**LIN28 Expression in Malignant Germ Cell Tumors Downregulates let-7 and Increases Oncogene Levels**


**Abstract**

Despite their clinicopathologic heterogeneity, malignant germ cell tumors (GCT) share molecular abnormalities that are likely to be functionally important. In this study, we investigated the potential significance of downregulation of the let-7 family of tumor suppressor microRNAs in malignant GCTs. Microarray results from pediatric and adult samples (n = 45) showed that LIN28, the negative regulator of let-7 biogenesis, was abundant in malignant GCTs, regardless of patient age, tumor site, or histologic subtype. Indeed, a strong negative correlation existed between LIN28 and let-7 levels in specimens with matched datasets. Low let-7 levels were biologically significant, as the sequence complementary to the 2 to 7 nt common let-7 seed "GAGGUA" was enriched in the 3′ untranslated regions of mRNAs upregulated in pediatric and adult malignant GCTs, compared with normal gonads (a mixture of germ cells and somatic cells). We identified 27 mRNA targets of let-7 that were upregulated in malignant GCT cells, confirming significant negative correlations with let-7 levels. Among 16 mRNAs examined in a largely independent set of specimens by quantitative reverse transcription PCR, we defined negative-associations with let-7 levels for six oncogenes, including MYCN, AURKB, CCNF, RRM2, MKI67, and C12orf5 (when including normal control tissues). Importantly, LIN28 depletion in malignant GCT cells restored let-7 levels and repressed all of these oncogenic let-7 mRNA targets, with LIN28 levels correlating with cell proliferation and MYCN levels. Conversely, ectopic expression of let-7e was sufficient to reduce proliferation and downregulate MYCN, AURKB, and LIN28, the latter via a double-negative feedback loop. We conclude that the LIN28/let-7 pathway has a critical pathobiologic role in malignant GCTs and therefore offers a promising target for therapeutic intervention. *Cancer Res; 73(15); 1–13. ©2013 AACR.*

**Introduction**

Germ cell tumors (GCT) are clinically and histopathologically complex. They present from early infancy through to late adulthood, occur at both gonadal and extragonadal sites, and comprise diverse histologic subtypes (1). Benign forms show somatic differentiation and are termed teratomas, whereas malignant GCTs are classified into germinomas (a collective term for testicular seminoma, ovarian dysgerminoma, and extragonadal germinoma) and non-germinomatous tumors, the main types of which are yolk sac tumors (YST) and embryonal carcinoma (1).

Although most patients with malignant GCTs have a good prognosis, some patients still have inferior outcomes, and testicular germ cell malignancy remains a leading cause of death in young men (2). Improved understanding of the molecular pathogenesis of malignant GCTs would represent an important step toward developing novel therapeutic agents with favorable toxicity profiles, which may improve survival for patients with high-risk disease and reduce toxicity for low-risk patients. It is particularly important to identify abnormalities that are shared across the diverse spectrum of malignant GCTs, as these are likely to be of fundamental significance in disease pathogenesis.

Using microarray profiling, we previously identified that all 9 members of the *letal-7* (let-7) microRNA family were significantly underexpressed in pediatric malignant GCTs, when compared with normalmalignant control tissues (3). MicroRNAs regulate gene expression via their 5′ seed region (nucleotides at positions 1–8; 1–8 nt), which binds the corresponding seed complementary region (SCR) located predominantly in the 3′ untranslated region (3′UTR) of mRNA targets (4). Within the seed, 2 to 7 nt are most critical for binding specificity (5). Importantly, all 9 let-7 microRNAs share the same 2 to 7 nt seed sequence (GAGGUA) and therefore share mRNA targets containing the 3′UTR SCR "TACCTC."
Let-7 microRNAs are important tumor suppressor genes (6) that regulate cell proliferation (7). The let-7 microRNA family is negatively regulated by the RNA-binding proteins LIN28 homolog-A (LIN28) and LIN28 homolog-B (LIN28B; refs. 8, 9), with LIN28 depletion resulting in specific increases in all let-7 family members (8, 10). The LIN28 proteins bind let-7 primary transcripts (pre-let-7) and precursor hairpins (pre-let-7), preventing processing by DROSHA and Dicer1, respectively (11). Binding of pre-let-7 by LIN28 occurs through a stem loop motif that includes "GGAG" (12, 13), leading to recruitment of the terminal-uridyl-transferase (TUTase) ZCCHC11 (10, 12), resulting in uridylation and subsequent degradation (14).

