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PRDM14 is expressed in germ cell tumors with constitutive overexpression altering human germline differentiation and proliferation

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ABSTRACT

Germ cell tumors (GCTs) are a heterogeneous group of tumors occurring in gonadal and extragonadal locations. GCTs are hypothesized to arise from primordial germ cells (PGCs), which fail to differentiate. One recently identified susceptibility loci for human GCT is PR (*P*RDI-BF1 and *R*IZ) domain proteins 14 (*PRDM14*). *PRDM14* is expressed in early primate PGCs and is repressed as PGCs differentiate. To examine *PRDM14* in human GCTs we profiled human GCT cell lines and patient samples and discovered that PRDM14 is expressed in embryonal carcinoma cell lines, embryonal carcinomas, seminomas, intracranial germinomas and yolk sac tumors, but is not expressed in teratomas. To model constitutive overexpression in human PGCs, we generated PGC-like cells (PGCLCs) from human pluripotent stem cells (PSCs) and discovered that elevated expression of PRDM14 does not block early PGC formation. Instead, we show that elevated PRDM14 in PGCLCs causes proliferation and differentiation defects in the germline.

1. Introduction

Germ cell tumors (GCTs) make up a heterogeneous group of tumors, encompassing five histologic subtypes. These are, germinomas, embryonal carcinoma, yolks sac, choriocarcinoma and teratomas. GCTS occur in gonadal and extra-gonadal locations, with extragonadal occurring primarily along the midline in the sacrum, mediastinum and pineal and/or suprasellar region of the brain. GCTs occur in a bimodal fashion with pediatric patients being most affected from birth to 3 years of age, and then again in adolescences into young adulthood. Although considered rare, GCTs account for 15% of malignancies in the adolescent to young adult population (15–40 years), with testicular GCTs being the most common malignancy of males in this age group (Calaminus and Joffe, 2016).

GCTs are hypothesized to arise from a common cell of origin, the embryonic progenitors of gametes called primordial germ cells (PGCs). Extragonadal GCTs are hypothesized to arise from PGCs that migrated inappropriately (Schmoll, 2002). PGCs are specified very early in the peri-implantation human embryo, between 2 and 3 weeks post-fertilization (De Felici, 2013). After specification, PGCs migrate through the hindgut, into the dorsal mesentery and begin colonizing the genital ridges starting at week 4–5 post-fertilization (De Felici, 2013). Specified human PGCs that are negative for the gonadal-stage germline markers VASA and deleted in azoospermia like (DAZL) are referred to as "early PGCs", whereas DAZL and VASA positive PGCs that have migrated into the dorsal mesentery and genital ridges are called "late PGCs" (Gkountela et al., 2015; Gkountela et al., 2013; Guo et al., 2015; Irie et al., 2015; Chen et al., 2017a). PGCs in the gonads are classically referred to as gonocytes. In humans, the PGC stage of germline development ends at around 8–10 weeks post-fertilization (De Felici, 2013). After this, PGCs in the gonad start to advance in differentiation, and transition into oogonia or pro-spermatogonia which ultimately become female and male gametes, respectively.

Much of what is known about mammalian PGC development comes from work in mouse models. However, recent studies revealed critical species-specific differences between mouse and human PGC development, including differences in the transcription factor network (Irie et al., 2015; Tang et al., 2016), and most importantly many of the GCT types found in humans, such as seminomas, yolk sac tumors, and intracranial germ cell tumors, do not occur in mouse models of the disease (Heaney et al., 2012; Irie et al., 2014). Therefore, understanding the cell and molecular origins of GCTs in humans requires developing new

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human cell-based models. Given that PGCs are embryonic progenitors that differentiate into more mature germline cell types at the end of the first trimester, it is not possible to isolate these cells from children or young adults at risk for GCT formation. Instead, we hypothesize that the use of pluripotent stem cells (PSC), and the generation of human PGC-like cells (PGCLCs), will allow for further investigation into the mechanisms of GCT formation in humans.

