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Genomic and Evolutionary Analyses of Asymmetrically Expressed Genes in Human Fetal Left and Right Cerebral Cortex

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In the human brain, the left and right hemispheres are anatomically asymmetric and have distinctive cognitive function, although the molecular basis for this asymmetry has not yet been characterized. We compared gene expression levels in the perisylvian regions of human left–right cortex at fetal weeks 12, 14, and 19 using serial analysis of gene expression (SAGE). We identified dozens of genes with evidence of differential expression by SAGE and confirmed these by quantitative reverse transcriptase–polymerase chain reaction. Most genes with differential levels of expression in the left and right hemispheres function in signal transduction and gene expression regulation during early cortical development. By comparing genes differentially expressed in left and right fetal brains with those previously reported to be differently expressed in human versus chimpanzee adult brains, we identified a subset of genes that shows evidence of asymmetric expression in humans and altered expression levels between chimps and humans. We also compared the coding sequences of genes differentially expressed between left and right hemispheres and found genes that show both asymmetric expression and evidence of positive evolutionary selection in the primate lineage leading to humans. Our results identify candidate genes involved in the evolution of human cerebral cortical asymmetry.

Keywords: brain asymmetry, evolution, human and chimpanzee, SAGE, differential gene expression, LMO4

Introduction

The human cerebral cortex is divided into left and right hemispheres, each governing the movement of the opposite half of the body. Interestingly, more than 90% of the human population is more highly skilled with the right hand than with the left (Corballis 2003), and there is a growing list of other cognitive functions that tend to be preferentially localized in one hemisphere. A unique function of the human brain is its capacity for spoken language, and this capability preferentially relies upon the left hemisphere in greater than 97% of the right-handed population. By contrast, this left-hemisphere dominance for language is observed in less than 60% of left-handed people (Galaburda and others 1978; Geschwind and Miller 2001). Further cognitive studies have revealed many other differences between the left and right cortex. For instance, the left hemisphere is dominant for mathematical and logical reasoning, whereas the right hemisphere excels at shape and spatial recognition, emotion processing, and musical and artistic functions (Gazzaniga 1998; Joseph 1998). Though the corpus callosum normally obscures these specializations because of the extensive interconnections of the 2 hemispheres, these differences become more readily apparent when lesions occur and damage either hemisphere alone or following "split-brain" operations in which the corpus callosum is severed for therapeutic reasons (Gazzaniga 2005).

Anatomical asymmetries of the cortex were first suggested based on postmortem studies and have more recently been analyzed by modern neuroimaging techniques like magnetic resonance imaging. For example, the posterior end of the Sylvian fissure, which separates the frontal and temporal lobes, is higher on the right hemisphere than on the left, whereas the left fissure has a more gentle slope (Rubens and others 1976; Toga and Thompson 2003). The planum temporale, a region on the posterior portion of the superior temporal sulcus, is larger on the left hemisphere than on the right in more than 65% of examined adult brains and 56%–79% of fetal or infant brains (Shapleske and others 1999; Hirayasu and others 2000). Recently, a new population-average, landmark-, and surface-based atlas approach has shown the most consistent asymmetries in and near the Sylvian fissure and the superior temporal sulcus (Van Essen 2005). Left–right asymmetries in the persylvian cortex have also been seen using microscopic histological techniques, such as Nissl staining (Hutsler 2003), though such analyses have been more limited.

There is increasing evidence that some of the hemispheric asymmetries that characterize the human cortex may be shared by nonhuman primates. For instance, asymmetries in Broca's area and the planum temporale have been described in chimpanzees and other great apes (Gannon and others 1998; Cantalupo and Hopkins 2001) but have not been conclusively demonstrated in other primates or nonprimate mammals (Geschwind and Miller 2001; Toga and Thompson 2003). However, most nonhuman primates do not show the consistent hand preference that characterizes humans (Fagot and Vauclair 1991; Mittra and others 1997), whereas chimpanzees show a preference for handedness that varies between left and right by community (Lonsdorf and Hopkins 2005).

How has the functionally asymmetric human cortex evolved that of our ancestors, such as chimpanzees, which show less consistent asymmetry? Three major molecular mechanisms of evolution have been widely proposed, including 1) the wholesale addition or removal of genes between species, 2) alterations in the amino acid coding sequence of proteins that alter biochemical function, and 3) changes in levels or patterns of gene expression, which is governed by noncoding promoter and enhancer elements in DNA. The recent completion of human and chimpanzee genome sequencing projects provides important opportunities to address which, if any, of these mechanisms

may be operating in the evolution of the unique features of the human cerebral cortex (2005; Hill and Walsh 2005).

