Intronic MiRNA MiR-3666 modulates its host gene FOXP2 functions in neurodevelopment and may contribute to pathogenesis of neurological disorders schizophrenia and autism

Abstract

MicroRNAs (miRNAs) are approximately 22-nucleotide-long, non-coding RNAs that bind to complementary mRNAs with inhibitory effect. An intronic miRNA is embedded in a particular gene called its host gene. Our study focuses on the Homo sapiens intronic miRNA-host gene pair, hsa-miR-3666 and FOXP2. Previous report of co-expression of miR-3666 and FOXP2 indicates possible regulation of FOXP2 functions by miR-3666. However, direct correlation has not been shown yet. Therefore, we took a computational approach to determine if and how such modulation occurs. ChIP-seq identified FOXP2 targets and putative miR-3666 targets showed a significant overlap of 574 common target genes. Functional enrichment analysis of common targets revealed over-representation of KEGG pathways and Gene Ontology modules associated with neurodevelopment. These modules, along with further literature mining and protein-protein interaction analysis of FOXP2 and miR-3666 identified several specific genes associated with neurodevelopment and finally integration of transcriptomic expressions data lead to the selection of four models depicting the mechanisms by which miR-3666 can modulate FOXP2 functions. Model 1 illustrates that during neurodevelopment, miR-3666 can directly modulate the functions of FOXP2 through regulation of common targets, such as IGF1 and EFN2, whereas model 2 shows miR-3666 can also indirectly modulate FOXP2 functions by considering targets that are not common for the intronic miRNA-host gene pair, for example CDH2 and LMO4. This direct and indirect regulation is necessary for precise spatial and temporal expression of genes during neurodevelopment. Models 3 and 4 exhibit mechanisms in which the interactions of miR-3666 and FOXP2 with target genes contribute to the pathogenesis of schizophrenia and autism respectively.

Keywords: FOXP2, miR-3666, target genes, interaction, co-regulation, neurodevelopment, schizophrenia, autism, autism spectrum disorder

Abbreviations: ASD, autism spectrum disorder; DN, differentiated neuron; FC, fold change; GEO, gene expression omnibus; GOBP, gene ontology biological process; GOCC, gene ontology cellular component; GOMF, gene ontology molecular function; HESC, human embryonic stem cell; IPC, intermediate progenitor cell; miRNA, microRNA; NE, neural ectoderm; NPC, neural progenitor cell; NSC, neural stem cell; NT2, Ntera2; PHS, pitt-hopkins syndrome; TF, transcription factor

Introduction

Mature microRNAs (miRNAs) are 18-22 nucleotides long and are known to repress translation by binding to the complementary 3′-UTRs of their target mRNAs. This binding results in inhibition of translation initiation or post-initiation translational block. Each miRNA can recognize multiple target mRNAs that may be related to one or more biological processes; hence miRNA can have diverse effects. Our study involves a type of miRNA called intronic miRNA that is embedded in the introns of its “host gene”. The definition of intronic miRNA depends on two parts: first, sharing the same promoter with their encoded genes and second, being spliced out of the transcript of their encoded genes and further processed into mature miRNAs.

These intronic miRNAs may support or counteract the functions of its host gene. Forkhead genes are a subgroup belonging to the helix-turn-helix class of proteins. FOXP2 (Forkhead box P2) protein contains a FOX DNA-binding domain and a large polyglutamine tract and is evolutionarily conserved, binding directly to 300 to 400 gene promoters in the human genome and hence can regulate a variety of genes. FOXP2 can form homodimers and heterodimers with FOXP1 and FOXP4; this dimerization is a requirement for DNA-binding. Heterodimers of FOXP2 with FOXP1 may have different transcriptional outcomes than their homodimers. Hence, situations may arise where lower levels of FOXP2 could repress transcription by heterodimerization with FOXP1 but as FOXP2 increases in amount, competition between FOXP2 homodimers and endogenous FOXP1 can lead to transcriptional activation. FOXP2 is said to have dual functionality, either repressing or activating gene expression. It generally works as a repressor, however, its overexpression has been shown to increase expression of some genes such as TAGLN and CER1, suggesting a role in transcriptional activation. FOXP2 hosts the intronic miRNA, miR-3666. Though not much work has been done on miR-3666, its targets have been predicted and deposited in various databases. A few recent experiments have shown the repression
activity of miR-3666 on targets such as MET and ZEB1 in thyroid carcinoma and cervical carcinoma cells, respectively.\textsuperscript{9,10}

