Distinct ontogenic and regional expressions of newly identified Cajal–Retzius cell-specific genes during neocorticogenesis

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Cajal–Retzius (CR) cells are early-generated transient neurons and are important in the regulation of cortical neuronal migration and cortical laminar formation. Molecular entities characterizing the CR cell identity, however, remain largely elusive. We purified mouse cortical CR cells expressing GFP to homogeneity by fluorescence-activated cell sorting and examined a genomewide expression profile of cortical CR cells at embryonic and postnatal periods. We identified 49 genes that exceeded hybridization signals by >10-fold in CR cells compared with non-CR cells at embryonic day 13.5, postnatal day 2, or both. Among these CR cell-specific genes, 25 genes, including the CR cell marker genes such as the reelin and calretinin genes, are selectively and highly expressed in both embryonic and postnatal CR cells. These genes, which encode generic properties of CR cell specificity, are eminently characterized as modulatory composites of voltage-dependent calcium channels and sets of functionally related cellular components involved in cell migration, adhesion, and neurite extension. Five genes are highly expressed in CR cells at the early embryonic period and are rapidly down-regulated thereafter. Furthermore, some of these genes have been shown to mark two distinctly different focal regions corresponding to the CR cell origins. At the late prenatal and postnatal periods, 19 genes are selectively up-regulated in CR cells. These genes include functional molecules implicated in synaptic transmission and modulation. CR cells thus strikingly change their cellular phenotypes during cortical development and play a pivotal role in both corticogenesis and cortical circuit maturation.

In the developing neocortex, the generation of distinct classes of cortical neurons is controlled by a hierarchical series of developmental events (1). In this process, postmitotic cortical neurons migrate along radial glial cell fibers from the ventricular zone. These cells form the cortical plate and subdivide the preexisting preplate into the superficial marginal zone (MZ) and the subplate (2). Cajal–Retzius (CR) cells represent the key neuronal subtype that regulates radial migration of cortical neurons and the laminar formation of the neocortex (2, 3). In mice, CR cells appear at embryonic days (E) 10 and 11, occupy a major cell population in the MZ throughout the entire cortex, and gradually decrease during the postnatal period. CR cells produce an extracellular matrix protein called reelin (4). Reelin is defective in the reeler mouse mutant and human congenital lissencephaly, both of which show altered cortical cell migration and an abnormal cortical layer formation (4, 5). CR cells also form synaptic connections with migrating cortical neurons, thus serving as a physiological scaffolding of cortical synaptic circuits during neocortical development (3, 6, 7). Despite great advances in the characterization of CR cells in neocortical development, neither the molecular entities that govern the CR cell identity nor the molecular mechanisms in which CR cells participate in the control of cortical organization were well understood.

In our previous studies, we reported that the membrane-anchored GFP transgene, when driven by the promoter function of metabotropic glutamate receptor subtype 2 (8), is specifically expressed by CR cells both in the MZ during the embryonic stage and in layer 1 during the postnatal period (7, 9). This specific expression of GFP in CR cells provided a unique opportunity to purify CR cells to homogeneity by fluorescence-activated cell sorting (FACS) and to investigate a genomewide expression profile of CR cells during neocortical development with microarray techniques. Here we report that CR cells not only acquire the generic properties of CR cell specificity from early development but also change their gene expression profiles markedly during cortical development. Furthermore, the identification of many functional molecules specific for CR cells suggests that CR cells play a pivotal role in the control of corticogenesis and cortical circuit maturation.

Methods

Cell Purification and FACS. Cerebral cortices from 10–24 embryos or newborns of the IG17 line of homozygous transgenic mice (8) were cut into small pieces in ice-cold L15 medium (Invitrogen). These pieces were treated with 10–20 units/ml papain (Nacalai Tesque, Kyoto) and 0.01–0.02% DNase I (Sigma) in a solution containing 0.02% BSA, 0.02% l-cysteine, and 0.5% glucose for 20 min at 37°C. Single cells were prepared by passing them through a plastic pipette 40 times. Dissociated cells were stained with propidium iodide (PI) (1 μg/ml, Sigma), and two-color cell sorting based on GFP and PI fluorescence was performed with a FACSVantage flow cytometer (BD Biosciences). Approximately 5 × 10⁵ purified GFP-positive, PI-negative cells and GFP-negative, PI-negative cells were isolated from cortices at E13.5 and postnatal day (P) 2 and used for microarray analysis.

