Introduction

Craniosynostosis is a common congenital defect (prevalence of 1: 2,500 born alive) characterized by the premature fusion of the neurocranium sutures [1,2]. Of all the craniosynostosis patients with genetic diagnosis, 32% have mutation in the FGFR2 (Fibroblast Growth Factor Receptor 2) gene [3]. Among the syndromic craniosynostosis caused by mutation in FGFR2, Apert syndrome (AS) and Crouzon syndrome (CS) can be highlighted representing the extremes of the spectrum of clinical variability caused by gain of function mutations in FGFR2.

Apert syndrome (OMIM #101200; prevalence of 1: 65,000 born alive) is considered one of the most severe forms of craniosynostosis. AS involves the bilateral premature fusion of the coronal sutures along with a midline calvarial bone agenesis, without formation of the metopic and sagittal sutures, and midfacial hypoplasia. Beside cranial defects, patients also have symmetrical syndactyly in upper and lower limbs and true megalencephaly, which allows to distinguish it from other syndromic craniosynostosis [4]. A range of skeletal abnormalities, mental deficiency, central nervous system (CNS) alterations and a variety of visceral malformations were also reported in AS patients [1,4]. AS inheritance is autosomal dominant and most cases are paternal origin de novo mutations [5]. On the other hand, Crouzon syndrome (OMIM # 123500; prevalence of 1:60,000 born alive) is clinically characterized by craniofacial abnormalities including premature fusion of coronal sutures but no limbs or other congenital malformation [6].

AS is mainly caused by the mutations S252W (the most prevalent one, accounting for approximately 64% of the patients) or P253R (33% of the patients) in FGFR2. Both are ligand-dependent gain-of-function mutations which elicit ligand-binding promiscuity of the receptor [7]. This is a distinct molecular mechanism as compared to FGFR2 mutations found in CS. The CS mutations found in different regions of the gene, constitutively activate FGFR2 by ligand-independent disulphide-mediated covalent receptor dimerization and activation [8].
We have previously described a specific gene expression signature of AS periosteal fibroblasts compared to wild-type (WT) fibroblasts [9]. The differentially expressed genes (DEGs) were associated with cell proliferation, nucleotide metabolism, gene expression regulation, cell adhesion and extracellular matrix organization, and PI3K-MAPK signaling cascades. More importantly, our results together with previous reports suggested that FGFR2S252W over-activates the normal molecular pathways stimulated by WT receptor [9,10]. Ligand-dependent and ligand-independent gain-of-function mutations in FGFR2 lead to a common bilateral coronal craniosynostosis but to very distinct abnormalities in AS and CS. Thus, we hypothesized that AS mutation in FGFR2 over stimulates downstream signaling pathways activated by WT receptor and further induces pathogenic novel molecular pathways, which may account for the AS abnormalities not seen in CS.

To test this hypothesis, we performed global gene expression analysis of WT and S252W periosteal fibroblasts stimulated with exogenous FGF2 in order to activate FGFR2 in both groups of cell populations. We also compared the DEGs in response to FGFR2 activation in both groups to CS periosteal fibroblasts harboring mutation C342Y in FGFR2. We finally investigated if DEGs found in this system were also altered in the brain of AS mouse model [11], which would explain CNS abnormalities seen in AS patients.

Methodology

An outline of the workflow for the performed experiments is shown in Figure S1.

Subjects

Coronal suture periosteal fibroblasts from three unrelated AS patients, three unrelated CS patients and from three age- and sex-matched control subjects were obtained as previously described [9,12]. The presence of the S252W and C342Y mutations were confirmed by direct DNA sequencing and expression of the mesenchyme-specific isoform of FGFR2 in the primary fibroblasts was examined by Western Blot and RT-PCR [9,12]. The project was approved by the Ethical Committee in Research of Human subjects (“Comitê de Ética em Pesquisa - Seres Humanos”) at the Institute of Biosciences University of Sao Paulo. All patients and controls were already enrolled for surgery and treatment by the Department of Plastic Surgery, School of Medicine, University of Sao Paulo, when we contacted them. Thus, those who declined to participate or otherwise did not participate were not disadvantaged in any other way by not participating in the study. Appropriate informed consent was obtained for the donation of the periosteum, a tissue that is usually discarded during surgical treatment, so that this procedure would represent no harm for any of the subjects. Because all the participants were under the age of 18, legal guardians gave written consent on behalf of them.

