Delayed Maturation and Differentiation of Neurons in Focal Cortical Dysplasia With

the Transmantle Sign: Analysis of Layer-Specific Marker Expression

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Supplemental digital content is available for this article. Direct URL citations appear in

the printed text and are provided in the HTML and PDF versions of this article on the

journal's Web site (www.jneuropath.com).

Abstract

Transmantle dysplasia is a rare type of focal cortical dysplasia (FCD) characterized by expansion of the cortex from the deep white matter to the surface and in which there is a FCD IIA or IIB pathologic pattern. To characterize possible mechanisms underlying this regional disorder of radial migrating cells, we studied the expression patterns of neocortical layer-specific markers using immunohistochemistry in surgical specimens from 5 FCD IIA and 4 FCD IIB cases in children. All neuronal cells expressed the mature neuron marker MAP2/2B but not the microglia markers Iba-1 and CD68. Some layer-specific markers showed distinct expression patterns. TBR1-positive, SATB2-positive, and FOXP1-positive cells were diffusely distributed in the cortex and/or the white matter. TBR1- positive and FOXP1-positive cells were generally more numerous in FCD IIB than in FCD IIA and were mostly in the cortical molecular and upper layers. FOXP1-, FOXP2-, and CUTL1-positive cells also expressed the immature neuron marker, Nestin/PROX1, whereas TBR1-, CTIP2-, and SATB2-positive cells only expressed MAP2/2B. These data highlight differences between FCD IIB and FCD IIA with more cells having the immature marker in upper layer markers in the former. By analyzing layer-specific marker expression patterns, we identified apparent neuronal

maturation differences between FCD IIA and FCD IIB in cases of transmantle

dysplasia.

KeyWords: Epilepsy, Focal cortical dysplasia, Layer-specific markers, Neural

maturation, Transmantle dysplasia, Transmantle sign.

INTRODUCTION

Abnormal maturation and differentiation of neurons result in the formation of cerebral cortical malformations associated with epilepsy. Focal cortical dysplasia (FCD) is a distinctive malformation of cortical development that is a common cause of intractable epilepsy in children who present for surgical treatment (1-4). Recently, the International League Against Epilepsy proposed a new classification of FCD pathologic subtypes based on the identification of isolated dyslamination of the neocortex (FCD I), the presence of characteristic dysmorphic neurons (FCD IIA) or balloon cells (FCD IIB), or a combination of dysplasia associated with tumor, hippocampal sclerosis, vascular malformation, or perinatal injury (FCD III) (5). FCD IIA and FCD IIB also show abnormal neocortical lamination. The pathogenesis of FCD is thought to involve abnormal differentiation of neuron and glial cell in the early embryonic period (6). In the magnetic resonance image, some FCDs exhibit a transmantle sign or are termed transmantle dysplasia (TD), a funnel-shaped lesion from the cortex that passes through the white matter (WM) to the superolateral ventricle (3, 7). It is thought that a genetic abnormality in earlyprogenitor cells forms funnel-shaped lesions of FCD (6). However, TD is a very rare, little-reported entity. A detailed pathologic examination of TD cases would enhance understanding of the pathogenetic mechanisms in FCD.

Laminar formation of the neocortex is known to be highly programmed by genetic control during the embryonic period. Using layer-specific markers, it is possible to identify cell birth timing and cell maturation lineage stage. At first, postmitotic projection neurons migrate into the cortical plate from the subventricular germinal zone with an inside-out pattern. At the neuronal migration stage, some molecules (e.g., reelin, Lis1, and doublecortin) are required to form the neocortex (2, 8). Finally, mammalian brains show a 6-layer neocortex, each layer with a specific function. Specific genes have important roles at each step; the molecular mechanisms are well known in rodent brains (9) and, to some extent, in the developing human brain (10). Special AT-rich sequence binding protein 2 (Satb2), COUP-TF interacting protein 2 (Ctip2), T-box brain 1 (Tbr1), Cut-like homeobox 1 (Cutl1), and Forkhead box protein P1 and P2 (Foxp1 and Foxp2) are well-known neocortical layer-specific markers. Other layer-specific molecules also play very important roles in forming cortical lamination, and disruption of their genes leads to profound cortical malformation (8, 11). SATB2 generates callosal projections in Layers II to IV and makes subcortical connections (9, 12). Orthodenticle homeobox 1 (Otx1) is expressed in Layers V and VI (13). Tbr1, a member of the T-box homeobox gene family, is expressed in preplate and Layer VI in fetal brain and makes corticocortical projection neurons (14). Cutl1, Drosophila

homeobox CUT-like 1, is expressed in pyramidal neurons of the upper layer (15).

Foxp1, a transcription factor of the winged-helix/forkhead family, is expressed in

neocortical Layers III to V (16).

In a previous study, we reported that the neocortical layer formation of human

developing brains is similar to that in mice using the layer-specific markers SATB2,

CTIP2, TBR1, CUTL1, and FOXP1 (10). In addition, we found that various

lissencephalies exhibited abnormal layer formation by random migration (10).

However, very little is known about the expression patterns of layer-specific markers in

human brain (10, 17), particularly in cortical dysplasia (18). To understand FCD

formation and the origin of the component neurons, we investigated the expression of

layer-specific markers from the deep WM to the cortex in FCD with TD.

