Detection of natural antisense non-coding RNAs transcribed from *Ncam1* in mice tissues at several developmental stages

MITSURU CHIBA

Department of Biomedical Sciences, Division of Medical Life Sciences, Hirosaki University
Graduate School of Health Sciences, Hirosaki, Aomori 036-8564, Japan

Abstract. Natural antisense ribonucleic acids (RNAs) are transcribed from a large number of genes in various species, including humans and mice. The expression of neural cell adhesion molecule 1 (*Ncam1*) antisense non-coding RNAs (ncRNAs) in mice has been demonstrated by functional annotation of the mammalian genome project, but the localization of *Ncam1* antisense ncRNAs has not been reported in mice tissues. In the present study, the localization of *Ncam1* antisense ncRNAs was examined in tissues at several developmental stages by *in situ* hybridization. At days 14 and 17 of embryonic development, *Ncam1* antisense ncRNAs were found in the heart, liver, lung, kidney, thymus and nerve regions including the brain (cerebral cortex, olfactory bulb, hippocampus and cerebellum) and spinal cord. In newborn mice, *Ncam1* antisense ncRNAs were detected in the brain, kidney and thymus, but was not detected in other tissues. In 8-week-old mice, *Ncam1* antisense ncRNAs were detected in the lung, kidney, thymus, pancreas, cornea, stomach and nerve regions including the brain. These results indicate that *Ncam1* antisense ncRNAs are expressed in mice tissues. Notably, *Ncam1* messenger RNAs (mRNAs), antisense ncRNAs co-localized in the Purkinje cells of the cerebellum and the levels of antisense ncRNAs appeared to be higher than those of mRNAs, suggesting that *Ncam1* antisense ncRNAs may regulate the expression of *Ncam1* mRNAs in the same cells.

Introduction

Natural antisense ribonucleic acids (RNAs) are transcripts that contain sequences complementary to other endogenous RNAs including messenger RNAs (mRNAs). Kiyosawa *et al* (1,2) identified ~2,500 complementary deoxyribonucleic acid (cDNA) pairs originating from opposite strands of the same genomic loci by mapping these cDNAs to the draft mouse genome sequence. The functional annotation of the mammalian genome (FANTOM) cDNA dataset contained numerous non-coding RNAs (ncRNAs) (1-3). More than 70% of the antisense pairs identified by Kiyosawa *et al* (1,2) included ncRNAs as one of the members in the pair. To date, a number of mammalian antisense ncRNAs have been found, indicating that antisense transcription may be a commonly employed mechanism to regulate gene expression in human cells (1-5). Faghihi *et al* (6) reviewed the functions of known antisense ncRNAs, such as transcriptional interference, genomic imprinting, X chromosome inactivation and alternative splicing. Certain investigators have reported that the gene expression of antisense ncRNAs was up- or downregulated under conditions of physiological change, such as the development of various types of cancer (7,8). However, it is difficult to elucidate the function of antisense ncRNAs via gene expression analyses, such as microarrays.

The neural cell adhesion molecule 1 (*Ncam1*, also known as CD56) gene encodes a cell adhesion protein and is a member of the immunoglobulin superfamily. The encoded protein is involved in cell-to-cell and cell-matrix interactions during development and differentiation, as well as nervous system development (9,10). Antisense ncRNAs from opposite strands of the *Ncam1* loci in mice have been registered in the NCBI database (http://www.ncbi.nlm.nih.gov/gene/100036537) and antisense viewer (http://genome.gsc.riken.jp/m/antisense). In addition, the expression of antisense ncRNAs from *Ncam1* loci in the brain has been proven using microarray analysis by Kiyosawa *et al* (2). However, the expression and localization of this ncRNAs in other tissues remains unclear.

It is necessary to examine the cells and/or tissues from which the ncRNAs are expressed in order to understand the role of *Ncam1* antisense ncRNAs. In the present study, the expression of *Ncam1* antisense ncRNAs was examined in mice tissues at different developmental stages by *in situ* hybridization.

