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The *RHOX* homeobox gene cluster is selectively expressed in human oocytes and male germ cells

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STUDY QUESTION: What human tissues and cell types express the X-linked reproductive homeobox (*RHOX*) gene cluster?

SUMMARY ANSWER: The RHOX homeobox genes and proteins are selectively expressed in germ cells in both the ovary and testis.

WHAT IS KNOWN ALREADY: The RHOX homeobox transcription factors are encoded by an X-linked gene cluster whose members are selectively expressed in the male and female reproductive tract of mice and rats. The *Rhox* genes have undergone strong selection pressure to rapidly evolve, making it uncertain whether they maintain their reproductive tissue-centric expression pattern in humans, an issue we address in this report.

STUDY DESIGN, SIZE, DURATION: We examined the expression of all members of the human *RHOX* gene cluster in 11 fetal and 8 adult tissues. The focus of our analysis was on fetal testes, where we evaluated 16 different samples from 8 to 20 weeks gestation. We also analyzed fixed sections from fetal testes, adult testes and adult ovaries to determine the cell type-specific expression pattern of the proteins encoded by *RHOX* genes.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We used quantitative reverse transcription–polymerase chain reaction analysis to assay human *RHOX* gene expression. We generated antisera against RHOX proteins and used them for western blotting, immunohistochemical and immunofluorescence analyses of RHOXF1 and RHOXF2/2B protein expression.

MAIN RESULTS AND THE ROLE OF CHANCE: We found that the *RHOXF1* and *RHOXF2/2B* genes are highly expressed in the testis and exhibit low or undetectable expression in most other organs. Using RHOXF1- and RHOXF2/2B-specific antiserum, we found that both RHOXF1 and RHOXF2/2B are primarily expressed in germ cells in the adult testis. Early stage germ cells (spermatogonia and early spermatocytes) express RHOXF2/2B, while later stage germ cells (pachytene spermatocytes and round spermatids) express RHOXF1. Both RHOXF1 and RHOXF2/2B are expressed in prespermatogonia in human fetal testes. Consistent with this, *RHOXF1* and *RHOXF2/2B* mRNA expression increases in the second trimester during fetal testes development when gonocytes differentiate into prespermatogonia. In the human adult ovary, we found that RHOXF1 and RHOXF2/2B are primarily expressed in oocytes.

LIMITATIONS, REASONS FOR CAUTION: While the average level of expression of *RHOX* genes was low or undetectable in all 19 human tissues other than testes, it is still possible that RHOX genes are highly expressed in a small subset of cells in some of these non-testicular tissues. As a case in point, we found that RHOX proteins are highly expressed in oocytes within the human ovary, despite low levels of *RHOX* mRNA in the whole ovary.

WIDER IMPLICATIONS OF THE FINDINGS: The cell type-specific and developmentally regulated expression pattern of the RHOX transcription factors suggests that they perform regulatory functions during human fetal germ cell development, spermatogenesis and

oogenesis. Our results also raise the possibility that modulation of *RHOX* gene levels could correct some cases of human infertility and that their encoded proteins are candidate targets for contraceptive drug design.

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Key words: testis / ovary / homeobox gene cluster / spermatogenesis / oogenesis

Introduction

The homeodomain is an evolutionarily ancient 60 amino-acid motif containing three alpha helices that binds to DNA (McGinnis and Krumlauf, 1992). Homeodomain-containing proteins, otherwise known as homeobox proteins, are transcription factors that regulate a wide variety of biological events in organisms that span the phylogenetic scale. Disruption of homeobox genes cause many types of developmental defects, including perturbed cell specification in limb, muscle and heart development (Panganiban and Rubenstein, 2002; Kim *et al.*, 2012; Makarenkova and Meech, 2012). In contrast to the well-established role of homeobox transcription factors in highly conserved biological events, little is known about their functions in rapidly evolving biological systems. A classic example of a rapidly evolving biological system is the reproductive tract. Not only do this system's properties typically differ greatly between closely related species, but the genes that regulate and mediate its functions are typically evolving at an extremely high rate (Swanson and Vacquier, 2002). This rapid evolution makes it challenging to identify regulatory factors and regulatory circuits that control the reproductive system. In this communication, we investigate a homeobox gene subfamily that is unique in being selectively expressed in the reproductive tract: the *Rhox* subfamily. All of the members of this *Rhox* homeobox subfamily are clustered together at a single site on the X chromosome, a chromosome particularly enriched for genes involved in spermatogenesis in mammals (MacLean and Wilkinson, 2010; Zheng *et al.*, 2010). In addition, two *Rhox*-like homeobox genes expressed in the reproductive tract, *Arx* and *Esx1*, are some distance away from the *Rhox* cluster on the X chromosome, probably as a result of ancient inversion events (MacLean and Wilkinson, 2010). The *Rhox* genes have mainly been studied in mice and rats, where they have been shown to be preferentially expressed in the testis, epididymis, ovary and placenta (Chun *et al.*, 1999; Takasaki *et al.*, 2000; Maclean *et al.*, 2005; MacLean and Wilkinson, 2010; Maclean *et al.*, 2012).

Even though all *Rhox* genes are expressed in reproductive-associated tissues, they are likely to have different functions, based on several lines of evidence. First, their spatial and temporal expression patterns are different. For example, individual *Rhox* genes exhibit peak expression at different time points during the first wave of spermatogenesis (Maclean *et al.*, 2005). Secondly, some *Rhox* genes differ in the cell types in which they are expressed. For example, in the adult testis, *Rhox10* and *Rhox13* are primarily (if not exclusively) expressed in germ cells (Geyer and Eddy, 2008; Song *et al.*, 2012), whereas *Rhox5* is only detectably expressed in Sertoli cells (Lindsey and Wilkinson, 1996; Rao *et al.*, 2003). In the fetal gonad, most *Rhox* genes are expressed in germ cells, whereas *Rhox8* is expressed in somatic cells (Daggag *et al.*, 2008; Song *et al.*, 2012). *Rhox* genes also exhibit sex-specific expression in the fetal gonad: *Rhox6* and *Rhox9* are specifically expressed in female germ cells, while *Rhox10* is specifically expressed in male germ cells (Daggag *et al.*, 2008; Song *et al.*, 2012). These differences in expression pattern suggest that rodent *Rhox* genes perform different functions. As further evidence for this, most *Rhox* genes encode proteins that have little amino-acid identity apart from the homeodomain region (Maclean *et al.*, 2005). Even the homeodomain region is quite divergent in sequence between most *Rhox* family members (Maclean *et al.*, 2005).

While the differences in amino-acid sequence and expression patterns of most *Rhox* family members suggests the possibility that they exert different functions in the reproductive tract, the functions of *Rhox* genes are only just beginning to be addressed. *Rhox5*-knockout male mice have increased numbers of apoptotic testicular germ cells, a reduced sperm count and decreased numbers of sperm with normal sperm motility, and are subfertile (Maclean *et al.*, 2005). *Rhox10*-knockout mice and transgenic mice specifically depleted of the *Rhox3* paralogs have dramatically impaired spermatogenesis (A.B., H.-W.S. and M.F.W., unpublished observations). In contrast, *Rhox9*-knockout mice do not exhibit any obvious defects, including in embryonic female germ cells where it is most highly expressed, perhaps as a result of redundancy with *Rhox6*, which has a nearly identical expression pattern and homeodomain region as *Rhox9* (Han *et al.*, 2000; Takasaki *et al.*, 2000, 2001).