LIN28 can reprogram human somatic cells into pluripotent stem cells, and is a putative cancer stem-cell marker (9, 11, 15). Of note, LIN28 is expressed at high levels in primordial germ cells (16), believed to be the cell of origin for malignant GCTs (1). Previous studies used immunohistochemistry (17–19) and RNA interference (RNAi; ref. 20) to investigate the expression and some aspects of LIN28 function in malignant GCTs. Here, we provide the first demonstration that low let-7 levels in malignant GCTs are directly attributable to LIN28 expression and are likely to contribute to significant upregulation of important cancer-associated protein-coding genes.

Materials and Methods

Tumor samples

Our study received ethical approval from Trent-MREC (ref: 02/4/071) and Cambridge-LREC (ref: 01/128). We analyzed the following tissue samples and datasets:

**Set 1.** Forty-eight samples of pediatric malignant GCTs and nonmalignant controls, which we previously used for global microRNA profiling (3) and for global mRNA profiling, a subset of 21 cases (3). Across the sample set, the controls \((n = 58)\) represented fetal yolk sac, fetal ovary, prepubertal testis, postpubertal testis, prepubertal ovary, and postpubertal ovary. These samples contain germ cells, with variable representation of somatic cells (21). One apparent teratoma sample (MT-34) was not included in any subsequent analysis as it was a component of a mixed malignant GCT and clustered with malignant cases on microRNA profiling (3). The remaining 20 samples with matched microRNA and mRNA profiles comprised 17 malignant GCTs (10 YSTs, 6 germinomas, and 1 embryonal carcinoma) and 3 normal gonadal controls (1 pre- and 1 postpubertal testis and 1 postpubertal ovary; ref. 3).

**Set 2.** A published dataset of global mRNA expression profiles of 25 samples from a study of adult testicular malignant GCTs (8 YSTs; 12 germinomas) and controls (5 normal adult testes, containing germ cells and somatic cells). Further details are available in the original publication (22) and our previous study (3).

**Set 3.** Thirty-two samples in which we measured levels of selected mRNAs and microRNAs by quantitative reverse transcription PCR (qRT-PCR). Full details are given in Supplementary Table S1. The malignant GCTs represented 9 YSTs, 9 germinomas, and 3 embryonal carcinomas, with all except 3 being from pediatric patients (<16 years). In addition, we used 6 malignant GCT cell lines and 5 benign teratomas. Twenty-four of the 32 samples overlapped with set 1 and had previously been used for microRNA profiling (3). However, only 6 of the 32 had undergone mRNA profiling (3; Supplementary Table S1), enabling set 3 to be used for independent qRT-PCR validation of findings from our mRNA microarray analyses of sets 1 and 2.

When combining sets 1 to 3, our study encompassed a total of 81 samples, comprising 54 different malignant GCTs (31 pediatric, 23 adult; 43 gonadal, 11 extragonadal), 6 malignant GCT cell lines, and 21 control samples (8 teratomas and 13 gonads/yolk sac).

MicroRNA and mRNA microarray expression analysis

We reanalyzed microRNA expression profiles for set 1 [obtained using the mirCURY-LNA array-v9.2 (Exiqon); ref. 3] and mRNA expression profiles for sets 1 and 2 [obtained using the HG-U133A GeneChip (Affymetrix); refs. 3, 22]. Differential gene expression was assessed using a moderated t statistic and \(P\) values adjusted for multiple testing using Benjamini and Hochberg method (23). MicroRNAs with adjusted \(P\) values less than 0.01 were considered to be significantly differentially expressed, whereas mRNAs with log_{2} fold-change 1.5 or more and adjusted \(P < 0.01\) were considered to be overexpressed (3).

**Sylamer bioinformatic algorithm**

Sylamer assesses enrichment and/or depletion of SCR nucleotide words of specific length in the 3' UTRs of genes within ranked lists (24). We used Sylamer to identify whether the let-7 downregulation in malignant GCTs was of biologic significance by causing shifts in expression of mRNAs with a 3' UTR let-7 SCR (3). We conducted 3 analyses of mRNA microarray data, examining the pediatric samples from set 1 with matched microRNA and mRNA profiles \((n = 20)\), the adult samples from set 2 \((n = 25)\), and both groups in combination \((n = 45)\). In Sylamer landscape plots, mRNA gene lists were ranked on the x-axis from downregulated (left) to upregulated (right). The y-axis showed log_{10} transformed and sign-adjusted enrichment \(P\) values for each SCR word, relative to \(P\) values of all other words. Consequently, an SCR showing a negative y-axis deflection on the right-hand side of each plot was enriched in upregulated genes. As previously (3), we calculated a single summed significance score and \(P\) value for each SCR. We only considered SCR elements that contained the core 2 to 7 nt sequence of the microRNA seed region, summatung data for 1 hexamer \((2–7\) nt), 2 heptamers \((1–7\) nt and 2–8 nt), and 1 octamer \((1–8\) nt). \(P\) values less than 0.01 were considered significant (3). We tested for enrichment in the upregulated genes of 3' UTR SCRs corresponding to the 1 to 8 nt seeds of all 126 microRNAs downregulated in the 48 set 1 pediatric malignant GCTs (3).

