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Epigenetics in ENS development and Hirschsprung disease

A. Torroglosa [a,b], M.M. Alves [c], R.M. Fernández [a,b], G. Antiñolo [a,b], R.M. Hofstra [c,d], S. Borrego [a,b,*]

[a] Department of Genetics, Reproduction and Fetal Medicine, Institute of Biomedicine of Seville (IBIS), University Hospital Virgen del Rocío/CSIC/University of Seville, Seville, Spain
[b] Centre for Biomedical Network Research on Rare Diseases (CIBERER), Seville, Spain
[c] Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands
[d] Stem Cells and Regenerative Medicine, Birth Defects Research Centre UCL Institute of Child Health, London, UK

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ABSTRACT

Hirschsprung disease (HSCR, OMIM 142623) is a neurocristopathy caused by a failure of the enteric nervous system (ENS) progenitors derived from neural crest cells (NCCs), to migrate, proliferate, differentiate or survive to and within the gastrointestinal tract, resulting in aganglionosis in the distal colon. The formation of the ENS is a complex process, which is regulated by a large range of molecules and signalling pathways involving both the NCCs and the intestinal environment. This tightly regulated process needs correct regulation of the expression of ENS specific genes. Alterations in the expression of these genes can have dramatic consequences.

Several mechanisms that control the expression of genes have been described, such as DNA modification (epigenetic mechanisms), regulation of transcription (transcription factor, enhancers, repressors and silencers), post-transcriptional regulation (3′UTR and miRNAs) and regulation of translation.

In this review, we focus on the epigenetic DNA modifications that have been described so far in the context of the ENS development. Moreover we describe the changes that are found in relation to the onset of HSCR.

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1. Introduction

The Enteric Nervous System (ENS) is the largest part of the peripheral nervous system and is composed by a network of neurons and glia within the wall of the bowel (Gershon, 2010; Furness, 2012). Formation of the ENS requires extensive cell migration, controlled cell proliferation, regulated differentiation, directed neurite growth, and establishment of a network of interconnected neurons. Each of those complex cellular events must be guided by specific molecular signals, with a major role of some specific pathways such as the RET/GFRα1/GDNF and EDNRB/EDN3/ECE1, some transcription factors as SOX10, PAX3, PHOX2B or ZFHX1B, or even some morphogens as SHH, netrins or semaphorins among others (Lake and Heuckeroth, 2013). Specific patterns of gene expression are required to control all such processes and alterations throughout them can lead to dramatic consequences, as evidenced by the aganglionosis observed in Hirschsprung disease (HSCR, OMIM 142623). There are several mechanisms regulating gene expression during ENS development, such as DNA modification (epigenetic mechanisms), regulation of transcription (mediated by transcription factors, enhancers, repressors and silencers), post-transcriptional regulation (with the involvement of 3´UTR regions and miRNAs) and regulation of translation. Among them we will focus on the epigenetic mechanisms.

Epigenetic events are defined as “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity state” (Bird, 2007). In some cases, epigenetic modifications are stable and passed on to future generations, but in other instances they are dynamic and change in response to environmental stimuli (Calvanese et al., 2012).

Despite increasing interest on this subject, there is not much known so far about the epigenetic changes that could contribute to ENS development, and to the onset of ENS related disorders, such as HSCR. Here, we review several epigenetic regulators that have been shown to play a role in ENS development and might well be key players for the development of HSCR.

1.1. The ENS, NCCs and HSCR

In the ENS there are many different types of enteric neurons that differ in their targets, inputs, direction of projections,

* Corresponding author at: Department of Genetics, Reproduction and Fetal Medicine, University Hospital Virgen del Rocío, Sevilla 41013, Spain.
E-mail address: salud.borrego.sspa@juntadeandalucia.es (S. Borrego).

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neurotransmitters, and electrophysiological characteristics. Most enteric neurons arise from neural crest cells (NCCs) that migrate from the neural tube adjacent to somites 1–7 (Le Douarin and Teillet, 1974). Previous studies in a variety of species, including humans, have shown that vagal NCCs enter the foregut and migrate caudally along the gut (Kapur et al., 1992; Wallace and Burns, 2005).

