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Cripto: Expression, epigenetic regulation and potential diagnostic use in testicular germ cell tumors

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ABSTRACT

Type II germ cell tumors arise after puberty from a germ cell that was incorrectly programmed during fetal life. Failure of testicular germ cells to properly differentiate can lead to the formation of germ cell neoplasia *in situ* of the testis; this precursor cell invariably gives rise to germ cell cancer after puberty. The Nodal co-receptor Cripto is expressed transiently during normal germ cell development and is ectopically expressed in non-seminomas that arise from germ cell neoplasia *in situ*, suggesting that its aberrant expression may underlie germ cell dysregulation and hence germ cell cancer. Here we investigated methylation of the *Cripto* promoter in mouse germ cells and human germ cell cancer and correlated this with the level of CRIPTO protein expression. We found hypomethylation of the *CRIPTO* promoter in undifferentiated fetal germ cells, embryonal carcinoma and seminomas, but hypermethylation in differentiated fetal germ cells and the differentiated types of non-seminomas. CRIPTO protein was strongly expressed in germ cell neoplasia *in situ* along with embryonal carcinoma, yolk sac tumor and seminomas. Further, cleaved CRIPTO was detected in media from seminoma and embryonal carcinoma cell lines, suggesting that cleaved CRIPTO may provide diagnostic indication of germ cell cancer. Accordingly, CRIPTO was detectable in serum from 6/15 patients with embryonal carcinoma, 5/15 patients with seminoma, 4/5 patients with germ cell neoplasia *in situ* cells only and in 1/15 control patients. These findings suggest that CRIPTO expression may be a useful serological marker for diagnostic and/or prognostic purposes during germ cell cancer management.

Abbreviations: GCNIS, Germ cell neoplasia *In Situ*; CH, choriocarcinoma; dcp, days *post coitum*; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorting; GCC, germ cell cancer; NS, non-seminoma; SE, seminoma; TE, teratoma; YST, yolk sac tumor.

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1. Introduction

Testicular germ cell cancers (GCCs), also known as Type II germ cell tumors (Oosterhuis and Looijenga, 2005), account for ~60% of all malignancies in men aged 20–40 (Adami et al., 1994; van de Geijn et al., 2009). The cell of origin or 'cancer stem cell', germ cell neoplasia *in situ* (GCNIS, according to the newest WHO classification, 2016), previously known as carcinoma *in situ* (CIS) and intratubular germ cell neoplasia unclassified (IGCNU), is considered to be an embryonic germ cell that has failed to differentiate into a prespermatogonium during development (Skakkebaek et al., 1987). Although GCNIS may be present before birth, it does not transform into GCC until after puberty when tumor pathology is classified into seminoma (SE) and non-seminoma (NS) (Sonne et al., 2009; van de Geijn et al., 2009). SE is characterized by fetal germ cell-like expression profile, and NS comprises both highly pluripotent/undifferentiated tumors (embryonal carcinoma; EC) and differentiated tumors: yolk-sac tumor (YST); choriocarcinoma (CH); teratoma (TE) and combinations of these.

The 'fetal origins hypothesis' of GCNIS predicts developmental pathways that control fetal germ cell pluripotency/differentiation contribute to their malignant potential. We recently discovered that the TGFβ signaling molecule Nodal and its obligate receptor Cripto are expressed at a critical point during fetal XY germ cell development in mice and that Nodal/Cripto signaling is active, apparently acting to maintain pluripotency and oppose differentiation (Spiller et al., 2012). We also found that Nodal/Cripto signaling is ectopically activated in NS and we therefore hypothesize that ectopic activation of Nodal signaling, or failure to silence it, contributes to GCC formation (Spiller et al., 2013).