Materials and methods

Sections of the embryo and tissues. C57BL/6J mice at different developmental stages (at day 14 of mouse embryo development, E14; day 17 of development, E17; and newborn

Correspondence to: Dr Mitsuru Chiba, Department of Biomedical Sciences, Division of Medical Life Sciences, Hirosaki University Graduate School of Health Sciences, 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan
E-mail: mchiba32@hirosaki-u.ac.jp

mice; 8-week-old mice) were obtained from the RIKEN BioResource Center (Ibaraki, Japan).

For *in situ* hybridization, tissues from mice at different developmental stages were first fixed *in situ* by perfusion with 4% (w/v) ice-cold paraformaldehyde solution in phosphate-buffered saline (PBS). The resulting tissues were excised and further fixed overnight in the paraformaldehyde solution. The fixed tissues were embedded in paraffin and 4 μm sections were cut. Sections were placed on glass slides and subjected to *in situ* hybridization.

Complementary RNA (cRNA) probe. For *Ncam1* antisense ncRNAs and mRNAs, probes with a specific sequence: 5'-ATC TGGTCAAGTACAGAGCGCTCGCCTCTGAGTGGAAAC CGGAAATCAGGCTCCCATCCGGCAGTGACGACC ACGTCATGCTCAAGTCCCTGGACTGGAACGCAGAGT ATGAAGTCTATG T-3'; and a size of 118 nucleotides were designed in the mRNAs of *Ncam1*. As a control RNA probe, a 120-nucleotide λ-phage sequence that had no similarity with any of the mammalian sequences registered in the DNA Data Bank of Japan (www.ddbj.nig.ac.jp/index-j.html) was used in all the *in situ* hybridization experiments to verify that the hybridization system did not emit any non-specific hybridization signals. Digoxigenin (DIG)-labeled cRNA probes were provided by Tsukuba GeneTech Laboratories (Ibaraki, Japan).

In situ hybridization. Sections of embryos and tissues on glass slides were deparrafinized with xylene and ethanol, washed in PBS and incubated in PBS containing 1.0 μg/ml proteinase K, at 37°C for 15 min. The slides were washed in PBS and treated with 0.1 M triethanolamine in 0.25% acetic anhydride for 15 min. Slides were subsequently washed with 0.1 M triethanolamine and 4X standard saline citrate (SSC), and incubated in prehybridization solution (50% formamide, 2X SSC) at 42°C for 30 min. Hybridization was performed in a solution containing 50% formamide, 2X SSC, 1.0 mg/ml transfer RNA (tRNA), 1.0 mg/ml salmon DNA, 1.0 mg/ml bovine serum albumin, 1.0% sodium dodecyl sulfate and 3.0 μg/ml DIG-labeled sense or antisense cRNA probes, at 42°C for 16 h.

The resulting slides were successively washed with prehybridization solution, 0.2X SSC and 0.1X SSC, at 42°C, followed by treatment with NT buffer [150 mmol/l NaCl and 100 mmol/l Tris-HCl (pH 7.5)] containing 10% fetal bovine serum, 1% blocking reagent (Roche Diagnostics, Basel, Switzerland) and 1 mg/ml tRNA, for 30 min. Subsequently, hybridization signals were visualized using the alkaline phosphatase-labeled anti-DIG antibody/nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl-phosphate system (Roche Diagnostics).

Results and Discussion

Recently, antisense ncRNAs from a number of genes have been identified in mice and humans using bioinformatic and microarray analyses. Antisense ncRNAs have been inferred to be involved in the control of trait expression in mammals, including humans, mice and livestock. Study of the sites of antisense ncRNA expression in tissues would provide important information regarding the functions of antisense ncRNAs

in mice. Therefore, mice were used in the present study to investigate the expression sites of previously described antisense ncRNAs from *Ncam1*.

