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Noggin4 expression during chick embryonic development

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ABSTRACT We describe here the expression pattern of *Noggin4* during the early development of the chick embryo *(Gallus gallus)*. The expression of this gene starts with the onset of gastrulation (stage HH4), in two bilateral bands along the primitive streak, with a local maximum around Hensen's node. By the end of gastrulation, *Noggin4* transcripts are distributed diffusely throughout the epiblast, with the highest concentration in the head ectoderm. Interestingly, the expression of *Noggin4* during the first half of gastrulation demonstrates a clear left-right asymmetry in Hensen's node, being much more intensive in its right anterior portion. During neurulation, *Noggin4* is expressed mainly in the neuroectoderm, with the most intensive expression in the head and lateral neural folds. In mesoderm derivatives, expression is seen in somites but not in the notochord. In general, primarily ectodermal and diffusive expression of *Noggin4* in chick embryo, with a maximum in the anterior neurectoderm, resembles that of its ortholog in *Xenopus*, which indicates a conservative function of this gene in evolution.

KEY WORDS: *Noggin, Noggin4, chick embryo*

During the last several years, at least three sub-families of *Noggin* genes, including *Noggin* (Smith and Harland, 1992), *Noggin2* (Fletcher *et al*., 2004) and *Noggin4* (Eroshkin *et al*., 2006), were identified in vertebrates. Among them, proteins of Noggin4 subfamily have primary structures very different from those of Noggin1 and Noggin2. This indicates that molecular functions of Noggin4 could be different from those of other Noggins. In support of this, the *Xenopus laevis* Noggin4, whose probable homologs were identified recently in planarians and named *Noggin-like genes* (*Nlg*) (Molina *et al*., 2009), has demonstrated no ability to induce neurogenesis in the *Xenopus laevis* embryonic ectoderm (Molina *et al*., 2011). It should be noted, however, that in contrast to the planarian Nlgs, vertebrates Noggin4 proteins have no long (50-60 amino acids) and quite conservative fragments between their 5th and 6th cysteine residues, the feature that sharply distinguishes planarian Nlgs from other Noggin family proteins (Eroshkin *et al*., 2006; Molina *et al*., 2009).

Since the expression of *Noggin4* was studied hitherto only in one vertebrate species (*Xenopus laevis*), it would be important to investigate expression of this gene in other organisms, preferably with maximally different type of embryogenesis. Such data could allow one to speculate on conservation or, conversely, variability, of Noggin4 functions during evolution. As no functional orthologs of *Noggin4* were found in mammals, we investigate now the expression pattern of *Noggin4* in chick (*Gallus gallus*), a traditional model of developmental biology, with early course of embryogenesis sharply different from amphibian but resembling that of mammals.

Results

The developmental stages of embryos were defined as described (Hamburger and Hamilton, 1992). The expression pattern of *Noggin4* was analyzed by RT-PCR and the whole mount *in situ* hybridization, beginning from Hamilton-Hamburger stage 1 (HH1) and up to HH12 stage.

A very weak expression signal was detected by RT-PCR already at HH1. Signal level gradually increased at later stages (Fig. 1). At the same time, no expression of *Noggin4* was revealed anywhere in the embryo by the whole-mount *in situ* hybridization until stage HH4 (Fig. 2 A,B). At this stage, the expression was observed along both sides of the primitive streak (PS), with the highest level of signal in the regions of Hensen's node (HN) and the posterior marginal zone (PMZ) (Fig. 2C). Interestingly, a slight left-right asymmetry of

Abbreviations used in this paper: F, foregut; HF, head fold; HH#, a developmental stage as it determined in [HAMBURGER, V. and HAMILTON, H.L. (1992); HN, Hensen's node; HP, head process; NF, neural folds; PMZ, posterior marginal zone; PN, presumptive neuroectoderm; PS, primitive treak; S, somites.

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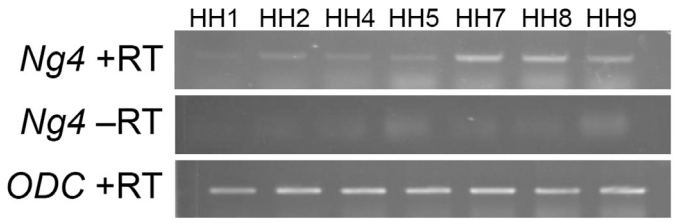

Fig. 1. Noggin 4 PCR *with or without reverse transcription (+RT or –RT respectively) of total RNA from embryos at the indicated stages with primers for* Noggin4 *and* ODC *as the loading control.*

the expression was detected at stage HH4 in HN of some embryos (Fig. 2C'). During HH5-HH6, this asymmetry can be clearly seen in all embryos (Fig. 2 D',E',F').