Although there is limited evidence for gain (Hayakawa and others 2005) or loss of genes in the human genes relative to other primates, changes in protein-coding sequence, and in patterns of expression, are considered to be the more important molecular evolutionary forces. The evolution of protein-coding sequences is commonly evaluated by comparing DNA sequence differences that alter amino acids in the coding region ("nonsynonymous" DNA changes, Ka) with DNA sequence changes that are silent either in the coding region ("synonymous" DNA changes, Ks) or in the intronic/intragenic regions (Ki). This evaluation is under the assumption that synonymous and/or intronic changes are usually evolutionarily neutral, whereas any change in the amino acid sequence is potentially subject to positive or negative evolutionary effects (Gilbert and others 2005). If the ratio of nonsynonymous changes to synonymous (Ka/Ks) or intronic (Ka/Ki) changes approaches or exceeds 1, the neural gene is likely to be under positive evolutionary selection (Gilbert and others 2005), whereas most genes are under negative evolutionary selection (meaning that any amino acids changes are likely to be deleterious evolutionarily) and have Ka/Ks or Ka/Ki $\ll 1$.

An increasing number of genes involved in human brain development show evidence of positive evolutionary selection based on having a high Ka/Ks ratio. For example, *FOXP2*, which is essential for normal human language; *AHI1*, which is essential for normal axon outgrowth and decussation (which causes Joubert syndrome when mutated); and *ASPM* and *MCPH1* (which cause a small cerebral cortex, called microcephaly when mutated in humans) have high Ka/Ks ratios and may play significant roles in human brain evolution (Hill and Walsh 2005; Mekel-Bobrov and others 2005).

The evolution of the human brain can also be regulated by changes in patterns or levels of gene expression (Enard and others 2002; Heissig and others 2005; Khaitovich and others 2005), though this has been more difficult to analyze systematically. Although it seems that neural genes change less than genes expressed in nonneural tissues, they have changed relatively more on the human lineage (Khaitovich and others 2005). Analyzing evolutionary selection of genes differentially expressed in human left–right hemispheres may shed light on evolution of human brain asymmetry.

In a previous study, we measured differential gene expression levels between human fetal left and right hemispheres using the serial analysis of gene expression (SAGE) technique (Sun and others 2005). We identified 27 genes highly expressed in either the left or the right hemispheres. Among them, transcription factor *Lim domain only 4 (LMO4)* displayed asymmetric expression in human and mouse brains (Sun and others 2005). In this paper, we classified the cellular functions of differentially expressed genes detected by SAGE in the left and right human fetal hemispheres. In addition, we analyzed genes differentially expressed in left–right human fetal brains for signs of evolutionary selection by comparing them with those genes differently expressed between human and chimpanzee adult brains and by performing Ka/Ki ratio analysis. The genes that significantly distinguish left–right brain during human fetal development are more constrained (conserved) in terms of protein divergence at 12 weeks than they are at 14 weeks. We have identified a set of genes that are candidates for the evolution of human hemispheric asymmetry.

Materials and Methods

Generating Human SAGE Libraries

Human fetal brain tissues at 12, 14, and 19 weeks were collected from the National Institutes of Health (NIH) funded "Brain and Tissue Bank" (University of Maryland). Total RNA was extracted from tissues dissected from distinct cortical regions using TRIzol (Invitrogen, CA). The 14 SAGE libraries were generated using an I-SAGE kit (Invitrogen) and then sequenced (Agencourt, Beverly, MA) (Sun and others 2005).

Statistical Analyses of SAGE Data

SAGE data (tag sequences and frequencies) were analyzed using the "SAGE 2000" software (Invitrogen, developed by Johns and Hopkins University) on a PC computer. The statistical significance of SAGE data was analyzed using the Monte Carlo test (SAGE 2000 software) and then the chi-square test. To perform the chi-square test using Microsoft Excel, the following algorithm was used: $X^2 = (I(a \times d - b \times c)^2)/(E \times F \times G \times H)$ (a and b: SAGE tag frequency in each sample, c and d: the sum of all other tag frequencies in each sample, E and F: $a + c$ and $b + d$, respectively, [a, c, E] and [b, d, F]: sets corresponding to 1 of 2 samples, G and H: $a + b$ and $c + d$, respectively, and I: $G + H$). Using the chi distribution with a degree of freedom of 1, only genes with chi value > 3.84 ($P > 95\%$) were chosen.

Gene Functional Grouping

The functions of genes detected by SAGE were grouped according to their cellular function, as suggested by the biological process ontology (http://www.geneontology.org). 1) Cell division (DNA synthesis/replication, apoptosis, cell cycle, and chromosome structure); 2) cell signaling/cell communication (cell adhesion, channels/transport proteins, effectors/modulators, intracellular transducers, metabolism, protein modification, and receptors); 3) cell structure/motility (cytoskeletal, extracellular matrix, and microtubule-associated proteins/motors); 4) cell/organism defense (homeostasis, DNA repair, carrier proteins/membrane transport, stress response, and immunology); 5) gene/protein expression (RNA synthesis, RNA polymerases, RNA processing, transcription factors, protein synthesis and processing, post transcriptional modification/targeting, protein turnover, ribosomal proteins, tRNA synthesis/metabolism, and translation factors); 6) metabolism (amino acid, cofactors, energy/tricarboxylic acid (TCA) cycle, lipid, nucleotide, protein modification, sugar/glycolysis, and transport); and 7) unclassified.

Comparing Differentially Expressed Genes in Human Fetal Brains with Those Differently Expressed between Human–Chimpanzee Adult Brains

Human–chimpanzee protein selection data (Ka/Ki values) were taken from the Broad Institute (Massachusetts Institute of Technology) web site: http://www.broad.mit.edu/ftp/distribution/papers/chimpanzee_genome/.

