Published in final edited form as:
Cancer Res. 2013 August 1; 73(15): 4872–4884. doi:10.1158/0008-5472.CAN-12-2085.

LIN28 expression in malignant germ cell tumors down-regulates *let-7* and increases oncogene levels

Matthew J. Murray[1,2], **Harpreet K. Saini**[3], **Charlotte A. Siegler**[1], **Jennifer E. Hanning**[1], **Emily M. Barker**[1], **Stijn van Dongen**[3], **Dawn M. Ward**[1], **Katie L. Raby**[1], **Ian J. Groves**[1], **Cinzia G. Scarpini**[1], **Mark R. Pett**[1], **Claire M. Thornton**[4], **Anton J. Enright**[3], **James C. Nicholson**[2], and **Nicholas Coleman**[1,5] **on behalf of the CCLG**

[1]Department of Pathology, Cambridge University, UK

[2]Department of Paediatric Oncology, Addenbrooke's Hospital, Cambridge, UK

[3]EMBL European-Bioinformatics-Institute, Hinxton, Cambridge, UK

[4]Department of Pathology, Royal Group of Hospitals Trust, Belfast, UK

[5]Department of Histopathology, Addenbrooke's Hospital, Cambridge, UK

Abstract

Despite their clinico-pathologic heterogeneity, malignant germ-cell-tumors (GCTs) share molecular abnormalities that are likely to be functionally important. In this study, we investigated the potential significance of down-regulation 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, since the sequence complementary to the 2-7nt common *let-7* seed 'GAGGUA' was enriched in the 3′untranslated regions of mRNAs up-regulated 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 up-regulated in malignant-GCT cells, confirming significant negative-correlations with *let-7* levels. Among 16 mRNAs examined in a largely independent set of specimens by qRT-PCR, we defined negative-associations with *let-7e* 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 down-regulate *MYCN, AURKB* and *LIN28,* the latter via a double-negative feedback loop. We concluded that the *LIN28/let-7* pathway has a critical pathobiological role in malignant-GCTs and therefore offers a promising target for therapeutic intervention.

Keywords

germ cell tumor; *let-7*; *LIN28*; microRNA

Correspondence: Dr Matthew Murray; Department of Pathology, Cambridge University, UK; telephone:0044-1223-765066; mjm16@cam.ac.uk.

Conflict-of-interest: none

Europe PMC Funders Author Manuscripts

Introduction

Germ-cell-tumors (GCTs) 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, while 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 (YSTs) and embryonal carcinoma (EC) (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 towards 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 nine members of the *lethal-7* (*let-7*) microRNA family were significantly under-expressed in pediatric malignant-GCTs, when compared with non-malignant control tissues (3). MicroRNAs regulate gene expression via their 5′-seed-region (nucleotides at positions 1-8; 1-8nt), which binds the corresponding seed-complementary-region (SCR) located predominantly in the 3′untranslated region (3′UTR) of mRNA targets (4). Within the seed, 2-7nt are most critical for binding-specificity (5). Importantly, all nine *let-7* microRNAs share the same 2-7nt 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*) (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 (*pri-let-7*) and precursor-hairpins (*pre-let-7*), preventing processing by Drosha and Dicer, 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 *pre-let-7* 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) (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 up-regulation 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 non-malignant-controls, which we previously used for global microRNA profiling (3), and for global mRNA profiling of a subset of 21 cases (3). Across the sample set, the controls (n=8) represented fetal yolk-sac, fetal ovary, pre-pubertal testis, post-pubertal testis, pre-pubertal ovary and post-pubertal ovary. These samples contain germ cells, with variable representation of somatic cells. 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, six germinomas, one EC) and three normal gonadal controls (one pre- and one post-pubertal testis and one post-pubertal ovary) (3).

Set-2—A published dataset of global mRNA expression profiles of 25 samples from a study of adult testicular malignant-GCTs (eight YSTs; 12 germinomas) and controls (five normal adult testes, containing germ cells and somatic cells). Further details are available in the original publication (21) 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 nine YSTs, nine germinomas and three ECs, with all except three being from pediatric patients (<16y). In addition, we used six malignant-GCT cell-lines and five benign teratomas. Twenty-four of the 32 samples overlapped with set-1 and had previously been used for microRNA profiling (3). However, only six 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 1-3, our study encompassed a total of 81 samples, comprising 54 different malignant-GCTs (31 pediatric, 23 adult; 43 gonadal, 11 extragonadal), six malignant-GCT cell-lines and 21 control samples (eight teratomas, 13 gonads/yolk-sac).

MicroRNA and mRNA microarray expression analysis

We re-analyzed microRNA expression profiles for set-1 [obtained using the miRCURY-LNA array-v9.2 (Exiqon, Vedbaek, Denmark)] (3) and mRNA expression profiles for sets-1 and -2 [obtained using the HG-U133A GeneChip (Affymetrix, Santa Clara, CA)] (3, 21). Differential gene expression was assessed using a moderated *t*-statistic and *p*-values adjusted for multiple testing using Benjamini and Hochberg's method (23). MicroRNAs with adjusted *p*-values <0.01 were considered to be significantly differentially-expressed, while mRNAs with $\log_2$ fold-change ≥1.5 and adjusted $p<0.01$ were considered to be over-expressed (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* down-regulation in malignant-GCTs was of biological significance by causing shifts in expression of mRNAs with a 3′UTR *let-7* SCR (3). We performed three 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 genelists were ranked on the *x*-axis from down-regulated (left) to up-regulated (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 up-regulated 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 2-7nt sequence of the microRNA seed-region, summating data for one hexamer (2-7nt), two heptamers (1-7nt, 2-8nt) and one octamer (1-8nt). *P*-values <0.01 were considered significant (3). We tested for enrichment in the up-regulated genes of 3′UTR SCRs corresponding to the 1-8nt seeds of all 126 microRNAs down-regulated 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, Crawley, UK), following reverse transcription of 1μg 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 (Ct) method (26) and normalized using four 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, Warrington, UK). 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, Warrington, UK), as described (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 microRNAs normalized to *RNU24* (3,28) and pri-microRNAs to *RPLO, GUS-B* and *18S*. Reference samples were as for mRNA qRT-PCR. In test amplifications using 0.01nM single-stranded *let-7e* RNA (Sigma Aldrich, Dorset, UK) Taqman qRT-PCR for *let-7b* and *let-7d* showed no cross-reactivity with *let-7e* (data not shown).

Western blotting

Western blots were performed for LIN28, LIN28B, AURKB and MYCN proteins using the antibodies ab46020 (1:10,000 dilution), ab119367 (1:1000), ab2254 (1:5,000) and ab16898 (1:250), respectively. Results were normalized to beta-tubulin (ab6046; 1:10,000). All antibodies were from Abcam (Cambridge, UK). Western blot densitometry was performed using FluorChem-9900 imaging system software (Alpha-Innotech, San Leandro, CA).

GCT cell-lines

We selected four cell-lines that reflected the range of malignant-GCT histologic subtypes commonly observed in clinical practice, namely EC [2102Ep (29)], YST (GCT44 and 1411H) and germinoma/seminoma [TCam2 (30)]. Cells were cultured at 37°C in 5% CO_2 in medium containing 10% fetal-calf-serum (FCS)/1% penicillin/streptomycin, and authenticated using short-tandem-repeat profiling (3).

