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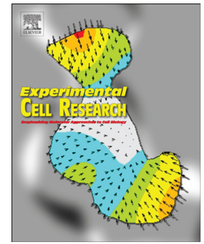

Research Article

Identification of the genes regulated by Wnt-4, a critical signal for commitment of the ovary

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ABSTRACT

The indifferent mammalian embryonic gonad generates an ovary or testis, but the factors involved are still poorly known. The Wnt-4 signal represents one critical female determinant, since its absence leads to partial female-to-male sex reversal in mouse, but its signalling is as well implicated in the testis development. We used the *Wnt-4* deficient mouse as a model to identify candidate gonadogenesis genes, and found that the *Notum*, *Phlda2*, *Runx-1* and *Msx1* genes are typical of the wild-type ovary and the *Osr2*, *Dach2*, *Pitx2* and *Tacr3* genes of the testis. Strikingly, the expression of these latter genes becomes reversed in the *Wnt-4* knock-out ovary, suggesting a role in ovarian development. We identified the transcription factor *Runx-1* as a Wnt-4 signalling target gene, since it is expressed in the ovary and is reduced upon *Wnt-4* knock-out. Consistent with this, introduction of the Wnt-4 signal into early ovary cells *ex vivo* induces *Runx-1* expression, while conversely *Wnt-4* expression is down-regulated in the absence of *Runx-1*. We conclude that the *Runx-1* gene can be a Wnt-4 signalling target, and that *Runx-1* and *Wnt-4* are mutually interdependent in their expression. The changes in gene expression due to the absence of *Wnt-4* in gonads reflect the sexually dimorphic role of this signal and its complex gene network in mammalian gonad development.

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Introduction

The early gonad in mammals is initially sexually indifferent, the primordium developed at around mid-gestation (E10) in the mouse and is derived from the intermediate mesodermal cells. Once formed, it attracts the germ cells which generate the primordial germ cells through migration from the base of the allantois towards the gonad. Around one day later (E11.0) the indifferent gonad is gradually being assigned to follow either the male or female sexual differentiation pathway, although the execution of this programme is still poorly characterized.

Certain genes which are critical for sex determination have been identified. For example, *Emx2* (*Empty spiracles homologue 2*), *Wt1* (*Wilm's tumour homologue 1*), and *Sf1* (*Steroidogenic factor 1*) [1] are critical for the differentiation of somatic cells. Once the key male determinant Sry (*Sex determining region of Y chromosome*) becomes activated at around E11.0 [2], this transcription factor triggers the testis development programme via its downstream target genes such as *Sox9* (*Sry-like HMG protein 9*), [3], which up-regulates an early response in the gene expression of *Fgf9* (*Fibroblast growth factor 9*), [4].

It was thought initially that the female sexual developmental pathway represents a default alternative for the situation in which Sry is not activated. The evidence obtained in recent years has indicated that female sexual commitment also depends on active cell–cell signalling that involves such secreted factors as Wnt-4 and R-spondin1 [5,6]. This conclusion has been based in part on the fact that the female embryo undergoes a partial female-to-male sex reversal in the absence of these factors [5,6]. Besides suppressing the fate of the Leydig cells, the associated testosterone synthesis genes [7] and the development of the Müllerian duct [5], *Wnt-4* also controls the initiation of meiosis in female germ cells [8].

Even though Wnt-4 is a critical signal for ovarian development, it may also have a role in the organogenesis of the testis [9]. In synergy with *Rspo1*, *Wnt-4* controls proliferation of the coelom epithelial cells in the early testis affecting the number of differentiated Sertoli cells [10]. Other than that the roles of Wnt-4 in male and female gonad development remain open.

To achieve a more comprehensive view of the molecular mechanisms of mammalian sex determination and early gonad organogenesis, genome-wide technologies such as microarrays have been used [11–20,21]. To study the roles of *Wnt-4* in the ovarian vasculature [18] and in the differentiation of granulosa cells [19] such method was carried on, although the molecular mechanisms by which *Wnt-4* controls early sexual development remain poorly characterized.

We adopted the genome-wide microarray approach and the *Wnt-4* knock-out model to describe a panel of genes which can be considered candidates for the control of early female and male sex organogenesis. We found that the *Notum*, *Phlda2*, *Runx1* and *Msx1* genes appear to be typical of early wild-type ovaries, while the *Osr2*, *Dach2*, *Pitx2* and *Tacr3* genes point to a testicular fate. The expression of these genes is notably reversed in the absence of Wnt-4 function in the ovaries. Our result also showed that *Runx-1* in particular represents a target gene for Wnt-4 signalling, but its function is also necessary for *Wnt-4* expression, depicting a positive feedback signalling loop between these factors that advances ovary development. This finding led us to conclude that a panel of genes regulated by Wnt-4 signalling is expressed in a sexually dimorphic manner during the early stages of female sexual development. Moreover, the data support the conclusion that *Wnt-4* has a separate role in males, that of controlling organogenesis in the testis.

Materials and methods

Mouse lines

The *Wnt-4* (W*nt-4*$^{-/-}$), *Runx-1*, and *Tie2Cre;Runx1*$^{Rev/Rev}$ knock-out mouse models were crossed, sexed and genotyped as reported [5,22,23]. The gonads of wild-type (WT, *Wnt-4*$^{+/+}$) littermate embryos served as controls throughout the study. All the experiments involving mice were approved as being in accordance with the Finnish national legislation, the European Convention ETS 123 and the EU Directive 86/609/EEC.

RNA extraction, microarray and quantitative real-time PCR

Ovaries and testes were prepared from embryos obtained from the W*nt-4*$^{+/-}$ heterozygous mouse intercrosses. Gonads derived from female or male embryos were used after sexing and genotyping the embryos. Only gonads derived from W*nt-4*$^{-/-}$ or WT embryos without the mesonephros were used. The ovaries and testes were pooled separately, frozen immediately in liquid nitrogen, and stored at −70 °C until used for RNA purification with an RNeasy kit (Qiagen). Eight pairs of gonads of each sex were prepared at E12.5 and six at E14.5. The probability to generate mutant males and females in a litter coming from two heterozygote crossing is of 25% (Mendellian ratio). Due to this reason, we pooled 8 mutants together from different litters to be able to have the correct amount of starting material at E12.5. The same was done for E14.5. Three sets of independent biological RNA samples were hybridized with microarray gene chips, which were performed according to the instructions in the Affymetrix Gene Chip technical manual. Embryonic gonad-derived RNA (8 μg) was used as the template for synthesizing double-stranded DNA generated from the RNA by means of a one-cycle cDNA synthesis kit (Affymetrix) and T7-(dT) 24 primers. This *in vitro* DNA was purified with the Gene Chip sample cleanup module (Qiagen).

Biotin-labelled *cRNA* probes were generated by *in vitro* transcription with the Affymetrix IVT labelling kit as suggested by the manufacturer were used for analyzing the microarray gene chips. The synthesized biotinylated *cRNA* molecules were subjected to the Gene Chip sample cleanup module (Qiagen), fragmented and hybridized with the Affymetrix Mouse Expression Set 430_2.0 arrays, which contain around 45,000 mouse transcripts. After washes and an amplification step with streptavidin–phycoerythrin (Molecular Probes) coupled with the biotinylated anti-streptavidin (Vector Laboratories) antibody, the bound probes were identified, their intensity on the microarray slides being estimated with the Gene Chip Scanner 3000 and quantified in relation to a reference target value of 500. The resulting microarray data sets were analyzed with the Microarray Suite 5.0 program and the AffyBio conductor package [24,25].

To normalize the data, an RMA function based on a robust multichip average expression measure [26] was applied. The statistical analysis was performed in a log base two with a fold change >1.5 and p-Value <0.05, which was considered to represent a significant change in the values. The fold change represents the calculated average value of the replicates of each of the

affymetrix gene data sets generated. The microarray data have been deposited in the Gene Expression Omnibus (GEO) with the accession number GSE52730.

For the qPCR, 1 μg of gonad-derived RNA was converted to *cDNA* by a reverse transcription reaction using the first strand *cDNA* synthesis kit (Fermentas) as suggested by the manufacturer. The qPCR assays were conducted in three biological replicates from pooled gonads from different litters.

To analyze expression of the *Fst*, *Notum*, *Phlda2*, *Runx1*, *Dkk1*, *Cbln1*, *Osr2*, *Dach2*, *Pitx2*, *Tacr3* and *Msx1* genes, specific oligonucleotide primers were designed and synthesized (see Supplementary data Table I). Two microliters of the cDNA and 0.3 μM of the respective primers were mixed with the Brilliant SYBRGREEN qPCR master mix (Stratagene) in a total volume of 10 μl for the PCR reaction cycles. The MX 3005 P sequence detection system (Stratagene) was used to analyze the data, and the *Gapdh* gene was taken as a reference in all cases. The values were analyzed with Student's *t*-test and the qPCR results are presented as means ± standard error. The standard error was around 0.2, and $P < 0.05$ was taken to denote statistical significance.

Pair-wise comparisons for identification of the *Wnt-4*-regulated genes

Although pair-wise comparisons between the genes were performed at the level of the transcripts, multiple entries were also found for individual genes since there were multiple probes for some of the genes depicted in the lists.

By analogy with the method used by Jameson [20], we adopted single or multiple pair-wise comparisons between the samples as a mean of extracting the genes of interest from the data sets. A *p*-Value of 0.05 and a cutoff of 1.5 or 2 for the fold change in the microarray were selected as criteria for significance. When multiple pair-wise comparisons were performed, the genes indicated as undergoing changes in many of the array data sets were selected for further studies.

