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BARHL1 homeogene, the human ortholog of the mouse *Barhl1* involved in cerebellum development, shows regional and cellular specificities in restricted domains of developing human central nervous system

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Abstract

The mouse homeobox gene *Barhl1* plays a central role in cerebellum development and its expression is activated by the transcription factor *Math1* which is involved in bone morphogenetic protein response pathways. We studied the human ortholog *BARHL1* and we found that human, mouse, monkey, rat, and zebrafish orthologs were highly conserved and are members of the *BarH* homeogene family, containing *Drosophila BarH1* and *BarH2*. The N-terminus of BARHL1 protein presents two FIL domains and an acidic domain rich in serine/threonine and proline, while the C-terminus contains a canonical proline-rich domain. Secondary structure analysis showed that outside the three helixes of the homeodomain, BARHL1 protein has essentially random coil structure. We isolated *BARHL1* and defined its expression pattern in human embryonic and fetal central nervous system (CNS) and compared it to the mouse *Barhl1* transcription. *BARHL1* mRNA was found exclusively in the CNS restricted to p1–p4 prosomeres of the diencephalon, to the dorsal cells of the mesencephalon, to the dorsal dl1 sensory neurons of the spinal cord, and to the rhombic lips yielding the cerebellar anlage. Detailed analysis of *BARHL1* expression in fetal cerebellar cell layers using our new optic microscopy technology showed *BARHL1* expression in external and internal granular cells and also in mouse adult granular cells, in agreement to *Barhl1* null mouse phenotype affecting the differentiation and migration of granular cells. These findings indicate that the regional and cellular specificities of *BARHL1* transcriptional control well correspond to the mouse *Barhl1* transcription and suggest a potential role of this gene in the differentiation of *BARHL1*-expressing neuronal progenitors involved in the pattern formation of human cerebral and cerebellar structures.

Keywords: *BARHL1* homeobox gene; Prosomeres p1–p4; dl1 dorsal sensory neurons; Cerebellar granular cells; Cell differentiation; BMP signaling pathway; Math1; Lhx2; Cerebellar pathology; Joubert syndrome

The central nervous system (CNS) is characterized by the most complex cellular organization, with the most abundant cellular types, which form a highly complex network. During CNS development, several complex cellular mechanisms are involved, characterized by important cellular migrations allowing the functional organization of the mature CNS. The cerebellum is relatively less complex than other brain regions, consisting in only five cellular types and a stereotyped regular organization. The largest part of the cerebellum corresponds to the cerebellar granular neurons, which are also the most abundant neuronal type in the CNS and have a central role in cerebellar function. The cerebellar granular precursor cells are generated in the rhombic lips, the most dorsal part of the rhombencephalic alar plate [1,2] yielding the external granular cell

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layer [3,4]. This cell layer generates the progenitors of the internal granular cells that proliferate, differentiate, and migrate during post-natal development to establish the internal granular cell layer under a monolayer of Purkinje cells.

Alterations of the proliferation, the differentiation, and the migration of the cerebellar granular cell progenitors determine abnormal foliation of the cerebellum. Interestingly, mutant mice for genes involved in these cerebellar developmental processes are generally viable and show no dramatic phenotypes. These genes can be studied relatively easier than genes involved in the development of the other brain regions and help us to better understand the molecular mechanisms controlling the CNS development. Recently, it has been demonstrated that *Barhl1* null mice showed deficiencies in the radial migration and increased cell death of the cerebellar granular cells determining attenuated foliation and hypotrophy of the cerebellum [5]. These findings demonstrated that *Barhl1* plays a crucial role in cell migration and in the inhibition of the apoptosis in cerebellar and precerebellar neurons during mouse cerebellar development [5].

The *Barhl1* gene encodes a transcription factor member of the *BarH* subgroup of homeobox genes including a limited number of proteins, namely the *Drosophila* BarH1 and BarH2 proteins. These *Drosophila* proteins are specifically coexpressed and functionally redundant for cell fate determination during eye and nervous system development, in response to decapentaplegic (Dpp) signals [6–8], the homolog of the vertebrate bone morphogenetic proteins (BMPs). In *Drosophila*, the intracellular responses to Dpp are mediated by interactions between BarH and different transcription factors. In the eye, complex interactions exist between BarH and atonal (ato), the homolog of the mammalian Math1: *BarH* expression is controlled by Ato and also controls *ato* expression by at least three different pathways, depending on the developmental time and the position in the eye disk [9]. In the leg imaginal disk, BarH proteins interact with a complex network of other transcription factors, such as Apterous and dLim1 (orthologs of the vertebrate proteins Lhx2 and Lim1, respectively), in response to Wingless (Wg) and Dpp signals [10,11]. In the pretarsus, *dlim1* expression represses *BarH* expression. Reciprocally, BarH represses *dlim1* expression while in tarsus four BarH activates the expression of *apterous* and the two proteins form a functional complex [10]. It has been demonstrated that most of the genes of the Dpp/BMP signaling have conserved sequences, developmental roles, and interactions in mammalian CNS [9–13].