RNA depletion and over-expression

Transcripts were depleted by RNAi in over-expressing malignant-GCT cell-lines using pools of four separate siRNAs in order 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, Lafayette, CO). Each pool was used at 66.7nM, which represented the minimal concentration of *LIN28* siRNAs that achieved >75% *LIN28* transcript depletion in test experiments (data not shown). All results were normalized to cells treated with a 66.7nM pool of four NTC siRNAs (D-001810-10-05, Dharmacon) (32). We confirmed the specific effects of the *LIN28*-targeting pool using two independent siRNAs (Hs_LIN28_7 and Hs_LIN28_8, both Qiagen; target sequences TAAAGACTTATTGGTACGCAA and CACGCTGTGAGATCACCGCAA, respectively). Differences between experimental observations were assessed using an unpaired, two-sample *t*-test (two-tailed with 95% confidence-intervals). *Let-7e* was replenished in under-expressing malignant-GCT cell-lines using *let-7e* miRIDIAN double-stranded RNA-mimic (C-300479-05) at 100nM (33), normalized to cells treated with 100nM 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 7×10^4 cells/well, 1411H and GCT44 at 1.0×10^5 cells/well and TCam2 1.5×10^5 cells/well. On d0, when cells were ~20% confluent, transfection was performed using Opti-MEM media and Lipofectamine-RNAiMAX (both Invitrogen, Paisley, UK). The optimal length of transfection, which maximized transfection efficiency and minimized toxicity, was 24h for 2102Ep cell-line, 6-8h for 1411H and GCT44, and 4-6h for TCam2. At least three biological replicates were performed 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 performed 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 biological replicates and then taking the mean of the resulting three values. Maximal growth rates were determined by plotting cell numbers at 24h 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, Menlo Park, CA). Test oligonucleotides were *let-7e*, non-targeting RNA (NT2; MIM9002) and a mutant *let-7e* (*let-7e-mutant*; sequence UGAGUGAGGAGGUUGUAUAGUU) in which the 2-7nt 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 performed twice in quadruplicate in 96-well plates, using >80% confluent cells, with 50ng of plasmid/well and 100nM oligonucleotides. Luminescence was quantified on a BioTek Synergy-HT multi-mode microplate reader (BioTek Instruments-Inc, Winooski, VT). 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 nine members of the tumor-suppressor *let-7* microRNA family were significantly down-regulated in pediatric malignant-GCTs, compared with non-malignant tissues (benign GCTs and

normal gonad) (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 under-expressed 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 Figure-S1A), or collectively (Figure-1A).

As individual *let-7* members are transcribed from multiple genomic loci, this observation suggested the possibility of a common post-transcriptional mechanism regulating *let-7* biogenesis in malignant-GCTs. We therefore sought to identify whether *let-7* down-regulation was associated with over-expression of *LIN28*. Using all available mRNA microarray data, we found that *LIN28* was highly expressed in 44/45 (97.8%) of malignant-GCTs from pediatric and adult patients (from sets-1 and -2 combined; Figure-1A), regardless of tumor site (gonadal/extragonadal) 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) (Figure-1B) and with levels of each individual *let-7* family member (all p<0.005) (Supplementary Figure-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 (Figure-1C). Twenty-four of the 27 also showed high expression of *LIN28B* (Figure-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 (Figure-1C). Using Taqman qRT-PCR, we confirmed that *let-7e* (the most significantly down-regulated *let-7* in our microarray analysis) showed low expression in all the malignant-GCT samples/cell-lines (Figure-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 Figure-S2), but not with *ZCCHC11*.

Biological significance of low *let-7* levels in malignant-GCTs

Sylamer showed that ‘TACCTC’ (complementary to the 2-7nt common *let-7* seed ‘GAGGUA’) was the top-ranking SCR in mRNAs significantly up-regulated 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* 1-8nt 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 Figure-S3) and the combined analysis (p=0.0013) (Figure-2A). In all analyses there was no significant enrichment in the up-regulated 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 over-expressed in all malignant-GCTs, for further validation in clinical samples and functional investigation *in vitro*. We identified 198 up-regulated 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 up-regulated 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, Supplementary Figure-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,*

RRM2, AURKB, MKI67, C12orf5, FZD5, KRAS, PGK1, SMAGP, RAB25, RAB15, MRS2, 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 up-regulated 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 over-expressed in some subtypes of malignant-GCT (YSTs and EC), it showed only minimal expression changes in another major subtype, germinoma (Supplementary Figure-S5A,-S5B). The lack of overall association between *HMGA2* and *let-7* levels was also confirmed in this qRT-PCR analysis (p=0.12) (Supplementary Figure-S5C).

Validation of *let-7* mRNA targets

By qRT-PCR analysis of the 32 samples in set-3, we confirmed over-expression of all 16 selected mRNAs in malignant-GCTs, compared with the control samples used (Figure-2B, Supplementary Figure-S6). We identified a negative-association with *let-7e* qRT-PCR levels for six mRNAs (*MYCN, AURKB, CCNF, RRM2, MKI67, C12orf5*) (Supplementary Table-S5, Figure-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, MRS2, PGK1, KRAS* and *LASP1* were over-expressed in malignant-GCTs of particular histologic subtypes (Supplementary Figure-S6), but did not show an association with *let-7e* levels across the whole sample set. The other five mRNAs (*FZD5, SMAGP, RAB15, SLC2A3* and *AGL*) showed no association with *let-7e* qRT-PCR levels (Supplementary Figure-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 non-specific observations. We tested for phenotypic and functional consistencies when: 1) depleting *LIN28* or *LIN28B* by RNAi, using panels of four siRNAs to minimize any off-target effects (31), with separate confirmation using independent siRNAs; 2) directly over-expressing *let-7e* (the *let-7* family member that showed the most significant under-expression *in vivo*; Supplementary Table-S3) using a double-stranded RNA mimic; and 3) depleting *MYCN*, a major *let-7e* target, by RNAi using a panel of four independent siRNAs. In this *in vitro* work, we used four representative malignant-GCT cell-lines, all of which showed *LIN28* up-regulation and *let-7* under-expression (Figure-1C).

In 2102Ep cells, a single treatment with pooled siRNAs depleted *LIN28* mRNA by >90% over a 7d period. There were parallel reductions in protein levels, which fell to <10% from d3 (Figure-3A and Supplementary Figure-S7A). There was no effect on *LIN28B* or *ZCCHC11* mRNA levels on d1-d3 (data not shown). The reductions in LIN28 protein levels were mirrored by changes in cell proliferation, which fell from d3 (Figure-3B). There was a 36% increase in mean population doubling time over the 7d time-course (33.9h *vs.* 24.9h, respectively).

In keeping with these observations, levels of *let-7e* started increasing from d3, with significant changes from d4 (Figure-3C). The specificity of the effects of the pooled *LIN28*

siRNAs on cell proliferation and *let-7e* levels was confirmed using two independent siRNAs (Supplementary Figure-S8). The pooled *LIN28* siRNAs also caused increases in other representative *let-7* family members (*let-7b* and *let-7d*), when assessed at d4 and d7 (Figure-3D). There was no difference in levels of the *pri-let-7e* precursor at d7 (data not shown). Of the selected *let-7* mRNA targets (Figure-2B), all six showed decreased levels on d4 and d7 (time-points at which growth-inhibition was observed), compared with d2 (when no growth-inhibition was seen). Most of the decreases were statistically significant (Figure-3D), with the most significant reduction being for *MYCN*, where transcript levels were lowered by 62% at d4 versus d2 ($p<0.0001$) (Figure-3D).