In a recent genome-wide association study of testicular and intracranial GCTs, the transcription factor *PRDM14* was identified as being a susceptibility locus for this disease (Ruark et al., 2013; Terashima et al., 2014). In mice, *Prdm14* is critical for PGC formation, being highly expressed from the time of specification until the end of the PGC period, which is embryonic (E) day E13.5 in the mouse (Nakaki and Saitou, 2014; Yamaji et al., 2008; Kurimoto et al., 2008). A homozygous null mutation in *Prdm14* in mice causes loss of PGCs by E12.5 due to a failure of *Prdm14* mutant PGCs to undergo germline reprogramming (Yamaji et al., 2008). In humans, the function of *PRDM14* in PGCs is unclear. RNA-Sequencing and immunofluorescence studies have found that human PGCs express low levels of *PRDM14* (Gkountela et al., 2015; Guo et al., 2015; Irie et al., 2015; Tang et al., 2015), and a knockdown of *PRDM14* has no effect on human PGCLC differentiation (Sugawa et al., 2015). Combined, these results suggest that the role of *PRDM14* in human PGCs may be different from mice, with one hypothesis being the repression of *PRDM14* is required for PGC differentiation.

In the current study, we used human GCT tissue samples, and the differentiation of PGCLCs from human PSC to address the hypothesis that *PRDM14* is expressed in human GCTs, and that over expression of *PRDM14* alters PGC differentiation.

2. Materials and methods

2.1. Cell lines and cell culture

Primed hESC lines were cultured on mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) in hESC media, per Pastor et al. (2016) with the addition of 50 ng/mL primocin (InvivoGen, ant-pm-2). All hESC lines were split every 7 days with Collagenase type IV (GIBCO, 17104-019). All hESC lines used in this study are registered with the National Institute of Health Human Embryonic Stem Cell Registry and are available for research use with NIH funds. Specifically, the following hESC lines were used in this study: UCLA2 (46XY), UCLA6 (46XY). The derivation and basic characterization of UCLA2 and 6 were previously reported (Diaz Perez et al., 2012). Experiments were performed between passage 15–25, two passages were performed between thaw and use in experiments. Human embryonal carcinoma cell (ECC) lines, GCT27 and NTERA2 were cultured in media containing 10% fetal bovine serum (FBS) (EDM Millipore, TMS-013-B), 1× Penicillin-Streptomycin-Glutamine (PSG) (Gibco, 10378-016), 1× Non-essential amino acids (NEAA) (Gibco, 11140-050), 50 ng/mL primocin (IvivoGen, ant-pm-2) in DMEM High Glucose (Gibco, 11960-069). GCT27 cell line was donated from Dr. Martin Pera (derivation described in (Pera et al., 1987)), NTERA2 cl.D1 (NT2) line was obtained from America Type Culture Collection (ATCC) (ATCC CRL-1973). All ECC lines were grown to 80–90% confluence prior to split with 0.05% Trypsin-EDTA (Gibson, 25300-054). Experiments were performed between passages 20–30, one passage was used between thaw and use in experiments. Human embryonic kidney (HEK) 293T cells were cultured in 10% FBS (ThermoFisher, SH3007003), 1× PSG (Gibco, 10378-016), 1× NEAA (Gibco, 11140-050), 55 μM Sodium Pyruvate (Gibco, 21985-023), and 50 ng/mL primocin (InvivoGen, ant-pm-2) in KnockOut DMEM (Gibco, 10829-018). Cells were cultured to 80–90% confluency prior to split with 0.05% Trypsin-EDTA. Experiments were performed between passage 8–15, one passage was used between thaw and use in experiments. All cell lines used in these experiments were Mycoplasma negative. Mycoplasma testing was performed every 6–9 weeks, using MycoAlert Detection Kit (Lonza, LT07-418).