Interestingly, other members of the *BarH* subfamily have been demonstrated to be involved in BMP signaling. The chick *Barx1* has also been demonstrated to respond to BMPs during face primordia development [14]. The *Xenopus Xvent-2* acts downstream to BMP4 to mediate its effects [15,16] and determines the posterior limit of neural plate differentiation, corresponding to the presumptive forebrain, by control of the *Xanf1* posterior expression limit [17]. *Xvent-2* belongs to a group of related genes which probably originated by amplification of an ancestral gene. All these *Xenopus* related genes, members of the BarH subfamily, are highly conserved and have been involved in the BMP responses [18].

In this work, we isolated and studied the human *BARHL1* gene and investigated its expression pattern directly in human central nervous system, during embryonic and fetal development. We also analyzed *Barhl1* expression in mouse embryos and adult cerebellum, and compared it to the *BARHL1* transcription. In addition, we used our new optic microscopy technology [19] allowing estimation of mRNA transcript steady state levels in different cell types and their direct comparison with cell density in the developing brain structures. We showed that *BARHL1* appeared specific of defined neuromeres with regional and cellular specificity in embryonic and fetal central nervous system. In cerebellum, *BARHL1* appeared specific of granular cells during all their development in the external granular cell layer, in the internal granular cell layer, and in the adult granular cells. Comparisons of the *BARHL1* expression pattern to those of *BMPs*, *Math1*, and *Lhx2* (the mammalian homologs of the *Drosophila atonal* and *apterous*, respectively), have been discussed.

Materials and methods

Human and mouse tissues. Human embryos at Carnegie stages 8, 12, 16, and 17, corresponding to 18, 26, 37, and 41 post-ovulatory days, respectively [20], and human fetuses at 17, 22, and 24 weeks of gestation, were obtained from legally approved medical abortions in the feto-pathological laboratory of the Robert Debré Hospital. The project was carried out with the approval of the French National Ethics Committee.

Human and mouse embryos, human fetuses, and adult mouse brains were fixed in 4% PFA, dehydrated by a series of ethanol washes of increasing ethanol concentration and xylene, and embedded in paraffin (Paraplast Plus, Sigma) following classical procedures. Ten micrometer serial sagittal and coronal sections were cut on microtome and stored at room temperature.

Protein sequence analyses. Homologous sequences to BARHL1 were identified by BLAST (Basic Local Alignment Search Tool) family of program (http://www.ncbi.nlm.nih.gov/blast/index.html) searches for similarity to sequences contained in GenBank.

Multiple sequence alignments were performed with BLAST 2 sequence program, for sequence pairs (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html), and the alignment CLUSTALW algorithm (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl). The output files were visualized using the Boxshade program (http://www.ch.embnet.org/software/BOX_form.html).

Physico-chemical characterization of protein sequences and primary structure analyses were performed using Infobiogen tools (http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html). The SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/) and the PFSCAN (http://hits.isb-sib.ch/cgi-bin/PFSCAN) programs were used to identify protein domain structures, Prosite profiles, and Pfam domains in the protein sequences.

Screening and cloning. A human fetal brain cDNA library was screened using a mouse *Barhl1* clone that we have previously isolated (unpublished data). Identified clones were sequenced using an ALF DNA sequencer (Pharmacia) with the ThermoSequenase protocol (Amersham). These clones corresponded to *BARHL1* gene and contained the homeobox domain.

We amplified about 700 bp of the 5′ of the *BARHL1* transcript, outside the homeodomain and we subcloned it in the pGEM-T Vector (Promega). Similarly, we subcloned about 700 bp of the mouse *Barhl1* gene sequence outside the homeodomain. These fragments did not show significant homology with other genes, as tested by BLAST searches, and have been used to generate riboprobes for in situ hybridization studies.

In situ hybridizations. To avoid cross-hybridization with other homeobox genes, we used the subcloned human and mouse cDNAs localized outside the homeodomain, as template to generate sense and antisense riboprobes for in situ hybridization, by Sp6 and T7 RNA polymerases (Promega) incorporating [^{35}S]UTP (Boehringer–Roche).

Paraffin sections were deparaffined in xylene and in decreasing ethanol series and finally, sections were incubated in PBS. Before hybridization with riboprobes, slides were treated with proteinase K, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, and pre-hybridized in hybridization buffer (50% formamide, 10% dextran sulfate, 50 mM DTT, 0.3 M NaCl, 30 mM Tris–HCl, 4 mM EDTA, 1× Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, and 0.5 mg/ml polyadenylic acid). Subsequently, sections were incubated overnight at 65 °C in the same hybridization buffer containing the radiolabeled probe.

