CBX2 and the Ovary: Novel Insights into Regulatory Networks in Humans

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Abstract

Background: *Chromobox protein homolog 2 (CBX2) is a DNA-interacting protein present in humans in two isoforms, CBX2.1 and CBX2.2. Isoform-1 promotes testis development, butthe role of both variants in the female gonad pathway remains unknown.*

Methods: *To understand its function in human ovaries, we evaluated expression of known female genes i.e.FOXL2, RSPO1 and WNT4 in human pre-granulosa cells after forced expression and RNA interference of CBX2 isoforms and vice versa.*

Results: *The two isoforms appeared to be functionally distinct. CBX2.1 has proved to be a pro-male by enhancing SOX9, SF1 and androgen receptor (AR) expression, whereas CBX2.2 might be partly pro-female most likely via interaction with RSPO1 and the regulation of ovary developmental markers (i.e. OCT-4, AMH and ERβ).*

Conclusions: *Both CBX2 isoforms might act as distinct regulatory agents protecting granulosa cells from uncontrolled growth and proliferation by adjusting WNT4 and RSPO1 signaling pathways, and participate to follicular development and fate by regulating the expression of SF1 and AR, thus potentially influencing fertility, menopause, ovarian cancer and perhaps polycystic ovary syndrome.*

Keywords: ovarian determination, CBX2, female markers, KGN

Introduction

As elegantly described by Sekido R, Lovell-Badge, "sex determination is a story of opposing forces and crucial alliances, where it is a matter of timing and expression level of sex determining factors that determines which pathway wins", resulting in either testes or ovaries [1]. Mutations in any of those specific gonadal genes can disrupt this balance to cause aberrant gonad determination associated with disorder/differences of sex development [2].

The identification of the mammalian testis-determining gene Sry (Sex determining region Y) provided an entry point to molecular studies of testis development, and major male-specific genes e.g. SOX9 has been revealed [3,4]. Since that time, much has been learned regarding the genetic networks responsible for orchestrating male gonads development, far more so than ovary development which remained partially unclear. Recently, pro-female markers were identified, andseem to work synergistically and in parallel to ensure properovary development. One of these female-specific genes is the winged helix/forkhead transcription factors (FOXL2) that has been described to be a master regulator in ovarian determination and differentiation,and

plays a continuous role in maintaining the ovary, presumably by suppressing the pro-testis action of SOX9 [5]. Mutations in FOXL2 in humans cause blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) associated to premature ovarian failure (POF) [6]. The signaling molecules RSPO1 (R-Spondin1) and WNT4 (wingless-type MMTV integration site family, member 4) have been reported to promote ovarian development and repress testis pathway in animals and humans. The actions of WNT4 and RSPO1 in the ovary appear to converge at the point of activation of β-catenin signaling to regulate fetal ovary to prevent the appearance of male-specific characteristics during the ovarian development [7,8]. Emerging data remarkably reported a 46, XY female patients with duplication of chromosome 1p including both WNT4 and RSPO1 resulting in testis development suppression while allowing ovarian pathway to dominate [7,8].

We recently identified mutations in Chromobox homolog 2 (CBX2), a polycomb (Pc) group protein important incell fate determination during developmental process, as the cause of DSD in 46, XY patients [9]. CBX2 is the human homolog of a mouse gene (originally called M33), which acts as a regulator of gene expression at the chromatin level. CBX2 ispresent in two alternatively spliced isoforms in humans: CBX2.1 and CBX2.2, the latter lacking the

Polycomb box. CBX2.1, although a member of the Pc family of transcriptional silencers, has been shown to increase expression of SF-1 (Steroidogenic Factor 1), known to play a number of roles in sex development in human and mice, including the establishment of the genital ridges and steroidogenesis in male and female pathways Cbx2 XY knockout mice showed defect in gonadal, adrenal, and splenic development as well as male-to-female sex reversal, and aberrant T-cell expansion [9-14]. Cbx2 XX knockout animals showed gonadal defects resulting in sterile female with either smaller or absent ovaries, suggesting that Cbx2 is also active in ovarian development [14,15]. These observations prompted us to study the relationship, at the molecular level between CBX2 isoforms and ovarian sex-determining genes (FOXL2, RSPO1, and WNT4). We investigated human pre-granulosa cell line, an in vitro model of human ovarian cells for its expression of the both CBX2 isoforms and their putative functions in the ovary network [16].

Materials and Methods
Cell culture
Human pre-granulosa cells (KGN) were provided by RIKEN BRC through the National Bio-Resource Project, Japan, and maintained in Dulbecco's essential medium/Ham's F12 medium supplemented with 10% fetal calf serum, 5% Penicillin/Streptomycin at 37°C in a 5% CO_2. Upon reaching 100% confluence, cells were routinely passaged. All molecular experiments were carried out with three dishes for each experiment.

Quantitative RT-PCR
Total RNA was isolated using Qiagen RNeasy extraction kit and treated with DNase I (Qiagen, Hilden, Germany). We included a minus Reverse Transcriptase (-RT) control to evaluate genomic DNA contamination. cDNA was synthesized from 2 µg total RNA using Omniscript RT Kit (Qiagen, Germany). We used human fetal ovary cDNA (BioChain, San Francisco USA) as acontrol to estimate CBX2.1 and CBX2.2 mRNA expression level comparing to cDNA of cultured KGN cells. Gene expression was evaluated by RT-PCR amplification using KAPA SYBR FAST qPCR Kit (KAPA BIOSYSTEMS). Gene-specific primers sequences are listed in supporting information (S1 table). Gene expression profiling analysis has been assessed after normalizing to an internal reference gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (S2 table). Statistical analyses were conducted using GraphPad Prism version 6 (California, USA). For each gene, data sets were analyzed for statistical significant differences between gene expressions levels using unpaired t-test with a confidence intervals set at 95%. For all our calculations, we used comparative Ct method ($2^{-\Delta\Delta Ct}$) to assess the relative gene expression level (Rel. Exp).

