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Review

MicroRNAs and the neural crest: From induction to differentiation

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ABSTRACT

MicroRNAs are small noncoding RNAs that can control gene expression by base pairing to partially complementary mRNAs. Regulation by microRNAs plays essential roles in diverse biological processes such as neural crest formation during embryonic development. The neural crest is a multipotent cell population that develops from the dorsal neural fold of vertebrate embryos in order to migrate extensively and differentiate into a variety of tissues. Gene regulatory networks that coordinate neural crest cell specification and differentiation have been considerably studied so far. Although it is known that microRNAs play important roles in neural crest development, posttranscriptional regulation by microRNAs has not been deeply characterized yet. This review is focused on the microRNAs identified so far in order to regulate gene expression of neural crest cells during vertebrate development.

1. Introduction

One type of small endogenous noncoding RNAs are microRNAs (miRNAs), which bind to target mRNAs and regulate protein expression by repressing translation, promoting degradation of the target mRNA, or enhancing translation at the post-transcriptional level through the RNA-induced silencing complex (Pasquinelli, 2012; Bartel, 2009; Vasudevan et al., 2007). MiRNAs comprise 1–2% of all genes in animals (Bartel, 2009), and because each miRNA is predicted to regulate hundreds of targets, half of protein-coding genes is thought to be under their control (Pasquinelli, 2012). The biological roles of miRNAs are so diverse that they become active players in developmental embryogenesis, cell differentiation, organogenesis, growth, and programmed cell death, as well as stem and germ cell maintenance, disease, and evolution (Kloosterman and Plasterk, 2006; Vidigal and Ventura, 2015).

The neural crest (NC) is a transient, multipotent stem cell-like population whose formation occurs early in development at the border of the developing neural tube. After closure of the neural tube, NC cells (NCC) experience an epithelial-to-mesenchymal transition (EMT) in order to delaminate and migrate away, undergoing some of the longest distant position of any embryonic cell type (Theveneau and Mayor, 2012). NCC differentiate into a variety of derivatives, including neurons and glia of the enteric, sensory, and autonomic nervous system, pigment cells, chromaffin cells, bone and cartilage of the face, endocrine cells, cardiac structures, smooth muscle cells, and tendons (Dupin and Le Douarin, 2014). Originally, by different experimental approaches that change the levels of transcription factors and signaling molecules in different animal models, several regulatory interactions within NCC have been largely documented. Recent advances in technology have increased the initial direct linkages within the genetic control of the sequential events that form the NC from a multipotent progenitor to a differentiated state given a highly complex gene regulatory network (GRN) (Martik and Bronner, 2017).

While much is known about NC key genes and networks, the relationship between miRNAs and genes involved in NC development is still relatively scarce. During development, miRNAs contribute to progressive changes in gene expression by fine-tuning protein levels, allowing for spatiotemporal protein downregulation, thereby shaping and diversifying the gene expression profiles of different cell types (Alberti and Cochella, 2017). In addition, miRNA-mediated protein downregulation can be useful to maintain target gene levels at required steady-state concentrations, thus providing reproducibility to processes occurring during accurate developmental stages (Cohen et al., 2006). The repressive effect of miRNAs on target expression is modest and is often limited to the level of translation with little effects on transcript abundance, considering that miRNAs do not act in isolation. Several cases show that miRNAs act in concert with other regulatory processes, such as transcriptional control, to regulate target gene expression at multiple levels and with greater strength (Vidigal and Ventura, 2015). Besides, a common idea among miRNAs is their positioning within GRNs, in particular within feedback and feed-forward loops (Tsang et al., 2007). Regarding this, one possibility is that the transcription of

Abbreviations: miRNA, microRNA; NC, neural crest; NCC, neural crest cells; EMT, epithelial-to-mesenchymal transition; GRN, gene regulatory network; DGS, DiGeorge syndrome; dpf, day post-fertilization

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the miRNAs and their targets is oppositely regulated by common upstream factors. For example, an upstream element could repress the transcription of a target gene and simultaneously activate the transcription of a miRNA that inhibits target gene translation (Tsang et al., 2007). The other possibility may be predominant, since genome-scale studies have shown that predicted target transcripts of several tissue-specific miRNAs tend to be at a lower concentration level in tissues where the miRNAs are expressed (Stark et al., 2005). Another key recurring function of miRNAs in GRN is to reinforce the gene expression program of differentiated cellular states (Tsang et al., 2007), as would be seen later in this review for NCC differentiation.

The aim of this work is to review the most relevant evidences reported so far regarding the participation of miRNAs in NC induction, specification, delamination, migration, and differentiation.

2. Dicer disruption in the NC

Functional mature miRNAs rise through several posttranscriptional processing steps that include cleavage by Drosha/Dgcr8 to pre-miRNA, nuclear export, and digestion by the RNase III endonuclease Dicer (Krol et al., 2010). Dicer is a large multi-domain protein, which not only is involved in the cleavage of pre-miRNAs but also participates in loading miRNAs into miRNA-induced silencing complexes (Krol et al., 2010). A number of non-canonical pathways for miRNA biogenesis have also been described, such as mirtrons that are able to bypass Drosha by using the splicing machinery instead (Abdelfattah et al., 2014). However, a common feature of all pathways is the cleavage of the intermediate precursor by Dicer, suggesting it is indispensable for the majority of miRNA biogenesis. One exception is the processing of miR-451, which has been shown to evade Dicer cleavage and be processed by Argonaute-2 (Yang and Lai, 2010).