Previous experiments\textsuperscript{11,12} have demonstrated how intronic miRNA can modulate host gene functions. Since little research has been done with miR-3666, its potential functions with respect to modulation of host gene activities are largely unknown. Furthermore, the co-expression of FOXP2 and miR-3666 has not only been computationally predicted; it has also been confirmed in vitro.\textsuperscript{13} We therefore sought to study if miR-3666 can play an antagonistic or synergistic role in regulation of FOXP2 functions and if it can, the mechanisms by which it does so. This regulatory function of intronic miRNA has critical implications in the designing of therapeutics or its role as biomarkers. miRNAs have been shown to be effective as therapeutics due to several reasons such as its small, conserved sequence; its high binding specificity and affinity; and overall desirable pharmacokinetic properties.\textsuperscript{14} Circulating miRNAs have also been considered good candidates as biomarkers.\textsuperscript{15} In this study, we took a “data-driven and knowledge-based approach” to find functional relations between intronic miRNA miR-3666 and its host gene, FOXP2. We hypothesized that the presence of common target genes for miR-3666 and FOXP2 mean that they are involved in regulation of a common pathway or biological function. Moreover, miR-3666 was also expected to have a synergistic or antagonistic effect on host gene function. Functional enrichment analysis of KEGG pathways and Gene Ontology (GO) on common targets from ChIP-seq experiments highlighted pathways, biological processes and sets of genes that are enriched for both miRNA and host gene.

Methods

Identification of hsa-miR-3666 (miR-3666) and FOXP2 targets

Several miRNA target databases were searched for putative targets of miR-3666 which includes TargetScan (www.targetscan.org) (Release 6.2: June 2012),\textsuperscript{16} TarBase (diana.imis.athena-innovation.gr/ DianaTools)\textsuperscript{17} PicTar (http://pictar.mdc-berlin.de)\textsuperscript{18} and miRecords (c1.accurascience.com/miRecords)\textsuperscript{19} (Figure 1). ChIP-seq experiment datasets of FOXP2 were downloaded from Encyclopedia of DNA Elements (ENCODE) ChIP-seq Experiment Matrix (human genome version hg19) dataset UCSC 2003-2012.\textsuperscript{19} The closestBed feature of BEDTools\textsuperscript{20} was used to identify nearest Ensembl (version 70)\textsuperscript{21} transcript from the ChIP-seq peak as the target gene.

Overlap analysis

Overlap analysis of the targets of FOXP2 and that of miR-3666 was carried out using the tool Venny (version 2.1.0).\textsuperscript{22} Significance of overlap analysis was based on Chi-square test.

Literature mining and expression data analysis

Extensive text-mining related to both FOXP2 and miR-3666 revealed their respective functions at the molecular level. Knowledge about the pathways and processes they are directly or indirectly involved; in their spatial and temporal expression patterns; and the disorders that may result due to perturbations in their function or expression, was used to create the basis upon which our models were developed. The expression profile of the selected genes across various stages of neurodevelopment were obtained from GEO series GSE28633.\textsuperscript{23} The data were log\textsubscript{2}-transformed and the expression values of multiple probes of the same gene were averaged, these values were then visualized as a heatmap using Matrix2png.\textsuperscript{24} For differential expression, the samples were placed into test and control groups and GEO2R analysis was carried out with default parameters. Log\textsubscript{2}FC (fold change) cut off was set to -0.5 and +0.5 for significant differential expression. OncoDrive analysis of Gitools\textsuperscript{25} was used for the detection of “driver genes”. The results of the driver gene analysis were visualized as a heatmap in Gitools using P value scale with corrected right p-value less than 0.05. To obtain expression profile of miR-3666, Gene Expression Omnibus (GEO)\textsuperscript{26,27} series “GSE15888”\textsuperscript{28} was subjected to GEO2R analysis. The samples in GSE15888 were placed into defined groups and the test was run using default parameters.

![Figure 1](image-url) Overview of process of developing models to depict the role of miR-3666 in modulation of host gene functions.

