Evaluation in mammalian oocytes of gene transcripts linked to epigenetic reprogramming

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Abstract

The mature mammalian metaphase II (MII) oocyte has a unique ability to reprogram sperm chromatin and support early embryonic development. This feature even extends to the epigenetic reprogramming of a terminally differentiated cell nucleus as observed in connection with somatic cell nuclear transfer. Epigenetic nuclear reprogramming is highly linked to chromatin structure and includes covalent modifications of DNA and core histone proteins as well as reorganization of higher-order chromatin structure. A group of conserved enzymes mediating DNA methylation, methyl-CpG-binding protein (MeCP), histone acetylation and methylation, and chromatin remodeling are extensively involved in epigenetic reprogramming in mammalian cells. Using the oligonucleotide microarray technique, the present study compared the expression levels of 86 genes associated with epigenetic reprogramming in murine *in vivo* matured MII oocytes with that of germinal vesicle oocytes. Correlation between biological replicates was high. A total of 57 genes with potential reprogramming effect were detected. In MII oocytes, four genes were significant up-regulated, whereas 18 were down-regulated and 35 unchanged. The significantly regulated genes were validated by real-time quantitative RT-PCR. For example, MII oocytes showed a significant down-regulation of oocyte-specific maintenance DNA methyltransferase, Dnmt1o, and up-regulation of MeCP transcript, methyl-CpG binding domain protein 2. Furthermore, histone acetyltransferases were proportionally overrepresented when compared with histone deacetylases. These data elucidate for the first time some of the mechanisms that the oocyte may employ to reprogram a foreign genome either in form of a spermatozoa or a somatic nucleus and may thus be of importance for advancing the fields of stem cell research and regenerative medicine.

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Introduction

The culmination of mammalian oogenesis is the creation of a mature metaphase II (MII) oocyte capable of sustaining fertilization, reprogramming the parental genomes, and supporting early embryonic development. The oocyte cytoplasm rapidly starts to modify the sperm genome upon fertilization resulting in decondensation of paternal chromatin, substitution of protamines with highly acetylated histones, and demethylation of DNA (Santos *et al.* 2002, Spinaci *et al.* 2004). By contrast, the oocyte-derived maternal chromatin seems more protected from this extensive reprogramming activity undergoing DNA demethylation at a more passive level during subsequent cleavages (Oswald *et al.* 2000). The observed asymmetry in parental genome reprogramming is believed to result from marked differences in the original nucleosomal structure of the maternal and paternal chromatin respectively (Mayer *et al.* 2000, Santos *et al.* 2002, Spinaci *et al.* 2004).

Several studies have approached the underlying molecular mechanisms that take place in connection with reprogramming, suggesting that the three-dimensional structure of chromatin is highly important in the regulation of gene expression. Generally, a condensed chromatin structure is associated with an inaccessible and gene repressive state, whereas more decondensed and open chromatin is more amenable to gene activation (Turner 2001).

Notably, transcription levels may be affected by covalent modifications of the DNA helix and core histones comprising the nucleosome, the core unit of chromatin. The complex mechanisms involved in the modifications of chromatin are mediated by a group of highly conserved enzymes, including DNA methyltransferases (DNMTs), methyl-CpG-binding proteins (MeCPs), histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases/deiminases (HDMs), and

chromatin remodeling complexes (ChRs). Covalent DNA methylation, which is the most abundant form of epigenetic modification, occurs at cytosines in the pyrimidine ring of CpG dinucleotides dispersed throughout the genome and is generally associated with gene silencing. The repressive effect of DNA methylation is mediated by the MeCPs, which again recruit a wide range of co-repressors, including HDACs. Nucleosome core histones are covalently modified at their N-terminal tails, which are highly charged and tightly associated with the DNA helix. In particular, acetylation and methylation have been associated with regulation of gene expression and it has been suggested that the vast number of combinations may actually form a specific histone code, which could determine the binding patterns of regulatory proteins. By contrast, ChRs do not interact covalently with DNA in a sequence-specific manner, but are rather recruited to promoters of target genes by sequence-specific transcription factors. Once recruited, the catalytic subunits of the ChRs (ChR-ATPases) cause an altered conformation and position of nucleosomes by ATP hydrolysis (Kingston & Narlikar 1999). This leads to weakened DNA–histone contacts resulting in histone octamers changing positions along the DNA helix (Bazett-Jones *et al.* 1999, Whitehouse *et al.* 1999), thus either increasing or reducing the binding of transcription factors to promoter regions. The groups of proteins important to epigenetic modifications and chromatin remodeling do not act independently, but are closely coordinated, often by forming large multi-protein complexes (Nan *et al.* 1998, Wade *et al.* 1999, Robertson *et al.* 2000, Rountree *et al.* 2000).