mRNA qRT-PCR

Relative mRNA transcript levels were measured in triplicate in clinical samples and cell lines using QuantiTect One-Step SYBR-Green qRT-PCR (Qiagen), following reverse transcription of 1 µg of total RNA using QuantiTect Reverse Transcription (Qiagen). Primers (Supplementary Table S2) were designed using Primer3 (25), ensuring that they crossed exon–exon boundaries. Expression ratios were calculated using the comparative threshold cycle \((C_{T})\) method (26) and
LIN28/let-7 Deregulation in Malignant Germ Cell Tumors

A

Median let-7 in pediatric samples (set 1)
LIN28 in pediatric samples (set 1)
LIN28 in adult samples (set 2)

B

Median let-7 microarray expression level
LIN28 microarray expression level

C

LIN28
LIN28B
let-7e
ZCCHC11

*Yolk sac tumor*  *Germinoma*  *Embryonal ca*  *GCT cell line*  *Teratoma*  *Normal gonad*
normalized using 4 housekeeping genes ACTB (Qiagen), YWHAZ, RPL13A, and HMBS (27). In analyses of clinical samples, results were referenced to a pooled normal gonadal control, using total RNA from human ovaries and testes (AM6974/AM7972, Ambion). It should be noted that these tissues contain both germ cells and somatic cells. For cell lines, results were referenced to cells treated with either non-targeting control (NTC) siRNA, or mimic negative control (MNC) RNA, as appropriate.

**qRT-PCR for microRNA and pri-microRNA**

MicroRNA levels were quantified in triplicate using TaqMan assays (Applied Biosystems), as described in references (3, 28). Levels of pri-microRNA were determined in quadruplicate, using TURBO DNA-free, TaqMan High Capacity RNA-to-cDNA, and TaqMan assays (all Applied Biosystems). Expression ratios were calculated using the comparative Ct method, with micro-RNAS normalized to RNU24 (3, 28) and pri-microRNAs to RPLP0, GUS-B, and 18S. Reference samples were as for mRNA qRT-PCR. In test amplifications using 0.01 nmol/L single-stranded let-7e RNA (Sigma Aldrich) TaqMan qRT-PCR for let-7b and let-7d showed no cross-reactivity with let-7e (data not shown).

**Western blotting**

Western blots were conducted for LIN28, LIN28B, AURKB, and MYCN proteins using the antibodies ab46020 (1:10,000) and -tubulin (ab6046; 1:10,000). All antibodies were from Abcam. Western blot densitometry was conducted using FluorChem-9900 imaging system software (Alpha Innotech). Results were normalized to b3-tubulin (ab6046; 1:10,000) and MYCN proteins using the antibodies ab46020 (1:10,000) and -tubulin (ab6046; 1:10,000). All antibodies were from Abcam. Western blot densitometry was conducted using FluorChem-9900 imaging system software (Alpha Innotech).

**GCT cell lines**

We selected four cell lines that reflected the range of malignant GCT histologic subtypes commonly observed in clinical practice, namely embryonal carcinoma (2102Ep; ref. 29), YST (GCT44 and 1411H), and germinoma/seminoma (TCam2; ref. 30). Cells were cultured at 37°C in 5% CO2 in medium containing 10% fetal calf serum and 1% penicillin/streptomycin, and were authenticated using short tandem repeat profiling (3).

**RNA depletion and overexpression**

Transcripts were depleted by RNAi in overexpressing malignant GCT cell lines using pools of 4 separate siRNAs to reduce any off-target effects (31). The probes targeted LIN28 (L-018411-01), LIN28B (L-028584-01), and MYCN (L-003913-01; all Dharmacon). Each pool was used at 66.7 nmol/L, which represented the minimal concentration of LIN28 siRNAs that achieved more than 75% LIN28 transcript depletion in test experiments (data not shown). All results were normalized to cells treated with a 66.7 nmol/L pool of 4 NTC siRNAs (D-001810-10-05, Dharmacon; ref. 32). We confirmed the specific effects of the LIN28-targeting pool using 2 independent siRNAs (Hs_LIN28_7 and Hs_LIN28_8, both Qiagen; target sequences TAAAGACTTTATTGGAGC and CACGCTGAGATGACCGG, respectively). Differences between experimental observations were assessed using an unpaired, 2-sample t test (2-tailed with 95% confidence intervals). Let-7e was repressed in underexpressing malignant GCT cell lines using let-7e miRIDIAN double-stranded RNA-mimic (C-300479-05) at 100 nmol/L (33) and was normalized to cells treated with 100 nmol/L miRIDIAN MNC (CN-001000-01, both Dharmacon).

