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Nodal/Cripto signaling in fetal male germ cell development: implications for testicular germ cell tumors

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ABSTRACT Testicular cancer is the most frequent cancer in young men aged 15-40 years and accounts for 1% of all cancer diagnosed in males. Testicular germ cell tumors (TGCT) encompass a broad group of cancers, each displaying different levels of pluripotency and differentiation as well as malignancy potential. The TGCT cell of origin is thought to be a fetal germ cell that failed to correctly differentiate during development: this is known as the 'fetal origins hypothesis'. This theory predicts that developmental pathways that control germ cell pluripotency or differentiation may be involved in the malignant transformation of these cells. Recently the Nodal/Cripto signaling pathway, known to control pluripotency and differentiation in embryonic stem (ES) cells, was implicated in regulating normal male fetal germ cell pluripotency. Although genes of this pathway are not normally expressed in germ cells during adult life, ectopic expression of this pathway was detected in several sub-groups of TGCTs. In this review, we consider the evidence for the fetal origins of TGCT and discuss the implications of Nodal/Cripto signaling in various aspects of germ cell development and cancer progression.

KEY WORDS: *germ cell, nodal, cripto, testicular germ cell tumor*

Introduction

During fetal development, primordial germ cells (PGCs) migrate to take up residence in the nascent gonads, and then respond to molecular cues from gonadal somatic cells that regulate their proliferation and sex-specific development. In a mouse fetal ovary, PGCs enter meiosis and commit to oogenesis, whereas in a fetal testis, they avoid entry into meiosis and instead undergo mitotic arrest and begin to differentiate towards spermatogenesis (Hilscher 1974; McLaren and Southee 1997). Recent studies have identified some of the key somatic factors involved in regulating fetal germ cell behavior appropriately (Barrios *et al.* 2010; Bowles *et al.* 2010; Bowles *et al.* 2006; Koubova *et al.* 2006).

It is currently hypothesized that dysregulation of PGC development can result in germ cell tumours. In mice, these can take the form of testicular teratomas that contain a wide range of differentiated cell types (Jiang and Nadeau 2001; Stevens 1967; Stevens 1984). Similarly, in humans, germ cells that are not controlled appropriately or that incompletely differentiate during fetal life has been linked to the development of testis cancer later in life. In the present review, we discuss the origins and characteristics of testicular cancer in more detail before focusing on the Nodal/Cripto signaling pathway that has recently been implicated in fetal germ cell pluripotency and development of the tumorigenic state.

Testicular germ cell tumors

Testicular germ cell tumors (TGCTs) are the most common solid tumor of young men aged between 15 and 45 years (Adami *et al.* 1994). The lifetime risk of testis cancer is estimated at approximately 0.5 – 1%; environmental and genetic factors, subfertility and abnormal testis development all contribute to susceptibility (Heimdal *et al.* 1997; Hemminki and Li 2004; Horwich *et al.* 2006). Various histological subtypes of testis cancer are observed, and most occur in the testicles although some tumors also arise in extra-gonadal locations. The majority of TGCTs (more than 95%) originate from the fetal germ cell population of the testis (Ulbright 1999).

Abbreviations used in this paper: CH, choriocarcinoma; CIS, carcinoma in situ; EC, embryonal carcinoma; EG, embryonic germ; EpiSC, epiblast stem cell; ES, embryonic stem; FGF, fibroblast growth factor; GCT, germ cell tumor; GWAS, genome wide association study; NS, non-seminoma; PGC, primordial germ cell; SE, seminoma; TE, teratoma; TGCT, testicular germ cell tumor; TGFβ, transforming growth factor beta; YST, yolk-sac tumor.

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Subtypes of TCGTs

Type I TGCTs (infantile germ cell tumors) arise from early PGCs and develop as teratomas and yolk-sac tumors (YST) in neonates and children. These tumors often arise at extra-gonadal locations such as the neck, midline brain and retroperitoneum, presumably because the transformation occurs as the PGCs are migrating towards the gonads during embryonic development (van de Geijn *et al.* 2009).

Type III germ cell tumors arise from differentiated spermatogonium/spermatocytes of the adult testis and develop as spermatocytic seminomas in elderly men. These tumors, along with Type I GCTs, are extremely rare and the incidence level has remained constant over several decades (Visfeldt *et al.* 1994).

