

## The Oncogenic Roles of *DICER1* RNase IIIb Domain Mutations in Ovarian Sertoli-Leydig Cell Tumors<sup>1,2</sup>

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### Abstract

DICER1, an endoribonuclease required for microRNA (miRNA) biogenesis, is essential for embryogenesis and the development of many organs including ovaries. We have recently identified somatic hotspot mutations in RNase IIIb domain of *DICER1* in half of ovarian Sertoli-Leydig cell tumors, a rare class of sex-cord stromal cell tumors in young women. These hotspot mutations lost IIIb cleavage activity of DICER1 *in vitro* and failed to produce 5p-derived miRNAs in mouse *Dicer1*-null ES cells. However, the oncogenic potential of these hotspot *DICER1* mutations has not been studied. Here, we further revealed that the global expression of 5p-derived miRNAs was dramatically reduced in ovarian Sertoli-Leydig cell tumors carrying DICER1 hotspot mutations compared with those without DICER1 hotspot mutation. The miRNA production defect was associated with the deregulation of genes controlling cell proliferation and the cell fate. Using an immortalized human granulosa cell line, SVOG3e, we determined that the D1709N-DICER1 hotspot mutation failed to produce 5p-derived miRNAs, deregulated the expression of several genes that control gonadal differentiation and cell proliferation, and promoted cell growth. Re-expression of let-7 significantly inhibited the growth of D1709N-DICER1 SVOG3e cells, accompanied by the suppression of key regulators of cell cycle control and ovarian gonad differentiation. Taken together, our data revealed that DICER1 hotspot mutations cause systemic loss of 5p-miRNAs that can both drive pseudodifferentiation of testicular elements and cause oncogenic transformation in the ovary.

*Neoplasia* (2015) 17, 650–660

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<sup>1</sup>Disclosure/conflict of interest: none.

<sup>2</sup>This work was supported by a Canadian Institute of Health Research grant (MOP-130244) and a Terry Fox New Frontiers Program Project grant (1021). Y.W.

was funded through fellowships from Canadian Institute of Health Research and Michael Smith Foundation for Health Research.

Received 4 June 2015; Revised 11 August 2015; Accepted 17 August 2015

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<http://dx.doi.org/10.1016/j.neo.2015.08.003>

## Introduction

Ovarian Sertoli-Leydig cell tumors (SLCTs) are a rare type of sex-cord stromal tumors (SCSTs) in the ovary, accounting for less than 0.5% of all ovarian tumors [1]. SLCTs, occurring in young women with the median age of diagnosis around 28 years old, are often associated with androgenic manifestations and pelvic mass [2,3]. Immunohistochemistry markers, such as EMA, Melan-A, and inhibin, are often useful in distinguishing SLCTs from other malignancies, although proper diagnosis of SLCTs can sometime remain challenging because of the lack of unique genomic features [3]. The prognosis of SLCTs correlates with the degree of histologic differentiation of the tumors [3,4]. Although surgery is the primary treatment for SLCT patients, intermediate and poorly differentiated SLCTs can recur and need effective postoperative treatment [2,4].

SLCTs of the ovary contain Sertoli cells and Leydig cells, both of which are somatic cells in male gonads. Thus, SLCTs of the ovary represents a pseudo-male gonadal genesis in the ovary. Using a laser capture microdissection method, Emerson et al. demonstrated that both Sertoli cells and Leydig cells in ovarian SLCTs shared common molecular features at several genomic loci, indicating that they are possibly derived from the same primitive cells during neoplastic transformation [5]. Significant ultrastructural and histologic similarities have been observed between Sertoli cells of SLCTs and neoplastic granulosa cells using electron microscopy and immunohistochemistry [6,7], suggesting that Sertoli cells in SLCTs may derive from primitive cells that normally differentiate into granulosa cells (pregranulosa cells) in the ovarian gonad [8,9]. However, it remains unclear how the differentiation of the primitive cells is rewired to stimulate the production of Sertoli and Leydig cells in the ovary. Studies of a few cases of SLCTs suggested that SRY-independent induction of SOX9 expression might contribute to the pseudogonadal biogenesis [7,10]. However, the significance of these studies needs to be determined in a large cohort of SLCTs.

Recently, we and others have discovered that more than 50% ovarian SLCTs harbor somatic heterozygous mutations at one of the five hotspot sites (E1705, D1709, E1788, D1810, or E1813) in the metal-binding catalytic cleft of the DICER1 RNase IIIb endoribonuclease domain [11,12]. DICER1 plays a crucial role in the maturation of microRNAs (miRNAs), a group of noncoding small RNA species that regulate gene expression posttranscriptionally [13]. Importantly, tumor cells with *DICER1* hotspot mutations often have loss of function defects in the other allele due to germline or other somatic events [11], suggesting that the allele with the hotspot mutation is the primary functional allele in miRNA biogenesis. Using *in vitro* DICER1 cleavage assays and *ex vivo* isogenic cell lines expressing DICER1 variants in *dicer1*-null mouse embryonic stem cells, we and others concluded that the hotspot RNase IIIb domain mutation of DICER1 leads to loss of RNase IIIb enzymatic activity and subsequent loss of miRNAs generated from 5p strand of miRNA precursors [11,14,15]. Similarly, biallelic mutations of *DICER1* have been identified in other tumors, such as a subset of Wilms tumors and pleuropulmonary blastoma [16,17]. However, whether hotspot mutations in the RNase IIIb domain of DICER1 alter miRNA and gene expression in SLCTs and how these hotspot mutations promote oncogenic transformation in specific tissue types are unknown.

In this study, we analyzed the global miRNA and gene expression in SLCTs with and without DICER1 hotspot mutations. We demonstrated that DICER1 hotspot mutations were associated with the global reduction of 5p-derived miRNAs in ovarian SLCTs as well

as with the deregulation of genes governing cell proliferation and differentiation in ovary. Using an immortalized human granulosa cell line, SVOG3e, we demonstrated that a DICER1 hotspot mutation promoted cell proliferation and regulated the expression of cell proliferation and differentiation genes, partially through silencing the expression of the let-7 miRNA family.

## Material and Methods

### Patient Samples and RNA Extraction

All the tumor samples were collected from the Ovarian Cancer Research Program tissue bank in Vancouver, British Columbia, Canada. Ethics approvals for collection and use of the patient samples were obtained from the University of British Columbia and BC Cancer Agency Research Ethics Board [11]. RNA was extracted using the miRNeasy or the miRNeasy formalin-fixed, paraffin-embedded (FFPE) kits (Qiagen) according to the manufacturer's protocols.

