Transposable elements contribute to the spatiotemporal microRNA landscape in human brain development

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Abstract

Transposable elements (TEs) contribute to the evolution of gene regulatory networks and are dynamically expressed throughout human brain development and disease. One gene regulatory mechanism influenced by TEs is the miRNA system of post-transcriptional control. miRNA sequences frequently overlap TE loci and this miRNA expression landscape is crucial for control of gene expression in adult brain and different cellular contexts. Despite this, a thorough investigation of the spatiotemporal expression of TE-embedded miRNAs in human brain development is lacking. Here, we identify a spatiotemporally dynamic TE-embedded miRNA expression landscape between childhood and adolescent stages of human brain development. These miRNAs sometimes arise from two apposed TEs of the same subfamily, such as for L2 or MIR elements, but in the majority of cases stem from solo TEs. They give rise to in silico predicted high-confidence pre-miRNA hairpin structures, likely represent functional miRNAs and have predicted genic targets associated with neurogenesis. TE-embedded miRNA expression is distinct in the cerebellum when compared to other brain regions, as has previously been described for gene and TE expression. Furthermore, we detect expression of previously non-annotated TE-embedded miRNAs throughout human brain development, suggestive of a previously undetected miRNA control network. Together, as with non-TE-embedded miRNAs, TE-embedded sequences give rise to spatiotemporally dynamic miRNA expression networks, the implications of which for human brain development constitute extensive avenues of future...
experimental research. To facilitate interactive exploration of these spatiotemporal miRNA expression dynamics, we provide the “Brain miRTExplorer” web application freely accessible for the community.

Introduction

Transposable elements (TEs) account for around half of the human genome and have contributed to the evolution of gene regulatory networks (Chuong et al. 2013, 2016, 2017; Garcia-Perez et al. 2016; Pontis et al. 2019; Turelli et al. 2020; Playfoot et al. 2021). The majority of TEs have lost their capacity to ‘copy and paste’ to new locations around the genome, instead being co-opted by the host organism to perform a plethora of regulatory homeostatic functions during normal development (Elbarbary et al. 2016; Chuong et al. 2017). One post-transcriptional regulatory mechanism in which TE-embedded sequences have been co-opted is the microRNA (miRNA) system (Smalheiser and Torvik 2005; Piriypapongsa et al. 2007; Roberts et al. 2014). Computational and experimental studies have shown different classes of TEs (LINE, SINE and LTR) can act as functional sources of miRNA in different cellular models. However, limited information exists for primary tissues, especially for tightly regulated spatiotemporal developmental processes such as human brain development (Piriypapongsa et al. 2007; Piriypapongsa and Jordan 2007; Ding et al. 2010; Frankel et al. 2014; Roberts et al. 2014; Spengler et al. 2014; Petri et al. 2019).

Recent studies in a small number of adult brains have highlighted the roles of TE-embedded miRNAs from the L2 family. These are functional in neurotypical adult brains and are differentially expressed in glioblastoma (Skalsky and Cullen 2011; Petri et al. 2019). Furthermore, miRNAs have critical roles in mammalian neuronal homeostasis, highlighting the fundamental nature of miRNAs in neurogenesis, alongside diverse roles in neurological disease and human evolution (Cao et al. 2007; Somel et al. 2011; Qureshi and Mehler 2012; Petri et al. 2014; Topol et al. 2016; Sambandan et al. 2017; Juźwik et al. 2019; Woods and Van Vactor 2021). miRNAs are spatially and temporally expressed in the developing human brain from birth to adolescence, however the contribution of TE-embedded sequences to this process has never been investigated (Ziats and Rennert 2014). Indeed, the years proceeding birth and
throughout childhood represent a crucial window in human brain development, characterized by extensive changes in size, cellular composition and functional processes such as synaptogenesis, myelination and synaptic pruning (Silbereis et al. 2016; Dyck and Morrow 2017).

We therefore aimed to determine the prevalence of spatiotemporally expressed, annotated TE-embedded miRNAs in the developing human brain by re-analysis of small RNA-seq data available from the BrainSpan Atlas of the Developing Human Brain from one year old to 19-year-old brains (Miller et al. 2014; Li et al. 2018). We computationally uncover dynamic spatiotemporal expression of numerous annotated TE-embedded miRNAs and a small number of previously undetected novel putative TE-embedded miRNAs, suggesting TE-sequence co-option as miRNAs may play a role in this important neurodevelopmental window. We provide the “Brain miRTExplorer” web application to facilitate interactive exploration of both annotated TE-embedded and non-TE-embedded miRNA spatiotemporal expression data, freely accessible for the community at https://tronoapps.epfl.ch/BrainmiRTExplorer/.

Results

TEs contribute to the annotated miRNA transcriptional landscape in the human brain

To determine spatiotemporal, small RNA expression in postnatal human brain development, we analyzed small RNA-seq data from 174 samples from one year to 20 years of age, encompassing 16 different brain regions, from 16 donors (9 male and 7 female) available through the BrainSpan Atlas of the Developing Human Brain (Supplemental Fig. S1) (Miller et al. 2014; Li et al. 2018). To enrich for different small RNA moieties, we separated sequencing reads into lengths of 18 - 25bp, 26 – 37bp and 38 – 50bp and intersected with Ensembl annotations, miRBase, the GtRNAdb database and our modified merged TE RepeatMasker data set (Kozomara and Griffiths-Jones 2014; Chan and Lowe 2016; Pontis et al. 2019; Turelli et al. 2020; Yates et al. 2019; Playfoot et al. 2021). As expected, the different read lengths enriched for annotated miRNAs, tRNAs and snoRNAs respectively (Fig. 1A; Supplemental
Fig. S2). By retaining the miRNA derived 18 – 25bp reads, we detected the expression of 543/1871 annotated miRNAs (Fig. 1B; Supplemental Table S1 & S2).

