Chapter 1

Single Cell RT-PCR on Mouse Embryos: A General Approach for Developmental Biology

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Abstract

Preimplantation development is a complicated process, which involves many genes. We have investigated the expression patterns of 17 developmentally important genes and isoforms in early mouse embryos as well as in single cells of the mouse embryo. The comparison is an excellent example for showing the importance of studying heterogeneity among cell populations on the RNA level, which is being increasingly addressed in basic research and medical sciences, particularly with a link to diagnostics (e.g. the analysis of circulating tumor cells and their progenitors). The ubiquitously expressed histone variant *H3f3a* and the transcription factor *Pou5f1* generated mRNA-derived products in all analyzed preimplantation embryos (up to the morula stage) and in all analyzed blastomeres from 16-cell embryos, indicating a rather uniform reactivation of pluripotency gene expression during mouse preimplantation development. In contrast, genes that have been implicated in epigenetic genome reprogramming, such as DNA methyltransferases, methylcytosine-binding proteins, or base excision repair genes revealed considerable variation between individual cells from the same embryo and even higher variability between cells from different embryos. We conclude that at a given point of time, the transcriptome encoding the reprogramming machinery and, by extrapolation, genome reprogramming differs between blastomeres. It is tempting to speculate that cells expressing the reprogramming machinery have a higher developmental potential.

Key words: Blastomere, Genome reprogramming, Mouse preimplantation embryo, Single cell analysis, RT PCR, Cell heterogeneity

1. Introduction

The genetic analysis of single cells to investigate heterogeneity of cell populations at the DNA and RNA level is a challenging field in biology and medicine. Single cell expression profiling is attracting an increasing number of researchers, and a plethora of cell types have been investigated (1, 2). Based on a new technology called AmpliGrid that amplifies the genetic content of very small amounts of biological material (including single cells), we describe

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a routine workflow for studying single cells by Reverse Transcription Polymerase Chain Reaction (RT PCR) in an easy and efficient way. The workflow consists of deposition of biological material onto a glass slide (the AmpliGrid), cell lysis by heating, specific RT of the mRNA species of interest, distribution of aliquots of the reaction volume onto other reaction spots, and finally end point PCR. The standard reaction volume for all reactions is one microliter enabling a very effective and reproducible workflow. Furthermore, the biological sample on the glass slide can be monitored by microscopy to confirm the investigation of a single cell and to provide a "What you see is what you amplify" process.

The set of genes for multiplex RT PCR has been designed based on the fact that genome reprogramming during preimplantation development must be regulated by maternal and/or embryonic expression of genes, controlling DNA demethylation and remethylation, chromatin modification, and pluripotency. DNA cytosine-5-methyltransferases (DNMTs) are the enzymes that are responsible for the establishment and maintenance of genomic methylation patterns (3). Inactivation of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* caused hypomethylation of the genome and embryonic lethality, indicating that these genes are essential for early mammalian development (4, 5). DNMT1 has a high affinity for hemimethylated sites that are generated transiently during DNA replication and is largely responsible for maintaining methylation patterns throughout cell divisions. DNMT3A and DNMT3B are thought to function in de novo methylation (6, 7). DNMT3L lacks the motifs for transmethylation 3 processes but stimulates de novo methylation through direct interactions with DNMT3A and DNMT3B (8). MECP2, MBD1, MBD2, MBD3, and MBD4 comprise a family of nuclear proteins, which include a 5-methyl-CpG binding domain (MBD), and with the exception of MBD3, all bind specifically to methylated DNA sequences (9). They recruit repressor and chromatin remodeling complexes to methylated DNA (i.e., promoters), causing gene silencing (10). Interestingly, MBD2 has been reported to function as a demethylase (11), but this could not be confirmed. Instead, active demethylation of nonreplicating DNA may be achieved by base excision repair, a highly conserved mechanism for the repair of damaged DNA bases (12). Type 1 DNA glycosylases remove modified (i.e. methylated) bases and leave an apurinic/apyrimidinic (AP) site which is then cleaved by the AP endonuclease APEX1 (13). The DNA backbone at the AP site is subsequently repaired by DNA polymerase(s) and ligase(s). It is noteworthy that MBD4 also functions as a mismatch glycosylase (14). Maternal cytoplasmic factors control active demethylation in the fertilized egg and can even reprogram a somatic cell nucleus that has been transferred into an oocyte (15). The mouse embryonic

