Differential Expression Patterns of occ1-Related Genes in Adult Monkey Visual Cortex

We have previously revealed that occ1 is preferentially expressed in the primary visual area (V1) of the monkey neocortex. In our attempt to identify more area-selective genes in the macaque neocortex, we found that testican-1, an occ1-related gene, and its family members also exhibit characteristic expression patterns along the visual pathway. The expression levels of testican-1 and testican-2 mRNAs as well as that of occ1 mRNA start of high in V1, progressively decrease along the ventral visual pathway, and end of low in the temporal areas. Complementary to them, the neuronal expression of SPARC mRNA is abundant in the association areas and scarce in V1. Whereas occ1, testican-1, and testican-2 mRNAs are preferentially distributed in thalamorecipient layers including “blobs,” SPARC mRNA expression avoids these layers. Neither SC1 nor testican-3 mRNA expression is selective to particular areas, but SC1 mRNA is abundantly observed in blobs. The expressions of occ1, testican-1, testican-2, and SC1 mRNA were downregulated after monocular tetrodotoxin injection. These results resonate with previous works on chemical and functional gradients along the primate occipitotemporal visual pathway and raise the possibility that these gradients and functional architecture may be related to the visual activity-dependent expression of these extracellular matrix glycoproteins.

Keywords: extracellular matrix, follistatin-related protein/TSC-36/FSTL1, in situ hybridization, monocular deprivation, RLCS

Introduction

Recent genome-wide analysis of gene expression patterns in the mouse brain has unveiled the variety of genes that are expressed in distinct parcellation of neuronal anatomical architecture (Lein et al. 2007). It has been suggested that these genes play roles in the formation or function of such structure (Pimenta et al. 1995; Job and Tan 2003; Hamasaki et al. 2004). Our own effort to identify area-selective genes in the adult monkey neocortex by differential display analysis has demonstrated that occ1 mRNA is preferentially expressed in the primary visual area (V1) (Tochitani et al. 2001). Examination of the occ1 mRNA expression by in situ hybridization (ISH) revealed the following anatomical features: occ1 mRNA expression 1) is dependent on neuronal activity, 2) is preferentially localized in thalamorecipient layers including “blobs” in layers II/III, and 3) is also localized in thalamorecipient layers of the extrastriate, somatosensory, and auditory cortices, although the expression levels are much lower than that in V1. These characteristics are restricted to the expression in excitatory neurons, whereas the expression is activity independent in parvalbumin (PV)-containing γ-aminobutyric acidergic (GABAergic) inhibitory interneurons that are broadly distributed throughout the neocortex (Tochitani et al. 2001; Komatsu et al. 2005; Takahata et al. 2006).

Our continued analysis is to search for area-selective genes using restriction landmark cDNA scanning (RLCS) technique, a large-scale analysis of differential gene expression. By this means, we identified testican-1 as a V1-enriched gene (Fig. 1A). The domain structure of the gene product of testican-1 is closely related to that of occ1. Both are characterized by the presence of one follistatin domain (F5 domain) followed by one extracellular calcium-binding domain (EC domain). At least 4 other genes are known to belong to this family (Yan and Sage 1999), the physiological roles of those genes in the central nervous system remain to be elucidated. Recent studies suggest that the ECM is involved in the maturation and plasticity of the synapse (Pizzorusso et al. 2002; Dityatev and Schachner 2006). Thus, occ1-related genes may have significant roles in regulating synaptic plasticity in the visual area in the primate neocortex. Here, we performed a comprehensive expression analysis of the members of this family, namely, testican-1, testican-2, testican-3 (also referred to as SPOCKs), secreted protein acidic and rich in cystein (SPARC: also referred to as BM-40 or osteonectin), and SPARC-like1 (SC1: also referred to as hevin). We found both similar and complementary expression patterns of the members of this family to that of occ1 in terms of area difference, particularly the V1-temporal association area (TE) gradient, lamina preference, blob preference, cell-type specificity, and sensory input dependence.

Materials and Methods

Animals and Sample Preparation

For RLCS analysis and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), postmortem brain tissues of African green monkeys (Cercopithecus aethiops) were obtained from the Japan Poliomyelitis Research Institute and processed as previously described (Watakabe et al. 2001). For ISH, ten Japanese macaque monkeys (Macaca fascicular, adult, either sex, 2.9-9.1 kg) were used. Six of the Japanese macaques were subjected to monocular deprivation (MD) during 5-21 days before sacrifice as follows: 10 μl of tetrodotoxin (4.7 μM in saline) was slowly microinjected into the left eyeball twice a week under ketamine anesthesia. The pupil of the injected eye was dilated after operation. A part of these samples were used in our previous studies (Tochitani et al. 2001; Takahata et al. 2006). Four of the 6 MD samples (5, 10, 14, and 21 days MD each) were used for quantification of MD effect on gene expression (Fig. 9). All the animals were administered an overdose of pentobarbital (at least 100 mg/kg per os) a week under ketamine anesthesia. The pupil of the injected eye was dilated after operation. A part of these samples were used in our previous studies (Tochitani et al. 2001; Takahata et al. 2006). Four of the 6 MD samples (5, 10, 14, and 21 days MD each) were used for quantification of MD effect on gene expression (Fig. 9). All the animals were administered an overdose of pentobarbital (at least 100 mg/kg per os) a week under ketamine anesthesia.
Differential gene expression among 4 cortical areas (V1, TE, the primary motor area [M1], and Brodmann’s area 46) of African green monkey was comprehensively identified by RLCS method as previously described (Suzuki et al. 1996; Shintani et al. 2004). Total RNAs were extracted separately from the cortical areas as described previously (Watakabe et al. 2001). Poly (A) mRNA was purified with BioMag Oligo (dT)20 (PerSeptive Biosystems, Framingham, MA) following manufacturer’s instructions. Double-stranded cDNAs were synthesized with an anchor primer targeting poly (A) tail and Superscript Choice System (Invitrogen, Carlsbad, CA). An overhang was created at the 5’-terminal of the cDNA using ApaLI, and the restriction cut site was radiolabeled by a full up reaction with [α-32P] deoxynucleotides. Labeled fragments containing the 3’-terminal were collected using Dynabeads Oligo (dT)20 (Dynal, Oslo, Norway) and cut away from the 3’-terminal by NotI digestion. The fragments were then subjected to agarose gel electrophoresis. After electrophoresis, the cDNA fragments in gel were digested with HinfI, in situ, and then subjected to acrylamide-gel electrophoresis. The cDNA fragments labeled and separated by the 2-dimensional (2D) electrophoresis were displayed as spots on X-ray films.