Care and use of mice for this study were in compliance with the relevant animal welfare guidelines approved by the Johns Hopkins University Animal Care and Use Committee and the Mount Sinai School of Medicine Animal Care and Use Committee. Mice were killed on P0 by inhalation anesthetics & cervical dislocation and weighed. The carcasses were fixed and whole brains were perfused in RNA later. Our sample consists of two litters imbed in different time, each consisting of two Fgfr2+/S252W and six WT littermates.

RNA extraction

Cells at a confluency of 80% in 25 cm² cell culture bottles were used for FGF2 treatment followed by microarray and qRT-PCR assays. After a 24 h starvation period S252W and WT fibroblasts were treated with DMEM High-Glucose without FBS supplemented with recombinant human FGF2 (Peprotech, Rocky Hill, NJ, USA – diluted in 1 x PBS – Phosphate Buffered Saline- to a final concentration of 2000 pM) or with DMEM High-Glucose, 0.5% FBS supplemented with 1 xPBS. It was reported that similar phosphorylation level of both WT and S252W FGF2 was observed when treated with 2000 pM of FGF2 [13]. Untreated and treated fibroblasts were harvested after 24 h of addition of FGF2, and had its total RNA isolated and purified as described below. When we first verified the expression level of genes up-regulated by FGFR2S252W, similar significant alterations in these genes in FGFR2 induced control fibroblasts was only observed after 24 h [9].

Apert Fgfr2+/S252W mice

The Apert Fgfr2+/S252W mice were generated in the laboratory of Dr. Ehlyn Wang Jabs [11]. They were inbred on a C57BL/6j background to minimize phenotypic variation due to genetic differences. Genotyping of tail DNA to distinguish mutant from wild-type progeny was carried out by polymerase chain reaction analysis. The primers for Fgfr2 were as described [11]. Care and use of mice for this study were in compliance with the relevant animal welfare guidelines approved by the Johns Hopkins University Animal Care and Use Committee and the Mount Sinai School of Medicine Animal Care and Use Committee. Mice were killed on P0 by inhalation anesthetics & cervical dislocation and weighed. The carcasses were fixed and whole brains were perfused in RNA later. Our sample consists of two litters imbed in different time, each consisting of two Fgfr2+/S252W and six WT littermates.

RNA extraction

Cells at a confluency of 80% in 25 cm² cell culture bottles were used for FGF2 treatment followed by microarray and qRT-PCR assays. After a 24 h starvation period S252W and WT fibroblasts were treated with DMEM High-Glucose without FBS supplemented with recombinant human FGF2 (Peprotech) or with DMEM High-Glucose without FBS supplemented with 1 xPBS. Total RNA was isolated from FGF2 treated and untreated cells using Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) after 24 h.

Mice whole brain RNA was extracted with RNasy Mini Kit (Qiagen) following manufacturer’s instruction. RNA quality and concentration were accessed by 1.5 percent agarose gel electrophoresis and Nanodrop ND-1000 (Thermo Scientific, Waltham, Massachusetts, USA) respectively.

Microarray Assays

For each cell line, cDNA was generated with the Affymetrix GeneChip WT cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, California) following the manufacturer’s instructions. cDNA was fragmented and end labeled with the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix,
efficiency (E) of each primer was calculated according to the Applied Biosystems, California, USA) and the amplification sample size was increased to 12 WT: 4 S252W from two litters. S252W littermates sample, if significant difference was observed, PLOS ONE | www.plosone.org 3 April 2013 | Volume 8 | Issue 4 | e60439

...fibroblast populations, whether WT or FGFR2 of the ligand, we compared the expression data of all three treated 

