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Comparative molecular portraits of human unfertilized oocytes and primordial germ cells at 10 weeks of gestation

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ABSTRACT Primordial germ cells (PGCs) are precursors of gametes and share several features in common with pluripotent stem cells, such as alkaline phosphatase activity and the expression of pluripotency-associated genes such as *OCT4* and *NANOG*. PGCs are able to differentiate into oocytes and spermatogonia and establish totipotency after fertilization. However, our knowledge of human germ cell development is still fragmentary. In this study, we have carried out genome-wide comparisons of the transcriptomes and molecular portraits of human male PGCs (mPGCs), female PGCs (fPGCs) and unfertilized oocytes. We detected 9210 genes showing elevated expression in fPGCs, 9184 in mPGCs and 9207 in oocytes, with 6342 of these expressed in common. As well as known germ cell-related genes such as *BLIMP1/PRDM1, PIWIL2, VASA/DDX4, DAZL, STELLA/DPPA3* and *LIN28*, we also identified 465 novel non-annotated genes with orthologs in the mouse. A plethora of olfactory receptor-encoding genes were detected in all samples, which would suggest their involvement not only in sperm chemotaxis, but also in the development of female germ cells and oocytes. We anticipate that our data might increase our meagre knowledge of the genes and associated signaling pathways operative during germ cell development. This in turn might aid in the development of strategies enabling better differentiation and molecular characterisation of germ cells derived from either embryonic or induced pluripotent stem cells. Ultimately, this would have a profound relevance for reproductive as well as regenerative medicine.

KEY WORDS: *PGC, oocyte, transcriptome, signaling pathway, meiosis, olfactory receptor*

Introduction

Primordial germ cells (PGCs) are the embryonic precursors of gametes. They could be regarded as the mother of all stem cells because of their unique ability to retain true developmental totipotency. But despite many similarities with embryonic stem cells (ESCs), they exhibit only temporary self-renewal capability and have distinct lineage-specific characteristics. In fact, under normal conditions, PGCs are believed to differentiate into germ cells only, which are oocytes in the female and prospermatogonia in the male, to ultimately produce eggs and sperm, respectively. It is not until the fertilization of the egg that the intrinsic germ cell program for totipotency is established.

Although restricted in developmental potency, PGCs share several molecular characteristics of pluripotent cells, these include high alkaline phosphatase activity (Ginsburg *et al.,* 1990; Goto *et al.,* 1999), expression of key pluripotency-related genes such as *POU5F1*/*OCT4, NANOG* and *ESG1* (Goto *et al.,* 1999; Western *et al.,* 2005; Sabour *et al.,* 2011). In addition, PGCs can give rise to embryonal carcinoma (EC) cells, the stem cells of testicular tumors, *in vivo*. Besides, when cultured under stimulation with

Abbreviations used in this paper: dpc, days post coitum; EC cells, embryonal carcinoma cells; EG cells, embryonic germ cells; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; mPGC/fPGC, male/female primordial germ cell; Oocs, oocytes; OR, olfactory receptor; rep, replicate.

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specific growth factors, PGCs can be converted into pluripotent embryonic germ (EG) cells. However, unlike EC and EG cells, PGCs cannot be cultured indefinitely, do not form embryoid bodies in culture and when injected into a host blastocyst cannot populate either the soma or the germline (Donovan and de Miguel, 2003).

PGCs are difficult to study because they are limited in number, deeply embedded within the embryo, and are known to migrate during development (Ginsburg *et al.,* 1990; McLaren and Southee, 1997), which limits the number of effective studies using these cells. Previous studies have demonstrated that around day 7 post coitum (dpc) in mice, PGCs can be detected at the base of the allantois within extraembryonic mesoderm. There, PGCs develop as a cluster of 40 – 50 cells. At 10 dpc the PGCs migrate to the genital ridge (Ginsburg *et al.,* 1990; Goto *et al.,* 1999; Anderson *et al.,* 2000; Molyneaux *et al.,* 2001; Saitou *et al.,* 2002), the sex determination starts and ends around 13 dpc. Female germ cells start entering meiosis, in contrast to male germ cells which pause at the mitotic stage and start meiosis at day 10 after birth (Ewing *et al.,* 1993).

In human, PGCs are identified in the hind gut at 4 weeks of gestation and then migrate to colonize the developing gonads by 7 weeks of gestation (Witschi, 1946; Gondos and Hobel, 1971; Motta and Makabe, 1986). At ~10 weeks of gestation, female PGCs (fPGCs) start to enter meiosis, while male PGCs (mPGCs) continue to divide mitotically until they are arrested in mitosis at 16–18 weeks of gestation (Gondos and Hobel, 1971; Goto *et al.,* 1999).

Many factors control the migration, proliferation and organisation of PGCs to developing gonads as well as the integration of somatic cells surrounding the PGCs. Bone morphogenetic proteins (BMPs) regulate gene expression and formation of PGCs (Ying *et al.,* 2000; Ying and Zhao, 2001; Pesce *et al.,* 2002). Recent successful studies have demonstrated that PGCs can be derived from pluripotent stem cells *in vitro*, these can then be further differentiated into oocyte-like cells or spermatogenic-like cells (Hubner *et al.,* 2003; Toyooka *et al.,* 2003; Geijsen *et al.,* 2004; Kee *et al.,* 2006; Nagano, 2007; Qing *et al.,* 2007; Wei *et al.,* 2008; Panula *et al.,* 2011).

Based on the complex control system during migration, proliferation and development of PGCs into gonads many disorders can arise if this developmental program is skewed. For example, different types of germ-cell tumors (GCTs) can result from mis-regulated expression of key PGCs-related genes. GCTs are a heterogeneous group of neoplasms and are classified into five groups (I-V). They arise in ovaries and testes as well as in different extragonadal regions (Oosterhuis and Looijenga, 2005; Looijenga, 2009).

