The transcription factor chicken Scratch2 is expressed in a subset of early postmitotic neural progenitors

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**A R T I C L E   I N F O**

Article history:
Received 13 March 2012
Received in revised form 19 March 2013
Accepted 21 March 2013
Available online 6 April 2013

Keywords:
Chicken SCRT2
Neural tube
Neural differentiation
NeuroM/Ath3/NEUROD4

**A B S T R A C T**

Scratch proteins are members of the Snail superfamily which have been shown to regulate invertebrate neural development. However, in vertebrates, little is known about the function of Scratch or its relationship to other neural transcription factors. We report the cloning of chicken Scratch2 (cScr2) and describe its expression pattern in the chick embryo from HH15 through HH29. cScr2 was detected in cranial ganglia, the nasal placode and neural tube. At all stages examined, cScr2 expression is only detected within a subregion of the intermediate zone of the neural tube. In the developing dorsal root ganglia from HH22–23 onwards and becomes limited to its dorsal medial domain at HH29. Phospho-Histone H3 and BrdU-labeling revealed that the cScr2 expression domain is located immediately external to the proliferative region. In contrast, cScr2 domain overlapped almost completely with that of the postmitotic neural transcription factor NeuroM/Ath3/NEUROD4. Together, these data define cScr2-positive cells as a subset of immediately postmitotic neural progenitors. Previous data has shown that Scr2 is a repressor of E-box-driven transcription whereas NeuroM is an E-box-transactivator. In light of these data, the co-localization detected here suggests that Scr2 and NeuroM may have opposing roles during definition of neural subtypes.

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Thus, to better resolve Scratch’s position in the neural differentiation transcriptional cascade in the vertebrate embryo, we cloned the full length coding sequence of the chicken Scratch2 homolog (cScrt2) and characterized in detail its expression pattern relative to other known markers in the trunk neural tube.

1. Results and discussion

The full-length chicken orthologue of Scr2 was cloned by RT-PCR using primers designed against a partial clone previously filed in the NCBI database (XM_426994) and clones obtained with

Fig. 1. Predicted protein sequence for chicken Scrt2 (Gallus gallus; AEW43643). Alignment with mouse Scrt1 (Mus musculus; NP_570963) and Scrt2 (NP_001153882) and zebrafish Scrt1a (Danio rerio; NP_001107073), Scrt1b (NP_001014369) and Scrt2 (NP_998802) and diagram showing positions of the previously described domains (Nieto, 2002) SNAG (aa 1–8), Scratch (aa 97–116) and 5 zinc fingers (Znf) (aa 127–150, aa 160–181, aa 185–207, aa 213–235 and 241–263) in the cScrt2 predicted amino acid sequence.

Fig. 2. Developmental expression of cScrt2. (A–E) Whole mount in situ hybridizations showing cScrt2 expression in HH17 (A and D), HH19 (B and E) and HH23 (C). Dashed lines in (B) and (C) indicate sectioning plans and levels for (F–H). Arrow and arrowhead in (D) indicate positive cells in hindbrain and nasal placode, respectively. Roman numerals in (E) indicate cranial ganglia that express cScrt2. (F) Section of HH19 embryo subjected to whole mount in situ hybridization showing expression in the trigeminal ganglion (arrowhead); arrow indicates the hindbrain. (G–I) In situ hybridization in trunk spinal cord cross sections showing cScrt2 expression in HH19 (G), HH23 (H) and HH29 (I). drg: dorsal root ganglia.

Thus, to better resolve Scratch’s position in the neural differentiation transcriptional cascade in the vertebrate embryo, we cloned the full length coding sequence of the chicken Scratch2 homolog (cScrt2) and characterized in detail its expression pattern relative to other known markers in the trunk neural tube.
RACE-PCR. The resulting full-length clone has 831 nucleotides (JN982016) and encodes for 276 amino acids. This complete coding sequence (CDS) has 99% identity (823/831) with a previously deposited cDNA sequence (FJ620691). The divergence between the two sequences is concentrated in the first 21 bases. Because the sequence of the primers we used for cloning the CDS were based in the sequencing of clones obtained with RACE-PCR that included both the 5' UTR and the 5' CDS in the same molecule, it is possible that the divergence with the previously deposited CDS results from alternative cloning procedures for the latter. To confirm the identity of our clone inside the Scratch family, we aligned the predicted translation product with other chordate Scratch orthologues. In the resulting phylogenetic tree, the clone grouped with the mammalian Scrt2 orthologues supported by the maximum bootstrap value (100%; data not shown), thus confirming the identity of the cloned gene as the chicken orthologue of \textit{Scrt2} (cScrt2).

The full sequence of the predicted protein shares identities of 59% with zebrafish Scrt2 and 68% with mouse Scrt2 (Fig. 1).