Functional enrichment analysis

Functional annotation of target genes is based on Gene Ontology (GO)\textsuperscript{29} as extracted from Ensembl\textsuperscript{30} and KEGG pathway database.\textsuperscript{10} Accordingly, all genes are classified into the ontology categories biological process (GOBP) and pathways when possible. We have taken only the GO/pathway categories that have at least 10 genes annotated. We used Gitools for enrichment analysis and heatmap generation.\textsuperscript{31} Resulting p-values were adjusted for multiple testing.
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(p-value less than 0.01) using the Benjamin and Hochberg’s method of False Discovery Rate (FDR). The candidate gene list for schizophrenia was collected from Schizophrenia Gene Resource whereas candidate genes for autism were collected from Autism KB and SFARI gene databases. Considering the candidate genes, we carried out enrichment analysis to find whether the disorders are significantly enriched when common target genes are considered.

Selection of genes and development of models

In order to construct a model to demonstrate how the intronic miRNA miR-3666 affects the function of its host gene, we selected a particular set of genes. These genes were selected based on their significance in neurodevelopment and potential in being directly or indirectly affected by FOXP2 and miR-3666. Protein-protein interaction studies were done using STRING (version 10.0) BioGRID (version 3.4) and IntAct databases. STRING database was used to build a protein-protein interaction network model keeping all parameters in default.

Results and discussion

Host and intronic miRNA targets overlap significantly

First we sought to find the target genes of miR-3666. Since miR-3666 has only recently been added to miRNA databases, experimentally validated targets are not available yet. The targets of miR-3666 downloaded from major miRNA target databases-Tarbase, TargetScan, PicTar, miRecords were combined to form a unique union set of 1028 target genes. This approach allowed a comprehensive method in determination of putative targets of miR-3666 and therefore an increased confidence in the results. Similarly, by analyzing FOXP2 ChIP-seq data in SK-N-MC cell line we identify total 7883 FOXP2 targets. In order to study the role of miR-3666 in modulating its host gene functions, we analyzed the target genes that are affected by the action of both host gene and intronic miRNA. Overlap analysis in a venn diagram showed that miR-3666 and FOXP2 have 574 common targets (Figure 2A). Chi-square test shows that the overlap is significant (p-value <10^-6 and Chi-square value 1817.16) and much higher than expected value (percentage deviation is +351.3%).

**FOXP2 and miR-3666 perform common role in neurodevelopment**

FOXP2 has widespread expression in humans (Supplementary Figure S1). It is expressed at high levels in the developing brain, with lower expression in various parts of the human brain. Besides the brain, it is expresses in the lungs and gut as well. Although FOXP2 has extensive expression in the developing brain, a quite low level in the adult brain suggests that the expression of FOXP2 is developmentally regulated. Mutational analysis in several studies have demonstrated the association of non-functional FOXP2 with motor dysfunction, cerebellar abnormalities and early postnatal lethality. In an experiment that studied miRNA expression during the process of neural differentiation using an RA (retinoic acid)-induced embryonal carcinoma Ntera2/D1 (NT2) cell line, miR-3666 was observed to continue being expressed in fully differentiated NT2-derived post-mitotic neurons and/or NT2-derived astrocytes. The differential expression of miR-3666 during the experiment indicated its role in regulation of neurodevelopment, particularly the peak in miR-3666 expression between 6 and 14 days of treatment implies a biological role in cell-fate determination (Supplementary Figure S2).

Above results and information indicates that miR-3666 and FOXP2 are involved in neurodevelopment so that our analysis focused on FOXP2 and miR-3666 co-regulation of neurodevelopmental processes.

