CHAPTER 20

The Role of Fanconi Anemia/BRCA Genes in Zebrafish Sex Determination

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Abstract

Fanconi anemia (FA) is a human disease of bone marrow failure, leukemia, squamous cell carcinoma, and developmental anomalies, including hypogonadism and infertility. Bone marrow transplants improve hematopoietic phenotypes but do not prevent other cancers. FA arises from mutation in any of the 15 *FANC* genes that cooperate to repair double stranded DNA breaks by homologous recombination. Zebrafish has a single ortholog of each human *FANC* gene and unexpectedly, mutations in at least two of them (*fancl* and *fancd1*(*brca2*)) lead to female-to-male sex reversal. Investigations show that, as in human, zebrafish *fanc* genes are required for genome stability and for suppressing apoptosis in tissue culture cells, in embryos treated with DNA damaging agents, and in meiotic germ cells. The sex reversal

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phenotype requires the action of Tp53 (p53), an activator of apoptosis. These results suggest that in normal sex determination, zebrafish oocytes passing through meiosis signal the gonadal soma to maintain expression of aromatase, an enzyme that converts androgen to estrogen, thereby feminizing the gonad and the individual. According to this model, normal male and female zebrafish differ in genetic factors that control the strength of the late meiotic oocyte-derived signal, probably by regulating the number of meiotic oocytes, which environmental factors can also alter. Transcripts from *fancd1*(*brca2*) localize at the animal pole of the zebrafish oocyte cytoplasm and are required for normal oocyte nuclear architecture, for normal embryonic development, and for preventing ovarian tumors. Embryonic DNA repair and sex reversal phenotypes provide assays for the screening of small molecule libraries for therapeutic substances for FA.

I. Introduction

A. Fanconi Anemia: A Disease of DNA Repair

Fanconi anemia (FA; MIM# 227650) is a rare recessive disorder found in all ethnic groups with an incidence ranging from one in 300,000 in some occidental countries to higher incidence in some populations, including Ashkenazi Jewish or Afrikaners (Rosendorff *et al.*, 1987; Whitney *et al.*, 1993). FA is characterized by catastrophic bone marrow failure, often by 5 years of age, an 800-fold increase in risk of acute myeloid leukemia (AML), and a 6000-fold increase in squamous cell carcinomas of the head and neck (Rosenberg *et al.*, 2008). In addition to hematopoietic phenotypes, FA is often accompanied by characteristic congenital anomalies, including slow growth, short stature, microcephaly, microphthalmia, as well as hypogonadism and infertility (Kee and D'Andrea, 2010). The most common congenital anomaly in FA is an abnormal or missing thumb and radius, but kidney and gonads are also frequently affected (De Kerviler *et al.*, 2000). Abnormal blood cell development is the main cause of morbidity and mortality (Bagby *et al.*, 2004; Tischkowitz and Hodgson, 2003). A gap in our knowledge is the mechanism by which FA leads to developmental anomalies in blood, skeleton, eyes, and gonads.

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only known cure for the bone marrow failure associated with FA (de la Fuente *et al.*, 2003; Dufour and Svahn, 2008; Huck *et al.*, 2008; Motwani *et al.*, 2005; Muller *et al.*, 2008; Wagner *et al.*, 2007). Suitable donors, however, are not available for many patients and transplant survivors still experience enormously increased susceptibility to cancers, especially squamous cell carcinomas of the head and neck (Rosenberg *et al.*, 2003, 2008). Although success of HSCT was initially low, improved chemotherapy strategies greatly increased the survival rate after HSCT from both related and unrelated donors (Bonfim *et al.*, 2007; Thakar *et al.*, 2011). Moreover, recent breakthroughs offer the possibility of genetically correcting defects in somatic cells from

FA patients, which can be reprogrammed to pluripotency to generate patient-specific induced pluripotent stem (iPS) cells that can give rise to phenotypically normal hematopoietic progenitors of the myeloid and erythroid lineages (Raya *et al.*, 2009). This advance should allow the production of a large number of genetically stable autologous hematopoietic stem cells that could potentially be transplanted into patients. Whether such cells would themselves progress to cancers or give rise to other problems is not yet known. Therefore, transplant survivors could still be exposed to an increased susceptibility to a variety of solid cancers. Thus, despite advances in transplantation therapies, a current problem is that we lack chemical therapies to ameliorate both the early hematopoietic and later tumorigenic phenotypes of FA.

B. Fanconi Anemia Genes

A total of 15 genes, when mutated, can lead to FA disease (FANCA, FANCB, FANCC, FANCD1(BRCA2), FANCD2, FANCE, FANCF, FANCG(XRCC9), FANCI, FANCJ(BRIP1), FANCL, FANCM, FANCN(PALB2)), FANCO (RAD51C), and FANCP(SLX4) (de Winter *et al*., 1998, 2000a, 2000b; Dorsman *et al*., 2007; Gurtan *et al*., 2006; Howlett *et al*., 2002; Kim *et al*., 2010; Levitus *et al*., 2005; Levran *et al*., 2005; Lo Ten Foe *et al*., 1996; Meetei *et al*., 2003, 2004a, 2004b, 2005; Reid *et al*., 2007; Sims *et al*., 2007; Smogorzewska *et al*., 2007; Strathdee *et al*., 1992; Timmers *et al*., 2001; Vaz *et al*., 2010; Wang, 2007; Xia *et al*., 2007). Because a few cases of FA are not assigned to any of these genes, additional Fanconi genes may remain to be identified.

Fanc proteins interact in three main groups to facilitate a DNA damage response leading to DNA repair. Cells exposed to DNA damaging agents or passing through the DNA synthesis phase of normal cell cycles activate the first group, a Nuclear Core Complex (NC complex) consisting of eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) and three proteins not yet shown to be mutated in human FA patients (FAAP100, FAAP24, and HES1) (Tremblay *et al.*, 2008, 2009; Wang, 2007). The NC complex functions as an E3 ligase, with Fancl providing the enzyme active site. The NC complex triggers the monoubiquitination of the second group of proteins, FANCI and FANCD2 (the ID complex) (Garcia-Higuera *et al.*, 2001; Sims *et al.*, 2007). In ways that are not fully understood, monoubiquitination of the ID complex causes it to translocate to sites of DNA damage where they interact with nuclear DNA repair foci. These nuclear foci constitute the third group of Fanc proteins, including FANCD1 (alias BRCA2), FANCJ (alias BRIP1), FANCN (alias PALB2), FANCO (alias RAD51C), FANCP (alias SLX4), BRCA1, FAN1, histone H2AX, and RAD51 (Crossan *et al.*, 2010; Garcia-Higuera *et al.*, 2001; Kim *et al.*, 2010; Kitao and Takata, 2011; Stoepker *et al.*, 2010; Wang *et al.*, 2004; Xia *et al.*, 2007). FA group 3 proteins then repair double strand DNA breaks by homologous recombination (Bagby, 2003).

C. The FA/BRCA Network

For some FA group 3 proteins, biallelic hypomorphic mutations result in FA, but monoallelic (heterozygous) severe mutations cause breast or ovarian cancer and biallelic severe mutations cause embryonic or fetal lethality (Crossan *et al.*, 2010; Kim *et al.*, 2010; Levy-Lahad, 2010; Neveling *et al.*, 2009; Stoepker *et al.*, 2010). Because of the involvement of Fanconi genes in both FA and breast cancer, this gene pathway is now called the FA/BRCA network (D'Andrea, 2003). Biallelic mutations in FA genes result in the loss or inactivity of nuclear foci and lead to genomic instability as reflected by hypersensitivity to DNA interstrand crosslinks (ICLs) caused by genotoxic agents such as cisplatin, mitomycin C (MMC), and diepoxybutane (DEB) (Auerbach, 1993; Shimamura *et al.*, 2002). The sensitivity of FA patients' cells to ICLs reflects defects in DNA repair mechanisms that likely contribute to aberrant apoptosis, genome instability, and cancer. A substantial gap in our knowledge is that, despite advances in understanding FA protein biochemistry, little is known about the mechanisms by which loss-of-function mutations in *FANC* genes impact the DNA damage response pathway and contribute to the clinical features of FA, including aplastic anemia, the predisposition to hematological malignancies, the susceptibility to insidious squamous cell carcinomas, the hypoplastic radius and thumb, and hypogonadism. These pleiotropic effects may arise from signaling roles that Fanc proteins play that are unrelated to their role in DNA repair and that we still do not understand well (Vanderwerf *et al.*, 2009).