Dicer mutants were first reported in 2003. In mice, *Dicer* deletion causes loss of the inner cell mass of the blastocyst, resulting in early embryonic lethality (Bernstein et al., 2003). In zebrafish, homozygous *dicer1*$^{-/-}$ mutants appear normal during the first week of development but undergo growth arrest at 8 days post fertilization (dpf) and dye at 14–15 dpf (Wienholds et al., 2003). To block maternal Dicer1, a zebrafish maternal-zygotic Dicer1 (MZ-Dicer1) mutant was generated (Giraldez et al., 2005). MZ-Dicer1 mutant embryos show the most severe phenotype; embryos display defective gastrulation, resulting in a reduced extension of the axis, as well as impaired brain, somite, heart, and ear development (Giraldez et al., 2005). Most of these defects are rescued by the injection of members of the miR-430 family, which also play a key role in the clearance of maternal mRNAs shortly after the activation of the zygotic genome (Giraldez et al., 2006), and avoid the formation of mixed states between different developmental stages (Schier and Giraldez, 2006).

Later on, mice with *Dicer* specific inactivation in the NC have been generated by the usage of a *Wnt1*-Cre transgenic line. *Dicer* homozygous mice with NC-specific inactivation are lethal while heterozygous mice are fertile and exhibit no apparent abnormalities, suggesting that one copy of the gene is sufficient for survival (T Huang et al., 2010; Zehir et al., 2010; Nie et al., 2011). The initial NCC induction and migration is not affected; however, as development progresses, massive cell death and complete loss of NCC-derived craniofacial structures are observed, leading to severe abnormalities during craniofacial morphogenesis (T Huang et al., 2010; Zehir et al., 2010; Nie et al., 2011). On the other side, migration and patterning of cardiac NCC, but not survival, are impaired. This results in a variety of cardiovascular abnormalities, including an interrupted aortic arch, double-outlet-right-ventricle, and ventricular-septal defects, which phenocopy certain forms of human congenital cardiac defects (ZP Huang et al., 2010). Deletion of *Dicer* in NCC also causes malformation of the dorsal root ganglia, enteric nervous system, and sympathetic ganglia (T Huang et al., 2010). Similar defects are observed in mice with a NCC-specific inactivation of *Dgcr8*, which codes for a double stranded RNA-binding protein that is central for miRNA biogenesis, acting as an essential cofactor for Drosha (Chapnik et al., 2012). Taken together, these results collectively establish the critical role of Dicer and miRNAs in NCCs to regulate development of craniofacial structures, as well as the neural and cardiovascular system.

It is worth mentioning that the *Wnt1*-Cre transgenic mice used to make *Dicer* mutants exhibit ectopic upregulation of Wnt1 in the midbrain, leading to a marked enlargement of the region (Lewis et al., 2013). However, authors did not detect grossly perturbations in NC development, especially in cranial and cardiac NC derivatives (Lewis et al., 2013). These results suggest that *Dicer* mutants generated with this transgenic line could be used to study NC development but nor midbrain formation.

3. NC induction and specification

NCC are induced in the ectodermal germ layer during gastrulation and initially reside in the neural plate border territory, which is positioned at the lateral edges of the neural plate. During neurulation, this border territory elevates as the neural plate closes to form the neural tube. Inductive waves of factors from neighboring tissues trigger the expression or repression of transcription factors, which specifies the neural plate border from which the NC arises by acting in hierarchical pathways (Steventon et al., 2005). Three major signaling pathways (BMP, WNT, and FGF) coordinately function driving the expression of neural plate border specifier genes (Steventon et al., 2005), which are *Tfap2*, *Msx1/2*, *Zic1*, *Gbx2*, *Pax3/7*, *Dlx5/6*, *Gata2/3*, *Foxi1/3*, and *Hairy2* (Simões-Costa and Bronner, 2015). These transcription factors engage in mutual cross-regulatory interactions leading to the stabilization of NC specification. The output of these signaling pathways, together with the transcription factors expressed at the neural plate border, establishes a novel scenario that sets apart presumptive NCC from the other cells belonging to ectodermal domains. This process, which is known as NC specification, concludes with the transcriptional activation of *FoxD3*, *Snai1/2*, *Tfap2*, *n-Myc*, and *Sox8/9/10*, typical marker genes of NC specification (Simões-Costa and Bronner, 2015). The task of these factors is to preserve multipotency, promote the EMT, initiate delamination and migration, and participate in cell proliferation, survival control as well as cell differentiation.

The regulatory mechanism that determines cell fate at the early stage of neural differentiation has been studied in mice embryonic stem cells in order to establish the distinction between neural tube epithelial cells and NCC lineage. Specifically, it has been found that miR-29b expression is upregulated in neural tube epithelial cells and downregulated in NCC, whereas miR-29a is downregulated in both cell types (Xi et al., 2017). The differential expression of miR-29b promotes neural differentiation and inhibits NCC lineage by targeting the epigenetic factor DNA methyltransferase 3a and Pou3f1, a critical transcription factor in neural differentiation (Fig. 1) (Xi et al., 2017). Further experiments will help to elucidate which NC target genes are regulated by this miRNA family and their effectors prior NCC induction.