Type II TGCTs arise from fetal gonadal germ cells, which, instead of differentiating into spermatogonia, develop as pre-invasive CIS (Skakkebaek 1972). TGCTs of Type II do not develop until after puberty and can be further divided into two sub-groups: seminomas (SE) and non-seminomas (NS), both of which display many markers of both fetal germ cells and pluripotency (Fig. 1) (Sonne *et al.* 2009; van de Geijn *et al.* 2009). Seminomas are characterised by germ cell-like gene expression and are the least-invasive Type II germ cell tumor. Conversely, non-seminomas comprise both highly pluripotent/undifferentiated pathologies (YST and embryonal carcinoma; EC) as well as differentiated pathologies containing cells from all three germ layers (choriocarcinoma; CH, mixed NS and teratoma; TE). Approximately 10-15% of GCTs are mixed tumors, containing both SE and NS histologies (Ulbright 1999). The incidence of Type II germ cell tumors has doubled over the last four decades without an obvious explanation (Giwercman *et al.* 1993; McGlynn *et al.* 2005; Richiardi *et al.* 2004; Shah *et al.* 2007; Swerdlow *et al.* 1998).

The TGCTs described above are distinct from somatic cancers for two important reasons: Firstly, NS can differentiate into somatic, germ and extra-embryonic cell lineages and therefore is considered the only known totipotent solid cancer (Honecker *et al.* 2006; van de Geijn *et al.* 2009). Secondly, the cell of origin or 'cancer stem cell', CIS, is commonly assumed to originate during early fetal development from a germ cell in an undifferentiated environment (as opposed to the differentiated, adult tissue that most solid somatic cancers arise within) (reviewed by (Kristensen *et al.* 2008)). Due to the latter point, and given the obvious difficulty of directly studying CIS in humans, we now discuss other model systems that have been sought to investigate this pre-cursor lesion and its subsequent transformation into type II GCTs.

Models of carcinoma *in situ* (CIS) and TGCT

In mice, only models of the type I GCT teratoma exist; teratomas have been observed in several mouse strains including the 129/Sv wildtype mouse strain, as well as *Kitl*, *Dead-end*, *Pten* and *Dmrt1* loss of function models (Heaney *et al.* 2008; Kimura *et al.* 2003; Krentz *et al.* 2009; Stevens 1967; Stevens 1984; Youngren *et al.* 2005). In each of these cases, susceptibility was increased on a 129/Sv background, suggesting strain-specific phenomena. Type III GCTs, spermatocytic seminomas, are believed to resemble canine TGCTs (Looijenga *et al.* 1994).

CIS, as well as type II TGCTs of SE and NS have not been observed in mice to date. As such, cell lines that resemble both SE and NS have largely been used for investigations into gene expression and behavior of these tumor sub-types. In addition to the lack of mouse models for CIS, CIS cells cannot be cultured and therefore no cell line exists for in vitro analysis.

The reason that mouse germ cells appear refractory to CIS and type II TGCT transformation is not yet known. Perhaps differences with respect to gene expression on the Y-chromosome have a bearing on the capacity of germ cells to transform: for example mouse *Tspy* (testis-specific protein Y-encoded) is non-functional but human *TSPY* is expressed in germ cells, CIS and SE (Li *et al.* 2007). Additionally, the timeframe of CIS transformation in human versus mouse is thought to play a significant role. In humans, both