# available in the R/Bioconductor package, both with p-value ≤0.05 adjusted by FDR (False Discovery Rate) correction factor. In order to minimize biological variations and focus on the effect of the ligand, we compared the expression data of all three treated fibroblast populations, whether WT or FGFR2<sup>+/S252W</sup> with the corresponding expression data of the same three untreated fibroblast populations. We extracted the genes that were commonly selected by the two different methods (RankProd and Limma) as significantly differentially expressed (DEGs) in order to minimize false positive occurrence. The Limma method performs statistical analysis similar to that used by SAM (Significance Analysis of Microarrays) [16], and is based on a moderate t-statistics to test the average difference in log expression levels between the treated and the control groups for each gene. The RankProd is a rank-based non-parametric method that uses geometric mean rank for each gene and its distribution is estimated by randomly permuting the observed ranks. The permutation principle partly alleviates the small sample sizes issue, enhancing the robustness against outliers [17]. To analyze the result, we used the IPA software for the analysis of gene interaction and functional classification of DEGs; DAVID for the enrichment of gene ontology and GT (GeneTrail) for analysis of over-or under representation of biological categories and pathways. Analysis of the promoter regions of DEGs was performed through “The IPA Upstream Regulator Analytic” function in IPA. IPA software was also used to study gene interactions and to perform functional classification of DEGs. Briefly, “The IPA Upstream Regulator Analytic” predicts which transcriptional regulators are involved with a set of genes and whether they are likely activated or inhibited.

Reverse Transcription Reactions and Quantitative Real Time PCR

Complementary DNA (cDNA) was produced from 1 μg of total RNA using Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA, USA). For human fibroblasts, qRT-PCR, assay was performed using approximately 20 ng of cDNA and SYBR Green PCR master mix in an ABI Prism 7500 system (Applied Biosystems, California, USA). For mouse brain qRT-PCR, experiments were run with 20 ng of cDNA and SYBR Green PCR master mix in an ABI Prism 7900 system (Applied Biosystems, California, USA). The PCR conditions for both were: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles. In the mouse brain study, first it was performed in a paired 2 WT: 2 S252W littersmate sample, if significant difference was observed, sample size was increased to 12 WT: 4 S252W from two litters.

Primers were designed with Primer Express software V.2.0 (Applied Biosystems, California, USA) and the amplification efficiency (E) of each primer was calculated according to the equation: 

\[ E = 10^{-\frac{1}{1+\text{slope}}} \]

The expression data of the studied transcripts was determined by relative quantification in comparison to endogenous controls (human controls: GAPDH, HMBS, HPRT1 and SDHA; mouse controls: Yicahz, Tbp, Tubb5 and Bn2).

We verified the gene expression stability of endogenous controls through geNorm VBA applet designed for Microsoft Excel. This tool calculates the most stable reference genes from a set of tested candidate reference genes in a given sample panel, and calculates the gene expression normalization factor for each target sample based on the geometric mean of a defined number of housekeeping genes [18]. The expression data is given by the ratio between each transcript ΔΔCt (E<sup>Ct</sup>) and a normalization factor. Samples from all cells analyzed previously in Microarray assay were run in technical triplicates, and the threshold suggested by the instrument software was used to calculate Ct. Primers used in this study are summarized in Table S1.

To assess the statistical significance of the correlation between microarray assay data and the qRT-PCR results we used the nonparametric two-tailed Spearman correlation test, with p-values of less than 0.05 considered to be statistically significant.

TCF19 immunostaining in human fibroblasts

Fibroblasts were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized in 0.05% Triton X-100 in PBS for 5 min. Nonspecific binding was blocked with 10% BSA in PBS for 1 h at room temperature. Cells were incubated with primary antibody against TCF19 (1:100, Sigma) overnight at 40°C. After several washes, cells were incubated with secondary (1:100, AlexaFluor 488, Invitrogen) antibodies against mouse IgG tagged with 2 h at room temperature. Slides were counterstained with DAPI (4′6-diamidino-2-phenylindole, Sigma). All images in the same set (treatments and controls) were obtained using the same photographic parameters of exposition and speed. Images were captured using the Axiovision 3.0 image analysis system (Carl Zeiss).

Strc immunostaining in mouse brain

Two anti-stereocilin antibodies were used, one previously described [19] and the other from Sigma (HPA015731). In our analysis they detected the same targets. Each antibody was tried on both paraffin and frozen sections of P0 WT and S252W brains for IHC, and with both chemical (DAB) and immunofluorescent (IF) visualization for the signal. The Sigma antibody was also used on frozen sections at E16.5 of WT and Apert mice, along with an antibody for Fgfr2 (Santa Cruz) on adjacent sections.