We used the following pairwise comparison criteria to identify the genes that were enriched in either of the sexes and common to both of the developmental stages: 1) the gene was expressed at a higher level in the *Wnt-4*$^{-/-}$ ovary than in the WT at E12.5, and 2) the values for relative expression at E14.5 were in the same range. Similar criteria were employed to identify the male genes that showed lineage-specific depletion.

Correspondingly, the following pairwise comparison criteria (with the cut offs depicted above) were used to identify genes enriched in a sex-specific manner: 1) the gene was expressed at a higher level in WT ovary than in the WT male at E12.5 (*i.e.* sex-specific expression), and 2) the gene was expressed at a higher level in the *Wnt-4*$^{-/-}$ female gonad than in the WT female embryonic gonad at E12.5 (*i.e.* female-specific expression).

The genes which overlapped between E12.5 and E14.5 were considered to represent the true Wnt-4 signalling targets. We adopted the same strategy to identify the genes which are enriched in the testes and included a *p*-Value > 0.05. To identify whether a gene identified as differentially expressed in the *Wnt-4*-deficient females and males represented either stromal or germ cell expression, the data sets generated at E12.5 were compared with those of Jameson et al.

In situ hybridization methods

The non-radioactive section *in situ* hybridization and the whole-mount *in situ* hybridization techniques were performed as described [8]. The *cDNA* probes were obtained as gifts from the following persons; *Fst* (Sergei Tevosian, USA), *Dkk1* (Sarah Millar, USA), *Pitx2* (Martin Blum, Canada), *Runx1* (Ditsa Levanon, Israel), *Osr2* (Jiang Rulang, USA), *Dach2* (Graeme Mardon, USA), *Tnfrsf19* (Marja Mikkola, Finland), *Msx1* (Image ID 4923403), *Cbln1* (Image ID 5684648), *Tacr3* (Image ID 30436441), *Notum* (Image ID 5125723) and *Phlda2* (Image ID 5042041). A minimum of three WT and three *Wnt-4*$^{-/-}$ gonad pairs were examined with each gene probe.

Ex vivo gonad organ culture and culture of their dissociated cells as reaggregates

A disaggregation/reaggregation assay was used to ascertain in more detail whether the Wnt-4 signal would regulate some of the genes identified as the major ones changed due to *Wnt-4* knockout. The cultures were set up according to Bowles et al. [27] on WT and Wnt4$^{-/-}$ mice. The signalling capacity of Wnt-4 was also analyzed in whole embryonic gonad cultures in the presence and absence of 100 ng/ml of the Wnt-4 recombinant protein (R&D system). The reaggregates were cultured for 48 h and frozen until used for RNA extraction as described above.

Nuclear extract and electrophoretic mobility shift assay (EMSA)

Mouse ovary-derived KK1 granulosa cell line was maintained in DMEM supplemented with antibiotics and serum (10% FBS, Sigma). Nuclear extracts were prepared from KK1 cell line using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific). The sequences of the *Runx-1* oligonucleotides were designed on the *Runx-1* mouse DNA and were used for the EMSA experiment: TCACTACCTCTTTTCTTCTCAAAGAGCCTGGGATGCTGACAGC (oligo 1), CAACTACTGAAGCTGATTTTCAAGGCTACTTAAAAAAAAAAAAAACTGC (oligo 2), AGCACGCGTGACTCTTATCTTCAAAGGTCTCCCCTGCATGGCAGA (oligo 3), TCACTCTGGATGCCTCCCTTCAAAGTAACCGTTGGAACAGTGAC (oligo 4), CTAAAGCTTTGGCTTACACGTCAAAGGGAAATTAGGCCAACTCCTG (oligo 5), AGTTCTCCCACTTTAGCAAATCAAAGACTCAGAGAGAAAGAACCTC (oligo 6). We also used oligonucleotides which contained mutations in the potential binding sites: CAACTACTGAAGCTGATT TTCTTGGCTACTTAAAAAAAAAAAAAACTGC (oligo 2 mutated) and AGC ACGCGTGACTCTTATCTTCTATGGTCTCCCCTGCATGGCAGA (oligo 3 mut ated). Equal amounts of complementary biotin-labelled oligonucleotides were annealed and used in LightShift chemiluminescent EMSA assay (Thermo Scientific). For competition analysis, a 200-fold molar excess of unlabelled annealed oligonucleotides was added. For supershift analysis, 2 μl of anti-Tcf-1 antibody (C63D9) (Cell Signalling) or 2 μl of anti-Sf-1 (Steroidogenic Factor 1) antibody (Abcam) were added to the EMSA reaction followed by incubation at RT for 40 min. Electrophoresis was then performed with a 6% non-denaturing polyacrylamide gel in TBE buffer. After electrophoresis the biotin-labelled DNA was electrophoretically transferred to a nylon membrane and then detected using a nucleic acid detection module (Thermo Scientific).

Immunohistochemistry

For the immunohistochemical studies, testes and ovaries at the stages indicated in the results section were prepared, dipped into a 30% sucrose solution at room temperature for 15 min, mounted

Table 1 – Expression of certain genes in the ovaries at two stages in development: microarray comparison between *Wnt-4*$^{-/-}$ females and WT females.

ID	FC E 12.5	FC E 14.5	Gene symbol	Gene name
1421365_at	−13.57	−6.07	Fst	Follistatin
1423635_at	−2.47	−2.68	Bmp2	Bone morphogenetic protein 2
1420425_at		−3.05	Prdm1	PR domain containing 1, with ZNF domain
1415854_at		−2.29	Kitl	Kit ligand

ID, identification; FC, fold change; E, embryonic stage.

to tissue freezing medium OCT, and frozen to generate cryostat sections. Thereafter the dried samples were stained with rabbit anti-Mvh antibody (Abcam, 1:200), rabbit anti-Runx-1 (Abcam, 1: 200), rabbit anti-Laminin (Abcam 1:250) or mouse anti-γH2AX antibodies (Millipore, 1:250). The corresponding Alexa Fluor-conjugated anti-mouse and anti-rabbit antibodies (Invitrogen) served to identify the bound primary antibody.

Bioinformatics analysis

The lists of up-regulated and down-regulated genes obtained from the microarray analysis at E12.5 and E14.5 were classified into specific categories using the Funnet program (http://www.funnet.info), which is an integrative functional genomics tool with annotations based on Gene Ontology and Kyoto Encyclopaedia of Genes and Genomes pathways.

We used the Ensemble programme to assess whether the *Runx-1* gene had TCF binding sites, as this enabled comparison and multiple sequence alignments. Four mammalian sequences were employed: *Mus musculus*, *Gorilla gorilla*, *Homo sapiens* and *Pan troglodyte*. The possible presence of putative conserved sequences mediating the binding of the Tcf-1 to the *DNA* (*TCAAAG*) and for Sf-1 (TCAAGGCTA) was screened from the *Runx-1* locus *in silico*.

Results

Absence of *Wnt-4* leads to changes in stage-specific gene expression in the embryonic gonad

The organogenesis of the mammalian gonad depends on the signals which are synthesized by the somatic, germ and mesonephric cells. *Wnt-4* is one crucial factor, since it is expressed in the somatic cells and its absence is characterized by a partial female-to-male sex reversal [5,7]. Thus *Wnt-4*$^{-/-}$ male and female embryos should provide a way of identifying a wealth of genes which are involved in the mammalian primary sex determination events and soon afterwards [9,6,28].

At E12.5, the fate of the gonad is determined to be either a testis, due to the Sry expression at E11.5, or an ovary due to *Wnt-4* or *Rspo-1* expression. At E14.5, the germ cells entered meiosis in the females, and they ceased mitotic cell divisions in the males. A deficiency in *Wnt-4* function may influence the somatic and germ cells of both sexes at E12.5 and E14.5 [5,7–9]. Given this, we set out to identify the putative Wnt-4 signalling target genes and prepared gonads from *Wnt-4*$^{+/-}$ litter mates at these two specific developmental stages. Total RNA was extracted, processed and subjected to affymetrix gene chips to assess potential changes in the transcriptome between the gonads of the *Wnt-4*$^{-/-}$ and WT embryos.

To test the feasibility of the approach, we set out first to determine from the microarray data whether any of the genes implicated in ovary development had been deregulated by the *Wnt-4* deficiency. As expected, the expression of *Fst* (*Follistatin*), a down-stream target of Wnt-4 signalling [29], *Bmp2* (*Bone morphogenetic protein 2*) [29], a regulator of *Fst*, and *Kitl* (*Kit ligand*) a survival factor for female primordial germ cells [30], were notably down-regulated relative to the controls (Table 1). We regard this as evidence that the approach is a feasible one for reliably detecting genes in the embryonic gonad which are regulated by *Wnt-4*.

Identification of candidate genes for female development by *in silico* subtraction of the microarray data

Of the 45,000 probes analyzed, the expression of only 101 genes at E12.5 and 93 genes at E14.5 was increased significantly by more than 1.5 fold as compared with the controls due to *Wnt-4* knockout (Fig. 1A, Supplementary Tables II–V). Similarly, 75 genes at E12.5 and 137 at E14.5 were down-regulated in the *Wnt-4*-deficient ovaries (Fig. 1A, Supplementary Tables II–V).

Interestingly, eleven out of the 176 genes which were up or down-regulated by more than 1.5-fold in the *Wnt-4*$^{-/-}$ ovary at E12.5 were to be found among the 220 such genes identified at E14.5. *Fst* was one of these [29], or the *Tnfrsf19* (*Tumour necrosis factor receptor superfamily member 19*) and *Wnt-4* genes, as expected (Fig. 1B).

