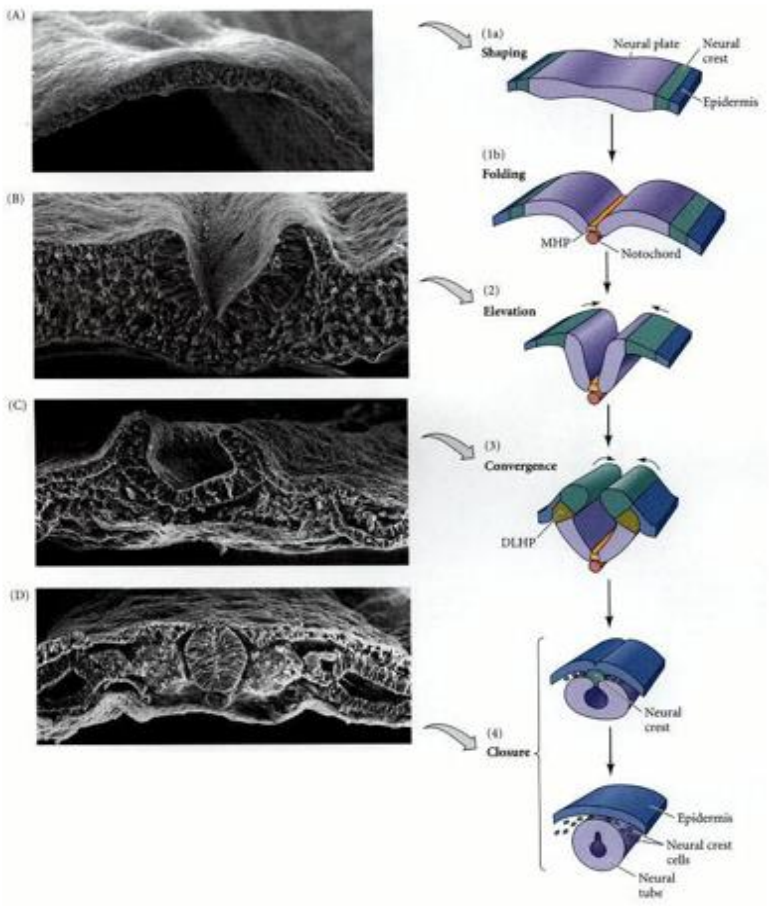

Figure 1. Neurulation in chicken embryo, cross-sectional view.

The formation of the neural tube begins with the neural plate, whose cells are taller than the surrounding ectoderm(1a). This plate has a middle neural hinge (MHP) in its center. The concomitant folding at the MHP and the elevation of the margins of this neural plate initiate the morphogenetic movements of neurulation (2, B). The convergence of the neural folds occurs at the dorsoventral hinge points (3, C). Finally, the neural tube closes leaving out the cells from the borders of the neural plate which will form the neural crest. (4, D). (Gilbert, 2000 based on Smith and Schoenwolf, 1997; and with electron micrograph by K. Tosney and G. Schoenwolf)

The neural tube is a pseudostratified epithelium. This is due the misalignment of the nuclei, caused by a process called interkinetic nuclear migration (NORDEN, 2017). As the number of cells increases, they begin to migrate to the outermost area of the neural tube, finally forming three areas denominated Ventricular Zone, Intermediate Zone and Mantle Zone. These zones are respectively characterized by the presence of cells in constant proliferation, post-mitotic cells and differentiated cells. The formation of these layers occurs gradually. As can be seen in Figure 2, in the earliest stages such as HH19 (HAMBURGUER; HAMILTON, 1951), a greater number of proliferative cells are observed. But, as the embryo develops, these begin to migrate to more external areas and begin to differentiate. In summary, the

Ventricular Zone (VZ) is made up of progenitor cells that divide constantly to increase the cell number in this tissue. In the next layer, the Intermediate Zone (IZ) harbors the early post-mitotic cells from the Ventricular Zone (HUTTNER; BRAND, 1997; GÖTZ; HUTTNER, 2005). After this stage, the outwardly migrating progenitors establish a more external and peripheral layer called the Mantle Zone, where cells already differentiated in neurons are found.(DIEZ DEL CORRAL; STOREY, 2001; MADARSZ, 2013)

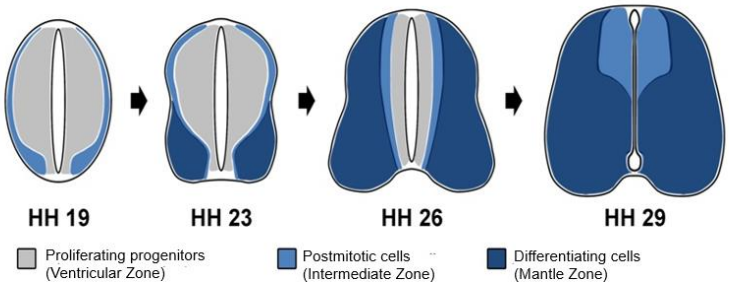

Figure 2. Progressive development of the neural tube.

