UNIVERSITÀ DEGLI STUDI DEL MOLISE

DIPARTIMENTO DI MEDICINA E SCIENZE DELLA SALUTE

DOTTORATO DI RICERCA IN MEDICINA TRASLAZIONALE E CLINICA

XXX CICLO

SETTORE SCIENTIFICO DISCIPLINARE BIO/09

MicroRNAs Profiling in Dopaminergic Neurons

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to my family

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1 – INTRODUCTION

1.1 Midbrain dopaminergic neurons

Neurons producing dopamine (DA, member of catecholamine) as neurotransmitter represent a heterogeneous group of cells involved in the control of different behaviors and physiological aspects of the mammal organisms.

In the mammalian central nervous system (CNS) dopaminergic nuclei have a broad distribution from the mesencephalon to the olfactory bulb described by Dahlstrom and Fuxe in 1964 (Dahlstroem et al., 1964) (fig. 1.1).

They are located in the area A16 of the olfactory bulbs (Gudelsky et al. 1976), area A17 of the retina (Djamgoz et al. 1992), areas A11-A15 of the diencephalon [e.g. hypothalamic arcuate nucleus (A12; Kizer et al. 1976) and sub-parafascicular thalamic nucleus (A13; Takada 1993)]. The areas identified as A8, A9 and A10 nuclei are usually indicated as midbrain dopaminergic neurons (mDA).

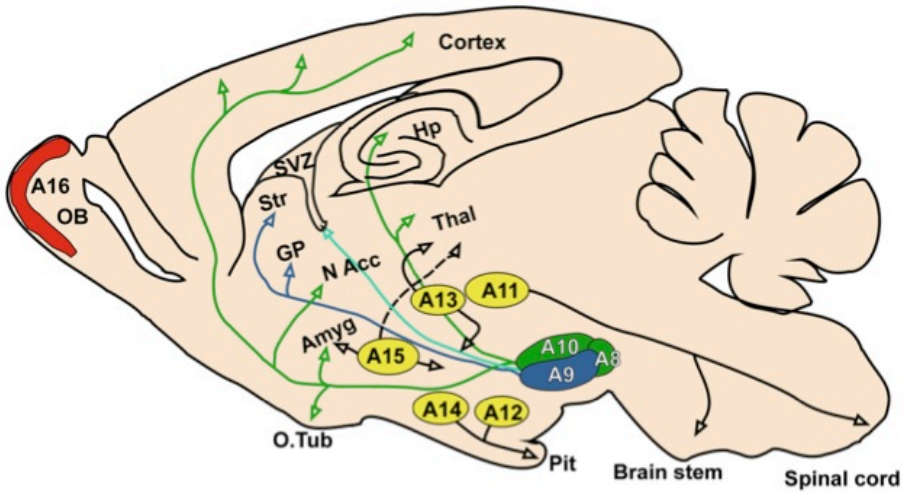

Figure 1.1 Schematic representation of anatomical localization of dopaminergic neurons in the adult brain of rodent. Dopaminergic neurons are distributed in 10 groups (A8 - A17) from the mesencephalon to the olfactory bulb described by Dahlstrom and Fuxe in 1964 (Dahlstroem et al., 1964). A8, A9 and A10 nuclei are usually indicated as midbrain dopaminergic neurons. Blu arrows represent nigro-striatal pathway; green arrows represents meso-cortico-limbic pathway. A8, Retrorubral field; A9, Substantia Nigra; A10, Ventral Tegmenta Area. Amyg, amygdala; DA, dopaminergic neurons; GP, globus pallidus; Hp, hippocampus; N Acc, nucleus accumbens; OB, olfactory bulb; O. Tub, olfactory tubercle; Pit, pituitary; Str, striatum; SVZ, subventricular zone; Thal, thalamus. (Rodríguez-Traver et al., 2015).

The mDA neurons in ventral midbrain (fig. 1.2) have been quantified, in rodents, as 20000 - 40000 neurons, while in human 400000 - 60000 (Björklund et al., 2007). However, it has been shown that environmental stimuli can modify their number and distribution (Tomas et al., 2015).

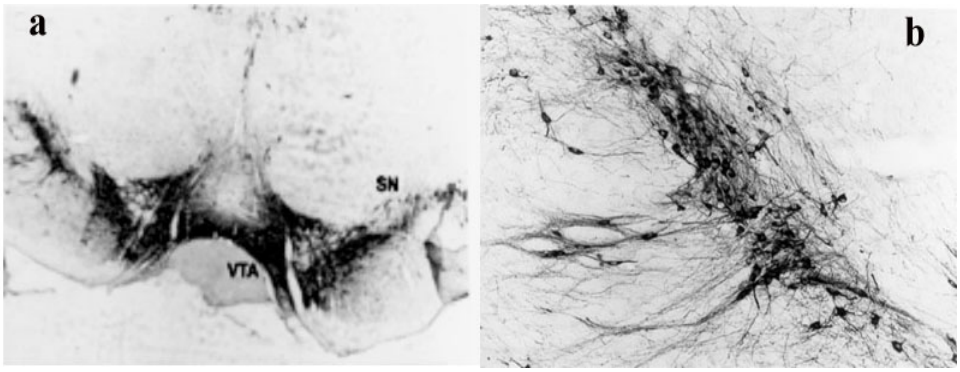

Figure 1.2 Midbrain Dopaminergic Nuclei. a) Immunostaining for Tyrosine hydroxilase (TH) on adult mouse ventral midbrain showing Substantia nigra (SN; laterally) and ventral tegmental area (VTA; medially). **b)** SN, enlargement. It is possible to distinguish SN pars compacta (SNc), where locate DA somata, and SN pars reticulata (SNr) with DA dendrites (di Porzio et al., 1990).

In detail the mDA neurons in ventral midbrain are located as described hereafter:

1) Retrorubral field (RRF, A8) is involved in the modulation of orofacial movements. The retrorubral DA neurons project mainly to Substantia nigra and ventral tegmental area (fig. 1.1) and probably coordinate the action of these two nuclei (Arts et al., 1996). Others projections of RRF are involved in the *arousal* (Simmons et al., 2011).

2) Substantia Nigra (SN, A9), the mDA fibers depart from SN *pars compacta* (SN_c) that contains DA cell bodies, while the dendritic extensions are located in the *pars reticulata* (SN_r) where they connect to the intrinsic GABAergic neurons and reach the *striatum* (corresponding to nuclei *caudate-putamen* in humans) to give rise to the Nigrostriatal pathway (NSp; fig. 1.1). NSp is responsible for decision and control of extrapyramidal motor functions, movement velocity and postural position (Kandel et al., 2000). Moreover, along with the other dopaminergic pathways, it is also partially involved in reward and in the memory consolidation (Wise, 2009).

The extrapyramidal pathway shown in Figure 1.3, imply the strict collaboration between the nuclei of basal ganglia and the cortex. Two different pathways called direct and indirect have been described. Both pathways reach the thalamus and go back to the cortex.

In the **direct pathway** (fig. 1.3), neurons from the cortex projects to the striatum. Here they remove inhibition of internal globus pallidus (GP_i) and SN_r on the thalamus, promoting the feedback signals of thalamus to the cortex. In the **indirect pathway** (fig. 1.3) neurons from the striatum project to the thalamus via the external globus pallidus (GP_e) the subthalamic nucleus and the globus pallidus again.

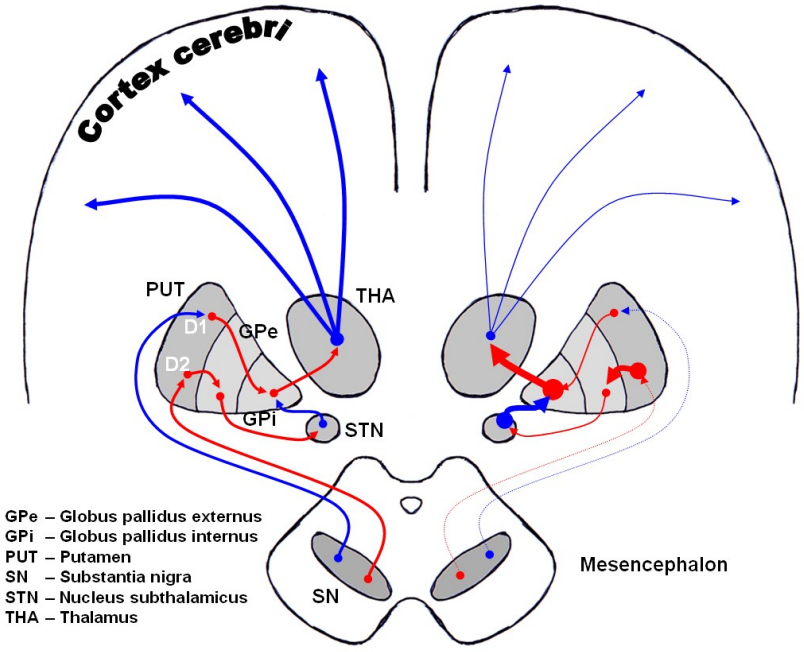

Figure 1.3 Extrapyramidal system neural networks in normal condition (left) and Parkinsons Disease (right). It is shown the modulatory action of *substantia nigra pars compacta* (SNc) dopaminergic neurons on the nuclei *caudate-putamen* (*striatum* in rodents). Excitatory pathways are in blu; inhibitory pathways are in red. SNr, Substantia Nigra *pars reticulata*; GPe, external Globus pallidus; GPi, internal Globus pallidus; STN, Subthalamic nucleus; D_1 and D_2 are the different types of DA receptor in the *striatum* (image from Dorland's Illustrated Medical Dictionary).

The NSp has a critic role in modulating this circuit so to determine, basing on experience, which are the most suitable adaptive behaviors. The loss of SN DA neurons causes Parkinson Disease (PD; Thomas et al., 2007), commonly a disorder with a late-onset. The degeneration of the NSp is responsible for an over-activation of GABAergic inhibitory neurons in the internal globus pallidus, with a subsequent inactivation of thalamic glutamatergic excitatory neurons direct to the cortex. This causes all the typical motor symptoms of PD, such as progressive loss of muscle control, tremor, loss of facial expressivity, rigidity and difficulties in completing simple tasks. It

was first described in 1817 by the famous neurologist James Parkinson in his "Essay on the shaking palsy" (Jenner et al., 1992).

3) Ventral Tegmental Area (VTA, A10), gives rise to a Meso-corticolimbic pathway (MCLp; 1.1), that is implicated in superior cognitive abilities such as reward, attention, and emotions, essential for social behaviors (sex, sociality, and aggression). MCLp neurons project to the nucleus accumbens, olfactory tubercle, cortical areas (prefrontal, cingulate and perirhinal cortex) as well as septum, amygdala, and hippocampus. The alteration of MCLp is the main cause of mood disorders (Zacharko et al., 1991, Martin-Soelch 2009), schizophrenia (Laviolette 2007), attention deficit hyperactivity disorder (ADHD; Ohno 2003), drug addiction and hallucinations (Morales et al., 2012).

Despite SN and MCL pathways are independent their connections overlap each other to promote higher cognitive processes. Indeed different studies have shown that the dysfunction of one can alter the function of the other (Jellinger 1991, Péron et al., 2012).

Moreover, DA neurons are also involved in the *working memory* formation by connecting basal ganglia with the prefrontal cortex. This specific kind of memory is well described in humans and is necessary to keep "active" in mind the acquired information for several seconds, to allow processes like reasoning, comprehension, problems resolution, planning and other complex cognitive functions (Lieberman 2009).

1.2 Dopamine metabolism

Dopamine (DA, a contraction of 3,4-dihydroxyphenethylamine) is neurotransmitter of the catecholamine family. Until of the 1950s, DA was considered only the intermediate for the synthesis of noradrenaline and adrenaline, but the studies conducted by of Von Euler and Lishajko first, and subsequently Bertler and Rosengren, demonstrated that DA is active by itself too. About ten years later, was discovered that the degeneration of DA neurons, in striatum, is a main cause of PD (Carlsson 1959, Ehringer and Hornykiewicz 1960) and the unique treatment for the symptoms of this disorder is use of DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA; Carlsson 1959, Ehringer et al., 1960). Then Carlsson and his collaborators identified in the ventral midbrain (Mb) the origins of the DA found in the striatum and also in the limbic system (Anden et al., 1964).

In the soma and in the presynaptic terminal of dopaminergic neurons, tyrosine is transformed into L-DOPA (fig. 1.4) by the action of tyrosine hydroxylase (TH), the limiting enzyme in the biosynthesis of catecholamines (dopamine, noradrenaline, and adrenaline). TH activity is modulated by its phosphorylation via the protein kinase cyclic adenosine monophosphate (cAMP)-dependent (PKA).

Then, L-DOPA is subsequently transformed into DA by the action of the Aromatic L-amino acid decarboxylase enzyme (AADC). In turn, DA is transferred in vesicles by the vesicular monoamine transporter 2 (VMAT-2), a protein consisting of 12 transmembrane domain. VMAT2 is coupled to the vesicular H+-ATPases (V-ATPases), which functions as ATP-driven proton pump keeping the internal milieu in synaptic vesicles acidic since DA oxidizes rapidly.

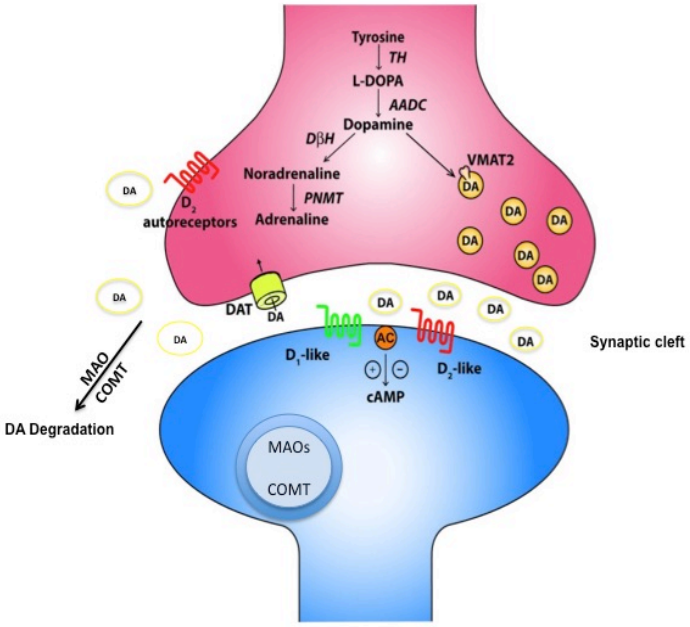

Figure 1.4 Schematic representation of dopamine metabolism and dopaminergic synapse. DA, dopamine; TH, *tyrosine hydroxilase*; L-DOPA, *L–3,4–dihydroxyphenylalanine*; AADC, *Aromatic L-amino acid decarboxylase*; VMAT2, *vesicular monoamine transporter*; MAO, *monoamine oxidase*; COMT, *catechol-O-methyltransferase;* DAT, *dopamine active transporter*; D_2-like dopaminergic inhibitory autoreceptors (DRD2); D_1-like dopaminergic postsynaptic excitatory receptors (DRD1); AC, adenyl cyclase; PNMT, Phenylethanolamine N-methyltransferase; DβH, Dopamine beta-hydroxylase (adapted from Sharples et al., 2014).

After exocytosis of the DA vesicles, DA binds to DA receptors on the postsynaptic membrane (D1-5), leading to the transduction of the signal in the postsynaptic neuron. There are two types of DA receptors (G-protein-coupled), constituted by 7 transmembrane domain receptors: DRD1 (D1 and D5) and the DRD2 (D2 - D4), that act in different and opposite manner. In detail: both types of DRDs modulate the cAMP/PKA transduction cascade and the intracellular Ca2+ levels, however, the DRD1 type promotes an increase in cAMP, conversely, DRD2 determines cAMP decrease (fig. 1.4). These receptors have selective agonists (Vallone et al., 1999) and specific anatomical and cellular distribution that can be pre-synaptic or post-synaptic. The D2 receptors are also localized on the DA neurons membranes (autoreceptors), regulating DA release as feedback inhibition of DA transmission (Bello et al., 2011).

The DA released into the synaptic cleft in part is recaptured by the dopamine transporter (DAT or SLC6A3) a protein of 12 transmembrane domain Na^+/Cl^- dependent transporter that is target of several drugs (cocaine, amphetamine, etc.) and mice knock-out show hyperactivity and insensibility to treatment by these drugs (Amara et al., 1993).

In the alternative, DA is catabolized by cathechol-O-methyltransferase (COMT) in homovanillic acid (HVA) or degraded to 3,4-dihydroxyphenylacetic acid (DOPAC) bys the action of the extracellular or mitochondrial monoamine oxidases (MAOs; fig. 1.4).

1.3 Development of midbrain DA neurons

Dopaminergic neurons in the brain are generated through the action of many transcription factors and endogenous molecules involved in their development in accurate spatiotemporal sequence. Intrinsic and extrinsic stimuli, such as environmental or soluble factors, electric activity, and cell-cell interactions coordinate specific developmental programs.

The complete generation of mDA neurons (fig. 1.5; Abeliovich et al., 2007; Perrone-Capano et al., 2008) is divided into four different phases: early Mb patterning, induction and specification of dopaminergic precursors, differentiation of post-mitotic mDA neurons, functional maturation of mDA neurons.

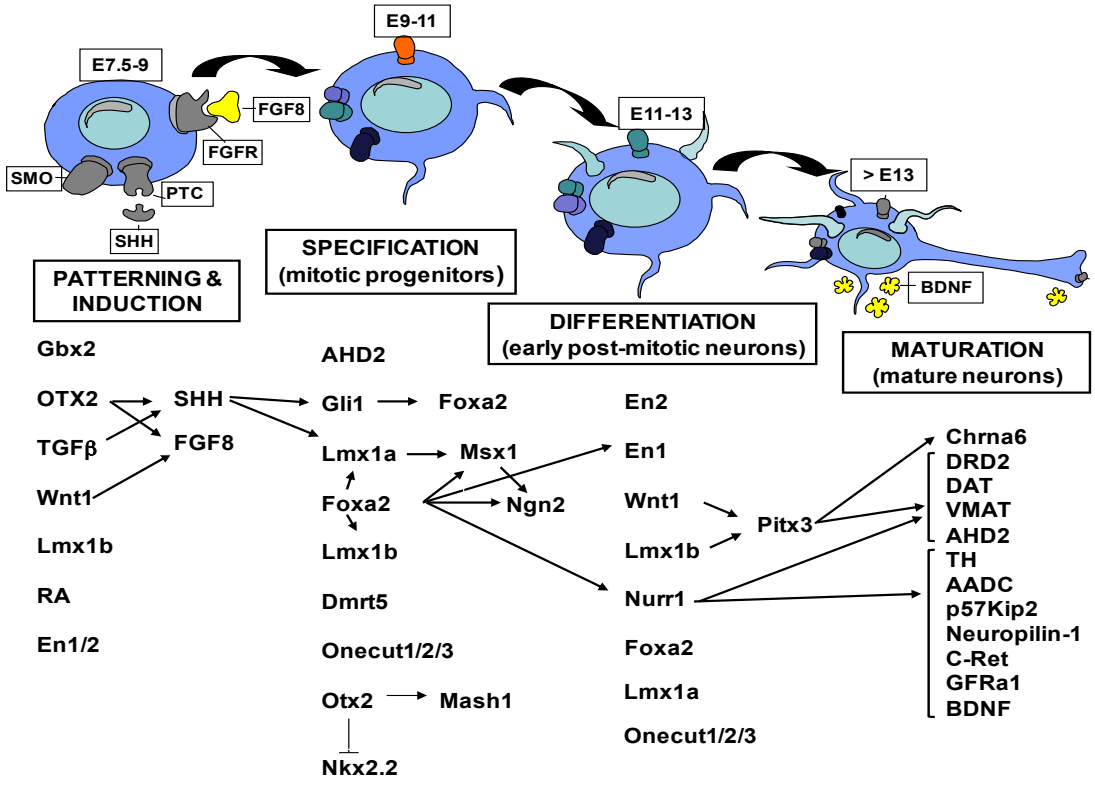

Figure 1.5 Schematic model of mDA neurons development. Diagram showing the main transcription or inductive factors involved in dopaminergic neurons development, divided into phases of the developmental stage in which they are involved (adapted from Perrone-Capano et al., 2008).