S1 Table

Primers of CBX2 isoforms and Female targets

Tragets	5'/3'sequences	Product size (bp)	Accession Number	Tm (Co)
CBX2.1	5'GGAACATGAGAAGGAGGTGCAG 3'GTCCAGCAGTTCCTCCTCTTC	153	Nm_005189.2	58
CBX2.2	5'GCGGGTGCACCTTAAATC 3'GATGTGCTTTGGCCTTCT	123	Nm_032647.3	55
FOXL2	5'ATGATGGCCAGCTACCCC 3'GCATCCGCCACAACCTCA	319	Nm_023064.3	56
RSPO1	5'CACCTGGATACTTCGACGCC 3'ACCATGGAGTGCAGTAGTCCT	197	Nm_001242910.1	56
WNT4	5'GCCATTGAGGAGTGCCAGTAC 3'GTGTACGCCATCTCTTCGG	133	Nm_030761.4	58
FST	5'AGCAGCCAGAACTGGAAGTCCA 3'CCTACTGTGTGACCTGTAATCGG	128	NM_006350.3	58
CYP19A	5'GACGCAGGATTTCCACAGAAGAG 3'TGATCGCAGCTCCTGACACCAT	145	NM_000103.3	55

Primers of Male targets

Tragets	5'/3'sequences	Product size (bp)	Accession Number	Tm (C°)
SF-1	5'CCGCAGCATTACCAACACCACA 3' 3'CAGCTCAGGATAAAGCACGGATG 5'	118	NM_004959.4	58
SOX9	5'AAGACATTTAAGCTAAAGGCAACTCGTAC 3'GAGGATGATGGAGAGGTCGTGTGATCA	197	NM_000346.3	58
DAX1	5'CCAAATGCTGGAGTCTGAACATC 3'TACATTCAGGGACTCCAGTGGG	120	NM_000475.4	58
WN1	5'GGTGTCTTCAGAGGCATTCAGGA 3'ATGTGTGCTTACCCAGGCTGC	117	NM_0001198552.1	58
SRY	5'CCCAGAATGCGAAACTCAGA 3'ATTACAGGCCATGCACAGAG	118	NM_003140.2	58
AR	5'ATGGTGAGCAGAGTGCCCTATC 3'ATGGTCCCTGGCAGTCTCCAAA			

Prime of Ovarian stages markers

Tragets	5'/3'sequences	Product size (bp)	Accession Number	Tm (Cº)
OCT-4	5'CCTGAAGCAGAAGAGGATCACC 3'CCAAACGACCATCTGCCGCTTT	106	NM_001285987.1	58
AMH	5'CGCTGCTTCACACGGATGACC 3'GGAACTCGAGGAGTCGCCACC	137	NM_000479.3	58
ER-BETA	5'ATGGAGTCTGGTCGTGTGAAGG 3'CCTGCCGACTTCGGAAGTGTTA	148	NM_001040275.1	58

S2 Table

PRIMERS	5'/3'Sequences	Product size (bp)	Tm (Cº)
GAPDH	5': GGA CCT GAC CTG CCG TCT AG 3': CTACAGCAACAGGGTGGTGG	106	58
VP1.5	5'-GGA CTT TCC AAA ATG TCG-3'	137	56
XL39	5'-ATT AGG ACA AGG CTG GTG GG	148	56

Transient Transfections

Pre-granulosa cells passage were seeded one day prior forcing expression and knocking down assays [15]. Cells were transfected in 10 cm dishes with 4 µg expression plasmids encoding for CBX2.1, C-Myc Tagged-CBX2.2, FOXL2, RSPO1, WNT4 and pCMV6-empty plasmid used as a negative control. Ratio of plasmids-Metafectene (Biotex, Germany) and oligos siRNA-Lipofectamine RNAiMax (Microsynth AG, Switzerland) was 1:4 for both reactions. CBX2 siRNA duplex final concentration was 80 nM, mixed with Opti-MEM serum free medium (Life Technologies, California, USA). Transfected cells were incubated for 48 hours. Data concerning all expression plasmids and Empty vector are presented in supporting information (S1 Text).

S1 Text: Expression plasmids used for KGN transfection

- untagged-Human chromobox homolog 2 (CBX2), transcript variant 1, (SC303599, OriGene)
- Myc-tagged-Human chromobox homolog 2 (CBX2), transcript variant2, (RC216313 OriGene)
- GFP-tagged - Mouse forkhead box L2 (Foxl2). (MG225684, OriGene)
- Untagged - Homo sapiens R-spondin 1 (RSPO1) (SC330056, OriGene)
- Untagged-Human wingless-type MMTVintegration site family, member 4 (WNT4),
- (SC109791, OriGene)
- Empty vector: pCMV6-AC-GFP, mammalian vector with C-terminal tGFP tag (PS100010)

si-RNA duplex altering endogenous CBX2.1 and CBX2.2 in pre-granulosa cells are:
CBX2.1–145: 5' –GGCUGGUCCUCCAAACAUATT–3'
CBX2.1–411: 5'–GGAUGACAGUGAGUUAGAUTT–3'
CBX2.2–770: 5'–AAA TCG AGG TGG CCA CGA AAG–3'
CBX2.2–796: 5'–AGG CAG GGC UGA CUG AAU A–3'.