### Small RNA Sequencing

Total RNA was quantified with the Qubit RNA BR Assay Kit (Life Technologies) and diluted to 200 ng/μl. Small RNA libraries were prepared using the TruSeq Small RNA Sample Preparation Kit from Illumina according to the manufacturer's instructions. Briefly, total RNA was ligated to 3' and 5' adapters and subsequently reverse transcribed to produce a single-stranded cDNA. cDNA was amplified, and index adapters were added by polymerase chain reaction (PCR). PCR products were pooled for gel purification and quantified. DNA was denatured and diluted to 10 to 12 pM before loading to the MiSeq V2 reagent cartridge (50 cycles). The fastq files generated from the MiSeq were analyzed using GenomeStudio data analysis software provided with the MiSeq instrument with alignment to the hg19 reference human genome. MiRBase 19 was used for miRNA annotation. Normalized miRNA reads (reads per million reads) were determined by the formula: normalized miRNA reads = (raw reads of individual miRNAs)/(the total number of aligned small RNA reads in the corresponding library)\*(one million).

### miRNA Real-Time PCR

Individual miRNAs were quantified using Exiqon's PCR system following the manufacturer's instructions. Briefly, 10 ng of total RNA was first converted to cDNA, which was then mixed with the PCR master mix and the PCR primer mix. PCRs were incubated in a 384-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. miRNAs were assayed in triplicate, and data were normalized to the endogenous small nuclear RNA RNU1A1.

### Illumina Whole-Genome DASL Microarray Analysis

RNA quality was determined by Taqman gene expression assay of RPL13A expression. SLCTs with and without DICER1 mutations (four for each subtype) were included for the analysis. A total of 200 ng of RNA was analyzed using the Illumina Whole-Genome DASL (cDNA-mediated annealing, selection, extension, and ligation) assay with the Human HT-12 v4 BeadChip following the manufacturer's instruction. Fluorescence intensities of the extracted images were read on a BeadArray Reader, which were analyzed by BeadStudio software followed by visualization in GeneSpring GX (v12, Agilent). Ingenuity pathway analysis was performed with differentially expressed genes (fold change > 2, *P* < .05). Raw data are available in the NCBI GEO database, Accession No. GSE71160.

### Gene Expression Analysis by Nanostring nCounter System

Multigene assay was applied to tumor or cell line samples using the Nanostring nCounter GX CodeSet Kit (NanoString Technologies). The total RNA (500 ng for FFPE samples, 100 ng for cell line samples) was hybridized with the available 45-gene code set for 18 hours at 65°C and processed according to manufacturer's instructions [18]. ACTB, GAPDH, and EDEM3 served as internal controls.

### Cell Culture and miRNA Mimic Transfection

SVOG3e cells, which derived from SV40 T-antigen-immortalized primary human granulosa cell line [19] by further introduction of hTERT, were maintained in Dulbecco's modified Eagle's medium-F12 medium (Hyclone) supplemented with 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub>. The synthetic hsa-let-7a mimic (Dharmacon) was transfected into cells with Lipofectamine RNAiMax reagent (Life Sciences) at 10 nM.

### Plasmid Construction and Virus Production

*DICER1* D1709N mutant was generated from pDONR\_wildtype (WT) [5] by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent). WT or D1709N *DICER1* sequences in pDONR221 vectors were shuttled into pLD-hygro-EnVM vectors [20] (Addgene # 24590) with Gateway LR-clonase reaction (Invitrogen) following the manufacturer's protocol. Sanger sequencing verified the *DICER1* mutations in the vectors. GFP-expressing vectors were used as the vector controls. A CRISPR gRNA (CCCCTATCGAGAAATTATGA) targeting the exon5/intron junction of human *DICER1* was cloned into LentiCRISPR vector [21] (Addgene #49535). To produce lentivirus expressing *DICER1* or *DICER1* gRNA/cas9, pLD-hygro-EnVM-*DICER1* (WT or D1709N) or LentiCRISPR-*DICER1* was cotransfected with packaging plasmids psPAX2 and pMD2.G into HEK293T cells using Mirus LT-1 reagent. The pMSCVpuro-let-7 sponge vector [22] (Addgene #29766) was cotransfected with Amphi-V packaging plasmid into HEK293T cells using Mirus LT-1 reagent. Supernatants were collected at 48 or 72 hours for retrovirus or lentivirus preparation, respectively.

### Cell Growth Assay

Cells were plated in 96-well plates at 2000 cells per well with 5 replicates. Cell growth was monitored for 7 days using the IncuCyte ZOOM Live Cell Imaging System (Essen BioScience). Percentage of cell confluence, the fractional area occupied by cells, was calculated to determine the cell growth rate.

### Statistical Analysis

All statistical analyses were performed using the independent *t* test in EXCEL or the one-way analysis of variance for multiple comparisons in PRISM 6. Results were considered statistically significant when *P* values were <.05.

## Results

### Deregulation of miRNA Biogenesis in Ovarian SLCTs

To reveal the impact of *DICER1* RNase IIIb domain mutation on miRNA production in tumors, we analyzed the miRNA expression profiles in ovarian SLCTs using the Illumina Small RNA Sequencing technology. Because the majority of our SLCTs were FFPE samples, we first compared the miRNA expression profiles in the frozen and FFPE tumor samples from two patients. MiSeq analysis of miRNA

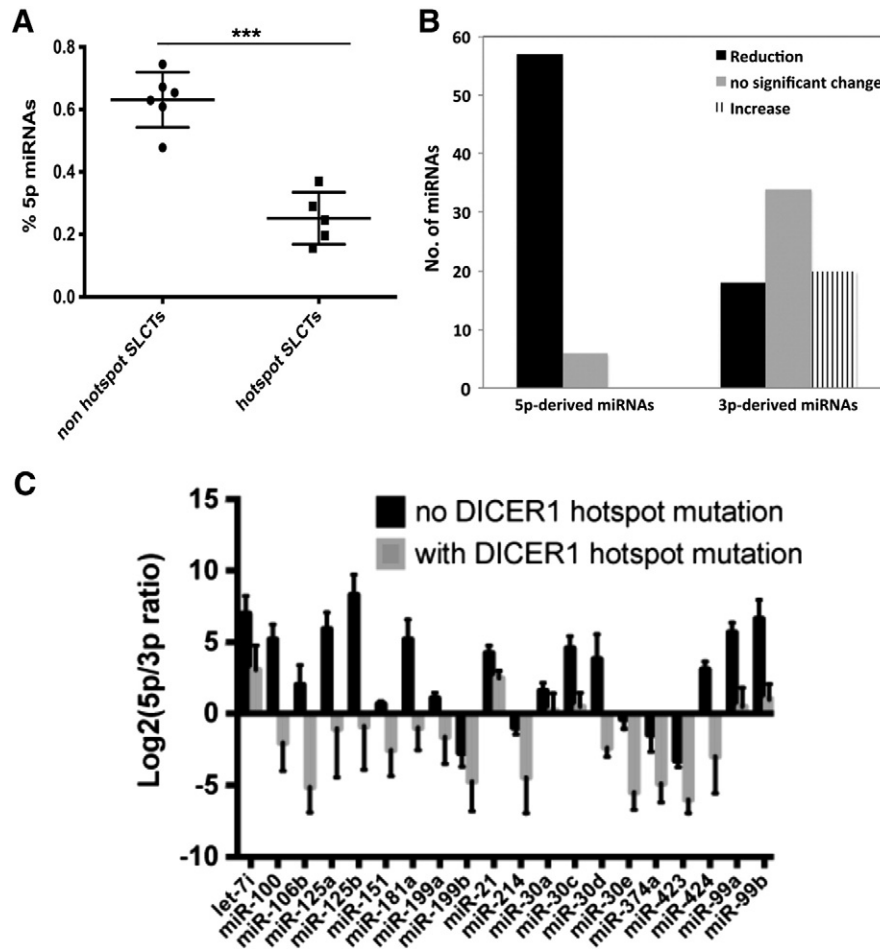
expression in matched frozen and FFPE samples showed good correlation between FFPE tumor samples and their frozen counterparts (Pearson correlation  $R^2 = 0.90$  and  $0.83$ , respectively; Supplemental Figure S1), indicating that the Illumina Small RNA Sequencing technology could reliably detect miRNA expression in FFPE samples.

