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Search for the genes involved in oocyte maturation and early embryo development in the hen

Sebastien Elis1, Florence Batellier1, Isabelle Couty1, Sandrine Balzergue2, Marie-Laure Martin-Magniette2, Philippe Monget1, Elisabeth Blesbois1 and Marina S Govoroun*1

Address: 1Physiologie de la Reproduction et des Comportements, UMR 6175 INRA-CNRS-Université F. Rabelais de Tours, Haras Nationaux, 37380 Nouzilly, France and 2Unité de Recherche en Génomique Végétale UMR INRA 1165 – CNRS 8114 – UIEVE 2 Rue Gaston Crémieux, CP 5708 91057 Every cedex, France

Email: Sebastien Elis - sebastien.elis@voila.fr; Florence Batellier - florence.batellier@tours.inra.fr; Isabelle Couty - isabelle.couty@tours.inra.fr; Sandrine Balzergue - balzerg@evry.inra.fr; Marie-Laure Martin-Magniette - martin@evry.inra.fr; Philippe Monget - philippe.monget@tours.inra.fr; Elisabeth Blesbois - elisabeth.blesbois@tours.inra.fr; Marina S Govoroun* - marina.govoroun@tours.inra.fr

* Corresponding author

Abstract

Background: The initial stages of development depend on mRNA and proteins accumulated in the oocyte, and during these stages, certain genes are essential for fertilization, first cleavage and embryonic genome activation. The aim of this study was first to search for avian oocyte-specific genes using an in silico and a microarray approaches, then to investigate the temporal and spatial dynamics of the expression of some of these genes during follicular maturation and early embryogenesis.

Results: The in silico approach allowed us to identify 18 chicken homologs of mouse potential oocyte genes found by digital differential display. Using the chicken Affymetrix microarray, we identified 461 genes overexpressed in granulosa cells (GCs) and 250 genes overexpressed in the germinal disc (GD) of the hen oocyte. Six genes were identified using both in silico and microarray approaches. Based on GO annotations, GC and GD genes were differentially involved in biological processes, reflecting different physiological destinations of these two cell layers. Finally we studied the spatial and temporal dynamics of the expression of 21 chicken genes. According to their expression patterns all these genes are involved in different stages of final follicular maturation and/or early embryogenesis in the chicken. Among them, 8 genes (btg4, chkmos, wee, zpA, dazL, cvh, zarl and ktfn) were preferentially expressed in the maturing oocyte and cvh, zarl and ktfn were also highly expressed in the early embryo.

Conclusion: We showed that in silico and Affymetrix microarray approaches were relevant and complementary in order to find new avian genes potentially involved in oocyte maturation and/or early embryo development, and allowed the discovery of new potential chicken mature oocyte and chicken granulosa cell markers for future studies. Moreover, detailed study of the expression of some of these genes revealed promising candidates for maternal effect genes in the chicken. Finally, the finding concerning the different state of rRNA compared to that of mRNA during the postovulatory period shed light on some mechanisms through which oocyte to embryo transition occurs in the hen.
Background

The activation of molecular pathways underlying oocyte to embryo transition (OET) depends exclusively on maternal RNAs and proteins accumulated during growth of the oocyte [1]. During OET and preimplantation development in mice, the embryo becomes almost autonomous, and may gradually eliminate maternal components. Indeed, by the two cell stage, the major pathways regulated by maternal mRNA are targeted protein degradation, translational control and chromatin remodelling [2]. The recruitment of maternal mRNA for translation has long been recognized as a widespread mechanism to generate newly synthesized proteins in maturing oocytes and fertilized eggs [3]. Conversely, RNA that is no longer needed is actively degraded in the early embryo [4]. Moreover, careful regulation of proteolysis during the same period is likely to be important in oocytes, which are predominantly transcriptionally inactive and must often wait for long periods before fertilization in different species such as Drosophila, Xenopus, Caenorhabditis and Zebrafish [5]. Maternal transcripts that are present in the early pre-implantation embryo can be subdivided into two classes according to whether they are re-synthesized soon after embryonic genome activation or not. The first is common to the oocyte and early embryo and is replenished after activation of the zygotic genome. The second consists of oocyte-specific mRNA that is not subsequently transcribed from zygotic genes in the embryo. This class of mRNA may be detrimental to early post-fertilization development [6].

Maternal effect genes have been found in several species ranging from invertebrates to mammals. Wide screening of mutants has been performed in invertebrates as Droso phila melanogaster [7] and Caenorhabditis elegans [8] where several mutations lead to arrest of early embryo development. Although females bearing this type of mutation are viable and appear to be normal, the development and survival of their embryos are compromised [9]. Maternal effect mutations have also been described in other vertebrates such as Danio rerio for the nebel gene [10], and Xenopus laevis for the af gene [11]. Despite the fact that maternal effect mutations are well known in lower organisms, only a few examples have been reported in mammals. All of them are based on knock-out experiments and concern three murine genes, i.e. Dnmt1, Hsf1 and Mater [9]. Mater (Maternal antigen that embryos require) is a single-copy gene that is transcribed in growing oocytes. Although its transcripts are degraded during meiotic maturation, MATER protein persists into the blastocyst. Female mice lacking this 125 kDa cytoplasmic protein produce no offspring because of an embryonic block at the early cleavage stage. Thus, Mater is one of few documented genes for maternal effect in mammalian development [12]. Mater has been found in bovine models but there is no report in the literature on maternal effect genes conserved between species.

No information has been available to date on maternal effect genes in birds. However, birds represent a good model to observe progressive accumulation of mRNA in the oocyte before ovulation. The embryonic genome of a model bird, i.e. the chicken, is activated when the embryo contains 30,000–50,000 cells [13] 24 h after fertilization. Proteins and mRNA, accumulated as the chicken oocyte matures, are essential not only for fertilization and first cleavage but also for supporting a high number of embryonic cell divisions before genome activation. By comparison, the embryonic genome is activated at the 8-cell stage in bovines [14] and at the 2-cell stage in the mouse [15]. The avian oocyte consists of a large amount of yolk and a structure called the germinal disc (GD) [16]. The GD is a white plaque of about 3−4 mm diameter on the top of the oocyte. It contains the nucleus and 99% of oocyte organelles although it occupies less than 1% of the cell volume [17]. Structurally, and therefore functionally, the GD is mostly equivalent to the mammalian oocyte. The ovary of the reproductively active hen consists of small pre-hierarchical follicles and maturing preovulatory follicles showing a hierarchy according to size (F6 to F1) [18]. Only a few studies have reported on gene expression in the oocyte and during early embryo development in the chicken. The dynamics of the overall RNA profile of the chicken oocyte through different maturation stages has been described by Olzanska et al. [13,19-22]. Chicken vasa homolog protein (CVH) was hypothesized to be maternally inherited in the chicken embryo, since it has been localized in chicken oocytes and during first cleavage [23]. Another protein, Epidermal Growth Factor, was found in F2 GD and its potential role in follicular development has also been investigated [24]. Since oocyte-specific genes expressed during follicular maturation and after ovulation are potentially involved in the fertilization process and in early embryo development, and almost no information is available on these genes in birds, the aim of this study was to identify avian oocyte-specific genes and then to investigate the temporal and spatial dynamics of their expression during follicular maturation and early embryogenesis. We chose initially to focus on oocyte-specific genes because the accumulation of their transcripts in the oocyte should have greater consequences on fertilization and OET. Two different strategies were used to identify avian genes potentially involved in oocyte developmental competence. The first was based on a candidate gene approach and consisted of a search for avian homologs of murine oocyte genes, previously identified by digital differential display [25]. The second strategy involved a global transcriptomic approach based
on chicken Affymetrix microarray. We report here several novel chicken genes with potential maternal effect identified using these two strategies. We also describe the spatial and temporal dynamics of the expression of some of these genes as well as some potential mechanisms in which they could be involved. We also compare chicken and murine orthologs in terms of their tissue specificity and their potential involvement in oocyte developmental competence and/or early embryogenesis.