Remarkably, the highly specialized ability of the MII oocyte cytoplasm to reprogram a sperm genome extends to a comparable level of dedifferentiation and reprogramming of a diploid somatic nucleus from adult tissue (Wilmut *et al.* 1997), i.e., somatic cell nuclear transfer (SCNT) or cloning. By contrast, fully grown germinal vesicle (GV) oocytes are not considered capable of adequate reprogramming (Fulka *et al.* 2002, Tesarik *et al.* 2003, Zhang *et al.* 2004). For example, when murine GV oocytes are enucleated and the remaining ooplasm subsequently is used for SCNT, nuclear reprogramming is severely reduced, without formation of pseudo-pronuclei or cleavage (Gao *et al.* 2002). The factors responsible for gamete and somatic reprogramming are likely to become available during the final stages of oogenesis during the transition from GV to MII stage, thus highlighting the MII oocyte cytoplasm as the key player in the reprogramming of a specialized genome into a totipotent state. However, the exact mechanisms by which the MII oocyte cytoplasm erases the established patterns of gene expression imposed on the sperm genome or a somatic nucleus remain largely unknown.

To elucidate some of the tools involved in early embryonic reprogramming, the levels of gene transcripts believed to be of importance to epigenetic modifications

and chromatin remodeling were detected by oligonucleotide microarrays in *in vivo* matured MII oocytes and compared with fully *in vivo* grown GV oocytes. Furthermore, real-time quantitative RT-PCR (qRT-PCR) was used to validate expression patterns of a number of significantly regulated reprogramming genes.

Results

Oocyte collection and validation of RNA

On average, 320 and 421 oocytes were pooled for each biological replicate of GV and MII oocytes respectively. An examination of total RNA on a 2100 Bioanalyzer confirmed the extraction of high-quality RNA (data not shown). Correlations between biological replicates were substantially high, indicating identical global transcription patterns and thus high reliability of the microarray gene chip procedure performed on minute amounts of oocyte RNA (Fig. 1 and Table 1). An unsupervised hierarchical cluster analysis using all detected probe sets showed that biological replicates representing the same developmental stage were independently grouped together as expected. Genes known to be preferentially expressed in oocytes such as the zona pellucida glycoproteins (*Zp1*, *Zp2*, and *Zp3*), growth differentiation factor 9 (*Gdf9*), bone morphogenetic protein 15 (*Bmp15*), moloney sarcoma oncogene (*Mos*), cyclin B1 (*Ccnb1*), spindlin (*Spin*), MATER (*Nalp5*), zygote arrest 1 (*Zar1*), STELLA (*Dppa3*), and oct4 (*Pou5f1*) were abundantly detected on the chip set (Supplementary Table 1, which may be viewed online at www.reproduction-online.org/supplemental). Although

Figure 1 Correlation plot illustrating the concordance in probe set expression values ($n=45\,101$) between two of the biological replicates. The high correlation coefficient (r) confirms the reliability of Affymetrix gene chip technology performed on minute amounts of mRNA extracted from mammalian oocytes.

Table 1 Characteristics for microarray chip samples. A high correlation coefficient (r) between biological replicates indicates almost identical global transcription patterns and thus high validity and reliability of the microarray procedure *per se* performed on minute amounts of RNA.

Develop- mental stage	Biological replicates (n)	Median (range) r between biological replicates	P value
GV oocytes	3	0.993 (0.992–0.995)	< 0.0001
MII oocytes	5	0.972 (0.955–0.992)	< 0.0001

GV, germinal vesicle; MII, metaphase II.

oocytes underwent careful denudation, we checked for potential contamination of heterolog RNA from cumulus-granulosa cells. The absence of cumulus transcripts such as hyaluronin synthase 2, pentraxin 3, and gremlin 1 confirmed the oocyte homogeneity of the collected samples.

Global oocyte gene expression and inclusion of genes for further analyses

After excluding probe sets that were 100% absent in both groups of replicates, the data were reduced from 45 101 to 24 919 probe sets. To minimize false positives, a probe set was then included for further analysis provided that a 100% present call was found in either one of the two groups of replicates, irrespective of its call percentage in the other replicate group. Accordingly, a total of 14 697 probe sets were expressed exclusively in GV oocytes, in MII oocytes, or both. A t -test for difference in expression level between GV and MII oocytes Benjamini–Hochberg corrected for multiple testing yielded that 5169 (35%) probe sets were significantly down-regulated, 609 (4%) probe sets were up-regulated, and 8919 (61%) probe sets were equally expressed. A complete list of up- and down-regulated genes is available at <http://www.ebi.ac.uk/arrayexpress/> (accession no. E-MEXP-1146).