NCCs are a population of multipotent stem/progenitor cells that are induced during gastrulation at the neural plate border, between the neural and non-neural ectoderm. Abnormal neural crest development is related with many of the most common human birth defects, since it can lead to craniofacial defects like cleft lip and palate, heart septation defects, and agangliogenesis of the colon (Jiang et al., 2006; Tennyson et al., 1986; Youn et al., 2003). In addition, NCCs are involved in a variety of diseases and syndromes such as HSCR, Waardenburg syndrome (OMIM 148820), CHARGE syndrome (OMIM 214800) and Williams Syndrome (OMIM 194050) (Ahola et al., 2009; Bajpai et al., 2010; Inoue et al., 2002; Kim et al., 2011; Yoshimura et al., 2009).

HSCR is the most common neurocristopathy in humans (1:5000 newborns), and is characterized by the absence of enteric ganglia along variable lengths of the distal gastrointestinal tract, resulting in severe intestinal dysfunction (Chakravarti and Lyonnet, 2001). It either appears with a familial basis or, most often, sporadically exhibiting a complex pattern of inheritance with low sex-dependent penetrance and variable expression.

2. Epigenetic mechanisms during NCC development

Transcriptional events are critical for the correct ENS development and there is growing evidence that epigenetic regulation is critical to control these events, most notably the timing of gene expression at different developmental stages (Hu et al., 2014; Portela and Esteller, 2010). Different epigenetic mechanisms are known, which include DNA methylation and Histone modifications, Polycomb repression, and ATP-dependent chromatin remodeling, (Liu and Xiao, 2011; Fujita et al., 2014). In this section we will go in detail into each of these events, and describe their involvement in ENS formation and neural crest development.

2.1. DNA methylation

DNA methylation involves the addition of a methyl group to DNA residues. The most extensively studied methylation event is the one that occurs at the fifth position of cytosine residues (5-methylcytosine, 5mC) localized at CpG islands, regions of cytosine and guanine pairs that extend for at least 200 bases. These islands are found in approximately 40% of all mammalian promoters and are usually unmethylated when gene expression occurs (Law and Jacobsen, 2010). DNA methylation is essential for mammalian development, X-chromosome inactivation, genomic imprinting, heritable silencing of transposable elements, regulation of gene expression and genomic stability (Bird, 2002; Ooi et al., 2009). This process is mediated by the family of DNA methyltransferases: DNMT1, DNMT3A and 3B. According to their function, the DNMTs can be categorized into two main groups, with DNMT1 representing the maintenance methyltransferase and DNMT3A and 3B acting as the *de novo* methyltransferases (Fig. 1). The *de novo* methyltransferases (DNMT3A and its paralog DNMT3B) are the major players in tissue specific gene regulation during development, establishing the initial CpG methylation patterns. They have been shown to be vital for normal mammalian development and were described to play an important role in the onset of neural crest related disorders (Ehrlich et al., 2008; Jaenisch and Bird, 2003; Yang et al., 2011). *Dnmt3A* homozygous knockout mice die several weeks after birth, and *Dntm3B* homozygous knockout embryos have rostral neural tube defects and growth impairment (Okano et al., 1999). Moreover, *DNMT3A* expression was detected in the neural crest territory of chicken embryos, and its loss of function results in down-regulation/loss of neural crest specific genes (*Pax3, Pax7, FoxD3, Sox10 and Snail2*) (Hu et al., 2012). *DNMT3B* has also been shown to be up-regulated during neural crest induction in chicken embryos (Adams et al., 2008), and in humans mutations in this gene have been found to cause the immunodeficiency-centromeric instability-facial anomalies syndrome (ICF), which is characterized by widened nasal bridge and hypotelorism, neurological dysfunction and other related defects (Ehrlich et al., 2008; Jin et al., 2008). In addition, knock-down of *Dnmt3B* in human embryonic stem cells (hESCs) accelerates neural crest differentiation and increases the expression of neural crest specific genes (Martins-Taylor et al., 2012).