In this study, we aim at deriving molecular portraits of human unfertilized oocytes, male and female PGCs using mRNAs previously isolated in earlier studies (Adjaye *et al.,* 1999; Goto *et al.,* 1999; Adjaye and Monk, 2000). We anticipate that this data will form the

TABLE 1

CANONICAL PATHWAYS ENRICHED IN FEMALE PGC AND OOCYTE TRANSCRIPTOMES

	fPGCs		Oocs	
IPA Canonical Pathways	**p-value**	**Ratio**	**p-value**	**Ratio**
EIF2 Signaling	3.98E-17	68%	5.01E-13	64%
Regulation of eIF4 and p70S6K Signaling	2.51E-14	63%	1.58E-11	60%
mTOR Signaling	7.94E-11	60%	4.90E-10	59%
ILK Signaling	1.74E-06	58%	4.90E-05	56%
Mitochondrial Dysfunction	3.31E-05	47%	7.08E-06	48%
Ephrin Receptor Signaling	3.39E-05	51%	4.47E-07	54%
Huntington's Disease Signaling	3.72E-05	52%	6.61E-05	52%
Wnt/β-catenin Signaling	6.31E-05	57%	3.31E-05	58%
Aminoacyl-tRNA Biosynthesis	1.07E-06	**37%**	2.04E-03	**30%**
PI3K/AKT Signaling	3.16E-06	55%	1.70E-04	51%
Pyrimidine Metabolism	8.13E-06	40%	1.45E-04	38%
Clathrin-mediated Endocytosis Signaling	9.77E-06	**56%**	1.70E-03	**51%**
Cell Cycle Regulation by BTG Family Proteins	1.05E-05	**78%**	2.29E-03	**67%**
Protein Ubiquitination Pathway	1.55E-05	54%	2.04E-04	52%
Role of CHK Proteins in Cell Cycle Checkpoint Control	1.62E-05	**70%**	1.35E-02	**57%**
Cyclins and Cell Cycle Regulation	2.69E-05	59%	1.74E-04	57%
Oxidative Phosphorylation	7.59E-05	54%	2.75E-04	52%
Role of BRCA1 in DNA Damage Response	8.13E-05	**63%**	1.22E-01	**48%**
AMPK Signaling	9.77E-05	48%	3.72E-04	46%
Integrin Signaling	1.55E-04	53%	1.00E-06	57%
Axonal Guidance Signaling	1.12E-04	48%	3.24E-06	50%
Germ Cell-Sertoli Cell Junction Signaling	1.17E-03	**53%**	3.47E-06	**58%**
Signaling by Rho Family GTPases	1.20E-03	49%	6.31E-06	53%
Rac Signaling	1.62E-03	**49%**	7.24E-06	**55%**
CDK5 Signaling	1.48E-03	**55%**	9.55E-06	**62%**
Molecular Mechanisms of Cancer	8.51E-04	47%	1.12E-05	49%
Mitotic Roles of Polo-Like Kinase	1.62E-03	**59%**	1.35E-05	**67%**
ERK/MAPK Signaling	6.17E-04	50%	1.86E-05	53%
Actin Cytoskeleton Signaling	3.24E-03	**47%**	2.09E-05	**52%**
RhoGDI Signaling	2.88E-03	47%	3.39E-05	51%
Ephrin B Signaling	2.63E-03	**55%**	3.47E-05	**61%**
Breast Cancer Regulation by Stathmin1	1.58E-03	50%	3.63E-05	53%
IL-8 Signaling	2.14E-03	49%	4.79E-05	52%

Summary of canonical pathways significantly enriched ($p < 0.0001$, highlighted in red (fPGCs) or green (oocytes)) in the set of genes expressed in fPGCs (9210) and oocytes (9207) as determined by IPA. The ratio describes the number of transcripts expressed in each data set that map to the pathway divided by the total number of genes that exist in the canonical pathway. To point out an additional level of enrichment bold numbers highlight differences between the fPGCs- or oocytes-specific ratios greater than or equal to 5%. The entire IPA output, including corresponding p-values and gene lists, is given in Supplementary Table 3.

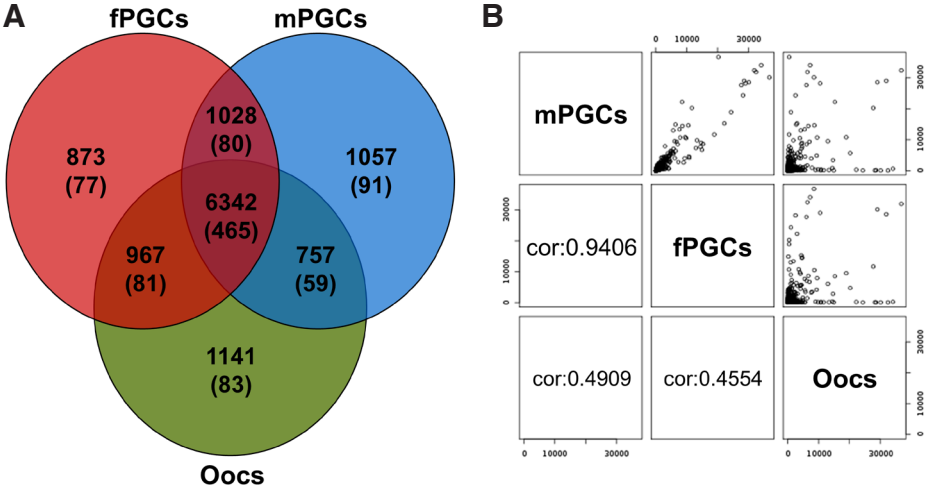
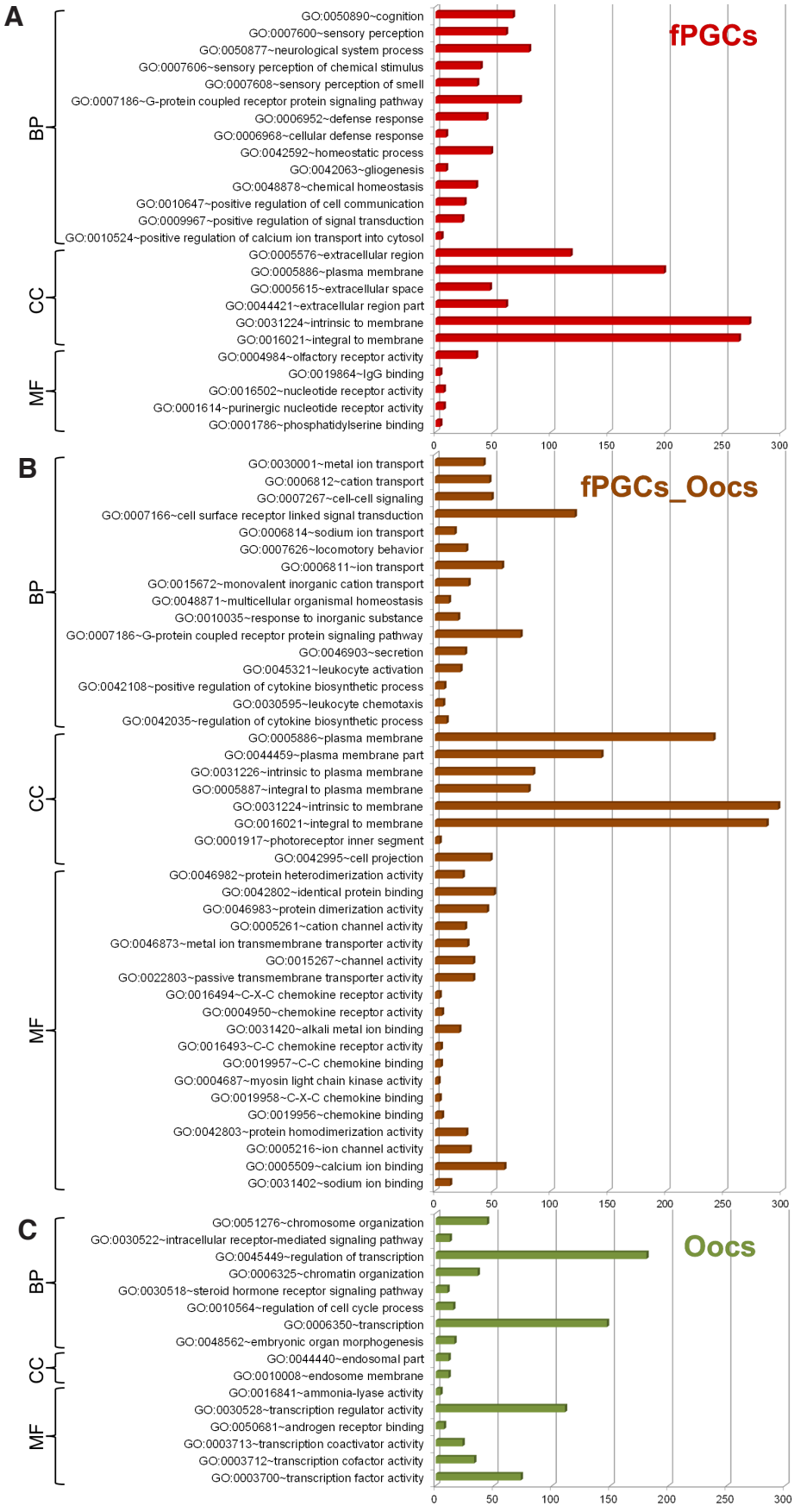