This data set contains data from 13 455 pairwise human–chimpanzee gene comparisons (Supplementary Table 23) and was matched to genes differentially expressed in the left–right human fetal brains based on Supplementary Table 1 and Supplementary Table 2 (12 and 14 week, confidence level $P > 99\%$) and Supplementary Table 3–12 (12, 14, and 19 week, confidence level $95\% < P < 99\%$) in Sun and others (2005).

Genes differently expressed in human–chimpanzee adult brains (Hsieh and others 2003), which is a reanalysis of data from Enard and others (2002), were compared with the differentially expressed genes in left–right human fetal brains (Supplementary Table 3 and Supplementary Table 12, Sun and others 2005). RefSeq and gene symbol identification of microarray "internal IDs" were obtained from the Affymetrix web site: http://www.affymetrix.com/support/technical/byproduct.affx?product=hgu95.

These RefSeq and gene symbol IDs were amended to the "brain-filtered" data set (Excel file "Primate Sig Genes") obtained from the Hsieh and others (2003) supplementary information on the Genetics web site: http://www.genetics.org/cgi/content/full/165/2/747/DC1.

Genes in the 2 data sets were matched using the statistics package JMP. All statistical analyses were also done using this software.

Human Fetal Brain Tissue In Situ Hybridization

The fresh human fetal brain tissues were collected from the NIH funded Brain and Tissue Bank (University of Maryland) and immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After cryostat protection in 20% sucrose in PBS, brain tissues were embedded in optimal cutting temperature compound (OCT) and kept at –80 °C until use. Freshly frozen human fetal brain tissues were sectioned and postfixed in 4% paraformaldehyde in PBS before in situ hybridization. About 16- to 20-μm serial coronal sections were collected on slides using a cryostat, dried, and then kept at –80 °C for in situ hybridization.

Digoxygenin (DIG)-labeled sense and antisense mRNA probes were produced by in vitro transcription. The in situ hybridization on sections were performed as described (Sun and others 1998). Briefly, the sections were hybridized at 65 °C overnight, washed, and then blocked for 2 h before being labeled with anti-DIG antibody (1:1500 dilution, Roche, Palo Alto, CA) at 4 °C overnight. Sections were washed and stained with BM purple (Roche) at room temperature until ideal intensity. The images of in situ hybridization on human brain sections were collected using a Spot digital camera under a dissection scope (Nikon, Melville, NY). The images of in situ hybridization on mouse brain sections were collected using a Spot digital camera under a microscope (Nikon). All images were stored in Photoshop.

Results

Low Frequency Genes May Play Essential Roles in Human Fetal Cortical Development

To comprehensively measure gene expression levels in the left and right hemispheres, we collected human fetal brain tissues at 12, 14, and 19 weeks from the NIH funded Brain and Tissue Bank. Because SAGE can detect all transcripts at even low expression levels and simultaneously compare gene expression levels among multiple samples, we decided to use the SAGE approach and generate SAGE libraries. Total RNA was extracted from separate tissues, and 14 SAGE libraries were generated (Sun and others 2005).

Each transcript that represents a gene is called "tag" in the SAGE library. To evaluate gene expression levels, we have sequenced at least 55 000 tags in each SAGE library. Because the frequency of a specific tag represents expression levels of this gene in a brain area, we analyzed tag frequency in SAGE libraries generated from the perisylvian regions of 12- and 14-week human left and right hemispheres (12L, 12R, 14L, and 14R). We found that the majority (about 88%) of transcripts have a low abundance with detectable 1–5 tag frequencies in these 4 SAGE libraries (Fig. 1). Evans and others (2002) also detected many low abundant genes in the rat hippocampus using the SAGE approach. Our study and others suggest that many genes playing essential roles in early brain development of humans and other mammals have low expression levels or are expressed in a small group of cells.

Functional Groups of Genes Detected by SAGE with Differential Expression Levels in the Left and Right Hemispheres

In order to detect genes with differential expression levels between the left and right (L–R) hemispheres, we compared tag frequency for each gene between 2 SAGE libraries generated from the perisylvian regions. To verify the statistical significance of differences in each comparison (e.g., left–right), we first did the Monte Carlo test using the SAGE 2000 software (Invitrogen, developed by the Johns and Hopkins University). We then verified the Monte Carlo test using the chi-square test (Sun and others 2005). Using the chi distribution with degree of freedom of 1 and confidence levels (P) of 95%, 99%, 99.5%, and 99.9%, we can sort genes within one comparison (e.g., left–right) according to different levels of statistical significance.