The spot intensity was carefully compared among the 4 cortical areas and the DNA fragments corresponding to the differential spot were eluted from the cutout gel and ligated with 2 adapters; one for the overhang site of ApaLI digestion and the other for the overhang site of NotI digestion. The amplified fragments were cloned as described previously (Komatsu et al. 2005). A total of 30 colonies per one cloned spot were picked up and subjected to PCR using the plasmid vector sequence. To select a positive clone corresponding to a differential spot, the fragments were subjected to restriction fragment length polymorphism (RFLP) analysis using a 4-base cutter, AluI. The inserted sequence that showed the most frequent pattern in RFLP was determined for each spot.

**RLCS**

**Real-Time RT-PCR**

To quantify the difference in the mRNA expression levels among areas, real-time RT-PCR was performed. Total RNA was isolated from tissues of 5 cortical areas; V1, TE, M1, the primary somatosensory area (S1), and area 46. In all, 20 µg of each was subjected to RNase-free DNase treatment, followed by another round of acid guanidinium thiocyanate-phenol-chloroform extraction to completely remove the contaminating genomic DNA. Two micrograms of such RNA was converted to cDNA using random nonamer and MMLV reverse transcriptase (Invitrogen) and used as the template for real-time RT-PCR. Prepared cDNAs were reacted with specific primers for each gene (Supplementary Table 1) and SYBR Premix Ex Taq (Takara, Otsu, Japan) under the control of OPTICON2 (Bio-Rad, Hercules, CA). Only single bands were obtained when each reacted sample was electrophoresed, and the sequences of those bands were confirmed as derived from the targeted sequences of each gene. Lines of diluted solutions of brain samples were examined to determine amplification efficiencies for each primer set. The amount of each mRNA in each area was calculated by cycle

![Figure 1](https://example.com/image1.png)

**Figure 1.** (A) The 2D electrophoresis in RLCS analysis in V1, TE, M1, and area 46. A more intense spot of the testican-1 gene was observed in V1 than in the other 3 areas (shown by a circle). (B) Schematic domain structures of occ1-related proteins. Testican-1 (*), which was identified as a V1-enriched gene, has a domain structure partially homologous to this group of proteins. They are characterized by one FS domain (FS) and a following EC domain (EC). Testicans have their unique domain both in the N-terminal (TES) and the C-terminal (C). TY denotes a thyroglobulin-like domain. Black arrowheads indicate the site of the glycosylated serine in Testicans (Alliel et al. 1999). Open bars represent unidentified unique domains for each protein (Maurer et al. 1995). (C) Quantification of area difference in mRNA expression of occ1-related genes among V1, TE, M1, S1, and area 46 by real-time RT-PCR analysis. The amount of each mRNA was normalized as a ratio to the amount of the internal standard, g3pdh mRNA. occ1, testican-1, and testican-2 mRNAs were most abundant in V1 among these 5 areas.
For colorimetric ISH, digoxigenin (DIG)-labeled antisense and sense riboprobes were prepared using a DIG-DUTP labeling kit (Roche Diagnostics, Indianapolis, IN). Riboprobes were newly prepared from the cDNA library of each animal by RT-PCR and conventional TA cloning techniques. The primer sequences are listed in Supplementary Table 1. The sense probes did not detect signals stronger than the background signal (data not shown). ISH was carried out as described previously (Tochitani et al. 2001; Takahata et al. 2006). Briefly, free-floating sections were soaked in 4% PFA/0.1 M PB (pH 7.4) overnight at 4°C and treated with 10 μg/ml proteinase K for 30 min at 37°C. After acetylation, the sections were incubated in the hybridization buffer (5x standard saline citrate [SSC], 1 x means 150 mM NaCl, 15 mM Na acetate, pH 7.0), 50% formamide, 2% blocking reagent, 0.1% N-lauroylsarcosine [NLS], 0.1% sodium dodecyl sulfate, 20 mM maleic acid buffer, pH 7.5) containing 1.0 μg/ml DIG-labeled riboprobe at 60°C overnight. Hybridized sections were washed by successively immersing in the washing buffer (2x SSC, 50% formamide, 0.1% NLS, 60°C, 20 min, twice), RNase A buffer (10 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid, 0.5 M NaCl, pH 8.0) containing 20 μg/ml RNase A (37°C, 15 min), 2x SSC/0.1% NLS (37°C, 20 min, twice), and 0.2x SSC/0.1% NLS (37°C, 15 min, twice). Hybridization signals were visualized by alkaline phosphatase (AP) immunohistochemical staining using a DIG detection kit (Roche Diagnostics). Because ISH signals were so strong for testican-2 and SCI, we shortened their reaction time to (6-8 h) with nitrro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) from the original reaction time (16-18 h) to prevent the saturation of ISH signals. Sections were mounted onto glass slides and dehydrated through a graded series of increasing ethanol concentration followed by xylene and then coverslipped with Entellan New (Merck, Tokyo, Japan). Fluorescence double-labeling ISH was performed as described previously (Takahata et al. 2006). Fluorescinc isothiocyanate (FITC)-labeled riboprobes for glutamic acid decarboxylase 67 (GAD67), vesicular glutamate transporter 1 (VGLUT1), and glial fibrillary acidic protein (GFAP) were prepared. Brain sections were hybridized with both the DIG-labeled and the FITC-labeled probes. The hybridization protocol was the same as that of colorimetric single ISH. DIG was detected by staining with AP-conjugated anti-DIG antibodies and using an RNPP Fluorescence Detection kit (Roche Diagnostics). FITC was detected by horseradish peroxidase-conjugated anti-FITC antibodies (Roche Diagnostics) followed by enhancement using a TSA-Plus DNP system (Perkin Elmer Life Sciences, Boston, MA) and staining with Alexa 488-conjugated anti-DNP antibodies (Molecular Probes, Eugene, OR). The sections were then counterstained with Hoechst 33342 (Molecular Probes) diluted in PBS to 1:1000. After mounting onto glass slides, sections were air dried and coverslipped with the PermaFluor Aqueous mounting medium (Thermo, Pittsburgh, PA).

