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A miR-372/let-7 Axis Regulates Human Germ versus Somatic Cell Fates

Nam D. Tran[1], **Michael Kissner**[1], **Deepa Subramanyam**[1], **Ronald J. Parchem**[1], **Diana J. Laird**[1,*], and **Robert H. Blelloch**[1,*]

[1]Departments of OB/Gyn and Urology, Center for Reproductive Sciences, and the Eli and Edythe Broad Center for Regeneration Medicine and Stem Cell Research, University of California, San Francisco, San Francisco, CA. USA

Abstract

The embryonic stem cell cycle (ESCC) and let-7 families of miRNAs function antagonistically in the switch between mouse embryonic stem cell (mESC) self-renewal and somatic differentiation. Here we report that the human ESCC miRNA miR-372 and let-7 act antagonistically in germline differentiation from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs). hESC and iPSC-derived primordial germ cell-like cells (PGCLCs) expressed high levels of miR-372 and conversely, somatic cells expressed high levels of let-7. Manipulation of miRNA levels by introduction of miRNA mimics or knockdown with miRNA sponges demonstrated that miR-372 promotes while let-7 antagonizes PGCLC differentiation. Knockdown of the individual miR-372 targets *SMARCC1*, *MECP2*, *CDKN1*, *RBL2*, *RHOC*, and *TGFBR2* increased PGCLC production, while knockdown of the let-7 targets *CMYC* and *NMYC* suppressed PGCLC differentiation. These findings uncover a miR-372/let-7 axis common to induced pluripotency and primordial germ cell (PGC) specification.

Graphical Abstract

Let-7 and miR-294 antagonistically regulate somatic versus germline fates through their influence on multiple cellular pathways.

Keywords

embryonic stem cells; primordial germ cells; microRNA; mir-372; let-7

Correspondence: DL: Diana.Laird@ucsf.edu, RB: Robert.Blelloch@ucsf.edu.

Supplementary Materials:
Materials and Methods
Supplementary References
Supplementary Figure Legends
Supplementary Figures

Author contributions:

Nam D Tran: Conception and design, collection and analysis of data, manuscript writing, final approval of manuscript
Michael Kissner: Collection of data
Deepa Subramanyam: Collection of data
Ronald Parchem: Collection of data
Diana Laird: Conception and design, financial support, data analysis, manuscript writing, final approval of manuscript
Robert Blelloch: Conception and design, financial support, data analysis, manuscript writing, final approval of manuscript

Introduction

The generation of human germ cells *in vitro* provides a promising avenue to study the molecular basis of their development as functional studies are not feasible. Successful differentiation of PGCLCs from ESCs has been reported in mouse [1] and human [2–4]. Importantly, *in vitro* differentiation recapitulates many major events observed *in vivo* [3–6], and mouse PGCLC function has demonstrated with successful spermatogenesis and oogenesis resulting in live births [7, 8].

miRNAs are short single-stranded RNAs that destabilize transcripts and repress translation primarily through partial complementation with the 3′UTRs of target mRNAs [9]. Several miRNAs have been implicated in PGC development [5, 10]. In particular, let-7 blocks the production of mouse PGCs both in vitro and in vivo, at least in part through the key PGC specification transcription factor, Prdm1 (Blimp1) [5]. The knockout of the miR-290 cluster in mice results in a subfertile phenotype with a reduction in PGCs but the specific targets remained to be investigated [11]. The miR-290 cluster (or miR-372 cluster in humans) consists of a combination of miRNAs, including members of the ESCC family, which have been shown to antagonize the let-7 family in the differentiation of embryonic stem cells [12]. Here, we aimed to dissect the roles of the let-7 and ESCC miRNAs and their targets in the production of human PGCs using an *in vitro* model of human PGCLC differentiation.