Immunoblotting analysis

Transfected cells were harvested 48h post-transfection and lysed cells were supplemented with protease inhibitor cocktail (Roche, Switzerland) to be subjected to SDS-gel electrophoresis and transferred to polyvinylidene (PVDF) membranes (Hybond ECL). Exogenous and endogenous proteins were detected using polyclonal antibodies (1:1000). Used antibodies were: polyclonal rabbit anti-CBX2 (ab80044, Abcam), mouse anti-C-Myc (Zymedlnc, San Fransisco USA),mouse anti-alpha-tubulin (GTX11323, GeneTex) and polyclonal anti-female markers (FOXL2, WNT4 and RSPO1) are from Abcam (Siwitzerland) (respectively: antibody ab68339, antibody ab91226 and antibody ab106556). Secondary HRP-conjugated anti-rabbit/anti-mice antibodies were from GE-Healthcare (Germany). For the immunoblotting analysis we loaded 10 µl of a total lysate cells (yield of 5.106cells) boiled in Laemmeli sample buffer.

Immuno-fluorescence analysis

KGN cells were grown on glass coverslips and fixed with 4% formaldehyde in phosphate saline buffer (PBS) for 10 min. Samples were permeabilized with 0.2% TritonX, for 5min and were incubated overnight at 4°C with primary antibodies (polyclonal rabbit anti-CBX2 (ab80044, Abcam), mouse anti-C-Myc (Zymedlnc, San Fransisco, USA). After 3 washes in PBS, cells were incubated with the Alexa Fluor-conjugated secondary antibodies (Goat Anti-Rabbit IgG H&L and Goat anti-Mouse IgG (H+L) secondary antibody Alexa Fluor® 488 conjugate) for 1 hour at room temperature. The cover slips were washed in PBS and were mounted with Vectashield containing DAPI (4', 6-diamidino-2-phenylindole) (Vector Laboratories). Cells were observed with Nikon Eclipse Ni-E Microscope System.