Fig. 1. Venn diagram-based analysis and correlation of the transcriptome of female PGCs, male PGCs and oocytes. (A) *The Venn diagram depicts the overlaps of genes with elevated expression levels in fPGCs, mPGCs and oocytes as determined by microarray-based transcriptome profiling. The numbers in brackets represent the number of "novel" genes in each section which have not been functionally annotated to date, including, e.g. "LOC389936", "C12orf12", "FAM10A6","FLJ31568","KIAA0895L","MGC35361". (B) Scatter plots depicting the correlation of complete gene expression data sets of mPGCs, fPGCs and oocytes. Raw data were normalized by quantile normalization and Pearson correlation coefficients (cor) calculated between the different samples.*



foundation for more extensive functional studies that might increase our meagre knowledge of the genes and associated pathways operative in these cells. This knowledge should also aid in our understanding of the aetiology of female germ cell derived cancers.

Results

Global gene expression analysis

Our aim was to profile and further analyse the transcriptomes and molecular portraits of distinct cell types of the female germ line, i.e. fPGCs and oocytes, using a microarray-based approach. We included mPGCs in the analyses to enable on the identification of genes specifically expressed in fPGCs and oocytes, i.e. female germ line-specific genes, in contrast to mPGCs-specific genes. Using our cut-off threshold, we detected 9210 genes showing elevated expression in fPGCs, 9207 genes for the oocytes sample and 9184 genes for the mPGCs as depicted in Fig. 1A. The majority of the 6342 germ cell-related genes, which includes housekeeping genes, are expressed in common between fPGCs, oocytes and mPGCs. Not surprisingly there are more genes in the overlap of fPGCs and mPGCs (1028) than in fPGCs and oocytes (967), or mPGCs and oocytes (757). Accordingly, the correlation coefficient between mPGCs and fPGCs is very high (0.94), whereas that between the PGCs and the oocytes are much lower (0.46 and 0.49, respectively, Fig. 1B).

As expected we have identified several non-annotated genes (depicted as “LOC…”, “C12orf…”, “FAM…”, “FLJ…”, “KIAA…” and “MGC…”) which we can assume as novel. This again highlights the novelty and usefulness of this study.

Functional annotation and enrichment analysis of putative female germ line-specific genes

As part of the DAVID functional annotation

Fig. 2. DAVID analysis of female PGC- and oocyte-specific genes. *As shown in Fig. 1, all genes exclusively expressed in either fPGCs (873, A) or oocytes (1141, C) or overlapping between fPGCs and oocytes (967, B) were assessed for functional enrichment of biological pathway-associated GOs (BP), cellular component-associated GOs (CC) and molecular function-associated GOs (MF) using the DAVID database. Significantly enriched terms ($p < 0.01$) are depicted. The terms are ordered by increasing p-values from top to bottom. The bars represent the number of genes that mapped to each term. The entire DAVID output, including corresponding p-values and gene lists, is given in Supplementary Table 2.*