Results and Discussions

To evaluate the roles of miRNAs in PGC development, we differentiated human ESCs and iPSCs in medium containing retinoic acid and then enriched for PGCLCs by fluorescence-based sorting using SSEA-1 and C-Kit [1, 2]. Differentiation of hESCs and iPSCs resulted in ~2.5–3.2% cells co-expressing both PGCLC markers, referred to as double-positive (DP) (Fig 1A–C). Somatic cells lacking these markers are referred to as double-negative (DN). DP cells expressed high levels of VASA and DAZL with concomitant up regulation of *PRDM1*, *DAZL*, *SYCP3*, *NANOG*, *POU5F1*, and *SOX17* (Fig 2A–E), similar to previous studies [3, 13–16]. However, SYCP3 immunolocalized to nuclear puncta without synaptonemal complex formation in 29% of PGCLCs, suggesting they have not entered meiosis (Fig 2F). High levels of H3K27me3 and H3K9me2 were observed in H9 derived DP and DN cells, respectively (Fig S1A), consistent with epigenetic reprograming of in vivo PGCs and somatic cells during development [17–20]. Partial demethylation of the imprinted *H19*, *PEG1*, and *SNRPN* loci was also observed in the PGCLCs (Fig S1B), as found in mouse and human PGCs at late-migratory, pre-meiotic stages [2–4, 14, 21].

Since the ESCC and let-7 families of miRNAs function antagonistically in the switch between mESC self-renewal and differentiation [12], we asked whether they function similarly in the reactivation of pluripotency during PGC specification. The ESCC miRNA miR-372 was highly expressed in H9 derived DP cells while let-7 was undetectable (Fig 3A); the converse was found in DN cells. Introduction of miR-372 mimics into H9 and H1 ESCs prior to differentiation increased the frequency of DP cells from 2.8% to 4.7% (1.7

fold) whereas let-7 mimics had the opposite effect 2.8% to 0.7% (0.25 fold) (Fig 3B), suggesting antagonizing roles of these miRNAs in PGCLC derivation.

miRNA sponges [22] were used to knockdown endogenous miR372 and let-7 function in hESCs and the specificity of the sponges was confirmed with target derepression as shown in Fig S2A–D. Sponge introduction did not alter the pluripotency of hESCs as they fully retain the capacity for multilineage differentiation as assessed by embryoid body formation (Fig S2E). Subsequently, H9 hESCs expressing these sponges were differentiated as described above. While the let-7 sponge significantly promoted PGCLC formation (1.7 fold), the miR-372 sponge abolished it (Fig 3C). The positive effect of the let-7 sponge on DP cell yield was recapitulated by the overexpression of LIN28, an inhibitor of let-7 biogenesis (Fig S3A–C). Combined knockdown of let-7 with miR-372 mimic addition further augmented the yield of DP cells (2.7 fold) (Fig 3C), supporting antagonistic roles of miR-372 and let-7 in PGCLC production.

In addition to modulating the frequencies of DP cells, miR-372 and let-7 also altered expression of germ cell genes. We found that miR-372 mimic increased, while let-7 decreased the levels of *PRDM11*, *VASA*, *DAZL*, and *SYCP3* in H9 derived DP cells (Fig 3D). In contrast, these mimics had the opposite effect on somatic markers *HOXA1* and *HOXB1* in the H9 derived DP cells (Fig 3E). The frequency of SYCP3-expressing PGCLCs increased from 29% in the control group to 51% in the miR-372 treated group (Fig 3F). miR-372 mimic also enhanced epigenetic reprogramming, as demethylation of the *H19*, *PEG1*, and *SNRPN* loci was more complete (Fig 3G, S1B). Thus, in addition to the overall frequency and efficiency of PGCLC differentiation, miR-372 and let-7 also impacted the degree of PGCLC development.

To distinguish between the possible function for miR-372 and let-7 in either specification or maintenance of PGCLCs, we introduced mimics at either day 0 or day 3 during differentiation on H9 hESCs. miR-372 enhanced germ cell marker expression within the DP cell population exclusively when introduced at day 0, but not day 3. In contrast, let-7 significantly suppressed *PRDM1, VASA, SYCP3* and *POU5F1* expression even when introduced three days after differentiation (Fig 4A),. Furthermore, miR-372 led to a small, but significant increase in phosphorylated histone H3 (PHH3) positive DP cells relative to control and let-7 mimic when transfected at day 0, suggesting a positive effect of miR-372 on PGCLC proliferation (Fig 4B). Together, these data implicate miR-372 in production and early expansion of PGCLCs, and let-7 as a suppressor of their specification and maintenance.