**Results**

**In silico search for chicken homologs of murine oocyte genes**

Differential digital display analysis performed on murine tissues provided a list of 101 potentially oocyte-specific murine genes [25-27]. Bioinformatic analyses were performed on this list of genes in order to find potentially oocyte-specific chicken orthologs. Genes with a blast score higher than 100 were localized using mapview [28] and blatsearch [29] tools. The syntenic regions were checked: chromosome localization of murine genes and chicken homologs were compared, in order to obtain the correct chicken ortholog of mice genes. Only genes with sufficient homology or whose localization was in accordance with syntenic regions were selected. Forty-one chicken genes were eliminated because of their poor homology with murine genes and 32 other genes were eliminated because they were localized outside the syntenic regions. Among the remaining 28 chicken genes the transcript of only 18 genes could be correctly amplified using real time or classic RT-PCR, of which the detailed study of two genes bmp15 and gdf9 has previously been reported [30]. Thus 16 avian genes were finally retained (Table 1). The homology with murine genes was strong for 11 of these genes (blast score between 288 and 2149) and was weak for 5 (blast score between 104 and 132). The last five chicken genes were nevertheless considered as potential orthologs of murine genes and kept as candidate genes because of their correct localizations with respect to the syntenic region. Eleven of the selected genes were localized in the expected syntenic region (btg4, chkmos, msh4, mtprd, mcmip, znfingerRIZ, discs5, trans fact 20, wee, zar1 and ktnf). Three other genes were localized in the vicinity of the expected syntenic region (dazl, fbox and mark3). Two genes were localized in the unexpected syntenic region, but they were identified with the same name as murine genes (zpA and zpC).

**Comparing oocyte and granulosa cells transcription profiles at final maturation steps using chicken Affymetrix microarrays**

The samples studied were: F1 GCs, corresponding to granulosa cells (GCs) of the largest follicles before ovulation (F1); F1 GDR, corresponding to the germinal disc region (GDR) of F1 follicles, and Ov GDR, corresponding to the germinal disc region of ovulated oocytes (Ov) (Fig. 1). Apart from the stage of maturation the main difference between these samples involved the presence of granulosa cells. They were not present in GDR from ovulated oocytes, they were slightly present in F1 GDR, and they constituted a major component of F1 GCs (see Materials and Methods). Statistical analysis of data obtained after Affymetrix microarray hybridization provided lists of genes differentially expressed in three comparisons: F1 GDR and Ov GDR, F1 GDR and F1 GCs, and Ov GDR and F1 GCs (accession number GSE7805). There were only a few differentially expressed genes in each comparison (fewer than 500 out of 28000 genes on the Chip) (Table 2). Indeed, the first comparison, between the F1 GDR and Ov GDR, showed 92 genes over-expressed at the F1 stage, including one of our in silico identified genes (zpC). In the second comparison, between F1 GDR and F1 GCs, 342 differentially expressed genes were identified. These genes involved 104 genes over-expressed in F1 GCs and 238 genes over-expressed in F1 GDR. Five of our in silico identified genes btg4, chkmos, dazL, zpA and ktnf were found among the latter genes over-expressed in F1 GDR (Table 2). The third analysis compared the expression of genes between F1 GCs and Ov GDR. We obtained a set of 448 genes that were differentially expressed between F1 GCs and Ov GDR, of which 392 genes were over-expressed in F1 GCs and 56 genes were over-expressed in Ov GDR. We found 1 of our in silico identified genes among these genes, (btg4) (Table 2). The Venn diagram (Fig. 2) shows overlapping differentially expressed genes between different dataset comparisons. Eighty-five differentially expressed genes were common for two comparisons (F1 GCs and Ov GDR, and F1 GCs and F1 GDR). Only one gene was common for the comparisons between F1 GDR and F1 GCs, and F1 GDR and Ov GDR and 1 gene was common for all three comparisons. F1 GDR samples contained a quantity of granulosa cells in contrast to Ov GDR samples which were free of granulosa cells. Analysis of the redundancy of over-expressed genes between different comparisons was therefore performed in order to distinguish between genes found over-expressed in F1 GDR samples due to granulosa cell contamination and those really over-expressed in the oocyte (Fig. 3). This analysis revealed that 85 of 92 genes over-expressed in F1 GDR were also over-expressed in F1 GCs, both compared to Ov GDR, indicating that these genes characterized granulosa cell expression rather than variation in the expression between F1 GD and Ov GD. Thus only 7 genes should be considered as overexpressed in F1 GD compared to Ov GD. Moreover, of the 104 genes overexpressed in F1 GCs compared to F1 GDR, 36 were also overexpressed in F1 GCs compared to Ov GDR. Consequently, taking into account the latter redundancy, a total of 460 genes was overexpressed in granulosa cells compared to oocytes. On the other hand 49 genes were redundant among genes
Table 1: Accession numbers of murine sequences used and of homolog chicken sequences found. Bold text represents chicken genes whose syntenic regions are conserved with the appropriate murine homologs.