2.2. Induction of PGCLCs though iMeLCs from primed hESCs

PGCLCs were induced from primed hESCs as described in Sasaki et al. (2015), with some modifications (Chen et al., 2017b). Day 7 hESCs were dissociated into single cells with 0.05% Trypsin-EDTA and plated onto Human Plasma Fibronectin (Invitrogen, 33016-015)-coated 12-well-plate at the density of 200,000 cells/well in 2 mL/well of iMeLC media, which is composed of 15% KSR, 1× NEAA, 0.1 mM 2-Mercaptoethanol, 1× PSG (Gibco, 10378-016), 1 mM sodium pyruvate (Gibco, 11360-070), 50 ng/mL Activin A (Peprotech, AF-120-14E), 3 μM CHIR99021 (Stemgent, 04-0004), 10 μM of ROCKi (Y27632, Stemgent, 04-0012-10), and 50 ng/mL primocin in Glasgow's MEM (GMEM) (Gibco, 11710-035). iMeLCs were dissociated into single cells with 0.05% Trypsin-EDTA after 24 h of incubation and plated into ultra-low cell attachment U-bottom 96-well plates (Corning, 7007) at the density of 3000 cells/well in 200 μL/well of PGCLC media, which is composed of 15% KSR, 1× NEAA, 0.1 mM 2-Mercaptoethanol, 1× PSG (Gibco, 10378-016), 1 mM sodium pyruvate (Gibco, 11360-070), 10 ng/mL human LIF (Millipore, LIF1005), 200 ng/mL human BMP4 (R&D systems, 314-BP), 50 ng/mL human EGF (R&D systems, 236-EG) 10 μM of ROCKi (Y27632, Stemgent, 04-0012-10), and 50 ng/mL primocin in Glasgow's MEM (GMEM) (Gibco, 11710-035).

2.3. Fluorescence activated cell sorting

Day 4 aggregates were dissociated with 0.05% Trypsin-EDTA for 10 min at 37 °C. The dissociated cells were stained with conjugated antibodies, washed with FACS buffer (1% BSA in PBS) and resuspended in FACS buffer accompanying with 7-AAD (BD Pharmingen, 559925). The conjugated antibodies used in this study are: INTEGRINa6 conjugated with BV421 (BioLegend, 313624), EPCAM conjugated with 488 (BioLegend, 324210). PGCLCs were either sorted 1000 cells in RLT buffer (QIAGEN) for RNA extraction or media for culture.

2.4. PGCLC culture on transwell membrane

Sorted day 4 PGCLCs were cultured in primed hESC media (Pastor et al., 2016), naïve hESC media (Pastor et al., 2016; Theunissen et al., 2014) or 7-factor media. 7-factor media was based on the formulation of Farini et al. (2005), without the addition of retinoic acid. Therefore, 7-factor medium contains the following: 15% Hyclone FBS (ThermoFisher, SH3007003), DMEM high glucose (Gibco, 11960-069), 1× NEAA (Gibco, 11140-050), 0.1 mM 2-mercaptoethanol (Gibco, 21985-023), 0.25 mM sodium pyruvate (Gibco, 11360070), 1× PSG (Gibco, 10378-016), 50 ng/mL SCF (PeproTech, 250-03), 10 ng/mL bFGF (R&D System, 233-FB), 10 ng/mL SDF1 (R&D Systems, 350-NS), 25 ng/mL human BMP4 (R&D Systems, 314-BP), 500 U/mL LIF (Millipore, LIF1005), 5 μM forskolin (Sigma, D6886), and 1 mg/mL *N*-acetyl-L-cysteine (Sigma, A9165). 1000 sorted PGCLCs were plated directly on 0.4 μM PET membranes (BD Falcon) in 24-well plates and cultured at 37 °C with 5% CO_2 with daily medium changes. For some downstream experiments, cultured PGCLCs were detached from the membrane with 0.05% trypsin for 5 min at 37 °C. Following trypsin cell numbers were counted with hemocytometer and trypan blue (Gibco, 15250061) for viability or placed in RLT buffer for RNA extraction (see below). For each experiment, three independent experiments were performed for each cell line UCLA2 and UCLA6.

2.5. Real time quantitative PCR

PGCLCs sorted or harvested from membranes were placed in 350 μL of RLT buffer (QIAGEN) and RNA was extracted using RNeasy micro kit (QIAGEN, 74004). cDNA was synthesized using SuperScript® II Reverse Transcriptase (Invitrogen, 18064-014). Real time quantitative PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, 4304437) and the expression level of genes-of-interest