MATERIALS AND METHODS

Tissue Samples

The use of all tissue samples for this research was approved by the patients' parents and

by ethical committees of the hospital and institute sources. The cases were selected

based on the presence of TD, that is, abnormal intensity from the lateral ventricle to the

cortex on the magnetic resonance image (7). Cerebral samples removed from patients

with drug-resistant epilepsy and TD during surgery were studied (Table 1 and Fig. 1).

The samples used were obtained from epileptogenic areas, as determined by analysis of

seizure semiology, ictal and interictal electroencephalography, magnetic resonance

imaging, fluorodeoxyglucose positron emission tomography, and interictal single

photon emission computed tomography. For the age-matched control study, we used the

same materials as previously described (10, 19). After resection, all samples were

fixed in 10% buffered formalin or 4% paraformalded embedded in paraffin. Serial

sections, 4 to 6 μm thick, were cut for histologic and immunohistologic examination.

For diagnosis of the resected tissues, the sections were stained with hematoxylin and

eosin and Klu¨ver-Barrera stains. The cases were diagnosed as FCD IIA or FCD IIB by

3 neuropathologists based on in the recent classification (5).

Immunohistochemistry

 Immunohistochemistry was performed as previously described (10, 20). The primary

antibodies were incubated at 4℃ for 24 to 72 hours. To investigate the presence of

layerspecific markers in the cortex and WM, we used monoclonal antibodies against

SATB2 (dilution of 1:100; Bio Matrix Research, Inc., Chiba, Japan), CUTL1 (1:1000;

Abnova, Taipei, Taiwan), CTIP2 (1:100; Abcam, Cambridge, UK), and polyclonal antibodies against TBR1 (1:100; Abcam), FOXP1 (1:200; Abcam), and FOXP2 (1:1000; Abcam). In addition, we performed double staining to confirm whether layer-specific marker cells were neuron or glial cells. We used antiYmicrotubule-associated protein 2 (MAP2, 1:100; Sigma, St. Louis,MO) or anti–MAP2B (1:100; BD Biosciences, San Jose, CA) as mature neuron markers. Antibodies to Nestin (1:100; Chemicon International, Inc., Billerica, MA) or Prospero homeobox protein 1 (PROX1, 1:100; Abcam) were used as immature neuron markers. Anti-glial fibrillary acidic protein (GFAP, 1:100; Cell Signaling Technology, Inc., Beverly, MA) or anti-GFAP (1:1000; Chemicon) were used as astrocyte markers. Antibodies against Iba-1 (1:100; Biocare Medical, Concord, CA) or CD68 (1:100; DAKO, Glostrup, Denmark) were used as microglia markers. 4',6'-Dimamidino-2-phenylindole was used as a cell nuclear marker.

Comparison of Immunopositive Cell Counts Between FCD IIA and FCD IIB with TD

  Variable cell staining was observed in all cases. Because the FCD neocortex showed random layer formation, we divided the cortex evenly into 4 parts: the molecular layer (CxMoL), cortical upper layer (CxUL, upper one-third), cortical middle layer (CxML,

middle one-third), and cortical lower layer (CxLL, other deep cortex). The WM was evenly divided into the subcorticalWM(ScWM; upper third), middleWM(MWM; middle third), and deep WM (DWM; periventricular region). Cells, excluding endothelial cells, were counted in each layer in 5 fields at a magnification of _200. The number was corrected per 100 nuclei in each layer for each case as the immunopositive cell density.

For statistical analyses, Student t-test was used for comparison between FCD IIA and FCD IIB in each region. The same test was applied using statistical software (SPSS Inc., Chicago, IL); p G 0.05 was considered significant.

RESULTS

The FCD samples showed abnormal lamination of the cortices and many ectopic neurons in the WM (Figs. 2 and 3). Our FCD series was composed of 5 FCD IIA and 4 FCD IIB, and the immunohistochemistry is summarized in Table 2. In previous studies, TBR1-, CTIP2-, SATB2-, FOXP1-, and CUTL1-containing cells appeared in the fetal period (10, 14, 21). After birth, SATB2-positive and CUTL1-positive cells are present until 3 months of age, although the other markers have already disappeared (10). In

addition, FOXP2 appeared predominantly in Layers IV to V before birth and in Layer

IV after birth (Figures: Supplemental Digital Content 1,

http://links.lww.com/NEN/A357; Supplemental Digital Content 2,

http://links.lww.com/NEN/A358; Supplemental Digital Content 3,

http://links.lww.com/NEN/A359; Supplemental Digital Content 4,

http://links.lww.com/NEN/A360). Distribution and Densities of Cells Expressing

Layer-Specific Markers In FCD IIA and FCD IIB, the layer-specific markerpositive

cells were diffusely localized in the cortex; there were few positive cells in

theWM(Figs. 2Y4). In FCD IIA, CUTL1- positive and FOXP2-positive cells were

generally more numerous than cells expressing the other markers (Fig. 2). Only TBR1-

positive cells were not in the CxMoL. In FCD IIB, all marker cells were also widely

distributed, although there were relatively few CTIP2-positive cells (Fig. 3). CUTL1-

positive, FOXP1-positive, and FOXP2-positive cells were numerous in both the cortex

and the WM. Densities of TBR1-positive and FOXP1-positive cells tended to be higher

in FCD IIB than those in FCD IIA (Figs. 4A, D). CTIP2-positive and SATB2-positive

cell densities were not different between FCD IIA and FCD IIB (Figs. 4B, C). In the

cortex, the densities of FOXP2-positive and CUTL1-positive cells in FCD IIA tended to

be higher than those in FCD IIB (Figs. 4E, F); however, in the WM, FOXP2-positive

and CUTL1-positive cell densities in FCD IIB were higher than those in FCD IIA (Figs. 4E, F). White matter densities of FOXP1-positive and FOXP2-positive cells in FCD IIB were greater than those in FCD IIA (Figs. 4D, E).