We attempted to deplete *LIN28* in three other malignant-GCT cell-lines, assessing the effects at d4 post-transfection, based on our findings in 2102Ep. While we could achieve 87% transcript depletion in TCam2, cell toxicity restricted the levels of depletion achieved in 1411H and GCT44 to 57% and 46%, respectively. There were parallel reductions in protein levels, as measured at d4 following siRNA treatment (Supplementary Figure-S7B), with a significant positive correlation between *LIN28* mRNA and protein levels across the four cell-lines ($R^2=0.88$, $p<0.0001$) (Supplementary Figure-S7C). The *LIN28* reductions observed in 1411H and GCT44 were not sufficient to affect cell numbers, compared with NTC-treated cells (Figure-4A and Supplementary Figure-7D). However, across all four cell-lines, there were significant positive-correlations between levels of *LIN28* and *MYCN* transcripts ($R^2=0.78$, $p=0.0001$) (Figure-4B) and between cell proliferation (quantifying cell numbers at d4) and transcript levels of *LIN28* ($R^2=0.85$, $p<0.0001$) and *MYCN* ($R^2=0.78$, $p<0.0001$) (Figure-4A).

We next depleted *LIN28B* in 2102Ep and TCam2, achieving >80% transcript depletion and >85% protein depletion by d4 (Figure-5A,-5B). In contrast to the effects of *LIN28* depletion (Figure-3D), we observed only modest increases in levels of *let-7b, let-7d* and *let-7e* (1.21-1.96-fold) (Figure-5C), with no effect on *MYCN* levels or cell proliferation (Figure-5A).

Restoration of *let-7* levels

Transfection with *let-7e* mimic produced the greatest increases in *let-7e* levels in 2102Ep and 1411H (Figure-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 $p\leq0.001$) (Figure-6A). Over a 3d time-course in 2102Ep, we observed reduced levels of *MYCN, AURKB, LIN28* and *LIN28B* transcripts (Supplementary Figure-S9A), with a significant negative correlation between mean transcript depletion over d1-d3 and the number of 3′UTR *let-7* SCRs ($R^2=0.97$, $p=0.0145$) (Supplementary Figure-S9B). Levels of all four proteins were reduced when assessed on d2 and d3 (Figure-6B,-6C). Overall, across the four malignant-GCT cell-lines examined, there was a significant negative-correlation between *let-7e* levels obtained following *let-7e* mimic transfection and cell proliferation ($R^2=0.94$; $p<0.0001$) (Figure-6D).

The *LIN28* and *LIN28B* depletion (Figure-6A, Supplementary Figure-S9A) were explained by the presence in 3′UTRs of the ‘TACCTC’ SCR for the common 2-7nt *let-7* seed, with one copy in *LIN28* (Supplementary Figure-S9C) and five copies in *LIN28B* (data not shown). In keeping with the reduced *LIN28* levels, we observed ~15-30 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) (Figure-6E). There was no increase in levels of a control microRNA (miR-492) lacking the LIN28 ‘GGAG’ binding site in its stem-loop (Figure-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 non-targeting oligonucleotides, in cells containing the 3′UTR for *LIN28* (p=0.0003), *MYCN* (p=0.011) or *AURKB* (p<0.0001), while there were no reductions with mutant *let-7e* (Figure-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 2-7nt 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 up-regulation of *MYCN* in both pediatric (set-1; n=20) and adult (set-2; n=25) malignant-GCTs, compared with the controls used. There was a significant positive correlation with *LIN28* levels in both sets (p<0.0001 and p=0.001, respectively) (Supplementary Figure-S10). In contrast, *CMYC* levels showed no elevation and no positive correlation with *LIN28* in either set (Supplementary Figure-S10). As *LIN28B* was not represented on the microarray used to profile mRNAs in the pediatric malignant-GCTs, we used qRT-PCR analysis of set-3 (n=32) to show significant positive correlations between *MYCN* and both *LIN28* and *LIN28B* in malignant-GCTs (p=0.0018 and p=0.0121, respectively). While depleting *MYCN* in 2102Ep and TCam2 reduced cell numbers, it had no consistent effects on levels of *LIN28* or *LIN28B* (Supplementary Figure-S11). Together, these data supported our evidence that *MYCN* is an important up-regulated *let-7* target in malignant-GCT cells, but argued against a significant effect of *MYCN* or *CMYC* upstream of *LIN28*/*LIN28B*.

Discussion

This study demonstrates 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 down-regulation (Supplementary Figure-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 pro-malignant 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 down-regulation of stem-cell markers (e.g. *OCT4/POU5F1* and *NANOG*) and induction of differentiation, although effects on *let-7* expression were not assessed (20). In our *LIN28* depletion experiments, protein levels fell to <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). Post-transcriptional effects of *LIN28/let-7* deregulation on *MYCN* levels (36) would explain the observations that *MYCN* is frequently over-expressed in malignant-GCTs (42) but shows copy-number-gain (at 2p23.4) in only ~1/3 of adult tumors (43) and <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.

As well as down-regulation of *let-7* by LIN28, we observed a reciprocal effect, with down-regulation of *LIN28* by *let-7e*, via a *let-7* SCR in the *LIN28* 3′UTR (45,46). In malignant-GCT cells, *let-7e*-mediated down-regulation of *LIN28* produced specific effects, by increasing other *let-7* family targets of LIN28 rather than producing a more generalized effect on microRNA biogenesis. Other microRNAs known to down-regulate *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 four were identified in our previous profiling study as being universally under-expressed in malignant-GCTs, compared to the control tissues used (3). As copy-number-gain at the *LIN28* locus (1p36.11) is not a feature of malignant-GCTs (44), down-regulation of these microRNAs is likely to be an important further contributor to *LIN28* over-expression *in vivo*.

Our data suggest that *LIN28*/*let-7* interactions are promising targets for novel therapies in malignant-GCTs. As well as directly depleting *LIN28*, it may also be possible to overcome the effects of over-expressed *LIN28* on microRNA maturation, for example, by protective small molecule targeting of *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* down-regulation, providing a molecular ‘switch’ effect that should result in a sustained reversion of cell phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Mr Alex Byford for technical assistance.

Financial support:

Cancer Research-UK programme grant (NC), Medical Research Council Fellowship (MJM) and Addenbrooke’s Charitable Trust (MJM).

References

1. Murray MJ, Nicholson JC. Germ Cell Tumours in Children and Adolescents. Paediatrics and Child Health. 2010; 20:109–16.
2. Huyghe E, Matsuda T, Thonneau P. Increasing incidence of testicular cancer worldwide: a review. J Urol. 2003; 170:5–11. [PubMed: 12796635]
3. Palmer RD, Murray MJ, Saini HK, van Dongen S, Abreu-Goodger C, Muralidhar B, et al. Malignant germ cell tumors display common microRNA profiles resulting in global changes in expression of messenger RNA targets. Cancer Res. 2010; 70:2911–23. [PubMed: 20332240]
4. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell. 2003; 115:787–98. [PubMed: 14697198]
5. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120:15–20. [PubMed: 15652477]

6. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004; 64:3753–6. [PubMed: 15172979]
7. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res. 2007; 67:7713–22. [PubMed: 17699775]
8. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008; 320:97–100. [PubMed: 18292307]
9. Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, et al. Lin28 promotes transformation and is associated with advanced human malignancies. Nat Genet. 2009; 41:843–8. [PubMed: 19483683]
10. Hagan JP, Piskounova E, Gregory RI. Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. Nat Struct Mol Biol. 2009; 16:1021–5. [PubMed: 19713958]
11. Viswanathan SR, Daley GQ. Lin28: A microRNA regulator with a macro role. Cell. 2010; 140:445–9. [PubMed: 20178735]
12. Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell. 2009; 138:696–708. [PubMed: 19703396]
13. Loughlin FE, Gebert LF, Towbin H, Brunschweiger A, Hall J, Allain FH. Structural basis of pre-let-7 miRNA recognition by the zinc knuckles of pluripotency factor Lin28. Nat Struct Mol Biol. 2012; 19:84–9. [PubMed: 22157959]
14. Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol Cell. 2008; 32:276–84. [PubMed: 18951094]
15. Peng S, Maihle NJ, Huang Y. Pluripotency factors Lin28 and Oct4 identify a sub-population of stem cell-like cells in ovarian cancer. Oncogene. 2010; 29:2153–9. [PubMed: 20101213]
16. West JA, Viswanathan SR, Yabuuchi A, Cunniff K, Takeuchi A, Park IH, et al. A role for Lin28 in primordial germ-cell development and germ-cell malignancy. Nature. 2009; 460:909–13. [PubMed: 19578360]
17. Cao D, Allan RW, Cheng L, Peng Y, Guo CC, Dahiya N, et al. RNA-binding protein LIN28 is a marker for testicular germ cell tumors. Hum Pathol. 2011; 42:710–8. [PubMed: 21288558]
18. Cao D, Liu A, Wang F, Allan RW, Mei K, Peng Y, et al. RNA-binding protein LIN28 is a marker for primary extragonadal germ cell tumors: an immunohistochemical study of 131 cases. Mod Pathol. 2011; 24:288–96. [PubMed: 21057460]
19. Xue D, Peng Y, Wang F, Allan RW, Cao D. RNA-binding protein LIN28 is a sensitive marker of ovarian primitive germ cell tumours. Histopathology. 2011; 59:452–9. [PubMed: 22034885]
20. Gillis AJ, Stoop H, Biermann K, van Gurp RJ, Swartzman E, Cribbes S, et al. Expression and interdependencies of pluripotency factors LIN28, OCT3/4, NANOG and SOX2 in human testicular germ cells and tumours of the testis. Int J Androl. 2011; 34:e160–74. [PubMed: 21631526]
21. Korkola JE, Houldsworth J, Chadalavada RS, Olshen AB, Dobrzynski D, Reuter VE, et al. Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. Cancer Res. 2006; 66:820–7. [PubMed: 16424014]
22. Mills, SE. Sternbergs Histology for Pathologists. Lippincott, Williams and Wilkins; New York: 2006. 3rd revised ed
23. Benjamini YHY. Controlling the false discovery rate – a practical and powerful approach to multiple testing. J R Stat Soc Ser B-Methodol. 1995; 57:289–300.
24. van Dongen S, Abreu-Goodger C, Enright AJ. Detecting microRNA binding and siRNA off-target effects from expression data. Nat Methods. 2008; 5:1023–5. [PubMed: 18978784]
25. Primer 3.[cited 26th August 2012]; Available from: http://frodo.wi.mit.edu/primer3/
26. Herdman MT, Pett MR, Roberts I, Alazawi WO, Teschendorff AE, Zhang XY, et al. Interferon-beta treatment of cervical keratinocytes naturally infected with human papillomavirus 16 episomes

promotes rapid reduction in episome numbers and emergence of latent integrants. Carcinogenesis. 2006; 27:2341–53. [PubMed: 16973673]

27. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002; 3:RESEARCH0034. [PubMed: 12184808]
28. Murray MJ, Saini HK, van Dongen S, Palmer RD, Muralidhar B, Pett MR, et al. The two most common histological subtypes of malignant germ cell tumour are distinguished by global microRNA profiles, associated with differential transcription factor expression. Mol Cancer. 2010; 9:290. [PubMed: 21059207]
29. Damjanov I, Andrews PW. Ultrastructural differentiation of a clonal human embryonal carcinoma cell line in vitro. Cancer Res. 1983; 43:2190–8. [PubMed: 6187445]
30. de Jong J, Stoop H, Gillis AJ, Hersmus R, van Gurp RJ, van de Geijn GJ, et al. Further characterization of the first seminoma cell line TCam-2. Genes Chromosomes Cancer. 2008; 47:185–96. [PubMed: 18050305]
31. Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, et al. 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Methods. 2006; 3:199–204. [PubMed: 16489337]
32. Muralidhar B, Winder D, Murray M, Palmer R, Barbosa-Morais N, Saini H, et al. Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. J Pathol. 2011; 224:496–507. [PubMed: 21590768]
33. Li Z, Wu F, Brant SR, Kwon JH. IL-23 receptor regulation by Let-7f in human CD4+ memory T cells. J Immunol. 2011; 186:6182–90. [PubMed: 21508257]
34. Pett MR, Alazawi WO, Roberts I, Dowen S, Smith DI, Stanley MA, et al. Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. Cancer Res. 2004; 64:1359–68. [PubMed: 14973079]
35. Boyerinas B, Park SM, Shomron N, Hedegaard MM, Vinther J, Andersen JS, et al. Identification of let-7-regulated oncofetal genes. Cancer Res. 2008; 68:2587–91. [PubMed: 18413726]
36. Buechner J, Tomte E, Haug BH, Henriksen JR, Lokke C, Flaegstad T, et al. Tumour-suppressor microRNAs let-7 and mir-101 target the proto-oncogene MYCN and inhibit cell proliferation in MYCN-amplified neuroblastoma. Br J Cancer. 2011; 105:296–303. [PubMed: 21654684]
37. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature. 2008; 455:58–63. [PubMed: 18668040]
38. Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, Dews M, et al. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. Proc Natl Acad Sci U S A. 2009; 106:3384–9. [PubMed: 19211792]
39. Cotterman R, Knoepfler PS. N-Myc regulates expression of pluripotency genes in neuroblastoma including lif, klf2, klf4, and lin28b. PLoS One. 2009; 4:e5799. [PubMed: 19495417]
40. Helland A, Anglesio MS, George J, Cowin PA, Johnstone CN, House CM, et al. Deregulation of MYCN, LIN28B and LET7 in a molecular subtype of aggressive high-grade serous ovarian cancers. PLoS One. 2011; 6:e18064. [PubMed: 21533284]
41. Molenaar JJ, Domingo-Fernandez R, Ebus ME, Lindner S, Koster J, Drabek K, et al. LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. Nat Genet. 2012 In Press.
42. Alagaratnam S, Lind GE, Kraggerud SM, Lothe RA, Skotheim RI. The testicular germ cell tumour transcriptome. Int J Androl. 2011; 34:e133–50. discussion e50-1. [PubMed: 21651573]
43. Kraggerud SM, Skotheim RI, Szymanska J, Eknaes M, Fossa SD, Stenwig AE, et al. Genome profiles of familial/bilateral and sporadic testicular germ cell tumors. Genes Chromosomes Cancer. 2002; 34:168–74. [PubMed: 11979550]
44. Palmer RD, Foster NA, Vowler SL, Roberts I, Thornton CM, Hale JP, et al. Malignant germ cell tumours of childhood: new associations of genomic imbalance. Br J Cancer. 2007; 96:667–76. [PubMed: 17285132]
45. Chen AX, Yu KD, Fan L, Li JY, Yang C, Huang AJ, et al. Germline genetic variants disturbing the Let-7/LIN28 double-negative feedback loop alter breast cancer susceptibility. PLoS Genet. 2011; 7:e1002259. [PubMed: 21912531]

46. Yang X, Lin X, Zhong X, Kaur S, Li N, Liang S, et al. Double-negative feedback loop between reprogramming factor LIN28 and microRNA let-7 regulates aldehyde dehydrogenase 1-positive cancer stem cells. Cancer Res. 2010; 70:9463–72. [PubMed: 21045151]
47. Zhong X, Li N, Liang S, Huang Q, Coukos G, Zhang L. Identification of microRNAs regulating reprogramming factor LIN28 in embryonic stem cells and cancer cells. J Biol Chem. 2010; 285:41961–71. [PubMed: 20947512]
48. Wu L, Belasco JG. Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. Mol Cell Biol. 2005; 25:9198–208. [PubMed: 16227573]
49. Li X, Zhang J, Gao L, McClellan S, Finan MA, Butler TW, et al. MiR-181 mediates cell differentiation by interrupting the Lin28 and let-7 feedback circuit. Cell Death Differ. 2012; 19:378–86. [PubMed: 21979467]
50. Trabucchi M, Briata P, Garcia-Mayoral M, Haase AD, Filipowicz W, Ramos A, et al. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature. 2009; 459:1010–4. [PubMed: 19458619]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