In the mesoderm, a weak expression of *Noggin4* was observed at stages HH5-HH6 in cells of the head process (HP). However, the expression is disappeared from HP by the time of the head fold (HF) formation at HH6+ (Fig. 2F). Beginning from this stage, *Noggin*4 expression is localized primarily in the presumptive neuroectoderm (PN) (Fig. 3A). No expression exceeding the background level was detected at this time in the notochord and in cells of surrounding mesoderm.

At HH7, the highest expression level was detected in the ectoderm of HF, and in the neural folds (NF) (Fig. 2G). By this stage, a weak expression of *Noggin4* can be also seen in the forming first somites, as well as in the un-segmented paraxial mesoderm. Simultaneously, the left-right asymmetry of the expression, which was seen before in HN, is disappeared by this stage (Fig. 2G').

As it may be seen on the transverse sections at the level of NF, the expression in the head region of embryo is localized almost exclusively in the neuroectoderm, rather than the underlying tissues (Fig. 3B). Similar localization of the expression was observed in this region at later stages (Fig. 3C). Beginning from this stage, the expression is characterized by a smooth anterior-posterior gradient, having the highest level at the rostral end of the embryonic body axis

Importantly, control hybridization of HH4 embryos with the sense *Noggin4* probe gave no signal at all (Fig. 2J). On the other hand, treatment of embryos with the probe to the homeobox gene Ganf, resulted in the distribution of hybridization signal strongly different from that generated by the anti-sense *Noggin4* probe (Fig. 2K). Obviously, both these results confirm specificity of the results obtained with the latter probe.

Discussion

The expression pattern of Noggin4 in the early development of chick embryo is generally similar to that of its *Xenopus laevis* homolog (Eroshkin *et al.*, 2006). In particular, in both species, the expression is revealed by the whole-mount *in situ* hybridization at the comparable stages (from stage HH4 and 10 in chick and *Xenopus*, respectively) primarily in PN and later in the neuroectoderm. From the very beginning, the expression is somewhat blur and tends towards wide spreading within the neuroectoderm, forming the anterior to posterior gradient. Moreover, in the mesoderm of both species *Noggin4* begins to be intensively expressed only in the forming somites, but no expression of this gene can be seen in the notochord. Thus, all this implies that functions of *Noggin4* have been retained in frog as well as chick.

Remarkably, the expression of *Noggin4* in early development appeared to be sharply different from that of two other representatives of *Noggin* family, *Noggin1* and *Noggin2*. In contrast to *Noggin4*, *Noggin1* is expressed during gastrulation exclusively in the presumptive chordamesoderm, and later on, together with *Noggin2*, in a thin stripe of cells corresponding to the presumptive dorsal forebrain (see Eroshkin *et al.*, 2006 for expression of Noggin1 and 2 in *Xenopus laevis* and Connolly *et al.*, 1997; Streit and Stern, 1999; Chapman *et al.*, 2002 and GEISHA database, University of Arizona, Tucson, AZ 85724; URL: http://geisha.arizona.edu/