Phenotypes of Cells Expressing Layer-Specific Markers

To investigate the phenotypes of cells expressing layerspecific markers, we performed double staining with mature neuron, immature or progenitor neuron, glial cell, and microglia markers. In both FCD IIA and FCD IIB, all TBR1- positive, CTIP2-positive, and SATB2-positive cells expressed MAP2 and/or MAP2B (MAP2/2B) but not Nestin, PROX1, or GFAP (Table 2). There were no Iba1- or CD68-positive cells that expressed the layer-specific markers (Figs. 5 and 6 and Table 2).

In FCD IIA, mean MAP2/2B-positive cell concentrations ranged from 20.0% to 30.3% among the layer-specific marker cells. FOXP1-positive, FOXP2-positive, and CUTL1-positive cells also expressed Nestin or PROX1 (Nestin/PROX1; Figs. 5JYL and Table 2). Mean Nestin/PROX1-positive cell concentrations ranged from 13.3% to 26.2%. FOXP1-positive and FOXP2-positive cells did not express GFAP, but some CUTL1-positive cells expressed GFAP (18.0% in all CUTL1- positive cells; Fig. 5R).

In FCD IIB, mean MAP2/2BYpositive cell concentrations were 32.2%, 23.5%, and

11.7%, respectively, for FOXP1-positive, FOXP2-positive, and CUTL1-positive cells.

Mean Nestin/PROX1-positive cell concentrations were 46.7%, 36.6%, and 59.1%,

respectively (Fig. 6 and Table 2). Thus, there were more Nestin/PROX1-positive cells

in FCD IIB than in FCD IIA. A few FOXP1-positive cells in FCD IIB expressed GFAP

but more numerous FOXP2-positive and CUTL1-positive cells expressed GFAP (Figs.

6PYR). The mean concentrations of GFAP-expressing cells were 23.2% and 28.3% of

cells, respectively.

DISCUSSION

Transmantle dysplasia is a very rare malformation of FCD, but its pathologic features

are the same as those of FCD IIA or FCD IIB. Although very little is known about TD,

this study may contribute to a new pathophysiologic understanding of FCD

malformations that extend from the ventricular wall to the cortical surface. Our results

indicate that cells expressing postnatal layer-specific markers are abnormally present

and continuously distributed from deep WM to the cortical surface in TD. As previously

described, neurons in these FCD lesions expressed layer-specific markers indicating

immaturity (19). Moreover, we identified some specific marker expression patterns that

differed between FCD IIA and FCD IIB pathologies.

 TBR1-positive, CTIP2-positive, SATB2-positive, and FOXP1-positive cells, which are

originally located in the deep cortex during the fetal period (10), were diffusely

observed in the cortex and/or WM. In particular, TBR1-positive and FOXP1-positive

cells in FCD IIB cases were mostly in the CxMoL, CxUL, and DWM, to a greater

degree than in FCD IIA cases. This suggests that the malformation and pathologic onset

of FCD IIB are more severe and earlier than those in FCD IIA. In mouse brain, Tbr1 is

identified from E11.5 and is expressed at a low level in the upper layer and at high

levels in the subplate and Layer VI, which extend axons to the thalamus and form

callosal projections (14). Satb2 serves to form callosal projections (12). The FCD IIB

malformation may be formed in the early fetal period and express immature markers.

SATB2 is also known as a repressor of CTIP2 (12). However, our data show that the

CTIP2-positive cell distribution is similar to that of SATB2. In the FCD brain, there

may be different molecular mechanisms between SATB2 and CTIP2. On the other

hand, the presence of many TBR1-positive and FOXP1- positive cells in DWM of FCD

IIB might indicate a neuronal migration arrest in the very early fetal period.

 The CUTL1-positive cell distribution pattern was similar to that of FOXP2. In the

cortex, concentrations of cells expressing these 2 markers tended to be larger in FCD

IIA than those in FCD IIB, whereas the pattern was reversed in WM. In FCD IIB, the

large numbers of CUTL1-positive and FOXP2-positive cells were in the ScWM, MWM

and DWM, although there were no significant differences between FCD IIA and IIB,

except for CUTL1 in MWM. In mouse brain, Cutl1 is expressed in the upper layer of

the cortex from E16 and regulates late neuronal differentiation, such as dendrite

development, spine formation, and synaptic function (15, 21). Persistent CUTL1

expression in FCD II suggests delayed synaptic maturation. Recently, FOXP2, a

causative gene of developmental verbal dyslexia, was reported to negatively regulate

sushi-repeat protein SRPX2 and plasminogen activator (uPAR), whose mutations lead

to Rolandic epilepsy or perisylvian polymicrogyria (22). We speculate that FOXP2

overexpression contributes to regional cortical dysplasia via abnormal downregulation

of the SRPX2/uPAR complex.

