Molecular mechanism of novel *FOXG1* variants in causing cortical malformations

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Abstract

FOXG1 (Forkhead-box Protein G1) is an important transcriptional repressor which binds to another transcription factors such as COUP-TF1, to regulate neuronal migration during cortical development. Recently, we found 5 novel FOXG1 variants in patients with FOXG1-related syndrome which has been reported to cause malformation of cortical development and epilepsy. However, the relationship between FOXG1 variants and clinical symptoms still remains unclear. We aim to elucidate how FOXG1 variants affect transcriptional regulation functions and lead to cortical malformation.

In conclusion, Disease causing FOXG1 variants not only affect transcriptional regulation of downstream transcription factors but also cause neuronal migration defects during corticogenesis. The correlation between *in vitro* and *in vivo* phenotypes and clinical symptoms may provide a reference in FOXG1 syndrome diagnosis.

FOXG1 Variants with Severe Symptom showed Low Protein Expression and Fail to Repress COUP-TF1

Fig.1 17 FOXG1 Variants in FOXG1 Gene.

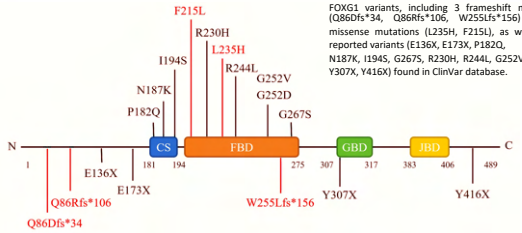

Fig.1 Among 17 FOXG1 variants, we identified 5 novel FOXG1 variants, including 3 frameshift mutations (Q86Dfs*34, Q86Rfs*106, W255Lfs*156) and 2 missense mutations (L235H, F215L), as well as 12 reported variants (E136X, E173X, P182Q, N187K, I194S, G267S, R230H, R244L, G252V, G252D, Y307X, Y416X) found in ClinVar database.

Fig.2 Protein Expression of WT FOXG1 and FOXG1 Variants.

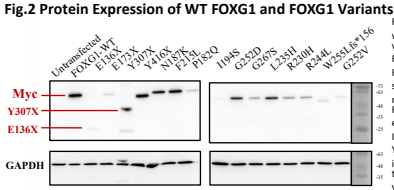

Fig.2 FOXG1 protein was analyzed by western blotting with Myc antibody to verify the changes of their expression. FOXG1 with severe variants (W255Lfs*156, R230H, R244L, G252V, E136X and I194S) showed a low protein expression. In moderate symptoms variants (L235H, F215L, G252D and G267S), protein expression relatively lower than WT FOXG1. In 5 mild variants (N187K, P182Q, E173X, Y307X and Y416X) protein expression were increased. These observations suggested that some of these disease-associated variants may lead to lower protein expression.

Fig.3 Luciferase Activity of WT FOXG1 and FOXG1 variants with its Downstream Target COUP-TF1.

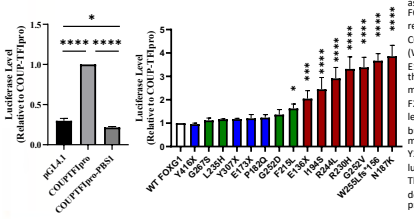

Fig.3 We utilized luciferase reporter assay to examine DNA binding of FOXG1 to the promoter and enhancer regions of its downstream target COUP-TF1. FOXG1 with severe variants (W255Lfs*156, R230H, R244L, G252V, E136X and I194S) showed defects in the repression of COUP-TF1. In moderate symptoms variants (L235H, F215L, G252D and G267S), luciferase level were lower than severe variants, but still higher than WT FOXG1. In 5 mild variants (N187K, P182Q, E173X, Y307X and Y416X), showed a similar luciferase level compared to WT FOXG1. These results further supported the defects of these mutant FOXG1 proteins fail to suppress COUP-TF1.

Overexpression of FOXG1 Variants with Severe Symptom altered Cell Fate of Ectopic Neurons