Results

S252W mutation alters the gene expression in response to FGFR2 activation

When WT FGFR2 was activated by FGF2, we found 79 DEGs, of which 48 were up-regulated and 31 were down-regulated (Table S2). There was an increased expression of genes involved in MAPK (DUSP6, MAPK4, RAS12 and ITGA2), PI3K/Akt (ITG42) and Jak-STAT (JL13RA2) signaling pathways. The most significant biological categories among these DEGS were cell growth (IPA: p = 0.0002; DAVID: p = 0.017, GT: p = 0.00024) and cell motility (IPA: p = 0.0002; GT: p = 0.00005). According to IPA analysis, 31 of the 79 DEGs (39.2%) are on the same gene network associated with movement and cell proliferation. Analysis of the promoter region of the DEGs showed enrichment for genes regulated by the transcription factors SP3 (p = 0.0002), SP1 (p = 0.0014), CLOCK (p = 0.0057), STAT3 (p = 0.01). Upon FGF2 stimulation, S252W fibroblasts significantly altered expression of 55 DEGs, up-regulating 21 genes and down regulating 34 genes (Table S3). Seven (12.7%) of the DEGs were associated with neurological diseases (B1T3, HSSST1, IFT14L, RFC3, RPS9, STRC and TCF19) according to the IPA analysis.
Validation of global gene expression analysis

To validate the gene expression microarray analysis, we conducted qRT-PCR of a set of DEGS identified between FGFR2 treatment and control groups. The mRNA expression of BDPI, CYP51A1, DUSP6, MAPK4 and STC1 were tested in WT fibroblasts treated with FGFR2; and BAT3, BDPI, CYP51A1, RFC3 and TCF19 in S252W fibroblasts treated with FGFR2. The differential expression of each gene between FGFR2 treatment and control groups was calculated as a fold-change value, and the correlation between these qRT-PCR fold-change and microarray analysis fold-change for each gene in each cell was evaluated by Spearman correlation test (Figure 1A). The correlation between the values of the two analysis in all cell lines and treatment groups was statistically significant ($r^2 = 0.853$, $p < 0.0001$). In conclusion, the values obtained from microarrays and qRT-PCR is consistent and therefore the DEGS selected by bioinformatics analysis are representative of the gene expression profiling experiments.

TCF19 was the only transcription factor in the FGFR2 induced S252W fibroblast DEG lists and we opted to further examine TCF19 protein levels through immunofluorescence staining in two S252W fibroblasts not previously included in the microarray experiment. TCF19 was only detectable when S252W fibroblasts were treated with FGFR2 (Figure 1B). Results are in agreement with global expression investigation, further validating statistical analysis used in Affymetrix experiment.

S252W and C342Y mutation affects FGFR2 signaling in different manners

To further delineate whether these gene expression circuitry modifications were consequence of altered ligand binding affinity of FGFR2 or of constitutively active intracellular signaling by the receptor, we sought for BAT3, BDPI, CYP51A1, TCF19 and RFC3 (DEGs in S252W fibroblasts treated with FGFR2) expression levels in a C342Y fibroblast through qRT-PCR. The correlation analysis of expression values between C342Y fibroblasts and S252W fibroblasts showed no significant correlation ($r^2 = 0.04$, $p = 0.904$) (Figure 1C). These difference in expression levels were confirmed in biological replicates among independently-derived C342Y and S252W fibroblasts.

CNS related gene Strc has increased expression in the brain of the Apert mouse model

Neuroanatomical abnormalities are a striking phenotype that is part of the wide range of abnormalities that characterize AS and are much more severe than the ones observed in other FGFR2-associated craniosynostosis [20]. These anomalies are also present in Fgfr2+/-/S252W mouse model at P0, and did not correlate with patterns of suture closure, suggesting that these alterations are a primary consequence of the mutation [21]. Remarkably, about 13% of the DEGs in FGFR2 treated S252W fibroblasts were associated with neurological diseases (BAT3, H6SS1, IFI44L, RFC3, RPS9, STRC and TCF19). Hence we evaluated the expression of the homologues of these 7 genes in P0 Fgfr2+/-/S252W mice whole brain (Figure S2) together with mRNA levels of the mutant receptor, Fgfr2, the epithelial isoform, Fgfr2b, the mesenchymal isoform, Fgfr2c, and of the Fgfr2 ligand gene as control of the experiment (Figure S2). After analysis of the 7 genes through qRT-PCR, we observed that only one gene, Strc, had differential expression in newborn AS mice brain with a 1.6 fold-change ($p = 0.006$) (Figure 2A).