Like most reproduction-associated genes (Swanson and Vacquier, 2002), the genes in the *Rhox* cluster are rapidly evolving. This is occurring at several levels. First, the *Rhox* genes encode proteins that are undergoing rapid changes in amino-acid sequence. This is not occurring as a result of random drift, but rather due to strong selection pressure, at least in the case of *Rhox5* and *RHOXF2* in rodents and primates, respectively (Sutton and Wilkinson, 1997; Niu *et al.*, 2011). The nature of the positive selection is not known, but a likely possibility is post-copulatory sexual selection in which mutations in *Rhox* genes confer an advantage in, for example, sperm–sperm competition. Secondly, the *Rhox* gene cluster exhibits large differences in copy number in different mammalian species. For example, the rat *Rhox* gene cluster contains 11 genes, while the mouse *Rhox* gene cluster has 33 genes by virtue of a series of recent mouse-specific duplication events that amplified the copy number of a trimer unit containing three *Rhox* genes (MacLean and Wilkinson, 2010; Maclean *et al.*, 2012). Primates appear to have different numbers of *RHOX* genes, depending on the species (Geserick *et al.*, 2002; Wayne *et al.*, 2002; Niu *et al.*, 2011). Finally, there is evidence that some *Rhox* genes have changed expression pattern over relatively short evolutionary time periods. Most notable is the recent finding that *Rhox5* and the subordinate *Rhox* genes under its control have undergone a

dramatic switch in their epididymal region-specific expression patterns since the divergence of mice and rats (Hammond, 2012; Maclean *et al*., 2012).

The rapid evolution of the *Rhox* gene cluster makes its relevance to the human reproductive tract uncertain. Thus, we elected to examine the expression pattern of the human *RHOX* gene cluster and the proteins it encodes. We found that the human *RHOX* gene cluster is most highly expressed in adult human testes and is developmentally regulated in fetal testes. Its members are expressed at lower levels in some other adult tissues, including the ovary, and in some fetal tissues. Using antibodies that we developed to distinguish between RHOX family members, we provide evidence that all RHOX family members are selectively expressed in germ cells in both the human testis and the ovary. Together, our results indicate that the *RHOX* gene cluster encodes transcription factors that have the potential to regulate human spermatogenesis and oogenesis.

Materials and Methods

Ethical approval

Human fetal tissues other than testes were collected courtesy of Planned Parenthood of the Pacific Southwest under UCSD IRB#081510X. Human adult testicular samples were collected from two healthy men (43- and 51-year-old) during reverse vasectomy surgery after informed consent obtained under IRB protocol #120471. A human adult epididymis sample was collected from a healthy man (63-year-old) during spermatocelectomy after informed consent obtained under IRB protocol #120471. For the placental RNA, informed consent was obtained and placental tissue was collected directly following repeat C-section at term under IRB# protocol 090652. Morphologically normal first- and second-trimester fetal testes (8–20 weeks gestation) were obtained after medical termination of pregnancy. Maternal consent was obtained and the study was approved by the Lothian Research Ethics Committee. Normal human ovarian tissues were obtained from a large bank of paraffin-embedded formalin-fixed tissue maintained by the UCSD Medical Center and Kaiser Hospital in San Diego under a protocol approved by the Human Research Protection Program at UCSD.

Tissue collection

The fetal tissues were collected, washed in PBS, snap-frozen in liquid nitrogen and stored at −80°C for RNA extraction. Total RNA was isolated using the mirVana kit (Ambion, Carlsbad, USA) following the manufacturer's instructions. Total cellular RNA from the placenta and epididymis were extracted using Trizol (Invitrogen, Carlsbad) following the manufacturer's instructions. The RNA from all other adult tissues was purchased from Agilent Technologies, Inc. (Santa Clara, USA).

The gestational age of fetal testes samples were determined by ultrasound scan and subsequent direct measurement of foot length. The sex of first trimester specimens was determined by PCR genotyping for the Y-linked *SRY* gene (Friel *et al*., 2002). Testes were dissected into sterile HBSS before either being snap-frozen and stored at −80°C for RNA extraction or fixed in formalin and processed into paraffin using standard methods for immunohistochemical analysis.

Adult human testicular biopsies were taken from a 36-year-old man who had a testicular tumor on one side of his testes. Histological evaluation revealed that the contralateral testis from this man had no tumors and that more than 90% of tubules showed qualitatively and quantitatively normal spermatogenesis. The ovarian tissues were surgically removed, fixed in 10% formalin, paraffin embedded and sectioned.

Quantitative reverse transcription–polymerase chain reaction analysis

cDNAs were generated from 0.5 to 1 μg total RNA using the Bio-Rad iScript reverse transcriptase kit, according to the manufacturer's protocol. For qPCR analysis, primer pairs were designed to amplify only spliced mRNA (by virtue of being complementary with sequences in separate exons). The primer pairs used were the following: *RPL32*, forward: 5′-CATCTCCTTCTCGGCATCA-3′, reverse: 5′-AACCCTGTTGTCAA TGCCTC-3′; *RHOXF1* (adult and fetal tissues), forward: 5′-GTGGA GGAGCTGGAAAGTGT-3′, reverse: 5′-TTCGGCAAGTTCCCTT CTT-3′; *RHOXF2/2B* (adult and fetal tissues), forward: 5′-CTGGCA AGAAGCATGAATGT-3′, reverse: 5′-CCCTCTGATGTCTCCTCC AT-3′; *RHOXF1* (fetal testis), forward: 5′-TGGAGGAGCTGGAAAG TGTT-3′, reverse: 5′-CTGGGTCAGCACGTAGTTC; *RHOXF2* (fetal testis), forward: 5′-GAGCAGTTCCCCAGTGAGTT-3′, reverse: 5′-ATG CCCTCTGATGTCTCCTC-3. The amplification efficiency for each primer pair was almost identical (the range was 102–110%). The cDNA and a set of primers were mixed with SYBR Green supermix (Bio-Rad, Hercules, USA) and PCR was performed using a StepOne real-time PCR machine, following the manufacturer's protocol (Applied Biosystems, Carlsbad). The production of the amplicon was measured by SYBR green fluorescence and the threshold cycle (Ct) values were calculated. The Ct values obtained were normalized to the *RPL32* housekeeping gene, which encodes the L32 ribosomal protein and is known to be nearly equally expressed in many different cell types (Zhang *et al*., 2005; Kriegova *et al*., 2008).

Antiserum generation and purification

Sequences encoding the N-terminal region of RHOXF1 (amino acids 1–103) and RHOXF2 (amino acids 1–140) were cloned into pGEX4T-1 (GE Healthcare, Little Chalfont, UK) to express GST-fusion proteins. Two rabbits were immunized with each of these fusion proteins and serum was collected by Proteintech Group, Inc (Proteintech, Chicago, USA). The IgG fraction from the final bleed was purified using protein G beads. The IgG fraction from preimmune serum was purified in the same way.

