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Essential but differential role of FOXL2^{wt} and FOXL2^{C134W} in GDF-9 stimulation of follistatin transcription in co-operation with Smad3 in the human granulosa cell line COV434

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ABSTRACT

The *FOXL2^{C134W}* mutation has been identified in virtually all adult granulosa cell tumors (GCTs). Here we show that the exogenous FOXL2 expression is necessary for GDF-9 stimulation of follistatin transcription in the human GCT cell line, COV434 that lacks endogenous FOXL2 expression. Interestingly, in the presence of Smad3 co-expression, FOXL2^{C134W} negated GDF-9 stimulation of follistatin transcription. However, mutation of the Smad binding element (SBE) located in the intronic enhancer elements in the follistatin gene restored normal FOXL2 activity to FOXL2^{C134W}, thus the altered activity of FOXL2^{C134W} is dependent on the ability of Smad3 to directly bind the SBE. Mutation of the FOXL2 binding element (FBE) or the FBE and SBE completely prevented GDF-9 activity, suggesting that the FBE is essential for GDF-9 stimulation in COV434. Overall, our study supports the view that altered interaction of FOXL2^{C134W} with co-factors may underlie the pathogenesis of this mutation in GCTs.

1. Introduction

Granulosa cell tumors (GCTs) represent around 5–10% of ovarian malignancies, with the majority (95%) of GCTs detected in adults (Kalfa et al., 2009). Although most GCTs are diagnosed at an early stage that can be treated with surgery, there is a high rate of recurrence, particularly in patients diagnosed at a later stage (Colombo et al., 2007). Approximately 80% of patients with advanced stage or recurrent tumors succumb to their disease, mainly due to the characteristic slow, indolent pattern of GCT progression (Amsterdam and Selvaraj, 1997). Since traditional chemotherapy is relatively ineffective in women with advanced-stage or recurrent GCTs a more effective therapeutic option for these patients is necessary. Unfortunately, the poorly understood etiology of GCTs has hampered the development of novel therapeutics. However, a recent breakthrough has surprisingly revealed that virtually all GCTs in adult women have a unique somatic *FOXL2* mutation (*FOXL2^{C134W}*) (Shah et al., 2009; Schrader et al., 2009; Jamieson et al., 2010; Kim et al., 2010a,b; Al-Agha et al., 2011). FOXL2, a member of the forkhead transcription factor family, is localized to GCs within the ovary and is essential for normal ovary development and function (Pisarska et al., 2011).

FOXL2 ablation in whole body knockout mouse models causes high perinatal mortality, which most likely reflects craniofacial defects (Schmidt et al., 2004; Uda et al., 2004). However, surviving mice confirm a critical role of FOXL2 in ovarian function, with a block in follicle development at the primary stage associated with a failure of GCs to complete the squamous to cuboidal transition (Schmidt et al., 2004; Uda et al., 2004). In contrast, GC-specific ablation of FOXL2 post-puberty in mice results in somatic sex reprogramming by induction of the transcription factor SOX9, with GCs becoming Sertoli cell-like and follicles changing into structures resembling seminiferous tubules (Uhlenhaut et al., 2009).

There are findings suggesting that *FOXL2^{C134W}* may promote GCT development at least in part by promoting cell cycle progression and helping cells evade apoptosis. In studies of human GCTs, a large proportion (58%) showed down-regulation of two members of the inhibitors of cyclin-dependent kinase 4 family (INK4A and INK4B), whose expression is altered in many different types of cancer (Arcellana-Panlilio et al., 2002). Consistent with this, in the GCT cell line COV434 that has undetectable *FOXL2* gene expression (Jamieson et al., 2010), overexpression of FOXL2^{wt} but not FOXL2^{C134W} induced transcriptional activity on *INK4A* (Benayoun et al., 2011). Evidence for a difference in apoptotic activity between FOXL2^{wt} and FOXL2^{C134W} comes from another *in vitro* study using

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KGN cells, heterozygous for *FOXL2*^{C134W}, which showed that FOXL2^{wt} elicited significant apoptosis, while the activity of FOXL2^{C134W} was impaired due to a failure to up-regulate the death receptors, TNF-R1 and Fas (Kim et al., 2010). These findings provide us with valuable information, although rather limited, to help understand the mechanisms underlying how FOXL2^{C134W} triggers GCT formation. Therefore, there is a need for further research on alternative cellular mechanisms that may be altered by FOXL2^{C134W}.