Depletion of miR-96 and miR-196a affects NC induction in *Xenopus laevis* embryos (Fig. 1) (Gessert et al., 2010). Nevertheless, putative target genes for these two miRNAs have not yet been identified. Recently, next generation sequencing from ectodermal explants of *Xenopus laevis* embryos has allowed the identification of novel miRNAs enriched in NC tissue (Ward et al., 2018). The induction of the NC tissue is performed by the injection of *Wnt-1* and *Noggin* mRNAs at one-cell stage embryos. Animal caps are left to develop until neural tube folding and tissue induction is validated by both qPCR and whole-mount *in situ* hybridization for NC markers. MiR-219, miR-218-2, miR-nov-12a-1, miR-338-3, miR-10b, miR-204a, miR-130b/c, miR-23, miR-24, and miR-196a are the ones upregulated in the NC against neural tissue (Fig. 1). The most abundant is miR-219, which targets platelet derived growth factor receptor A (Pdgfrα), a receptor for the ligand platelet derived growth factor (Pdgf), a migratory NCC marker gene (Dugas

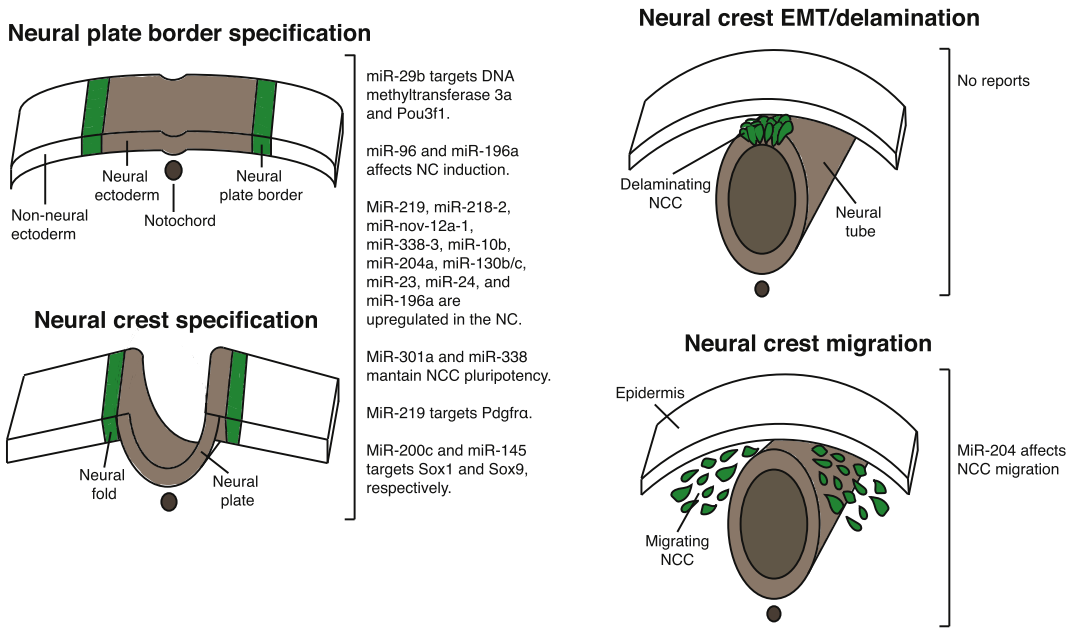

Fig. 1. Summary of NC miRNA-mRNA regulation. The NC forms by a series of important regulatory events: induction at the neural plate border, specification, delamination from the neural tube, and migration throughout the embryo. Experimentally elucidated miRNAs-mRNAs interactions are highlighted for each stage of NC development.

et al., 2010). *Pdgfrα/Pdgf* transcripts co-localize in migrating cranial NCC and their inhibition impairs EMT by preventing N-cadherin expression (Bahm et al., 2017). Apparently, miR-219 restrains Pdgfrα expression in pre-migratory NCC to prevent premature migration (Fig. 1). In addition, possible miRNAs contributing to NC multipotency enriched in both the NC and blastula have been identified. Among them, miR-301a and miR-338 are highly expressed in both tissue types suggesting a role for these miRNAs in maintaining the stem cell-like phenotype of NCC (Fig. 1) (Ward et al., 2018). Further experiments will help to elucidate the possible target genes for these miRNAs that are involved in multipotency.

On the other hand, by employing murine embryonic stem cells, it has been shown that metformin, a popular antidiabetic drug, interferes NCC determination by deregulating canonical Wnt axis and a set of miRNAs (Banerjee et al., 2016). When murine embryonic stem cells are differentiated into NCC and treated with metformin, miR-200c and miR-145 show more than 5-fold upregulation over other miRNAs. Further loss- and gain-of-function studies confirm that transcripts of the NC specifier genes *Sox1* and *Sox9* are direct targets of miR-200c and miR-145, respectively (Fig. 1) (Banerjee et al., 2016). Given that metformin is a widely used drug, even during pregnancy, authors alert that its administration could affect NCC formation by interfering with canonical Wnt signaling, mysregulation of miR-145 and miR-200c, and consequently the expression of NC specifier genes.

4. NC delamination and migration

Once the NC is specified, it undergoes drastic gene regulatory changes that enable NCC to engage in EMT and gain migratory behavior and diversification. NCC separate from neighboring neuroepithelial cells by delamination, which involves a partial or complete EMT (Theveneau and Mayor, 2012). A BMP/canonical Wnt pathway, involving *Bmp4*, *Wnt1*, *Msx1* and *c-Myb*, triggers NCC delamination. This pathway promotes EMT *via* the transcriptional activation of *Snail2*, *FoxD3* and members of the *SoxE* family, which participate in the switching of Cadherin profiles and in the modulation of gap junction and integrin proteins (Simões-Costa and Bronner, 2015). At the end of migration, the expression of most NC specifier genes is downregulated, except for some factors like *Sox10* and *FoxD3*, which remain active in a subset of NCC, contributing to terminal differentiation (Kelsh, 2006).

