NEOPLASIA
www.neoplasia.com

Volume 17 Number 8 August 2015 pp. 650–660 650

The Oncogenic Roles of *DICER1* RNase IIIb Domain Mutations in Ovarian Sertoli-Leydig Cell Tumors[1,2]

Yemin Wang[*,†,‡], Jiamin Chen[*], Winnie Yang[‡], Fan Mo[§], Janine Senz[‡], Damian Yap[*,‡], Michael S. Anglesio[*,†,‡], Blake Gilks[*], Gregg B. Morin[¶,#] and David G. Huntsman[*,†,‡]

[*]Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada; [†]Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, BC, Canada; [‡]Department of Molecular Oncology, British Columbia Cancer Agency Cancer Research Centre, Vancouver, BC, Canada; [§]Vancouver Prostate Cancer Centre, Vancouver, BC, Canada; [¶]Michael Smith Genome Sciences Centre, British Columbia Cancer Agency, Vancouver, BC, Canada; [#]Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

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

Address all correspondence to: Gregg B. Morin, 7112-675 West 10th Avenue, Michael Smith Genome Sciences Centre, BC Cancer Agency, Vancouver, BC, V5Z 1 L3. Or David G. Huntsman, 3427-600 West 10th Avenue, BC Cancer Agency, Vancouver, BC, V5Z 4E6.
E-mail: gmorin@bcgsc.ca

[1] Disclosure/conflict of interest: none.

[2] 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

1476-5586
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_2. 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 Ampho-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 1*A*, $P < .001$; 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 1*B* 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 1*B* 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 significant reduced in SLCTs with *DICER1* mutation versus those without *DICER1* hotspot mutations (Figure 1*C*, $P < .01$), 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 2*A*, $P < .001$), 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 2*B*). The decrease of 5p-derived miRNAs did not depend on the specific site of RNase IIIb mutation present (Figure 2*C*). 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 3*A*). 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 3*B*), suggesting that SLCTs