D. FANC Genes do More than Repair DNA

Several clinical manifestations of FA are not readily explained purely from defective DNA cross-link repair, including developmental phenotypes such as radial defects of the forelimb (70% of patients), microphthalmia and short stature (60%), and endocrine abnormalities (70%), including deficiencies in growth hormone, thyroid hormone, and diabetes (Giri *et al.*, 2007; Wajnrajch *et al.*, 2001). We still do not understand the relationship of defective DNA repair to progressive bone marrow failure, even though we know that this phenotype arises from impaired self-renewal of hematopoietic stem cells (Carreau *et al.*, 1999). Additional defects not easily understood from defects in DNA repair, include poor resistance to oxidative damage, interaction with inflammation pathways, and hyperactivation of the MAPK pathway leading to overproduction of TNF-α (Bagby, 2008; Briot *et al.*, 2008; Li *et al.*, 2007; Sejas *et al.*, 2007; Uziel *et al.*, 2008).

At least some of these difficult to explain phenotypes may involve key functions of FANCs distinct from the NC complex. For example, FANCC, FANCD2, and FANCG help activate STAT5A, which facilitates replication and survival of hematopoietic stem and progenitor cells. FANCC modulates proapoptotic double-stranded RNA-dependent protein kinase (PKR), which makes FANCC-deficient hematopoietic cells hypersensitive to the apoptotic effects of the cytokines TNFα and IFNγ (Haneline *et al.*, 1998). Perfidiously, FANCC-deficient cells also overproduce

TNFα, which depends upon TLR8 (Vanderwerf *et al.*, 2009). Thus, *FANCC*-deficient cells inappropriately express, and are hypersensitive to, extracellular apoptotic cues (Li *et al.*, 2007), which likely contributes to bone marrow failure. In addition, the exposure to TNFα creates a selective pressure that purges TNFα-hypersensitive *FANCC* hematopoietic stem cells while allowing somatically mutated preleukemic stem cell clones to flourish (Vanderwerf *et al.*, 2009). These and other mechanisms that generate the clinical features of FA that are not obviously linked to DNA repair remain to be more fully investigated.

II. Results and Discussion

A. The FA/BRCA Gene Network is Conserved between Humans and Zebrafish

Zebrafish has become a prominent animal model to study human genetic disease because it shares with humans conserved genetic mechanisms of development, biochemistry, and physiology; it is amenable to forward and reverse genetic screens for mutations; and it possesses optically clear embryos. All 15 genes shown to be mutated in human FA patients are conserved in zebrafish *FANCA* (AY968592), *FANCB* (AY968593), *FANCC* (AY968594), *FANCD1* (EF088196), *FANCD2* (NM_201341), *FANCE* (AY968595), *FANCF* (AY968596), *FANCG* (AY968597), *FANCI* (FJ032296), *FANCJ* (EF088194), *FANCL* (AY968598), *FANCM* (EF088195), *FANCN* (FJ032295), *FANCO* (ENSDARG00000068919), and *FANCP* (ENSDARG00000093798) (Blom *et al.*, 2004; Leveille *et al.*, 2006; Liu *et al.*, 2003; Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010; Titus *et al.*, 2006, 2009).

The identification of zebrafish orthologs of all 15 human FANC genes shows that the full complement of FA genes had a more ancient origin than had originally been assumed and supports the notion that the zebrafish is a suitable model for the investigation of the FA/BRCA network. Screens in other nonmammalian, forward-genetic models, including the fruit fly *Drosophila melanogaster* and the nematode worm *Caenorhabditis elegans*, have shown that parts of FA group 2 and 3 are present, but the upstream, regulatory components of the NC complex, group 1 are absent (Collis *et al.*, 2006; Dequen *et al.*, 2005; Fei *et al.*, 2005; Marek and Bale, 2006; Youds *et al.*, 2008, 2009).

Although a whole genome duplication event (the teleost genome duplication, TGD) occurred in the lineage of teleost fish after it diverged from the tetrapod lineage (Amores *et al.*, 1998; Jaillon *et al.*, 2004; Naruse *et al.*, 2004; Postlethwait *et al.*, 1998, 2002; Taylor *et al.*, 2003) and about 30% of human genes remain duplicated in zebrafish (Postlethwait *et al.*, 2000), no duplicates of any of the 15 FA genes have been retained from the TGD event. This unlikely (1.4×10^{-8}) occurrence suggests that selective forces may have acted on FA/BRCA genes to cause them to revert to single copy. This situation might be explained if FA/BRCA proteins were required in precise stoichiometric amounts to perform the functions of the FA/BRCA pathway (i.e., the protein interactions necessary to form the FA core

complex). Alternatively, FA/BRCA genes may possess few subfunctions, a situation that, according to theory, will reduce the likelihood that a gene is retained in duplicate copy (Force *et al.*, 1999).

Gene expression analyses in zebrafish suggest that FA/BRCA genes may indeed have few regulatory subfunctions. Gene expression studies show that FA gene transcripts accumulate in developing zebrafish oocytes and are present in 1–2 cell stage embryos (Titus *et al.*, 2009), before the initiation of zygotic expression (Kane and Kimmel, 1993); this maternal message could then provide Fanconi proteins to repair DNA damage produced during DNA replication in the rapid cleavage cell divisions during the early stages of embryonic development.

Zygotic expression of FA/BRCA genes in developing zebrafish embryos is broad but is especially strong in tissues that are affected in FA patients, such as the central nervous system, eyes, and hematopoietic tissues, which is consistent with the microcephaly, microphthalmia, and bone marrow failure observed in FA patients (Titus *et al.*, 2009). Interestingly, FA genes are also expressed in the apical ectodermal ridge of the fin bud, a signaling center for fin/limb development, as well as in the oral epithelium, expression patterns that correlate, respectively, with the radius/thumb anomaly and the oral squamous cell carcinomas observed in FA patients (Titus *et al.*, 2009). Overall, FA genes are expressed in rapidly dividing tissues (i.e., proliferative regions of the intestine and the brain), expression patterns that are expected for the functioning of the FA/BRCA network in DNA repair (Titus *et al.*, 2009). Fanconi patients show other non-life-threatening phenotypes such as hypogonadism and infertility, suggesting that Fanconi proteins are involved in gonadogenesis in humans. Accordingly, ovaries and testes of mature zebrafish express Fanconi genes in specific stages of oocyte and spermatocyte development, which may be related to DNA repair during homologous recombination in meiosis and to infertility in human patients.

B. Zebrafish Fanconi Mutants and Sex-Determination

Mutations in two of the 15 FA/BRCA genes have been isolated and characterized in zebrafish. The first zebrafish Fanconi mutant characterized was mutated in the *fanconi anemia complementation group L* gene (*fancl*, MIM# 608111), and was generated by insertional mutagenesis in a Tol2 transposon-mediated enhancer trap screen (Nagayoshi *et al.*, 2008; Rodriguez-Mari *et al.*, 2010). Two mutations in the *fanconi anemia complementation group D1* gene, also known as *breast cancer 2* (*fancd1*(*brca2*), MIM# 605724, MIM# 600185), have been generated, one by ENU-mutagenesis (Shive *et al.*, 2010) and one by retroviral insertion (Amsterdam and Hopkins, 2006; Rodríguez-Marí *et al.*, 2011).

The characterization of the *fancl* zebrafish mutant line revealed an unexpected phenotype: all homozygous mutants developed exclusively as males. This all-male phenotype was also observed in the two *brca2* zebrafish mutant lines, suggesting a common role of FA/BRCA genes in sex determination in zebrafish.