2.2. Histone modifications

Histones are the main chromatin binding proteins and their association with a compacted DNA strand results into a structural component called nucleosome. Each nucleosome contains eight histones, two of each of the core histones (H2A, H2B, H3 and H4), forming an octameric structure called nucleosome core around which DNA is wrapped with unstructured tails (Gibney and Nolan, 2010). The core histone proteins are highly conserved throughout evolution and their tails are subjected to post-translational modifications such as methylation, acetylation, deacetylation, phosphorylation, ubiquitination, and sumoylation (Fig. 2; Berger, 2007; Kouzarides, 2007). So far, only histones methylation, acetylation and deacetylation have been described to play a role in NCCs development and in the onset of neurocristopathies. Therefore, these three histone modifications will be described in more detail in this review.

2.2.1. Histone methylation and demethylation

Histone methylation is associated with both active and repressive transcription. Histone methyltransferases (HMTs) add methylation marks, whereas histone demethylases remove methylation marks. The histone modification H3K4me3 (Histone 3 Lysine 4 trimethylation) is indicative of a transcriptionally permissive chromatin state, and is mostly found in the promoter regions of genes (Akkers et al., 2009; Barski et al., 2007; Cheung et al., 2010; Pan et al., 2007). H3K36me3 is also present in

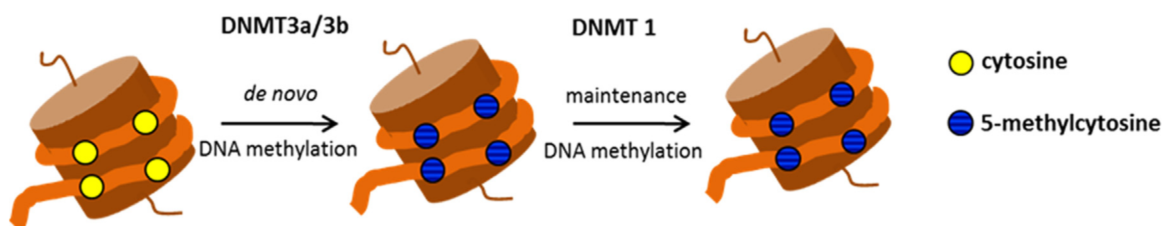

Fig. 1. Representation of the three enzymes responsible for establishment (DNMT3A/B) and maintenance (DNMT1) of DNA methylation.

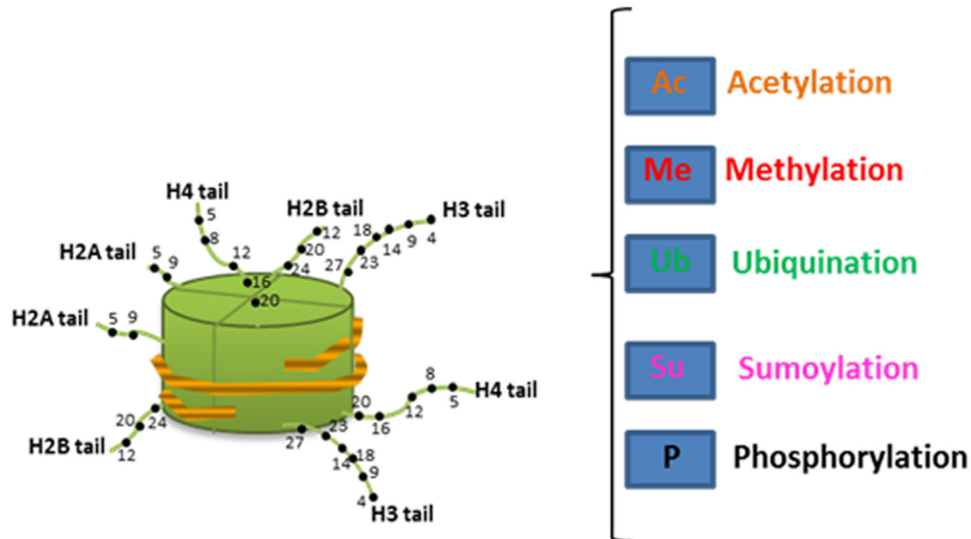

Fig. 2. Nucleosome model and major post-translational modifications that play essential roles in gene expression regulation and disease processes. Adapted from http://www.integratedhealthcare.eu/1/en7histones_and_chromatin/1497.

