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Unique Expression and Localization of Aquaporin- 4 and Aquaporin-9 in Murine and Human Neural Stem Cells and in Their Glial Progeny

CHIARA CAVAZZIN,[1,†] DANIELA FERRARI,[1,†] FLORIANA FACCHETTI,[2] ANNA RUSSIGNAN,[2] ANGELO L. VESCOVI,[1] CATERINA A.M. LA PORTA,[2,*] AND ANGELA GRITTI[1,*]
[1]*Institute for Stem Cell Research, DIBIT, Fond San Raffaele, Milan, Italy*
[2]*Department of Biomolecular and Biotechnology Science, University of Milan, Italy*

KEY WORDS
neural stem cells; water channels; aquaporins; glia; CNS; subventricular zone

ABSTRACT
Aquaporins (AQP) are water channel proteins that play important roles in the regulation of water homeostasis in physiological and pathological conditions. AQP4 and AQP9, the main aquaporin subtypes in the brain, are expressed in the adult forebrain subventricular zone (SVZ), where neural stem cells (NSCs) reside, but little is known about their expression and role in the NSC population, either in vivo or in vitro. Also, no reports are available on the presence of these proteins in human NSCs. We performed a detailed molecular and phenotypical characterization of different AQPs, and particularly AQP4 and AQP9, in murine and human NSC cultures at predetermined stages of differentiation. We demonstrated that AQP4 and AQP9 are expressed in adult murine SVZ-derived NSCs (ANSCs) and that their levels of expression and cellular localization are differentially regulated upon ANSC differentiation into neurons and glia. AQP4 (but not AQP9) is expressed in human NSCs and their progeny. The presence of AQP4 and AQP9 in different subsets of ANSC-derived glial cells and in different cellular compartments suggests different roles of the two proteins in these cells, indicating that ANSC-derived astrocytes might maintain in vitro the heterogeneity that characterize the astrocyte-like cell populations in the SVZ in vivo. The development of therapeutic strategies based on modulation of AQP function relies on a better knowledge of the functional role of these channels in brain cells. We provide a reliable and standardized in vitro experimental model to perform functional studies as well as toxicological and pharmacological screenings. © 2005 Wiley-Liss, Inc.

INTRODUCTION

Aquaporins (AQPs) are a family of water transport tissue-specific proteins with 11 different subtypes in mammals. Some of these channels (AQP3, AQP7, and AQP9) are also permeable to small noncharged solutes. Disregulation of water balance in the central nervous system (CNS) underlies focal edemas following brain tumors, ischemia, and traumatic injuries (King and Agre, 1996; Badaut et al., 2003). AQP4 and AQP9 are the most abundant brain AQPs (Venero et al., 1999; Saadoun et al., 2002). AQP4 is expressed in ependymal cells lining the ventricular system and subarachnoidal spaces as well as in glial endfeet contacting the blood-brain barrier (BBB), where it is thought to regulate water permeability and cerebrospinal fluid reabsorption (Amiry-Moghaddam et al., 2003, 2004). AQP9 is expressed in astrocytic cell bodies and processes that do not directly contact vascular structures (Elkjaer et al., 2000; Nicchia et al., 2001; Badaut and Regli, 2004). It might participate in regulating water redistribution after osmotic gradients and might play a buffer role around neurons. Also, its role in the regulation of post-ischemia edema has been proposed (Badaut et al., 2001).

Neural stem cells (NSCs) compartments are present in the subventricular zone (SVZ) of the forebrain lateral ventricles (Doetsch et al., 1999; Alvarez-Buylla et al., 2002) and in the dentate gyrus of the hippocampus (Palmer et al., 1997; Gage et al., 1998; Song et al., 2002), the main neurogenetic areas of the adult mammalian brain. Adult murine NSCs (mANSC) show morphological and immunogenic features of glial cells in vivo (Doetsch et al., 1999; Seri et al., 2001). They can be epigenetically isolated and maintained in vitro as undifferentiated cells, or they can be driven to differentiate into neurons and glia (Reynolds and Weiss, 1992; Palmer et al., 1997; Gritti et al., 1999). Progenitors displaying stem cell features have also been isolated from the fetal (Vescovi et al., 1999a,b; Svendsen et al., 1999; Carpenter et al., 1999) and adult (Sanai et al., 2004) human CNS. Both AQP4 and AQP9 expression is found in the adult forebrain periventricular region (Venero et al., 1999; Badaut et al., 2002), where the NSC pool resides. However, little is known about the expression of these proteins in the NSC population, either in vivo or in vitro. Also, no reports are available on the presence of these proteins in human NSCs. Interestingly, many neurodegenerative

*Correspondence to: Angela Gritti, Institute for Stem Cell Research (SCRI), DIBIT, Fondazione S. Raffaele del Monte Tabor, Via Olgettina 60, 20132 Milan, Italy. E-mail: gritti.angela@hsr.it, and Caterina A.M. La Porta, Dept. of Biomolecular and Biotechnology Science, University of Milan, Via Celoria 26, 20133 Milan, Italy. Email: caterina.laporta@unimi.it

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†C.C. and D.F. contributed equally to this work.

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disorders in which the beneficial effect of NSC-based therapy has been proposed or demonstrated are characterized by inflammation, BBB breakdown, water homeostasis disregulation, and alteration in neuronal excitability, pathological processes in which aquaporins play a critical role. Since the presence of NSCs in the SVZ of the adult human brain has been recently demonstrated (Sanai et al., 2004), studying the potential role of AQPs in human NSC and their potential involvement in the development of pathological conditions in which water homeostasis is compromised is of most importance for the development of potential therapeutic strategies.

In the present study, we performed a detailed characterization of different AQPs, and particularly AQP4 and AQP9, in murine and human NSC cultures at predetermined stages of differentiation. We demonstrated that AQP4 and AQP9 are expressed in mANSCs and that their levels of expression and cellular localization are differentially regulated upon mANSC differentiation into neurons and glia. Also, AQP4 (but not AQP9) is expressed by human NSCs. The presence of AQP4 and AQP9 in different subsets of NSC-derived glial cells and in different cellular compartments suggests different roles of the two proteins in these cells and point to the notion that ex vivo isolated ANSC-derived astrocytes might maintain the heterogeneity that characterize the astrocyte-like cell populations in the SVZ in vivo. Thus, we provide a reliable in vitro experimental model to study the role of AQPs in brain cells.

MATERIALS AND METHODS

SVZ-Derived Primary Cultures

Three-month-old C57/BL6 mice (Charles River, Calco, LC, Italy) were anesthetized by intraperitoneal injection of 4% chloral hydrate (0.1 ml/10 mg body weight) and killed by cervical dislocation. The brains were removed and tissue containing the subventricular zone (SVZ) of the forebrain lateral ventricles was dissected out. Tissues derived from seven mice were pooled to generate each culture. Dissected tissues were transferred to Earl's Balanced Salt Solution (Invitrogen, San Diego, CA) containing 1 mg/ml papain (22.9 U/mgP; Worthington, Lakewood, NJ), 0.2 mg/ml cysteine (Sigma, St. Louis, MO), and 0.2 mg/ml EDTA (Sigma) and incubated for 45 min at 37°C on a rocking platform. Tissues were then transferred to DMEM-F12 medium (1/1 v/v; Invitrogen) containing 0.7 mg/ml ovomucoid (Sigma) and mechanically dissociated.

Primary cells were plated on 10-mm Matrigel-coated coverslips (100,000 cells/cm^2) in a chemically defined, serum-free medium (control medium) (Gritti et al., 2002) in the presence of 2% fetal calf serum (FCS) and cultured for 20 days. Cultures were then fixed with paraformaldehyde 4% and processed for indirect immunofluorescence assay, as described below.

Isolation and Culture Propagation of mANSC

Mouse adult neural stem cells (mANSC) were established as previously described (Gritti et al., 1999, 2002). Briefly, primary cells isolated as above were plated in control medium containing basic fibroblast growth factor (FGF2) and epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ; 10 and 20 ng/ml, respectively; growth medium) (Gritti et al., 2002). Under these culture conditions, a fraction of cells proliferate forming clonal spheres that float in suspension (neurospheres). Primary neurospheres were collected, mechanically dissociated to a single cell suspension and replated in growth medium (3,500 cells/cm^2). This procedure was repeated twice; bulk cultures were then generated by replating cells in growth medium at a density of 10^4 cells/cm^2. Bulk cultures were routinely assessed for self-renewal and multipotentiality as previously described (Gritti et al., 1999). In addition, individual primary and serially passaged neurospheres were plated in differentiated conditions and processed for indirect immunofluorescence assay, as described below.

Isolation and Culture Propagation of hFNSC

Multipotential neural stem cell lines were originally established from the diencephalons (DE) of the 10.5-week post-conception human brain, as previously described (Vescovi et al., 1999b).

Homogeneous batches of cells between passages 15 and 20 were used in this study. Cells were grown and expanded in growth medium by plating 10^4 cells/cm^2 at each subculturing passage in untreated tissue culture flasks.

Cultures of mANSC and hFNSC at Different Stages of Maturation

The first cell preparation is obtained by dissociating 5- or 10-day-old neurospheres for mANSC and hFNSCs, respectively, and growing them for 24 h in growth medium, and consists of an enriched population of highly undifferentiated and proliferating cells (precursor cells). The second cell preparation (committed progenitors) consists of cells grown for three days in the presence of an adhesion substrate, and in serum-free medium containing FGF2. These cultures, that contain glial and neuronal progenitors at different stages of commitment, are exposed to serum-free medium containing 2% FCS and grown for additional 5/7 days, to achieve terminal differentiation of neural progenitors into neurons, astrocytes and oligodendrocytes (differentiated cells). The extent of neuronal and glial differentiation/maturation in the different cell fractions has been assessed by using antibodies against lineage- and stage-specific markers, as described below.