To determine the overlap of annotated miRNAs with TEs, we intersected their genomic coordinates with those from our curated RepeatMasker data set (Turelli et al. 2020; Playfoot et al. 2021). 17% of annotated miRNAs were derived from TEs, in either sense and antisense orientation to the miRNA and belonged to all known classes of elements, with representatives from various subfamilies and evolutionary ages (Fig. 1B & C; Supplemental Table S1). Only 36/543 expressed miRNAs were annotated as mirtrons (Da Fonseca et al. 2019), none of which were TE-embedded. This indicates that TEs do not contribute to mirtrons in this context.

L2 family members of 105 - 177 million-year-old (MYO) contributed the most to annotated detectably expressed miRNAs in the child and adolescent brain (Fig. 1C), with detection of all L2-embedded, annotated miRNAs previously noted in adult brain and glioblastoma (Piriyapongsa et al. 2007; Petri et al. 2019), pointing to their likely roles in earlier stages of brain development (Supplemental Table S1). The previously described 43.2 MYO MADE1 elements and the 177 MYO MIR family elements also heavily contributed to expressed miRNAs (Fig. 1C) (Piriyapongsa and Jordan 2007; Shao et al. 2010; Borchert et al. 2011; Spengler et al. 2014).

To determine the potential importance of TE-embedded miRNAs versus non-TE-embedded miRNAs we plotted the mean expression of all miRNAs in descending order (Supplemental Fig. S3A). One quarter (24/94) of TE-embedded miRNAs were in the top 200 most expressed miRNAs (Supplemental Fig. S3A).
Figure 1. TEs contribute to annotated miRNAs in the child and adolescent human brain. (A) Stacked bar chart indicating the percentage of 18-25bp reads overlapping different annotated genomic features for samples from the dorsolateral frontal cortex (DFC). If a TE overlaps an annotated feature (miRNA, tRNA etc.) the feature takes preference. (B) Pie charts indicating the number of miRBase annotated miRNAs overlapping at least one TE (top), and their relative orientations (bottom). (C) Bar chart indicating the number of TEs overlapping miRBase annotated miRNAs and their class and age in million years old (MYO). (D) Expression in log2 counts per million+1 (CPM+1) of mature TE-embedded
miRNA in 399 cell types and tissues from FANTOM5 (De Rie et al. 2017). * denotes miRNAs highlighted in the text. Samples comprise largely primary cells such as epithelial, fibroblast, endothelial, connective tissue, smooth muscle, immune, neural stem, dendritic and pluripotent stem cells, among others (full list available in De Rie et al., 2017). (E) Pie charts indicating the number of human TE-embedded miRNAs detected in syntenic locations in macaque and mouse miRBase annotations (see also Supplemental Table S3 and methods for detail).

Four of these were in the top 50 most expressed and have described roles in neurogenesis and glioma (Zhang et al. 2012; Huang et al. 2015; Ruan et al. 2015). Despite this, TE-embedded miRNAs were significantly less expressed than non-TE-embedded miRNAs (Supplemental Fig. S3B), however even TE-embedded miRNAs with low expression levels (e.g., hsa-mir-326_Arthur1B - 529th in the expression list) have roles in neurogenic diseases like glioma (Kefas et al., 2009). This indicates that TE-embedded miRNAs are expressed at generally lower levels than non-TE-embedded miRNAs but still play functional roles in the brain.

We next aimed to determine if TE-embedded miRNAs were produced in other cell types and tissues by analyzing miRNA expression data from 399 human samples comprising largely primary cells such as epithelial, fibroblast, endothelial, connective tissue, smooth muscle, immune, neural stem, dendritic and pluripotent stem cells, among others (De Rie et al. 2017). Mature TE-embedded miRNAs were broadly expressed in the majority of cell types, with relatively ubiquitous, high levels for MIRc-embedded hsa-miR-378a-3p, L2d2-embedded hsa-miR-28-3p, L2c-embedded hsa-miR-151a-3p/5p and MamRTE1-embedded hsa-miR-130a-3p and lower expression for other TE-embedded miRNAs (Fig. 1D). A similar ubiquitous expression was detected for mature non-TE-embedded miRNAs (Supplemental Fig. S4). Together, these data indicate that a multitude of TE-embedded miRNAs are broadly expressed in the child and adolescent human brain, with appreciable expression in other cell types.