Immunohistochemical analysis

Immunohistochemical analysis was performed on paraffin-embedded sections that were fixed in 10% neutral-buffered formalin (fetal testis and ovary) or in Bouin's fixative (adult testis), as previously described (Song *et al*., 2012). Sections were then deparaffinized two times in xylene, followed by serial dilutions of ethanol. After heat-induced antigen retrieval in antigen unmasking solution (Vector Laboratory, Inc., Burlingame, USA), the internal peroxidase activity was quenched by incubation with 3% hydroperoxide in methanol for 15 min. Sections were blocked in Avidin solution for 15 min, in Biotin solution for 15 min (Vector Laboratory, Inc.), then in 5% bovine serum albumin, and 20% goat serum for 1 h at room temperature. Sections were then incubated overnight at 4°C with the primary antibodies against the following proteins: RHOXF1 (1:500 dilution for adult testis and ovary; 1:1000 dilution for fetal testis), RHOXF2 (1:500 dilution) and VASA (Abcam, Cambridge, UK; 1:500 dilution). To test the specificity of the RHOXF1 and RHOXF2/2B antisera, each was preincubated with its corresponding antigens (GST-RHOXF1 and GST-RHOXF2, respectively) for 2 h at 4°C before being applied onto sections. The antibodies were detected using secondary antibodies in the ABC kit (Vector Laboratory, Inc.), following the manufacturer's instructions, and incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratory, Inc.) substrate. Nuclei were counterstained with hematoxylin (Sigma, St. Louis, USA). The

sections were viewed with a Leica DMI6000 B inverted microscope and the images were recorded with a Leica DFC425C color camera (Leica Inc., Voorburg, The Netherlands).

Double immunofluorescence analysis

The sections generated and deparaffinized as described above were made accessible to antibody (i.e. antigen retrieval) by heating in citric acid buffer, quenched for internal peroxidase activity by incubation with 3% hydroperoxide in methanol for 15 min. The sections were then blocked as described above, then incubated overnight at 4°C with primary rabbit antibodies against RHOXF1 (1:1000 dilution) and RHOXF2 (1:2000 dilution). The antibodies were detected using secondary antibodies in the ABC kit (Vector Laboratory, Inc.), following the manufacturer's instructions, and incubation for 10 min with cyanine 3 labeled Tyramide (PerkinElmer, Waltham, USA). For double staining, sections were then incubated with a primary antibody against the pre-spermatogonia marker, MAGE-A4 (a gift from Dr Spagnoli, 1:50 dilution) overnight at 4°C, followed by incubation with Alexa 488-conjugated anti-mouse IgG (1:1000 dilution) for 1 h at room temperature. All slides were mounted using Vectashield containing DAPI (Vector Laboratory, Inc.). The images were viewed using an Olympus Gemini Fluoview confocal microscope (Olympus, Tokyo, Japan).

Results

Tissue-specific expression pattern of the human *RHOX* gene cluster

There are three genes in the human *RHOX* gene cluster (Fig. 1A and B). Two of these genes, *RHOXF2* and *RHOXF2B*, are 99.8% identical (based on comparison of both their exons and introns using BLAST analysis) and their encoded proteins differ at only two amino-acid positions (Fig. 1C and data not shown). Because of their high similarity, these two genes and their encoded protein products cannot be easily distinguished and thus we will refer to them as *RHOXF2/2B* and RHOXF2/2B, respectively, in this report. In contrast, *RHOXF1* has little sequence similarity with *RHOXF2/2B*, with almost no sequence identity at the gene level and proteins that only exhibit ~20% sequence identity (based on BLAST analysis). Even the homeodomain (DNA-binding) region of RHOXF1 and RHOXF2/2B share only 45% sequence identity (Fig. 1C). Of the four amino acids in homeodomains of known structure that make base-specific contacts with DNA (Gehring *et al.*, 1994), two differ between RHOXF1 and RHOXF2/2B (Fig. 1C), raising the likely possibility that RHOXF1 and RHOXF2/2B regulate different subsets of target genes (see the Discussion).

To elucidate the expression pattern of the *RHOXF1* and *RHOXF2/2B* genes, we performed quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis on RNA prepared from 11 human fetal tissues and 8 adult human tissues. This revealed that both *RHOXF1* and *RHOXF2/2B* are most highly expressed in adult testis (Fig. 1D). *RHOXF1* is also expressed in adult ovary, brain, placenta and epididymis, as well as some fetal tissues (albeit at ~1–3% of the level in adult testes). *RHOXF2/2B* is also expressed at low levels in non-testicular tissues, including the adult ovary. While the low expression of *RHOXF1* and *RHOXF2/2B* in non-testicular tissues could indicate these genes have no functional role in these organs, it is also possible that these homeobox genes are highly expressed in a small subset of cells in these organs, where they may exert important functions.

Germ cell-specific expression of RHOX proteins in the human testis

We elected to focus our subsequent analysis on the testis and ovary for two reasons: (i) these two organs are where gametogenesis takes place, (ii) the rodent *Rhox* genes are predominantly expressed in these two organs (Pitman *et al.*, 1998; Maclean *et al.*, 2005). To determine which cell type(s) in the testis and ovary express RHOXF1 and RHOXF2/2B, we generated antiserum against these two proteins in rabbits. Because homeodomains are highly conserved in structure (Duboule, 1994) and thus are likely to generate cross-reacting antibodies, we chose to use the non-homeodomain portion of each protein (the N-terminal half) as an immunogen. To test the specificity of the antiserum, we performed western blot analysis on extracts from freshly collected human testicular tissues from two normal individuals. We found that the RHOXF1 antiserum detected three bands migrating at ~25–30 kDa in testis extracts. Pre-incubation with recombinant GST-RHOXF1 protein drastically reduced the level of the ~27-kDa band (Fig. 2A, marked with an arrow; compare lanes 1 and 2 with lanes 3 and 4) but not the other 2 bands (marked with a [*]), suggesting that only the ~27-kDa band is RHOXF1. While the ~27-kDa band migrated to a position consistent with its being larger than RHOXF1's predicted size (20.5 kDa), this was not unexpected since another RHOX protein, mouse RHOX5, migrates at ~35 kDa even though its predicted size is 23.0 kDa (Hu *et al.*, 2008), presumably due to post-translational modifications and/or a stable protein conformation that is resistant to standard denaturation conditions. In contrast to RHOXF1, RHOXF2 migrated close to its predicted molecular weight. The RHOXF2/2B antiserum detected a ~33 kDa band, a size closely matching RHOXF2/2B's predicted molecular weight of 31.6 kDa, in testes extract (Fig. 2A, arrow). The specificity of this ~33 kDa band was demonstrated by finding that pre-incubation with recombinant GST-RHOXF2 protein eliminated it (compare lanes 5 and 6 with lanes 7 and 8), whereas non-specific bands (marked with a [*]) were not reduced in level by this pretreatment.