 Putative upper layer marker cells, FOXP1, FOXP2, and CUTL1, expressed the

immature neuron marker Nestin/PROX1, whereas cells expressing the putative lower

layer markers TBR1, CTIP2, and SATB2 only expressed MAP2/2B. This suggests that

the cells expressing the putative upper layer markers may maintain their neuronal

immaturity. Many CUTL1-positive, FOXP1-positive, and FOXP2-positive cells

contained GFAP. Although we do not know the relationship of these markers to glial

cell development, these findings may indicate abnormal neuronal progenitor cell differentiation into both the neuron and glial cells.

In conclusion, we demonstrate that neurons of FCD with TD had delayed maturation and abnormal differentiation. The remaining abnormal expression of various layerspecific markers in TD is evidence of immature neuronal differentiation in these cortical malformations. The present study may make it possible to clarify differences between FCD IIA and FCD IIB based on their layer-specific marker expression patterns.

ACKNOWLEDGMENTS

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TABLE 1. Clinicopathological profile of FCD with TD patients

Case	Sex	Age at Seizure Onset	Age at Surgery	Seizure Type	Intelligence (DQ or IQ)	TD Location on Imaging	Pathologic Diagnoses
1	F	1mo	6mo	GTC	53	P+O	FCD IIA
2	F	1mo	8mo	GTC+WS	86	T+P+O	FCD IIA
3	M	0mo	1mo	WS	100	Fr	FCD IIA
4	F	2y1mo	6y4mo	GTC	83	Fr	FCD IIA
5	F	1mo	2y9mo	GTC+WS+MS	10	T+P+O	FCD IIA
6	M	0mo	6mo	GTC+MS	95	Fr	FCD IIB
7	F	3y0mo	10y9mo	CPS	111	T+P	FCD IIB
8	M	1y0mo	1y1mo	CPS	91	Fr	FCD IIB
9	M	4mo	2y1mo	WS+CPS	100	P	FCD IIB

M, male; F, female; GTC, generalised tonic-clonic seizure; WS, West syndrome; MS, myoclonic seizure; CPS, complex partial seizure; IQ, intelliget quotient; DQ, developmental quotient; Fr, frontal lobe; T, temporal lobe; P, parietal lobe; O, Occipital lobe; TD, transmantle dysplasia; FCD IIA and FCD IIB, focal cortical dysplasia IIA and IIB. Pathological diagnoses are based on the international classification.
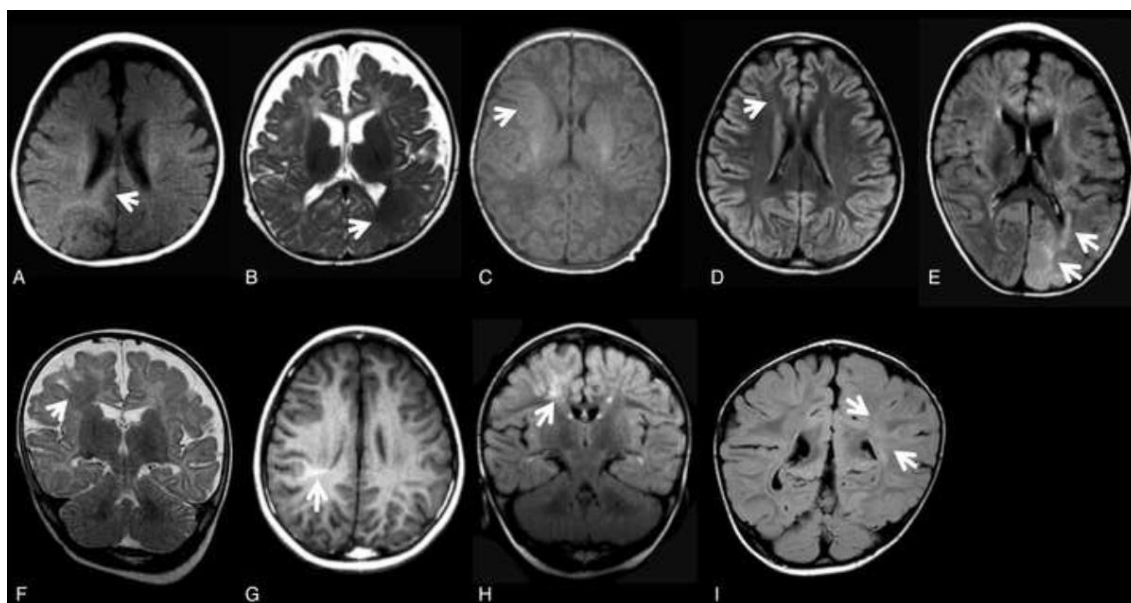

FIGURE 1. Head magnetic resonance images of all patients showing the ''transmantle sign'' of focal cortical dysplasia (arrows). (A) Axial view of a T1-weighted image of Case 1. (B) Axial view of a T2-weighted image of Case 2. (C) Axial view of a T1-

weighted image of Case 3. (D) Axial view of a FLAIR image of Case 4. (E) Axial view

of a FLAIR image of Case 5. (F) Coronal view of a T2-weighted image of Case 6. (G)