Different zones are formed as the neural tube develops. The number and type of cells from these layers varies depending on the embryo stage. In stage 19 it can be found more cells in proliferative state than in differentiate state. As the neural tube develops, the number of differentiate cells increases, leaving few proliferative cells.

Each region of the neural tube described above is regulated by different genes that directly influence its cells, directing their behavior (GÖTZ; HUTTNER, 2005).This will be addressed here later.

Cell cycle (nuclear inter kinetic migration), cell cycle exit, differentiation

Nuclear interkinetic migration was first described in 1935 (SAUER, 1935). The nuclei of the neuroepithelium migrate apical-basically and occupy different levels depending on the phase of the cell cycle. When the cell is going through the M phase, the nucleus will be found exclusively in the most apical region of the neural tube. As it progresses to the other phases of the cycle, this nucleus moves to the most basal areas of the tube where phase S occurs. On the way from the apical region to the basal region of the tube phase-G1 takes place while the nucleus migrates in a basal-apical way, the G-2 phase occurs (Figure 3).

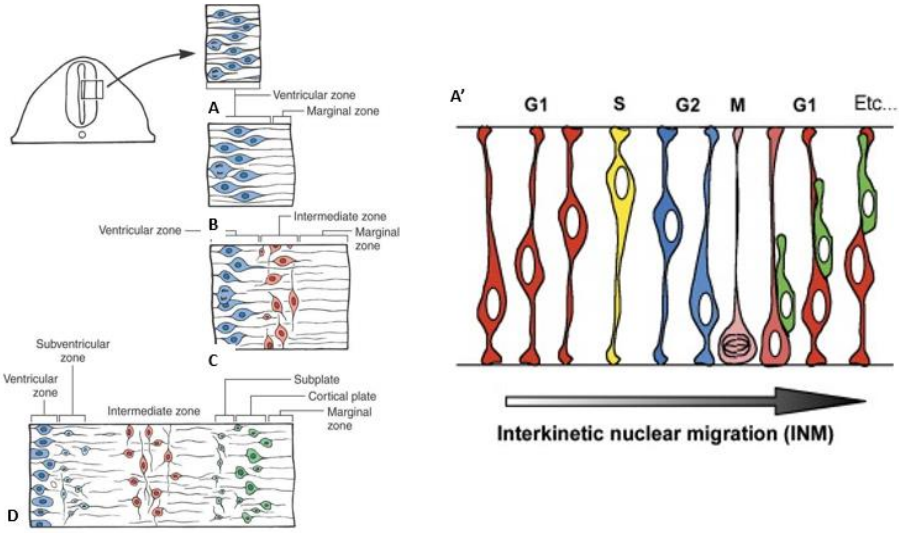

Figure 3. The progenitor cells of the neural tube proliferate at the ventricular zone.

(A) The proliferation is coupled with interkinetic nuclear migration, where the cell body is positioned in specific niches along the apical-basal axis at different stages of the cell cycle (A'). The asymmetric division in this progenitor pool generates radial glial cells and postmitotic neural progenitors (respectively the red and green cells in M –G of A'). Radial glial cells guide the migration of the postmitotic populations to the intermediate zone (B). Thereafter, as differentiation proceeds, these cells migrate further towards external areas to form the marginal zone (C) (Adapted from Preston, M. et al 2012).

Neural progenitors in ventricular zone can undergo two types of mitosis: symmetric and asymmetric. In symmetric division the daughter cells are both identical to the mother, thus maintaining the undifferentiated state of the ventricular zone. The maintenance in this proliferative niche requires the integrity of adherent bonds, like the cell adhesion proteins catenins and cadherins. These proteins hold neural progenitors together and keep them in the notch-positive ventricular zone (NEWGREEN et al., 1997; OTERO et al., 2004; CHALASANI; BREWSTER, 2011; MIYAMOTO; SAKANE; HASHIMOTO, 2015). In turn, the subcellular localization of the adherent bonds depends on the apical-basal polarity of the cells. Also, the participation of different genes that maintain these undifferentiated states are necessary (PATTHEY; GUNHAGA, 2014). Once these cells exit the cell cycle, they form the Intermediate zone. Thus, the intermediate zone harbors neural progenitors that are initiating their differentiation program (LOWELL, 2000).