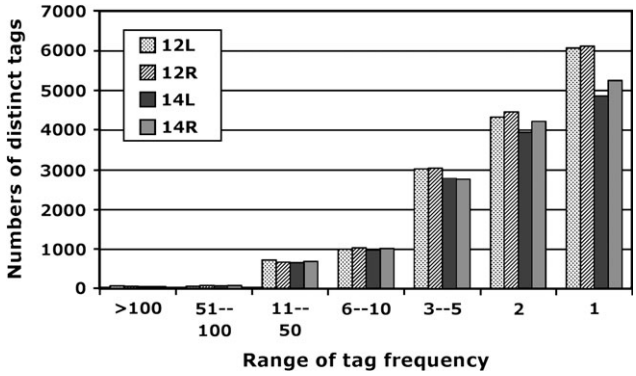

Figure 1. Characterization of gene abundance using SAGE tag frequency in SAGE libraries. The identified SAGE tags are divided into the "range of tag frequency" based on SAGE tag counts. SAGE libraries from the human fetal left (12L, 14L) and right (12R, 14R) hemispheres at 12 and 14 weeks were analyzed. Because SAGE can detect genes with low abundance in the developing cortex, genes having fewer than 5 tags were also collected. The majority of tags per library have a frequency of 5 or less.

Using standard gene ontology, we examined functions of genes detected by SAGE analysis. We focused on genes showing significant statistical differences (P > 99%) between the left–right hemispheres at human fetal 12 and 14 weeks (Sun and others 2005). We divided genes into 6 groups according to their cellular function (see Materials and Methods). We found that the majority of genes differentially expressed in the left–right hemispheres are genes regulating cell signaling or cell communication and genes controlling gene or protein expression (Fig. 2). The unclassified genes are those either with unknown function or with expressed sequence tags (ESTs). We did not find a particular signaling pathway among detected genes (data not shown). Additionally, we did not observe obvious change of functional groups between the human brains at fetal 12 and 14 weeks. Therefore, if there is a molecular basis of cortical asymmetry at these stages, it is largely controlled by genes functioning in signal transduction and gene expression regulation, though we cannot rule out other patterns of expression at earlier stages.

Left–Right Cortical Asymmetry May Occur by Early Fetal Stages

It has been suggested that the critical time of human cortical regionalization is likely between 6 and 20 weeks (Geschwind and Miller 2001). To evaluate the timing of human cortical asymmetry, we examined numbers of genes displaying differential expression levels between the left–right hemispheres at fetal 12, 14, and 19 weeks. Though the chi-square test identifies genes with significant expression differences, compared with the Monte Carlo test, it also detects genes with very low tag frequencies (fewer than 4) (data not shown). Because we found that gene expression levels measured by SAGE with high tag frequencies matched well with those measured by real-time reverse transcriptase–polymerase chain reaction (Sun and others 2005), we concentrated on this study based on genes detected by the Monte Carlo test.

The numbers of genes showing significant expression differences were compared in SAGE libraries generated from the left and right perisylvian regions in the human cortex at early brain developmental stages (fetal 12 and 14 weeks, active neuronal proliferation and migration stages) and late stage (fetal 19

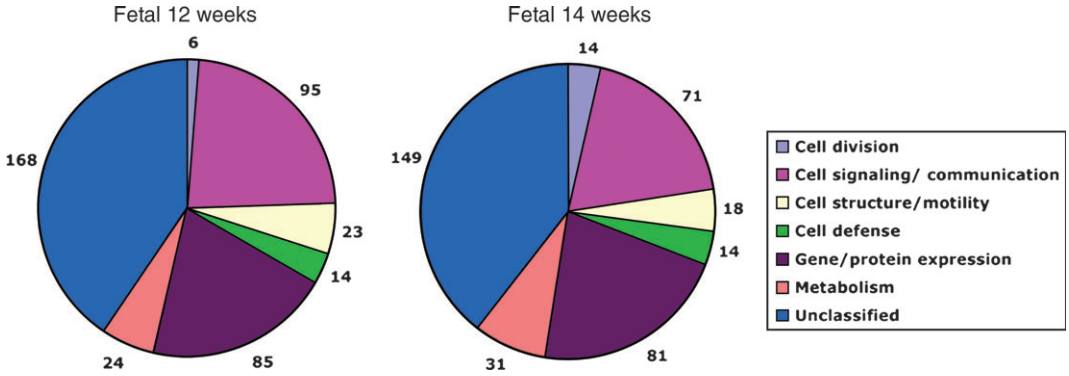

Figure 2. Functional groups of differentially expressed genes in the left and right human fetal brains that were detected by SAGE. Genes showing statistical significance of differential expression levels between the left and right hemispheres of the human fetal 12 and 14 weeks brains were analyzed using the Monte Carlo test and the chi-square test. These genes were divided into 6 groups according to their cellular functions. Numbers of genes in each functional group are listed.