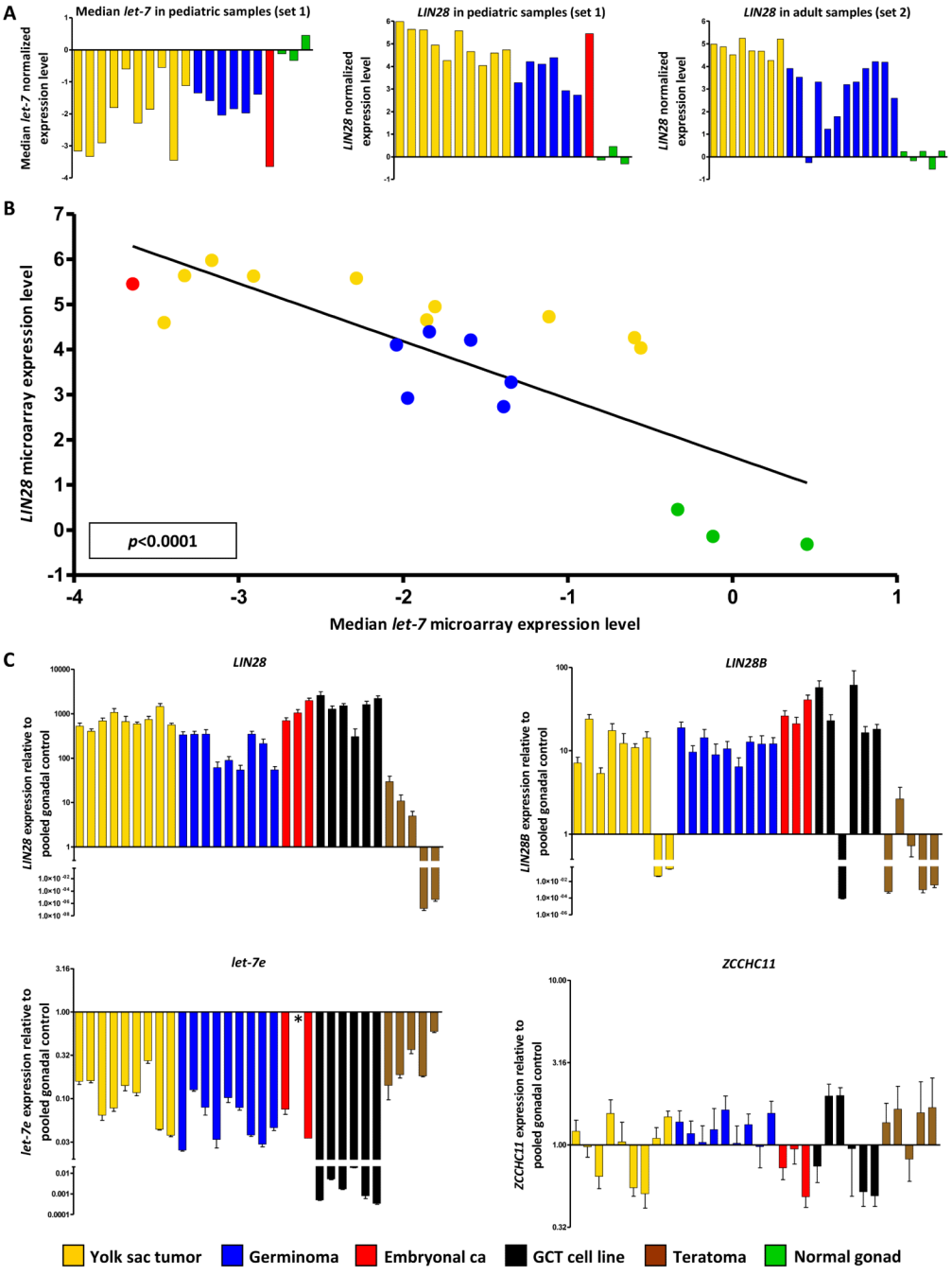

Figure-1. *Let-7* and *LIN28* expression in malignant-GCTs

A) The left and middle panels respectively show median levels of all nine *let-7* family members and levels of *LIN28* in the 20 samples from set-1 with matching microRNA and mRNA data. The right panel shows levels of *LIN28* in sample set-2. B) Linear-regression analysis of median *let-7* family levels versus *LIN28* levels in the set-1 samples with matching data. In A) and B), all values are referenced to the mean of the normal gonadal samples. C) qRT-PCR validation in sample set-3, referenced to the pooled normal gonadal control sample. Error-bars= standard error of the mean (SEM). For one sample (asterisked) there was insufficient RNA for *let-7e* quantification. The color-code for all panels is shown in the key. For details of the normal gonadal controls, see Materials and Methods.