Results

To gain deeper insights into human ovary pathway, we firstly investigated human pre-granulosa cells for their expression of genes specifically important for ovarian development, i.e.FOXL2, RSPO1 and WNT4 and genes implicated in sex development in general, such as SF-1 and DAX 1 by means of RT-qPCR. In a first step, we confirmed femaleness of our cell system by validating the expression of the female specificgenes. Further, we checked expression of genes involved in male sex pathway SRY, SOX9 and WT1 (expected to be suppressed). As expected, pro-male markers showed a low expression level in pre-granulosa cells, SRY and WT1 being completely absent in these cells. The identity of PCR products was confirmed by agarose gel-electrophoresis for correct size and by sequencing (data not shown). KGN characterization was performed in every passage from passage P (10) to passage P (29). Our results showed that KGN cells seem to change expression pattern in a passage-dependent way (S1 Fig). Based on the expression pattern we opted to use the optimal passage 15 for all our experiments. RT-qPCR analysis showed that in KGN, endogenous expression of CBX2.2 was around 2.5 times less than CBX2.1, but 5 fold and 10 fold respectively compared to their expression in fetal ovary cells (used as the control set as 1) (Fig 1A).
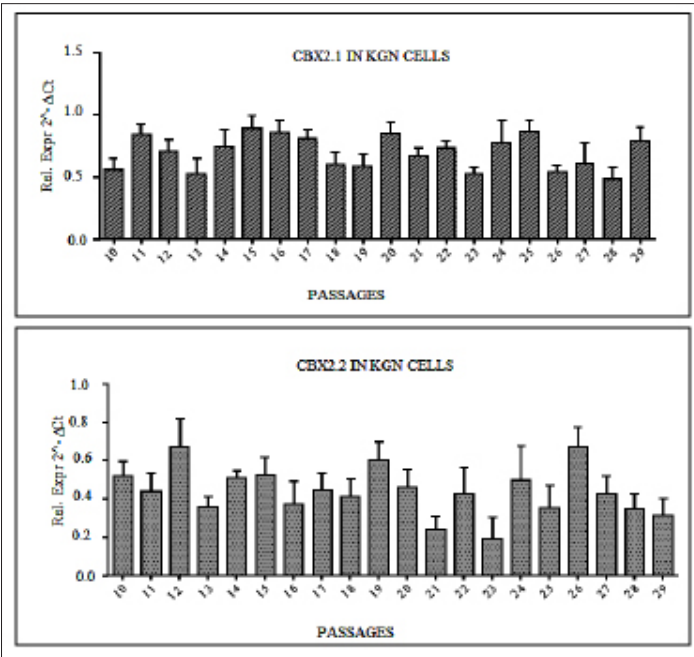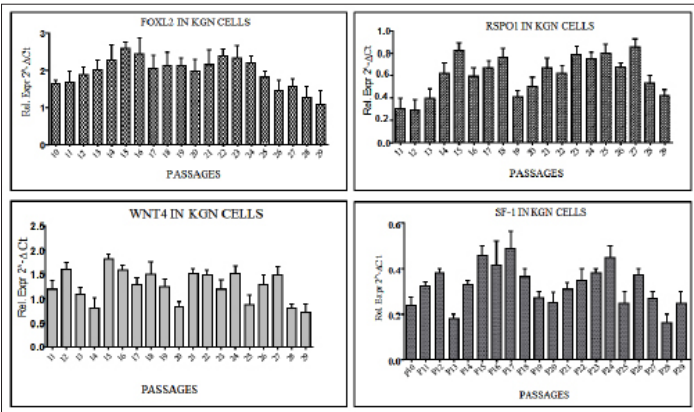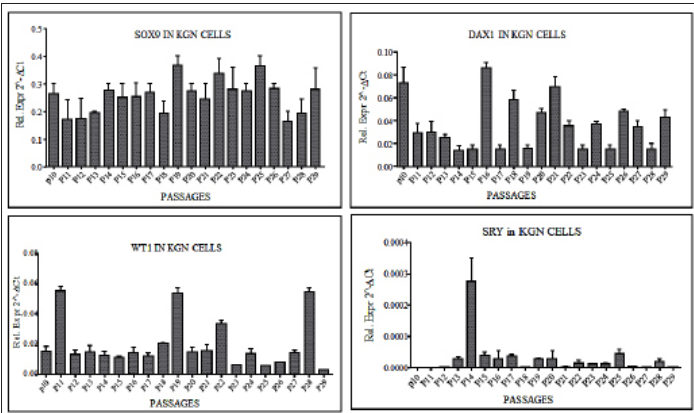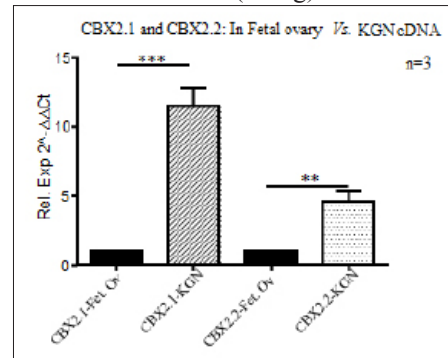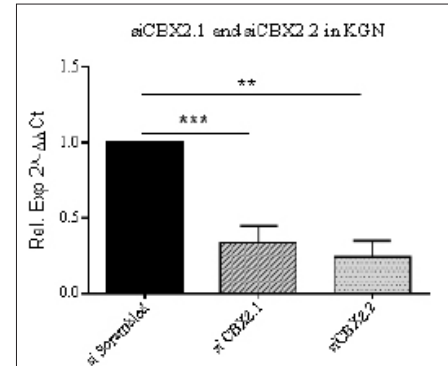
S1 Figure: Gene expression profiling in KGN: we checked expression profile of CBX2 isoforms (CBX2.1 and CBX2.2), FOXL2, and RSPO1 and WNT4, crosswise KGN passages (P10 to P29). The figure shows also the gene expression profiling of SF-1, SOX9, WT1, DAX1 and SRY through KGN passaging. Male specific genes were very low expressed according to their fold change ($2^{-\Delta Ct}$). SRY and WT1 were not expressed in KGN. The profiles showed instability of genes expression. In this experiment, statistical significance was not calculated due to the difficulty in obtaining human ovarian tissue to use as a control due to ethical limitations.
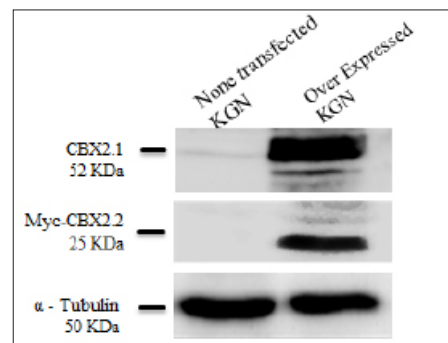
We then checked the expression level of FOXL2, RSPO1 and WNT4 under CBX2.1 and CBX2.2 overexpression and siRNA silencing assays. Downregulation was about -70 to -75% compared to the non-targeting siRNA (si-scrambled) for both isoforms (CBX2.1 and CBX2.2) (Fig 1B). Western blot detected the expected protein bands at approximately 52 kDa and 25 kDa corresponding respectively to the over expressed CBX2.1 and Myc tagged- CBX2.2 (Fig 1C). In spite of several optimization assays of transfection conditions and dilution of anti-CBX2 (N-Ter and C-Ter) antibodies, we were unable to detect the endogenous short isoform, most probably because of the lack of a specific CBX2.2 antibody. Transfected CBX2.1 and CBX2.2 were found to be localized in the nucleus of the KGN cells (Fig 1 D and E) as to be expected for a chromatin modifier and transcription factor. Female markers (FOXL2, RSPO1 and WNT4) have been checked after forced expression and corresponding correct bands size have been observed (S2 fig).
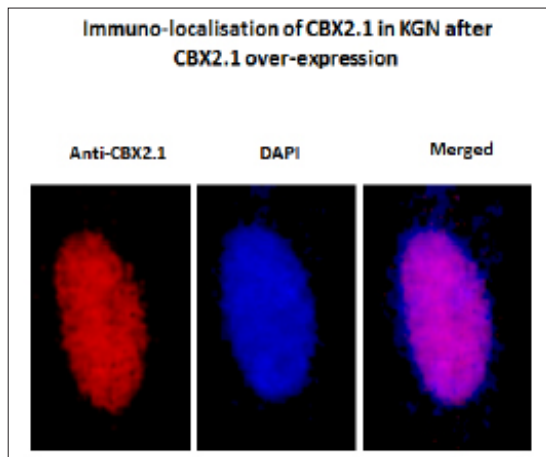
Immuno-localisation of CBX2.1 in KGN after CBX2.1 over-expression