General characteristics of reprogramming genes

A comprehensive list of 86 candidate transcripts involved in epigenetic modifications and chromatin remodeling was constructed from available literature and the Nuclear Protein Database (<http://npd.hgu.mrc.ac.uk>, Accessed April 2, 2006). Supplementary Table 1 (which may be viewed online at www.reproduction-online.org/supplemental) shows the average expression indices, fold changes, and corrected P values supplemented with values for a number of transcripts preferentially expressed in the oocyte. In total, 57 genes (70%) were detected in GV and/or MII oocytes on the chip set. Within the 33 genes detected as down-regulated, 14 had fold changes from -1 to -2 , eight had fold changes from -2 to -5 , three had fold changes from -5 to -10 , and eight had

fold changes < -10 . A total of 24 genes were detected as up-regulated with 16 genes having fold changes from 1 to 2 and eight genes having fold changes ranging from 2 to 5. However, when only statistically significant fold changes were considered, four genes were up-regulated and 18 genes were down-regulated, whereas 35 genes indicated an unchanged level of gene expression. The dendrogram in Fig. 2 illustrates the final clustering tree, where significantly regulated genes close to each other have high similarity in their standardized expression values across all eight samples.

Figure 2 Dendrogram illustrating the final hierarchical clustering tree of 22 significantly up- or down-regulated reprogramming genes (for gene symbols, see Supplementary Table 1, which may be viewed online at www.reproduction-online.org/supplemental) in MII oocytes when compared with GV oocytes (five and three biological replicates respectively). The expression values for a gene across all eight samples are standardized to have mean 0 and s.d. 1 by linear transformation. The distance between two genes is defined as $1 - r$, where r is the standard correlation coefficient between the eight standardized values of two genes. Two genes with the closest distance are first merged into a supergene, connected by branches with length representing their distance, and deleted for future merging. The expression level of the newly formed supergene set is the average of standardized expression levels of the two genes (average linkage) for each sample. Then, the next pair of genes (supergene) with the smallest distance is chosen to merge and the process is repeated until all genes are merged into one final cluster. Red and blue color indicates up- and down-regulation respectively relative to the mean expression of the eight samples combined.

DNMTs and MeCPs

The gene of the oocyte variant form of the maintenance DNMTs, *Dnmt1o*, was abundantly expressed in GV oocytes and then significantly down-regulated in MII, whereas both the *de novo* DNMTs *Dnmt3a* and *Dnmt3b* indicated unchanged level of expression. Of the seven known MeCPs that bind to methylated DNA, two were significantly up-regulated (*Mbd2*, *Mbd4*), one was down-regulated (*Mbd1*), one (Kaiso/*Zbtb33*) showed no change, while *Mecp2*, *Zbtb4*, and *Zbtb38* were not detected on the chip.

HATs

HATs can be classified into five subgroups according to sequence homology and internal protein domains. All five members of the MYST subgroup were detected in oocytes. TIP60 (*Httatip*), MOF (*Myst1*), and MORF (*Myst4*) showed significant down-regulation, while HBO1 (*Myst2*) and MOZ (*Myst3*) were unchanged. Of the members belonging to the GNAT subgroup, elongation protein 3 homolog (*Elp3*), and GCN5 (*Gcn5l2*) were significantly down-regulated, histone aminotransferase 1 (*Hat1*) was unchanged and p300/CBP-associated factor (*Pcaf*) was absent. The two transcriptional 'coactivators' E1A-binding protein p300/p300 (*Ep300*) and CREB-binding protein/CBP (*Crebbp*), which are almost functionally equivalent (p300/CBP subgroup), were both expressed in oocytes at an unchanged level as were the three nuclear hormone-receptor cofactors (SRC subgroup) SRC-1 (*Ncoa1*), GRIP1 (*Ncoa2*), and ACTR (*Ncoa3*). The basal transcription factors TAF_{II}250 (*Taf1*) and TFIIC (*Gtf3c*) were also expressed at an unchanged level. The more recently identified HATs, *Cdyl*, *Atf2*, and *Ogt* were all expressed in oocytes at an unchanged level.

HDACs

HDACs are subdivided into four classes according to phylogenesis. Class I members *Hdac1*, *Hdac2*, and *Hdac3* were expressed in oocytes, whereas *Hdac8* was absent. *Hdac1* was down-regulated and *Hdac2* and *Hdac3* were unchanged. No class II HDAC was detected, except *Hdac6* and *Hdac9*, which were down-regulated and unaffected respectively. Except *Sirt2*, all sirtuins or class III HDACs were expressed in oocytes of which *Sirt4* was up-regulated, *Sirt3*, *Sirt5*, and *Sirt7* were down-regulated, and *Sirt1* and *Sirt6* showed no change. The single class IV member, *Hdac11*, was not detected.