genome is already activated at the two-cell stage (16). One key factor for the control of early embryonic development is the transcription factor POU5F1 (OCT4). The fertilized mouse oocyte and early cleavage stages contain residual maternal *Pou5f1* mRNA. Embryonic *Pou5f1* expression is initiated at the four to eight-cell stage (17). *Pou5f1* appears to be required in blastomeres throughout all preimplantation stages to maintain pluripotency (18). The histone variant H3F3A can replace histone H3 and thus epigenetically mark chromatin. It shows an asymmetric distribution between male and female pronucleus in the mouse zygote and is present in the nuclei of all preimplantation stages up to the blastocyst (19).

2. Materials

2.1. Mouse Embryos and Single Embryonic Cells

1. Six to eight week old female C57BL6/J mice
2. Male C57BL6/J mice
3. Hormones: 7.5 IU of eCG(equine chorionic gonadotropin) and hCG (human chorionic gonadotropin)
4. Acid Tyrode's solution (Sigma-Aldrich)
5. PBS containing PVP (1 L): 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, adjust pH to 7.4; Polyvinylpyrrolidone is added to a final concentration of 1 mg/ml; aliquots are prepared on the day of use
6. Glass micropipettes
7. Bunsen burner, flame polished glass pipettes

2.2. Reverse Transcription of RNA, Amplification (RT PCR) and Primers

1. OneStep RT PCR Kit (Qiagen)
2. Multiplex PCR kit (Qiagen)
3. Primers, oligonucleotides
4. Staining solution: 1% $AgNO_3$ in double distilled H_2O
   (a) Developer: 0.1% formaldehyde solution, alkaline, containing NaOH (3 pellets/100 ml of solution)
   (b) AmpliGrid 480F (Beckman Coulter)
5. AmpliSpeed Cycler, for running PCR in the 1 µl regime on AmpliGrid (Beckman Coulter)
6. Covering solution for running PCR (Beckman Coulter)
7. Double distilled H_2O

2.3. Gel Electrophoresis of Amplicons

1. 8% polyacrylamide/bis solution (37:1), containing TEMED (N,N,N,N′-Tetramethylethylenediamine, toxic)

2. 50×TBE: 540 g of Tris(hydroxymethyl)methylamine, 275 g of Orthoboric acid, 100 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0, combined in 1 L of double distilled H_2O
3. Loading dye: 0.25% bromphenolblue, 30% glycerol in H_2O

3. Methods

In principle, the single cell platform (AmpliGrid glass slide) allows for the optical inspection of the sample (cell or cells, sperm, etc.) that is to be amplified. Quality and integrity of the biological material can be monitored during the preparation process under the microscope even after staining procedures or other kinds of manipulation (see Note 1).

3.1. Preparation of Single Cells and Embryos

1. Superovulate 6–8 week old female C57BL6/J mice by intraperitoneal injection of 7.5 IU eCG and 44–48 h later by injection of 7.5 IU hCG.
2. Mate the hormone treated females with C57BL6/J males.
3. Flush one-cell, two-cell, four-cell, and 16-cell stage embryos from the oviducts at 10–12 h, 33 h, 41 h, and 60 h after fertilization, respectively (see Note 2).
4. Isolate individual blastomeres by washing the 16-cell embryos in calcium- and magnesium-free PBS containing 1% PVP followed by incubation in acid Tyrode's solution (pH 2.3) for about 10 s at 37°C.
5. When thinning of the zona pellucida is observed, transfer the embryos into a drop of fresh PBS/PVP. Remove the residual zona by gentle pipetting with a glass micropipette (see Note 3).
6. Flush zona-free embryos with PBS/PVP and then incubate in a new drop of PBS/PVP for 5 min at 37°C.
7. Disaggregate blastomeres by pipetting up and down with a flame-polished glass micropipette (see Note 4).
8. Collect blastomeres in a 15-µl drop of PBS medium at 37°C (Fig. 1a, b).

3.2. RT PCR on AmpliGrid Chips

1. Transfer either individual preimplantation embryos or individual blastomeres in approximately 0.3 µl of PBS each onto an AmpliGrid slide (Fig. 1c) (see Note 5).
2. Carry out reverse transcription with RT primer mixture (Table 1) using a final primer concentration of 0.3 µM each, using buffer and polymerases of the OneStep RT PCR Kit according to the kit's instructions (see Note 6).