To determine the cell types in the human testis that express RHOX proteins, we used these antisera for immunohistochemical analysis. Specificity was demonstrated by finding that pre-incubation of the RHOXF1 or RHOXF2 antisera with recombinant GST-RHOXF1 and GST-RHOXF2 protein, respectively, resulted in a drastic reduction of staining (compare left with right panel in Fig. 2B and C). The RHOXF1 antisera stained pachytene spermatocytes (non-basal germ cells with large nuclei harboring highly condensed chromatin) and round spermatids (non-basal germ cells with small round nuclei; Fig. 2D, arrowheads and insets). The RHOXF2/2B antisera stained B-spermatogonia (basally located germ cells with spherical nuclei harboring slightly condensed chromatin), preleptotene spermatocytes (basally located germ cells with condensed chromatin) and leptotene spermatocytes (located near the Sertoli cell barrier; Fig. 2E, arrowheads and insets). While the RHOXF2/2B-specific antiserum stained B-spermatogonia, it did not

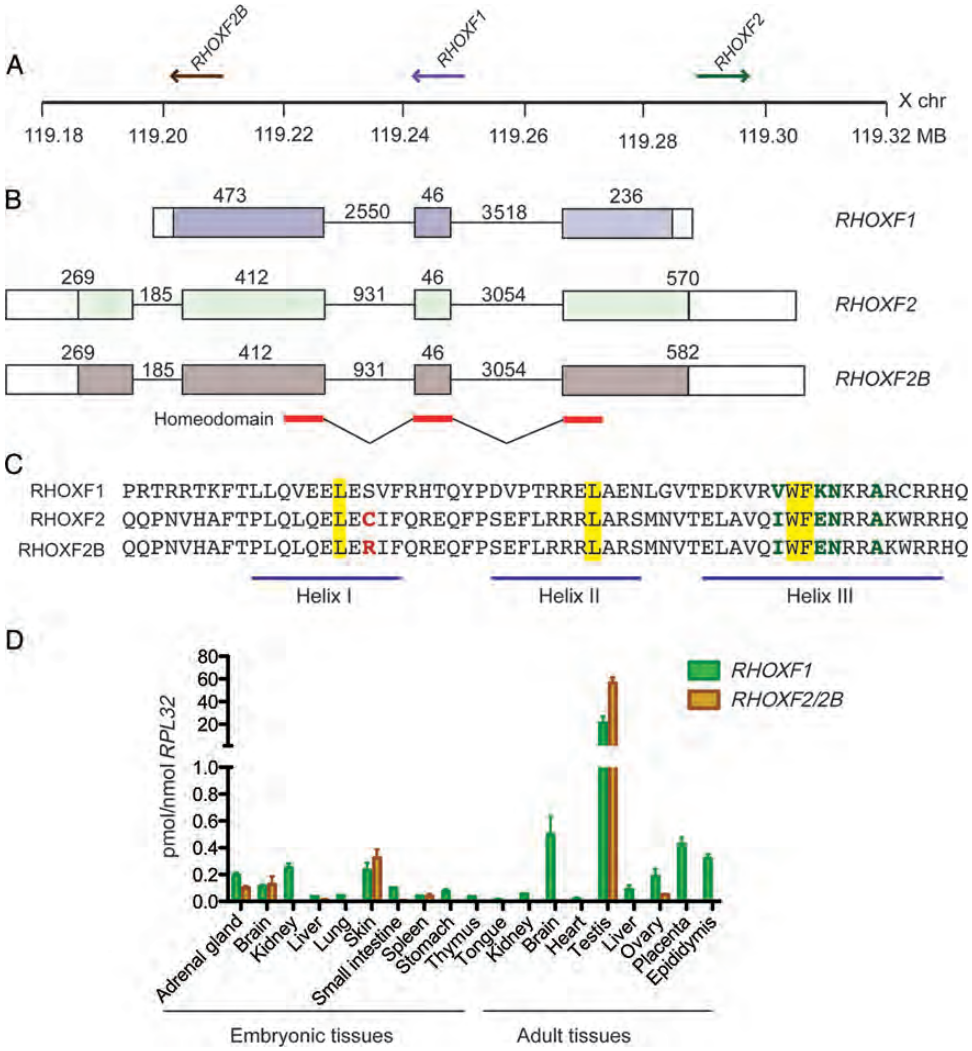

Figure 1 (**A**) The human *RHOX* cluster. The map position on the X chromosome is in accordance with the high coverage assembly GRCh37 from the Genome Reference Consortium (Ensembl.org). (**B**) The exon–intron structure of the human *RHOX* genes. The numbers indicate the nucleotide length of the exons and introns. The colors denote protein-coding regions. (**C**) Alignment of the predicted amino-acid sequences in the homeodomain region of the human RHOX proteins. The four amino acids that mediate base-specific binding to DNA (Gehring *et al*., 1994) are indicated in green. The single amino-acid difference between RHOXF2 and RHOXF2B in the homeodomain region is indicated in red. The yellow-shaded residues are the most conserved amino acids in all known homeodomains; they serve as crucial anchor points to appropriately fold the homeodomain structure (Duboule, 1994). (**D**) qRT–PCR analysis of total cellular RNA from the indicated human tissues. Values were normalized to *RPL32* mRNA level and denote the mean fold change ± standard error of the mean (SEM).

detectably stain the most primitive germ cells–A-dark-spermatogonia–which contain dark ovoid nuclei adjacent to the basement membrane). Most, if not all, A-pale spermatogonia (harboring pale ovoid nuclei adjacent to the basement membrane) were also negative (Fig. 2E). The most mature germ cells (elongated spermatids), as well as somatic cells (Leydig cells, peritubular myoid cells and Sertoli cells), were also not significantly stained with either the RHOXF1 or RHOXF2/2B antiserum (Fig. 2D and E). We conclude that both RHOXF1 and RHOXF2/2B are expressed specifically in germ cells within the human testis, and each of these proteins is present in distinct germ cell subsets.

Developmentally regulated RHOX expression in fetal testicular germ cells

Our finding that RHOXF2/2B protein is expressed in spermatogonia in the adult human testes (Fig. 2E) raised the possibility that it is also expressed in fetal testes, where it might control human gonadal development. As a first test of this possibility, we examined whether the *RHOXF2/2B* gene is actively transcribed in human fetal testes. In support of this, qRT–PCR analysis demonstrated that *RHOXF2/2B* mRNA is present in human fetal testes. This analysis also revealed that the level of *RHOXF2/2B* mRNA is developmentally regulated in