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NCCs DEVELOPMENT
Neural Tube

	EPIGENETIC MECHANISMS	REFERENCES
NCCs	**DNA methylation**	
	De novo methyltransferase (BNMT3B/A)	Ehrlich et al., 2008; Jaenisch and Bird, 2003; Yang et al., 2011; Adams et al., 2008; Jin et al., 2008; Okano et al., 1999; Martins-Taylor et al., 2012
	Histone modifications	
	Histone demethylation	
	JMJD2A (H3K9me3)	Strobl-Mazzulla et al., 2010
	PHF8 (H4K20me1 and H3K9me1)	Phillips et al., 2006; Qi et al., 2010; Matsukawa et al., 2015: Maxson and Ishii, 2008; Monsoro-Burq et al., 2005; Takahashi et al., 2001
	Histone deacethylation (HDCA)	Bonn et al., 2012; Cotney et al., 2012; Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011; Hatta et al., 1987; Nakagawa and Takeichi, 1995; Taneyhill et al., 2007
	HDCA4	Ignatius et al., 2008; DeLaurier et al., 2012;Park et al., 2006; Alsdorf and Wyszynski et al., 2005;Williams et al., 2010
	HDAC1	Wang et al.,2015; Cunliffe, 2004; Ignatius et al., 2008; Nambiar and Henion, 2004; Stadler, 2005; Yamaguchi et al., 2005; Ignatius et al., 2013
	HDAC3	Singh et al., 2011
	HDAC8	Haberland et al., 2009
	HDAC1 and HDAC2	Jacob et al., 2014;Wang et al., 2009
	Polycomb repressive (PRC)	
	PCR2	Schwarz et al., 2014; Kim et al., 2015; Tien et al., 2015
	PCR1	Van der Velden et al., 2013
	ATP-dependent chromatin remodeling	
	CHD7-PBAF	Muchardt and Yaniv, 2001; Bajpaiet al., 2010; Zentner et al., 2010
	WSFT (WINAC and WICH)	Barnett and Krebs, 2011; Barnett et al., 2012; Yoshimura et al., 2009

FG
MG
HG

PROLIFERATION
MIGRATION
DIFFERENTIATION

2
ENS
DEVELOPMENT

	EPIGENETIC MECHANISMS	REFERENCES
HSCR	**DNA methylation**	
	De novo methyltransferase (BNMT3B)	Torroglosa et al., 2014; Linhart et al., 2007
	DNA methylation/Gene expression	
	RET	Munnes et al., 1998; Angrisano et al., 201; Draht et al., 2016
	EDNRB	Tang et al., 2013a
	PHOX2B	DePontual et al., 2007
	PAX6	Enguix-Riego et al., 2016
	miRNA -144	Tang et al., 2013b
	MeCP2	Zhou et al., 2013
	Polycomb repressive (PCR)	
	AEBP2 (PCR2)	Ahola et al.,2009; Inoue et al., 2002; Kim et al., 2011; Kim et al., 2011

NCCs
Enteric precursors derived from Truncal NCCs
Enteric precursors derived from Vagal NCCs
Enteric precursors derived from Sacral NCCs

Fig. 3. Diagram showing the epigenetic mechanisms involved in ENS development. Adapted from Heanue and Pachnis (2007).

euchromatic regions that are associated with active transcription, and are primarily found within gene bodies. In contrast, H3K27me3 catalyzed by the Polycomb repressive complex (Schwartz et al., 2006; Simon and Kingston, 2009; Swigut and Wysocka, 2007; Tolhuis et al., 2006), and H3K9me3 (Allan et al., 2012; Nielsen et al., 2001; Shi et al., 2003), are associated with

transcriptional repression.

