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# Comprehensive analysis of microRNA expression provides mechanistic insights into transcriptomic alteration in primary and metastatic testicular germ cell tumors

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MicroRNA (miRNA) dysregulation is implicated in testicular germ cell tumor (TGCT) pathogenesis. Here, we characterized miRNA expression profiles across TGCT histologic subtypes using miRNA-sequencing on 43 formalin-fixed paraffin-embedded (FFPE) tissue samples (31 primary, 12 metastases) from 29 patients to identify diagnostic markers and their regulatory functions. From 20 seminomas (SEM), 14 non-seminomatous germ cell tumors (N-SEM), and 9 teratomas, we profiled a total of 2606 miRNAs. Compared to teratomas, 154 miRNAs (targeting 657 genes) were enriched in SEM, and 141 miRNAs (targeting 358 genes) in N-SEM. miR-200-3p, targeting the DNA methyltransferase DNMT3B, was enriched in N-SEM versus SEM. Our findings showed high concordance with The Cancer Genome Atlas (TCGA)-TGCT data (Pearson R > 0.66,  $p < 1e-10$ ). miRNA expression was largely similar between primary and metastatic tissues and between chemotherapy-treated and untreated teratomas, reflecting teratoma chemo-resistance. Using novel candidates, miRNA-based logistic regression classifiers distinguished viable GCT (SEM/N-SEM) from teratoma (Area Under the Curve [AUC] > 0.96) and SEM from N-SEM (AUC = 0.81), outperforming well-known miRNA markers. Target gene analysis implicated FOXO and RUNX1 regulation, somatotroph signaling, and height-related pathways. Overall, our comprehensive tissue-level miRNA profiling in TGCTs identified potential diagnostic biomarkers for histologic subtypes, offering insights into miRNA-mediated transcriptional dysregulation.

Testicular germ cell tumors (TGCTs) are the most common malignancy in young men, accounting for 95% of all testicular cancers, with increasing incidence globally<sup>1,2</sup>. These tumors are highly heterogeneous, encompassing distinct histologic subtypes including pure seminomas (SEM) and non-seminomas. Despite high cure rates with current treatment protocols, challenges remain in diagnosing and managing certain subtypes, particularly teratomas and other chemo-resistant tumors. Advances in understanding the molecular underpinnings of TGCT pathogenesis could improve diagnostic precision and therapeutic decision-making, especially for cases with ambiguous histology or atypical clinical presentations.

MicroRNAs (miRNAs) are small non-coding RNAs that fine-tune gene expression by binding to and suppressing target messenger RNA (mRNA) transcripts<sup>3</sup>. They have emerged as key regulators of cellular processes such as proliferation, apoptosis, differentiation, and stress responses<sup>4</sup>. Dysregulation of miRNA networks is a hallmark of many cancers, including TGCTs, where they contribute to tumorigenesis, metastasis,

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and treatment resistance. miRNAs influence oncogenic and tumor suppressive pathways, often functioning as molecular switches that alter cellular behavior. In cancers such as breast, lung, colorectal, and prostate cancer, miRNA profiling has provided critical insights into tumor biology, identifying promising biomarkers for early detection, prognosis, and treatment stratification.

In the context of TGCTs, the miR-371–373 cluster has gained prominence as a highly specific and sensitive biomarker for detecting non-teratomatous TGCTs<sup>5</sup>. Serum miR-371a-3p levels, in particular, have shown exceptional diagnostic accuracy, correlating with tumor burden and treatment response<sup>6</sup>. This marker is emerging as a promising, non-invasive tool for diagnosing and monitoring TGCTs. However, most existing studies have focused on circulating miRNAs, and there is limited knowledge of tissue-level miRNA expression patterns across different TGCT subtypes. This is a critical gap, as tumor tissues harbor the primary molecular alterations that drive tumorigenesis, and their miRNA profiles may reflect subtype-specific regulatory networks.

Moreover, while teratomas are known to be resistant to chemotherapy, the molecular mechanisms underlying this resistance remain poorly understood. Distinguishing teratoma from fibrosis radiographically following chemotherapy remains an additional challenge, and circulating miRNAs have exhibited poor performance in identifying teratoma<sup>7,8</sup>. Tissue-level miRNA expression in teratoma compared to other TGCT subtypes could reveal miRNAs associated with chemoresistance and tumor viability. Beyond miR-371a-3p, few miRNAs have been validated as reliable markers for distinguishing among TGCT histologies, and their roles in key processes such as metastasis and differentiation remain to be elucidated.

Studies of miRNA expression in other cancers have demonstrated their potential to uncover novel regulatory networks and therapeutic targets. For example, in lung cancer, the let-7 family is known to suppress oncogenic RAS signaling<sup>9</sup>, while in prostate cancer, miR-21 promotes tumor progression by targeting tumor suppressor genes such as PTEN and PDCD4<sup>10–13</sup>. These studies underscore the power of miRNA profiling to identify critical pathways and actionable targets, insights that can be leveraged to improve TGCT management.

In this study, we sought to comprehensively analyze tissue-level miRNA expression across different TGCT histologic subtypes in primary and matched metastatic tumors. Leveraging a cohort that includes major TGCT subtypes (SEM, non-teratomatous non-seminomas (N-SEM), and teratoma), we performed differential expression analyses, identified candidate miRNA biomarkers, and inferred regulatory networks to uncover novel mechanistic insights into TGCT pathogenesis. By integrating these findings with known miRNA functions and pathway enrichment analyses, we aim to provide a detailed survey of miRNA dysregulation in TGCTs and identify novel biomarkers and pathways that could improve diagnostic precision and therapeutic targeting.

## Materials and methods

### Sample collection

All methods were carried out in accordance with relevant guidelines and regulations. Approval was obtained from the Johns Hopkins Medicine Institutional Review Board (IRB), and informed consent was obtained for participating subjects. We obtained 51 formalin-fixed paraffin-embedded (FFPE) tissue samples. Following QC and exclusion of non-TGCT samples, we leveraged 43 samples from primary testicular tumors (N=31) and matched retroperitoneal lymph node metastases (N=12) from 29 patients treated for TGCT between 2017 and 2021 at our institution. All patients were annotated with clinical and pathologic data, including treatment course and oncologic outcomes. We stratified samples by histologic subtype, tissue of origin, and receipt of chemotherapy prior to acquisition.