Early spinal cord differentiation and development

All the cells in the neural tube undergo proliferation and thereafter, cell cycle arrest and differentiation. The differentiation fate is tightly coupled with the anatomical coordinates of the embryonic axis. In other words, neural cell fate is coupled to its localization through patterning events. Several signaling and gradients have been identified (ULLOA; BRISCOE, 2007; ALAYNICK; JESSELL; PFAFF, 2011; KICHEVA; BRISCOE, 2015) For example, the different gradients of Shh and BMP morphogens, secreted from the ventral and dorsal poles respectively, during the beginning of the development of the spinal cord set the primary basis for the formation of its first 14 domains. These domains are composed of transcriptionally different neural progenitors which will later differentiate into neurons. (ZHOU; ANDERSON, 2002; ULLOA; BRISCOE, 2007; ALAYNICK; JESSELL; PFAFF, 2011)The transcription factor code that generates the diversity of neural fates is complex and not completely identified.

Dorso-ventral patterning

The initial patterning of the neural tube is induced by signals from its neighboring tissues. The ectoderm overlying the dorsal region of the neural tube secretes BMP while notochord under the neural tube secretes Shh.(LIEM; TREMML; JESSELL, 1997; LEE; PFAFF, 2003). This generates a bipolar gradient: in the dorsal region BMP concentrations are high and that of Shh low, whereas in the ventral region BMP concentrations are low and Shh high. The transcriptional output of these proliferating progenitor cells is sensitive to these gradients. Different ratios of BMP/Shh induce the expression of a distinct set of transcription factors and proneural genes (Figure 4A). As a consequence, the transcriptome of post-mitotic precursor cells in different dorsal-ventral coordinates is different. This difference, in turn, will lead them into different neuronal fates. In the posterior neural tube, the effect of this dorsal-ventral morphogenetic gradient is clear in the anatomy of the spinal cord. The dorsal-most region houses sensitive interneurons whereas the ventral region is enriched with motoneurons (LAI; SEAL; JOHNSON, 2016).

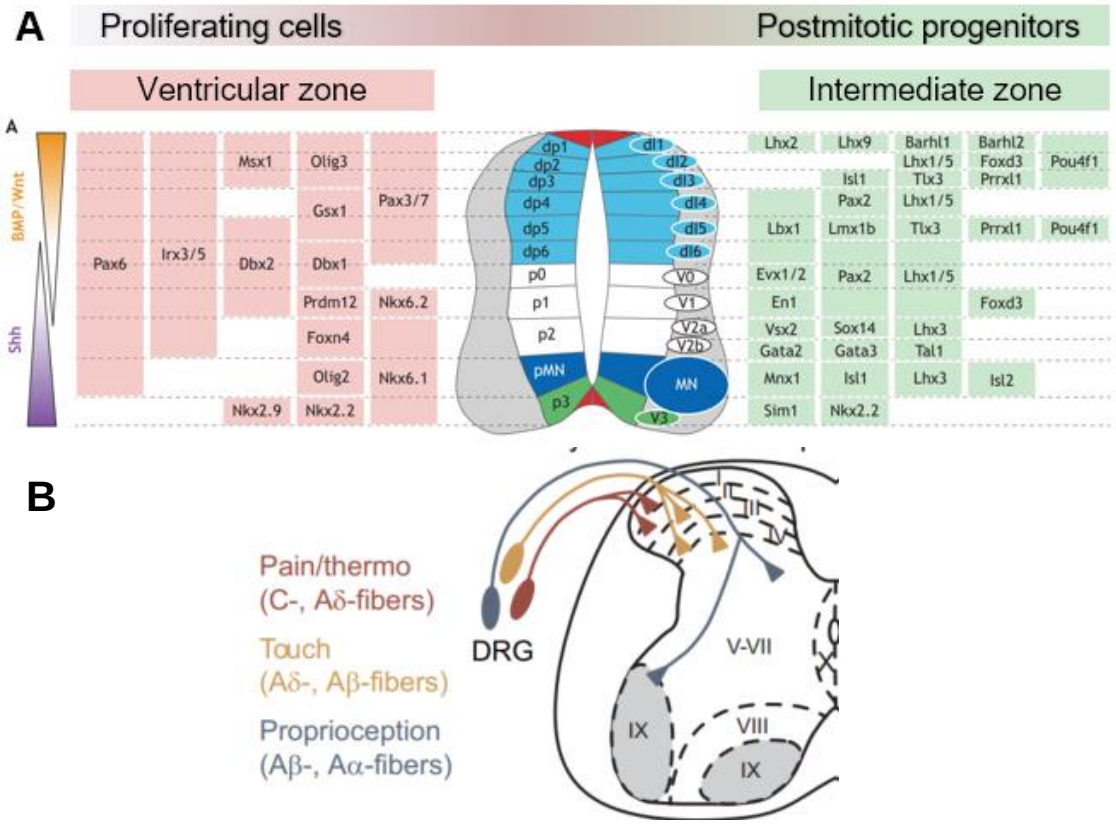

Figure 4. Expression of transcription factors that regulate the formation of progenitor and neuronal diversity in the spinal cord.