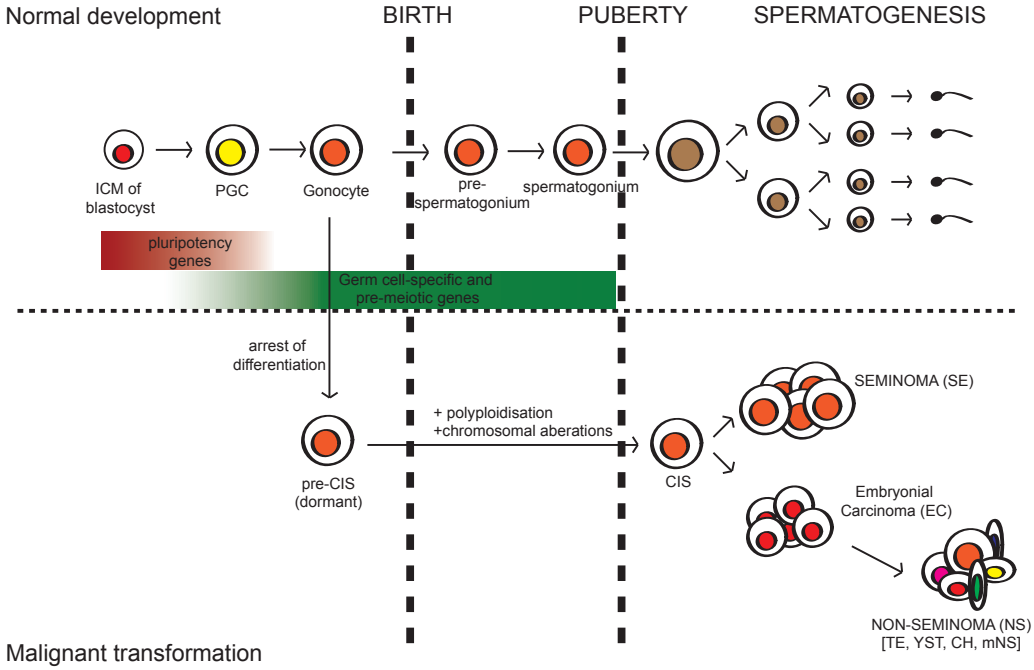

Fig. 1. Developmental origin of human type II TGCT. *During normal germ cell development pluripotency genes are turned off as fetal germ cells differentiate into spermatogonia. After puberty spermatogenesis begins and sperm is produced. Under pathological conditions, germ cells that fail to udergo correct spermatogenic differentiation and instead retain expression of pluripotency markers, develop into the precursor lesion carcinoma in situ (CIS). CIS cells remain dormant until puberty when they begin transformation into either seminoma (SE) or non-seminomas (NS), or both. Seminomas display fetal germ cell characteristics. NS include the most common subtype, embryonal carcinoma (EC), as well as yolk sac tumours (YST), teratomas (TE), choriocarcinomas (CH) or a mixture of these subtypes (mixed non-seminomas; mNS). Modified from Rajpert-De Meyts, 2006.*

germ cells and CIS remain in a quiescent state for over a decade before proliferation resumes during puberty, presumably in response to hormonal triggers (Rajpert-De Meyts and Skakkebaek 1993). In mice, the period of germ cell quiescence lasts for little more than one week, making it unlikely that the environmental/niche cues that direct CIS transformation (along with the accumulation of substantial chromosomal abnormalities) have sufficient time to induce transformation.

TGCT fetal origins hypothesis

The fetal origins hypothesis asserts that CIS arises from fetal germ cells that have failed to differentiate correctly and that CIS is the precursor cell for type II TGCTs that arise after puberty (Skakkebaek *et al.* 1987). There is now abundant indirect evidence to support this hypothesis, discussed below. For a comprehensive reviews see (Kristensen *et al.* 2008; Rajpert-De Meyts *et al.* 2003).

CIS cells resemble fetal germ cells (morphologically and transcriptionally)

There is a strong morphological resemblance between CIS cells and gonocytes; both are large round cells with distinct nucleoli and similar ultrastructural characteristics (Nielsen *et al.* 1974; Sigg and Hedinger 1984; Skakkebaek *et al.* 1987). At the transcriptional level many markers are shared between CIS and fetal germ cells (Almstrup *et al.* 2005). Some of these include: placental-like alkaline phosphatase (Manivel *et al.* 1987), the proto-oncogene kit (Jorgensen *et al.* 1995), OCT3/4, NANOG (Hoei-Hansen *et al.* 2004a) AP-2γ (Hoei-Hansen *et al.* 2004b), DCN, IGFBP6, SFRP1, SALL1 and SOX17 (Hoei-Hansen *et al.* 2004a).

CIS cells arise at the right place and time to originate from fetal germ cells

After specification in the epiblast, the germ cells migrate through the hindgut toward the developing genital ridges. Incidences of extra-gonadal germ cell tumors (usually YST) often arise along the midline, consistent with the migratory path of fetal germ cells (Oosterhuis *et al.* 2007). CIS cells have been observed during fetal development also: mid-trimester fetuses with trisomy 21 were positive for markers of CIS (Jacobsen and Henriques 1992; Satge *et al.* 1996). Based on shared marker expression between CIS and germ cells, and because some genes are expressed only transiently during normal germ cell development, it has been estimated that CIS arises before the 9th week of gestation in humans (Jorgensen *et al.* 1995).

CIS cells display pluripotent 'stem cell' characteristics

In addition to sharing a transcriptional profile similar to germ cells, CIS cells also display stem cell characteristics, consistent with them being the precursor cell to TGCTs. Transcriptional analysis of CIS and ES cells revealed almost 50% shared gene transcription between the two populations (Hoei-Hansen *et al.* 2004a).

CIS will usually transform to testicular cancer

While CIS is commonly observed in parenchyma adjacent to TGCTs (in 90% of cases) (Jacobsen *et al.* 1981), in multiple cases previous identification of CIS has also been associated with patients subsequently developing TGCT. As such, identification of CIS results in a 50% risk of developing TGCTs within 5 years and a 70% chance within 7 years (Linke *et al.* 2005; von der Maase *et al.* 1986).

Predictions of the TGCT fetal origins hypothesis

The fetal origins hypothesis was described over three decades ago and is now well accepted due to the vast body of supportive evidence described above. Based on this model, we can therefore predict that at least some of the molecular pathways affecting germ cell fetal development must be relevant to CIS transformation and adult testicular cancer. In the next section we outline signaling pathways involved in fetal germ cell development, which have also been implicated in TGCTs.

