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Novel action of FOXL2 as mediator of *Col1a2* gene autoregulation

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ABSTRACT

FOXL2 belongs to the evolutionarily conserved forkhead box (FOX) superfamily and is a master transcription factor in a spectrum of developmental pathways, including ovarian and eyelid development and bone, cartilage and uterine maturation.

To analyse its action, we searched for proteins that interact with FOXL2. We found that FOXL2 interacts with specific C-terminal propeptides of several fibrillary collagens. Because these propeptides can participate in feedback regulation of collagen biosynthesis, we inferred that FOXL2 could thereby affect the transcription of the cognate collagen genes. Focusing on COL1A2, we found that FOXL2 indeed affects collagen synthesis, by binding to a DNA response element located about 65Kb upstream of this gene. According to our hypothesis we found that in *Foxl2*$^{-/-}$ mouse ovaries, *Col1a2* was elevated from birth to adulthood.

The extracellular matrix (ECM) compartmentalizes the ovary during folliculogenesis, (with type I, type III and type IV collagens as primary components), and ECM composition changes during the reproductive lifespan. In *Foxl2*$^{-/-}$ mouse ovaries, in addition to up-regulation of *Col1a2*, *Col3a1*, *Col4a1* and *fibronectin* were also upregulated, while *laminin* expression was reduced. Thus, by regulating levels of extracellular matrix components, FOXL2 may contribute to both ovarian histogenesis and the fibrosis attendant on depletion of the follicle reserve during reproductive aging and menopause.

1. Introduction

Since its discovery more than 25 years ago (Weigel et al., 1989) the conserved forkhead DNA binding domain (DBD) has been identified in 50 human genes. They are now classified as FOX (Forkhead bOX) and subdivided alphanumerically into subfamilies based on evolutionary conservation (Kaestner et al., 2000; Jackson et al., 2010). Their fundamental importance in regulating numerous pathways involved in development, signalling, metabolism and human disease, has been increasingly recognized (Benayoun et al., 2011); but the molecular mechanisms under control of the forkhead factors are only partially understood.

FOXL2 plays a major role in early ovarian development and its expression in granulosa cells affects their differentiation, follicle formation and sex determination. It was first identified as mutated in the autosomal dominant Blepharophimosis /Ptosis/Epicanthus inversus Syndrome (BPES, MIM #605597; Crisponi et al., 2001). Heterozygous mutations in FOXL2 lead to Primary Ovarian Insufficiency (POI), an index of insufficient ovarian follicle reserve in females, coupled to a complex eyelid malformation in both males and females (Crisponi et al., 2001). Knock-out mouse models have shown that homozygous ablation of *Foxl2* expression results in a complete block of ovarian follicle formation and derepression of male-specific genes during development, postnatally and even during adulthood (Uda et al., 2004; Ottolenghi et al., 2007; Garcia-Ortiz et al., 2009; Uhlenhaut et al., 2009). In addition to several mutations in FOXL2 leading to ovarian insufficiency by granulosa cell and follicle depletion, a recurrent heterozygous somatic mutation (c.C402G; p.C134W) has been found in 97% of adult onset granulosa cell tumors (GCTs), associated with unregulated granulosa cell proliferation in 5–10% of all ovarian cancers (Shah et al., 2009; Jamieson et al., 2010; Gershon et al., 2011).

FOXL2 is also implicated as a master transcription factor in a

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range of developmental pathways that include growth and bone, cartilage and uterine maturation (Shi et al., 2014; Heude et al., 2015; Marongiu et al., 2015; Bellessort et al., 2015). In the pituitary gland, where it has been further analysed, it is expressed mainly in thyreotrope and gonadotrope cells (Ellsworth et al., 2006), and it has been reported that FOXL2 along with SMAD proteins in activin A induction, regulates *Fshb* expression in gonadotrope cells (Lamba et al., 2009; Tran et al., 2011). However, most BPES patients do not have a recognizable pituitary phenotype or lesions in bone, etc., suggesting that other systems are less sensitive to FOXL2 dosage than are the developing eyelids and ovary.

FOXL2 clearly has a considerable degree of transcriptional regulation activity, with cell-type specific actions as both an activator and repressor of promoter action in target genes. This differential activity is likely dependent on specific FOXL2 binding partners that constitute the transcriptional complex leading to gene regulation (Fleming et al., 2010; Pisarska et al., 2010, 2011; Georges et al., 2013) as well as post-translational modifications (PTMs) and variant alternative FOXL2 binding sites (see below). A variety of protein binding partners of FOXL2 have been reported, including DEAD box-containing protein (DP103; Lee et al., 2005), SMAD3 (Blount et al., 2009), ER alpha, (Kim et al., 2009), Steroidogenic factor-1 (SF-1; Park et al., 2010), UBC9, PIAS1 (Marongiu et al., 2010), LATS1 (Pisarska et al., 2010), FOXL2 itself (Kuo et al., 2011), CXXC4, CXXC5, CREM, GMEB1, NR2C1, SP100, RPLP1, BANF1, XRCC6, SIRT1 L'Hôte et al., 2012), GSK3β, MDM2 (Kim et al., 2014) and NOBOX (Bouilly et al., 2014).

As PTMs, sumoylation, phosphorylation and acetylation, have all been shown to modulate FOXL2 activity (Kuo et al., 2009; Marongiu et al., 2010; Pisarska et al., 2010; Georges et al., 2011; Kim et al., 2014). Recent evidence indicates that such modifications can even drive FOX factors to bind to particular target genes in response to environmental signals (Benayoun et al., 2011).