The intricate GRN of miRNAs and transcription factors governing EMT of non-NCC has been extensively characterized (reviewed in Nieto et al., 2016). However, only one work has addressed the role of a miRNA in NCC migration. By using single-cell live-imaging in a GFP medaka transgenic line, embryos treated with a Morpholino for miR-204 show a decreased of NCC migration with respect to control embryos (Avellino et al., 2013). Further experiments confirm that miR-204 controls the migration and localization of mesenchymal NCC, although the target gene/s have not been identified yet (Fig. 1).

It is largely known that NC delamination and migration processes are mirrored in cancer metastasis, where a primary tumor will undergo an EMT before migrating and invading other cell populations to create a secondary tumor site. In recent years, EMT of NCC and tumor cell migration has been profoundly studied (Barriga et al., 2018; Taneyhill and Schiffmacher, 2017), as well as important new insights into tumorigenesis and metastasis have also been achieved (Gallik et al., 2017; Yang and Weinberg, 2008). These discoveries have been driven by the observation that many cancers misregulate developmental genes to reacquire proliferative and migratory states (Powell et al., 2013). Besides, other studies have shown that dysregulation of several miRNAs greatly impacts cancer angiogenesis and metastasis (Lou et al., 2017). Surprisingly, there is no link between all those miRNAs expressed in cancer metastasis and NC EMT and/or migration. Consequently, it should be important to decipher the identity of miRNAs involved in NC and cancer processes in a comparative manner, since it will expand our knowledge of metastasis and NC development.

5. NC differentiation

The process of NC diversification initiates with the activation of differentiation pathways in subpopulations of migratory cells. These pathways operate under a positive feed-forward loop where initial NC regulators function together with locally activated differentiation effector genes (Simões-Costa and Bronner, 2015). In order to arrive at the proper target region, NCC have to interpret multiple environmental signals that directly influence the place where they have to migrate in

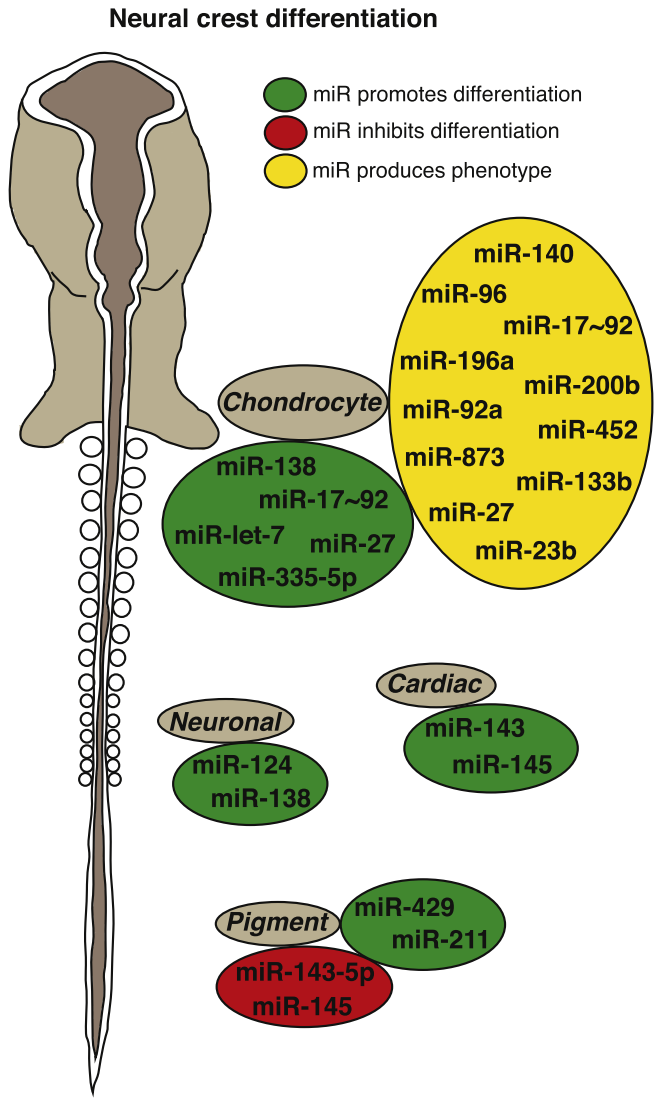

Fig. 2. Summary of NC miRNA regulation during differentiation. Experimentally elucidated miRNAs that affect NCC differentiation processes of cranial, heart, neuronal, and pigment lineages.

order to settle and differentiate. Sox9 and Sox10 transcription factors are major players regulating effector genes that give specific characteristics to cells. These transcription factors are first expressed during NC specification in premigratory (Sox9), early delaminating, and migrating (Sox10) NCC (Simões-Costa and Bronner, 2015; Theveneau and Mayor, 2012). Following, the evidences reported so far regarding miRNAs during the differentiation processes of chondrocyte, cardiac, neuronal, and pigment lineages are described in sub-sections (Fig. 2 and Table 1).

5.1. Chondrocyte lineage

The role of miRNAs in chondrocyte NC lineage differentiation has been largely documented. *Dicer* homozygous mutant mice with chondrocyte-specific inactivation (*Dicer*-deleted gene under the control of *Col2a1* promoter) display severe skeletal defects and premature death due to progressive reduction in chondrocyte proliferation and precocious differentiation to hypertrophic chondrocytes (Kobayashi et al., 2008). The blockage of cell proliferation and the acceleration of chondrocyte differentiation in cells lacking miRNAs suggest that one or more miRNAs act over genes that normally control the passage from cell division to chondrocyte maturation. More recently, another Dicer conditional knockout mice model in which the *Dicer* alleles are deleted through Cre-mediated recombination following *Pax2* expression has been generated in order to specifically analyze mammalian palatogenesis development (Barritt et al., 2012). It has been shown that secondary palatal development becomes morphologically arrested prior to mineralization with a significant increase in the expression levels of apoptotic markers, such as Caspase3, p53, and p21. Besides, miR-101b, miR-140, and miR-145 are significantly downregulated in these *Dicer* mutant mice during palatal development when compared with wild-type embryos (Barritt et al., 2012).