The developing spinal cord originates from the posterior neural tube and its spatial coordinates are established by a double gradient of morphogens in the dorsal-ventral (DV) axis. The different concentrations of these morphogens create an extracellular signaling matrix where the position of an individual cell determines its intracellular events. As a result, the tube expresses a combinatorial pattern of transcription factors in the ventricular zone that varies along the DV axis and determines 11 domains. In turn, the unique combination of transcription factors in each compartment results in the expression of different transcription factors in the postmitotic progenitors in the intermediate zone. The different DV domains harbor the progenitor's specific subsets of spinal cord interneurons (DI1-V3) and motoneurons (MN). (Modified from Sagner and Briscoe, 2019) (B) the mature spinal cord is subdivided into 10 laminae. The more dorsal layers are contacted by the dorsal root ganglia (DRG). In general, laminae I-V receive distinct sensorial input. The motoneurons are in laminae IX (Modified from Lai et al., 2016)

In the dorsal region of the embryonic spinal cord the interneurons are divided into two classes. Class A is made up of dorsal interneurons 1, 2, and 3. These neurons are at the dorsal-most portion of the embryonic spinal cord. Accordingly, they depend on the initial input high levels of BMP to differentiate. Class B neurons are ventral to class A neurons, and do not depend on high levels of BMP for its initial establishment. Class B neurons are made up of interneurons 4, 5 and 6.(JESSELL, 2000; GROSS; DOTTORI; GOULDING, 2002; HELMS; JOHNSON, 2003)

In the ventral region, the high levels of Shh induces the expression of transcription factors such as Nkx6.1. The precursors closest to the notochord are exposed to the highest levels of Shh will express Nkx2.2 which will later

differentiate into ventral floor plate neurons (V3). At slightly more dorsal region of the ventral neural tube, the concentration of Shh decreases, and other transcription factors such Pax6 are expressed. These cells which will later differentiate into motoneurons (LEE; PFAFF, 2001; MUHR et al., 2001; GILBERT, 2010).

Dorsal interneurons

The dorsal-ventral patterning of the early neural tube is maintained as the early postmitotic progenitors evolve into neuronal subtypes. The postmitotic progenitors are initially divided into 11 populations. Six of these (DI1-D16) are dorsally located interneuron precursors. The remaining five (V0-V3 and Motoneurons-MN) are ventral to these (Figure 4A). Each of these populations express transcription factors that have been identified as required for the establishment and maintenance of their lineage. For instance, MATH1-expressing progenitors give rise to dI1 cells; while the dI2 group is formed from progenitors that express NEUROG1. For the differentiation of the dI3 and dI5 groups, the expression of ASCL1 is fundamental (reviewed in (LEE; PFAFF, 2001)). Once differentiated, many of the factors that were expressed when they were progenitors will be inhibited and very few will continue to be expressed in later stages. Differentiation of these 11 original subpopulations divides them further into neuronal subtypes that vary in their axonal projections and neurotransmitter expression. The mature spinal cord is dorsal-ventrally organized in 10 laminae (laminae I-X). A general principle, dorsal layers I-II receive pain and thermosensitive input. Laminae II-V are contacted by touch afferents and proprioceptive afferents target more ventral laminae and the ventrally-located motoneurons (laminae IX) (Figure 4B).

Besides undergoing fine-tuning of their cell fate, these differentiating precursors also migrate between the DV compartments, intermingling with the other subpopulations. The final histo-physiological organization of the spinal cord is a complex mixture of embryological origin and functional segregation. Thus, the developmental details that guide the evolution from early postmitotic

precursor to each neuronal subtype is quite unclear. However, it is clear that is relies heavily on the interplay of different transcription factors.

The postmitotic neuronal precursors in DI1 are marked by the transcription factors Lhx2, Barhl1 and Lhx9 (SAGNER; BRISCOE, 2019). DI2 progenitors mostly express the transcription factors Lhx1/5 and Foxd3. Finally, DI3 progenitors express the transcription factors Isl1 and Tlx3. The DI1, DI2 and DI3 group will differentiate into somatosensory glutamatergic neurons (LIEM; TREMML; JESSELL, 1997; JESSELL, 2000; GÖTZ et al., 2015).