The overall census of FOXL2-regulated loci has also grown progressively. In the specific instance of the ovary, a number of candidates have been identified in gonadal cells (and also have been detected in many cases in pituitary), including genes involved in steroidogenesis (e.g. *STAR*, *CYP17* and aromatase; Pisarska et al., 2004; Park et al., 2010; Pannetier et al., 2006), inflammation (e.g. *NFAT* and *PTGS2/COX2;* Batista et al., 2007*;* Kim et al., 2009*)* and apoptosis or detoxification (e.g., *MNSOD*; Benayoun et al., 2009).

To further analyse the spectrum of FOXL2 action, we have used two-hybrid screening to look directly for binding partners of the protein. We find a novel interaction with C-terminal portions of collagen proteins, in particular COL1A2. Because these propeptides can participate in feedback regulation of collagen biosynthesis, we inferred that by virtue of this binding FOXL2 could thereby affect the transcription of the cognate collagen genes. Focusing on COL1A2, we found that FOXL2 indeed affects collagen synthesis, by binding to a DNA response element located about 65Kb upstream of this gene and that in *Foxl2*$^{-/-}$ mouse ovaries, *Col1a2* was elevated from birth to adulthood.

2. Materials and methods

2.1. Yeast two hybrid assay

A yeast two-hybrid screen was performed using the Matchmaker™ Two-Hybrid System 3 system (Clontech). *Foxl2* full-length cDNA was cloned into pGBKT7 vector and transformed into AH109. Mouse embryo 17.5 dpc MATCHMAKER cDNA Library (containing potential interaction partners as preys) cloned in the pGADT7 vector was transformed into AH109 cells already containing *Foxl2* as a bait, and co-transformants were screened on selection plates according to the Clontech protocol. Both bait plasmid pGBKT7, containing the *Foxl2* full-length cDNA fused in-frame to the DNA binding domain of GAL4, and prey plasmids PGADT7, containing a 17.5 dpc mouse embryo cDNA library fused to the GAL4 activator domain, were co-transformed into AH109 yeast cells. A total of 1.6×10^5 independent clones were screened for the expression of four reporter genes (*ADE2, HIS3, MEL* and *LacZ*). The strength of the interactions was also assessed by liquid X-α-Gal assay, which also allowed false positives reduction.

2.2. Constructs, cell culture, DNA transfections, cell lysis, immunoblotting and immunoprecipitation

Mouse *Col1a2* C-propeptide cDNAs, obtained from pGADT7 vector isolated by yeast two hybrid screening, was cloned into the pCRUZ-HA vector (Santa Cruz biotechnology, inc). Likewise, the *Foxl2* full-length cDNA, isolated from genomic DNA mouse has been cloned in the pCRUZ-Myc vector (Santa Cruz biotechnology, inc). These constructs have been transfected using Lipofectamine 2000 (Invitrogen) into Alpha T3-1 cell lines grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. After 48 h, cells were lysed with modified RIPA buffer (140 mM NaCl, 10 mM TrisHCl pH 7.5, 1 mM EDTA, 1% Triton x-100, 0.1% Sodium deoxycholate) containing Complete Protease Inhibitor Cocktail (Roche) and 40 mM of N-ethylmaleimide (NEM), according to Hattori et al., 2006. After brief sonication and 30 min on ice, the lysates were centrifuged at 4 °C, 12,000 rpm, for 20 min. Protein concentrations were measured by Bio-Rad Protein Assay. For immunoprecipitation, the lysates were diluted 5-fold in co-immunoprecipitation buffer (140 mM NaCl, 10 mM TrisHCl pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate) and immunoprecipitated using the Profound™ HA-Tag IP/Co-IP kit as per manufacturer's specifications (Thermo Scientific). Samples were fractionated on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, cat. No. NP0321 Box), then transferred to PVDF membranes (HYBOND-P, Amersham Biosciences), hybridized with the primary antibody overnight, then with a horseradish peroxidase-conjugated secondary antibody that was detected by enhanced chemiluminescence (ECL, Amersham Biosciences). The primary antibodies used were mouse anti-Myc (Santa Cruz sc40, 1:200 dilution), rabbit anti-HA (Santa Cruz sc805, 1:200 dilution). Secondary antibodies were purchased by Santa Cruz biotechnology inc, goat anti-mouse IgG (Santa Cruz sc2005, 1:100,000 dilution), goat anti-rabbit IgG (Santa Cruz sc2004, 1:100,000 dilution).

2.3. Ethics statement

Mice were manipulated and housed according to the European Community Council Directive (EEC/609/86) and to the Italian guidelines DL 116/1992. The experimental protocol and the detailed application form that focuses on how the animals have been used, have been approved by the Italian National Institute of Health, in particular by the Service for Biotechnology and Animal Welfare, and by the University of Cagliari.

Foxl2$^{-/-}$ mice were created by deleting the entire *Foxl2* coding region as previously described (Uda et al., 2004). C57B6 mice were used for this study. Mice were housed conventionally at constant temperature (20–24 °C) and humidity (50–60%) in an animal facility with a 12 h light–dark cycle, with free access to food and water. Mice were sacrificed by CO2 asphyxiation. PCR genotyping reactions with specific primers according to Uda et al. (2004) were used to detect the WT and mutant alleles.

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