| Murine genes | Chicken genes |
|--------------|---------------|
| Accession number | Name | Localisation | Accession number | Name | tblastn score | Localisation | abbreviation |
| | Chromosome | Position (kb) | | Chromosome | Position (kb) | |
| XM_205433.2 | Oog2-like | 4 E1 | 142170 | XM_417634 | similar to zinc finger protein RIZ. partial | 100 | 21 | 4843 | znfingerRIZ |
| XM_357175.2 | TRAF-interacting protein | 2A1 | 6081 | XM_416727 | similar to mtprd protein – mouse | 102 | 1 | 100734 | mtprd |
| AK054339.1 | FBXO12A | 9F2 | 109192 | XM_419103 | similar to hypothetical protein | 104 | 2 | 91887 | fbox |
| AY351591.1 | Msh4 | 3H3 | 154505.7 | XM_422549 | similar to MutS homolog 4 | 107 | 8 | 29983 | msh4 |
| XM_138939.3 | Speer-like | 14A3 | 20635 | XM_421604 | similar to Discs. large homolog 5 | 132 | 6 | 12497.5 | discs5 |
| AK018361.1 | Zfp393 | 4D2 | 116103.6 | XM_422416 | similar to Kruppel-like transcription factor | 288 | 8 | 20700.7 | ktfn |
| NM_172481.1 | Nalp9E | 7A1 | 5473.6 | XM_420951 | similar to mast cell maturation inducible protein I | 473 | 5 | 3008 | mcmip |
| BC066811.1 | Btg4 | 9A5 | 51279 | XM_417919 | similar to p30 B9.10 | 599 | 24 | 5212.7 | btg4 |
| XM_355960.1 | PAR-1Alike | 7A3 | 11760.3 | XM_421385 | similar to MAP/microtubule affinity-regulating kinase 3 long isoform | 643 | 5 | 47042.5 | mark3 |
| AY191415.1 | Zar1 | 5 | 72968 | XM_424318 | Gallus gallus similar to zygote arrest I | 643 | 4 | 68326.1 | zar1 |
| XM_139155.2 | Dazl | 14D3 | 71005 | XM_416218 | similar to transcription factor 20 isoform I | 688 | 1 | 46012.9 | transfact20 |
| NM_010021.2 | Dazl | 17B1 | 48475 | NM_204218 | deleted in azoospermiar-like | 863 | 2 | 33589.4 | dazL |
| NM_020021.1 | Mos | 4A1 | 3798.4 | M19412 | Chicken c-mos proto-oncogene | 955 | 2 | 110418 | chkmos |
| NM_011775.2 | ZP2 | 7F2 | 107432.5 | XM_424608 | similar to zona pellucida A | 1146 | 6 | 15797.3 | zpa |
| NM_201370.1 | VWE1hu | 6B1 | 40383 | XM_425491 | similar to VWE1A kinase | 1218 | 1 | 54018.4 | wee |
| NM_011776.1 | ZP3 | 5G2 | 133429.5 | D89097 | zona pellucida C protein | 2149 | 10 | 188.600 | zpC |
overexpressed in the Ov GDR and F1 GCs, and F1 GDR and F1 GCs comparisons.

Based on GO annotation the genes upregulated in GCs were mostly related to metabolic processes, transport, proteolysis, regulation of transcription, immune response and cell adhesion, whereas genes preferentially expressed in GDR were preferentially involved in cell cycle, chromosome organization, phosphorylation of proteins, regulation of transcription, multicellular organism development and DNA metabolic processes (Fig. 4).

**Tissular pattern of gene expression**

On the basis of the *in silico* and microarray approaches, 17 genes were retained for further study. Among these, 16 genes were found using the *in silico* approach as described above (Table 1), of which 6 genes (*chkmos, daZL, btg4, zpA, ktfn* and *zpC*) were also found using the microarray approach (Table 2) and 1 gene (*zpD*) was found only in the microarray approach. The latter gene was chosen because of its strong involvement in the fertilization process [31]. In contrast to *zpA* and *zpC*, the *zpD* gene was not found in the mouse [32], explaining why it was absent from the list of murine potentially oocyte-specific genes. Four other genes already known for their involvement in final follicular maturation or early embryo development in the chicken or in other vertebrates were added (*foxl2* [33], *igf2* [34,35], *hsf1* [36], and *cvh* [23]) (Table 3). Finally 21 chicken genes were further studied. Real time RT-PCR performed on 11 adult tissue samples (total ovary, spleen, intestine, gizzard, liver, heart, skin, brain, pectoralis muscle, lung and pituitary gland) revealed differences in the specificity of their tissular expression patterns. Tissular expression profiles of some of these genes are presented on Fig. 4. Seven of these genes (*daZL, wee, zar1, zpA, btg4, zpC* and *chkmos*) were specifically expressed in the ovary (Fig 5A). The specificity of *zpC* and *chkmos* has previously been described and our results concerning these genes (data are not shown) were in accordance with the literature ([37] and [38], respectively). Eight genes were preferentially and strongly expressed in the ovary (Fig 5B). Three genes from last group were slightly expressed in another tissue: *fbox* and *zpD* in the pituitary gland and *ktnf* in the muscle. We also confirmed preferential ovarian expression of *cvh* [23] and *foxl2* [33], as well as a low expression of the latter in the pituitary gland (data not shown). Three other genes from this group (*igf2, mark3* and *znfingerRIZ*) were slightly expressed in other tissues, in addition to the ovary. The last 6 genes (*trans fact 20, msh4, mitprd, mcnp, discs5* and *hsf1*) were expressed as highly in the ovary as in other tissues (data not shown).
RNA state during follicular maturation and early embryo development

Analysis of total RNA, assessed with Agilent RNA nano chips (Fig. 6A), showed an atypical state of RNA from GDR between ovulation and oviposition. In our conditions, rRNA seemed degraded from ovulation until oviposition. With three different RNA extraction methods (tri reagent (Euromedex), RNeasy kit (QIAGEN) and MasterPure™ RNA Purification Kit (Epicentre Biotechnologies)), the RNA profile of GDR from the ovulation stage and from the following embryonic stages remained degraded (data not shown). Moreover, we assessed rRNA 18S and 28S by real time PCR in order to confirm this atypical RNA state (Fig. 6B and 6C). These results confirmed our previous observations; 18S and 28S rRNA expression showed a huge decrease from ovulation until the oviposition. We then performed labelled reverse transcription to investigate whether mRNA was also degraded. We compared three samples, i.e. the RNA from GDR of F1 stage, from a whole adult ovary that had a normal rRNA profile and GDR of ovulation stage with had degraded rRNA. Electrophoresis profiles of labelled cDNA in denaturing agarose gel were almost identical for the three samples investigated and the smear corresponding to the reverse transcribed mRNA was still present in all these samples (Fig. 6D).

Gene expression during follicular maturation and early embryo development

A detailed study of the expression profiles during follicular maturation and early embryo development using real time RT-PCR was performed for 21 genes, for which tissue specificity of the expression was characterized. First unsupervised hierarchical clustering of our data was performed in order to confirm the biological appropriateness of the selected genes and samples (Fig 7). Two other previously studied genes (gdf9 and bmp15) were added to this analysis in order to facilitate the clustering process because of their already known oocyte-preferential localization [30]. This analysis discriminated two major groups of samples. The first included all granulosa samples from F6 to F1 (correlation threshold 0.58). The second group corresponded to GDR from F6 to the ovulation stage and to all embryo stages (correlation threshold 0.60). Several subgroups could be distinguished within each group (correlation threshold 0.69–0.94). Each subgroup corresponded to a different physiological state, indicated on Fig 6A, suggesting that the genes selected were pertinent. Moreover, unsupervised clustering arranged samples in the perfect chronological order.

