Developmental Biology 390 (2014) 273–287

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Genomes and Developmental Control

Auto and cross regulatory elements control *Onecut* expression in the ascidian nervous system

Maria Rosa Pezzotti [a,*], Annamaria Locascio [a], Claudia Racioppi [a], Laura Fucci [b], Margherita Branno [a,*]

[a] Cellular and Developmental Biology Department, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy
[b] Biology Department, University of Naples Federico II, Via Cinthia, 80126 Napoli, Italy

ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form
28 February 2014
Accepted 19 March 2014
Available online 26 March 2014

Keywords:
Onecut enhancer
Photoreceptor cells
Nervous system
Ciona intestinalis
Neurogenin

ABSTRACT

The expression pattern of *Onecut* genes in the central and peripheral nervous systems is highly conserved in invertebrates and vertebrates but the regulatory networks in which they are involved are still largely unknown. The presence of three gene copies in vertebrates has revealed the functional roles of the *Onecut* genes in liver, pancreas and some populations of motor neurons. Urochordates have only one *Onecut* gene and are the closest living relatives of vertebrates and thus represent a good model system to understand its regulatory network and involvement in nervous system formation. In order to define the *Onecut* genetic cascade, we extensively characterized the *Onecut* upstream *cis*-regulatory DNA in the ascidian *Ciona intestinalis*. Electroporation experiments using a 2.5 kb genomic fragment and of a series of deletion constructs identified a small region of 262 bp able to reproduce most of the *Onecut* expression profile during embryonic development. Further analyses, both bioinformatic and *in vivo* using transient transgenes, permitted the identification of transcription factors responsible for *Onecut* endogenous expression. We provide evidence that Neurogenin is a direct activator of *Onecut* and that an autoregulatory loop is responsible for the maintenance of its expression. Furthermore, for the first time we propose the existence of a direct connection among Neurogenin, Onecut and Rx transcription factors in photoreceptor cell formation.

Introduction

Onecut is a transcription factor belonging to the class of homeoproteins containing a Cut domain. This domain, consisting of about 70 amino acid residues, was discovered in *Drosophila* (Johnston et al., 1998) and has properties very different from classical homeodomains, attributable to the sequence divergence at the level of the third helix responsible for DNA binding (Catt et al., 1999; Iyaguchi et al., 2006; Lannoy et al., 1998; Lemaigre et al., 1996). Onecut proteins have been identified in both invertebrates and vertebrates where different numbers of gene copies have been isolated. In particular, except for *Caenorhabditis elegans* (Burglin and Cassata, 2002; Lannoy et al., 1998), a single gene copy is present in almost all invertebrates and lower chordates, for example the fruit fly *Drosophila melanogaster* (Nguyen et al., 2000), the sea urchin *Strongylocentrotus purpuratus* (Otim et al., 2004; Poustka et al., 2004) and the ascidian *Ciona intestinalis* (http://www.aniseed.cnrs.fr, D'Aniello et al., 2011). During chordate evolution this gene duplicated, leading to the presence of three homologs (OC1/HNF6, OC2 and OC3) in vertebrates (humans: (Jacquemin et al., 2001); mice (Vanhorenbeeck et al., 2002); zebrafish *Danio rerio* (Matthews et al., 2004)). This gene family encodes highly conserved transcription factors, acting as key regulators in multiple developmental processes involved in cell differentiation and morphogenesis.

In invertebrates Onecut is predominantly expressed in the nervous system. In *D. melanogaster* it has been demonstrated that D-Onecut has a direct role in the central and peripheral nervous systems and also in the formation of photoreceptors (Nguyen et al., 2000). Initially *D-Onecut* was isolated as a potential regulator of the gene coding for the rhodopsin in the photoreceptor cells (R) suggesting its involvement in the regulation of R cell differentiation during the final stages of development of the eye. The overexpression of a dominant negative form of D-Onecut specifically interferes with R cell differentiation in the eye, but not with the determination of their cell fate (Nguyen et al., 2000).

Five *Onecut* genes have been characterized in *C. elegans*, but their functions *in vivo* are unknown (Lannoy et al., 1998). The Ceh-21 and Ceh-39 factors are able to recognize the same binding site as vertebrate OC1/HNF-6, suggesting that these factors

* Corresponding authors.
E-mail addresses: mariarosa.pezzotti@szn.it (M.R. Pezzotti), margherita.branno@szn.it (M. Branno).

http://dx.doi.org/10.1016/j.ydbio.2014.03.011
0012-1606/

may play a similar role to that of OC1/HNF-6 in mammals. Another peculiar gene is *Ceh-38*, which is expressed in different tissues during development, particularly in the endoderm derivatives and in some types of neurons, similar to the Onecut genes in mammals (Cassata et al., 1998).

In the sea urchin *S. purpuratus, Sphnf6* is a maternal transcript that is distributed in a uniform manner until gastrulation, and is required for the activation of genes involved in the differentiation of primary mesenchyme cells (PMC). After gastrulation Sphnf6 participates in the regulation of ectodermal oral genes and in the formation of the neural ciliate band. The formation of the oral ectoderm and of the ciliate band are abnormal in the absence of the Sphnf6 factor (Otim et al., 2004).

A single Onecut gene has been identified in Urochordates. In two ascidian species, *Halocynthia roretzi* and *C. intestinalis*, the spatial–temporal expression profile of the *OC1*/*HNF-6* gene coding for a Onecut protein was defined. The expression of this gene begins at neurula stage and is localized in various regions of the central nervous system (CNS) until the tailbud stage and, in particular, in the sensory vesicle, visceral ganglion and some cells of the posterior nerve cord (http://www.aniseed.cnrs.fr, D'Aniello et al., 2011; Sasakura and Makabe, 2001). The role of Onecut as a transcriptional regulator has been partly described in *H. roretzi*, where the OC1/HNF-6 protein is responsible for the restriction of *Pax*258 gene expression in the regionalization of the neural tube (Sasakura and Makabe, 2001), although it is still unknown if OC1/HNF-6 directly controls *Pax*258 regulatory elements or acts *via* other intermediate genes.

