their developmental potential within the extraembryonic lineage

ation for the comparisons between ES cells and pMEFs and ES and TS cells, only 14 are common to both lists (Figure 5A, Table S4). This indicates that the differentiation pathways that are epigenetically inactivated in embryonic and extraembryonic lineages differ substantially from each other. We also analysed the developmental expression profiles of the Sequenom-validated genes on the ES cell versus pMEFs list using GNF SymAtlas. Expression data were available for 33 of the genes which are hypomethylated in ES cells and hypermethylated in pMEFs. Interestingly, the most common expression profile is one of predominant expression in either blastocysts, or in oocytes and fertilised eggs, or both (Table 2).

Genes that are hypomethylated in ES cells and hypermethylated in pMEFs or TS cells are potentially regulators of pluripotency. It is known that Oct4 and Nanog are key transcription factors which regulate pluripotency and self-renewal of ES cells; we therefore analysed our meDIP data for those genes found in a recent genome-wide study to be bound in ES cells by Oct4 or Nanog [43]. Significantly, genes bound by Oct4 or Nanog in ES cells become methylated in pMEFs and in TS cells (Figure 5B). Since Oct4 and Nanog are not expressed in either pMEFs or TS cells, this strong correlation suggests that DNA methylation may control the repression of the Oct4/Nanog regulatory network when pluripotency is lost.

Polycomb group (PcG) proteins are required for the maintenance of ES cell pluripotency and developmental plasticity [44–47]. To determine whether PcG complex occupancy is associated with DNA methylation, we compared our meDIP results to a global study of PcG-targeted genes in mouse ES cells [45] (Figure 5B). Genes occupied by key PRC1 and PRC2 proteins in ES cells were not found to be hypermethylated in pMEFs. This suggests that most of the genes targeted by PcG are silenced during embryonic development independently of DNA methylation. However, we did find a significant enrichment of genes that are hypermethylated in TS cells amongst genes occupied by PRC2 but not PRC1 complex in ES cells (Figure 5B).

To reveal any correlation between histone modifications and DNA methylation, genes with specific histone modifications in ES cells [48] were compared with our meDIP data (Figure 5B). Genes hypomethylated in ES cells (compared to pMEFs and TS cells) were found to be significantly enriched within those genes marked by trimethylated lysine 4 of histone H3 (H3K4me3). We found no significant correlations between either the repressive histone mark (H3K27me3), or the bivalent mark (H3K4me3 and H3K27me3) compared to differential DNA methylation.

[illegible]

Our analysis has shown that the majority of promoters that are hypomethylated in ES and EG cells are also hypomethylated in sperm. However, there are a small number of exceptions to this rule which are interesting and important. The promoters of Nanog

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the ES cells versus pMEFs comparison. The ubiquitin cycle is a part of the process of posttranslational protein modification and includes both deubiquitination and ubiquitination of proteins, including histones [54]. Of note is the presence of Ring2 (Ring1B), a member of the polycomb repressive complex 1 (PRC1), which mediates the monoubiquitination of histone H2A lysine 119 [55] and has recently been shown to have an important role in repressing developmental control genes in ES cells [56]. Epigenetic regulation through histone ubiquitination may be an, as yet, unexplored facet of pluripotent cell types. Differences in expression and activity of genes related to the ubiquitin cycle may also be related to a different rate of protein degradation in ES cells and pMEFs. The pluripotent nature of ES cells involves their ability to rapidly respond to stimuli such as differentiation signals. Therefore, they would be predicted to have a higher rate of protein turnover than differentiated cells, and indeed such a

is a highly differentiated cell type with a specialised function, its promoter methylome resembles that of other cell types of the pluripotency-germline cycle. Importantly this suggests that promoters in sperm, on a genome-wide scale, do not need to undergo extensive reprogramming by demethylation at fertilisation. This is in agreement with recently published work analysing differentially methylated regions specific to the testis [21], which tended not to be found in typical pendee21.4xtensivethe

correlation has been found in the myogenic differentiation pathway [57].