We next used the microarray data to highlight those genes which were enriched in the individual key cell types of the gonad: supporting interstitial/stromal cells, germ cells and endothelial cells [20]. The aim was to reveal in what cell types *Wnt-4* would regulate the genes characterized here. *In silico* comparison revealed that Wnt-4 affects substantially different gonadal gene sets at E12.5 and E14.5 (Fig. 1B).

We went on to classify by means of the Funnet program those genes that were most notably changed by the absence of Wnt-4 signalling based on the function demonstrated for them. The approach showed that at the developmental stages studied here, compromised Wnt-4 signalling largely influenced genes in categories such as nuclear factors, extracellular matrix (ECM) proteins and plasma membrane associated proteins (Supplementary Fig. 1A and B). Hence Wnt-4 signalling has a profound effect on its target cells, from the ECM all the way to the nucleus, when its function is impaired.

One of our goals was to find novel candidate functional genes for the female sexual developmental pathway. This was done by means of pair-wise comparisons, focusing first on those genes whose expression was higher in the WT ovaries than in the WT testes at E12.5 and E14.5 (Fig. 1C and D). This comparison was expected to reveal the genes which are enriched in the early ovary. In addition,

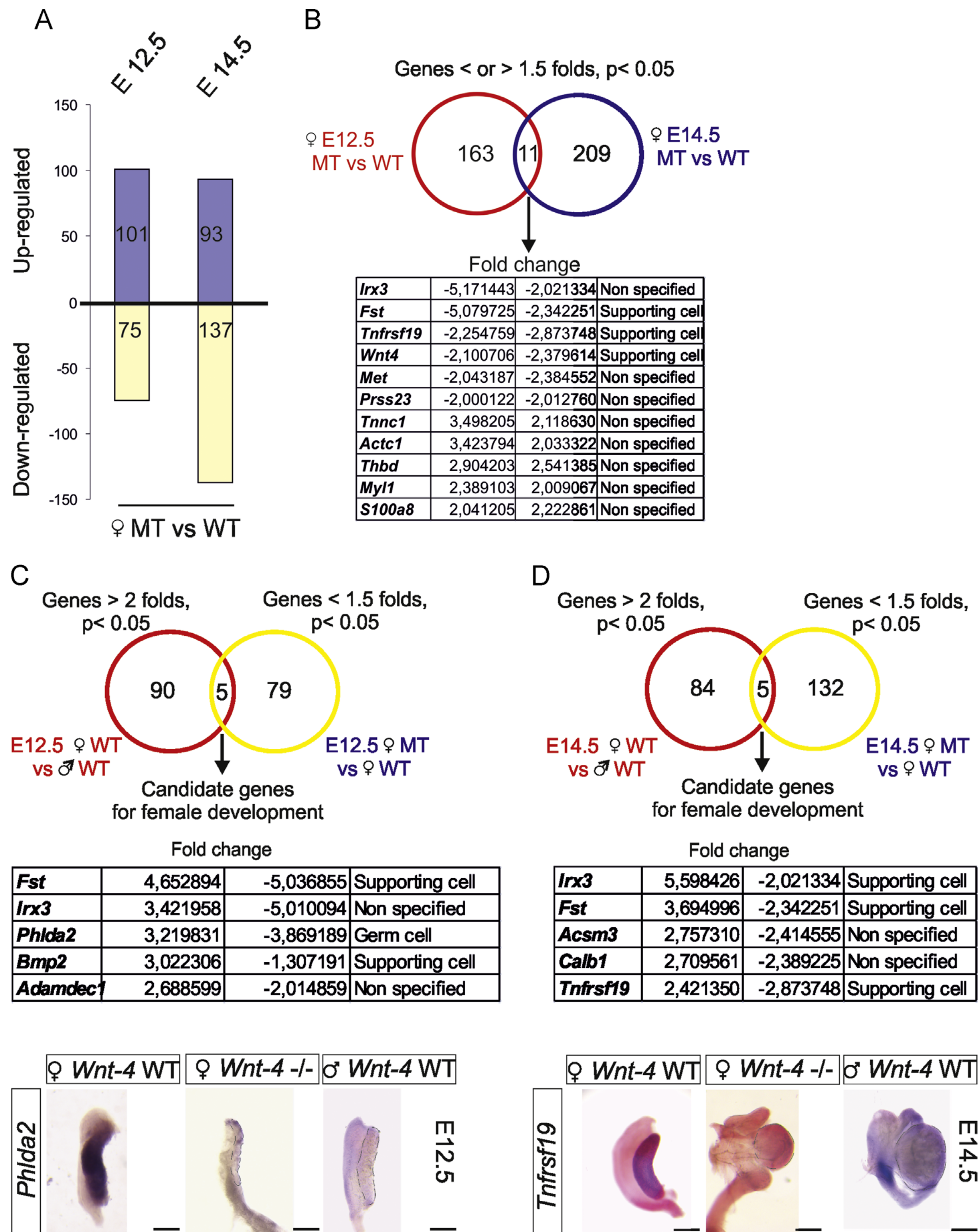


Fig. 1 – The selected pool of genes which are regulated specifically in the embryonic ovary. (A) Number of genes expressed in ovaries which have an expression signal that shows a statistically significant increase or decrease of 1.5 fold between the *Wnt-4*$^{-/-}$ and WT ovaries at stages E12.5 and E14.5. (B) Venn diagram illustrating the overlap in the number of significant genes that are differentially expressed between *Wnt-4*$^{-/-}$ and WT ovaries at stages E12.5 and E14.5. (C) Venn diagram illustrating the overlap in the number of significant differentially expressed genes between WT ovaries and testes as compared with *Wnt-4*$^{-/-}$ and WT ovaries at E12.5. *Phlda2* whole mount *in situ* hybridization for *Wnt-4*$^{-/-}$ and WT ovaries and WT testes confirmed the results of the comparison. (D) Venn diagram illustrating the overlap in the number of significant differentially expressed genes between WT ovaries and testes as compared with *Wnt-4*$^{-/-}$ and WT ovaries at E14.5. *Tnfrsf19* whole mount *in situ* hybridization for the *Wnt-4*$^{-/-}$, WT ovaries and WT testes confirmed the results of the comparison. (B, C, D) The common genes summarized in the tables were compared as described in Material and Methods and with previous data [20] in order to determine the specific cell type in which the gene is expressed. Non-specified depicts herein that the gene has not been signed to a specific lineage. WT, wild type; MT, mutant. Scale bar (C–D) 100 μm.

we compared those genes which were reduced by more than one and half-fold at E12.5 and E14.5 with those that were expressed differentially between the *Wnt-4*$^{-/-}$ and WT ovaries (Fig. 1C and D). Such *in silico* subtraction would highlight better those genes which may prove relevant for ovary development in the end.

Comparison of the transcriptome between the WT ovaries and testes identified a set of 95 genes whose expression was increased more than two-fold at E12.5 and 89 genes at E14.5 (Fig. 1C and D; red circles). When the transcriptome of the *Wnt-4* deficient ovaries was compared with the one derived from the WT ovaries, only 75 and 137 down-regulated genes (1.5-fold) were identified, respectively (Fig. 1C and D; yellow circle).

We went on to compare the data obtained from the two computed data sets at E12.5 and E14.5 (Fig. 1C and D; intersections of the red and yellow circles). Interestingly, these comparisons revealed only five genes at E12.5 and five genes at E14.5 whose expression had altered significantly between the data sets. One of these was the above-mentioned Wnt-4 signalling target *Fst* and the others were *Irx3* (*Iroquois 3*), *Phlda2* (*Pleckstrin homology-like domain, family A, member 2*), *Bmp2*, and *Adamdec1* (*ADAM-like, decysin1*) at E12.5, and *Irx3*, *Fst*, *Acsm3* (*acyl-CoA synthetase medium-chain family member 3*), *Calb1* (*Calbindin-1*) and *Tnfrsf19* at E14.5. The WT testis did not express the *Phlda2* and *Tnfrsf19* genes, but the WT ovarian cells did. Expression was reduced in the absence of *Wnt-4* in the female, as expected, which is in line with the microarray data depicted also with whole mount *in situ* hybridization analysis (Fig. 1C and D).