The ESCC miRNAs promote dedifferentiation of somatic cells back to induced pluripotent stem cells (iPSCs) through repression of multiple targets including cell cycle regulators (*CDKN1A, RBL2, CDC2L6*), epithelial-mesenchymal transition regulators (*RHOC, TGFBR2*), and epigenetic regulators (*MECP2, SMARCC2*) [23]. To test a potentially parallel role of miR-372 in the specification of PGCLCs, which involves reactivation of the pluripotency program in mice [18], we evaluated eleven targets of the ESCC miRNAs previously tested in human iPSC production. [23]. Knockdown of six of the eleven targets increased the fraction of PGCLCs to from the baseline of 1% to the range of 1.97–2.8%,

representing an increase of 1.8–2.5 fold (Fig 4C). These findings suggest that ESCC miRNAs function through similar pathways to promote PGCLC production during hESC differentiation and iPSC production during human somatic cell dedifferentiation.

Previous studies demonstrated that the let-7 target *Prdm1* inhibits the mouse ESC to PGC transition [4, 5]. Introduction of let-7 similarly suppressed *PRDM1* in human cells (Fig 3D). Knockdown of *PRDM1* led to near abolishment of DP cells (Fig S3D–E). Since let-7 has been shown to target MYC genes in ESCs promoting ESC differentiation [12], we asked whether MYC ablation similarly affects PGCLC production. Individual knockdown of *CMYC* and *NMYC* significantly decreased DP cell frequency suggesting that MYC activity enhances hPGCLC specification (Fig 4D), similar to mouse PGCs [24]. Therefore, like the ESCC miRNAs, the let-7 miRNAs share common targets in the regulation in PGCLC production and ESC self-renewal.

Conclusion

These results support a model by which the ESCC and let-7 miRNAs have opposing effects on the production of hPGCLCs from pluripotent cells (Fig 4E). Knockout of the miR-290 cluster in mouse, which contains both ESCC miRNAs orthologous to human miR-372 and non-ESCC miRNAs, results in a depletion of PGCs, although the specific miRNAs targets and mechanisms involved remained unclear [11]. As shown here, miR-372 augments PGC numbers through the suppression of multiple pathways including cell cycle (*CDKN1A, RBL2, CDC2L6*), EMT (*RHOC, TGFBR2*), and epigenetic regulators (*MECP2, SMARCC2*). Suppression of these targets can also enhance human iPSC formation [23]. In contrast, let-7 inhibits PGCLC formation in part through the suppression of *MYC*, a target which also promotes ESC differentiation [12, 25]. Thus, these miRNA-regulated networks are common to the production of iPSCs and PGCs, both of which involve a reactivation of pluripotency. Given the success of miRNA manipulation in improving the efficiency of iPSC production, our studies suggest that similar strategies will be successful in rapidly evolving protocols for *in* vitro PGC derivation [3, 4]. The expression of miRNAs in such new protocols and potential synergy with miR-372/let7 is an important area of future experimentation.

MATERIALS AND METHODS

hESC culture and differentiation conditions

hESC lines, H1 (XY) and H9 (XX), and iPS lines, BJ3 (XY) and iPS BJ4 (XY) were cultured and passaged as described [1, 2]. Cells were differentiated on CF-1 irradiated mouse embryonic fibroblasts (MEFs) in KnockOut DMEM media supplemented with 20% KnockOut serum replacement (KSR), nonessential amino acids, L-glutamine, beta-mercaptoethanol, and 4ng/ml of recombinant human FGF-2 (Invitrogen) as described by West and colleagues [1] in the presence of 10^{4}–10^{-9} M of trans-retinoic acid (RA) (Sigma-Aldrich) without passaging for 4–10 days. In the 20% FBS group, FGF-2 was withheld and 20% KSR was replaced with 20% FBS (Hyclone). ESCs were transfected with miRNA mimics at final concentration of 50 nM using Dharmafect 1 (ThermoFisher) as described [3] on day 0 prior to differentiation. All mimics were purchased from ThermoFisher. For

miRNA sponges, ESC colonies were treated with Accutase (Invitrogen) and plated on DR4MEFs at 50,000 cells per 6-well plate and transduced with lentiviral vectors in the presence of polybrene (Sigma). Media was changed 24 hours after transduction. Puromycin at 1ng/ml was used to select for transduced cells 72 hours post-transduction.