**Over-represented Gene Ontologies and KEGG pathways of common targets are related to neurodevelopment, which are not modulated by miR-3666 lone targets**

Enrichment analysis allows us to understand and quantitatively measure whether there are statistically significant changes in a set of biological annotations. We sought to determine the host gene functions that are modulated by intronic miRNA, miR-3666. Hence we have done enrichment analysis using Gitools to obtain significantly over-represented Gene Ontology Biological Process (GOBP) terms (Figure 2B) and KEGG pathways (Figure 2C) and on the common and unique targets of FOXP2 and miR-3666. Although functional enrichment analysis showed enrichment for a diverse set of processes (Figure 2B & 2C) at a very low p-value of 0.01, there is a clear predominance of functions such as “neuron differentiatation”; neurological disorders; and signaling pathways involved in neurodevelopment, neurogenesis or gliogenesis in commonly regulated targets of FOXP2 and miR-3666. All of the enriched KEGG pathways and GO Biological Processes were then analyzed for related enriched genes. By combining all the enriched genes obtained from both KEGG and GOBmodules, we hence obtained 249 common target genes that were significantly enriched for all pathways and processes related to neural development (Supplementary Table S3).

Language disorder and cognitive deficiency is commonly considered as one of the principal symptoms in several disorders, such as schizophrenia and autism. Hence, FOXP2 is likely to be a good candidate gene since its mutation has been reported to be associated with speech and language deficits. FOXP2 has also been reported to be associated with schizophrenia and autism. FOXP2 may therefore directly cause the disease or affect its downstream targets that lead to the development of such disorders. We see that, when considering candidate genes of the disorders and common targets of FOXP2 and miR-3666, both schizophrenia and autism are significantly enriched (Figure 2D).

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Supplementary Table S3 List of FOXP2 and miR-3666 target genes that are enriched for neural development related terms in GOBP and KEGG pathways

| AAK1 | CDC73 | ERBB4 | KIT | NDEL1 | PRKD3 | SOX4 | TRIB1 |
|------|-------|-------|-----|-------|-------|------|-------|
| ABCC4 | CEPE | EREG | LDLRAD3 | NEDD4L | PRNP | SOX5 | TRIM2 |
| ACVR1 | CEPI20 | FBXW11 | LDLR | NEUROD1 | PSD3 | SPI | TRPC3 |
| ADCY1 | CHRM2 | FERMT2 | LIN28A | NFB | PTEN1 | SPATA2 | TSC1 |
| AGFG1 | CHST11 | FMR1 | LMLN | NPN | PTFRD | SPEN | TTPA |
| AHR | CLTC | FNBP1 | LRPI2 | NPTN | QKI | SPHK2 | TXNP |
| AK4 | COL19A1 | FOXP1 | LRPIB | NR3C1 | RAB5B | SPOCK1 | UHMK1 |
| APCDD1 | COL6A3 | FRMD6 | LRP6 | NRP1 | RACGAP1 | SPRED1 | ULK2 |
| ARHGAP24 | COX7A2L | FRZB | LRP8 | NRP2 | RALBP1 | SRGAP3 | UNCI3A |
| ARHGEF12 | CPEB2 | FZD3 | LRRK2 | NRSN1 | RAPGEF4 | STAT3 | VANGLI |
| ARID5B | CPEB4 | GAB1 | MAFB | NJUS1 | RNF41 | STAT6 | VAPA |
| ARL4A | CREB1 | GCLC | MAGI1 | OTX2 | ROBO2 | STIM2 | VAV2 |
| ARX | CREG5 | GDA | MAML3 | PDE4D | ROCK2 | STK4 | WASL |
| ATP2A2 | CUL3 | GJA1 | MAPK3 | PDE5A | RP56KA2 | SULF1 | WHSC1L1 |
| ATP6V1B2 | CXCL12 | GNA12 | MAP4 | PDEB7 | RRAGD | SYT2 | WNT10A |
| ATXN1 | DCBLD2 | GRIK2 | MAP7 | PDEBA | RTNI | TACCI | WNT2B |
| BAG5 | DENND1A | HBPI | MAPK10 | PI4KA | RUNX1T1 | TAF4B | XYL1T |
| BCL11A | Dicer1 | HECA | MAPK1 | PIK3CA2 | RFXFP2 | TAF4 | YES1 |
| BHLHE41 | DLC1 | HEG1 | MBD2 | PIK3IP1 | S1PR1 | TANCI | ZBTB7B |
| BMPR1B | DLG5 | HESI | MBNL1 | PLEKHS5 | S1PR2 | TAOK1 | ZEB1 |
| BMP2 | DNM2 | HOMER1 | MDFRC | PLEKHS5 | SBF2 | TBL1XR1 | ZEB1 |
| BPTF | DPYSL2 | HSPA8 | MDGA2 | PLLP | SETD7 | TP61 | ZFAND5 |
| BTG1 | EDA | IGF1 | MEOX2 | PMEPA1 | SH2B3 | TCF4 | ZFPM2 |
| CABLES1 | EFNB2 | IGFBP5 | MET | POU3F2 | SHANK2 | TENM1 | ZIC5 |
| CALB1 | EGR3 | INHBB | MIL | POU4F1 | SHC3 | TFDP2 | ZNF3 |
| CALM1 | EIF2C1 | ITGAI | MLTK | PPARGC1A | SIK1 | TGF2 | ZNR3F |
| CALM2 | EMX2 | ITGB8 | MYB | PPIA2 | SIPA1L2 | TGFB2 | ZNR3F |
| CANX | ENAH | ITPKB | MOY10 | PPP1R9A | SMAD2 | THSD7A | |
| CAPRIN2 | EPB41L1 | JARID2 | MYT1L | PRICKLE2 | SMAD5 | TIMP2 | |
| CAV2 | EPDR1 | KCNND3 | NAV1 | PRKAA1 | SNAP25 | TNRC6B | |
| CCDC88A | EPHA7 | KIAA1462 | NCOA1 | PRKAB1 | SNX2 | TNRC6C | |
| CCND3 | EPS15 | KIF13A | NCOA3 | PRKacb | SOX21 | TP63 | |
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Figure 2: Identification of FOXP2 and miR-3666 common targets and functional overrepresentation analysis of targets.