The brain regions of macaques were identified using brain atlases (Paxinos et al. 2000). Images of the ISH sections were captured with an HNPP Fluorescence Detection kit (Roche Diagnostics). Because ISH signals were so strong for the deprived or nondeprived ocular dominance column. We mainly used tangential/semitangential sections or coronal sections that showed wide ocular dominance columns for the analysis, in order to identify regions of each ocular dominance column. The shapes of each ocular dominance column were delineated with reference to the occ1 ISH staining pattern in V1 tangential or coronal sections from 4 MD monkeys and applied to adjacent ISH sections for the other genes (see Fig. 10). The gray level of ISH signal for each gene and each column was converted into ROD using the following equation, ROD = log10 (255/observed gray levels).

The background ROD was subtracted from each ROD. The value of each ROD was then expressed as mean ± standard error of the mean, and the difference between deprived and nondeprived columns was determined by paired Student’s t-test (P < 0.05 was considered significant).

Results

Identification of testican-1 as a V1-Enriched Gene by RCLS

To systematically identify area-specific molecules in the monkey neocortex, we carried out large-scale screening by RCLS. In this analysis, mRNAs were purified from 4 distinct cortical areas V1, TE, M1, and area 46, converted to cDNA by reverse transcription, and digested with a pair of restriction enzymes for 2D analysis. In ApaLI and HindIII enzyme combination, approximately 850 spots were obtained, and we found 86 apparent differential spots among the 4 cortical areas. Among them, one spot was particularly intense in the visual area (Fig. 1A). This spot was cloned and identified as the testican-1 gene, a member of the gene family to which occ1 belongs (Fig. 1B).

Expression Patterns of occ1-Related Genes in Macaque Neocortex

The identification of testican-1 as a V1-enriched gene prompted us to carry out the comparative expression analysis of all the family genes of occ1 in the monkey neocortex. Besides occ1 and testican-1, there are 4 genes reported to belong to this family, namely, testican-2, testican-3, SPARC, and...
Expression Patterns of *occ1*-Related Genes in the Macaque Neocortex

SC1 (Vannahme et al. 1999; Nakada et al. 2001) (Fig. 1B). To estimate the differences in expression levels of those genes among different cortical areas, we carried out quantitative real-time RT-PCR using specific primers for *occ1*-related genes on cDNAs prepared from cortical samples of V1, TE, M1, S1, and area 46. We confirmed that *occ1* mRNA is particularly abundant in V1, compared with other areas (Fig. 1C), with *occ1* mRNA in V1 being about 3 times as abundant as that in M1 and association areas (TE and area 46). The expression level of *occ1* mRNA in S1 was approximately 1.5 times higher than those in M1 and association areas. The *testican-1* mRNA was also most abundant in V1, and its level was 2 times higher than that in association areas, validating the result of the RLCS analysis. Similarly, we observed that *testican-2* mRNA was also most abundant in V1, and its level was 1.2–1.4 times higher than that in other areas. No clear area difference was observed for *testican-3*, *SPARC*, and *SC1* in this analysis.

We further examined their mRNA expression in detail using ISH histochemistry. Figure 2 shows their expression patterns in 4 coronal sections of the macaque neocortex in low-magnification photographs. Consistent with the RT-PCR analysis results, we confirmed that both the *occ1* and *testican-1* ISHs exhibited a V1-enriched pattern. Similarly, *testican-2* mRNA signals were also observed to be abundant in V1, although the area difference was less conspicuous than those of *occ1* and *testican-1*. Both in S1 and A1, the mRNA signals of *occ1*, *testican-1*, and *testican-2* were also relatively strong. The mRNA signals of *testican-1* and *testican-2* were moderate in the higher association areas compared with those in V1 and other primary sensory areas. To our surprise, the staining pattern of ISH for *SPARC* showed clear area difference. Complementary to those of *occ1*, *testican-1*, and *testican-2*, the mRNA signals of *SPARC* were weak in V1 and strong in the higher association areas. The *SPARC* mRNA signals were also scarce in S1 and A1. Both *testican-3* and *SC1* ISH exhibited moderate or strong signals in the macaque neocortex, respectively, but the area differences in their expression were not observed.