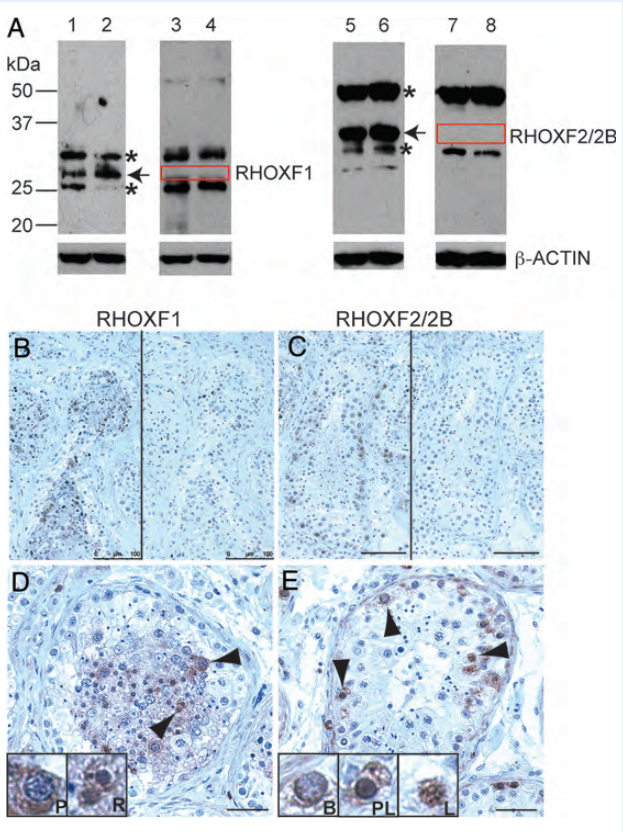

Figure 2 Cell type-specific expression of RHOX transcription factors in adult human testes. (**A**) Western blot analysis to validate the specificity of the antiserum we generated against RHOXF1 and RHOXF2/2B. Representative figures out of three replicates are shown. Loaded are extracts from human testicular tissue from two individuals (lanes 1, 3, 5 and 7 are from one individual, while lanes 2, 4, 6 and 8 are from another individual). These extracts were incubated with purified RHOXF1 antiserum (lanes 1 and 2), purified RHOXF1 antiserum preincubated with recombinant GST-RHOXF1 protein for 2 h (lanes 3 and 4), purified RHOXF2/2B antiserum (lanes 5 and 6), or purified RHOXF2/2B antiserum preincubated with recombinant GST-RHOXF2 protein for 2 h (lanes 7 and 8). The arrows and asterisks indicate specific and non-specific bands, respectively. Red rectangles denote the position of bands largely abolished by preincubation with recombinant RHOX proteins. β-actin serves as a loading control. (**B**–**E**) Immunohistochemical analysis performed on adult human testis sections with purified RHOXF1 antiserum (B left and D), purified RHOXF1 antiserum preincubated with GST-RHOXF1 protein (B right), purified RHOXF2/2B antiserum (C left and E) and purified RHOXF2/2B antiserum preincubated with GST-RHOXF2 protein (C right). Nuclei were counterstained with hematoxylin. Representative figures out of three replicates are shown. Arrowheads denote selected cells stained with the RHOXF1 or RHOXF2/2B antiserum. Inset boxes show enlarged views of RHOXF1- and RHOXF2/2B antisera stained cells. P, pachytene spermatocytes; R, round spermatids; B, type B spermatogonia; PL, preleptotene spermatocytes; L, leptotene spermatocytes. Scale bar: 20 μm (C, E) and 100 μm (B, D).

fetal testes: it is low during the first trimester of gestation (8–9 weeks) and dramatically increases during the second trimester (Fig. 3B), which coincides with the differentiation of gonocytes into prespermatogonia (Gaskell *et al.*, 2004).

We also assessed whether the *RHOXF1* gene was expressed in the fetal testis. We considered this unlikely given that the RHOXF1 protein is only expressed in meiotic and later stage germ cells in human adult testes (Fig. 2C). Contrary to this prediction, we found that *RHOXF1* mRNA is present in human fetal testes. However, our qRT–PCR analysis indicated that *RHOXF1* mRNA levels were ~10-fold lower than that of *RHOXF2/2B* in 17–20-week fetal testes (Fig. 3A). While this is only an approximate fold-difference value since it is not possible to precisely compare expression levels determined using different primer pairs, it is likely to be reasonably accurate since our standard curve analysis indicated that the primer pairs recognizing *RHOXF1* and *RHOXF2/2B* have virtually identical amplification efficiencies (data not shown).

To determine which cell type(s) in fetal testes expresses the RHOXF1 and RHOXF2/2B proteins, we performed immunohistochemical analysis on 18- and 19-week human fetal testes sections. This is an ideal developmental window for such analysis, as human testes from these fetal ages contain several stages of male germ cells, including gonocytes, intermediate cells and prespermatogonia, all in the same cord (Gaskell *et al.*, 2004). Consistent with our qRT–PCR analysis (Fig. 3A and B), immunohistochemical analysis revealed that both RHOXF1 and RHOXF2/2B protein are expressed in 18-week fetal testes (Fig. 3C and D). Like adult testes, fetal testes express both RHOXF1 and RHOXF2/2B largely (if not exclusively) in germ cells. We noted that the germ cells positive for RHOXF1 and RHOXF2/2B had the morphology of VASA-positive prespermatogonia (Fig. 3D; Gaskell *et al.*, 2004; Anderson *et al.*, 2007). To directly test whether RHOXF1 or RHOXF2/2B are expressed in prespermatogonia, we performed double immunofluorescence staining with the prespermatogonia marker, MAGE-A4, which is recognized by a mouse monoclonal antibody that can be distinguished, using appropriate immunofluoresence-labeled secondary antibodies, from the rabbit antibodies we generated against the RHOX proteins. Double immunofluoresence analysis revealed that both RHOXF1 and RHOXF2/2B are largely expressed in the same cells as those that express MAGE-A4 (Fig. 4). This confirmed that these two homeobox proteins are expressed in prespermatogonia in human fetal testes.

RHOXF1 and RHOXF2/2B are expressed in oocytes

As described above, we found that both the *RHOXF1* and *RHOXF2/2B* genes are expressed at low levels in the adult human ovary (Fig. 1D). One possibility is that this reflects low expression of *RHOX* genes throughout the ovary. An alternative possibility is that this reflects high expression of *RHOX* genes in one or more specific cell subsets in the ovary. To distinguish between these possibilities, we performed immunohistochemical analysis of adult human ovary sections with the RHOXF1- and RHOXF2/2B-specific antiserum that we generated. We observed strong staining with the RHOXF1-specific antiserum in oocytes (Fig. 5). Staining in oocytes was observed in all follicles, regardless of stage (Fig. 5). Oocytes in primordial, primary and antral follicles also stained with the RHOXF2/2B-specific antiserum, although the staining was much weaker than with the RHOXF1 antiserum, particularly in the more immature follicles. We observed little or no RHOXF2/2B antiserum staining in the few secondary follicles we were able to identify. We also detected low levels