Axial view of a T1-weighted image of Case 7. (H) Coronal view of a FLAIR image of
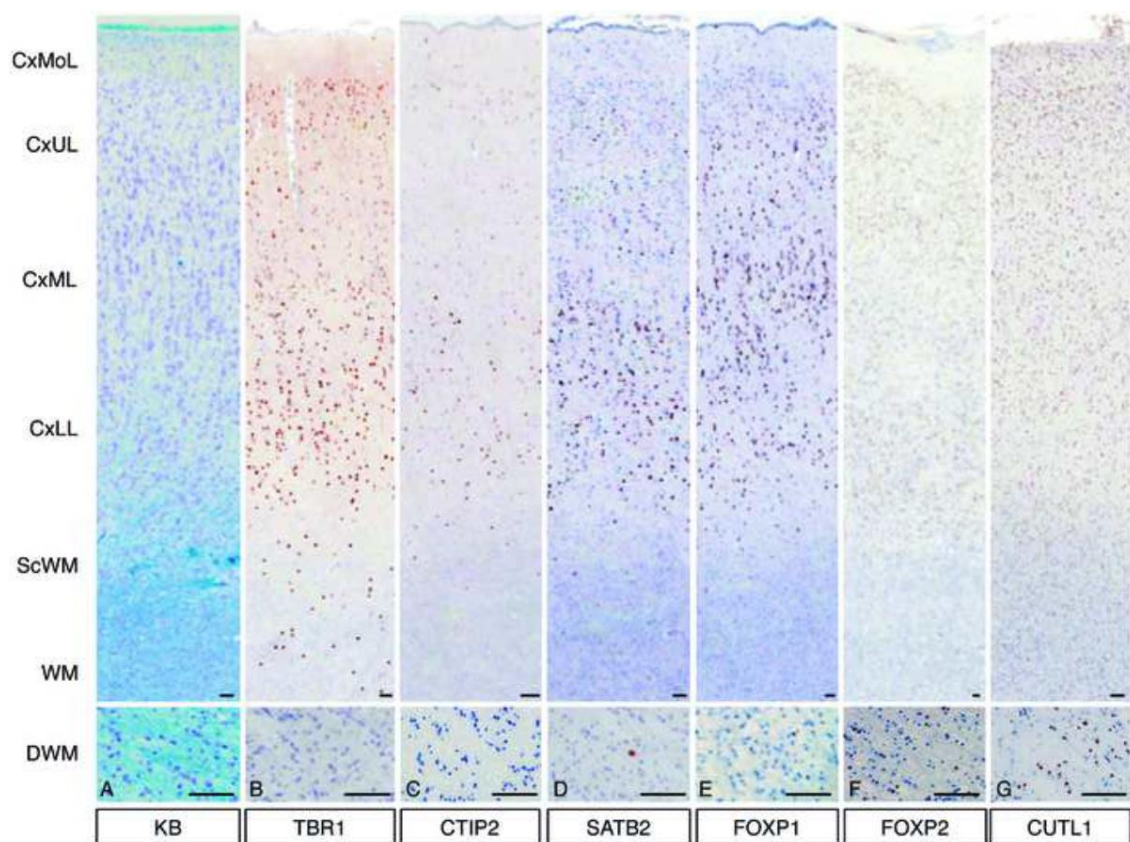
Case 8. (I) Coronal view of a FLAIR image of Case 9.

FIGURE 2. Expression patterns of layer-specific markers in focal cortical dysplasia

(FCD) IIA. (A) FCD IIA shows cortical dyslamination, a high cell density, and many

heterotopic neurons in the white matter. (B) TBR1-positive cells are distributed

diffusely, particularly in the cortical upper (CxUL) and lower (CxLL) layers. (C, D)

CTIP2-positive cells and SATB2-positive cells are predominantly in the CxLL. (E)

FOXP1-positive cells are in the cortical middle layer (CxML) and CxUL. (F) FOXP2-

positive cells are diffusely distributed in the cortex and white matter; there are a few in

the deep white matter (DWM). (G) CUTL1-positive cells are also diffusely distributed.

(A) KB indicates Klüver-Barrera. (B) TBR1 indicates T-box, brain, 1. (C) CTIP2

indicates COUP-TF interacting protein 2. (D) SATB2 indicates special AT-rich

sequence binding protein 2. (E) FOXP1 indicates Forkhead box protein P1. (F) FOXP2

indicates Forkhead box protein P2. (G) CUTL1 indicates Cut-like homeobox 1. CxMoL

indicates molecular layer of cortex; CxUL, upper layer (upper one third of the cortex);

CxML, middle layer (middle one-third of the cortex); CxLL, lower layer (lower one-

third of the cortex); ScWM, subcortical white matter; MWM, middle white matter;