Kiyosawa *et al* (2) examined sense/antisense expression using a custom microarray with different cDNA priming methods. *Ncam1* antisense ncRNAs in the brain were more strongly expressed than those mRNAs in the microarray analysis using oligo(dT) or random nonamer primers (Fig. 1). Microarray analysis using oligo(dT) primers is assumed to detect RNAs (transcripts) with poly(A) tails, whereas microarray analysis using random nonamer primers is assumed to detect RNAs with and without poly(A) tails. This information suggests that *Ncam1* antisense ncRNAs may have poly(A) tails.

To date, the open reading frame, or protein-coding region, of *Ncam1* antisense RNAs has not been confirmed in several databases, including FANTOM. Therefore, *Ncam1* antisense RNAs are ncRNAs. To examine the expression and localization of *Ncam1* antisense ncRNAs at different developmental stages, *in situ* hybridization was performed. *In situ* hybridization is known to detect RNAs with and without poly(A) tails. Therefore, it is generally considered that the signal intensity patterns of *in situ* hybridization are the same as those of microarray analysis using random nonamer primers.

In E14 embryos, *Ncam1* antisense ncRNAs were uniformly detected in the heart, liver and nervous system regions, including the brain, and were detected at high levels in certain regions of the lung, kidney and thymus (Fig. 2). In E17 embryos, *Ncam1* antisense ncRNAs were detected at high levels in specific cells of the brain, whereas they were uniformly detected in other tissues (heart, liver, lung, kidney and thymus). In newborn mice, *Ncam1* antisense ncRNAs were weakly detected in the brain, kidney and thymus. The transcription pattern of NB mice was similar to that of E17 embryos, except that the signals in organs other than the brain were weaker than those of the E17 embryos (Figs. 2 and 3). In the 8-week-old mice, *Ncam1* antisense ncRNAs were detected at high levels in limited regions of the brain, stomach, cornea and all the regions of the pancreas. Contrastingly, they were detected at low levels in the remaining organs that were examined, excluding the liver. With regards to the liver, negligible amounts of *Ncam1* antisense ncRNA expression was detected (Fig. 3). These results indicate that *Ncam1* antisense ncRNAs are expressed in mice tissues.