Progenitors in DI4 and DI5 form sensory interneurons. DI4 neurons express the transcription factors Pax2 and Lhx1/5 in the postmitotic progenitor cells. They will differentiate into GABAergic inhibitory neurons. (BETLEY et al., 2009; FINK et al., 2014) Post-mitotic DI5 neural progenitors mostly express the transcription factors Lbx1, Tlx3, Pou4f1. They will form somatosensory glutamatergic interneurons (SZABO et al., 2015).

TRANSCRIPTION FACTORS bHLH AND ZN FINGER IN NEURAL DEVELOPMENT

The transcriptomic profile of neural progenitors evolves dynamically until neuronal subtypes are established. Transcriptomic changes rely on the modulation of different cis-regulatory element by the transcription factor code contained in each cell (TF). In turn, the transcription factor code also evolves with the remainder of the transcriptome, thus forming a complex network with various feedback points. TF play an important role both in activation and repression of gene transcription by associating with short segments of DNA called enhancers (100 base pairs). These enhancers contain specific short sequences (6 to 12 bp) called binding motifs that are bound to TF (HUGHES, 2011; MINCHIN; BUSBY, 2013). These principles are the basis for regulating neural TFs expression sequentially in the form of gene cascades or networks (SPITZ; FURLONG, 2012).

So far, several types of transcription factors have been described and selected based on the structural similarities they share, thus forming different families from which we will focus on the role of two types of TFs in neural

development: bHLH (basic helix-loop-helix) and Zn fingers (Zinc fingers) (DURHAM et al., 2016).

bHLH (basic helix-loop-helix) transcription factor

Basic helix-loop-helix transcription factors are characterized by the presence of two alpha helices of different sizes that are found in each of the terminals of the protein. Is the amino-terminal basic domain that contains the DNA binding region. This domain is rich in lysine and arginine and confers great affinity to its specific recognition site, which is the E-box consensus hexanucleotide sequence (CANNTG) (FAIRMAN et al., 1993). The HLH domain is located adjacent to the DNA binding region and due to the flexibility of the non-conserved loop that connects the two helices, allows the interaction of the HLH protein with other proteins. These interactions can form homodimers or heterodimers that modulate transcriptional regulation differently (Figure 5) (CHAUDHARY; SKINNER, 1999; JONES, 2004).

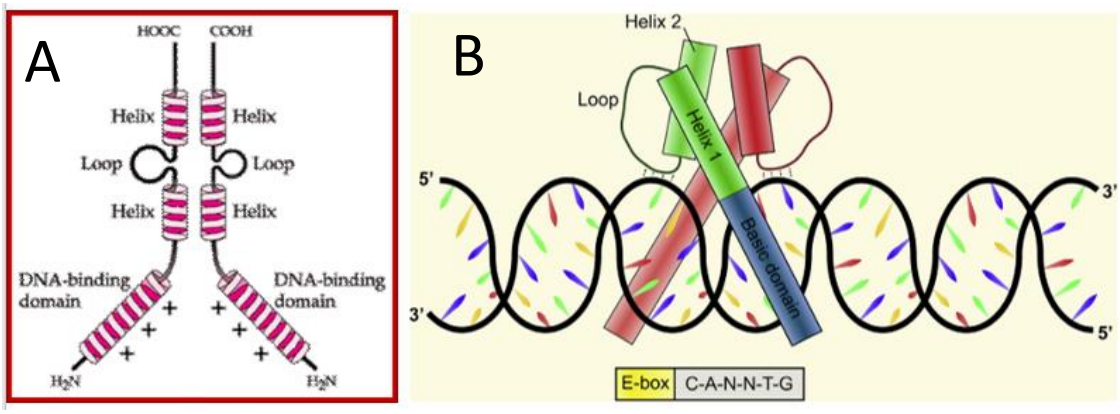

Figure 5. Structure of bHLH transcription factors.
(A)The bHLH transcription factors are characterized by the presence of the helix-loop-helix structure in the C-terminal of the protein, while in the N-terminal is the DNA-binding domain or basic domain with a positive charge conferred by lysine and arginine aminoacids, which can recognize the consensus sequence E-box (B) (Adapted of Gilbert, S. 2017 and Dennis, D. et al, 2019)

The bHLH proteins are divided mainly into two classes: I and II. The first refers to the proteins that are ubiquitously expressed throughout the body; the second, to the proteins that are tissue-specifically expressed and participate actively in various developmental process (DENNIS; HAN; SCHUURMANS, 2019). Spinal cord development depends on the different combinations of class

II bHLH proteins and homeodomain-containing TFs. (reviewed in (LAI; SEAL; JOHNSON, 2016)). Here we will focus on the main objective of our study: the bHLH TF NEUROD4.