Europe PMC Funders Author Manuscripts

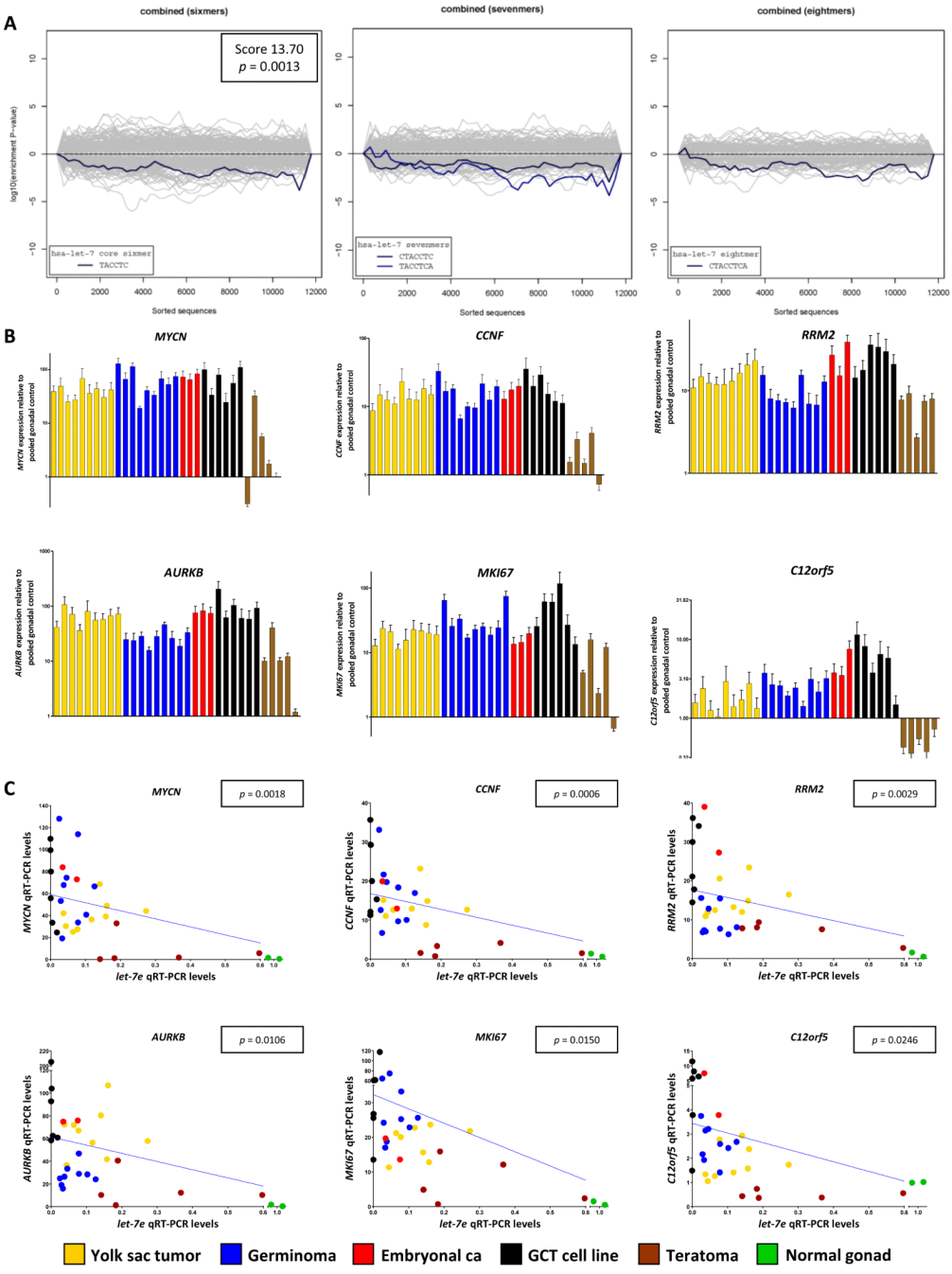

Figure-2. Significance of *let-7* down-regulation in malignant-GCTs

A) *Sylamer* landscape plots for SCR words corresponding to the common seed of the nine *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 up-regulated genes. The left-hand plot shows data for the hexamer complementary to the core 2-7nt component of the common seed-region, the central plot the two heptamers (1-7nt; 2-8nt), and the right-hand plot the octamer (1-8nt). The single summed significance score and *p*-value for all four SCR words is shown. B) Levels of the six 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.

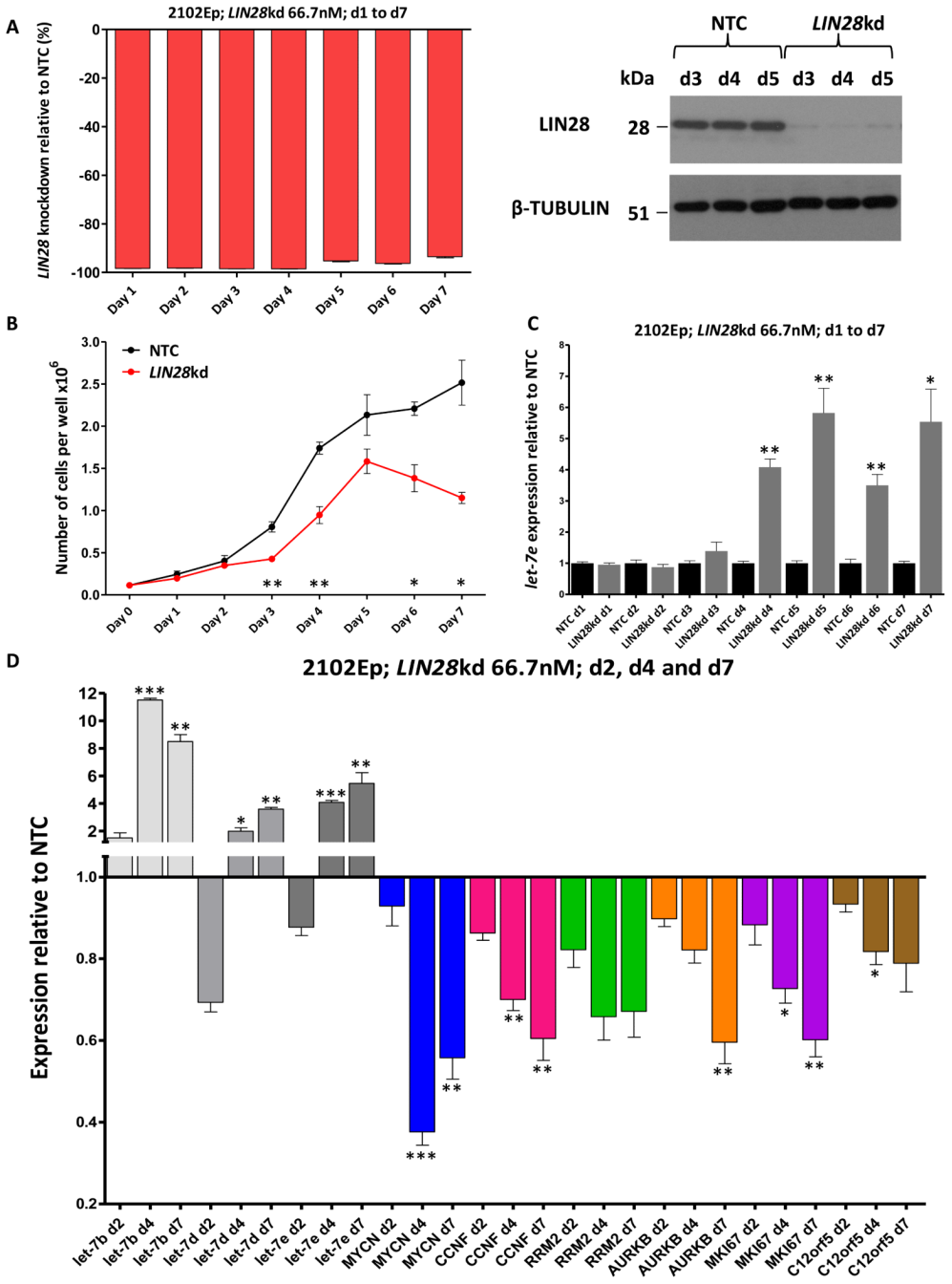

Figure-3. *LIN28* depletion in 2102Ep malignant-GCT cells

A) Depletion of *LIN28*, measured by qRT-PCR over d1-d7 (left) and by Western blot over d3-d5 (right). NTC=non-targeting-control siRNA, kd=knockdown. B) Cell numbers following *LIN28* depletion. C) Levels of *let-7e* over d1-d7 following *LIN28* depletion. In A) to C), statistical comparisons are versus NTC-treated cells. d=day. D) Levels of *let-7* family members (*let-7b, let-7d* and *let-7e*) and the six selected *let-7* mRNA targets at d2, d4 and d7 following *LIN28* depletion. Expression values are referenced to NTC-treated cells. Statistical comparisons are for *LIN28*kd cells at d4 and d7, versus *LIN28*kd cells at d2. Error-bars=SEM. In panels B, C and D, *=$p<0.05$;**=$p<0.005$;***=$p\leq0.0001$.