**Cell transfection and proliferation assays**

Following optimization of transfection conditions (data not shown), cells were seeded in 6-well plates, with 2102Ep at 7 × 10^3 cells per well, 1411H and GCT44 at 1.0 × 10^5 cells per well, and TCam2 1.5 × 10^5 cells per well. On day 0 (d0), when cells were approximately 20% confluent, transfection was conducted using Opti-MEM media and Lipofectamine RNAiMAX (both Invitrogen). The optimal length of transfection, which maximized transfection efficiency and minimized toxicity, was 24 hours for 2102Ep cell line, 6 to 8 hours for 1411H and GCT44, and 4 to 6 hours for TCam2. At least three biologic replicates were done for each treatment. For RNA and protein quantification, the replicate samples were pooled prior to further analysis. For qRT-PCR, at least three technical replicates were done per analysis.

Cell numbers were quantified using Trypan blue on a Countess automated cell counter (Invitrogen), determining the mean of two values for each of three biologic replicates and then taking the mean of the resulting three values. Maximal growth rates were determined by plotting cell numbers at 24-hour time-points on a logarithmic scale and calculating population doubling-time from the linear section of the curve, as described (34).

**Luciferase reporter assays**

We studied let-7 effects on target genes using GoClone luciferase reporter plasmids containing the full-length 3'UTR for LIN28 (S813978), MYCN (S807230), or AURKB (custom-made), plus a control luciferase plasmid containing no 3'UTR (S890005; all plasmids from SwitchGear Genomics). Test oligonucleotides were let-7e, non-targeting RNA (NT2; MIM9002), and a mutant let-7e (let-7e-mutant; sequence UGAGUGAGGA-GGUUGUAUAGUU), in which the 2 to 7 nt seed was mutated to “GAGUGA.” The latter sequence did not correspond to any known human microRNA seed, thereby avoiding seed-like off-target effects in transfected cells. All experiments were done twice in quadruplicate in 96-well plates, using more than 80% confluent cells, with 50 ng of plasmid per well and 100 nmol/L oligonucleotides. Luminescence was quantified on a BioTek Synergy-HT multi-mode microplate reader (BioTek).
LIN28/let-7 Deregulation in Malignant Germ Cell Tumors

A

Combined (sixmers)

Score 13.70

P = 0.0013

Combined (sevenmers)

Combined (eightmers)

B

MYCN

CCNF

RRM2

AURKB

MKI67

C12orf5

MYCN expression relative to pooled gonadal control

CCNF expression relative to pooled gonadal control

RRM2 expression relative to pooled gonadal control

AURKB expression relative to pooled gonadal control

MKI67 expression relative to pooled gonadal control

C12orf5 expression relative to pooled gonadal control

C

MYCN

CCNF

RRM2

AURKB

MKI67

C12orf5

MYCN qRT-PCR levels vs let-7e qRT-PCR levels

CCNF qRT-PCR levels vs let-7e qRT-PCR levels

RRM2 qRT-PCR levels vs let-7e qRT-PCR levels

AURKB qRT-PCR levels vs let-7e qRT-PCR levels

MKI67 qRT-PCR levels vs let-7e qRT-PCR levels

C12orf5 qRT-PCR levels vs let-7e qRT-PCR levels

P = 0.0018

P = 0.0006

P = 0.0029

P = 0.0106

P = 0.0150

P = 0.0246

Yolk sac tumor

Germinoma

Embryonal ca

GCT cell line

Teratoma

Normal gonad
OF6
Cancer Res; 73(15) August 1, 2013

Murray et al.

Instruments Inc.). After background correction, the means for the test let-7e/let-7e-mutant oligonucleotides were normalized to values for NT2-treated cells, then referenced to cells containing the no 3'UTR control reporter that had also been treated with let-7e/let-7e-mutant, as appropriate.

Results

Let-7 and LIN28 expression in malignant GCTs

In our previous microRNA profiling study of sample set 1, we identified that all 9 members of the tumor-suppressor let-7 microRNA family were significantly downregulated in pediatric malignant GCTs, compared with nonmalignant tissues (benign GCTs and normal gonad; ref. 3), which contain a variable representation of germ cells and somatic cells. The fold changes observed for each let-7 family member are given in Supplementary Table S3, with let-7e being the most significantly underepressed by P value. For the subset of 20 pediatric samples with matched microRNA and mRNA profiles, the malignant GCTs again showed significant reductions in let-7 microRNAs, when assessing each family member individually (Supplementary Fig. S1A), or collectively (Fig. 1A).