1.3.1 Early midbrain patterning: regionalization

During the neural tube development, many inductive factors are released from specific zones, named "organizers" to give rise to different early brain structures: telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. The midbrain generation is guided by signalling of the floor plate (FP) and the mid-hindbrain boundary (MHB or IsO, isthmic organizer). In this process are involved several transcription factors (FTs) such as: Otx2 and Gbx2, which are responsible for MHB formation and their expression is mutually exclusive (Hidalgo- Sánchez et al., 1999). In particular, Gbx2 is expressed more caudally and it is essential for the correct development of hindbrain and cerebellum. Conversely, Otx2 is crucial in the specification and regionalization of telencephalon and mesencephalon (Simeone et al., 2002). Otx2 is also involved in the regulation of some proneural genes like Ascl1 (or Mash1) and Neurogenin2 (Ngn2), implicated into proliferation phases of the mesencephalic progenitors (Vernay et al., 2005).

After MHB formation (E8 in mice) other factors are secreted, indeed it promotes the expression of engrailed transcription factors (En1/2), releases the fibroblast growth factor 8 (FGF8) and guides the correct regionalization along the anteroposterior axis of the developing CNS (1.6). Gene expression analysis and in vitro studies shown that different factors are implicated to correct positioning of the MHB such as the transforming growth factor β (TGFβ; Farkas et al., 2003), the LIM-homeodomain factor Lmx1b (Smidt et al., 2000) and the morphogenetic factor Wnt1 (Schulte et al., 2005).

In detail, Wnt1 is a member of Wnt protein family (19 members), a class of secreted glycoproteins, associated with the transmembrane G-protein-coupled receptors frizzled (Fz) determining the activation of the cytoplasmic protein Dishevelled (Dsh) that regulates transcription of Wnt target genes through its intracellular transducer β-catenin. Numerous members of the Wnt/β-catenin pathway seem to be involved in specification, proliferation, and neurogenesis in the ventral Mb (Prakash et al., 2006). In fact, null mutations for frizzled3 (Fz3) and frizzled6 (Fz6) result in a reduction of mDA neurons (Sousa et al., 2010, Stuebner et al., 2010). Importantly, the activation of Wnt pathway is stronger in the hindbrain.

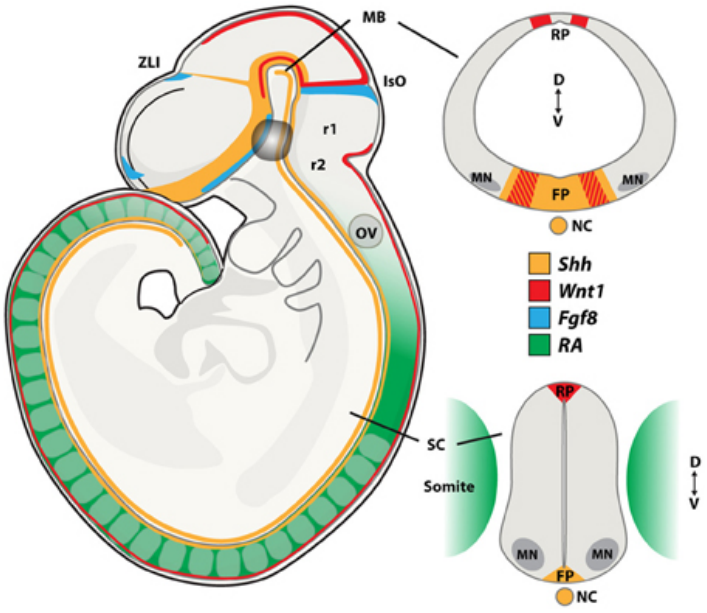

Figure 1.6 Morphogen signaling during neural tube development. Schematic representation of sagittal (left side) and coronal (right side) views of the midbrain (top) and spinal cord (bottom) with the expression pattern for the morphogenes SHH, Wnt1, FGF8 and Retinoic Acid (RA) at E9.5. FP, floor plate; IsO, isthmic organizer; MB, midbrain; NC, notochord; OV, otic vesicle; RP, roof plate; MN, Spinal motor neurons; SC, spinal cord; ZLI, zona limitans intermedia; r1, rhombomer 1; r2, rhombomer 2; D, dorsal; V, ventral. Image from Allodi et al., 2014.

Moreover, Lmx1b is expressed in the MHB at the early stage (E7.5) of mouse development (Adams et al., 2000, Guo et al., 2007), but it is not detectable until E10.5 in mDA precursor (Smidt et al., 2000). Studies conducted on mutant mice for Lmx1b reveal defects in MHB structure, therefore mDA neurons deficiency and altered genes regulation, such as: Fgf8, Engrailed 1 and 2 (En1/2), Pax2, Gbx2 and Wnt1. (Guo et al., 2007). Indeed specific inactivation of Lmx1b in mDA progenitors and not in the MHB does not alter the differentiation of these neurons (Yan et al., 2011). Surprisingly, Lmx1b expression disappears around E11.5 but reappears again at E16 in postmitotic mDA progenitors to be maintained until the adulthood in co-expression with Pitx3 and TH (Dai et al., 2008). Although the loss of Lmx1b leads to a reduction of mDA neurons (Smidt et al., 2000), in *Lmx1b* null mice neural precursors express Nurr1 (Nuclear Receptor-Related 1 protein or *Nr4a2*) and TH^+ dopaminergic markers but fail to express Pitx3. These TH^+ neurons lacking Pitx3 expression are lost around birth suggesting a role for Lmx1b in *Pitx3* regulation and in mDA neurons survival. Interestingly, a similar phenotype is observed for the *Wnt1* null mutant. In this case, the few TH^+ neurons generated lack Pitx3 expression and are lost by E12.5 (Prakash et al., 2006).

The similar effects caused by the loss of these two factors suggest the existence of a regulatory loop between Wnt1 and Lmx1b (Adams et al., 2000, Guo et al., 2007), in addition Lmx1b and Lmx1a cooperation contributes to Wnt1 regulation in MHB and mDA progenitors proliferation (Panhuysen et al., 2004).

1.3.2 Induction and specification of DA precursors

While MHB is essential to determinate the anteroposterior axis, at the same time the notochord and then the floor plate (FP) determinate the dorsoventral axis by releasing the morphogen sonic hedgehog (SHH; before E9.5 in mice). SHH binds patched 1 receptor (Ptc1) preventing its inhibition on Smoothened protein (Smo) and triggering the activation of the Gli family transcription factors (Gli-1/3; Stone et al., 1996, Taipale et al., 2002). FGF8 and SHH are necessary for induction and proliferation of mDA precursors, establishing the proper inductive signals for the correct differentiation (Hynes et al., 1999). The ectopic expression of SHH and FGF8 generate mDA neurons and for that reason, they are usually used for the *in vitro* strategies of mDA differentiation (Lee et al., 2000).

Lmx1a, as well as **Lmx1b**, is a LIM homeobox conserved transcription factor (TF). LIM TFs have two zinc finger motifs specialized in the interaction with cofactors in order to form transcriptional-regulator complexes (Doucet-Beaupré et al., 2015). Lmx1a is expressed early in mice brain (E8.5) in the dorsal midline (roof plate) of the neural tube and then in progenitor zone of ventral midbrain and in optic vesicles (Failli et al., 2002, Millen et al., 2004, Andersson et al., 2006). Loss of Lmx1a (Dreher mice) completely abolishes roof plate induction in the spinal cord (Millonig et al., 2000), the specification of dopaminergic neurons (Andersson et al., 2006) and retina formation in Drosophila (Wang et al., 2016). Thank Wnt1/SHH and FoxA1/FoxA2 pathways are possible to express Lmx1a, the former is regulated by Lmx1a generating an autoregulatory loop (Chung et al., 2012). Lmx1a is co-expressed with Lmx1b also during the post-mitotic maturation (Zou et al., 2009). The loss of one can be compensated from the other one as confirmed by single KO mice (Ono et al., 2007). Both can ectopically generate mDA neurons (Nakatani et al., 2010) and their structures are homologs at 80%. In a recent work, it has been hypothesized that during the specification stage, Lmx1b is required and influences the differentiation of several neuronal subtypes in the Mb, including ocular motor and red nucleus neurons, while Lmx1a functions seem to be more restricted to the mDA fate (Deng et al., 2011).

FoxA1 and **FoxA2** are two additional TFs involved in embryonic development and in tissue specification of mDA by regulating *Ngn2* and later *Nurr1* and *TH* (Ferri et al., 2007, Lin et al., 2009).

Dmrt5 (*doublesex and mab-3-related transcription factor 5*) is a zinc-finger TF, identified from a differential expression screening within the ventral Mb cell populations, for this reason, it is considered crucial in mDA fate specification (Gennet et al., 2011).

1.3.3 Post-mitotic maturation of mDA neurons

In mice, during the post-mitotic stage (E9.5 - E13.5), immature neurons exit of the cell cycle and migrate radially from the ventricular surface (Bayer et al., 1995, Abeliovich et al., 2007). As previously observed, starting from E9.5 radially migrating cells express TH. The first TH^+ cells and fibers appear close to the ventricular ependymal layer (di Porzio et al., 1990). Many TFs are involved in post-mitotic mDA, such as: En1/2, Nurr1, Pitx3, and Lmx1a, but none of these transcription factors are sufficient to induce the mDA phenotype in Embryonic Stem Cells (ESc)

in vitro, suggesting that a more intricate regulatory network is required for proper DA development (Abeliovich et al., 2007).

Nurr1 (Nr4a2) is a member of orphan receptor subfamily of the steroid nuclear hormone receptors, with Nur77 (Nr4a1) and Nor-1 (Nr4a3) (Zhao et al., 2010). It is considered an orphan nuclear receptor since its ligands have not been identified yet (Mangelsdorf et al., 1995). Nurr1 is largely expressed in the SN, VTA, limbic system and olfactory bulbs (Zetterström et al., 1997). In mice, its starts being expressed at E10.5, before TH (Volpicelli et al., 2004, Jankovic et al., 2005). Mice Nurr1$^{-/-}$ born with a normal frequency but they die within two days; they are deprived of TH^{+} cells in the Mb (SN and VTA) and are characterized by loss of striatal innervations and DA markers (Zetterström et al., 1997). Nurr1 overexpression is typically used to induce different cell type toward DA phenotype (Wagner et al., 1999, Chung et al., 2002). Indeed, it is involved in the regulation of several DA markers, such as: TH (Zhou et al., 1995), Dat (Giros et al., 1996), Vmat2 (Colebrooke et al., 2006), p57Kip2 (Joseph et al., 2003) and c-Ret (Jain et al., 2006, Kramer et al., 2007), Bdnf (Volpicelli et al., 2007). Furthermore, genetic studies prove that mutations in Nurr1 are a cause of the rare familial form of PD (Le et al., 2003, Sleiman et al., 2009, Decressac et al., 2013).

Pitx3 (Pituitary homeobox 3) is expressed in several tissues during embryonic development, but after the birth, it is detectable only in mDA (Smidt et al., 1997). Aphakia mutant mice (*ak*), harboring a spontaneous mutation in the gene coding for Pitx3, shown motor impairment due to specific loss of mDA neurons in SN while VTA appears unaffected (Smidt et al., 2007). Pitx3 expression in SN precedes that of TH while in VTA it occurs simultaneously (Maxwell et al., 2005). These data strongly suggest that specific differentiation programs must take place in the two DA subpopulations, thus explaining their different susceptibility to the loss of Pitx3.

A recent study demonstrated that Nurr1, Pitx3, and Lmx1a are key of mDA generation (Hong et al., 2014). Both Lmx1a and Lmx1b persist during maturation of mDA. **Lmx1a** inhibits non-dopaminergic destinies (Deng et al., 2011) and regulates axonal guidance promoting *Slit2* expression (Yan et al., 2011); while **Lmx1b** is involved in the maturation of post-mitotic cells by regulating Pitx3. Indeed, Lmx1b KO mice show the specific loss of mature mDA neurons (Simeone 2005).

TFs **En1** and **En2**, like Lmx1b, are important during the first phase of MHB formation (Liu et al., 2001) but their expression is not detectable anymore till E11.5 when En1 and En2 start to be expressed again in ventral mDA differentiating neurons. This expression is maintained into and throughout the adulthood (Simon et al., 2001, Albéri et al., 2004). En1 and En2 are required to prevent apoptosis suggesting a role in maintenance and survival of ventral mDA (Albéri et al., 2004).

1.3.4 Functional maturation and survival of mDA neurons

The last stage of mDA neurons generation takes place in the striatum (E15.5 in mice), where various FTs and molecules are involved in neurons maturation, maintenance, and guidance.

EphrinB2 and its receptor EphB1, respectively express in the striatum and mDA neurons, enrich SN DA striatal innervation (Yue et al., 1999). *In vitro* EphrinB2 overexpression in mesencephalic primary cultures, increases *Nurr1* transcript (Calò et al., 2005). mDA circuits formation are also regulated by the semaphorins and netrin (Torre et al., 2010, Xu et al., 2010). mDA projections undergo also axonal growth inhibitions by the diffusible chemorepellents. For e.g. **Slit2** and its receptors Robo, play a major role in guiding developing axons towards their correct targets by preventing them from entering or steering them away from certain regions (Lin et al., 2005, Dugan et al., 2011).

After correct formation of dopaminergic circuits, there are two moments around postnatal day 2 (P2) and around P14, when mDA neurons undergo towards naturally-occurring cell-death (Burke 2003). In this moment, mDA need to receive growth and neurotrophic factors by post-synaptic cells to survive. The most well-established target-derived neurotrophic factor for VM DA neurons is the Glial cell line-derived neurotrophic factor (**GDNF**; Lin et al., 1993, Beck et al., 1995, Akerud et al., 1999). Other neurotrophic factors identified for VM DA neurons include the brain-derived neurotrophic factor (**BDNF**; Hyman et al., 1991), and the more recently identified dopamine neurotrophic factor (**CDNF**; Krieglstein 2004, Lindholm et al., 2007). Both GDNF and BDNF show a protective role on mDA neurons, following a number of experimental lesions. They also promote neuronal survival and differentiation *in vitro* (Hyman et al., 1991, Feng et al., 1999, Consales et al., 2007).

Many neurodegenerative diseases depend on the absence of neurotrophic signals, for this reason, these molecules could be therapeutic drugs for the treatment of DA-associated neurological disorders.

1.4 *In vitro* generation of mDA neurons

In the last years, several studies have been dedicated to the identification of mechanisms underlying development and function of dopaminergic neurons. The final aim is being able to generate, *in vitro,* mDA neurons useful for both transplantation approaches and modeling DA-related pathologies.

Several studies have shown that Embryonic Stem Cell (ESC) and Neural Stem Cell (NSC) cultures can generate TH^+ cells expressing mDA phenotypic markers and that the *in vitro* developmental program appears to recapitulate the temporal course of normal mDA development (Kim et al., 2002, Barberi et al., 2003, Martinat et al., 2004, Sonntag et al., 2004, Andersson et al., 2006). The embryonic stem cells (ESC) are pluripotency and generate every cell type. Conversely, multipotent and pluripotent stem cell lines can generate fewer cell types but can be isolated also from adult tissues and for these reasons, they are useful for the generation of cellular models from affected patients.

A useful alternative for modeling DA neurons in vivo are Epiblast Stem Cells (epiSCs), pluripotent cells isolated from mouse post-implantation epiblasts (around E9, E10). An interesting feature of epiSCs is that they show patterns of gene expression and signaling responses more similar to human ESC (hESC) then to mouse ESC (mESC) (Chenoweth et al., 2010). Previous works showed mESC and hESC are distinct in their epigenetic state and in the signalling guiding their differentiation. Moreover, mESC and hESC use also different signalling pathways to maintain their pluripotent status.

EpiSCs maintain OCT4 and SOX2 expression, but they downregulate expression of most of the other pluripotency factors, including NANOG, ESRRβ, KLF2 and KLF4 (Hackett et al., 2014). Moreover, EpiSCs have not undergone differentiation, but they upregulate lineage commitment factors such as homeobox protein OTX2, Brachyury and zinc-finger protein ZIC2 (Buecker et al., 2014).

In classical growth condition, epiSCs are able to proliferate and self-renew in presence of Activin and bFGF, to form teratomas following in vivo injection (Guo et al., 2009).

The main advantages in using epiSC rather than ESC are that epiSC are more homogenous in the undifferentiated state and they seem to be primed to differentiate, as demonstrated they are a more rapid and efficient tool for the obtainment of DA neurons in vitro (Jaeger et al., 2011).

As described following in Materials and Methods, it is possible to differentiate epiSCs toward the neuronal phenotype, removing Activin and bFGF. With this differentiation protocol, both neuroectodermal and neuronal markers appear earlier when compared to ESC. Interestingly, by a short treatment with the FGF/ERK pathway inhibitor PD0325901 (PD03), we could induce an earlier expression of Lmx1a and Foxa2, two of the main TFs implied in the early phases of mDA development. Addition of SHH and FGF8 is necessary to address differentiation toward the DA phenotype. Using this protocol, nearly 40% of TH^+ neurons generated from epiSCs co-express Pitx3 while in the case of ESC derived neuronal populations, $Pitx3^+$ neurons were rarely observed (Jaeger et al., 2011).

Starting from epiSCs differentiated toward DA phenotype, the goal of this thesis was to discover microRNAs (miRNAs) expressed in mDA neuron and potentially involved in their differentiation.