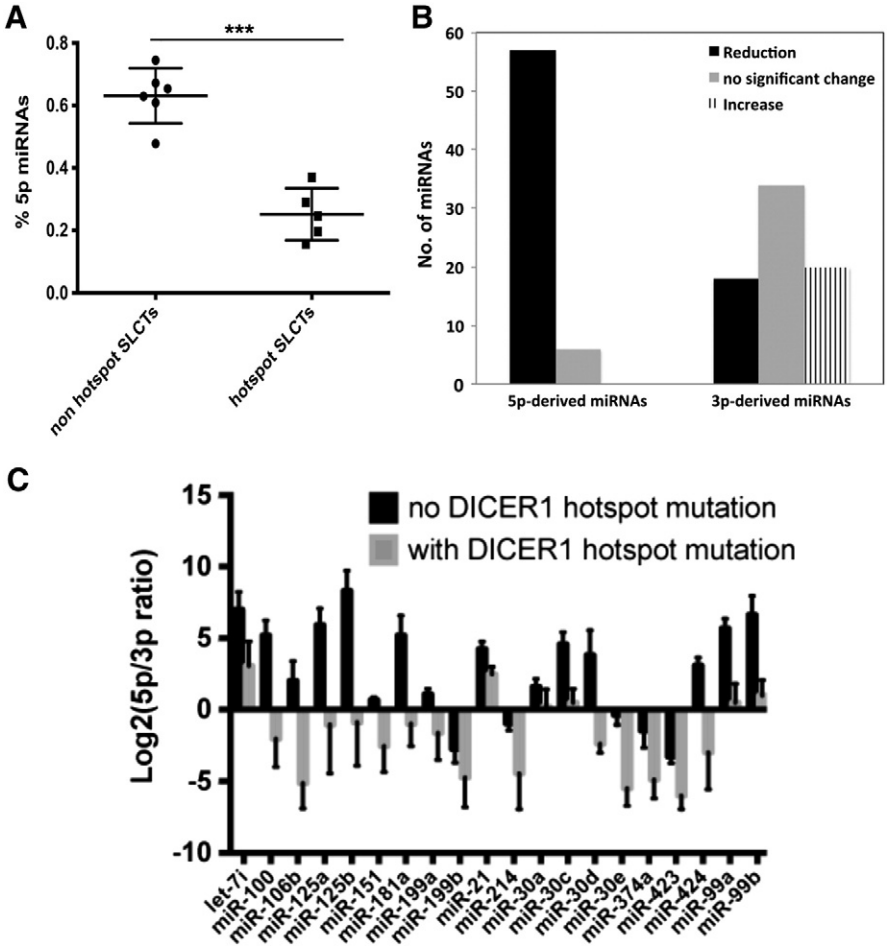

Figure 1. Global loss of 5p-derived miRNAs in SLCTs with DICER1 hotspot mutations. (A) Percentage of total 5p-derived miRNA reads was determined by calculating the ratio of the total reads of 5p-derived miRNAs to that of all miRNAs. (B) The number of significantly altered 5p- and 3p-derived miRNAs between 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 3*C*), in agreement with the substantially lower amount of let-7 in those tumors (Figure 2*A*). 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 Genesis 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

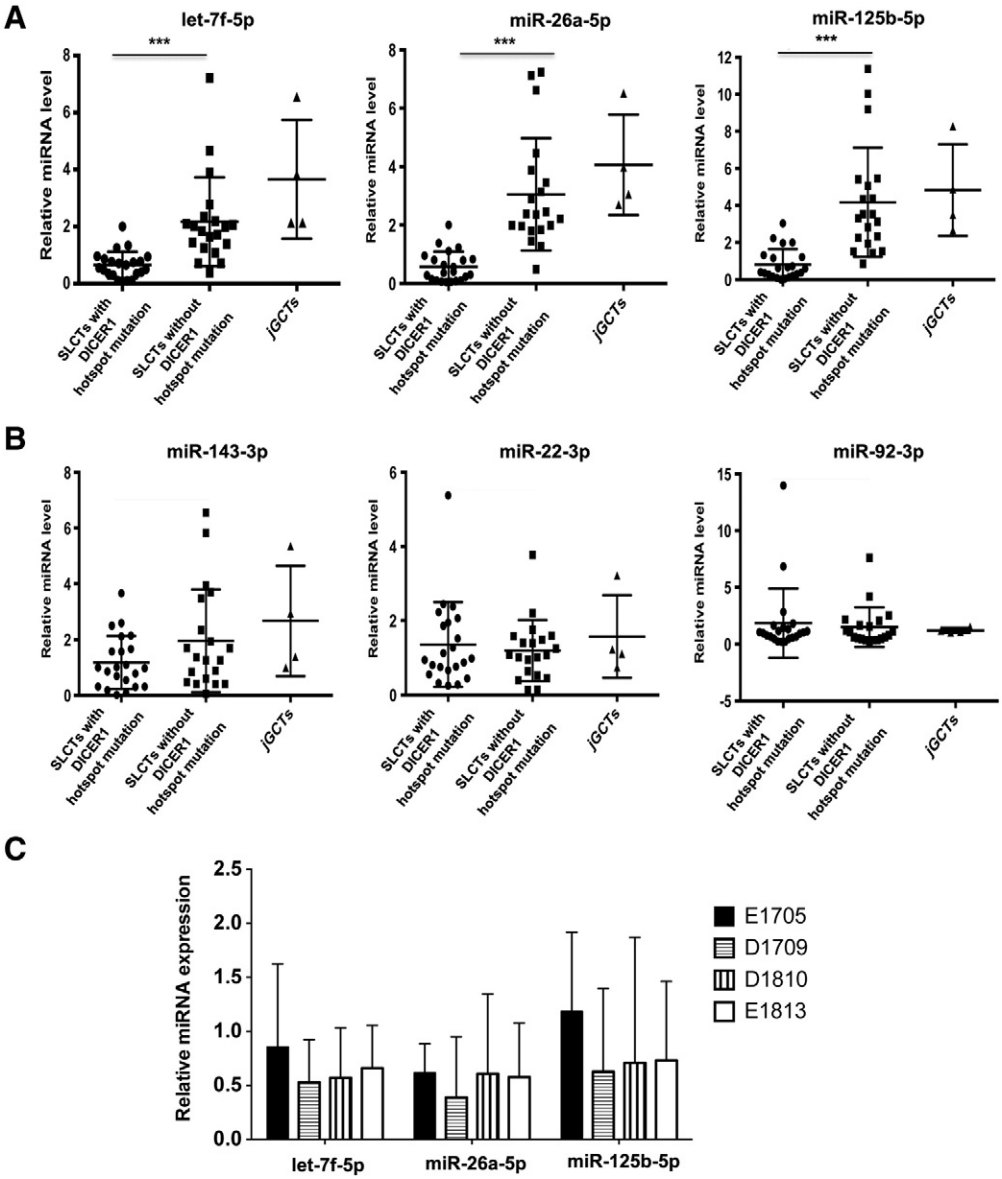

Figure 2. Reduced expression of 5p-derived miRNAs (let-7, miR-26a, and miR-125b) in SLCTs with DICER1 hotspot mutations. Real-time reverse transcriptase PCR was used to quantitate the expression of selected miRNAs in SLCTs and jGCTs. The expression of selected 5p-derived (A), but not 3p-derived (B), 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 4*A*), 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 4*C*). 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