To investigate if TE-embedded miRNAs are conserved and in syntenic locations among mammals, we performed a series of lift-overs from the human (hg19) to the macaque (rheMac8) and mouse (mm10) genomes. We next downloaded annotated miRNA coordinates from miRBase for macaque and mouse
Of the 92 TE-embedded miRNAs detected in human (Fig. 1B), 88 remained after lift-over to macaque, whereas the last four had no syntenic homologue. An intersect of these syntenic coordinates in macaque with the annotated miRNA coordinates from miRBase detected 45/88 TE-embedded miRNA loci which were largely annotated with the same miRNA name (Fig. 1E; Supplemental Table S3). Intersecting these with all TE coordinates from rheMac8 also showed that 40/45 overlapped the same or closely related TE subfamilies in macaque (Supplemental Table S3).

In mouse, 53 loci remained when TE-embedded miRNA coordinates in hg19 were lifted over to mm10. Of these, 28/53 overlapped coordinates from miRBase for mm10, again largely with the same name (Fig. 1E; Supplemental Table S3). The subsequent intersect with all TE loci for mouse resulted in 19/28 TE-embedded miRNA loci in human also overlapping the same or closely related TE subfamily in mouse (Supplemental Table S3). These were the older, more conserved TE subfamilies such as L2 (105 – 177 MYO), MIR (177 MYO) and some DNA elements (105 – 177 MYO) (Supplemental Table S3). Together, these analyses indicate that a large subset of TE-embedded miRNAs expressed in the human brain are conserved in mammalian lineages.

**TE-embedded miRNAs exhibit spatiotemporal expression patterns**

To investigate the temporal dynamics of TE-embedded miRNAs in brain development, we compared their expression from childhood (1 to 5 years) to adolescence (9 to 20 years) (Supplemental Fig. S1). We initially combined samples of forebrain (FB) origin, representing 124 samples from 16 donors, with 66 and 58 samples representing childhood and adolescence respectively (Supplemental Fig. S1). Data was normalized using the trimmed mean of the log expression ratios (TMM) method which excludes the top and bottom expressed miRNAs prior to computing library sizes to ensure very high or low expressed miRNAs did not dominate the library size normalization (Robinson and Oshlack 2010). Counts per million (CPM) values were generated after correcting by the TMM library size. There was no difference in the number of reads, percentage of reads aligned to the genome or assigned to a feature between childhood and adolescence categories (Supplemental Fig. SSA-C).
16% and 5.5% of TE-embedded miRNAs were significantly more highly expressed in childhood or adolescence respectively, whilst 78% were continually expressed (Fig. 2A). Differentially expressed miRNAs, again represented a suite of TE subfamilies and evolutionary ages (Supplemental Table S1). There was no difference in the ages of differentially expressed or continual TE-embedded miRNAs (Supplemental Fig. S6A).

To determine the relevance of TE-embedded miRNAs versus non-TE-embedded miRNAs, we compared the fold changes of each. Indeed, non-TE-embedded miRNAs were differentially expressed to a comparable extent and in similar proportions as the TE-embedded miRNAs (Supplemental Fig. S6B-D). Of note, the TE-embedded miRNA, hsa-mir-548ba_MADE1, was the most upregulated miRNA in childhood compared to adolescence, indicating the relevance of TE-embedded miRNAs in the temporal neurodevelopmental context (Supplemental Fig. S6B & C). This indicates that temporal expression is not restricted to TE-embedded miRNAs but is a broad feature of this class of post-transcriptional regulators (Ziats and Rennert 2014).
Figure 2. TE-embedded miRNAs are temporally expressed between child and adolescent human brains. (A) Volcano plot highlighting TE-embedded miRNAs significantly differentially expressed in FB (adjusted P-value ≤ 0.05, 1.5-fold change). (B) Dot plots showing the correlation of expression and age for specific TE-embedded miRNAs. Shaded area represents the variance. (C) (Top) Integrated genome
viewer (IGV) visualization of four childhood (Child; blue) and four adolescent (Ado; orange) BAM files from DFC small RNA-seq data. (Middle) IGV visualization of three AGO2 RIP-seq (RIP; green) and three input (In; grey) BAM files from adult brain from Petri et al. 2019 (GSE106810). Read count is shown within square brackets. (Bottom) miRBase annotation, TE annotation and gene annotations for hg19. (D) miRNA hairpin schematics from miRNAfold (Tempel and Tahi 2012; Tav et al. 2016) for the DNA sequences in C. Each hairpin structure exhibits 90% of verified miRNA hairpin features as previously defined (Tempel and Tahi 2012; Tav et al. 2016). 22bp peak sequences are highlighted by the black bars on arms of the hairpin. (E) Heatmaps showing regional expression in log2 counts per million (CPM), alongside differential expression results (black and grey bar). For differential expression, a linear model was generated for every expressed miRNA, with miRNA expression as response variable and stage as explanatory variable. TMM normalized expression estimates were used as input for the modelling. Region abbreviations are defined in Supplemental Fig. S1.

In order to confirm our differential expression results, we next matched the expression of TE-embedded miRNAs in the FB with donor age. Of the 20 differentially expressed TE-embedded miRNAs, 12 also exhibited significant correlations or anti-correlations with this parameter (Supplemental Fig. S7; Supplemental Table S4).