TABLE 2

BIOLOGICAL FUNCTIONS ENRICHED IN FEMALE PGC AND OOCYTE TRANSCRIPTOMES

Cancer	fPGCs p-value	Oocs p-value
tumorigenesis	6.72E-18	7.22E-17
neoplasia	7.48E-15	1.15E-13
cancer	1.29E-14	3.36E-13
solid tumor	2.31E-11	1.36E-10
carcinoma	4.10E-11	4.99E-10
infection of tumor cell lines	3.36E-07	3.82E-08
infection of cervical cancer cell lines	4.30E-07	7.17E-10
cell transformation	7.28E-07	2.54E-08
mammary tumor	2.40E-06	9.87E-06
colon tumor	2.63E-06	2.63E-06
colon cancer	6.54E-06	4.26E-06
gastrointestinal tract cancer	1.13E-05	7.93E-05
colorectal cancer	1.24E-05	2.13E-05
colorectal tumor	8.40E-06	1.33E-04
intestinal cancer	9.14E-06	7.93E-05
hematological neoplasia	3.10E-05	2.20E-03
benign tumor	5.46E-05	2.25E-03
Cell Cycle		
cell cycle progression	4.38E-09	7.46E-15
M phase	5.81E-08	1.56E-10
mitosis	1.17E-05	8.78E-12
cytokinesis	1.56E-05	8.78E-08
interphase	8.87E-05	2.88E-08
modification of chromatin	2.11E-03	2.40E-07
remodeling of chromatin	#N/A	7.05E-06
senescence of cells	1.28E-03	1.40E-05
G2/M phase	#N/A	2.53E-05
G2 phase	1.74E-03	3.63E-05
ploidy of cells	4.08E-03	6.63E-05
arrest in interphase	1.97E-03	8.19E-05
metaphase	#N/A	8.36E-05
aneuploidy of cells	2.29E-03	9.12E-05
segregation of chromosomes	2.17E-04	9.31E-05
Cell Death		
cell death	3.59E-22	2.08E-24
apoptosis	5.60E-16	2.59E-17
necrosis	1.11E-13	9.63E-13
cell death of tumor cell lines	6.55E-09	3.42E-09
apoptosis of tumor cell lines	9.86E-07	1.21E-05
cell survival	1.09E-06	1.52E-05
neuronal cell death	1.49E-06	4.76E-05
cell death of connective tissue cells	2.85E-06	4.17E-06
cell viability	3.10E-06	1.72E-04
fragmentation of DNA	2.45E-05	1.41E-03
cell viability of tumor cell lines	7.70E-05	#N/A
cell death of epithelial cells	9.88E-05	2.28E-03
cell death of cervical cancer cell lines	4.56E-03	3.59E-06
Cellular Movement / Interaction		
cytokinesis	1.56E-05	8.78E-08
binding of cells	6.73E-05	#N/A
cell movement	1.14E-04	8.86E-06
cell movement of tumor cell lines	5.52E-04	3.85E-05
Development / Morphology		
morphology of reproductive system	8.82E-07	1.31E-05
abnormal morphology of embryonic tissue	1.75E-06	1.19E-05
morphology of embryonic tissue	2.36E-06	4.99E-06
size of embryo	5.98E-06	2.23E-09
proliferation of tumor cell lines	4.15E-05	2.63E-05
morphology of genital organ	2.69E-05	2.94E-04
abnormal morphology of genital organ	5.89E-05	4.13E-04
proliferation of embryoblast	6.48E-05	2.06E-04
fibrogenesis	7.61E-05	1.15E-04
formation of embryonic tissue	1.84E-03	9.76E-06
abnormal morphology of extraembryonic tissue	6.59E-04	2.79E-05
growth of embryo	7.44E-03	3.30E-05
morphology of extraembryonic tissue	1.23E-03	3.69E-05
morphology of gonad	1.16E-04	5.19E-05

Selection of functional enrichment of diseases and disorders, molecular and cellular functions as well as physiological system development and function of all genes expressed in either fPGCs (9210) or oocytes (9207) using the IPA software. The complete IPA output, including p-values and the gene sets corresponding to each term, is available in Supplementary Table 4 ($p < 0.0001$ highlighted in red (fPGCs) or green (oocytes)).

analysis we derived biological pathway, cellular component and molecular function Gene Ontology (GO) and KEGG pathway terms for subsets of genes solely expressed in either fPGCs (873) or oocytes (1141) or overlapping between fPGCs and oocytes (967). We considered terms with a p-value < 0.01 to be significantly enriched.

Regarding genes solely expressed in fPGCs we found the KEGG pathways 'hsa04740:Olfactory transduction', 'hsa00140:Steroid hormone biosynthesis' and 'hsa00150:Androgen and estrogen metabolism' being significantly enriched. Concerning the GO terms we would like to emphasize the 'extracellular region' – terms for cellular component GO, 'sensory perception' connected terms for biological process GO and 'olfactory receptor activity' as highest enriched term for molecular function GO. These results indicate that within the genes exclusively expressed in fPGCs there is a significant association with olfactory-related processes and maybe function (Fig. 2A).

With respect to genes exclusively expressed in oocytes we found an association with the KEGG pathway 'hsa04810:Regulation of actin cytoskeleton' as enriched. Furthermore we found 'chromatin organization', 'cell cycle', 'transcription regulation' and 'endosomal' – associated biological property, cellular compartment and molecular function GO terms, respectively, thus indicative of transcriptional control as an active process in oocytes (Fig. 2C).

Regarding genes overlapping between fPGCs and oocytes we found, for example, the KEGG pathways 'hsa04510:Focal adhesion' and 'hsa04020:Calcium signaling pathway' as significant. Furthermore, we found molecular function GO terms associated with 'protein formation', 'chemokine receptor' and 'metal ion binding', cellular compartment GO terms associated with'(plasma-) membrane' and biological pathway GO terms associated with'metal ion transport', 'cell-cell-signaling' and 'secretion' (Fig. 2B).

Ingenuity Systems canonical pathway analysis of all genes expressed in female PGCs and oocytes

In order to acquire greater insights into pathways and functions associated with the total number of elevated transcript levels found in either fPGCs or oocytes, we conducted an IPA Ingenuity-based analysis. We compared transcription regulators, biological functions and canonical pathways most highly enriched in fPGCs and oocytes.

Regarding the canonical pathways output we found 'EIF2-', 'Regulation of eIF4 and p70S6K-', 'mTOR-', 'ILK-', 'Ephrin Receptor-', 'Huntington's Disease-', 'Wnt/β-catenin-' signaling pathways as well as 'Mitochondrial Dysfunction' being most enriched in both fPGCs and oocytes. Notable enrichment and ratio differences were observed in 'Aminoacyl-tRNA Biosynthesis', 'Cell Cycle Regulation by BTG Family', 'Role of CHK Proteins in Cell Cycle' and 'Role of BRCA1 in DNA Damage Response', which are more highly enriched in fPGCs. Whereas 'Germ Cell-Sertoli Cell Junction Signaling', 'Rac Signaling', 'CDK5 Signaling', 'Mitotic Roles of Polo-Like Kinase', 'Actin Cytoskeleton Signaling' and 'Ephrin B Signaling', are more highly enriched in oocytes (Table 1).

With respect to the output associated with biological function we found mammary-, colon- and colorectal tumor/cancer terms

TABLE 3

PUTATIVE CRUCIAL TRANSCRIPTION FACTORS ACTIVE IN FEMALE PGCs AND OOCYTES

Transcription Regulator	fPGCs p-value	Oocs p-value
HNF4A	1.78E-18	8.60E-18
MYCN	3.94E-14	1.59E-10
MYC	3.77E-08	4.10E-05
E2F4	7.48E-06	1.08E-04
HTT	6.05E-05	9.06E-03
IRF4	1.17E-04	> 0,05
NFE2L2	1.88E-04	8.49E-04
TP53 (includes EG:22059)	2.45E-04	7.81E-03
SREBF2	2.46E-04	3.40E-03
HSF2	4.29E-04	7.86E-03
XBP1 (includes EG:140614)	5.57E-04	1.92E-03
CCNE1	1.65E-03	1.75E-03
E2F1	2.12E-03	> 0,05
TFEB	2.14E-03	4.82E-02
KDM5B	2.41E-03	5.00E-03
ATF4	2.60E-03	> 0,05
NOTCH4	2.81E-03	> 0,05
E2F3	3.92E-03	8.90E-03
CTNNB1	4.39E-03	1.00E-02
SREBF1 (includes EG:176574)	4.58E-03	> 0,05
NPAS2	4.77E-03	4.94E-03
ASCL2	4.77E-03	4.94E-03
SOX11	6.92E-03	4.82E-02
TBX2	7.20E-03	2.03E-03
CDKN2A	1.21E-02	7.00E-04
PPARGC1B	1.21E-02	2.96E-02
TFAP2A	1.56E-02	2.84E-03
SKI	1.57E-02	4.45E-03
SPDEF	2.62E-02	> 0,05
ELK3	3.45E-02	6.27E-03
SKIL	3.91E-02	2.50E-04
HEXIM1	4.30E-02	4.94E-03
SIRT2	4.53E-02	4.70E-02