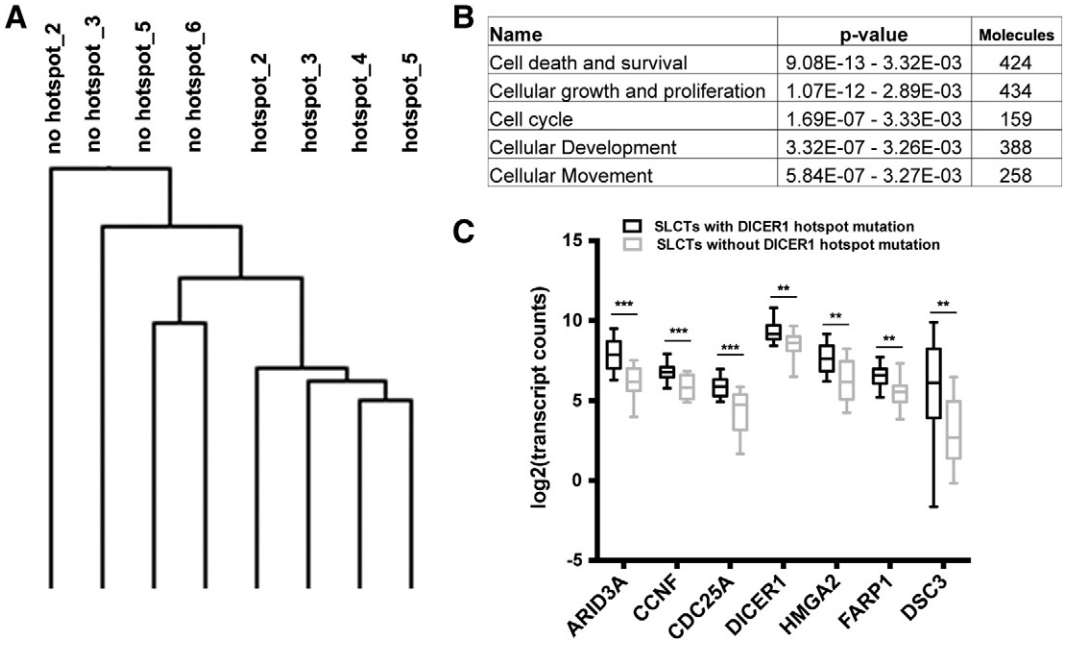

Figure 3. SLCTs with hotspot DICER1 mutations have similar global gene expression landscapes. (A) Clustering of SLCTs based on their global gene expression landscape. (B) Top molecular and cellular functions affected by DICER1 hotspot mutations. (C) Increased expression of let-7 targets in SLCTs with 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 5*A*). 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 5*B*). 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 4*A*) 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 5*C*). 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 5*C*). The effect of DICER1 D1709N on the increased expression of CDC25A and SOX9 was further confirmed by Western blotting (Figure 5*A*). Furthermore, the SVOG3e-D1709N cells proliferated significantly faster than the SVOG3e-WT cells (Figure 5*D*), in agreement with the induction of cell growth–promoting genes ARID3A, CDC25A, and PLAGL1 [26–28] (Figure 5*C*). 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 6*A*); and suppressed the proliferation of SVOG3e-D1709N cells (Figure 6*B*), 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 6*C*), 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 6*D*), supporting a physiological role for let-7 miRNAs in controlling the proliferation of ovarian granulosa cells.

