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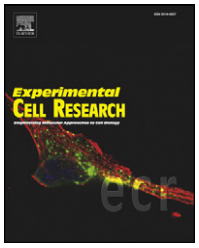

Research Article

Loss of *Dnd1* facilitates the cultivation of genital ridge-derived rat embryonic germ cells

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ABSTRACT

Pluripotent cells referred to as embryonic germ cells (EGCs) can be derived from the embryonic precursors of the mature gametes: the primordial germ cells (PGCs). A homozygous mutation (*ter*) of the *dead-end homolog 1* gene (*Dnd1*) in the rat causes gonadal teratocarcinogenesis and sterility due to neoplastic transformation and loss of germ cells. We mated heterozygous *ter*/+ WKY-*Dnd1*^{ter}/Ztm rats and were able to cultivate the first genital ridge-derived EGCs of the rat embryo at day 14.5 post coitum (pc). Genotyping revealed that ten EGC lines were *Dnd1* deficient, while only one wild type cell line had survived in culture. This suggests that the inactivation of the putative tumor suppressor gene *Dnd1* facilitates the immortalization of late EGCs *in vitro*. Injection of the wild type EGCs into blastocysts resulted in the first germ-line competent chimeras. These new pluripotent rat EGCs offer an innovative approach for studies on germ cell tumor development as well as a new tool for genetic manipulations in rats.

Introduction

The gametes of adult vertebrates, oocytes and sperm, develop from their embryonic precursors, the primordial germ cells (PGCs), and are responsible for the transmission of genetic information from one generation to the next. During ontogenesis, the PGCs migrate through various tissues before finally reaching the genital ridge. PGCs themselves are highly specialized cells restricted in their developmental potency [1]. Pluripotent cells referred to as embryonic germ cells (EGCs), can be obtained from PGCs *in vitro*. Culture of PGCs leads to this not well-understood process of epigenetic reprogramming which transforms the unipotent PGCs to pluripotent EGCs [2]. The EGCs proliferate indefinitely and, unlike PGCs [3], can result in germ-line competent chimeras upon blastocyst injection [4,5]. EGCs have been made from PGC in the mouse before and during migration, as well as after entry in the genital ridge [3,6]. Only recently, the first EGCs were obtained from pre-migratory PGCs of the rat. Culture conditions similar to those for rat ESCs, using the two inhibitors (2i) and LIF on feeder cells, could procure these rat EGCs [7–9].

Loss of *Dnd1* can lead to the formation of germ cell tumors and infertility. Dnd1 is an RNA-binding protein that competes with

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miRNAs for the binding of specific target gene mRNAs. In doing so, Dnd1 inhibits the miRNA-mediated repression and enables the expression of certain target genes even though miRNAs are present. The persistence of mRNAs from the tumor suppressor genes *Lats2* and *p27* as well as *Nanos1*, *Tdrd7* and *Connexin 43* in the germ cells depends on expression of *Dnd1* [10,11]. Dnd1 has also been shown to interact with the multifunctional protein APOBEC3 [12]. The *Ter* mutation in the 129/Sv mouse strain was traced back to a point mutation leading to a premature stop codon in *Dnd1* [13,14]. This mutation causes decreased fertility in *Ter/Ter* females and infertility in *Ter/Ter* males and increases the teratoma incidence in the testis to 17% in *Ter/+* and 94% in *Ter/Ter* males, compared to the 1% in wild type 129/Sv [15,16]. It is supposed that the Dnd1 protein is involved in the mitotic arrest to G0 of male PGCs [17]. Loss of PGCs during ontogenesis was first detectable in *Ter/Ter* mouse embryos of both genders at day 8.5 post coitum (E8.5pc) [18].

A spontaneous mutation with a recessive mode of inheritance leading to teratomas in ovaries and testis also occurred in the WKY/Ztm rat strain and was named *ter* [19]. This *ter* mutation was identified as a point mutation in exon 4 of the *Dnd1* gene that introduces a premature stop codon. Resulting loss or truncation of the Dnd1 protein in the homozygous *ter/ter* rats leads to infertility due to the ontogenetic death of germ cells and initiates gonadal teratoma formation in all animals through neoplastic transformation of surviving germ cells. The onset of teratocarcinogenesis and the teratoma progression were influenced by gender. While tumors were detected between 21 and 66 days of age in female *ter/ter* rats with a mean survival of 34 days, the tumorigenesis was delayed in *Dnd1*-deficient males where 61% survived longer than day 70 and 10% longer than day 125 with a mean survival of 58 days. Furthermore, both ovaries were affected in the females whereas 50% of the males had unilateral tumors in the testis ([19], Zschemisch et al., submitted).