Several genes have been described to encode regulatory proteins for histone methylation. Of special notice for the content of this review are *JMJD2A* and *PHF8*. *JMJD2A* was the first epigenetic gene discovered to regulate neural crest specification, and encodes for a demethylase that modulates H3K9me3 of neural crest genes. Specifically, down regulation of *Sox8*, *Sox10*, *Fox3D* and *Snail*, as well as up-regulation of *Wnt1* and *Twist1,* have been described when *JmjD2A* was knock-down in fertilized chicken eggs. *In vivo* Chromatin Immunoprecipitation (ChIP) assays revealed direct interaction of *JmjD2A* with *Sox10* and *Snail2* promoter regions that are occupied by H3K9me3 (Strobl-Mazzulla et al., 2010). Thus, JmjD2A is required to demethylate histones occupying specific promoter regions at a proper time and place to allow neural crest specification to occur. *PHF8* also encodes for a histone demethylase, and mutations in this gene have been identified in patients with craniofacial deformities. PHF8 is capable of demethylating the repressive marks H4K20me1 and H3K9me1 around the transcription start site to activate transcription. In zebrafish, phf8 was found to directly regulate the expression of the homeodomain transcription factor *msxB* during craniofacial development, especially of the lower jaw (Phillips et al., 2006; Qi et al., 2010). Moreover, it has also been previously implicated in the regulation of neural crest development in many vertebrate models such as *Xenopus* and mice (Matsukawa et al., 2015; Maxson and Ishii, 2008; Monsoro-Burq et al., 2005; Takahashi et al., 2001).

2.2.2. Histone acetylation and deacetylation

Histone acetylation is associated with active transcription, and histone deacetylation silences transcription (Jenuwein and Allis, 2001). Histone acetyltransferases (HATs), and histone deacetylases (HDACs) are two classes of enzymes that antagonize each other. HATs transfer acetyl groups to lysines, and their binding is correlated with active transcription (Carrozza et al., 2003; Shahbazian and Grunstein, 2007). HATs have also been identified as co-transcriptional activators (Roth et al., 2001; Yang, 2004). In contrast, HDACs deacetylate lysine residues and one of their major functions is to remove acetyl groups added by HATs (Wang et al., 2002). Therefore, HDACs have been identified as transcriptional co-repressors (Kadosh and Struhl, 1997; Rundlett et al., 1998), whose function is required for chromatin to reset its tightly packed state (Hsieh et al., 2004).

Since histone acetylation is closely associated with enhancer activity, it is a new, powerful way to identify neural crest cis-regulatory regions. For example, H3K27ac is associated with active enhancers (Bonn et al., 2012; Cotney et al., 2012; Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011). The HDAC repression complex has been shown to promote trunk crest cells specification (Murko et al., 2013), and plays an essential role in controlling neural crest migration (Hatta et al., 1987; Nakagawa and Takeichi, 1995; Taneyhill et al., 2007). Later in development, and HDCA4 are required for distinct developmental processes and their activity is present in a broad range of cell types during neural crest derived differentiation (Ignatius et al., 2008; DeLaurier et al., 2012). In zebrafish, *hdac1* is required for the development of the eye, CNS and neural crest cell populations (Wang et al., 2015; Cunliffe, 2004; Nambiar and Henion, 2004; Stadler, 2005; Yamaguchi et al., 2005). Therefore, there seems to be a specific requirement for *hdac1* in ENS formation, at least in this animal model (Ignatius et al., 2013). HDCA4 is also highly associated with neural crest related diseases and syndromes (Park et al., 2006; Alsdorf and Wyszynski, 2005). In human development, haploinsufficiency of *HDAC4* causes brachydactyly mental retardation syndrome, characterized by craniofacial and skeletal abnormalities (Williams et al., 2010). Other HDACs important for neural crest development include HDAC3 and HDAC8. Hdac3 has been shown to be crucial for the regulation of smooth muscle differentiation and cardiac outflow tract formation during cardiac neural crest development in mouse (Singh et al., 2011). HDAC8 controls skull morphogenesis by repressing the homeobox transcription factors Otx2 and Lhx1, in cranial neural crest-derived cells (Haberland et al., 2009).