### Sample preparation and miRNA sequencing

miRNAs from each sample were isolated using the miRNeasy FFPE kit (Qiagen, Germantown, MD) according to manufacturer protocols. 500 ng of total RNA was used as input for the Illumina TruSeq Small RNA Sample Kit according to the manufacturer's instructions. This approach uses a naturally occurring miRNA specific structure with a 5'-phosphate and a 3'-hydroxyl to ligate adapter sequences exclusively to miRNA species. After adapter ligation, reverse transcription, PCR amplification and polyacrylamide gel electrophoresis were performed to generate a library. Library quality and quantity were assessed with the Agilent Bioanalyzer using the High Sensitivity Chip according to manufacturer's instructions. Sequencing was performed using the Illumina Novaseq X using 150 bp by 150 bp paired end chemistry at the Experimental and Computational Genomics Core (ECGC) within the Sidney-Kimmel Comprehensive Cancer Center at Johns Hopkins University. Depth of coverage was targeted for 50 million total reads per sample.

### miRNA-sequencing data analysis

We performed raw read QC using FastQC, followed by adapter trimming using Trim Galore (version 0.6.3). We filtered reads from likely contamination, and the adaptor was trimmed from the 3' end. Because miRNA length is defined as being between 17 and 22 bp, we discarded all the reads with a length < 15 bp. Flash version 1.2.11 was used to merge the read1 and read 2 and fastx\_toolkit version 0.0.14 was used to convert the merged fastq file to fasta format. We performed sequence alignment against miRBase mature miRNAs using blast version 2.2.30 and performed alignment against a snRNA, snoRNA, tRNA, MTncRNA database using blast version 2.2.30. For each sample, we calculated the number of reads that aligned to any of the microRNA. Only the hits with 100% identity and matching the whole length of microRNA were used for downstream analysis.

To summarize read count for each miRNA species, we applied featureCounts from the Subread package (release 2.0.6). We filtered miRNAs which have low reads across all samples, requiring each species to have at least 5 reads in at least 2 samples, resulting in a total of 2,606 miRNAs included in the downstream analysis.

### Statistical analysis of miRNA data

We performed a regularized log transformation of count data for variance stabilization (`rlog` function in `DESeq2`) prior to performing principal component analysis (PCA) to visualize sample distances and replication between samples from the same histologic group. For statistical analysis, we only focused on histologic groups with more than 3 samples, including pure seminoma (SEM), non-teratoma non-seminomatous elements (N-SEM), and teratoma. To identify marker miRNAs, we applied `DESeq2` with Bonferroni-adjusted  $p < 0.05$  and log fold-change  $> 2$  for each pair of histologic subtypes (SEM vs. teratoma, N-SEM vs. teratoma, and SEM vs. N-SEM) using all samples in each comparison. For subgroup analysis, we followed the same process but only focused on comparisons in which each group had at least 3 samples; therefore, we performed four additional differential analyses, including pairwise comparison between orchietomy samples and retroperitoneal lymph node dissections (RPLND) samples, and comparison between teratoma samples that were exposed to versus naïve to chemotherapy. Here, to test differential effect between the subgroups, we applied a linear model with an interaction term between tissue source and histology group to identify miRNA species with significant different effects stratified by subgroup. For each differential analysis, we then applied logistic regression and receiver-operating curve analyses to quantify the performance of marker miRNAs in distinguishing TGCT subtypes from each pairwise analysis.

To infer functional implications of identified marker miRNAs, we applied the `multiMiR` R package (v1.28.0) to infer gene targets for each miRNA. We used default parameters and retrieved all validated miRNA-target interactions with at least two independent supporting experiments. For each target gene retrieved, we further required that they were supported by at least two marker miRNAs to be included in the downstream enrichment analysis, and we only included target genes not shared by miRNA markers in more than one histology group. We used the `enrichR` R package (v3.2) to perform enrichment of target genes on GO terms (molecular function, cellular component, biological process, all v2023), WikiPathways (v2023), Reactome (v2022), IDG drug targets (v2022), and GWAS catalog (v2023).

### Validation using data from the cancer genome atlas (TCGA)

We obtained miRNA-seq data from  $N = 156$  TGCT samples from the TCGA (accessed October 20, 2024). We followed the process for performing differential expression by `DESeq2` for each pair of histology groups (SEM vs. teratoma, N-SEM vs. teratoma, and SEM vs. N-SEM). Due to sequencing differences, we identified a total of 128 miRNA species shared between our study and TCGA. We assessed the correlation of the log fold-change for each miRNA between TCGA and our institutional data in each pairwise comparison to evaluate concordance between our data and published datasets.

To further assess the degree of congruence between our inferred miRNA targets and the TCGA dataset, we obtained RNA-seq data from the same set of TCGA-TGCT samples and performed `DESeq2` analysis following the same approach to identify differential genes between each pair of histology groups. We classified each mRNA as a target for either histology group if it was a validated target of miRNA markers for the corresponding group, or as belonging to both or neither of the groups if it was a target of both or neither of the groups, respectively. We then compared the distribution of RNA-seq changes for each category of genes to assess whether target mRNAs demonstrated lower expression in the corresponding histologic group.