Of note, *Ncam1* mRNAs and antisense ncRNAs co-localized in Purkinje cells of the cerebellum and the levels of antisense ncRNAs appeared to be higher than those of mRNAs (Fig. 4). Based on the patterns of the *in situ* hybridization signals for *Ncam1* antisense ncRNAs detected in tissues, some potential functions were identified for *Ncam1* antisense ncRNAs. In the analysis of the adjacent sections, *Ncam1* mRNAs were detected in some of the brain cells found to produce *Ncam1* antisense ncRNAs. This indicates that *Ncam1* antisense ncRNAs may control the function of *Ncam1* mRNAs, as previously proposed in reviews on other antisense ncRNAs (6,11). Currently, the exact function of *Ncam1* antisense ncRNAs in each tissue is unknown. However, the expression patterns of *Ncam1* antisense ncRNAs may provide information for understanding their function. In future studies, the interactions between *Ncam1* mRNAs and antisense ncRNAs should be examined.
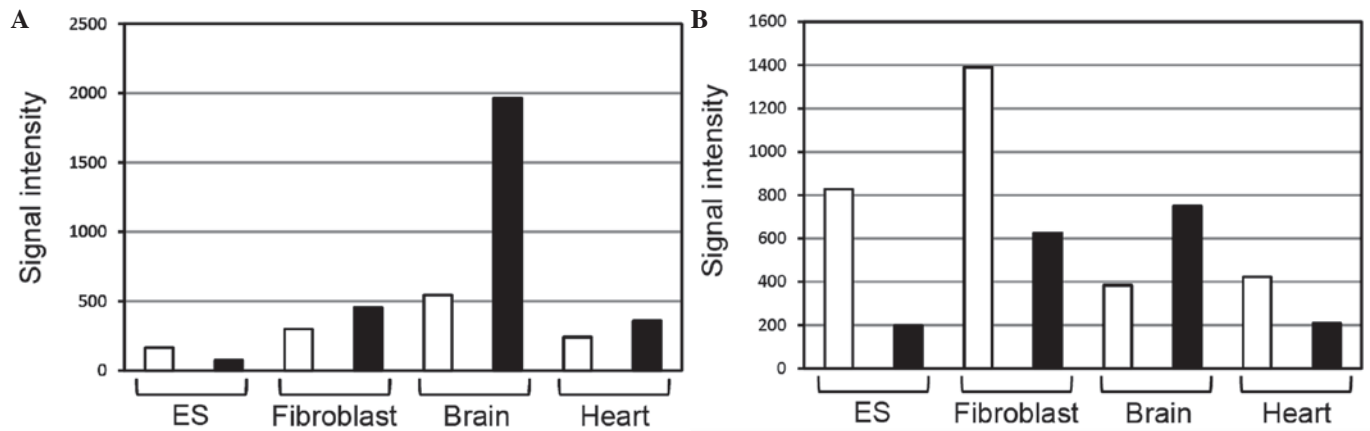
Figure 1. Expression of neural cell adhesion molecule 1 (*Ncam1*) messenger RNAs (mRNAs) and antisense non-coding RNAs (ncRNAs), according to micro-array analysis. (A) Microarray signal intensity obtained with samples labeled by oligo(dT) priming. (B) Microarray signal intensity obtained with samples labeled by random nonamer priming. White bars represent the signal intensity of *Ncam1* mRNAs, whereas black bars represent the signal intensity of *Ncam1* antisense ncRNAs.
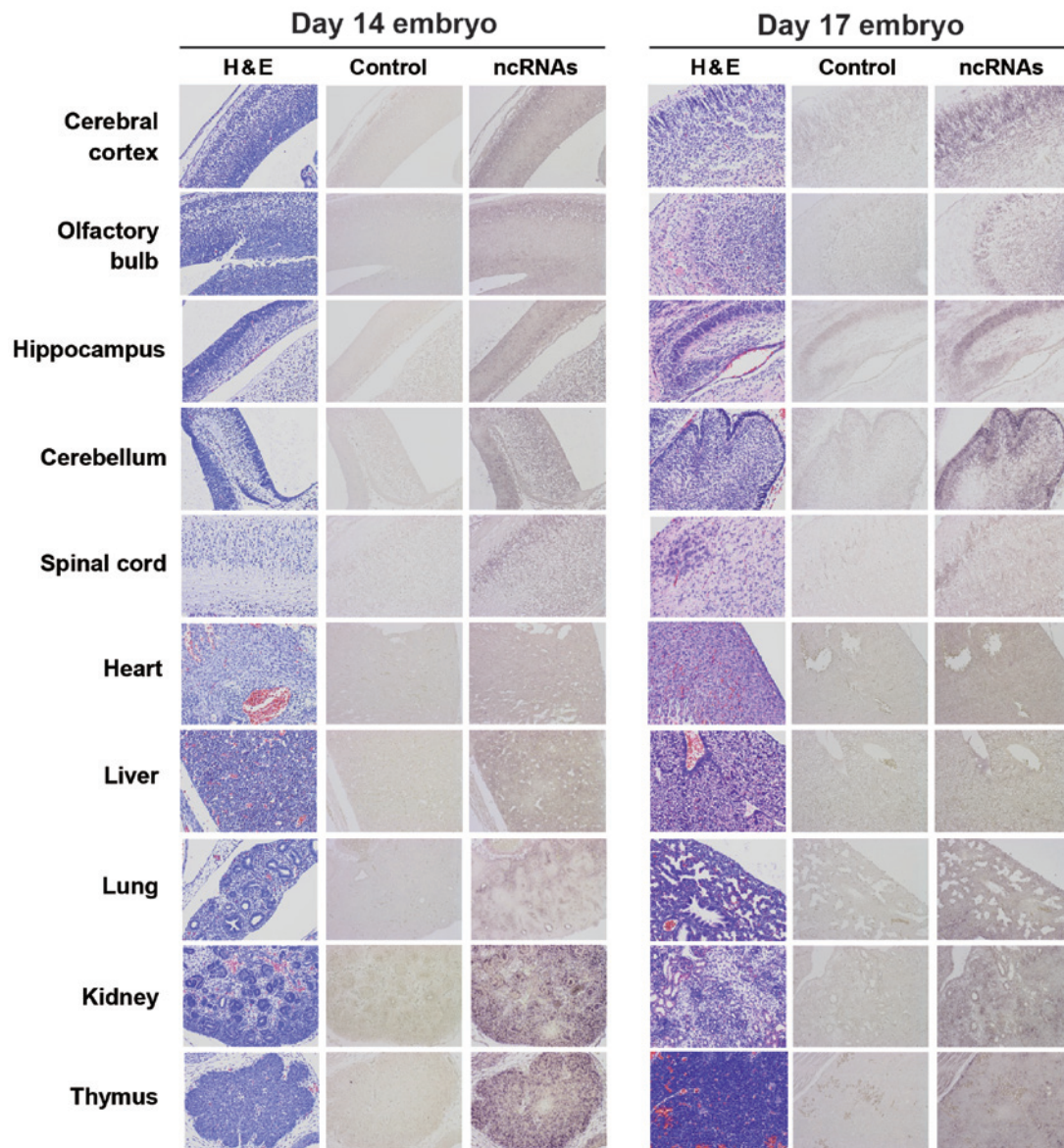