Signaling pathways involved in germ cell development and tumorigenesis

Over the past 2 decades several studies, including a recent genome wide association study (GWAS), have identified genetic pathways that account for about 15% of the genetic risk for TGCT. These include pathways known to control various aspects of germ cell development: kit signaling (KIT, KITL, SPRY4, BAK1), telomerase regulation (TERT, ARF7IP) and sex determination (DMRT1) (Turnbull and Rahman 2011).

Kit - Kitl signaling

Spontaneous mutations in kit ligand (Kitl; SCF) and its receptor (Kit) were implicated in germ cell development many years ago; homozygous mutations in either affect fetal germ cell migration and survival and result in infertility (Nishimune *et al.* 1980; Roskoski 2005). Kit protein, normally expressed by fetal germ cells, is detectable in CIS (Biermann *et al.* 2012; Rajpert-De Meyts and Skakkebaek 1994) and SE, but not in NS (Rajpert-De Meyts and Skakkebaek 1994). Interestingly, heterozygous deletion of *Kitl* in mice increases the TCGT susceptibility on the 129/Sv background (Heaney *et al.* 2008).

In humans, amplifications of genomic region containing KIT (chromosome 4q12) are associated with seminomas (Biermann *et al.* 2007; Looijenga *et al.* 2003; Murty *et al.* 1992) while deletions of the *KITL* genomic region (chromosome 12q22) are associated with NS. In all cases, mutations to *KIT/KITL* genomic regions are not identified within the precursor lesion CIS, suggesting that kit signaling determines the tumor progression after CIS initiation (Heaney *et al.* 2008).

Signaling pathways downstream from KIT/KITL have also been associated with TGCT susceptibility: two genes *SPRY4* (*Sprouty 4*; chromosome 5q31) and *BAK1* (*BCL2*–agonist/killer 1; chromosome 6p21) regulate mitogen-activated kinase signaling and pro-apoptotic pathways, respectively (Sasaki *et al.* 2003; Turnbull and Rahman 2011; Yan *et al.* 2000). These findings suggest that the larger network of KIT signaling is involved in the TGCT pathology.

Telomerase function

The extension of terminal chromosomal sequences by the enzyme telomerase occurs during every cell division. Reduced telomere function has been associated with genome instability (Hackett *et al.* 2001) whilst reactivation favors extended replicative lifespans of malignant cells (Fernandez-Garcia *et al.* 2008). The *TERT* gene encodes the catalytic subunit of the telomerase complex which, although normally absent in adult somatic tissues,

is highly expressed in germ, stem and tumor cells. As such, high *TERT* expression was detected in undifferentiated TGCTs (SE) but absent from differentiated teratomas (Schrader *et al.* 2002). More recently, amplification of the genomic region harboring *TERT* (chromosome 5p15) was associated with TGCT predisposition (Turnbull and Rahman 2011). In the same study, ATF71P (activating transcription factor 7 interacting protein; chromosome 12p13), a protein that regulates expression of TERT, was also implicated in TGCT susceptibility (Turnbull and Rahman 2011).

DMRT1

DMRT1 (doublesex and mab-3 related transcription factor 1) is a transcription factor involved in testis differentiation during development in multiple species (Smith *et al.* 1999). Homozygous deletion of *Dmrt1* on a 129/Sv background increases teratoma formation to 90% compared to 1% in wildtype male mice (Krentz *et al.* 2009). In a human GWAS study, the genomic region harboring *DMRT1* (chromosome 9p24) was found to be associated with TGCT susceptibility (Turnbull and Rahman 2011) and deletions of this region have previously been associated with impaired gonad development and TGCT formation (Barbaro *et al.* 2009; Livadas *et al.* 2003).

As mentioned, the above genetic pathways account for only 15% of heritable TGCT risk, and as such, new pathways regulating this process remain to be discovered. Recently, the Nodal signaling pathway has been shown to control mouse fetal germ cell pluripotency and was also found to be overexpressed in human TGCTs (Spiller *et al.* 2012). We now examine this signaling pathway in more detail and discuss the implications of this work.

