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

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ABSTRACT

The embryonic stem cell cycle (ESCC) and let-7 families of miRNAs function antagonistically in the switch between mouse embryonic stem cell 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 whereas let-7 antagonizes PGCLC differentiation. Knockdown of the individual miR-372 targets *SMARCC1*, *MECP2*, *CDKN1*, *RBL2*, *RHOC*, and *TGFBR2* increased PGCLC production, whereas knockdown of the let-7 targets *CMYC* and *NMYC* suppressed PGCLC differentiation. These findings uncover a miR-372/let-7 axis regulating human primordial germ cell (PGC) specification. STEM CELLS *2016;34:1985–1991*

SIGNIFICANCE STATEMENT

This study deciphers molecular mechanism underlying the production of human germ cells. It shows how a class of noncoding RNAs called microRNAs can regulate the number and quality of primordial germ cells produced during human embryonic stem cell differentiation. In particular, two families of microRNAs are shown to have opposing effects on the number of primordial germ cells produced. The microRNAs function through the suppression of multiple downstream targets, which are themselves key regulators of germ cell specification. These results give important new insight into basic molecular mechanisms regulating differentiation of early embryonic cells into germ cells. The resulting knowledge also has practical value as it should help increase the production of germ cells that eventually could be used to model germ line diseases and the treat infertility.

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 primordial germ cell-like cells (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 been 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 primordial germ cell (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 embryonic stem cell cycle (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.

Materials and Methods

Human embryonic stem cell (hESC) lines (H1, H9), and induced pluripotent stem cell (iPSC) lines (BJ3, BJ4) were differentiated as previously described [2] with slight modifications. See supplementary materials for all methods including: cell culture; flow cytometry; RT-qPCR; western blot analysis; bisulfite sequencing; design and introduction of siRNAs, miRNA mimics, Lin28, Prdm1 shRNA, and miRNA sponges; luciferase reporter assays; and statistical methods.

Results and Discussion

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–1C). 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–2E), 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 (Supporting Information Fig. S1A), consistent with epigenetic reprogramming 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 (Supporting Information 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 whereas let-7 was undetectable (Fig. 3A); the converse was found in DN cells. Introduction of miR-372 mimics into H9 and H1 hESCs 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 (2.8–0.7%, 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 Supporting Information Fig. S2A–S2D. Sponge introduction did not alter the pluripotency of hESCs as they fully retained the capacity for multilineage differentiation as assessed by embryoid body formation (Supporting Information Fig. S2E). Subsequently, H9 hESCs expressing these sponges were differentiated as described above. Although 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–S3C). Combined knockdown of let-7 with miR-372 mimic