In order to group genes with similar expression patterns supervised clustering was performed with samples arranged first according to the nature of the sample (GCs or GDRs) and then according to chronological order (Fig. 8). Five clusters (threshold > 0.64) were identified (C1 to C5). A representative example of the expression profile characterizing each cluster is shown on Fig. 9. The graphical representation of expression profiles of all other genes with the corresponding statistical analysis of variation of their expression is supplied in additional files (see Additional file 1, 2, 3 and 4). The chkmos, big4, wee, zpA, dazL, hsf1, fbox and bmp15 genes, forming cluster C1, were preferentially expressed in GDR. These genes showed a significant increase in mRNA expression in GDR during follicular maturation and a steady decrease after ovulation, becoming nearly undetectable at 36 h after fertilization. The cvh, ktfn and zar1 genes included in Cluster C2 were also preferentially expressed in GDR but, in contrast

Table 3: Accession number of chicken genes selected on the basis of the literature

| Bibliographic reference | Accession number | Name | Abbreviation |
|-------------------------|------------------|------|--------------|
| Govoroun et al. 2004    | NM_001012612     | forkhead box L2 | foxl2 |
| Aegerter et al. 2005, Heck et al. 2005 | XM_421026 | Insulin growth factor 2 | IGF 2 |
| Tsunekawa et al. 2000   | NM_0204708       | Gallus gallus DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4) | cvh |
| Hsf1 Anckar et al. 2007 | L06098           | chicken heat shock factor protein 1 | hsf1 |
to genes from cluster C1, they were constantly expressed in the early embryo. Moreover, *ktfn* and *zar1* genes displayed a significant increase in expression from 24 to 48 hours after fertilization. The genes from cluster C3 were expressed in GCs, in GDRs and in the embryo. Moreover, *mark3*, *igf2*, *gdf9* and *transfact20* genes showed a significant decrease in expression during the last stages of follicular maturation in both GDR and GCs. Cluster C4, including *discs5*, *znfingerRIZ*, *foxL2* and *mtprd* genes, was characterized by expression that was fairly similar to that of cluster C3. The difference consisted of less pronounced variations in gene expression in GCs and more pronounced variations in gene expression in GDR for cluster C4 compared to genes in cluster C3 through the stages investigated. Genes belonging to cluster C5 were expressed in GCs and GDR but, in contrast to the clusters C3 and C4, their expression dropped dramatically at ovulation, especially for *zpC* and *zpD* whose transcripts showed increasing expression in GCs during the last stages of follicular maturation and a less marked increase in the expression in GDR. In contrast, the levels of *mcmip* decreased progressively during the same period. The last gene (*msh4*) was not clustered. Its expression profile was fairly similar to that of genes belonging to cluster C3, but its expression increased significantly 36 h after fertilization.

**Localization of gene expression in the ovary by in situ hybridization**

We performed in situ hybridization on mature and immature ovarian sections. All the probes were assessed on the two stages, but only the most significant results are shown.
| Affymetrix reference | Accession number | Gene name found by Blast search | Abbreviation of genes studied | Fold change | Comparison | Number of overexpressed genes |
|----------------------|------------------|---------------------------------|-------------------------------|-------------|------------|-----------------------------|
| Gga.305.1.S1_at      | NM_204783.1      | Gallus gallus wingless-type MMTV integration site family, member 4 | GgaWnt4 | 150 | F1 GCs and Ov GDR | 56 |
| GgaAffx.13126.1.S1_at| AJ71023          | Gallus gallus mRNAs for hypothetical protein, clone 3202 | 129 |
| Giga.331.1.S1_at     | BU265377         | Finished cDNA, clone CHEST7468b | 105 |
| Giga.7980.1.S1_at    | CD730241         | similar to interferon regulatory factor 6 | 102 |
| Giga.4536.2.S1_at    | BXX9466.1        | aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase) | 94 |
| Giga.9136.2.S1_at    | CD740066         | Gallus gallus tumor-associated calcium signal transducer 1 | 92 |
| GgaAffx.11525.1.S1_at| AJ719422         | Gallus gallus claudin 1 | 91 |
| GgaAffx.20379.1.S1_at| CR524236.1      | Finished cDNA, clone CHEST91e03 | 90 |
| Giga.9936.1.S1_at    | BXX94602.6       | Amyotrophic lateral sclerosis 2 chromosomal region candidate gene | 83 |
| Giga.5591.1.S1_at    | BU450115         | B-cell translocation gene 4 | 52 |
| Giga.14454.1.S1_at   | NM_213576.1      | zona pellucida protein D | zpD | 890 | F1 GCs and Ov GDR | 392 |
| Giga.12391.1.S1_at   | BXX934169.2      | Gallus gallus similar to adrenodoxin homolog | 718 |
| Giga.1824.1.S1_at    | BUX350625        | Gallus gallus finished cDNA, clone CHEST97618 | 703 |
| Giga.6358.1.S1_at    | BXX265773        | Gallus gallus similar to chromosome 9 open reading frame 61 | 687 |
| Giga.596.1.S2_at     | NM_205118.1      | Gallus gallus 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase | 667 |
| Giga.1706.1.S1_at    | CR388473.1       | Gallus gallus finished cDNA, clone CHEST591g1 | 579 |
| Giga.3095.1.S1_at    | BXX932425.2      | similar to hypothetical protein FLJ22662 | 561 |
| Giga.13065.1.S1_at   | BU424424         | Gallus gallus finished cDNA, clone CHEST373f21 | 559 |
| GigaAffx.5954.1.S1_at| ENSGALT0000015374.1 | Gallus gallus similar to LRT5841 | 557 |
| GigaAffx.24194.1.S1_at| ENSGALT0000021874.1 | Gallus gallus similar to CG8947-PA | 534 |
| Giga.305.1.S1_at     | NM_204783.1      | Gallus gallus wingless-type MMTV integration site family, member 4 | GgaWnt4 | 90 | F1 GDR and F1 GCs | 238 |
| Giga.7980.1.S1_at    | CD730241         | similar to interferon regulatory factor 6 | 80 |
| Giga.4510.1.S1_at    | BUX350625        | Gallus gallus similar to adrenodoxin homolog | 75 |
| Giga.4901.1.S1_at    | BUX264477        | Gallus gallus similar to carbonic anhydrase 9 | 72 |
| Giga.9136.2.S1_at    | CD740066         | Gallus gallus tumor-associated calcium signal transducer 1 | 66 |
| Giga.5333.1.S1_at    | NM_204218.1      | Gallus gallus deleted in azoo sperma-like | dazL | 34 |
| Giga.5591.1.S1_at    | BU450115         | B-cell translocation gene 4 | btg4 | 33 |
| Giga.8089.1.S1_at    | BU258896         | Gallus gallus similar to Kruppel-like transcription factor neptune | ktnf | 18 |
| Giga.5714.1.S1_at    | BXX268842        | Gallus gallus similar to zona pellucida A | zpA | 12 |
| GigaAffx.9819.1.S1_at| ENSGALT0000001451 | oocyte maturation factor Mos | chimos | 10 |
| Giga.9254.1.S1_at    | CF384921         | Gallus gallus similar to CG8947-PA | 120 |
| GigaAffx.96.1.S1_at  | ENSGALT0000001451 | Gallus gallus similar to CG8947-PA | 68 |
| Giga.12454.1.S1_at   | BUX35007         | similar to relaxin 3 preproprotein | 68 |
| Giga.12454.1.S1_at   | BU450054         | Gallus gallus finished cDNA, clone CHEST599k2 | 56 |
| GigaAffx.24194.1.S1_at| ENSGALT0000021874.1 | Gallus gallus similar to CG8947-PA | 31 |
| Giga.572.1.S1_at     | NM_205078.1      | Gallus gallus nuclear receptor subfamily 5, group A, member 2 | 27 |
| Giga.10434.1.S1_at   | BXX93835.1       | Gallus gallus similar to chromosome 9 open reading frame 61 | 19 |
| Giga.4510.1.S1_at    | NM_205261.1      | Gallus gallus finished cDNA, clone CHEST159c8 | 17 |
| Giga.7212.1.S1_at    | BUX26436         | Gallus gallus similar to Ephi1 protein | 14 |
| Giga.3667.1.S1_at    | NM_204839.1      | Gallus gallus reversion-induced LIM protein | 13 |
| Giga.14454.1.S1_at   | NM_213576.1      | Gallus gallus zona pellucida protein D | zpD | 602 | F1 GDR and Ov GDR | 92 |
| Giga.12391.1.S1_at   | BXX934169.2      | Gallus gallus zona pellucida protein D | 321 |
| Giga.596.1.S2_at     | NM_205118.1      | Gallus gallus 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase | 317 |
| Giga.1706.1.S1_at    | CR388473.1       | Finished cDNA, clone CHEST591g1 | 307 |
| GigaAffx.5954.1.S1_at| ENSGALT0000015374.1 | Gallus gallus similar to LRT5841 | 247 |
| Giga.6358.1.S1_at    | BUX265773        | weak similarity to HUMAN Putative protein X123 | 240 |
| Giga.1824.1.S1_at    | BU424424         | Gallus gallus similar to chromosome 9 open reading frame 61 | 213 |
| Giga.13065.1.S1_at   | BU424424         | Gallus gallus finished cDNA, clone CHEST778f4 | 195 |
| Giga.3095.1.S1_at    | BXX934225.2      | similar to hypothetical protein FLJ22662 | 183 |
| Giga.7210.1.S1_at    | NM_204839.1      | Gallus gallus zona pellucida glycoprotein 3 | zpC | 64 |
on Fig. 10. For all the genes studied we detected homogeneous signals of mRNA expression in oocytes similar to those we have previously described for *bmp15* and *gdf9* [30]. The mRNA of four of eight genes localized by *in situ* hybridization (*btg4*, *dazL*, *cvh* and *fbox*) were detected with a high intensity signal in the oocytes from follicles of 300 µm – 600 µm from both immature (Fig. 10A, B, C and 10D) and mature (data not shown) ovaries. No significant expression was detected in somatic cells. For four other genes (*chkmos*, *zpA*, *zpC* and *zpD*) a signal was found in oocytes from the largest follicles of 500 µm-6 mm of mature ovaries (Fig. 10E, F, G and 10H). The signal was particularly high for *chkmos* and *zpA* (Fig. 10E and 10F), whereas *zpC* and especially *zpD* had weaker signals in both oocyte and somatic cells (Fig. 10G and 10H). No significant signal was detected with the corresponding sense probes.