Nodal signaling in germ cells and cancer

The Nodal signaling pathway

Nodal, a member of the TGFβ family, signals by binding to Activin receptors (serine/threonine kinase receptors, predominantly Alk4 (Acvr1b) and ActRIIA/B (Acvr2a/b)) in the presence of the obligate co-receptor, Cripto (also known as teratocarcinoma derived growth factor 1; TDGF-1) (Fig. 2). Binding of Nodal to Activin receptors causes them to phosphorylate (activate) the transcription factor Smad2 that, together with Smad4, regulates transcription of target genes (Chang *et al.* 2001; Schier and Shen 2000). Nodal up-regulates its own expression as well as the expression of two other TGFβ molecules, Lefty1 and Lefty2, both of which act as dose-dependent feed-back inhibitors of the pathway (reviewed by (Schier 2009)). Nodal and its secreted Lefty inhibitors (Hamada *et al.* 2002) have been studied intensively for key roles in mesoderm generation, establishment of left-right asymmetry during gastrulation and specification of ventral cell identity during patterning of the nervous system (Shen 2007). All of these functions are Cripto-dependent.

Although most studies have focused on its roles during embryogenesis and in vitro stem cell generation, Cripto is emerging also as a regulator of normal tissue growth and remodeling in various tissues including mammary epithelial cells (Bianco *et al.* 2002), adipose tissue (Andersson *et al.* 2008), endometrium (Papageorgiou *et al.* 2009) and myoblasts (Kemaladewi *et al.* 2012), reviewed by (Gray and Vale 2012). Transient activation of Cripto is required for stem cell self-renewal and pluripotency, but continuous activation is associated with initiation or progression of cancer in many tissues including skin, pancreas, intestine and breast (reviewed by (Bianco *et al.* 2005)). Although Cripto is an obligate co-receptor for Nodal, it is also required for signaling by two other TGFβ molecules: growth derived factor 1 and 3 (GDF1, GDF3) (Chen *et al.* 2006; Cheng *et al.* 2003).

Nodal regulates fetal germ cell pluripotency

Recently Nodal signaling was shown to be active in XY, but not XX, fetal germ cells during the period of sexual fate determination in the mouse embryo (Souquet *et al.* 2012; Spiller *et al.* 2012). Nodal, Cripto and the downstream modulator of Nodal signaling, SMAD2, were activated in XY germ cells, but not somatic cells, indicating autocrine Nodal signaling in those cells (Souquet *et al.* 2012; Spiller *et al.* 2012). Although all XY germ cells appear to express *Cripto*, the sub-population of germ cells with greatest potential to form embryonic germ cells at this stage expressed *Cripto*, *Nodal*, *Lefty1* and *Lefty2* at the highest levels. In purified germ cell culture, *Cripto* expression was induced by the male fate-promoting factor, FGF9 (Bowles *et al.* 2010) suggesting that this is how it comes to be upregulated in XY germ cells during fetal development. In a similar way, FGF2, one of the factors required for dedifferentiation of germ cells to EG cells (Durcova-Hills *et al.* 2006; Durcova-Hills and Surani 2008; Matsui and Tokitake 2009; Matsui *et al.* 1992; Resnick *et al.* 1992), was able to induce *Cripto* expression in vitro.

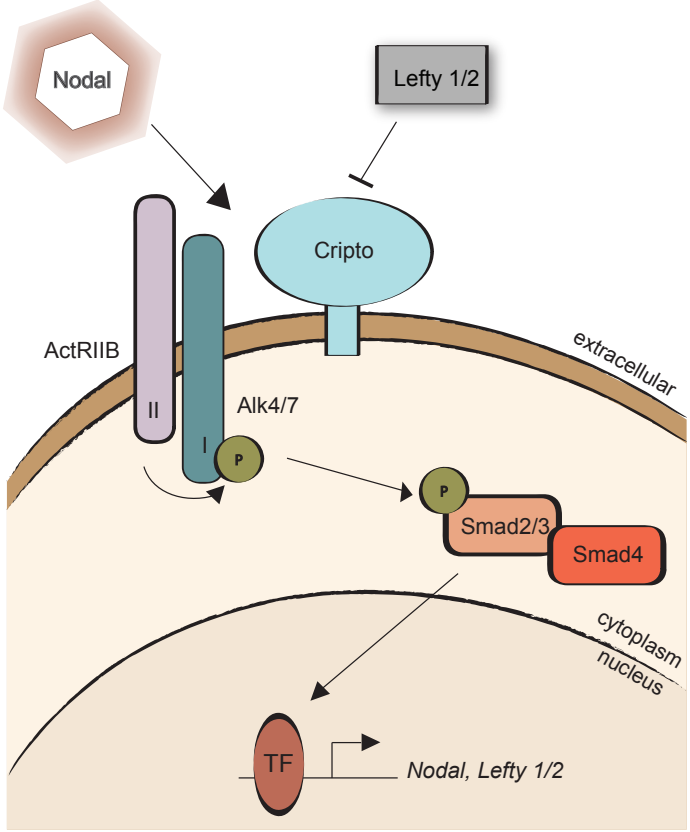

Fig. 2. Model of Nodal signaling. *Nodal, a member of the TGFβ family, signals by binding to Activin receptors (predominantly ActRIIB, ALK4) in the presence of the obligate co-receptor, Cripto. Binding of Nodal to Activin receptors causes them to phosphorylate (activate) SMAD2 to regulate transcription of target genes. Lefty1 and Lefty2, also TGFβ molecules, repress the pathway in a dose-dependent manner. Nodal up-regulates its own expression as well as the expression of its inhibitors, Lefty1 and Lefty2.*