In 2008, Eberhart et al. demonstrated that miR-140 affects cranial NCC dispersion and modulates palatogenesis in zebrafish. Authors show that the array of facial defects detected in miR-140 duplex-injected embryos precisely phenocopied those ones observed in Pdgf-receptor alpha (*pdgfrα*) mutants. Besides, the 3′UTR of *pdgfrα*-mRNA contains a miR-140 binding site that negatively regulates its translation *in vivo* (Table 1). Therefore, miR-140 seems to inhibit Pdgf signaling and modify oral ectodermal gene expression, causing alteration in the shape of the palatal skeleton (Fig. 2). Curiously, Pdgfrα was previously described as being regulated by miR-219 before NCC delamination (see Section 3 of this article), suggesting that several miRNAs contribute to control its expression at different stages of NC development.

The miR-17–92 family has been linked to Feingold syndrome in human patients (Tassano et al., 2013). Mice-targeted knockouts specific for those miRNAs show hypoplasia of most skull bones, including reduced ossification and cleft palate, similarly to human abnormalities observed in patients (Ventura et al., 2008). At molecular level, miR-17–92 mutant mice display expanded *Tbx1* and *Tbx3* expression in craniofacial structures. Other authors have found functional miR-17–92 seed sequences in *Tbx1* and *Tbx3*-3′UTR mediating their gene repression (Table 1) (Wang et al., 2013). Besides, the analysis of miR-17–92 promoter regulatory regions has revealed conserved and functional recognition elements for Tfap2α, the master regulator of cranial NC development (Wang et al., 2013). Recently, a more detailed study developed in mice has shown that the inhibition of miR-17 and miR-18 leads to arrest of palate formation prior to palatal shelf elevation, while the inhibition of miR-17, miR-18, miR-19, and miR-92 leads to arrest at a later stage of palatal development and extension (Fig. 2) (Ries et al., 2017). Further experiments performed in transgenic mice suggest that the cleft-palate phenotype is partially attributable to aberrations in the Tgfb signaling, by interactions between Tgfbr2 and miR-17–92 (Ries et al., 2017). More experiments showing miRNA-target interactions would help to clear the proper regulations occurring in this pathway.

In *Xenopus laevis*, the depletion of miR-96, miR-196a, and miR-200b results in abnormal cranial cartilage structures (Fig. 2) (Gessert et al., 2010). Although authors cannot identify putative target genes for these miRNAs, other experiments performed in cell culture have revealed that miR-96 represses Tbx1 expression and Tbx1 represses miR-96 expression, thus working in a regulatory loop (Table 1) (Gao et al., 2015). Both *Tbx1* conditional knockout and over-expression in mice recapitulate craniofacial defects associated with *Tbx1* deletion or gene duplication. Altogether, results from cell culture and knockout mice suggest that a regulatory loop between Tbx1 and miR-96 is implicated in craniofacial development (Gao et al., 2015). DiGeorge syndrome (DGS; MIM#188400) involves a deletion within chromosome 22 with an occurrence estimated of 1 in 4000 live births, and the phenotype include craniofacial malformations, cleft palate, as well as congenital heart defects (Lindsay, 2001). Since *TBX1* is located within the deleted region of DGS, it is tempting to speculate that *TBX1* is a candidate gene for DGS phenotype. *DiGeorge critical region 8* gene (*DGCR8*) maps to the common deletion region of DGS and encodes a cofactor of Drosha. In mice, the conditional loss of *Dgcr8* causes a wide spectrum of malformations in cardiac NCC, suggesting that this cofactor is also involved in DGS defects (Chapnik et al., 2012). Thereby, craniofacial alterations in DGS could be attributed to both TBX1 and miR-96 while to DGCR8 for defects in cardiac NCC development.

Table 1
Summary of experimentally elucidated direct interactions between miRNAs and their targets during NCC differentiation.

NC differentiation				
	miR family	Targets	Experiments	Reference
Chondrocyte	miR-140	Pdgfra	*in vivo* (zebrafish): 3′UTR GFP reporter + low protein levels	Eberhart et al., 2008
	miR-17–92	Fgf10, Shox2, Tbx3 and Osr1	*in vitro*: 3′UTR luciferase reporter + mutation in binding site	Wang et al., 2013
	miR-96	Tbx1	*in vitro*: 3′UTR luciferase reporter + low protein levels	Gao et al., 2015
	miR-138	Oct4	*in vitro*: 3′UTR luciferase reporter	Mohanty et al., 2016
	miR-let-7	Trim71	*in vitro*: 3′UTR luciferase reporter	Mohanty et al., 2016
	miR-873	Zic2	*in vitro*: 3′UTR luciferase reporter	Koufaris et al., 2015
	miR-92a	Nog3	*in vivo* (zebrafish): 3′UTR GFP reporter + mutaton in binding site	Ning et al., 2013
	miR-27	Ptk2aa	*in vivo* (zebrafish): 3′UTR GFP reporter + mutation in binding site + low protein levels	Kara et al., 2017
	miR-141/miR-200a	Dlx5	*in vitro*: 3′UTR luciferase reporter + mutation in binding site	Itoh et al., 2009
	miR-452	Wnt5a	*in vitro*: 3′UTR luciferase reporter + mutation in binding site + low protein levels	Sheehy et al., 2010
	miR-335-5p	Dkk1	*in vitro*: 3′UTR luciferase reporter + mutation in binding site	Zhang et al., 2011
Cardiac	miR-143	Elk1	*in vitro*: 3′UTR luciferase reporter + mutation in binding site + low protein levels	Cordes et al., 2009
	miR-145	Klf4 and CAMkII	*in vitro*: 3′UTR luciferase reporter + mutation in binding site + low protein levels	Cordes et al., 2009
Neuronal	miR-138	Ccnd1, Sox2, and Jun	*in vitro*: 3′UTR luciferase reporter	Yun et al., 2010
Pigment	miR-143-5p	TGF-β-activated kinase 1	*in vitro*: 3′UTR luciferase reporter + mutation in binding site	Ji et al., 2018
	miR-145	Myo5a	*in vitro*: 3′UTR luciferase reporter + mutation in binding site	Dynoodt et al., 2012
	miR-429	FoxD3	*in vitro*: 3′UTR luciferase reporter + mutation in binding site | *in vivo*: low protein levels	Yan et al., 2013
	miR-211	TGF-ß receptor 2	*in vitro*: 3′UTR luciferase reporter + mutation in binding site	Dai et al., 2015