## Results

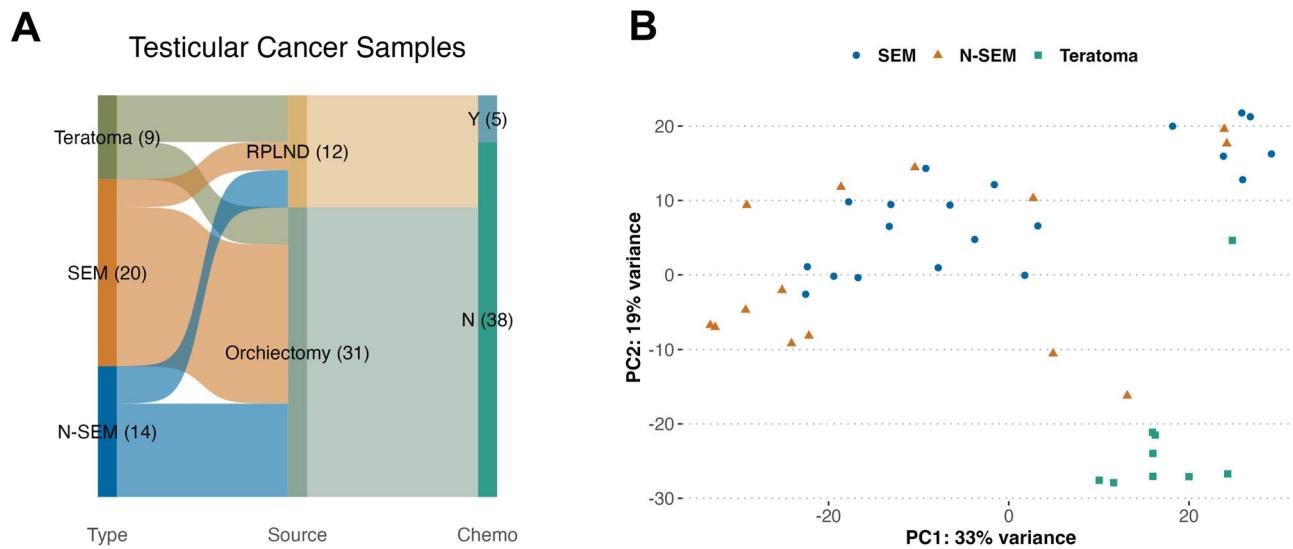
### miRNA-seq from testicular tissues distinguishes TGCT subtypes

We analyzed 43 formalin-fixed paraffin-embedded (FFPE) tissue samples from 32 patients treated for TGCT at our institution between 2017 and 2021, including 30 primary tumors from radical orchietomies, and 13 matched retroperitoneal lymph node metastases from primary or post-chemotherapy retroperitoneal lymph node dissections (RPLND). The histologic distribution included 20 SEM, 9 teratomas, and 14 N-SEM (9 embryonal carcinoma, 1 yolk sac tumor, and 4 mixed N-SEM elements excluding teratoma). Six of the teratoma samples were acquired following systemic treatment with cisplatin-based chemotherapy, while the remaining samples were all chemotherapy-naïve (Fig. 1A, Supplementary Table 1).

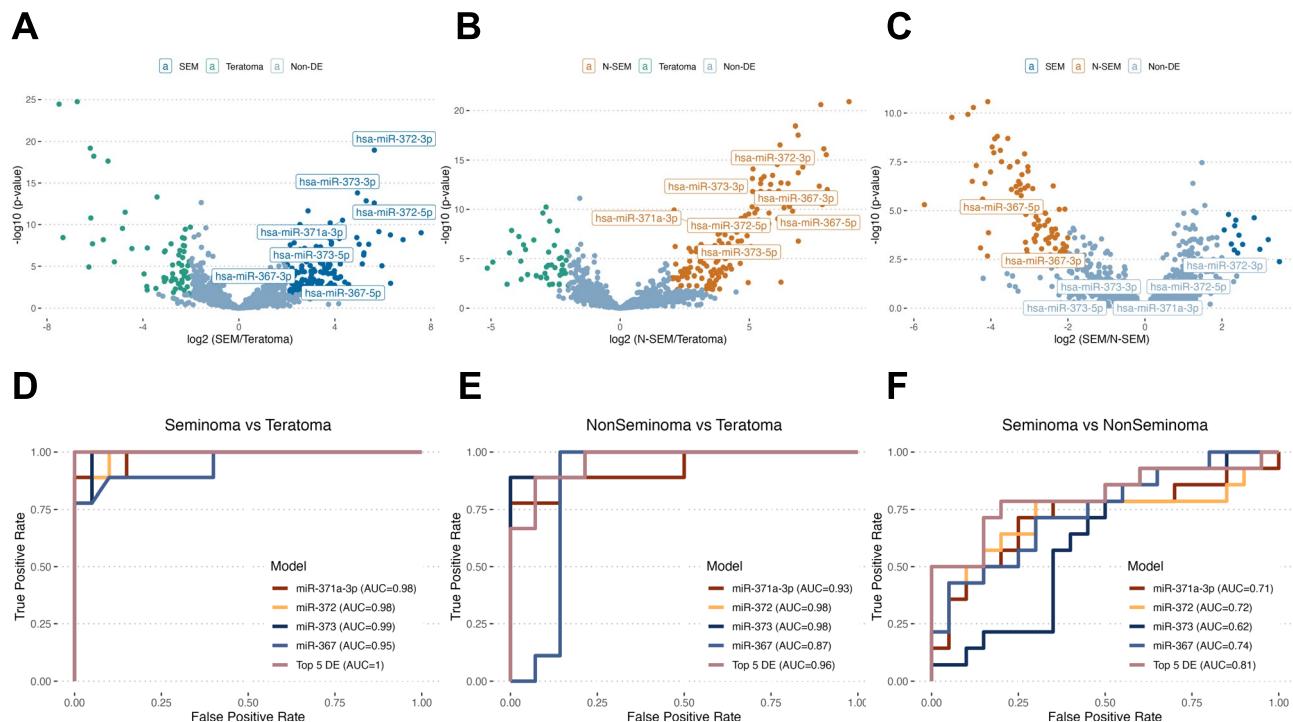
We performed miRNA-sequencing across all 43 samples. Although teratoma samples had lower total sequencing reads mapped per sample, there was no statistical difference in the number of reads mapped to miRNA species across the three cancer types (Kruskal-Wallis test,  $p = 0.059$ , Figure S1). After performing quality control and excluding miRNA species with low expression across all samples, we identified 2,606 miRNAs, including both the 3p and 5p arm of mature miRNAs. This significantly expands the number of miRNAs catalogued in The Cancer Genome Atlas (TCGA) for TGCT, which includes 983 mature miRNAs<sup>14</sup>. Principal component analysis revealed that samples belonging to the same histologic group, particularly teratoma, cluster more closely in the high-dimensional miRNA-seq space. However, there is considerable variability within the miRNA-seq profiles of SEM and N-SEM (Fig. 1B). Overall, these findings suggest that SEM and N-SEM showed higher similarity compared to teratoma.