1.5 MicroRNAs

MicroRNAs (miRNAs) are a class of small non-coding single-strand RNA (~22 nucleotides) evolutionary conserved and encoded within the genomes of almost all eukaryotes (Fabian et al., 2012). Organisms express hundreds of miRNAs involved in the regulation of many biological processes, such as embryonic development, cell differentiation and growth, cell proliferation, apoptosis, and regulation of metabolic processes (He et al., 2004). For this reason, altered miRNAs levels might be involved in the onset of pathological conditions (Im et al., 2012, Junn et al., 2012). In mammals, miRNAs act as the post-transcriptional regulators of gene expression, by targeting partially complementary sequences in the 3' untranslated regions (UTRs) of the target messenger RNA (mRNAs) that are in turn directed to degradation or translational repression (Bartel et al., 2004). About 60% of all human transcripts contain known or predicted miRNA target recognition sites (Friedman et al., 2009). A single type of miRNA may have up to

thousands of targets and each mRNA can be targeted by several microRNAs, suggesting a really strong role for miRNAs in the complex landscape of gene expression but at the same time giving rise to a huge complexity in its understanding and characterization. Additionally, many miRNAs have multiple paralogs throughout the genome (McCreight et al., 2017).

The first miRNA called lin-4, was discovered in 1993 in *Caenorhabditis elegans (C. elegans),* as a regulator of lin-14 protein expression (Lee et al., 1993). Later another miRNA, let-7, was identified in *C. elegans* (Reinhart et al., 2000) and surprisingly was found to be extremely conserved among a wide range of species, human included (Pasquinelli et al., 2000). One year later were identified other tens of them (Lagos-Quintana et al., 2001, Lau et al., 2001, Lee et al., 2001). At that point was clear that a new class of gene expression regulators had been identified and so they were called microRNA, but the widespread effects of miRNAs were not fully recognized until the early 2000s (Berezikov 2011).

1.5.1 miRNAs biogenesis

MicroRNAs genes, transcribed from the genome result in a primary miRNA transcript that may include a single miRNA or a cluster of miRNAs (Berezikov 2011). Several miRNA coding genes are located in regions of the genome relatively distant from previously annotated protein coding genes. They are transcribed as independent units with their own promoter (Bartel et al., 2004). However, almost 40% of miRNA genes have been found to be in introns of protein coding or non-coding RNA and in this case they are generally found in a "sense orientation" leading to think they are regulated together with their host transcript (Rodriguez et al., 2004, Kim et al., 2007). More rarely miRNAs can be found in exons of protein coding genes (Rodriguez et al., 2004). miRNAs can be transcribed as monocistronic transcripts or polycistronic transcripts (miRNA cluster) originating by local duplication of an existing miRNA locus (Altuvia et al., 2005). At the same time is not rare to find miRNA families with paralogues located in different genomic loci in monocistronic units or even clusters containing a wide variety of miRNA families (Olena et al., 2010).

Most miRNAs have a protein coding gene-like promoter and are usually transcribed by RNA polymerase II (Lee et al., 2004, Zhou et al., 2007). The product of transcription is a long RNA,

called primary-miRNA (primiRNA), with one or more stem and loop structures, whose length depends on the miRNA gene type, while for the intronic miRNA it is the protein coding mRNA itself and the pri-miRNA origins from the splicing process (Lee et al., 2002, Cai et al., 2004). Pri-miRNAs are generally 5'capped and 3' poly-adenylated (Cai et al., 2004).

In figure 1.7 is shown the canonical miRNA processing pathway, the pri-miRNA is bound by the Di George syndrome critical region 8 (DGCR8; Pasha in invertebrates). Regions of a primary miRNA form hairpin structures that are recognized by the ribonuclease type III (RNAaseIII) enzyme Drosha (Gregory et al., 2006), which cleaves the doubles tranded stem region of the hairpin to produce an approximately 83 nucleotide (nt) precursor miRNA (pre-miRNA) (Fang e al. 2013). The resulting pre-miRNA, because of the typical RNAaseIII cut, will have two nucleotide overhang at its 3'end with a 3' hydroxyl and a 5' phosphate terminal groups (Basyuk et al., 2003, Lee et al., 2003). After that, Pre-miRNA is exported into the cytoplasm by the nucleocytoplasmatic shuttle protein Exportin-5 (fig. 1.7). This protein is able to recognize and bind the 2nt 3'overhang of the precursor and to transport it across the nuclear membrane, in a RanGTP dependent manner (Bohnsack et al., 2004). After being exported to the cytoplasm, pre-miRNA is further processed by a second endonuclease, Dicer, that acts in a complex with the Transactivating response RNA binding protein (TRBP) and other cofactors, to remove the terminal loop of the pre-miRNA (Grishok et al., 2001, Hutvágner et al., 2001). The result of this enzymatic cleavage is an approximately 22 nt double-stranded RNA duplex that contains the mature miRNA and its complement, called the star strand (*) (Hutvagner et al., 2001). The duplex is then separated, the strand with the less thermodynamically stable 5' end becomes the mature sequence, while the star sequence is degraded. The mature miRNA is loaded together with Argonaute (Ago 2) proteins onto RNA-induced silencing complex (RISC) (Bartel et al., 2004; Winter et al., 2009). The Ago protein contains two RNA-binding domains: the PAZ domain binds the 3'end of the mature miRNA while the PIWI domain interacts with the 5'phosphate group on the 5'end (Pratt et al., 2009). Once the miRISC is assembled, the miRNA drives it to silence target mRNA via mRNA cleavage, translational repression or deadenylation (Nilsen 2007, Winter et al., 2009).

1.5.2 miRNAs: Mechanism of action and bioinformatics identification

miRNAs may have a negative or a positive regulatory effect (Ambros 2001). In animals, they usually bind with partial complementarity to 3'UTR regulatory elements on mRNAs called 'seed sequences', or to miRNA response elements (MREs) that causes translational repression (Ambros 2004). A major silencing mechanism of miRNAs in animals results in target mRNA destabilization through a cleavage-independent process, affecting transcript level (Lim et al., 2005; Pillai et al., 2004). A small number of miRNAs also show decoy activity by binding directly to proteins such as RNA-binding proteins, inhibiting interaction with their target RNAs (Eiring et al., 2010). In some cases, miRNAs also regulate gene expression at the transcriptional level (Kim et al., 2008) by binding directly to DNA regulatory elements. In certain cases and cell types, they can enhance translation (Vasudevan et al., 2007).

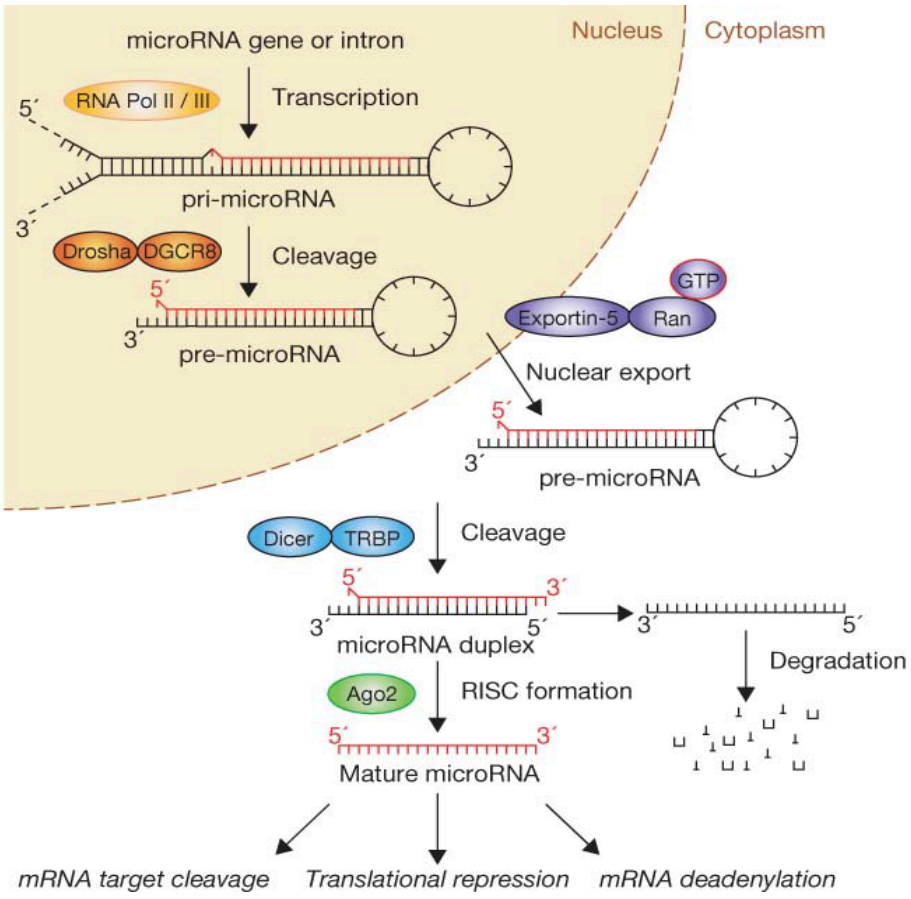

Figure 1.7 miRNAs biogenesis and action. miRNA processing pathway. The main steps for miRNA maturation are shown: Transcription of the pri-miRNA by RNApolII or III; cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus; export to the cytoplasm by Exportin-5–Ran-GTP; cleavage of the pre-miRNA by Dicer in complex with TRBP; miRNA duplex functional strand recruitment, together with Ago2, into the RISC complex, where it guides RISC to silence target mRNA. (Image from Winter et al., 2009).

However, there are varying degrees of complementarity between a miRNA and its mRNA target, binding is most highly dependent on positions 1 through 8 of the 5' end of the mature miRNA, known as the seed region (Berezikov 2011). While 75% of down-regulated mRNA have canonical seed sites in their 3' UTR, the seed region is not always sufficient for causing down-regulation (Kim et al., 2008). The 3' end of the mature miRNA can also have an effect: positions 13±16 are highly conserved, and their proper complementary base pairing to a mRNA target is associated with down-regulation (Grimson et al., 2007).

A number of bioinformatic tools and databases have been devised to manage the growing body of data and at present, there are 129 miRNA tools currently used in miRNA research (Akhtar et al., 2015).

A well-known database for miRNAs is miRBase (http://www.mirbase.org/). It has emerged as a definitive repository of miRNA sequences as well as an authoritative source of miRNA nomenclature that is a valuable resource for miRNA profiling studies (Pritchard et al., 2012). Indeed miRBase was established with the aim to annotate the continue increasing number of miRNA being discovered and to provide a trustworthy resource for any researcher interested in looking after a miRNA sequence, miRNA cluster, family composition or miRNA genome localization (Kozomara et al., 2011). This resource provided for each miRNA a unique name in agreement with the scientific community (Ambros et al., 2003). miRNAs name consist of "miR-" preceded by a three-letter-code, identifying the species (the first one corresponding to the genus and the following two to the species. e.g. mmu for Mus Musculus) and followed by the numeric identifier for the specific miRNA. Variants of the same miRNA, present in multiple copies into the genome, can often be followed by an extra letter the distinguish them (e.g mmu-miR-148a) while paralogues miRNAs (transcribed by different promoters; often, in this case, the mature miRNA sequence is identical) have a dash followed by the paralogue number (e.g. mmu-mir-218-1). The name of the mature form of the miRNA is followed by the suffix -3p or -5p depending on the strand of the pre-miRNA from which the mature one comes from (e.g. mmu-218-1-5p).

To predict how miRNA-mRNA interaction occurs, several prediction tools are currently used. The most used are TargetScan *(*www.targetscan.org/) (Grimson et al., 2007, Friedman et al., 2009) and miRanda (http://www.microRNA.org/) (Enright et al., 2003, John et al., 2004). The majority of algorithms at the base of these software packages use a similar set of parameters to

identify and classify each candidate target site. These usually analyze seed region complementarity between the miRNA and the target site, free energy of the RNA duplex formed, with some of the methods also taking into account regions surrounding the target site. To increase the prediction reliability, a "conservation" filter can be applied with the assumption that the biological relevant loci are more likely to be conserved during the evolution. It is important to underline that experimental procedure is necessary to support predictions and discover really the interaction between miRNA-mRNA targets.

1.5.3 miRNAs & diseases

Approximately 2200 miRNA genes have been reported to exist in the mammalian genome, the abnormal expression of miRNAs has been proven to be extensively involved in the pathogenesis of numerous types of diseases, such as cardiovascular, inflammatory, autoimmune, neurodevelopmental, skeletal and skin, liver diseases and cancers (Ardekani et al., 2010). miRNAs such as miR-9, miR-124a/b, miR-135, miR-153, miR-183, and miR-219, among others, have also been shown to play critical roles in the development and function of the brain where they have been found specifically expressed in differentiating neurons (Sempere et al., 2004). They are crucial players in several aspects of brain development such as neurogenesis, neuronal maturation, synapses formation, axon guidance and neuronal plasticity (Kapsimali et al., 2007; McNeill et al., 2012). The brain enriched of miR-137 has been shown is an essential regulator in controlling the dynamics between neural stem cell proliferation and differentiation during neural development (Sun et al., 2011). Furthermore, cellular and animal models show that miR-218 is essential in motoneurons development and disease (Thiebes et al., 2015).

In particular, there are many studies that underline how an alteration of a specific miRNA is cause of DA neuron deprivation and Parkinson's disease (Kim et al., 2007; Miñones-Moyano et al., 2011; Saba et al., 2012; Tobon et al., 2012; Yang et al., 2012). Regarding more specifically the dopaminergic neurons, nowadays very few miRNAs have been identified as involved in their development or function. miR-133b is enriched in human Mb, where it is thought to regulate the maturation and function of mDA through a negative feedback circuit involving the transcription factor Pitx3 (Kim et al., 2007). miR-132 is expressed in ESC-derived TH^+ cells and regulates Nurr1 mRNA (Yang et al., 2012). Studies on miRNA expression in neuronal diseases suggest a

role of these molecules in neurodegeneration (Kim et al., 2007, Hébert et al., 2009, Doxakis et al., 2010). miR-142-3p has been shown to suppress D1 type DA receptor expression both during development and in cell culture (Tobón et al., 2012). miR-181a is induced by dopamine signalling in primary neurons, as well as by cocaine and amphetamines, in a mouse model of chronic drug treatment (Saba et al., 2012). Recent works underline the importance of miR-135a2 in determining midbrain size and the allocation of prospective mDA precursors by modulating the extent of the Wnt signaling (Joksimovic et al., 2014; Nouri et al., 2015). miR-34b and miR-34c have been found to be significantly decreased in PD patients (Miñones-Moyano et al., 2011). miR-218 has been found to be expressed in the ventral midbrain of E12.5 mouse embryos, and specifically lost in the *Wnt1-Cre* conditional knock out (Huang et al., 2010).

The increasing knowledge about the role played by miRNAs in the dopaminergic system will provide important insights into molecular mechanisms involved its alterations and could eventually generate novel targets for therapeutic care.

1.6 Aim of PhD thesis work

MicroRNAs, are key regulators of gene expression, can influence many biological processes and are biomarkers for diseases. For these reasons, miRNAs expression profiling is gaining increasing popularity (Pritchard et al., 2015).

The aim of this thesis is to individuate miRNAs expressed in dopaminergic neurons and potentially involved in their development and function. Several studies suggest that miRNAs are involved in Dopaminergic neurons development, function and disease, despite details regarding their mechanism of action are still missing (Kim et al., 2007; Miñones-Moyano et al., 2011; Saba et al., 2012; Tobon et al., 2012; Yang et al., 2012).

To identify which miRNAs are expressed in mDA neurons, I analyzed, through a bioinformatics approach, microarray data, obtained from EpiSCs differentiation to DA phenotype, available in my host Lab. Each miRNA were evaluated for their capacity to induce DA phenotype. This approach allowed the identification few miRNAs able to promote mDA neurons differentiation and which are selectively expressed in the midbrain. The most interesting candidates I identified are miR34b/c and miR-218. Interestingly miR-218 was known being expressed in motoneurons where it is essential to generate correct establishment of the neuromuscular junction (Amin et al., 2015; Thiebes et al., 2015). However, its role in dopaminergic neurons is still not clear. To further understand its role in dopaminergic neurons I have generated conditional KO mice (cKO) for miR-218. By mating these mice with mice expressing the Cre Recombinase under the control of specific tissue promoter, we are currently able to evaluate the contribution of miR-218 to the development of specific neural subpopulations. Our preliminary data show that miR-218 is important for proper motor function and suggest its potential role in DA neurons.

Because is still unclear the non-coding RNAs role in the DA neurons, any additional information will be important to clarify and establish their function in DA system development, a phenomenon remained elusive till now.

In addition, this new knowledge appears to have a major role in the practice of personalized medicine or the treatment of DA system-linked diseases in the near future.

2 - MATERIALS AND METHODS

2.1 Cell Cultures

Materials for tissue culture, like multiwell plates, serological pipettes or pipette tips, were purchased from Corning, BD Biosciences or Nunc. All steps were carried out inside a laminar flow sterile hood (Jupiter) to avoid contamination. All the equipment was sprayed with Ethanol (70%) before use. Cells were incubated at 37°C with 5% CO_2 in a humidified incubator (Thermo Forma). Dissections were performed with horizontal flow hood (Hermes II). All the factors and reagents mentioned in the below described methods are listed in Table 2.1.

2.1.1 Animals and dissections

Timed pregnant wild-type C57BL/6 (Charles-River) or C57BL-6-Tg.pTH-GFP (Dr. Hideyuki Okano) mice were sacrificed in accordance with Society for Neuroscience guidelines and Italian law. The embryonic age (E) was determined by considering the day of insemination (as confirmed by vaginal plug) as day E0. Embryos from day 12.5 (E12.5) or E14.5 of gestation were quickly removed and placed in phosphate buffered saline (PBS), without calcium and magnesium and supplemented with 33 mM glucose. The brain structures (midbrain, striatum, and cortex) were carefully dissected under a stereoscope in sterile conditions and processed for cell cultures. Tissues were pooled and triturated with a mechanical dissociation.

2.1.2 Mesencephalic primary cultures (mE12.5-PCs)

Single cells were obtained from embryonic midbrain as previously described (Prochiantz et al. 1979, di Porzio et al. 1980). Briefly, the tissues were transferred into a 15 mL tube and mechanically dissociated with a sterile pipette in a solution containing 0,01% pancreatic DNAse. The cell suspension was centrifuged 5' at 100 g and resuspended in plating medium and counted. For the viable count, the cell suspension was diluted 1:10 with 0,1% Trypan blue and loaded into a Burker's counting chamber slide. Cell concentration was determined on the basis of the total cell count, the dilution factor, and the trypan blue exclusion.