Recent studies have shown that FOXL2 and Smad3 cooperatively regulate activin stimulation of follistatin, FSH ß and GnRH receptor transcription in mouse pituitary cells (Ellsworth et al., 2003, 2006; Lamba et al., 2009; Blount et al., 2009; Corpuz et al., 2010). As FOXL2 and Smad3 are similarly expressed in GCs (Pangas and Matzuk, 2004; Shimasaki et al., 2004; Knight and Glister, 2006), this led us to ask whether a similar cooperation between FOXL2 and Smad3 occurs in GCs. The mutated Cys^{134} residue is within the C-terminal region of the forkhead domain, required for both DNA-binding (Benayoun et al., 2010) and FOXL2 interaction with Smad3 (Blount et al., 2009). Disruption of DNA- and/or Smad-binding capacity by this mutation would likely result in altered transcriptional activity and could ultimately result in tumor formation. As activation of Smad2/3 has been implicated in the pathogenesis of juvenile GCTs in humans (Middlebrook et al., 2009), it is therefore plausible that known GC mitogens such as GDF-9, which signal through Smad2/3, may play a role in the pathogenesis of GCTs.

Follistatin is highly expressed in GCs of developing follicles (Shimasaki et al., 1988, 1989; Nakatani et al., 1991; Roberts et al., 1993) and binds activin (Nakamura et al., 1990, 1992; DePaolo et al., 1991; Xiao and Findlay, 1991; Xiao et al., 1992; Cataldo et al., 1994; Erämaa et al., 1995) and some BMPs including BMP-7, and BMP-15 (Yamashita et al., 1995; Iemura et al., 1998; Otsuka et al., 2001). In so doing, follistatin inhibits the actions of each of these GC mitogens (Miró and Hillier, 1996; Otsuka et al., 2000; Lee et al., 2003). Thus, in doing so follistatin acts as an anti-proliferative factor. Our current data suggest that FOXL2 may act as a cell growth inhibitor by stimulating production of follistatin, an anti-proliferative factor. This hypothesis is additionally supported by the *in vivo* observation that follistatin expression was severely compromised in *Foxl2* null mouse ovaries (Schmidt et al., 2004). Dysregulation of follistatin by the FOXL2 GCT mutation could result in increased GC proliferation.

COV434 cells are a well-established immortalized human GC line derived from a 27-year-old patient with a metastatic GCT (van den Berg-Bakker et al., 1993). They possess certain morphologic and physiologic characteristics in common with normal GCs; in the presence of FSH and androstenedione COV434 cells secret estradiol and cAMP levels are increased indicating that the FSH receptor and P450 aromatase are present in these cells. In contrast to KGN cells used by other laboratories in recent studies (Schrader et al., 2009; Benayoun et al., 2010) that heterozygously express the *FOXL2*^{C134W} mutation, COV434 cells lack the FOXL2 mutation and FOXL2 mRNA and protein are undetectable(Jamieson et al., 2010). Thus, COV434 cells provide a platform for direct comparison of FOXL2^{C134W} and FOXL2^{wt}.

The ultimate purpose of our research is to illuminate the molecular and cellular mechanisms underlying altered GC function triggered by acquisition of the somatic *FOXL2*^{C134W} mutation and gain a better understanding of what drives GCT formation. Towards this goal, the aim of the current study was to determine (i) whether FOXL2 is necessary for follistatin transcription regulated by GDF-9 in COV434 cells, (ii) whether FOXL2^{C134W} has altered activities as compared with FOXL2^{wt} and (iii) whether FOXL2 and Smad3 coordinately regulate follistatin transcription in the ovary.