### Differential expression analysis between pairs of histology groups reveals well-known and novel miRNA markers in TGCT

To identify marker miRNA species in each histologic group, we applied `DESeq2` on each pair of groups (SEM vs. teratoma, N-SEM vs. teratoma, SEM vs. N-SEM) for all 2606 miRNAs. We found that teratoma samples were distinct from SEM and N-SEM samples, with a large number of differentially expressed miRNAs. Notably, previously identified TGCT markers, such as the miR-371-373 cluster and miR-367-3p, were expressed at a significantly higher level in SEM and N-SEM compared to teratoma (Fig. 2A,B). Between SEM and N-SEM, we found a smaller number of differentially expressed miRNAs. Notably, the miR-371-373 cluster did not exhibit differential expression between SEM and N-SEM, while miR-367-3p and miR-367-5p were both more abundant



**Fig. 1.** miRNA-seq from testicular tissues distinguishes TGCT subtypes. (A) Sankey diagram showing the relationship among 43 miRNA-seq samples by their histologic group (SEM, N-SEM, teratoma), tissue source (primary tumor [orchietomy] or retroperitoneal lymph nodes [RPLND]), and whether systemic chemotherapy was administered prior to tissue acquisition (Y – received chemotherapy; N – did not receive chemotherapy). (B) Principal component analysis (PCA) plot showing the relationship among samples as quantified by  $n = 2606$  miRNA species. Colors indicate histologic group for each sample.

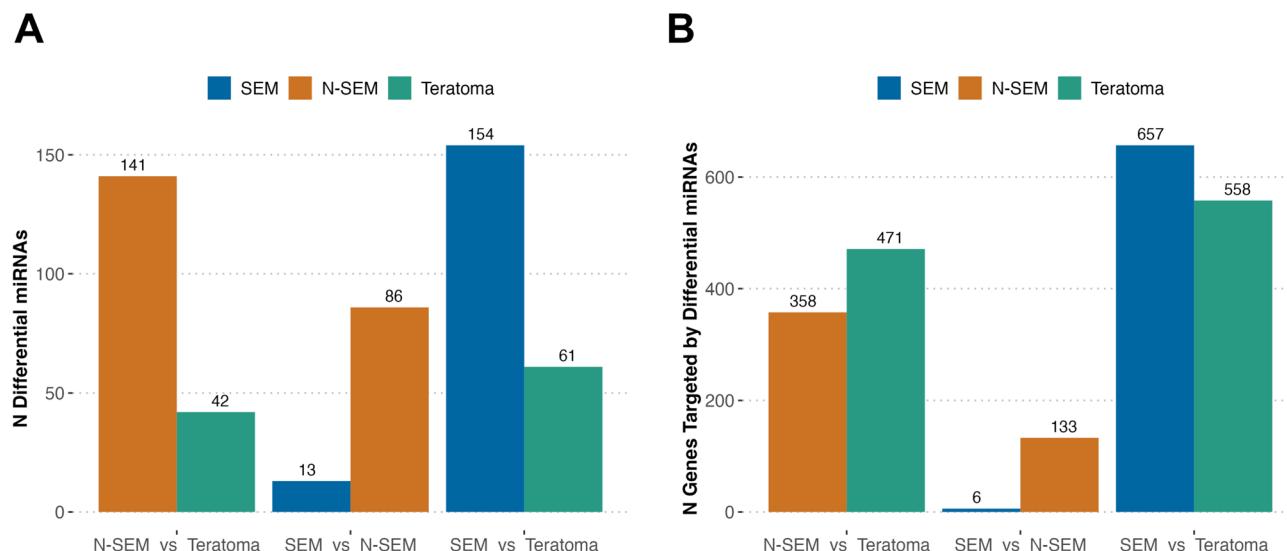


**Fig. 2.** Differential expression analysis between pairs of histology groups reveals well-known and novel miRNA markers in TGCT. (A–C) Volcano plot showing the  $\log_2$  fold-change (x-axis) between SEM and teratoma (A), N-SEM and teratoma (B), and SEM and N-SEM (C), and the  $-\log_{10} p$ -value of corresponding differential analysis. Differentially expressed miRNAs were identified by Bonferroni-adjusted  $p < 0.05$  with  $\log_2$  fold-change  $> 2$ . Previously described miRNAs implicated in TGCT (miR-371a-3p, miR-372, miR-373, miR-367) were labeled, as did miR-200b-3p in (C). Colors indicate miRNAs differentially expressed in each tumor type in each pairwise comparison. (D–F) Receiver operating curves for distinguishing histology group pairs ((D) SEM vs. teratoma, (E) N-SEM vs. teratoma, (F) SEM vs. N-SEM), using only the previously described markers and the top 5 most differentially expressed miRNAs identified in our study (see Supplementary Table 2 for details). AUC: area under the receiver-operating curve.

in N-SEM compared to SEM (Fig. 2C). Although our analysis included almost twice as many miRNAs compared to published studies, we sought to replicate our findings using the TCGA-TGCT miRNA-seq dataset ( $N=153$  samples, 29,898,407). In total, we found 128 shared miRNAs between studies. After performing DESeq2 on the same three pairwise comparisons, we found that the log fold-change values obtained using our cohort are highly correlated with those obtained using TCGA data (Pearson correlation  $R>0.66$ ,  $p<2.2e-16$  for all comparisons, Figure S2). These findings validate our dataset in recapitulating known miRNA markers in TGCT and suggest that we had sufficient power to detect novel miRNAs, as our effect size (log fold change) remained consistent with that observed in the larger TCGA dataset.

To directly demonstrate the utility of our candidate miRNA markers, we next developed linear classifiers based on the top miRNAs from each differential analysis to distinguish between histologic groups. Briefly, in each comparison, we selected the top 5 differentially expressed miRNAs by their absolute log fold change, none of which were miRNA markers previously described in TGCT. These candidates include miR-202-3p, miR-429, miR-934, miR-548x-3p, and miR-200b-3p for SEM vs teratoma, miR-520a-3p, miR-520f-5p, miR-518a-3p, miR-520c-3p, and miR-520e for N-SEM vs teratoma, and miR-448, miR-518a-3p, miR-520d-3p, miR-1298-5p, and miR-516b-5p for SEM vs N-SEM (Supplementary Table 2). We then performed a multinomial logistic regression analysis using histologic group as the dependent variable and the expression level of the five miRNAs as independent variables. We subsequently constructed the linear classifier using coefficients obtained from the logistic regression model and assessed its performance using receiver operating curve (ROC) analysis against true histology group labels. We found that the top 5 novel miRNA markers consistently achieve  $AUC>0.96$  in distinguishing teratoma from SEM and N-SEM (Fig. 2D-E), and  $AUC=0.81$  in distinguishing SEM from N-SEM (Fig. 2F), achieving comparable performance compared to previously proposed serum miRNA markers for TGCTs (including miR-371, miR-372, miR-373, and miR-367). These findings suggest that the miRNAs identified from our differential analyses could refine our understanding of miRNA expression and regulatory networks in distinct TGCT subtypes.