Next, we sequenced small RNAs in five SLCTs with *DICER1* RNase IIIb hotspot mutations and six SLCTs without *DICER1* hotspot mutations, all of which were FFPE samples. Consistent with our data in isogenic mouse embryonic stem cells expressing *DICER1* variants [14], the average percentage of 5p-derived miRNAs in the whole miRNA population decreased dramatically from 63.1% in tumors without *DICER1* hotspot mutation to 25.1% in tumors with *DICER1* hotspot mutations (Figure 1A,  $P < .001$ ; and Supplemental Table S1). Among the 135 miRNAs whose average reads were at least 10 per million reads across all the samples, the expression of 90.5% (57/63) 5p-derived miRNAs and 25% (18/72) 3p-derived miRNAs decreased dramatically in SLCTs with *DICER1* hotspot mutations compared with those without hotspot mutations (Figure 1B and Supplemental Table S2). Interestingly, the expression of 26% (19/72) 3p-miRNAs was significantly increased in SLCTs with *DICER1* hotspot mutation versus those without *DICER1* hotspot mutation (Figure 1B and Supplemental Table S2). The expression ratio of the 5p- and 3p-derived miRNAs (5p/3p ratio) of 20 miRNA pairs whose precursors produce miRNAs from both 5p and 3p strands in SLCTs was significantly reduced in SLCTs with *DICER1* mutation versus those without *DICER1* hotspot mutations (Figure 1C,  $P < .01$ ), further supporting that there is a defect in producing mature miRNAs from the 5p-strand of pre-miRNAs.

Next, we validated the expression of selected 5p- and 3p-derived miRNAs by real-time PCR in 41 SLCT FFPE samples (21 SLCTs with *DICER1* hotspot mutations and 20 without *DICER1* hotspot mutations, Supplemental Table S3). The expressions of miR-26a-5p, let-7f-5p, and miR-125b-5p were all dramatically reduced in tumors with *DICER1* hotspot mutations compared with those in tumors without *DICER1* hotspot mutations (Figure 2A,  $P < .001$ ), whereas no significant difference was observed for the expression of miR-143-3p, miR-92a-3p, and miR-22-3p between SLCTs with or without *DICER1* hotspot mutations (Figure 2B). The decrease of 5p-derived miRNAs did not depend on the specific site of RNase IIIb mutation present (Figure 2C). Therefore, both the miRNA sequencing and real-time PCR data support a miRNA biogenesis defect in SLCTs with *DICER1* RNase IIIb hotspot mutations.

### Association Between *DICER1* Hotspot Mutations and Altered Expression of Cell Growth and Development Genes

MiRNAs fine-tune gene expression largely through causing mRNA degradation [23]. To understand the biological impact of *DICER1* hotspot mutation-mediated miRNA biogenesis defects in SLCTs, we compared the mRNA expression profiles of four SLCTs with hotspot *DICER1* mutation and four SLCTs without hotspot *DICER1* mutation using the Illumina DASL whole transcriptome analysis. Clustering analysis revealed that the gene expression profiles of four SLCTs with *DICER1* hotspot mutations tightly clustered together, deviating away from those of four SLCTs without *DICER1* hotspot mutations (Figure 3A). Ingenuity pathway analysis of 1396 differentially expressed genes (fold change > 2,  $P < .05$ , Supplemental Table S4) revealed an enrichment of genes regulating cell survival, proliferation, and development (Figure 3B), suggesting that SLCTs



**Figure 1.** Global loss of 5p-derived miRNAs in SLCTs with DICER1 hotspot mutations. (A) Percentage of total 5p-derived miRNA reads was determined by calculating the ratio of the total reads of 5p-derived miRNAs to that of all miRNAs. (B) The number of significantly altered 5p- and 3p-derived miRNAs between SLCTs with and without DICER1 hotspot mutations. (C) The expression ratio of 5p- and 3p-miRNAs derived from the same precursor was calculated in each SLCT. \*\*\* $P < .001$ .

with *DICER1* hotspot mutations have distinct cellular behaviors from those without *DICER1* hotspot mutations. Further analysis of 699 upregulated genes identified an enrichment of genes (115 genes) that are either validated or predicted targets of let-7 family miRNAs (Supplemental Table S5), a collection of miRNAs that constitute the most abundantly expressed miRNAs in both normal human granulosa cells [24] and SLCTs without *DICER1* hotspot mutations (Supplemental Figure S2), indicating that loss of let-7 miRNAs may have important roles in SLCT development.