Our meDIP data show that genes with the most pronounced methylation differences between ES cells and pMEFs have a preference for expression in early development. This suggests that early transcriptional competence is retained as hypomethylation within the cells of the ICM, and thus ES cells where expression may be reduced by other mechanisms, and subsequently permanently repressed by hypermethylation in differentiated cells. Through this comparison, we identified genes that include pluripotency factors and early patterning genes such as *Nanog* [58], *Tdgf1* [59], and *Lefty1* [60], genes involved in RNA transport with a function in germ cells such as *Aksap1* [61,62], the regulator of apoptosis, *Bcl2l10* [63], and the tumour suppressor gene *Mia2* [64]. Of particular interest are the nucleosome remodelling factor *Smarcd1* [65], and the putative bromodomain gene *Brd1* [66]. Additionally, when this comparison was evaluated against the ES versus TS cell comparison, 14 genes were found to be overlapping on the lists (Table S4). Presumably this comparison is also enriched for genes with functions in the germline, early embryogenesis, and the regulation of pluripotency. A role in these processes can therefore also be envisaged for the genes that came out of these comparisons whose function is yet to be determined.

We find that promoter methylation in sperm is strikingly similar to that in ES and EG cells. This means that the sperm genome, on the whole, has not acquired promoter methylation that would need to be erased after fertilisation to enable zygotic gene expression from the paternal genome. Thus, while the sperm itself

F 6. E *Nanog* . (A)
Nanog n mf n m Nanog
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embryo, presumably to enable loss and re-establishment of pluripotency in a cyclical fashion [3]. Demethylation of the promoters of these genes is thus critical for the pluripotent part of the germline cycle, while re-methylation is crucial for the differentiation part of this cycle. Although it has been reported that a pluripotent state can be induced in differentiated cells by forced expression of a small number of key transcription factors, the efficiency of reprogramming is low and requires a long selection process [70–72]. Our genome-wide methylation study might thus help to identify additional factors as well as targets with a role in reprogramming and to improve the efficiency of the process.

M M

Cell culture

ES cells (129/Sv×129/Sv-CP) F1 were cultured on γ-irradiated pMEF feeder cell layer with ES medium (500 ml knockout DMEM, 90 ml knockout serum replacement (Hyclone), 6 ml 100x non-essential amino acids, 6 ml 100x pen/strep, 6 ml 100x glutamine, 4.6 μl β-mercaptoethanol, 1000 units/ml ES-

concentration and quality were determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (Ultrospec 3100 pro, Amersham Bioscience).

DNA

Genomic DNA from three biological replicates of each sample was prepared as described above. Before sonication 20 µg of RNase were added to 60 µg of DNA in a total volume of 700 µl to digest RNA. Genomic DNA was incubated on ice and sonicated with 20% amplitude, 4 pulses with 10 s sonication and 30 s pause. 35 µl of sonicated DNA were run in 1% agarose gels to check the size of DNA fragments was in the range of 300 to 1000 bp. Sonicated DNA of the correct size was subsequently recovered by ethanol precipitation.

MeDIP was performed as described previously [33]. Briefly, 4 µg restriction enzyme digested (for subsequent PCR analysis) and 100 µg of sonicated (for genome-wide promoter array analysis) DNA was denatured for 10 min at 95°C. The denatured DNA fragments were immunoprecipitated using a monoclonal antibody against 5-methylcytidine (5meC) (Eurogentec) for 2 h at 4°C with 500 µl IP buffer (10mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100). Subsequently the mixture was incubated with 30 µl of Dynabeads coated with M-280 sheep anti-mouse IgG antibody (Dynal Biotech) for 2 h at 4°C and washed three times with 700 µl of IP buffer. After recovering the pull-down methylated DNA by proteinase K digestion for 3 h at 50°C, the methylated DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. The pellet was dissolved in nuclease free water (Ambion).

Chip

Genomic profiling was done by NimbleGen Systems. Arrays are composed of 1.5 kb of promoter regions for a minimal set of 26,275 mouse genes containing tiling 50-mers with 100 bp spacing (NimbleGen Systems, Inc.). Three successive early passages of R1 ES, E11.5 EG, E12.5 EG, TS cells, pMEFs, and sperm from three independent male mice older than 9 weeks were used as independent biological replicates. Six rounds of MeDIP were performed for every sample in order to obtain sufficient amounts of immunoprecipitated (methylated) DNA fragments for hybridization. We provided 3 µg of sonicated DNA as input and 4 µg of 5meC antibody pull-down DNA samples to NimbleGen Systems for differential labelling by random priming with Cy3 or Cy5 and hybridization to the mouse promoter arrays. Dye-swapping was done for one replicate of every tissue type to reduce signal error due to dye bias. Initial data preparation was performed using the in-house developed software ChIPMonk (http://www.bioinformatics.bbsrc.ac.uk/projects /chipmonk/). The raw array data were subjected to a Lowess normalisation.