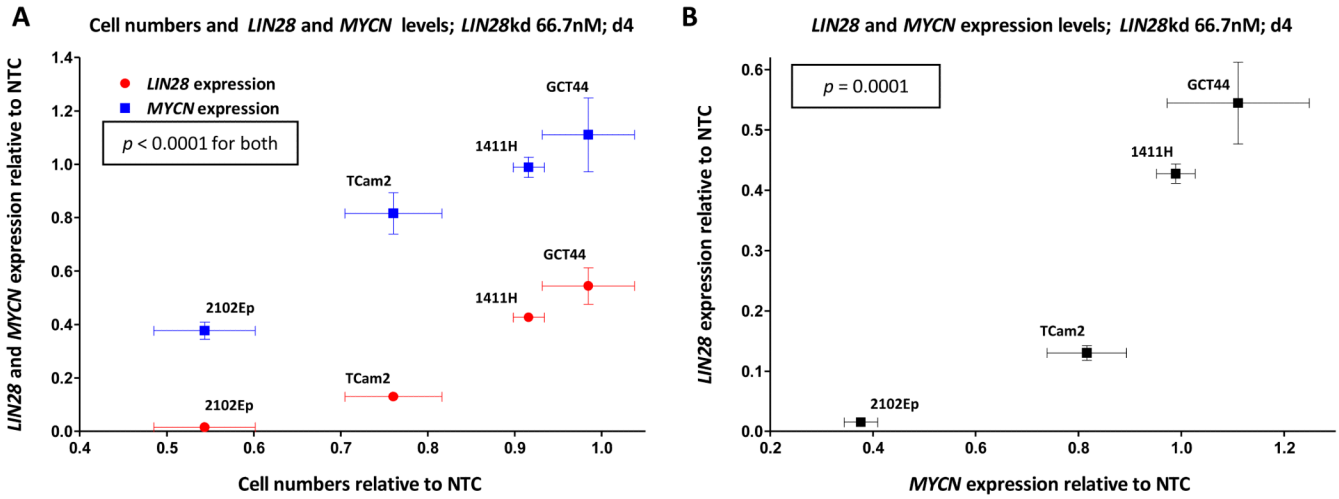

Figure-4. Correlations between *LIN28*, *MYCN* and cell numbers following *LIN28* depletion

The graphs show data for all four malignant-GCT cell-lines at d4 following *LIN28* depletion, compared with NTC-treated cells. Panel A shows cell numbers versus the levels of *LIN28* (red) and *MYCN* (blue), while panel B shows levels of *MYCN* versus *LIN28*. NTC=non-targeting-control siRNA, kd=knockdown. Correlation *p*-values were determined by linear-regression. Error-bars=SEM.

Europe PMC Funders Author Manuscripts

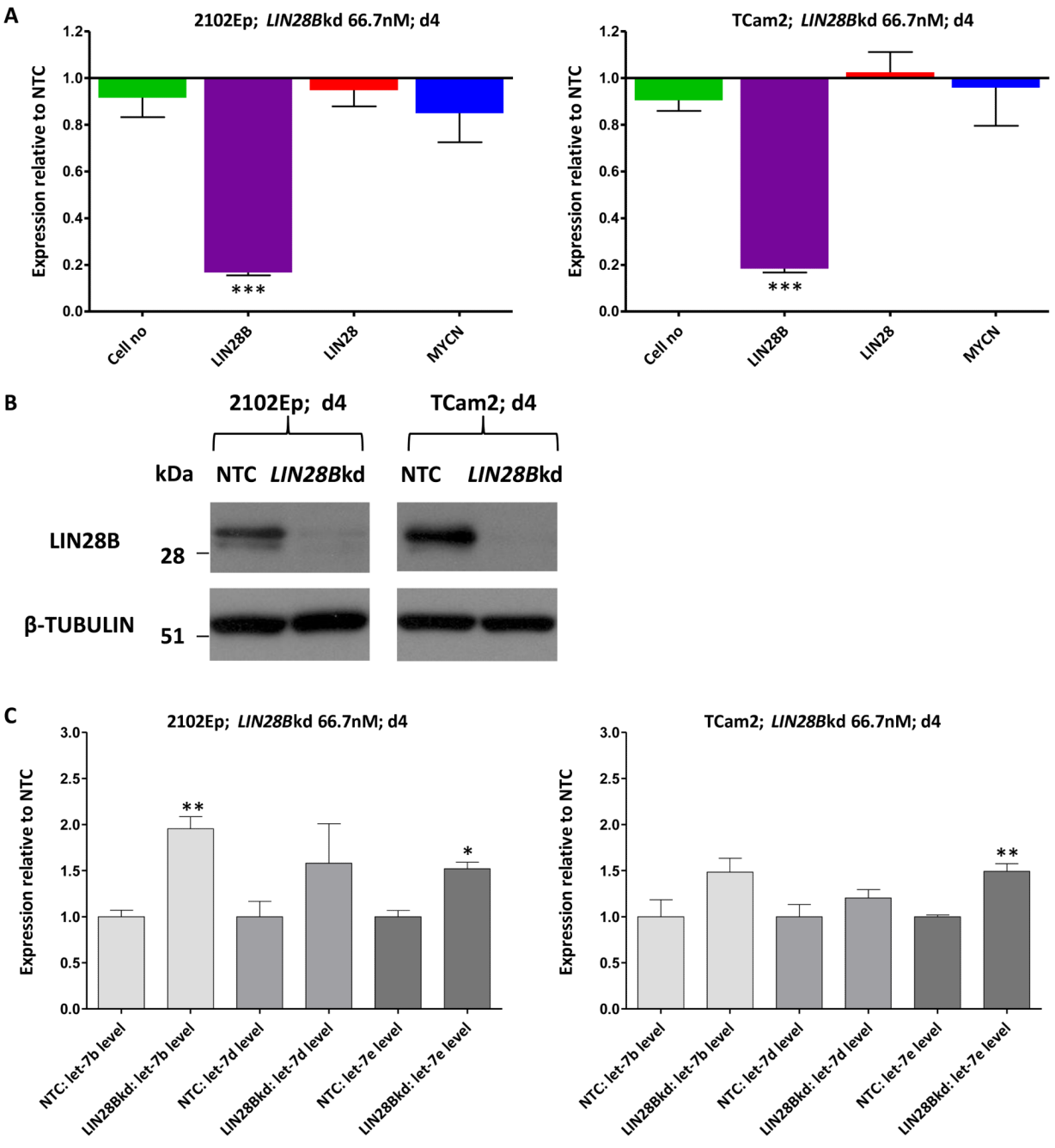

Figure-5. *LIN28B* depletion in malignant-GCT cells

A) Cell numbers and qRT-PCR expression levels of *LIN28B, LIN28* and *MYCN* on d4 following *LIN28B* depletion in 2102Ep (left) and TCam2 (right). B) Western blots showing expression of LIN28B on d4 following *LIN28B* depletion in 2102Ep (left) and TCam2 (right), compared with NTC-treated cells. C) Levels of representative *let-7* family members (*let-7b, let-7d* and *let-7e*) on d4 following *LIN28B* depletion in 2102Ep (left) and TCam2 (right). All values are referenced to NTC-treated cells. NTC=non-targeting-control siRNA, kd=knockdown, error-bars=SEM. *=$p<0.05$;**=$p<0.005$;***=$p\leq0.0001$.