Figure 3 shows magnified views of ISH sections for the *occ1* family genes in V1 and extrastriate visual cortices. As reported previously (Tochitani et al. 2001; Takahata et al. 2006), layer IVC/B was most strongly labeled by the *occ1* probe among the layers in V1. Strong signals were also observed in layers II/III of V1. Due to the strong V1 expression of *occ1* mRNA, the V1/V2 boundary was distinct (arrow in Fig. 3A). In extrastriate cortices, the *occ1* mRNA signals were localized mainly in pyramidal neurons that were present in the deeper layer of layer III, and the signal intensity reduced along the visual ventral pathway (asterisks in Fig. 3A–D). Only faint *occ1* mRNA signals were observed in TE. The expression patterns of *testican-1* and *testican-2* mRNAs were quite similar to each other, and their area preferences were similar to that of *occ1*; that is, the *testican-1* and *testican-2* mRNA signals were strongest and densest in layer IV of V1 (Fig. 3E–I) and thus, the V1/V2 boundary was clearly observed in their ISH. Pyramidal neurons in the deeper part of layer III were strongly labeled by the *testican-1* and *testican-2* probes in V2, and the signal intensity gradually decreased along the visual ventral pathway (Fig. 3E–I). Compared with those of *occ1*, the *testican-1* and *testican-2* mRNA signals in the visual cortices were stronger and more broadly distributed to other layers. Layer Vla also showed strong *testican-1* and *testican-2* mRNA signals and layers II, upper III, IV, and V of extrastriate cortices exhibited moderate *testican-1* and *testican-2* mRNA signals as well. The difference in the expression pattern between *testican-1* and *testican-2* was that the laminar specificity and area preference of the *testican-1* mRNA signal distribution were clearer than those of *testican-2* (Fig. 3E–I). The *testican-3* mRNA signals were sparsely distributed all across the visual cortices without apparent difference among layers and areas, except for layer I, which showed a low level of *testican-3* mRNA signal. The granule layer (layer IV) showed slightly strong *testican-3* mRNA signals (Fig. 3M–P). *SPARC*-mRNA-positive cells were observed on the surface of layer II and deeper layer VI (Vlb) in V1, and little signal was observed in the other layers (Fig. 3Q). The *SPARC* ISH pattern in V2 was similar to that in V1, and thus, the V1/V2 boundary was not apparent in the ISH section for *SPARC*. The only difference in the *SPARC* mRNA expression between V1 and V2 was that the width of the visual boundary was clearly observed in their ISH.
signals in layer II was slightly broadened into deeper layers in V2 compared with that in V1. Along the visual ventral pathway, the width of the SPARC mRNA signals in the supragranular layers further extended into deeper layers, and it almost covered the entire layers II/III in TE (brackets in Fig. 3Q–T). The SPARC mRNA signals also gradually increased along the pathway in layer V of extrastriate visual cortices. This expression pattern of the SPARC mRNA is very reminiscent of that of retinal-binding protein (Rbp), which we have identified as an association area–enriched gene in the adult monkey neocortex (Komatsu et al. 2005). The abundance of the SPARC mRNA signals in layer VIb was not apparently different throughout the visual cortices. In addition, the SPARC mRNA signals were most strongly observed on the pial surface throughout the visual cortices, where neuronal cells are not present. The SCI mRNA signals were very strong throughout the neocortex and layers, including layer I (Fig. 3U–X). The SCI mRNA signals were more intense in layers IVC and VI of V1 than the other layers in V1, and signals in layers III, IV, and VI of extrastriate visual cortices were also strong.

We have already reported that the patchy distribution of the occ1 mRNA signals in layers II/III of V1 coincides with CO-dense puffs or blobs (Takahata et al. 2006). We examined in V1 tangential sections whether other genes of this family were also enriched in blobs. We found that both the testican-1 and testican-2 mRNA signals were broadly distributed in tangential sections of layers II/III, but their expression levels were slightly higher in CO-rich blobs than in the remaining (interblob) regions (Fig. 4A–D). Notably, the distribution of the SCI mRNA signals exhibited clear enrichment in the CO-dense blobs (Fig. 4E,F). The testican-3 and SPARC mRNA signals showed no such correlation with blobs (data not shown).

As ISH for occ1-related genes showed characteristic expression patterns in the visual circuit, we also examined their ISH in
a distinct area of the primate visual system, the middle temporal visual area (MT), which is a highly myelinated compartment and the center of motion processing in the visual dorsal pathway (Kaskan and Kaas 2007). However, the expression patterns of \( \textit{occ1} \)-related genes in MT were similar to those in extrastriate visual cortex and did not show clear demarcation with surrounding areas (Fig. 5).