**Discussion**

In the present study we identified and characterized for the first time several genes expressed in the chicken oocyte during follicular maturation and/or in early embryo. Moreover, we showed that our candidate gene approach and microarray approach were complementary in finding new avian genes potentially involved in follicular maturation and/or early embryo development. Five genes preferentially and highly expressed in the oocyte (*btg4*, *chkmos*, *dazL*, *zpA* and *ktfn*) were identified using both microarray analysis and digital differential display on murine genes. Moreover, 2 genes (*zpC* and *zpD*), identified as overex-
pressed in GCs by microarray analysis, were also confirmed by real time PCR analysis to be highly preferentially expressed in chicken GCs compared to GDR. Microarray analysis identified a total of 245 genes upregulated in the hen F1 and ovulated oocytes both compared to F1 GCs. Among these, 49 overexpressed genes were common to Ov GDR and and F1 GDR, both compared to F1 GCs, and therefore represent particular interesting candidate oocyte genes for further exploration of their potential role in oocyte maturation, fertilization and OET. The fact that we found almost five times fewer genes overexpressed in the oocyte at the ovulation stage (comparison between Ov GDR and F1 GCs) than in the oocyte at F1 stage (comparison between F1 GDR and F1 GCs), compared to granulosa cells from F1 follicles, means that for some genes mRNA expression in the oocyte decreased between F1 and ovulation stages. This change in the mRNA expression levels between F1 and ovulated oocytes is probably insufficient to be detected in the comparison between Ov GDR and F1 GDR by microarray hybridization, since only 7 differentially expressed genes were identified in this comparison. On the other hand microarray analysis enabled us to detect the presence of GCs in F1 GDR samples, revealed by the redundant overexpressed

**Figure 6**

Ribosomal and messenger RNA profiles during oocyte maturation and early embryo development. Total RNA was extracted from the GDR of an oocyte or from the embryo. RNA quality was assessed using nanochips (Agilent technologies) as described in the Materials and Methods. This analysis represents rRNA 18S and 28S subunit profiles in the samples (A). Ribosomal RNA 18S and 28S subunit profiles were then confirmed by real time PCR analysis (B and C, respectively) using the TaqMan kit (Eurogentec) as described in the Materials and Methods. Labelled 32P reverse transcription was performed to investigate the quality of mRNA extracted from F1 GDR, Ov GDR and from the ovary. The radioactive signal for samples and ethidium bromide staining for the ladder are shown (D).
genes in F1 GDR and in F1 GCs compared to Ov GDR. The functions of overexpressed genes in the various comparisons according to GO categories revealed clear differences between GCs and mature oocytes. For GCs overexpressed genes these functions were mostly related to metabolic processes, transport, proteolysis, regulation of transcription, immune response and cell adhesion, whilst for the mature oocyte they were mostly related to cell cycle, chromosome organization, phosphorylation and dephosphorylation of proteins, multicellular organism development and DNA metabolic process. These presumed functions of genes overexpressed in the oocyte are consistent with the physiological processes that it must undergo: i.e. fertilization, cleavage, chromatin remodeling, and supporting early embryo development.

The use of both bioinformatics and microarray approaches provided information on the molecular mechanisms through which OET is driven in the hen. The expression of several oocyte-specific genes increased during final follicular maturation, suggesting that transcription was still effective. After ovulation, despite the fact that 18S and 28S ribosomal RNA subunits were degraded, we showed by both labelled reverse transcription and microarray analysis that the integrity of mRNA was almost unaffected. In fact, mRNA levels were nearly the same for many genes because only 92 of 28000 genes were differentially expressed between GDRs before and after ovulation, of which only 7 really corresponded to the oocyte genes. The high number of replicates performed and the different extraction methods used strongly indicate that the difference in quality between oocyte rRNA and mRNA after ovulation is not the artifact of the experiment but reflects a real physiological feature of chicken OET, consisting probably of the arrest and degradation of the oocyte translational machinery. It could thus be hypothesized that the maternal translational system has to be replaced by the embryonic translational system. Indeed, the maternal ribosome in the embryo must be degraded before activation of the genome, in other words, before the beginning of transcription, and translation, when new embryonic ribosomes are required. This suggests that, because there are no maternal ribosomes at the stage between ovulation and oviposition, there is probably no translation or only translation of a few specific genes. If this is the case, maternal proteins should be the major essential components that support early embryo development after fertilization. This is supported by the fact that, based on GO annotation, a considerable number of the genes overexpressed in the mature oocyte are related to protein phosphorylation. Further investigation is required onto whether accumulated proteins have such an important role during these early stages of development in birds or if de novo protein synthesis still occurs and is dependent on the oocyte pool of ribosomes as in mammals [39].