Like zebrafish, FA patients often show problems with gonadogenesis, including hypogonadism and infertility. Similarly, FA gene knockout in mouse leads to hypogonadism, impaired gametogenesis, and infertility as the most consistent phenotypes (e.g., *Fancc, Fancg, Fanca, Fancd1(Brca2),* and *Fancd2*; reviewed in Parmar *et al.*, 2009). FA gene knockout mice, however, do not show the all-male phenotype observed in zebrafish, a difference likely related to the high developmental plasticity of sex determination in fish compared to the strong genetic sex-determining mechanism of mammals.

C. Mechanisms of Sex Determination

The all-male phenotype of zebrafish *fancl* and *fancd1* mutants provides a tool to investigate the mechanisms of sex determination in zebrafish. The existence of two differentiated sexes is common among animals, and yet the mechanisms that determine sex are amazingly diverse. Sex-determining mechanisms can be divided mainly into two categories: genetic sex determination (GSD) and environmental sex determination (ESD).

In GSD, genetic elements specify the sex of the individual independent of the environment. GSD includes monogenic as well as polygenic systems. In monogenic systems, a major sex-determining gene is usually found on a sex chromosome that evolved from a pair of autosomes after it acquired a novel sex-determining allele (reviewed in Marshall Graves, 2008). Most sex chromosome systems can be subdivided into XX/XY or ZZ/ZW systems. In the XX/XY system, males are the heterogametic sex, as in mammals, fruit flies, medaka, and many other species. In the ZZ/ZW system, females are the heterogametic sex, as in birds, snakes, and turbot among others (reviewed in Ezaz *et al.*, 2006; Martnez *et al.*, 2009). In ESD, environmental factors such as temperature specify an individual's sex. For example, in crocodilians and marine turtles, egg incubation temperature determines sex (reviewed in Western and Sinclair, 2001).

GSD and ESD have both been shown to control sex among the more than 24,000 species of fish (Baroiller and Guiguen, 2001; Nelson, 1994). GSD mechanisms described in fishes are highly diverse and range from monogenic to polygenic, including systems with dominant sex-determining factors mixed with influences from autosomal chromosomes (reviewed in Devlin and Nagahama, 2002). Some fishes have highly evolved sex chromosomes with XX/XY (e.g., medaka, *Oryzias latipes*) or ZZ/ZW (e.g., in tilapia, *Oreochromis aureus*) systems. ESD mechanisms range from temperature to behavioral effects on sex determination (reviewed in Devlin and Nagahama, 2002; Volff and Schartl, 2001).

Although GSD and ESD mechanisms have long been thought of as distinct, recent data show regulation by both genetic and environmental factors within a single species (Barske and Capel, 2008). In such species, the integration of genetic and environmental factors ultimately tips the bipotential gonad towards the male or the female fate (reviewed in Baroiller *et al.*, 2009). This is clearly exemplified in tilapia, which has a ZZ/ZW system in which the sex ratio can be modified by temperature,

and moreover, in which autosomal chromosomes can influence the definitive sex of the fish (reviewed in Devlin and Nagahama, 2002). Another example is medaka, a teleost fish widely used as a model organism in developmental biology, which has an XX/XY sex determination system that can be modified by high temperatures, which cause XX females to develop into sex-reversed males (Sato *et al.*, 2005).

1. Master Sex-Determining Genes

Despite the vast diversity of primary sex-determining mechanisms, genes downstream in the sex determination pathway appear to be broadly conserved among vertebrates. Genes upstream in the pathway have often changed during evolution, sometimes relatively recently as species recruited different downstream genes to be their major sex-determining loci. Thus, changes at the top of the sex-determining pathway appear to be better tolerated than changes lower in the pathway because the top ones are less likely to have deleterious effects (Marin and Baker, 1998).

In mammals, the Y chromosome gene *Sry* (*Sex-determining region Y*) is at the top of the sex determination cascade (Gubbay *et al.*, 1990; Koopman *et al.*, 1991; Sekido and Lovell-Badge, 2009; Sinclair *et al.*, 1990). *SRY* acts as a genetic switch that triggers the bipotential gonad to initiate the male pathway (reviewed in Brennan and Capel, 2004). *Sry,* however, does not appear to exist beyond therian mammals (Wallis *et al.*, 2007). In several animal groups, including mammals, *dmrt1* (*double-sex and mab-3 related transcription factor 1*) is a downstream gene in the male sex-determination pathway, but in medaka (*Oryzias latipes*), a duplicated copy of *dmrt1* (called *DMY* or *dmrt1by*) is the major sex-determining gene (Matsuda *et al.*, 2002; Nanda *et al.*, 2002) and recent work has shown that *dmrt1* is required for testis development in chickens (Smith *et al.*, 2009). Interestingly, *dmrt1by* is present in a few species of the *Oryzias* genus and is absent in all other fishes, including zebrafish (Kondo *et al.*, 2003). This finding rules out *dmrt1b* as the universal sex-determining gene in fishes and shows that the upstream regulators of sex determination can change rapidly. Thus, the main sex-determining gene remains to be discovered in the vast majority of fishes, including zebrafish.

A polygenic sex-determining system may guide zebrafish gonad development (reviewed in Orban *et al.*, 2009; Siegfried, 2010), and a recent study using methyltestosterone treatments to control sex determination has suggested the presence of female-dominant genetic factors in zebrafish (Tong *et al.*, 2010). Interestingly, among the several microarray-based transcriptional studies of zebrafish gonads (Li *et al.*, 2004; Santos *et al.*, 2007; Small *et al.*, 2009; Sreenivasan *et al.*, 2008; Wen *et al.*, 2005), some have pointed to opposite conclusions: either a masculinization (Small *et al.*, 2009) or a feminization of the zebrafish transcriptome (Santos *et al.*, 2007). Therefore, it is likely that new methods and approaches based on high-throughput sequencing will be required to definitively solve this basic biological question in zebrafish as well as in other fish species in which the master sex-determining genes remain unknown. It is possible that different species may have recruited any of several different downstream genes as major sex-determinants

while maintaining basic elements of the pathway. Gene expression analyses in zebrafish have already revealed the conservation of structure, function, and expression of a large number of mammalian genes downstream in the sex determination pathway, including *cyp19a1a, sox9a, sox9b, amh, ff1, dmrt1,* and *r-spondin1* (Chiang *et al.*, 2001; Guo *et al.*, 2005; Jorgensen *et al.*, 2008; Rodriguez-Mari *et al.*, 2005; von Hofsten *et al.*, 2005a, 2005b; Wang and Orban, 2007; Yan *et al.*, 2002, 2005; Zhang *et al.*, 2011).

2. Gonad Development in Fish

Fish show a striking plasticity of the sex determination process, which is reflected in a wide range of gonad differentiation mechanisms, and includes gonochoristic species as well as hermaphroditic species. Zebrafish is a gonochoristic species, in which each mature individual possesses only ovaries or testes. Hermaphroditic species can initially mature either as males (protandrous) or females (protogynous), or in contrast, can be synchronous hermaphrodites that simultaneously contain functional male and female gonadal tissues (reviewed in Devlin and Nagahama, 2002). Zebrafish are undifferentiated gonochoristic fishes and all individuals initially develop ovarian tissue (Yamamoto, 1969), in contrast to differentiated gonochoristic species in which an indifferent gonad proceeds to develop as a testis or as an ovary directly.

D. Sex Determination and Gonad Development in Zebrafish

Although zebrafish has been used extensively as a model organism in developmental biology and biomedical research, the genetic mechanisms of sex determination in zebrafish remain poorly understood. Zebrafish has no detectable heteromorphic sex chromosomes (Amores and Postlethwait, 1999; Pijnacker and Ferwerda, 1995; Schreeb *et al.*, 1993), which hinders the study of the molecular mechanisms leading to a sex-specific program of gonadogenesis. In contrast to early juvenile stages, in which sexes cannot be distinguished externally, the two sexes as adults show different external characteristics that makes them easily distinguishable: males show a brighter orange coloration of the anal fin (Fig. 1A) and females have an enlarged belly engorged with ovaries full of eggs (Fig. 1B). Depending on rearing conditions, zebrafish take an average of about 3 months to become reproductively mature adults.