HMTs and HDMs

HMTs either activate or repress gene function depending on the residue of the modified amino acid. Histone methylation at positions *H3K4*, *H3K36*, or *H3K79* is associated with gene activation. Of the activating HMTs,

DOT1-like (*Dot1l*) was undifferentially expressed, SET and MYND domain containing 3 (*Smyd3*) was down-regulated, and SET domain containing 1A (*Setd1a*), myeloid/lymphoid or mixed-lineage leukemia 1 (*Mll1*), SET7/9 (*Setd7*), and Meisetz PR domain containing 9 (*Prdm9*) were absent. Of the HMTs associated with repression (*H3K9*, *H3K27*, and *H4K20*), suppressor of variegation 3-9 homolog 1 (*Drosophila*) (*Suv39h1*) and ESET SET domain, bifurcated 1 (*Setdb1*) were down-regulated; SUV39h2 (*Suv39h2*), Eu-HMTase1/GLP1 (*Ehmt1*), ENX-1 (*Ezh2*), PR-SET7/SET8 (*Setd8*), SUV4-20h1 (*Suv420h1*), SUV4-20h2 (*Suv420h2*), and RIZ (*Prdm2*) were unchanged; and G9a (*Ehmt2*) was absent. NSD1 (*Nsd1*), which can act both as activator and repressor, was not detected.

Of the arginine HMTs, one was slightly up-regulated (*Prmt3*), one was down-regulated (*Prmt7*), two were undifferentially expressed (*Prmt1*, *Prmt2*), and three were absent (*Prmt4*, *Prmt5*, and *Prmt6*).

All currently known HDMs were absent in oocytes, except lysine-specific demethylase 1 (*Aof2*), which was expressed at an unchanged level.

ChR-ATPases

The ChR-ATPases are classified into subfamilies according to the presence of conserved domains outside their catalytic region. The two bromodomain SNF2-subfamily ATPases hBRM (*Smarca2*) and BRG1 (*Smarca4*) were both expressed in oocytes and down-regulated. The SANT domain ISWI-subfamily members SNF2L (*Smarca1*) and SNF2H (*Smarca5*) were absent or unchanged respectively. Among the four chromodomain CHD-subfamily ATPases, only Mi-2 β (*Chd4*) was expressed at unchanged level, while *Chd1*, *Chd2*, and Mi-2 α (*Chd3*) were absent.

Real-time quantitative RT-PCR

The four transcripts having statistically significant up-regulation and the 18 transcripts being down-regulated as indicated by the present microarray data were selected for further analysis by real-time quantitative RT-PCR. To select an appropriate internal reference gene, we tested the expression of the three housekeeping genes β -actin (*Actb*), porphobilinogen deaminase (*Hmbs*), and TATA box-binding protein (*Tbp*) using equivalent amounts of total RNA converted into cDNA. A statistically insignificant relationship existed between oocyte developmental stage and expression of *Actb*, which was confirmed by using NormFinder. Accordingly, *Actb* was used as internal reference gene for further analyses.

The RT-PCR expression data for the 22 reprogramming gene transcripts are presented in Fig. 3. The absence of

Figure 3 Bar chart showing fold changes of 22 reprogramming genes measured by real-time quantitative RT-PCR. Mean fold changes are expressed on a $\log_2$ scale as metaphase II oocyte values relative to germinal vesicle stage oocytes. Whiskers represent 95% confidence intervals.

non-specific amplified products was confirmed by melting curve analysis. When comparing RT-PCR fold changes with the microarray data, we observed some discrepancy regarding direction and amplitude. To test whether such differences could be explained by the use of random hexamer primers during cDNA synthesis, we repeated the RT-PCR using oligo(dT) primers (Fermentas, Helsingborg, Sweden). In this case, there was a significantly higher correlation between array data and qRT-PCR data regarding direction and amplitude, suggesting the occurrence of selective deadenylation during meiotic maturation.

Discussion

To our knowledge, this is the first study that specifically investigates the expression levels of a larger group of genes currently believed to be involved in epigenetic modifications and chromatin remodeling. We show that 57 out of these 86 gene transcripts are present in mouse oocytes. We also validated 22 reprogramming gene transcripts that indicated statistically significant up- or down-regulation from GV to MII stage using real-time quantitative RT-PCR. Of particular interest, we observed that the oocyte-specific maintenance DNMT, *Dnmt1o*, is down-regulated and that the *Mbd2* gene showed a significant fivefold up-regulation. Furthermore, we observed that HATs were proportionally overrepresented when compared with the antagonistic HDACs. Taken together, these data suggest that the reprogramming machinery of the oocyte correlates with developmental state in late oogenesis.

In SCNT, the success of epigenetic nuclear reprogramming is thought to depend on tightly coordinated

successive events (Gurdon 2006). First, the epigenetic constraints once imposed on the foreign nucleus need to be erased. Active genes composing the phenotype must be repressed, whereas inactive or repressed genes must become accessible to regulatory protein complexes. Similar to the sperm genome, these changes include decondensation of higher-order chromatin structure, covalent histone modifications, and DNA demethylation. Subsequently, genes required for early embryonic development need to be correctly imposed on the genome and expressed, including stem cell genes associated with pluripotency (Simonsson & Gurdon 2004, Gurdon 2006).