Europe PMC Funders Author Manuscripts

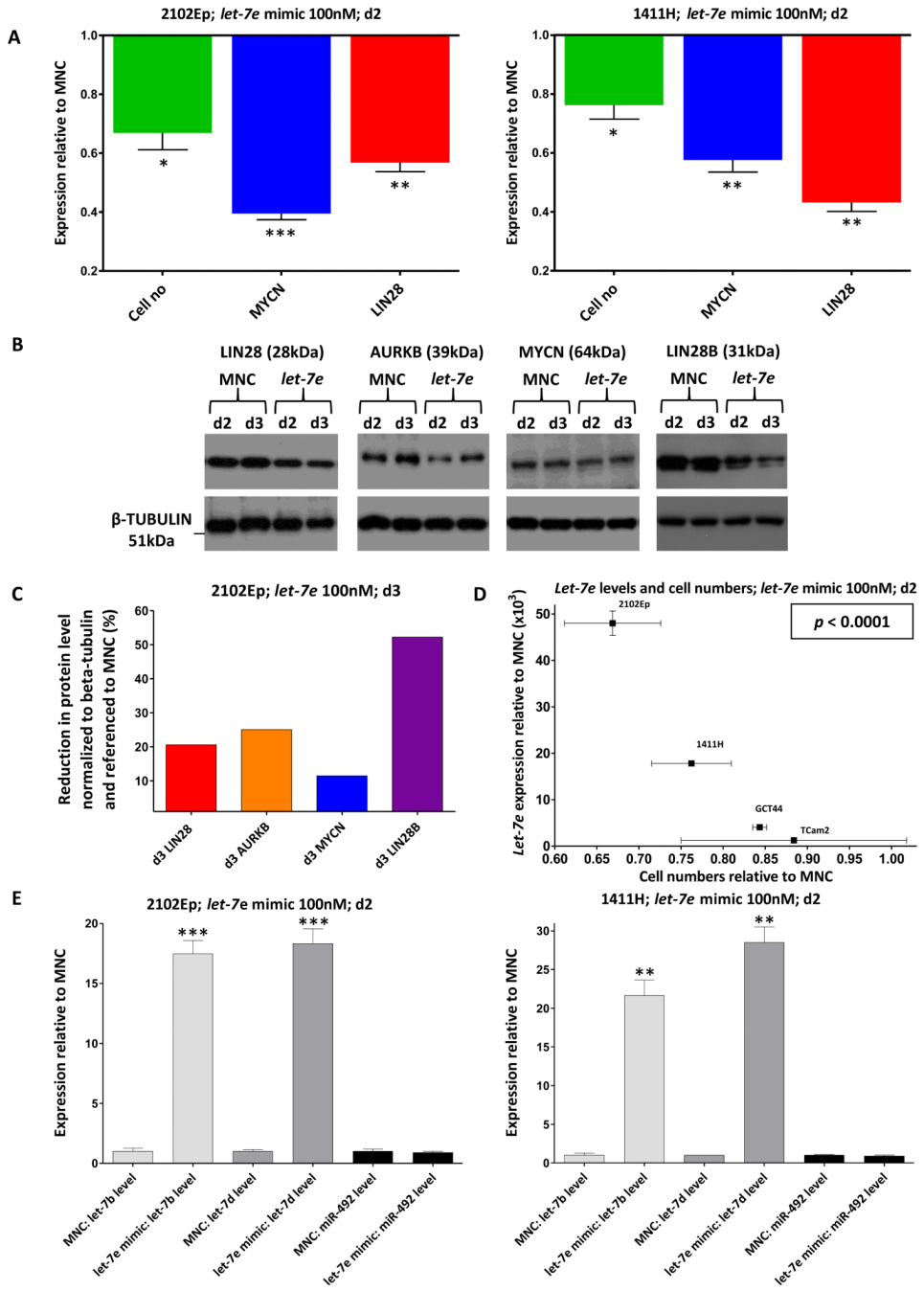

Figure-6. Effects of *let-7e* mimic in malignant-GCT cells

A) Cell numbers and expression levels of *MYCN* and *LIN28* in 2102Ep (left) and 1411H (right), at d2 post-transfection of *let-7e* mimic, relative to cells treated with mimic-negative-control (MNC) RNA. B) Western blots showing expression of LIN28, AURKB, MYCN and LIN28B proteins at d2 and d3 following *let-7e* mimic transfection of 2102Ep cells, corresponding to Supplementary Figure-S9A. The lower row shows the beta-tubulin loading-control. C) The graph shows protein levels at d3, as determined by densitometry of the western blots shown in B), normalized to beta-tubulin and referenced to MNC-treated cells. D) Cell numbers versus *let-7e* levels at d2 in four different malignant-GCT cell-lines, compared to MNC-treated cells. The *p*-value was determined by linear-regression. E) Levels

of other representative *let-7* family members (*let-7b* and *let-7d*) and control miR-492, at d2 following *let-7e* transfection in 2102Ep (left) and 1411H (right), relative to MNC-treated cells.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

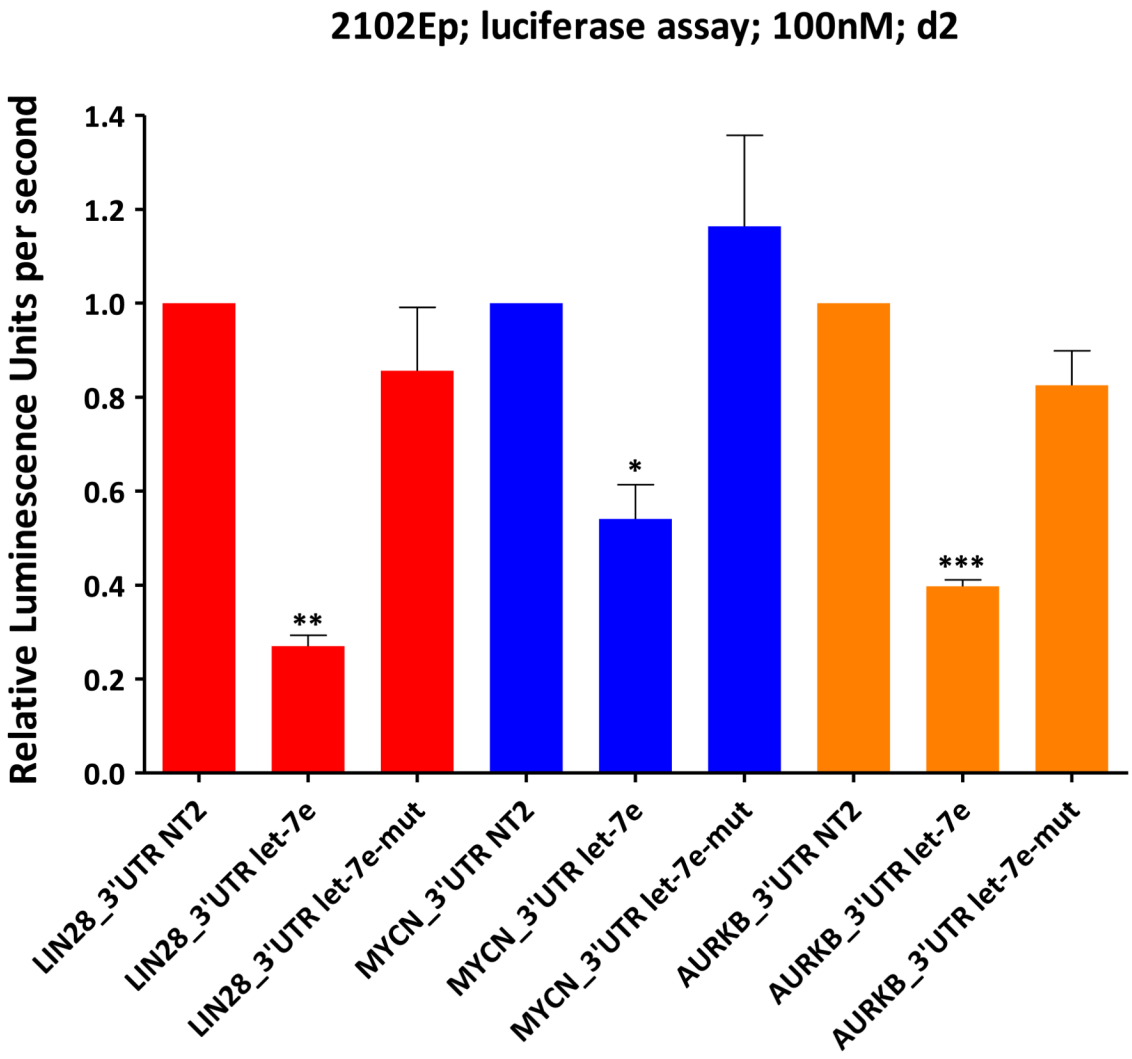

Figure-7. Luciferase assay confirmation of *let-7* targets in malignant-GCT cells

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 non-targeting 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. *=$p<0.05$;**=$p<0.005$;***=$p\leq 0.0001$.