One of the most significantly differentially expressed TE-embedded miRNAs in the FB was the cancer- and cell proliferation-associated hsa-mir-378a, which displayed higher expression in childhood and a significant anti-correlation with donor age in the small RNA-seq data (Li et al. 2015; Velazquez-Torres et al. 2018; Guo et al. 2019)(Fig. 2A, B left & C left top). To confirm our detection of this TE-embedded miRNA, we reanalysed a publicly available Argonaute2 RNA-immunoprecipitation sequencing (AGO2 RIP-seq) data set from three adult human brains (Petri et al. 2019; GSE106810). AGO2 directly binds to mature processed miRNAs for incorporation into the RISC complex for targeting of mRNA (Kobayashi and Tomari 2016; Michlewski and Cáceres 2019), therefore AGO2-bound elements are likely to represent bona fide miRNAs rather than mere degradation products. Enrichment of reads in one out of three AGO2 RIP-seq samples was observed compared to the input sample, with a peak residing over the same sequence as the small RNA-seq data (Fig. 2C left middle). This hsa-mir-378a miRNA is embedded in two intronic, MIRc elements arranged in opposite orientations, facilitating high confidence pre-miRNA hairpin precursor formation as determined by in silico miRNA folding analyses to detect hairpins with 90% of verified miRNA hairpin features (Tempel and Tahi 2012; Tav et al. 2016) (Fig. 2C bottom left & D left). The glycolysis-, cancer- and cell proliferation-associated hsa-mir-5683
was also significantly more expressed in childhood, with a significant anti-correlation with donor age and was detectable in at least one AGO2 RIP-seq sample, however was embedded in a solo 105 MYO MERT5A1 element which also facilitated pre-miRNA hairpin formation (Miao et al. 2020; Rong et al. 2020) (Fig. 2A, B right, C middle & D middle). One TE-embedded miRNA which was continually expressed in childhood and adolescent brains from small RNA-seq data and detectable in all samples of the AGO2 RIP-seq data was the cancer- and neuron- associated hsa-mir-582 (Fig. 2C right) (Fang et al. 2015; Zhang et al. 2015; Ding et al. 2019). This miRNA is embedded in two apposed L3 elements, again leading to an in silico predicted high-confidence precursor hairpin structure (Fig. 2D right). Indeed, 18/92 miRNAs overlapped at least two TEs, with varying genomic orientations (Supplemental Table S5), although the majority of expressed TE-embedded miRNAs overlapped only one TE.

**Different regions exhibit diverse miRNA temporal expression patterns**

The temporal TE and gene expression profile of the human brain varies by region, notably with the cerebellum (CB) displaying a different transposcriptional and transcriptional landscape when compared to FB (Playfoot et al. 2021). We therefore next determined the temporal expression profile of miRNAs in childhood and adolescence in different individual brain regions (Fig. 2E, Supplemental Table S1, see methods). hsa-mir-378a MIRc exhibited significantly higher expression in childhood, not only in combined FB samples, but also in individual FB regions such as the dorsolateral prefrontal cortex (DFC), the inferior temporal cortex (ITC), the medial prefrontal cortex (MFC) and the superior temporal cortex (STC), along with non-FB regions such as the mediodorsal nucleus of the thalamus (THA) (Fig. 2E left). Similarly, hsa-mir-5683 MERT5A1 had significantly higher expression in childhood versus adolescence in the FB combined, along with other individual FB regions, but also in the CB (Fig. 2E middle). In both instances, expression in the CB was higher than for any other individual region. In contrast, the L3-embedded hsa-mir-582 exhibited continual high expression across childhood and adolescence for all regions, except the CB where hsa-mir-582 L3 expression was largely absent in adolescence and restricted to childhood (Fig. 2E right). This provides a striking example of
spatiotemporal control of the miRNA transcriptional landscape. Overall, these data demonstrate that
the TE-embedded miRNA transcriptional landscape exhibits diverse spatiotemporal dynamics, with
sometimes overt differences between childhood and adolescence for FB and non-FB regions.

**TE-embedded miRNAs are spatially expressed**

Due to the temporal nature of miRNA expression in multiple brain regions, we next aimed to determine
spatial differences in TE-embedded miRNA expression, regardless of age. We performed 120
differential expression analyses, comparing each region to each other independent region. We fitted
a linear model for every expressed miRNA, with miRNA expression as response variable and region as
explanatory variable. Stage and patient were used as covariates to the statistical model. TMM
normalized expression estimates were used as input for the modelling. miRNAs with fold change larger
than 1.5 and \( P\text{-value} \leq 0.05 \) were considered as differentially expressed.

Of these comparisons, the region with the largest number of differentially expressed TE-embedded
miRNAs was consistently the CB (Fig. 3A). The CB was responsible for half of the top 30 comparisons
with the highest number of differentially expressed TE-embedded miRNAs (Fig. 3A). The CB versus the
hippocampus (HIP) had the highest number of differentially expressed TE-embedded miRNAs, followed
by the CB versus striatum (STR), amygdala (AMY) and many regions of the FB (Fig. 3A). These data
suggest that the CB exhibits, not only different TE and gene expression compared to other brain regions
as previously described (Playfoot et al. 2021; Li et al. 2018), but also differences in TE-embedded
miRNA expression.

To determine the relevance of TE-embedded miRNAs compared to non-TE-embedded miRNAs in the
spatial context, we ordered all differentially expressed miRNAs on the basis of their fold change in the
cerebellum versus hippocampus (Supplemental Fig. S8A & B). The differential expression of TE-
embedded miRNAs was spread between small and large fold changes, similar to non-TE-embedded
miRNAs. For example, mir-1298_X24_DNA was the second most highly upregulated miRNA in the
hippocampus when compared to cerebellum (Supplemental Fig. S8A). Together, these results indicate
that TE-embedded miRNAs exhibit similar expression differences to non-TE-embedded miRNAs in regional comparisons.
Figure 3. TE-embedded miRNAs exhibit spatial expression with major differences in the cerebellum.