**Cell-Type Specificity in Expression of \textit{occ1}-Related Genes in Visual Cortex**

To identify the type of the cells that express \( \textit{occ1} \)-related genes in the macaque neocortex, we carried out fluorescence double-labeling ISH with an excitatory neuron marker, \( \textit{VGluT1} \), and a GABAergic neuron marker, \( \textit{GAD67} \), as we had done to analyze \( \textit{occ1} \)-mRNA-positive neurons in the previous study (Takahata et al. 2006). Furthermore, we used in this study an astrocyte marker, \( \textit{GFAP} \), because SPARC and SC1 proteins are reported to localize in glial cells in the brain (Johnston et al. 1990; Mendis et al. 1995; Mendis, Ivy, and Brown 1996). Below we first describe the double-labeling ISH experiments on \( \textit{occ1} \)-related genes in V1. The \( \textit{testican-1} \) mRNA signals colocalized with \( \textit{VGluT1} \) (Fig. 6A) and \( \textit{GAD67} \) mRNA signals (Fig. 6B) but not with the \( \textit{GFAP} \) mRNA signals (Fig. 6C). This suggests that \( \textit{testican-1} \) mRNA is expressed in both excitatory neurons and GABAergic interneurons but not in astrocytes in V1. Similarly, the \( \textit{testican-2} \) mRNA signals colocalized with both \( \textit{VGluT1} \) and \( \textit{GAD67} \) mRNA signals but not with the \( \textit{GFAP} \) mRNA signals in V1 (data not shown). In contrast to those of \( \textit{testican-1} \) and \( \textit{testican-2} \), the \( \textit{testican-3} \) mRNA signals predominantly colocalized with the \( \textit{GAD67} \) mRNA signals, and colocalization with the \( \textit{VGluT1} \) mRNA signals was rare, although low levels of \( \textit{testican-3} \) mRNA signals colocalized with the \( \textit{VGluT1} \) mRNA signals in layer IV (Fig. 6D,F). Colocalization of the \( \textit{testican-3} \) mRNA signals with the \( \textit{GFAP} \) mRNA signals was not observed (Fig. 6G). The \( \textit{SPARC} \) mRNA signals in layer VIb predominantly colocalized with the \( \textit{VGluT1} \) mRNA signals (Fig. 7A). The strong \( \textit{SPARC} \) mRNA signals on the pial surface corresponded to the \( \textit{GFAP} \) mRNA signals (Fig. 7C). Given that there were few \( \textit{SPARC} \)-mRNA-positive cells, little colocalization of the \( \textit{SPARC} \) mRNA signals with any markers was observed in the remaining layers of V1 (Fig. 7A–C). The \( \textit{SC1} \) mRNA signals colocalized with both the \( \textit{VGluT1} \) and \( \textit{GAD67} \) mRNA signals in the granular and infragranular layers of V1 (Fig. 7D,E). The \( \textit{SC1} \) mRNA signals also colocalized with the \( \textit{GFAP} \) mRNA signals in superficial layers (Fig. 7F), although they appeared a different population from the \( \textit{SPARC} \)--\( \textit{GFAP} \)-double-positive cells.

In higher visual cortices, the cell-type specificity of \( \textit{testican-1} \) (Fig. 8A–C), \( \textit{testican-2} \), \( \textit{testican-3} \), and \( \textit{SC1} \) (data not shown)
mRNA expression was essentially the same as that in V1, with the exception of SPARC. SPARC mRNA signals were rarely observed in layers II--V of V1; however, there were high levels of SPARC mRNA signals in layers II/III and V of TE. They colocalized well with both VGluT1 and GAD67 mRNA signals (Fig. 8D,E). This indicated that SPARC mRNA expressions in excitatory and GABAergic neurons are similar for a particular cortical area. The SPARC mRNA signals on the pial surface in TE colocalized with the GFAP mRNA signals and those in layer IVb colocalized with the VGluT1 mRNA signals (Fig. 8D,F) as well as in V1.

To calculate the proportion of either testican-1- or SPARC-mRNA-positive neurons to excitatory or GABAergic neurons of V1 and TE, we counted the number of cells positive for each mRNA (Table 1). The proportions of testican-1-mRNA-positive cells to VGluT1-mRNA-positive cells were high in layers of V1 and TE (>75%), except for infragranular layers of V1 (41.8%), where the expression is low in layers V and VIb. The proportions of testican-1-mRNA-positive cells to GAD67-mRNA-positive cells were approximately half (42.9--69.2%) in each layer of V1 and TE. On the other hand, the proportions of SPARC-mRNA-positive cells to VGluT1-mRNA-positive cells were very low in each layer of V1 (<15%), whereas those were remarkably higher in TE, especially in supragranular layers (3.6% in V1 and 71.0% in TE), which is consistent with our observation described above. The proportions of SPARC-mRNA-positive cells to GAD67-mRNA-positive cells were also higher in TE (19.8--38.5%) than in V1 (1.1--24.1%) throughout all the layers.

Immunohistochemical Identification of OCCI-Related Proteins
We examined IHC using anti-Testican-1 and anti-SPARC antibodies to reveal their protein localizations. In Testican-1 IHC, a consistently similar pattern to the ISH pattern was observed (Fig. 9A--F). There were abundant Testican-1 signals in layers II--IVA, IVC, and V of V1 (Fig. 9A,F). IHC signals were also abundantly observed in layers deeper III, IV, and VI of extrastriate visual cortices and consistent with ISH data, Testican-1 protein signals in the thalamorecipient layers progressively decreased along the occipitotemporal visual pathway (Fig. 9A,B). At higher magnification, Testican-1 protein signals were detected in the neuronal cell bodies and proximal dendrites (Fig. 9C--F). It may be noted that a secreted form of

Figure 6. Coronal sections for double-labeling fluorescence ISH for occ1-related genes (red) and cell-type marker genes (green) in V1. (A--C) The testican-1 mRNA signals colocalized well with both VGluT1 (A) and GAD67 (B) mRNA signals but not with GFAP mRNA signals (C). (D--F) The testican-3 mRNA signals more predominantly colocalized with GAD67 mRNA signals (E) than with VGluT1 mRNA signals (D). They did not colocalize with GFAP mRNA signals (F). Small squares indicate the region where higher magnification photos, which are shown under each panel, were taken. Scale bar = 200 μm.
Testican-1 protein, if it exists, may be difficult to detect using IHC. In SPARC IHC, intense signals were detected only in the pial surface in all the cortical areas that were examined, which most likely corresponded to SPARC mRNA signals detected in astrocytes of the pial surface. At higher magnification, IHC signals were observed in glia-like small cells in the pial surface, and processes that are considered to be derived from astrocytes (Fig. 9).