Immunostaining of NSC Cultures

Double-labeling immunofluorescence was performed as previously described (Gritti et al., 1999, 2002). Briefly, cultures were fixed in 4 % paraformaldehyde, rinsed with phosphate-buffered saline (PBS) and incu-

bated for 90 min at 37°C in PBS containing 10% normal goat serum (NGS), 0.3% Triton X-100 (omitted when antibodies against membrane-bound antigens were used), and appropriate primary antibodies. After washing, cells were reacted for 1 h at room temperature (RT) with the appropriate secondary antibodies. Samples were rinsed three times with PBS, once with distilled water, and mounted with Fluorsave (Calbiochem, La Jolla, CA). Primary antibodies used: mouse monoclonal anti-GFAP (Chemicon, Temecula, CA; 1:200), anti-β-tubulin type III (Tuj1; 1:400; Covance, UK), anti-O4 (Chemicon; 1:50), anti-GalC (Chemicon; 1:200), anti PSA-NCAM (AbCys, Paris, France; 1:1,000), anti-KDEL (Abcam, Cambridge, UK; 1:300); affinity-purified rabbit polyclonal antibodies anti-AQP 1, 4, 8 and 9 (Alpha Diagnostic International, San Antonio, TX; IgG, 1:100 for AQP1, AQP 9, and AQP8; 1:500 for AQP4). Secondary antibody used: Alexafluor 546- and Alexafluor 488-conjugated goat anti-mouse or goat anti-rabbit IgG (1:2,000; Molecular Probes, Eugene, OR). To label the Golgi apparatus a monoclonal TRITC-conjugated anti-WGA (wheat germ lectin, Sigma; 1:200) was used. Samples were examined and photographed using a Nikon Eclipse 3000 fluorescence microscope. Negative control samples included NSC cultures in which primary antibodies were omitted and cultures of murine myoblasts (C2C12 cells) and human epithelial HeLa cells, which do not express the two AQPs, treated with primary and then secondary antibodies against AQPs. In both cases, no positive signal was observed. Nuclei were counterstained with 4,6-diamine-2-phenylindole dihydrochloride (DAPI; Sigma; 50 μg/ml in PBS) for 15 min at RT. Immunoreactive cells were counted in at least 5 nonoverlapping fields in each sample (>500 cells/sample) and expressed as a percentage of the total number of nuclei. Confocal images were taken with a three-laser confocal microscope (Leica TCS SP2). Fluorescent signals from single optical sections were sequentially acquired and analyzed by Photoshop 7.0 (Adobe). Data are the mean ± SD of two to three samples/experiments in three independent experiments.

Tissue Processing and Immunohistochemistry

Anesthetized mice were perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA). Brains were equilibrated for 24 h in 30% sucrose in PBS and were quick-frozen in optimal cutting temperature compound. 20-μm coronal cryostatic sections were blocked in PBS/10% goat serum/0.1% Triton X-100/1% bovine serum albumin (BSA), incubated overnight with primary antibodies at 4°C: mouse monoclonal anti-nestin (Chemicon; 1:200), anti-Tuj1 (Covance; 1:800), anti GFAP (Chemicon; 1:200); affinity-purified rabbit polyclonal antibodies, anti-AQP4 and anti-AQP9 (1:400 and 1:100, respectively), and then with Alexafluor-546 or Alexafluor-488 (Molecular Probes; 1:2,000) in blocking solution at room temperature for 2 h, washed, coverslipped in permanent mounting medium, and examined under a Leica confocal microscope.

RT-PCR

Total RNA was prepared from murine- and human-derived neurospheres, precursor cells, committed progenitors, and differentiated cells using the RNeasy Mini Kit from Qiagen. Brain, liver, and intestine samples obtained from syngenic mice were used as positive controls. A murine myoblast cell line (C2C12) were used as negative control. In this study, 1 μg of total RNA was reverse transcribed and amplified by means of an Enhanced avian hs reverse transcription-polymerase chain reaction (RT-PCR; Sigma) according to the manufacturer's instructions. To avoid the possibility of a genomic amplification of AQPs, control RT-PCR reactions were performed by omitting the reverse transcriptase or the RNA templates. β-Actin was used as housekeeping gene:

Primers used for mouse AQPs: sAQPO 5′′-ATGTGGGAACTTCGGTCTGC-3′, rAQPO 5′-TTACAGGGCCTGAGTCTTCA-3′ (792 bp); sAQP1 5′-GCCAGCATGGCCAGTGAAAT-3′, rAQP1 5′-GTGGAGATGAAGCCCAAATAG-3′ (815 bp); sAQP2 5′-GCAGCATGTGGG- AACTCCGG-3′, rAQP2 5′-AGACCCTTCTTGAGGCTCAC-3′ (955 bp); rAQP3 5′-TAGCTACTTTGCACTCGTAC-3′, sAQP3 5′-ACACAGTCAGTGGACACTCA-3′ (869 bp); sAQP4 5′-AAAACCCCTTACCTGTGGAC-3′, rAQP4 5′-TCCTCCACCTCCATGTAGCTC-3′ (657 bp); sAQP5 5′-AT CTACTTCACCGGCTGTTCC-3′, rAQP5 5′-GTCAGCTCGA TGGTCTTCTTC-3′ (260 bp); sAQP7 5′-AGACACAGAGCACTTCAGAG-3′, rAQP7 5′-ATTGCAGTACGGTTGTGTT-3′ (1153 bp); sAQP8 5′-GGTGGACACTTCAACCCTGC 3′, rAQP8 5′-CCCAGCCAGTAGATCCAATG 3′ (437 bp); sAQP9 5′-CACTCAATGATGACGCTGAG-3′, rAQP9 5′-TTTGACTCCAGAAACCTGGG-3′, (355bp).

Cell cycle conditions were for AQP0, 1, 2, 3, 7, 8, and 9: 95°C 1 min, 55°C 1 min, 72°C 1, 3 min, 33 cycles and for AQP4 and AQP5: 94°C 45 s, 50°C 1 min, 72°C 1 min, 35 cycles.

Primers used for human: sAQP4 5′-GGAATCCTCTATCTGGTCACA-3′, rAQP4 5′-TGTTTGCTGGGCAGCTTTGCT-3′ (429 bp); sAQP5 5′-GGTGTGCTCCGTGGCCTTCCT-3′, rAQP5 5′-CTTCCGCTCTTCCCGCTGTCC-3′ (759 bp); sAQP8 5′-CCTGCTTGTTGGACTGCTC-3′, rAQP8 5′-AAATGGGGTGCGGGAAATGAG-3′ (419 bp); sAQP9 5′-GCCTAGAGCCCATTGCCATCG-3′, rAQP9 5′-TGGTTTGTCCTCAGATTGTTC-3′(289 bp). Cell cycle conditions: AQP4: 92°C 30 s, 65°C 30 s, 72°C, 30 s, 35 cycles; AQP5 and AQP8: 92°C 30 s, 65°C 30 s, 72°C 30 s, 35 cycles; AQP9: 94°C 60 s, 58°C 60 s, 72°C 60 s, 30 cycles.

PCR products were visualized by ethidium bromide staining followed by electrophoresis on 1.5% agarose gel. The identity of murine and human AQPs sequences amplified by RT-PCR was verified by sequencing the related complementary DNA.

RESULTS

AQP4 and AQP9 Expression in the Periventricular Region of the Adult Forebrain

Previous work reported the specific presence of AQP4 and AQP9 in periventricular areas (Elkjaer et al., 2000;