TABLE 1

EXPRESSION OF NODAL PATHWAY GENES IN TYPE II TGCTs

Tumor		*NODAL*	*CRIPTO*	*LEFTY1*
Carcinoma in situ (CIS)		+	+	+
Seminoma (SE)		-	-	-
	Embryonal carcinoma (EC)	+	+	
	Mixed non-seminoma (mNS)	+/-	+/-	+/-
Non-seminoma (NS)	Yolk-sac tumor (YST)	+	+	+
	Choriocarcinoma (CH)	-	-	-
	Teratoma (TE)	-	-	-

Suppression of Nodal/Cripto signaling using the hypomorphic *Nodal*$^{flox/flox}$ mouse line (Lowe *et al.* 2001) led to a reduction in germ cell pluripotency makers and a decreased ability to form EG cells (Spiller *et al.* 2012).

These results suggested that the function of cell-autonomous Nodal/Cripto signaling in XY germ cell development is to maintain germ cells in a state of pluripotency, thereby avoiding differentiation for a time (Spiller *et al.* 2012), a role akin to that seen in human ES cells (James *et al.* 2005). In contrast to these findings however, another study concluded that disruption of Activin/Nodal signaling affected fetal germ cell meiosis (although pluripotency was not investigated) (Souquet *et al.* 2012). In that study, Activin/Nodal inhibitors disrupted Nodal signaling in germ cells but also Activin signaling in somatic cells, so it is possible that the observed meiosis is the result of inadvertent disruption of the gonadal environment: Activin is normally required for the induction of germ cell mitotic arrest and differentiation (Mendis *et al.* 2010). Complete Nodal deletion in germ cells may be required to clarify whether the main role of Nodal signaling is to maintain pluripotency or to prevent meiosis. In the hypomorphic *Nodal*$^{flox/flox}$ mouse line, no ectopic entry into meiosis was observed (Spiller *et al.* 2012)

Nodal signaling pathway genes are over-expressed in TGCTs

Having established a role for endogenous Nodal signaling during normal germ cell pluripotency, and based on the fetal origins hypothesis, it was hypothesized that this pathway may be mis-regulated in cases of human TGCT (Spiller *et al.* 2012). Consistent with a role in pluripotency maintenance in fetal germ cells, *CRIPTO*, *NODAL* and downstream target *LEFTY1* expression positively correlated with CIS presence in testis biopsies and in the undifferentiated NS (EC and YST) (summarized in Table 1; (Spiller *et al.* 2012)). The finding that a developmental pathway that controls pluripotency in fetal germ cells is mis-regulated in cases of human TGCTs provides further support for the fetal origins hypothesis.

Implications for normal germ cell development

As discussed, during fetal development in the testis, XY germ cells express *Nodal*, *Cripto* and also *Lefty1* and *Lefty2* (Souquet *et al.* 2012; Spiller *et al.* 2012). *Lefty1* and *Lefty2* are known direct downstream targets of Nodal signaling and their transcription in germ cells confirms that these cells are responding to Nodal signaling (Branford and Yost 2002; Feldman *et al.* 2002; Meno *et al.* 1999). During gastrulation and neurogenesis, Leftys play important roles in limiting the range of Nodal signaling and fine-tuning the Nodal concentration gradient via a reaction-diffusion mechanism (Schier 2009; Shen 2007). Hence, the question arises as to the function of Lefty during testicular germ cell development, given that only germ cells express the co-receptor Cripto and that positional information does not seem to be essential for determining germ cell behavior in the developing testis. It is possible that Leftys act to prevent non-Cripto dependent Nodal action outside of the germ cell compartment of the testis. It will be of interest to test the consequences of genetically or pharmaceutically manipulating Lefty activity during gonadal development. It will also be informative to determine whether any Cripto-independent role for Nodal exists, and vice versa, using *Cripto* deletion in germ cells (Liguori *et al.* 2008). Given the early requirement for both Nodal and Cripto during gastrulation, conditional deletion of these genes within fetal germ cells will be required.