The gonadal primordium in juvenile zebrafish contains centrally located germ cells surrounded by somatic cells. Regardless of their definitive sex, normally developing zebrafish juveniles initially form a gonadal primordium that develops as an ovary containing immature oocytes (Maack and Segner, 2003; Selman *et al.*, 1993; Takahashi, 1977). In approximately half of the population, oocytes begin to degenerate between 20 and 30 days post-fertilization (dpf); this period lasts several days and varies among individuals and among rearing conditions (Maack and Segner, 2003; Rodriguez-Mari *et al.*, 2005, 2010; Siegfried and Nusslein-Volhard, 2008; Takahashi, 1977; Uchida *et al.*, 2002; Wang and Orban, 2007). In these

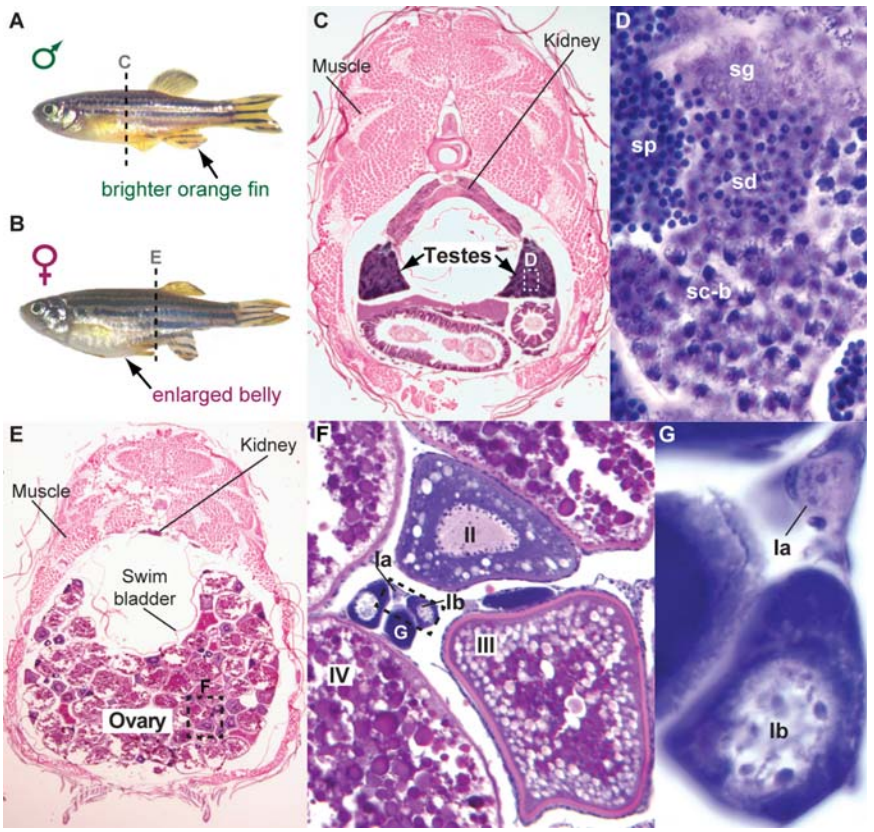

Fig. 1 Sexual dimorphism of external characters, spermatogenesis, and oogenesis in adult zebrafish. Zebrafish adult males (A) show a brighter orange coloration of the anal fin while zebrafish adult females (B) have an enlarged belly engorged with ovaries full of eggs. (C) Cross-section of an adult male stained with hematoxylin and eosin showing the position of muscle and kidney as well as the bilateral position of testes. (D) Higher magnification of adult testes shown in (C, dashed box) reveals germ cells at various stages of spermatogenesis: spermatogonia (sg), spermatocytes at bouquet stage (sc-b), spermatids (sd), and sperm (sp). (E) Cross-section of an adult female stained with hematoxylin and eosin showing the position of muscle, kidney, swim bladder, and ovary. (F) Higher magnification of the adult ovary shown in (E, dashed box) displaying germ cells at various stages of oogenesis: stage Ia, Ib, II, III, and IV oocytes. (G) Higher magnification of stage Ia oocytes residing in a nest surrounded by prefollicle cells and a perinucleolar stage IB oocyte outside the nest. Oocyte staging according to Selman *et al.* (1993). (See color plate.)

animals, gonads begin to acquire testis morphology, germ cells enter into spermatogenesis, mature testes form, and the animals become mature males (Figs. 1C and D). In the other half of the population, oocytes progress through oogenesis, mature ovaries form, and the animals become mature females (Figs. 1E–G) (Maack and Segner, 2003; Takahashi, 1977; Uchida *et al.*, 2002). The mechanisms that drive juvenile zebrafish gonads toward the definitive female or male developmental pathways, however, are not yet known.

1. Gonad Differentiation Determines Sex in Zebrafish: Animals Lacking Germ Cells Develop Testes and Become Sterile Males

The essential role of gonad differentiation in determining the final sex in zebrafish has been demonstrated by experiments knocking-down the function of *dead end* (*dnd*), a gene that is essential for primordial germ cell (PGC) migration and survival in zebrafish (Ciruna *et al.*, 2002; Weidinger *et al.*, 2003). Zebrafish embryos injected with *dnd* antisense morpholino (MO) at the 1-cell stage develop gonads completely depleted of PGCs; such gonads develop into testes, which causes the individual to become an infertile male (Siegfried and Nusslein-Volhard, 2008; Slanchev *et al.*, 2005) (Fig. 2). This finding is significant first, because it provides evidence that the alteration of gonad development is sufficient to alter the sex of the individual, thereby showing that gonad sex drives definitive sex in zebrafish. Second, this result suggests that germ cells signal the somatic cells of the gonads to select sexual fate in zebrafish (Siegfried and Nusslein-Volhard, 2008; Slanchev *et al.*, 2005). Therefore, it was postulated that germ cells control female fate in zebrafish (Siegfried and Nusslein-Volhard, 2008). Studies comparing zebrafish *fancl* homozygous mutants, which develop exclusively as males that produce functional sperm, to *dnd* knock down animals, which develop as sterile males without sperm, suggested that it is specifically oocyte survival through meiosis, rather than the mere presence of germ cells, which is required for female gonad differentiation in zebrafish (Rodriguez-Mari *et al.*, 2010).

2. Mutations in FA Genes Induce Female-to-Male Sex Reversal in Zebrafish

Zebrafish *fancl* homozygous mutants, like germ cell-depleted animals, develop exclusively as males even though their gonads are not germ cell deficient (Rodriguez-Mari *et al.*, 2010). Sex ratios in test crosses revealed that the failure of homozygous *fancl* mutants to become females is not due to female-specific lethality, but to female-to-male sex reversal: animals that otherwise would have become females instead develop as males (Rodriguez-Mari *et al.*, 2010). This finding was confirmed and extended to another gene in the FA/BRCA network by studies of two different *fancd1*(*brca2*) alleles, a nucleotide substitution mutation and an insertional mutation, which also result in exclusively male development due to female-to-male sex reversal (Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010).