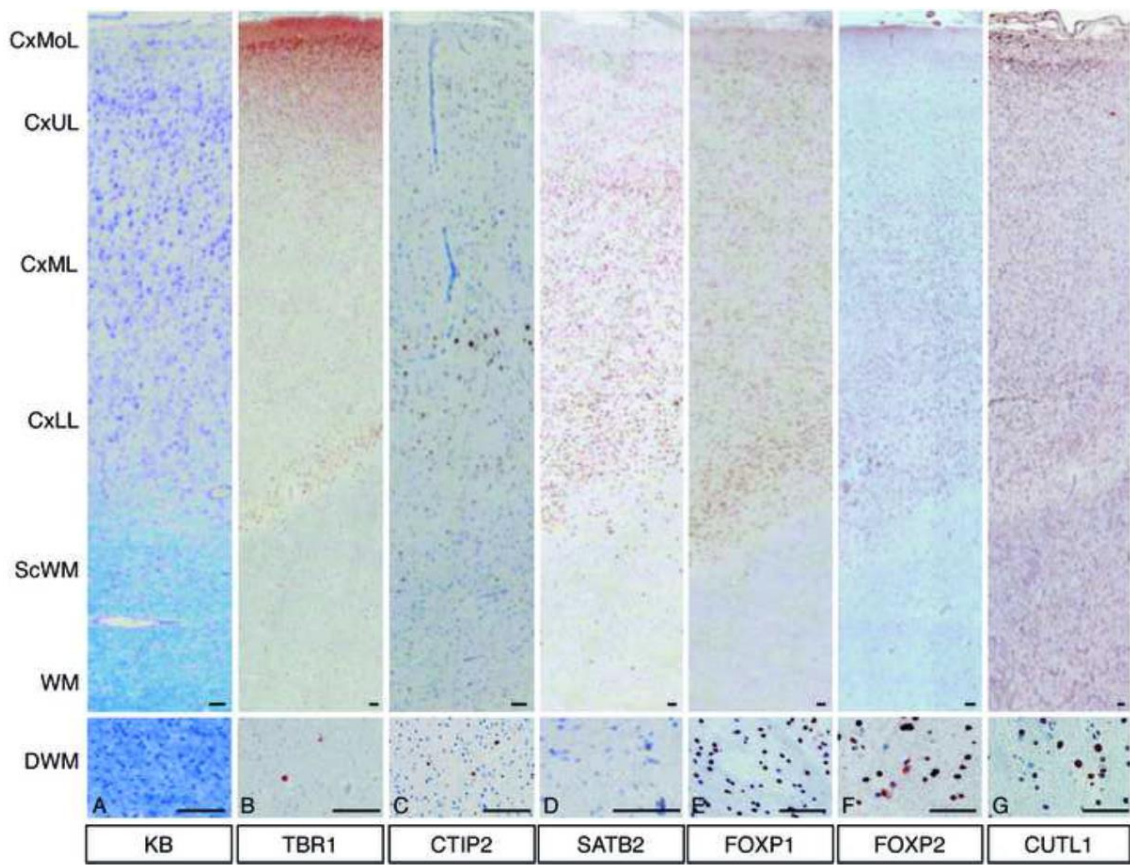DWM, deep white matter (near ventricular wall). Bars = 100 μm.

FIGURE 3. Expression patterns of layer-specific markers in focal cortical dysplasia

(FCD) IIB. (A) FCD IIB also shows cortical dyslamination, high cell density, and many

heterotopic neurons in the white matter. (B) TBR1-positive cells are diffusely

distributed in the cortex and white matter. (C) CTIP2-positive cells are in the CxML and

CxLL. (D) SATB2-positive cells are diffusely distributed in the cortex, but there are

only a few in the white matter. (E) FOXP1-positive cells are in the cortex and DWM.

(F) FOXP2-positive cells are also in the cortex and white matter. (G) CUTL1-positive

cells are diffusely distributed; they are particularly numerous in the white matter. (A)

KB indicates Klüver-Barrera. (B) TBR1 indicates T-box, brain, 1. (C) CTIP2 indicates

COUP-TF interacting protein 2. (D) SATB2 indicates special AT-rich sequence binding

protein 2. (E) FOXP1 indicates Forkhead box protein P1. (F) FOXP2 indicates

Forkhead box protein P2. (G) CUTL1 indicates Cut-like homeobox 1. CxMoL indicates

molecular layer of cortex; CxUL, upper layer (upper one-third of the cortex); CxML,

middle layer (middle one-third of the cortex); CxLL, lower layer (lower one-third of the

cortex); ScWM, subcortical white matter; MWM, middle white matter; DWM, deep

white matter (near ventricular wall). Bars = 100 μm.

Table 2 Cell-Type and Layer-Specific Marker Coexpression

	Cell-Type Marker	Layer-Specific Marker					
		TBR1	CTIP2	SATB2	FOXP1	FOXP2	CUTL1
FCD ⅡA	MAP2/2B	+	+	+	+	+	+
	Nestin/PROX1	—	—	—	+	+	+
	GFAP	—	—	—	—	—	+
	Iba-1/CD68	—	—	—	—	—	—
FCD ⅡB	MAP2/2B	+	+	+	+	+	+
	Nestin/PROX1	—	—	—	+	+	+
	GFAP	—	—	—	+	+	+
	Iba-1/CD68	—	—	—	—	—	—