Flow cytometry

Colonies were trypsinized and stained in 1% BSA with antibodies directed against human SSEA-1 and c-Kit (R&D Systems) for 30 minutes at 25°C. For VASA, SSEA-1+, c-Kit+ cells were permeabilized with 0.25% Triton X-100 for 10 minutes and stained with anti-VASA (R&D Systems). FITC-anti goat IgG was used as secondary antibody for VASA studies. SSEA-1-, c-Kit-cells were used as controls. Cells were analyzed and sorted using FACS AriaII (BD Biosciences) and Flow Jo cytometry analysis software (Tree Star, Ashland, OR).

Immunocytochemistry

Sorted cells were placed on slides using a cytospin at 800 rpm for 5 minutes, fixed with 4% paraformaldehyde for 15 minutes, permeabilized in 0.25% Triton X-100 for 10 minutes, blocked with 5% BSA for 1 hour and incubate with primary antibody overnight at 4°C. Primary antibodies against VASA, SCP3 (Abcam), TriM-H3K27 (H3K27me3) and DiM-H3K9 (H3K9me2) (Millipore) were used at 1:200 dilutions. FITC or PE secondary antibodies were used as 1:500 dilutions. Cells were counter stained with DAPI. Confocal microscopy was performed using the Leica TCS SP5 system (Leica).

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Cells were sorted directly into tubes for RNA extraction using RNeasy kits (Qiagen). RNA was treated with DNAse (Invitrogen) prior to reverse transcription. cDNA synthesis was performed using SuperScript III (Invitrogen) and real-time PCR and relative quantification were performed with Perfecta SYBR Green Fastmix (Quanta Biosciences) using the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). 75 cells were used for each reaction. Each experimental sample was run in triplicate. RT-qPCR for miRNAs was performed as described previously [4]. For relative fold changes, normalization was first done against GAPDH using the ΔΔCt method of quantification. Average fold changes from 3–5 independent experiments were calculated as $2^{-\Delta\Delta Ct}$.

Western blot analysis

Cells were sorted directly into cold PBS plus 2X protease inhibitors (Complete Mini, Roche). The cells suspension was spun at 2500g for 5 minutes. The cell pellet was resuspended with RIPA buffer plus 2X protease inhibitors. Protein lysates were denatured at 1:1 ratio with 2X Laemmli buffer at 95C for 5 min, then loaded on a 10% SDS-PAGE gel, blotted onto polyvinylidene fluoride (PVDF) membrane using the Bio-Rad electrophoresis unit. Blots were blocked with LI-COR blocking buffer for 2 hours followed by incubation with anti-DAZL antibodies (Abcam) at 1:500 dilutions. The blots were washed and incubated with secondary antibodies at the appropriate concentrations in the blocking buffer. Blots were imaged using the LI-COR Odyssey scanner.

Bisulfite Sequencing

Genomic DNA was extracted using ZR Genomic DNA II Kit (Zymo Research, Orange, CA) and procedure was performed as described [2, 5]. Amplified PCR products were cloned into pCR4-TOPO vector (Invitrogen) and 10 clones were sequenced for each sample.

siRNA, miRNA sponge, Lin28 expression, and shRNA *Prdm1* constructs

siRNA against Smarcc2, Mbd2, MeCP2, Rab11Fip5, Cdc2L6, CDKN1A, Rbl2, Aktl, RhoC, RGFBR2, nMyc, cMyc were obtained from ThermoFisher and introduced as previously described [3]. To build miRNA sponges, 7 target sites for let-7 or miR-372 were cloned into the 3′UTR of the eGFP gene (fig S4a) of the lentiviral based Psin vector as previously described [6]. The plasmid constructs together with packaging vectors were transfected into HEK293. Media was harvested 60 hours later and used to transduce hESC lines. The Lin28 gene was cloned into the Psin backbone replacing EGFP. shRNA constructs for *Prdm1* knockdown in the pLKO.1-pura vector system were obtained from Open Biosystems.

Luciferase reporter assays

Luciferase reporter constructs were previously reported [7, 8]. 5000 cells were plated per well in a 96-well plate and grown for 24 hours prior to transfection. Transfection was performed as described [7] along with Psin-let-7 or Psin-miR-372 vectors. Cells were lysed at 12 hours post transfection and processed for luciferase assay using Dual-Luciferase Reporter Assay System (Promega). We measured luciferase activity by Mithras LB 940 (Berthold Technologies).