Genes expressed in either fPGCs (9210) or oocytes (9207) were analysed to predict transcriptional regulators significant in the two gene expression datasets using the IPA software. Significant results are highlighted with decreasing colour gradients (fPGCs in red, oocytes in green) ranging from $p < 0.0001$ over $p < 0.001$ and $p < 0.01$ to $p < 0.05$.

enriched in both of fPGCs and oocytes. 'Hematological neoplasia' and 'benign tumor' are slightly more enriched within fPGCs. The gene list associated with these cancer-related terms can be found in Supplementary Table 4. All of the 'Cell Cycle' terms are much more enriched in oocytes, furthermore we found 'binding of cells' and 'cell viability of tumor cell lines' being by far more enriched in fPGCs in contrast to 'cell death of tumor cell lines' which is enriched at a similar level in both fPGCs and oocytes. Concerning 'Development and Morphology' we found several 'embryonic tissue' related terms enriched in both cell types. The terms 'morphology of genital organ' and 'fibrogenesis' are slightly more enriched within fPGCs whereas 'formation of embryonic tissue', '(abnormal) morphology of extraembryonic tissue' and 'growth of embryo' are slightly more enriched within oocytes (Table 2). The list of genes associated with each of these terms can be found in Supplementary Table 4.

Regarding the transcription regulators output we found HNF4A, MYCN, MYC, E2F4 and HTT being the five most enriched transcription regulators in fPGCs. Most of transcription regulators being enriched at a higher level show a smaller p-value for fPGCs than for oocytes (Table 3).

Comparison of the transcriptomes of human and mouse female PGCs

To further confirm independently the germ cells-specific transcriptome of our fPGCs we analysed the overlaps between our fPGCs with that of the transcriptomes of PGCs isolated from female mouse embryos at distinct stages of development (embryonic days 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, and 18.5) (Sabour *et al.*, 2011). As shown in Fig. 3A, the highest overlap is between human fPGCs and 14.5F replicate (rep) 2 mouse fPGCs (4269 + 635 = 4904). However, Fig. 3B highlights that the overlap between the intersection of 13.5F rep1 and 13.5F rep2 mouse fPGCs (which actually contains less but more conserved genes than each of 13.5F replicate 1 and 2) and the human fPGCs contains more genes than all of the other overlaps of mouse fPGCs intersections and human fPGCs (4482). We identified 9210 genes being expressed in our human fPGCs (Fig. 1A). Of these, 6746 genes belong to the 8876 known orthologous genes mentioned above; therefore 6746 is the maximum number possible for the numbers in the lower parts of

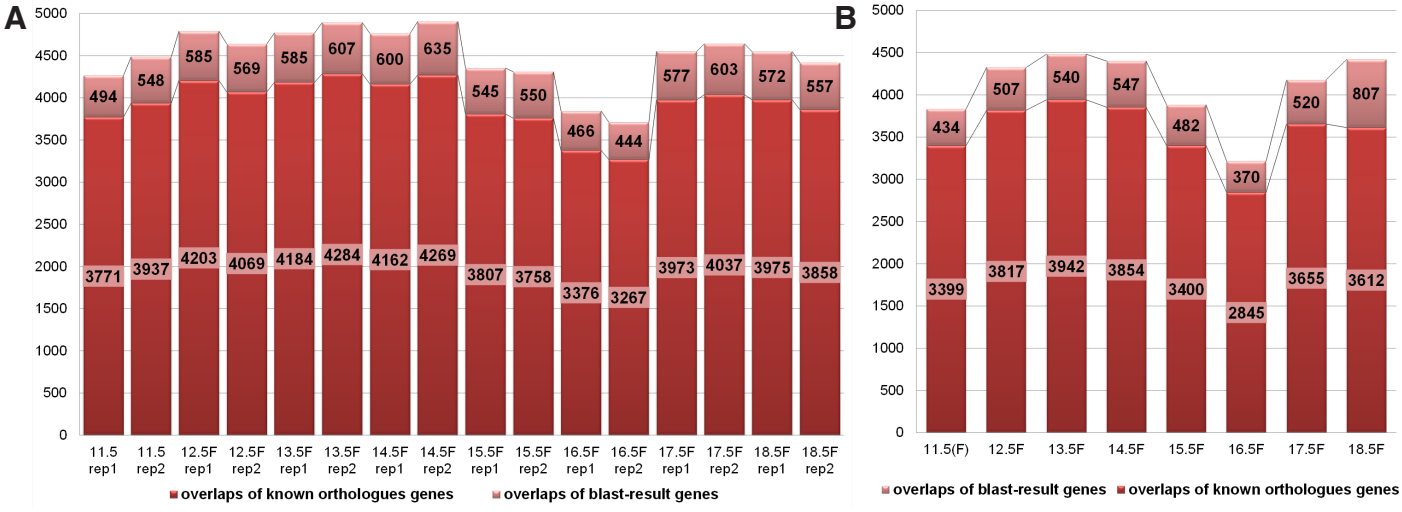


Fig. 3. Comparison of the transcriptomes of mouse and human female PGCs. *Known orthologous genes (8876) and putative orthologous genes resulting from our nucleotide BLAST search (1632) between human and mouse fPGCs (Sabour D* et al.*, 2011) were analysed to identify overlaps.* **(A)** *Comparison of separate mouse fPGCs samples (replicates 1 and 2) at distinct developmental stages (embryonic day 11.5 – 18.5) with our human fPGCs gene expression data set.* **(B)** *Comparison of the intersection of mouse fPGCs replicates 1 and 2 with our human fPGCs transcriptome data.*

TABLE 4

DISTINCT AND OVERLAPPING EXPRESSION OF MEMBERS OF THE OLFACTORY RECEPTOR GENE FAMILY IN FEMALE PGCS, MALE PGCS AND OOCYTES