Implications for embryonic stem (ES) and embryonic germ (EG) cells

Activin/Nodal signaling is now well established in the maintenance of pluripotency of human and rabbit ES cells (Honda *et al.* 2009; James *et al.* 2005; Vallier *et al.* 2004) and mouse and pig epiblast stem cells (EpiSC), which are derived from the epiblast of post-implantation, pre-gastrula embryos (Alberio *et al.* 2010; Brons *et al.* 2007; Tesar *et al.* 2007). EpiSCs, like human and rabbit ES cells, require the presence of FGF2 and Activin in order to maintain pluripotency (Brons *et al.* 2007; Chou *et al.* 2008; Tesar *et al.* 2007). They express *Oct4*, *Sox2, Nodal* and *Nanog* as well as low levels of the ES cell and germ cell marker *Stella*, presumably due to their epigenetic status. In such contexts Nodal is known to regulate expression of the homeodomain transcription factor Nanog (Chambers *et al.* 2003; Mitsui *et al.* 2003). *Oct4*, another target of Nodal signaling and activator of *Cripto* transcription (Watanabe *et al.* 2010), together with *Sox2* and *Nanog* comprise the gene regulatory network sufficient for pluripotency maintenance in ES cells. In contrast, mouse ES cell maintenance only requires the cytokine LIF, a member of interleukin-6 family that signals via the gp130 receptor to control the Jak-Stat pathway (Loh *et al.* 2006). Given that endogenous Nodal signaling maintains pluripotency in fetal germ cells (Spiller *et al.* 2012), it seems that these cells are more similar to human and rabbit ES cells and mouse and pig EpiECs, than to mouse ES cells. Such properties may be important when considering re-programming of ES cells to germ cells and vice-versa.

The generation of EG colonies from primordial germ cells is testament to their stem cell potential as these colonies closely resemble ES cells and can contribute to all three embryonic germ layers. EG derivation is induced by the presence of three factors: LIF, FGF2 and KITL (Durcova-Hills *et al.* 2006; Durcova-Hills and Surani 2008; Matsui and Tokitake 2009; Matsui *et al.* 1992; Resnick *et al.* 1992). Interestingly, FGF2 is able to upregulate *Cripto* expression in isolated XY and XX germ cells (Spiller *et al.* 2012): since FGF2 is required only during the first 24 hours of EG cell derivation (Durcova-Hills *et al.* 2006) it is possible that its major role is to prime the germ cells for reprogramming by triggering *Cripto* expression. The male-specific endogenous expression of *Cripto* may underlie the slightly greater propensity of XY germ cells to generate EG cells when compared with XX germ cells, as has been reported in some studies (Kimura *et al.* 2008; Labosky *et al.* 1994).

It is important to note that only small populations of germ cells are able to respond to the signaling molecules that induce EG cell derivation (Durcova-Hills and Surani 2008). This phenomenon is being reflected in many recent findings highlighting the heterogeneity of the fetal germ cell population with respect to gene and cell-surface marker expression (Matsui and Tokitake 2009; Spiller *et al.*

2012). Sub-populations of fetal germ cells with differing potentials for pluripotency and/or differentiation would also suggest that inherent differences in these cells contribute to the potential for CIS transformation, in addition to disturbances in the local niche environment.

Implications for germ cell transformation to CIS

The findings of Spiller *et al.* (2012) led us to hypothesize that during human CIS formation, *CRIPTO* expression is maintained or re-activated, triggering upregulation of Nodal signaling, aberrant expression of pluripotency markers and subsequent progression to the oncogenic state. It is possible that ectopic expression of CRIPTO makes such germ cells refractory to normal differentiation cues of the TGFβ signaling pathway as has been observed in other systems (Gray *et al.* 2003; Gray *et al.* 2006). In human cancers, CRIPTO has emerged as an oncogenic growth factor, controlling proliferation, migration and survival (Bianco *et al.* 2005; Wechselberger *et al.* 2005). CRIPTO functions as a dominant transforming gene when over-expressed in the NOG-8 mouse mammary epithelial cell line or NIH/3T3 fibroblasts (Ciccodicola *et al.* 1989). Despite the multi-hit hypothesis for tumor formation, our understanding of the biology of Nodal/Cripto signaling in germ cells so far suggests that expression of Cripto may be sufficient to endow germ cells with sufficient tumorigenic capacity to trigger CIS.

Implications for CIS transformation into seminoma vs. non-seminoma

CIS cells have the potential to develop down multiple pathways: germ-cell like tumor (SE), pluripotent tumors (EC) and differentiated teratomas and extra-embryonic components (CH, YST). The mechanism by which a CIS cell is induced to develop into either SE or NS (or both) is unclear. Over-expression of Nodal signaling genes was detected in biopsies containing CIS and the NS tumors EC and YST (Spiller *et al.* 2012). This finding is somewhat unusual given that genes expressed in CIS are often also expressed in SE given their close resemblance to fetal germ cells. The finding that CRIPTO is expressed by CIS but is switched off during progression into SE suggests that Nodal signaling in this context is highly dependent on the stem cell niche of the CIS cell environment. It is possible that, as seems to be the case with the generation of EG cells in vitro, expression of CRIPTO/NODAL makes human germ cells more susceptible to signals that trigger complete dedifferentiation and tumor formation. Perhaps the CIS cells that lack such dedifferentiation factors in their environmental niche are the ones that progress to SE, a cancer characterised by expression of fetal germ cell genes.