Statistics

Two-tailed *t*-Tests were performed to evaluate significance, defined as $p < 0.05$, between two groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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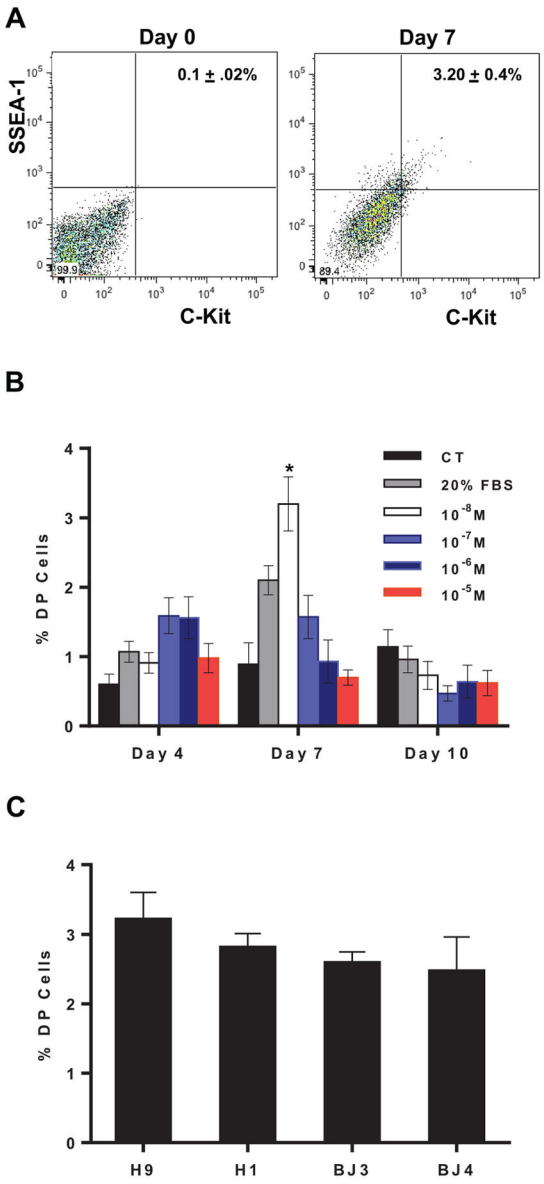

Figure 1.

Modeling human PGCLC formation *in vitro*. (A) Differentiation of representative H9 hESCs into PGCLCs as shown with representative flow cytometry analysis at days 0 and 7 following differentiation. Percentage of double positive (DP) cells ± SD cells expressing both SSEA-1 and C-Kit shown in top right corner (n=5). (B) H9 hESCs were either cultured in media alone, media with 20% FBS, or media with increasing concentration of RA for 4–10 days. Highest rate of SSEA-1+/c-Kit+ cells (PGCLCs), DP cells, was detected in the 10^{-8}M RA group by flow cytometry analysis after 7 days of differentiation. * $p<0.05$ relative to all groups. (C) Comparison of percent DP cells resulting from differentiation of various pluripotent human stem cell lines in retinoic acid for 7 days: H1 (XY) hESC, H9 (XX) hESCs, and two independent iPSC lines (BJ3 and BJ4), derived from BJ fibroblasts (XY), shown as mean ± SD (n=5).

