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Parent-of-origin dependent gene-specific knock down in mouse embryos

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Abstract

In mice hemizygous for the Oct4-GFP transgene, the F1 embryos show parent-of-origin dependent expression of the marker gene. F1 embryos with a maternally derived OG2 allele ($OG2^{mat}/-$) express GFP in the oocyte and during preimplantation development until the blastocyst stage indicating a maternal and embryonic expression pattern. F1-embryos with a paternally inherited OG2 allele ($OG2^{pat}/-$) express GFP from the 4- to 8-cell stage onwards showing only embryonic expression. This allows to study allele specific knock down of GFP expression. RNA interference (RNAi) was highly efficient in embryos with the paternally inherited GFP allele, whereas embryos with the maternally inherited GFP allele showed a delayed and less stringent suppression, indicating that the initial levels of the target transcript and the half life of the protein affect RNAi efficacy. RT-PCR analysis revealed only minimum of GFP mRNA. These results have implications for studies of gene silencing in mammalian embryos.

Keywords: Oct4-GFP transgene; RNA interference; Maternal inheritance; Paternal inheritance; Short interfering RNA; Preimplantation development; Gene expression

Development of early mammalian embryos is characterized by a switch from a maternal to an embryonic expression program [1]. Maternally derived transcripts and proteins are deposited in the oocyte and consumed after fertilisation at least until the embryonic genome is activated. The RNA binding protein Msy2 has been proposed to stabilize maternal mRNAs in the oocyte [2].

The timing of the major onset of embryonic genome transcription is species specific. In mouse embryos it occurs at the 2-cell stage, in human embryos at the 4-cell stage, and in bovine embryos at 8–16-cell stage. A ''minor'' genome activation has been observed prior to the major genomic activation. Some genes show a biphasic expression pattern, in that maternally derived mRNAs and proteins are present in the oocyte and that the gene are also actively transcribed after embryonic activation.

It has been shown that RNA interference (RNAi)-mediated gene knock down works in mammalian embryos for several genes including E-cadherin, Mos, Plat, Oct4, Cox5a, Cox5b or Cox6b1 [3–9]. However, it is unknown whether RNAi is equally effective for maternal and embryonic transcripts. Maternal transcripts have a long half-life in the range of days, whereas embryonic transcripts last at most for minutes. In the present study, a breeding schedule was applied to produce hemizygous embryos carrying a marker transgene that permits a comparison of gene silencing of the same allele expressed either maternally or after embryonic genome activation. The zygotes analysed in this study were hemizygous for a GFP transgene driven by the promoter of the POU transcription factor Oct4 [10,11]. It has been previously shown that the Oct4 promoter activates transcription in early blastomeres from the 4- to 8-cell stage onward, in the inner cell mass (ICM) of preimplantation embryos, in the epiblast and eventually in the gametes [12]. The Oct4-GFP transgene has been shown to be active in oocytes and both mRNA and GFP protein are maternally stored in MII oocytes and zygotes. Terminally differentiated spermatozoa, however, do not express Oct4. Mating of homozygous OG2 males or females with non-transgenic mice, produced hemizygous OG2 F1 embryos with either embryonic GFP

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expression after the 4-cell stage ($OG2^{pat}/-$), or zygotes with maternal deposits of GFP mRNA and protein ($OG2^{mat}/-$), i.e. maternal expression. Thus knock down of one of two alleles of the target gene located at the same integration site, sharing identical regulatory and structural elements, and identical secondary structure of the transcripts could be studied in two different modes of expression.

Materials and methods

Production of zygotes hemizygous for the OG2 transgene. OG2 transgenic mice (tg; Oct4 promoter-GFP) [10,11] were maintained as an inbred homozygous line by sib matings. For the production of hemizygous OG2 zygotes, OG2 animals were mated with non-transgenic NMRI animals. OG2 and NMRI females were superovulated by intraperitoneal injections of 10 IU PMSG (pregnant mare serum gonadotropin, Intervet, Unterschleißheim, Germany), followed by injections of 10 IU hCG (human chorion gonadotropin, Intervet) 48 h later and mating with OG2 or NMRI males, respectively. Zygotes were collected by flushing the oviducts of plugged females 22–24 h after hCG injection with phosphate buffered saline (PBS) containing 0.3% (w/v) bovine serum albumin (BSA). Cumulus cells were completely removed by a short treatment with 0.03% hyaluronidase and zygotes were collected in PBS/0.3% BSA on a warming plate. Animals were maintained and handled according to German guidelines.

Injection of siRNA and in vitro culture. The lyophylised siRNAs were dissolved at a concentration of 20 μM in a buffer consisting of 30 mM HEPES/KOH (pH 7.4), 100 mM K-acetate, and 2 mM Mg-acetate and stored frozen at −20 °C in 10 μl aliquots. Denuded zygotes were washed in PBS/0.3% BSA and transferred to a micromanipulation unit in a droplet of PBS/0.3% BSA. Individual zygotes were fixed by suction to a holding capillary, approximately 10 pl of siRNA solution was injected into the cytoplasm using an Eppendorf transjector 5246 (Eppendorf, Hamburg, Germany). Rhodamine conjugated siRNAs (Qiagen, Hilden, Germany) with 3′-TT overhangs against GFP (upper strand: 5′-GCAAGCUGA CCCUGAAGUUCAU) or with a random sequence (upper strand: 5′-UU CUCCGAACGUGUCACGU) with no known homology to mammalian genes were used for injection. Successful injections were verified by fluorescence microscopy.