On the basis of in silico and microarray approaches and analysis of the literature, 21 chicken genes were chosen in this study for further investigation of their expression using real time PCR and in situ hybridization. All these genes showed state-specific and/or cell type-specific expression patterns throughout the period, beginning from the first stages of final follicular maturation until embryonic genome activation. This suggests that these genes have different functions and have a role at the different physiological stages investigated. The observed decrease in the mRNA expression of almost all genes studied between ovulation and oviposition, which corresponds to late genome activation in chicken embryo [13], is consistent with a potential arrest of transcription and progressive maternal mRNA degradation occurring during meiotic maturation [40]. However, the rate of maternal mRNA degradation in the chicken seems to be considerably lower than that of rRNA, as demonstrated by the present study. Five genes (chkmos, bgd4, wee, dazl, and zpa) belonging to cluster C1 (Fig. 6B) were no longer expressed after activation of the embryonic genome, and thus transcripts of these genes are only maternally inherited. Moreover, chkmos, bgd4 and wee genes are known to be involved in the cell cycle in other species. In our study these maternally inherited genes were increasingly expressed during follicular maturation and thus are probably used during the last stages of final follicular maturation and/or early embryo development. Chkmos is the chicken homolog of...
mos [38], protein kinase required for meiotic maturation in vertebrates [41,42] and for mitosis in *Xenopus laevis* [43]. Meiotic maturation is brought about by steroids using redundant pathways involving synthesis of Mos, which regulates the activity of MPF (M-phase promoting factor). The Mos-MAPK pathway has long been implicated in the arrest of mitosis in vertebrate eggs [43]. The B cell translocation gene 4 (*btg4*) belongs to a family of cell cycle inhibitors. In the mouse and bovine, *btg4* is preferentially expressed in the oocyte [44,45] where it exerts a marked antiproliferative activity, [46]. *Wee* is a conserved gene from invertebrates to mammals and regulates meiotic maturation during oocyte development [38,41-43]. The transcripts of *dazL* are also maternally inherited in the medaka embryo [47], and in adult medaka fish the expression of *dazL* was detected exclusively in the ovary and in the testis [48].

ZpA, ZpC and ZpD, that belong to the ZP (zona pellucida) gene family, are known to be involved in oogenesis, fertilization and preimplantation development [49]. In our study the expression pattern of zpA was different from that of zpD and zpC, using both real time PCR analysis, where they were distributed in different clusters (C1 for zpA and C5 for zpD and zpC respectively), and *in situ* hybridization analysis. In contrast to chicken zpD and zpC, which were expressed in oocytes and somatic cells, chicken zpA was found to be specific to the oocyte, as is the case in the

**Figure 8**

*Supervised hierarchical clustering of candidate genes during follicular maturation and early embryo development.* Real time PCR analysis was performed in duplicate on samples at 18 different stages with two biological replicates. Total GDR and GC RNA from different preovulatory follicles (F1 to F6), and total GDR RNA from just ovulated oocytes and early embryos at 6.5 h, 12 h, 24 h, 36 h and 48 h post ovulation were extracted as described in the *Materials and Methods*. Supervised hierarchical clustering of genes was performed using Cluster 3.0 software as described in the *Materials and Methods*. Five clusters are shown (C1 to C5). Node correlation thresholds are indicated for each cluster.
mouse [50,51], and expressed earlier than zpD and zpC. In the mouse the expression of zpA also precedes that of zpC [50,51]. Indeed in our in situ hybridization experiments zpA expression detected in small follicles of the mature ovary was oocyte-specific, whereas that of zpC and zpD was weaker and found in oocytes and in the somatic cells of the same follicles. Real time RT-PCR, showed increasing expression of zpC and zpD in both GCs and GDR from F6 to F1. Our finding on the dynamics of zpC in GCs are in accordance with a previously reported study [52].

Moreover at the F1 stage both zpC and zpD were significantly more highly expressed in GCs than in GD and this was consistent with our results for microarray hybridization, but expression decreased dramatically after ovulation. In contrast to the cellular expression pattern found for chicken zpC, in several mammals (murines, bovines and porcines) zpC (zp3) is specifically expressed in the oocyte. However, in the equine species, ZPC protein synthesis is completely taken over by cumulus cells [53]. These findings indicate species specificity of zpC distribu-
tion inside the follicle. ZPC protein plays a crucial role in the fertilization process in mammals and birds, [31,37,49].

As zpA other genes (zar1, kifn and cvh) were preferentially expressed in the oocyte and might play role in fertility (zar1, kifn) or in germ cell specification (cvh). Both zar1 and kifn were expressed at higher levels after activation of the embryonic genome. We could therefore hypothesize that these genes might be involved not only in oocyte maturation, but also in early embryo development, just after maternally inherited genes. Of these 2 genes, only zar1 has been studied in reproduction. It is one of the few known oocyte-specific maternal-effect genes essential for OET in mice. In mammals and humans it is hypothesized to be involved in the initiation of embryo development and fertility control [54,55]. CVH protein has been previously proposed to be a part of the mechanism for germ cell specification in birds [16,23]. Our results concerning the spatio-temporal expression of cvh mRNA during follicular maturation and early embryo development are consistent with previously reported studies on the CVH protein.

The genes belonging to clusters C3 and C4 were all preferentially expressed in the ovary in both GCs and GDR and had quite similar expression patterns. Except for foxL2, their expression declined during follicular maturation in

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**Figure 10**

Localization of candidate genes mRNA in the hen ovary. Localization of dazL(A), btg4 (B), vasa (C), fbox (D), chkmos (E), zpA (F), zpC (G) and zpD (H) by in situ hybridization in immature ovaries (A, B, C and D) or in mature ovaries (E, F, G and H) as described in the Materials and Methods. Bright fields are on the left, and dark fields are on the right. The control sections were hybridized with sense probes. The hybridization with only one sense probes is represented because no signal was observed after hybridization with any sense probes. Oocytes (Oo), granulosa cells (GCs) and somatic cells (SCs) are indicated. Scale bar = 100 µm.
GCs and less in GDR and persisted at low levels in the early embryo. This suggests that they are especially involved in the first stages of final follicular maturation as well as in oocyte maturation. The chicken homolog of the mouse par-1a-like gene, i.e. mark3, is required for oocyte differentiation and microtubule organization in the Drosophila [56], and its role in cell polarity and Wnt signaling is conserved from invertebrates to mammals [57,58]. The expression of another gene (msh4), which did not belong to any cluster, also decreased during follicular maturation in GCs and in GD but, as for zar1 and ktfn, it showed a significant increase after embryonic genome activation. Msh4 is known to be involved in mediating recombination of homologous chromosomes and DNA mismatch repair in the mouse [59]. These events occur during the meiotic prophase, the stage where oocytes are blocked for a long time before meiotic maturation.