Folate receptor alpha (FRα) upregulates pluripotency markers, such as *Oct4*, *Sox2*, and *Klf4*, in cranial NCC by binding to their promoter regions. Simultaneously, FRα downregulates miR-138 and miR-let-7 (Mohanty et al., 2016). MiR-138 targets *Oct4* and miR-let-7 its effector *Trim71*, thus controlling the proliferation of cranial NCC (Table 1, Fig. 2). These regulatory interactions explain how pre-migratory NCC maintain their multipotent phenotype and proliferation potential prior to differentiation (Mohanty et al., 2016).

Bioinformatic analysis and experimental approaches indicate that miR-873 is involved in the regulation of the Hedgehog signaling, an essential pathway for craniofacial patterning and differentiation (Koufaris et al., 2015). Data have been generated from a patient displaying several craniofacial abnormalities likely due to a not previously reported genome deletion including the miR-873 and miR-876 genes (Koufaris et al., 2015). The human factor ZIC2, involved in normal craniofacial development, has been identified as a possible miR-873 target (Table 1) (Koufaris et al., 2015). Further studies, such as the miRNAs embryonic expression patterns and the generation of suitable genetically modified animal models, are needed to clarify and confirm the role of the miR-873/miR-876 cluster in development and disease.

Microarray analysis has detected differentially expressed miRNAs in murine orofacial tissues at three different gestation days. Moreover, by using a program to generate relevant and interacting biological networks, it has been found a list of miRNA-target genes involved in cell proliferation, cell adhesion, differentiation, apoptosis, as well as in EMT, all critical processes for normal orofacial development (Mukhopadhyay et al., 2010). Additional experimental approaches should be carried out to specifically address miRNA-mRNA target gene regulation during such processes.

In zebrafish, the inactivation of miR-92a increases *Nog3* levels in the pharyngeal region, resulting in the loss of pharyngeal cartilages due to a significant reduction in cell proliferation, differentiation, and survival of pharyngeal chondrogenic progenitors (Ning et al., 2013). On the other hand, the knockdown of miR-27 causes severe defects in the neurocranium by impaired proliferation and differentiation of chondrogenic progenitors (Kara et al., 2017). miR-27 targets the focal adhesion kinase Ptk2aa, a key regulator in integrin-mediated extracellular matrix adhesion proposed to function as a negative regulator of chondrogenesis (Table 1) (Kara et al., 2017). It has been shown that miR-27 downregulates *Ptk2aa* in pharyngeal arches, promoting chondrogenic differentiation (Fig. 2) (Kara et al., 2017).

Studies in mouse pre-osteoblast cell culture evidence that miR-141 and miR-200a modulate BMP-2-stimulated pre-osteoblast differentiation by targeting Dlx5, one of the osteogenic master transcriptional factors (Itoh et al., 2009). Moreover, miR-452 has been found to be the most enriched miRNA in mouse NCC and reported to regulate epithelial-mesenchymal interactions in the mandibular region of pharyngeal arch 1 (Sheehy et al., 2010). Specifically, the knockdown of miR-452 decreases Dlx2 expression in the mandibular component of pharyngeal arch 1, leading to craniofacial defects (Fig. 2) (Sheehy et al., 2010). MiR-452 targets *Wnt5a* (Table 1), which downregulates Shh signaling and indirectly promotes Dlx2 expression in the neighboring NCC-derived mesenchyme (Thomas et al., 2000). Thus, miR-452 might upregulate Dlx2 expression through this complex epithelial-mesenchymal interaction. Considering these results, it would be interesting to study the role of miRNAs in regulating the expression of Dlx homeobox transcription factors; since they are a highly conserved family between vertebrates and critical to establish the axes in pharyngeal arch 1 (Cobourne and Sharpe, 2003).

At least 170 differentially expressed miRNAs have been found by next-generation sequencing and computational annotation approaches, showing a remarkably dynamic regulation of miRNA expression during chicken, duck, and quail cranial NCC before and after species-specific facial distinctions take place (Powder et al., 2012). Data suggest that differential proliferation rates can influence the depth, width, and curvature of the beak, being those miRNAs involved in the different cellular transitions (Wu et al., 2006). More recently, a high-throughput miRNA sequencing study carried out in mouse developing-facial structures has detected hundreds of miRNAs potentially involved in craniofacial development. By *in situ* hybridizations and functional analysis, miR-23b and miR-133b have been involved in craniofacial development (Ding et al., 2016). A broadening of the ethmoid plate and aberrant cartilage structures in the viscerocranium has been observed when overexpressing miR-23b in zebrafish embryos, whereas a reduction in the ethmoid plate size and a significant midfacial cleft phenotype has been detected when overexpressing miR-133b (Fig. 2). These results suggest that both miRNAs are important for a proper craniofacial development, although they seem to act over different genes or networks. Further studies need to be performed in order to shed light on the corresponding target genes.