Although histone deacetylation was originally thought to function by silencing genes via local compaction of the chromatin structure, it has also been shown to activate or maintain the active transcription state (Wang et al., 2009) by acting in concert with HATs. Recent studies have showed that HDAC1 and HDAC2 are able to bind to promoter regions and promote differentiation of NCCs to peripheral glia (Jacob et al., 2014).

2.3. Polycomb repressive (PRC)

The PRC is composed by a group of proteins that are able to epigenetically silence the transcription of their target genes by catalyzing trimethylation of histone 3 lysine 27 complex (H3K27me3) (Mikkelsen et al., 2007; Schuettengruber et al., 2007). These polycomb proteins are involved in many events during embryonic development in a stage and time dependent manner. PRC1 and PRC2 are both involved in the differentiation of neural crest-derived craniofacial structures. It has been described that EZH2 (the enhancer of zeste homolog 2), one of the four core subunits of PCR2, and similarly Ring 1b/Rnf2 (the single E3 ubiquitin ligase) in PRC1, regulate cranial neural crest differentiation in chondrocytes (Schwarz et al., 2014; van der Velden et al., 2013). Recently, it has been described the opposite function of the two isoforms of AEBP2 (DNA-binding repressor for the aP2) on PCR2 expression and, consequently, on neural crest cell migration during development (Kim et al., 2015). Likewise, it has been also shown that Snail/Slug cooperates with PRC2 in the regulation of NCCs development (Tien et al., 2015).

2.4. ATP-dependent chromatin remodeling

The ATP-dependent chromatin remodeling is executed by protein complexes such as SWI/SNF (mating-type switch/sucrose nonfermenting), ISWI (imitation switch), and CHD (chromodomain helicase DNA-binding), which regulate gene expression by changing the position or structure of higher order chromatin in an ATP-dependent manner. They create nucleosome-free regions to facilitate access of DNA to transcription factors and regulatory proteins (Kwon and Wagner, 2007; Wu et al., 2009). It has been reported that CHD7, an ATP-dependent helicase, cooperates with the PBAF (SWI/SNF) chromatin remodeling complex (Muchardt and Yaniv, 2001) to promote neural crest specification in hESCs induced to become NCCs (Bajpai et al., 2010). In this sense, the malfunction of CHD7 in early neural crest development is related to CHARGE syndrome, another neurocristopathy (Zentner et al., 2010). On the other hand, the Williams syndrome transcription factor (WSFT), a major subunit of two distinct ATP-dependent chromatin remodeling complexes: WINAC and WICH (Barnett and Krebs, 2011), has also been associated with neural crest defects, since mutations in this gene have been found in patients diagnosed with Williams syndrome, a developmental disorder characterized by cardiovascular defects, developmental delays and learning disabilities (Barnett et al., 2012; Yoshimura et al., 2009).

3. Epigenetics in an ENS related disease: HSCR

Several epigenetic mechanisms have been described for NCCs development that, when disturbed, lead to a number of pathologies in humans (Yang, 2004; Webb et al., 2009; Yang et al., 2011;

van der Velden et al., 2013; Williams et al.,2010; Liu and Xiao, 2011; Ordog et al., 2012). One of these pathologies is HSCR. In this section, we focus on a set of specific genes implicated in HSCR development, and describe the epigenetic mechanisms known to date, to regulate their expression.

3.1. *RET*

The *RET (REarranged-during Transfection)* proto-oncogene (OMIM 164761) encodes a receptor tyrosine kinase essential for ontogenesis of the ENS and kidney (Pachnis et al., 1993; Schuchardt et al., 1994). Mutations in *RET* have been found to explain approximately 20% of all HSCR cases, and therefore, *RET* is considered to be the main gene implicated in this disease (Amiel et al., 2008; Borrego et al., 2013; Alves et al., 2013).