Recent studies also demonstrate a functional connection between *Onecut* and *Rx* genes during the development of photosensitive structures. Double *in situ* hybridizations and transgenic experiments demonstrated that in *C. intestinalis* Onecut recognizes two *Rx* regulatory elements and functions as a direct activator of *Rx* gene expression in photoreceptor cells (D'Aniello et al., 2011).

During vertebrate evolution this gene duplicated and acquired new functions. The involvement of the vertebrate *Onecut* genes in the formation of endoderm derivatives and of various structures of the nervous system is indicated by their expression profiles and by their mutant phenotypes. In adult mice the *OC1/HNF*-6 and *OC*3 genes show common and specific territories of expression. They are both expressed in the brain, but *OC1/HNF*-6 is also specifically present in liver and pancreas while *OC*3 in intestine and stomach. Furthermore, the expression of *OC1/HNF*-6 in liver and pancreas also overlaps with that of OC2, although these two transcription factors control different target genes (Jacquemin et al., 1999; Vanhorenbeeck et al., 2002). Single or double mutant mice for *OC1/HNF*-6 and *OC*2 evidenced morphogenetic alterations during the development of the liver and pancreas (Clotman et al., 2005; Simion et al., 2010).

Concerning the neural expression of these genes it is notable that OC1/HNF-6 is expressed in the brain and different areas of the central nervous system, while OC2 and OC3 are expressed only in the brain (Rausa et al., 1997). Despite this broad neural expression, gene inactivation experiments in mice did not show evident alterations in the formation of the nervous system. Experiments performed on mice mutants for *OC*1/*HNF*-6 and/or OC2 indicate that they control a genetic program for motor neuron differentiation. Onecut factors directly control Isl1 gene expression in specific motor neuron subpopulations (Roy et al., 2012), coordinates the formation of hindlimb neuromuscular junctions (Audouard et al., 2012) and, in particular, the organization of the Purkinje cells of the cerebellum (Audouard et al., 2013). It has been recently demonstrated in mouse that OC1/HNF-6 and OC2 have very similar expression patterns throughout retinal development and they may regulate the formation of retinal ganglion cells (RGCs) and also have a function in the genesis and maintenance of horizontal cells (Wu et al., 2012).

In humans, Onecut-2 (hOC-2) is expressed in melanocytes and regulates the MITF gene (Microphthalmia-associated transcription factor), which encodes a transcription factor essential for the differentiation of melanocytes (Jacquemin et al., 2001).

Among the three *Onecut* genes identified in zebrafish there is a neural Onecut member specifically expressed only in neural cells that shows a highly dynamic expression in primary neurons of the brain and spinal cord during embryogenesis (Hong et al., 2002).

It would therefore appear that in the course of evolution, the expression profile of *OC*1/*HNF*6 has been progressively extended to new regions, indicating a possible extension of the functions of this factor. Its wide areas of expression, including the nervous system and territories arising from the endoderm, clearly indicate that this factor plays a key role in embryonic development. The tight cooperation existing among the three gene copies is suggested not only by their partly overlapping territories of expression but also by the cross-regulatory loop between *OC*1/*HNF*-6 and *OC*3. OC1/HNF-6 expression in mice is required for the activation of its ortholog OC3 (Pierreux et al., 2004). Unfortunately, Onecut factor co-expression represents a complication in terms of elucidating their specific functions and individual gene regulatory networks.

Urochordates occupy a key position in the evolutionary tree. They are located at the base of vertebrate origin before the wide genome duplication typical of vertebrates.

C. intestinalis may therefore represent a good model system to understand the Onecut genetic pathway and the function of this gene in neural development, avoiding the problem of masked gene function due to the presence of more than one gene copy. The identification of transcription factors responsible for its activation could greatly contribute to understanding *Onecut* gene regulation in more complex organisms.

Here we describe the analysis of a 2.6 kb non-coding sequence upstream of the *Ciona Onecut* gene. Using deletion analysis we identified a 262 bp region (-935 to -674 bp) able to recapitulate the *Onecut* endogenous expression pattern in transient transgenic embryos. Bioinformatic analysis indicated that there are putative Neurogenin and HNF-6/Onecut binding sites within this region of the promoter. We go on to provide evidence that Neurogenin is directly involved in *Onecut* activation and maintenance *in vivo* and that, by an autoregulatory loop, Onecut itself maintains its expression.

Our results illustrate the involvement of *Neurogenin*, *Onecut* and *Rx* in the same regulatory network controlling central nervous system development and in particular photoreceptor cell formation.

Materials and methods

Animals and embryos

Adult *C. intestinalis* was collected from the Gulf of Naples, Italy. Gametes were collected from the gonoducts of several animals and used for *in vitro* fertilization. Animal handling and transgenesis *via* electroporation have been carried out as previously described (D'Aniello et al., 2011).

Construct preparation

pBlueScript II KS 1230 (gift of R. Krumlauf, Stowers Institute, Kansas City, USA), which contains the *LacZ* reporter gene and SV40 polyadenylation sequence with the human β-globin basal promoter, was used for all the constructs containing the *Onecut* promoter fragments. All fragments were obtained by PCR using specific primers designed using sequence information from the *C. intestinalis* genome