Dickkopf-related protein 1 (Dkk1) is an extracellular antagonist of Wnt signaling, essential for osteogenic differentiation by maintaining skeletal homeostasis. A study has demonstrated that miR-335-5p decreases *Dkk1* expression by binding to its 3′UTR in osteoblasts (Table 1), and promotes osteogenic differentiation by activating the Wnt signal pathway (Fig. 2) (Zhang et al., 2011). Transgenic mice specifically overexpressing miR-335-5p in the osteoblast lineage display higher bone mass and increased parameters of bone formation, in correlation with enhanced expression of osteogenic differentiation markers (Zhang et al., 2017). Upon osteogenic induction in these transgenic mice, Dkk1 expression is downregulated and Wnt signaling upregulated, in accordance with results from cell culture. It is worth noting that bone marrow stromal cells from transgenic mice are able to repair craniofacial bone defects by inducing osteogenic differentiation *ex vivo*, supporting the potential application of miR-335-5p-modified cells in craniofacial bone regeneration (Zhang et al., 2017).

5.2. Cardiac lineage

As was mentioned, a specific inactivation of *Dgcr8* was performed in mice NCC (Chapnik et al., 2012). Mutant *Dgcr8* mice died prenatally with major cardiovascular defects at E18.5, including persistent truncus arteriosus and ventricular septal defect. This phenotype could be attributed to apoptosis of a significant portion of cardiac NCC, causing a decrease in the pool of progenitors required for cardiac outflow tract remodeling (Chapnik et al., 2012). Although *Dicer* and *Dgcr8* mutant mice exhibit profound defects in cardiac cell differentiation, only a pair of miRNAs was found regulating NCC differentiation into smooth muscle cells (Fig. 2). The co-transcription of miR-143 and miR-145 cooperatively targets a network of transcription factors, including Klf4, Myocardin, and Elk1, promoting differentiation and repressing the proliferation of vascular smooth muscle cells *in vitro* (Cheng et al., 2009; Cordes et al., 2009). Remarkably, the introduction of miR-145, but not of miR-143, into NC stem cells is sufficient to guide specific differentiation into vascular smooth muscle cells, suggesting a role of miR-145 in smooth muscle fate (Cordes et al., 2009). Mechanistically, miR-143 targets *Elk1* (encoding an activator of vascular cell proliferation), and miR-145 targets *Myocardin* (encoding an activator of vascular cell differentiation) as well as *Klf4* and *calmodulin kinase II-D* (positive regulators of cell proliferation) (Table 1) (Cordes et al., 2009). Altogether, miR-145 and miR-143 are strongly comprised into a central transcriptional network involved in smooth muscle differentiation and proliferation, being miR-145 a critical switch in stimulating cell differentiation.

5.3. Neuronal lineage

In developing Schwann cells, Dicer expression is crucially involved in peripheral myelination both in mammalian cell culture and conditional knockout mice (Bremer et al., 2010; Pereira et al., 2010; Verrier et al., 2010; Yun et al., 2010). The cellular processes affected by specific *Dicer* deletion on Schwann cells include differentiation, myelination (initiation of myelination from the promyelinating state and myelin growth), cell survival, demyelination, and ultimately axonal integrity (Pereira et al., 2010; Verrier et al., 2010). At the molecular level, the promyelinating transcription factor Krox20 and several myelination-related genes, including *Egr2*, are strongly reduced in *Dicer* mutant cells. In contrast, the expression of genes characterizing the undifferentiated state, such as *Sox2*, *Notch1*, *Hes1*, *Jun*, and *Ccnd1* are increased, providing an additional potential basis for impaired myelination (Pereira et al., 2010; Yun et al., 2010). Similar results have been found when conditionally ablating *Dgcr8* in Schwann cells during development, which leads to a cell differentiation arrest (Lin et al., 2015). Analysis by developmental profiling, bioinformatics, and *in vitro* assays identify miR-138 as a potential repressor of *Sox2*, *Jun*, and *Ccnd1* genes (Table 1, Fig. 2) (Yun et al., 2010). Recently, it has been shown that Sox10 and Egr2 bind to an active enhancer near the mir-138-1 locus in mice Schwann cells and upregulate miR-138 expression during myelination (Lin et al., 2018). It has been hypothesized that miR-138 helps Egr2 to downregulate inhibitor myelinating factors, thus promoting the transition between undifferentiated and myelinating Schwann cells (Lin et al., 2018). By using microarrays, 225 miRNAs have been identified as differentially expressed during mouse peripheral myelination (Gokey et al., 2011). Between them, miR-138 is highly expressed throughout development and strikingly elevated in adult samples (Gokey et al., 2011), consistent with its previous reported role in repressing negative regulators of myelination (Yun et al., 2010). Moreover, authors find 9 miRNAs that are positively regulated by Sox10 transcription factor (Gokey et al., 2011). All these reports have contributing to get insight into the roles of miRNAs in the control of premyelinating development, as well as into the initiation of myelination by Schwann cells. While some important miRNA targets have begun to be identified, the developmental regulation of many of these miRNAs remains to be characterized.