Microarray Analysis. An Affymetrix (Santa Clara, CA) MOE430A mouse expression microarray consisting of 22,690 probe sets was used in all microarray experiments. cRNA probes were synthesized according to a two-cycle in vitro transcription labeling protocol (Affymetrix), and four sets of biotinylated cRNA probes were prepared from total RNAs isolated from CR and non-CR cells at E13.5 and P2. Hybridization and scanning were performed according to the manufacturer’s instructions (Affymetrix). Hybridization signals were calculated by analyzing raw data with MICROARRAY SUITE 5.0 (Affymetrix) and were further analyzed with GENESPRING 6.1 (Silicon Genetics). The data were normalized to the 50th percentile for per-chip normalization.

Abbreviations: CMWT, caudomedial wall of the telencephalic vesicle; CR, Cajal–Retzius; En, embryonic day; FACS, fluorescence-activated cell sorting; MZ, marginal zone; PI, postnatal day; PI, propidium iodide; VDCC, voltage-dependent calcium channel.

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Results

Purification of CR Cells by FACS. We focused on CR cells at E13.5 and P2, because the expression of the GFP transgene is not only specific to CR cells within the developing neocortex but also sufficiently appears at both stages (7, 9). The neocortices were prepared from the brains of E13.5 and P2 transgenic mice, and cells were dissociated into single cells by papain treatment. Trypan blue staining, a marker of dead cells, showed that almost all and approximately four-fifths of purified GFP-positive cells were viable in the E13.5 and P2 cell populations, respectively. To remove nontiable cells, dissociated cells were stained with PI, a fluorescent marker of dead cells, and GFP-positive and PI-negative viable CR cells were purified with FACs with GFP and PI fluorometry (Fig. 1A). In this sorting, GFP-negative, PI-negative cells were also pooled and used as a cell population of non-CR cells. Each set of cRNA probes was subjected to microarray hybridization analysis. As a criterion to select candidate genes, we used hybridization signals of >1,000 in E13.5 and/or P2 CR cells, a level sufficiently higher than that of the background signal. On the basis of this criterion, we identified 3,657 and 3,275 probe sets from data analysis of E13.5 and P2 CR cells, respectively. Hybridization signals of the well known CR cell marker reelin, transformation related protein 73 (p73), and calretinin genes (2, 12) were prominently higher in CR cells compared with non-CR cells at both E13.5 and P2 (Fig. 2A), and this expression profile was in marked contrast to the intense hybridization signals of the housekeeping GAPDH and β-actin transcripts in both CR and non-CR cells (Fig. 2A). This examination validated that our microarray data are reliable for further characterization of a gene-expression profile characteristic of CR cells.

We first examined developmental changes of gene expression in CR cells per se by comparing the 3,657 probe sets at E13.5 and the 3,275 probe sets at P2 with the corresponding probe sets at P2 and E13.5, respectively. Upon this comparison, we identified 150 and 121 genes that increased by more than three times in E13.5 and P2 CR cells, respectively. The functions of 131/150 genes at E13.5 and 111/121 genes at P2 were annotated on the basis of available databases and literature and classified into eight categories (Tables 1 and 2, which are published as supporting information on the PNAS web site). For convenience, the 131 genes characteristic of E13.5 CR cells and the 111 genes of P2 CR cells are termed “E13.5 genes” and “P2 genes” hereafter.

When the functions of the 131 E13.5 genes and the 111 P2 genes are inspected, transcription regulators/nuclear proteins are strikingly prominent in E13.5 CR cells, accounting for almost half (45%) of the E13.5 genes (Fig. 2B). By contrast, extracellular signaling molecules, cytoskeletal proteins, and metabolic enzymes are up-regulated at P2 CR cells, as compared with E13.5 CR cells (Fig. 2B). Furthermore, the E13.5 gene products are more closely related to cell proliferation, cell differentiation, neurogenesis, and cell morphogenesis, whereas the P2 gene products are implicated in synaptic organization, synaptic transmission, and transmission-linked signal transduction (see Discussion). CR cells thus dramatically change their cellular phenotypes from the embryonic period to the postnatal period.