Wnt-4 deficiency deregulates expression of a panel of genes during female sexual development

To confirm the feasibility of the microarray data, we selected those genes which appeared to be enriched in the ovary and were illustrative of the female sexual differentiation pathway. We

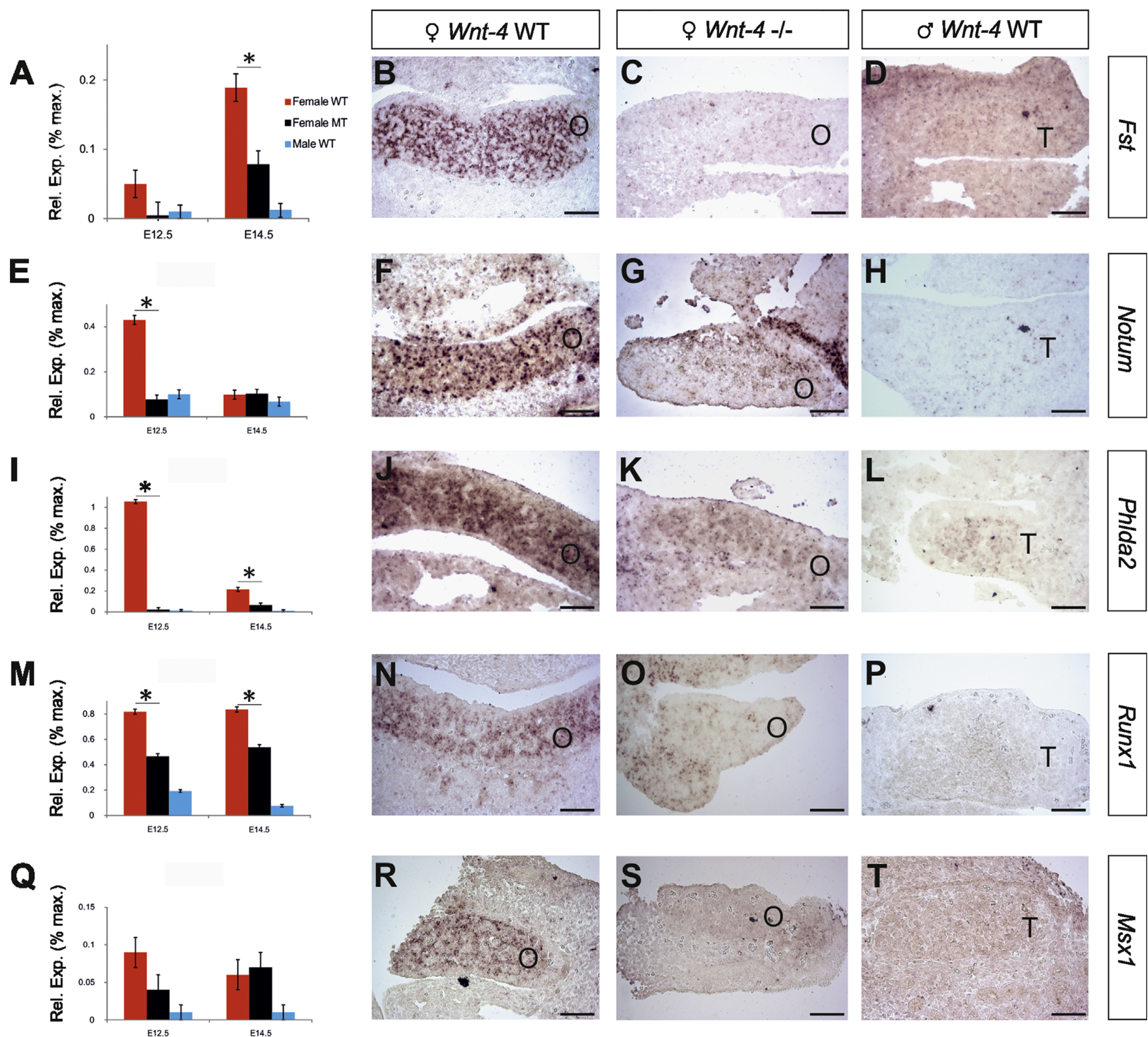
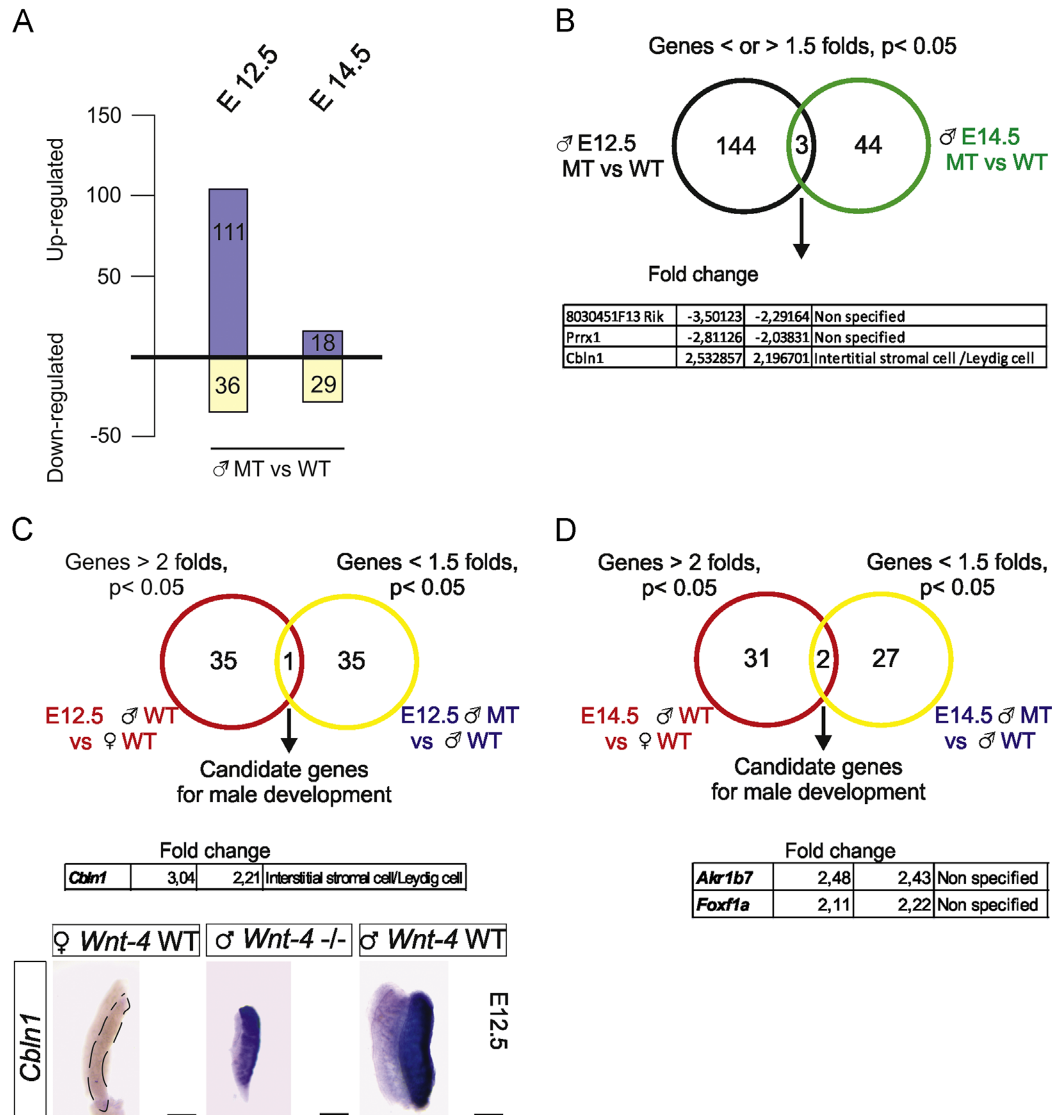


Fig. 2 – Expression profiles of candidate genes involved in the early steps of female sexual development. (A–T) *Fst*, *Notum*, *Phlda2*, *Runx1* and *Msx1* gene expression was analyzed in WT ovaries and testes and *Wnt-4*$^{-/-}$ ovaries at E12.5 and E14.5, by qPCR and at E12.5 by section *in situ* hybridization. Expression of all these genes was down-regulated in the *Wnt-4*$^{-/-}$ ovaries. O, ovary; T, testis. Scale bar (A–Y) 100 μm. WT wild type; MT mutant.

subjected them to qPCR, and also to section and whole-mount *in situ* hybridization studies (Fig. 2, Supplementary Fig. 2). The *Fst* gene was chosen as a positive control since its expression was high in the WT ovaries but low in the WT testes (Fig. 2A). Indeed, *Wnt-4* deficiency notably reduced *Fst* expression as judged by qPCR and *in situ* hybridization, which was consistent with earlier findings [29] (Fig. 2A–D).

We then picked the *Notum* [31], *Phlda2*, *Runx1* (*Runt-related transcription factor 1*, [32]), and *Msx1* (*Homeobox, msh-like 1*, [33]) genes for closer studies. The β-catenin/TCF complex, for example, interacts with the *Notum* promoter to regulate gene expression [31], making it an interesting putative Wnt-4 target. *Notum* is indeed expressed normally in the embryonic ovary, but its expression was down-regulated at E12.5 (Fig. 2E) in the absence of Wnt-4 function (Fig. 2E and F–H). Similarly, the ovarian expression of the *Phlda2* and *Runx1* genes was down-regulated in the absence of Wnt-4 function (Fig. 2I and M), as also shown by *in situ* hybridization (Fig. 2J–L and N–P).

Of the genes studied here, *Msx1*, which encodes a transcription factor, is implicated in meiosis [33] and is expressed in the ovary



8030451F13 Rik	-3,50123	-2,29164	Non specified
Prrx1	-2,81126	-2,03831	Non specified
Cbln1	2,532857	2,196701	Intertitial stromal cell /Leydig cell

Cbln1	3,04	2,21	Interstitial stromal cell/Leydig cell

Akr1b7	2,48	2,43	Non specified
Foxf1a	2,11	2,22	Non specified

Fig. 3 – The selected pool of genes which are regulated specifically in the embryonic testes. (A) Number of genes expressed in testes which have an expression signal showing a statistically significant increase or decrease of 1.5 fold between *Wnt-4*$^{-/-}$ and WT testes at stages E12.5 and E14.5. (B) Venn diagram illustrating the overlap in the number of significant differentially expressed genes between *Wnt-4*$^{-/-}$ and WT testes at stages E12.5 and E14.5. (C) Venn diagram illustrating the overlap in the number of significant differentially expressed genes between WT testes and ovaries as compared with *Wnt-4*$^{-/-}$ and WT testes at E12.5. *Cer* whole mount *in situ* hybridization for *Wnt-4*$^{-/-}$ and wild-type testes and wild type ovaries confirmed the results of the comparison. (D) Venn diagram illustrating the overlap in the number of significant differentially expressed genes between WT testes and ovaries as compared with *Wnt-4*$^{-/-}$ and WT testes at E14.5. (B, C) The common genes summarized in the tables were compared as described in *Material and methods* and with previous data [20] in order to determine the specific cell type in which the gene is expressed. Non-specified depicts herein that the gene has not been signed to a specific lineage. WT, wild type; MT, mutant. Scale bar (C) 100 μm.

at E12.5 and E14.5. Consistent with our microarray data, *Msx1* expression was lower in the *Wnt4*$^{-/-}$ ovary, as judged from the *in situ* hybridization studies (Fig. 2Q–T).