NEUROD4

This transcription factor, is also known as NeuroM or ATH3, is a bHLH-type neural transcription factor. This proneural gene -defined as the genes that encode bHLH transcription factors and required to initiate neurogenesis- (HUANG; CHAN; SCHUURMANS, 2014) is expressed during the development of the neural tube in the intermediate zone. Specifically, between the boundary of the region where proliferative and non-proliferative cells are found, which have not yet migrated to the outer layers and are not yet differentiated. This location indicates that it acts on the transition between mitotic and early postmitotic states (ROZTOCIL et al., 1997; LEE; PFAFF, 2003; VIECELI et al., 2013). Consistent with this, NEUROD4 is regulated by NGN2, which is also bHLH TF with a central role in primary neurogenesis. Together, these data strongly suggest that NEUROD4 controls the passage from proliferation to differentiation (PERRON et al., 1999). NEUROD4 activity is regulated by phosphorylation and dephosphorylation. It has been shown that phosphorylation on six proline-directed kinase sites (SP/TP) of this factor promotes neurogenesis and dephosphorylation promotes differentiation through increased protein stability, prolonging the expression of NEUROD4 target genes such as xNeuroD1 (HARDWICK; PHILPOTT, 2015).

Zinc Finger transcription factors

In 1985, the transcription factor IIIa (TFIIIa) protein from *Xenopus laevis* gave us the first panorama on a new type of transcription factor denominated as Zinc Finger transcription factor (MILLER; MCLACHLAN; KLUG, 1985). This type of transcription factors is widely studied because it makes up approximately 3% of the total human genome (KLUG, 2010).

From the extensive work carried out in *Drosophila melanogaster*, it was possible to biochemically describe the conformation of this type of transcription

factors (THUMMEL, 1995; ZHU et al., 2000; MAGLICH et al., 2001; CHUNG et al., 2002; KING-JONES; THUMMEL, 2005). The most prominent feature in this group is the presence of a Zinc ion which is linked to a pair of cysteines and histidines that stabilize the so-called “zinc fingers” which also have an internal structural hydrophobic core (CASSANDRI et al., 2017). Additionally, the crystal structure showed that each finger is composed of two antiparallel β-sheet, and an α-helix (MILLER; MCLACHLAN; KLUG, 1985; ZHANG et al., 2011).The interaction of this transcription factor with the DNA occurs through the alpha helices. They bind to the major groove of the DNA of the consensus sequence of an E-box. The E-box sequence is the same that is also recognized by the bHLH transcription factors as mentioned above (PAVLETICH; PABO, 1991) (Figure 6).

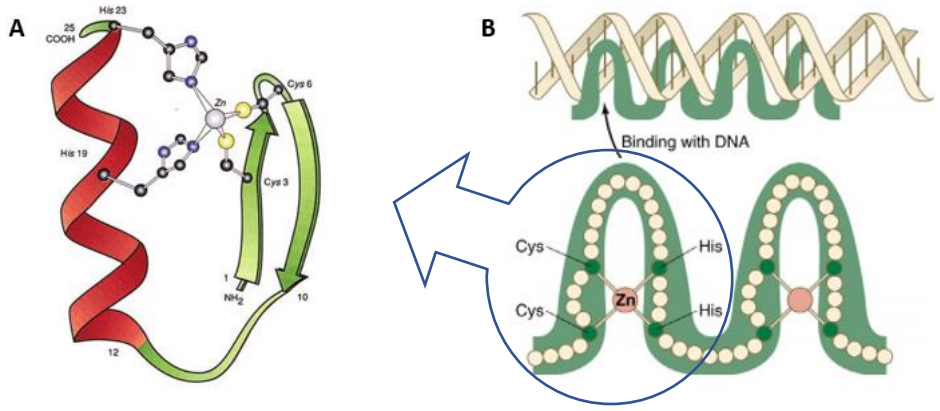

Figure 6. General structure of the zinc finger family of transcription factors.

(A) The Zinc finger motif consists of a short antiparallel -sheet formed by two strands and hairpin turn, followed by an -helix (adapted from Lee et al., 1989). This motif contains 2 cysteine and 2 histidine residues bonded tetrahedrally to a Zn ion which help maintains its 3D structure. (B) The alpha helices bind to the major groove of the DNA with the consensus sequence E-box. (Adapted from Ganss,B. et al 2004)

Within this group of transcription factors we will focus on the SNAIL/SLUG superfamily, whose homologs have been found in chordates, vertebrates, mollusks, nematodes and humans, and have an evolutionary conserved role in epithelial-mesenchymal transition (GRIMES et al., 1996; NAKAKURA et al., 2001).

