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CNBP modulates the transcription of Wnt signaling pathway components

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ABSTRACT

Background: Cellular nucleic acid binding protein (CNBP) is a small and highly conserved protein with nucleic acid chaperone activity that binds single-stranded nucleic acids. Data collected so far suggests that CNBP is required for proper craniofacial development. Despite the advances achieved in the last decade, the identity of the molecular targets of CNBP responsible for its role in rostral head development remains elusive.
Methods: In this work we used the CNBP single-stranded DNA-consensus binding sequence to find out putative CNBP target genes present in the human, mouse, chicken, *Xenopus* and zebrafish genomes.
Results: Most of the identified genes are associated with embryonic developmental processes, being three of them (*cdk14*, *ptk7* and *tcf7l2*) members of the Wnt signaling pathway. This finding, along with previous one showing that CNBP down-regulates the transcription of *Wnt5*, aimed our work to address the role of CNBP on the WNT signaling players and pathway regulation. Experiments carried out in zebrafish developing embryos revealed that craniofacial morphology was more adversely affected as CNBP abundance decreased. Furthermore, we observed that CNBP up-regulated in a dose-dependent fashion the transcription of *cdk14*, *ptk7* and *tcf7l2*, which in turn was reflected in *c-myc*, *ccnd1* and *axin2* expression.
Conclusions: Results reveal a role of CNBP in transcriptional control of components of the Wnt signaling pathway, which might explain its requirement for proper craniofacial development.

1. Introduction

The face is the anatomical feature which is truly unique to each human, though the basis of its general development is identical for all humans and similar to other species. Apparently, the wide-spectrum of facial shapes depends on distant-acting enhancers that fine-tune craniofacial morphology. More than 4000 candidate enhancer sequences predicted to be active in developing craniofacial complex were identified. One of them is an enhancer sequence upstream the *cnbp* transcription start site [1].

Cnbp codes for the cellular nucleic acid binding protein (CNBP[1]), also called zinc-finger protein 9 (ZNF9), a single-stranded nucleic acid-binding protein strikingly conserved among vertebrates [2,3]. CNBP binds single-stranded nucleic acid and acts as nucleic acid chaperone fine-tuning transcriptional processes throughout chromatin remodeling, which finally modulates the action of specific trans-acting factors [3]. Retroviral insertional mutation of CNBP resulted in anterior patterning and craniofacial defects in mice. Heterozygous mutant mice display short snout, smaller lower jaw and reduced or lacking eyes. Homozygous mutants lack rostral head structures, including the entire forebrain and are smaller than their wild-type littermates [4]. Similar data were observed in chicken [5] and zebrafish [6]. Although zebrafish CNBP morpholino-knockdown embryos (morphants) showed severe defects in brain morphology at 24 hpf, early anterior–posterior brain gene patterning was normal [6]. No differences were observed in the expression patterns for telencephalon, forebrain, midbrain, midbrain–hindbrain border (mhb), rhombomeres 3 and 5, and hindbrain typical marker genes in morphants from late segmentation to early pharyngula stages [6]. Further researches showed that CNBP mediates skeletogenic but not non-skeletogenic cranial neural crest (CNC) [7], a population of cells that migrates dorsolaterally to produce the craniofacial mesenchyme which differentiates into the cartilage, bone, odontoblasts of the tooth primordia, and the bones of middle ear and jaw [8,9]. How CNBP fulfills its biological function in CNC development remains elusive; likely, because of the scant information available on its molecular targets. None of the genes typically related to the development of the CNC were found out as potential targets of CNBP in a one-hybrid

Abbreviations: CNBP, cellular nucleic acid binding protein; ZNF9, zinc-finger protein 9; CNC, cranial neural crest; CNBP-CBS, CNBP DNA-consensus binding sequence; *cdk14*, cyclin-dependent kinase 14; *tcf7l2*, T-cell specific, HMG-box transcription factor 7-like 2; *ptk7*, protein tyrosine kinase 7; MEME, multiple EM for motif elicitation; MAST, motif alignment and search tool; GO, gene ontology; hpf, hours post-fertilization; BiNGO, biological networks gene ontology; MO, morpholino; dpf, days post-fertilization; eGFP, enhanced green fluorescent protein; PFA, paraformaldehyde; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription and real-time quantitative PCR; *ef1α*, eukaryotic translation elongation factor 1 alpha; *rpl13α*, ribosomal protein L13 alpha; ChIP, chromatin immuno precipitation; *actb2*, actin beta 2; WISH, whole-mount in situ hybridization; *myca*, myelocytomatosis oncogene a; *ccnd1*, cyclin D1; *sp5l*, Sp5 transcription factor-like

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assay in yeast. However, this assay, followed by in silico studies and in vivo analyses on zebrafish developing embryos, revealed that CNBP modulates *tbx2b*, *smarca5*, and *wnt5b* transcription [10]. A CNBP DNA-consensus binding sequence (CNBP–CBS) consisting of a single-stranded 14-nucleotide motif comprised by a duplication of a central core of five guanosines flanked by one adenine residue was also retrieved from the mentioned one-hybrid assay [10].

Wnt5 belongs to the large Wnt family of secreted glycoproteins acting as short or long range signaling molecules. To trigger a cellular response and to activate intracellular signal transduction, Wnt proteins bind to receptors of the Frizzled family and several co-receptors such as Lipoprotein Receptor-related Protein (LRP)-5, LRP-6, Related to tyrosine kinase (Ryk), Receptor tyrosine kinase-like Orphan Receptor (ROR) or protein tyrosine kinase 7 (PTK7) [11]. In certain cases, the signal transducing capability depends on the phosphorylation of these co-receptors [12]. Based on different biological readouts, Wnt ligands, as well as Frizzled receptors, Wnt signaling pathways have been characterized in canonical and non-canonical pathways. However, the term 'Wnt signaling' does not imply a single-purpose signal transduction system. Rather, it refers to a diverse collection of signals triggered by Wnt ligand–receptor interactions that direct cell behavior in multiple ways: cell polarity, movement, proliferation, differentiation, survival and self-renewal. A large body of evidence gathered during the past few years supports a crucial role for Wnt signaling in multiple steps of CNC formation. These steps include induction, maintenance of presumptive CNC cell fate, proliferation of progenitors, both proliferation and specification of differentiated cell types, as well as cell delamination and migration of cells [13–15].