Overall, compared to teratoma, we identified 154 miRNAs that were more abundant in SEM and 141 miRNAs that were more highly expressed in N-SEM, while there were 61 and 42 miRNAs enriched in teratoma compared to SEM and N-SEM respectively. Comparing SEM with N-SEM, we identified 13 miRNAs increased in SEM and 86 miRNAs enriched in N-SEMs (Fig. 3A, Supplementary Table 2). We hypothesized that these miRNAs can explain gene expression changes observed in each TGCT histologic group; therefore, to better understand the functional consequences of these miRNAs, we queried the target genes for each miRNA using MultiMiR. We implemented a conservative filtering strategy to prioritize gene targets supported by at least two miRNAs in each analysis, identifying mRNA targets most likely to exhibit altered expression in each histologic group. In total, compared to teratoma, we found 657 and 358 genes targeted by miRNAs in SEM and N-SEM, while 558 and 471 genes were targeted by miRNAs in teratoma compared to SEM and N-SEM, respectively. Comparing SEM and N-SEM, we only found 6 (SEM) and 133 (N-SEM) genes targeted by their respective miRNAs (Fig. 3B, Supplementary Table 3), suggesting that these miRNAs may play only a modest role in modulating the transcriptomic differences between SEM and N-SEM. Among the relatively few miRNAs differentially expressed between SEM and N-SEM, miR-200-3p was more highly expressed in N-SEM. Intriguingly, miR-200-3p targets DNMT3B, a DNA methyltransferase critical for epigenetic regulation, which was previously reported to underlie epigenetic differences between N-SEM and SEM<sup>15</sup>.



**Fig. 3.** Pairwise DESeq2 analysis reveals miRNAs and gene targets differentially expressed in each histology type. (A) Barplot showing the number of differentially expressed miRNAs in three pairwise tests, where colors indicate miRNA count enriched in the corresponding histologic group. (B) Barplot showing the number of target genes inferred by MultiMiR based on the differentially expressed miRNAs shown in (A).

To evaluate whether our inferred mRNA targets demonstrate altered expression in TGCT, we curated RNA-seq data from the same set of TGCT samples in TCGA and performed differential gene expression analysis using DESeq2. We then compared RNA-seq log fold changes across gene groups predicted to be targeted by either histologic group, neither, or both. We found general congruence in the direction of effects, where genes targeted by miRNA markers tended to exhibit lower expression in RNA-seq data, although not always reaching statistical significance (Figure S3). This weak correlation between inferred miRNA targets and expected RNA expression changes may stem from the heterogenous cell composition of the TGCT tumor samples, since miRNAs can regulate different genes depending on cell and tissue type<sup>16</sup>. This may be further compounded by the potential compensatory effects through which gene targets may achieve normal levels of expression via other mechanisms. Our observations highlight the complexities in dissecting the functional consequence of miRNA species from complex tissues.

### Enrichment analysis of inferred target genes reveals diverse biological processes and related complex traits targeted by marker miRNAs in each subtype

Despite the modest correspondence between miRNA-seq and RNA-seq on the TGCT samples, we sought to investigate the genes and biological pathways directly targeted by miRNAs in each histologic group. To this end, we performed enrichment analysis using four well-curated databases (WikiPathways, Reactome, GWAS catalog, and GO terms) on the inferred miRNA target genes from each pair of histology groups (Fig. 4). We found that the FOXO-mediated transcription pathway was highly enriched in SEM and N-SEM, when compared with teratoma. This pathway regulates cell survival, growth, and adaptation to environmental changes<sup>17,18</sup>. We also found that the transcription factor RUNX1, which was known to be extensively targeted by miRNAs<sup>19–24</sup> and strongly implicated in hematopoiesis and hematological malignancies<sup>25,26</sup>, was more active in both SEM and N-SEM compared to teratoma. Further, we found that the core transcription machinery (RNA polymerase II) and transcription factor binding pathways were enriched in SEM and N-SEM, suggesting that miRNAs in SEM and N-SEM may exert their functions by specifically targeting the transcription factors in the core promoters, as opposed to miRNAs in teratoma, which were more likely to target post-translational modification pathways such as protein phosphatase binding (Fig. 4A,B). Additionally, we found the somatotroph signaling pathway and anthropometric traits such as height were significantly more active in SEM and N-SEM when compared to teratoma. These trends support previous studies on the relationship between height and TGCT risk from genome-wide association studies<sup>27</sup>. Finally, despite the small number of target genes inferred from miRNAs differentially expressed between SEM and N-SEM, we found the ATM-dependent DNA damage response pathway and IL-24 mediated signaling pathway were enriched in N-SEM (Fig. 4C). These results suggest that distinct immunomodulatory functions of miRNAs may underlie the chemoresistant nature of in N-SEM when compared to SEM<sup>28</sup>. Overall, our findings provide new mechanistic insights into the transcriptional networks targeted by miRNAs in each histologic subtype.