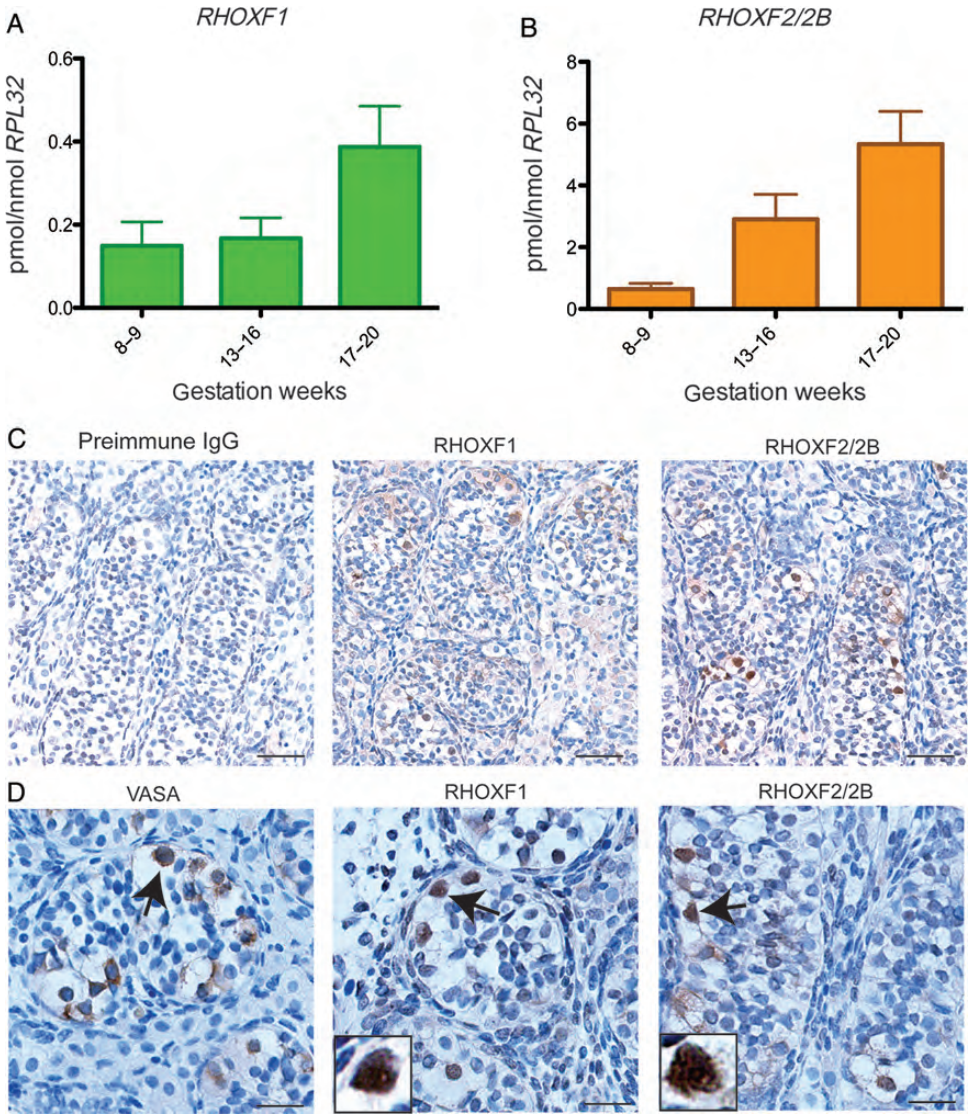

Figure 3 RHOX expression in human fetal testes. (**A**, **B**) qRT–PCR analysis of total cellular RNA from human fetal testes of the indicated gestation weeks (from 6, 8 and 5 pooled fetal testes derived from 8–9-, 13–16- and 17–20-week-old fetuses, respectively). Values were normalized to *RPL32* mRNA level and denote the mean fold change ± SEM. (**C**, **D**) Immunohistochemical analysis performed on 18–19 week fetal testes sections with purified antiserum against the indicated protein or preimmune IgG at low magnification (C) or high magnification (D). Representative figures out of three replicates are shown. Nuclei were counterstained with hematoxylin. Arrows denote selected cells expressing the protein recognized by the antisera. Inset boxes show enlarged views of RHOXF1- and RHOXF2/2B antisera stained cells. Scale bar: 20 μm (C) and 100 μm (D).

of RHOXF1- and RHOXF2/2B-antiserum staining in some granulosa cells in a small subset of antral follicles (Fig. 5D and data not shown). We did not detect reproducible staining in theca cells in any follicular stage with the RHOXF1 or RHOXF2/2B antiserum (data not shown). We conclude that both RHOXF1 and RHOXF2/2B are primarily expressed in oocytes in the adult human ovary.

Discussion

In this communication, we report the expression pattern of the human X-linked *RHOX* homeobox gene cluster, which encodes transcription factors that have been extensively studied in rodents, but not humans. We demonstrate that the genes in this cluster are most abundantly expressed in the adult human testis (Fig. 1D). This confirms the finding of an earlier study using the insensitive technique, northern blot analysis (Wayne *et al.*, 2002). While this earlier study did not detect *RHOX* gene expression in non-testicular tissues, we found, using qRT–PCR analysis, that *RHOX* genes are actually expressed at low levels in several adult and fetal tissues, including the adult human ovary (Fig. 1D). In the case of the adult ovary, we provide evidence that the reason that *RHOX* genes are expressed at low level is because of their selective expression in limited cell types: oocytes and a small proportion of granulosa cells (Fig. 5). Likewise, we found that *RHOX* gene products are largely confined in their expression to germ

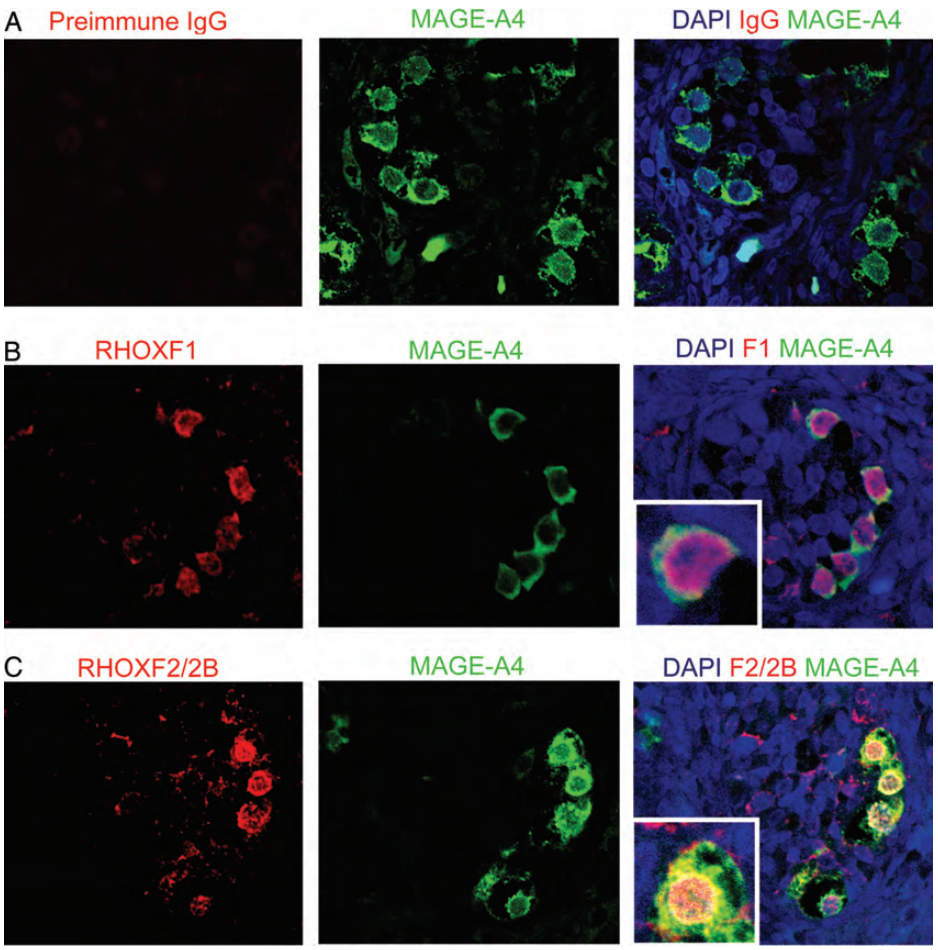

Figure 4 RHOX proteins are specifically expressed in prespermatogonia in human fetal testes. Double immunofluorescence analysis performed on 18-week human fetal testes sections first incubated with preimmune IgG (red, **A**), purified RHOXF1 antiserum (red, **B**) or purified RHOXF2/2B antiserum (red, **C**), followed by incubation with purified MAGE-A4 antiserum (green, A–C). Representative figures out of three replicates are shown. Enlarged views of MAGE-A4-stained and RHOXF1- or RHOXF2/2B-co-stained cells are shown in the inset boxes. F1, RHOXF1; F2/2B, RHOXF2/2B.

cells in adult and fetal human testes. In the adult testis, one of the human RHOX proteins, RHOXF1, is specifically expressed in pachytene spermatocytes and round spermatids (Fig. 2D). This expression pattern is reminiscent of that of the mouse *Rhox3* gene paralogs, a set of eight highly related genes that are also highly expressed in pachytene spermatocytes and round spermatids, and whose knockdown by RNAi *in vivo* elicits a dramatic reduction in number of these two cell types (A.B., H.-W.S. and M.F.W., unpublished observations). Thus, both human *RHOXF1* and the mouse *Rhox3* paralogs may encode transcription factors that regulate target genes crucial for late meiotic events and haploid germ cell differentiation events. In contrast to RHOXF1, RHOXF2/2B is expressed in immature human male germ cells. It is highly expressed in B spermatogonia, a proliferative cell that is committed to differentiate into meiotic spermatocytes, as well as early (preleptotene and leptotene) spermatocytes (Fig. 2E). Thus, RHOXF2/2B has an expression pattern similar to mouse RHOX10 and RHOX13 (Geyer and Eddy, 2008; Song *et al.*, 2012), which do not have established roles as of yet, but presumably function in early stages of spermatogenesis.