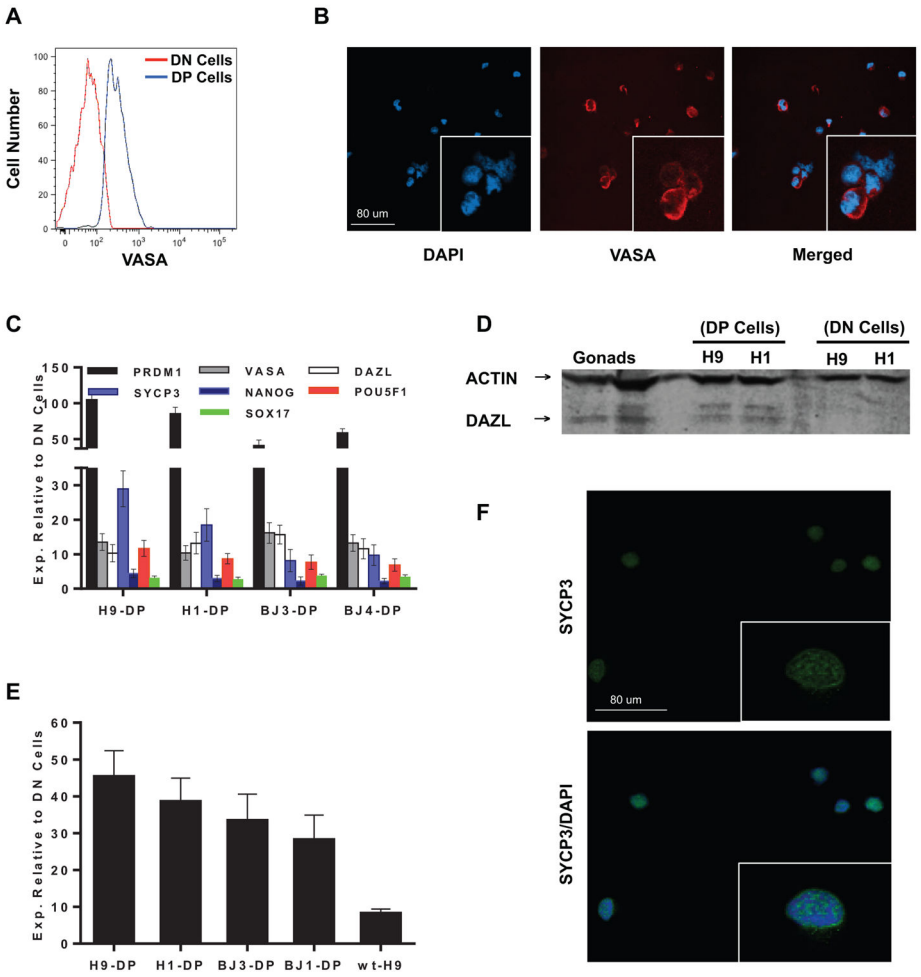

Figure 2.

PGCLCs express known markers of germ cells. (A) Flow cytometric analyses of intracellular VASA expression of representative H9 derived PGCLCs (n=3) presented as histogram. (B) VASA expression of in representative H9 derived DP cells assessed by confocal microscopy. DAPI (blue) and VASA (red). (C) qRT-PCR analysis of germ cell expressed genes in DP cells differentiated from hESC (H9, H1) and iPSC (BJ3, BJ4) lines (n=3). Expression levels are shown as mean ± SD relative to DN (somatic-like) cells. All are statistically significant against DN cells, $p<0.05$. (D) Western blot for DAZL comparing H9 and H1 derived DP to DN cells. Gonads = human testicular tissue. (E) qRT-PCR analysis for *SYCP1* expression in DP cells derived from H9, H1, BJ3, and BJ4 cells shown as mean ± SD (n=3). (F) SYCP3 expression in H9 derived DP cells assessed by confocal microscopy. DAPI (blue) and SYCP3 (green).