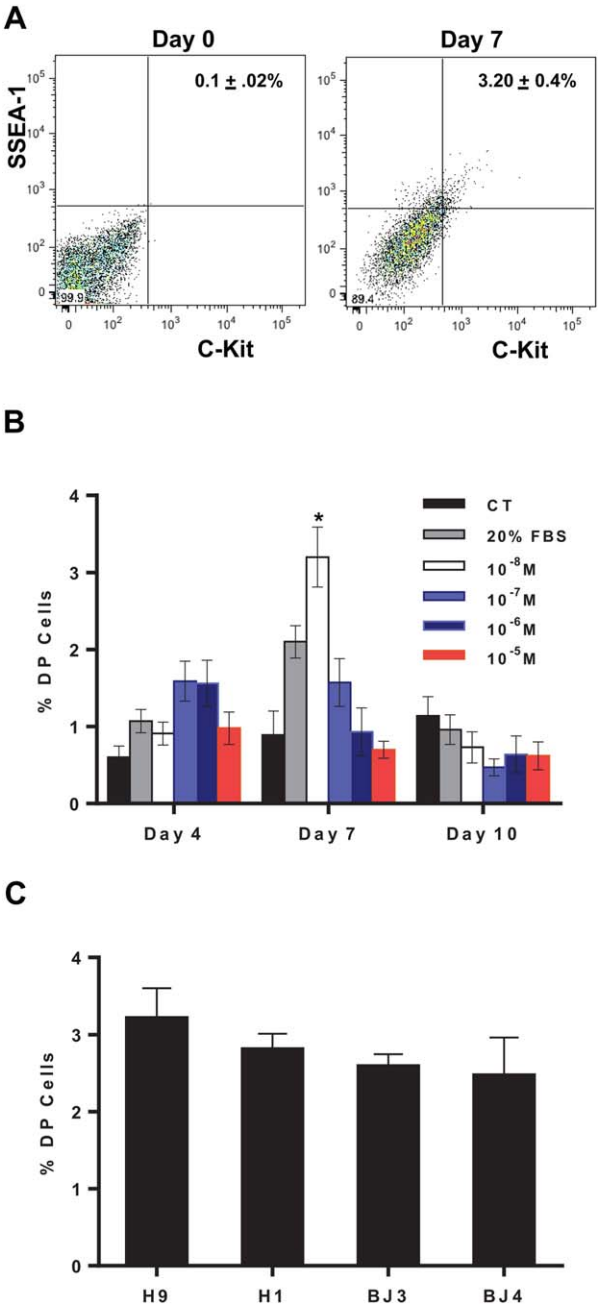

Figure 1. Modeling human primordial germ cell-like cells (PGCLC) formation in vitro. **(A)**: Differentiation of representative H9 human embryonic stem cells (hESCs) into PGCLCs as shown with representative flow cytometry analysis at days 0 and 7 following differentiation. Percentage of DP cells ± SD 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% fetal bovine serum, 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. *, $p < .05$ 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 ($n = 5$). Abbreviation: DP, double positive.

