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The Dlx5 and Foxg1 transcription factors, linked via miRNA-9 and -200, are required for the development of the olfactory and GnRH system

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ABSTRACT

During neuronal development and maturation, microRNAs (miRs) play diverse functions ranging from early patterning, proliferation and commitment to differentiation, survival, homeostasis, activity and plasticity of more mature and adult neurons. The role of miRs in the differentiation of olfactory receptor neurons (ORNs) is emerging from the conditional inactivation of *Dicer* in immature ORN, and the depletion of all mature miRs in this system. Here, we identify specific miRs involved in olfactory development, by focusing on mice null for *Dlx5*, a homeogene essential for both ORN differentiation and axon guidance and connectivity. Analysis of miR expression in *Dlx5*$^{-/-}$ olfactory epithelium pointed to reduced levels of *miR-9*, *miR-376a* and four *miRs* of the *-200* class in the absence of *Dlx5*. To functionally examine the role of these miRs, we depleted *miR-9* and *miR-200* class in reporter zebrafish embryos and observed delayed ORN differentiation, altered axonal trajectory/targeting, and altered genesis and position of olfactory-associated GnRH neurons, i.e. a phenotype known as Kallmann syndrome in humans. *miR-9* and *miR-200*-class negatively control *Foxg1* mRNA, a fork-head transcription factor essential for development of the olfactory epithelium and of the forebrain, known to maintain progenitors in a stem state. Increased levels of *z-foxg1* mRNA resulted in delayed ORN differentiation and altered axon trajectory, in zebrafish embryos. This work describes for the first time the role of specific miR (-9 and -200) in olfactory/GnRH development, and uncovers a Dlx5–Foxg1 regulation whose alteration affects receptor neuron differentiation, axonal targeting, GnRH neuron development, the hallmarks of the Kallmann syndrome.

1. Introduction

During mammalian embryonic development, olfactory receptor neurons (ORNs) are specified and differentiate within two distinct neuroepithelial regions: the main olfactory epithelium (OE) and the Vomero-Nasal (VN) epithelium. Although similar, OE- and VN-derived mature neurons express distinct classes of odorant receptors and carry out different odour-transducing functions. Such molecular and physiological distinctions are apparently maintained in all vertebrates.

During their early differentiation, immature ORNs extend their axons to reach the anterior forebrain and contact projection neurons. In the mouse embryo, overt axonal extension begins around E9–E10, and is accompanied by pools of migratory cells including the GnRH neurons, which reach the olfactory bulbs (OB) around E12–E13 and subsequently reach their final destination in the medial preoptic area and other areas of the hypothalamus (Astic et al., 1998; Cariboni et al., 2007; Forni et al., 2011; Tarozzo et al., 1995; Whitlock et al., 2006; Wray et al., 1989). Defects in olfactory development and GnRH neuron migration are thought to be the primary cause of the congenital disorder known as Kallmann syndrome (KS); this notion is supported by various mutant mouse phenotypes (see below) and by the observation of a single human foetus affected by KS (Schwanzel-Fukuda and Pfaff, 2002). KS is characterized by central hypogonadotropic hypogonadism (CHH) combined with a varying degree of anosmia and other disturbancies. Several protein-coding genes are known to be mutated in KS and/or in normosmic CHH (nCHH) patients, including *KAL1*, *FGFR1*, *FGF8*, *PROK-*

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2, *PROKR-2*, *Kiss1R/GPR54*, *NELF*, *CHD7*, *GnRH-R*, *GnRH-R*, *HS6ST1*, *TAC3*, *TACR3*, *SOX10*, *SEMA3a* and 5 members of the "FGF8-synexpressome" (Bonomi et al., 2012; Cadman et al., 2007; Cariboni and Maggi, 2006; Dode and Hardelin, 2009; Hardelin and Dode, 2008; Hu et al., 2003; Miraoui et al., 2013; Semple and Topaloglu, 2010; Topaloglu and Kotan, 2010). However, mutations in these genes account for less than 40% of the cases. It is expected, therefore, that more KS and CHH disease genes remain to be identified. Likewise, several mutant mouse strains display a KS-like phenotype (Berghard et al., 2012; Cariboni et al., 2011; Corradi et al., 2003; Hanchate et al., 2012; Hardelin and Dode, 2008; Hirata et al., 2006; Ikeda et al., 2007; Laub et al., 2006; Levi et al., 2003; Long et al., 2003; Matsumoto et al., 2006; Merlo et al., 2007; Ng et al., 2005; Shimizu and Hibi, 2009; Watanabe et al., 2009; Yoshida et al., 1997), but these all represent loss-of-function mutations in protein-coding genes.

It is increasingly being recognized that biological processes are governed by complex regulatory modules and networks of molecular interactors, rather than simplistically by individual genes with individual functions. In these networks, non-coding RNAs (miR, lncRNAs, lincRNAs, anti-sense RNAs and pseudogenes) play an important role (Arora et al., 2013; Esteller, 2011; Konopka, 2011; Mayanil, 2013; Ng et al., 2013; O'Brien et al., 2012; Salmena et al., 2011; Satoh, 2012; Schonrock et al., 2012). Thus, it is conceivable that mutations or misexpression of non-coding RNAs could participate in the molecular pathogenesis of KS/nCHH. Gaining knowledge on the RNA networks and regulations underlying olfactory differentiation, neuronal connectivity and guidance would be of great importance.