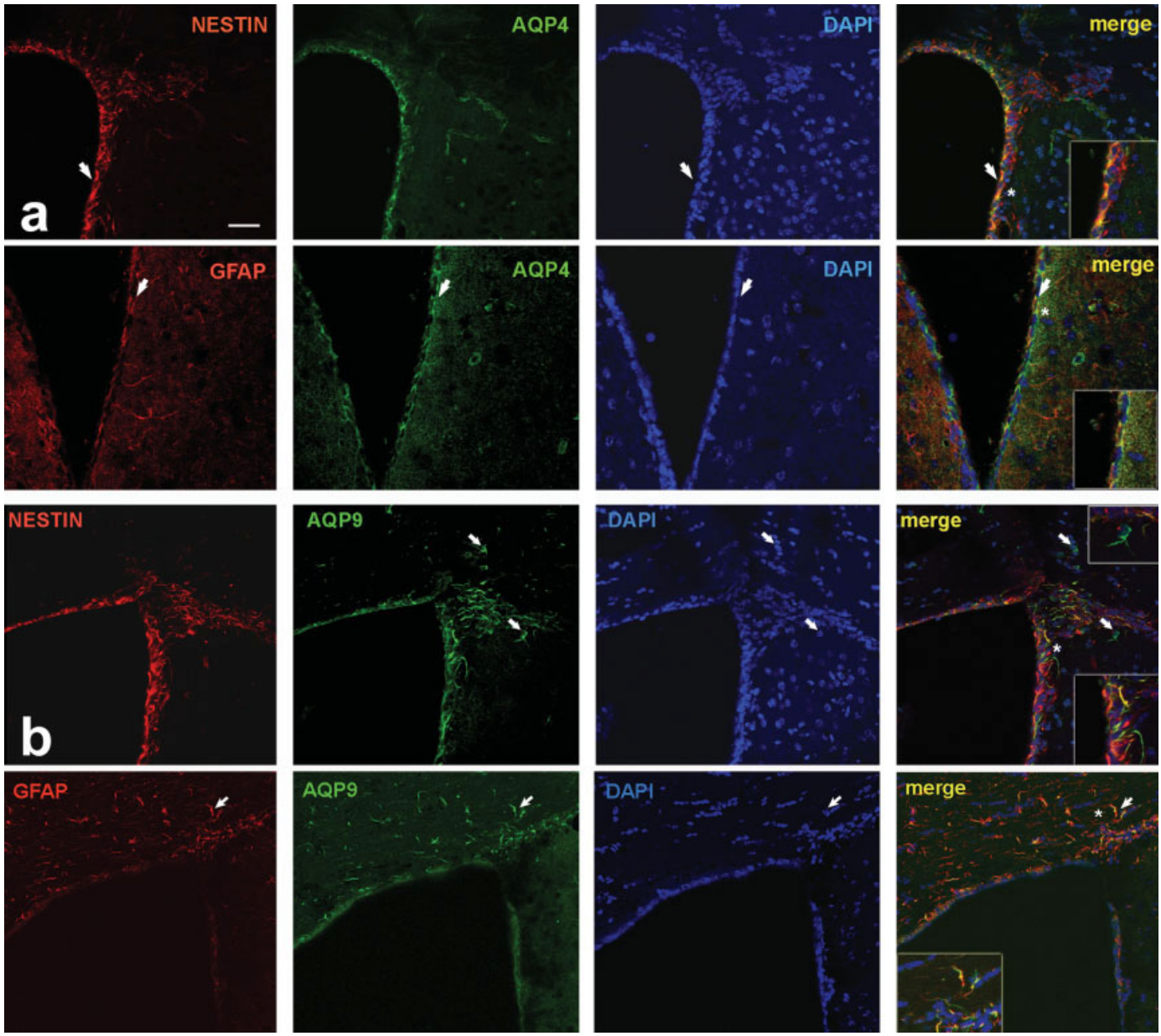

Fig. 1. AQP4 and AQP9 are expressed in different subsets of glial cells in the adult forebrain periventricular region. Confocal analysis of tissue sections comprising the forebrain lateral ventricles demonstrate that AQP4- (**a**) and AQP9-expressing cells (**b**) are present in the ependymal and subependymal layer, and that some of them co-express nestin or GFAP. Green, AQPs; red, nestin, and GFAP; blue, cell nuclei visualized by DAPI. Arrows identify cells in the fields. Asterisks identify cells shown at higher magnification in insets. Note two cells that express AQP9 but are negative for nestin (b, top and inset). Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). Original magnification ×630. Scale bar = 500 μm.

Badaut et al., 2002). By indirect immunohistochemistry followed by confocal analysis, we found the expression of both AQP4 and AQP9 in the periventricular region surrounding the forebrain lateral ventricles and in the adjacent parenchyma (Fig. 1). $AQP4^+$ cells mainly lined the ventricular walls with an ependymal-like distribution and co-expressed nestin, a marker of precursor cells in the SVZ (Doetsch et al., 1997, 1999) that is also expressed by adult ependymal cells (Mignone et al., 2004). Some $AQP4^+$ cells also expressed GFAP (a marker of SVZ astrocytes) (Fig. 1a). AQP9 labeling was present mainly in the subventricular region, on cell bodies and processes (Fig. 1b). Most of $AQP9^+$ cells expressed nestin, many of them displayed a glial-like morphology and co-expressed GFAP. We never found $AQP4^+$ or $AQP9^+$ cells co-expressing TUJ1 or PSA-NCAM (markers of SVZ neuroblasts) (see Supplementary Fig. 1). These results indicate that, in the adult brain, distinct subsets of periventricular glial cells express AQP4 and AQP9.

AQP4 and AQP9 Expression in SVZ-Derived Primary Cultures

To test whether the cell type specificity of expression of AQPs was retained in vitro, we prepared primary cultures derived from SVZ tissue. Since SVZ comprises both neuronal and glial precursors, we were expecting to obtain differentiated cultures containing astrocytes, neurons, and oligodendrocytes. Three weeks after plating, we found large numbers (30–40%) of TUJ1$^+$ cells in our cultures, some of them still appearing in clusters most likely derived from the proliferation of restricted numbers of neuroblasts (Fig. 2A,D). Some of the TUJ1$^+$ cells also expressed MAP2, a marker of mature neurons, which was also present in a fraction of TUJ1$^-$ cells displaying long, thin processes with evident varicosities (not shown). Primary cultures also contained large numbers of GFAP$^+$ cells (60–70%), displaying variable morphology, but mainly resembling polygonal astrocytes (Fig. 2B,E), as well as a minor proportion of oligodendrocytes, identified by GalC immunoreactivity (Fig. 2C,F). We did not observe TUJ1$^+$ or MAP2$^+$ cells co-expressing AQP4 or AQP9 (Fig. 2A,D). On the contrary, both proteins were expressed by GFAP$^+$ cells, although in different proportions and with a different cellular localization. AQP4 labeling was present in a large fraction (>60%) of GFAP$^+$ cells and the signal was apparently localized in the cell membrane (Fig. 2B), while a smaller subset of GFAP$^+$ cells expressed AQP9, with a typical intracellular localization resembling the basket-like appearance of cytoskeleton proteins (Fig. 2E). AQP4 and AQP9 were also expressed by some GalC$^+$ cells (Fig. 2C,F).

AQP4 and AQP9 Expression in SVZ-Derived ANSCs and in Their Progeny at Different Stages of Differentiation and Maturation

To evaluate whether AQP4 and AQP9 were indeed expressed in adult SVZ cells endowed with stem cell potential, we established cultures from the forebrain periventricular region under conditions in which only a minor fraction of cells proliferate, forming primary neurospheres, and generated serially passaged, nontransformed NSC lines by epigenetic stimulation (Gritti et al., 1999). We then evaluated the expression of different AQPs (AQP0, 1, 2, 3, 4, 5, 7, 8, and 9) in primary as well as in serially passaged neurospheres (subculturing passages 4, 9, and 14) by means of RT-PCR. Tissues obtained from brain, liver, or intestine of syngenic mouse and the murine myoblast cell line C2C12 were used as positive and negative controls, respectively. We found the transcripts for AQP4, AQP8, and AQP9 in both primary (not shown) and serially passaged neurospheres (Fig. 3A, lane 1), with lower levels of expression of AQP8 with respect to the other two AQPs, while expression of AQP5 and of AQP0, 1, 2, 3, and 7 (data not shown) was not detected. These results showed that ANSCs and their progeny in the neurospheres expressed the same AQPs that are mainly expressed in the brain,

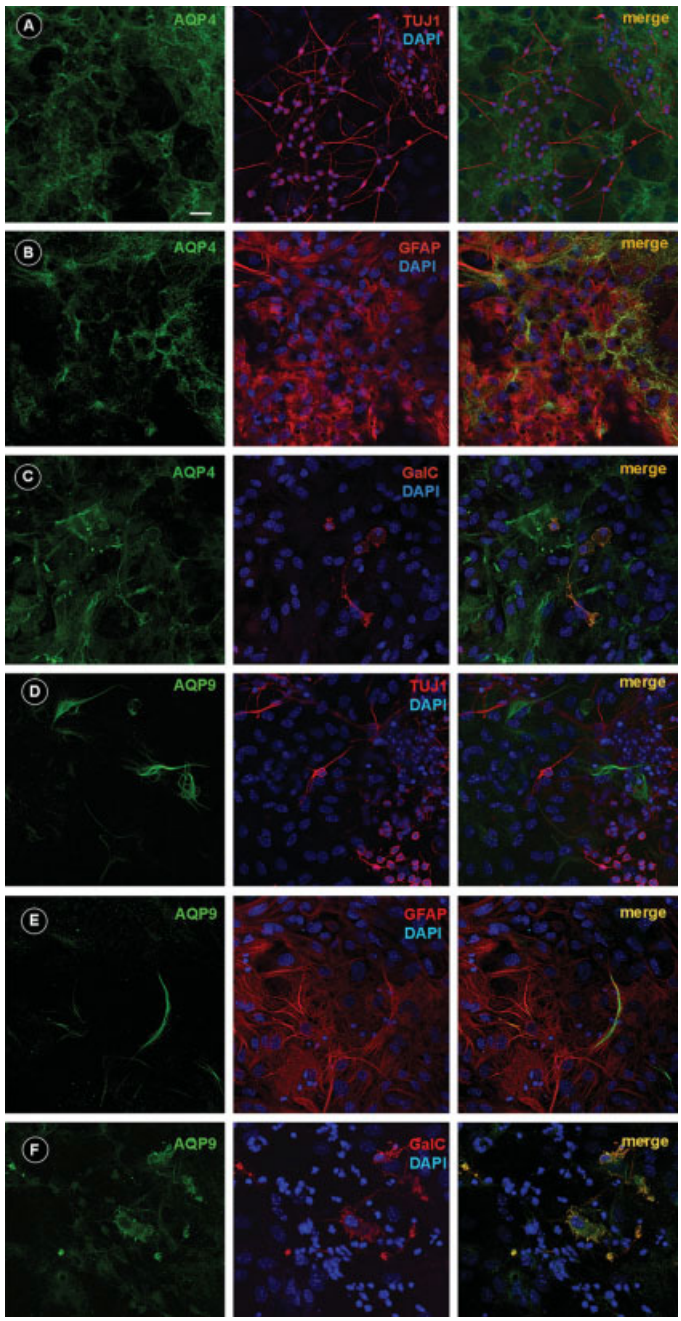

Fig. 2. AQP4 and AQP9 are expressed in astrocytes and in a fraction of oligodendrocytes in SVZ-derived primary cultures. Confocal analysis on 20-day-old primary cultures established from SVZ tissue revealed no AQP4 and AQP9 expression in TUJ1-expressing neuronal cells (**A,D**). As shown in the merged pictures, a large fraction of GFAP$^+$ astrocytes co-expressed AQP4 (**B**), while only a fraction of them co-expressed AQP9 (**E**). The two AQPs were expressed also by some GalC$^+$ oligodendrocytes (**C,F**). Green, AQP4; red, TUJ1, GFAP, and GalC; blue, cell nuclei visualized by DAPI. Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). Original magnification ×400. Scale bar = 15 μm.