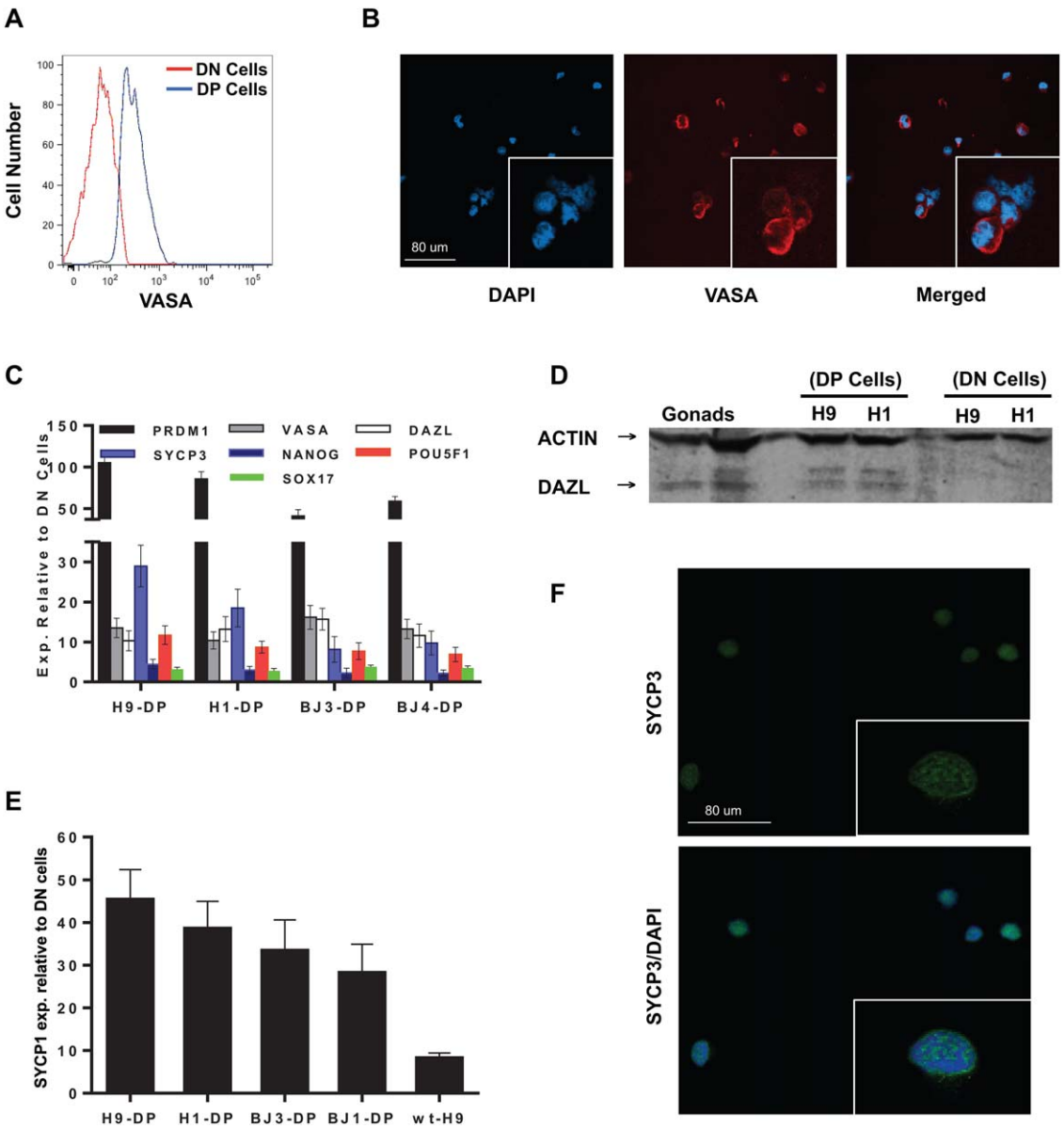

Figure 2. Primordial germ cell-like cells (PGCLCs) express known markers of germ cells. **(A)**: Flow cytometric analyses of intracellular VASA expression in representative H9 derived PGCLCs ($n = 3$) presented as histogram. **(B)**: VASA expression in representative H9 derived DP cells assessed by confocal microscopy. DAPI (blue) and VASA (red). **(C)**: qRT-PCR analysis of germ cell expressed genes in DP cells differentiated from human embryonic stem cells (hESC) (H9, H1) and induced pluripotent stem cells (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, $p < .05$. **(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). Abbreviations: DN, double-negative; DP, double-positive.

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, whereas let-7 decreased the levels of *PRDM1*, *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, Supporting Information S1B). Thus, in addition to the overall frequency and efficiency of PGCLC differentiation, miR-372 and let-7 also impacted the quality 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 of 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*