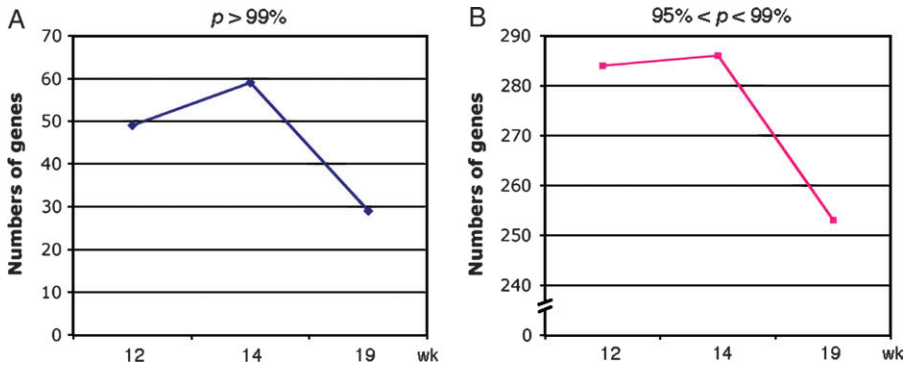

Figure 3. Human cortical left and right asymmetry may occur at early fetal stages. (*A–B*) Genes differentially expressed in the perisylvian regions in the left and right hemispheres were analyzed using the Monte Carlo test at the human fetal 12, 14, and 19 weeks. Numbers of genes displaying statistical significance of differences were analyzed at 3 developmental stages with confidence levels $P > 99\%$ (*A*) and $95\% < P < 99\%$ (*B*). There are more differentially expressed genes at early stages (12 and 14 week) than those at late stages (19 week) in the human fetal left and right cortices.

weeks, the cortical regionalization is likely finished) (Sidman and Rakic 1973). We compared differentially expressed genes with high (Fig. 3*A*) and relatively low (Fig. 3*B*) statistical significance ($P > 99\%$ and $95\% < P < 99\%$, respectively). There are about 2-fold more differentially expressed genes between the left and right hemispheres at early developmental stages (fetal 12 and 14 weeks) than late stages (fetal 19 week) using the $P > 99\%$ criteria (Fig. 3). On the other hand, when the lower criteria ($95\% < P < 99\%$) was used, the developmental stage difference became modest (Fig. 3*B*). We did not generate SAGE libraries from human brains earlier than fetal 12 weeks or later than 19 weeks. Nevertheless, our results suggest that human cortical asymmetry may be an early developmental event.

Comparisons of Differentially Expressed Genes in Human Fetal Brains with Genes Differently Expressed between Adult Human and Chimpanzee Brains

Gene expression differences in human fetal brains can be viewed through the lens of differences in expression by species. This approach is useful because some left–right asymmetries in human fetal brains may be relatively ancient (i.e., present in other primate species), whereas other asymmetries may be new to humans. Currently, there are no comparative studies of human and chimpanzee fetal brain gene expression. However, data are available on adult brain expression differences from the 2 species. Admittedly, the majority of important expression differences between species may occur at the fetal stage and be short lived. Nonetheless, it is instructive to investigate whether some fetal gene expression asymmetries persist into adulthood and also distinguish species.

Evolution at the molecular level can be viewed from 2 complementary perspectives. First, as genes evolve, they accumulate DNA sequence differences that can lead to amino acid replacements (Gilbert and others 2005). This protein divergence can be measured by the number of DNA substitutions leading to amino acid changes per amino acid-altering site (Ka) normalized by a measure of the neutral substitution rate, in this case, the local intergenic/intronic substitution rate per site (Ki). Most proteins have Ka/Ki values close to zero because natural selection does not tolerate amino acid change and instead acts to maintain unchanged structures and functions over time. Therefore, high values of Ka/Ki ($\gg 1$) are relatively rare and interpreted as evidence for adaptation (positive selection) at the molecular level. Second, genes accumulate DNA substitutions in their regulatory regions as they evolve, leading to expression divergence, which includes altered mRNA levels. Protein divergence and expression divergence are complementary aspects of the evolutionary process.

Protein Divergence

We first analyzed Ka/Ki values of genes differentially expressed in human fetal 12 weeks brains (Sun and others 2005). Genes having significant differential expression between left–right fetal brains show significantly less protein divergence between

species than does the average gene compared between humans and chimpanzees (Ka/Ki = 0.14 vs. 0.27; the latter value is from the Chimpanzee Sequencing and Analysis Consortium, 2005). Thus genes involved in fetal hemispheric asymmetry as a class are relatively conserved in protein evolution compared with other genes (Fig. 4). For instance, for *LMO4*, there is only one silent change from humans to chimpanzees, and the protein is conserved with the mouse, as well as the macaque and the chimpanzee. Nonetheless, some individual genes showing left–right fetal brain differences have an elevated Ka/Ki value, indicating that they may have undergone selection (Fig. 4 and Supplementary Table 1). Furthermore, the degree to which genes exhibit differential hemispheric difference in expression is not related to their degree of protein divergence. This demonstrates that as protein-coding differences accumulate over time, they are neither coupled to gene expression changes nor reflective of the extent of regulatory change.

We next compared Ka/Ki values of genes differentially expressed in human fetal 12 and 14 weeks brains. The genes showing the most significant left–right hemispheric expression differences in human fetal brains are more constrained in terms of protein divergence between species at an earlier fetal stage (12 weeks) than at a later stage (14 weeks). Differentially expressed genes at 12 weeks have an average Ka/Ki value of 0.08, significantly lower ($P < 0.02$) than for those at 14 weeks with Ka/Ki value of 0.20. This trend is not observed for genes exhibiting a less significant ($95\% < P < 99\%$) left–right differences in brains at fetal 12, 14, or 19 weeks. Moreover, no human fetal brain region stands out as uniquely different in terms of adaptational change at the protein level. Specifically, there was no significant difference among the classes of genes differentially expressed in different fetal brain regions in terms of Ka/Ki.