Nodal, a member of the TGFβ family, signals by binding to Activin receptors in the presence of the receptor Cripto (also known as teratocarcinoma derived growth factor 1; Tdgf1). Nodal signaling is absent in normal adult tissues, but is critical for patterning events during embryogenesis (Shen, 2007). Cripto is also essential during embryogenesis, and plays additional roles in stem cell self-renewal and pluripotency in human embryonic stem cells (Bianco et al., 2010; Wei et al., 2005). Its continuous activation is associated with initiation or progression of cancer in many tissues including skin, pancreas, intestine, breast, bladder and brain (Klauzinska et al., 2014). As a cell-surface receptor for Nodal, Cripto must remain tethered to the cell membrane via its glycosylphosphatidylinositol (GPI) anchor at its carboxy terminal (Watanabe et al., 2007b). Cleavage of Cripto at the GPI anchor by GPI-phospholipase D produces a shorter, biologically active form of Cripto that can promote endothelial cell migration, independent of Nodal signaling (Watanabe et al., 2007a). Detection of cleaved Cripto in serum has been identified as a promising diagnostic for breast, colon and brain cancer (Bianco et al., 2006; Pilgaard et al., 2014).

Hypomethylation of oncogenes and hypermethylation of tumor-suppressor genes are commonly seen in cancer, therefore it is possible that dysregulation of Cripto expression in GCC may reflect aberrant methylation of regulatory sequences. In this study we investigated the methylation status of the Cripto promoter during normal fetal germ cell development in mice and contrasted this to human GCC. We also assessed Cripto protein expression in GCNIS and GCC of different histologies. Lastly we used ELISA to quantitate levels of Cripto protein present in conditioned media from GCC cell lines and serum from patients with GCC.

2. Materials and methods

2.1. Mouse strains

Protocols and use of animals in these experiments were approved by the Animal Ethics Committee of the University of Queensland. Embryos were collected from timed matings of the CD1 strain and Oct4ΔPE:eGFP (OG2) strain (Szabo et al., 2002), with noon of the day on which the mating plug was observed designated 0.5 days post coitum (dpc).

2.2. Gonadal collection, germ cell isolation

Gonads without mesonephros were dissected at 11.5–17.5 dpc from CD1 embryos. At 11.5 dpc embryos were genotyped by PCR for genetic sex (McFarlane et al., 2013); later stages were sexed visually by gonad morphology. For each stage three pools of ≥4 gonads were processed for qRT-PCR. For germ cell isolation, sexed gonadal tissue was collected from Oct4Δ PE:eGFP matings. Tissue was dissociated and germ cell (GFP+) populations isolated by fluorescence-activated cell sorting (FACS) using a FACSAria Cell Sorter (BD Biosciences). For each sex and timepoint approximately 100,000 cells were collected and processed for methylation profiling.

2.3. Cell line culture and 5-azacytidine treatment

TCam-2 and JKT-1 were maintained in RPMI (Life Technologies), and NT2 and TERA-1 cells in DMEM (Life Technologies) with the addition of 10% fetal calf serum (GE Healthcare Life Sciences, HyClone Laboratories), 100 U/ml penicillin and 100 ug/ml streptomycin in a humidified 5% CO_2, 37 °C incubator. Treatment with 5-azacytidine (AG scientific) was used at a final concentration of 10 μM (Wermann et al., 2010).

2.4. Human GCC samples

Use of tissue samples for scientific reasons was approved by an institutional review board (MEC 02.981 and CCR2041) in The Netherlands and by the Human Research Ethics Committee at the University of Queensland. Samples were used according to the "Code for Proper Secondary Use of Human Tissue in The Netherlands" developed by the Dutch Federation of Medical and Scientific Societies (FMWV) (Version 2, update 2011). Tissues were collected as described (Looijenga et al., 2003) and diagnosed according to WHO standards by an experienced pathologist (J.W Oosterhuis, Erasmus MC, Rotterdam, The Netherlands). The testicular parenchyma samples investigated have been described previously (Mosselman et al., 1996) and comprised of tissue microarrays as well as single biopsies. Samples (paraffin blocks,

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