After hybridization, sections were washed with increasing stringency in standard saline citrate solutions (SSC), twice in 5× SSC plus 50 mM β-mercaptoethanol for 30 min, once at 50 °C for 30 min, and once in 2× SSC plus 50 mM β-mercaptoethanol at 55 °C for 30 min. Then, they were treated with RNase A (50 μg/ml) for 30 min at 37 °C, and washed twice with 2× SSC for 30 min at 37 °C and twice in 0.1× SSC at 65 °C for 30 min. Specimens were then dehydrated, air-dried, and exposed to Kodak Biomax X-ray film for 3–5 days. For cellular resolution, selected slides were subsequently dipped in photographic emulsion Kodak NTB2, incubated for 3–4 weeks, and then developed in Kodak D-19, fixed, mounted in the Permount, and observed on a Zeiss photomicroscope equipped with bright-field and dark-field optics.

The specificity and effectiveness of the probe were tested performing negative controls on adjacent paraffin embedded sections hybridized with sense riboprobes or digested with RNase before hybridization. No signal was obtained in such negative controls.

Optical technology. Human embryonic and fetal brain tissues were analyzed using our new optical technology [19], an oblique illumination that permits us to distinguish between cell bodies and extracellular matrix. Microscopy observations using this technique can be carried out directly on the in situ hybridization treated samples, without additional staining that normally decreases or loses the radioactive signal. In addition, this optic technology is able to directly compare cell density in the analyzed region and the in situ hybridization signal intensity.

This technique uses the property of the oblique illumination and the resulting image appears as if the specimen were being viewed from an angle; this gives an impression of relief with more details than phase contrast microscopy. This effect is created by slightly moving the bulb laterally and by using the position of the phase contrast condenser where the light field is the largest.

Results

We identified human cDNA clones corresponding to the human *BARHL1* by screening a human fetal brain cDNA library using a murin *Barhl1* cDNA. The human *BARHL1* gene is localized in 9q34 band on chromosome 9 and its genomic sequence spans about 8 kb and consists in three exons. The 1593 bp *BARHL1* mRNA encodes a putative protein of 327 amino acids (gi:55958522).

Mouse and rat ortholog *Barhl1* genes are known (gi:33417237 and gi:16923956, respectively). To identify other vertebrate *Barhl1* ortholog genes and to analyze conservation of their sequences, we searched translated genomic sequences showing homologies with the BARHL1 protein sequence. We identified two predicted genes in monkey (*Pan troglodytes*, gi:55632543) and zebrafish (*Danio rerio*, gi:46399182). Using global alignment of the five protein sequences (Fig. 1A), we found complete sequence identity between human and monkey, and between mouse and rat. Comparison between human–monkey and mouse–rat sequences showed 97.9% of identity, corresponding to seven different amino acids of which six are located in the N-terminus and only one in the C-terminus whereas no differences were found in the homeodomain (Fig. 1A). Comparison between human and zebrafish sequences showed 86.2% of identity, corresponding to 46 different amino acids of which 41 are located in the N-terminus, only one in the 30 amino acid region preceding the homeodomain, and four in the C-terminus. The complete

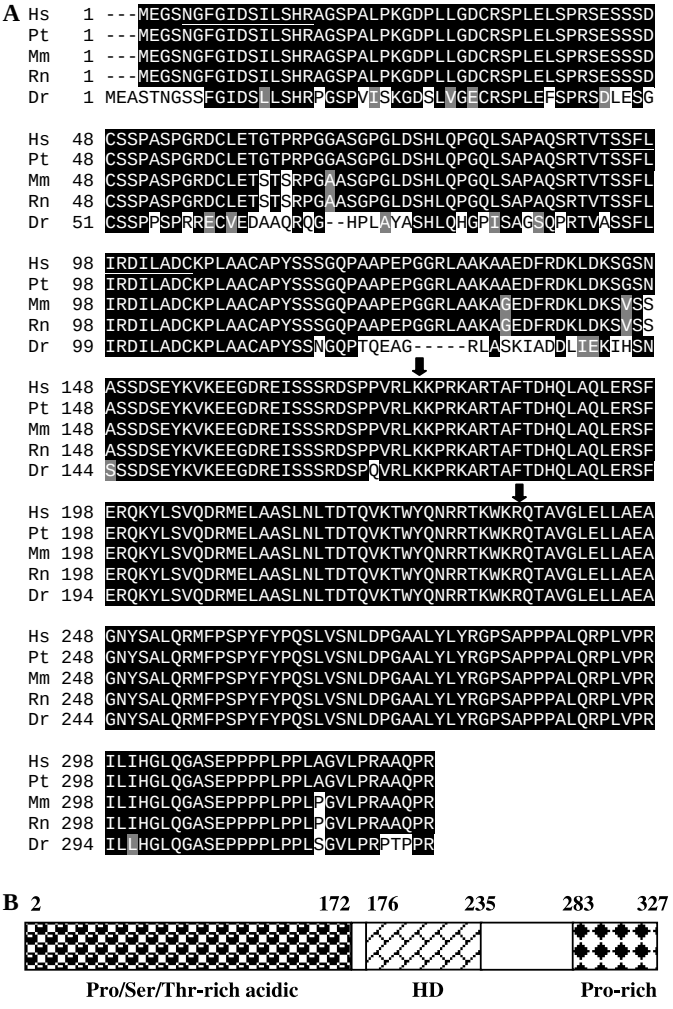

Fig. 1. Sequence analysis of BarH proteins. (A) Multiple sequence alignment of the human *BARHL1* (Hs) and monkey (Pt), mouse (Mm), rat (Rn), and zebrafish (Dr) orthologs. Identical amino acids were indicated in black. Similar conserved amino acids were indicated in grey. The two FIL domains were underlined. The arrows indicated the homeodomain. (B) Schematic representation of BARHL1 protein. The position of the amino acids is indicated by numbers. *Abbreviations:* Pro/Ser/Thr-rich acidic, acidic domain rich in proline and serine-threonine residuals; HD, homeodomain; Pro-rich, proline-rich domain.