During oocyte maturation, we observed a significant down-regulation of *Dnmt1o* in both array data and qRT-PCR, which is consistent with earlier observations (Ratnam *et al.* 2002, Vassena *et al.* 2005). This oocyte-specific form has a nuclear localization at the earliest stages of oocyte growth, but then becomes localized in a cytoplasmic shell just within the GV oocyte plasma membrane prior to ovulation (Carlson *et al.* 1992). It remains here – potentially without effect on the chromatin – during pre-implantation development except at the eight-cell stage where it briefly enters and exits the nuclei, perhaps to maintain methylation patterns of imprinted genes (Carlson *et al.* 1992, Mertineit *et al.* 1998). Maternal *Dnmt1o* re-enters the nucleus after implantation and is soon replaced by its somatic counter form, *Dnmt1s*, expressed by the embryonic genome. The nuclear absence of *Dnmt1o* during late oogenesis and early embryonic development is in accordance with the lack of maintenance methylation and hence global hypomethylation of zygotic genome during early cleavage. The attenuated

transcription of *Dnmt1o* observed during meiotic maturation suggests that the cytoplasmic storage reaches adequate levels in the fully grown GV oocyte. The poor success rates and developmental abnormalities observed in offspring derived by SCNT may be attributed to the counteracting presence of Dnmt1s in somatic nuclei used as donors. Notably, the rapid genome-wide DNA demethylation of the paternal genome observed within hours after fertilization is believed to be an active process and not a result of nuclear lack of Dnmt1o, since it occurs before the first DNA replication and cleavage in the newly formed zygote (Oswald *et al.* 2000). Thus, DNA methylation is a reversible process, which strongly indicates the presence of specific demethylases residing in the mature MII cytoplasm.

The identity of specific DNA demethylases responsible for paternal genome reprogramming has remained a subject of controversy. Similar to methylated DNA, methylation of core histones was previously considered to be irreversible, but a growing number of histone demethylases/deiminases have recently been identified and characterized (Shi *et al.* 2004, Cloos *et al.* 2006). Among the reprogramming genes yielding up-regulation in MII oocytes, we observed that *Mbd2* yielded a particularly high fold change in comparison to the small fold changes otherwise observed. Notably, meiotic maturation is characterized by general transcriptional silence and considerable mRNA degradation. Earlier, Szyf and co-workers reported to have purified a *bona fide* demethylase able to catalyze active demethylation by hydrolytic cleavage releasing the methyl group from cytosine in the form of methanol (Ramchandani *et al.* 1999). The enzyme was suggested to be identical to Mbd2 (Bhattacharya *et al.* 1999, Cervoni & Szyf 2001, Detich *et al.* 2002), and has shown to be processive (Cervoni *et al.* 1999), a critical feature for the global demethylation event following fertilization. It has also been reported that overexpression of *Mbd2* results in histone acetylation and global demethylation in non-transformed NIH 3T3 cells and cancer cell lines (D'Alessio & Szyf 2006). However, other investigators have been unable to demonstrate a demethylating activity of Mbd2. In fact, Mbd2 was reported to associate with HDAC1 in the gene repressing *MECP1* complex (Ng *et al.* 1999) and, importantly, *Mbd2*-deficient oocytes retain the capacity to demethylate paternal genomes at the same level as wild-type controls (Santos *et al.* 2002). The latter findings suggest that Mbd2 does not have a DNA demethylating effect in early embryonic development or that other compensatory mechanisms may exist. However, an alternative explanation might be the presence of an as yet unidentified oocyte-specific *Mbd2* transcript ('Mbd2o') accounting for the demethylating activity. Oocyte-specific isoforms have been reported for other factors involved in chromatin remodeling (Mertineit *et al.* 1998, Tanaka *et al.* 2001),

and a truncated alternatively spliced Mbd2 transcript exists in the testis (Hendrich & Bird 1998).

The discovery that methylated DNA causes its gene repressive effect through the binding of MeCPs and recruitment of chromatin-condensing HDAC complexes (Nan *et al.* 1998), initiated the view that the relationship between methylated DNA and histone modifications is unidirectional. However, a growing body of evidence suggests that the relationship between chromatin structure and DNA methylation may be more bidirectional than previously thought and that chromatin itself can influence the methylation status of DNA (D'Alessio & Szyf 2006, Milutinovic *et al.* 2006). For example, the HDAC-inhibitor sodium butyrate can trigger replication-independent DNA demethylation (Szyf *et al.* 1985) and inhibitors of histone acetylation inhibit DNA demethylation (Cervoni *et al.* 2002). Histone acetylation induced by external mediators, including HDAC inhibitors, initiates demethylation of ectopically methylated genes, suggesting that histone acetylation precedes DNA demethylation (Cervoni & Szyf 2001, Detich *et al.* 2003). Interestingly, it has been demonstrated that HAT activity is associated with Mbd2 *in vivo* (Angrisano *et al.* 2006) and that Mbd2 co-localizes with the HAT, CBP (*Crebbp*) (D'Alessio & Szyf 2006). We observed that 18 out of 19 known HATs, including *Crebbp*, were abundantly expressed in oocytes, as opposed to 11 out of the 18 known HDAC members. We hypothesize that a yet unidentified oocyte-derived active DNA demethylase, which acts during fertilization, may be facilitated by a cellular environment characterized by the preponderant presence of HATs.