In order to understand how increased expression of Strc could be contributing to AS brain phenotype, we performed immunofluorescent staining of P0 mouse brain. Endothelial cells of blood vessels were the only cells positive for Strc in the p0 brain (white arrows in Figure 2B). This was made more evident by the robust staining observed in the basilar artery that runs ventral to the pons. Moreover, there is a strong positive Strc domain next to the basilar artery, in the ventral portion of the pons (dashed line in Figure 2B) which is wider in the Fgfr2+/-/S252W compared to WT littermates. Image analysis of 7 sections at different brain levels of Fgfr2+/-/S252W and WT p0 mouse brain revealed that mutant mice displayed an average of 1.33±0.17 fold more Strc positive blood vessels than control animals. This is in accordance with our previous qRT-PCR results.

We next compared Fgfr2 to Strc expression sites to understand the role of S252W mutation in the overexpression of Strc in the brain. At E16.5, Strc signal did not match the major sites of Fgfr2 expression at the ventricular zones in both Fgfr2+/-/S252W and WT embryos (arrows in Figure 2C). On the other hand they matched in endothelial cells (arrowhead in Figure 2C). Thus, it is possible that mutant Fgfr2 expressed in endothelial cells may lead to increased Strc expression and affects angiogenesis and vascularization of the developing brain.

Discussion

Transmission of extracellular signals via plasma membrane proteins to the intracellular compartment is crucial for the cell to recognize and interrelate with neighboring cells and extracellular structures. Fibroblast growth factor receptors (FGFR) mediate the signaling from FGFs into the cell. The amplitude of cell response to FGFR signaling is allowed by both alternate mRNA splicing and binding specificity. In the presence of the S252W mutation, FGFR2 loses normal isoform ligand specificity for most of the ligands. We earlier reported a unique expression profile of S252W coronal suture periosteal fibroblasts and showed that the mutation leads to excessive FGFR2 signaling [9]. Although part of the AS phenotype caused by S252W mutation can be explained by increased downstream signaling, we hypothesized that the mutation also leads to abnormal novel signaling in the cell.

S252W mutation causes a unique response to FGF stimulation

In order to test this premise, we first performed global gene expression analysis in response to FGFR2 activation in WT or S252W periosteal fibroblasts through microarray experiments. We found that WT fibroblasts stimulated by FGFR2 activated the transcription of genes involved in cell proliferation and migration, most particularly those involved with the activation of MAPK and JAK-STAT signaling pathways, all part of the known canonical FGF-FGFR signaling pathway, thus consistent with the extensive literature in this field [22–26]. Importantly, even though activated by the same FGF, cells that have the mutant FGFR2 receptor is
capable of carrying a different response. This was highlighted by the observation that DEGs of S252W periosteal fibroblasts were not only different from the DEGs of WT fibroblasts, but they were also regulated by a different set of transcription factors. Therefore, activation of the mutant receptor leads to new signaling circuitries that activate different gene regulatory networks.

The only differentially expressed transcription factor activated by S252W FGFR2 was TCF19. It has been suggested that TCF19 plays a role in the regulation of expression of other genes necessary for the later stages of cell cycle progression (Ku et al. 1991; Hystad et al. 2007). Since we previously reported that S252W periosteal fibroblasts have enhanced proliferation compared to WT fibroblasts (Fanganiello et al., 2007; Yeh et al., 2011) further experiments will be important to determine its function as a potential mediator of the increase in proliferation in mutant fibroblasts.

Additionally, expression of DEGs in FGFR2\textsuperscript{C342Y} fibroblasts treated with FGF2 did not correlate with the expression levels obtained in FGFR2\textsuperscript{AS} fibroblasts under the same treatment. C342Y mutation in FGFR2 (CS) leads to ligand-independent activation of the receptor [27], while S252W mutation in FGFR2 (AS) leads to an unspecific ligand affinity of the receptor [7,13]. However, CS patients have milder phenotype compared to AS individuals, which indicates that these two mutations have different molecular and cellular consequences. It is likely that S252W mutation leads to conformational modifications in FGFR2 upon activation, which may affect downstream secondary messenger recruitment. Our results confirm that two different types of gain-of-function mutation in the same gene result in distinct molecular signaling in the same cell type and in the presence of a same ligand.