To determine the temporal and spatial pattern of expression of cScrt2, we performed \textit{in situ} hybridization. We did not detect any hybridization or qPCR signal in embryos from HH5 to HH14 (data not shown). In whole mount embryos, cScrt2 expression was first detected in few cells of the hindbrain and nasal placode of HH15 embryos (data not shown). This expression domain expanded significantly by HH17 and could be clearly detected in the mesencephalon and the nasal placode (Fig. 2A and D). In stage HH19 embryos the expression domain at the mesencephalon, mesencephalon and posterior neural tube (Fig. 2B and E). In the latter region, cScrt2 expression occurred as a continuous domain concentrated in the intermediate region of the trunk neural tube. This domain was thinner in the dorsal region of the neural tube and wider in ventral regions by HH19 (Fig. 2G), and this relative pattern is progressively inverted in later stages (Fig. 2H and I). In addition, we observed clear staining in the cranial ganglia (Fig. 2E). cScrt2 expression in the trigeminal ganglia was more widespread and weaker than in the neural tube (Fig. 2F). At HH23, subtle staining was detected in the prospective dorsal root ganglia (DRG) (Fig. 2C and H). cScrt2 expression in the DRG was stronger in E6 (embryonic day 6; HH28–29) embryos and was clearly enriched in the dorsomedial domain (Fig. 2I).

Our \textit{in situ} hybridization results differ slightly from the pattern reported for the mouse Scrt2 orthologue. Two forms of Scratch have been identified in mouse: mScrt1 and mScrt2. Here, we show that cScrt2 expression is uninterrupted throughout the dorsoventral axis. In contrast, mScrt2 is absent from a small region of the dorsoventral axis, corresponding to the V2 interneuron column, whereas mScrt1 expression domain is continuous (Marín and Nieto, 2006). Thus, in this aspect, cScrt2 expression pattern is more similar to mScrt1 than to mScrt2, and may reflect a form of paralogue exchange across species.

On the other hand, cScrt2 and both forms of mouse Scrt are excluded from the ventricular zone of the posterior neural tube suggesting that all are expressed in the early postmitotic progenitor cells. Furthermore, the ventral to dorsal wave of expression of cScrt2 in the trunk neural tube recapitulates the reported overall cell pattern of proliferation in the neural tube, which stops ventrally on embryonic day 5 (E5) and continues dorsally until E8 (Langman and Haden, 1970). Indeed, in the mouse anterior neural tube labeling for PCNA (Proliferating Cell Nuclear Antigen) and mScrt2 are mutually exclusive (Marín and Nieto, 2006).
To clarify the relationship between cScrt2 and regions of cell proliferation in the chick, we investigated the expression domain of cScrt2 relative to the ventricular proliferative zone by double-labeling for cScrt2 expression and BrdU-incorporation or the presence of phospho-Histone H3 (pHH3) as markers for S and M phase, respectively. As expected, cScrt2 expression domain in the chick embryo does not contain mitotic cells either in the posterior or anterior neural tube (Fig. 3 and data not shown). Rather, cScrt2 is expressed in a region immediately external to the zone harboring S-phase cells in the proliferation zone. Therefore, as in other vertebrates, cScrt2 is also associated with postmitotic neural progenitors.

In various developing neural tissues, the homeobox gene Islet1 is one of the earliest markers for post-mitotic neuroblasts (Ericson et al., 1992; Avivi and Goldstein, 1999). Thus, to determine if cScrt2 is indeed expressed in early post-mitotic cells, we detected cScrt2 transcripts and Islet1 protein through double-labeling. In the posterior neural tube, Islet1 is expressed in the ventral-most regions of the spinal cord where motor neuron progenitors differentiate (Ericson et al., 1992). The overlap between Islet1 and cScrt2 is most obvious in the medial border of the motor neuron cluster, where newborn motor neurons reside (Fig. 4A-C; Hollyday and Hamburger, 1977). Interestingly, more mature cells, located ventro-laterally, display higher levels of Islet1 and lower levels of cScrt2. In the DRG, cScrt2 expression coincides completely with that of Islet1 (Fig. 4D-F). In this tissue, Islet1 is also restricted to postmitotic cells and is one of the earliest markers for neural differentiation (Avivi and Goldstein, 1999; Cui and Goldstein, 2000). The DRG dorsal pole and perimeter are devoid of both Islet1 and cScrt2 signals (Fig. 4D-F). Interestingly, these two sites harbor most of the proliferative cells at this stage (George et al., 2010). In the trigeminal ganglion, the cScrt2 expression domain overlaps with Islet1 at the ventral-medial domain (Fig. 4G-I). However, unlike in the DRG, in this case the cScrt2 domain is significantly larger than that of Islet1 and