2. Materials and methods

2.1. Plasmids and reagents

Recombinant mouse GDF-9 was purchased from R & D systems (Minneapolis, MN). The mouse monoclonal anti-Flag M2 antibody and anti-Flag M2 antibody conjugated agarose beads were purchased from Sigma–Aldrich Co. (St. Louis, MO), the mouse monoclonal anti-Myc antibody was from BD Biosciences (San Jose, CA), and the α-tubulin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

The rat follistatin luciferase rFS(0.3ex45)-luc reporter plasmid, N-terminal Flag-tagged human FOXL2^{wt} and N-terminal Myc-tagged human Smad3 were kindly provided by Dr. Louise Bilezikjian of the Salk Institute (Blount et al., 2009). The rFS(0.3ex45)-luc plasmid contains the +1784/+1912 section of intron 1 downstream of a −312/+136 minimal promoter. The +1784/+1912 section of intron 1 contains a forkhead-binding element (FBE) just downstream of a Smad-binding element (SBE). It is of note that the DNA sequences at and surrounding the SEB and FBE of the rat follistatin gene are identical to those of the human gene. Mutant versions of the rFS(0.3ex45)-luc reporter were generated with mutations in the SBE (mutant 1), FBE (mutant 2), or SBE and FBE (mutant 3) by site directed mutagenesis in a two-step PCR. DNA fragments containing the mutations were generated using the common primers 5′-AATCGCGCGGGCGGCCGGTGGCG-3′ and 5′-GGAATGCCAA GCTTAGTCCTAGG-3′ and the following specific primers to introduce the mutations: 5′-CAAGCTGCACGTGTTGTAATTGGGTCACTG GTAACTGACATTGATATGGCTAGGCGCAGCGGCTGCTGCTC-3′ and 5′-GAGCAGCAGCCGCTGCGCCTAGCCATATCAATGTCAGTTACCAGTG ACCCA ATTACAACACGTGCAGCTTG-3′ for mutant 1; 5′-CA AGCT GCACGTGTTGTGTCTGGGTCACTGGTAACTGTCGAACTCTTGGCTAGG CGCAGCGGCTGCTGCTC-3′ and 5′-GAGCAGCAGCCGCTGCGCCTA GCCAAGAGTTCGACAGTTACCAGTGACCCAGACACAACACGTGCAGC TTG-3′ for mutant 2; and 5′-CAAGCTGCACGTGTTGTAATTGGGT CACTGGTAACTGTCGAACTCTTGGCT AGGCGCAGCGGCTGCTGCTC-3′ and 5′-GAGCAGCAGCCGCTGCGCCTAGCCAAGAGTTCGACAGTTAC-CAGTGACCCAATTACAACACGTGCAGCTTG-3′ for mutant 3. The resulting DNA fragments were cloned into to the rFS(0.3ex45)-luc plasmid using EagI and HindIII restriction sites. From the Flag-FOXL2^{wt} expression plasmid we generated the Flag-FOXL2^{C134W} mutant expression plasmid by site directed mutagenesis as explained above. Briefly, primers A (5′-TACGTGGCGCTC ATCGCCATGGCGATC-3′) and D (5′-AGCGCCATGCTCTGCACGCG TGTGTAC-3′) contained Sty I and Mlu I restriction sites respectively, while primers B (5′-CTCGAACATGTCTTCCCAGGCCGGGTC-CAG-3′) and C (5′-CTGGACCCGGCCTGGGAAGACATGTTCGAG-3′) included the 402 C to G mutation that corresponds to the C134W amino acid change. The resulting mutant FOXL2 was ligated to the pFlag-FOXL2/pCS2+ construct using standard procedures. A β-galactosidase reporter plasmid driven by the Herpes virus thymidine kinase promoter was used as internal control for transfection efficiency in all luciferase reporter experiments.

2.2. Harvest and culture of primary rat granulosa cells

Female Sprague Dawley rats (24 days old) were implanted with silastic implants (Dow Corning, Corp., Midland, MI) containing 10 mg diethylstilbestrol (DES) to stimulate GC proliferation. 4 days after DES implantation GCs were harvested from the ovaries as previously described (Otsuka and Shimasaki, 2002). Briefly, ovarian follicles were punctured with needles to release GCs and oocytes, and oocytes were subsequently removed using a 40 μm cell strainer. GCs were cultured in serum-free McCoys 5A culture media containing antibiotics. The Institutional Animal Care and Use Com-