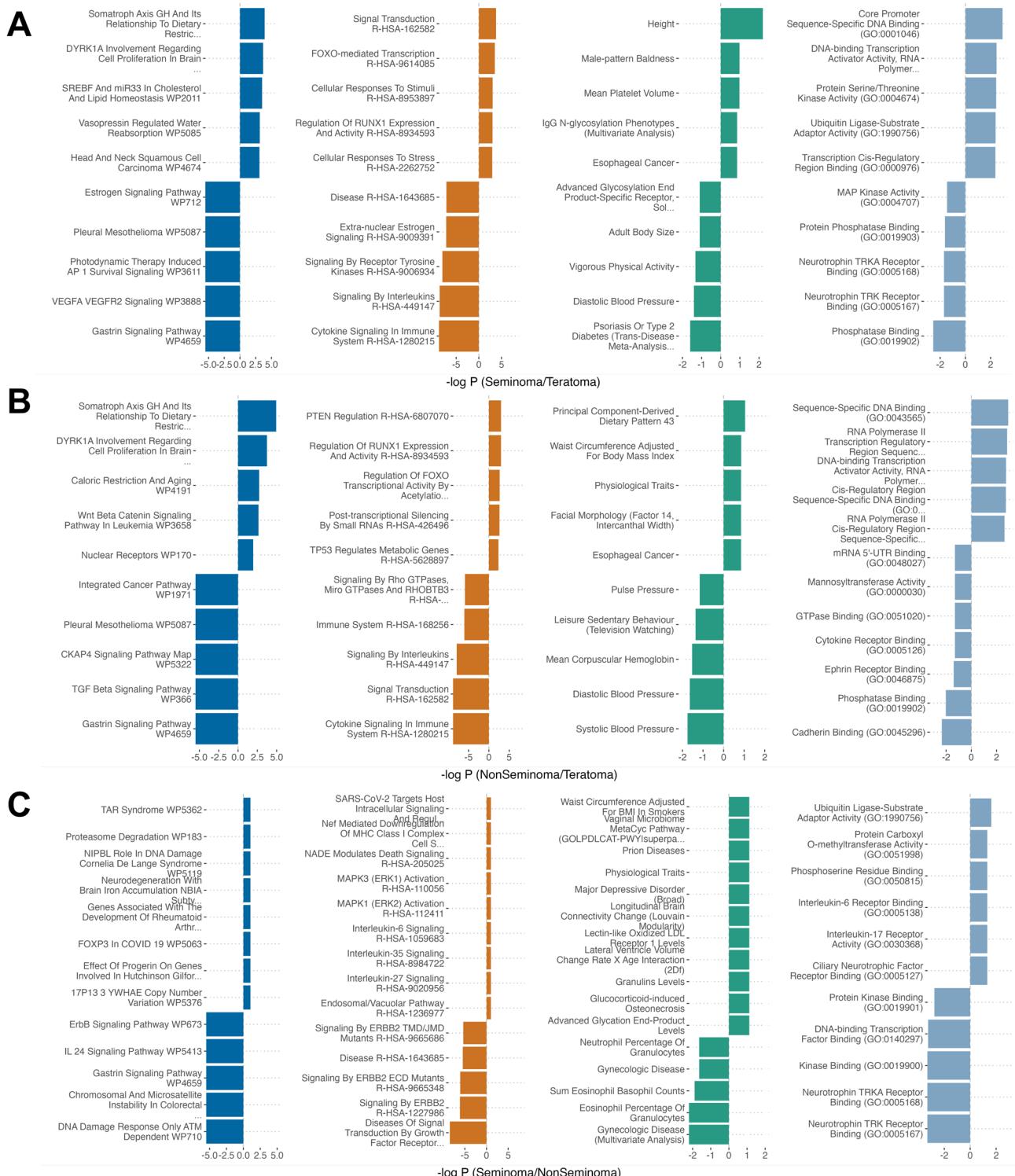
### Subgroup analysis in teratoma reveals minimal miRNA expression differences between tissue source and treatment groups

As our cohort consisted of different tissue sources (orchiectomy and RPLND specimen) with variable exposure to systemic cisplatin-based chemotherapy prior to tissue acquisition, we sought to explore whether tissue source and chemotherapy exposure influence transcriptomic differences by histologic group. To minimize the impact of small sample size and reduced power, we only focused on comparisons in which there were at least three samples in each group. We found that miRNA expression in SEM and N-SEM remained largely consistent between primary tumors and metastatic retroperitoneal lymph nodes, with few differentially expressed miRNAs and a much smaller effect size compared to differences between histologic subtypes (Fig. S4). Comparing orchiectomy and RPLND samples in teratoma, we found that the miR-371–373 cluster and miR-367-3p were highly expressed in primary tumors, but the vast majority of the remaining miRNAs did not exhibit differential expression between primary and metastatic tumors (Fig. 5A, Supplementary Table 4). To systematically test differential effects by tissue of origin for each miRNA, we conducted an additional regression analysis with an interaction term to test if the observed differential effects between orchiectomy and RPLND samples were significant. Here, we only found a total of 53 miRNAs demonstrating such differential effects, representing 2.0% of the whole repertoire of miRNAs.

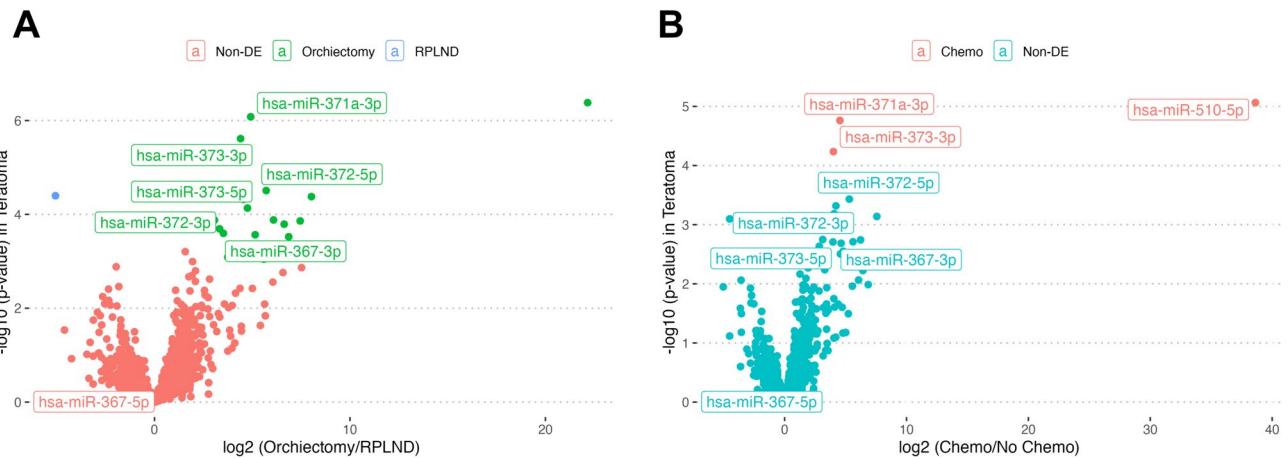
Lastly, we conducted subgroup analysis comparing teratoma samples acquired following treatment with chemotherapy ( $n=6$ ) versus those acquired in the chemotherapy-naïve setting ( $n=3$ ). We found that only three miRNAs were more highly expressed in the chemotherapy-treated samples (miR-510-5p, miR-371a-3p, and miR-373-3p) (Fig. 5B, Supplementary Table 5). Although our subgroup analysis was limited by low sample size, the relatively conserved miRNA profile in teratomas following cisplatin-based chemotherapy are consistent with their inherent chemoresistant properties<sup>29</sup>.