A. Venn representation of common and unique targets genes of host genes and intronic miRNA. The left circle represents FOXP2 target genes, where the number in the blue circle corresponds to genes exclusive for FOXP2. The right circle represents miR-3666 target genes, where the number in the yellow circle corresponds to genes exclusive for miR-3666. The intersection area of the two circles represents the number of target genes shared by both FOXP2 and miR-3666. Enriched heatmap of:

B. Gene Ontology Biological process (GOBP) terms.

C. KEGG pathways and

D. Enriched neurological disorders schizophrenia and autism. Multiple test corrected p-values are represented in color coded heatmaps. The higher the color intensity towards red, the greater is the significance, while higher color intensity towards yellow represents lower significance. Gray represents insignificant p-value.

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**Supplementary Table S4** List of selected 30 genes full name and symbols

| Gene name                                                      | Gene symbol |
|---------------------------------------------------------------|-------------|
| Achaete-Scute Family BHLH Transcription Factor 1              | ASCL1       |
| Brain-Derived Neurotrophic Factor                             | BDNF        |
| Cyclin D3                                                     | CCND3       |
| Cadherin 2                                                    | CDH2        |
| Contactin Associated Protein-Like 2                          | CNTNAP2     |
| CAMP Responsive Element Binding Protein 1                     | CREB1       |
| DiGeorge Syndrome Critical Region Gene 2                      | DGCR2       |
| Disrupted In Schizophrenia I                                  | DISC1       |
| Ephrin B2                                                     | EFNB2       |
| Empty Spiracles Homeobox 2                                    | EMX2        |
| Erb-B2 Receptor Tyrosine Kinase 4                             | ERBB4       |
| Forkhead Box P1                                               | FOXP1       |
| Hes Family BHLH Transcription Factor 1                        | HES1        |
| Insulin Like Growth Factor 1                                  | IGF1        |
| LIM Domain Only 4                                             | LMO4        |
| MET Proto-Oncogene, Receptor Tyrosine Kinase                   | MET         |
| NudE Neurodevelopment Protein 1 Like 1                        | NDEL1       |
| Neuronal Differentation 1                                     | NEUROD1     |
| Neurogenin 1                                                  | NGN1        |
| Neurogenin 2                                                  | NGN2        |
| Neuregulin 1                                                  | NRG1        |
| Paired Box 6                                                  | PAX6        |
| POU Class 3 Homeobox 2                                        | POU3F2      |
| Protein Tyrosine Phosphatase, Non-Receptor Type II            | PTPN11      |
| Protein Tyrosine Phosphatase, Receptor Type J                 | PTPRJ       |
| Synaptosome Associated Protein 25kDa                          | SNAP25      |
| SRY (Sex Determining Region Y)-Box 2                          | SOX2        |
| SRY (Sex Determining Region Y)-Box 21                         | SOX21       |
| SRY (Sex Determining Region Y)-Box 4                          | SOX4        |
| Transcription Factor 4                                        | TCF4        |