identity in the homeodomain sequence (Fig. 1A) indicated that the identified zebrafish gene corresponds effectively to the *BARHL1* ortholog.

BARHL1 protein and the other members of the BarH homeobox subfamily are characterized by the presence of a threonine residue at position 47 of the homeodomain rather than the more common isoleucine. This subclass of homeogenes includes the *Drosophila* BARH1 and BARH2 proteins [6,7], of which the subfamily name derived. The *Drosophila* proteins showed high sequence conservation in the homeodomain sequences (data not shown).

The N-terminus of the BarH homeobox proteins presents peptide motifs (twelve amino acids) designated as FIL domains [21], FIL1 and FIL2 (Fig. 1A), which contains a consensus sequence composed of phenylalanine (position 3), isoleucine (position 5), and leucine (position 9). The *Drosophila* members, BarH1, BarH2, and Om1D proteins, have only one FIL domain. In the N-terminus of BARHL1, a highly acidic domain was found in the first 175 amino acids, conferring at this domain a theoretical p*I* of 4.79, against a p*I* of 9.20 for the global protein. This acidic domain appeared rich in serine/threonine (35 amino acids, corresponding to 20% of the domain sequence, against the 51 serine/threonine residual in the global protein, corresponding to 15.6%) and also rich in proline, containing 19 proline residual, corresponding to 10.9%. The C-terminus contains a canonical proline-rich domain from amino acid 283 to 326 (Fig. 1B). Secondary structure analysis showed that outside the homeodomain, consisting in three helixes, BARHL1 protein has essentially random coil structure.

The human *BARHL1* cDNAs that we identified contained the homeobox domain. To avoid cross-hybridization with other homeobox genes, in particular with the *BARHL2* gene, also a member of the *BarH* homeobox subfamily, we subcloned an amplified fragment of the 5′ gene sequence, outside the homeodomain. We used this new clone to generate riboprobes and we performed in situ hybridization on human embryos and fetuses.

We showed that *BARHL1* transcription was limited to the CNS, while no expression was detected elsewhere (Fig. 2A). Similar to the most homeobox genes, *BARHL1* showed a specific expression pattern in the CNS, restricted to some domains of the forebrain, the midbrain, the hindbrain, and the spinal cord. This gene is not transcribed in earliest embryonic stages.

In the forebrain, *BARHL1* transcription was delimited to the diencephalon, whereas no expression has been detected in the telencephalon during development (Figs. 2A and C). In the diencephalon, *BARHL1* is expressed in a region corresponding to the basal plate of the anlages of the prosomeres (neuromeres of the forebrain) p1–p4 (Figs. 2A and C), defined by the Prosomeric model [22,23]. This Prosomeric model subdivides the CNS into longitudinal (e.g., basal and alar plates) and transverse domains (neuromeres) determined by longitudinal and transverse patterning mechanisms. The prosomere p1 corresponds to the pretectum, the prosomeres p2 and p3 to the dorsal and ventral thalamus, and the prosomere p4 to the mammillary area [24]. In these regions of the embryonic diencephalon, *BARHL1* transcription was concerned with the entire basal plate thickness (Figs. 2A and C). In the midbrain, *BARHL1* expression was confined to a dorsal cell layer in the cortical plate (Figs. 2A and C), indicating a *BARHL1* cell specific expression in a dorsal cell subset. In the hindbrain, *BARHL1* transcript was found in the more dorsal part of the alar plate of the rhombic lips (Figs. 2C and E), containing the progenitor cells of the external granular cells and of the deep nuclei. In addition, *BARHL1* expression was found also along the neural tube in a caudal domain which extends from the rhombencephalon to the entire spinal cord. In these structures, this gene is expressed as a longitudinal strip along the most dorsal area of the alar plate, but not in the roof plate (Fig. 2F). We also analyzed *Barhl1* expression in mouse embryos showing that the expression pattern of *BARHL1* during human embryonic development well corresponds to the transcriptional profile observed in mouse (Fig. 2D).