The promoter of *RET* has a 5′-CG-3′ rich region susceptible to be methylated, and it has been demonstrated that in some human adult tissues, including peripheral white blood cells from HSCR patients, the expression of *RET* is regulated by DNA methylation. Moreover, it has been observed that *RET* expression can be occasionally activated in specific adult tissues (Munnes et al., 1998). In this sense, retionic acid has been also demonstrated to regulate the expression of *RET* by DNA methylation on this 5´-CG-3´ rich enhancer region (Angrisano et al., 2011). Moreover, recently it has been described that the *RET* promoter CpG island methylation is a potential prognostic marker in stage II colorectal cancer patients (Draht et al., 2016). *RET* expression is regulated by DNA methylation changes and therefore this mechanism seems to be implicated in the onset of HSCR.

3.2. *EDNRB*

EDNRB encodes for the Endothelin receptor type B. The interaction between EDNRB and its ligand, EDN3 (Endothelin 3), is essential for normal development of enteric ganglia and NC-derived pigment cell populations (McCallion and Chakravarti, 2001). EDNRB is also considered to be crucial for HSCR development. Mutations in *EDNRB* have been identified in HSCR patients (Puffenberger et al., 1994). Moreover, an up-regulated expression of this receptor has been recently found in tissues collected from HSCR patients, when compared with controls. In these patients, a significant lower level of methylation of *EDNRB* was detected demonstrating that epigenetic inactivation of *EDNRB* may also play a role in HSCR development (Tang et al., 2013a).

3.3. *PHOX2B*

PHOX2B (Paired-like homeobox 2B) encodes a highly conserved homeotic transcription factor that, together with SOX10, is responsible for maintaining the processes of neuronal and glial differentiation of NCCs (Pattyn et al., 1999). *PHOX2B* has been identified as a disease-causing gene in some dysautonomic disorders including Congenital Central Hypoventilation Syndrome (OMIM 209880), Neuroblastoma (OMIM 256700) and HSCR. Although no direct connection has been found between epigenetic control of this gene and HSCR development, an aberrant CpG dinucleotide methylation of the 500 bp promoter region of *PHOX2B* was found in 4/31 neuroblastoma tumors and cell lines (12.9%). Since a neuroblastoma is an embryonic tumor originating from NCCs, this result suggests that the methylation status of this gene could play a role in the regulation of NCCs processes (de Pontual et al., 2007), and therefore could also be involved in HSCR.

3.4. *DNMT3B*

DNMT3B encodes for one of the *de novo* methyltransferases, and as mentioned before, is required to establish the initial CpG methylation patterns. Recently, it has been described to play a role in the development of the ENS and in the onset of HSCR disease (Torroglosa et al., 2014). A decreased expression of DNMT3B was observed in enteric precursors isolated from HSCR patients, as a result of DNA hypomethylation in patients *versus* controls. *DNMT3B* mutational analysis in a cohort of HSCR patients has also revealed the presence of 3 potentially pathogenic missense mutations (p.Gly25Arg, p.Arg190Cys, and p.Gly198Trp). These results showed that DNMT3B may regulate ENS development through DNA methylation in NCCs, and suggest that aberrant methylation patterns could play a relevant role in HSCR. Moreover, it is possible that a synergistic effect of mutations in both *DNMT3B* and other HSCR–related genes may contribute to a more severe phenotype in terms of length of aganglionosis, in HSCR patients. Moreover, it has been described the role of DNMT3B as a promoter of the tumorigenesis in colon cancer by gene-specific de novo methylation and transcriptional silencing (Linhart et al., 2007).

3.5. *PAX6*

PAX6 (Paired box 6) encodes for a transcription factor that is highly involved in the development of the eye and CNS (Halder et al., 1995; Paridaen and Huttner, 2014). Recently, *PAX6* has also been identified as a candidate gene for HSCR. A differential expression study using Neurosphere-like bodies, revealed a significant lower expression level of PAX6 in HSCR patients *versus* controls. Since Pax6 was identified as a target of the Dnmt3B methyltransferase in mice (Enguix-Riego et al., 2016), it has been hypothesized that the reason underlying PAX6 decreased expression in humans might be an aberrant DNMT3B-dependent methylation. It has also been suggested that this decrease in PAX6 expression may influence the activation of signalling pathways involved in ENS development, with the confluence of additional genetic factors for the manifestation of the HSCR phenotype.