+ indicate appearance of merged cells; -, no merged cell appearance.
MAP2/2B indicates microtubule-associated protein 2/2B; GFAP, glial fibrillary acidic protein; TBR1, T-box, brain, 1; CTIP2, COUP-TF interacting protein 2; SATB2, special AT-rich sequence binding protein 2; CUTL1, Cut-like homeobox 1, FOXP1 and FOXP2, Forkhead box P1 and P2; PROX1, Peospero homeobox protein 1.
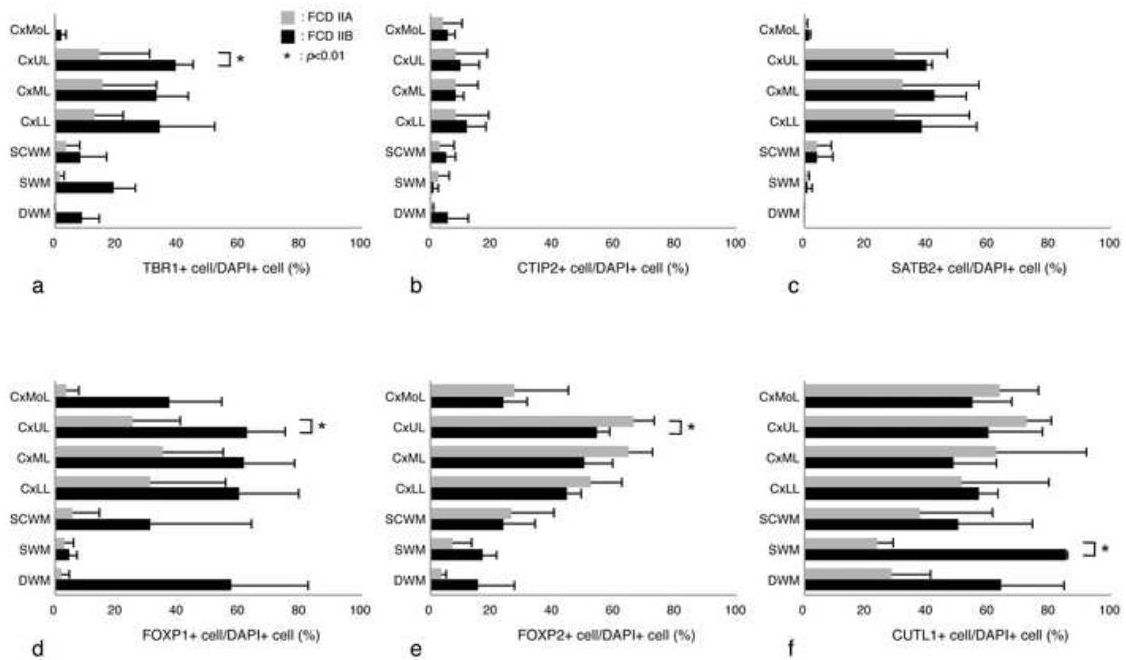
FIGURE 4. Distribution of layer-specificmarker cell ratios (marker-positive cell number/total 4',6'-dimamidino-2-phenylindoleYpositive cell number) in regions of focal cortical dysplasia (FCD) IIA and IIB. (A-F) Compared with FCD IIA, FCD IIB has a higher TBR1 ratio (A) and FOXP1 ratio (D) in the cortical upper layer (CxUL), and CUTL1 ratio in the middle white matter (MWM) (F). On the contrary, FCD IIA shows higher FOXP2 ratio in the CxUL (E). Other regionmarker ratios are not significantly different between FCD IIA and FCD IIB. TBR1 indicates T-box, brain, 1; CITP2, COUP-TF interacting protein 2; SATB2, special AT-rich sequence binding protein 2; FOXP1 and 2, Forkhead box protein P1 and P2; CUTL1, Cut-like homeobox 1.
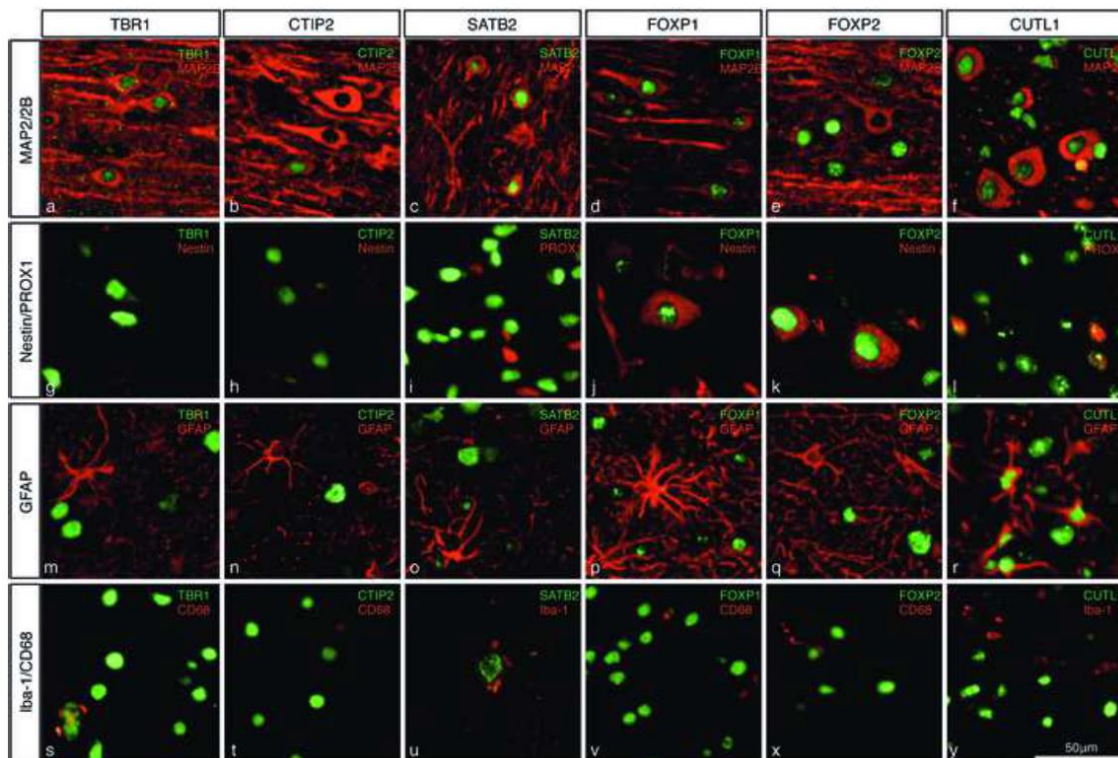
FIGURE 5. Types of cells expressing layer-specific markers in focal cortical dysplasia