and that this feature was maintained through serial passages in cultures. However, this analysis did not give any information on the cell type/s actually expressing

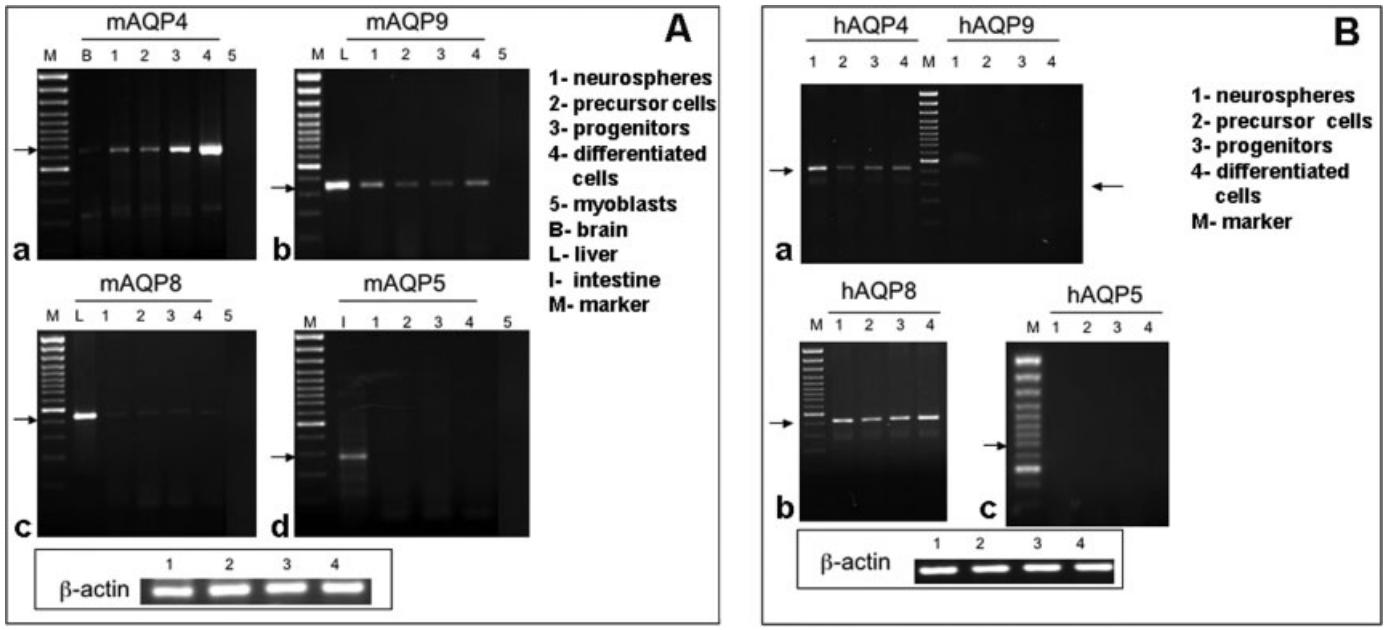

Fig. 3. AQP4 and AQP9 are expressed in NSCs and their differentiated progeny. **A:** Semiquantitative RT-PCR shows high levels of AQP4 and AQP9 messenger in SVZ-derived, serially passaged (passage 4) murine adult neural precursor cells (**lane 2**) as well as in NSC-derived passage 4 neurospheres (**lane 1**), committed progenitors (**lane 3**), and differentiated cells (**lane 4**). Upregulation of AQP4 (a) but not of AQP9 (b) was found following NSC differentiation. Low levels of AQP8 expression could be detected in all cell fractions (c), while no expression of AQP5 was found (d). Similar results were found in primary neurospheres, and in mANSCs-derived cell preparations analyzed at passages 9 and 14. **B:** The same analysis made on human neural stem cells (passage 18) shows AQP4 (a) and AQP8 (b) expression in all the cell fractions. Again, no AQP5 expression is found (c). Quantitative comparison was made with the housekeeper gene β-actin.

those AQPs. In fact, neurospheres represent a mixed neural population, containing stem cells as well as committed progenitors and differentiated glial and neuronal cells (see below). It is known that plating stem cell progeny onto an adhesion substrate and removing mitogens promotes a progressive differentiation process, resulting in the production of mature neuronal and glial cells (Vescovi et al., 1999b; Gritti et al., 2002) (see Materials and Methods). We evaluated the presence of AQP4- and AQP9-expressing cells in SVZ-derived differentiated primary (not shown) and serially passaged neurospheres (Fig. 4). We found that none of the two proteins was expressed in $TUJ1^+$ neuronal cells, whereas AQP4 was expressed virtually in all astrocytes and in some oligodendrocytes, while AQP9 was present in a fraction of $GFAP^+$ astrocytes and oligodendrocytes.

To evaluate whether the expression of AQPs was regulated during the process of NSC differentiation and to investigate the expression of AQPs in mANSC-derived glial and/or neuronal progeny we generated from neurospheres (subculturing passages 4, 9, 14) three types of cultures: (1) a cell population in which virtually all the cells express the neuroepithelial marker nestin, incorporate S-phase markers such as BrdU and [^{3}H]thymidine, are immunoreactive for proliferating cellular nuclear antigen (PCNA), and in a serial subcloning assay are clonogenic, self-renewing, and multipotent (precursor cells); (2) a cell population enriched in committed neuronal and glial progenitors (committed progenitors) that begin to express markers of differentiation (i.e., TUJ1, O4, and GFAP; for quantitative analysis, see Table 1); of note, neuronal and oligodendroglial markers appear first, while markers of mature astrocytes are still scarcely represented in this cell fraction; and (3) the differentiated and mature progeny of precursor and progenitor cells, consisting of cells expressing high levels of either neuronal or glial markers (differentiated cells). The extent of cell differentiation/maturation in the different fractions was assessed by lineage-specific markers (see also Materials and Methods and Table 1 for quantitative analysis). On these cell fractions we performed RT-PCR analysis by using the same paradigm described for the analysis of neurospheres (see above). The results are summarized in Figure 3A and show that AQP4, 8, and 9 are expressed in all cell fractions (lanes 2, 3, and 4). No expression has been detected for AQP5 and for AQP0, 1, 2, 3, and 7 (data not shown). A progressive upregulation of AQP4 expression was detected in committed progenitors and in differentiated cells with respect to their undifferentiated counterpart and to neurospheres, as shown by comparing the relative levels of β-actin, while no striking differences in the levels of expression of other AQPs in relation to cell differentiation were found (Fig. 3A).

To confirm the data obtained by RT-PCR analysis, we then evaluated and quantified the presence of AQP4 and AQP9 proteins in the same cell fractions by indirect immunofluorescence (see Table 1 for quantitative analysis). We found that AQP4 and AQP9 are expressed in a variable but consistent number of cells in the undifferentiated cells and committed progenitors (Fig. 5). Upon terminal differentia-

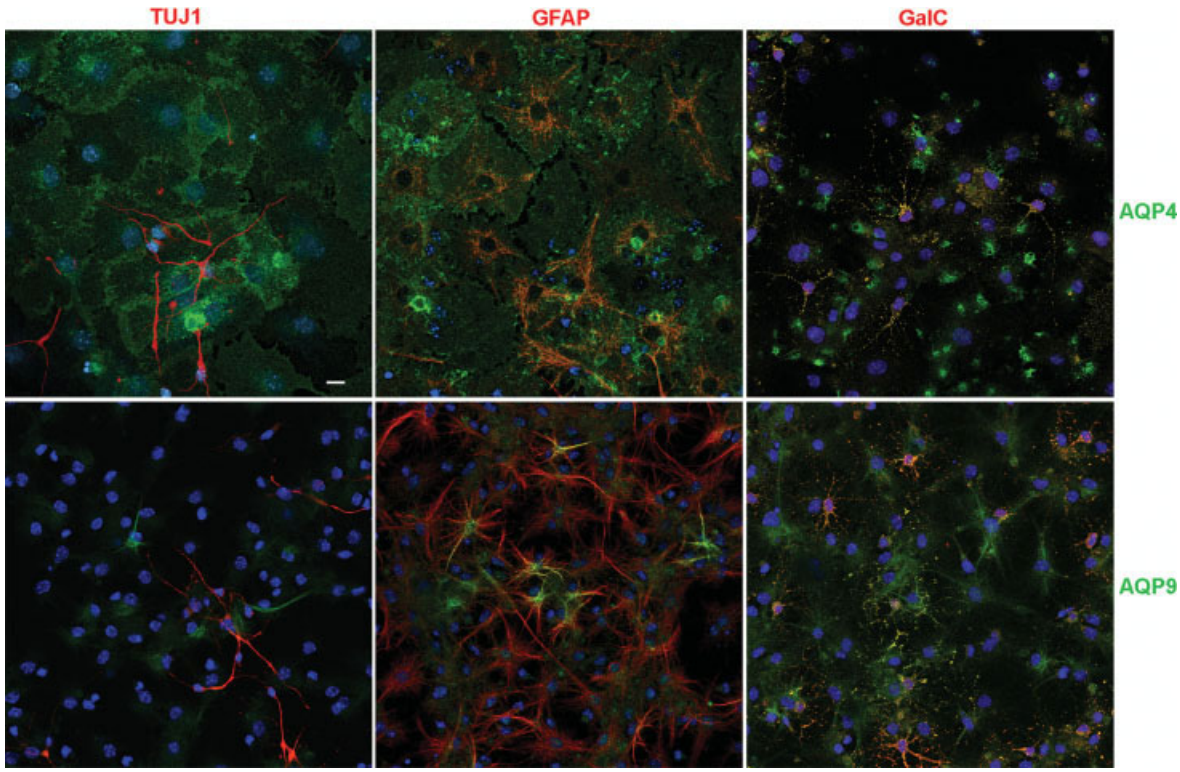

Fig. 4. Glial cells in SVZ-derived neurospheres express AQP4 and AQP9 proteins. Serially passaged (passage 4) ANSC-derived neurospheres were plated in differentiating conditions (see Materials and Methods) and the presence of AQPs in the different cell types was evaluated by means of double labeling immunofluorescence followed by confocal analysis. Green, AQP4 and AQP9; red, GFAP, TUJ1, and GalC; blue, cell nuclei visualized by DAPI. About 70% and 30% of $GFAP^+$ cells in the spheres expressed AQP4 and AQP9, respectively, and the two AQPs were localized in different cell compartments (cell membranes and cytoplasm for AQP4 and AQP9, respectively). Both AQPs were expressed also in a fraction of $GalC^+$ oligodendrocytes, but were absent in $TUJ1^+$ neuronal cells. The same analysis performed on primary neurospheres gave similar results. Fluorescent signals were sequentially acquired from single optical sections and are shown after merging. Original magnification ×400. Scale bar = 15μm