Among the ChR-ATPases, BRG1 (*Smarca4*) attracts particular interest. Our array data indicated down-regulation of *Smarca4* in MII oocytes, whereas qRT-PCR indicated unchanged expression or slight increase. The apparent presence of BRG1 in mature oocytes confirms that BRG1 plays an important role in early embryonic development, since BRG1-depleted oocytes are arrested at the two-cell stage in mice (Bultman *et al.* 2006). Further depletion of BRG1 has been shown to inhibit the reprogramming capacity in egg cell extracts, while its overexpression is associated with extended reprogramming activity (Hansis *et al.* 2004).

Our data revealed that ~14 000 probe sets were detected in oocytes, which is a number very similar to that found by others using Affymetrix oligonucleotide microarray gene chips on oocytes (Pan *et al.* 2005). Global expression profiles between the biological replicates of GV oocytes were very similar, while those between MII replicates were slightly more varied. Such differences in similarity between MII populations comply with observations seen in human oocytes (Goto *et al.* 2002), and may be attributed to the general mRNA degradation during meiotic maturation.

The detection of an abundant expression of oocyte-specific genes combined with the absence of cumulus

granulosa cell transcripts increased the validity of the microarray procedure performed on minute amounts of mammalian oocyte RNA. Considerably high correlation coefficients and group clustering between biological replicates supports experimental reliability. Furthermore, we aimed at optimizing microarray data processing by implementing quantile normalization method, which is especially suitable for pairwise comparisons (Bolstad *et al.* 2003). We also used the GCRMA method to calculate gene expression indices, since it has less variance at low RNA concentrations, better precision for lower expression values, and more consistent estimates of fold change when compared with other normalization models (Irizarry *et al.* 2003a).

Validation of 22 reprogramming genes by RT-PCR confirmed the presence of reprogramming gene transcripts in mammalian oocytes. However, discordance in some cases was observed when results were compared with the microarray data. These differences are likely to be attributed to the use of different types of primers during the first-strand synthesis in the RT step. Following standard protocols, T7-oligo(dT) primers were used in the microarray experiments, whereas random hexamer primers were used for RT-PCR. During mammalian oocyte maturation, some poly(A) mRNAs are deadenylated, but not degraded (El Mouatassim *et al.* 1999, Lequarre *et al.* 2004). The subsequent translation of mRNA is linked to the extent and timing of its adenylation status during the transition from GV to MII oocyte stage. Accordingly, the use of oligo(dT) primers for RT may increase the risk that some deadenylated transcripts with a too short or completely absent poly(A) tail will not be detected in an equal abundance in MII oocytes, while this should not affect the binding of random hexamer primers. Accordingly, the apparent down-regulation in the array data for some gene transcripts may therefore represent deadenylation instead of degradation. This particular outcome has been verified in a study comparing the use of random hexamer and oligo(dT) primers in mammalian oocytes undergoing meiotic maturation (Lequarre *et al.* 2004). When the RT-PCR procedure was repeated using oligo(dT) primers, we also observed a significantly higher agreement between microarray and RT-PCR data. Nonetheless, some inconsistency in reproducibility is still to be expected due to statistical variation at low copy numbers for genes that are expressed at very low initial amounts (Peccoud & Jacob 1996). Currently, no validated method exists for the adequate quantification of mammalian MII oocyte transcripts using microarrays. Even with the implementation of specific primers that initiate cDNA synthesis at multiple steps along the mRNA during the Affymetrix amplification steps, some discordance is still observed between microarray and RT-PCR data (Su *et al.* 2007).

In conclusion, a total of 57 out of 86 known genes of importance to epigenetic modifications and chromatin remodeling were detected in murine oocytes. Taken together, these observations support the notion that the reprogramming machinery is fully functional in the mature MII oocyte, possibly including specific active DNA demethylating activity facilitated by preceding HAT activity. However, the precise extent of the translation of these transcripts remains to be evaluated since transcription rates do not necessarily correlate with protein activity. The scarcity of biological material seriously impedes proteomic investigations in mammalian oocytes at present.

The presented list of reprogramming genes may be useful for future investigations in areas involving epigenetic modifications, such as developmental biology and regenerative medicine.