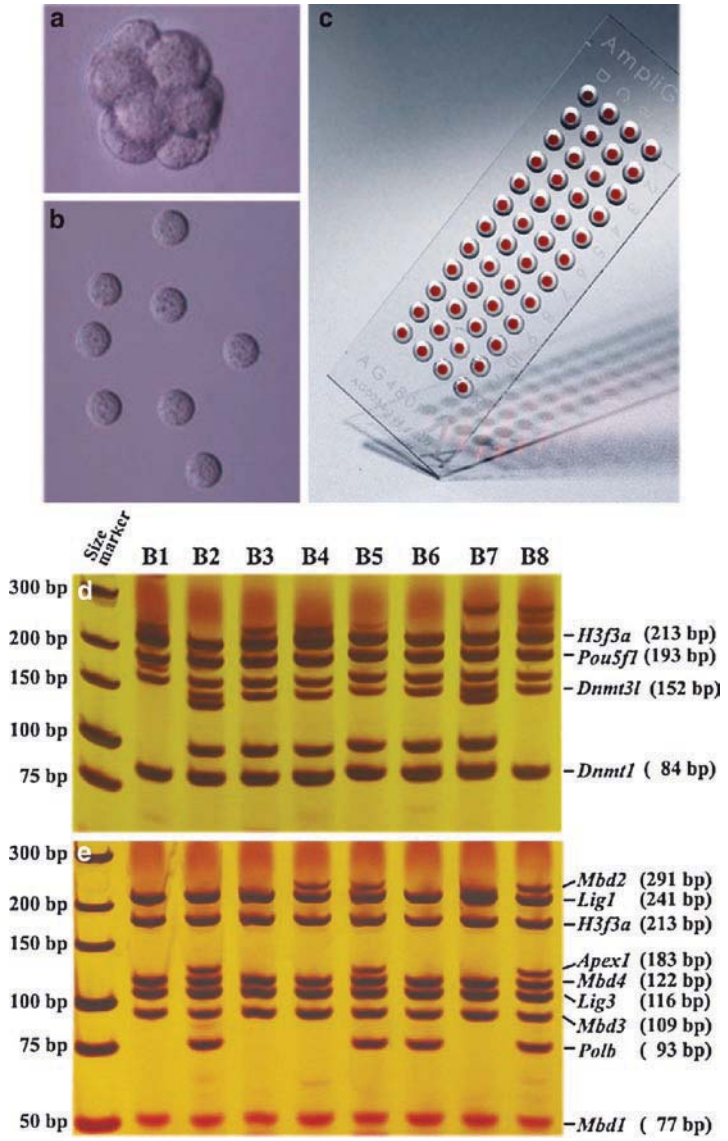

Fig. 1. Overview of single cell expression analysis with AmpliGrid technology. A mouse embryo (**a**) is disaggregated into individual blastomeres (**b**) which are placed onto discrete reaction sites of the AmpliGrid (**c**) for multiplex RT PCR. The PCR products from individual blastomeres (B1 to B8) are then resolved by gel electrophoresis (**d**, **e**). To facilitate the resolution of gene products on minigels, PCR was performed in two different reactions, each using one quarter of the RT product as starting material and amplifying products of the reference gene *H3f3a* and 8 study genes/isoforms. In the top gel (**d**), the bands representing *H3f3a*, *Pou5f1*, *Dnmt3l,* and *Dnmt1* are indicated on the right side. The unlabeled bands represent different amplicons of *Dnmt3a* and *Dnmt3b*. In the bottom gel, each band represents an individual gene, as indicated on the right. Please note that *H3f3a* gene products are detected in each cell, whereas most other gene products, i.e. *Polb* are only present in a subset of cells

Table 1
Primer mixtures for RT and PCR reactions. This primer set facilitates investigation of the expression of the DNA methyltansferase genes: *Dnmt1*, *Dnmt3a* (two isoforms), *Dnmt3b* (four isoforms), and *Dnmt3l*, the methycytosine-binding protein genes *Mbd1*, *Mbd2*, *Mbd3*, and *Mbd4*, the base excision repair genes *Apex1*, *Lig1*, *Lig3*, and *Polb*, the pluripotency marker *Pouf51*, and the histone variant *H3f3a*. The oligonucleotide sequences of the primers have been published elsewhere (20)