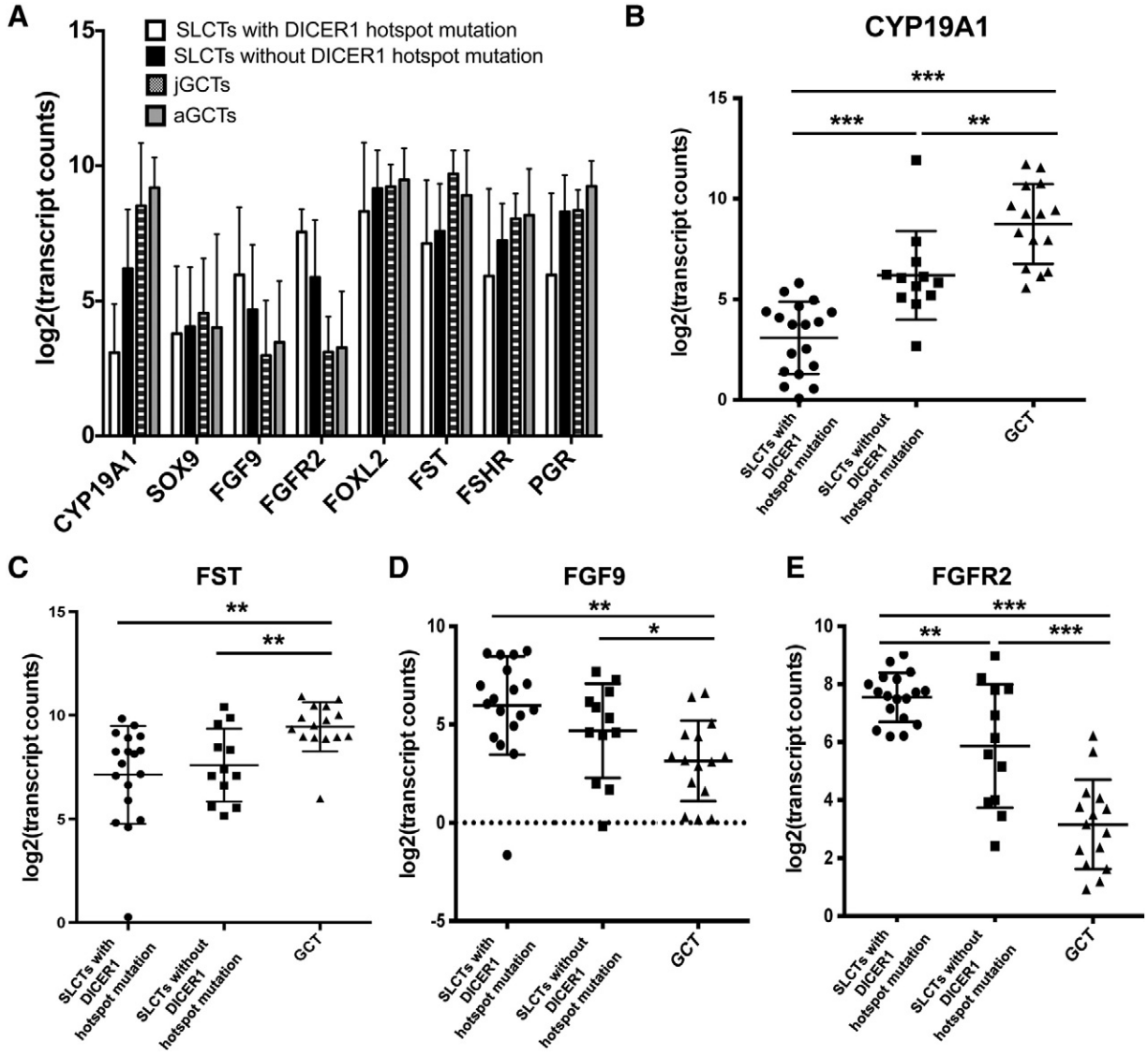

Figure 4. Expression of gonadal genesis genes is changed in SLCTs and GCTs with and without hotspot DICER1 mutations. Nanostring nCounter system was used to determine the expression of a selected panel of gonad genesis genes in SLCTs or GCT FFPE samples (A–E). The transcript counts of each gene in the 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 gonad genesis. 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 pathogenomic 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