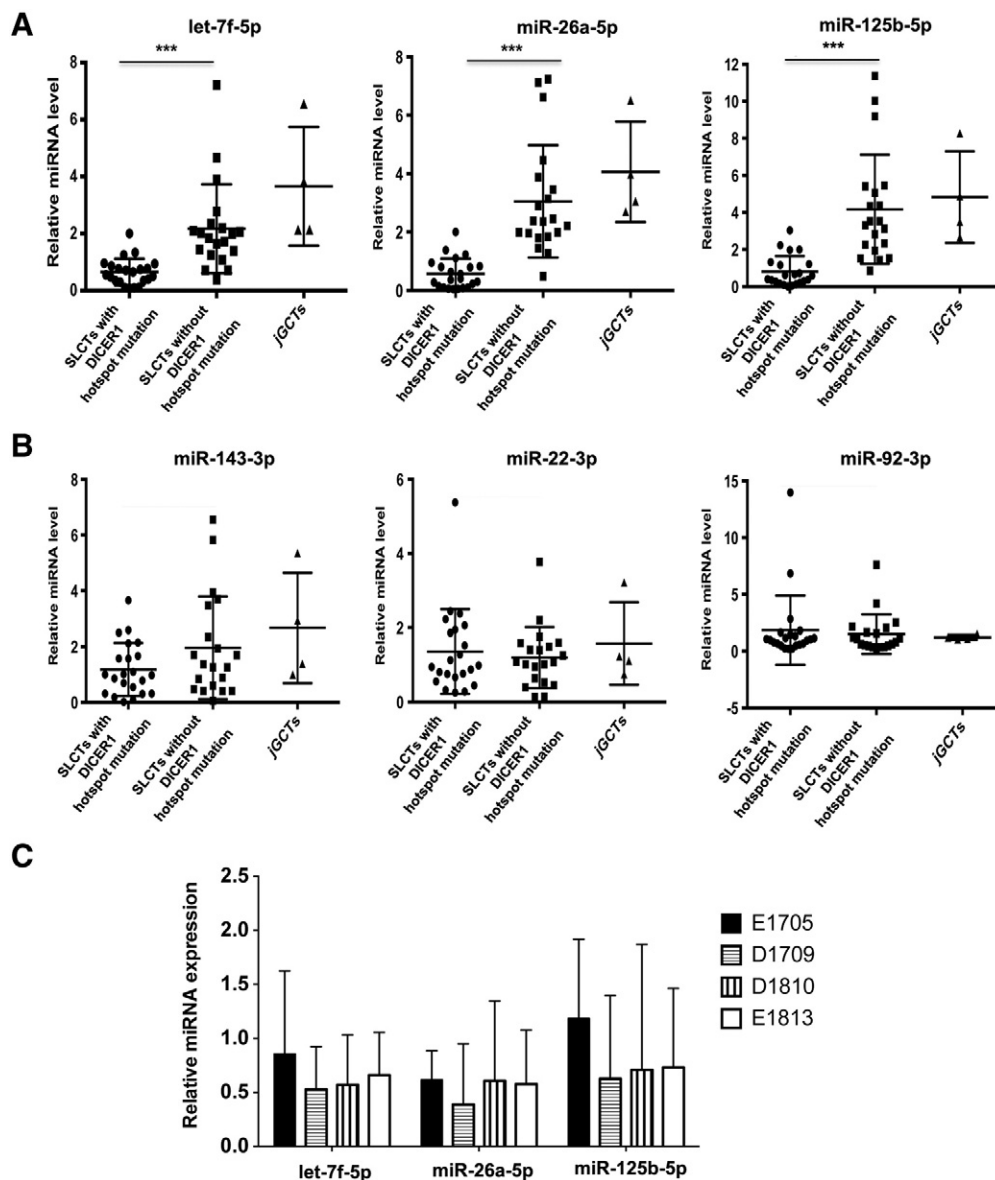
Next, the expression of a subset of genes ( $n = 34$ , Supplemental Table S6), including the top 20 upregulated, top 8 downregulated genes, and 7 selected predicted targets of let-7 from DASL whole transcriptome analysis, was verified using the Nanostring nCounter system in 41 FFPE SLCT tumor samples (Supplemental Table S3). About 82% (27/34) of the selected genes exhibited significantly differential expression between SLCTs with and without *DICER1* hotspot mutations (Supplemental Table S6), indicating a high correspondence with the DASL whole transcriptome analysis. In particular, all the seven selected targets of let-7 were expressed at significantly higher levels in SLCTs with hotspot *DICER1* mutation compared with those without *DICER1* hotspot mutations (Figure 3C), in agreement with the substantially lower amount of let-7 in those tumors (Figure 2A). Therefore, the differential gene

expression between SLCTs with and without *DICER1* hotspot mutations may occur as a result of defective miRNA biogenesis.

#### Association Between *DICER1* Hotspot Mutations and Deregulation of Gonadal Genes

Only a few genes crucial for gonadal genesis, such as CYP19A1 (aromatase), had differential expression between SLCTs with and without *DICER1* hotspot mutations in the microarray analysis (Supplemental Table S3). However, because SLCTs of the ovary represents a pseudo-male gonadal genesis in the ovary, deregulation of gonadal genes may occur despite the status of *DICER1* mutations in SLCTs. Thus, to ascertain the effect of *DICER1* hotspot mutations on gonadal genes, we reexamined the expression of selected gonadal genes using the Nanostring nCounter system and compared them with that of juvenile and adult granulosa cell tumors (jGCTs and aGCTs, respectively), none of which had *DICER1* hotspot mutations (data not shown). We validated that the expression of CYP19A1, encoding aromatase which controls estrogen synthesis from testosterone and differentiation of somatic cell lineages in ovarian gonad [25], was remarkably lower in SLCTs with *DICER1* hotspot mutations than that in SLCTs without *DICER1* hotspot mutation (-7.8-fold,  $P < .001$ ; Figure 4, A and B). In comparison to jGCTs and aGCTs, the expression of CYP19A1 was significantly lower in either





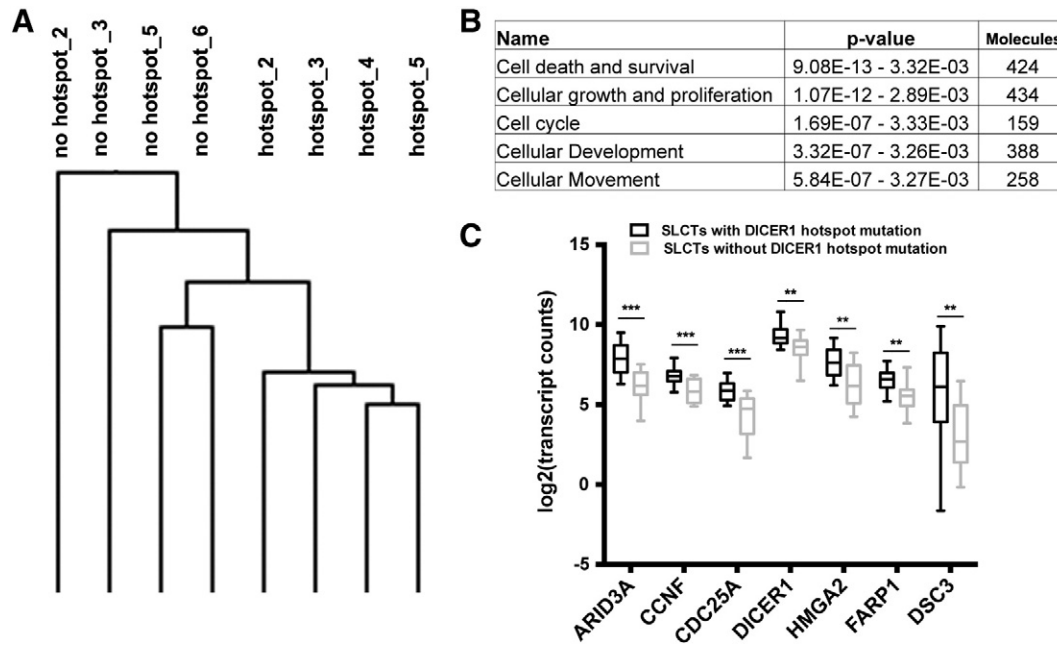
**Figure 2.** Reduced expression of 5p-derived miRNAs (let-7, miR-26a, and miR-125b) in SLCTs with *DICER1* hotspot mutations. Real-time reverse transcriptase PCR was used to quantitate the expression of selected miRNAs in SLCTs and jGCTs. The expression of selected 5p-derived (A), but not 3p-derived (B), miRNAs was significantly reduced in SLCTs with *DICER1* hotspot mutations. The decrease of selected 5p-derived miRNAs did not depend on the specific site of RNase IIIb mutation present (C). \*\*\* $P < .001$ .