Fertilized embryos of both inheritance patterns were microinjected with either the GFP-siRNA or the control random-siRNA, untreated embryos were used as culture controls. Groups of 10 embryos were cultured for 4.5 days in 50 μl microdrops of KSOM medium overlaid with silicone oil at 37 °C in an atmosphere of 5% CO_2 in air. For fluorescence microscopy, a Zeiss Axiovert 35 M microscope equipped with fluorescence optics for Hoechst 33342, GFP and rhodamine was used [13]. Images were recorded on Ektachrome 320 T film (Kodak) with a Contax167MT camera. The GFP fluorescence signals were normalized by using a fixed exposure time of 15 s. 4-cell stage embryos and blastocysts were collected for RT-PCR and stored frozen at −80 °C. At the end of the in vitro culture period developmental stage, nuclei number and GFP mRNA levels were determined. For determination of the number of nuclei per blastocyst, embryos were stained with 1 μg/ml Hoechst 33342 for 5 min.

Determination of GFP transcript levels by RT-PCR. Poly(A)$^+$ RNA was isolated from single embryos using a Dynabeads mRNA DIRECT Kit (Dynal, Norway, Product number 610.11) as described [14]. The mRNAs were reverse transcribed in a reaction mixture consisting of 1× RT buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, Perkin-Elmer), 50 mM $MgCl_2$, 1 mM dNTPs (Amersham, Brunswick, Germany), 20 IU RNase inhibitor (Perkin-Elmer) and 50 U/μl reverse transcriptase (RT, Perkin-Elmer) in a total volume of 20 μl using 2.5 μl of 50 μM random hexamer primers (Perkin-Elmer Vaterstetten, Germany). The RT reaction was carried out at 25 °C for 10 min and then 42 °C for 1 h followed by denaturation at 99 °C for 5 min. The polymerase chain reaction (PCR) was performed with the reverse transcribed product using 0.2 embryo equivalents. The final volume of the PCR mixture was 50 μl (1× PCR buffer, 20 mM Tris buffer–HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.25 μM of each primer). The GFP primers were 5′-AGC ACG ACT TCT TCA AGT CC and 5′-TGA AGT TCA CCT TGA TGC CG (annealing temperature: 58 °C, 35 cycles). For poly(A) polymerase (also known as Papola; Mouse Nomenclature Page, www.informatics.jax.org/mgihome) amplification, the primers were 5′-CCT CAC TGC ACA ATA TGC CGT CTT CAC CTG and 5′-AGC CAA TCC ACT GTT CTC CCA CCT TTA CTC (annealing temperature 54 °C, 35 cycles). The PCR cycle numbers were optimized to ensure that the reactions are in the linear range of amplification. PCR amplicons were separated by electrophoresis on a 2% agarose gel, and were recorded using a CCD camera (Quantix, Photometrics, Munich Germany). Sequence identities of amplicons were identified by sequencing.

Results

Hemizygous embryos with a maternally inherited Oct4-GFP allele ($OG2^{mat}/-$) expressed GFP in oocytes and zygotes; while embryos with a paternally inherited Oct4-GFP allele initiated transcription of GFP after embryonic activation and displayed GFP fluorescence at the 4–8-cell stage (Fig. 1A). Pronuclear stage zygotes of both inheritance patterns were used for siRNA injection, the siRNAs were conjugated with rhodamine, thus successful injection and metabolism of the siRNAs could be followed by fluorescence microscopy (Fig. 1B and C). The majority of embryos had reached the blastocyst stage at the end of the culture period at day 4.5. Apparently the injection procedure did not reduce the blastocyst rate compared to the untreated control group (Table 1).

After injection of GFP-siRNA, green GFP fluorescence was significantly suppressed in embryos with paternally inherited Oct4-GFP during the entire observation period (Fig. 2A) and 95% of blastocysts showed down-regulation of GFP fluorescence (Table 1). On average, the GFP mRNA levels were reduced by 97.4% in siRNA treated ($OG^{pat}/-$)-blastocysts as determined by RT-PCR analysis (Fig. 3A and B).

In contrast, embryos with a maternally inherited GFP allele ($OG2^{mat}/-$) showed a delayed reduction of GFP fluorescence after siRNA injection. All stages from zygote to morula continued to display GFP fluorescence; a significant knock down of GFP fluorescence became was only apparent at the blastocyst stage (Fig. 2B).

The remaining GFP transcript levels in blastocyst stages were low and not different between embryos with a maternally ($OG^{mat}/-$) or a paternal allele ($OG^{pat}/-$) inherited allele (Fig. 3C) indicating that maternal and embryonic transcripts were equally susceptible to RNAi. To determine whether maternally derived GFP transcripts were degraded slower after RNAi injection, $OG^{mat}/-$ zygotes were injected with GFP-siRNA and early 4-cell stages were analysed by RT-PCR. At this time point, embryonic transcription of the Oct4-GFP transgene had yet not started. RT-PCR indicated only minimum levels of GFP transcripts (data not shown), suggesting that predominantly