Few transcription factors have so far been identified as being expressed in germ cells in the human testis. One of the best-studied transcription factors in this regard is cAMP response element modulator (CREM), which, like RHOXF1, is expressed in pachytene spermatocytes and round spermatids in human testes (Vouk *et al.*, 2005). CREM is likely to have important roles in human spermatogenesis, based on two observations. First, mutations in the human *CREM* gene are associated with azoospermia and round spermatids arrest in human males (Vouk *et al.*, 2005). Secondly, targeted disruption of the *Crem* gene in mice revealed that its germ cell-specific isoform, CREM-τ, is essential for male fertility and that it executes important roles in post-meiotic germ cell differentiation by regulating the expression of several key post-meiotic genes, including those encoding the transition proteins and protamines (Blendy *et al.*, 1996; Nantel *et al.*, 1996; Sassone-Corsi 1998). In contrast to late-stage

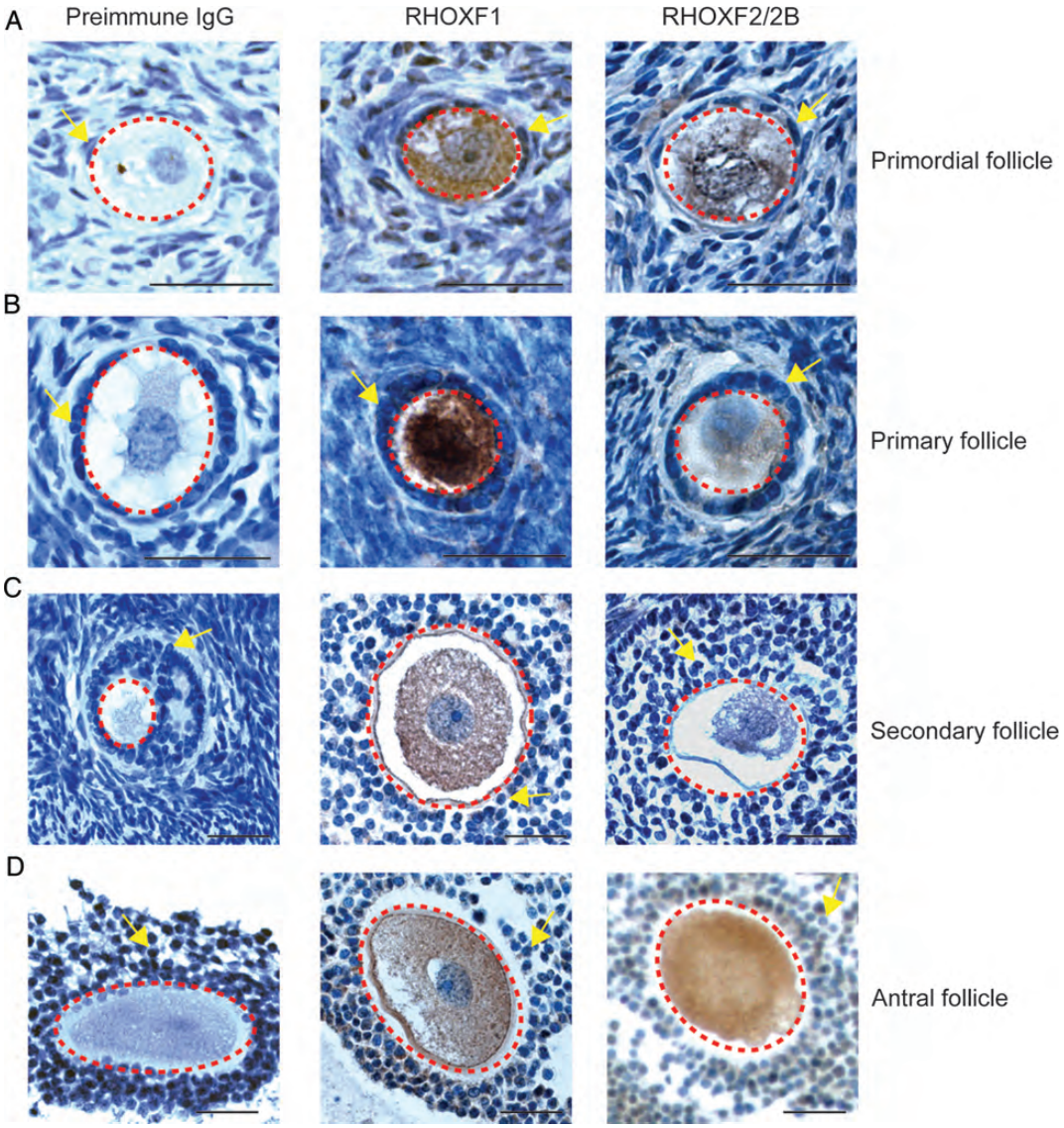

Figure 5 RHOX proteins are expressed in the oocytes in adult human ovaries. Representative figures are shown from immunohistochemical analysis of human adult ovary sections from nine individuals using purified RHOXF1 antiserum, purified RHOXF2/2B antiserum or preimmune IgG. Representative staining of primordial follicles (**A**), primary follicles (**B**), secondary follicles (**C**) and antral follicles (**D**). The red dotted lines outline oocytes and the yellow arrows demarcate granulosa cells. Scale bar: 20 μm.

transcription factors, to our knowledge, no early stage transcription factors that have roles in human male fertility have been identified. Good candidates are SOHLH1 and SOHLH2, which are expressed in mouse spermatogonia and have been shown to act cooperatively to promote germ cell differentiation in mice by repressing genes involved in spermatogonial stem cell maintenance and inducing genes important for spermatogonial differentiation (Toyoda *et al.*, 2009; Suzuki *et al.*, 2012). If the human versions of these transcription factors are shown to be expressed in spermatogonia, it will be interesting to determine whether they also have a functional relationship with RHOXF2/2B, which we found is highly expressed in human spermatogonia (Fig. 2E). Evidence that SOHLH1 has a role in human male fertility is the finding that mutations in the human *SOHLH1* gene that generate a non-functional protein are associated with non-obstructive azoospermia (Choi *et al.*, 2010).