This superfamily is characterized by the presence of a well-preserved C-terminal region that contains four Zinc finger domains that recognize the conserved E-box sequence (CAGGTG) (NAKAKURA et al., 2001; REECE-HOYES et al., 2009). Also, in the N-terminal region there is a SNAG domain,

which extends to the first nine aminoacids and is conserved in vertebrates, cephalochordates and echinoderms (NIETO, 2002; LIN et al., 2010).

The Scratch family is part of the SNAIL/SLUG superfamily, and is further divided into Scratch1 and 2; and the Snail family, is divided into snail 1 and 2 (NIETO, 2002). What sets apart one family to another is the presence of the SCRATCH and SLUG domains in each one respectively (Figure 7). Both domains are located between the SNAG domain and the Zinc fingers. The SNAG domain was characterized as important for the repression of the E-cadherin promoter through its interaction with the co-repressor NcOR (MOLINA-ORTIZ et al., 2012). Also, this domain interacts with histone lysine-specific demethylase 1 (LSD1). In turn, an LSD1-CoREST complex (REST co-repressor 1 protein) is formed which allows each of the members of this complex to be stabilized and prevent its degradation. The formation of the Snail1-LSD1-CoREST complex results in the demethylation of H3K4me2 from the E-cadherin promoter which represses its transcriptional expression. On the other hand, the function of the SCRATCH domain is still unknown (GRIMES et al., 1996; NIETO, 2002; LIN et al., 2010).

The members of the Snail family are both known for repressing the expression of the *Cdh1*(E-cadherin) gene by directly binding to its promoter, and so, inducing cell delamination because reduction of E-cadherin protein expression destabilizes adherens junctions (ITOH et al., 2013). The Scratch genes are expressed specifically in the developing nervous system (ROARK et al., 1995; NAKAKURA et al., 2001).

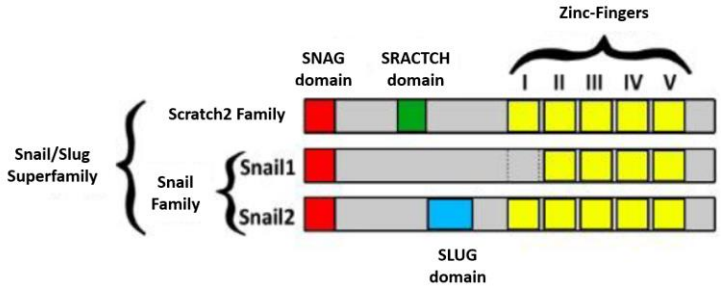

Figure 7. Schematic representation of the members of the Snail / Slug Superfamily.

All members of this superfamily have the SNAG domain in the N-terminal region, while in the C-terminal region they have 4 to 6 zinc fingers. The Snail family is divided in Snail 1 and Snail 2, the latter is differentiated from the other member of the family because it presents a Slug domain. The Scratch family stands out from the other members of the superfamily because it presents a Scratch domain between the SNAG domain and the 5 Zinc fingers. (Modified from Vieceli, 2009)

SCRATCH2 (SCRT2)

SCRATCH2 is expressed in neural progenitors that recently exited the cell cycle (MÜHLFRIEDEL et al., 2007). Described for the first time in *Drosophila melanogaster*, SCRATCH was associated with a significant reduction in the number of retinal cells in knockout flies. In the same work it is highlighted that no significant morphological change was observed in the embryonic neural nervous system once this transcription factor was eliminated by itself. But, when its removal occurs in conjunction with the pan-neural *deadpan* (dp) gene, a reduction in the number of neurons was observed. The ubiquitous expression of SCRATCH has an opposite effect, the number of neurons increased. With all these data, it was suggested that this gene is related to neural differentiation by repression of other genes (ROARK et al., 1995).

In other species such as *Caenorhabditis elegans*, the orthologous gene of SCRATCH called CES-1, represses programmed cell death during the asymmetric division of neural progenitors that generated NSM cells (motor neurons) (METZSTEIN; HORVITZ, 1999). During this division two daughter cells are formed of which one of them follows its course of differentiation while the other enters apoptosis. Ces-1 gene interacts with E-box sites and represses pro-apoptotic genes such as egl-1 (REECE-HOYES et al., 2009; YAN et al., 2013; WEI et al., 2017).