CR Cell-Specific Gene Expression in the Developing Neocortex. We next analyzed a gene-expression profile specific for CR cells by comparing the above 3,657 and 3,275 probe sets of CR cells with the corresponding sets of non-CR cells. We identified 69 and 96 probe sets that were more than five times higher in hybridization signals of CR cells at E13.5 and P2 than those of non-CR cells at the corresponding periods, respectively. The functions of 63/69 genes at E13.5 and 87/96 genes at P2 were annotated on the basis of available databases and literature (Tables 3 and 4, which are published as supporting information on the PNAS web site). Among these 150 genes, 49 had a signal >10 times higher in CR cells compared with non-CR cells at E13.5, P2, or both. These 49 genes, hereafter termed “CR genes,” can be classified into three groups according to the difference in expression levels between E13.5 and P2 (Fig. 2A). Group 1 CR genes showed high levels of expression in both E13.5 and P2 CR cells, with the relative ratios of P2 to E13.5 CR cells within the range from 0.33 to 3.0. This group includes not only the CR cell marker genes (reelin, calretinin, and p73) but also 22 other genes. The reelin gene showed the highest hybridization signals, but hybridization signals of several other genes (Zic1, Cacna2d2, and Scnb3) were as high as those of the p73 and calretinin genes at both E13.5 and P2. The functions of the group 1 genes are diverse, suggesting that diverse functional molecules are necessary for inducing and maintaining cellular phenotypes characteristic of CR cells. Group 2 of the CR genes comprises five genes that exhibited a marked reduction (>3.0-fold) in expression levels from E13.5 to

Fig. 1. Purification of CR cells by FACS. (A) GFP-positive, PI-negative cells (fraction b) and GFP-negative, PI-negative cells (fraction a) at P2 were sorted by FACS as CR cells and non-CR cells, respectively. The PI fluorescence of viable CR cells was slightly higher than that of viable non-CR cells because of a slight overlap of GFP fluorescence in PI fluorescence measurement. (B and C) Purified GFP-positive CR cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and detected with microscopy. (Scale bar, 50 μm.)
P2. Group 3 CR genes showed a prominent elevation (>3.0-fold) in expression levels from E13.5 to P2. The relationship between the CR cell specificity and the ontogenic expression profile was further examined by analyzing ontogenic changes in expression levels of the CR genes listed in Fig. 2A. In this comparison, the CR genes exhibiting a 10-fold increase in expression levels at either E13.5 or P2 CR cells, termed "E-CR genes" and "P-CR genes," respectively, were...
CR genes up-regulated during the postnatal period. Cells acquire distinct properties that can be characterized by the proteins, and intracellular signaling molecules. These findings extracellular signaling molecules, membrane-integral receptor Moreover, the majority of these up-regulated genes encode D/H11015 genes also were highly expressed at E13.5 CR cells (Fig. 2 C and D). Importantly, the remaining ~60% (19/33) of the P-CR genes showed a dramatic increase in expression levels at P2 (Fig. 2D). Moreover, the majority of these up-regulated genes encode extracellular signaling molecules, membrane-integral receptor proteins, and intracellular signaling molecules. These findings demonstrate not only that many genes other than the well known CR cell marker genes are selectively and highly expressed in both embryonic and postnatal CR cells but also that postnatal CR cells acquire distinct properties that can be characterized by the CR genes up-regulated during the postnatal period.

Spatial and Temporal Expression Patterns of CR Genes. To confirm the spatially restricted expressions of the CR genes, we performed in situ hybridization analysis of 30 CR genes, as well as double in situ hybridization analysis of 18 CR genes, by using a digoxigenin-labeled GFP probe and radioisotope (R1)-labeled probes for CR genes (Fig. 3A). Hybridization signals of the group 1 genes (those shown in Fig. 3A and Ebf3, Lhx5, Zic1, Siat7e, Unc5h2, and Mab21l1) were detected both in the MZ at E13.5 and in layer 1 at P2. The signals of these transcripts were mostly confined to the MZ at E13.5 but in some cases were distributed not only in layer 1 but also in other cortical layers at P2 (Fig. 3A) (for example, see reelin, Cxcr4, and tau), and these data are consistent with other reports (13–15).