As individual let-7 members are transcribed from multiple genomic loci, this observation suggested the possibility of a common posttranscriptional mechanism regulating let-7 biogenesis in malignant GCTs. We therefore sought to identify whether let-7 downregulation was associated with overexpression of LIN28. Using all available mRNA microarray data, we found that LIN28 was highly expressed in 44 of 45 (97.8%) of malignant GCTs from pediatric and adult patients (from sets 1 and 2 combined; Fig. 1A), regardless of tumor site (gonadal/extra gonadal) or histologic subtype. For the 20 pediatric samples with matched microRNA and mRNA data, LIN28 showed a highly significant negative correlation with median let-7 levels ($R^2 = 0.63; P < 0.0001$; Fig. 1B) and with levels of each individual let-7 family member (all $P < 0.005$; Supplementary Fig. S1B).

We validated these microarray findings using the independent technique of qRT-PCR in a panel of 32 samples (set 3). Compared with pooled normal gonadal control RNA, all 27 of the malignant GCT samples and cell lines showed high LIN28 expression (Fig. 1C). Twenty-four of the 27 also showed high expression of LIN28B (Fig. 1C), which is located at a different chromosomal locus (6q16.3; vs. 1p36.11 for LIN28). In contrast, levels of the TUTase ZCCHC11 were not elevated either in malignant GCT samples or teratomas (Fig. 1C). Using TaqMan qRT-PCR, we confirmed that let-7e (the most significantly downregulated let-7 in our microarray analysis) showed low expression in all the malignant GCT samples/cell lines (Fig. 1C). Linear regression using the qRT-PCR data confirmed that let-7e levels were significantly negatively correlated with LIN28 and LIN28B ($P = 0.017$ and $P = 0.036$, respectively; Supplementary Fig. S2), but not with ZCCHC11.

Biologic significance of low let-7 levels in malignant GCTs

Sylamer showed that “TACCTC” (complementary to the 2–7 nt common let-7 seed “GAGGAUA”) was the top-ranking SCR in mRNAs that significantly upregulated in malignant GCTs (compared with the normal gonadal control tissues), irrespective of patient age. There were highly significant $P$ values for the summed significance scores of the let-7 1 to 8 nt SCR in the datasets for the pediatric malignant GCTs (set 1; $P = 0.00057$), the adult malignant GCTs (set 2; $P = 0.00026$; both Supplementary Fig. S3), and the combined analysis ($P = 0.0013$; Fig. 2A). In all analyses, there was no significant enrichment in the upregulated mRNAs of SCRs for any of the other 126 microRNAs tested.

We used Sylamer to produce a list of let-7 mRNA targets that were overexpressed in all malignant GCTs, for further validation in clinical samples and functional investigation in vitro. We identified 198 upregulated genes from the pediatric mRNA dataset (set 1), of which 50 (25.3%) had at least one “TACCTC” 3’UTR sequence. For the adult dataset (set 2), we identified 428 upregulated genes, of which 106 (24.8%) contained at least one 3’UTR TACCTC. These values compared with an overall frequency of 19.8% for the TACCTC sequence in the 3’UTR of all annotated genes on the array. Thirty-six let-7 mRNA targets were common to both datasets, with 27 having a significant negative correlation with median let-7 levels in the 20 pediatric tissue samples from set 1 that had matched microRNA and mRNA microarray data (Supplementary Table S4 and Supplementary Fig. S4). We selected 16 of these 27 genes for further interrogation, based on their reported functions in human disease, including cancer. The genes were: MYCN, CCNF, BRM2, AURKB, MKI67, C12orf5, FZD5, KRAS, PGK1, SMAGP, RAB25, RAB15, MBR2, SLC2A3, LASP1, and AGL.

HMGA2, a known let-7 target in carcinoma cells (35), was included in the initial list of 36 mRNAs, as it was upregulated in both pediatric set 1 (rank 40/50) and adult set 2 (rank 63/106). However, HMGA2 showed no significant correlation with median let-7 levels across these datasets ($P = 0.12$). To investigate these observations further, we measured HMGA2 levels in set 3 using qRT-PCR. This showed that while HMGA2 was overexpressed in some subtypes of malignant GCT (YSTs and embryonal carcinoma), it showed only minimal expression changes in another major subtype, germinoma (Supplementary Fig. S5A and S5B). The lack of overall association between HMGA2 and let-7 levels was also confirmed in this qRT-PCR analysis ($P = 0.12$; Supplementary Fig. S5C).