We extended our study of *BARHL1* expression pattern in the developing fetal brain. This gene continues to be not transcribed in the telencephalic structures (Figs. 3A and B) whereas in the fetal cerebellum, *BARHL1* was highly transcribed (Figs. 3C and D). To better characterize *BARHL1* transcription in the more differentiated cerebellar cell layers, we used our optical microscopy technology [19] allowing direct observation at cellular levels of the in situ hybridization sections without staining. This approach showed that *BARHL1* is transcribed in the external granular cell layer, containing also the precursor cells of the internal granular cells, and in all internal granular cells (Figs. 3C and D). At this fetal stage, the Purkinje cells are not well identified since they cross the internal granular cells, which leave the external granular cell layer, to migrate under the external granular cell layer. The analysis of the fetal cerebellum hybridized with the *BARHL1* probe, using the optical technology, indicated that the Purkinje cells did not express this gene during fetal cerebellar development (Figs. 3C and D). We performed in situ hybridization on serial sections of human fetal cerebellum using a *SIM2* cDNA that we recently demonstrated to be expressed in external granular cells, internal granular cells, and Purkinje cells (Fig. 3F) [25]. The *SIM2* signal in the fetal cerebellum displayed two highly labeled layers corresponding to the external granular cells and the Purkinje cells, while the internal granular cells showed a relatively lower expression because of their low cell density (Fig. 3F). In the adjacent fetal cerebellar section hybridized with *BARHL1*, only the external granular cell layer and the internal granular cells were labeled, while the forming Purkinje cell layer was not labeled (Fig. 3E). Thus, the comparison of *BARHL1* and *SIM2* expression also indicated that the Purkinje cells did not express *BARHL1*. The gene continues to be expressed in the adult cerebellar granular cells, as we showed in the adult mouse cerebellum (Figs. 3G and H),