Our finding that RHOXF1 and RHOXF2/2B are expressed in germ cells in second trimester male gonads (Figs 3 and 4) suggests that they also may have roles in early germ cell development. The second trimester human fetal testis contains three populations of germ cells reflecting different stages of development: gonocytes ($OCT4^{pos}$/$C\text{-}KIT^{pos}$/$MAGE\text{-}A4^{neg}$), intermediate germ cells ($OCT4^{low/neg}$/$C\text{-}KIT^{neg}$/$MAGE\text{-}A4^{neg}$), and prespermatogonia ($OCT4^{neg}$/$C\text{-}KIT^{neg}$/$MAGE\text{-}A4^{pos}$) (Gaskell *et al.*, 2004; Anderson *et al.*, 2007). Gonocytes and intermediate cells are proliferative (PCNA-positive) cells, while prespermatogonia are non-proliferative (PCNA-negative) cells (Schlatt and Weinbauer, 1994). Gonocytes,

which are the most abundant cells in the first trimester, are largely replaced by prespermatogonia in the latter phase of the second trimester. This timing may explain why *RHOXF2/2B* mRNA level increases in human fetal testes during the second trimester (Fig. 3B), as we found that RHOXF2/2B protein is primarily expressed by prespermatogonia (Fig. 4). RHOXF1, which we found is also primarily expressed in prespermatogonia (Fig. 4), is expressed from an mRNA that undergoes a modest increase in level during human testes development (Fig. 3A).

Interestingly, RHOXF1 and RHOXF2/2B were detected in both the nucleus and the cytoplasm of most of the germ cells we examined (Figs 2–5). One possibility is that RHOXF1 and RHOXF2/2B might equilibrate in both of these cellular compartments, since their molecular weights are under that required for active transport across the nuclear pore (Gorlich and Kutay, 1999). The second possibility is that they might be actively localized in the cytoplasm. Transcription factors are, by definition, proteins that regulate transcription in the nucleus, but some, including homeobox transcription factors, also function in the cytoplasm. For example, there are some homeobox transcription factors that directly regulate the translation of specific target mRNAs in the cytoplasm while others regulate translation indirectly through RNA-binding proteins that they interact with in the cytoplasm (Niessing *et al*., 2002; Cho *et al*., 2005). We recently reported that the mouse RHOX10 protein shifts its subcellular localization during male germ cell development and that it is localized exclusively in the cytoplasm of non-proliferative mouse fetal germ cells (Song *et al*., 2012). While we do not know the functional significance of this, it is intriguing to speculate that the conserved presence of RHOX proteins in the cytoplasm of fetal germ cells in mammals indicates these transcription factors have a conserved alternative function in the cytoplasm.

Meiotic sex chromosome inactivation (MSCI) strongly represses the transcription of genes on the X chromosome after the zygotene-to-pachytene transition (Turner, 2007). Since the *RHOX* gene cluster is on the X chromosome, it should be subject to MSCI. In apparent contradiction to this, we found that RHOXF1 is first detectably expressed in late pachytene spermatocytes (Fig. 2C), a stage of male germ cell development when MSCI is in full force. Thus, the *RHOXF1* gene may escape MSCI, which would be of considerable interest given that there have been no reports of other protein-coding genes that escape MSCI. In further support of the possibility that *RHOXF1* might escape MSCI, it was recently reported that MSCI in humans might be more relaxed than in mice, based on analysis of RNA polymerase II binding, variant histone eviction, uridine analog incorporation and repeat RNA abundance in sex chromosome in human pachytene spermatocytes (de Vries *et al*., 2012). While our finding that RHOXF1 protein is first detectably expressed when MSCI is operative suggests the possibility that the *RHOXF1* gene escapes MSCI, we note that there are alternative explanations. For example, *RHOXF1* mRNA may be transcribed before the pachytene stage (when MSCI has not yet been engaged) and then stored until it is translated during the pachytene stage (when MSCI is engaged). We recently investigated the mechanism by which another *Rhox* gene, the mouse *Rhox10* gene, is expressed in pachytene spermatoctyes when MSCI is active (Song *et al*., 2012). While we found that both *Rhox10* mature mRNA and RHOX10 protein are present in pachytene spermatocytes, the *Rhox10* gene is not actively transcribed in these cells, based on their having very low levels of *Rhox10* pre-mRNA. Instead, we found that the *Rhox10* gene is only strongly transcribed in type-A spermatogonia, which suggests that *Rhox10* mRNA and/or RHOX10 protein are stabilized to such a large extent in these proliferative cells that these molecules persist until after these cells differentiate into spermatocytes, when MSCI is operative.

Our finding that RHOX proteins are expressed in human oocytes (Fig. 5) raises the possibility that they function in oogenesis. Their expression in human oocytes also has potential clinical importance; for example, it indicates that *RHOX* genes are candidates for involvement in non-syndromic ovarian failure, largely idiopathic abnormalities that affect 1–2% of women. While considerable effort has been directed towards identifying oocyte-specific transcription factors possessing functional roles in oogenesis using mouse models, little is known about transcription factors selectively expressed in human oocytes (Pangas and Rajkovic, 2006). In the mammalian ovary, oocytes arrested at the diplotene stage of propase I in meiosis are initially located in the primordial follicles, a small proportion of which proceed to go through a series of follicular developmental events to produce a functional oocyte. We found that both RHOXF1 and RHOXF2/2B are present in oocytes at most of the developmental stages that we examined: from those in primordial follicles and gonadotropin-independent pre-antral follicles to fully grown oocytes in antral follicles (Fig. 5). Likewise, the homeobox transcription factor, Newborn Ovary Homeobox (NOBOX), is expressed in human oocytes in all follicular stages (Huntriss *et al*., 2006). Interestingly, loss-of-function mutations in human *NOBOX* are associated with 6.2% of primary ovarian insufficiency cases, all of which result in female infertility (Bouilly *et al*., 2011). While there is no direct evidence that NOBOX has a role in normal human oogenesis, targeted mutation of the *Nobox* gene in mice causes follicular arrest at the primordial stage (Rajkovic *et al*., 2004).

In conclusion, we provide evidence that the RHOXF1 and RHOXF2/2B homeobox transcription factors are preferentially expressed in germ cells in the human male and female reproductive tract. The dynamic and stage-specific expression pattern of these RHOX proteins in developing male germ cells suggests functional roles at specific stages of human spermatogenesis. Their constitutive expression in female germ cells, regardless of follicular stage, suggests stage-independent roles in human oogenesis. Future studies will be devoted to determining the specific roles of RHOX transcription factors in the development of male and female germ cells from human embryonic stem cells *in vitro*. It will also be important to assess whether mutations in *RHOX* genes are associated with human infertility, a reasonable possibility given that many single nucleotide polymorphisms that lead to non-synonymous amino-acid substitutions have been identified in the *RHOXF1*, *RHOXF2* and *RHOXF2B* genes (Niu *et al*., 2011). If indeed the human *RHOX* genes are shown to have roles in human infertility and/or gametogenesis, this will open up investigation of whether their encoded proteins or downstream gene products are viable targets for the treatment of infertility and contraceptive drug design.

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Authors' roles

H.W.S. designed this project and performed the immunohistochemical, immunofluoresence and immunoblotting analysis and some of the qRT–PCR analysis. R.A.A. and R.A.B. did the qRT–PCR analysis on human fetal testis samples and prepared human fetal testis sections. J.G. prepared human adult testis sections. S.S. and R.J.C prepared human adult ovary sections and were involved in data analysis. M.M.P. prepared human placenta samples. L.C.L prepared human fetal tissue samples. T.C.H obtained human adult testis and epididymis samples. D.G.D.R. helped to analyze the cell types expressing RHOX proteins in adult testes. M.F.W. helped to design this project. H.W.S. and M.F.W. wrote the manuscript with help from the other authors.

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Conflict of interest

None declared.

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