Dissociated cells were plated at a density of 4×10^4 cells/cm^2 on multiwells previously coated with 15 μg/mL poly-D-lysine for 1 h at 37°C and washed three times with sterile H_2O. Cells were grown in NBM, supplemented with B27, 0.5 mM L-glutamine, Pen/Strep, bFGF (20 ng/mL) FGF8 (10 ng/mL) and SHH (50 ng/mL) to induce mDA phenotype. After 3 days in culture, (*days in vitro*, DIV) half of the medium was replaced and inducible lentiviruses were added at respective dilutions. At DIV6, the proliferative medium was replaced with a differentiating medium, NBM supplemented with B27, 100 mM L-glutamine, Pen/Strep, Ascorbic acid (200 μM), and 1 mM dibutyryl cyclic adenosine 3', 5'-monophosphate (cAMP). From DIV6 the expression of *trans*genes was induced by the addition of doxycycline (4μg/mL) to the medium. At DIV12 cells were fixed or collected for further analyses.

2.1.3 Embryonic Stem Cells (ESCs)

$R26^{CreER/+}$ mouse embryonic stem cells culture (Omodei et al. 2008) were performed in Glasgow Minimal Essential Medium (GMEM, SIGMA) plus 12% FBS (Hyclone) and LIF (300U/ml Millipore). To induce DA differentiation mES were plated at low density on the gelatin-coated plate in N2B27 medium (day 0). Four days after plating cells were passaged on Poly-L-lysin (15μg/ml in PBS)/Laminin (20μg/ml in PBS). From the day next until the day 9 SHH (200 ng/ml) and FGF8 (100ng/ml) were added to the medium. Cells were cultured until day 14.

2.1.4 Epiblast Stem Cells (EpiSCs)

EpiSCs have been derived as described by Guo and colleagues (Guo et al. 2009). They are cultured in *epiSC medium* containing half DMEM/F12 and half Neuralbasal medium, supplemented with N2, retinol-free B27, 2mM L-Glutamine, 0.05 mM β-mercaptoethanol, 10ng/mL bFGF and 20 ng/mL Activin. Cells were split every 2-3 days as epiSC by using mechanical dissociation with a 2mL serological pipet and plated in multiwell plates, coated with FCS for 30' at 37°C. For DA differentiation epiSCs were plated one day before the bFGF and Activin withdrawal in 12-wells plate, previously coated with a solution of 15 μg/mL Fibronectin for 30' at 37°C. One day later (DIV1), cells should reach 60 to 80% of confluence and at this point is possible to switch the medium from *epiSC medium* to simple *N2B27 medium*, that is *epiSC medium* but without bFGF and Activin. During these phases, cells were infected with

lentiviral particles as described in single experiments. At DIV2 cells were split and diluted on a new 12-wells plate previously coated with Fibronectin. From DIV5 to DIV9 cells were maintained in *N2B27 medium* supplemented with SHH (100 ng/mL or SAG 0.4 μM) and FGF8 (100 ng/mL). Usually, at DIV9 *N2B27* is supplemented with doxycycline (4 g/mL) and ascorbic acid (200 μM). Cells were cultured until DIV16.

Description	**Manufacturer**	**Catalogue number**
Fibronectin	Millipore	FC010
Gelatine	Sigma-Aldrich	G1393
Laminin	Sigma-Aldrich	L2020
Poly-D-lysine	Sigma-Aldrich	P7405
DMEM	Invitrogen	11995065
DMEM/F12	Invitrogen	21331-020
F12	Invitrogen	21700-026
GMEM	Sigma-Aldrich	G5154
IMDM	Invitrogen	31980-030
Neurobasal NBM	Invitrogen	21103-049
FBS	Euroclone	ECS0180L
FCS	Biosera	1810-500
B27	Invitrogen	17504-044
B27 wo vit. A	Invitrogen	12587-010
N2	Invitrogen	17502-048
βmercaptoethanol	Invitrogen	31350-010
HEPES	Invitrogen	15630-106
L-glutamine	Euroclone	ECB3000D
Na-piruvate	Invitrogen	11360-039
Pen/Strep	Sigma-Aldrich	P0781
Trypsin	Sigma-Aldrich	T4799
DNase	Sigma-Aldrich	DN25
Activin	R&D	338-AC-025
bFGF	Sigma-Aldrich	F0291
EGF	Sigma-Aldrich	E9644
cAMP analog	Sigma-Aldrich	D0627
Doxycycline	Clontech	631311
FGF8	Sigma-Aldrich	F6926
L-ascorbic acid	Sigma-Aldrich	A4544
LIF	Millipre	ESG1107
SHH	R&D	1845-SH-100
SAG	Sigma-Aldrich	SML1314

Table 2.1 Factors and reagents used for cell cultures.

2.1.5 HEK293T cells

Human embryonic kidney 293T (HEK293T) cells were generated from human embryonic kidney cells obtained from a single apparently healthy foetus legally aborted. In the early 70s, these cells were genetically transformed with Adenovirus 5 DNA to obtain a stable cell line (Graham et al. 1977). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator, in DMEM with 10% foetal bovine serum (FBS), Pen/Strep and 25 mM Hepes.

2.1.6 HeLa cells

HeLa is an immortalized epithelial human cell line derived from cervical cancer cells (Scherer, Syverton et al. 1953). HeLa cells were maintained at 37°C with 5% CO2 in a humidified incubator, in DMEM (Invitrogen) with 10% foetal bovine serum (FBS; Euroclone) and 100 unit/ml Streptomycin (Sigma) and 100 μg/ml Penicillin (Sigma).

2.2 Lentiviral production

Last generation lentiviruses have been constructed in order to contain less than 10% of the original viral genome. They express only the sequences needed for reverse transcription and integration of the gene expression cassette into the host genome. To further increase the security level, these genes are cloned in three different vectors to minimize the risk of the recombination event. To further minimize the possibility to generate functional auto-replicant viruses, the Psi (ψ) sequence, responsible for the viral genome packaging, is localized exclusively on the *exogenous gene*-containing transfer vector. The gene expression is under a control of a doxycycline-inducible promoter (Tet-ON). The used vectors for the production of the lentiviral particles were: a Gag/Pol containing pMDL vector, a pRev vector, a pVSV-G vector and the exogenous gene-containing transfer vector Tet-O-FUW. Moreover, a prtTA vector, expressing the reverse tetracycline transactivator (rtTA) protein, in combination with pMDL, pRev and pVSV-G was necessary to induce gene expression in the presence of the antibiotic tetracycline or one of its derivatives (e.g. doxycycline; rtTA vector was supplied by Dr. Caiazzo, IGB, Naples). For all these vectors Gigapreps were made using the EndoFree Plasmid Gigaprep (Qiagen) following the manufacturer instructions.

2.2.1 PCR for cloning reactions and vector construction

RNA extracted from an E14 embryonic mesencephalon was used for the cDNA preparation as template to perform PCR for the intended target 3'UTRs to amplify and clone into the pmiR-Report vector. While, In the case of pre-miRNA coding regions amplicons to clone in the Tet-O-Fuw vector, genomic DNA from E14 embryonic mesencephalon was used as template. Oligos were designed in a way to amplify specific gene containing specific restriction sites (Table 2.2).

Oligos used to amplify several genes 3'UTRs were designed with an 11nt long 5'end tail containing the SpeI (forward oligo) and the HindIII (reverse oligo) restriction sites. Oligos used to amplify pre-miRNA coding regions were designed both with an 8 nt long 5'end tail containing the EcoRI restriction site.

For 3'UTR amplicons, DNA band extraction from the agarose gel was performed using the PureLink® Quick Gel Extraction Kit (Invitrogen) according to the provided instructions. Eluted DNA was incubated for 4h at 37°C with the specific restriction enzymes (SpeI and HindIII for 3'UTR amplicons).

For pre-miRNA amplicons instead, Amplicons were directly cloned into a pCR®2.1-TOPO® TA (Invitrogen) vector following the TOPO® TA Cloning Kit (Invitrogen) subcloning protocol. In order to isolate the insert on each gene-containing TOPO TA plasmid was restricted with a specific enzyme (4 h at 37°C). Insert band was extracted from the agarose using the PureLink® Quick Gel Extraction Kit (Invitrogen) according to the provided instructions.

For the ligation step, 50 ng of the vector were used. The amount of insert to use for the ligation reaction was calculated using following formula:

$$ng\ insert = \frac{(ng\ vector \times kbp\ insert)}{kbp\ vector} \times 3$$

A control reaction was performed without adding the insert to the reaction. The enzyme I used was a T4 ligase (NEB) and the reaction buffer was the one provided by the company. Ligation was conducted over-night at 16°C in a final volume of 10 µl with 400 units enzyme. 5 µl of this reaction were used to transform 50 µl of competent DH5α cells (Invitrogen) following the standard transformation protocol: 30' (minutes) on ice, heat shock at 42°C for 30'' (seconds), 2' on ice, cells suspension in 250 µl SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), followed by 1 h growth in

agitation at 37°C. Finally, the cells were seeded on Lysogeny broth (LB)-Agar plates (10 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract, 20 g Bacto-agar) containing ampicillin (50 ng/ml) and growth over-night at 37°C. Single colonies were screened by PCR. Positive colonies were grown in 5 mL LB (10 g NaCl, 10 g Bacto-tryptone, 5 g yeast extract) containing ampicillin (50 ng/ml). Plasmidic DNA was isolated using the PureLink Quick Plasmid Miniprep (Invitrogen) and later sequenced. miR-target sequence was mutated with the Quickchange II XL site-direct mutagenesis kit (Agilent) accordingly with manufacture instruction and later sequenced. Oligos designed in a way to modified seed sequences of 3'UTR of Nurr1 and Wnt1 are reported in Table 2.2.

2.2.2 Transfection and lentiviral production

For the production of the viral particles (Tiscornia et al. 2006), 8.2 million HEK293T cells were plated in 150 mm × 25 mm dishes in DMEM (Invitrogen) supplied with 10% FBS (Euroclone), 25 mM Hepes (Invitrogen), 100 U/mL Streptomycin and 100 µg/mL Penicillin (Pen/Strep, Sigma-Aldrich). 24 h later an 80% confluence is generally obtained. The Medium was replaced by IMDM medium (Invitrogen), 10% FBS, Pen/Strep. 3 h later the 4 vectors were co-transfected following the calcium phosphate transfection protocol.

In details, for each dish, a solution was prepared to contain 270 mM $CaCl_2$, 6.25 µg pRev, 9 µg pVSVG, 14.6 µg pMDL and 32 µg of the insert containing transfer vector. After 5' of incubation at room temperature, drop-by-drop, in low agitation, a 2xHBS pH 7.12 (NaCl 280 mM, Na_2HPO_4 1.5 mM, HEPES 50 mM) solution was added to the vectors mix and incubated for 15' at room temperature.

Amplified cDNA	**Oligo sequences with restriction sites**	**Cloning Enzyme**
3xFlagNurr1 Fw	GCGC**CAATTG**ATGGACTACAAAGACCATGA	**MfeI**
3xFlagNurr1 Rev	GCGC**CAATTG**TTAGAAAGGTAAGGTGTC	**MfeI**
mmu-miR-27a-3p(687 bps) Fw	CC**GAATTC**GTGTTCAGCTATGTGAGACC	**EcoRI**
mmu-miR-27a-3p (687 bps) Rev	CC**GAATTC**CCCATCTATCTGCTTTGGG	**EcoRI**
mmu-miR-29a-3p (336 bps) Fw	CC**GAATTC**TAAGCCTTCTCTGGAAGTGG	**EcoRI**
mmu-miR-29a-3p (336 bps) Rev	CC**GAATTC**TTAACCATGCTGTTGCTGG	**EcoRI**
mmu-miR-34b/c-5p (983 bps)Fw	CC**GAATTC**GGCTTGCGGGAAGAAGGAC	**EcoRI**

mmu-miR-34b/c-5p (983 bps)Rev	CC**GAATTC**TAGCAGCTAAGGGCTAGCGG	**EcoRI**
mmu-miR-132-3p (562 bps) Fw	CC**GAATTC**GCTGGGACATCTTTGACG	**EcoRI**
mmu-miR-132-3p (562 bps) Rev	CC**GAATTC**CTCTTGCTCTGTATCTGCC	**EcoRI**
mmu-miR-148a-3p (294 bps) Fw	CC**GAATTC**TCTTCTTTGCCTTCACTGG	**EcoRI**
mmu-miR-148a-3p (294 bps) Rev	CC**GAATTC**TCAGGTTCTTCACAAAGCC	**EcoRI**
mmu-miR-204-5p (309 bps) Fw	CC**GAATTC**CCGGAGAATCAAGATGAGC	**EcoRI**
mmu-miR-204-5p (309 bps) Rev	CC**GAATTC**GTTATGGGCTCAATGATGG	**EcoRI**
mmu-miR-210-3p (306 bps) Fw	CC**GAATTC**AGGGGGATATGGGTATTGG	**EcoRI**
mmu-miR-210-3p (306 bps) Rev	CC**GAATTC**CACCCTGTCTATCTGAATCC	**EcoRI**
mmu-miR-218-1-5p (374 bps) Fw	CC**GAATTC**GATCATACACAATCTGCGGGAAG	**EcoRI**
mmu-miR-218-1-5p (374 bps) Rev	CC**GAATTC**GGACATTTGTTATTCTCCCCTC	**EcoRI**
mmu-miR-219-1-5p (358 bps) Fw	CC**GAATTC**CATTCACTCGTGTGCTCC	**EcoRI**
mmu-miR-219-1-5p (358 bps) Rev	CC**GAATTC**CCCAACTTCTCTCAAGCC	**EcoRI**
mmu-miR-370-3p (313 bps) Fw	CC**GAATTC**GTGGGTGTGGCTTTGAGG	**EcoRI**
mmu-miR-370-3p (313 bps) Rev	CC**GAATTC**CCCTTTCACAAATCTTTGCCC	**EcoRI**
mmu-miR-375-3p (346 bps) Fw	CC**GAATTC**CGCCACTGCCGCCGACGTG	**EcoRI**
mmu-miR-375-3p (346 bps) Rev	CC**GAATTC**GGCGGGGCCTGATGGGAACC	**EcoRI**
mmu-miR-494-3p (322 bps) Fw	CC**GAATTC**GTCTCAGGCAATTCTGTGG	**EcoRI**
mmu-miR-494-3p (322 bps) Rev	CC**GAATTC**ATGCCATACTCCCATGTCC	**EcoRI**
Nurr1 3'UTR Fw (1326bps)	TCCAA**ACTAGT**CCAAGCACGTCAAAGAACT	**SpeI**
Nurr1 3'UTR Rev (1326bps)	CTTAA**AAGCTT**ATCTCTAACTGTCGTACACC	**HindIII**
Wnt1 3'UTR Fw (933 bps)	TCCAA**ACTAGT**CGCTCTCTTCCAGTTCTC	**SpeI**
Wnt1 3'UTR Rev (933 bps)	CTTAA**AAGCTT**ATAGATATTTTATTCCTCAGAGGAAG	**HindIII**
Nurr1 3'UTRΔ34 Fw	TCGTACACCATAGAAAAAAAACTCATCTCATGTGCCGTAC	**Not used**
Nurr13'UTRΔ34 Rv	GTACGGCACATGAGATGAGTTTTTTTTCTATGGTGTACGA	**Not used**
Nurr1 3'UTRΔ204Fw	GTACATTGGAAAATCCTGACACACATAGTGTTTGTAACACCG	**Not used**
Nurr1 3'UTRΔ204Rv	CGGTGTTACAAACACTATGTGTGTCAGGATTTTCCAATGTAC	**Not used**

Wnt1 3'UTRΔ34Fw	GGCCAAATTGGGGAAAGGAGTCTCCCTCAAA GAG	**Not used**
Wnt11 3'UTRΔ34Rv	CTCTTTCAGGGAGACTCCTTTCCCCAATTTG GCC	**Not used**
Wnt1 3'UTRΔ148Fw	GGCCAAATTGGGGAAAGGAGTCTCCCTCAAA GAG	**Not used**
Wnt1 3'UTRΔ148Rv	CTCTTTCAGGGAGACTCCTTTCCCCAATTTG GCC	**Not used**
Wnt1 3'UTRΔ27Fw	GGAGCCATTGAACAGCCATGCCTCCCTCAG	**Not used**
Wnt1 3'UTRΔ27Rv	CTGAGGGAGGCATGGCTGTTCAATGGCTCC	**Not used**

Table 2.2. Oligos for lentiviral and luciferase vectors construction.

2.3 Dual fluorescent reporter sensor

DFRS plasmids were kindly provided by Prof. Wieland B. Huttner. Cloning strategy was performed as previously described (De Pietri Tonelli et al. 2014). Annealing of synthetic oligonucleotides to prepare the sensor cassette for a given miRNA. In an appropriate PCR tube, set up a 100 µl reaction as follows: add 76.8 µl of H_2O; 10 µl of 10X T4 ligase buffer (NEB); 5 µl of forward oligo (from 100 µM stock); 5 µl of reverse oligo (from 100 µM stock); 0.5 µl of PNK (NEB, stock 10 U/µl).

Incubate the reaction in a conventional thermal cycler programmed as follows: temperature (Time— Reaction Step): 37 °C (1 h— oligo phosphorylation); 94 °C (5 min— PNK inactivation); followed by a controlled oligonucleotide annealing:

decrease by 0.1 °C/s to 90 °C; incubate at 90 °C (3 min);

decrease by 0.1 °C/s to 70 °C; incubate at 70 °C (3 min);

decrease by 0.1 °C/s to 50 °C; incubate at 50 °C (3 min);

decrease by 0.1 °C/s to 25 °C; incubate at 25 °C (3 min).

Annealing of oligos can be controlled on a conventional 4 % agarose gel for DNA: load 2–5 µl of the annealing reaction on the gel; as control load 0.5–1 µl of a corresponding single- strand (either forward or reverse) oligo from stock 100 µM, which runs faster than the double-strand

annealed DNA.

The fragment is ligated (without purification) into the pDSV3 vector cut with PacI and NdeI and dephosphorylated. To set up the ligation reaction add 5 µl of the annealed fragment in a 10 µl final ligation reaction, incubate the reaction +4 °C overnight. Later plasmids were sequenced.

Plasmids were transfected into control (basal) and differentiating mouse Embryonic Stem Cell (mES) by Lipofectamine. Fluorescences were monitored every day until 72 hours post-transfection. In Table 2.3 is listed oligos used for annealing reaction.