Comparison of our data sets with those of Jameson et al. (2012) indicates that *Phlda2* and *Msx1* represented the germ cell genes while *Fst*, *Notum* and *Runx1* were typical of the somatic cells (Fig. 2A–T and Supplementary Fig. 2). In conclusion, our data indicate that Wnt-4 signalling controls not only the genes in gonadal somatic cells, but also those in germ line cells.

Wnt-4 signalling also controls the genes associated with the male sexual development pathway

Wnt-4 was characterized initially as a female sex determinant which inhibits the male developmental programme in female embryos [5]. Besides this key role, however, *Wnt-4* has also been considered to take part in the control of testis organogenesis [9,10,34,35]. Thus we made use of the microarray data to extract putative *Wnt-4*-dependent genetic changes in the male embryonic gonad as well.

The genes expressed in the testes of the *Wnt4*$^{-/-}$ embryos were compared with those in WT testes at E12.5 and E14.5, focusing on those genes which are induced or repressed by more than 1.5-fold.

The *in silico* comparison highlighted 111 genes that were upregulated at E12.5 and 18 at E14.5, while 36 and 29 genes, respectively, were repressed in the early testis (Fig. 3A, Supplementary Tables VI–IX). The *Igfbp3* (*Insulin-like growth factor binding protein 3*, [36]) and *Col14a1* (*Collagen 14a1*, [15]) genes represent ones that are induced (Supplementary Tables VI–IX) and are involved in testis development [36,15]. Categorization of the *Wnt-4* regulated genes to functional classes revealed similar groups to those noted in the female embryos (Supplementary Fig. 1, compare D with C).

We then assessed whether any of the genes were common to the sets depicted at E12.5 and E14.5, so that they could be seen to have been either up or down-regulated (Fig. 3B). Unexpectedly, the comparison revealed only three genes: *8030451F13 Rik*, *Prrx1* (*Pair-related homeobox 1*) and *Cbln1* (*Cerebellin 1 precursor protein*) [18] (Fig. 3B, Supplementary Tables VI–IX).

We also made pair-wise comparisons to extract in more depth data that might identify other possible candidates for testis organogenesis and point to genes which were two-fold higher in the WT testes than in the WT ovary. We also looked for genes with one and half-fold reduced expression in the *Wnt-4*$^{-/-}$ testes than in the WT testes at E12.5 and E14.5 (Fig. 3C).

The analyses highlighted only *Cbln1* as a differentially expressed gene at E12.5 (Fig. 3C), and two genes *Akr1b7* and *Foxf1a* at E14.5 (Fig. 3D). Consistent with this finding, the *Cbln1* gene was expressed in the WT testis but not in the WT ovary, and its expression was reduced in the *Wnt-4* deficient testes (Fig. 3C). We conclude that the genetic programming behind testis and ovary development involves genes that are stage-specifically regulated by *Wnt-4*.

To substantiate our findings, we selected a panel of genes which appeared to be differentially expressed on the basis of the microarray data for qPCR and the *in situ* hybridization analysis. *Dkk1* (*Dickkopf homologue 1*) served as a control as it is enriched in the embryonic testis [37,38]. It was in fact robustly expressed in the WT embryonic testes, not detected in the WT ovaries, and reduced in the *Wnt-4*-deficient testes (Fig. 4A–D, Supplementary Fig. 2K and L). We speculate that *Dkk1* may suppress the female properties of Wnt-4 in the embryonic testis.

We also showed that the *Cbln1* [18], *Osr2* (*Oscar-2*), *Tacr3* (*Tachychin receptor 3*), *Pitx2* (*Paired-like homeodomain transcription factor 2*), *Dach2* (*Dachshund homologue 2*) [39–42] genes were enriched in WT embryonic testes. *Cbln1,* for example, which is expressed in interstitial cells (Fig. 4E–H) was reduced in the absence of *Wnt-4* (Fig. 4H), as it is also the case with the *Osr2* and *Tacr3* genes (Fig. 4I–L and M–P).

We then examined those genes which were absent from the WT ovaries but highly expressed in the WT testes, with reduced expression in the absence of *Wnt-4* function in the male. *Pitx2* (Fig. 4Q–T) and *Dach2* serve as examples of such genes in the seminiferous tubules (Fig. 4U–X). Taken together, we consider our findings to support the conclusion that Wnt-4 is indeed functional in the testis, having an intrinsic role in the coordination of its organogenesis.

Wnt-4 and *Runx-1* expression patterns are mutually interdependent in the embryonic ovary

Given the important role of *Runx-1* in the development of the aorta-gonad-mesonephros (AGM) region [22],its expression in granulosa cells [43,44] and its reduced expression in *Wnt-4*$^{-/-}$ ovaries (Fig. 2M–P, Supplementary Table III), we focused our attention on *Runx-1*.

The data indicate that the *Runx-1* gene is expressed in the early ovary and is not detected in the testis (Fig. 2M–P). The *in situ* hybridization analysis highlighted *Runx-1* expression in the ovarian somatic cells and a considerable decrease occurred relative to the controls in the absence of *Wnt-4* (Fig. 5A–C). Thus *Runx-1* expression depends on Wnt-4 function and could indeed represent a true Wnt-4 signalling target and was examined directly.

We first performed an *in silico* analysis to determine whether the *Runx-1* gene contained any conserved binding sites for the TCF transcription factor, a key mediator of the canonical Wnt signal transduction pathway. Comparison of four mammalian *Runx-1* sequences revealed five potential conserved sites (Fig. 5D) in the locus, in line with the possibility that *Runx-1* might indeed be regulated by the Wnt signalling pathway. Also one steroidogenesis factor 1 (SF-1) binding site was found. To confirm the potential Tcf-1 and binding sites, we used a mouse ovarian granulosa cell line (KK1) which is immortalized luteinizing granulosa cells [45].

The binding of nuclear proteins to oligonucleotide probes spanning the potential TCF1-binding sites was examined by EMSA. The oligonucleotides 1, 4, 5 and 6 did not demonstrate any signal shifts when they were combined with nuclear extracts from KK1 cell line, whereas oligonucleotides 2 and 3 formed protein–DNA complexes (S1 and S2) (Fig. 5E). Mutated oligonucleotides, which contained mutations in the potential binding sites 2(Sf-1)and 3(Tcf-1), were used in EMSA reactions. The nuclear protein binding to the oligonucleotide 3 did not depend on the presence of intact TCF1 binding site. On the other hand, mutated variant of oligonucleotide 2 did not bind any transcription factors in EMSA assay. The production of oligonucleotide 2-specific complex was prevented by competition from an excess of corresponding non-labelled DNA probe. Moreover, anti-Tcf-1 antibody almost completely prevented the formation of the DNA–protein complexes, indicating that Tcf-1 may bind the *Runx-1* gene directly at T2 site (Fig. 5E). We also used an antibody against