Most of the group 1 CR genes also were detected at the MZ of the caudomedial wall of the telencephalic vesicle (CMWT), including at the cortical hem and at the cortical surface of the pallium (data not shown), where newly generated CR cells from origins are supposed to be transiently accumulated (9, 16). Importantly, signals of p21 and Gdn at E13.5 were abundantly seen at the CMWT possessing massive GFP-positive CR cells, whereas the signal of Ebf2 was considerably detected at the MZ adjacent to the ventral pallium (Fig. 4). These group 2 CR genes are thus distinctly expressed at the regions corresponding to the plausible CR cell origins at the early stage of corticogenesis (9, 16–18).

Fig. 3. Analysis of in situ hybridization and temporal expression patterns of CR genes. (A) In situ hybridization analysis of CR genes was conducted in neocortical sections at E13.5 and P2, and the results are displayed in Top and Middle, respectively. CP, cortical plate; I-VI, layers 1–6; SP, subplate; DG, digoxigenin. In Bottom, double in situ hybridization analysis in neocortical sections at E13.5 (Cacna2d3, tau, and p21) and at P2 (others) is indicated. (Scale bars: Top and Middle, 100 μm; Bottom, 10 μm.) (B) Temporal changes in expression levels of indicated CR genes were analyzed at five to six time points from E13.5 to P8. Expression levels at P2 were taken as 1 in all analyses.that group 3 CR genes are up-regulated in CR cells during development but in distinct temporal fashions.

Discussion

In this investigation, a large number of CR genes were newly identified, and their functions appear to be diversified. However, sets of the CR genes are functionally closely related, and several possible functional roles of the CR genes can be envisaged in relation to the characteristic features of CR cells.

Origins of CR Cells. With respect to the CR cell origins, many sources have been proposed, including the pallium (17), the subpallium (18), the retrobulbar area (16), and the cortical hem region (9). The Ebf2 gene and the Gdn and p21 genes of the
group 2 CR genes are preferentially expressed at the plausible CR cell origins (Fig. 4). Ebf2 is a member of the helix-loop-helix transcription factors (19) and serves as an upstream transcription factor in the helix-loop-helix transcriptional cascade (see below), whereas p21 is a downstream signaling molecule of the transforming growth factor β (TGF-β) signaling pathway (20). The TGF-β signaling enhances the formation of the Smad-Foxo3 signaling complex (20). Interestingly, Foxo3 (Table 3, No. 12) is selectively expressed in E13.5 CR cells, whereas Tgfb1 and AcvrII, which are the members of TGF-β receptor subtypes I and II, respectively, also are highly expressed in E13.5 CR cells and then down-regulated at P2. The selective expressions of Ebf2, Gdn, and p21 at two distinct regions corresponding to the plausible CR cell origins suggest that prospective CR cells are heterogeneous and are distributed through the MZ by tangential migration.

Transcriptional Regulation of CR Cells. We identified seven transcription regulators/nuclear proteins as the group 1 or group 2 CR genes (Fig. 2A). In the Xenopus nervous system, Ebf2, NeuroD, and Ebf3 serve as a transcriptional cascade in this sequential order and regulate neural cell development and differentiation (19). Ebf2 is expressed at the very early stage of CR cells and is down-regulated thereafter. NeuroD (Table 1, No. 4) is highly expressed in both CR and non-CR cells at E13.5 and also is down-regulated at P2. In contrast, Ebf3 is prominently expressed in CR cells at both E13.5 and P2. The Ebf2–NeuroD–Ebf3 transcriptional cascade may thus participate in the regulation of CR cell development and differentiation. Lhx1 and Lhx5, another family of the CR genes, belong to the LIM family of the homeobox genes (21). The expression of Lhx1 is restricted at the MZ at E13.5. Lhx2 (Table 1, No. 27) and Lhx9 (Table 1, No. 11) also are highly expressed in CR cells at E13.5 and down-regulated at P2. In contrast, Lhx5 is persistently high in CR cells throughout development. Gene targeting of Lhx5 results in the disappearance of hippocampal CR cells and hippocampal malformation (21). Zic1 also was identified as a group 1 CR gene, and its family, Zic3 (Table 1, No. 43) and Zic5 (Table 3, No. 9), are highly and relatively selectively expressed in CR cells at E13.5 and down-regulated at P2. Zic1 was originally isolated as a specific transcription factor of embryonic cerebellar granule cells that secrete reclin during cerebellar development (4, 22). These findings suggest that several distinct transcriptional cascades coordinately regulate the fate and cellular phenotypes of CR cells.