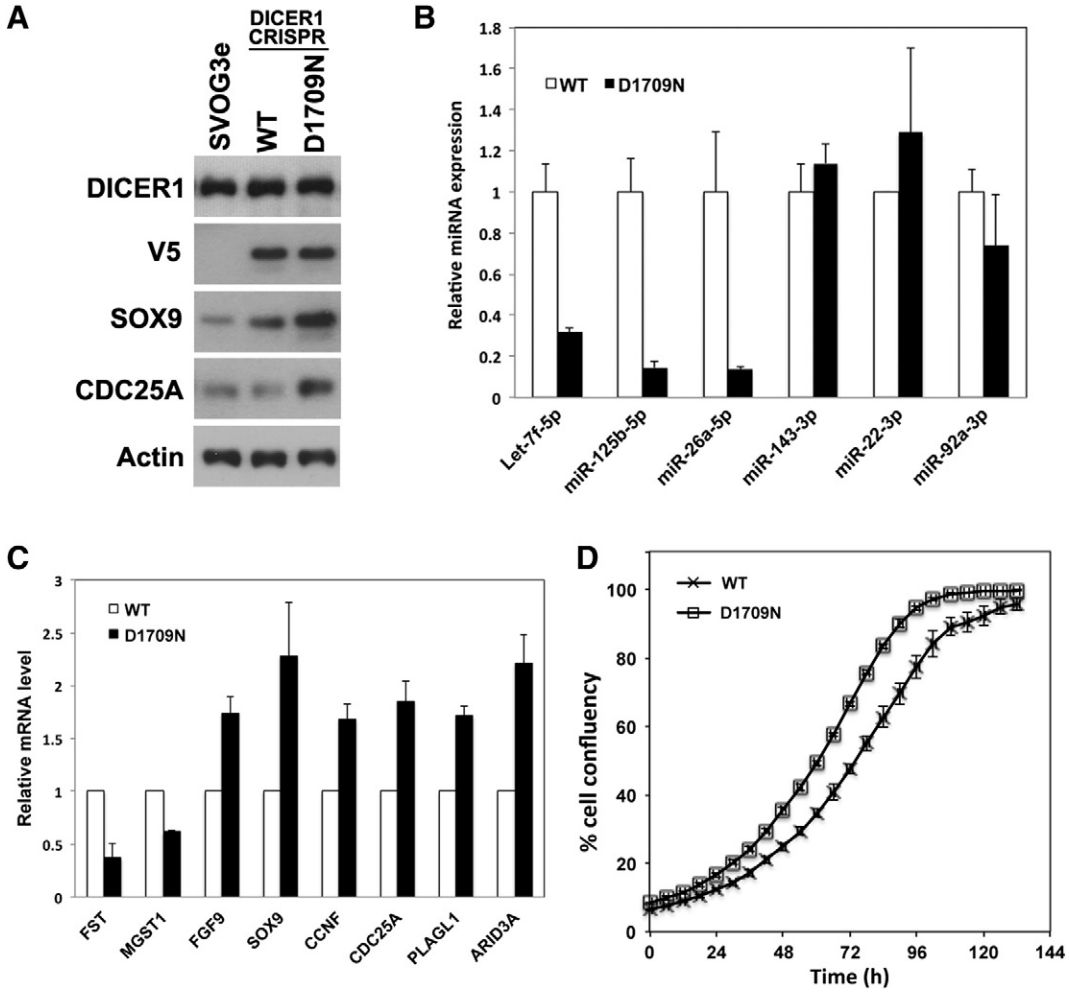

Figure 5. A DICER1 hotspot mutation affects miRNA and gene expression and cell proliferation in primary granulosa cells. Isogenic SVOG3e cell lines expressing ectopic V5-tagged DICER1 (WT versus D1709N) with simultaneous ablation of the endogenous DICER1 allele by CRISPR/Cas9 technology were established 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 retiform 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 3*A*). 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. Noteworthily, 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