tion of mANSCs, expression of AQP4 protein was found in the vast majority of cells in the cultures (75.39% ± 9.08%), and the expression was mainly present in the cell membranes of the $GFAP^+$ astrocytic fraction (Fig. 6a). However, cultures also contained $AQP4^+GFAP^-$ cells (13.8% ± 11.52%) as well as $AQP4^-GFAP^+$ cells (6.57% ± 2.93%), and we could observe different levels of expression of AQP4 and GFAP among the double positive cells (Fig. 6a,b,c). Conversely, only a subpopulation of NSC-derived differentiated cells expressed AQP9 (23.44% ± 9.13%) and all of them expressed GFAP (Fig. 6d). AQP9 labeling was intracellular, with the same pattern previously observed in astrocytes of primary cultures. Immunoreactivity for AQP4 and AQP9 was present in a variable fraction (up to 50%) of oligodendroglial ($GalC^+$) cells (Fig. 6c,f). Interestingly, a weak immunoreactivity for both AQPs was occasionally found also in mANSC-derived $TUJ1^+$ cells (Fig. 6b,e).

*TABLE 1. Regulation of AQP Expression in mANSC and in Their Glial and Neuronal Progeny at Different Stages of Differentiation**

	% of immunoreactive (IR) cells		
	Precursor cells[a]	Committed progenitors[a]	Differentiated cells[a]
Marker[b]			
TUJ1	2.16 ± 1.81	7.59 ± 4.11	9.72 ± 4.15
PSA-NCAM	ND	ND	6.85 ± 2.33
GFAP	4.30 ± 3.11	8.70 ± 2.54	73.80 ± 9.73
GalC	ND	ND	9.17 ± 4.58
O4	1.60 ± 2.26	4.20 ± 5.94	4.95 ± 0.63
AQP4	78.77 ± 19.67	88.74 ± 17.76	75.39 ± 9.08
AQP9	84.87 ± 19.98	79.32 ± 27.32	23.44 ± 9.13

ND, not determined.
[a]The three cell fractions were prepared from undifferentiated mANSCs, as described in the text in Materials and Methods and Results.
[b]The extent of cell differentiation was evaluated by quantifying the number of cells immunoreactive (IR) for neuronal (TUJ1, PSA-NCAM), astroglial (GFAP) and oligodendroglial (GalC, O4) markers.
*Data are expressed as number of IR cells/total number of cells in the cultures: mean ± SD, two to three coverslips/experiment, three or four independent experiments.

AQP4 and AQP9 Expression in hFNSCs

In a manner similar to that of their murine counterpart, NSCs isolated from the human fetal brain can be grown extensively in culture in the presence of EGF and FGF2, maintaining stable proliferation and self-renewal capacity over time (Vescovi et al., 1999b). Also, removal and/or substitution of growth factors drives a progressive restriction of their lineage potential, resulting in the generation of terminal differentiated neuronal and glial cells (Galli et al., 2002). We thus applied the same experimental paradigm described for ANSCs to obtain

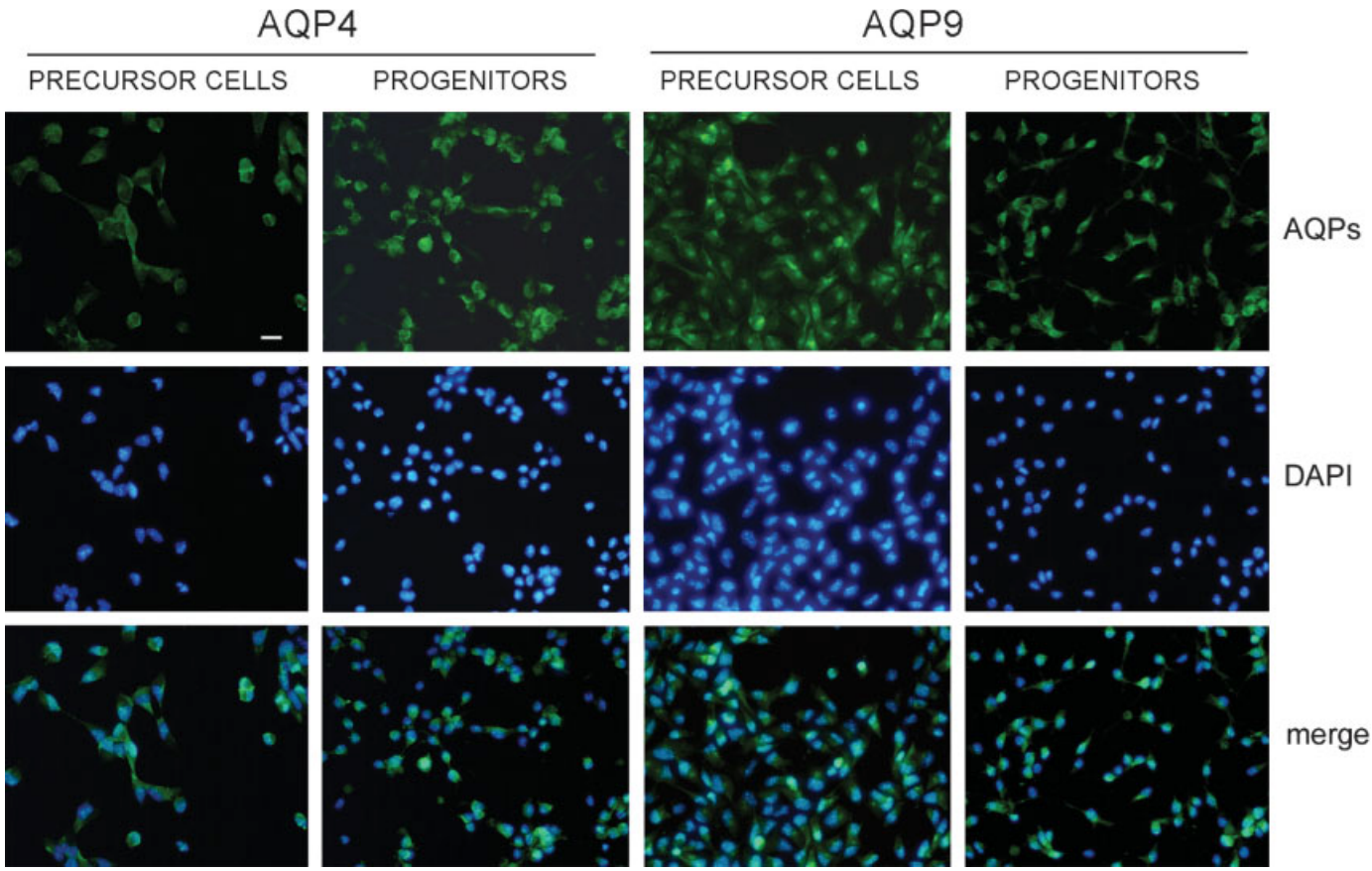

Fig. 5. AQP4 and AQP9 proteins are expressed in consistent numbers of undifferentiated mANSCs and their committed progenitors. The expression of both AQPs was evaluated by immunofluorescence analysis on the different cell fractions prepared as described in Materials and Methods. The vast majority of cells in the stem cell and in the progenitor cell fractions expressed AQP4 and AQP9, as shown in the merged images (green, AQPs; blue, cell nuclei counterstained with DAPI). See Table 1 for quantitative analysis. The picture shows NSCs at subculturing passage 9. Cells analyzed at subculturing passages 4 and 14 gave similar qualitative and quantitative results. Scale bar = 15μm.

hFNSC-derived cultures at specific stages of differentiation (precursor cells, committed progenitors, and differentiated cells). The extent of differentiation/maturation in the cell fractions was assessed by lineage-specific markers, as described for ANSCs. On these cell fractions, we performed RT-PCR analysis and immunocytochemistry to look for the expression of different AQPs. We found that AQP4 and AQP8 are expressed in all cell fractions while no expression has been detected for AQP5, AQP9 (Fig. 3B) and AQP0, 1, 2, 3, 7 (data not shown). As opposed to mANSCs, no detectable changes in the levels of AQP4 expression was detected in progenitors and in differentiated cells with respect to their undifferentiated counterpart and to neurospheres. The presence of AQP4 and AQP9 was evaluated in precursor cells, progenitors, and differentiated cells by means of indirect immunofluorescence, followed by confocal analysis. We found expression of AQP4 in a consistent fraction (>70%) of precursor cells and progenitors (Fig. 7a,b). Upon terminal differentiation of hFNSCs, expression of AQP4 protein was found in the vast majority (>80%) of GFAP-immunoreactive (IR) astrocytes (Fig. 7c), but no AQP4-IR was found in $TUJ1^+$ neuronal cells (Fig. 7d). In differentiated hFNSC-derived cultures

Fig. 6. Distinctive expression and localization of AQP4 and AQP9 in mANSC-derived differentiated cells. Serially passaged (passage 9) mANSCs were plated in differentiating conditions (see Materials and Methods) and the presence of AQPs in the different cell types was evaluated by means of double labeling immunofluorescence followed by confocal analysis. **a–c:** Variable numbers of $AQP4^+GFAP^+$ cells were present in the cultures, as shown in the merged pictures. Cells expressing low levels of AQP4 (arrowheads in b,c) and displaying high levels of GFAP ($AQP4^-GFAP^+$; arrow in a) were present, together with cells expressing high levels of AQP4 and low levels of GFAP ($AQP4^+GFAP^-$; arrowhead in a). **d–f:** AQP9-IR was present in a smaller proportion of cells and was localized mainly intracellularly with respect to the widespread and membrane-bound AQP4-IR, but all $AQP9^+$ co-expressed GFAP (d). AQP4- and AQP9-IR were also present in $GalC^+$ oligodendrocytes (c, f) and, occasionally, in $TUJ1^+$ neurons (b,e). See Table 1 for quantitative analysis. Cells analyzed at subculturing passages 4 and 14 gave similar qualitative and quantitative results. Green, AQP4 and AQP9; red, GFAP, TUJ1, and GalC; Fluorescent signals were acquired from single optical fields and are shown individually and after merging (merge). Original magnification ×400. Scale bars = 15μm in d–f; 20μm in a–c.