	Olfactory receptors expressed in				
fPGCs_mPGCs_Oocs	**fPGCs**	**Oocs**	**mPGCs**	**fPGCs_Oocs**	**fPGCs_mPGCs**
OR10G7	OR10AD1	OR11A1	OR10A7	OR10R2	OR5D13
OR10J1	OR10G2	OR1A2	OR10V1	OR13G1	OR9I1
OR10S1	OR11H4	OR2A5	OR1B1	OR1D2	
OR10W1	OR13A1	OR4C11	OR2A9P	OR1N1	
OR11G2	OR1A1	OR51A7	OR2D3	OR2AE1	
OR11H12	OR1E2	OR51B5	OR2T6	OR2L1P	
OR12D2	OR1L8	OR51Q1	OR4F15	OR2M1P	
OR13C2	OR1N2	OR52N4	OR52W1	OR2M2	
OR13C4	OR2AT4	OR5AK2	OR5M11	OR2M3	
OR13C9	OR2T10	OR5K4	OR6A2	OR3A1	
OR14I1	OR2T12	OR5M3	OR6C76	OR4C12	
OR1J2	OR4C15	OR7D4	OR6K6	OR4F4	
OR1J4	OR4C3	OR8A1	OR7E37P	OR4N2	
OR2A1	OR4D11	OR8B2		OR4S1	
OR2A2	OR4D2	OR8B4		OR51I2	
OR2A20P	OR4D9	ORC1		OR5W2	
OR2A25	OR4K14			OR6C65	
OR2AG1	OR4K17			OR6C74	
OR2B2	OR4K2			OR7C2	
OR2C3	OR4S2			OR7G3	
OR2G3	OR4X1			OR8G1	
OR2L3	OR51L1			OR8G5	
OR2M4	OR52L1			OR8K1	
OR2T11	OR52N5			OR8K3	
OR2T33	OR56A3			OR8U9	
OR2W3	OR56B1				
OR2W5	OR5AR1				
OR2Z1	OR5AS1				
OR4A15	OR5H2				
OR4B1	OR5H6				
OR4C45	OR5K1				
OR4C46	OR6W1P				
OR4F3	OR6X1				
OR51D1	OR8U8				
OR51E2	OR9G1				
OR51M1	ORC4				
OR51T1					
OR52A5					
OR52B2					
OR52B6					
OR52E6					
OR52B6					
OR52E6					
OR52H1					
OR52J3					
OR52K1					
OR52N2					
OR56B4					
OR5AN1					
OR5AU1					
OR5C1					
OR5K2					
OR5R1					
OR5T1					
OR5V1					
OR6B3					
OR6C6					
OR6F1					
OR6V1					
OR7A10					
OR7A17					
OR7E156P					
OR7G1					
OR8D1					
OR8G2					
OR8H3					
OR8J3					
ORAOV1					
ORC3					
ORC5					
ORC6					
ORM1					
ORMDL2					
ORMDL3					

Genes highlighted in red are those with orthologs in mouse PGCs. Also refer to Supplementary Table 5.

the columns in Fig. 3. For 1242 of the fPGCs genes, we found a possible orthologous gene by nucleotide BLASTN and as such 1242 is the maximum number possible for the numbers in the upper parts of the columns in Fig. 3. Novel orthologous genes either human or mouse are highlighted in blue in Supplementary Table 5.

As a result there are 1242 fPGCs genes remaining which are not comparable to any of the mouse genes detectable using the mouse Illumina BeadChip neither by official gene symbol nor our BLASTN result. We identified 4904 genes for the fPGCs-14.5F rep2 overlap and 4482 for the 13.5F rep1/rep2-intersection – fPGCs overlap of a maximum of 6746 + 1222 = 7968 genes.

Distinct and overlapping expression of members of the olfactory receptor gene family

Olfactory receptor (OR) proteins are members of a large family of G-protein-coupled receptors (GPCR) arising from single exon-coding genes and are the largest in the genome (Feldmesser *et al.,* 2006). In an earlier study, we identified expression of OR-encoding genes expressed in mPGCs (Goto *et al.,* 1999), to further validate this finding, we interrogated our current dataset for the presence of these genes. In total, we detected expression of 164 OR-encoding genes. Of these 72 are common between fPGCs, mPGCs and oocytes, 36 in fPGCs, 16 in oocytes, 13 in mPGCs, 25 in both fPGCs and oocytes and finally 2 in common between fPGCs and mPGCs. Of these human OR-encoding genes, we identified 33 mouse orthologs highlighted in red (Table 4).

Discussion

To date the *in vivo* derived transcriptomes of both male and female PGCs have been restricted to the mouse, where the identification and isolation of such cells is feasible using the promoter sequences of PGCs-lineage markers such as *Oct4*, *Blimp1* and *Prdm14* serving as reporters (Sabour *et al.,* 2011; Saitou, 2009). To circumvent this drawback, *in vitro* differentiation models both human and mouse (Hubner *et al.,* 2003; Toyooka *et al.,* 2003; Geijsen *et al.,* 2004; Kee *et al.,* 2006; Nagano, 2007; Qing *et al.,* 2007; Wei *et al.,* 2008; Hayashi *et al.,* 2011; Panula *et al.,* 2011) have been established using pluripotent stem cells based on embryonic and induced pluripotent stem cells. Although differentiation of human ESCs and iPSCs into PGCs-like cells have been performed in mouse (Geijsen *et al.,* 2004; Kee *et al.,* 2006; Panula *et al.,* 2011) transcriptome analysis of the derived PGCs have been restricted to known PGCs-specific genes such as *BLIMP1/PRDM1, c-KIT STELLA/DPPA3, VASA/DDX4,* and *FRAGILIS/IFITM5*. The lack of genome-wide transcriptome analyses of these human ESCs- and iPSCs-derived PGCs further precludes in-depth knowledge and analyses of key mechanisms underlying the complex and intricate biological processes of human germ cell development and subsequent gametogenesis.

In an attempt to overcome some of the afore-mentioned shortfalls associated with current *in vitro* models of human PGCs, we have carried out in this study extensive transcriptome-based comparative analyses of the molecular portraits of SMART-generated T7 promoter-linked double-stranded cDNA samples derived from 4 unfertilized oocytes, 200 mPGCs and 500 fPGCs previously generated as described in (Adjaye *et al.,* 1999; Goto *et al.,* 1999). To enrich for transcripts expressed in female germ cells, we included mPGCs as a means of excluding Y-linked genes. Confirmation of the success of this subtraction approach is the detected expression

of *ZFY* solely in mPGCs and the Zona Pellucida genes *ZP4* in both fPGCs and oocytes, whilst *ZP1* and *ZP3* were detected in oocytes only (Supplementary Table 1).