Fig.4 Overexpressed FOXG1 Variants resulted in Cell Fate Changes

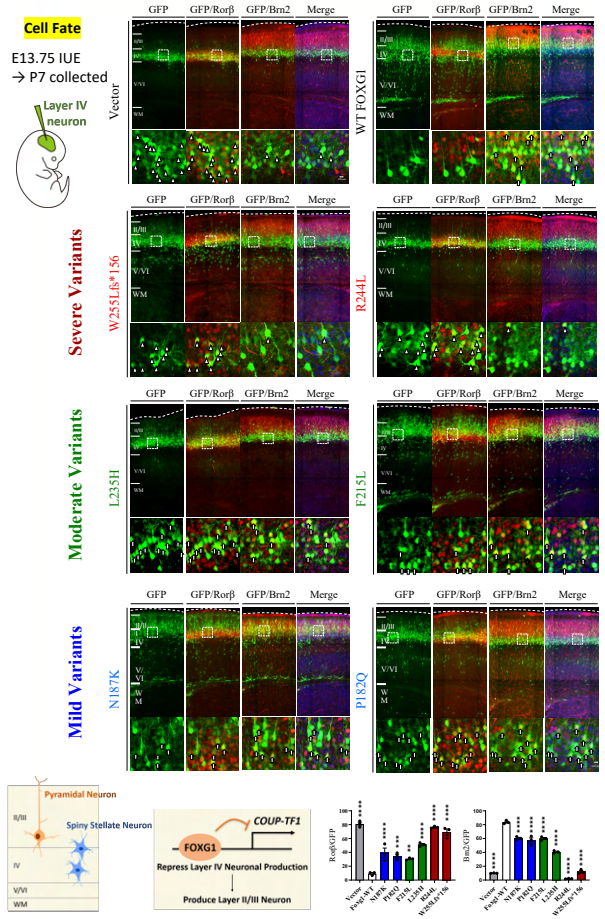

Fig.4 To investigate the potential effects of FOXG1 variants in embryonic brain development, we conducted *in utero* electroporation (IUE) of constructs overexpressing WT FOXG1 or its variants along with GFP plasmid into neural progenitors in the ventricular zone (VZ) of mouse developing brain at embryonic day 13.75 (E13.75). The brains were harvested and sectioned at postnatal day 7 (P7). By utilizing specific layer markers, including layer IV marker Rorβ and layer II/III marker Brn2 to label different types of neurons. In brains electroporated with empty vector, we found that most cells have reached layer IV specifically and expressed Rorβ but not Brn2, indicating that most control GFP+ cells were layer IV interneurons. In contrast, most cells overexpressing WT FOXG1 were distributed in layer II/III, V and VI with more Rorβ+ cells compared to empty vector. At P7, the GFP+ cells expressing severe variants (W255Lfs*156, R230H, R244L, G252V, E136X and I194S) showed Rorβ+ in layer IV rather than layer II/III. The GFP+ cells expressing moderate variants (L235H, F215L, G252D and G267S) were located in layer II/III and IV with partially Rorβ+ and Brn2+. In 5 mild variants (N187K, P182Q, E173X, Y307X and Y416X), the electroporated cells were located in layer II/III with more Brn2+ cells. These results overexpression of different FOXG1 variants caused different severity of cell fate changes compared to WT FOXG1 overexpression.

Overexpressed FOXG1 variants resulted in Morphological Changes and altered Callosal Projection

Fig.5 Overexpressed FOXG1 Variants resulted in Morphological Changes.

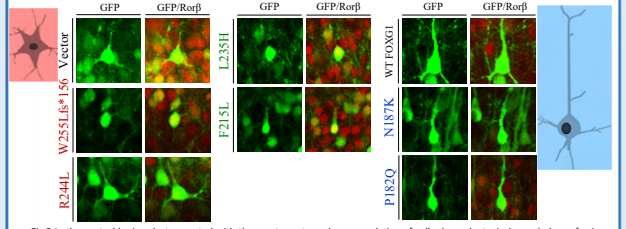

Fig.5 In the control brains electroporated with the empty vector, a large population of cells showed a typical morphology of spiny stellate neurons, with basic property of a short dendritic tree and without apical dendrite. In contrast, cells electroporated with WT FOXG1 showed a classic pyramidal neuron morphology with a triangular cell body extending a long and thick apical dendrite. Cells expressing severe variants (W255Lfs*156, R230H, R244L, G252V, E136X and I194S) showed classic morphology of spiny stellate neurons. Cells expressing moderate variants (L235H, F215L, G252D and G267S) showed partially spiny stellate and pyramidal neuron. In 5 mild variants (N187K, P182Q, E173X, Y307X and Y416X), the morphology of electroporated cells showed as pyramidal neurons.

Fig.6 Overexpressed FOXG1 Variants changed the Connectivity of Corpus Callosum.