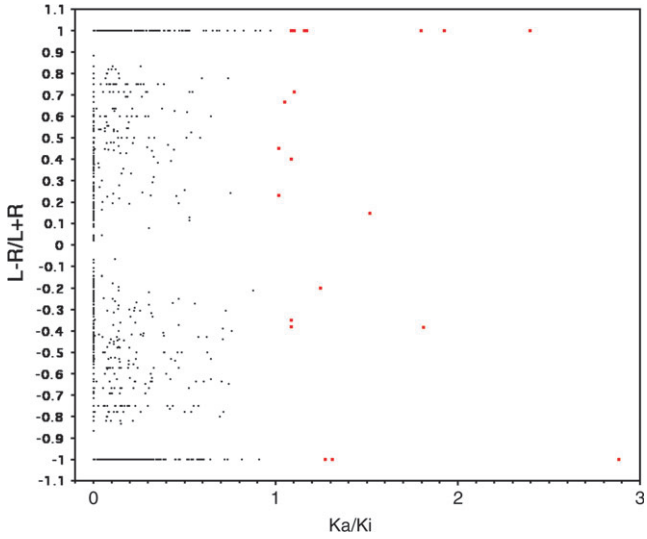

Figure 4. Comparisons of Ka/Ki values of genes differentially expressed between human fetal left–right brains. The *y* axis distinguishes between genes more highly expressed in the left fetal brain than in the right (positive values, L–R) and genes more highly expressed in the right than the left (negative values). All values are normalized by the sum of their expression values in the left plus right hemispheres (L + R). Ka/Ki values are from the Chimpanzee Sequencing and Analysis Consortium. A list of genes with pairwise human–chimpanzee Ka/Ki values above one (in red) can be found in Supplementary Table 1.

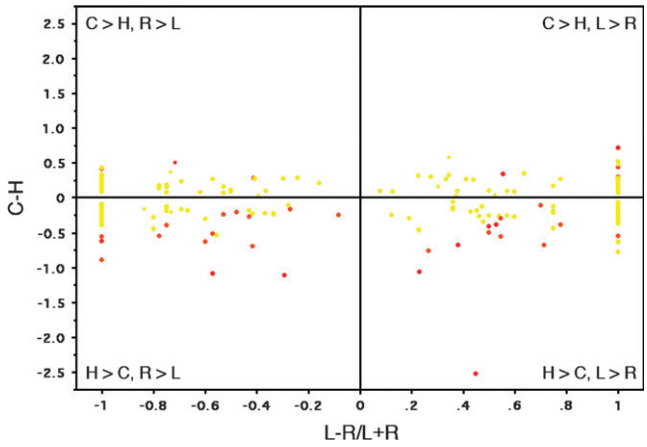

Figure 5. Comparisons of genes different between human fetal left–right brains with those different between human–chimpanzee adult brains. The colors correspond to levels of statistical significance in Hsieh and others (2003) (significance levels: Bonferroni = –log $P > 5.4$ red; 0.001 = –log $P > 3.0$ orange; 0.05 = –log $P > 1.301$ yellow). A list of genes can be found in Supplementary Table 2. H, Human; C, Chimpanzee; L, Left; R, Right.

Expression Divergence

We then analyzed differentially expressed genes ($P > 95\%$) in human left–right fetal brains (Sun and others 2005) that are also significantly differentially expressed in human versus chimpanzee adult brains (Enard and others 2002; Hsieh and others 2003). Genes displaying significant expression differences both between human fetal hemispheres (left–right) and between species (human–chimpanzee) are shown in Figure 5 and Supplementary Table 2. Some of these genes are implicated in neurological diseases that have been claimed to be unique to humans and absent in chimpanzees, for example, PIN1 (peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1) in Alzheimer's disease and PRDX2 (peroxiredoxin 2) (Olson and Varki 2003).

Asymmetric Expression of Transcription Factor LMO4 in a Human Fetal 15 Weeks Cortex

In our previous work (Sun and others 2005), we focused on expression patterns of the transcription factor *LMO4* in human fetal brains because 1) *LMO4* was detected in SAGE libraries generated from human brains at both fetal 12 and 14 weeks; 2) the mouse *Lmo4* plays critical roles in mouse cortical development (Hahm and others 2004; Tse and others 2004; Lee and others 2005).

In this study, we looked at *LMO4* expression in a human 15-week fetal brain using in situ hybridization on freshly frozen sections. Similar to its expression in human fetal 14 and 16 weeks brains, the human *LMO4* at 15 weeks was expressed in essentially all layers of the cortical plate through wide regions of the cortex. In contrast, *LMO4* was not expressed in the ventricular zone, suggesting that *LMO4* expression at this stage is highest in postmitotic neurons (Fig. 6). *LMO4* was also expressed in a few noncortical telencephalic structures, notably the putamen and claustrum (Fig. 6*C*).

Although *LMO4* expression was quite symmetric in the cortical plate of dorsal and medial regions of the cortex (Fig. 6), it was highly expressed in the ventrolateral cortical plate of the right hemisphere whereas corresponding regions of the left ventrolateral cortical plate expressed low levels (Fig. 6). In contrast to the asymmetric cortical expression, *LMO4* expression in the putamen and claustrum, immediately beneath the perisylvian cortex, was not obviously asymmetric (Fig. 6*C*).