Calcium Signaling of CR Cells. Cacna2d2 and Cacna2d3 of group 1 CR genes encode subunit composites of voltage-dependent calcium channels (VDCCs) (23). In addition, Cacng4 (Table 3, No. 39), Cacng5 (Table 2, No. 38), and Cacna2d1 (Table 4, No. 45) other VDCC composites, are relatively selectively expressed in CR cells. These VDCC composites per se have no channel activity and modulate the VDCC activity (23). Sclh3, identified as a group 1 CR gene, also encodes a modulatory composite of sodium channels (24). The mutant of Cacna2d2 called ducky shows a reduction of a current density of VDCCs in Purkinje cells and impairs the dendritic formation of these cells (23). In addition, several intracellular calcium signaling molecules, including Dscr1l1, calretinin, Rii2, and Pcep4 (25–27), are selectively expressed in CR cells (Fig. 2A). The CR cell-specific Ca²⁺ signaling components could thus play an important role in development and functions that are characteristic of CR cells.

Cell Migration, Adhesion, and Neurite Extension. CR cells migrate along the most apical layer and dramatically change their morphology. This layer also is filled with growing dendrites, axons, and extracellular matrix proteins during development. Many functional molecules involved in cell migration, cell adhesion, neurite/axon extension, and extracellular matrix maturation are specifically expressed in CR cells. These include Cxcr4, Dsp, Mmp2, Siat7e, and Siat9 of the group 1 CR genes and Cxcl12, Nptn, and Thy-1 of the group 3 CR genes (28–31) (Fig. 2A). Particularly interesting is the CR cell-specific expression of chemokine signaling molecules. Cxcr4 is the receptor for the Cxcl12 chemokine and is persistently up-regulated in embryonic and postnatal CR cells (Fig. 2A). In the developing neocortex, Cxcl12 is continuously produced at adjacent meningeal cells (15). Furthermore, this chemokine is strikingly up-regulated in CR cells per se at the postnatal period (Fig. 3B). In addition, chemokine orphan receptor 1 (Cmklr1; Table 3, No. 37) also is selectively and highly expressed in CR cells at E13.5. Although gene targeting of either Cxcr4 or Cxcl12 has been shown to keep CR cells at the MZ, it is possible that the chemokine signaling may regulate the migration of immature CR cells and/or axonal responsiveness to a variety of guidance cues (15, 32). Another interesting feature is the selective expression of Siat7e and Siat9. In the MZ/layer 1, the neural cell adhesion molecule is preferentially polysialylated, and this polysialylation attenuates the adhesive property of the neural cell adhesion molecule, thereby facilitating neural cell migration (2, 33). Polysialylation requires the preceding α2,3- or α2,6-linked siaiylation reaction (34). Siat7e and Siat9 are α2,6- and α2,3-sialyltransferases, respectively (35). The CR cell-specific expression of these sialyltransferases could be important for sialylation-mediated regulation of cell–cell interactions and neurite extension.

Induction of CR Cell-Specific Genes During Cortical Circuit Maturation. Many functional molecules involved in synaptic transmission and modulation are selectively up-regulated at the late developmental stage (Figs. 3A and 4B). P2X5 is a purinergic receptor and is capable of exciting neuronal cells by means of cation permeation (36). Acetylcholinesterase (Ache) may also regulate the cholinergic transmission to layer 1 γ-aminobutyric acid (GABA)-ergic interneurons, because these interneurons receive cholinergic inputs by means of muscarinic cholinergic receptors (6). The selective expression of prepronociceptin (Phnc) is also interesting because the nociceptin receptor is expressed in cortical pyramidal neurons from early development to the adult stage.
(37). Furthermore, Psek1n is an inhibitor of proteolytic cleavage of many peptide precursors, whereas Nep, also called enkaphalinase, inactivates biologically active peptides such as enkephalins and substance P (38, 39). Another interesting biological relevance is the relationship between Prdc and Gdf5. Gdf5 is rapidly regulated Prdc at the late stage (Fig. 3B) up-regulated and thus could be controlled by the slowly up-regulated Prdc at the late stage (40). Interestingly, Gdf5 is rapidly up-regulated and thus could be controlled by the slowly up-regulated Prdc at the late stage (40). Conclusion, this investigation strongly indicates that CR cells are involved more actively than previously envisioned in corticogenesis and cortical circuit maturation and contribute as a key signaling center to regulating dynamic cortical development.

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