3. Zebrafish FA Mutants Show that the Mere Presence of Germ Cells is not Sufficient to Feminize the Gonads

The total loss-of-function of *dead end*, *nanos*, *ziwi*, or *zili* results in exclusively male development in zebrafish due to the lack of germ cells (Draper *et al.*, 2007; Houwing *et al.*, 2007, 2008; Siegfried, 2010; Slanchev *et al.*, 2005). In contrast, germ cells are present throughout the entire life of all individuals homozygous for *fancl* or *fancd1*(*brca2*) mutations; these findings reveal that sex reversal of homozygous

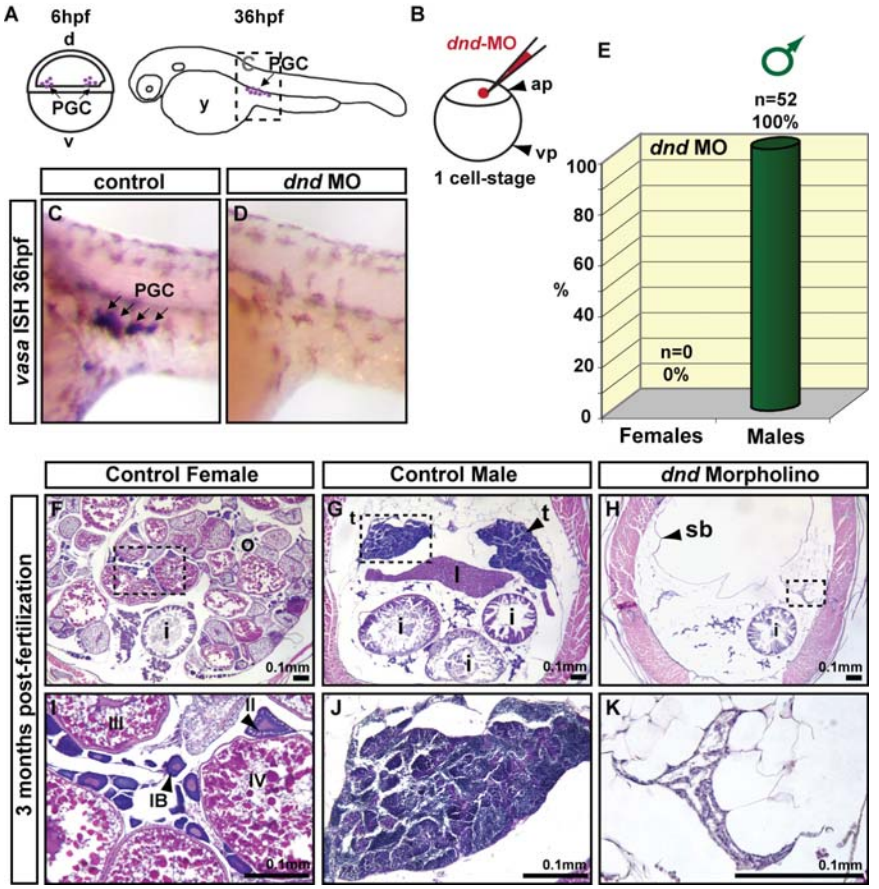

Fig. 2 Zebrafish depleted of germ cells by *dead end* knockdown morpholino develop testes and become sterile males. (A) Schematic representation of the position of PGC in normal animals at two different stages of development. At 6 h post fertilization (hpf), PGCs are found in four clusters (arrows show two clusters in lateral view); these PGCs migrate and reach the location of the future gonad by 24 hpf; by 36 hpf, all PGCs have reached their definitive location (Yoon *et al.*, 1997). (B) Schematic representation of the injection of *dnd* morpholino (*dnd*-MO) into 1-cell stage wild-type embryos. (C, D) *In situ* hybridization (ISH) of noninjected controls and *dnd*-MO injected embryos, using *vasa* probe as a PGC marker, confirms the total depletion of PGC in *dnd*-MO animals. (E) As adults, all animals depleted of PGC using *dnd*-MO develop as phenotypic males ($n = 52$; 100%). (F–K) Cross-sections of animals at 3 months post fertilization at lower (F, G, H) or higher (I, J, K; dashed rectangles in F, G, H) magnification shows that controls had mature ovaries (F, I) or testes (G, J) filled with different stages of oocytes or spermatocytes respectively. In contrast, animals injected with *dnd*-MO developed as phenotypic males and had testes consisting solely of somatic tissue depleted of PGC (H,K), revealing that the differentiation of the gonads drives sex determination in zebrafish (Siegfried and Nusslein-Volhard, 2008; Slanchev *et al.*, 2005). Abbreviations: ap, animal pole; d, dorsal; i, intestine; l, liver; o; ovary; sb, swim bladder; t, testis; v, ventral; vp, vegetal pole; y, yolk; IB, II, III, IV, oocyte stages. (See color plate.)

Fanconi mutants that otherwise would have become females is not due to lack of germ cells (Rodriguez-Mari *et al.*, 2010; Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010). Thus, in contrast to female-to-male sex-reversed animals that lack germ cells, *fancl* and *fancd1*(*brca2*) mutants show that the mere presence of germ cells

throughout development and adult stage is insufficient to feminize the gonads (Rodriguez-Mari *et al.*, 2010; Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010).

4. Oocytes Passing Through Meiosis are Essential to Support the Differentiation of Ovaries in Zebrafish

Zebrafish *fancl* mutants initially develop bipotential juvenile ovaries containing early oocytes and express both female (*cyp19a1a*) and early male (*amh*) somatic markers as in wild types (Rodriguez-Mari *et al.*, 2010). In contrast to wild-type control gonads, which contain prerecombinational oocytes (early stage IB) that progress through meiosis, complete recombination, and reach the diplotene stage of meiosis (late stage IB oocytes), most *fancl* mutants lack late stage IB oocytes (Rodriguez-Mari *et al.*, 2010). This finding indicates that in *fancl* mutants, early oocytes fail to progress through meiosis beyond the pachytene stage, when recombination occurs, and do not enter diplotene. Consistent with this finding, in wild-type gonads, *fancl* (and also *fancd1*(*brca2*)) gene expression upregulates in oocytes transitioning from prerecombinational stages to diplotene late stage IB during the critical stages for sexual fate decision in zebrafish (Rodriguez-Mari *et al.*, 2010; Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010). Because the FA/BRCA system is involved in the repair of DNA double-strand breaks by homologous recombination, including those occurring in meiosis, *fancl* mutants are likely deficient in DNA repair after homologous recombination, and unrepaired DNA damage may impact oocyte survival (Rodriguez-Mari *et al.*, 2010). Gonads of *fancl* mutants, which have germ cells but lack oocytes, do not maintain the expression of somatic female-specific genes that were initiated in the juvenile bipotential gonad (e.g., *cyp19a1a*) and upregulate early male-specific genes that were initiated in the juvenile bipotential gonad (e.g., *amh*) (Rodriguez-Mari *et al.*, 2010). This expression profile masculinizes gonads of *fancl* mutants, which become fertile testes. Like *fancl* mutant gonads, *fancd1*(*brca2*) mutant gonads contain germ cells but lack oocytes (Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010). Overall, these results further support the finding that the presence of germ cells is not sufficient to feminize the gonads and instead suggest that oocytes surviving through meiosis are essential to support the differentiation of ovaries.

5. The Role of Meiotic Oocytes in Preventing Female Somatic Cells (Pregranulosa) from Transdifferentiating into Somatic Male Cells (Sertoli-Like Cells)

A few *fancl* mutant gonads that had retained several meiotic oocytes at the time of examination had, adjacent to the oocytes, somatic cells that expressed *cyp19a1a* (Rodriguez-Mari *et al.*, 2010), which encodes aromatase, the enzyme that converts testosterone to estrogen (Hu *et al.*, 2001). This observation led to the suggestion that oocytes are essential to maintain *cyp19a1a* expression in somatic cells, which can be understood if oocytes that have progressed through meiosis produce a signal that is essential for the maintenance of pregranulosa cells or for their differentiation into

mature granulosa cells (Rodriguez-Mari *et al.*, 2010). The finding that *fancl* mutants that lack meiotic oocytes also lack *cyp19a1a* expression and upregulate *amh* expression led to the hypothesis that meiotic oocytes provide a signal that prevents *cyp19a1a*-expressing pregranulosa cells from transdifferentiating into *amh*-expressing Sertoli-like cells (Rodriguez-Mari *et al.*, 2010). This hypothesis is supported with findings in other species, including medaka and mouse, in which granulosa cells (normally found only in ovaries) and Sertoli cells (normally found only in testes) arise from a common somatic precursor and can trans-differentiate (Albrecht and Eicher, 2001; Guigon and Magre, 2006; McLaren, 1991; Nakamura *et al.*, 2008).