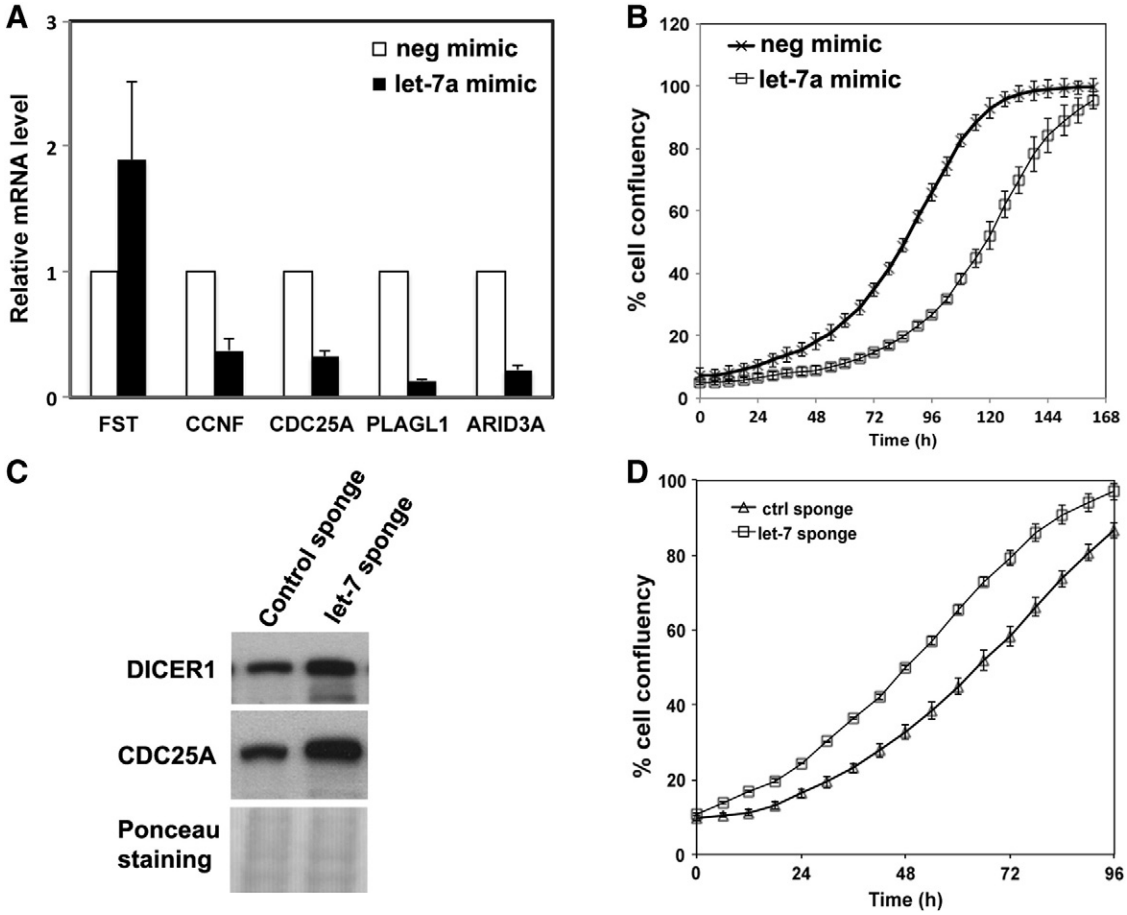

Figure 6. Loss of let-7 promotes cell proliferation of primary granulosa cells harboring a DICER1 hotspot mutation. (A, B) SVOG3e cells expressing D1709N were transfected with either synthetic negative control mimic (neg mimic) or let-7a mimic. Forty-eight hours later, cells were harvested for gene expression 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-drived 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 6*A*), 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.

References

[1] Lantzsch T, Stoerer S, Lawrenz K, Buchmann J, Strauss HG, and Koelbl H (2001). Sertoli-Leydig cell tumor. *Arch Gynecol Obstet* **264**, 206–208.

[2] Gui T, Cao D, Shen K, Yang J, Zhang Y, Yu Q, Wan X, Xiang Y, Xiao Y, and Guo L (2012). A clinicopathological analysis of 40 cases of ovarian Sertoli-Leydig cell tumors. *Gynecol Oncol* **127**, 384–389.

[3] Young RH and Scully RE (1985). Ovarian Sertoli-Leydig cell tumors. A clinicopathological analysis of 207 cases. *Am J Surg Pathol* **9**, 543–569.

[4] Sigismondi C, Gadducci A, Lorusso D, Candiani M, Breda E, Raspagliesi F, Cormio G, Marinaccio M, and Mangili G (2012). Ovarian Sertoli-Leydig cell tumors. a retrospective MITO study. *Gynecol Oncol* **125**, 673–676.

[5] Emerson RE, Wang M, Roth LM, Zheng W, Abdul-Karim FW, Liu F, Ulbright TM, Eble JN, and Cheng L (2007). Molecular genetic evidence supporting the neoplastic nature of the Leydig cell component of ovarian Sertoli-Leydig cell tumors. *Int J Gynecol Pathol* **26**, 368–374.

[6] Hittmair A, Zelger BG, Obrist P, and Dirnhofer S (1997). Ovarian Sertoli-Leydig cell tumor: a SRY gene-independent pathway of pseudomale gonadal differentiation. *Hum Pathol* **28**, 1206–1210.