(A) Bar chart showing the number of differentially expressed TE-embedded miRNAs per regional comparisons ($P$-value $\leq 0.05$, 1.5-fold change up or down). Only the top 30 comparisons are shown. For differential expression, a linear model was generated for every expressed miRNA, with miRNA expression as response variable and region as explanatory variable. Stage and patient were used as covariates to the statistical model. TMM normalized expression estimates were used as input for the modelling. miRNAs with fold change larger than 1.5 and $P$-value $\leq 0.05$ were considered as differentially expressed. (B) Heatmap comparing the fold change of region X (center diagonal) to region Y (left and top) for two TE-embedded miRNA loci described in Fig. 2. Only regions with significant fold changes are colored ($P$-value $\leq 0.05$, 1.5-fold change).
As hsa-mir-378a MIRc exhibited distinct temporal expression (Fig. 2) we next assessed its potential spatial expression. Indeed, the CB exhibited significantly higher expression of hsa-mir-378a MIRc when compared to most other regions (Fig. 3B). Conversely, hsa-mir-582 L3 exhibited significantly lower expression in the CB compared to all other regions, suggestive of diverse regulatory control of different miRNAs (Fig. 3B). A multitude of other examples of spatial miRNA expression suggests widespread spatial regulation of not only TE-embedded miRNAs, but also non-TE-embedded miRNAs. These dynamics can be interactively explored for all miRNAs with our Brain miRTExplorer application.

**TE-embedded miRNAs target neurogenesis-associated genes**

In order to determine possible functional relevance, we extracted predicted genic targets of TE-embedded miRNAs from the TargetScan database (Supplemental Table S6) (Agarwal et al. 2015; McGeary et al. 2019). We specifically focused on conserved predicted target sites (defined by conserved branch lengths such as an 8mer or 7mer) of conserved miRNA families (defined by multiple-sequence miRNA alignments), as annotated in TargetScan (Agarwal et al. 2015; McGeary et al. 2019).

Using this stringent list, we used two different gene ontology (GO) enrichment analysis tools (clusterProfiler, Yu et al. 2012; and PantherDB, Mi et al. 2021). Biological process analyses indicated that many target genes of TE-embedded miRNAs are enriched in neurogenesis-associated functions, alongside other enriched pathways such as regulation of transcription and metabolic processes, indicating the diversity of miRNA targets (Fig. 4A, C & E). For example, the L2c-embedded hsa-mir-374b targets all four genes involved in striatal medium spiny neuron differentiation (GO:0021773) and three out of four genes associated with glial cell fate specification (GO:0021780) and oligodendrocyte cell fate specification (GO:0021778) (Supplemental Table S7). Similarly, the L2b-embedded hsa-mir-493 was enriched in positive regulation of synaptic vesicle exocytosis (GO:2000302) and neurotransmitter receptor transport to plasma membrane (GO:0098877), among others such as actin polymerization-dependent cell motility (GO:0070358) (Supplemental Table S7).
These two analyses also revealed significant enrichments in GO cell component terms for hsa-mir-374b such as synapse (GO:0045202), golgi apparatus (GO:0005794) and transcription regulator complex (GO:0005667) (Fig. 4B; Supplemental Table S7). hsa-mir-493 also exhibited diverse cellular component GO enrichment terms such as NMDA selective glutamate receptor complex (GO:0017146), glial cell projection (GO:0097386) and integral component of postsynaptic specialization membrane (GO:0099060), among other neurogenesis and non-neurogenesis terms such as chromatin silencing complex (GO:0005677) (Fig. 4D; Supplemental Table S7). The aforementioned continually expressed, L3-embedded hsa-mir-582, was also broadly enriched in neurogenesis and non-neurogenesis-associated biological process and cellular component terms such as astrocyte end-foot (GO:0097450), main axon (GO:0044304) and glycoprotein complex (GO:0090665) (Fig. 4E; Supplemental Table S7).

Similar results were obtained for non-TE-embedded miRNAs such as the highly expressed hsa-mir-191, enriched in neurogenesis processes such as synapse assembly (GO:0007416) and non-neurogenesis such as pancreas development (GO:0031016) and cellular component terms such as spindle midzone (GO:0051233) and chromatin (GO:0000785) (Supplemental Fig. S9A & B). The non-TE-embedded miRNA with the largest fold change between childhood and adolescence, hsa-mir-211, also exhibited similar GO enrichments for neurogenesis-associated biological processes and cellular components as TE-embedded miRNAs (Supplemental Fig. S9C & D). Together, the GO enrichments detected for different TE-embedded and non-TE-embedded miRNA predicted targets highlight the specialized miRNA target networks in human neurogenesis and other diverse processes.
Figure 4. TE-embedded miRNA predicted targets are enriched in neurogenesis and other diverse gene ontology terms. ClusterProfiler emap network plots showing the top ten enriched biological process (A, C & E) and cellular component (B, D & F) GO terms for specific TE-embedded miRNAs. The
number of genes associated with each term is shown by point size and the adjusted *P-value* as the
indicated color. Edges connect overlapping gene sets which cluster together, indicating the relatedness
of terms. See also Supplemental Table S7 for GO enrichment from Panther DB.