Activity Dependence in Expression of occ1-Related Genes in V1

We observed strong expression of testican-1 and testican-2 mRNAs in layer IVC and in blobs of V1 and in the deeper layer III of the extrastriate cortex. SC1 mRNA expression was also strong in blobs. These observations raised the possibility that the transcriptions of those genes are driven by neuronal activity because those regions show more activity, as revealed by CO enzymatic reactivity, than others (Horton and Hubel 1981; Wong-Riley et al. 1989). Thus, we examined whether the expression of occ1-related genes, as with occ1, is dependent on sensory activity in V1 (Tochitani et al. 2001). ISH for occ1 revealed periodical light and dark columns in the coronal sections of V1 in macaques of 5–21 days MD (Fig. 10A). In the adjacent coronal sections of ISH for testican-1, testican-2, and SC1, there was a partial decrease of the signal intensities in apparent deprived ocular dominance columns (Fig. 10B–D). To confirm that this reduction occurred in the deprived column, we examined ISH in the tangential sections of V1 in MD macaques. In both layers II/III and IVC, the decrease in the occ1 mRNA expression was clearly observed, and the characteristic pattern of ocular dominance column staining was observed (Fig. 10E,I). In the ISH for testican-1, testican-2, and SC1 in adjacent tangential sections of around layer IVC, the same pattern of ocular dominance column staining to that of occ1 ISH was observed, presumably because of a significant decrease in their signals in the deprived columns (Fig. 10F–I). To quantify the effects of MD on their expression, we measured their RODs in deprived and nondeprived columns and compared them between the 2 (Fig. 10J–P). The occ1 mRNA expression showed a large and significant decrease in the ROD of the deprived columns both in layers III and IVC (23.5 ± 5.2%, P < 0.05 and 48.0 ± 2.9%, P < 0.005, respectively; n = 4 each). The testican-1 and testican-2 mRNA expressions also exhibited large and significant decreases in RODs owing to MD in layer IVC (16.4 ± 1.7%, P < 0.05 and 20.3 ± 1.7%, P < 0.05, respectively; n = 4 each). The reduction in the SC1
mRNA signals was small (5.7 ± 1.3%) but statistically significant ($P < 0.05; n = 4$) in layer IVC. Significant reduction in mRNA expression of testican-1, testican-2, and SC1 was not observed in layer III (4.2 ± 3.1%, $P = 0.41$, 5.7 ± 2.9%, $P = 0.15$ and 2.0 ± 1.9%, $P = 0.48$, respectively; $n = 4$ each). The effects of MD in the mRNA expression of testican-3 and SPARC were not detected (data not shown).

Discussion
In this study, we examined the expression of occ1-related genes in cortical areas, particularly in the visual areas of macaque monkeys. We found that the area and laminar preferences of the testican-1 and testican-2 mRNA expressions were similar to that of occ1, whereas that of SPARC mRNA was complementary to them. Neurons in the blob regions exhibited high levels of testican-1, testican-2, and SC1 mRNA expressions. These suggested that their expressions are linked to neuronal connectivity. The activity-dependent regulation of testican-1, testican-2, and SC1 mRNA expression in V1 also raised the possibility of a functional involvement in synaptic plasticity. In earlier studies, we reported unique area- and lamina-associated expression of occ1 and Rbp and their complementary patterns in the monkey neocortex (Yamamori and Rockland 2006). The novelty of the present study is the finding that a particular paralogous group of genes exhibit similar or complementary expressions, in relation to the anatomical architecture of the sensory and association cortex.
Expression Patterns of occ1-Related Genes

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The occ1, testican-1, and testican-2 genes are abundantly expressed in layer IVc of V1, blobs and deeper layer III of the extrastriate visual cortex, and scarce in higher-order areas. This graded distribution pattern resembles the immunoreactivities of PV, which reveal the distribution of thalamocortical projection fibers (Hashikawa et al. 1995; Levitt et al. 1995; Rockland et al. 1999). The same kind of graduation is also exhibited by CO enzymatic activity (Wong-Riley 1994). On the other hand, the expression of SPARC, as well as Rbp, seems to avoid the thalamorecipient layers, and thus, this expression pattern also resembles the graded distribution of zinc-enriched terminations, which represent a subset of nonthalamocortical glutamatergic synapses (Ichinohe and Rockland 2005). Similar increasing chemical gradients of GluR2/3 and calbindin D-28K outside the granular layer along the visual pathway have also been reported (Kondo et al. 1994; Xu et al. 2003).

Overall, there seems to be 2 opposing patterns of neurochemical gradient between V1 and TE, which are represented by the occ1 and Rbp patterns. It has been reported that there are gradients in timing of maturation, dendritic field size of pyramidal neurons, and susceptibility of synaptic plasticity among occipito-temporal visual pathways (Murayama et al. 1997; Elston and Rosa 1998; Bourne and Rosa 2006). These features may be due to the molecules that exhibit gradual distribution along the visual pathway of the neocortex, by regulating cell maturation, arbor extension, and physiological properties.

Unlike the correspondence of occ1 mRNA expression with blobs to which we described as activity-dependent regulation (Takahata et al. 2006), this is not the case for SC1. Interestingly, SC1 mRNA was also selectively expressed in the koniocellular layers of the dorsal lateral geniculate nucleus (dLGN) (Takahata and Yamamori, unpublished data), which presumably send thalamocortical axons to blob regions (Ding and Casagrande 1998). The occ1 mRNA is only weakly expressed in the magnocellular layers of dLGN (Tochitani et al. 2001), which mainly send axons to layer IVc of V1 (Callaway 1998). Thus, the SC1 mRNA expression may reflect a specific type of projections rather than neuronal activity.

Cell Type Specificity in Expression of occ1-Related Genes

We have already demonstrated that the pronounced area-specific expressions of occ1 and Rbp are primarily due to their

Table 1

<table>
<thead>
<tr>
<th>Testican-1</th>
<th>SPARC</th>
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<tr>
<td>V1</td>
<td>TE</td>
</tr>
<tr>
<td>II/III</td>
<td>II/III</td>
</tr>
<tr>
<td>IVC</td>
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<td>V/V</td>
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<td>Layer Double/GluT1</td>
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<td>SPARC</td>
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<td>Layer Double/GluT1</td>
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<td>V/V</td>
<td>V/V</td>
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</table>

Note: Figures in the brackets indicate numbers of counted cells those are double positive for testican-1 or SPARC and cell-type marker (GluT1 or GAD67) out of numbers of counted cells those are positive for particular cell-type marker (GluT1 or GAD67).