[7] Papanastasopoulos P, Repanti M, Damaskou V, Bravou V, and Papadaki H (2008). Investigating differentiation mechanisms of the constituent cells of sex cord-stromal tumours of the ovary. *Virchows Arch* **453**, 465–471.

[8] Pask AJ, Calatayud NE, Shaw G, Wood WM, and Renfree MB (2010). Oestrogen blocks the nuclear entry of SOX9 in the developing gonad of a marsupial mammal. *BMC Biol* **8**, 113–123.

[9] Ross AJ and Capel B (2005). Signaling at the crossroads of gonad development. *Trends Endocrinol Metab* **16**, 19–25.

[10] Wilhelm D, Martinson F, Bradford S, Wilson MJ, Combes AN, Beverdam A, Bowles J, Mizusaki H, and Koopman P (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev Biol* **287**, 111–124.

[11] Heravi-Moussavi A, Anglesio MS, Cheng SW, Senz J, Yang W, Prentice L, Fejes AP, Chow C, Tone A, and Kalloger SE, et al (2012). Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. *N Engl J Med* **366**, 234–242.

[12] Witkowski L, Mattina J, Schonberger S, Murray MJ, Choong CS, Huntsman DG, Reis-Filho JS, McCluggage WG, Nicholson JC, and Coleman N, et al (2013). DICER1 hotspot mutations in non-epithelial gonadal tumours. *Br J Cancer* **109**, 2744–2750.

[13] Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297.

[14] Anglesio MS, Wang Y, Yang W, Senz J, Wan A, Heravi-Moussavi A, Salamanca C, Maines-Bandiera S, Huntsman DG, and Morin GB (2013). Cancer-associated somatic DICER1 hotspot mutations cause defective miRNA processing and reverse-strand expression bias to predominantly mature 3p strands through loss of 5p strand cleavage. *J Pathol* **229**, 400–409.

[15] Gurtan AM, Lu V, Bhutkar A, and Sharp PA (2012). In vivo structure-function analysis of human Dicer reveals directional processing of precursor miRNAs. *RNA* **18**, 1116–1122.

[16] Wu MK, Sabbaghian N, Xu B, Addidou-Kalucki S, Bernard C, Zou D, Reeve AE, Eccles MR, Cole C, and Choong CS, et al (2013). Biallelic DICER1 mutations occur in Wilms tumours. *J Pathol* **230**, 154–164.

[17] Pugh TJ, Yu W, Yang J, Field AL, Ambrogio L, Carter SL, Cibulskis K, Giannikopoulos P, Kiezun A, and Kim J, et al (2014). Exome sequencing of pleuropulmonary blastoma reveals frequent biallelic loss of TP53 and two hits in DICER1 resulting in retention of 5p-derived miRNA hairpin loop sequences. *Oncogene* **33**, 5295–5302.

[18] Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, and Grogan T, et al (2008). Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol* **26**, 317–325.

[19] Lie BL, Leung E, Leung PC, and Auersperg N (1996). Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen. *Mol Cell Endocrinol* **120**, 169–176.

[20] Mak AB, Ni Z, Hewel JA, Chen GI, Zhong G, Karamboulas K, Blakely K, Smiley S, Marcon E, and Roudeva D, et al (2010). A lentiviral functional proteomics approach identifies chromatin remodeling complexes important for the induction of pluripotency. *Mol Cell Proteomics* **9**, 811–823.

[21] Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, and Doench JG, et al (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343**, 84–87.

[22] Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, and Jacks T (2008). Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci U S A* **105**, 3903–3908.

[23] Guo H, Ingolia NT, Weissman JS, and Bartel DP (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **466**, 835–840.

[24] Velthut-Meikas A, Simm J, Tuuri T, Tapanainen JS, Metsis M, and Salumets A (2013). Research resource: small RNA-seq of human granulosa cells reveals miRNAs in FSHR and aromatase genes. *Mol Endocrinol* **27**, 1128–1141.

[25] Pask AJ (2012). A role for estrogen in somatic cell fate of the mammalian gonad. *Chromosome Res* **20**, 239–245.

[26] Rezvani G, Lui JC, Barnes KM, and Baron J (2012). A set of imprinted genes required for normal body growth also promotes growth of rhabdomyosarcoma cells. *Pediatr Res* **71**, 32–38.

[27] Suzuki M, Okuyama S, Okamoto S, Shirasuna K, Nakajima T, Hachiya T, Nojima H, Sekiya S, and Oda K (1998). A novel E2F binding protein with Myc-type HLH motif stimulates E2F-dependent transcription by forming a heterodimer. *Oncogene* **17**, 853–865.