The NC-derived sympathoadrenal cell lineage gives rise to sympathetic neurons and to endocrine chromaffin cells of the adrenal medulla. Recently, it has been shown that miR-124 is detectable in developing sympathetic neurons but absent in chromaffin cell precursors (Fig. 2), likely contributing to the establishment of specific neuronal features in developing sympathoadrenal cells (Shtukmaster et al., 2016). Further experimental results should be gathered to specifically address the role of miRNAs and to identify their mRNA targets during sympathoadrenal cell development.

5.4. Pigment lineage

Through deep sequencing, several hundreds of known and novel miRNAs have been detected in small RNA libraries from human cell cultures of melanoblasts, melanocytes and nevocytes, as well as from diverse melanoma cell cultures (Stark et al., 2010). Further experimental findings need to be achieved in order to shed light on the corresponding target genes. Upon melanocyte differentiation in mice, the microphtalmia-associated transcription factor Mitf, the key melanocyte transcription factor, binds and activates a conserved regulatory element located to the *Dicer* promoter region (Levy et al., 2010). *Dicer* mutant mice targeted to the melanocyte lineage are white from birth and have a profound loss of both stem cells and differentiated melanocytes (Levy et al., 2010). Cell lethality is likely due to abrogation of the Dicer-dependent processing of the pre-miRNA-17–92 cluster since miR-17–92 targets BIM, a known pro-apoptotic regulator of melanocyte survival (Levy et al., 2010).

By using a microarray screen, 10 miRNAs have been identified in the skin of alpacas showing different coat color (Zhu et al., 2010). Interestingly, an inverse relationship of relative transcripts abundance between *Mitf* and miR-25 is observed in skin samples collected from alpacas displaying diverse coat color. Although the *Mitf*-miR-25 relationship has been also validated in melanocyte cell culture, mRNA target genes have not been discovered yet (Zhu et al., 2010). Though not formerly identified in the microarray screening (Zhu et al., 2010), high levels of miR-143-5p have been detected by *in situ* hybridization in the cytoplasm of alpaca melanocytes (Ji et al., 2018). By luciferase reporter assays, it has been shown that miR-143-5p regulates *TGF-β-activated kinase 1* (*TAK1*) expression (Table 1). The overexpression of miR-143-5p in alpaca melanocytes decreases TAK1 expression, increases melanocyte migration and proliferation, and diminishes Mitf levels (Fig. 2) (Ji et al., 2018). Altogether, results suggest that different miRNAs are involved in controlling Mitf levels, producing the wide spectrum of alpaca coat color.

In another study, 16 differentially expressed miRNAs have been detected in mouse melanocytes stimulated with solar-simulated UV and forskolin, a known stimulator of the cAMP pathway. In particular, miR-145 overexpression causes a reduction in the protein levels of the

pigmentation enzymes Tyr, Myo5a, Rab27a, and Fscn1 (Table 1, Fig. 2). Furthermore, downregulation of miR-145 results in an increased level of the same enzymes, highlighting the importance of miR-145 during the pigmentation process (Dynoodt et al., 2012).

In tilapia and common carp fish, 13 miRNAs are differentially expressed between red and white skin (Yan et al., 2013). Bioinformatics analysis and luciferase reporter assays show that miR-429 directly regulates *FoxD3* expression by targeting its 3′UTR (Table 1). The daily injection of an antagomiR for miR-429, leads to an increase of *FoxD3* levels and a repression of *Mitf* transcription with the consequent downregulation of the enzyme melanogenic genes (Fig. 2) (Yan et al., 2013). This work provides clear evidence regarding the role of miRNA in the determination of skin color in fish.

MiR-211 has been associated with melanoma proliferation and invasiveness by promoting pigmentation in mouse melanoblasts and melanocytes (Dai et al., 2015). By using immortalized cell lines and primary NCC explants, it has been shown that miR-211 expression is regulated by Mitf and controls pigmentation by targeting TGF-ß receptor 2 (Table 1, Fig. 2) (Dai et al., 2015), involved in pigmentation by diminishing Pax3 and Mitf levels (Yang et al., 2008). Apparently, miR-211 influences the balance between Mitf-mediated pro-melanogenic and TGF-ß receptor 2-mediated anti-melanogenic pathways (Dai et al., 2015). Altogether, pigmentation process seems to be regulated by the master transcription factor Mitf, which leads to miRNAs maturation by activating Dicer. Finally, a diverse set of mature miRNAs will contribute to fine-tune Mitf as well as other factors allowing melanocyte survival, differentiation, and also giving a wide array of color pattern.

6. Conclusions

This review gathers major information regarding the participation of miRNAs in different stages of NC development, connecting elucidated miRNAs and potential targets whose disruption might cause malformations or deformities in NC derivatives. Figures and Table 1 summarizes all the reports for miRNAs and their experimentally corroborated targets. Interestingly, NCC identity is intimately coupled to the expression of miRNAs at all steps of its development. However, much more evidence of miRNA action has been collected from the NC differentiation stage (Table 1 and Fig. 2). This observation is in accordance with results from NC-specific *Dicer* mutants, where the initial steps of specification and migration are not affected, but massive cell death and broad loss of NCC derivatives have been observed. As a consequence, differentiation effector factors, like SoxE proteins, influence the expression of interactive partners, either directly or *via* miRNAs. These interactions induce regulatory pathways that allow, for example, Sox proteins not only to maintain cells in a developmental stage, but also to promote transition from one state to the next one during lineage progression. Altogether, miRNAs may promote NC differentiation through targeting genes that function in commitment, differentiation, and/or signaling. Although some miRNAs-targets have not been identified yet, future research revealing the role of these molecules during NC development will be extremely valuable to adjust a complete GRN.

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