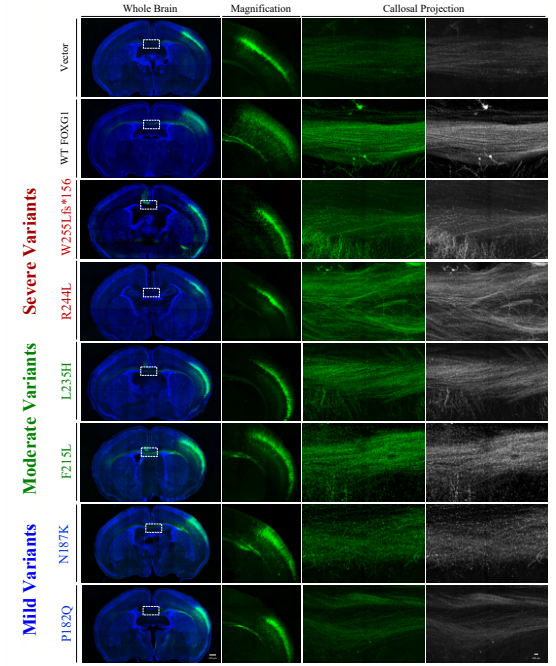

Fig.6 In the control brains electroporated with the empty vector, only a few axons extending from GFP+ cells can be observed in the corpus callosum. On the contrary, cells electroporated with WT FOXG1 projected high density of axons through the corpus callosum. Cells expressing severe variants showed the intensity of callosal projections decrease. The cells expressing moderate variants showed partially unorganized within the corpus callosum. In 5 mild variants, the morphology of electroporated cells showed unorganized and less dense. These observations suggesting that FOXG1 variants did hamper the connectivity of callosal projection.

Overexpression of Severe FOXG1 Variants cause Neuronal Migration Defects due to Loss of Function

Fig.7 Overexpression of FOXG1 Variants changes Neuronal Cell Distribution during Cortical Development.

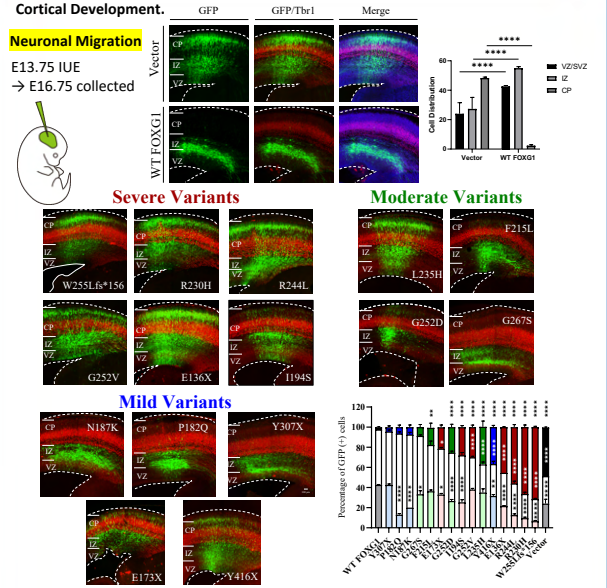

Fig.7 To investigate the potential effects of FOXG1 variants in embryonic brain development, we conducted IUE at E13.75 and collected. 3 days after IUE, the brains were harvested and sectioned and stained with Tbr1 as a marker for deep cortical plate (CP). In control brains electroporated with empty vector into cortex, about half of GFP+ cells have migrated from the ventricular zone (VZ) to the CP, with some cells still migrating in the IZ (intermediate zone) and VZ. Remarkably, when electroporated WT FOXG1 into the mouse cortex, the majority of electroporated GFP+ cells were distributed in the IZ and VZ with very few cells reaching the CP. Cells with severe variants were mostly located in CP due to FOXG1 loss-of-function. The cells of moderate symptoms variants were located in lower CP and upper IZ. In 5 mild variants, the electroporated cells were majorly located in IZ compared to WT FOXG1.

Conclusion

Diagnostic Tools of FOXG1 Variants
1. Protein Stability
2. Transcription Repression Ability
3. Neuronal Migration/Differentiation

		Severe	Moderate	Mild
In Vitro Assays	Protein Expression	Low		High
	Luciferase Level	High		Low
IUE	Cell Fate	Rorβ+	Partial Rorβ+ and partial Brn2+	Brn2+
	Neuronal Morphology	Spiny Stellate Neurons	Both Spiny Stellate and Pyramidal Neurons	Pyramidal Neurons
	Callosal Projection	Severe CC abnormality	Moderate CC abnormality	Mild CC abnormality
	Neuronal Migration	CP	Lower CP Upper IZ	IZ
Clinical Symptoms	Cortical & CC anomaly	✓	✓	✓
	Pachygyric Frontal Cortex	✓		
	Deficient Myelination	✓		
Molecular Level	FOXG1 Variants	W255Lfs*156, R230H, R244L, G252V, E136X, I194S	L235H, F215L, G252D, G267S	N187K, P182Q, E173X, Y307X, Y416X
	Domain	N-terminus FHD	FHD	CS, GBD, C-terminus

Acknowledgement

Special thanks to my professor Jin Wu Tsai who gave the golden opportunity to do join the lab and work in this project to fulfill my scientific research career. Water for experiment was obtained from Milli-Q purification system. Thanks again to all people who supported me during my master degree.