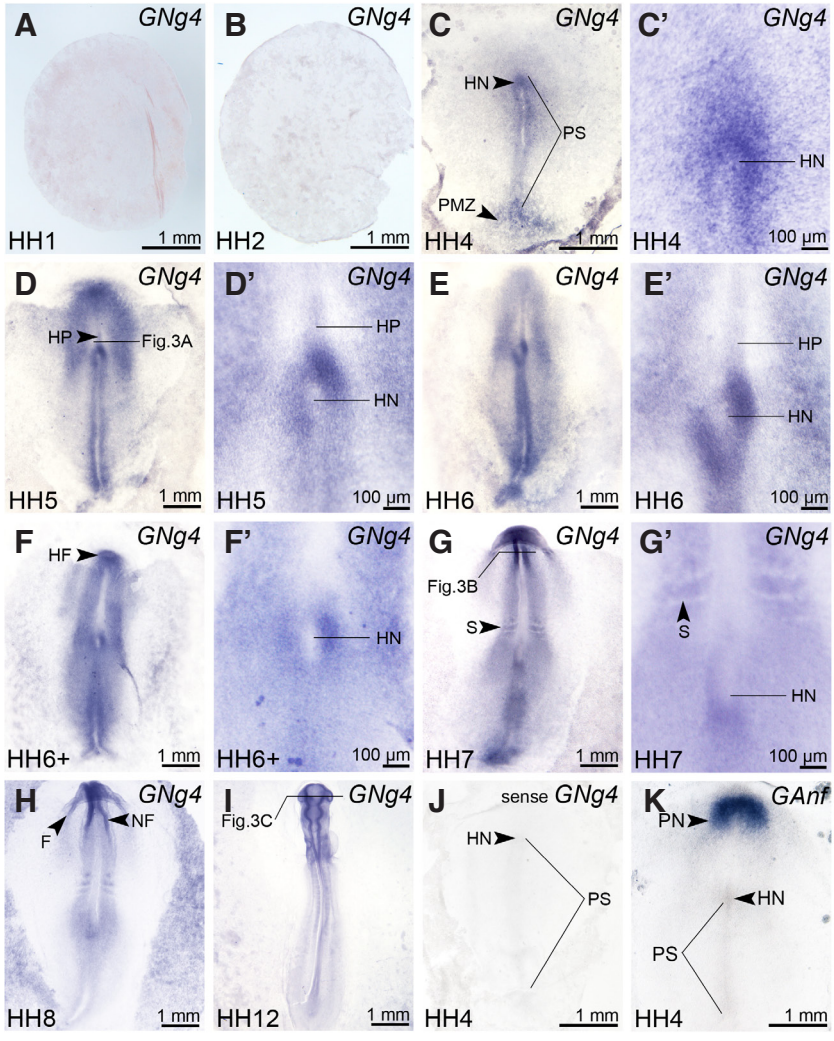

Fig. 2. Expression of *Noggin4* in chick embryos at stages HH1-HH12. *Enlarged images of Hensen's node region of embryos shown in (C-G) are presented in (C'-G'.) All embryos are shown from the dorsal side, anterior to the top. Abbreviations: F, foregut; HF, head fold; HN, Hensen's node; HP, head process; NF, neural folds; PMZ, posterior marginal zone; PS, primitive streak; S, somite.*

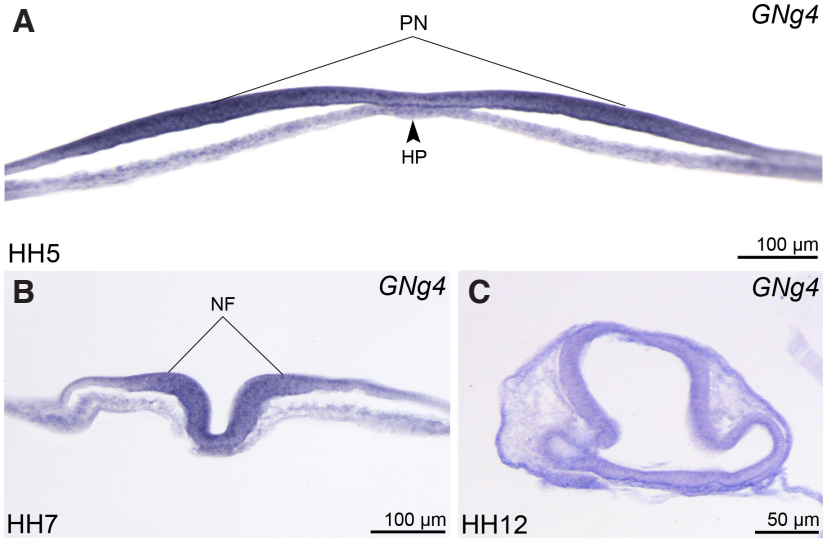

Fig. 3. Histological cross-sections of chick embryos at stages HH5 (A), HH7 (B) and HH12 (C) *at the levels indicated in Fig. 2 D,G,I. N, notochord; NF, neural folds; PN, presumptive neuroectoderm. Dorsal side to the top.*

geisha/search.jsp?entrez_gene=459378 for expression of Noggin1 in chick). This indicates that physiological, as well as molecular, function of Noggin4 might be also different from those of Noggin1 and Noggin2. In support of this, no ability to inhibit BMP signaling was reported recently for *Xenopus laevis* Noggin4 (Molina *et al.*, 2011). Instead, the authors of this paper have found out that this protein can slightly ventralize the *Xenopus* embryo by an unknown mechanism. Given all these, it would be important to test whether Noggin4 retains two other functions recently demonstrated for Noggin1 and Noggin2, namely, the ability to bind and antagonize non-BMP TGFbeta proteins and Wnt (Bayramov *et al.*, 2011).