**TEs contribute to novel putative miRNAs**

Most studies rely on mapping small RNA-seq reads directly to miRNA annotations provided in miRBase.

As a large proportion of annotated miRNAs are embedded in TEs, we reasoned that other TE loci could
be contributing to previously undetected, novel miRNAs expressed in the brain. We therefore further
investigated our unbiased, unique mapping to the whole genome used for detection of annotated TE-
embedded miRNAs. To ensure robustness and to limit false positives, we used our custom
RepeatMasker annotation (Turelli et al. 2020; Playfoot et al. 2021), alongside manual curation by
inspecting BAM files from childhood and adolescent samples of the DFC to detect a characteristic
~22bp peak. These candidates were further refined by intersecting with genomic coordinates of TEs
with at least one read from the AGO2 RIP-seq data. As TEs are inherently repetitive and have many
thousands of copies, we next aimed to ensure that the sequences residing below putative miRNA peaks
were indeed novel by searching for the sequences in miRBase. This resulted in a stringent list of eight
novel non-annotated TE-embedded miRNA candidates (Supplemental Table S8). We next focused on
two of these which met our strict criteria. The first was embedded in two apposed head-to-head,
intronic MER3 elements and was confirmed with peaks detectable in the AGO2 RIP-seq data,
suggestive of processed miRNA (Fig. 5A & B left). Indeed, the 200bp sequence covering the miRNA
locus facilitated *in silico* hairpin structure formation with 90% of verified features and the 22bp 3p and
5p peak sequences contributing to each arm of the hairpin (Fig. 5C left). The same was observed for a
novel putative miRNA embedded in a single MER5A element, however AGO2 RIP-seq peaks overlapped
the probable miRNA star sequence (the peak with fewer reads in the Brainspan samples) (Fig. 5A, B &
C right). AGO2 alternative strand loading can lead to shifts in the target profile of the miRNA-Induced
Silencing Complex and may account for this (Medley et al. 2020).
To determine the evolutionary history of these two loci, we assessed the 22bp sequence using MULTIZ alignments (Blanchette et al. 2004). Indeed, the MER3-embedded miRNA is present in rhesus macaque but absent from mouse, whereas the MER5A element is present in rhesus macaque but with a deletion in the seed region in mouse (Fig. 5D). To determine their novelty, the 22bp sequence of these candidates were searched in miRBase and did not match any sequences. These two TE loci therefore represent robust, novel TE-embedded miRNAs, the function of which remains to be elucidated. Together, these data highlight the dynamic spatiotemporal nature of annotated and novel TE-embedded miRNAs in the developing human brain and provides scope to investigate the disease and functional relevance of TE sequence co-option as miRNAs throughout evolution.
Figure 5. Novel, non-annotated TE-embedded miRNAs are present in child and adolescent brains. (A) IGV visualization of non-annotated TE-embedded miRNAs with classical 22bp peaks in four childhood (blue) DFC BAM files and four adolescent (orange) DFC BAM files, alongside TE and gene annotations for hg19. (B) IGV visualization of three AGO2 RIP-seq (RIP; green) and three input (In; grey) BAM files from adult brain (Petri et al. 2019; GSE106810) for the corresponding region in A. (C) miRNA hairpin schematics from miRNAfold (Tav et al. 2016) for the DNA sequences in A. 22bp peaks are highlighted by the black bars on both arms of the hairpin. (D) MULTIZ alignment from the UCSC genome browser of the 22bp miRNA sequence beneath the largest peak in A (Blanchette et al. 2004; Navarro Gonzalez et al. 2021). The putative seed region and orientation are indicated.
Discussion

Human brain development is a dynamic and highly regulated spatiotemporal process, however the contribution of TEs to the miRNA mechanism of regulatory control has never been formally investigated in this context. We show that the postnatal TE-embedded miRNA landscape is indeed spatially and temporally dynamic, with alterations in TE-embedded miRNA expression from childhood to adolescence, similar to non-TE-embedded miRNAs. Our previous work highlighted a distinct TE expression switch during late prenatal and early postnatal developmental timepoints, accompanied by coordinated reduction in expression of their controlling transcription factors, the KRAB-zinc finger proteins (KZFPs) (Playfoot et al. 2021). Furthermore, we determined spatiotemporal TE-mediated alternative promoter usage leading to novel mRNA transcript isoforms, indicative of direct TE-dependent transcriptional innovation (Playfoot et al. 2021). Here, we expand the role of TEs in human brain development to that of miRNAs; a more indirect, but no less important method of transcriptional innovation.