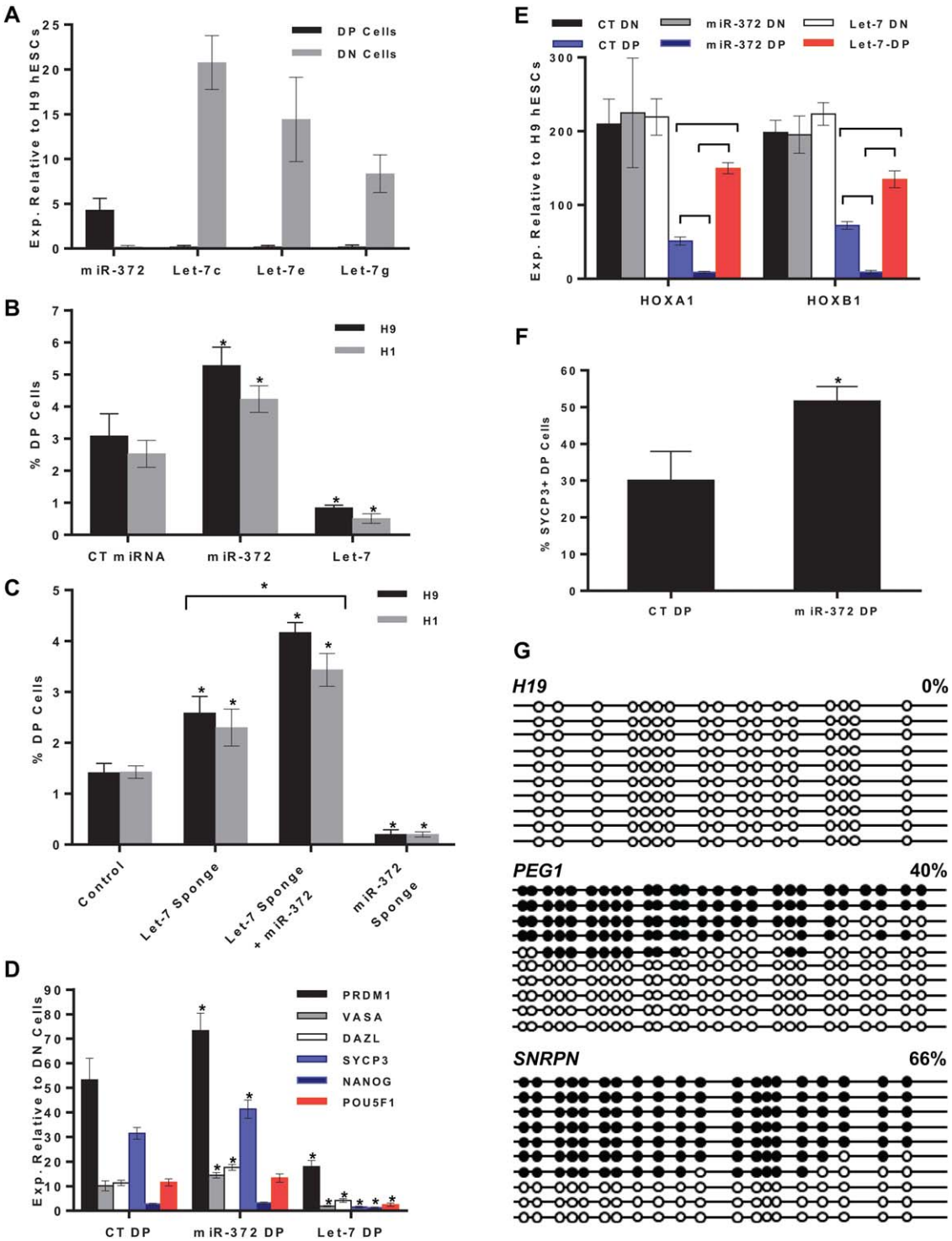

Figure 3. miR-372/let-7 axis in human primordial germ cell-like cell (PGCLC) formation. **(A)**: qRT-PCR of mature miRNAs, miR-372 and let-7 family members in H9 derived DP and DN populations shown as mean ± SD, normalized over H9 hESCs ($n = 3$). Let-7 was not detected in DP cells. **(B)**: Percent 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. *, $p < .05$ 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 ($n = 3$). *, $p < .05$ 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 ($n = 3$). *, $p < .05$ 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 ($n = 3$). *, $p < .05$ **(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 (Supporting Information Fig. S1B). Abbreviations: DN, double-negative; DP, double-positive; hESCs, human embryonic stem cells.