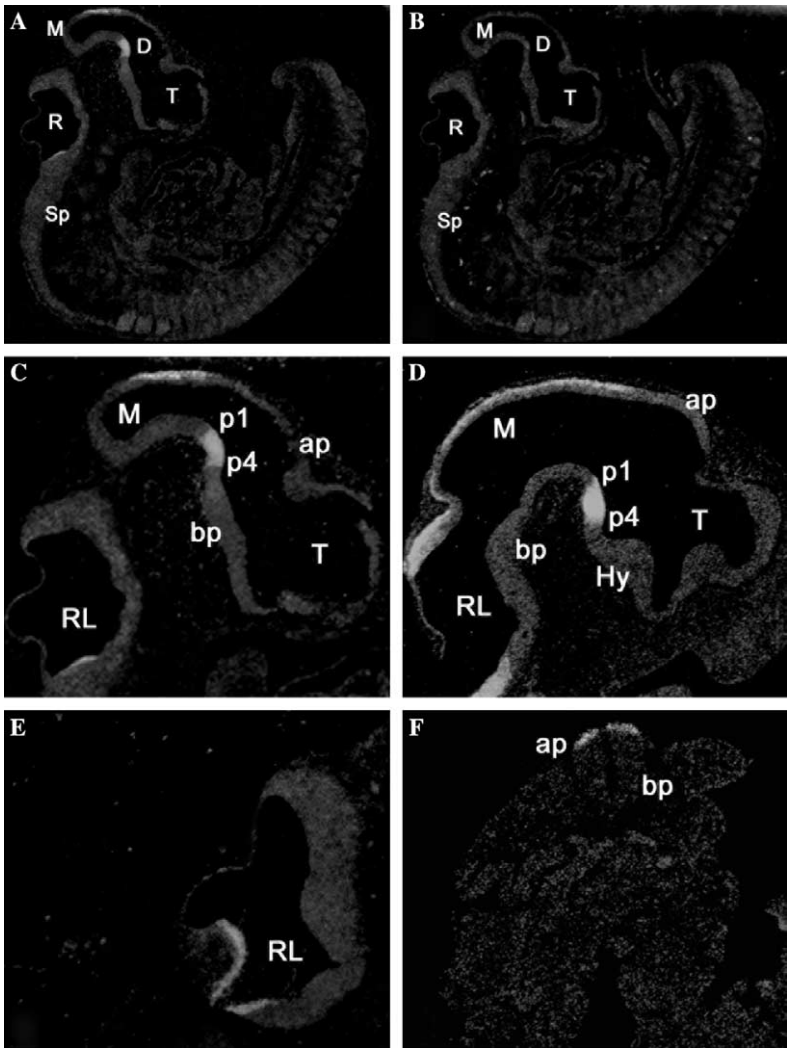

Fig. 2. *BARHL1* expression pattern during human and mouse embryonic development. (A) Sagittal section of a human embryo at Carnegie stage 16, corresponding to 37 p.o. days, estimated according to the Carnegie staging system [20] showing *BARHL1* expression in basal plate of the diencephalon (D), in the alar plate of the mesencephalon (M), and in the rhomboncephalon (R). (B) Note the absence of silver label in the negative control hybridization on an adjacent section at stage 16. (C) Detail of the head of a human embryo at Carnegie stage 16 showed *BARHL1* expression in the basal plate (bp) of the diencephalic p1, p2, p3, and p4 prosomeres (p1 and p4), in a dorsal cell layer in the alar plate (ap) of the mesencephalon (M), and in the rhomboncephalic lips (RL). (D) Detail of the head of a mouse embryo at E11.5 showed *Barhl1* expression in the basal plate (bp) of the diencephalic p1, p2, p3, and p4 prosomeres (p1 and p4), in a dorsal cell layer in the alar plate (ap) of the mesencephalon (M), and in the rhomboncephalic lips (RL). Note that the mouse *Barhl1* and the human *BARHL1* expression patterns are highly comparable. (E) Sagittal section of a human embryo at Carnegie stage 17, corresponding to 41 p.o. days, shows gene expression in the rhomboncephalic lips (RL). (F) Transverse section of a human embryo at Carnegie stage 12, corresponding to 26 p.o. days, showing gene transcription in a dorsal cell subset of the alar plate (ap) of the spinal cord. Abbreviations: Hy, hypothalamus; Sp, spinal cord; T, telencephalon. Original magnifications: (A,B) 25×; (C–E), 50×; (F) 75×.

where the mature Purkinje cells are well identifiable in a cell monolayer and they are clearly not labeled by *Barhl1* probe (Figs. 3G and H).

The external granular cell layer showed a higher expression steady state level than in the internal granular cells (Figs. 3C–E). Using our optical technology, we can evaluate variations in gene transcription level during the development by comparison of the signal intensity to the cell density. Thus, we showed that the external granular cell layer was characterized by a higher cell density than the internal granular cells (Fig. 3D), indicating that *BARHL1* was expressed at a similar level in both external and internal granular cells.

Discussion

The human *BARHL1* is a member of the *BarH* homeodomain subfamily, with known orthologs in mouse and rat. By search in vertebrate genome database, we identified new *Barhl1* orthologs in monkey and zebrafish, showing a complete identity with human *BARHL1* homeodomain sequence.

BARHL1 protein and the other members of the BarH homeobox subfamily are characterized by the presence of a threonine residue at position 47 of the homeodomain rather than the more common isoleucine, as found in the Antennapedia class [26]. This substitution in the