Figure 9. Immunohistochemical analysis of Testican-1 (A–F) and SPARC (G–J) expression in the monkey neocortex. (A, B) IHC for Testican-1 in V1 (A) and TE (B) showed patterns very similar to those obtained by ISH at a glance. The arrow in (A) shows the V1/V2 boundary. (C–F) High power images of Testican-1-positive neurons in layer III of V2 (C) and layer V of V2 (D) and TE (E). The immunoreactivity was mainly observed in the cell body and proximal neurite. (F) Immunostaining with anti-Testican-1-antiserum in V1. (G, H) IHC for SPARC in V1 (G) and TE (H). In this staining, SPARC signals were only observed in the astrocyte-like cells. (I, J) High power images of SPARC immunoreactivity in the pial surface (I) and layer III (J) of V1. Scale bar in (A, B) = 400 μm, scale bar in (C–F) = 10 μm, scale bar in (F) = 80 μm, scale bar in (G, H) = 80 μm, and scale bar in (I, J) = 10 μm.
expressions in excitatory neurons (Komatsu et al. 2005; Takahata et al. 2006). The pronounced expression of occ1 is observed in excitatory neurons of thalamorecipient layers in V1, whereas the occ1 expression in inhibitory neurons is observed throughout all the layers of the neocortical areas examined (Takahata et al. 2006). As we have shown above, testican-3 mRNA, which does not show clear area difference or activity dependence, is expressed predominantly in GABAergic inhibitory interneurons. This difference supports our idea that the gene expression in excitatory neurons mainly comprises of area-selective and activity-related patterns.

The proportions of testican-1 mRNA expression to either excitatory or GABAergic neurons were not evidently different between V1 and TE, which suggest that it is the difference in the level of expression per cell, rather than in the probability whether or not a cell is determined to express the gene, is a main factor in the area selectivity of testican-1 mRNA expression. In this regard, it is unclear whether the area selectivity of testican-1 and testican-2 mRNA expressions is exclusively due to the expression in excitatory neurons. In case of SPARC, it is obvious that the expressions in excitatory and GABAergic neurons both contribute to the overall area selectivity of SPARC mRNA expression. This observation was unexpected because excitatory glutamatergic neurons and the majority of GABAergic interneurons are presumably different in their developmental origins (Kornack and Rakic 1995; Tan et al. 1998). Therefore, the expression pattern of SPARC mRNA may be established after the formation of cortical areas and the tangential migration of GABAergic interneurons. Alternatively, SPARC-mRNA–containing GABAergic neurons may have similar origin to excitatory neurons. In humans, it is reported that more than half of GABAergic neurons originate from progenitors in the neocortical ventricular and subventricular zones of the dorsal forebrain (Letinic et al. 2002). In either case, the establishment of area-selective gene expression might be initially regulated by several differential mechanisms and finally tuned in a coordinated manner.

Consistent with previous studies (Johnston et al. 1990; Mendis et al. 1995; Mendis, Ivy, and Brown 1996; Liu et al. 2005; Vincent et al. 2008), we found that SPARC and SC1 mRNA expressions are observed not only in neurons but also in glial cells. We have demonstrated that their heterogeneous distributions in the
neocortex are only observed in the neurons and that no particular area preference in glial expression was found for these genes. Their expressions in glial cells would obscure the regional difference of neuronal expressions both in the real-time PCR and histochemistry. The SPARC or SC1 mRNA expressions in neurons showed clearer structure-related boundaries than the overall appearance that includes those of glial cells.

IHC revealed that the protein localization of Testican-1 parallels that of testican-1 transcripts. This observation evidently demonstrates the existence of the same type of heterogeneity in the macaque neocortex in the protein level as in the mRNA level. This is inconsistent with the report that the Testican-1 protein exclusively localizes in the postsynaptic region of neurons in the mouse cortex (Bonnet et al. 1996). Although the exact reason for this discrepancy is not known, one possible explanation is that posttranscriptional and/or posttranslational modifications of testican-1 gene are different between mice and macaques. We also confirmed the reduction of immunoreactivity of Testican-1 after MD (data not shown). SPARC IHC only showed signals in the astrocytes and their processes in the pial surface and not in cortical neurons. This inconsistent observation of IHC signals with ISH signals is likely attributable to the differential detection sensitivity between the 2 methodologies, that is, the neuronal expression of SPARC is too weak to be detected in IHC (also note that testican-1 ISH signals are much stronger than Testican-1 IHC signals). The strength of IHC signals can be weakened due to low specificity of antibody, inappropriate fixation, and diffusion of targeted protein. Nonetheless, it is possible that the neuronal translation of SPARC is indeed considerably weak or SPARC proteins are substantially secreted from neurons, and they are too diffusely localized to be sensitive to IHC.

Possible Function of OCC1-Related Proteins in Visual Processing and Plasticity

The OCC1-related proteins are all multifunctional secreted glycoproteins and considered to be components of the ECM (Yan and Sage 1999). They are classified on the basis of their FS glycoproteins and considered to be components of the ECM. The OCC1-related proteins are all multifunctional secreted proteins. Nonetheless, it is possible that the neuronal translation of SPARC is indeed considerably weak or SPARC proteins are substantially secreted from neurons, and they are too diffusely localized to be sensitive to IHC.