An intriguing feature of the *Noggin4* expression pattern in chicks, which was not reported for *Noggin4* in *Xenopus*, is its asymmetrical expression in HN. Interestingly, the expression of *Noggin4* in HN at HH5 in chicks corresponds to that of two right-expressing genes – *cAct-RIIa* (Levin *et al.*, 1995) and *MID1* (Granata and Quaderi, 2003). As protein products of the latter two genes are involved in the early patterning along the left-right body axis, one may suppose that Noggin4 may also participate in the process of the left-right asymmetry establishment.

Materials and Methods

Cloning of Noggin4 cDNA and RT-PCR

Using the fact that all *Noggin* family genes contain no introns, we obtained the *Noggin4* coding sequence by PCR from *Gallus gallus* genomic DNA with the following primers designed on the base of a sequence deposited in Gene Bank (AY779059):
forward: 5'- GCACCGCTCGCCATGCAGGACCCCT;
reverse: 5'- CCCCCTCAGCGGCAGGA. The purified *Noggin4* cDNA was cloned into the *pGEM-T* vector (Promega) and sequenced.

Samples of the total RNA were prepared from the whole embryos at the appropriate stages (5 embryos for each sample) as described (Zaraisky *et al.*, 1992). PCR was performed with the following primers: 5′- GGGATGGAGCTGCCCCCTGA and 5′- CCAGCAGCTTGAGGTGAGCGA. The 25 PCR cycles were made. As the internal control the expression of *ODC* was monitored in the same samples with primers: 5′-CGGCGGAGGGTTTCGGGGTT and 5′-AGGCCCCGGACCCAGGTTACT for 18 cycles of PCR.

Whole-mount in situ hybridization

The digoxigenin-UTP-labeled anti-sense and sense RNA probes were synthesized with either T7- or SP6-RNA polymerase (Promega) respectively from DNA fragment obtained from *pGEM-T-Noggin4* plasmid by PCR with M13-direct and M13-reverse primers. The probe for Ganf mRNA was obtained as described (Kazanskaya *et al.*, 1997). Eggs were collected not later than 3 hours after oviposition. An incubation of the eggs was performed during proper time for embryos to reach certain stage. After incubation, embryos were removed from yolk, washed in PBT (phosphate-buffered saline with 0.1% Tween-20) and fixed with ice-cold MEMFA fixative (paraformaldehyde 3.7%, EGTA 2mM, MsSO4 1mM, MOPS 0.1M) at room temperature for two hours. After fixative was removed by washing in PBT, embryos were treated with 50% and 96% EtOH and stored in 96% EtOH at -20°C overnight. Embryos were rehydrate through a graded EtOH series, rinsed triply in PBT and digested in Proteinase K (10 μl/ml) 5 to 10 mins. Digestion was stopped by washing in 0.1M triethanolamine with 0.1% acetic anhydride. After that, embryos were rinsed in PBS twice and fixed in MEMFA over 20 mins. Fixative was removed by two washings in PBT, prehybridized in 300 μl of prehybridization solution (50% formamide, 5x SSC, Torula RNA (1mg/ml), Denhardt's solution, 0.1% Tween-20, 0.1% Chaps and 10mM EDTA) over 120 mins. The solution was replaced by 300 μl of digoxygenin-labeled RNA probe, and hybridization was performed overnight at 60°C. After that, embryos were washed by 500 μl of prehyb over 60 mins at 60°C, 2x SSC (300mM NaCl, 30mM sodium citrate) twice each of 60 mins at 60°C, 0.2x SSC over 30 mins at 60°C and 0.2x SSC over 30 mins at room temperature. After that, embryos were rinsed twice with MAB (100mM maleic acid, 150mM NaCl) at room temperature and treated with 2% blocking reagent in MAB over 60 mins, 20% heat treated lamb serum in 2% blocking reagent in MAB over 60 mins and 1:1000 anti-Digoxigenin-AP Fab fragments (Roche cat# 11 093 274 910) in 20% heat treated lamb serum in 2% blocking reagent in MAB overnight at 4°C. Antibodies were removed by five washings in MAB at 4°C, then overnight. After that, embryos were treated with alkaline phosphatase buffer (Chapman *et al.*) (100mM Tris-HCl, 50mM MgCl2·6H2O, 100mM NaCl, 0.1% Tween-20) containing 2mM levamisol twice and the color reaction was performed in BM Purple AP Substrate (Roche cat# 11 442 074 001) over several days until the desired coloration was reached. After that, embryos were rinsed with AP twice, fixed and stored in MEMFA.

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