3.6. miRNAs

MicroRNAs (miRNAs) are small, non-coding RNA molecules that are about 19–25 nucleotides long (Spiegel et al., 2011). They regulate target genes by triggering mRNA degradation or translational repression through complementary binding to the 3′-untranslated regions of target mRNAs (Lee et al., 2011). MiR-141, a member of the miR-200 family, is highly associated with various human diseases, especially malignancies such as gastric, colon, prostate and pancreatic cancer (Du et al., 2009). MiR-141 has been also shown to be down-regulated in colon tissues collected from HSCR patients when compared with controls, whereas the expression of its target genes, *CD47* and *CUL3*, was increased in the same set of patients. The increased levels of *CD47* and *CUL3* induced by miR-141 were observed to reduce proliferation and migration of 293T and SH-SY5Y (human neuroblastoma cell line) cell lines, suggesting that an aberrant reduction of miR-141 may play an important role in the pathogenesis of HSCR by inhibiting migration and proliferation of NCCs. Furthermore, hypermethylation of a CpG island in the promoter region of miR-141 was found in HSCR patients, confirming that the expression levels of miR-141 are regulated by the methylation status of its promoter (Tang et al., 2013b).

3.7. *MeCP2*

MeCP2 (Methyl-CpG binding Protein 2) encodes for a member of the methyl-CpG-binding domain protein complex that associates with HDACs and HMTs to form a stable repressor complex (McGinty, 2012). *MeCP2* has been implicated in many human diseases, especially in neurological disorders (Webb et al., 2009;

Belligni et al., 2010; Banerjee et al., 2012; Dichter et al., 2012). It has been also found to exhibit reduced expression levels in tissues of HSCR patients when compared with controls and this reduced expression suppressed the proliferative ability of the SH-SY5Y cells. However, no significant difference in MeCP2 methylation levels was found between HSCR patients and controls. Similarly, no difference was found between HSCR patients and controls in the levels of miRNA-34b, the predicted regulator of MeCP2 expression through complementary binding to its 3′-untranslated region. Nevertheless, it is still possible that the aberrant decreased levels of MeCP2 may be involved in HSCR pathogenesis, since it has been suggested that this could probably occur through regulation of histone modifications (Zhou et al., 2013).

3.8. AEBP2

AEBP2 encodes for a component of the polycomb repressive complex 2 (PRC2), and is expressed in the neural crest territory (Kim et al., 2009). Heterozygous mutant *Aebp2*$^{+/\beta\text{-Geo}}$ mice present a pot shaped belly and a reduction of density of ganglia in the section between the anus and cecum. Moreover, they show white spotting at the tail tip and white toes at the hind limbs, and do not have a brisk acoustic startle response to clapping sounds, suggesting potential hearing defects. The three phenotypes observed in *Aebp2*$^{+/\beta\text{-Geo}}$ mice are similar to those found in patients diagnosed with Waardenburg syndrome Type 4 (WS4), a developmental disorder characterized by megacolon, hypopigmentation, and auditory defects (Ahola et al., 2009; Inoue et al., 2002). Expression levels of key neural crest genes were found to be modified in *Aebp2* heterozygous mutants. In particular, *Sox10* was consistently down-regulated, which is concordant with the reduced *SOX10* dosage frequently observed in WS4 patients. Therefore, it was suggested that *AEBP2* deregulation contributes to the development of HSCR and WS4 via improper epigenetic regulation of neural crest genes, such as *SOX10* (Kim et al., 2011).

4. Conclusions

HSCR is a human birth defect caused by disturbed ENS development due to abnormal migration, proliferation, differentiation or survival of NCCs. Epigenetic events have been described to be involved in the incorrect development of the ENS, and although they have been suggested to contribute to HSCR, very little is known about their involvement in the onset of this disease. In this review, we have shown that there are many epigenetic mechanisms implicated in NCCs and ENS development, confirming that aberrant epigenetic patterns may contribute to the development of neurocristopathies. We have aimed to review the specific levels at which epigenetic mechanisms are acting during embryonic ENS development (Fig. 3). Nevertheless, further investigation is required to completely dissect the role of epigenetic changes in the pathogenesis of HSCR.

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