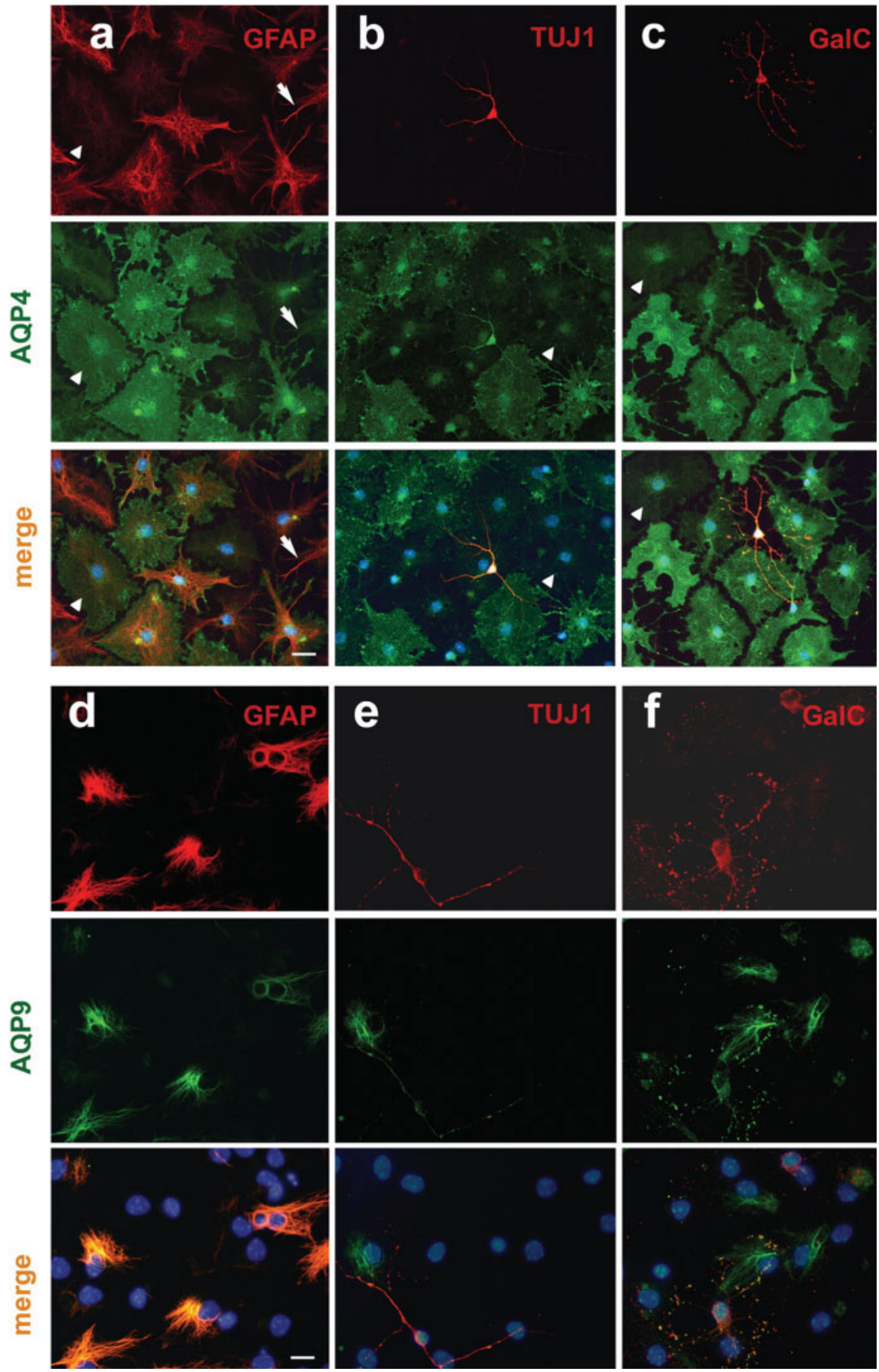

Figure 6.

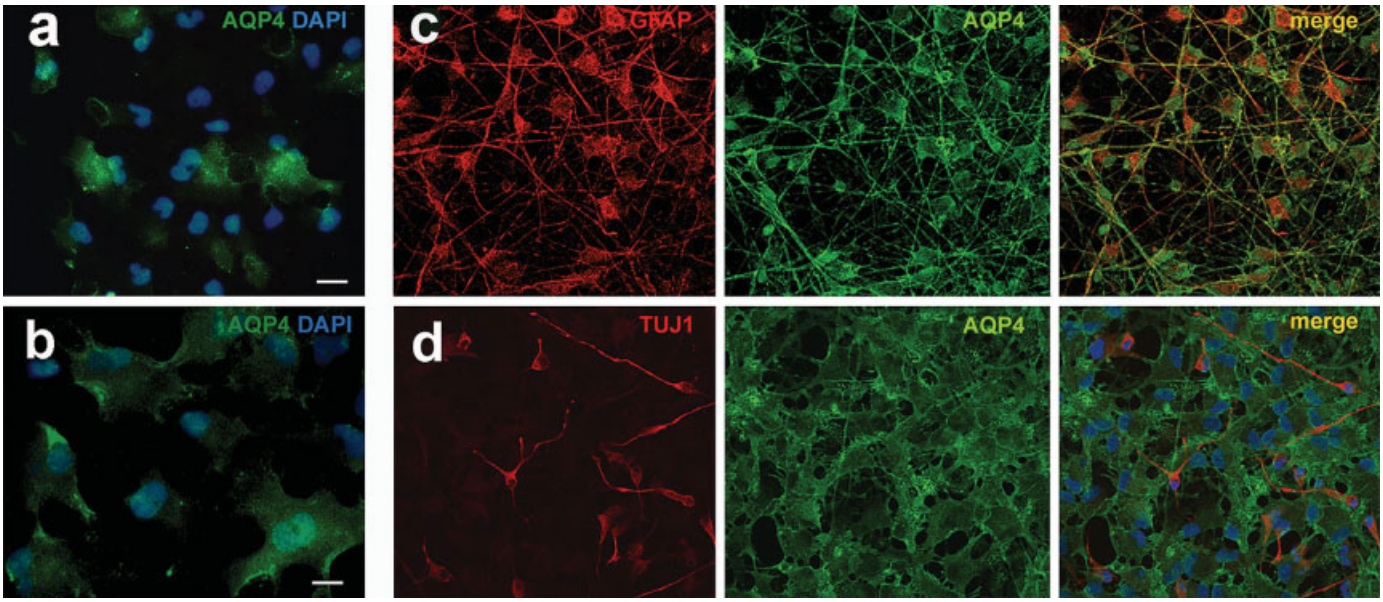

Fig. 7. Human NSCs and their progeny express AQP4. The expression of AQP4 was evaluated by immunofluorescence analysis followed by confocal analysis on the different cell fractions prepared from serially passaged (passage 18) hfNSCs, as described in Materials and Methods. **a,b:** The vast majority of precursor cells and progenitors expressed AQP4, as shown in the merged images (green, AQPs; blue, cell nuclei counterstained with DAPI). In differentiated cultures, AQP4 was expressed by $GFAP^+$ cells (**c**), but not by $TUJ1^+$ neuronal cells (**d**). Green, AQP4; red, GFAP (c) and TUJ1 (d). Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). Original magnifications ×400 in a; ×630 in b–d. Scale bars = 15μm in a; 5μm in b.

the proportion of $GalC^+$ oligodendrocytes is low (<3%); thus, it was difficult to detect any co-localization of $AQP4^+GalC^+$ cells. We did not find AQP9 expression in any of the different hFNSC-derived cell preparations.

Subcellular Localization of AQP4 and AQP9 in mANSCs and hfNSCs

The intracellular localization of AQP4 and AQP9 in NSC-derived differentiated progeny was characterized by double-labeling immunofluorescence followed by confocal microscopy analysis using antibodies specific for different subcellular compartments. For the endoplasmic reticulum (ER) we used an antibody recognizing the sequence Lys-Asp-Glu-Leu (KDEL) present at the C-terminus of soluble resident proteins and some membrane proteins. The presence of C-terminal KDEL appears to be necessary for ER retention and appears to be sufficient to reduce the secretion of proteins from the ER. Lectins are highly specific carbohydrate binding proteins. Anti-wheat germ lectin (WGA) is commonly used to detect proteins in the Golgi apparatus. We found expression of AQP4 and AQP9 in the ER (Fig. 8a,c) and of AQP4 (but not AQP9), in the Golgi apparatus (Fig. 8b) of ANSC-derived differentiated cells. Additional pictures showing Z-analysis and high-resolution confocal images are included as supplementary material (see Supplementary figures 2–4). In hFNSC-derived differentiated cells we found AQP4 expression in the ER (Fig. 8d), but not in the Golgi apparatus (not shown).