**Conclusion**

In conclusion, the findings of the present study on spatio-temporal expression of 8 chicken oocyte specific genes (chkmos, big4, wee, zpA, dazL, cvh, zar1 and ktfn) were consistent with our hypothesis that oocyte-specific genes in the chicken should play a major role in oocyte maturation, fertilization and early embryo development as in the mouse [12]. Other genes, whose mRNA expression were found in our study to be more specific for GCs or detected in both GCs and GDR depending on the stage, seem to be involved in follicular maturation (foxl2, transfac 20, mark3, ...) and fertilization (zpD and zpC) rather than in early embryo development. Moreover, the microarray approach provided allowed the discovery of a set of new potential chicken mature oocyte and chicken granulosa cell markers for future studies. Interestingly, 40% of these genes had no homologs in the gene databases and some of them probably correspond to specific chicken mechanisms such as hierarchical follicular maturation or rapid yolk accumulation.

**Methods**

**Animals**

Laying breed hens aged 60–70 weeks (ISA Brown, egg layer type, Institut de Selection Animale, Saint Brieuc, France) were housed individually in laying batteries with free access to feed and water and were exposed to a 15L:9D photoperiod, with lights-on at 8.00 pm. Individual laying patterns were monitored daily. For in situ hybridization, these hens and younger ones of (10 weeks old) were used to provide mature and immature ovaries, in order to study follicles at each stage. Hens used to provide fertilized eggs were bred in the same conditions and inseminated once a week.

**Collection of tissues, oocytes and embryos**

Hens aged 60 weeks were used. Tissue samples were collected from the ovary, spleen, intestine, gizzard, liver, heart, skin, brain, pectoralis muscle, lung and pituitary gland. Germinal disc regions (GDR) and granulosa cells (GCs) surrounding the GDR (Fig. 1) were collected from different preovulatory follicles (F1 to F6), just ovulated oocytes and early embryos at 6.5 h, 12 h, 24 h, 36 h and 48 h post ovulation. The GDR and GCs surrounding the GDR were carefully dissected in the same way under a binocular microscope using fine forceps and scissors (World Precision Instruments) as previously described for quail oocytes [60]. After washing in phosphate buffer saline (PBS, Gibco, Cergy Pontoise, France) GDR and GCs were frozen in liquid nitrogen and then were stored at -80°C until use. For the last two stages, eggs were incubated at 37.8°C for 12 and 24 h, respectively. The 6.5 h, 12 h, and 24 h stages of embryo development correspond to stages I, V and X of the Eyal-Giladi and Kochav classification, respectively [61]. The 36 h and 48 h stages correspond to stages 3 and 6 of the Hamburger-Hamilton classification, respectively [62]. During follicular maturation the germinal disc is closely associated with its overlying granulosa cells (GCs) and forms a structure called the germinal disc region (GDR) (Figure 1). The GDR from F6-F1 follicles used for these studies consisted not only of the germinal disc but also of the overlying layer of GCs, because GD and overlying GCs cannot be completely separated [60,63,64] and the number of GCs in GDR preparations could not be counted.

**Bioinformatic analysis**

A differential digital display analysis has already been performed with mouse ESTs [25-27], providing a list of murine oocyte-specific genes. Using this murine gene list, we systematically searched for chicken orthologs of these genes in international public databases pubmed [65]. Blast bit scores higher than 100 were retained. Moreover, the physical localization of genes identified on chicken chromosomes was retrieved from both mapview [28] and from blat search [29]. We also verified that chicken homologs were localized in the syntenic genomic regions conserved with that of mouse species to have a better chance that true orthologs were studied with ensembl [66].

**RNA isolation and Microarray Analysis**

Total RNA was extracted from GDR of F1 and ovulated oocytes, and from GCs of F1 follicles as described above. We thus had 3 samples with a biological replicate of each sample. The RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions. The tissues (GDR or GC) from 25 hens were pooled for each stage investigated to obtain enough RNA for probe synthesis. Two such a pools were constituted for
each sample in order to achieve two biological replicates of microarray hybridization. All RNA samples were checked for their integrity on the Agilent 2100 bioanalyzer according to Agilent Technologies guidelines (Waldbronn, Germany). Two micrograms of total RNA were reverse transcribed with the One-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA), according to the manufacturer's procedure. Clean up of the double-stranded cDNA was performed with Sample Cleanup Module (Affymetrix, Santa Clara, CA) followed by in vitro transcription (IVT) in the presence of biotin-labelled UTP using GeneChip™ IVT labelling Kit (Affymetrix). The quantity of the cRNA labelled with Ribogreen™ RNA Quantification Reagent (Turner Biosystems, Sunnyvale, CA) was determined after cleanup by the Sample Cleanup Module (Affymetrix). Fragmentation of 15 µg of labelled-cRNA was carried out for 35 minutes at 94°C, followed by hybridization for 16 hours at 45°C to Affymetrix GeneChip™ Chicken Genome Array, representing approximately 32,773 transcripts, corresponding to over different 28,000 Gallus gallus genes. After hybridization, the arrays were washed with 2 different buffers (stringent: 6X SSPE, 0.01% Tween-20 and non-stringent: 100 mM MES, 0.1 M [Na+] 0.01% Tween-20) and stained with a complex solution including Streptavidin-Phycocyrthrin conjugate (Invitrogen/molecular probes, Carlsbad, CA) and anti Streptavidin biotinylated antibody (Vectors laboratories, Burlingame, CA). The washing and staining steps were performed in a GeneChip™ Fluidics Station 450 (Affymetrix). The Affymetrix GeneChip™ Chicken Genome Arrays were finally scanned with the GeneChip™ Scanner 3000 7G piloted by the GeneChip™ Operating Software (GCOS).

All these steps were performed on Affymetrix equipment at INRA-URGV, Evry, France.

The .raw CEL files were imported in R software for data analysis. All raw and normalized data are available from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) [67], accession number GSE7805. Gene Ontology annotations were performed with NetAffx.

**RNA extraction and reverse transcription**

Total RNA was extracted from whole adult tissues (ovary, spleen, intestine, gizzard, liver, heart, skin, brain, pectoralis muscle, lung and pituitary gland) using Tri-reagent (Euromedex, Mundolsheim, France) according to the manufacturer's procedure. RNA quality and quantity were then assessed by using RNA nano chips and Agilent RNA 6000 nano reagents (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. Samples were stored at -80°C until use. Reverse transcription (RT) was performed to test the expression of candidate genes in different tissues and at different stages of follicular maturation and embryo development using polymerase chain reactions (PCR). One microgram of total RNA extracted from tissues or GDR was digested by RQ1 DNase (Promega, Madison, WI, USA) and reverse transcribed to first-strand cDNA using Moloney Murine Leukemia Virus reverse transcriptase I with an oligo dT-random primer mix (Promega, Madison, WI, USA) according manufacturer’s instructions.

Labelled RT was performed in order to assess mRNA quality in GDR of F1 stage and just ovulated oocytes. Ten µCi αP32 dCTP was added to the reverse transcription mix in order to label cDNA. Labelled cDNA was then separated on 1.2% denaturing agarose gel (50 mM NAOH, 1 mM EDTA) by electrophoresis. A storage phosphor screen (Amersham Biosciences, Bucks, UK) was placed on the gel in an exposure cassette (Amersham Biosciences, Bucks, UK). The signal was detected one hour later with a STORM 840 (Molecular Dynamics), a phosphor screen imaging system.