class of SLCTs (Figure 4, A and B), in agreement with the fact that patients with SLCTs are often associated with elevated testosterone levels [2]. Consistently, the mRNA levels of *FSHR* and *PGR*, two estrogen-responsive genes crucial for ovulation, were significantly lower in SLCTs with *DICER1* hotspot mutations compared with their counterparts ( $P < .05$ ; Figure 4A), although it remained possible that their expression might be suppressed by some upregulated miRNAs in SLCTs with *DICER1* hotspot mutation (Supplemental Table S7). Furthermore, the expression of *FST*, the gene encoding follistatin that binds to activin and functions downstream of *Wnt4* to control ovarian development [9], was significantly lower in SLCTs compared with granulosa cell tumors (GCTs) ( $P < .01$ ; Figure 4C). The expression of both *FGF9* and *FGFR2*, two genes crucial for Sertoli cell differentiation [9], was also significantly higher in SLCTs than that in GCTs (Figure 4, D and E). Therefore, our

transcriptome analysis suggests that *DICER1* hotspot mutations may have important contributions to the pseudo-male gonadal development in the ovary of SLCT patients.

#### Regulation of Cell Proliferation and Differentiation Genes by *DICER1* Hotspot Mutations in Primary Granulosa Cells

To understand how the hotspot mutations of *DICER1* promote tumorigenesis in the ovary, we first engineered an immortalized human primary granulosa cell line, SVOG3e, to express either WT or D1709N *DICER1*. This cell line model was chosen because no ovarian SLCT cell line has been established and the Sertoli and Leydig cells in ovarian SLCTs are believed to originate from primordial cells that normally differentiate into granulosa cells [6–9]. Because *DICER1* is an essential gene, we stably overexpressed either WT or



**Figure 3.** SLCTs with hotspot DICER1 mutations have similar global gene expression landscapes. (A) Clustering of SLCTs based on their global gene expression landscape. (B) Top molecular and cellular functions affected by DICER1 hotspot mutations. (C) Increased expression of let-7 targets in SLCTs with DICER1 hotspot mutations.  $**P < .01$ ,  $***P < .001$ .

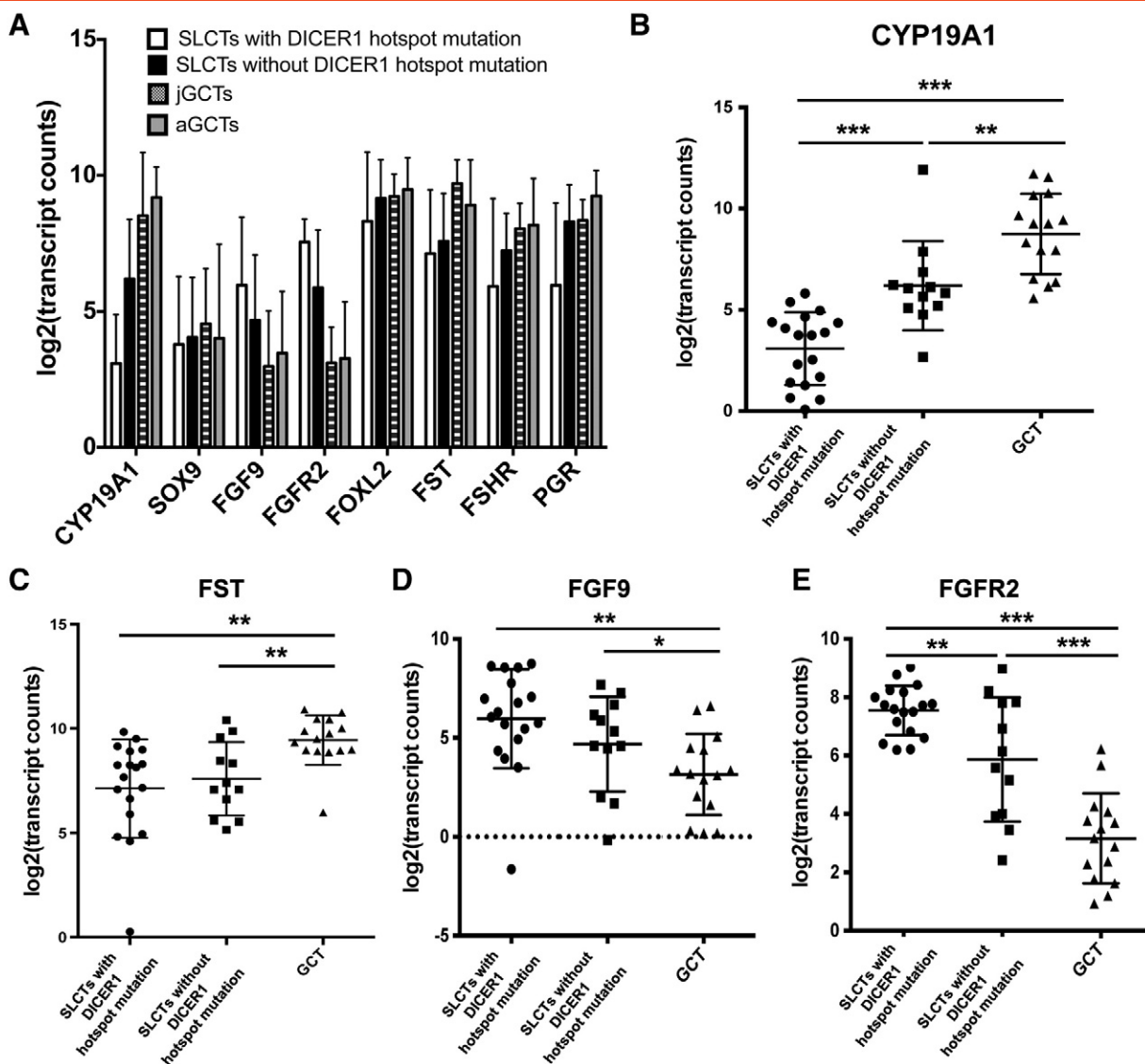
D1709N DICER1 in SVOG3e cells followed by depleting the endogenous DICER1 gene using a CRISPR gRNA (LentiCRISPR-DICER1) that targets the exon5-intron5 boundary (Supplemental Figure S3). This strategy led to the generation of isogenic SVOG3e cell lines that predominantly expressed either V5-tagged WT or D1709N DICER1 (named SVOG3e-WT or SVOG3e-D1709N, respectively) (Supplemental Figure S4 and Figure 5A). The expression of several 5p-derived miRNAs, such as let-7f-5p, miR-26a-5p, and miR-125b-5p, was significantly reduced in SVOG3e-D1709N DICER1 cells compared with SVOG3e-WT DICER1 cells, whereas the expression of several 3p-derived miRNAs was largely unaffected (Figure 5B). Interestingly, the expression of miR-181a (5p) and miR-181a-2-3p was significantly reduced and increased (Supplemental Figure S5), respectively, supporting the observation that the expression of a portion of 3p-derived miRNAs was increased in SLCTs with DICER1 hotspot mutations (Supplemental Table S7). Thus, our isogenic DICER1 variant SVOG3e model confirmed that hotspot mutations in the RNase IIIb domain of DICER1 are defective in the maturation of miRNAs originated from 5p strands of pre-miRNAs.