Anti-CBX2.1   DAPI   Merged

D

Immuno-localisation of CBX2.2 in KGN after CBX2.2 over-expression
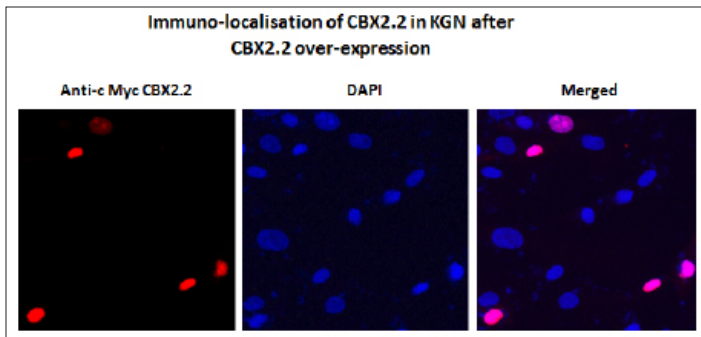
Anti-c Myc CBX2.2   DAPI   Merged

E

Figure: 1(A) Comparison between CBX2.1 and CBX2.2 endogenous expression level: CBX2.1 seems to be 2.5 fold more expressed than CBX2.2 in KGN cells. When compared to human fetal ovarian sample (Fet. Ov), CBX2.1 and CBX2.2 expression appear to be highly expressed in KGN cells. **(B) siRNA inhibition effect on CBX2.1 and CBX2.2 in KGN**. RT-qPCR showed that silencing effect was estimated to be around -70 to -75% for the two isoforms. All graphs represent the average of 3 independent experiments, error bars represent SD, and values are expressed as relative to control (fetal ovary =1); ***: P<0.001; **: P<0.01. **(C) Western blotting analysis of CBX2.1 and Myc-CBX2.2**: Exogenous and endogenous CBX2.1 has been detected using polyclonal anti-rabbit CBX2 antibody and showed the expected

size of 52 kDa. Tagged C-Myc CBX2.2 (25 kDa) was detected with mouse anti-Myc antibody. **(D and E) immune-localization of CBX2.1 and CBX2.2 in the nucleus of KGN**:
both CBX2 isoforms were visualized by indirect immunofluorescence using anti CBX2 and anti-C Myc antibodies and showed to have a nuclear localization in KGN cells.

S2 Fig
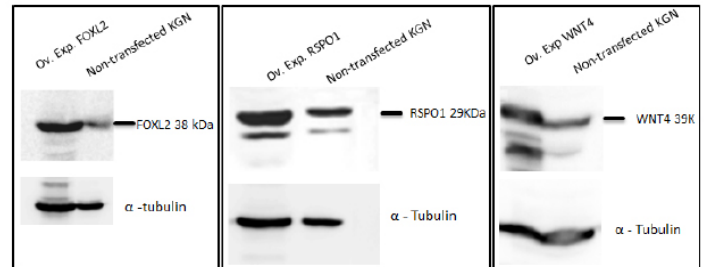
S2 Fig Transfected KGN representing over-expression offemale markers samples
FOXL2 (38kDa), RSPO1 (29kDa) and WNT4 (39 kDa). Non-transfected KGN is the sample hosting the empty vector used as a negative control and the tubulin (50 kDa) was used as loading control.

The influence of CBX2 isoforms on the expression of female markers (FOXL2, RSPO1 and WNT4) and male factors (SOX9 and SF-1) was studied by quantitative Real-Time PCR. Under CBX2.1 overexpression, FOXL2,RSPO1 and WNT4 expression appeared to be significantly repressed when compared to the control. Downregulation was about 40% for FOXL2 and WNT4 and approximately 40% for RSPO1 (Fig 2A). Reciprocally, after CBX2.1 knocking-down FOXL2 and RSPO1 were found to be significantly upregulated (50% and 30% respectively) (Fig 2A). Since the maintenance of female phenotype in the gonad is also partly dependent on the repression of "maleness" and to validate the CBX2.1 forced expression effect, we looked at gene expression of CBX2.1 downstream targets SOX9 and SF-1 male markers expression [5]. These genes were found to be increased approximately 2- and 1.5-fold respectively following CBX2.1 overexpression, which is in agreement with previous results and we observed a slight but significant reduction of SF-1 and SOX9 upon si CBX2.1 (Fig 2 B1) [10,17].
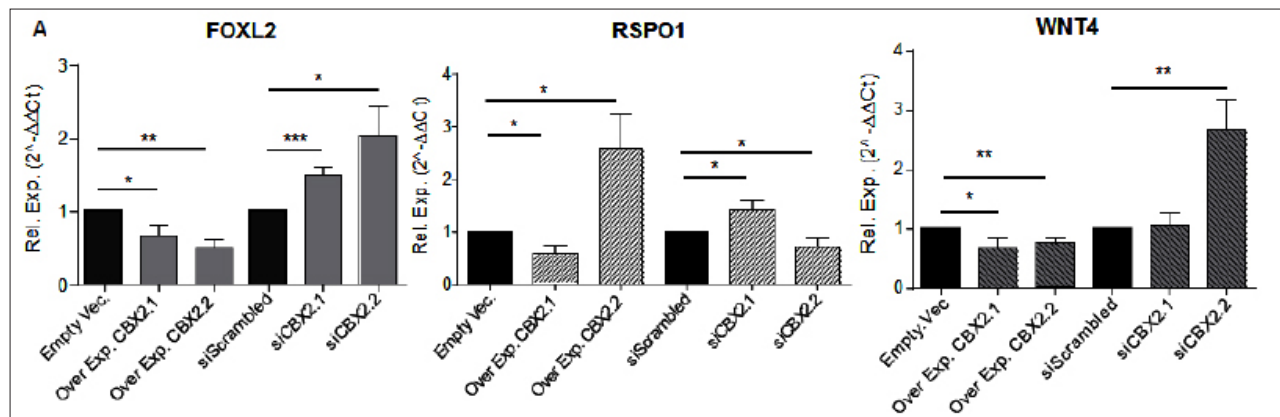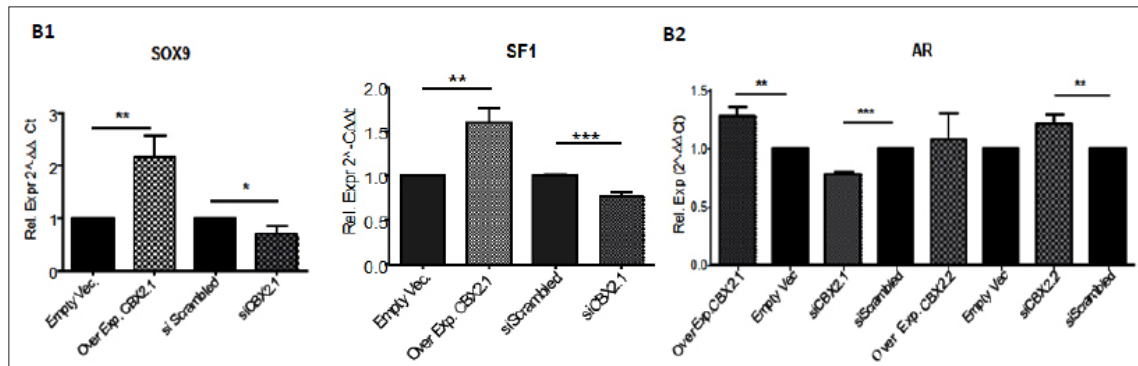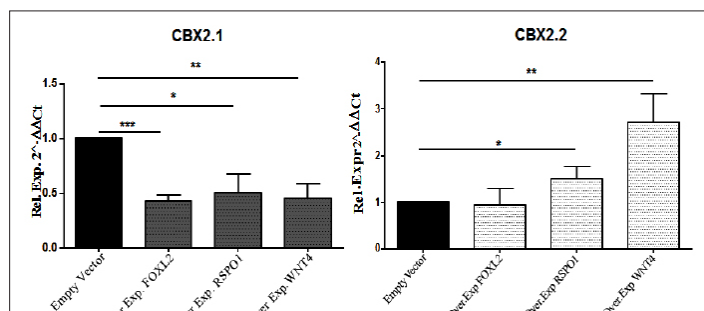