## Discussion

It has been well established that miRNAs play crucial roles in gene regulation, and the dysregulation of this mechanism has been strongly implicated in a variety of cancers<sup>30</sup>. In testicular cancer, the miR-371–373 cluster represents the first evidence that miRNAs can serve as sensitive biomarkers for diagnosing TGCT and monitoring treatment response, while the promise of circulating miRNA biomarkers in other cancer types have faced challenges in specificity and reproducibility<sup>31,32</sup>. However, the differences in miRNA expression among different histologic subtypes of TGCT remain poorly characterized, which hinders our understanding of the genes and molecular pathways targeted by subtype-specific miRNA transcriptomes to inform precise therapeutic strategies. By performing comprehensive miRNA-sequencing on a heterogeneous collection of TGCT samples, we significantly expanded the number of miRNAs analyzed in each histologic group (SEM,



**Fig. 4.** Enrichment analysis of inferred target genes reveals diverse biological processes and related complex traits targeted by marker miRNAs in each subtype. Shown are  $-\log P$ -value of enrichment of five classes biological processes (WikiPathways, Reactome, IDG drug targets, risk genes for complex traits in the GWAS catalog, and GO terms), for each of the three pairwise comparisons (A—SEM vs. teratoma, B—N-SEM vs. teratoma, C—SEM vs. N-SEM). Positive values indicate enrichment in the first histology group (A: SEM, B: N-SEM, C: SEM), and negative values indicate elevated expression in the second histology group (A: teratoma, B: teratoma, C: N-SEM).



**Fig. 5.** Subgroup analysis in teratoma reveals minimal miRNA expression differences between tissue source and treatment groups. (A) Volcano plot showing the log2 fold-change (x-axis) between orchectomy and RPLND samples, and the  $-\log_{10} p$ -value of each miRNA (y-axis). (B) Volcano plot showing the log2 fold-change (x-axis) between chemotherapy-treated and chemotherapy-naïve samples, and the  $-\log_{10} p$ -value of each miRNA (y-axis). Differentially expressed miRNAs were identified by Bonferroni-adjusted  $p < 0.05$  with log2 fold-change  $> 2$ . Previously described miRNAs implicated in TGCT are labeled.

N-SEM, and teratoma), discovered novel marker miRNAs to better differentiate among histologic types, and identified important biological pathways targeted by these marker miRNAs to provide new mechanistic insights into the distinct pathophysiology of TGCT.

The observation that serum levels of miR-371–373 are elevated in individuals with TGCTs<sup>33</sup> led to several subsequent studies supporting their unique potential for serving as circulating biomarkers for TGCT<sup>5,34–37</sup>. The largest prospective study on serum miRNA-371a-3p (M371 test) revealed  $> 90\%$  sensitivity and specificity in diagnosing TGCT, significantly outperforming conventional serum markers<sup>6,38</sup>. Recent studies also reported the utility of the M371 test in the surveillance of TGCT recurrence and prediction of chemotherapy response with successful application in real-life scenarios, although with a smaller number of patients included in these cohorts<sup>35,39,40</sup>. Earlier studies also reported unique miRNA expression patterns in testicular tissues derived from patients with TGCT compared to those derived from patients without TGCT, confirming the restricted expression of miR-371–373 in TGCT<sup>41–44</sup>; however, few studies reported the relative miRNA expression between different TGCT histologic subtypes using high-resolution miRNA-sequencing. Here, we successfully recapitulated enrichment of the described miRNA-371–373 cluster and miRNA-367-3p in TGCT, with remarkable consistency of differential analysis results between our study and the TCGA dataset. Importantly, for each histologic group, we identified several novel marker miRNAs that, taken together, demonstrated significant improvement in discriminative capacity among TGCT histologies. Our comprehensive analysis of the miRNA transcriptome represents a valuable resource to further elucidate the pathophysiological mechanisms and clinical significance of these novel miRNA markers to improve TGCT histology-specific diagnosis and management.

Although teratoma is a subset of non-seminomatous germ cell tumor, it does not exhibit high expression of candidate biomarker miRNAs previously described for SEM and N-SEM, such as miR-371a-3p<sup>45</sup>, and thus using miRNAs as reliable biomarkers for teratoma has posed a significant challenge<sup>7,46–48</sup>. Clinically, identifying teratoma is important to guide management, as it is inherently both chemoresistant and radioresistant, and treatment requires surgical resection. Radiographically, cross-sectional anatomic imaging (e.g., contrast-enhanced CT or MRI scans) and functional imaging (e.g., FDG-PET scans) are also inadequate to differentiate teratoma from fibrosis or necrosis following chemotherapy. Thus, patients with residual masses  $> 1$  cm following chemotherapy for non-seminomatous germ cell tumors require surgical resection for diagnostic confirmation, yet up to 50% of these patients exhibit no residual malignancy and may be subjected to avoidable surgical risks and morbidity<sup>49,50</sup>. Hence, there is a critical need to uncover novel biomarkers capable of accurately identifying teratoma. Lobo et al. nominated several miRNAs, including miR-885-5p, miR-448 and miR-197-3p, as putative markers for teratoma based on a small group of 12 patients<sup>51</sup>. Although these miRNAs demonstrated higher expression in teratoma compared to SEM and N-SEM in our cohort, they did not reach statistical significance or log fold-change threshold. It is possible that miRNAs have distinct expression profiles in serum and in tissue. On the other hand, we identified several candidate miRNAs, such as miR-143, miR-194, miR-199b, miR-200b, and miR-490 (both 3p and 5p species), that were highly enriched in teratoma samples (Bonferroni-adjusted  $p < 1e-4$  in both SEM vs teratoma and N-SEM vs teratoma comparisons). Future studies with better powered and matched serum and tissue samples would be warranted to validate the clinical utility of these candidate miRNAs to serve as potential biomarkers in diagnosing and managing teratoma<sup>52</sup>.