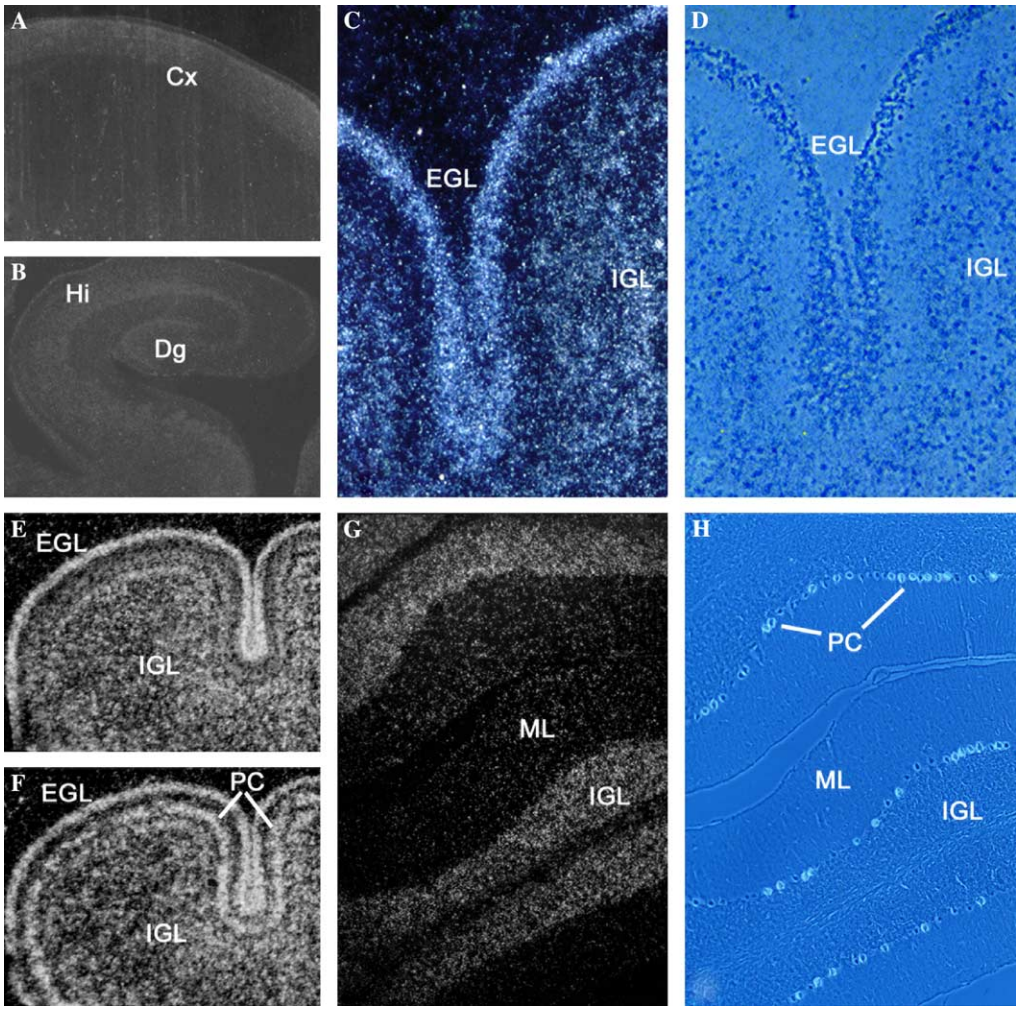

Fig. 3. *BARHL1* expression pattern during human fetal development and in mouse adult cerebellum. (A,B) Note the absence of silver label in the fetal cortex (Cx), hippocampus (Hi), and dentate gyrus (Dg) at 22 and 17 weeks of gestation, respectively. (C,D) Human fetal cerebellum at 22 weeks of gestation observed using (C) dark field and (D) our optical microscopy technology [19] showed high *BARHL1* expression in the external granular cells (EGL) and in the internal granular cells (IGL). (E) Human fetal cerebellum at 24 weeks of gestation observed using dark field showed high expression of *BARHL1* in the external granular cells (EGL) and in the internal granular cells (IGL). (F) Adjacent section of the human fetal cerebellum at 24 weeks of gestation observed using dark field showed high expression of *SIM2* in the external granular cells (EGL), in the internal granular cells (IGL), and in the Purkinje cells (PC). (G) Adult mouse cerebellum showed *Barhl1* expression in the granular cell layer. (H) The same section in (G) observed using our optical technology shows that the Purkinje cell layer (PC) is not labeled. ML, molecular layer. Original magnifications: (A,B) 25×; (E,F), 75×; (C,D,G,H), 200×.

recognition third helix has been shown to alter the DNA binding specificity of HOX11 and Lbe from TAAT to TAAC motif [27–29]. In addition, the N-terminus of the human protein contains two FIL (phenylalanine, isoleucine, and leucine) domains [21] and a highly acidic domain rich in proline and serine/threonine. In the C-terminus, a canonical proline-rich domain was found that may be involved in transcription activation [30–32].

To study the potential role of the *BARHL1* during human development, we analyzed its expression directly in human embryos and fetuses, to avoid mere extrapolations of human knowledge from mouse findings. The *BARHL1* expression pattern was exclusive to the CNS and appeared to well correspond to the mouse *Barhl1* as confirmed in this work, by other authors [5,33] and expression databases (http://www.informatics.jax.org/; http://genome.ucsc.edu/index.html; http://symatlas.gnf.org/SymAtlas/).

In the spinal cord, *BARHL1* was detected in two subtle cell strips in the dorsal part of the alar plate. The spinal cord is composed of different cell types which are generated and localized in different positions along the dorso-ventral axis of the neural tube [34]. The motor neurons are generated lateral to the ventral floor plate and cells lateral to the dorsal roof plate differentiate into different types of dorsal sensory interneurons that integrate incoming sensory information and transmit it to higher brain centers [35,36]. In the dorsal sensory interneurons, six different cell subsets have been defined by combination of expression pattern of the transcription factors involved in their differentiation [37–39]. The *BARHL1*-expressing cells in the neural cord correspond to the dI1 or D1 subset of dorsal neurons.

In mouse, it has been determined that the *Barhl1*-expressing dorsal neurons migrated laterally and in the later development they were found in a subtle strip of migrating cells in the middle of the spinal cord corresponding to the mantle zone cells localized in the ventral part of the alar plate. This region corresponds to the final localization of the migrating dl1 dorsal sensory neurons [5,33,39].

In the forebrain, *BARHL1* transcription is limited to the basal plate of the anlages of the prosomeres p1–p4 of the diencephalon, corresponding to the pretectum, the dorsal and ventral thalamus and the mammillary area [24]. During late mouse development, *Barhl1* expression became restricted to the mammillary area of the hypothalamus [33]. The primordium of the mammillary region corresponds to the mammalian ventral midline, localized between the infundibular area (the future neurohypophysis) and the floor of the mesencephalon [40]. The adult mammillary area is located above the mammillary body in the hypothalamus and is involved in learning and memory processes.

In the midbrain, the *BARHL1*-expressing cells, detected in a dorsal cell layer in the cortical plate, correspond to the progenitor cells of the superior and inferior colliculi. The superior colliculi control the visual reflexes in the coordination of the head and eye movements, and the inferior colliculi connect the auditive receptors of the ear and the cerebral cortex. In the hindbrain, *BARHL1* was detected in the rhombic lips, which contain the progenitor cells of the cerebellum [1,2,4,41] known as the primary motor coordination center of the CNS and also involved in cognitive processing and sensory discrimination.