Figure: 2A

Figure: **B1**

Figure 2: **(A) Effects of CBX2.1 and CBX2.2 overexpression (Ov. Exp) and knock-down assay (si) on female markers (FOXL2, RSPO1 and WNT4)**. All female specific genes have shown a significant decrease after CBX2.1 forced expression. FOXL2 and RSPO1 were found to be up-regulated after siCBX2.1. Following CBX2.2 silencing, we observed elevation of FOXL2 and WNT4 however RSPO1 showed a decrease in mRNA level. **(B1) Effects of overexpression (Ov.Exp) and silencing (si) of CBX2.1 on SOX9 andSF-1**: In KGN both male markers (SOX9 and SF-1) seem to be dependent on CBX2.1 effect. An upregulation has been shown after CBX2.1 overexpression comparing the control (Empty Vec), whereas down-regulation has been observed following CBX2.1 RNA interfering. **(B2) effects CBX2.1 and CBX2.2 on AR**: After CBX2.1 forced expression, AR was found to be slightly but significantly upregulated. However, silencing assay showed a significant down-regulation.

CBX2.2 seems to not influence AR, whereas following siCBX2.2 we observed an expression increase relatively to the negative control (Empty Vec.). All graphs represent the average of 3 independent experiments, error bars represent SD, and values are expressed as relative to control (control =1); ***: P<0.001; **: P<0.01; *: P<0.005.

CBX2.2 forced expression seemed to significantly repress endogenous FOXL2 and WNT4, with a down-regulation effect of -50% and -30% respectively less than the negative control sample (Fig 2A). si-CBX2.2 treatment was found to remarkably enhance FOXL2 (1.5 fold) and WNT4 (2.5 fold).Contrary toCBX2.1, CBX2.2 overexpression was found to increase 2.5-fold RSPO1 mRNA levels. After CBX2.2 downregulation in pre-granulosa cells, RSPO1 expression showed around 30% reduction compared to the scrambled siRNA (Fig 2A). This suggested a possible distinct role of isoform-2 in ovarian cells.

Next, to explore whether the connection between CBX2 isoforms and female regulators goes both ways, we investigated the effect of FOXL2, RSPO1 and WNT4 overexpression on CBX2 isoforms by the mean of RT-qPCR. Our results showed that CBX2.1 endogenous expression was reduced to about 50% under FOXL2, RSPO1 and WNT4 forced expression (Fig 3), Unlike CBX2.1, RSPO1 and WNT4 were found to stimulate CBX2.2 expression level (Fig 3). Upregulation was estimated to be 1.5- and 2,5- fold respectively, whereas FOXL2 did not show any effect on CBX2 isoform-2.

Figure 3: **Effects of FOXL2, RSPO1 and WNT4 over expression (Over Exp) on CBX2.1 and CBX2.2**: CBX2.1 appeared to be diminished by all forced expressed female markers (FOXL2, RSPO1 and WNT4). CBX2.2 showed significant augmentation under RSPO1 and WNT4 over-expression, whereas FOXL2 seemed to have no effect on the expression of isoform-2.

To better understand the apparent differences in function between the two CBX2 isoforms, we assessed the effect of CBX2.1 and CBX2.2 on early and late ovarian developmental stage markers expression: OCT-4 (early fetal development), AMH (early and late folliculogenesis indicator) and ERβ (early and late marker) expressed in granulosa cell's nucleus of growing follicles at all stages from primary to mature follicles, and germinal epithelium cells, whereas the other subtype ERα was not tested being poorly expressed in granulosa cells and located in theca internal and interstitial cells [18].