6. Increased Tp53-Dependent Germ Cell Apoptosis Causes Sex Reversal in *fancl* Mutants by Compromising Oocyte Survival

Zebrafish *fancl* mutants have an abnormal increase of Caspase-3 mediated germ cell apoptosis during the period of gonad fate decision (25 dpf) (Rodriguez-Mari *et al.*, 2010). More importantly, this abnormal increase in germ cell apoptosis is the cause of the sex-reversal phenotype observed in *fancl* mutants, because introduction of homozygous mutations in *tp53* (*p53*), an important activator of apoptosis (Fridman and Lowe, 2003), rescues the sex-reversal in *fancl* mutants by reducing germ cell apoptosis, which allows oocyte survival, ovary differentiation, and female development in *fancl* mutants (Rodriguez-Mari *et al.*, 2010). These studies show that Tp53-mediated germ cell apoptosis is a cellular mechanism contributing to the female-to-male sex-reversal phenotype observed in *fancl* zebrafish mutants (Rodriguez-Mari *et al.*, 2010). The mutation of *tp53* also rescues the female-to-male sex reversal phenotype of *fancd1*(*brca2*) mutants, although germ cell apoptosis was not investigated in *fancd1;tp53* doubly homozygous mutants (Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010).

7. A Threshold Number of Developing Oocytes could be a Determinant of Ovarian Fate

In zebrafish, all juveniles begin oogenesis regardless of their definitive sex, whereas in medaka, only XX females start oogenesis while XY males suppress meiosis and germ cells delay differentiation (reviewed in Saito and Tanaka, 2009). Despite this developmental difference, a threshold number of developing oocytes might be a key factor that tips undifferentiated gonads towards ovarian fate in both species (Rodriguez-Mari *et al.*, 2010; Saito and Tanaka, 2009; Siegfried and Nusslein-Volhard, 2008). In medaka *hotei* mutants, oocyte development is aberrant (Morinaga *et al.*, 2007), gonads fail to maintain *cyp19a1a* expression, and individuals develop testes, a situation similar to that found in zebrafish *fancl* mutants. These comparisons support the hypothesis that the sexual fate of the gonad tips towards the female pathway when the number of oocytes exceeds some threshold; alternatively, when the oocyte number falls under this threshold, the sexual fate of the gonad tips towards the male pathway, as happens in zebrafish *fancl* mutants.

The oocyte-derived signal hypothesized to maintain female gene expression in gonadal somatic cells in juveniles could provide a mechanism for the oocyte-threshold hypothesis. A greater number of meiotic oocytes would provide a greater amount of the hypothesized oocyte-derived signal and thus maintain sufficient *cyp19a1a*-expressing cells to provide substantial amounts of estrogen to promote female development. Alternatively, if the number of meiotic oocytes falls under the critical threshold, the level of *cyp19a1a* would fall and the level of estrogen would not rise sufficiently to maintain ovary development and inhibit testis development. In support of this model, the depletion of PGCs can reduce the number of developing oocytes below a threshold necessary for female development in medaka (Kurokawa *et al.*, 2007). Likewise, the transplantation of single PGCs into PGC-depleted zebrafish embryos produces almost exclusively male fish that can develop reliably into females after estrogen treatments (Ahsan *et al.*, 2008; Higaki *et al.*, 2010; Kawakami *et al.*, 2010). Accordingly, zebrafish *fancl* mutants suffer an abnormal increase of germ cell apoptosis during the gonadal fate decision period (25dpf) (Rodriguez-Mari *et al.*, 2010), likely reducing the total number of germ cells in the still bipotential gonads. Therefore, it is plausible to hypothesize that a higher number of undifferentiated germ cells results in a higher number of oocytes passing through meiosis, which increases the level of an oocyte-derived signal that is necessary to maintain aromatase expression and direct the gonad towards the ovarian differentiation pathway.

8. A Model for Zebrafish Sex Determination: Tp53-Mediated Germ Cell Apoptosis can Regulate Oocyte Survival through Meiosis and alter Gonad Fate

The results summarized above suggest the following model for zebrafish sex determination (Fig. 3). According to this model, somatic cells of the bipotential gonad express early male genes, including *amh* and *sox9a*, as well as female genes, like aromatase and *foxl2* (Rodriguez-Mari *et al.*, 2010; Siegfried and Nusslein-Volhard, 2008). In normal gonad development (Fig. 3A), all zebrafish bipotential juvenile gonads contain oocytes. When oocytes progress through meiosis, they make double strand DNA breaks, which they repair by homologous recombination, then enter in diplotene stage where they arrest. According to our model, a signal arising from diplotene oocytes induces the soma to maintain or perhaps upregulate aromatase expression and/or activity. Aromatase converts testosterone to estrogen, which negatively impacts germ cell differentiation along the spermatogenic pathway, downregulates early male genes like *amh*, and maintains female fate of somatic gonadal cells. As a consequence, the gonad develops into an ovary and the individual becomes a female.

In contrast to wild types, *fanc* mutant oocytes passing through meiosis suffer DNA breaks of meiotic recombination but inadequately repair these breaks due to a defect in the FA system (Fig. 3B). We hypothesize that the cell detects the persistence of unrepaired DNA breaks and initiates an apoptotic response that involves the action of Tp53 to destroy the defective cell. Without diplotene oocytes, gonads lack the

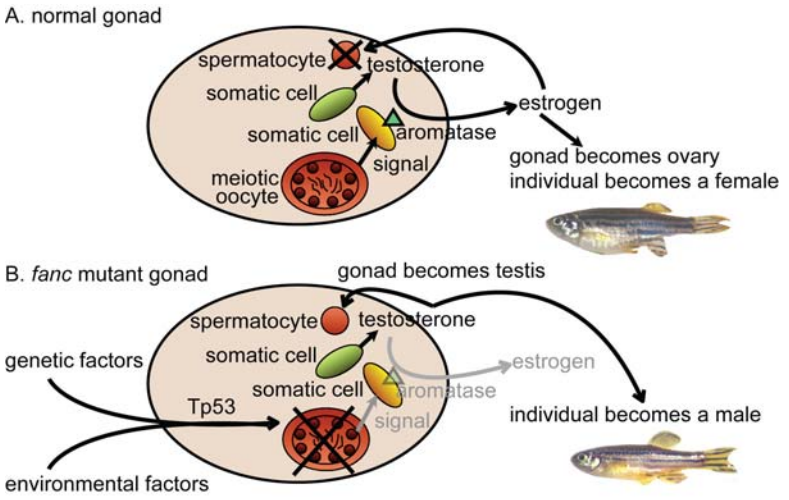

Fig. 3 A model for zebrafish sex determination: Tp53-mediated germ cell apoptosis can regulate oocyte survival through meiosis and alter gonad fate. Bipotential gonads express both aromatase and enzymes that produce testosterone. (A) In a normal gonad, diplotene stage oocytes signal somatic cells to maintain aromatase expression, and with sufficient signal, aromatase converts testosterone to estrogen, which suppresses spermatogenesis, maintains ovary development, and causes individuals to become females. If the oocyte-derived signal is weak, testosterone masculinizes the gonad and the individual. (B) In *fanc* mutant gonads, double strand DNA breaks of homologous recombination are not repaired, oocytes die by Tp53-mediated apoptosis, the oocyte-derived signal is weak or missing, aromatase expression is not maintained, testosterone is not converted to estrogen, spermatogenesis ensues, the gonad becomes a testis, and the individual becomes a male. (For color version of this figure, the reader is referred to the web version of this book.)

oocyte-derived signal that maintains aromatase expression in gonadal somatic cells, testosterone is no longer converted to estrogen, spermatogenesis is not repressed, the gonad becomes a testis, and the individual becomes a male. In *fanc;tp53* double mutants, while oocytes still do not repair the DNA damage of meiosis, they cannot commit apoptosis efficiently because they have reduced Tp53 activity; surviving diplotene oocytes then produce the oocyte-derived signal, somatic cells maintain aromatase, and double mutants become females.