MicroRNAs (miRs) represent a class of short non-coding RNAs that act as negative post-translational regulators on longer coding and non-coding RNAs (Bartel, 2004). Annealing of complementary sequences enables miR to induce degradation or inhibit translation of target mRNAs (Plasterk, 2006). The neuronal functions of miR range from patterning and cell differentiation during embryonic development to physiology of more mature and adult neurons, including their survival, homeostasis, activity and plasticity (Agostini et al., 2011; Aranha et al., 2011; Bian and Sun, 2011; Brett et al., 2011; Fiore et al., 2011; Gao, 2010; Gaughwin et al., 2011; Li et al., 2011; Luikart et al., 2011; Olde Loohuis et al., 2012; Shi et al., 2010). More specifically, a role of miRs in the development of sensory neurons, including olfactory sensory neurons, is beginning to emerge. In *Drosophila*, *miR-7* has been implicated in the differentiation of photoreceptor cells via regulation of the EGF receptor signalling (Li and Carthew, 2005). In *Caenorhabditis elegans* *miR-273* and *lsy-6* have been shown to be required for asymmetric expression of taste receptors in chemosensory neurons (Chang et al., 2004; Johnston and Hobert, 2003). In *Danio rerio* (zebrafish) the *miR-200*-class is required for the proliferation, differentiation and survival of ORNs (Choi et al., 2008). In *Xenopus laevis miR-124* regulates changes in the sensitivity of retinal ganglion cells' growth cones to the guidance signal SEMA3A (Baudet et al., 2011), implicated in the pathogenesis of KS (Cariboni et al., 2011; Hanchate et al., 2012). In the mouse, the conditional disruption of *Dicer* in the developing olfactory system results in impaired ORN differentiation and reduced survival (Choi et al., 2008), indicating that mature miRs are required for these processes; however, without revealing their identity.

Since the activity of single miR is context- and time-specific, their functions should be examined within these contexts. With this in mind we generated high-throughput data from the developing olfactory system, focusing on the *Dlx5* homeogene: its targeted inactivation leads to a fully penetrant KS-related defects consisting in delayed ORN differentiation, impaired axonal connectivity and failure of GnRH neurons to reach the forebrain (Levi et al., 2003; Long et al., 2003; Merlo et al., 2007). We screened for miR expression in ORNs, comparing wild-type vs *Dlx5* mutant tissues, and identified *miR-9* and *miR 200*-class as the molecular link between Dlx5 and Foxg1. Using reporter zebrafish strains to visualize the embryonic olfactory axons (Miyasaka et al., 2005; Sato et al., 2005; Yoshida et al., 2002) or the GnRH + neurons (Abraham et al., 2008, 2009, 2010), we show that *miR-9* and *miR-200*-class play a role in ORN differentiation and axonal organization. We also show that these miRs are required for early GnRH neuron genesis and position. Thus we have identified a novel miR-based regulation that participates in the control of olfactory development, axon connectivity and GnRH neuron development.

2. Materials and method

2.1. Dlx5 mutant mouse strain

All procedures using mice were approved by the Ethical Committee of the University of Torino, and by the Italian Ministry of Health. Mice with targeted disruption of *Dlx5* have been previously reported (Acampora et al., 1999). The null allele, denominated $Dlx5^{lacZ}$, allows for detection of the *Dlx5*-expressing cells by staining for β-galactosidase (β-gal) expression. The olfactory phenotype has been previously characterized (Levi et al., 2003; Long et al., 2003; Merlo et al., 2007). $Dlx5^{+/-}$ (heterozygous) males and females were crossed, and generated the expected Mendelian ratios of embryos with genotype WT, $Dlx5^{+/-}$ and $Dlx5^{-/-}$. Pregnant females were sacrificed at the chosen embryonic age by cervical dislocation.

2.2. Collection of embryonic olfactory epithelia and RNA extraction

Embryos were collected clean of extra-embryonic tissues (used for genotyping) by manual dissection, transferred in RNAse-free PBS, and further dissected to separate the head. This was then included in 3% low-melting agarose (Sigma-Aldrich) in PBS, let harden and vibratome-sectioned at 250 μm thickness. Sections were transferred in RNase-free PBS, and manually dissected with fine pins to collect the OE or the VNO epithelia. For the *Dlx5* mutant tissues, the entire epithelial lining of the nasal cavity was collected, since it is not possible to discriminate the olfactory vs. the respiratory epithelium. The excised tissues were individually collected in RNA-later (Life Technologies AM7020) and stored at -20 until extraction. The collected tissues with the same genotype were pooled into three samples, used to extract total RNA with the TRIzol reagent (Life Technologies), following the manufacturer's instructions. Correct pooling was further verified by RT-PCR for the *Dlx5* mRNA. RNA samples were quantified using a NanoDrop1000 spectrophotometer (Nanodrop Technologies, Inc.), and analysed by capillary electrophoresis on an Agilent Bioanalyzer. Only samples showing an RNA Integrity Number > 5 were further processed. The quality of small RNAs was further assayed by using a small RNA Chip Bioanalyzer (Agilent).

2.3. miR profiling and data analysis

RNA samples were labelled using the one-colour method, with the miRNA Complete Labelling and Hybridization kit (Agilent). For the profiling, the mouse 8x15K arrays were used (Agilent) on a mouse V2 microarray platform. These arrays comprise probes for 627 mature mouse miRs and 39 viral miRs. Data were extracted using conventional spot-recognition and significantly-above-background tools; the signal intensity was normalized across samples using the Lowess cyclic normalization algorithm. Differentially expressed miRs (DEM) were detected using the SAM two-class unpaired statistical tool, using a FDR = 5%. Of the 627 mouse miR probes present on the arrays, 118 miRNA were found to be expressed significantly above the background.

2.4. Softwares and databases used

For preliminary Gene Ontology (GO) analyses we used DAVID (http://david.abcc.ncifcrf.gov/) and KEGG (http://www.genome.jp/kegg/pathway.html). For improved categorization and visualization, we used ClueGO (Bindea et al., 2009). To examine the embryonic

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