Our data showed that CBX2.1 forced expression resulted in about 50% reduction of OCT-4, AMH and ERβ less than the negative control (Fig 4). Inversely, after CBX2 isoform-2 overexpression, the expression level of developmental stage markers (OCT-4, AMH and ERβ) was significantly increased (6-, 2.8-and 4-fold respectively). These findings suggest an opposite effects of the two CBX2 isoforms and probably reflecting diverse functions in the ovary and might translate into an important biological function of the short isoform CBX2.2 during ovarian determination and maintenance. In this view, we also checked the effect of CBX2 isoforms on androgen receptors (AR), described to be a key factor for follicles growth and woman fertility. AR expression level was estimated by RT-PCR assay following CBX2.1 forced expression and silencing conditions (Fig2 B2). The rationale was based on the AR mRNA found to be increased significantly 20% more than the non-transfected KGN cells while it showed 23% downregulation in the CBX2.1 silencing sample. On the other side CBX2.2 overexpression seems to not influence AR expression, whereas a 21% upregulation has been observed after CBX2.2 knock down.
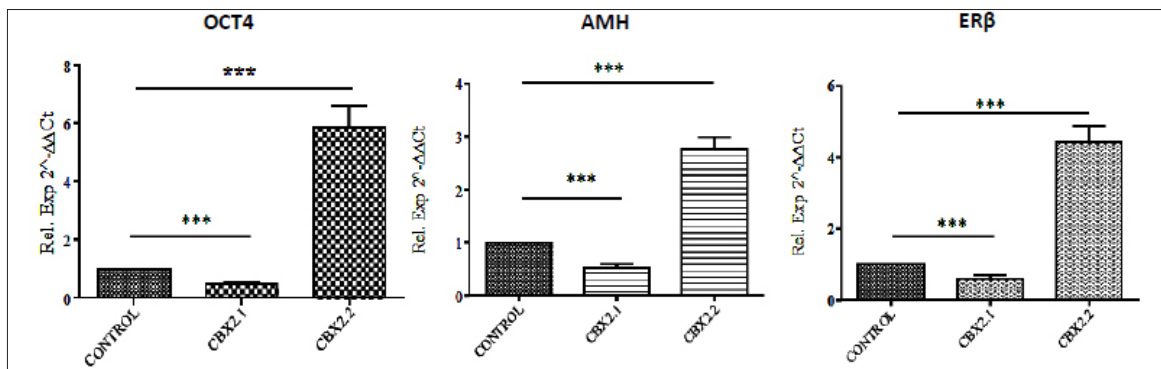
Figure 4. **Effects of CBX2 isoforms over-expression on developmental stage markers:** OCT-4, AMH, and ERβ. Developmental markers seem to be differently regulated by CBX2.1 and CBX2.2. We found that isoform-1 inhibited OCT-4, AMH and ERβ (ESR2), however CBX2.2 activated their expression. All graphs show the average of 3 independent experiments, error bars represent SD, and values are expressed as relative to the control (control =1); ***: $P<0.001$.

Discussion

In sex development, CBX2 has been revealed to be essential for proper malegonadal development in humans and mice by enhancing SF1 and SOX9 expression [10,15]. Its role in the human ovary is unknown, but knockout Cbx2 XX mice showed abnormalities of the reproductive system with gonadal growth retardation and germ cells loss resulting in small ovaries and infertility prompting us to investigate the role of CBX2 in human ovaries [9]. Since Homo sapiens seem to be the only mammalian species to have two CBX2 isoforms the use of animal model system was precluded to us. Thus, human ovarian pre-granulosa cell lines represented the most suitable alternative to ascertain the role CBX2.1 and CBX2.2 in human ovaries.

Our findings demonstrated that CBX2.1 forced expression in pre-granulosa cells resulted in the repression of endogenous level of FOXL2, RSPO1 and WNT4,with a concomitant increase of the traditional male markers SOX9, SF-1 and AR. Therefore, similarly to what has been shown in testicular cells, CBX2.1 seems to act as an anti-female regulatory factor [10,17]. In fact, SF1 is involved in regulating adrenal development, gonad determination/differentiation, and in the hypothalamic-pituitary control of reproduction and metabolism. A heterozygote mutation in SF-1 are one of the most common causes of 46, XY Disorders/differences of Sex Development (DSD) due to gonadal dysgenesis. Also, all androgens exert their function via activation of the AR in target tissues. Hemizygote mutations in 46, XY patients also cause an X-linked form of DSD called Androgen Insensitivity Syndrome (AIS). It is intriguing to attempt to explain the physiological role ofa pro-male/anti-female factor in ovarian cells.

In recent years studies in humans and mice revealed a role of both AR and SF-1 in maintaining ovarian function [19]. SF-1 dysfunction may affect the ovary at multiple levels,including reduced germ cell number, impaired stromal integrity, defective steroidogenesis and abnormal folliculogenesis which may go through a stage of decreased ovarian reserve (preantral and dominant antral follicles) engendering then premature ovarian failure [11,20,21]. Given the influence of CBX2.1 on SF-1, we speculated on a putative maintaining role for CBX2.1 in later stages of ovarian life could be considered in the adult ovaries through the activation of SF-1. Consistently, Cbx2 was confirmed to regulate gonadal size determination through Sf-1 and Lhx9 activation in XX mice during adulthood [14]. Alas,

experimental data supporting these hypotheses are difficult to obtain due to ethical (and legal) limitations in obtaining human fetal tissue.