In normal zebrafish juveniles, we speculate that both genetic and environmental factors affect the number of oocytes passing through meiosis, either by affecting the proliferation rate of PGCs or by increasing or decreasing Tp53-dependent germ cell apoptosis during the sexual fate decision period. Genetic and environmental factors that increase the number of oocytes passing through meiosis increase the likelihood that the individual will become a female. In contrast, genetic and environmental factors that decrease the number of oocytes that pass through meiosis increase the likelihood that the individual will become a male. Identification of the genetic and environmental factors that affect the strength of the hypothesized oocyte-specific signal is an important research goal. Studies conducted in FA zebrafish mutants point toward Tp53-dependent germ cell apoptosis regulation as a crucial determinant for the definitive sex in zebrafish.

E. Zebrafish as a Model for Understanding the Molecular Basis of FA Disease and Gonadal Cancers

Studies of zebrafish *fancl* and *fancd1*(*brca2*) genes have not only provided a better understanding of the molecular and cellular mechanisms that control zebrafish gonad differentiation and sex determination but have also provided information on the molecular basis of FA and gonadal cancers. FA patients often show phenotypes in the gonads, including hypogonadism, impaired gametogenesis, defective meiosis, and sterility (Auerbach, 2009; Wong *et al.*, 2003). Because zebrafish have a complete set of FA genes that are expressed in rapidly proliferating tissues (Titus *et al.*, 2009) and that are often affected in FA patients, zebrafish offers an attractive experimental system to help unravel the mechanisms of FA disease. In addition, given the involvement of the FANC/BRCA pathway in ovarian and breast cancer (reviewed in Bogliolo *et al.*, 2002; D'Andrea, 2010; Levy-Lahad, 2010; Taniguchi *et al.*, 2003), study of zebrafish is likely to contribute to our understanding of these cancers.

1. Alteration of the FA/BRCA Network Abnormally Activates Tp53-Dependent Apoptosis in Zebrafish as it does in Humans

The finding that *fancl* is expressed in germ cells during gonad differentiation in zebrafish (Rodriguez-Mari *et al.*, 2010) and mouse (Agoulnik *et al.*, 2002; Lu and Bishop, 2003) suggests a conserved role in vertebrate germ cell development. Hypogonadism, impaired gametogenesis, and infertility are among the most consistent FA phenotypes in murine FA gene knockout models (e.g., *Fancc, Fancg, Fanca, Fancd1*, and *Fancd2*) (reviewed in Parmar *et al.*, 2009). Likewise, zebrafish *fancl* mutants also show problems with gonad development, as all gonads develop into testes, but not into ovaries. Because zebrafish *fancl* mutant males and *fancl;tp53* double mutant rescued females are fertile, we conclude that Fancl function is not essential for the differentiation of spermatogonia and oogonia into sperm or oocytes, respectively. Rather, Fancl activity is required to prevent apoptotic cell death cues (Rodriguez-Mari *et al.*, 2010). Increased germ cell apoptosis in zebrafish *fancl* mutants mimics the increase of apoptosis in a variety of cell types reported in FA gene knockout mice. For instance, knockout mice for $Fanca^{-/-}$, $Fancc^{-/-}$, and $Fancg^{-/-}$ show increased apoptosis of neuronal and hematopoietic cells that leads to a progressive loss of stem and progenitor cells (Freie *et al.*, 2003; Rio *et al.*, 2002; Sii-Felice *et al.*, 2008). Excessive apoptosis and subsequent failure of the hematopoietic stem cell compartment can cause bone marrow failure in children with FA disease (reviewed in Parmar *et al.*, 2009). Interestingly, $Fanca^{-/-}$ knockout mice also show increased male germ cell apoptosis (Wong *et al.*, 2003), which suggests that a role in germ cell apoptosis might be a conserved feature of the FA/BRCA network in fish and mammals. Likewise, genetic deletion of Tp53 rescues the TNF-α (Tumor Necrosis Factor) dependent apoptosis caused by accumulation of the proapoptotic protein kinase PKR that results from a mutation of the human FANCC gene

(Freie *et al.*, 2003). Thus, an inappropriate activation of Tp53-dependent apoptosis seems to be a common mechanism that affects cell survival in both zebrafish and human when the FA network is altered.

2. Postembryonic Roles of *fancd1*(*brca2*) in Zebrafish Gametogenesis and Oocyte Nuclear Architecture Provide Novel Clues to help Understand Human Gonadal Cancers

People carrying one mutant null-allele of *FANCD1*(*BRCA2*) have an elevated risk of breast and ovarian cancer, and the possession of two hypomorphic mutant alleles leads to the development of FA (Neveling *et al.*, 2009). Because humans and mice homozygous for null activity alleles are embryonic lethal (Neveling *et al.*, 2009), only limited information on the adult roles of *FANCD1*(*BRCA2*) is available. The zebrafish *fancd1*(*brca2*) gene shares functions with its human ortholog, including protection from DNA damaging agents and maintenance of genome stability (Rodríguez-Marí *et al.*, 2011). As in humans, *fancd1*(*brca2*) mutation in zebrafish leads to gonadal tumors, although *fancl* mutation has not been shown to increase tumor risk in zebrafish. The alteration of germ cell development in *fancd1*(*brca2*) mutants is more severe than that in *fancl* mutants because adult *fancd1*(*brca2*) mutants are sterile but *fancl* mutants are fertile. This suggests the hypothesis that the overproliferation of the gonadal soma in *fancd1*(*brca2*) mutants could be related to the failure of germ cell development. Spermatogenesis in zebrafish *fancd1*(*brca2*) mutants arrests in late-zygotene/early pachytene during or after meiotic recombination followed by spermatocyte death. Although mutation of *tp53* rescues the *fancd1* sex-reversal phenotype, it does not rescue fertility in *fancd1;tp53* double mutant males or females. Double mutant males have empty testis tubules surrounded by somatic cells in the posterior testes, which normally contains only mature sperm; these empty mutant tubules eventually initiate neoplastic proliferation (Fig. 4A) (Rodríguez-Marí *et al.*, 2011). Zebrafish depleted of germ cells by *dnd*-knockdown also lack sperm and form testicular neoplasias (Fig. 4B) similar to those found in germ cell-depleted regions of *fancd1* and *fancd1;tp53* mutants (Rodríguez-Marí *et al.*, 2011). This finding suggests that neoplastic growth in the testes of *fancd1*(*brca2*) mutants could be an indirect consequence of the absence of germ cells rather than a direct effect of the lack of Fancd1(Brca2) activity. These observations further suggest the hypothesis that germ cells in some way signal the gonadal soma to regulate proliferation (Figs. 4D and E) (Rodríguez-Marí *et al.*, 2011).

Transcripts from *fancd1*(*brca2*) localize tightly to the animal pole of the oocyte cytoplasm in wild-type females, which suggests a possible role in either the oocyte or the early developing zygote (Rodríguez-Marí *et al.*, 2011). Because transcripts of *fancd1*(*brca2*) as well as *oct4* are also tightly localized to the animal pole in *fancd1;tp53* double mutant females, *fancd1*(*brca2*) does not appear to be necessary to localize transcripts in the ooplasm (Rodríguez-Marí *et al.*, 2011).