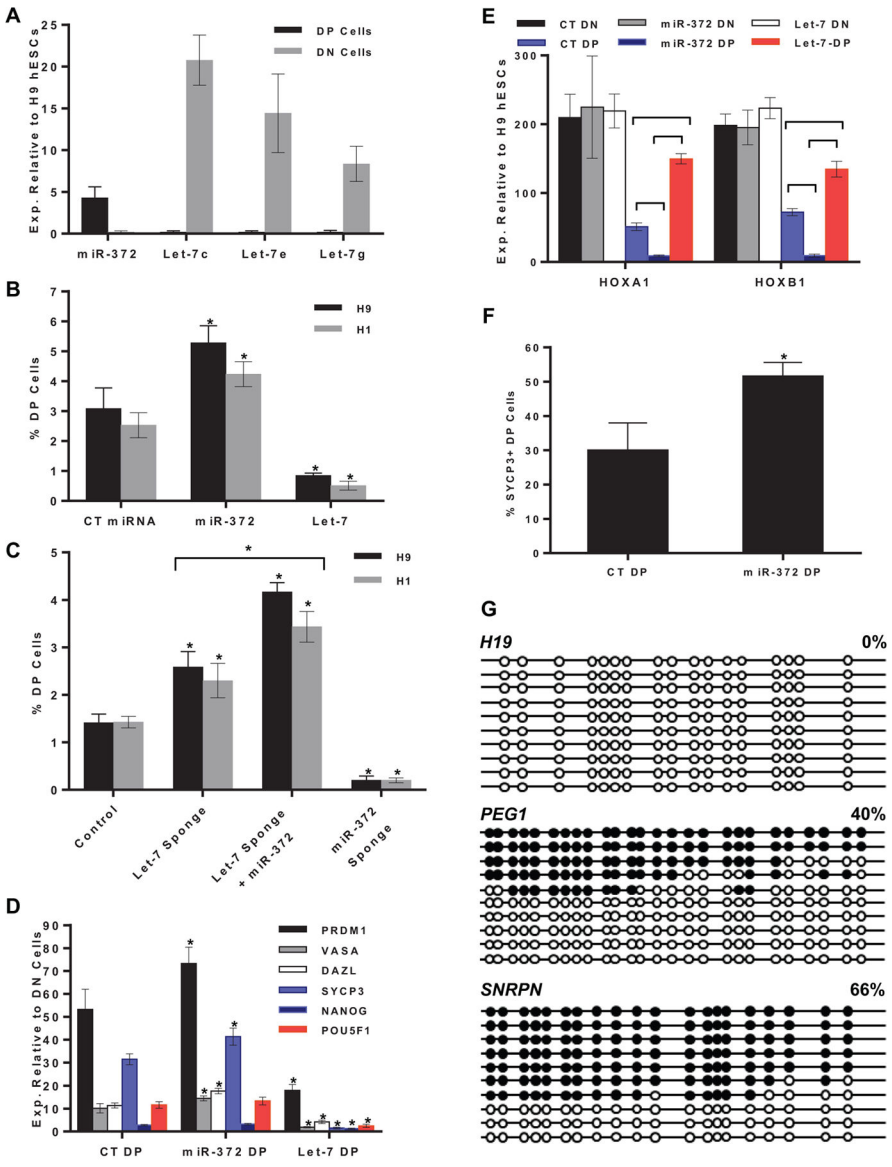

Figure 3.

miR-372/let-7 axis in human PGCLC formation. (A) qRT-PCR of mature miRNAs, miR-372 and let-7 family members in H9 derived DP and DN populations shown as mean ± SD, normalized over H9 hESCs (n=3). Let-7 was not detected in DP cells. (B) Percent DP cells obtained from differentiation of H9 and H1 hESCs (day 7) following introduction of miR-372 and let-7 mimics at day 0 of differentiation shown as mean ± SD (n=3). Mutant miR-294 was used as negative control. * $p<0.05$ relative to control (CT). (C) Percent DP cells obtained from differentiation of H9 and H1 hESCs transduced with control, let-7 sponge, miR-372 sponge, or let-7 sponge followed by transfection with miR-372 mimics at day 0 shown as mean ± SD (n=3). * $p<0.05$ relative to control. (D) qRT-PCR for germ cell markers in DP cells derived from H9 hESCs transfected with control, miR-372, or let-7 at day 0. Levels shown relative to DN cells as mean ± SD (n=3). * $p<0.05$ relative to control. (E) qRT-PCR for *HOXA1* and *HOXB1* genes in DN and DP cells derived from H9 hESCs

transfected with mimics as in (D). Levels shown relative to wt-H9 hESCs as mean ± SD (n=3). * $p<0.05$ (F) Fraction of 100 H9 derived DP cells that stained positively for SYCP3 following differentiation in presence of control miRNA or miR-372 mimic shown as mean ± SD (n=3). (G) Bisulfite sequencing of differentially methylated regions (DMRs) at the *H19*, *PEG1*, and *SNRPN* loci in H9 derived DP cells following introduction of miR-372. Compare to no miRNA control in (Fig S1B).