One critical limitation of our study is the restriction to postnatal timepoints. As major gene and TE expression changes occur during prenatal to postnatal transitional stages, future work should aim to generate small RNA-seq data covering the whole timeframe of human brain development. miRNAs were previously demonstrated to play critical roles in mouse prenatal brain development (Petri et al. 2014), and we found here that many human TE-embedded miRNAs were more highly expressed in childhood when compared to adolescence. As many neurological disorders appear to have origins in early development (Short and Baram 2019), it would be imperative to investigate both TE-embedded and non-TE-embedded miRNA expression at prenatal stages. To date, the limited number of human studies aiming to address this point were restricted by sample number, developmental stages and regions (Nowakowski et al. 2018). Future work should also focus on the spatiotemporal control of TE-embedded miRNA expression in other human developmental tissues, however current availability of relevant data sets preclude this analysis.
Another limitation of our study is that we could not assess the effect of miRNA expression on their mRNA targets. First, the effect size of miRNAs on target mRNAs is usually very small and the frequent redundancy of miRNAs acting on the same mRNA target can mask the influence of a single effector (Friedman et al. 2009; McGeary et al. 2019). In addition, brain samples comprising multiple different cell types were analyzed here by bulk small RNA- or mRNA-sequencing, precluding a straightforward interpretation of the relationship between miRNA and target mRNA expression. Advances in human embryonic stem cell differentiation protocols have enabled in vitro study of different neurological cell types and cerebral organoids, hence will facilitate this type of exploration, although these approaches still fail to recapitulate the wide cellular diversity or maturity found in tissue samples.

The detection of novel, non-annotated TE-embedded miRNAs is suggestive of a previously undetected TE-originating miRNA landscape. The volume of data assessed may have allowed the detection of these, however computational limitations of using only uniquely mapping reads is especially acute for young, more homogenous TE subfamilies which have accumulated less mutations. This can be further compounded by the absence of unique molecular identifiers (UMI) in sequencing reads, leading to misinterpretation of PCR duplications. Future work should experimentally assess putative, young repetitive TE-embedded miRNAs, as they have the potential to expand significantly the RNA-based regulome. Their repetitive nature likely facilitates post-transcriptional control of mRNA targets containing the same TE subfamilies in their 3’ UTRs, as has been shown for the annotated L2-embedded miRNAs (Petri et al. 2019). These results also suggest a multifactorial role for TEs, whereby some TEs give rise to mature miRNAs but are also targets of the miRNA microprocessor machinery themselves, thus acting to restrict their movement when the TEs remain retrotransposition competent (Heras et al. 2013, 2014). Indeed, brain-specific data sets determining direct targets of the microprocessor would be useful to determine if these TE-embedded miRNAs are bona fide targets of the miRNA processing machinery. Furthermore, it may be possible that the expression of TEs themselves may contribute to the expression of miRNAs, again creating a regulatory feedback loop.
In summary, the spatiotemporal expression of TE-embedded miRNAs from childhood to adolescence suggests a role for TEs in the fine tuning of transcriptional networks at the post-transcriptional level throughout human brain development. Although these dynamics are not restricted to TE-embedded miRNAs, these analyses provide a novel insight into a crucial understudied developmental window, as the role of TE-embedded miRNAs has only been previously investigated in adult or disease contexts.

**Materials and Methods**

**Dataset download and preprocessing**

Raw small RNA-seq FASTQ files from the BrainSpan Atlas of the Developing Human Brain (phs000755.v2.p1 provided by Dr. Nenad Sestan), were downloaded from the dbGaP-authorized access platform (Miller et al. 2014; Li et al. 2018) (Supplemental Acknowledgements). The reads were first trimmed to remove Illumina small RNA 3’ sequencing adapters (TGGAATTCTCGGGTGCCAAGG) using FLEXBAR (version 3.5.0) with parameters --adapter-trim-end RIGHT --min-read-length 18 (Dodt et al. 2012). Trimmed reads were then divided by read length ranges of 18-25 nucleotides, 26-37 nucleotides and 38-50 nucleotides. Reads were then mapped to the human hg19 genome (GRCh37.p5) using Bowtie (version 2.3.4.1) with parameter --very-sensitive-local (Langmead et al. 2009). Read counts on different genomic features were quantified using featureCounts (version 1.6.2 of the subread package) (Liao et al. 2014). Uniquely mapped reads were quantified with parameters -t exon -g gene_id -Q 10 and multimapped reads with parameters -M -fraction -t exon -g gene_id -Q 0. We used the parameters -s 1 and -s 2, to quantify sense and antisense reads respectively which were subsequently merged, keeping only the strand with the most reads. To confirm specific read lengths were enriching for specific RNA moieties, the annotation of snoRNA, snRNA, miscRNA, scRNA and genes from Ensembl (GRCh37.p5, release 100) were used. For miRNAs and tRNAs, miRBase version 20 (Kozomara and Griffiths-Jones 2014) and tRNA annotations from GtRNAdb (release 19) were used respectively (Chan and Lowe 2016). For repetitive sequences, a previously described in-house curated version of the RepeatMasker database was used (where fragmented LTR and internal segments
belonging to a single integrant were merged) (Turelli et al. 2020; Playfoot et al. 2021). Exons of genes and TEs overlapping small RNAs in the same orientation were removed using BEDTools intersect (version 2.27.1) with default parameters, to prioritize reads falling on small RNAs (Quinlan and Hall 2010). To determine which expressed annotated miRNAs overlapped TEs, we used BEDTools to intersect the miRBase and our custom RepeatMasker merged TE annotations with a minimum of one base pair overlap. TE subfamily age estimates were obtained from DFAM (Hubley et al. 2016). BAM files were visualized using the Integrative Genome Viewer (Robinson et al. 2011).