Bioinformatics

The data used were the subtracted average $\log_2$ ratios from two tissues for the 900 bp upstream of genes on autosomal chromosomes. CpG content for a region is calculated as the proportion of the region +/-300bp which comprises CG dinucleotides. Only promoters with a CpG content of 2-9% and which contained at least 5 probes were used for these analyses. Firstly, for the correlation analysis, R-values were compared for significant correlation both within and between groups. Secondly, for the GO analysis, all GO categories of level 2 were tested. A dataset of subtracted $\log_2$ ratios for each gene in the category was constructed and this was tested for significant deviation from a mean of 0 using a 2-tailed t-test. T-test p-values were adjusted using Bonferoni multiple testing correction. Interesting categories were judged to be those with a corrected p-value of < 0.01. Where multiple nested categories were present only the most specific category was kept.

MeDIP

Only promoters with a CpG content of 2–9% and which contained at least 5 probes were used, it is therefore likely that genome-representation has not been reached in this study. For the algorithm we set limits on the Log2 values to define regions we considered to be methylated and unmethylated. Regions were selected by using a 500 bp sliding window to identify areas where the methylation state consistently and significantly changed between the two tissues being compared.

Bisulphite sequencing

Genomic DNA from R1-ES, pMEF (passages 1 and 5), TS cells and sperm were bisulphite treated using the Zymo EZ DNA methylation kit (Zymo research). Candidates were selected randomly for the TS vs. ES cell comparison, and by a predominantly hierarchical approach based on the predicted methylation status in pMEFs for the pMEF vs ES cell comparison. Promoter regions were selected based on the position of the oligonucleotides on the NimbleGen promoter array and primer pairs were designed using the MethPrimer program (http://www.urogene.org/methprimer/index1.html). A complete list of primers used for analysis is available on request. Amplification of the bisulphite converted DNA, preparation of PCR products for quantitative analysis of promoter methylation detected by the Mass Array system was according to the protocol provided by the manufacturer. An example of methylation analysis using this method is shown (Figure S1).

RT-PCR

Total RNA was purified from 3 cell types, R1-ES, pMEFs (passages 1) and TS cells, using the RNeasy kit (QIAGEN) to eliminate contaminating genomic DNA; this was followed by DNase treatment of eluted RNA. cDNA was synthesized using SuperScript II reverse transcriptase and Oligo (dT) primers (Invitrogen) in a 20 µl reaction volume according to the manufacturer's protocol. For the PCR reactions we used Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) using the MX3005P machine (Stratagene). Reactions were done in triplicate using 1 µl of cDNA as a template in a 25 µl reaction volume. The amount of starting cDNA was normalized to three

Transfection

PMEF feeder cells were seeded at 1.5×10^5 cells/well in six-well tissue culture plates coated with gelatin and incubated for 24 h. The growth medium was removed and R1 ES (1.5×10^5) cells were plated in ES medium with LIF one day before transfection. Plasmids for co-transfection, including Gal4-Dnmt3a WT and Mut (6 μg/well), pdsRed2-C1 (1 μg/well), Nanog promoter GFP reporter plasmids including LR/Nanog-GFP, 3xUAS-NanogGFP and 6xUAS-NanogGFP (1 μg/well) were diluted with Opti-MEM I Reduced Serum Medium without serum. Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Samples were analysed 2 days after transfection.

PCR

Primers were designed to specifically amplify the bisulphite-converted DNA region of interest. Nested PCR was performed with PCR conditions: 94°C for 2 min followed by 10 cycles consisting of 94°C for 30 s, 50–55°C for 2 min, 72°C for 2 min, 20 cycles consisting of 94°C for 30 s, 50–55°C for 1.5 min, 72°C for 2 min plus 5 extra s for each cycle, with a final 72°C extension for 5 minutes. Primers used for nested PCR were: Nanog promoter F 5′-AATAGAGATT TTGGTAGTAAGGTTTG, R 5′-ACCCACACTCATATCAATATAATAAC; Nanog promoter nested F 5′-TTAGGGTTTGGAGGTGTAGT, R 5′ -CCCA-CACTCATATCAATATAATAAC; Nanog-GFP F 5′-AAATA-GAGATTTTGGTAGTAAGGTTT, R 5′ -ACAAATAAACTT-CAAAA TCAACTTA; Nanog-GFP nested F 5′-TAGAAAGAA-TGGAAGAGGAAATTTAG, R 5′ -AATA ATAAAACAACA-CAATAACCAAC. Lefty1 nested PCR primers and conditions are available on request. 1–3 μl of the first PCR product was used for setting up the second nested PCR reaction.

[illegible]

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