![Fig. 4. cScrt2 and Islet1 are coexpressed in the embryonic CNS and PNS. (A–I) Double labeling for cScrt2 by in situ hybridization (A, D, G) and immunofluorescence for Islet1 (B, E, H) showing the relationship of their expression domains in the spinal cord (A–C) and trigeminal ganglion (G–I) in HH19 and in the DRG in HH25 (D–F). Arrows in (A–C) show a site in the motorneuron region where a stronger cScrt2 signal overlaps with a weaker Islet signal. (C), (F) and (I) are merged images for each set. The yellow signal is generated from autofluorescent blood cells in the tissue.](image-url)
Fig. 5. Comparison between cScrt2 expression and markers for different stages of neurogenesis and differentiation in the trunk spinal cord. (A–J) In situ hybridization in HH25 trunk spinal cord cross sections showing expression of Notch1 (A), Sox3 (C), Ngn2 (E), NeuroM (G) and SCG10 (I) or cScrt2 combined with Notch1 (B), Sox3 (D), Ngn2 (F), NeuroM (H) or SCG10 (J). (B', D', F', H', J') are magnifications of the delimited regions in (B, D, F, H, J), respectively. (K) Summary diagram representing a possible timeline of expression for Notch1, Sox3, Ngn2, NeuroM, Scrt2, Islet1 and SCG10 during neurogenesis and differentiation in the spinal cord. The left edge corresponds to the ventricular zone while the right edge corresponds to the marginal region of the neural tube.
If Scrt2 participates in the neural differentiation program of postmitotic cells, most likely it does so in conjunction with other genes involved in the early steps of differentiation. Scrt2 is a bona fide E-box-binding repressor (Nakakura et al., 2001a; Reece-Hoyes et al., 2009; Rodríguez-Aznar and Nieto, 2011; Paul et al., 2012). It is quite well established that a variety of E-box-binding proteins heterodimerize with transcription factors to regulate gene expression (Castro et al., 2006; Powell and Jarman, 2008). To further refine the localization of cScrt2-expressing cells in the developing neural tube relative to the neural differentiation transcription factor cascade and to identify potential binding partners, we performed double in situ hybridizations in HH25 embryos with transcription factors that are involved in cell cycle and in differentiation. As expected from our data above, the proliferative marker Notch1 expression domain abuts but does not overlap with cScrt2 (Fig. 5B). cScrt2 expression domain is external to and overlaps partially with the external perimeter of the neural progenitor cell markers Sox3 and Ngn2/NEUROG2 (Fig. 5D and F and Supplementary Figure 1). However, cScrt2 expression domain overlaps considerably with that of NeuroM (Fig. 5H and Supplementary Figure 1), which is expressed in early postmitotic neural progenitors (Roztocil et al., 1997). Similarly, in the DRG, cScrt2 expression domain also coincides with that of NeuroM and Ngn2 (Fig. 6A-C).

In the murine embryonic cortex Scrt2 expression also coincides with that of Ngn2 in the apical region of the ventricular zone (Paul et al., 2012). These expression data, together with data showing that Scrt2 overexpression decreases Ngn2 expression in Xenopus neurulas (Paul et al., 2012) suggested that Scrt2 could downregulate Ngn2 expression. In light of this, we performed overexpression of loss-of-function cScrt2 in chick neural tube using a truncated cScrt2 and a fusion protein with the activator domain of VP16. Neither of the two forms expanded Ngn2 expression in chick neural tube (Supplementary Figure 2). A similar result was reported with Scrt2 gain-of-function in the mouse pallium, which also failed to alter local Ngn2 expression (Paul et al., 2012).

What then could be Scrt2 function? In the mouse, Scrt2 expression overlaps with that of neuronal differentiation marker beta-III tubulin in the pallium’s intermediate zone. Consistent with a possible role in regulating neural differentiation, gain of Scrt2 function enhances mitotic exit and increases beta-III tubulin expression in the cortex (Paul et al., 2012). On the other hand, in the chick embryo the Scrt2 expression domain does not overlap with that of the other late differentiation marker SCG10/STMN2 in the trunk neural tube (Fig. 5J and K). Furthermore, loss of cScrt2 function did not alter SCG10 expression (Supplementary Figure 2). Finally, gain of cScrt2 function also did not alter the expression of the pan-neuronal markers HuC/D and TuJ1 (data not shown). Together, these data suggest that either cScrt2 is not directly involved in the later steps of neural differentiation in the chick spinal cord or that it is involved with neuronal subtype specification, the variations of which cannot be detected by variations in pan-neuronal markers expression.