**Filtering and normalization**

Samples were sequenced with a read length of 51bp and samples with less than 1 million reads mapped were removed. Features where the sum of the counts over all the samples was lower than the total number of samples were removed. TEs overlapping gene exons were also removed using BEDTools closest (Quinlan and Hall 2010). Normalization for the sequencing depth was performed for all features on the sense and antisense with the trimmed mean of the log expression ratios (TMM) method as implemented in the R package limma (version 3.46.0) (Ritchie et al. 2015). The TMM method excludes the top and bottom expressed miRNAs prior to computing library sizes to ensure very high or low expressed miRNAs did not dominate the library size normalization (Robinson and Oshlack 2010). Counts per million (CPM) values were generated after correcting by the TMM library size. The subsequent total number of mapped reads was used as library size.

**Differential expression analysis**

Samples from one year to five years were considered as childhood and nine years to 20 years as adolescence (Supplemental Fig. S1). To perform the aggregated temporal FB differential expression, the following brain regions were considered as FB: dorsolateral prefrontal cortex, inferior temporal cortex, medial prefrontal cortex, orbital prefrontal cortex, posterior inferior parietal cortex, primary auditory (A1) cortex, primary somatosensory (S1) cortex, primary visual (V1) cortex, superior temporal cortex, ventrolateral prefrontal cortex and primary motor (M1) cortex (Supplemental Fig. S1).
Independent temporal comparisons were performed without aggregations of multiple regions. For
differential expression between regions, all samples regardless of age were used.

Differential gene expression analysis was performed using voom (Law et al. 2014) as it has been
implemented in the R package limma (version 3.46.0), with TMM normalized counts as input and using
staging and patient information as covariates when fitting the linear models. \( P\)-values were corrected
for multiple testing using the Benjamini-Hochberg’s method (Benjamini and Hochberg 1995). A feature
was considered to be differentially expressed when the fold change between the groups compared
was higher than 1.5 and the adjusted or non-adjusted \( P\)-value \( \leq 0.05 \) as stated in figure legends.

**Correlation analysis**

Correlation between age and miRNA expression was assessed using spearman correlation and \( P\)-values
were adjusted using the Bonferroni correction.

**Expression of TE-embedded miRNAs in other tissues**

Processed CPM expression data of mature miRNAs in 399 human samples (De Rie et al. 2017; file:
human.srna.cpm.txt) was downloaded and log2 CPMs of all annotated mature miRNAs non-overlapping
and overlapping-TE annotations were plotted with addition of a pseudo-count of 1.

**miRNA precursor secondary structure analyses**

To predict in silico miRNA precursor hairpin structures, the DNA sequence of a 200 to 300 bp window
around consistent 22bp peaks observed in BAM files was inputted to miRNAfold (Tempel and Tahi
2012; Tav et al. 2016). A stringent threshold of 90% of verified features was used, to ensure only robust
hairpins with a very low false positive rate were returned (Tempel and Tahi 2012; Tav et al. 2016).

**AGO2 RIP-seq data**

Three publicly available neurotypical adult brain AGO2 RIP-seq data sets (Petri et al. 2019; GSE106810)
were processed using the same pipeline as for the small RNA-seq analysis.
Evolutionary conservation

MULTIZ tracks from the UCSC genome browser were used to determine the presence of non-annotated TE-embedded miRNA sequences in different species (Blanchette et al. 2004; Navarro Gonzalez et al. 2021). TE-embedded miRNA coordinates were lifted over from the human genome (hg19) to that of the macaque (rheMac8) and the mouse genome (mm10) using the UCSC lift over tool (Hinrichs et al. 2006). BEDTools intersect (version 2.27.1) (Quinlan and Hall 2010) was used with default parameters to intersect lift-over coordinates with miRNA coordinates from miRBase for macaque (rheMac8) and mouse (mm10) (Kozomara and Griffiths-Jones 2014).

miRNA target prediction

miRNA target predictions were downloaded from TargetScan Human (Release 8.0) (Agarwal et al. 2015; McGeary et al. 2019; File: Predicted_Targets_Context_Scores.default_predictions.txt). We utilized only the conserved target predictions for conserved miRNAs as defined in TargetScan (Agarwal et al. 2015; McGeary et al. 2019). GO analysis and visualization was performed using clusterProfiler (Yu et al., 2012) with default options and PantherDB (Release: 20210224) (Mi et al., 2021), with GO ontology database (Release: 2021-08-18) and enrichment was assessed using Fisher’s exact test followed by false discovery rate adjustment using all human genes as background (Mi et al. 2013).

Brain miRTExplorer application

The Brain miRTExplorer application was implemented in R using the Shiny app package (Chang et al., 2017). A description and example of usage is provided as Supplemental Fig. S10.

Availability of data and materials

No new data was generated during the course of this study. Processed data can be interactively visualized using our “Brain miRTExplorer” application at https://tronoapps.epfl.ch/BrainmiRTExplorer/.

Competing interests
The authors declare that they have no competing interests

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**Author contributions**

C.P. conceived the study, performed bioinformatic analyses, interpreted the data and wrote the manuscript. S.S. and E.P. performed bioinformatics analyses. C.P. and D.T. edited the manuscript. All authors reviewed the manuscript.

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Transposable elements contribute to the spatiotemporal microRNA landscape in human brain development

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