Identifying key oocyte factors involved in epigenetic modifications and chromatin remodeling will provide further insight into how the highly differentiated parental genomes are restored to totipotency. These profound rearrangements are likely to be similar to the reprogramming event observed during SCNT. Hence, the characterization of one or more dedifferentiating 'master genes' may have important implications for human stem cell research, ultimately providing a route towards autologous cell replacement without the need for human eggs or embryos (Oliveri 2007).

Materials and Methods

Collection of mouse oocytes

Oocytes were obtained from 3-week-old B6D2F1 mice (Taconic Europe, Ejby, Denmark) and were carefully processed to avoid human contamination. Mice were handled under standard laboratory conditions and experiments were conducted following national guidelines on animal care (Danish Ministry of Justice; approval 2003/561-713).

For the collection of GV oocytes, mice were each injected with 15 IU menotropin (Menopur; Ferring, Copenhagen, Denmark) and killed by cervical dislocation 42 h later. Ovaries were isolated and antral follicles were punctured by 27-ga needles. Cumulus-enclosed GV oocytes were released into α -MEM (Invitrogen) supplemented with 4 mmol/l hypoxanthine (Sigma) to inhibit GV breakdown during handling. Only spherical GV oocytes with a distinct GV and attached cumulus cells were collected and immediately underwent mechanical denudation with a fine bore glass pipette.

For the collection of MII oocytes, mice were first primed with 15 IU menotropin (Menopur; Ferring) followed by injection of 5 IU of choriongonadotropin 44 h later (Pregnyl; Organon, Oss, The Netherlands), and were then killed 15 h later. Collected oviducts were punctured in α -MEM to yield the cumulus-oocyte complexes (COCs). COCs were briefly exposed to 80 IU/ml hyaluronidase (SynVibro Hyadase; MediCult, Jyllinge, Denmark) at 37 °C to remove attached

cumulus granulosa cells. Only spherical MII oocytes with a distinct first polar body were selected.

After denudation, all oocytes were briefly exposed to 10 mg/ml protease (Sigma) at 37 °C to remove the zona pellucida and remaining attached cumulus cell debris. Oocytes were washed several times and collected for immediate RNA extraction.

RNA extraction and quality assessment

Oocyte RNA was obtained using the RNeasy Micro Kit (Qiagen), which enriches mRNAs from small samples by selectively excluding shorter RNAs such as rRNAs and tRNAs. Briefly, pools of oocytes were lysed with guanidine isothiocyanate-containing buffer and were applied to spin columns for adsorption of the RNA. Carrier was included to improve the yield of low-abundance mRNAs further (Ståhlberg *et al.* 2004). Pellets were washed with 80% ethanol, centrifuged, and air-dried. Subsequently, the RNA was eluted in nuclease-free water and immediately stored at –80 °C until use.

To ensure that high-quality RNA was hybridized to the gene expression arrays, isolated RNA was assessed on a 2100 Bioanalyzer using a RNA 6000 Pico LabChip (Agilent Technologies, Palo Alto, CA, USA).

The protocol used for microarray chip hybridization required at least 100 ng total RNA for each biological replicate. Due to limited sample quantities of oocytes, pooling of total RNA from repeated experiments was necessary, so with an expected average amount of 0.3–0.5 ng total RNA in a murine oocyte, each replicate was constructed from pooled RNA of oocytes from ~20 mice. We isolated sufficient oocyte RNA for the generation of three and five biological replicates of GV and MII oocytes respectively.

Amplification and hybridization to microarray chips

Due to the small amounts of initial RNA, the process required an amplifying two-cycle target labeling assay step to obtain sufficient amounts of labeled cRNA target for analysis with arrays. A minimum of 100 ng total RNA was used to synthesize double-stranded cDNA with the Superscript Choice system (Invitrogen) with an oligo(dT) primer containing a T7 RNA polymerase promoter (GenSet). The cDNA was used as the template for an *in vitro* transcription reaction to synthesize antisense cRNA (BioArray high yield RNA transcript labeling kit; Enzo, Farmingdale, NY, USA). The signal was then amplified in a second cDNA synthesis step using random primers for first-strand synthesis and T/oligo(dT) primer for the second-strand synthesis. The amplified cDNA was used as template for a second *in vitro* transcription to synthesize biotin-labeled antisense cRNA (BioArray high yield RNA transcript labeling kit; Enzo).

The labeled cRNA was fragmented at 94 °C for 35 min in fragmentation buffer (40 mM Tris, 30 mM magnesium acetate, and 10 mM potassium acetate) and was hybridized for 16 h to the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA), which covers transcripts and variants from 34 000 well-characterized mouse genes. Probe sets on this array are derived from sequences from

GenBank, dbEST, and RefSeq. The arrays were washed and stained with phycoerythrin–streptavidin using a Fluidics Station 450 (Affymetrix), and the arrays were scanned in a GeneArray 3000 scanner (Affymetrix). Data were digitalized and converted into CEL-format (v. 3) for data analysis.