**Supplementary Table S5** List of selected genes linked to neurodevelopmental disorders schizophrenia and/or autism

| Schizophrenia candidate genes | Autism candidate genes |
|-------------------------------|------------------------|
| BDNF                          | BDNF                   |
| DGCR2                         | CNTNAP2                |
| ERBB4                         | ERBB4                  |
| FOXP2                         | FOXP1                  |
| NRG1                          | FOXP2                  |
| MET                           | MET                    |
| PAX6                          | PAX6                   |
| PTPN11                        | SNAP25                 |
| TCF4                          |                        |

**Figure 3** STRING network displaying

a. FOXP2 interactions and
b. Interactions among 30 selected genes.

The network nodes represent proteins whereas the edges represent protein-protein associations. Small nodes represent protein of unknown 3D structure whereas large nodes mean some 3D structure is known or predicted. The colored nodes are for query proteins and first shell of interactors whereas white nodes are second shell of interactors.

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**FOX2 and miR-3666 may be responsible for the pathogenesis of schizophrenia**

Schizophrenia (SCZ) is a severe and chronic neuropsychiatric disorder; it is reported to have a lifetime prevalence of approximately 1%–3%. This neurodevelopmental disorder involves multiple genes that may be directly or indirectly modulated by FOX2 and miR-3666. As model 3 (Figure 5C) shows, the candidate gene ERBB4, being a common target gene, may be directly regulated by miR-3666 and FOX2. However, miR-3666 and FOX2 can regulate the levels of candidate genes BDNF (Brain-Derived Neurotrophic Factor), DGCR2 (DiGeorge syndrome critical region gene 2), and NRGI through common target CREB1. This relation is evident from the GEO2R analysis of GSE17612 expression data where up regulation of FOX2 (log_FC=0.54360366) and downregulation of BDNF (log_FC=-0.54217872) is observed in these cells (Figure 7). We assume the upregulation of FOX2 may be responsible for low BDNF levels; FOX2 and miR-3666 may indirectly repress BDNF by inhibition of CREB1. In the BDNF expression profile, one sample shows much higher expression level compared to the rest, which may be due to age-related differences or the use of anti-psychotic drugs. Moreover, FOX2 probably directly inhibit DISC1 (Disrupted-In-Schizophrenia 1), whereas miR-3666 may indirectly inhibit it by repressing NDEL1 and hence disrupting DISC1-NDEL1 interaction.

**FOX2 and miR-3666 may be responsible for the pathogenesis of autism and ASD**

Autism and ASD are developmental disorders with three core symptoms: “deficits in social interactions and understanding; aberrant communication and/or language development; and restricted interests and repetitive, stereotyped behaviors”47. Autism candidate genes ERBB4 and BDNF have been associated with ASD. As shown in model 3 (Figure 5C), ERBB4 may be directly co-regulated by miR-3666 and FOX2 since it is a common target; whereas BDNF levels may be indirectly regulated via common target CREB1. Therefore, these interactions have not been shown in model 4 (Figure 5D) again. FOX1 (forkhead box protein P1) deletions and increase have both been associated with ASD. Both FOX1 and FOX2 can downregulate CNTNAP2 (Contactin-associated protein-like 2), another candidate gene of ASD. Chien et al. hypothesized that enhanced FOX1 expression can increase the expression of FOX2 through a feedback mechanism, which in-turn may then lead to the reduction of CNTNAP2 levels and result in ASD. Hence, interactions among FOX1, FOX2 and CNTNAP2 genes may be responsible for the pathogenesis of syndromic and non-syndromic ASD. As model 4 (Figure 5D) shows, miR-3666 can play an “enemy” role to FOX2 by repressing FOX1 and removing its inhibitory effect on CNTNAP2 or it may inhibit FOX1 expression and affect the modulatory roles of FOX2 that requires FOX1-FOX2 dimerization. FOX2 and miR-3666 can also jointly affect the levels of common targets MET (MET receptor tyrosine kinase), TCF4, SNAP25 (synaptosomal-associated protein of 25 kDa) and PTEN, which are candidate genes for ASD. PAX6 regulation by miR-3666 is not only important to maintain the levels of FOX2 but also to prevent the development of autism and related disorders, as PAX6 is also a candidate gene for ASD.