Next, the expression of the 34 top deregulated genes or let-7 targets and 8 selected gonadal genes (Figure 4A) was determined in isogenic DICER1 SVOG3e cell lines using the Nanostring platform. Among the 42 genes, only 16 genes were expressed in SVOG3e cells, with half of them displaying differential expression between the isogenic pairs (Figure 5C). In agreement with the differential expression between SLCTs with or without DICER1 hotspot mutations, SVOG3e-D1709N cells had significantly lower expression of FST and MGST1 genes than SVOG3e-WT cells but expressed higher amount of FGF9, SOX9, CCNF, CDC25A, PLAGL1, and ARID3A than their counterpart (Figure 5C). The effect of DICER1 D1709N on the increased expression of CDC25A and SOX9 was further confirmed by Western blotting (Figure 5A). Furthermore, the

SVOG3e-D1709N cells proliferated significantly faster than the SVOG3e-WT cells (Figure 5D), in agreement with the induction of cell growth-promoting genes ARID3A, CDC25A, and PLAGL1 [26–28] (Figure 5C). Therefore, our data support a causal role for DICER1 hotspot mutations in mediating the pseudo-male gonadal development and oncogenesis in the ovary.

### The Role of Let-7 in DICER1 Hotspot Mutation-Mediated Differentiation and Oncogenesis

The highly conserved let-7 family miRNAs have important roles in differentiation and proliferation [29,30]. Particularly, substantially higher levels of let-7 were found in sheep ovary versus sheep testis [31], suggesting a potential role of let-7 miRNAs in ovary development. Furthermore, the let-7 family miRNAs constitute the most abundant miRNAs in normal human granulosa cells [24] and SLCTs without DICER1 hotspot mutations (Supplemental Figure S2). Thus, we investigated whether loss of let-7 miRNAs contributed to DICER1 hotspot mutation-mediated phenotypic changes. Expression of a let-7 synthetic miRNA mimic robustly reduced the expression of CCNF, CDC25A, PLAGL1, and ARID3A, four published or predicted targets of let-7; upregulated the expression of FST (Figure 6A); and suppressed the proliferation of SVOG3e-D1709N cells (Figure 6B), suggesting that deregulation of let-7 miRNAs may be a crucial event in DICER1 hotspot mutation-mediated oncogenesis in the ovary. Furthermore, functional inhibition of let-7 miRNAs with a miRNA sponge [22] upregulated the expression of two known let-7 targets, CDC25A and DICER1 [32,33], by Western blotting in the parental SVOG3e cells (Figure 6C), suggesting an efficient suppression of the let-7 function by the let-7 sponge. Accordingly, the SVOG3e-let-7 sponge-expressing cells proliferated faster than the control cells (Figure 6D), supporting a physiological role for let-7 miRNAs in controlling the proliferation of ovarian granulosa cells.



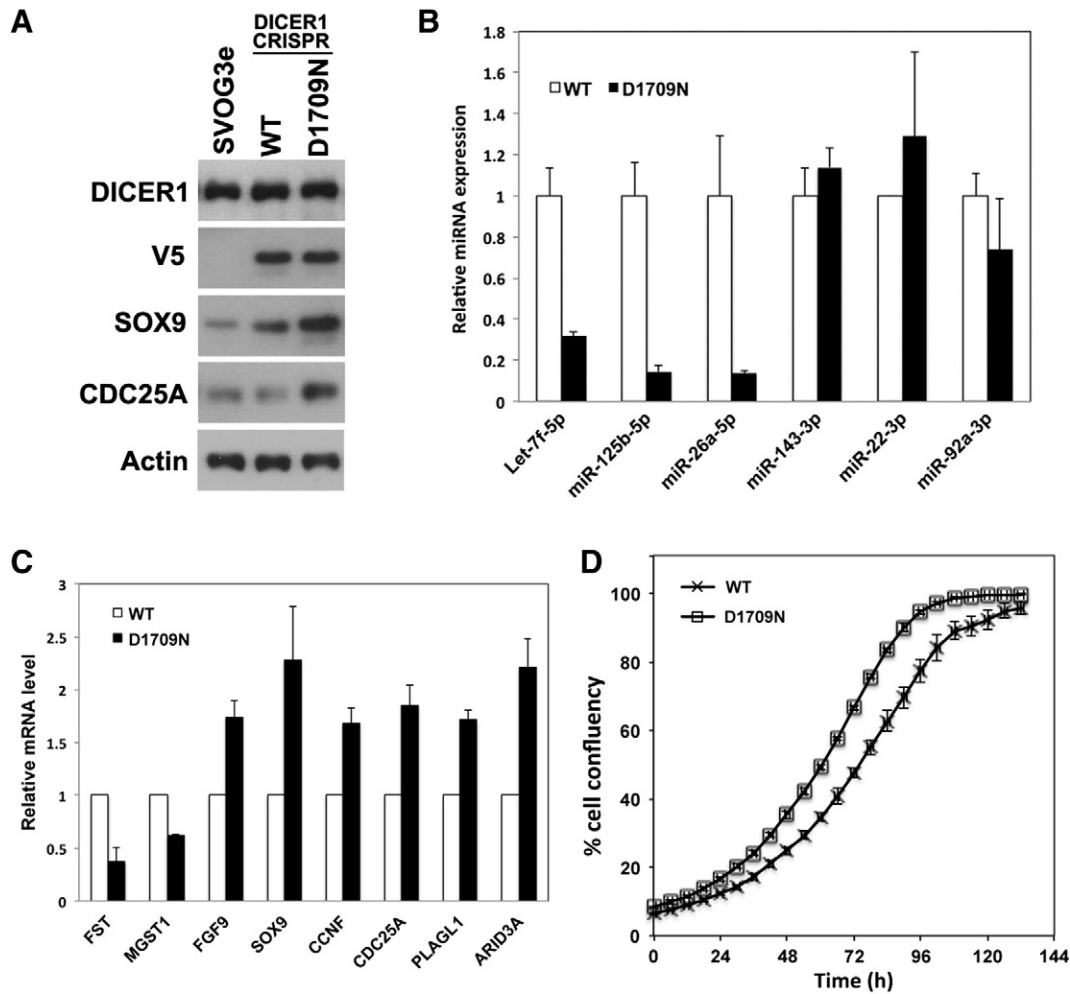
**Figure 4.** Expression of gonadal genes is changed in SLCTs and GCTs with and without hotspots *DICER1* mutations. Nanostring nCounter system was used to determine the expression of a selected panel of gonad genes in SLCTs or GCT FFPE samples (A–E). The transcript counts of each gene in the 500 ng of RNA samples were normalized to those of a panel of control genes in Material and Methods. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ .

## Discussion

*DICER1* controls the biogenesis of miRNAs and is essential for embryogenesis and development of many organs. Although early mouse models suggested that *DICER1* functioned as a haploinsufficient tumor suppressor, recent genomic studies by others and our own laboratory suggest a distinct role of *DICER1* in the oncogenesis of specific tumor types. Germline mutations of *DICER1* have been well documented in a wide range of tumors in a cancer predisposition syndrome, the so-called *DICER1* syndrome [34–37]. Since our first report of somatic hotspot mutations in the RNase IIIb domain of *DICER1* in nonepithelial ovarian tumors (particularly SLCTs of the ovary) [11], several groups have described that missense mutations of the *DICER1* RNase IIIb domain occur frequently in *DICER1* syndrome patients, indicating a “two-hit” tumor-suppressive model of *DICER1*. However, unlike classic “two-hit” models, our previous [11,14] and present studies determined that the second hit of *DICER1* mutations generates a partially functional allele that elicits a

miRNA biogenesis bias, leading to systemic loss of 5p-derived miRNAs in SLCTs and the maintenance or increase of 3p-derived miRNAs. Importantly, *DICER1* hotspot mutations lead to aberrant cell proliferation and deregulation of genes regulating cell growth and gonadogenesis. Therefore, we have provided evidence that hotspot mutations in the RNase IIIb domain of *DICER1* produce an oncogenic allele.

Ovarian sex-cord stromal cell tumors include a collection of distinct subtypes with variable clinical presentations and prognosis, such as GCTs and SLCTs. Proper diagnosis of SCSTs is often very challenging because of the lack of specific markers; however, this is improving because of our recent findings of the overwhelming presence of the pathogenic *FOXL2* C134W mutation in >95% GCTs [38,39] and the occurrence of *DICER1* hotspot mutations in about 50% of SLCTs [11]. In the current study, we demonstrate that although there is no correlation between the histologic grades and *DICER1* mutational status of SLCTs, SLCTs with *DICER1* hotspot



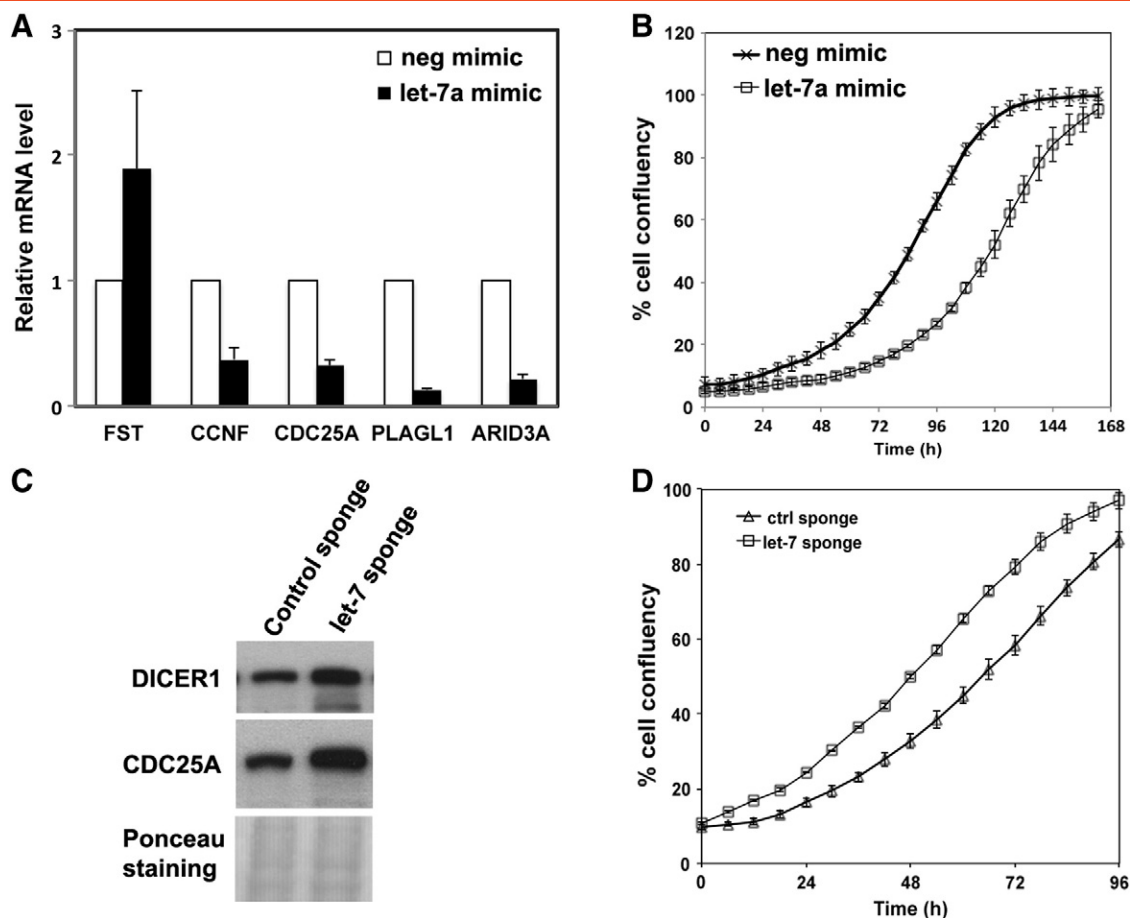
**Figure 5.** A DICER1 hotspot mutation affects miRNA and gene expression and cell proliferation in primary granulosa cells. Isogenic SVOG3e cell lines expressing ectopic V5-tagged DICER1 (WT versus D1709N) with simultaneous ablation of the endogenous DICER1 allele by CRISPR/Cas9 technology were established and subjected to protein expression analysis by Western blotting (A), miRNA expression analysis by quantitative real-time PCR (B), gene expression analysis by NanoString nCounter system (C), and cell proliferation analysis by Incucyte Live Cell Imaging monitor (D).