Thus, *Dnd1* fulfils different roles in PGC migration, development and survival, depending on the species. So far the molecular mechanisms involved in the rat remain entirely unknown. We cultured rat EGCs *in vitro* derived from embryonic PGCs of the genital ridge as a tool to investigate the mechanisms of gender-dependent germ cell differentiation and germ cell tumor development.

Materials and methods

Animals

All animals were bred and maintained at the Institute of Laboratory Animal Science, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany (subline code: Ztm: http://www.mh-hannover.de/einrichtungen/tierlabor). The experiments were in accordance with the German Animal Welfare Legislation (Tierschutzgesetz 2002), approved by the local Institutional Animal Care and Research Advisory Committee and permitted by the Animal Welfare Service of the Lower Saxony State Office for Consumer Protection and Food Safety (Az.10/0226; Az.10/0209).

Strain origin

The rat strain WKY-*Dnd1*^{ter}/Ztm traces back to a spontaneous mutation that occurred in the Institute of Laboratory Animal Science, Hannover Medical School in the inbred generation F73 of the WKY/Ztm rat strain [19]. The *ter* mutation leads to the development of teratomas and infertility with an incidence of 100% in homozygous animals, while no effects are seen in heterozygous animals. WKY-*Dnd1*^{ter}/Ztm colony is maintained as a segregating, coisogenic inbred strain.

Husbandry

Animals were maintained under standardized conditions in the following environment: temperature of 22 ± 2 °C, relative humidity of approximately 55%, and artificial light for 14 h. The commercial softwood granulate bedding was sterilized (Lignocel, Altromin; Lage, Germany). They received an autoclaved commercial pelleted diet (Altromin 1314) (protein 22%, fat 5%, raw fiber 4.5%, ash 7%, utilizing energy 3.1 kcal/g) and water *ad libitum*.

The rats were kept under conventional housing conditions, as pairs or in sibling groups. Mice were housed under specified pathogen free (SPF) conditions, separated by gender and in individually ventilated cages according to ETS123. Microbiological status was monitored according to FELASA recommendations [20]. The mice were free of the listed microorganisms. The DA.1 M rats were positive for parvovirus, helicobacter hepaticus and apathogenic protozoa and the WKY/Ztm rats were positive for parvovirus and apathogenic protozoa.

EGC derivation

WKY-*Dnd1*^{ter}/Ztm rats with a *ter/+* genotype were mated and the pregnant females were sacrificed at E14.5pc. Embryos were collected and post-migratory EGCs (late EGCs) were isolated as previously described by Cooke and colleagues [21] with minor modifications: The genital ridge containing the PGC was dissected free from the embryo without the mesonephros and a single-cell suspension was obtained by mechanical disruption and digestion with accutase (Sigma, St. Louis, MO). Heterozygous *ter/+* WKY-*Dnd1*^{ter}/Ztm were also mated and sacrificed at E10.5pc for the isolation of pre-migratory EGCs. These early PGCs were collected as described by Leitch and colleagues [9].

EGC culture

EGCs were maintained on γ-irradiated TRF-O3 cells as feeder in N2B27 media supplemented with 2i-LIF. 2i-LIF includes the MEK-inhibitor PD032591 (1 μM; Axon, Groningen, The Netherlands), the GSK3 inhibitor CHIR99021 (3 μM; Axon) and rat LIF (1000 U/ml; Millipore, Billerica, MA). The TRF-O3 cells stem from a teratoma that developed in the ovaries of a female *ter/ter* WKY-*Dnd1*^{ter}/Ztm rat and are maintained in DMEM media with FCS. Between 7 and 10 days of primary culture all visible genital ridge-derived EGC (late EGC) colonies were picked and expanded. The EGCs that stem from embryos at day 10.5pc (early EGCs) were picked between 9 and 12 days of culture. The primary EGC colonies were identified based on their morphology and the fact that they were easy to detach from the feeder cells. To maintain the cells, medium was changed every other day and cells were split with accutase every 3–4 days. Cells were cryopreserved in 2i-LIF medium supplemented with 10% DMSO and survived multiple freeze–thaw cycles.

Immunocytofluorescence and alkaline phosphatase staining

Immunostaining was performed by fixation of the cells in 4% paraformaldehyde for 2 h. Cells were then made permeable using