Figure 2. Detection of neural cell adhesion molecule 1 (*Ncam1*) antisense non-coding RNAs (ncRNAs) in mice tissues at embryonic development day 14 (E14) and 17 (E17). *Ncam1* antisense ncRNAs were detected by *in situ* hybridization with a digoxigenin (DIG)-labeled sense complementary-RNA probe. As a negative control, DIG-labeled control probes were used for *in situ* hybridization. Hematoxylin and eosin (H&E) staining was performed to visualize the fine structures of the tissues.
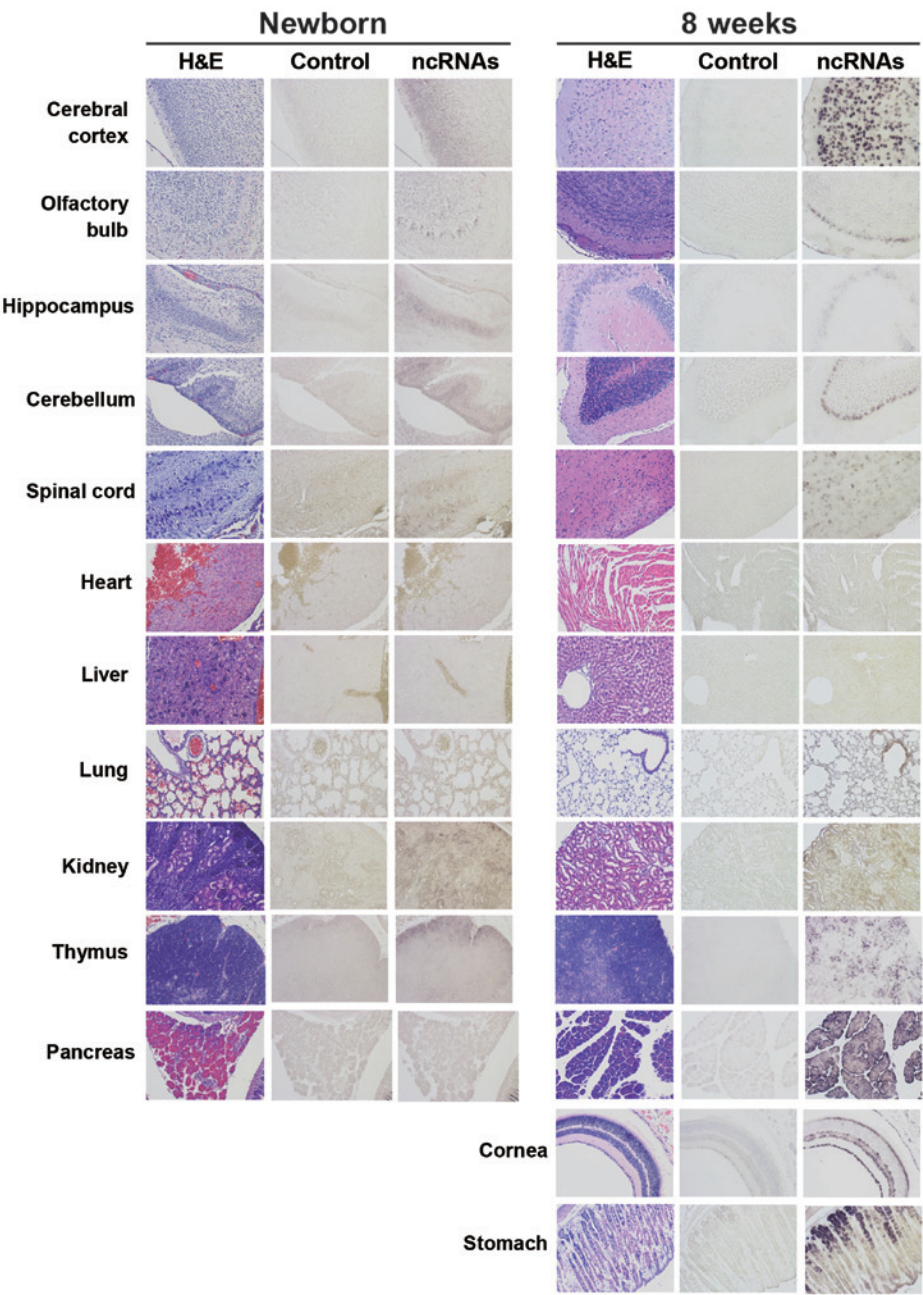
Figure 3. Detection of neural cell adhesion molecule 1 (*Ncam1*) antisense non-coding RNAs (ncRNAs) in newborn and 8-week-old mice. *Ncam1* antisense ncRNAs were detected by *in situ* hybridization with a digoxigenin (DIG)-labeled sense complementary-RNA probe. As a negative control, DIG-labeled control probes were used for *in situ* hybridization. Hematoxylin and eosin (H&E) staining was performed to visualize the fine structures of tissues.
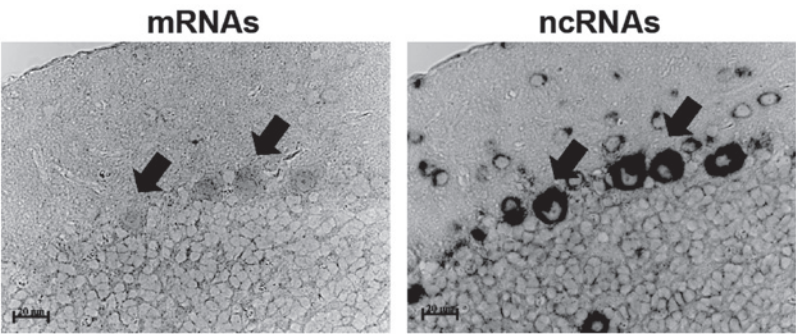

Figure 4. Co-localization of neural cell adhesion molecule 1 (*Ncam1*) messenger RNAs (mRNAs) and antisense non-coding RNAs (ncRNAs) in Purkinje cells of 8-week-old mice. Murine cerebellum tissue embedded in paraffin was serially sectioned at 1.5-µm, which allowed one Purkinje cell to be split into ≥2 consecutive sections. *In situ* hybridization was performed using a digoxigenin-labeled sense complementary-RNA probe. *Ncam1* mRNAs and antisense ncRNAs existed in the same Purkinje cells, as indicated by black arrows. Scale bar represents 20 µm.

Acknowledgements

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