RT primer mixture	PCR primer mixture 1		PCR primer mixture 2	
Dnmt3a 1er	*Dnmt3a* 1el+r	270 bp	*Lig1* 1cl+r	241 bp
Dnmt3a 2gr	*Dnmt3a* 2gl+r	165 bp	*Lig3* 1fl+r	116 bp
Dnmt3b 1br	*Dnmt3b* 1bl+r	102 bp	*Mbd2* 1el+r	291 bp
Dnmt3b 2br	*Dnmt3b* 2bl+r	138 bp	*Mbd1* 1dl+r	77 bp
Dnmt3b 3pr	*Dnmt3b* 3pl+r	145 bp	*Mbd4* 1dl+r	122 bp
Dnmt1 1gr	*Dnmt1* 1gl+r	84 bp	*Mbd3* 1dl+r	109 bp
Dnmt3l 1fr	*Dnmt3l* 1fl+r	152 bp	*Apex1* 1bl+r	183 bp
Pou5f1 1cr	*Pou5f1* 1cl+r	193 bp	*Polb* 1bl+r	93 bp
Lig1 1cr	*H3f3a* l+r	213 bp	*H3f3a* l+r	213 bp
Lig3 1fr				
Mbd2 1er				
Mbd1 1dr				
Mbd4 1dr				
Mbd3 1dr				
Apex1 1br				
Polb 1br				
H3f3a r				

3. Prepare a master mix as described above calculating 1.05 × number of 1 µl reactions that are to be run on the AmpliGrid (see Note 7).
4. Add 1 µl of master mix to each reaction spot (negative control, positive control, respective biological material such as single cell).
5. Cover each sample with 5 µl of covering solution to prevent evaporation.
6. Place the prepared slide on the AmpliSpeed Cycler.

7. Run the appropriate thermal profile for reverse transcription on a preheated cycler, e.g., 60°C for 30 min.
8. Remove the 1 µl reaction volume (containing cDNA) from the chip, dilute 1:4 in double distilled H_2O and pipette 1 µl onto empty spots of an AmpliGrid.
9. Evaporate template cDNA to dryness on two different reaction sites of a second AmpliGrid (see Note 8).
10. Carry out two PCR reactions with the multiplex PCR kit and primer mixture 1 and 2 respectively (Table 1). Prepare a master mix with all buffer substances, Taq polymerase and either PCR primer mix 1 or 2 at a final primer concentration of 0.3 µM each according to the kit's manual. Use the Q solution at 0.3-fold concentration.
11. Apply 1 µl each of master mix 1 and 2 to the two different reaction sites with the template and cover with 5 µl of sealing solution.
12. Carry out PCR on a slide cycler with an initial denaturation step of 95°C for 10 min and 40 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s, and a final 10 min extension step at 72°C (see Note 9).

3.3. Gel Electrophoresis of Amplicons and Gel Staining

1. Add 4 µl of loading dye onto each reaction site by carefully pipetting on top of the mineral oil.
2. Let the aqueous phase of 1 µl combine with the loading dye.
3. Pipette the resulting 5 µl from underneath the mineral oil and transfer to one slot of the polyacrylamide gel.
4. Load the remaining samples to be analyzed in this way.
5. Run electrophoresis at 10 V/cm for 30 min.
6. Remove gel from gel electrophoresis chamber.
7. Stain the gel by soaking in staining solution for 5 min.
8. Wash the gel twice in ddH_2O.
9. Visualize the Amplicons by shaking the gel in developer for 3–5 min.
10. Stop developing by washing the gel two times in ddH_2O.

3.4. Documentation

1. Transfer results in the presence or absence of transcripts onto the result table (see Note 10).

4. Notes

1. This feature is unique to the AmpliGrid technology and is the key for reliable results for single cell RT PCR. Whereas in a conventional tube, an optical control of the target cell to be

amplified is impossible by means of a standard microscope; this is possible on the AmpliGrid platform. Depending on the specific application, it will be extremely useful to check for the integrity of the nucleus, the total morphology of the cell, stained parts of the cell, etc.