**MiR-3666 directly or indirectly regulates FOX2 functions in neurite outgrowth and cortical patterning**

As model 1 (Figure 5A) proposes, miR-3666 regulates neurite outgrowth, axon guidance and synaptic plasticity by regulating the gene EFNB2, a well-validated ChIP-Seq target of FOX2. miR-3666 can regulate proper cortical patterning through regulation of the common target gene EMX2 (empty spiracles homolog 2) and miR-3666 target PAX6 (paired box 6). Additionally, model 2 (Figure 5B) demonstrates miR-3666 may enhance FOX2 expression of LMO4 (LIM domain-only 4) by inhibiting the common target gene, CREB1 (cAMP responsive element binding protein 1). LMO4 gene shows asymmetric expression in the embryonic brain possibly due to repression by FOX2 and hence plays important roles in cortical patterning. Analysis of GSE28633 revealed the expression changes of genes of models 1 and 2. Log-transformed and median centered expression values were visualized as a heatmap using a color coded scale. FOX2 shows relatively higher expression in neuroectodermal stage (NE) and in differentiated neurons (DN) and lower expression in human embryonic stem cells (hESCs). As expected, during differentiation, we see the upregulation of genes EFNB2, PAX6 and EMX2 in NE and S0X4 in DN (Figure 6). Besides regulating FOX2 functions by binding to its targets, model 1 (Figure 5A) shows miR-3666 may also regulate the expression of FOX2 itself. POU3F2 (POU class 3 homeobox 2, aka Brn-2) has been known to bind and activate FOX2. Besides activating NEUROG2, PAX6 can also induce the expression of POU3F2 and FOX2. Hence, we infer that miR-3666 can therefore regulate the expression of FOX2 by regulating its targets, the FOX2 activators PAX6 and POU3F2.

**Figure 4** Venn diagrams representation of common genes between a. Autism candidate genes and our list of selected genes and b. Autism candidate genes and our list of selected genes. The left circle represents disease candidate genes, where the number in the blue circle corresponds to genes exclusive for the disease. The right circle represents genes exclusive for the selected genes. The intersection area corresponds to genes exclusive for the selected genes. The right circle corresponds to genes exclusive for the disease.

![Figure 4](image-url)
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revealed the upregulation of TCF4 (log2 FC=0.629) (Figure 8A) and downregulation of SNAP25 (log2 FC=−0.828) (Figure 8B). GEO2R analysis of GSE29691 also revealed the up regulation of TCF4 (log2 FC=0.55079923) (Figure 8C) and down regulation of PTPN11 (log2 FC=−0.53875346) (Figure 8D). Even though low levels of TCF4 has resulted in Pitt-Hopkins Syndrome (PHS)104,111 a disease related to autism with common symptoms, high levels of TCF4 has been observed in patients afflicted with SCZ.112,113 Since SCZ and autism are closely related, it may be deduced that high levels of TCF4 may lead to development of autism or related disorders. GEO2R analysis of GSE6575114 did not show any significant differential expression of our selected genes, however, driver gene analysis of GSE6575 (Figure 9) revealed significant (p<0.05) down regulation of CNTNAP2 and TCF4 but no upregulated genes. These observations are in concordance to our models’ suggestion of the levels of candidate genes associated with the disorders. It is not surprising that the expression data used as evidence for autism candidate gene levels did not show differential expression of FOXP2 levels in autism patients. This may be due to the use of blood samples in the experiment related to GSE29691, as FOXP2 is not significantly expressed in blood.115 Also, since post-mortem brains are used, the expression may be too low for detection. A previous study attempted to measure the mRNA level of FOXP2 in lymphoblastoid cell lines using RT-qPCR, but the mRNA levels were too low to be detected.100

Figure 5 Models depicting the possible role of miR-3666 in modulation of FOXP2 functions in neurodevelopment in pathogenesis of neurological disorders.