Oligos for annealing	Oligo sequences with restriction sites
NdeI_34bs_**PacI** Fw	**TATG**ACAATCAGCTAATTACACTGCCTGGCGCGCCCGCAATCAGCTAACTACACTGCCT**TTAAT**
PacI_34bs_ **NdeI** Rv	**TAA**AGGCAGTGTAGTTAGCTGATTGCGGGCGCGCCAGGCAGTGTAATTAGCTGATTG**TCA**
NdeI_34MUT_**PacI** Fw	**TATG**ACAATCAGCTAATTACGGCCGGTGGCGCGCCCGCAATCAGCTAACTACGGCCGGTT**TAAT**
PacI_34MUT_ **NdeI** Rv	**TAA**ACCGGCCGTAGTTAGCTGATTGCGGGCGCGCCACCGGCCGTAATTAGCTGATTG**TCA**

Table 2.3 Oligos for annealing of synthetic oligonucleotides

2.4 Molecular biology methods

2.4.1 miRVana RNA extraction and TaqMan® MicroRNA Assays

Usual RNA purification strategies rely on organic extraction, followed by alcohol precipitation. These strategies, because of the alcohol precipitation, are inefficient in recovering small RNA forms. The miRVana miRNA isolation (Ambion) strategy uses organic extraction followed by purification on a glass fiber filter (GFF) under specialized binding and washed conditions. With this strategy, all RNAs are recovered, from large mRNAs to ribosomal RNAs down to 10-mers small RNAs. 1 volume of cold sterile PBS was added to samples in RNA later. Samples were centrifuged, RNA later/PBS solution was removed and the cell pellet was resuspended in the provided lysis buffer. Later, RNA was extracted following the manufacturer instructions. As for the last step, RNA was eluted in 100 µl of RNAase-free water, previously warmed at 95°C and

then quantified by spectrophotometric analysis. For the TaqMan® MicroRNA Assays sample were further diluted to 2 ng/µl.

TaqMan® MicroRNA Assays (Applied Biosystem) is the most commonly used strategy to quantify miRNAs. This assay is based on a two-step process. First, a reverse transcription is performed starting from a miRNA specific stem&loop primer. Secondly, target amplification is obtained using a miRNA specific forward primer and a reverse primer able to bind the opened loop. Amplification levels detection is based on a TaqMan® miRNA specific probe conjugated with a fluorescent dye (FAM™ dye) on its 5'end and a non-fluorescent quencher on its 3'end. Moreover, a minor groove binder (MGB) is conjugated too on the 3'end. It is a particular tripeptide that allows stabilizing the probe specific binding without increasing probe length. Briefly, reverse transcription (RT) for miR- miR-9-1-5p, miR-218-5p, miR-124a, miR-34b/c, miR-204, miR-148a and the reference snoRNA-202 was performed using the TaqMan® microRNA Assay provided RT primers for each of the listed miRNAs. The RT reaction was carried out with the suggested TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystem Cod. 4366596) using reagents and enzymes provided with the kit and following the protocol provided by the company. qPCR was performed using primers and probe provided with the miRNA specific TaqMan® microRNA Assay e with the TaqMan® Universal Master Mix II, no UNG (Applied Biosystem Cod. 4440040). To perform qPCR I followed the protocol provided by the manufacturer. The instrument was the 7900 HT Fast Real-time PCR system (Applied Biosystems).

2.4.2 High-Content imaging

Screening of miRNAs involved in DA induction was performed plating mE12.5-PCs in an optical 384-wells (Thermo Scientific) as described above. The number of TH^+ cells was automatically analyzed by cell-based High Content Screening (HCS) confocal microscope Opera® Phenix (Perkin Elmer). HCS is an analysis tool used to acquire, manage, and search multi-parametric information regarding the composite phenotype of cells. In my experiments, I acquired at least 270 areas per condition.

2.5 Luciferase assay

Usually, in biological research luciferase assays are performed in order to characterize transcriptional activation from a specific promoter cloned upstream to the luciferase coding region. In my experiments instead, I used the pmiRReport vector (Applied Biosystem) specifically designed to allow the characterization of the regulatory potential of specifics 3'UTRs cloned downstream to the luciferase coding region. In general, this assays is based on the luciferase capacity to catalyze a specific reaction, having luciferin as a substrate, able to release a photon of light as a product of the reaction. Light can be detected by a specific instrument called luminometer and the amount of luciferase protein inferred. The 3'UTR-containing pmiR-Report was co-transfected both with the TET-OFuw miRNA over-expressing vector and the rtTA expressing vector in HeLa cells. In addition, to quantify the transfection efficiency a pRL-SV40 Renilla/Luciferase Reporter Vectors (Promega) was also used. For each assay, controls with the empty pmiR-Report vector or without over-expressing any miRNA were conducted. 24 hours following transfection cells were washed in PBS and lysed in passive lysis buffer (PLB; 25mM Tris-phosphate pH 7.8; 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton-x-100) (Promega). The assay was made using the Luciferase™ Reporter Assay System (Promega) on 10µl of cell extract and following the protocol provided with the kit. The Firefly luciferase luminescent signal was normalized to the Renilla luciferase signal. Data shown in the graphs (section Results) represent mean ± s.e.m. from triplicate cultures of a single experiment.

2.6 *In Situ Hybridization* & Immunohistochemistry

2.6.1 *In Situ Hybridization* on Paraffin-embedded brain sections

Embryonic brains of mice from day 11.5 to P0 of gestation were quickly dissected and placed in phosphate buffered saline (PBS), later were placed in 4% paraformaldehyde (PFA) overnight (O.N.) at 4°C.

The day after, the brains were washed as follows: - Ethanol 50% in PBS for 30-60 minutes (depends to the stage); - Ethanol 70% twice for 30-60 minutes (depends to the stage); - O.N. at 4°C in Ethanol 70% in shaking.

As follow the brains were dehydrated (all washes 30-60 minutes): - Ethanol 85%; - Ethanol 95%; - Ethanol 100% twice; - O.N. at 4°C in Ethanol 100% in shaking.

Finally the brain can paraffin-embedded as described: - Xylene twice (30 minutes); - Xylene: paraffin (1:1) at 60°C (30 minutes); - Paraffin three times at 60°C (30 minutes). In The end, the brains were placed and oriented in mold O.N. at 4°C.

Paraffin-embedded brains tissue samples were cut in coronal sections by using microtome (6-8 μm). For *In Situ Hybridization* special attention must be given to limiting RNase contamination by wearing gloves and using RNase depleted water, use autoclaved or sterile buffers and heat-treated glassware.

I placed sections in Xylene three times for 5 minutes to remove paraffin after the sections were washed with decreasing volumes of Ethanol (from 100% to 25%) and one wash with PBS.

I performed Proteinase-K treatment 10 μg/ml in PK-buffer (5 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM NaCl, autoclaved) at 37°C for 10 min was performed. The sections were washed in 0.2% Glycine and twice in sterile PBS immediately. The slices were fixed in 4% PFA, washed again in PBS. I proceeded with Acetylation by using 0.1 M Triethanolamine pH 8.0 (10 min); 0.1 M Triethanolamine/0.25% acetic anhydride (10 min) and PBS twice.

After for Hybridization, the stock probe was diluted (1:1000) in Hybridization Buffer (5X SSC, 50% Formamide 5X Denhardt's solution, 500 μg/ml salmon sperm DNA; 250 μg/ml tRNA) was then denatured by heating to 65°C for 5 min and placed on ice. Then 100 μl of the probe was applied on a slide at a temperature 21-26 °C below LNA's Temperature melting (for miRCURY™ LNA Detection Exiquon probes miR-218 was 45°C). Post-Hybridization washes were performed as described: 5X SSC wash at RT 5 min., 50% Formamide/2X SSC wash at 45°C 30 min., 2X SSC wash at RT 5 min., TBS-T wash (0.1% Tween-20 in TBS) 5 min.

After the sections were blocked in 100 μl of 10% sheep serum/TBST 30 min. and finally were applied 100 μl of anti-DIG-AP at 1:500 dilution in Blocking buffer, then coverslip and incubate in a humidified (water) chamber at 4°C for 2-3 hour.

At last, I have performed Staining by use NBT/BCIP ready to use solution (SIGMA) by incubating slices from 5h to O.N. at Room Temperature (RT).

Staining buffer is alkaline (pH 9.5) for DIG coupled Alkaline Phosphatase (AP) to be functional, BCIP is the AP-substrate which reacts further after the dephosphorylation to give a dark-blue dye as an oxidation product. NBT serves as the oxidant and gives also a dark-blue dye. It intensifies the color further. When staining was completed I washed sections in TBS-T and mounted using 20% Mowiol.

2.6.2 Immunohistochemistry on Paraffin-embedded brain sections

Adjacent sections were then processed either for RNA hybridization and for immunohistochemistry. For immunostaining, the initial passages are same to *in situ hybridization* but after washes with decreasing volumes of Ethanol, the sections were brought to a boil in 100 mM sodium citrate and 100 mM citric acid buffer (pH 6.0) and then maintain at a sub-boiling temperature for 8-10 minutes. I have washed the sections by immersing them in water and PBS for 5 minutes. The slices were incubated in PBS and 0.1% Triton three times for 5 min. and after in blocking solution (2% BSA, 5% NGS) for 2h at RT. I have added primary antibody (Table 2.4) diluted in blocking solution and incubated at RT for 1-2 hours. In particular, Pitx3 primary antibody (used 1:500) was kindly provided by Prof. Marten Smidt. Then stored overnight at 4°C in humidified chamber. The day after, the sections were washed with PBS and 0.1% Triton 3-4 times to remove primary antibody and were incubated with secondary antibody (Alexa Fluor-Invitrogen) diluted in blocking solution for 1-2h at RT in dark site. Finally, the sections were washed same previously and mounted using 20% Mowiol.

2.6.3 Microscopy

Images for *in situ hybridization* (in the bright field) and immunohistochemistry (in fluorescence) were acquired using a Leica DMI6000 inverted microscope at 10× and 20×. In order to compare pictures from the same set of experiments, images were taken keeping the same settings on the acquisition software (Leica Application Suite AF). For cell counting, the number of positive cells was quantified using a custom macro disposable in the ImageJ software.

Epitopes	Host species	Applications	Working dilution	Manufacturer	Catalogue number
TH	Rabbit	IF	1:500	Millipore	AB152
Lmx1a	Rabbit	IF	1:500	Millipore	AB10533
Isl1	Mouse	IF	1:100	Millipore	MABD131

Table 2.4 List of antibodies.

2.7 FACS Analysis

Pitx3-GFP^{+} cells were trypsinized, centrifuged, and resuspended in ESC medium containing 2% FBS and 2 mM EDTA. The positive cells fraction was sorted using a BD FACSAria III (Becton Dickinson) into a 6-well plate. The data were analyzed using the BD FACSDiva software.

2.8 Generation of miR-218-2 conditional knock-out mouse model

The conditional knock-out mouse model was generated starting from generation of targeting vector containing miR-218-2 sequence flanked by LoxP sites.

Briefly, the Slit3 genomic sequence was obtained from Ensembl database, repetitive elements were mapped by using repeat masked software and the restriction map was obtained with the ape tool. Three pairs of oligonucleotides with selected restriction enzyme sites (Table 2.5) were designed to amplify three consecutive genomic regions: Long arm (6274 bps), Floxed arm (978 bps) containing miR-218-2 sequence and Short arm (1467 bps). The PCR products were obtained from bacterial artificial chromosomes (BACs A19) and purified. All products were sequentially cloned in the pFlrt3W vector (5115 bps) as shown in figure 2.1, the Long arm was cloned upstream to LoxP site, the Floxed arm between LoxP sites, the short arm downstream to Neo cassette (a selectable marker through intermediate steps of cloning and sequencing).

The sequence was verified to exclude the presence of undesired mutations. The construct was linearized with NotI restriction enzyme and after was introduced in the E14Tg2a4 ESC line by electroporation. The screening of homologous recombinant ESC clones was performed by PCR with a specific couple of oligos (Table 2.5), followed by Southern blot with three different designed probes: 3' Probe, 5'Probe and Neo Probe to confirm the identification of positive ES cell clones (see in Results).

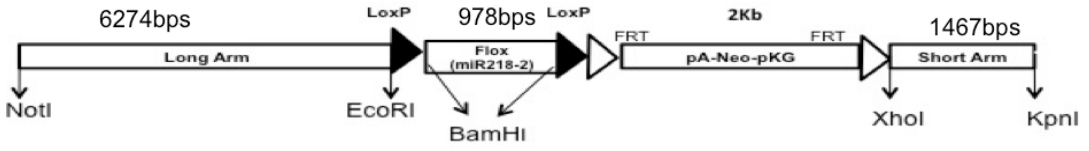

Figure 2.1 Schematic representation of targeting vector

I have obtained Neo probe by restriction with NcoI and BglII on the pGND vector. The 3' and 5' probes were obtained by PCR with specific oligos (table 2.5) Purified products were cloned in StrataClone cells by using standard protocols of StrataClone PCR Cloning Kit (Agilent). Plasmidic DNA was isolated using the PureLink Quick Plasmid Miniprep (Invitrogen) and later I have verified positive cloning cells by cutting with EcoRI enzyme. DNA band extraction from the agarose gel was performed using the PureLink® Quick Gel Extraction Kit (Invitrogen) according to the provided instructions. Eluted DNA was ligated O.N. on themselves to generate probe.

Southern Blotting was performed as described in the literature (Southern 2006), to detect signal I have used Deoxycytidine triphosphate, labeled on the alpha phosphate group with ^{32}P (NEG513H; PerkinElmer).

The ES cells containing a mutated allele (miR-218-2^{flox}) were injected into the blastocyst and implanted into the foster mouse (pseudopregnant mouse) by standard procedures (Acampora et al. 1995). We have mated Chimeric mice with wild-type mice, and their mutated allele was checked for germline transmission to gain heterozygous mice in F1. The Neo-cassette was removed by mating heterozygous mice with Flippase mice, the screening was performed by PCR analysis, by using listed oligos in table 2.5.

To inhibit miR-218-2 expression only in the dopaminergic field, we have mated conditional miR-218-2 mice with specific Cre-lines: Cre^{En1}, that have Cre recombinase expression directed by the En1 to obtain miR-218-$2^{fl/fl}$; $En1^{Cre/+}$ mice.

Oligos for cloning target vector	**Oligo sequences with restriction sites**
Long NotI Fw	TTT**GCGGCCGC**GCTCCAGGGAGTTCTCTGAATC
Long EcoRI Rv	TTT**GAATTC**CACAACTCGAGAGGCTCCTGGGGACCTT
Floxed BamHI Fw	TTTA**GATC**TTGTTGAAGGGAGGATTTGCCTGG
Floxed BamHI Rv	TTTA**GATC**TCTCACCCTCTGCATTAGCTACC
Short XhoI Fw	TTT**CTCGAG**GTCGCTCTTGACTTTCACCACTCTTG
Short KpnI Rv	TTT**GGTACC**GATAGGGTGGCTGCTGATGTTG
Oligos for PCR screening	**Oligo sequences**
Wal_FL Fw	GGTCTCTGCAGTGTCGTCGATCGA

S3RV1Est Rv	TGGATGGTCTAGGATCAGAAGTAG
Oligos Probes Southern Blot	**Oligo sequences**
3'Probe Fw	CAGGTGACATCAGATCCTCTGAGG
3'Probe Rv	CCTCTGCAGTGGAACCGCGAC
5' Probe Fw	TGGCAGCAGTGCCCATTACTATCTG
5'Probe Rv	TGGAGTGACCTGTGGTACCTCG
Oligos for Neo-cassette screening	**Oligo sequences**
FwflpF	GAGTAGCAGCTAGGTTCCAACTTC
RVflpS	ACCCATGTCTGTTCTACCCATTC
PgK1A	ACTTGTGTAGCGCCAAGTGCCAGC
FlpF	CACCTAAGGTCCTGGTTCGTCAGT
FlpR	CCAGATGCTTTCACCCTCACTTAG

Table 2.5 Oligos to generate target vectors and to screen homologous recombination

The project for generation of a miR-218 cKO mouse model was approved by Italian Health Ministry, Department of Veterinary, Public Health Nutrition, and Food Safety, Directorate-General for animal health and veterinary medicinal products

2.9 Statistical analysis

For all experiments, analysis of variance was carried out, followed by post hoc comparison (ANOVA, Dunnett's or Newmann-Keuls test). A value of $p \leq 0.05$ was considered significant. Data were expressed as mean ± SEM and show p values with special symbols. At least three independent replicates were used for RT-PCR, Luciferase Assay and for cell counts.

Analysis of High-Content data was performed on R software with one-way analysis of variance and Benjamini-Hochberg's post hoc test, after standardization of value.

3 - RESULTS

3.1 miRNAs upregulated in dopaminergic cells *in vitro*

Midbrain DA neurons development is a complex and still not fully understood phenomenon. In the last years, it becomes clear that also miRNAs play important roles in promoting mDA differentiation and survival (Kim et al., 2007; Miñones-Moyano et al., 2011; Saba et al., 2012; Tobon et al., 2012; Yang et al., 2012).

Previous studies performed in my host laboratory have identified miRNAs expressed during *in vitro* dopaminergic differentiation (De Gregorio et al., unpublished data).

This goal has been achieved by using EpiSCs, isolated from Pitx3-GFP mice expressing the GFP in the Pitx3 locus. EpiSCs were maintained in culture for 15 or 19 days in the N2B27 medium after Activin and bFGF withdrawal and differentiation as a monolayer. The FGF/ERK inhibitor PD0325901 (PD03) was added to the medium from day 0 to day2 and the inductive factors SHH and FGF8 were added from day5 to day9.

Terminal differentiation was promoted by the addition of GDNF, BDNF and Ascorbic Acid from day9 to the end of the *in vitro* differentiation protocol (optimized from Jaeger et al., 2011)(Fig. 3.1).

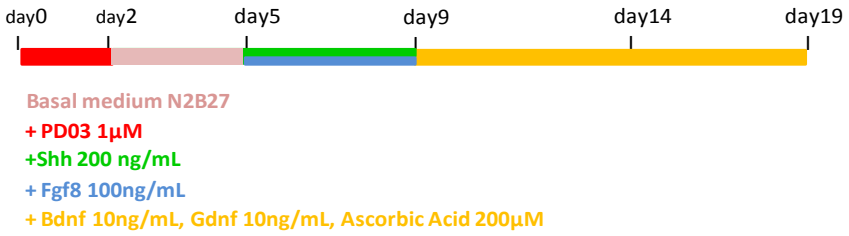

Figure 3.1 Schematic representation of the protocol used to profile miRNAs expression during DA differentiation of mouse epiSCs.

Samples were collected at the end of the differentiation protocols and processed for mRNA and microRNAs on an Agilent microarray platform.

A bioinformatic approach allowed the identification of two groups of microRNAs that behave with opposite trends: the first group was represented by those miRNAs which expression increased during the entire process of differentiation while the second contains those that were progressively down-regulated (De Gregorio et al., unpublished) (Table 3.1).

miRNAs up-regulated in DA differentiation	**miRNAs down-regulated in DA differentiation**
mmu-miR-370-3p	mmu-miR-128-3p
mmu-miR-494-3p	mmu-miR-153-3p
mmu-miR-375-3p	mmu-miR-129-1-3p
mmu-miR-218-1-5p	mmu-miR-137-3p
mmu-miR-219-1-5p	mmu-miR-326-3p
mmu-miR-34b-5p	mmu-miR-129-5p
mmu-miR-34c-5p	mmu-miR-138-5p
mmu-miR-212-3p	mmu-miR-181a-5p
mmu-miR-132-3p	mmu-miR-709
mmu-miR-148a-3p	mmu-miR-383-5p
mmu-miR-152-3p	mmu-miR-671-5p
mmu-miR-210-3p	mmu-miR-3285p
mmu-miR-27a-3p	mmu-miR-331-3p
mmu-miR-29a-3p	
mmu-miR-204-5p	
mmu-miR-211	

Table 3.1 Lists of selected miRNAs. The two lists distinguish between miRNAs found to be upregulated or downregulated in the DA protocol. miRNAs were selected on the base of their fold change compared to the generic neuronal protocol and on their predicted target genes.