We also investigated the correlation between AR and CBX2 expression in pre-granulosa cells. AR expression is present in all mammalian ovaries and in every stage of follicular development, which strongly supports a universal role for AR-mediated androgen actions in female gonads [22]. Genetic experiments in mice demonstrated a role of AR in the control of ovulation and follicular atresia. This follicular atresia regulates the rate of follicular pool depletion and therefore the time of onset of menopause. Accordingly, targeted deletions of the AR in animals decreased ovulation rate and accelerated atresia leading to earlier cessation of reproductive function [22-24].

In our hands, CBX2 seemed to increase AR expression suggesting that it might be involved in local control mechanisms during follicle development (preantral follicles as well small and large antral follicles) via the activation of AR at different follicular developmental stages within the ovary and therefore in the regulation of the onset of menopause [25-27]. CBX2.1 loss-of-function might therefore be correlated to lower levels of AR expression in the ovary, infertility and a shorter reproductive age, i.e. premature ovarian failure.

On the other hand, CBX2 isoforms might also have, through their repression of WNT4,RSPO1 and FOXL2, supplemental action on the ovary through the control of the growth and prevention of ovary tumor formation and/or progression. WNT4, RSPO1 and FOXL2 were already described to be associated to multiple malignancies, including ovarian, pancreatic, colon, breast, and lung cancer [28,29]. In particular, Chromobox homolog proteins have been designated as a tumor suppressors and allele loss of polycomb protein in human was associated to neoplasia [30,31]. CBX2.1 forced expression showed a decrease of the endogenous level of FOXL2, RSPO1 and WNT4, confirming the anti-female aspect of CBX2.1 in pre-granulosa cells [10,17]. The repression of WNT4, FOXL2 and RSPO1 expression by CBX2.1 was reciprocated by these very same factors.We therefore propose the existence of a bidirectional negative loop relating CBX2.1 and the known ovarian regulatory genes FOXL2, RSPO1 and WNT4, reminiscent of the known repressive relationship between SOX9 and FOXL2 [19]. Whether this inhibitory action is performed directly by CBX2.1 or indirectly through the activation of downstream factors, e.g. SOX9, has still

to be elucidated. Therefore, we hypothesized that CBX2 isoform-1 might protect ovarian granulosa cells from uncontrolled proliferation and growth, by fine-tuning WNT4 and RSPO1 expression.

Unlike CBX2.1, CBX2.2, seemed to be partly involved in stimulating the ovarian specific-gene RSPO1, through a positive regulatory loop RSPO1-CBX2.2, although it still repressed WNT4 and FOXL2.Thus it appeared that the two isoforms are, at least in part, functionally divergent in pre-granulosa cells. Several mechanisms underlying this difference might be hypothesized.

Firstly, difference in composition of Pc complexes might play of the main roles. Both isoforms have been reported to interact with different partners. For instance, CBX2.1 specifically recognizes tri-methylated lysine 27 on histone H3 and its Pc box recruits diverse PRC1 (Polycomb Repressive Complex 1) components involved in gene repression, whereas CBX2.2 self-associates to prevent transcriptional machinery recruitment [32].

Secondly, CBX2.1 and CBX2.2 functional diversity might be explained by distinct differential splicing regulation of the two transcripts. Although the molecular basis of these differences remains unclear, previous data revealed that alternative splicing differences affect diverse functions including gene expression, signal transduction, cell death, and susceptibility to diseases [33,34]. Notably, diverse transcriptional regulations including different polyadenylation sites and alternative splicing of CBX2 isoforms described by Volkel et al . underlie most likely a divergence of proteins functionality resulting in a short and less active CBX2.2 isoform lacking the polycomb domain comparing to the longer isoform-1 [32].

Thirdly, diverse action of CBX2.1 and CBX2.2 might also be due to a different timing of expression. We explored the possibility that the two isoforms might act during distinct developmental stages. Given the limitations in directly analyzing the expression of CBX2 isoforms in human fetal ovaries, we opted for an indirect approach. We analyzed the impact of CBX2 isoforms on specific ovarian developmental stage markers correlated with early embryonic stage such as OCT-4 (octamer-binding transcription factor 4), an early marker expressed in the undifferentiated fetal ovary and essential for germ cells survival and development, AMH (Anti-MüllerianHormone) a later developmental marker and a folliculogenesis regulator (primary and preantral follicles) during advanced fetal development and ERβ (Estrogen receptor beta), a marker associated to follicles growth at all ovarian developmental stages [35-38].

Remarkably, in pre-granulosa cell model, CBX2 isoform-1 was found to repress OCT4, ERβ and AMH mRNA expression, whereas CBX2.2 seemed to significantly increase the same factors. Considering these data, isoform-2 might be required during the very early stages in bipotential gonadal determination but also perhaps implicated in early folliculogenesis through the activation of AMH and ERβ genes and, as previously discussed, of RSPO1 that is expressed across early human fetal gonadal development stage, 7 to 8 weeks post-conception [39].

In conclusion, with the limitations of a cell culture system, it appears that both isoforms of CBX2, although seemingly anti-female/pro-male factors, might play a dual role in ovarian life. On one hand they fine-tune granulosa cell growth by regulating expression of WNT4, RSPO1 and FOXL2, and on the other hand they might be important in regulating follicular fate, ovulation and follicle atresia by stimulating SF1 and AR expression. They seem to do so in a distinct fashion and possibly in different times of ovarian development as suggested by the differentially regulated expression of stage specific markers OCT4, AMH, ERβ and RSPO1. The implications for woman's health, albeit speculative, might involve the understanding of infertility, premature ovarian failure, ovarian cancer and perhaps polycystic ovarian syndrome.

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