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An intriguing hypothesis, the activity of matrix metalloproteinases (MMPs), which play crucial roles in neuronal plasticity through degradation of ECM and synapse apparatus in brains (Dzwonek et al. 2004; Monea et al. 2006), may be regulated in different ways among areas by OCC1-related proteins. OCC1-related proteins have a Kazal-like motif that is associated with serine protease inhibition in their FS domains (Alliel et al. 1993). More directly, the N-domains of Testican-1 and Testican-3 are shown to inhibit protease activity of MT1-MMP in cancer tissues (Nakada et al. 2001). Conversely, Tremble et al. (1993) suggested that SPARC induces MMP expression in fibroblasts. If OCC1-related proteins regulate the MMP activity in macaque neocortex, then they may play a role in stabilizing synapses through the suppression of MMPs in active neurons of V1 and allow plasticity to occur in higher-order areas. Once retinal activity is silenced, the expressions of occ1-related genes may be downregulated, allowing neuronal remodeling of visual areas where the silenced retina had projected. Additionally, the idea that OCC1-related proteins are involved in stabilization of ECM may also explain the fact that the dendritic field area of layer III pyramidal neurons, as well as their horizontal axon field size, progressively increases along the visual ventral pathway (Elston and Rosa 1998; Tanigawa et al. 2005) because ECM can limit the neurite extension and MMP can release it (Hayashita-Kinoh et al. 2001). Consistently, Testican-1 and Testican-2 have been shown to inhibit neurite outgrowth in cultured cells (Marr and Edgell 2003; Schnepf et al. 2005).

Glial expressions of SPARC and SC1 are well studied in rodents. Their expressions are similar to those in macaques as described here, suggesting that their function has been conserved through the course of evolution. Brown’s group suggested the possibility that the pial SPARC regulates angiogenesis from the pia, interacting with platelet-derived growth factor, thrombospondin, basic fibroblast growth factor, and others (Mendis and Brown 1994). In addition, they have also hypothesized that SC1 is involved in synaptogenesis, based on its expression pattern and protein localization in post-synapse apparatus and perisynaptic glial processes, and its anticlell adhesion ability (Lively et al. 2007; Lively and Brown 2008). Both SPARC and SC1 expressions in the brain are induced under stressful conditions such as seizure, brain injury, morphine intoxication, and axon deafferentation (Mendis et al. 1998, 2000; Ikemoto et al. 2000; Liu et al. 2005; Lively and Brown 2007), suggesting that they have protective roles against harmful assaults to the brain. This kind of glial expression
under gliosis has also been reported for human Testican-1 (Marr et al. 2000).

The significance of the differential expression among occ1-related genes is still unclear. As such, questions include: 1) Why are the expression patterns of occ1, testican-1, and testican-2 genes alike in spite of their functional differences? That is, it has been suggested that Testican-2 interacts with other Testican family proteins (Nakada et al. 2003), and OCC1 has lost its ability to bind extracellular calcium in the course of molecular evolution (Hambrock et al. 2004). 2) Why is testican-3 mRNA predominantly expressed in GABAergic interneurons? 3) Why is SC1 mRNA expression in blobs not downregulated after MD? Modification of the basic functions may result in significantly heterogeneous extracellular environments among distinct cortical areas. Detailed functional assays are required to elucidate the roles of OCC1-related proteins in the cerebral cortex.

**Significance of occ1-Related Genes in Brain Evolution**

One of our primary motivations in the investigation of area-specific genes is what they can tell us about the evolution of the primate neocortex. We previously showed that the characteristic V1-enriched expression pattern of occ1 is conserved between macaques and marmosets but is absent in mice, rabbits, and ferrets (Takahata et al. 2006). Moreover, the expression of the orthologue gene of occ1, occ1/Frp, in mouse sensory domains is not downregulated by sensory ablation (Takahata et al. 2008). Thus, we currently consider that the V1-selective and activity-dependent occ1 mRNA expression was uniquely acquired by the primate lineage during the course of evolution.

The testican-1 and testican-2 mRNA expressions have been shown to be distributed strongly and evenly throughout the adult mouse neocortex (Bonnet et al. 1996; Vannahme et al. 1999). According to Mendis and Brown (1994; Mendis, Shahin, et al. 1996), the expression intensities of SPARC and SC1, at both the mRNA and protein levels, do not differ among cortical areas in mice, although the SPARC expression shows an anterior–posterior gradient in the entire brain. Consistent with these reports, there is no evidence for area-related differential expression of occ1-related genes in adult mice (either from the database [http://www.brain-map.org/welcome.do] or from our preliminary experiments). However, the results of RNA dot blot analysis in the human neocortex suggested that testican-1 mRNA is most abundantly expressed in the occipital lobe and most scarcely expressed in the frontal lobe (Marr et al. 2000) (ca. 1.4 times richer in the occipital lobe than in the frontal lobe). Our preliminary experiment suggested that testican-1 and testican-2 mRNAs are preferentially expressed in V1 of both marmosets and squirrel monkeys (data not shown). These are consistent with our present data in macaques and in view of these findings, we consider that the area-selective expression patterns of occ1-related genes may also be present exclusively in the primate lineage.

All the members of the occ1 family of genes are present in the genome of various vertebrates and their amino acid sequences are well conserved, suggesting an ancient origin and a functional importance of these genes. Nevertheless, their transcriptional regulatory systems in neuronal cells appear markedly altered in the cerebral cortex in the primate lineage. We suggest that this alteration is tightly and specifically correlated with the evolution of the architecture in primates.

In this sense, further studies on occ1-related genes will provide molecular clues to understanding the basis of the anatomical heterogeneity of the primate cerebral cortex.

**Supplementary Material**

Supplementary Table 1 can be found at: http://www.cercor.oxfordjournals.org/.

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**References**


Yan Q, Sage EH. 1999. SPARC, a matricellular glycoprotein with important biological functions. J Histochem Cytochem. 47:1495-1506.