To identify which miRNAs can promote dopaminergic differentiation, each candidate (table 3.1) was screened for its ability to promote the expression of the tyrosine hydroxylase. To this purpose, I cloned the cDNA for eleven microRNAs into an inducible lentiviral vector (TET-O-Fuw; see in Materials and methods for details).

In particular lentiviral particles for the following microRNAs have been generated: miR-34b and 34c cluster, miR-218-1, miR-148a, miR-210, miR-27a, miR-29a, miR-132, miR-204, miR-219, miR-370, miR-375, and miR-494.

Mesencephalic primary cultures, isolated from TH-GFP mice at stage E12.5 have been infected with lentiviral particles expressing each microRNAs in combination with Nurr1, a transcription factor essential to promote tyrosine hydroxylase expression.

The ability of each microRNA to promote DA phenotype was investigated by following the GFP expression and represent as the ratio of $Nurr1^{+}/TH^{+}$ cells. The results are shown in Figure 3.2.

Only miR-34b/c and miR-218-1, when combined with Nurr1, were able to increase the number of GFP positive cells, compared to Nurr1 alone.

Taking advantage of this data, I have further investigated the importance of miR-34b, miR-34c and miR-218 in the dopaminergic differentiation.

MiR-34b-5p and miR-34c-5p (called hereafter miR-34b/c) are two microRNAs expressed in a unique gene cluster. The mature forms are almost identical since they differ for only one nucleotide. Both belong to a large family of miRNAs that include miR-34a-5p and the miR-449 cluster (449a-5p, 449b-5p and 449c-5p), and it has been hypothesized their possible role in the etiopathogenesis of Parkinson Disease (Miñones-Moyano et al., 2011).

Also, miR-218 is present in two copies in mammalian genome named miR-218-1 and miR-218-2. It has been mainly associated with motor neurons but recent data also suggest a possible role in dopaminergic differentiation (Baek et al., 2014; Rivetti et al., 2017).

None of the others candidates miRNAs were indeed able to promote DA differentiation, despite we cannot exclude theirs contribute to dopaminergic neurons development, function, and survival.

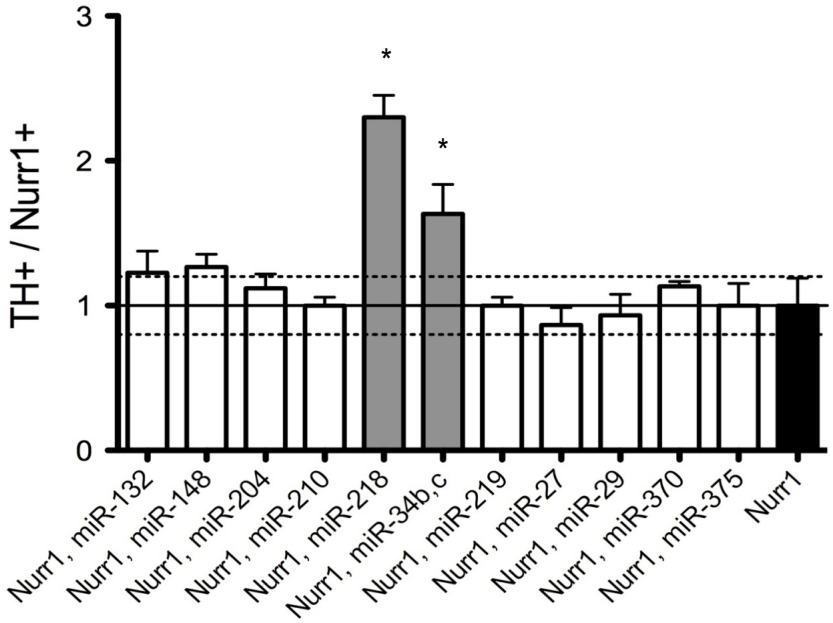

Figure 3.2 miRNAs 34b/c and miRNA-218-1 promote DA phenotype. High-content screening of miRNA's activity on the number of TH^+ cells after over-expression of Nurr1 in mE12.5-PCs derived from TH-GFP mice. GFP^+ cells were counted at automated confocal microscope Opera and normalized on $3xFlag^+$ cells. Means ± SEM, n = 270 ± 80. * $p \leq 0.05$ of the samples respect to the control.

3.2 Functional evaluation of miRNAs

miRNAs regulate the expression of target genes by binding to specific sequences located at the 3' untranslated regions (3'UTRs) of the target mRNA. The binding sequences can be unique or repeated, in such case, a strong enhancement of the downregulation may be expected. Similarly, different microRNAs may cooperate to regulate the same transcript by binding specific adjacent sequences.

Thus by using available miRNA target prediction tool (targetscan.org/mmu_71/) (Agarwal et al., 2015), I screened the selected miRNAs, previously identified, for their ability to target mRNAs involved in DA differentiation.

In particular, I searched for mRNAs containing 2 or more binding sites for the short group of microRNAs we previously identified. Following this scheme, I focused on Nurr1 and Wnt1 since both were targeted by at least two different microRNAs.

3.2.1 miR-34b/c suppresses Wnt1 expression via targeting at the 3′UTR

Wnt1 3'UTR is 933 bps long and hundreds of miRNAs are predicted to potentially bind and regulate it. Three microRNAs identified previously were potentially able to target it. These are miR-34b/34c, miR-148a, miR-27a. To verify their ability to downregulate Wnt1 I performed Luciferase assays.

This approach, associated with miRNAs over-expression, allows detecting possible miRNA effect on the chosen 3'UTR simply by looking at the luminescence levels. Indeed if a miRNA regulates the cloned 3'UTR, the luciferase mRNA will be repressed or degraded, and a lower luminescent signal will be detected.

Luciferase assay has been performed by transfecting miRNA over-expressing vectors and Wnt1-3'UTR-pmiR-Report in HeLa cells.

Almost the entire sequence corresponding to the Wnt1 3'UTR was cloned into a pmiR-Report luciferase reporter vector (Ambion) downstream to the luciferase coding sequence (see in Materials and Methods) and then transfected in combination with a plasmid expressing the microRNA.

3'UTR Wnt1position /miRNA	**Predicted consequential pairing of target region (top) and miRNA (bottom)**
Position 241-247 of Wnt1 3' UTR mmu-miR-34b/c-5p	5' ...GGGAGACUCCUUUUGCACUGCCC... | | | | | | | 3' CGUUAGUCGAUUGAUGUGACGGA
Position 239-245 of Wnt1 3' UTR mmu-miR-148-3p	5' ...GAGGGAGACUCCUUUUGCACUGC... | | | | | | | 3' UGUUUCAAGACAUCACGUGACU
Position 572-578 of Wnt1 3' UTR mmu-miR-27a-3p	5' ...GGAGCCAUUGAACAGCUGUGAAC... | | | | | | 3' CGCCUUGAAUCGGUGACACUU

Table 3.2 Consequential pairing shows miRNA-3'UTR of Wnt1 complementarity, whether inside or outside the seed region, that is predicted to influence the efficacy of targeting (Grimson et al., 2007). (Adapted from Targetscan).

In all wells were added also Tetracycline-controlled transcriptional activation vector (rtTA), this transfection was necessary in order to trans-activate expression from the TET-O-Fuw inducible promoter, following doxycycline addition to the medium.

All candidate miRNAs were able to reduce Luciferase activity, miR-27a of 53%, miR-34b/c of 35% and miR-148a of 23%.

To further confirm this data I mutated the binding site for each microRNA I examined (3'UTR Wnt1Δ34, 3'UTR Wnt1Δ148, 3'UTR Wnt1Δ27 in Table 3.2).

Interestingly, the negative regulation of miRNAs was abolished after mutation of the predicted binding site for miR-34b/c but not for miR-148a-3p and miR-27a on Wnt1 3'UTR sequence (fig. 3.3).

Thus suggesting that only miR- 34b/c effectively binds to its predicted site at the Wnt1-3'UTR. The empty pmiR-Report vector was used as an additional control. All luciferase data have been normalized to the Renilla (RL-SV40) activity.

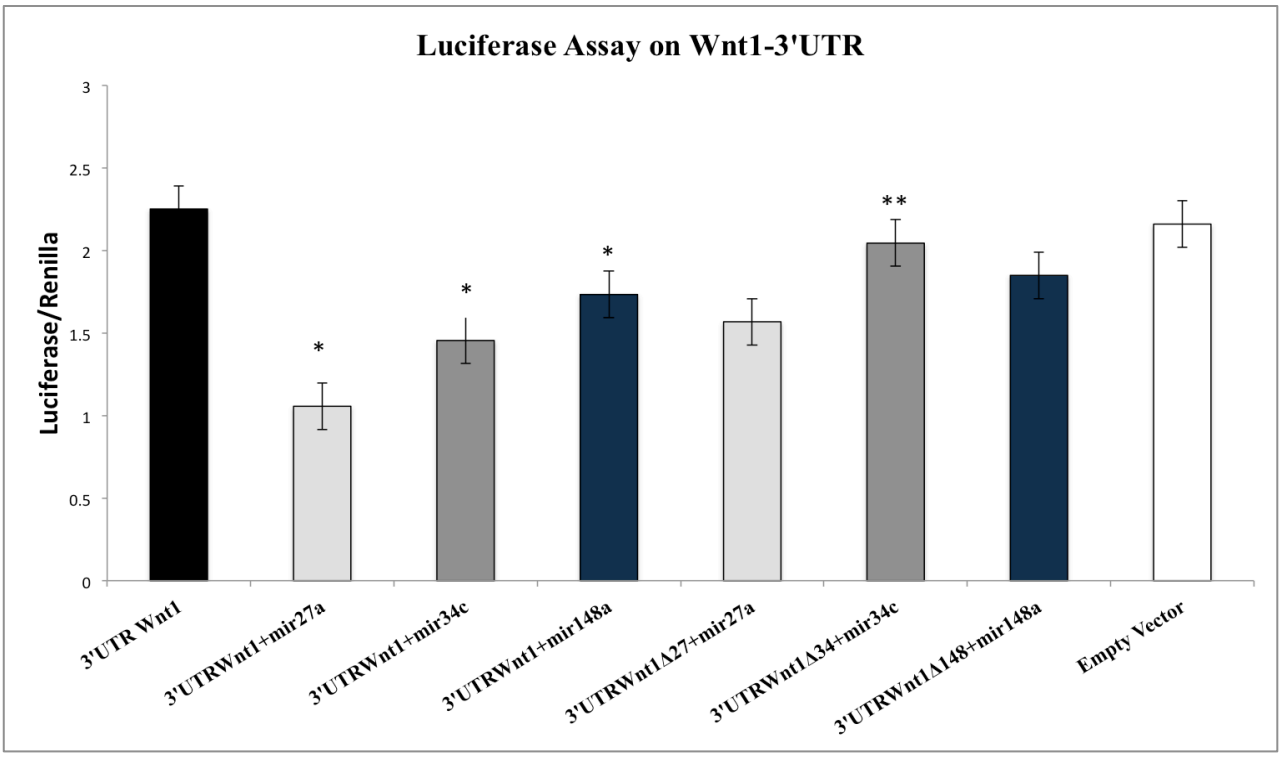

Figure 3.3 Luciferase Assay on Wnt1-3'UTR in HeLa cells. pmiR-Reports containing the wild-type (3'UTR Wnt1) or mutated (3'UTR Wnt1 Δ) 3'untranslated sequence for Wnt1 were co-transfected with Tet-O-FUW-miR-34b/c plus rtTA (miR-34b/c), Tet-O-FUW-miR-148a-3p plus rtTA (miR-148a) Tet-O-FUW-miR-27a plus rtTA (miR-27a). The empty pmiR-Report vector was used as a additional control. All luciferase data have been normalized to the Renilla (RL-SV40) activity. Data represent mean± s.e.m. from three independent experiments. *, P<0.01, **, P<0.5 (Student's t-test).

3.2.2 miR-204-5p and miR-34b/c-5p inhibit Nurr1 expression by targeting 3'UTR

The same approach was used to identify candidate miRNAs able to bind Nurr1-3'UTR and regulate its expression. The entire 3'UTR for Nurr1 mRNAs corresponding to 1326 bps, was cloned downstream the pmiR-Report luciferase reporter vector.

The experimental procedure that I used was identical to previously described for Wnt1 3'UTR. Two microRNAs we previously identified were potentially able to bind Nurr1-3'UTR. These are miR-34b/c and miR-204, the predicted binding sites are reported in Table 3.3.

HeLa cells were transfected with Nurr1-3'UTR-pmiR-Report luciferase vector in presence of expressing plasmids for 34b/c and miR-204-5p miRNA.

I was able to detect a significant reduction of the luciferase signal as a result of the inhibitory action of the both miR-204 and 34b/c on Nurr1 3'UTR, corresponding to 33% for miR-34b/c and 36% for miR-204 compared to control vector (Fig. 3.4).

3'UTR Nurr1position /miRNA	**Predicted consequential pairing of target region (top) and miRNA (bottom)**
Position 870-876 of Nurr1 3' UTR long mmu-miR-204-5p	5' ...UGGAAAAUCCUGACAAAAGGGAC... | | | | | | | 3' UCCGUAUCCUACUGUUUCCCUU
Position 1205-1211 of Nurr1 3' UTR long mmu-miR-34b/c-5p	5' ...GCACAUGAGAUGAGUCACUGCCU... | | | | | | | 3' UGUUAGUCGAUUAAUGUGACGGA

Table 3.3 Consequential pairing shows miRNA-3'UTR of Nurr1 complementarity, whether inside or outside the seed region, that is predicted to influence the efficacy of targeting (Grimson et al., 2007). (Adapted from Targetscan)

Mutation of the predicted binding sites (3'UTR Nurr1Δ34, 3'UTR Nurr1Δ204) abolishes the effect of both miRNAs suggesting that the binding was specific and that both microRNAs were, indeed, able to modulate Nurr1 expression (fig. 3.4).

The empty pmiR-Report vector was used as an additional control. All luciferase data have been normalized to the Renilla (RL-SV40) activity.

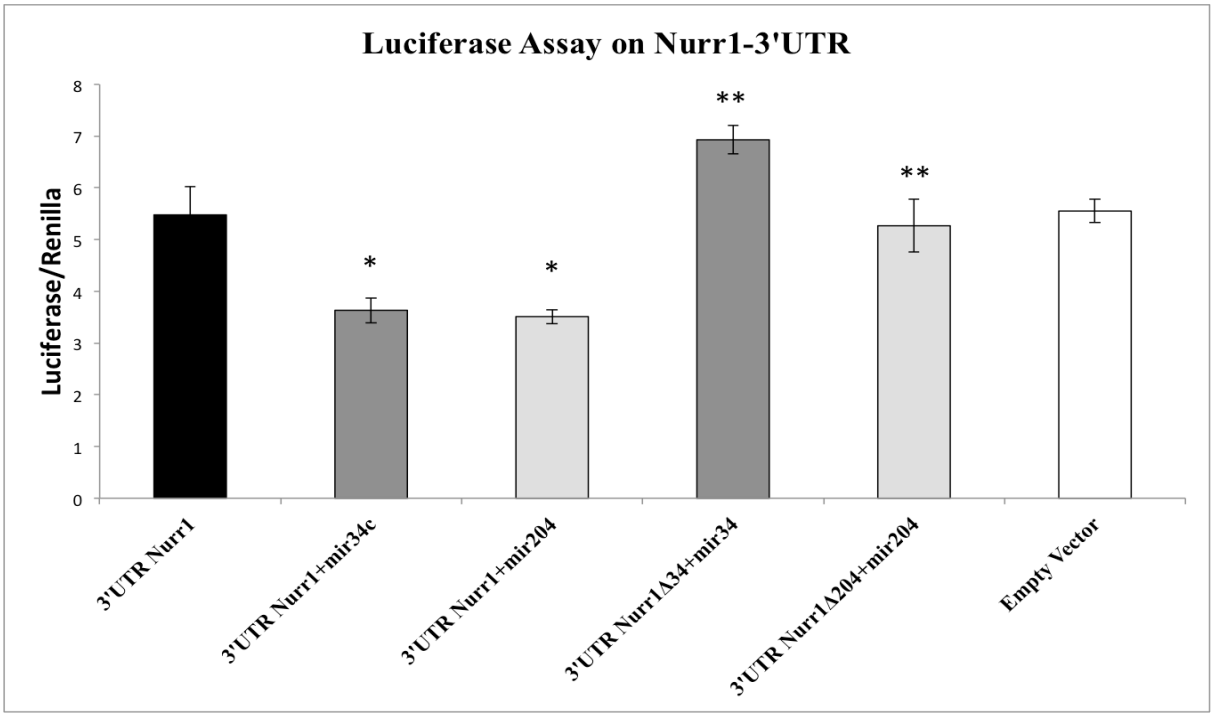

Figure 3.4 Luciferase Assay on Nurr1-3'UTR in HeLa cells. pmiR-Reports containing the wild type (3'UTR Nurr1) or mutated (3'UTR Nurr1 Δ) 3'Untranslated sequence for Nurr1 were co-transfected with Tet-O-FUW-miR-34b/c plus rtTA (miR-34b/c), Tet-O-FUW-miR-204-5p plus rtTA. The empty pmiR-Report vector was used as a additional control. All luciferase data have been normalized to the Renilla (RL-SV40) activity. Data represent mean± s.e.m. from three independent experiments. *, P<0.01, **, P<0.5 (Student's t-test).

3.3 Wnt1 is a target of miR-34b/c and is expressed during DA neurons differentiation

In this context, I choose to further investigate the role of miR-34b/c cluster on Wnt1 expression. The final aim was to better understand their implication in dopaminergic fate. To this purpose, I used a dual-fluorescent plasmid (DFSP): green fluorescent protein (GFP)-reporter/monomeric red fluorescent protein (mRFP)-sensor (De Pietri Tonelli et al., 2006), which allows the detection of miRNAs at single cell resolution. I cloned a tandem cassette complementary to miR-34b/c in the 3'UTR of the mRFP sensor (pDSV3-34) or mutated in the region corresponding to the "seed sequence" of miR-34b/c (pDSV3-34mut).