Alternatively (or additionally), it is intriguing to speculate that levels of CRIPTO expression in a given CIS cell are instructive, rather than passive, and determine the fate of CIS transformation into SE versus NS. Indeed, such heterogeneous regulation of CRIPTO (at both the level of promoter methylation and protein expression) in the EC cell line NTERA2, correlated Cripto^{High}-expressing cells with an undifferentiated state and greatest tumorigenic potential (Watanabe *et al.* 2010).

Looking at the broader Nodal signaling pathway, it is possible that *NODAL* and *CRIPTO* act as oncogenes during development of CIS and TGCTs whilst *LEFTY* genes may encode tumor-suppressors. This hypothesis is supported by the observation that *NODAL, CRIPTO* and *LEFTY* expression are elevated in the YST subtype of NS but only *NODAL* and *CRIPTO* expression are elevated in the more dangerous form, EC. If Nodal signaling is triggered but the negative regulator of the pathway, *LEFTY*, is not expressed, a highly pluripotent phenotype should result (Postovit et al. 2008).

Given that Nodal/Cripto signaling is a key developmental pathway essential for gastrulation, it is unlikely that gene mutations will be discovered within this pathway in CIS and TGCTs. Rather, levels of gene expression are likely altered during the process of transformation. Investigation of the methylation status of Nodal/Cripto pathway gene promoters in CIS and TGCT pathologies may shed some light on the regulation of gene expression in these contexts: hypomethylation of oncogenes and hypermethylation of tumor-suppressor genes are commonly seen in cancer. Additionally, analysis of the *CRIPTO* promoter during normal fetal germ cell development may provide clues as to how CRIPTO expression is regulated under normal circumstances.

Future directions

Confirmation that the Nodal/Cripto pathway is active during normal human fetal germ cell development and that the Cripto protein is expressed on the surface of CIS cells should now be sought. Importantly, investigation of Cripto expression in the rare instance when fetal CIS biopsies are available would assist in confirming whether CRIPTO expression is maintained or instead re-activated during CIS development. If these studies suggest that CRIPTO expression is maintained, the developmental window for fetal gonocyte transformation into CIS could be narrowed to the period of endogenous Cripto expression during development (ie. 12.5 – 14.5 dpc in the mouse). A better understanding of how Nodal/Cripto signaling is regulated during fetal germ cell development, including FGF control of this process, will help us to understand how Cripto is retained or re-activated during malignant transformation.

Conclusions

We rely on germ cells for fertility - the propagation of our species. Because these cells are generally specified and matured correctly, the potential they hold for reversion to pluripotent and tumorigenic states is perhaps not often fully appreciated. A fine balance between XY germ cell pluripotency, proliferation and quiescence must be achieved within a relative short window during human gestation: uncontrolled proliferation results in cancer whereas failure to differentiate can result in infertility. We currently know little of how these processes are balanced, although we predict, based on the fetal origins hypothesis, that developmental pathways controlling germ cell development are involved. The discovery that the Nodal/Cripto signaling pathway controls normal germ cell pluripotency but is activated ectopically in the most deadly forms of TGCTs in humans may have provided a starting point for further investigations into this process. Indeed, only 15% of TGCTs can be explained by gene amplifications/deletions so far, and so the search must continue for new mechanisms of regulation. Understanding such molecular pathways and factors that control or maintain germ cell differentiation or stemness will have important implications for both fertility and cancer treatment in the future.

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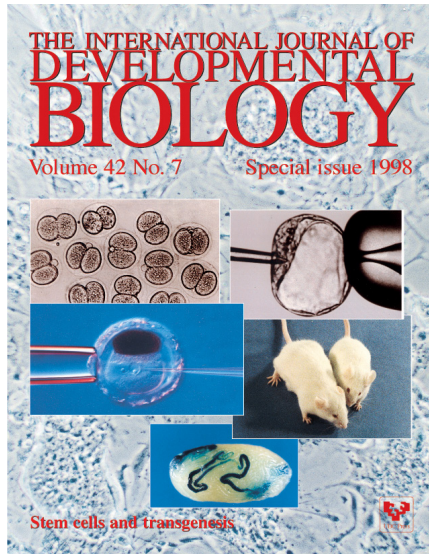

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