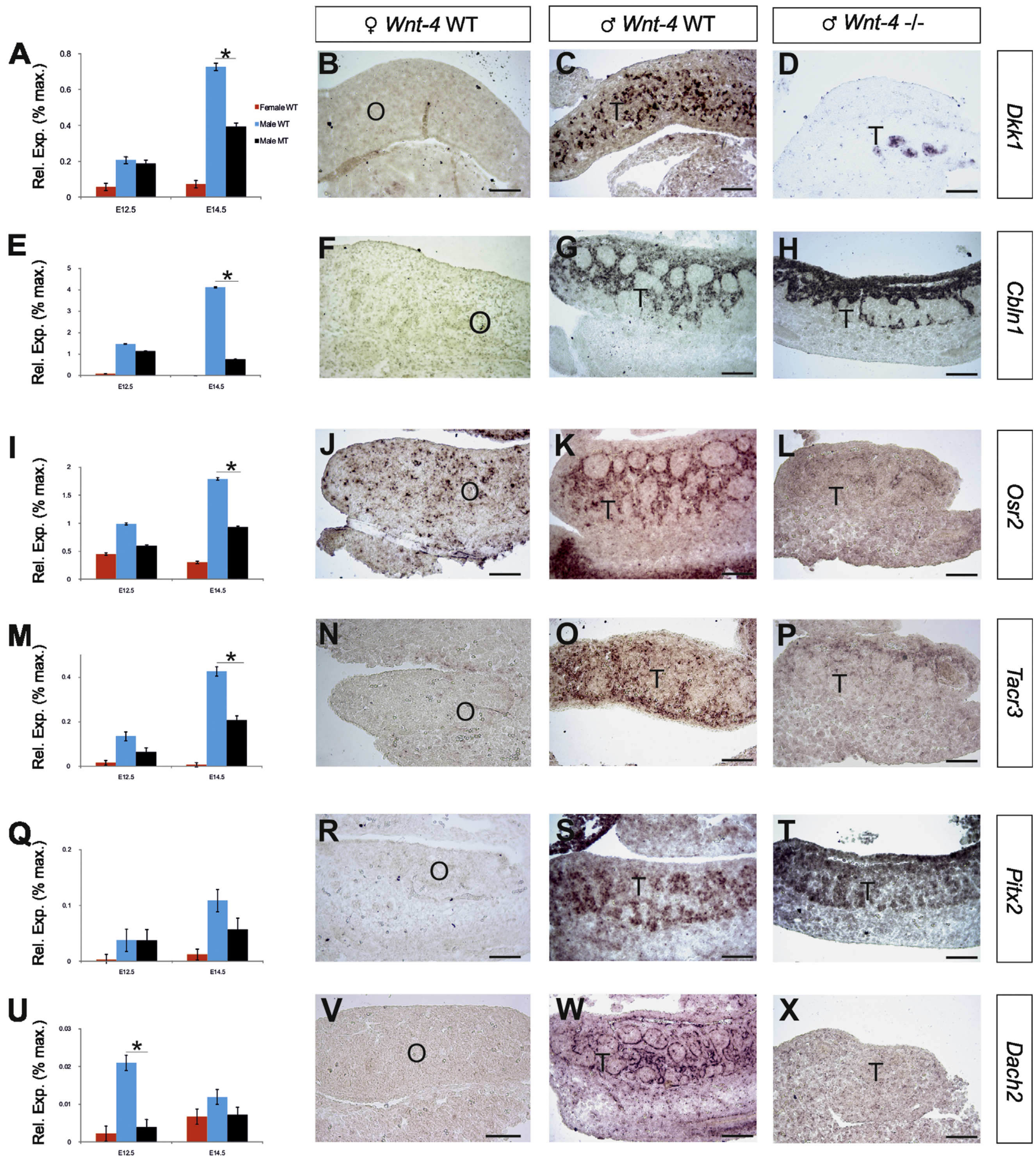


Fig. 4 – Developmental changes in the expression of a panel of genes during early male sexual development. (A–X) Changes in expression of the *Dkk1*, *Cbln1*, *Osr2*, *Tacr3*, *Pitx2* and *Dach2* genes were analyzed in embryonic WT ovaries and testes and *Wnt-4*$^{-/-}$ testes at E12.5 and E14.5 by qPCR and at E12.5 by *in situ* hybridization. O, ovary; T, testis. Scale bar (A–X) 100 μm. WT, wild type; MT, mutant.

Sf-1 and observed that a shift band appeared, but when both antibodies were combined the complex was completely reduced preventing the formation of DNA–protein complex with oligonucleotide 2 (Fig. 5E). This result indicates that Tcf-1 and Sf-1 might work synergistically to bind on the *Runx-1* gene.

After obtaining these data, we went on to the *in vivo* studies with *Runx-1* knock-out mice. *Runx-1* knock-out leads to anaemia and is lethal at around E12.5 [22], precluding any straightforward use of this model to address the roles of Runx-1 in the control of *Wnt-4* expression. Prior to the *in uteri* death of the *Runx-1*$^{-/-}$ embryos, however, *Wnt-4* expression in the somatic embryonic ovarian cells was similar to the WT ovary (Fig. 6, compare B with A), but no *Wnt-4* expression was observed in embryonic male cells (Fig. 6C).

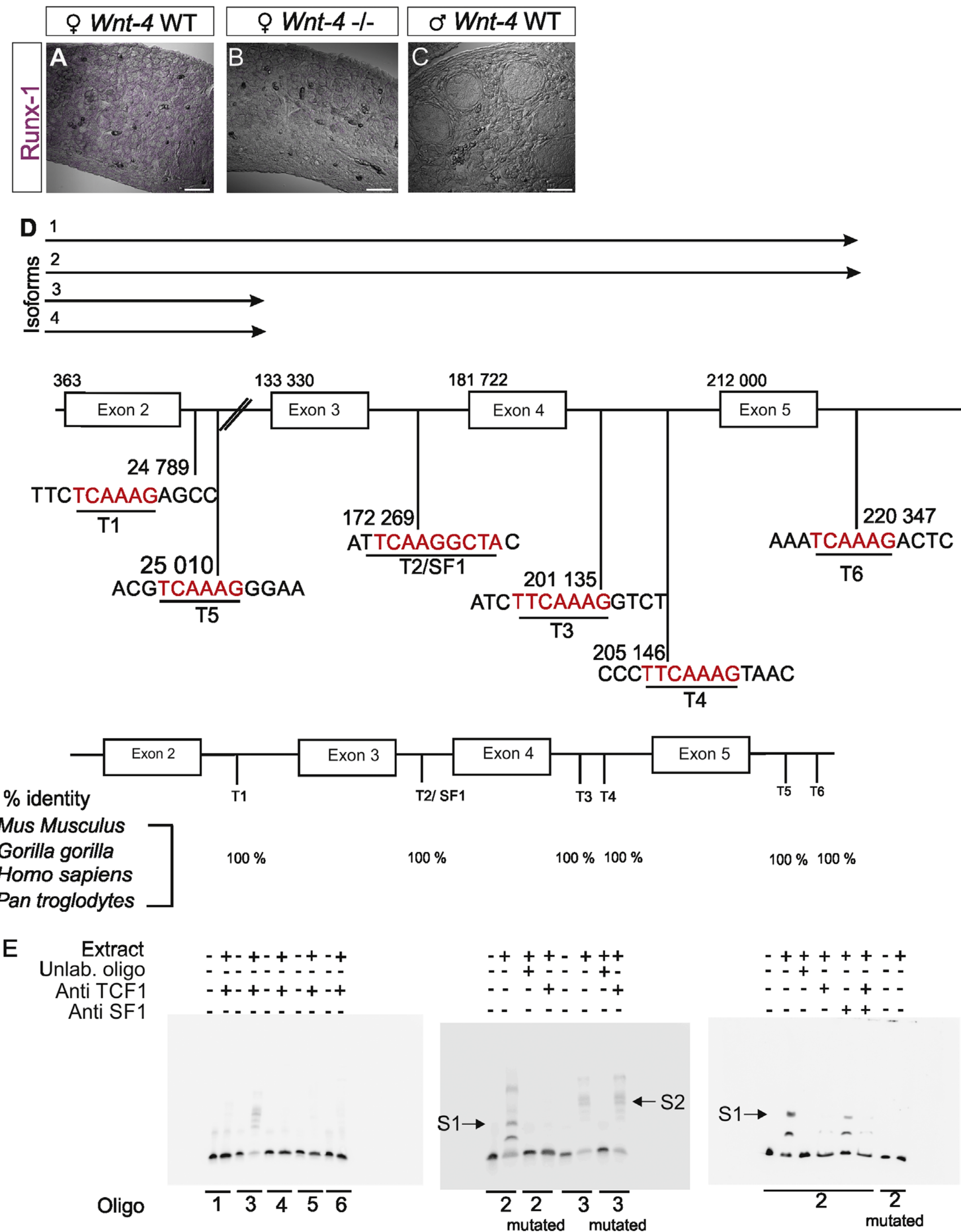


Fig. 5 – *In silico* analysis of TCF binding sites on the mammalian *Runx-1* gene. (A, B, C) Immunohistochemistry of Runx-1 in WT and *Wnt-4*$^{-/-}$ ovaries and WT testes. (D) *Runx1* contains four isoforms (splice variants 1, 2, 3 and 4) which are common to the four selected mammalian sequences. We found five conserved TCF and one SF1 binding sites in the *Runx-1* gene. (E) EMSA analysis of the potential binding sites on the *Runx-1* gene. Nuclear extracts, unlabelled control oligonucleotides, anti-Tcf-1 and anti-Sf-1 antibodies were added to the binding reactions as indicated. S1 and S2 – shifted bands for oligonucleotides 2 and 3, correspondingly. Scale bar (A–C) 100 μm.

To overcome the embryonic lethality caused by *Runx-1* deficiency, we made use of yet another mouse model in which the *in utero* deficiency is rescued by targeted expression of the *Runx-1* gene in the embryonic endothelial cells [22]. We crossed *Runx-1* floxed mice with *Tie2-Cre* ones, thereby rescuing this *Tie2Cre*; *Runx1*$^{Rev/Rev}$ cross from the embryonic lethality of the *Runx-1* knock-out [23].