Microarray data analyses

To adjust the overall chip brightness of the arrays to a similar level and thus make the individual chips comparable with each other, the quantile normalization method was used (Bolstad *et al.* 2003). To summarize the probe level data for each probe set, an expression index was calculated by the guanine–cytosine corrected robust multiarray analysis (GCRMA) method (Irizarry *et al.* 2003b). Expression values were estimated on logarithmic scale (base 2) instead of raw intensities because of the variance-stabilizing effect of this transformation.

To remove the ambiguity of multiple and less specific probe sets, we retained only transcripts with an Affymetrix oligonucleotide probe set corresponding to a single gene ('_at' suffices) or with anticipated oocyte-specific splice variants ('_a_at' suffices) only allowing less specific probe sets if a unique probe set for a gene was missing on the gene chip at the time of chip manufacturing. Probe sets were then cross-examined in the ADAPT database (Leong *et al.* 2005) to ensure that only probe sets representing mRNAs remained, thus avoiding regions outside exons and 3' UTR. Eventually, if a gene was represented by more than one unique probe set, we selected the probe set with most present calls or positioned in the most distal 3' end of the transcript.

Hierarchical clustering was performed only on genes showing statistically significant up- or down-regulation as identified by *t*-test and with a *P* value corrected for multiple testing with the Benjamini–Hochberg method to avoid erroneous result by less variant or absent genes (Benjamini & Hochberg 1995). Significance level was 0.05, distance metric was $1 - r$, and linkage method was centroid. To validate the overall quality of the experiment, hierarchical clustering was also performed on sample level, since biological replicates representing the same condition should to be grouped together because of identical global expression pattern. Accordingly, they should be separated from samples representing other conditions.

Normalization and calculation of gene expression indices were performed with the GCRMA package (Wu *et al.* 2004), comparisons of samples were performed with the *simpleaffy* package (Wilson & Miller 2005), and Benjamini–Hochberg corrected *P* values were calculated using the *multtest* package (Pollard *et al.* 2004). All packages were run in the *R* statistical software (<http://www.r-project.org>). Hierarchical clustering was performed in DNA-Chip Analyzer (dChip) 2005 (Li & Wong 2003). Microarray analyses are reported according to the MIAME statement (Brazma *et al.* 2001).

Real-time quantitative RT-PCR

Oocytes were collected and total RNA was extracted as described above. cDNA was synthesized with Superscript III (Invitrogen) or iScript cDNA Synthesis Kit (Bio-Rad Laboratories) using oligo(dT) or random hexamers as first-strand

primer. Equal amounts of total RNA (24 ng) were converted into cDNA for GV and MII samples respectively. Subsequently, the cDNA elute was diluted 1:20 before downstream application.

For PCR amplification, the gene-specific primers were constructed using Primer3 (Rozen & Skaletsky 2000) or PrimerBank (Wang & Seed 2003). Primers were carefully chosen or designed to eliminate the risk of amplifying unspecific targets, such as primer dimers or genomic DNA. Primer sequences, exon complementarity, melting temperature, GenBank accession number, and size of the amplified product are presented in Supplementary Table 2, which may be viewed online at www.reproduction-online.org/supplemental. Expression levels were quantified in real time on a LightCycler Instrument (Roche Diagnostics) using LightCycler FastStart DNA Master PLUS SYBR Green I (Roche Applied Science) or SYBR Premix Ex Taq kit (Takara, Madison, WI, USA) as detection chemistry. The PCR mixture consisted of 2 µl diluted cDNA, 1 µl gene-specific primers, and 2 or 5 µl SYBR Green I master mix (depending on manufacturer), and added water to a total volume of 10 µl. First, cDNA was denatured and pre-incubated by heating for 10 s at 95 °C. Template was then amplified by 45 cycles consisting of denaturation for 5 s at 95 °C, annealing for 20 s at 55 °C, and extension for 10 s at 72 °C. At least three replicates were used for each reaction, and a minus RT and a minus template served as negative controls. To verify RT-PCR product identity, melting point curves were generated following amplification. Samples were heated to 95 °C followed by immediate cooling for 15 s at 65 °C and then slowly heating by 0.1 °C/s up to 95 °C, while monitoring fluorescence continuously.

To determine the effect of meiotic maturation on the expression of a candidate internal reference gene, we evaluated a range of housekeeping genes. Briefly, identical amounts of input RNA from GV and MII oocytes were used for each cDNA reaction. Putative candidate reference genes were amplified and the gene showing the lowest variability was chosen as internal reference using NormFinder (Andersen *et al.* 2004).

All target genes were normalized against the chosen internal reference gene within the log-linear phase of the amplification curve, and relative quantification was assessed using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = (C_{T\text{-target}} - C_{T\text{-reference}})_{\text{MII}} - (C_{T\text{-target}} - C_{T\text{-reference}})_{\text{GV}}$ (Pfaffl 2001). All fold changes are presented as means with 95% confidence intervals.

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