Figure 2. Significance of let-7 downregulation in malignant GCTs. A, Sylamer landscape plots for SCR words corresponding to the common seed of the 9 let-7 microRNA family members in the combined analysis of pediatric (set 1) and adult (set 2) malignant GCTs. Log$_{10}$-transformed $P$ values for each SCR word are plotted on the y-axis, against the ranked gene list on the x-axis. A negative y-axis deflection on the right-hand side of the plot signifies SCR enrichment in upregulated genes. The left-hand plot shows data for the hexamer complementary to the core 2 to 7 nt component of the common seed region, the central plot the 2 heptamers (1–7 nt; 2–8 nt), and the right-hand plot the octamer (1–8 nt). The single summed significance score and $P$ value for all 4 SCR words is shown. B, levels of the 6 selected let-7 mRNA targets in sample set 3 determined by qRT-PCR. Error bars, SEM. C, correlations between each mRNA and let-7e in set 3. $P$ values were determined by linear regression.

Published OnlineFirst June 17, 2013; DOI: 10.1158/0008-5472.CAN-12-2085

Downloaded from cancerres.aacrjournals.org on April 11, 2017. © 2013 American Association for Cancer Research.
Validation of let-7 mRNA targets

By qRT-PCR analysis of the 32 samples in set 3, we confirmed overexpression of all 16 selected mRNAs in malignant GCTs, compared with the control samples used (Fig. 2B, Supplementary Fig. S6). We identified a negative association with let-7e qRT-PCR levels for 6 mRNAs (MYCN, AURKB, CCNF, RBM2, MKE67, and C12orf5; Supplementary Table S3; Fig. 2C). It should be noted that the associations were only significant when including the control samples, in which there was a mixture of germ cells and somatic cells. Accordingly, these findings should be viewed with caution in the absence of follow-up functional data (see below). On the other hand, there was no significant association for the other 10 of the 16 genes, when the control samples were included. Of these other 10 mRNAs, RAB25, MBS2, PGK1, KRAS, and LASP1 were overexpressed in malignant GCTs of particular histologic subtypes (Supplementary Fig. S6), but did not show an association with let-7e levels across the whole sample set. The other 5 mRNAs (FZD5, SMAGP, RAB15, SLCA23, and AGL) showed no association with let-7e qRT-PCR levels (Supplementary Fig. S6), suggesting that their expression is regulated by additional factors, which may include other microRNAs.

Depletion of LIN28 and LIN28B

We next tested the functional significance of our observations in vitro, using multiple complementary experimental approaches to minimize the possibility of nonspecific observations. We tested for phenotypic and functional consistencies when: (i) depleting LIN28 or LIN28B by RNAi, using panels of 4 siRNAs to minimize any off-target effects (31), with separate confirmation using independent siRNAs; (ii) directly overexpressing let-7e (the let-7 family member that showed the most significant underexpression in vivo; Supplementary Table S3) using a double-stranded RNA mimic; and (iii) depleting MYCN, a major let-7e target, by RNAi using a panel of 4 independent siRNAs. In this in vitro work, we used 4 representative malignant GCT cell lines, all of which showed LIN28 upregulation and let-7e underexpression (Fig. 1C).

In 2102Ep cells, a single treatment with pooled siRNAs depleted LIN28 mRNA by more than 90% over a 7-day period. There were parallel reductions in protein levels, which fell to less than 10% from d3 (Fig. 3A and Supplementary Fig. S7A). There were parallel reductions in protein levels, which fell to less than 10% from d3 (Fig. 3A and Supplementary Fig. S7A). It should be noted that the associations were only significant when including the control samples, in which there was a mixture of germ cells and somatic cells. Accordingly, these findings should be viewed with caution in the absence of follow-up functional data (see below). On the other hand, there was no significant association for the other 10 of the 16 genes, when the control samples were included. Of these other 10 mRNAs, RAB25, MBS2, PGK1, KRAS, and LASP1 were overexpressed in malignant GCTs of particular histologic subtypes (Supplementary Fig. S6), but did not show an association with let-7e levels across the whole sample set. The other 5 mRNAs (FZD5, SMAGP, RAB15, SLCA23, and AGL) showed no association with let-7e qRT-PCR levels (Supplementary Fig. S6), suggesting that their expression is regulated by additional factors, which may include other microRNAs.