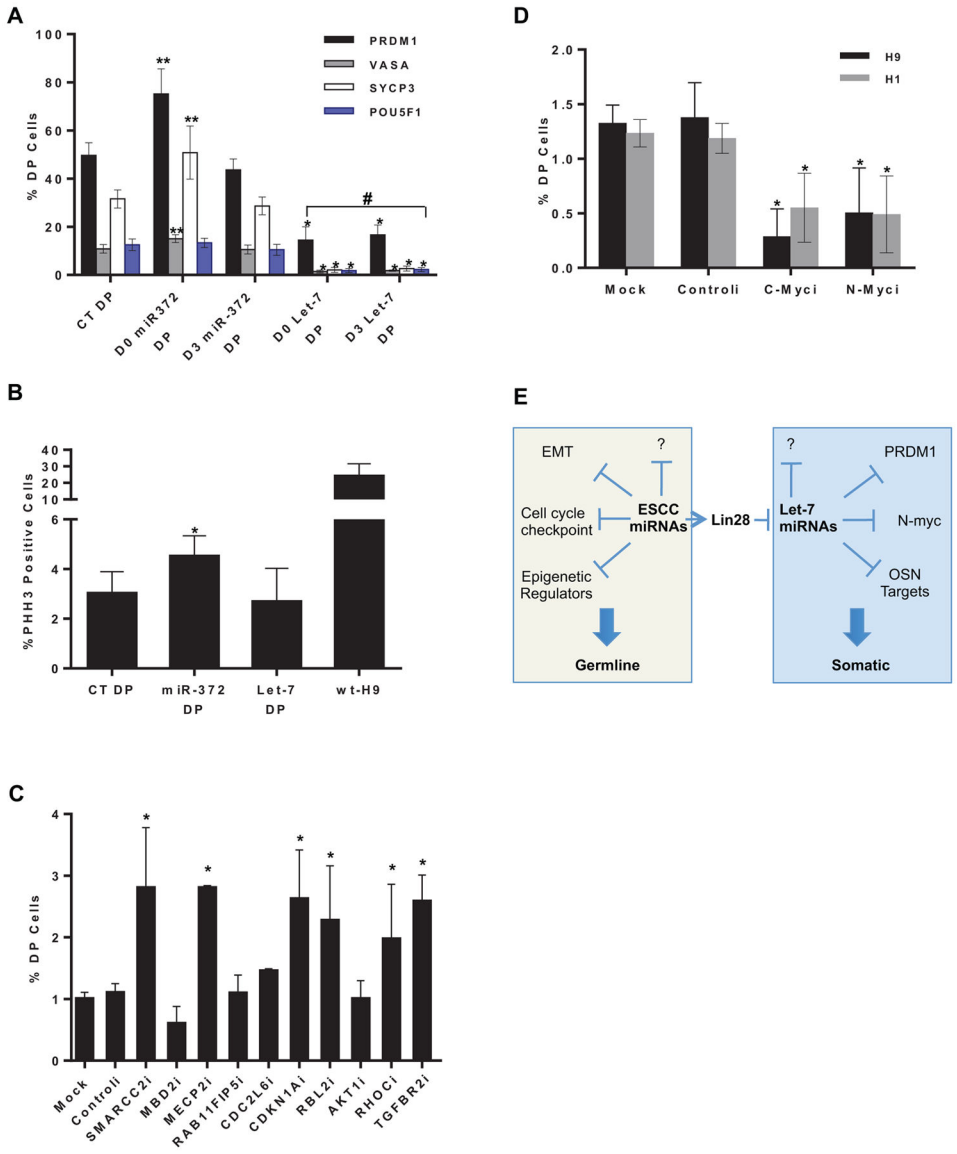

Figure 4.

Effects of miR-372 and let-7 in human PGCLC formation. (A) qRT-PCR of germ cell markers as in Fig 3D, but following introduction of miRNAs at different time points of differentiation H9 hESCs: day 0 (D0) or day 3 (D3) shown as mean ± SD (n=3). * $p<0.05$ relative to control (CT) DP cells, ** $p<0.05$ relative to both control and D3 miR-372 DP cells. # $p>0.4$ between the two groups. (B) Percent of H9 derived DP cells expressing phospho-Histone H3 (PHH3) as assessed by immunofluorescence, shown as mean + SD (n=6). *$p<0.05$ relative to both CT and let-7 derived DP cells. (C) Percent of H9 derived DP cells following transfection of siRNAs to indicated miR-372 target mRNAs at D0 of differentiation shown as mean ± SD (n=3). * $p<0.05$. (D) Percent of H9 derived DP cells following transfection of siRNAs to indicated let-7 target mRNAs, C*MYC* and *NMYC*

shown as mean ± SD (n=3). * $p<0.05$. (E) Model showing how ESCC and let-7 miRNAs modulate the differentiation of pluripotent cells to hPGCLCs.