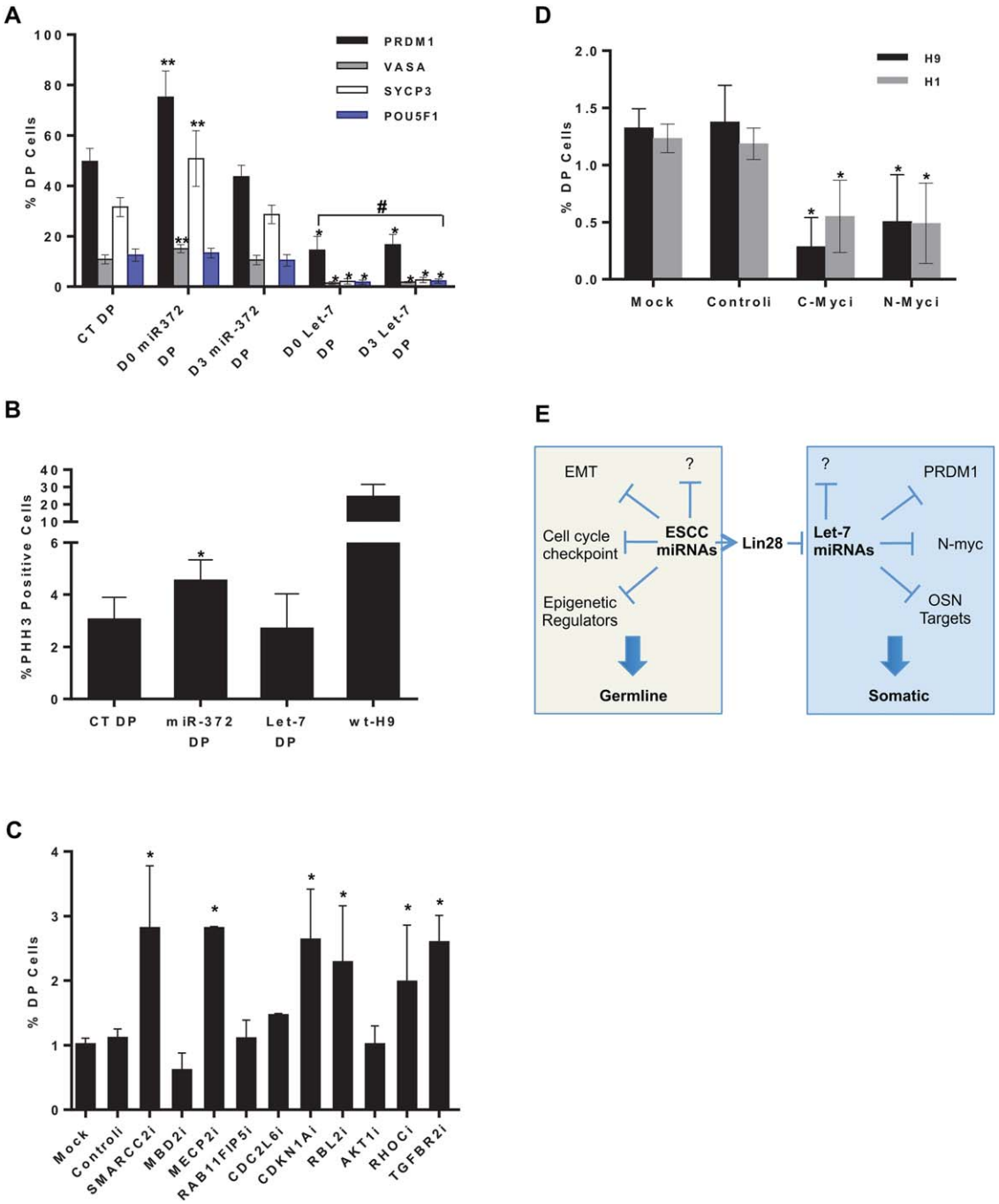

Figure 4. Effects of miR-372 and let-7 in human primordial germ cell-like cell (PGCLC) formation. **(A)**: qRT-PCR of germ cell markers as in Fig. 3D, but following introduction of miRNAs at different time points of differentiation H9 human embryonic stem cells (hESCs): day 0 (D0) or day 3 (D3) shown as mean ± SD ($n = 3$). *, $p < .05$ relative to control (CT) DP cells, **, $p < .05$ relative to both control and D3 miR-372 DP cells. #, $p > .4$ between the two groups. **(B)**: Percent of H9 derived DP cells expressing phospho-Histone H3 (PHH3) as assessed by immunofluorescence, shown as mean ± SD ($n = 6$). *, $p < .05$ 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 ($n = 3$). *, $p < .05$. **(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 ($n = 3$). *, $p < .05$. **(E)**: Model showing how ESCC and let-7 miRNAs modulate the differentiation of pluripotent cells to hPGCLCs. Abbreviation: DP, double-positive.

expression even when introduced 3 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 11 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 (Supporting Information Fig. S3D–S3E). 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). 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 remaine 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, 24]. 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.

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Author Contributions

N.D.T.: Conception and design, collection and analysis of data, manuscript writing, final approval of manuscript; M.K.: Collection of data; D.S.: Collection of data; R.P.: Collection of data; D.L.: Conception and design, financial support, data analysis, manuscript writing, final approval of manuscript; R.B.: Conception and design, financial support, data analysis, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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