In DFRS plasmids, both GFP and mRFP are under the control of identical constitutive promoters (SV40), the GFP-reporter was used to identify the cells actually expressing the plasmid, given that the sensor-based strategy relies on the silencing of a transcript. The mRFP-sensor contained 3'UTR with a tandem cassette complementary to the miRNA of interest (see

detail in Materials and Methods). Mouse Embryonic Stem Cell (mESc) were transfected with pDSV3-34 and fluorescence was monitored during differentiation.

This approach has been described as very efficient for monitoring the endogenous expression of miRNAs both *in vitro* and *in vivo* and allows monitoring miRNA expression in defined cell lineages during development. In differentiating mESc transfected with the pDSV3-34 the expression of the mRFP-sensor was strongly reduced 72 hours after transfection (fig. 3.5). This effect was abolished with pDSV3-34mut (Figure 3.5), thus suggesting that miR-34b/c is expressed in vitro during the dopaminergic differentiation of mES (unpublished data).

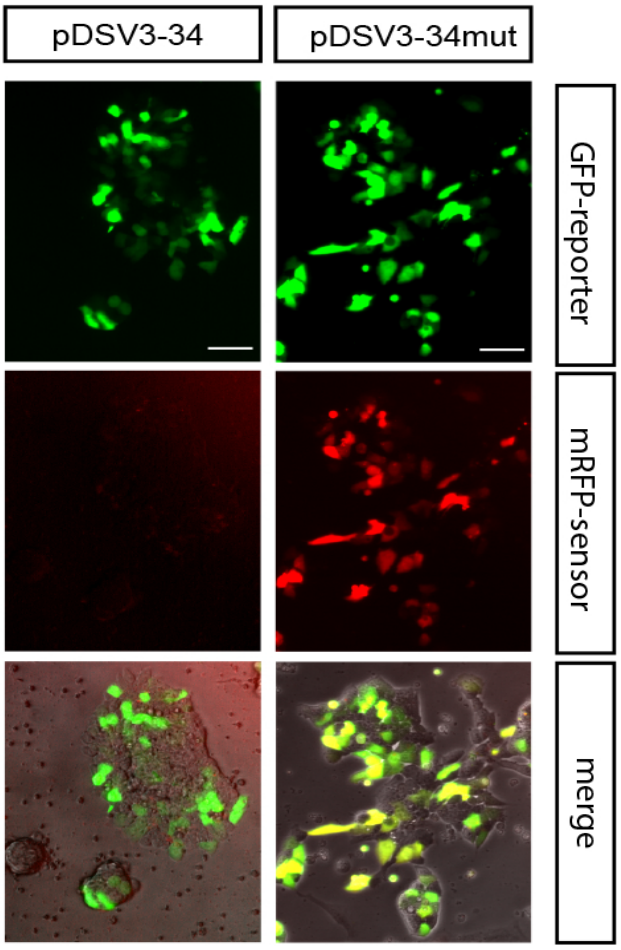

Figure 3.5 miR-34b/c Dual fluorescence reporter assay. mES cells transfected with a plasmid containing a complementary sequence to the miR-34b/c downstream the CDS for the mRFP sensor (pDSV3-34) or with a plasmid containing a sequence mutated in the region corresponding to the "seed" for miR-34b/c (pDSV3-34mut). Images were acquired 72 hours after transfection. Scale bar is 50 μm.

3.4 miRNAs enriched in Midbrain during development

We performed quantitative expression analysis of miRNAs in Midbrain, Striatum, and Cortex to compare the expression of selected miRNAs in the different brain regions.

I focused this analysis on the three microRNAs described above. These are miR-34b/c, miR-204, miR-218. All these microRNAs resulted enriched in the midbrain at E14 but not in tissues obtained from adult mice (fig. 3.6 A). Interestingly miR-34b/c, miR-204, and miR-218 were also enriched in FACS sorted GFP$^+$ cells obtained from E13.5 Pitx3-GFP mice embryos when compared to the negative sorted cells (CTRL) (fig. 3.6 B). At the opposite miR-124a and miR9, two well known microRNAs expressed in the brain but not enriched in our dopaminergic protocols indeed were not expressed in E14 midbrain tissues (fig. 3.6 A, B).

All these data confirm support our hypothesis concerning their involvement in dopaminergic development.

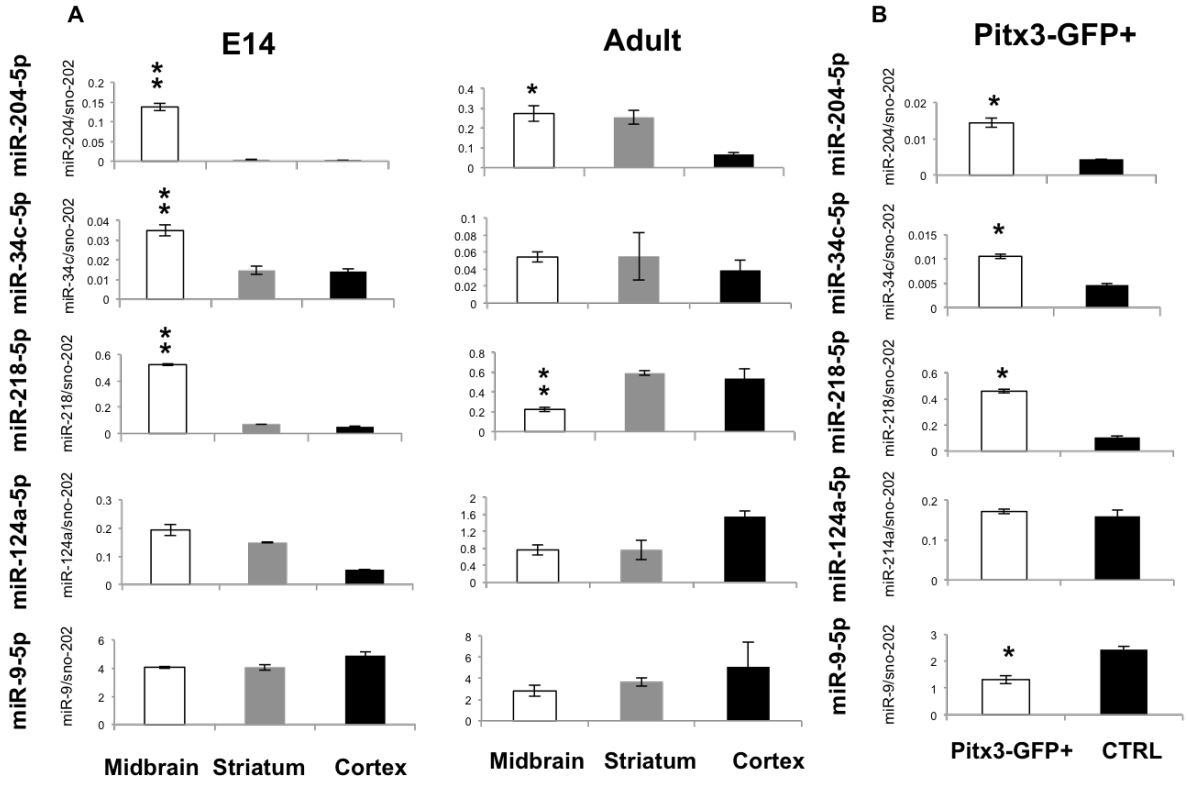

Figure 3.6 qPCR analysis A) Expression of mature miR-204, miR-34c-5p, miR-218-5p, miR-124a-5p and miR-9-5p on specific microdissected developing (E14.5) and adult brain areas (midbrain; striatum; cortex). **B)** Expression of mature miR-204, miR-34c-5p, miR-218-5p, miR-124a-5p and miR-9-5p on E13.5 Pitx3-GFP$^+$ sorted cells (Pitx3-GFP$^+$) compared with negative sorted control cells (Ctrl). All qPCR data have been normalized to the average of the reference sno-202. Data represent mean± s.e.m. from triplicate of dissected samples from different animals.*, P<0.05 relative to the other brain areas or to ctrl (Student's t-test).

3.5 miR-218 is expressed in the Midbrain

Despite we didn't find any target gene among those important for dopaminergic differentiations miR-218 was among the most upregulated genes in Pitx3-GFP positive cells FACS purified at E14. For this reasons we further analyzed its expression by *in situ hybridization*.

To this purpose, I analyzed its expression the midbrain of mice, starting from E11.5 until Postnatal 0 (P0). As shown in figure 3.7 miR-218 was detectable in Mb of mice starting from E12.5.

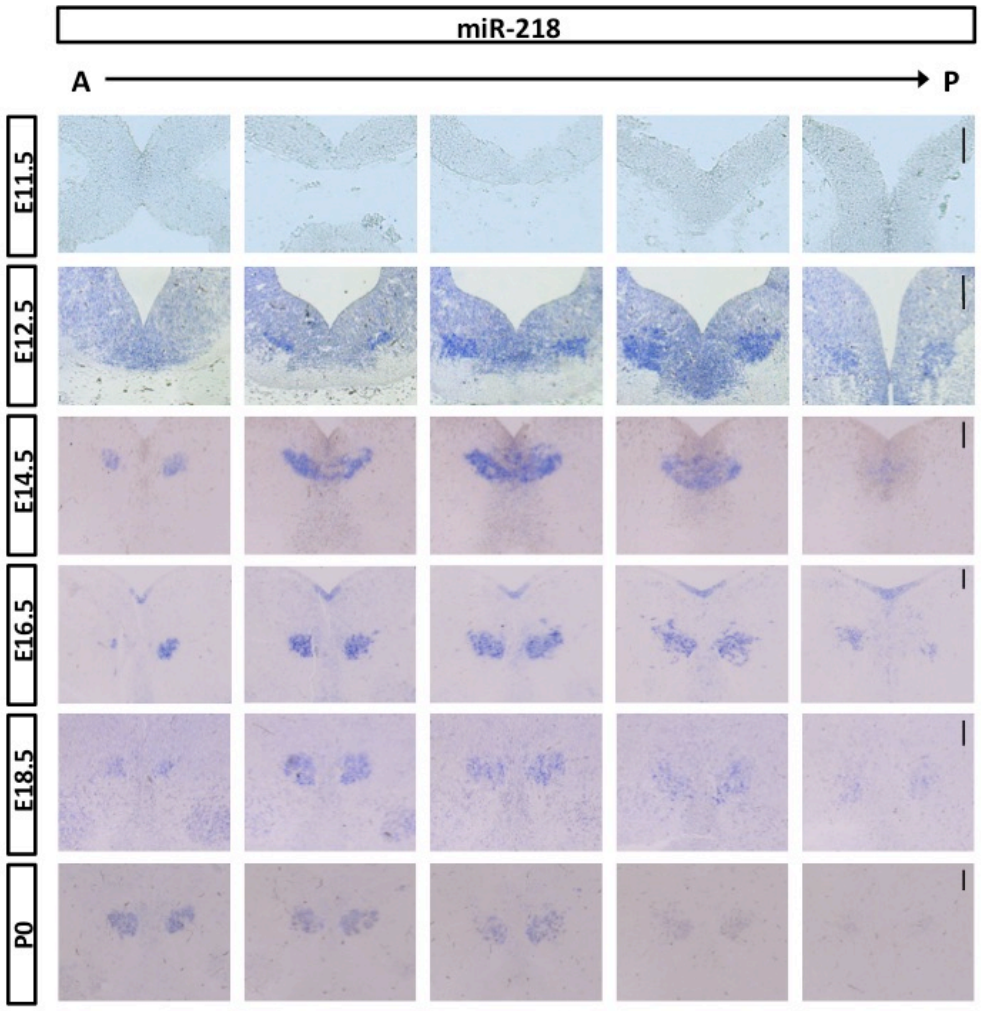

Figure 3.7 ***In situ hybridization*** to detect expression of miR-218 in paraffin embedded slides of midbrain mice from E11.5 to P0. Scale bar= 100um.

To understand if the expression profile of miR-218 was similar to that observed for typical dopaminergic markers, I performed immunohistochemistry (IHC) on adjacent slices of Mb at both E12.5 and E14.5. To this purpose, I used the dopaminergic markers Lmx1a, TH, Pitx3. Moreover, since the Mb is enriched also of mesencephalic motor neurons, I also used Isl1 as a specific marker of mesencephalic motor neurons (Liang et al. 2011).

As shown in figure 3.8 the expression of miR-218 co-localizes partially with that of dopaminergic and motoneuron markers such as Lmx1a/ TH/Pitx3 and Isl1 respectively.

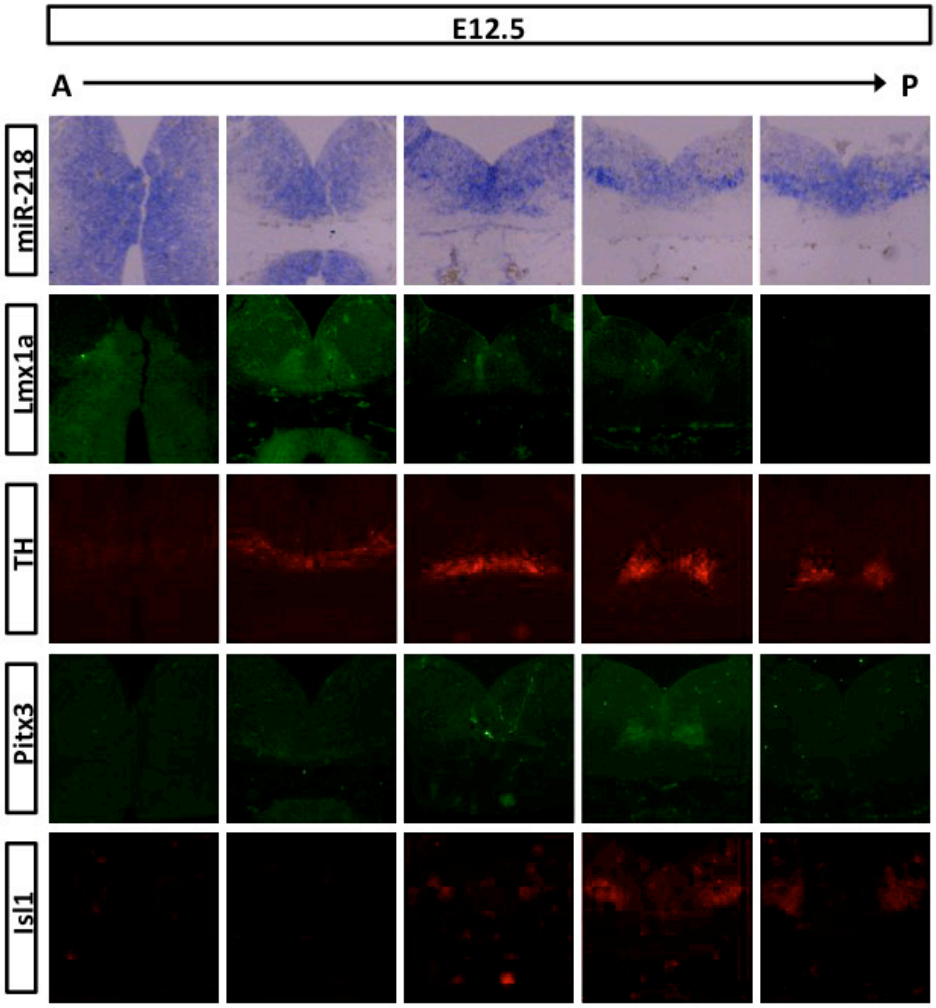

Figure 3.8 miR-218 is expressed in E12.5 midbrain. Panel of miR-218 in situ hybridization and IHC for dopaminergic markers: Lmx1a, Th, Pitx3 and motor neuron marker: Isl1. Scale bar= 100um

This data is very clear at E14.5 (fig. 3.9) where miR-218 overlaps with Isl1 in rostral slices, while with dopaminergic markers more caudally.

These data are in line with our hypothesis that miR-218 is required in dopaminergic neurons generation and with data shown in the literature of miR-218 implication in motor neuron (Thiebes et al. 2014; Amin et al. 2015).

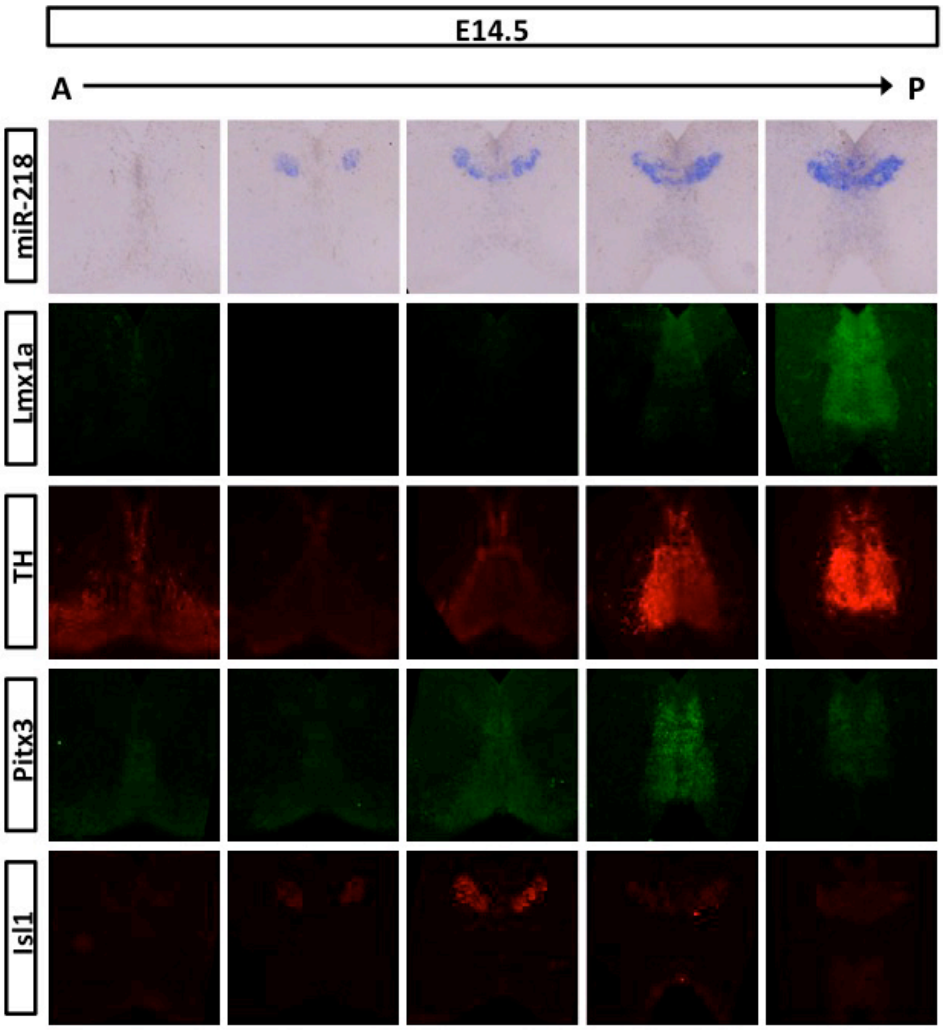

Figure 3.9 miR-218 is expressed in E14.5 midbrain. Panel of miR-218 in situ hybridization and IHC for dopaminergic markers: Lmx1a, Th, Pitx3 and motor neuron marker: Isl1. Scale bar= 100um

3.6 miR-218-2 conditional KO mouse model

To deeply understand the role of miR-218 in midbrain development I generated a conditional knock-Out (cKO) mouse model for miR-218. miR-218 is present in two identical copies in the genome: miR-218-1 and miR218-2, encoded by the intronic region Slit2 and Slit3 genes, respectively.