In this work, we used the identified CNBP–CBS for finding out novel and conserved transcriptional targets of CNBP likely involved in vertebrate developmental processes. Bioinformatics analyses and in vivo studies performed in zebrafish developing embryos revealed that CNBP affects the transcription of *tcf7l2* (formerly *tcf4*), *ptk7* and *cdk14* (formerly *pftk1*), three genes involved in Wnt signaling pathways [16–19]. This finding along with a previous one showing a transcriptional control of *wnt5* by CNBP [10] led us to suggest that this protein plays a role in modulating the Wnt signaling in vertebrates, with possible consequences on the 'fine-tuning' of rostral head development.

2. Material and methods

2.1. Promoters sequence retrieval

Promoter regions were arbitrarily defined as the region spanning 1 kbp upstream from reported transcription start sites. Promoter sequences were downloaded using Ensembl Biomart tool (http://www.ensembl.org/biomart/martview) and genome versions GRCh37.p10 (*Homo sapiens*), GRCm38.p1 (*Mus musculus*), WASHUC2 (*Gallus gallus*), JGI4.2 (*Xenopus tropicalis*), and Zv9 (*Danio rerio*).

2.2. Searching for putative CNBP binding-sites inside promoters

Putative CNBP-binding sites were searched in sequences using MEME/MAST (Motif Alignment and Search Tool) [20] and the CNBP–CBS as reported elsewhere [10]. Parameters were: mast meme.xml mart_export.txt -oc. -nostatus -remcorr -ev 10 -mev 1e-05. Only those sequences with E-value $\leq 1e^{-05}$ were kept for further studies.

2.3. Gene data analysis

Functional information of the identified genes was obtained from Pubmed (http://www.ncbi.nlm.nih.gov/pubmed). Gene Ontology (GO) data and GO term enrichment was analyzed with BiNGO plugin using Cytoscape v2.8 (http://www.cytoscape.org/) [21]. GO term statistical significance was analyzed using a hypergeometric test and a Benjamini & Hochberg false discovery rate correction (FDR). Gene list intersection was made using BioVenn (http://www.cmbi.ru.nl/cdd/biovenn/) [22].

2.4. Animal handling and ethics statement

This study was carried out in strict accordance with relevant national and international guidelines. Protocols were approved by the Committee on the Ethics of Animal Experiments of the Facultad de Cs. Bioquímicas y Farmacéuticas — Universidad Nacional de Rosario, which had been accepted by the Ministerio de Salud de la Nación Argentina (http://www.saludinvestiga.org.ar/comites.asp?num_prov=13); Expedient No. 6060/132; Resolution No. 298/2012.

2.5. Fish and embryo rearing

Adult zebrafish were maintained at 28 °C on a 14 h light/10 h dark cycle as previously described [34]. Embryos were staged according to development in hours post-fertilization (hpf) at 28 °C [20].

2.6. Microinjection of zebrafish embryos

Embryos were obtained by natural mating and injected at the one-cell stage into the yolk immediately below the blastomeres using a gas-driven microinjection apparatus (MPPI-2 Pressure Injector, Applied Scientific Instrumentation; Eugene, OR, USA). Embryos were injected with 5 nL of 0.4 μg/μL Spl-MO or mis-MO solutions prepared in Danieau 1× as described elsewhere [7,10]. Injection of capped-mRNA coding for the wild type zebrafish CNBP fused to the eGFP was performed as previously described [6].

2.7. Cartilage staining and image analysis

Four days post-fertilization (dpf) larvae were fixed for 24 h in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline 1× (PBS) containing 0.1% (v/v) Tween-20 (PBT 1×). Larvae were then washed in PBT 1× four times and stained as described elsewhere [23]. Morphant and control embryos were observed with a MVX10 Olympus Microscope and recorded with a MVXTV1XC Olympus digital camera. Quantitative parameters were determined using the ImageJ software [24,25].

2.8. Primer design

All oligonucleotides used in this study (Supplementary Table 1) were purchased from GenBiotech (http://www.genbiotech.com.ar/). Specific oligonucleotide primers for each gene under study were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and their specificity checked using MFE primer 2.0 (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/).

2.9. Reverse transcription and real-time quantitative PCR (RT-qPCR) assays

Total RNA from 24-hpf embryos was obtained using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. Purified RNA was incubated with RQ1 DNAse (Promega) and oligo dT retrotranscribed with MMLV reverse transcriptase (Promega). Quantification reactions were performed using three different RNA purifications from three independent microinjection experiments using an Eppendorf Realplex2 apparatus and SYBR green I (Invitrogen) chemistry. Each reaction tube (20 μL) consisted of 0.5× SYBR green I, 0.2 μM of each primer (Supplementary Table 1), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U Platinum Taq DNA polymerase (Invitrogen) and 2 μL of template/negative controls. Templates were 1:20 diluted cDNA samples. After an initial denaturation step (94 °C for 5 min), 40 amplification cycles were performed, with each cycle consisting of a denaturing step of 20 s at 94 °C, an annealing step of 30 s at 63 °C and an extension step of 30 s at 68 °C, and a final extension step of 10 min at 68 ° C. *Ef1α*