mutations more frequently presented with various levels of retransform foci (8/21), a structure frequently seen in moderately and poorly differentiated SLCTs [40], in comparison to SLCTs without DICER1 hotspot mutations (2/20) (Supplemental Table S3). Furthermore, SLCTs with DICER1 hotspot mutations displayed distinct gene expression landscapes. Thus, SLCTs with and without DICER1 hotspot mutations may possibly represent different diseases with different patient outcomes. Notably, SLCTs with DICER1 hotspot mutations have significantly lower amount of CYP19A1, the enzyme catalyzing the conversion of testosterone into estrogen [25], suggesting that the function of estrogen is more severely attenuated in this group of tumors. Given that SLCTs can be categorized into three groups based on endocrine functions (androgen excess, estrogen excess, and no endocrine function) [2], DICER1 hotspot mutations may be more prevalent in patients with androgenic symptoms. Thus, it will be interesting to determine whether the testosterone level in SLCT patients correlates with mutational status in the RNase IIIb domain of DICER1. Furthermore, SLCTs with DICER1 hotspot mutations resembled each other at the transcriptional level regardless of the specific site of where mutation occurred, whereas SLCTs without DICER1 hotspot mutations were more heterogeneous

(Figure 3A). We predict that continued efforts to identify the leading genomic alternations in SLCTs without DICER1 hotspot mutations and other SCSTs will eventually lead to the reclassification of SCSTs based on genomic features and improve diagnosis accuracy.

The molecular mechanism underlying the unusual differentiation of Sertoli and Leydig cells in the SLCTs of the ovary is largely unknown. In this study, we demonstrated that DICER1 hotspot mutation could alter the expression of key genes controlling the fate determination of ovarian pregranulosa cells. These include the downregulation of several key ovarian development genes, such as FST and CYP19A1 [9,25], and the upregulation of two key Sertoli cell differentiation genes, FGF9 and FGFR2, that are known to stimulate Sertoli cell differentiation by sustaining the expression of SOX9 [9,41]. The imbalance of gonadal gene expression may therefore shift the differentiation of the primordial gonadal cells towards a pseudotesticular cell differentiation in the ovary. Noteworthy, although FGF9 and FGFR2 are known to stimulate Sertoli cell differentiation by sustaining the expression of SOX9 [9,41] and ablation of CYP19A1 promoted differentiation of Sertoli-like cells with SOX9 expression in mouse ovary [42], we found that the SOX9 gene expression remained at low levels at both





**Figure 6.** Loss of let-7 promotes cell proliferation of primary granulosa cells harboring a *DICER1* hotspot mutation. (A, B) SVOG3e cells expressing D1709N were transfected with either synthetic negative control mimic (neg mimic) or let-7a mimic. Forty-eight hours later, cells were harvested for gene expression analysis (A) or replated for measuring cell proliferation using Incucyte Live Cell Imaging monitor (B). (C, D) SVOG3e cells stably expressing control or let-7 sponge were collected for Western blotting analysis of let-7 target genes (C) or reseeded for determining cell proliferation with Incucyte Live Cell Imaging system (D).

mRNA and protein level (Immunohistochemistry staining, not shown) in the majority of SLCTs. Interestingly, the expression of SOX9 can be significantly upregulated by the D1709N *DICER1* hotspot mutation in SVOG3e cells. These data favor a model in which transient activation of SOX9 may be sufficient to initiate Sertoli-like cell differentiation in ovarian pregranulosa cells and sustained high level of SOX9 may be harmful for SLCT development in some cases. Because both SLCTs with and without *DICER1* mutation reflect a pseudo-male gonadgenesis in the ovary (i.e., reduced CYP19A1 expression), we speculate that SLCTs without *DICER1* mutation may arise from mutations in genes that govern the developmental fate decision of primordial granulosa cells.

Global loss of 5p-driven miRNAs is believed to be the primary driving force for *DICER1* hotspot mutation-mediated tumorigenesis in SLCTs and other *DICER1* syndrome-associated tumors, which are mostly primitive tumors. In particular, the let-7 family miRNAs, the most abundant miRNA in normal human granulosa cells [24] and SLCTs without *DICER1* hotspot mutations (Supplemental Figure S2), are important regulators of cell proliferation and differentiation [29,43]. In agreement with the tumor-suppressive role of let-7, we demonstrated that inhibition of let-7 promoted granulosa cell proliferation and restored expression of let-7 retarded the growth of SVOG3e-D1709N

cells. Importantly, we also report that the expression of FST was increased by let-7 mimic expression (Figure 6A), suggesting that, in addition to the role in embryonic stem cell differentiation, let-7 may play specific roles in regulating ovarian pregranulosa cell differentiation. However, some issues associated with the immortalized granulosa cell line SVOG3e, such as nondetectable transcript and protein levels of CYP19A1 and inability to express CYP19A1 upon cAMP stimulation (data not shown) in SVOG3e cells, prevented us from testing the relationship between any steroidogenic change or other differentiation phenotypes and *DICER1* mutation status or let-7 function. Thus, some better granulosa cell line models [44] or a transgenic animal model that tightly expresses *DICER1* hotspot mutation in ovarian granulosa cells will help understand the oncogenic role of *DICER1* hotspot mutations.

Interestingly, the expression of a subset of 3p-derived miRNAs was significantly upregulated in SLCTs with *DICER1* hotspot mutations versus those without *DICER1* hotspot mutations (Supplemental Table S7). Some of them, such as miR-125b-1-3p, were shown to be upregulated in the serum of a pleuropulmonary blastoma patient with biallelic *DICER1* mutations [45]. Although the mechanisms warrant further study, the interruption of the negative feedback loop between *DICER1* and let-7 (and other 5p-derived miRNAs, such as miR-130a [46]) may have stimulated the expression of the mutant *DICER1*

allele and may thus promote the production of some 3p-miRNAs. Alternatively, 3p-miRNAs normally degraded may be stabilized in the absence of their more stable cognate 5p-miRNA. These 3p-derived miRNAs are predicted to target some mRNAs that were differentially expressed in hotspot and nonhotspot SLCTs (Supplemental Table S7), suggesting that the selective upregulation of certain 3p-miRNAs in SLCTs may also contribute to the tumorigenesis of SLCTs in the ovary.

Taken together, our present study revealed that the selective accumulation of hotspot mutations in the DICER1 RNase IIIb domain in SLCTs causes defective miRNA biogenesis and gene expression changes in key gonad development genes, and potentiate proliferation, which may ultimately rewire differentiation and drive oncogenesis in ovarian gonads.

## Acknowledgements

We thank Vancouver Prostate Cancer Centre Laboratory for Advanced Genome Analysis for Illumina Whole-genome DASL gene expression analysis. We thank all members of Huntsman and Morin lab for invaluable technical assistance and discussions. This work was supported by a Canadian Institute of Health Research grant (MOP-130244) and a Terry Fox New Frontiers Program Project Grant (1021). Y.W. was funded through fellowships from Canadian Institute of Health Research and Michael Smith Foundation for Health Research.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2015.08.003>.

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