2. Only "high quality" embryos (by morphological criteria) were used for further analysis. Embryos with developmental delay or signs of fragmentation were discarded.
3. For its optical properties, glass pipettes are used for micromanipulation rather than plasticware.
4. It is important to work with micropipettes that are free of any nucleic acid or protein (like RNAses). In order to avoid contamination in one of the subsequent reaction steps, it is recommended to flame-polish the micropipette for a few seconds using a Bunsen burner.
5. This is a glass chip with a surface structure for the specific positioning of one single microliter on each of 48 discrete reaction sites. For a detailed description of the structuring, see product description on www.advalytix.com.
6. The Q solution was used at onefold concentration.
7. As in conventional tube-based reactions, the total volume of mastermix should be 5% more compared to the exact calculated volume to ensure that the number of reactions can definitely be processed. This is especially true for very low volumes like 1 µl PCR, and we recommend to take into account a factor of 1.05 × number of 1 µl reactions.
8. The end concentration of the reaction mix must not be diluted by additional liquid that might have stayed on the reaction sites. Carefully monitor the drying process of cDNA that will take a few minutes at room temperature and normal humidity.
9. Success rates of single cell PCR will strongly depend on the set up of the total workflow for single cell manipulation as well as on the assay optimization. The total workflow for analyzing single cells under optical control is a new technology that enables routine investigation. For an illustration of the workflow, see Fig. 1. Secondly, PCR conditions (time and temperatures during PCR) will influence the outcome dramatically. For a technical evaluation of sensitivity, some of the genes of interest should be cloned into expression vectors and the amount of synthetic gene sequences that can be detected should be known (see Fig. 2).
10. In principle, the analysis of gene expression in single cells results in the presence/absence of data transcripts in a respective single cell when carrying out end-point-PCR. Analysis of this kind of data has been shown to result in meaningful findings

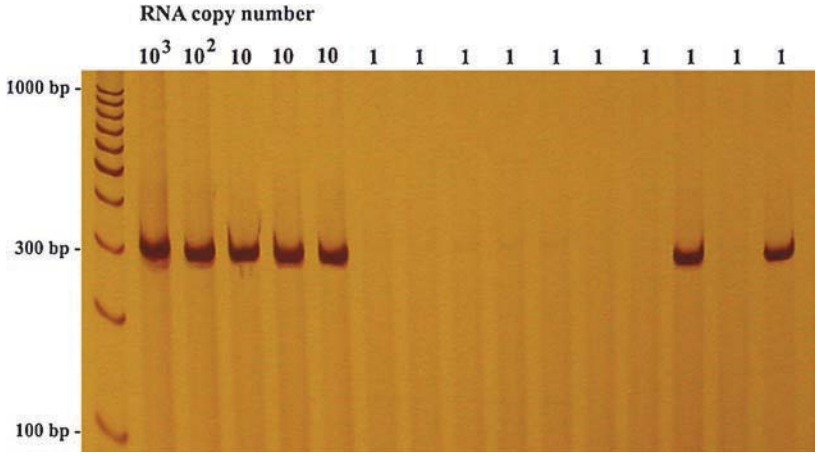

Fig. 2. RNA dilution series of a test RNA, showing the sensitivity of AmpliGrid technology. On this particular gel, the expected amplification product was detected in all trials with 1,000, 100, and 10 RNA copies and in two of ten trials with one RNA copy. When we generated RNAs of four of our study genes by in vitro transcription of *Apex1*, *Lig1*, *Lig3,* and *Mbd2* cDNA clones, a gene-specific product was detected in all trials with 1,000 or 100 RNA copies, in 68–89% of trials with ten copies, and in 4–24% of trials with one copy. The water controls were always negative. When working with limiting dilution (on average one copy per reaction), approximately 30% of the reactions may not contain any RNA molecule, whereas another 30% may contain two or more RNA molecules. Thus, the detection rate with one to two RNA copies is around 20%, with ten copies around 80%, and with 100 or more copies 100%

after Principal Components Analysis (20). Of course we are aware of the fact that real-time PCR can be regarded as state-of-the-art technology and can also be applied on single cells. Both methods are comparable in sensitivity (20). However, routine analysis demands technologies that are easy to handle and reveal a high level of accuracy in quality control. The optical properties of the AmpliGrid make this an excellent tool for achieving this purpose. In addition, one could speculate that gene expression in the early stages of the developing embryo might be characterized by large differences in mRNA level of the respective transcripts that are relevant for cell programming. Binary signatures of these cells are easy to interpret and can be generated in high throughput.

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