[28] Karlsson-Rosenthal C and Millar JB (2006). Cdc25: mechanisms of checkpoint inhibition and recovery. *Trends Cell Biol* **16**, 285–292.

[29] Bussing I, Slack FJ, and Grosshans H (2008). let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med* **14**, 400–409.

[30] Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, and Shingara J, et al (2007). The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res* **67**, 7713–7722.

[31] Torley KJ, da Silveira JC, Smith P, Anthony RV, Veeramachaneni DN, Winger QA, and Bouma GJ (2011). Expression of miRNAs in ovine fetal gonads: potential role in gonadal differentiation. *Reprod Biol Endocrinol* **9**, 2–12.

[32] Huang JC, Babak T, Corson TW, Chua G, Khan S, Gallie BL, Hughes TR, Blencowe BJ, Frey BJ, and Morris QD (2007). Using expression profiling data to identify human microRNA targets. *Nat Methods* **4**, 1045–1049.

[33] Tokumaru S, Suzuki M, Yamada H, Nagino M, and Takahashi T (2008). let-7 regulates Dicer expression and constitutes a negative feedback loop. *Carcinogenesis* **29**, 2073–2077.

[34] Slade I, Bacchelli C, Davies H, Murray A, Abbaszadeh F, Hanks S, Barfoot R, Burke A, Chisholm J, and Hewitt M, et al (2011). DICER1 syndrome: clarifying the diagnosis, clinical features and management implications of a pleiotropic tumour predisposition syndrome. *J Med Genet* **48**, 273–278.

[35] Schultze-Florey RE, Graf N, Vorwerk P, Koscielniak E, Schneider DT, and Kratz CP (2013). DICER1 syndrome: a new cancer syndrome. *Klin Padiatr* **225**, 177–178.
[36] de Kock L, Sabbaghian N, Druker H, Weber E, Hamel N, Miller S, Choong CS, Gottardo NG, Kees UR, and Rednam SP, et al (2014). Germ-line and somatic DICER1 mutations in pineoblastoma. *Acta Neuropathol* **128**, 583–595.
[37] de Kock L, Sabbaghian N, Plourde F, Srivastava A, Weber E, Bouron-Dal Soglio D, Hamel N, Choi JH, Park SH, and Deal CL, et al (2014). *Acta Neuropathol* **128**, 111–122.
[38] Shah SP, Kobel M, Senz J, Morin RD, Clarke BA, Wiegand KC, Leung G, Zayed A, Mehl E, and Kalloger SE, et al (2009). Mutation of FOXL2 in granulosa-cell tumors of the ovary. *N Engl J Med* **360**, 2719–2729.
[39] Kommoss S, Anglesio MS, Mackenzie R, Yang W, Senz J, Ho J, Bell L, Lee S, Lorette J, and Huntsman DG, et al (2013). FOXL2 molecular testing in ovarian neoplasms: diagnostic approach and procedural guidelines. *Mod Pathol* **26**, 860–867.
[40] Young RH (1993). Sertoli-Leydig cell tumors of the ovary: review with emphasis on historical aspects and unusual variants. *Int J Gynecol Pathol* **12**, 141–147.
[41] Eggers S and Sinclair A (2012). Mammalian sex determination-insights from humans and mice. *Chromosome Res* **20**, 215–238.
[42] Britt KL and Findlay JK (2003). Regulation of the phenotype of ovarian somatic cells by estrogen. *Mol Cell Endocrinol* **202**, 11–17.
[43] Boyerinas B, Park SM, Hau A, Murmann AE, and Peter ME (2010). The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer* **17**, F19–F36.
[44] Havelock JC, Rainey WE, and Carr BR (2004). Ovarian granulosa cell lines. *Mol Cell Endocrinol* **228**, 67–78.
[45] Murray MJ, Bailey S, Raby KL, Saini HK, de Kock L, Burke GA, Foulkes WD, Enright AJ, Coleman N, and Tischkowitz M (2014). Serum levels of mature microRNAs in DICER1-mutated pleuropulmonary blastoma. *Oncogenesis* **3**, e87 [1-6].
[46] Kovaleva V, Mora R, Park YJ, Plass C, Chiramel AI, Bartenschlager R, Dohner H, Stilgenbauer S, Pscherer A, and Lichter P, et al (2012). miRNA-130a targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells. *Cancer Res* **72**, 1763–1772.