To perform a more detailed analysis of *BARHL1* expression in the more differentiated fetal cerebellum, we used our new optical microscopy technology [19] allowing direct observation at cellular levels of the in situ hybridization sections without staining. Interestingly, we found that the external and internal granular cells highly expressed *BARHL1* while the future Purkinje cells do not seem to transcribe it. This result was confirmed by two additional data: first, by the expression pattern of the *Barhl1* in the adult mouse cerebellum, where the well identifiable monolayer of mature Purkinje cells does not transcribe *Barhl1*; second, by the expression pattern of the human *SIM2*, which is transcribed in both cerebellar granular and Purkinje cells [25]. Therefore, external and internal granular cell layers expressed *BARHL1* during all their development: from their initial determination in the rhombic lips, during their determination in the external granular cells and precursors of the internal granular cells, and during their migration to constitute the internal granular cell layer and in the adult cerebellum.

These findings indicated that *BARHL1* transcriptional control mechanisms were characterized by a regional and cellular specificity. This specific spatio-temporal expression pattern of *BARHL1* suggests a potential role of this gene in the development of the CNS regions and cell subsets that expressed it.

Interestingly, several members of the *BarH* homeogene family have been demonstrated to be involved in BMP responses: *Barx1* expression pattern corresponds to the *BMP-4* expression in face primordia in chick and its transcription is reduced by BMPs [14]; the *Xenopus* members *Xvent-2* and *Xvent-1* and their related genes also depend from BMP signals [15,16,42–45]; the *Drosophila BarH1* and *BarH2* [6,7], from which the subfamily name derived, are involved in eye and nervous system development, in response to decapentaplegic (dpp, the *Drosophila* BMP homolog) signals [8,46].

The regions that express *BARHL1* correspond to CNS domains where the BMPs play important roles in their development. In the developing mesencephalon, BMPs are expressed in the floor plate [47]. In the developing spinal cord, BMPs are produced in the roof plate. The different subsets of dorsal sensory interneurons differ in their dependence on roof plate BMP signals for their proliferation, migration, and differentiation [39]. Among them, the dl1 sensory interneurons have a completely BMP-dependent differentiation [39]. In the cerebellar anlage, the dorsal midline cells adjacent to the rhombic lip express BMP proteins that induce granular cell precursor differentiation [48,49].

Interestingly, it has been demonstrated that the mouse *Barhl1* expression in cerebellar granular cells and dorsal sensory interneurons of the spinal cord depends on Math1, a transcription factor involved in BMP response pathways [50]. *Math1* is also expressed in the inner ear [50,51] where it has been demonstrated that *Barhl1* is expressed and plays an important role for ear sensory cell development [52]. Math1 is the homolog of the *Drosophila* atonal (ato), which interacts with BarH in different pathways during the development of the eye disk [9]. In addition, the *Barhl1*-expressing dl1 dorsal interneurons also express *Lhx2*, the vertebrate homolog of *Drosophila apterous* gene [12,13], which interact to BarH proteins in response to Wg and Dpp signals during leg development [10,11].

The highly specific *BARHL1* expression pattern that we found in CNS regions depending on BMP action during CNS development, its correspondence to the expression pattern of BMP signaling genes, and previous functional data of *Barhl1* and other *BarH* subfamily members suggest a role of BARHL1, similarly to MATH1, in the mediation of specific responses to BMPs in the *BARHL1*-expressing regions. BARHL1 protein could effect its molecular function interacting to different pathways involved in BMP transduction signals and the differentiation of specific cell subsets may be determined by specific combination of transcription factors [9,12,13,39]. On this basis, it appears that the molecular function of the *BarH* homeogene family is conserved from *Drosophila* to human.

Barhl1 has been demonstrated to be involved in cerebellar development [5]. *Barhl1* null mice showed attenuated foliation and hypotrophy of the cerebellum caused by aberrant radial migration and increased granular cell death [5]. Moreover, in these mice, a decreased expression of

neurotrophin 3 (*NT-3*) gene in cerebellar granular cells has been demonstrated indicating that *Barhl1* plays a crucial role in cell migration and in the inhibition of apoptosis in cerebellar cells by activation of *NT-3* during cerebellar development [5]. Interestingly, BMP interactions with neurotrophin-3 (NT-3) is important for neuron survival [53–56] and neurite outgrowth and survival [57]. The demonstration that Barhl1 induces *NT-3* expression in mouse cerebellum [5] is in agreement to a potential role of Barhl1 in transduction pathways coordinating BMP and NT-3 signals.

The human *BARHL1* expression pattern and previous functional studies of the mouse *Barhl1* suggest an important role of this gene during CNS development, particularly in the cerebellum. Therefore, *BARHL1* mutations could be responsible of neurological pathologies, in particular at the cerebellar level.

The human *BARHL1* gene is localized in 9q34 band on chromosome 9. Interestingly, this region has been linked to some cases of the Joubert syndrome [58]. This pathology is characterized by agenesis or dysgenesis of the cerebellar vermis with accompanying brainstem malformations and the molar tooth sign of the midbrain–hindbrain junction [59] and abnormal eye movements [60]. To date no mutations have been found in *BARHL1* in analyzed Joubert syndrome patients [61,62], but in these studies, only the coding region has been analyzed. Therefore, on the basis of our and functional findings, *BARHL1* could remain a good candidate gene for Joubert syndrome.

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