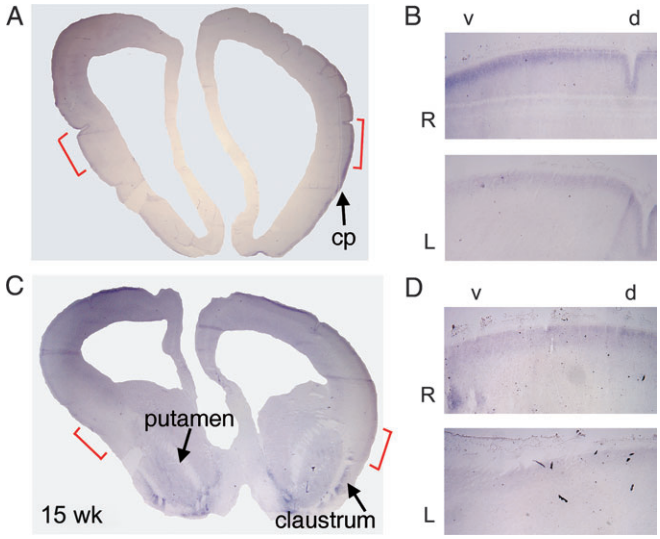

Figure 6. *LMO4* is highly expressed in the right hemisphere than the left in a human fetal 15 weeks brain. Serial coronal sections were collected from the frontal lobe (*A*) and perisylvian regions (*B*) in the cortex for in situ hybridization. The dorsal cortex is on the top. (*A*) Human *LMO4* was more highly expressed in the cortical plate (cp) in the right (R) hemisphere than that in the left (L) on coronal sections of the frontal lobe in a human fetal 15 weeks brain. (*B*) High power view of selected areas in panel (*A*). *LMO4* expression in the matched ventral lateral areas (red) is higher in the right hemisphere than that in the left. The dorsal (d) cortex is on the right side and the ventral (v) cortex is on the left. (*C*) Human *LMO4* was expressed in the cortical plate in the perisylvian regions in the human fetal 15 weeks brain. Similarly to its expression in the frontal cortex, *LMO4* is highly expressed in the right hemisphere. *LMO4* expression in the putamen and claustrum was detected without obvious difference. (*D*) High power view of selected areas in panel (*C*). The dorsal cortex is on the right side.

Overall, the asymmetric expression of *LMO4* in ventrolateral cortex was quite reproducible in more than 20 coronal sections examined. The asymmetries of expression suggest that a large zone of ventral lateral perisylvian neocortex expresses higher levels of *LMO4* in the right hemisphere and lower levels in the left, whereas there is symmetric expression in the medial and dorsal cortical regions.

Discussion

Although many lines of evidence have shown the left–right anatomical and functional asymmetry in the human cortex, the genetic regulation of this asymmetry remains unclear. Identifying genes differentially expressed in the left–right hemispheres may shed light on the molecular mechanisms of cortical asymmetry. Using the SAGE approach, we have generated comprehensive data of gene expression levels in the human fetal left and right hemispheres. We have found that genes involved in signal transduction and expression regulation may play essential roles in human fetal brain development and asymmetry. Applying genomic tools, such as SAGE, and comparing human and nonhuman primate genomes may allow us to study the molecular and evolutionary mechanisms regulating cortical asymmetry.

The Regulation of Cortical Asymmetry and Anterior–Posterior Patterning

Recent studies of the anterior–posterior (A–P) pattern formation in the cortex have enriched our knowledge of how distinct anatomical and functional cortical regions are formed during embryonic development (Rakic 1988; Eagleson and Levitt 1999; Rubenstein and others 1999; O'Leary and Nakagawa 2002; Grove and Fukuchi-Shimogori 2003). Molecules secreted from the patterning centers in the cortex establish concentration gradients of gene expression levels and define distinct cortical regions (Grove and Fukuchi-Shimogori 2003). Though the cortical left–right asymmetry has been noticed for a long time, an interesting question of whether this asymmetry is also regulated at molecular genetic levels remains unknown (Geschwind and Miller 2001; Toga and Thompson 2003). There is still no genetic evidence of the existence of patterning centers that controls the establishment of left and right hemispheres.

Applying a quantitative genome-wide screen approach, we have measured gene expression levels between the left and right human fetal hemispheres. At least at the transcriptional level, we have detected a large number of genes with left and right differential expression in developing human cortices (Sun and others 2005). The majority of these genes detected by SAGE function in signal transduction and gene expression regulation, suggesting a transcriptional regulation of establishing human cortical asymmetry (Fig. 2). Mapping expression patterns of *LMO4* in human and mouse cortices also demonstrates a molecular basis of cortical asymmetry (Sun and others 2005).