In the Zebrafish *Danio rerio*, SCRATCH2 is related to the control of the cell cycle in neurons during the development of this animal's embryonic neural tube. In *Danio rerio*, SCRATCH2 maintains postmitotic neural progenitors out of the cell cycle. In this context, SCRATCH2 maintains high levels of p57, a cell cycle inhibitor, through the downregulation of miR-25 (RODRÍGUEZ-AZNAR; BARRALLO-GIMENO; NIETO, 2013).

In mice, SCRATCH2 is associated with cell migration of post-mitotic neural progenitors in the neocortex. SCRATCH2 represses E-cadherin transcription and contributes with postmitotic migration to the outer layers of the cortex (ITOH et al., 2013; PAUL et al., 2014).

SCRATCH2 expression in the chicken embryo (*Gallus gallus*) was characterized by Felipe Vieceli in 2013 (VIECELI et al., 2013), SCRATCH2 is expressed in the neural tube and dorsal root ganglia at different stages of embryonic development. Similar to NEUROD4, in the neural tube, SCRATCH2 is expressed in the intermediate zone, in cells that left the cell cycle and prepare to differentiate themselves. Indeed, the expression domain of SCRATCH2 overlaps with NEUROD4 (Figure 8). Further, since both TFs recognize the E-box domain, it is possible that they share target genes. In this sense, as SCRATCH2 has been described mainly as a transcriptional repressor and NEUROD4 as an activator, they might have opposite effects on these shared target genes. Thus, the transcriptional outcome would depend on the balance of SCRATCH2 and NEUROD4 activities.

Therefore, here, our aims are to characterize the effect of neural transcription factors SCRATCH2 and NEUROD4 individually and together on the cell cycle and transcriptional regulation.

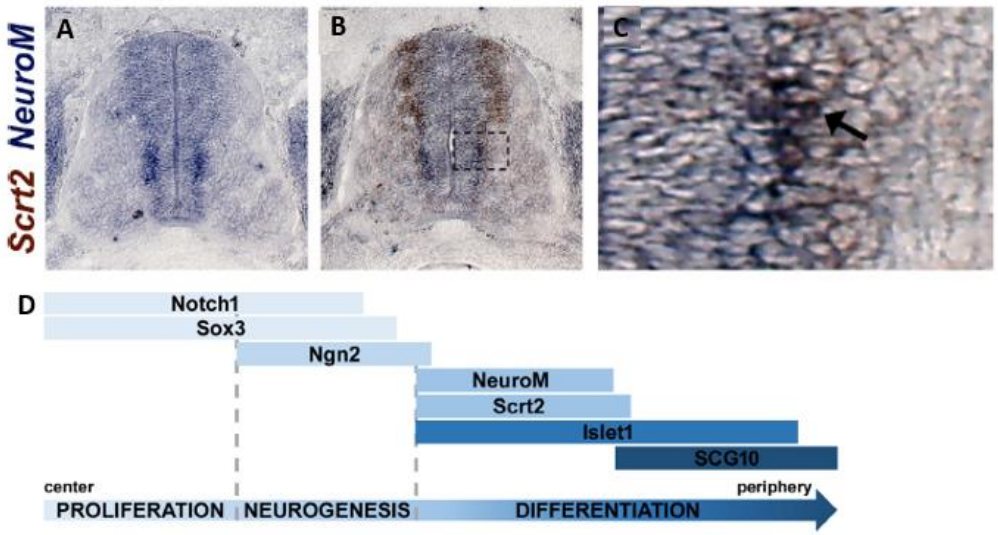

Figure 8. Developmental expression pattern for SCRATCH2 and NEUROD4 in the neural tube

(A) NEUROD4 is expressed in the intermediate zone of the neural tube. (B) SCRATCH2 is in the intermediate zone. (C) is a higher magnification image of the dotted square in (B). The black arrow indicates the region where SCRATCH2 and NEUROD4 are co-expressed in the intermediate zone. (D) The graph represents the expression of various transcription factors during the various stages of proliferation, neurogenesis and differentiation. (Adapted from Vieceli et al, 2013)

CONCLUSIONS

Thus, we conclude:

1) NEUROD4 but not SCRATCH2 reduces the number of proliferative neural progenitors in the neural tube.
2) The transcriptional activity of SCRATCH2 is repressive while that of NEUROD4 is activating in HEK293T cells. Both recognize E-box sequences.
3) Our bioinformatic analysis suggest that SCRATCH2 regulates the expression of LHX9 and BARHL1 in partnership with bHLH proneural transcription factors.
4) SCRATCH2 could maintain the identity of dorsal interneurons class 4 and 5 through direct repression of ISLET1

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