Amongst the well characterised PGCs-specific genes, we detected for example, expression of *BLIMP1/PRDM1* and *PIWIL2* solely in the fPGCs and mPGCs cells, *VASA/DDX4* in fPGCs and oocytes, *DAZL* and *STELLA/DPPA3* in the oocytes only (Supplementary Table 1). Interestingly, *LIN28A* is expressed in all cell types and it has been shown in the mouse that *Lin28*, a negative regulator of Let-7, is essential for PGCs development and also associated with germ cell malignancy (Rybak *et al.,* 2008; West *et al.,* 2009).

BMP signaling has been shown to be essential for PGCs specification (Ying *et al.,* 2000; Ying and Zhao, 2001; Pesce *et al.,* 2002) and in line with this we detected expression of *BMP2, 3, 4, 6, 7* and *GDF1, 2, 3, 9* and *15* and the receptors *BMPR1A* and *BMPR2* (Supplementary Table 1).

The observed variable expression patterns, for example, *VASA/DDX4* in fPGCs and oocytes, *DAZL* and *STELLA/DPPA3* in the oocytes can be attributed to the PCR-based SMART-generated T7 promoter-linked double-stranded cDNA from the reduced cell numbers (4 unfertilized oocytes, 200 mPGCs and 500 fPGCs). Nonetheless, our approach has provided for the first time a snapshot of the transcriptomes of these rare human cells.

The comparison of the human fPGCs transcriptomes with the transcriptomes of PGCs isolated from mouse embryos at distinct stages (E11.5 – E18.5) of development (Sabour *et al.,* 2011) resulted in the identification of 1632 mouse orthologous genes with nucleotide identities within the coding regions ranging from 100 – 22%. The highest number of overlapping genes was with the transcriptomes derived from E14.5 mice (Fig. 3), the significance of this is at present unknown. Furthermore, of the 11 genes identified by Sabour *et al.,* as expressed exclusively in male and female PGCs both *in vitro* and *in vivo* but not in ESCs, we identified 4 of these (*4930432K21Rik, Mov10l1, Tex13, Hba-a1*) as expressed in human fPGCs, thus further confirming the quality of our dataset which highlights the conservation of key PGCs-associated genes potentially involved in the development and specification of PGCs in both species.

With the transcriptome data at hand we could associate expressed genes to signaling pathways. For example, we identified key signaling pathways such as mTOR, WNT/β-catenin, ERK/MAPK, PI3K/AKT, estrogen receptor and androgen receptor, cell cycle regulation by BTG family proteins, Rac, CDK5, Actin cytoskeleton, Ephrin B and germ cell-Sertoli cell junction signaling as active in fPGCs and oocytes.

Cytological analysis in mice and human suggests that both female and male PGCs are equally capable of entering meiosis (12 dpc in mice and 10 weeks in humans) and that the decision of cell fate of germ cells, either meiosis or mitosis takes place in the gonad (McLaren and Southee, 1997; Suzuki and Saga, 2008; Bowles and Koopman, 2010). It is still unknown and controversial whether entry into meiosis is induced in the female gonad or whether it is intrinsically programmed in both male and female PGCs and inhibited in the male gonad and permissive in the female gonad, or whether it is due to a combination of regulatory factors. Indeed, we do see differential expression of a host of transcription factors (for example, zinc finger containing transcription factors-ZNFs) in fPGCs and mPGCs, as potential regulators of this decision making process (Supplementary Table 1).

Furthermore, expression of several genes associated with components of the meiotic machinery was observed, for example, the synaptonemal complex (*SYCP2, SYCP3, TEX11, TEX13B, TEX15*), meiosis-specific cohesins (*SMC1B, STAG3*), meiotic recombination machinery (*DMC1, MSH4, TRIP13*), piRNA pathway (*PIWIL2, TDRD1, MAEL*) and other meiosis-associated genes (*HSPA4L, HSF2BP, HORMAD2, INTS6, CREBL2, ATF7IP2, MTL5, ZFP473*) (Su *et al.,* 2011). In contrast, in mPGCs we detected expression of *NODAL*, which has been shown in the mouse to have an autocrine signaling role during the specification of male PGCs (Souquet *et al.,* 2012).

An unexpected observation in our study is the expression of a plethora of olfactory receptor-encoding genes in fPGCs, mPGCs and unfertilized oocytes (Table 4). Olfactory receptors (ORs) which are G-protein-coupled receptors (GPCR) share a 7-transmembrane domain structure with many neurotransmitter and hormone receptors and are responsible for the recognition and G protein-mediated transduction of odorant signals. In an earlier study using the same fPGCs and mPGCs mRNAs (differential display based-analyses) as employed in this study, we identified OR-encoding genes expressed in mPGCs cells (Goto *et al.,* 1999). Although OR-encoding genes have so far been detected in mPGCs, the detected expression in fPGCs and oocytes in the current study is not an artefact as it has recently been shown that there is widespread expression of olfactory-related genes in tissues as diverse as testis, muscle, liver and skin, although some of these might be pseudogenes (Feldmesser *et al.,* 2006). Of the 164 expressed OR-encoding genes, *OR10J1, OR11A1, OR1D2*, *OR52K1* and *OR7A17* have been detected as expressed in human testis (Feldmesser *et al.,* 2006) and of these only *OR10J1* has so far been detected as expressed in mPGCs (Parmentier *et al.,* 1992). Additionally, of the 164 human OR-encoding genes differentially expressed between mPGCs, fPGCs and oocytes, we have identified 33 mouse orthologs (for example, *OR51M1*/*Olfr7*8, *OR4D11*/*Olfr1423* and *OR1B1*/*Olfr362*) highlighted in red (Table 4). Though unexpected, this is the first description of a plethora of OR-encoding genes showing overlapping and distinct expression patterns in human unfertilized oocytes, male and female PGCs at 10 weeks of gestation. Based on our findings, the current hypothesis that OR-encoding genes are involved in sperm chemotaxis should be extended to include the development of female germ cells and oocytes.

In summary, we anticipate that our current dataset will provide insights into the design of more extensive functional studies that might increase our meagre knowledge of the genes and associated signaling pathways operative during germ cell development and hence in turn lead to the development of strategies enabling better differentiation and molecular characterisation of germ cells derived from either ESCs or iPSCs. Ultimately, this would have a profound relevance to reproductive as well as regenerative medicine.

Materials and Methods

The isolation of the human germ cells and also failed fertilised oocytes are as described in detail in Goto *et al.,* 1999 and Adjaye *et al.,* 1999 respectively.

Fetal samples

The human fetal samples were obtained from the Human Embryonic Tissue Bank maintained at the Institute of Child Health (ICH) in collaboration with the Department of Obstetrics and Gynaecology, University College London (UCL), UK, and funded by the Medical Research Council. The collection, deposition and use of human fetal samples were approved by the Joint UCL/UCLH Committees on the Ethics of Human Research and

the Ethical Committee of the ICH, and were carried out in accordance with the Polkinghorne report. Gonads were obtained from a male and a female fetus, at 10 weeks gestation, for isolation of PGCs. The age of the fetus was the anatomical (embryonic) age, as determined by limb development, and not the age from the last menstrual period (LMP). The fetal samples were kept on ice in Leibovitz's L15 medium (Gibco BRL, UK) and the gonads were dissected from the fetuses within 2–3 h after the surgical termination of pregnancy.