In normally developing oocytes, nucleoli distribute around the periphery of the oocyte nucleus while chromosomes occupy the center (Selman *et al.*, 1993)

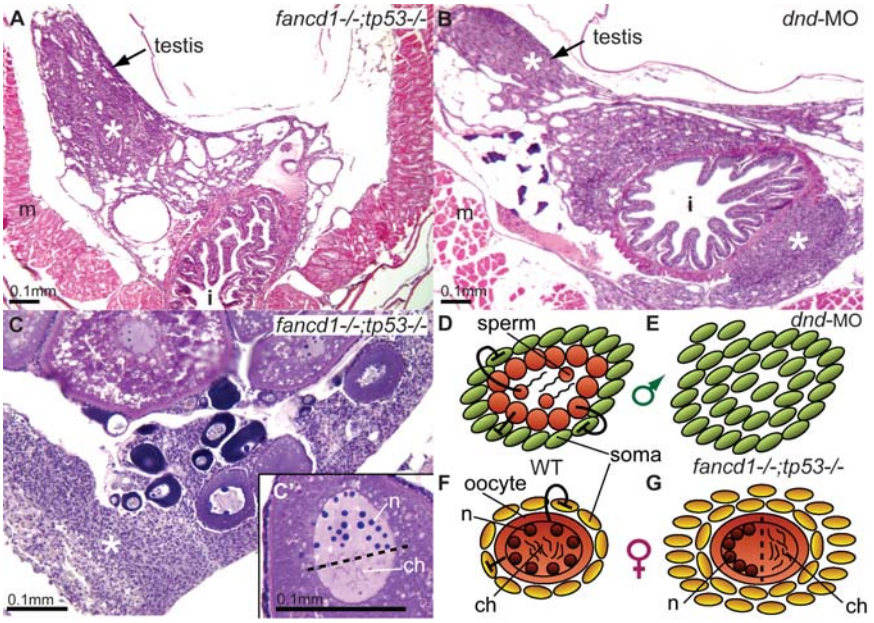

Fig. 4 Abnormal somatic cell proliferation in gonads of *fancd1* and *fancd1;tp53* double mutants and in *dnd*-knockdown animals. (A) Adult *fancd1;tp53* double mutant male cross-section stained with hematoxylin and eosin showing the posterior part of the testes, which in wild-type animals normally contains sperm. In double mutant males the posterior part of the testes is devoid of germ cells and undergoes abnormal proliferation of gonadal somatic cells (asterisk). (B) Cross-section of an adult *dead end* (*dnd*) knockdown animal stained with hematoxylin and eosin showing germ cell-depleted testes undergoing abnormal proliferation of gonadal somatic cells (asterisk) as observed in *fancd1;tp53* double mutant testes. (C) Adult *fancd1;tp53* double mutant female cross-section stained with hematoxylin and eosin showing a region of the ovary containing oocytes surrounded by abnormally proliferating gonadal somatic cells (asterisk). (C') Higher magnification of a *fancd1;tp53* double mutant oocyte showing the abnormal architecture of the nucleus with nucleoli accumulated on one side and chromosomes accumulated at the opposite pole. By contrast, the wild-type oocyte nucleus is radially symmetric with peripheral nucleoli and central chromosomes (see wild-type stage II oocyte in Fig. 1F for comparison). (D–G) A model of germ cell signaling for the control of somatic cell proliferation in male (D,E) and female (F,G) gonads. (D) Wild-type testes are organized in cysts containing germ cells at different stages of spermatogenesis: spermatogonia and spermatocytes (red circles) or sperm (smaller red circles with tails) can signal the soma to control the proliferation of surrounding somatic cells (green ellipses; i.e., Sertoli, Leydig, interstitial cells). (E) In the absence of germ cell/soma signaling, somatic cells can overproliferate (green ellipses) as observed in the posterior region of *fancd1;tp53* mutant testes and *dnd*-MO animals that lack germ cells. (F) Radially symmetric wild-type oocyte (large red circle) with peripheral nucleoli (n) and central chromosomes (ch) signals the soma to control proliferation of surrounding somatic cells. (G) *fancd1;tp53* mutant oocytes show abnormal nuclear architecture and nucleoli accumulate to one side of the nucleus and chromosomes (ch) accumulate to the opposite pole. These aberrant oocytes do not signal the soma correctly, and surrounding somatic cells can overproliferate and produce ovarian tumors, such as the ones observed in *fancd1;tp53* double mutant females. (See color plate.)

(Fig. 1F). Surprisingly, in the oocytes of *fancd1*(*brca2*);*tp53* mutant females, nucleoli occupy one pole of the nucleus and chromosomes occupy the opposite pole (Figs. 4C and C′), a phenotype not observed in *tp53* homozygous mutant females (Rodríguez-Marí *et al.*, 2011). This finding reveals a role for *fancd1* (*brca2*) in establishing or maintaining oocyte nuclear architecture (Rodríguez-

Marí *et al.*, 2011). Similar observations have not been made in mouse or human due to the lethality of null alleles in these species, which further validates the zebrafish as a suitable model for the discovery of novel vertebrate functions of Fancd1(Brca2).

In addition to their abnormal location, oocyte chromosomes are more decondensed in *fancd1*(*brca2*);tp53 double mutant females than in wild types and show abnormal extensions, which would be expected if the DNA breaks of meiosis cannot be repaired.

Doubly heterozygous embryos from homozygous *fancd1*(*brca2*)*;tp53* double mutant females develop into aberrant embryos that fail to survive past early segmentation stages (Rodríguez-Marí *et al.*, 2011). In contrast, the genetically identical doubly heterozygous embryos produced by *fancd1*(*brca2*)*;tp53* double heterozygous females develop normally. These findings show that maternally provided Fancd1(Brca2) activity is required for normal embryonic development, presumably to repair chromosome damage that likely occurs during rapid cell divisions, as has been shown in *fancd1*(*brca2*) tissue culture cells (Rodríguez-Marí *et al.*, 2011).

As in *fancd1* and *fancd1;tp53* mutant males, *fancd1*;*tp53* mutant females show abnormal somatic cell proliferation in their gonads (Fig. 4C) (Rodríguez-Marí *et al.*, 2011; Shive *et al.*, 2010). Although *tp53* mutants also develop tumors, these develop later than in *fancd1*;*tp53* females, are less frequent, and affect a different spectrum of tissues (Berghmans *et al.*, 2005; Parant *et al.*, 2010; Rodríguez-Marí *et al.*, 2011). The early appearance and unique ovarian location of tumors in *fancd1;tp53* double mutants suggests a specific association of ovarian tumors with Fancd1(Brca2) activity, potentiated by impaired *tp53* function. Similarly, loss of *TP53* function potentiates ovarian tumors in women heterozygous for *BRCA2* mutations (Easton, 1997). The discovery that Brca2 is required for oocyte nuclear architecture may lead to new perspectives in understanding the mechanisms of BRCA2 in meiotic progression and in ovarian cancers. As in *fancd1* males, aberrant germ cell development reflected in the abnormal oocyte nuclear architecture of *fancd1;tp53* female mutants may alter oocyte–soma signaling and enable the abnormal proliferation of surrounding somatic gonadal cells that leads to ovarian tumors (Figs. 4F and G).

3. Zebrafish FA Mutants as Useful Tools for Drug Screening

Therapeutics for FA patients are desperately needed, and the zebrafish might contribute to the identification of therapeutic compounds that could ameliorate some of the symptoms of FA patients. Zebrafish are easy to maintain and breed year-round, have a short generation time (3 months), high fertility rate (hundreds of eggs are laid per female each week), and the embryos are optically transparent and develop synchronously and rapidly (most organs develop within the first 48 h postfertilization (hpf). These characteristics make zebrafish a suitable model to use in drug screening (e.g., Peterson *et al.*, 2004). Because studies in zebrafish have revealed

that the basic cellular mechanisms of the FA/BRCA pathway is conserved between the human and zebrafish program, zebrafish mutations in genes belonging to the FA/BRCA network become useful tools for the screening of small molecule libraries to identify potentially therapeutic compounds that can ameliorate the symptoms of FA patients.

III. Summary

The FA system in zebrafish is remarkably similar to that in mouse in that hematopoietic phenotypes are subtle to detect, suggesting that some of the mechanisms of human hematopoiesis may be absent in these animal models. On the other hand, the FA/BRCA networks in zebrafish, mouse, and human all share a caretaker role for genome stability and cancer. The investigation of FA/BRCA zebrafish mutants shows that oocyte survival is essential in zebrafish sex determination and suggests the existence of a diplotene oocyte-derived factor that maintains aromatase expression in the somatic cells of bipotential gonads as a key event in zebrafish sex determination. This conclusion provides a compelling argument for future investigations that focus on the molecular nature of the hypothesized signal and on identification of genetic and environmental factors that affect its strength. Furthermore, investigations of zebrafish mutants have revealed the novel finding that *fancd1* (*brca2*) is required to establish or maintain oocyte nuclear architecture and to insure the survival of offspring. Zebrafish are especially suitable for initiating a whole-animal screen of small molecules for substances with specific phenotypic effects. The features of the zebrafish FA system discussed here suggest potential screens for therapeutic substances for FA disease.

Acknowledgments

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