FGF2 induced differential expression of genes important for development and maintenance of the CNS only in S252W fibroblasts. Of these genes, two – BAT3 and RFC3 – were validated through qRT-PCR, attesting the reliability of the microarray assay. These findings are not surprising since the most abundant and widely distributed FGF in the central nervous system is FGF2 [28,29], which is localized in neurons and glial cells and is expressed in the CNS both during development and postnatally [30–32].
Expression of stereocilin is increased in Fgfr2$^{S252W/+}$ mice brains

Megaencephaly and benign distortion ventriculomegaly are landmarks of Apert syndrome [1,33–36]. Other common CNS alterations observed in Apert syndrome patients are agenesis of the corpus callosum [33,34,37], anomalies in limbic structure [33,35,37–39], and in gyral patterning [33,37]. Although brain size is not increased in Fgfr2$^{+/S252W}$ mouse model at P0, other CNS anomalies (e.g., asymmetry of cerebral hemispheres and enlarged ventricles) in these animals were found to be highly correlated to the human phenotype [21]. However, molecular signaling that links FGFR2 mutation to these malformations remains unclear.

Given species-specific and tissue-specific differences, we surprisingly found up-regulation of Strc (stereocilin) in Fgfr2$^{+/S252W}$ mouse brains, consistent with human S252W fibroblast microarray analysis. Though it is also expressed in brain, eyes, testis and lungs, the role for stereocilin is better established in sensorial hair cells in the cochlea [40]. It is localized at the apical end of kinocilium and is thought to be responsible for the establishment of interaction between stereocilia (specialized motile cilia) and tecta membrane [41]. Loss of function mutations in human STRC gene are causative of autosomal recessive deafness [40] and Strc knockout mice also show hearing impairment [19]. It is possible that upregulation of STRC can explain hearing impairment in AS, a deficiency present in more than 90% of the patients [42]. Although no difference in spatial expression of Strc in the brain, there were more Strc positive blood vessels in Apert mice brains in association with FGFR2 expression. Our results indicate that there is an unknown role for Strc in endothelial cells in AS CNS probably through biomechanical forces response.

Conclusion

Although part of the phenotype caused by S252W mutation can be explained by over-activation of the normal molecular pathways elicited by WT receptor, the mutation also induces novel molecular pathways. This characteristic distinguishes AS from other FGFR2-related syndromic craniosynostosis, such as CS. Moreover, our data suggests that abnormal signaling elicited by mutant FGFR2 induces differential expression of genes important for development and maintenance of the CNS. Among these genes, we validated Strc in newborn Apert mouse brain, suggesting a role for endothelial cells in the establishment of landmark CNS abnormalities of AS. These results also suggest that STRC is in the same circuitry as FGF/FGFR2. Further investigation of the vascularization in the CNS in AS is required for a better understanding not only of the clinical manifestations but also of the role of FGF signaling in brain development.

Supporting Information

Figure S1 Experiments workflow.

Figure S2 Quantitative RT-PCR results for CNS related DEGs in p0 Fgfr2$^{+/+}$ (WT) and Fgfr2$^{+/S252W}$ littermates whole brain RNA. A–K shows each of these DEGs.

Table S1 Primers used for quantitative real time PCR.

Table S2 Differentially expressed transcripts in FGF2 -treated WT fibroblasts compared to the same samples without treatment. In each treatment, transcripts are ordered
by average Fold change ratio (treated vs non-treated) of the replicates.

(DOXY)

Table S3 Differentially expressed transcripts in FGFR2-treated S252W fibroblasts compared to the same samples without treatment. In each treatment, transcripts are ordered by average Fold change ratio (treated vs non-treated) of the replicates.

(DOXY)

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Author Contributions

Conceived and designed the experiments: EY RDF YW EM MP-B. Performed the experiments: EY RDF YW EM MP-B. Analyzed the data: EY RDF YW EM MP-B. Contributed reagents/materials/analysis tools: EY RDF YW EM MP-B. Wrote the paper: EY RDF YW EM MP-B.