While SEM is homogeneous in cellular composition, N-SEM (excluding teratoma as defined herein) is a group of highly heterogeneous tumors which can contain either a single histological component (pure) or multiple distinct histological components (mixed), including potential admixture with seminomatous subcomponents<sup>53</sup>; these non-teratomatous non-seminoma histologic elements include embryonal carcinoma, yolk sac tumor, and

choriocarcinoma. Different risk factors have been implicated in the differentiation of SEM versus N-SEM from their common precursor (germ cell neoplasia in situ), and patient outcomes have also differed between the two entities<sup>54,55</sup>. While we observed smaller differences of the miRNA transcriptome between SEM and N-SEM, we nevertheless identified cancer-associated miRNAs and target genes predicted to be regulated by these miRNA markers. Consistent with previous studies, we found DNA methyltransferase DNMT3B, a miR-200-3p target and important regulator of epigenetic states, to be inversely associated with miRNA markers in SEM and N-SEM, suggesting that the observed differences in histopathology between SEM and N-SEM may in part be explained by differences in epigenetic remodeling pathways targeted by histology-specific miRNAs<sup>15,56</sup>. Importantly, epigenetically regulated genes have long been implicated in TGCT pathophysiology<sup>57</sup>, with strong genetic evidence suggesting increased risk of developing TGCT in patients with genetic variants perturbing normal epigenetic programming<sup>58</sup>. Clinical studies have also suggested the involvement of epigenetic dysregulation in developing cisplatin resistance, particularly in N-SEM<sup>59,60</sup>. Our study provides fundamental insights into epigenetic perturbations in TGCT mediated by miRNAs, which warrant mechanistic validation.

In addition to epigenetic remodeling, several other mechanisms have been proposed to underlie the functional impact of miRNA expression changes in TGCT. miR-372 and miR-373 have been found to directly target tumor suppressor LATS2 to counteract P53-P21-CDK signaling, thus promoting TGCT proliferation<sup>61</sup>. However, little is known about additional biological pathways targeted by ss in TGCT<sup>62</sup>. Here, we performed comprehensive enrichment analysis using inferred target genes from miRNA species and discovered several important transcription factors, such as RUNX1, FOXO, and PTEN, suggesting specific targeting in SEM and N-SEM compared to teratoma. Additionally, we found that in SEM and N-SEM, miRNA target transcription factor binding in the core promoters were dysregulated, further supporting a model in which miRNAs exert their effects by disrupting key transcription factors in TGCT. Future studies characterizing the transcription factor regulatory networks perturbed by these miRNAs are critical to elucidate the functional impact of miRNAs on the transcriptome and to nominate potentially actionable therapeutic targets.

Surprisingly, we also found that somatotroph signaling is enriched in SEM and N-SEM compared to teratoma, and many genes targeted by marker miRNAs were linked to height by GWAS<sup>27</sup>. Although increased height has been described as a risk factor for TGCT<sup>63</sup>, there is conflicting evidence regarding the mechanism of this association. A recent Mendelian randomization study did not support a causal link between height and TGCT risk<sup>64</sup>, although it has not been revisited in the context of new genetic loci identified from more recent and better-powered GWAS<sup>65</sup>. Our data suggests that the association between height and TGCT may be in part explained by common miRNA species targeting genes involved in both the development of TGCT and linear growth; therefore, transcriptomic modulation by shared miRNAs rather than by shared genetic variants may be better suited to delineate this association. In this instance, miRNA can play an important role in the interpretation of genetic association with complex traits, which can have profound impact on research into the genetic architecture of human diseases.

Our study does have important limitations. Most notably, we collected data and performed miRNA-sequencing at a single institution from limited FFPE tissue samples. Given the potential variation in sample preparation and miRNA quantification, we recognize the urgent need to establish standardized protocols for the collection and reporting of miRNA-sequencing data to facilitate interpretation and clinical implementation of findings<sup>66</sup>. We were not able to replicate our top candidate miRNAs in published resources such as the TCGA because of our inclusion of many more miRNA species, although we achieved excellent validation when comparing miRNAs present in both our dataset and the TCGA dataset. Additionally, we had a relatively modest sample size with a few individuals contributing to more than one sample, and therefore were limited by insufficient power to perform subgroup analyses on chemotherapy-induced miRNA expression changes and further subgroup analyses on distinct histologic entities within the N-SEM umbrella. Exploration of our candidate miRNAs in the circulatory compartment will be important to provide additional non-invasive serum markers that may augment the discriminatory capacity and performance characteristics of leading biomarker candidates, such as miR371a-3p. By presenting our key findings and sharing our dataset with the community, we are hopeful that future studies can build on this resource to improve power for miRNA discovery and mechanistic investigation.

In summary, we present a comprehensive survey of tissue-level miRNA expression patterns across different TGCT histologies from matched primary and metastatic tumors. We discovered novel miRNA species with improved discriminative capacity among histologic subgroups and identified specific transcription factors and biological pathways targeted by miRNAs. Our data provides an important resource to elucidate the mechanisms of miRNA regulation in testicular cancer with the goal of informing discovery of novel diagnostic biomarkers and precise therapeutic strategies.

## Data availability

Raw and processed miRNA-seq data was deposited in the GEO database (accession number GSE301954).

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## Author contributions

N.S. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: N.S., S.E.L. Acquisition of data: N.S., T.L., A.Y., S.B., E.B., S.E.L. Analysis and interpretation of data: N.S., T.L., S.B., C.R.L., J.C., Y.G., E.B., S.E.L. Drafting of the manuscript: T.L., S.B., S.E.L., N.S. Critical revision of the manuscript for important intellectual content: all authors. Statistical analysis: T.L., J.C. Obtaining funding: N.S. Administrative, technical, or material support: N.S., S.E.L. Supervision: N.S., S.E.L.

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## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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