A. Model 1 depicts direct regulation by miR-3666 of FOXP2 and common targets.
B. Model 2 depicts indirect regulation by miR-3666 of FOXP2 functions.
C. Model 3 represents co-regulation of miR-3666 and FOXP2 in pathogenesis of schizophrenia and
D. Model 4 represents co-regulation of miR-3666 and FOXP2 in pathogenesis of autism and related disorders. miR-3666 is shown in blue whereas FOXP2 is shown in yellow.

The common target genes are shown in green whereas targets that are not shared by miR-3666 and FOXP2 are shown in red. The green arrows represent directional activation and the blunt-ended red lines show directional inhibition. The black lines with circled ends represent interaction and the blue lines represent binding (or dimerization). The orange block arrows associated with the genes show the function of the particular genes and the purple block arrows show levels of candidate genes found in diseased individuals.

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Figure 6 Heatmap visualization of the expression profile of model 1 and 2 genes during neural differentiation.
Log2 expression values of each gene is subtracted from row median expression and represented in color coded heatmap. The rows represent genes whereas the columns represent samples corresponding to:

I. hESC: human embryonic stem cell
II. NI: neural induction
III. NE: neural ectoderm and
IV. DN: differentiated neurons

A color coded scale from 2 to 12 represents expression values where color intensity towards red corresponds to higher expression from median and color intensity towards green corresponds to lower expression from median expression value and yellow corresponds to median expression.

Figure 7 Expression profile of
A. FOXP2 and
B. BDNF in GSE17612.
The title shows GEO accession number (GSE17612), platform ID (GPL570) and the probe ID (1555648_at for FOXP2 and 239367_at for BDNF). The groups shown are (1) control and (2) SCZ (schizophrenia). Each group contains particular samples as designated by their GSM IDs. The red bars represent the expression measurement extracted from the MAS5.0 signal intensity values of the samples.

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The profile diagram is titled in the format “GEO accession number/platform ID/probe ID”. The groups shown are (1) control and (2) autism-related. Each group contains particular samples as designated by their GSM IDs. The red bars in GSE38322 represent the expression measurement extracted from the quantile normalized, variance stabilized, signal intensity values of the samples whereas the red bars in GSE29691 represent the expression measurement extracted from the Log2 GCRMA signal intensity values of the samples.

**Conclusion**

This study demonstrates that intronic miRNA, miR-3666 and its host gene, FOXP2, coincides with a functional relation in neurodevelopment, as deduced from literature mining, expression data analysis and functional enrichment analysis of the common target genes. Further literature mining and interaction studies show how miR-3666 may regulate FOXP2 functions as an “enemy” or “partner”; based on which four models were developed. Neurodevelopment being a complex biological process requires precise regulation; these models suggest mechanisms in which miR-3666 can modulate FOXP2 functions by directly or indirectly affecting the expression of FOXP2 target genes to ensure the precise spatial and temporal regulation of genes associated with neurodevelopment. The models also show how miR-3666 and FOXP2 may be associated with the neurodevelopmental disorders schizophrenia and autism. Microarray expression data were analyzed which support some interactions between FOXP2 and its targets as portrayed in the models. Further validation of these models by in vitro and in vivo experiments would help in the development of more effective stem cell therapies, which is especially attractive due to limited regenerative capacity of neurons in mammals. Besides stem-cell therapies, an understanding of the function of miR-3666 may be useful for the designing of miRNA-based therapeutics. For example, in our study we find that miR-3666 and FOXP2 inhibit MET expression. Since reduced MET levels in the brain have been associated with autism, anti-miR-3666 may be a potential drug to offset the inhibitory effects of FOXP2 and return MET to normal levels. Additionally, the results may be implicated in development of therapeutics against neurodevelopmental disorders.

**Figure 8** Expression profile of
A. TCF4
B. SNAP25 in GSE38322
C. TCF4 and
D. PTPN11 in GSE29691.

**Figure 9** Heatmap of selected down regulated driver genes.

Driver gene analysis from GEO expression dataset GSE65755. P-value of significance as candidate driver gene is represented in a color coded scale in the heatmap. Color towards red indicates more significantly down regulated driver genes, where as color towards yellow indicates less significantly down regulated genes and grey indicates non-significant genes. Our selected genes are highlighted in square box.
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Conflict of interest
The author declares no conflict of interest.

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