The targeted endothelial *Runx-1* gene expression in the *Tie2Cre; Runx1*$^{Rev/Rev}$ knock-out background did not, however, rescue the *Wnt-4* gene expression in the embryonic ovary (Fig. 6, compare E with D), looking similar to the WT testes (Fig. 6F). Since meiosis entry is compromised in the absence of Wnt-4 signalling, we assessed the fate of meiosis in these embryos using the γH2AX protein as a meiosis marker [8]. *γH2AX* protein expression was reduced to some extent in

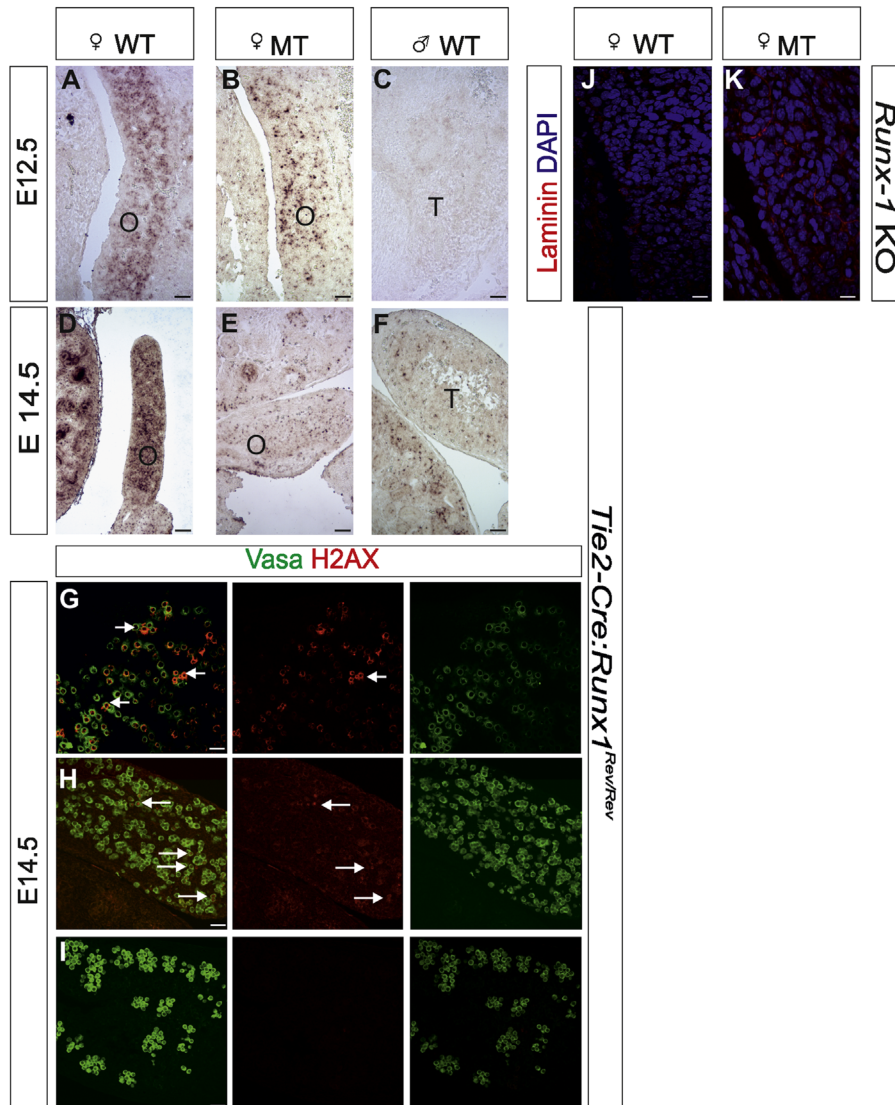


Fig. 6 – Expression of *Wnt-4* in the *Runx-1*-deficient ovary. (A–F) *Wnt-4* expression was down-regulated at E12.5 of the *Runx-1* knock-out female embryos and at E14.5 in the ovaries of the *Tie2-Cre;Runx1$^{Rev/Rev}$* crossing. (G-I) Germ cells are identified with anti Mvh antibodies (in green) and meiosis with anti γH2AX antibodies (in red), which denote the germ cells of the wild-type and *Tie2-Cre; Runx1$^{Rev/Rev}$* deficient embryonic ovaries. Note that the germ cells of normal testes do not express γH2AX. (J, K) Depiction of Laminin expression pattern in WT and *Runx-1*$^{-/-}$ ovaries. Scale bar (A–F) 250 μm, (G–K) 200 μm. O, ovary; T, testis. WT, wild type; MT, mutant.

the *Tie2Cre*; *Runx1$^{Rev/Rev}$* embryos, and was not rescued even in part by endothelial retargeted expression of the *Runx-1* gene. The anti-Vasa immunostaining suggested that germ cells were present in related intensities in the ovaries of the *Tie2Cre;Runx1$^{Rev/Rev}$* and WT embryos (Fig. 6G–I). We conclude that the Runx-1 and the Wnt-4 signalling pathways are mutually interdependent in the ovary. To further analyze the *Runx-1* ovary fate, laminin staining was performed. The ovaries of the Runx-1$^{-/-}$ females demonstrated seminiferous-like peritubular laminin expression and the ovary appeared more round in shape and differed in this respect from WT ovary at this stage (Fig. 6J and K).

Wnt-4 signalling enhances *Runx-1* expression in the embryonic gonad *ex vivo*

Given the mutual dependence of *Runx-1* and *Wnt-4* expression, we applied the *ex vivo* experimental approach to determine in such a setting whether Wnt-4 would regulate *Runx-1* expression. The classic embryonic gonad cell dissociation and reaggregation technology was employed for this purpose [46]. In this case the Wnt-4 signal, which normally shows poor diffusion in tissues, due in part to Wnt binding to the heparan sulphate of the extracellular matrix and the cell surface proteoglycans, is exposed to gonadal cells in its close proximity.

We dissected the embryonic ovaries out at E12.0 and dissociated the constituent cells after treatment with a mild enzyme, washed them and reaggregated them in the presence or absence of the Wnt-4 recombinant protein. Dissociation and reaggregation of the primary embryonic gonadal cells with or without the Wnt-4 protein, together with a 48-hr sub-culture of the reconstituent caused some degree of recovery of the gonadal structure, since germ cells, presumptive germ-line nest in female and seminiferous tubule-like structures in male were noted (Fig. 7A and data not shown).

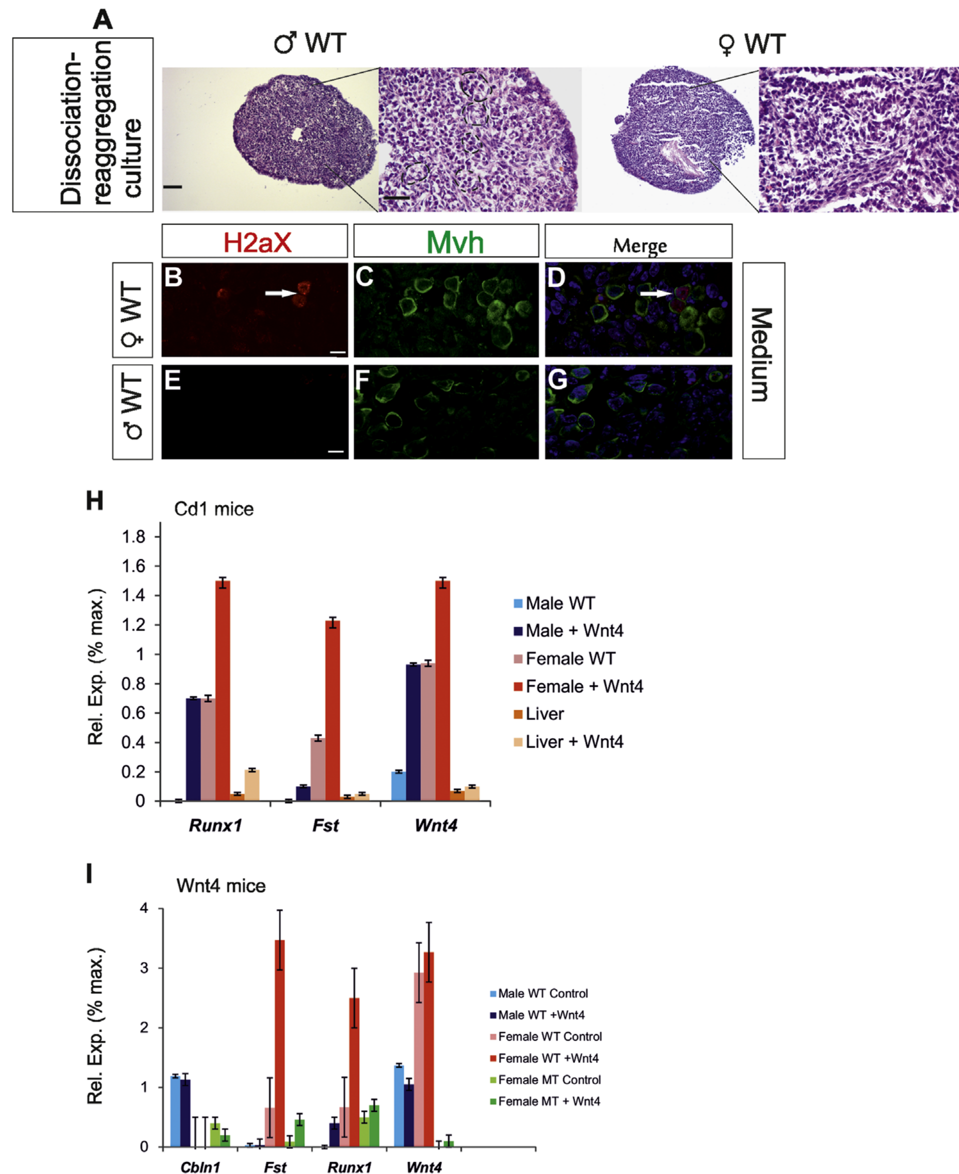


Fig. 7 – Recovery of candidate *Wnt-4* target genes upon Wnt-4 gain of function in the reconstituted organotypic gonadal model. (A) Histology of the reaggregates after prior dissociation of gonadal cells obtained from wild-type female and male embryos at E12.5, cultured for 48 h and shown in the form of a high power micrograph of a pellet of reaggregated cells. (B–G) Anti-Mvh antibody-mediated staining of a germ cell (in green). The anti-γH2AX antibodies (in red), indicating meiosis in the germ cells, are expressed throughout the ovaries of the wild-type embryos cultured for 48 h, but γH2AX is not expressed by the embryonic testicular germ cells. (H) Histograph illustrating *Runx1*, *Fst* and *Wnt-4* gene expression in CD1 gonadal and liver cells which had been dissociated and reaggregated alongside the Wnt-4 recombinant protein and subcultured for 48 h. (I) Histograph illustrating *Cbln1*, *Runx1*, *Fst* and *Wnt-4* gene expression in *Wnt-4* gonadal cells which had been dissociated and reaggregated alongside the Wnt-4 recombinant protein and subcultured for 48 h. Scale bars: (A) 50 μm and (B–G) 100 μm. WT, wild type; MT, mutant.