Restoration of let-7 levels

Transfection with let-7e mimic mimicked the greatest increases in let-7e levels in 2102Ep and 1411H (Fig. 6), which were selected for further investigations. In both cell lines, let-7e transfection resulted in significant reductions in mRNA levels of MYCN and LIN28 at d2, when compared with MNC-treated cells (all P ≤ 0.001; Fig. 6A). Over a 3d time-course in 2102Ep, we observed reduced levels of MYCN, AURKB, LIN28, and LIN28B transcripts (Supplementary Fig. S9A), with a significant negative correlation between mean transcript depletion over d1 to d3 and the number of 3’UTR let-7i SCR (R² = 0.97, P = 0.0145; Supplementary Fig. S9B). Levels of all 4 proteins were reduced when assessed on d2 and d3 (Fig. 6B and C). Overall, across the 4 malignant GCT cell lines examined, there was a significant negative correlation between let-7e levels obtained following let-7e mimic transfection and cell proliferation (R² = 0.94; P < 0.0001; Fig. 6D).

The LIN28 and LIN28B depletion (Fig. 6A, Supplementary Fig. S9A) were explained by the presence in 3’UTRs of the “TACCTC” SCR for the common 2 to 7 nt let-7 seed, with one copy in LIN28 (Supplementary Fig. S9C) and 5 copies in LIN28B (data not shown). In keeping with the reduced LIN28B levels, we observed approximately 15- to 30-fold increases in other let-7 family members examined (let-7b and let-7d) in both 2102Ep (P = 0.0001 for both) and 1411H (P = 0.0006 and P = 0.0002, respectively; Fig. 6E). There was no increase in levels of a control microRNA (miR-192) lacking the LIN28 “GGAG” binding site in its stem-loop (Fig. 6E).
We confirmed let-7e effects on LIN28, MYCN, and AURKB using quantitative luciferase reporter assays. Let-7e produced a significant reduction in luminescence relative to nontargeting oligonucleotides, in cells containing the 3’UTR for LIN28 (P = 0.0003), MYCN (P = 0.011) or AURKB (P < 0.0001), whereas there were no reductions with mutant let-7e (Fig. 7). These findings are supported by evidence from other cell types showing direct targeting of MYCN by let-7e (36) and of AURKB by let-7b (37), which has an identical 2 to 7 nt seed to that of let-7e.

**Effects of MYCN depletion**

We tested whether LIN28/LIN28B levels in malignant GCTs might be regulated by MYCN and the related protein CMYC, similar to findings in other tumor types (38, 39). Our microarray data showed upregulation of MYCN in both pediatric (set...
nant GCTs have known promalignant effects, such as increased expression experiments. The potential by our functional data from protein-coding gene targets, a possibility that was supported regulating let-7 expression may contribute to increased expression of let-7. It is likely that levels of pri-let-7 are low even in the presence of abundant LIN28, for example due to degradation after LIN28 binding. As well as downregulation of let-7 by LIN28, we observed a reciprocal effect, with downregulation of LIN28 by let-7e, via a let-7SCR in the LIN28 3’UTR (45, 46). In malignant GCT cells, let-7e-mediated downregulation of LIN28 produced specific effects, by increasing other let-7 family targets of LIN28 rather than producing a more generalized effect on microRNA biosynthesis. Other microRNAs known to downregulate LIN28 in embryonic stem-cells and cancer cells through 3’UTR SCRs include miR-9 (47), miR-30 family (47), miR-125 (47, 48), and miR-181 (49). Interestingly, all 4 were identified in our previous profiling study as being universally underexpressed in malignant GCTs, compared with the control tissues used (3). As copy number gain at the LIN28 locus (1p36.11) is not a feature of malignant GCTs (44), downregulation of these microRNAs is

Figure 4. Correlations between LIN28, MYCN, and cell numbers following LIN28 depletion. The graphs show data for all 4 malignant GCT cell lines at d4 following LIN28 depletion, compared with NTC-treated cells. A and B, cell numbers versus the levels of LIN28 (red) and MYCN (blue; A), whereas B shows levels of MYCN versus LIN28 (B). NTC, nontargeting control siRNA; kd, knockdown. Correlation P values were determined by linear regression. Error bars, SEM.

Discussion

This study shows that LIN28-homolog A (LIN28) is abundantly expressed in all malignant GCTs, regardless of patient age, histologic type or anatomic site, thereby extending published reports describing predominantly or exclusively tumors of adults (17–20). Importantly, we identify the functional significance of the observed LIN28 expression, which results in let-7 family downregulation (Supplementary Fig. S12). Our qRT-PCR analysis of tissue samples suggested that let-7 under-expression may contribute to increased expression of let-7 protein-coding gene targets, a possibility that was supported by our functional data from LIN28 depletion and let-7e over-expression experiments. The potential let-7 targets in malignant GCTs have known promalignant effects, such as increased proliferation and reduced apoptosis (Supplementary Table S5).

While LIN28B is also highly expressed in malignant GCTs, our data do not indicate an important role for the protein in regulating let-7 microRNAs.