(FCD) IIA. (A-F) All cells expressing layerspecific markers express microtubule-

associated protein (MAP) 2 or 2B. (G-L) Forkhead box protein P1 (FOXP1)-positive,

Forkhead box protein P2 (FOXP2)-positive, and Cut-like homeobox 1

(CUTL1)Ypositive cells also express the immature neuron markers, Nestin or Prospero

homeobox protein 1 (PROX1) (J-L), whereas cells expressing the other markers do not

(G-I). (M-R) Only CUTL1-positive cells express glial fibrillary acidic protein (GFAP)

(R). (S-Y) Layer-specific marker-positive cells do not express Iba-1 or CD68.
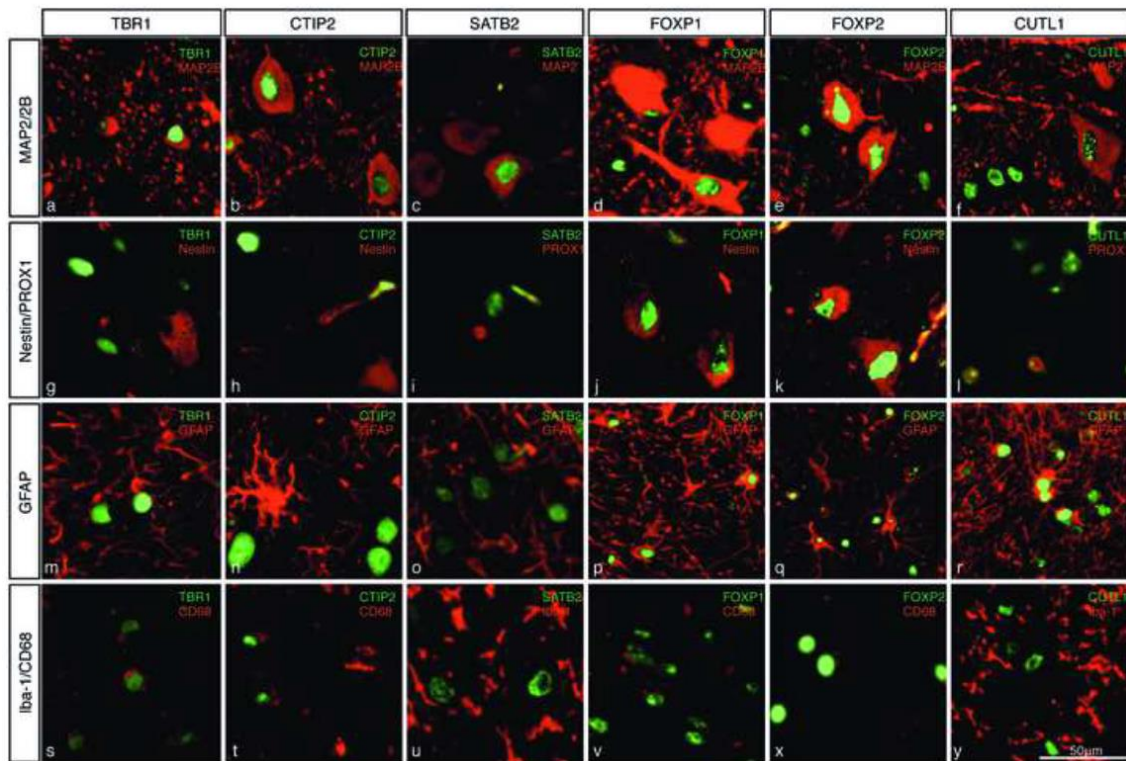
FIGURE 6. Types of cells expressing layer-specific markers in focal cortical dysplasia (FCD) IIB. (A-F) All cells expressing layerspecific markers express microtubule-associated protein (MAP) 2 or 2B. (G-L) Forkhead box protein P1 (FOXP1)-positive, Forkhead box protein P2 (FOXP2)-positive, and Cut-like homeobox 1 (CUTL1)-positive cells also express the immature neuron markers, Nestin or Prospero homeobox protein 1 (PROX1) (J-L), whereas cells expressing the other markers do not (G-I). (M-R) FOXP1-positive, FOXP2-positive, and CUTL1-positive cells express glial fibrillary acidic protein (GFAP) (P-R). (S-Y) Layer-specific markerpositive cells do not express Iba-1 or CD68.