Isolation of germ cells from the gonad

Germ cells were isolated from the gonad following the protocols described by Buehr and McLaren, 1993. The gonads, dissected from the fetus, were freed from the attached mesonephric tissue under the dissecting microscope. They were then incubated in 1 mmol/l EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 5 min at room temperature to loosen the germ cells from somatic cells. The gonads were washed briefly in PBS, transferred to fresh PBS and gently squeezed by watchmaker's forceps to release the germ cells. The germ cells could be distinguished from somatic cells by their size, round shape and bright appearance. The germ cells were manually collected by a finely-drawn Pasteur pipette, and 200 (male) and 500 (female) germ cells placed in 30 μl of ice-cold lysis buffer [0.8% IGEPAL (Sigma, UK), 1 U of RNase inhibitor (Gibco BRL, UK), 5 mM dithiothreitol (DTT; Gibco BRL)], snap-frozen in liquid nitrogen and stored at -70°C until RNA extraction.

Oocyte samples

Human oocytes derived by *in vitro* fertilization (IVF), and forming part of a research project on embryo culture conducted by V. Bolton, were donated for research with consent by patients attending the Assisted Conception Unit, King's College Hospital (KCH) London. Pituitary suppression, ovarian stimulation and oocyte retrievals were carried out as described previously (Waterstone and Parsons, 1992). IVF was performed as described previously (Bolton *et al.,* 1989) except that the culture medium used was a commercial preparation of Earle's balanced salt solution (Medi-Cult, Imperial Laboratories, Hampshire, UK). The four unfertilized oocytes collected on day 1, were failed-fertilization oocytes selected from cohorts placed with poor quality sperm such that none of the oocytes developed a second pronucleus. Great care was taken to remove contaminating cumulus cells surrounding the oocytes. The oocytes were anonymized and lysed at KCH before transfer to the Institute of Child Health. Oocytes in 0.5 μl of PBS were added to 1.5 μl of lysis buffer [0.8% IGEPAL (Sigma), 1 U/μl of RNAsin (Gibco BRL), 5 mM DTT (Gibco BRL)], centrifuged briefly at 12 000×g and overlayed with one drop of mineral oil (Sigma) and stored at -70°C.

mRNA extraction and cDNA amplification

T7 promoter–linked double-stranded cDNA samples derived from 4 unfertilized oocytes, 200 mPGCs and 500 fPGCs were previously generated as described (Adjaye *et al.,* 1999; Goto *et al.,* 1999; Adjaye *et al.,* 2005). Briefly, mRNA was extracted from thawed lysed cells using Oligo-dT magnetic beads (Dynabeads). cDNA was generated using T7 promoter-linked oligo-dT primers for the reverse transcription (RT) step, and whole-transcriptome amplification was executed using a modified SMART amplification protocol (BD Biosciences, San Jose, CA, http://www.bdbiosciences. com). Finally, concentrations were evaluated on the Agilent 2100 Bioanalyzer.

Illumina bead chip hybridization and data analyses

Global gene expression analysis was carried out employing the Illumina microarray platform. SMART generated T7 promoter-linked cDNA were used as input for the T7 polymerase-mediated *in vitro* transcription to produce biotin-labeled cRNA (Illumina TotalPrep RNA Amplification Kit, Ambion, Austin, TX, USA). 500 ng of purified cRNA was hybridized onto Illumina HumanRef-8 v3 Expression BeadChips (Illumina, San Diego, CA, USA) on the Illumina Beadstation 500 platform followed by washing and blocking of the samples, staining with streptavidin-Cy3 and quantitative detection of the resulting fluorescent array image.

Raw data were obtained using the manufacturer's software GenomeStudio V2010.2 (Gene Expression module v.1.7.0). Subsequently, raw data were imported into the Bioconductor environment (Gentleman *et al.,* 2004) and quantile normalized using the beadarray package (Dunning *et al.,* 2007). In order to test for global gene expression similarities, pairwise Pearson correlation coefficients were calculated for all samples. Pearson correlation co-efficients, scatter- and boxplots, and hierarchical clustering were calculated using the R environment (http://www.r-project.org). We examined the distribution of normalized gene expression signals in each sample separately. Due to the low overall signal intensities we defined the quantile of 0.5 (arbitrary selection) as a threshold for identifying genes showing elevated expression levels in order to define these as "expressed" (Supplementary Fig. 1 for the distribution of raw and normalized gene expression signals). To identify overlapping and distinct genes expressed within oocytes, male and female PGCs we generated Venn Diagrams using the 'VENNY' online tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Functional annotation and enrichment analyses of particular gene sets resulting from the Venn Diagram analysis were performed using the DAVID platform version 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (Dennis *et al.,* 2003; Huang da *et al.,* 2009). Human official gene symbols were used as input against DAVID's 'homo sapiens' background; analyses were executed based on DAVID default parameter settings unless stated otherwise. We further analysed the data employing IPA (Ingenuity Systems, www.ingenuity.com) to gain further information on potential important transcription factors and alternative surveys on enriched biological functions and canonical pathways. For each sample of interest we used official gene symbols of the total number of expressed genes to do the IPA analysis based on the entire active cellular transcriptome.

Finally, we performed a nucleotide blast (Perl version 5.12.4-4, 2011-09-06, www.perl.org; Bioperl version 1.6.901-1, 2011-06-17, www.bioperl.org; BLAST (Altschul *et al.,* 1990)) between genes we found expressed in human fPGCs and within one of several datasets of mouse fPGCs derived from mouse embryos at distinct stages of gestation (Sabour *et al.,* 2011). We compared the non-redundant union of all human genes expressed in either fPGCs, mPGCs and oocytes, amounting to 12165 genes, to the non-redundant list of all mouse genes detectable as expressed by the Illumina MouseRef-8 v2.0 expression BeadChip used by Sabour D. *et al.,* which is a total of 17957 genes. This analysis led to the identification of 8876 known orthologous genes bearing the same official gene symbols. The remaining (12165 – 8876 =) 3289 human genes were used to perform a nucleotide blast search against the remaining (17957 – 8876 =) 9081 mouse genes to identify additional matching genes. Applying an arbitrary expectation value threshold of 1E-10 resulted in the identification of 1632 pairs of human and mouse genes showing significant sequence overlap and are thus also declared as orthologous genes (see Supplementary Table 5).

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