A previous study showed that LIN28 depletion in malignant GCT cells led to downregulation of stem-cell markers (e.g., OCT4/POU5F1 and NANO1) and induction of differentiation, although effects on let-7 expression were not assessed (20). In our LIN28 depletion experiments, protein levels fell to less than 10% from d3, a change that coincided with reduced cell growth from d3 and increased let-7 levels from d4. These findings are consistent with the observation that LIN28 depletion in carcinoma cells in vitro was not associated with significant increases in let-7 levels until d4 after transfection (40). In addition, the let-7e targets identified in the present study resonate with those seen in other malignancies, suggesting molecular parallels between disparate tumor types (40, 41). Posttranscriptional effects of LIN28/let-7 deregulation on MYCN levels (36) would explain the observations that MYCN is frequently overexpressed in malignant GCTs (42) but shows copy number gain (at 2p23.4) in only approximately 1/3 of adult tumors (43) and less than 1/5 of pediatric cases (44). Interestingly, we found that LIN28 depletion led to increased levels of mature let-7 without reducing levels of pri-let-7. It is likely that levels of pri-let-7 are low even in the presence of abundant LIN28, for example due to degradation after LIN28 binding.

Published OnlineFirst June 17, 2013; DOI: 10.1158/0008-5472.CAN-12-2085

www.aacrjournals.org
Cancer Res; 73(15) August 1, 2013
OF9

Downloaded from cancerres.aacrjournals.org on April 11, 2017. © 2013 American Association for Cancer Research.
likely to be an important further contributor to \textit{LIN28} overexpression \textit{in vivo}.

Our data suggest that \textit{LIN28}/\textit{let-7} interactions are promising targets for novel therapies in malignant GCTs. As well as directly depleting \textit{LIN28}, it may also be possible to overcome the effects of overexpressed \textit{LIN28} on microRNA maturation, for example, by protective small molecule targeting of \textit{pre-let-7} stem-loop binding motifs, inhibition of the TUTase ZCCHC11,
or induction of the stem-loop binding protein KSRP, which promotes maturation of a subset of microRNAs that includes let-7 (50). These indirect interventions would not counteract LIN28 effects on primary transcript processing and may not restore adequate levels of mature let-7 molecules if used in isolation. An alternative strategy is direct replacement of let-7 using mature let-7 mimics. Our data indicate that administering a single member of the let-7 family should restore...
levels of other family members in malignant GCTs by targeting LIN28. The other let-7 members would lead to further reinforcement of LIN28 downregulation, providing a molecular “switch” effect that should result in a sustained reversion of cell phenotype.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.J. Murray, C.A. Siegler, J.E. Hanning, J.J. Groves, C.G. Scarpini, A.J. Enright, J.C. Nicholson, N. Coleman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Murray, C.A. Siegler, J.E. Hanning, D.M. Ward, K.L. Raby, N. Coleman
Writing, review, and/or revision of the manuscript: M.J. Murray, H.K. Saini, J.E. Hanning, J.J. Groves, C.G. Scarpini, A.J. Enright, J.C. Nicholson, N. Coleman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.J. Murray, E.M. Barker, D.M. Ward, K.L. Raby, C.M. Thornton, A.J. Enright
Study supervision: M.J. Murray, J.C. Nicholson, N. Coleman

Acknowledgments
The authors thank Mr. Alex Byford for technical assistance.

Grant Support
This work was supported by Cancer Research-UK programme grant (N. Coleman), Medical Research Council Fellowship (M.J. Murray), and Addenbrooke’s Charitable Trust (M.J. Murray).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 25, 2012; revised May 8, 2013; accepted June 10, 2013; published OnlineFirst June 17, 2013.

References

Figure 7. Luciferase assay confirmation of let-7 targets in malignant GCT cells. Luciferase assay data at d2 for 2102Ep cells transfected with a reporter containing the full-length 3’UTR for LIN28 (red), MYCN (blue), or AURKB (orange). Cells were also transfected with either let-7e or let-7e-mutant (let-7e-mut). Luminescence values were normalized to cells treated with nontargeting oligonucleotides (NT2), then referenced to cells containing a no 3’UTR control reporter and treated with let-7e/let-7e-mutant, as appropriate. Error bars, SEM. All correlation P values were determined by linear regression. *, P < 0.05; **, P < 0.005; ***, P ≤ 0.0001.
Malignant germ cell tumours of the testis: new associations of familial/bilateral and sporadic.


**LIN28 Expression in Malignant Germ Cell Tumors**

**Downregulates let-7 and Increases Oncogene Levels**


*Cancer Res* Published OnlineFirst June 17, 2013.

---

**Updated version**

Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-2085

**Supplementary Material**

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/06/18/0008-5472.CAN-12-2085.DC1

---

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.