Since it has been shown that miR-218-1 KO mice are viable and not present any phenotype (Amin et al. 2015), I have generated cKO mice only for miR-218-2.

The targeting vector I have generated hosted the miR-218-2 sequence flanked by LoxP sites and Neomycin (Neo)-cassette (figure 3.10 A). The vector was then electroporated in ESCs and the clones screened for its integration by PCR analysis (data not shown) and Southern Blot.

To identify those clones where the homologous recombination occurs in the desired locus I performed Southern Blot Analysis by using three independent probes. The 3' Probe (scheme in figure 3.10 A) allowed for identifying positive clones where homologous recombination effectively occurred from negative ones (previously positive by PCR) where vector integration occurs unspecifically into the genome. Six clones on eight were positive showing two different bands corresponding to the targeted allele of 7Kb, and the wild type of 5.5Kb (figure 3.10 B). The 5' and the Neomycin probes further confirm proper integration.

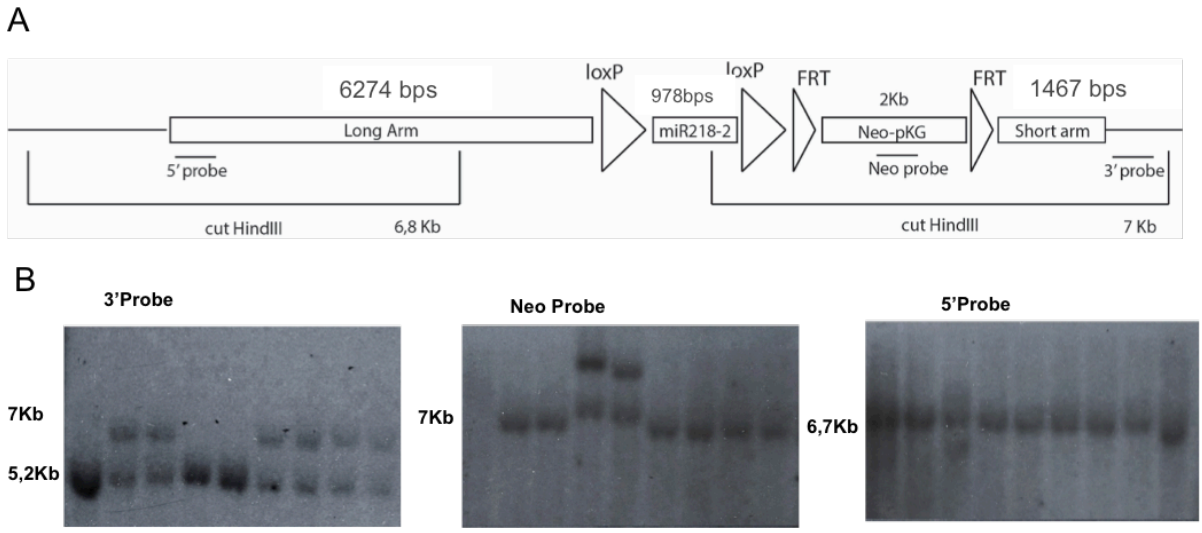

Figure 3.10 Homologous Recombination of miR-218-2. A) Schematic representation of targeting vector cKO miR-218-2 within design probes to discriminate positive clones. **B)** Southern Blot analysis that shown six positive clones on eight.

The ES cells containing the knockout mutation were injected into the blastocyst and implanted into the foster pseudopregnant female mouse. Chimeras mice were obtained at the F1 and germline transmission tested in the F2. At this stage, I obtained the first heterozygous mouse. Female mice were then mated with a Flippase male in order to remove the Neomycin cassette and obtain a clean conditional allele (miR-218-2$^{fl/fl}$).

In order to inhibit miR-218-2 expression only in dopaminergic field, we have mated conditional miR-218-2 with specific Cre-lines: Cre^{En1}, that have Cre Recombinase expression directed by the En1, this is an essential TF express in ventral Mb from E11.5 and persist also in adult brain (Simon et al. 2001, Albéri et al. 2004).

Preliminary data obtained on miR-218-2$^{fl/fl}$; En1$^{Cre/+}$ mice show motor impairment phenotype, but to confirm this data I'm currently performing behavior tests and in vivo analysis.

4 - DISCUSSION

Rita Levi Montalcini, speaking about the brain said: "It is imperfection - not perfection - that is the end result of the program written into that formidably complex engine that is the human brain, and of the influences exerted upon us by the environment and whoever takes care of us during the long years of our physical, psychological and intellectual development**."**

In recent decades, the scientific community has focused its attention on the direct and indirect involvement of Non-coding RNA in the development and function of neurons. Non-coding RNAs are emerging as important regulators of gene function and it has been shown that the vast majority of transcribed RNA corresponds to noncoding RNA (Consortium, 2004).

MicroRNAs constitute a class of small non-coding single-strand RNA (~22nucleotides) that act as post-transcriptional regulators of gene expression via the recognition of complementary sequences in the 3' untranslated regions (UTRs) in target messenger (m)RNAs that are thus directed to degradation or translational repression (Bartel, 2004).

Current knowledge suggests that miRNAs are fine-tuning regulators of gene expression acting in a wide range of biological processes, from development to cancer. Moreover, they are involved in embryonic development, cell differentiation and growth, cell proliferation, apoptosis and regulation of metabolic processes (He et al., 2004)

In neurons, the function of individual miRNAs are just beginning to emerge, and recent studies have elucidated roles for neural miRNAs at various stages of neuronal development and maturation, including neurite outgrowth, dendritogenesis, and spine formation (Olde Loohuis et al., 2012).

These findings emphasize that gene regulatory networks based on miRNA activities may be particularly important to brain function, and that perturbation of these networks may result in abnormal brain function (Wang et al., 2012). The enigma is that each microRNA targets numerous messenger RNAs (mRNAs) and each mRNA is targeted by many microRNAs. It has been estimated that a single microRNA may target up to hundreds of mRNAs.

In this context, I have performed a miRNAs profiling in order to understand which one is expressed in DA neurons and is potentially required in their development.

DA neurons development is intricate network and consists of four different spatial-temporal stages: induction, specification, differentiation, and maturation. Each phase is regulated by various transcription factors, morphogens, and other molecules.

In the literature, many studies propose the role of miRNAs in mDA neuron differentiation and related diseases such as Parkinson's. miR-133b was found to be downregulated in post-mortem Parkinson disease (PD) patient brains supporting its role in the pathogenesis of the disease (Kim et al., 2007); miR-34b/c cluster is significantly decreased in PD patient brains, even in the premotor stage of the disease. Moreover, its downregulation was associated to decrease expression of DJ1 and Parkin (Miñones-Moyano, Porta et al., 2011). Similarly miR-142-3p inhibit D1 type DA receptor expression both during development and in-vitro in cell culture (Tobón et al., 2012) while miR-181a is expressed upon dopamine signalling in primary neurons, as well as by cocaine and amphetamines, in a mouse model of chronic drug treatment (Saba et al., 2012). Many other microRNAs regulate the expression of several key factors for dopaminergic differentiation. miR-132 regulates the expression of Nurr1 (Yang et al., 2012) and miR-135a2 plays a crucial role in determining midbrain size and the allocation of prospective mDA precursors through the modulation of the Wnt signaling (Joksimovic et al., 2014, Nouri et al., 2015).

In line with the literature, we hypothesized that Dopaminergic System development and function are also regulated by the action of microRNAs that are potentially relevant for diagnostic purpose.

To address this topic, we have analyzed, through a microarray platform which miRNAs are upregulated during mDA differentiation. Indeed, we found few miRNAs involved in this process.

The data I obtained are further confirmed by *in vitro* and *in vivo* approaches. To summarise I show that:

- Overexpressing of miR-34b/c cluster and miR-218, in combination with Nurr1, enhances the number of TH^+ cells derived from mE12.5-PCs.

- miR-34b/c cluster and miR-148a bind Wnt1-3'UTR as predicted in TargetScan, but only miR-34b/c targets Wnt1-3'UTR and represses luciferase activity.

This finding differs from a previous report (Shi et al., 2015) and could be explained either by the different cellular context or the cloning strategy we used. Indeed instead of the 8bp corresponding to the precise miRNA's seed complementary region, we cloned almost the entire Wnt1 3'UTR sequence (~900 bp) downstream the luciferase reporter coding sequence. Therefore, our result could reflect a more in vivo like situation representing the real difference in targeting efficiency for both miR-34b/c and miR-148a-3p.

-The expression of miR-34b/c during mDA differentiation was further confirmed by using the dual fluorescence reporter assay. miR-34b/c here controls also the expression of the transcription factor Wnt1 through the binding to its 3'UTR-sequence.

- miR-34b/c cluster and miR-204 bind 3'UTR-Nurr1 and inhibit its expression in the Luciferase assay in HeLa cells.

The role of miR-204 is still nor clear in mDA neurons, for this reason, in future I would like to examine its implication in Dopaminergic System.

- In addition, miR-204, miR-218, and miR-34b/c, are also expressed in the midbrain of E14 mice embryos and in Pitx3-GFP positive cells, but not in the adult stage.

Altogether these results confirm the involvement of microRNAs in dopaminergic neurons development.

- Interesting the *in situ hybridization* analysis confirmed that miR-218 is mainly expressed at E12.5 and E14.5 in the midbrain where it partially colocalizes with motorneuron and dopaminergic markers.

These data fit with the previous reports indicating that miR-218 is motor neurons related microRNA (Amin et al. 2015, Thiebes et al. 2015). In these cells, it represses alternative fate, mainly GABAergic facilitating terminal differentiation of motor neurons. Similarly, it was also shown that miR-218 is important to reprogram astrocytes into dopamine neurons (iDANs) (Rivetti et al., 2017).

KO animals for miR-34b/c and miR-204 are already available but they show the limited phenotypic effect at the level of the midbrain. The lack of phenotypical anomalies in KO mouse model for microRNAs is a "typical" feature often reported. Several possible explanations have

been proposed. The most accepted are related either to the redundancy of miRNAs that compensate from a functional point of view the absence of a single member of the family either by the idea that miRNAs regulate target genes expression in cooperation with others molecules (miRNAs). Thus removing a single player might be inefficient in term of alteration of a biological process.

Surprisingly, this is not the case for miR-218. Here constitutive KO mice are perinatal lethal while conditional deletion in the midbrain results in important behavioral anomalies. For this reason, these animals represent an extremely valuable tool to study the role of miR-218 and will help to understand how miRNAs participate in neuronal function and development.

I believe we just begin to understand how non-coding RNAs participate in the generation and degeneration of the Central Nervous System. In future miRNA profiling will represent useful molecular diagnostic tools to early identify neuronal disorders. There are significant obstacles for a complete understanding the biological variability related to the phenotype of interest. Once we will have a complete picture of how miRNAs work, we will be able to use our knowledge for possible diagnostic and prevention approaches in translational medicine.

5- APPENDIX

During my PhD I have focused my attention also on a small project about *"A meta-analytic approach to genes that are associated with impaired and elevated spatial memory performance"* (under revision in Psychiatric Research).

Spatial memory deficits are a common hallmark of psychiatric and neurodegenerative conditions (for e.g. PD disease), possibly due to a genetic predisposition. Thus, unravelling the relationship between genes and memory might suggest novel therapeutic targets and pathogenetic pathways. Genetic deletions are known to lead to memory deficits (post-deletion "forgetfulness" genes, PDF), or, in few instances to improve spatial memory post-deletion "hypermnesic" genes, PDH). To assess this topic, I performed a meta-analytic approach on spatial memory behavior in knock-out mice. I screened 300 studies from PubMed and retrieved 87 genes tested for possible effects on spatial memory. This database was crossed with the Allen Brain Atlas (brain distribution) and the Enrichr (gene function) databases. The results show that PDF genes have higher expression level in several ventral brain structures, particularly the encephalic trunk and in the hypothalamus. Moreover, part of these genes is implicated in synaptic function. Conversely, the PDH genes are associated with G-protein coupled receptors downstream signalling. Some candidate drugs were also found to interfere with some of the PDH genes, further suggesting that this approach might help in identifying drugs to improve spatial memory performance in neuro disorders.

6 – BIBLIOGRAPHY

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7 - LIST OF ABBREVIATIONS

A

A*(n)*	Catecholaminergic nuclei
AADC	Aromatic L-amino acid decarboxylase
ADHD	Attention deficit hyperactivity disorder
Ahd2	Aldehydedehydrogenase2
AK	Aphakia mice
Ascl1	Achaete-scute homolog 1

B

BDNF	Brain deriven neurotrophic factor
bFGF	Basic fibroblast growth factor (FGF2)

C

cAMP	Cyclic Adenosine Mono-phosphate
CDNF	Cerebral dopamine neurotrophic factor
cKO	conditional Knock-out
CNS	Central nervoussystem
COMT	Catechol-O-methyltransferase
c-Ret	GDNF receptor

D

Δ	Deletion/mutation
DA	Dopamine / Dopaminergic

DBH	Dopamine ß-hydroxylase
DRD	DA receptor
DAT	Dopamine active transporter (Slc6a3)
DIV or d	Days *in vitro*
Dmrt5	Doublesex and mab-3related transcription factor 5
DOPAC	3,4-dihydroxyphenylacetic acid
Dox	doxycyxline
Dsh	Dishevelled

E

E	Embryonic day
En1/2	Engrailed 1 and 2
epiSC	Epiblast stem cells
Eph	Ephrines
ERK	Extracellular signal–regulated kinase
ESRRβ	Estrogen-related receptor beta
ESC	Embryonic stem cells

F

FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGF8	Fibroblast growth factor 8
FP	Floor plate
FoxA1/2	Forkhead box protein A1/A2
Fz	Frizzled
Fz3	Frizzled 3

Fz6	Frizzled 6

G

GABA	γ-Aminobutyric acid
Gbx2	Gastrulation Brain Homeobox 2
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPe	external Globus pallidus
GPi	internal Globus pallidus

H

HEK293T	Human embryonic kidney cell line 293T
hESC	Human embryonic stem cells
HVA	Homovanillic acid

I

IF	Immunofluorescence analysis
IGB-ABT	Institute of genetics and biophysics Adriano Buzzati Traverso
iPSC	Induced pluripotent stem cells
ISH	*In situ hybridization*
IsO	Isthmic organizer

K

KFL2	Krüppel-like Factor 2
KFL4	Krüppel-like Factor 4
KO	Knock-out

L

L-DOPA	L–3,4–dihydroxyphenylalanine (levodopa)
LIF	Leukemia inhibitory factor
Lmx1a	LIM-homeodomain factor1a
Lmx1b	LIM-homeodomain factor1b
LV	Lentiviral particles

M

MAO	Monoamine oxidase
mDA	Midbrain dopaminergic neurons
MHB	Mid-hindbrain boundary
Mb	Midbrain
MCLp	Mesocorticolimbic pathway
mESc	Mouse Embryonic Stem Cell
mE12.5-PCs	Mouse embryonic primary cultures from E12.5 midbrains
miRNA	microRNA
mRNA	messanger RNA
Msx1	Msh homeobox 1

N

NC	Notocord
NEAA	Non-essential aminoacids
Ngn2	Neurogenin 2
Nkx2.2	NK2 Homeobox 2
Nkx6.1	NK6 Homeobox 1
Nr4a1	Alias for Nurr77

Nr4a2	Alias for Nurr1
Nr4a3	Alias for Nor-1
NSC	Neural stem cells
NSp	Nigrostriatalpathway
Nurr1	Nuclear Receptor Related 1 protein

O

OB	Olfactory bulbs
Otx2	Orthodenticle Homeobox 2
Otx4	Orthodenticle Homeobox 4
OV	Otic vescicle

P

$p57^{Kip2}$	Cyclin-dependent kinase inhibitor 1C
PD	Parkinson's disease
PDH	Post-deletion "Hypermnesic" genes
PDF	Post-deletion "Forgetfulness" genes
PD03 (PD0325901)	Selective inhibitor of mitogen-activated protein kinase kinase
Pen/Strep	Streptomycin and Penicillin
PhD	Doctor of Philosophy
Pitx3	Pituitary homeobox 3
PNMT	Phenylethanolamine N-methyltransferase
pre-miRNA	Precursor-miRNA
pri-miRNA	Primary-miRNA
PSF	Splicing factor PTB-associated
PTB	Polypirimidine–tract-binding-protein

Ptc1	Patched 1
PKA	Protein Kinase A

R

RA	Retinoic acid
RP	Roof plate
RRF	Retrorubral field
RT-PCR	*real time* PCR

S

SAG	Smoothened Agonist
SC	Spinal cord
SEM	Standard error of the mean
SHH	Sonic Hedgehog
Slit2	Slit Guidance Ligand 2
Smo	Smoothened
SN	Substantia Nigra
SNc	Substantia Nigra *pars compacta*
SNr	Substantia Nigra *pars reticulata*
Sox2	SRY (sex determining region Y)-box 2
STN	Subthalamic nucleus

T

TF	Transcription factors
TGFβ	Transforming growth factor *β*
TH	Tyrosine Hydroxylase
TIGEM	Telethon Institute of Genetics and Medicine

TPH2	Tryptophan hydroxylase 2

U

UTR	Untranslated regions

V

Vmat2	Vesicular monoamine transporter
VTA	Ventral Tegmental Area

W

Wnt	Wingless-Type MMTV Integration Site Family
wt	Wilde type

Z

Zic2	Zic Family Member 2

8 – ACKNOWLEDGEMENTS

I would like to thank my Tutor Prof. Davide Viggiano and Co-Tutor Dr. Gian Carlo Bellenchi, for their constant support during my PhD.

I want to express my sincere gratitude to Dr. Umberto di Porzio and all the collaborators of the Lab: Prof. Carla Perrone Capano, Prof. Floriana Volpicelli, Luisa Speranza, Teresa Giuliano, Valerio Piscopo.

Special thanks to Roberto De Gregorio & Salvatore Pulcrano for sharing their knowledge and for their contribution to this work.

Thanks to IRCCS Neuromed, in particular, Prof. Antonio Simeone, for their financial support.

Thanks to the Mouse modeling facility, the Animal House, the Integrated Microscopy and the FACS Facilities at the IGB-ABT CNR. For the high-content imaging and statistical analysis, I have to thank also Dr. Diego Medina (TIGEM) and Dr. Claudia Angelini (IAC-CNR).