DISCUSSION

We present a detailed molecular and phenotypical characterization of AQP4 and AQP9 expression, in murine and human NSC cultures at specific stages of differentiation. We demonstrated that AQP4 and AQP9 are expressed in adult murine SVZ-derived NSCs (mANSCs) and that their levels of expression and cellular localization are differentially regulated upon mANSC differentiation into neurons and glia. In addition, we show that AQP4 (but not AQP9) is expressed in human NSCs and their differentiated progeny.

AQP4- and AQP9-expressing cells in the forebrain periventricular region, the region from which we isolated and cultured NSCs, were localized in different cell subsets. AQP4- expressing cells mainly lined the ventricle with an ependymal-like pattern and were scarcely represented in the dorsolateral part of the SVZ. Cells co-expressing AQP4 and either nestin (an intermediate filament expressed in several SVZ cell types; Doetsch et al., 1997) or GFAP were found in this region. On the contrary, AQP9-expressing cells lined the ventricles but showed a more pronounced subependymal-like distribution, with a clear presence in the dorsolateral part of the SVZ, which harbored many AQP9-positive cells co-expressing nestin and GFAP. Although not all the AQP^+ cells co-expressed GFAP, we did not find co-expression with neuronal markers, thus confirming the previously reported glial-restricted expression of AQP4 and AQP9 (Venero et al., 1999; Badaut et al., 2004). AQP4 expression in brain astrocytes has been correlated with the development, function and integrity of the BBB (Wen

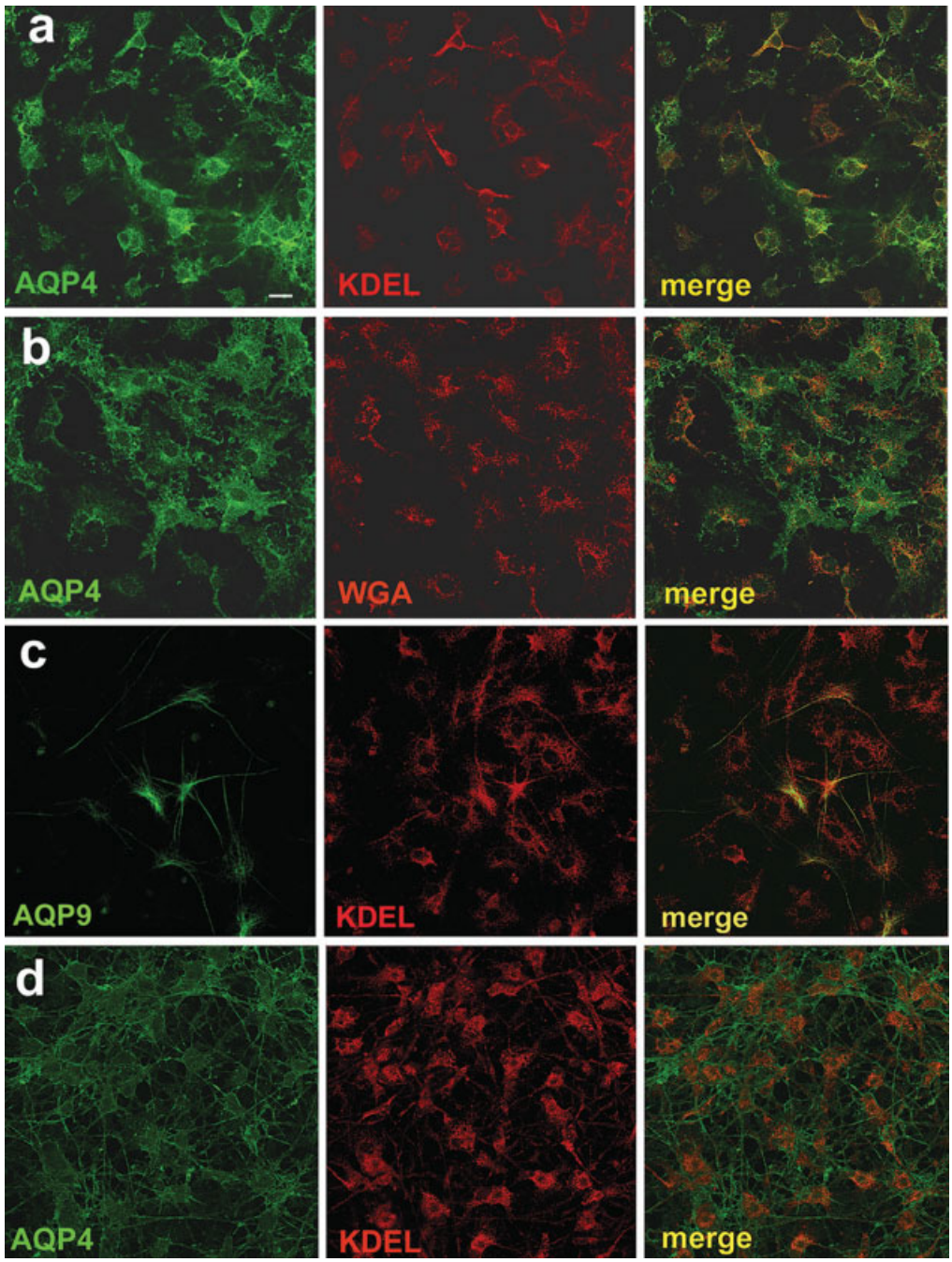

Fig. 8. A cytoplasmic pool of AQP4 and AQP9 is present in murine and human NSCs. **a–c:** Double-labeling immunofluorescence followed by confocal analysis showed a partial distribution of AQP4 and AQP9 in the endoplasmic reticulum (ER) of mANSC-derived differentiated cultures (a,c), while only AQP4 was found in the Golgi apparatus (Ga) (b). In hfNSCs, AQP4 was found only in the ER (**d**). Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). Green, AQPs; red, KDEL (ER) and WGA (Ga). Original magnification ×400. Scale bar = 15 μm.

et al., 1999; Nicchia et al., 2004). The adult neurogenetic regions (Alvarez-Buylla et al., 2002) share a peculiar environment (niche) that differs considerably from that found in the adjacent parenchyma (Doetsch, 2003). Recently, a subset of GFAP-expressing astrocytes have been described as the primary precursors in vivo (Alvarez-Buylla et al., 2002) and, interestingly, endothelial cells have been recognized as critical components for the maintenance of the stem cell niche (Palmer et al., 2000; Shen et al., 2004). For this reason, it is conceivable that regulation of water balance by aquaporins, which is critical during CNS development, might also be important during adult neurogenesis in adult neural stem cells (NSCs) and in their progeny.

The adult SVZ is enriched in stem cells as well as in neuronal and glial precursors. When freshly isolated cells from SVZ tissue are plated in differentiating conditions, they proliferate for a limited period of time and then differentiate, generating a mixed neuronal/glial primary culture. Appearance and accumulation of AQP4 protein have been correlated to glial cell maturation in the cerebellum (Wen et al., 1999) and AQP4 levels were described to parallel those of GFAP during retinoic acid-induced astrocytic differentiation of embryonal carci-

noma cells in culture (Yoneda et al., 2001). In our primary cultures, AQP4 protein levels were not directly related to GFAP levels, since we found different proportions of $AQP^-/GFAP^+$, $AQP^+/GFAP^-$, and $AQP^+/GFAP^+$ cells. Of note, AQP4 expression was always found in cells bearing the morphology of protoplasmic astrocytes, even in the absence of GFAP immunoreactivity (IR). The presence of this miscellaneous pattern of AQP4 and GFAP expression was similar to that observed in brain tissue. Conversely, all the $AQP9^+$ cells in our cultures expressed GFAP. These results suggest that different populations of astrocytes are generated from SVZ-derived NSCs and that they are present in culture at different stages of maturation, reinforcing the notions that ex vivo isolated ANSC-derived astrocytes might maintain the heterogeneity that characterize the astrocyte-like cell populations in the SVZ in vivo. Interestingly, we report for the first time the presence of both AQPs in $GalC^+$ oligodendroglial cells.

SVZ-derived primary cultures reflect the cellular composition of the original tissue. Plating adult SVZ-derived cells in a serum-free, chemically defined medium containing appropriate mitogens allows for the selection, maintenance, and expansion of a population of actively proliferating early precursors (Gritti et al., 1999; Galli et al., 2002). We found that AQP4 and AQP9 are highly expressed in mANSCs and in their committed progenitors. Following cell differentiation there was an increase in the overall levels of AQP4 mRNA and a concomitant redistribution of the protein, which localized in a high proportion of differentiated glial cells and clearly displayed membrane localization. Again, AQP4-IR was not necessarily linked to GFAP-IR, suggesting that expression and increasing levels of this water channel protein might precede the appearance of markers typical of astrocytic maturation. Virtually all the GFAP-expressing astrocytes in our differentiated cultures are postmitotic and nonproliferating. However, radial glia can be generated from ANSCs (Gregg and Weiss, 2003) and subpopulations of ANSC-derived astrocytes retain markers of immature glia, such as nestin and vimentin (A. Gritti, unpublished results). It would be interesting to investigate the potential correlation between AQP4 expression and the maintenance of an immature glial phenotype by adult astrocytes in vitro, also in view of the fact that adult SVZ stem cells have been shown to derive from radial glia (Merkle et al., 2004) and that putative stem cells have been described in the human adult brain that display glial morphology and antigenic properties (Sanai et al., 2004). These periventricular glia-like neural stem cells and their progeny might be implicated in the generation of brain tumors (Rubinstein et al., 1984; Recht et al., 2003), as well as in the process of endogenous brain repair (Fallon et al., 2000; Nakatomi et al., 2002). Thus, a better characterization of these cells in terms of their water homeostasis dynamics might be critical for the development of potential therapeutic approaches for CNS disorders.