It was concluded from the occurrence of γH2AX expression that the germ cells had regenerated their capacity to initiate meiosis even after dissociation and reaggregation of the ovarian cells (Fig. 7B–D) [47]. The γH2AX meiosis marker was not expressed in similar reaggregates produced from embryonic testis, as expected (Fig. 7E–G). We conclude that this set-up also provided a way of addressing the roles of Wnt-4 signalling in the control of *Runx-1* and *Fst* expression.

The presence of Wnt-4 protein in the dissociated and reconstituted gonad auto-induced its own gene expression, in line with findings in the embryonic kidney [48] (Fig. 7H). The presence of Wnt-4 signalling in the reaggreates induced expression of the *Fst* and the *Runx-1* genes as compared with the controls (Fig. 7H). The liver, which also contains *Runx1,* served as a negative control here [22] (Fig. 7H). To confirm the data obtained from the *CD1* mice, we generated similar reaggregates using the *Wnt-4* mice (Fig. 7I). One male gene *Cbln1* was added in the assay which is expressed in WT males and *Wnt-4*$^{-/-}$ females. By supplementing Wnt4 protein into the medium, *Cbln1* expression was reduced in both WT males and *Wnt-4*$^{-/-}$ females, suggesting that Wnt4 signals rather directly to control the expression of these selected genes (Fig. 7I). *Runx-1* and *Fst* expression was increased in the

reaggregates in all genotypes (Fig. 7I). These data reinforce the conclusion that the functions of Runx-1 and Wnt-4 are well coordinated by their own activities to advance the developmental programme that leads to assembly of the ovary.

Discussion

Mammalian sex determination and the putative *Wnt-4* gonadal target genes

The mammalian gonad is initially indifferent, but around mid-gestation it is directed to follow either the male or the female sexual development pathway, *i.e.* to form testes or ovaries. Consequently, considerable interest has been shown in identifying the genes involved during these critical developmental stages. Since *Wnt-4*-deficient female embryos undergo a partial sex reversal and adopt certain critical male characteristics, Wnt-4 serves as good model for depicting the genes involved in the early sexual development of female and male embryos.

Applying microarray technology, we did indeed identify Wnt-4-dependent changes in *Fst* and *Dkk1* expression. *Fst* has been shown to take part in the Wnt-4-regulated early female sexual development steps [29], while *Dkk1* takes part in the control of organogenesis in the testes [37,38]. We therefore considered that the approach selected here faithfully depicted those genes that are relevant for early gonad development.

The strategy of comparing genes which are differentially expressed between wild type and the *Wnt-4*$^{-/-}$ ovaries, and correspondingly the testes, at E12.5 and E14.5, revealed a panel of candidate *Wnt-4*-related genes. Based on these results, we speculate that, apart from having a role in female sex organogenesis, Wnt-4 signalling is also involved in early testis development, a fact which warrants further investigation.

Out of the list of 50 genes that were altered most in the absence of Wnt-4 function, we focused on eleven genes for which earlier data had suggested an association with Wnt signalling, sex determination/differentiation and the hormone synthesis pathways. Among these, the *Fst*, *Phlda2*, *Dkk1*, *Cbln1*, *Osr2* and *Pitx2* genes were of particular interest, since they have been considered in the light of earlier microarray studies to be potential gonad development genes [18,32,12,11,49]. The *Notum*, *Msx1*, *Dach2*, *Tacr3*, and *Runx1* genes were selected for further study due to the robustness of the changes in their expression in the absence of Wnt-4 signalling. These genes may be considered to represent part of the characteristic signature of ovary and testis development and as such may serve to highlight the developmental programme that directs the indifferent gonad to construct either an ovary or a testis. Whether the identifiable parts of such gonadal signature genes indeed function directly as a cascade in the female and male *Wnt-4*-controlled gonad development programmes remains to be seen.

The *Notum* and *Dach2* genes encode proteins that serve as antagonists of the Wnt signalling pathway in the fly and the planarians [50,51]. *Notum* expression was reduced by *Wnt-4* knock-out and it may fine-tune *Wnt-4* signalling in the ovary and serve as a *Wnt-4*-dependent antagonist to the Wnt-4 signal.

In the fly, the Dach2 and Wnt-4 functions are connected [42]. Dach2 expressed in the endothelial cells signals to the adjacent dorsal ectodermal cells to regulate *Wnt*-4 expression during myogenesis. As *Dach2* expression is down-regulated in the absence of *Wnt-4* in the mammalian gonad, this may suggest the existence of a *Wnt-4*-dependent antagonist which would normally express *Dach2*.

Our earlier work demonstrated that the absence of Wnt-4 signalling changes the expression of the *Sypc1*, *Sypc3*, *Msh5*, *Spo11* and *Stra8* genes and that the entry of germ cells into meiosis was severely compromised [8]. The *Msx1* gene triggers meiosis in female germ cells in synergy with retinoid acid and *Stra8*, a key meiosis entry factor [33]. Since *Wnt-4* provides a critical signal for meiosis control [8], the identification of *Msx-1* as a putative *Wnt-4* signalling target gene may represent a hallmark of how Wnt-4 signalling contributes to meiosis control in mammals.

Of the Wnt-4-regulated genes, the *Tacr3* gene product is connected to the tachykinin pathway. Since Tacr3 function is involved in the hypothalamic-pituitary-gonadal axis, which also involves Wnt-4 function, we selected *Tacr3* for further study. Tacr3 is a G protein-coupled receptor which is expressed by the Leydig cells in the testis [52]. Since *Wnt-4* deficiency induces ectopic androgen-producing Leydig cells [7], *Tacr3* expression in the *Wnt-4*-deficient gonad may be another indication of the ectopic activation of the male programme for controlling genes which are normally inhibited by Wnt-4 signalling in the female gonad. Thus the Wnt-4 signal may normally inhibit *Tacr3* gene expression in the early ovary to promote the female sex determination process regulated by *Wnt-4* [5].

Runx-1 as a target gene for Wnt-4 signalling with putative roles in ovary development

The *Runx* genes encode critical transcription factors in several biological processes [53]. *Runx1*, also known as *Acute Myeloid Leukaemia 1* (*AML1*), regulates haematopoietic stem cells in the aorta-gonad-mesonephros (AGM) region [22]. The *Runx1* gene is present in the chromosomal region that undergoes translocation in association with leukaemia, for example. This suggests that *Runx-1* regulates cell differentiation and transformation when it is hit by a mutation [53]. Besides the AGM, the *Runx-1* has also been localized to the granulosa cells [54], and changes in its expression correlate with the maturation of the female follicle during the menstrual cycle, with a peak during ovulation [44].

Since *Runx-1* transcripts appear in the mouse gonad after the sex determination stages [11], we speculated that Runx-1 would be a suitable Wnt-4 signalling target. *Runx-1* controls cell proliferation and cell differentiation during intra-embryonic haematopoietic system development in the mouse [55] and Wnt-8 similarly induces Runx-1 in the haematopoietic stem cells of the zebrafish [56]. We found here that *Wnt-4* expression is substantially reduced in the *Runx-1*-deficient ovary, and that *Runx-1* expression was reduced in the absence of Wnt-4 function. We found one Tcf-1 and one Sf-1 binding sites on the *Runx-1* gene. ChIP-seq data from Wu et al. [57] showed that TCF-7, also known as Tcf-1, bind onto the *Runx-1* gene at several places in the mouse multipotential hematopoietic cell line, demonstrating interactions between TCF-1 factor and *Runx-1* gene. Moreover, Wnt-4 gain-of-function induced *Runx-1* expression in embryonic gonadal cell reaggregates in WT and in Wnt4$^{-/-}$ female. Collectively these data reinforce the conclusion that these two components are mutually dependent on each other's activity. Indeed, TCF1 (Lef1) binds to the *Runx2* regulatory sequences in osteoclasts that bring about promoter regulation in a ß-catenin-dependent manner [58]. Based on these findings and the presence of functional Tcf and Sf-1 binding sites in the *Runx-1* gene, we

speculate that Tcf-1 may indeed synergize to coordinate *Wnt-4* gene regulation in the gonad and advance ovary development. The various *Wnt-4* mouse models generated in recent years, such as *Wnt-4Cre*, *Wnt-4 floxed* and *Wnt-4mCherry* may serve as good models for addressing the potential roles of direct target of secondary Wnt-4 signalling in the control of gonad development.

To summarize, we have identified a panel of candidate genes which may contribute to mammalian sex determination, somatic and germ cell differentiation and the formation of the ovary and testis. This study has revealed a panel of candidate genes subject to Wnt-4 signalling that may be essential for forming the mammalian gonad.

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F.N. and S.V. conceived and designed the study. A.L. provided the samples. F.N., R.K., A.S., Q.X. performed the experiments and F. N. collected the data. W.Y., Z.S., B.S. performed the data analysis of the microarray and A.M., S.Q. gave important expertise. F.N. and S. V. wrote the draft manuscript. All authors contributed to the final version of the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.01.010.

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