**Real time RT-PCR**

Specific sets of primer pairs (Sigma Genosis), designed using Vecteur NTI software to amplify fragments of 21 different transcripts, are shown in Table 4. Real time PCR reactions were carried out in 25 µl containing primers at a final concentration of 150 nm of each, 5 µl of the RT reaction diluted 1/30 and qPCR Mastermix Plus for Sybr Green 1 (Eurogentec) according to the manufacturer’s instructions. Real time PCR was performed using an ABI Prism 7000 (Applied Biosystems). After incubation at 50°C for 2 min, and 95°C for 10 min, the thermal cycling protocol was as follows: 40 cycles at 95°C for 15 sec and 60°C for 1 min. The amounts of 18S rRNA and 28S rRNA were measured in the RT reactions diluted 1/5000 using 28S rRNA and 18S rRNA control kits (Eurogentec), respectively, according to the manufacturer’s instructions. PCR amplification without cDNA was performed systematically as a negative control. 18S rRNA was used as a reporter gene in the study of the mRNA tissular expression pattern, whereas β actin, gapdh and ef1α were used as reporter genes in the mRNA expression study during follicular maturation and early embryo development, the expression of these three genes being similar in GCs and in GDR (data not shown). In both cases (tissular expression study or temporal dynamic study) the samples were analyzed in duplicate on the same plate for a given gene. Real time RT-PCR was performed in the temporal dynamic study with two biological replicates of each sample. Melting curve analysis was systematically performed for all genes in order to verify the specificity of the PCR product. Real time PCR efficiency (E) was measured in duplicate on serial dilutions of cDNA (pool of reverse-transcribed RNA from GDR and GC samples at the same stages as used for the temporal dynamic study) for each
primer pair and was calculated using the following equation: \( E = \left(10^{1/slope}\right)^{-1} \). In the tissular expression study the relative amounts of gene transcripts (R) were calculated according to the equation:

\[
R = \frac{E_{\text{Ct gene}}}{E_{\text{Ct gene}} - E_{\text{Ct 18S rRNA}}}
\]

where \( E \) is a cycle threshold. In the temporal dynamic study \( R \) was calculated using following equation:

\[
R = \frac{E_{\text{Ct gene}}}{\text{Mean}(R_{\text{ef1\(\alpha\)}}R_{\beta\text{actin}}R_{\text{gapdh}})}
\]

where \( R_c \) is corrected relative reporter gene expression calculated as explained below. In order to take into account only fold changes in the expression levels of reporter genes between samples but not the differences in the expression levels of reporter genes in the sample, the expression levels of reporter genes in each sample (sample\(_i\)) was adjusted against the relative amount of ef1\(\alpha\) in the F1GDR sample according to the equation:

\[
R_{\text{reporter}} = \frac{E_{\text{Ct reporter in F1GDR}}}{E_{\text{Ct reporter in sample}}}
\]

Hierarchical clustering

The hierarchical classification of data obtained using real time RT-PCR was performed with the Cluster 3.0 program.
using unsupervised single linkage or supervised complete linkage clustering in order to classify biological samples or to group together genes with a similar expression pattern, respectively [68].

**In situ hybridization**

Female mature chickens were sacrificed at different stages of sexual development. Two types of tissue were used, i.e. mature ovaries, containing follicles of different sizes (50 \( \mu \)m-7 mm) from 60-week-old hens (most follicles being larger than 300 \( \mu \)m) and immature ovaries, containing a majority of small follicles (25–500 \( \mu \)m) from 10-week-old hens (most follicles being smaller than 100 \( \mu \)m). Mature and immature ovaries were then collected and included in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Frozen ovaries were serially sectioned with a cryostat (thickness 10 \( \mu \)m) to perform in situ hybridization experiments using \(^{35}\)S-labeled chicken gene cRNA. The gene antisense and sense constructs used for in situ hybridization were generated by inserting 700 – 800 bp fragments of chicken cDNA into the pGEM-T vector (Promega, Madison, WI, USA), and selecting a clone with the appropriate antisense or sense orientation. The gene cDNA fragments were generated by RT-PCR from chicken ovary mRNA using forward and reverse primers (Table 4). The in situ hybridization was performed as previously described [69]. Hybridization specificity was assessed by comparing signals obtained with the cRNA antisense probe and the corresponding cRNA sense probe.

**Statistical analysis**

Data obtained after Affymetrix microarray hybridization analysis were normalized with the gcrrma algorithm [70], available in the Bioconductor package [71]. Differential analysis was performed with the varmixt package of R [72]. A double-sided, unpaired \( t \)-test was computed for each gene between the two conditions. Variance of the difference in gene expression was split between subgroups of genes with homogeneous variance [72]. The raw P values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate (FWER) [73]. A gene is declared differentially expressed if the Bonferroni-corrected P-Value is less than 0.05.

All other experimental data are presented as means ± SEM. One-way analysis of variance (ANOVA) was used to test differences. If ANOVA revealed significant effects, the means were compared by Fisher’s test, with \( P < 0.05 \) considered significant. Different letters indicate significant differences.

**Authors’ contributions**

SE performed the experiences, the sequence alignment and the microarray analysis. FB participated in the design of the study and coordinated oocytes and embryos collection. IC participated in the experiences. SB carried out the microarrays hybridization. MLMM performed the statistical analysis of microarray data. PM participated in the design of the study and provided the list of murine oocyte genes identified in silico. EB participated in the design of the study. MSG conceived of the study, and participated in its design and coordination. SE and MSG wrote and revised the manuscript. All authors read and approved the final manuscript.

**Additional material**

**Additional file 1**

Real time RT-PCR analysis of expression of chkmos, btg4, wee, zpa, dazL and cvh transcript during follicular maturation and early embryo development. The real time RT-PCR data provided represent the related expression of the 6 genes chkmos, btg4, wee, zpa, dazL and cvh during follicular maturation and early embryo development, in granulosa cells and in germinal disc region. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-9-110-S1.png]

**Additional file 2**

Real time RT-PCR analysis of expression of ktf1, zar1, zpC, zpD and mcmip transcript during follicular maturation and early embryo development. The real time RT-PCR data provided represent the related expression of the 5 genes ktf1, zar1, zpC, zpD and mcmip during follicular maturation and early embryo development, in granulosa cells and in germinal disc region. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-9-110-S2.png]

**Additional file 3**

Real time RT-PCR analysis of expression of mark3, igf2, trans fact 20, znfingerRIZ, msh4 and foxL2 during follicular maturation and early embryo development. The real time RT-PCR data provided represent the related expression of the 6 genes mark3, igf2, trans fact 20, znfingerRIZ, msh4 and foxL2 during follicular maturation and early embryo development, in granulosa cells and in germinal disc region. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-9-110-S3.png]

**Additional file 4**

Real time RT-PCR analysis of expression of discs5, mtrprd, fbox and hsf1 during follicular maturation and early embryo development. The real time RT-PCR data provided represent the related expression of the 4 genes discs5, mtrprd, fbox and hsf1 during follicular maturation and early embryo development, in granulosa cells and in germinal disc region. Related expression measured by real time RT-PCR (as described in materials and methods). Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-9-110-S4.png]
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