Overexpression of NeuroM in the chick embryo also does not affect the expression of pan-neuronal markers. Instead, NeuroM indirectly induces expression of GDNF receptor alpha-1 (GFRalpha1), suggesting that NeuroM biases progenitor cells towards a specific subtype (Shimada et al., 2012). The expression pattern of GFRalpha1 and that of GFRalpha4 are very similar to that of both NeuroM and cScrt2. The original report on the dynamic pattern of NeuroM expression strongly suggests that its overlap with cScrt2 occurs at other stages and tissues as well. Firstly, similar to cScrt2, NeuroM expression in the neural tube is first detected at HH17 in an intermediate domain between the ventricular zone and the outer mantle region and is excluded from BrdU-incorporating proliferating cells. Moreover, NeuroM expression follows a ventral to dorsal wave with the highest level of labeling around HH21 in the motor neuron domain, where it declines slowly and disappears in E6 embryos (Roztocil et al., 1997). In the developing DRG, NeuroM is also first detected around HH21, and becomes progressively restricted to the dorsomedial region (Roztocil et al., 1997). Finally, NeuroM is also present in the condensed trigeminal ganglia (Roztocil et al., 1997; Ohsawa et al., 2005).

Given their similarity in expression pattern, we investigated if cScrt2 regulated NeuroM expression. Reduction of cScrt2 activity did not affect NeuroM expression, suggesting that NeuroM is not under cScrt2 control (Supplementary Figure 2). Instead, it is very likely that both cScrt2 and NeuroM are regulated by the homeodomain transcription factor Brn3a. Brn3a binds directly to NeuroM enhancer locus and represses NeuroM expression (Lanier et al., 2007; Dykes et al., 2011). Likewise, an in silico search for Brn-binding motifs in the mouse genome identified Scrt2 as a possible target gene (Castro et al., 2006).
In contrast to their similarity in expression pattern, Scrt2 and NeuroM have been ascribed with opposing molecular actions. While Scrt2 represses E-box-driven expression (Nakakura et al., 2001a; Reece-Hoyes et al., 2009; Rodríguez-Aznar and Nieto, 2011; Paul et al., 2012), NeuroM transactivates E-box-containing promoters (Roztocil et al., 1997). These data, taken together with the co-expression of cScrt2 and NeuroM in a subset of neural progenitors shown here, raise the intriguing possibility that Scrt2 and NeuroM may act at similar levels but in an opposing manner in the neural differentiation hierarchy of neural subtype specification.

2. Experimental procedures

2.1. Cloning of chicken Scrt2

When we started this project, the chicken Scrt2 homologue available in the NCBI database (XM_426994) was partial and lacked a portion of the 5’ coding sequence. Thus, we cloned the Scrt2 5’ UTR and coding terminal using nested RACE-PCR to amplify this sequence from RACE cDNA libraries from HH8, HH19 and HH24 (GeneRacer; Invitrogen). Gene-specific primers used in the nested RACE-PCR were cScrt2-Rv2: GAAGTAGCGGGCGGAGAGTGGGA and cScrt2-Rv3: CCTGCTTCTTGCGGTTTGTAG.

The primers for full coding sequence were cScrt2-Fw1: CCGCGAGATCCCCGCTCTT and cScrt2-Rv1: CTAGTCTCCATTGGCA-CAGCTGTTGTTG. Expected amplicon was cloned in pGEM-T (Promega) and then subcloned in the expression vector pMES-GRP (Swartz et al., 2001). The final full-length clone was confirmed by sequencing (JN982016).

2.2. RNA in situ hybridization and immunohistochemistry

Embryos between developmental stages HH5 and HH28–29 were fixed in 4% paraformaldehyde in PBS overnight at 4 °C or BM-Purple (Roche). The following probes were used: anti-BrdU (Accurate, 1:100), anti-pHH3 (Ser10, Millipore; 1:100) and anti-Islet1 (clone 39.4DS, DSHB; 1:50). The same sections were first captured in bright field for the hybridization signal and then in fluorescent microscopy. Afterwards, the two images were overlaid to facilitate visualization of the two domains. The contrast levels in the fluorescent images were heightened for better visualization and these were overlaid using the Blend tool in Adobe Photoshop.

2.3. BrdU labeling

Chicken eggs were incubated for 5 days and 20 μl of 2 mg/ml BrdU in Ringer’s saline containing 0.05% Fast Green were injected in the space surrounding the embryo and delimited by the vitelline membrane. Eggs were closed and reincubated for 1 h. Thereafter, embryos were collected and fixed overnight in 4% PFA in PBS at 4 °C before being processed for cryosectioning. With this protocol, the BrdU-positive region was clearly distinct from the phospho-Histone H3-positive region.

Acknowledgments

We thank Dr. Shankar Srinivas for sharing the protocol for in situ hybridization in paraffin sections and Dr. Cristóvão de Albuquerque for critically reading this manuscript. This work was supported by a grant from FAPESP (2009/50544-1) and CNPq to CYL. FMV was supported by a fellowship from FAPESP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gep.2013.03.004.

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