We have detected more differentially expressed genes between the left and right hemispheres at earlier human fetal stages (12 and 14 weeks) than later stage (19 weeks). *LMO4* expression also displayed more striking asymmetry in fetal brains at 12 and 14 weeks than those at 15–19 weeks by in situ hybridization (Sun and others 2005). It is likely, similar to cortical A–P patterning, that there is an intrinsic control of cortical asymmetry that occurs at very early developmental stage (Fig. 3, Rakic 1988; Grove and Fukuchi-Shimogori 2003). Therefore, cortical asymmetric patterning might be a very early developmental event. Comparing gene expression levels between the left and right hemispheres at early human embryonic stages, such as 8–10 weeks, might reveal more strikingly asymmetrically expressed genes.

Lmo4 Function in Asymmetric Cortical Patterning

Asymmetric *LMO4* expression was detected largely in the cortical plates in human fetal brains from 12 to 17 weeks (Sun and others 2005). It was expressed in most postmitotic cells but not in the ventricular zone, containing progenitor cells. These results suggest that LMO4 might maintain the asymmetry initiated by other molecules, for instance, the morphogens secreted in patterning centers in the cortex. We propose that, superimposed on a generally symmetric A–P gradient, differentially expressed morphogens may induce the transcriptional cortical asymmetry. The sources of morphogens regulating transcription factors, such as *Lmo4*, and how morphogens function in establishing left–right cortical asymmetry are not known. However, the recently identified cortical antihem that is laterally positioned as a mirror image of the cortical hem might include potential laterally placed signaling information centers, like the lateral neocortex (Assimacopoulos and others 2003).

The asymmetric expression of *Lmo4*, which in mice is expressed in broad regions of the cortical plate that roughly correspond to broad functional areas (i.e., high in frontal motor cortex, low in presumptive parietal sensory cortex, and high in occipital visual cortex), suggests highly asymmetric patterning of cerebral cortical functional or cytoarchitectonic areas. The embryonic lethality and severe defects in the neural tube (anencephaly or exencephaly) in the *Lmo4* mutant further

suggest an essential role of *Lmo4* in brain development (Hahm and others 2004; Tse and others 2004; Lee and others 2005).

Modest anatomical asymmetries in quantitative aspects of cortical organization and functional specialization have been reported in individual rodent brains (Lipp and others 1984; Riddle and Purves 1995; Chen-Bee and Frostig 1996). In particular, anatomical asymmetry in somatosensory cortex may correlate with paw preference in mice, analogous perhaps to handedness (Barneoud and Van der Loos 1993). The regional specific expression of *Lmo4* in the P1 cortex suggests that Lmo4 may play an essential role in the development of the somatosensory cortex (Sun and others 2005). Generating viable conditional knockout mouse for *Lmo4* may help us to gain insight into the mechanisms of cortical asymmetric regional patterning.

Evolutionary Mechanisms Regulating Cortical Asymmetry

Human brains are larger and more complex than those of nonhuman primates. An intriguing question is how handedness and specified asymmetric brain functions are evolved in humans. In our previous work, we have shown that whereas *LMO4* was consistently asymmetrically expressed in human fetal cortices, its expression was also asymmetric in mouse cortices, but this asymmetry is not biased to either the left or the right hemispheres (Sun and others 2005). Evolutionary mechanisms that bias or entrain this apparently random asymmetry of rodents may have allowed the development of more consistent functional asymmetries in the primate cortex (Palmer 2004). The identification of markers asymmetrically expressed in the cortex may allow us to begin to understand the genetic and evolutionary control of the human cortical asymmetry.

In this study, we have identified some genes that show both left–right differences in human fetal brains and human–chimpanzee expression differences in adult brains. Simultaneous consideration of both sources of expression differences can help tease apart ancient from more recent evolutionary changes. After all, chimpanzees are likely to exhibit some of the same left–right brain differences that humans do for particular genes (even if not to the same extent), whereas other expression asymmetries may be species specific. Because the human species has the unique capacity for language, and because much of that capability is centered in the left hemisphere, it is intriguing from an evolutionary perspective to identify genes differentially expressed between left and right hemispheres that may be unique to humans. Indeed, some differentially expressed genes detected by SAGE in left–right human fetal brains have high Ka/Ki values, indicating that these genes may be under positive evolutionary selection (Fig. 4 and Supplementary Table 1). Studying the functions of these genes might be significant in evolutionary or phenotypic terms, that is, how they are related to brain evolution. To confirm the signal of selection and to determine which evolutionary lineage (human vs. chimpanzee) has undergone the most molecular evolutionary change, further primate DNA sequences should be part of additional analyses.

Genes showing significant expression differences both between human fetal hemispheres (left–right) and between species (human–chimpanzee) are also very interesting (Fig. 5 and Supplementary Table 2). These genes might play critical roles in human brain evolution. And during the process of evolution, these genes might also function in controlling or maintaining brain asymmetry in humans. Analyzing Ka/Ki values for these genes between humans and other nonhuman primates may enrich our understanding of their general roles in brain evolution.

Furthermore, evolutionary selection undoubtedly works on different aspects of protein expression and structure. We also compared the Ka/Ki values of differentially expressed genes between left–right human fetal hemispheres detected by SAGE. This kind of analysis allows us to highlight genes that may have undergone multiple rounds of selection at different levels. Though this analysis should be considered preliminary, as more expression and genomic data become available, it will have interesting implications for molecular evolution and selection of human brain asymmetry.

Supplementary Material

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

Notes

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