In contrast to AQP4, only 30% of the cells in the differentiated cultures showed AQP9-IR, which was strongly correlated to GFAP-IR, and displayed a typical cytoplasmic localization, two characteristics that we already observed in primary cultures. The abundance and specific cell distribution of AQP4 and AQP9 in our NSCs-derived cultures reflect the situation found in the adult rodent brain, with AQP4 being the most abundant water channel and AQP9 expression being restricted in a subset of astrocytes and in a different cellular compartment. The presence of AQP4 and AQP9 has not been described in cells of the oligodendroglial lineage, neither in vivo (Venero et al., 1999) nor in vitro (Yamamoto et al., 2001; Yoneda et al., 2001). Interestingly, we described the expression of both AQPs in a subset of oligodendrocytes, both in SVZ-derived primary cultures and NSC-derived differentiated cultures. In our culture conditions, and particularly in differentiated cultures derived from serially passaged NSCs, immunoreactivity for GalC identify postmigratory, nonproliferating oligodendrocytes that have not yet reached functional maturation and expression of myelin proteins, often co-expressing NG2 or O4, markers of oligodendrocyte precursors. Thus, the presence of AQPs in these cells might indicate the persistence of immature features and an ongoing process of differentiation/maturation.

Overall, the peculiar and miscellaneous expression described for AQP4 and AQP9 in NSC-derived glial cells not only underlies the notion of adult glia heterogeneity in vitro but might be viewed as an indication of a newly identified trait between glial cells and their precursors. These results and comments are summarized in the cartoon in Figure 9.

Levels of expression and cellular localization of AQP4 in hFNSCs were similar to those found in their murine counterpart; conversely, neither AQP9 mRNA nor proteins were detected. This finding is consistent with previous reports indicating that this water channel is not expressed in human brain tissue, while it is expressed in leukocytes, spleen, bone marrow, lung and liver (Tsukaguchi et al., 1999). This supports the existence of specie-specific expression of this protein and of a distinct pattern of tissue distribution, most likely due to different requirements of metabolites between rodents and humans.

Previous reports described the absence of AQPs in neurons, both in vivo and in primary cultures (Elkjaer et al., 2000; Nagelhus et al., 2004). Recently, AQP9 expression was found in catecholaminergic neurons in vivo, suggesting that neuronal AQP9 may be implicated in brain energy metabolism (Badaut and Regli, 2004). The almost complete absence of both AQP4 and AQP9 in mouse and human NSC-derived neuronal cells is likely explained considering that nearly all neurons are γ-aminobutyric acid (GABA)ergic and glutamatergic in our culture conditions (Gritti et al., 1996), few or none of them expressing tyrosine hydroxylase. Since AQP9 and AQP4 are expressed in near all undifferentiated cells and in the vast majority of neuronal and glial progenitors, our results suggest that protein levels are progressively downregulated in the NSC postcommitment phases. The occasional finding of AQP4- and AQP9-IR in $TUJ1^+$ cells derived from serially passaged mANSCs is

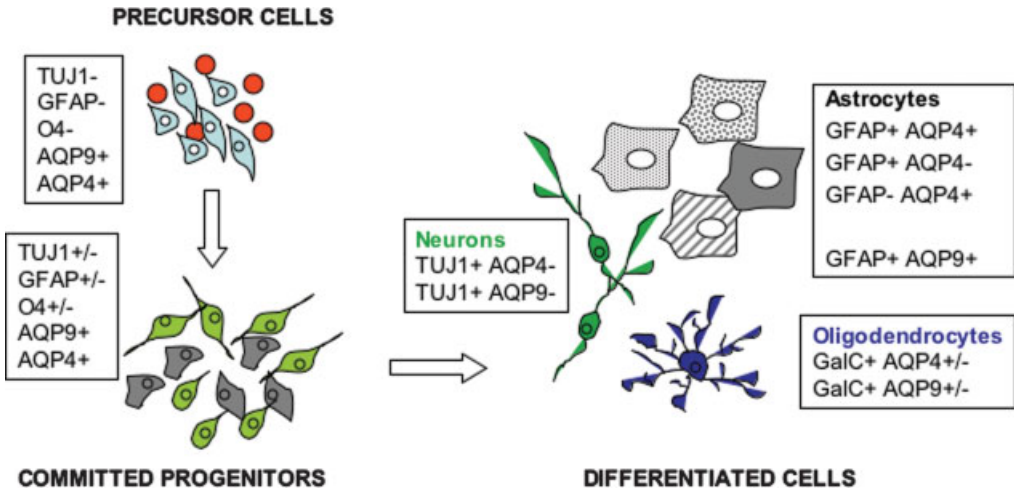

Fig. 9. AQP expression underlies heterogeneity in mANSC-derived differentiated glial cells. The three cell fractions (precursor cells, committed progenitors, and differentiated cells) were prepared as described in Materials and Methods and Results. The extent of cell differentiation/maturation was evaluated by quantifying the number of cells immunoreactive for neuronal (TUJ1), astroglial (GFAP) and oligodendroglial (GalC, 04) markers. Refer to Table 1 for quantitative analysis. Precursor cells (stem cells, red; immature progenitors, light blue) are highly proliferative and lack differentiation markers ($TUJ1^{-}$, $GFAP^{-}$). The large majority of these cells express AQP4 and AQP9. By varying the epigenetic factors to which they are exposed (see Materials and Methods) we can induce their lineage commitment and progressive differentiation, obtaining a mixed cell population of neuronal and glial progenitors (represented as green and gray cells, respectively) that start to express lineage-specific differentiation markers ($TUJ1^{+/-}$; $GFAP^{+/-}$; $O4^{+/-}$). At this stage of commitment most of these cells still express AQP4 and AQP9. In the continuous presence of differentiation signals these progenitors mature, acquire functional competence and maximum levels of specific protein expression (differentiated cells; neurons, astrocytes, oligodendrocytes). During this time window $AQP4^{-}$ and AQP9 expression is almost completely down-regulated in neuronal and oligodendroglial cells but remain, although at different levels and in variable proportions, in astrocytes. At least three subsets of astrocytes can be identified based on different combinations of GFAP and AQP4 expression ($GFAP^{+}$ $AQP4^{+}$, $GFAP^{-}$ $AQP4^{+}$, $GFAP^{+}$ $AQP4^{-}$; represented in the picture with different patterns on a gray background), and in each subset the levels of expression of the two proteins may vary. On the contrary, all AQP9-expressing cells also expressed GFAP, indicating an additional astroglial cell population that might be distinct from the others. The occasional presence of AQP9 and AQP4 in $GalC^{+}$ oligodendroglial and, more infrequently, in $TUJ1^{+}$ neuronal cells, might be an indication of the persistence of in these cells of immature traits. Overall, our data show heterogeneity in the NSC-derived glial cell population in vitro and suggest that AQP expression might be a newly recognized common attribute shared by astroglial and precursor cells.

probably due to the persistence in these cells of immature neuronal features (see also Fig. 9).

AQP4 is enriched in the basolateral membranes of brain cells (Nagelhus et al., 2004; Frigeri et al., 1995; Nielsen et al., 1997) and specific targeting of AQP4 has been described both in vivo (Frigeri et al., 1995; Fujita et al., 1999) and in vitro (Madrid et al., 2001). NSC-derived astrocytes in our cultures are nonpolarized cells; thus, the AQP4 pool is distributed homogeneously in the plasma membrane. The presence of a cytoplasmic pool of AQP4 in the endoplasmic reticulum and in the Golgi apparatus of both mANSC- and hFNSC-derived astrocytes might reflect the different steps of the biosynthetic pathway and sorting of this water channel protein but may also suggest a more specific function of this cytoplasmic pool, as it has been shown for AQP2 (Hendriks et al., 2004). Conversely, AQP9 is enriched in the perikaria of tanycytes lining the third ventricle (Elkjaer et al., 2000) and in astrocytic processes and cell bodies in the forebrain periventricular region, but no polarization on astrocytic endfeet is observed (Badaut and Regli, 2004; Nagelhus et al., 2004). We observed an intracellular localization of AQP9 in glial cells, both in primary cultures and in mANSC-derived astrocytes, but weak or no co-localization with the endoplasmic and the Golgi compartments, respectively. This reinforces the notion that differences in intracellular trafficking and function exist between the two water channel proteins.

The expression of AQP5 and AQP8 has been reported in brain tissue and in cultured rat astrocytes (Yamamoto et al., 2001). We did not find mRNA for AQP5 (as well as for AQP0, 1, 2, 3, 4, and 7) in our NSC-derived cultures. On the contrary, both mANSCs and hFNSC expressed AQP8 by RT-PCR. We did not further investigate on AQP8, since we focused on this AQP in a different work (La Porta et al., personal observations).

The similarities and differences in the localization, cellular distribution, and some regulatory mechanisms between AQP9 and AQP4 in physiological as well as pathological conditions (Gunnarson et al., 2004) suggest similar functions of the two proteins as water channels but different functions as metabolic channels. Further insight into the mechanisms regulating brain AQPs will be of utmost clinical importance, since perturbed water flow via brain AQPs has been implicated in many neurological diseases. We provide a reliable in vitro experimental model in which the expression and function of AQP4 and AQP9 may be studied in murine and human NSCs and in their differentiated progeny, both in physiological conditions and, most interestingly, following insults that reproduce pathological conditions occurring in vivo. The possibility to modify NSCs genetically to

overexpress AQP genes, and the possibility to use NSC cultures derived from AQP-knockout mice, could provide new tools for the functional study of these water channel proteins in mammalian brain cells.

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