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The *Pou5f1* distal enhancer is sufficient to drive *Pou5f1* promoter-EGFP expression in embryonic stem cells

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Abstract

The POU5F1 transcription factor is the gatekeeper of the pluripotent state in mammals. It is essential for epigenetic reprogramming events and also for germ cell viability. The *Pou5f1* gene's expression is tightly controlled during embryogenesis, but its regulatory regions are not fully deciphered. The GOF18ΔPE-EGFP transgene, harboring the enhanced green fluorescence protein reporter gene inserted into an 17- kilobase long mouse *Pou5f1* genomic sequence, has been widely used to visualize pluripotent embryonic cells and primordial germ cells in the mouse and other mammalian species. This construct includes the *Pou5f1* promoter under the control of the distal enhancer and also includes the *Pou5f1* gene body and flanking sequences. In search of the essential regulatory regions of *Pou5f1* we generated four shorter forms of this construct. We found that the shortest form, containing the *Pou5f1* promoter and distal enhancer but lacking the gene body and upstream flanking sequences, correctly expressed EGFP in transiently transformed undifferentiated ES cells, correctly switched it off upon ES cell differentiation, and correctly kept it silenced in differentiated Hep3B cells. Similarly to the original GOF18ΔPE-EGFP, this shortest form was expressed in the fetal mouse gonad. Our data suggest that the *Pou5f1* distal enhancer and proximal promoter may be sufficient to specify transgene expression in pluripotent cells.

Keywords

Pou5f1; Oct4; EGFP transgene; pluripotency

INTRODUCTION

The *Pou5f1* gene (also termed *Oct3*, *Oct4* or *Oct3/4*) encodes a member of the Pic-1, Oct1,2, Unc-86 (POU) transcription factor family (Yeom *et al.*, 1996). Pou5f1 protein is present in the totipotent zygote as a maternal factor. The *Pou5f1* gene is activated during cleavage stages and remains active in the inner cell mass (ICM) and epiblast. After gastrulation, *Pou5f1* is exclusively expressed in the developing germ line. POU5F1 transcription factor is essential for the pluripotency of ICM cells in vivo (Nichols *et al.*, 1998) and for the viability

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of primordial germ cells (PGC) (Kehler *et al.*, 2004). Introduction of POU5F1 and few other transcription factors can reprogram somatic cells into an induced pluripotency state or even can induce trans-differentiation, altering cell fate (Sterneckert *et al.*, 2012). Therefore, POU5F1 is not only a gatekeeper in the early mammalian development, but also a gatekeeper for reprogramming expressway (Pesce and Scholer, 2001, Sterneckert *et al.*, 2012). Because of its strict regulation during development, reporter genes under the control of the mouse *Pou5f1* regulatory elements provide suitable tools for identifying pluripotent cell types (Yeom *et al.*, 1996). A reporter transgenic construct, GOF18ΔPE-EGFP, consisting of a 7.5 kb promoter and distal enhancer region upstream of the *Pou5f1* gene, an enhanced green fluorescent protein (EGFP) gene, and the five exons of *Pou5f1*, has been used as a powerful tool to visualize pluripotent cells in mouse development (Yoshimizu *et al.*, 1999). The GOF18ΔPE-EGFP transgene provided an EGFP expression pattern that was faithful to the endogenous Pou5f1 expression in other mammals including pigs (Kirchhof *et al.*, 2000, Nowak-Imialek *et al.*, 2011). Transgenic mouse strains made with GOF18ΔPE-EGFP, such as the TgOG2 line (Szabó *et al.*, 2002, Yoshimizu *et al.*, 1999) have been very useful for isolating PGCs and fetal germ cells using flow cytometry based on EGFP expression. We decided to find the essential components of the GOF18ΔPE-EGFP transgene that are sufficient to specify reporter expression in pluripotent embryonic stem (ES) cells and in fetal germ cells and specify its silencing in differentiated cell types. We constructed four shorter forms of GOF18ΔPE-EGFP and analyzed their expression patterns in cultured cells and mouse gonads. To this end, we found that the shortest form consisting of the *Pou5f1* distal enhancer and promoter is sufficient to drive EGFP expression in undifferentiated ES cells and in the 14.5 days post coitum (dpc) fetal gonad and is also sufficient to be silenced in differentiated Hep3B cells.

RESULTS

Considerations for shortening the GOF18ΔPE-EGFP construct

The Schöler laboratory has characterized the regulatory regions of the *Pou5f1* gene in great detail using LacZ reporter transgenic constructs (Figure 1.) From these analyses we concluded that in order to keep the specific expression pattern of the GOF18ΔPE-EGFP in the shortened construct, we must keep at least two essential regions, the proximal promoter (PP) and the distal enhancer (DE) together with the EGFP reporter. The 230 bp long PP is essential for gene activity in pluripotent cells, because the promoterless GOF18ΔPP-LacZ construct is completely silent in ES cells (Figure 1). The *Pou5f1* PP is also essential for restricted germ cell-specific expression after gastrulation. GCNF orphan nuclear receptor binds and represses the *Pou5f1* PP upon differentiation, restricting its activity to germ cells (Fuhrmann *et al.*, 2001). The DE is required for activating the PP during preimplantation development and in ES cells, as well as in the germ cells (compare GOF9 and GOF6; compare GOF18ΔPE-LacZ and GOF18ΔDE-LacZ). We hypothesized that we could shorten the GOF18ΔPE-EGFP construct from the 3’ end: it might be possible to remove the downstream sequences and the gene body without compromising specificity. The downstream part was not essential for expression in pluripotent cells (compare GOF18 and GOF13). The gene body may not be required, but this deletion hasn’t been tested before.

Additionally, we considered that the upstream region 5' to the DE might also be shortened, because GOF9, that lacks this region is expressed in pluripotent cells.

Four shorter forms of GOF18ΔPE-EGFP were expressed in mouse ES cells

We generated four shorter forms GOF18ΔPE-EGFP, each containing the EGFP gene (Figure 2) fused to variable lengths of *Pou5f1* sequences (Table 1). GOF18ΔPE-EGFP S1 retained the 7.5 kb of promoter/enhancer region and the first exon of *Pou5f1* gene. GOF18ΔPE-EGFP S2 contained 5.5 kb of the promoter/enhancer region and the first exon. GOF18ΔPE-EGFP S3 contained 4.5 kb of the *Pou5f1* enhancer/promoter region and five exons. GOF18ΔPE-EGFP S4, the shortest form, only harbored the 3.5 kb DE-PP to drive EGFP expression. To analyze if these shorter versions of GOF18ΔPE-EGFP retain the expression specificity of the original transgene, we transfected them into mouse ES cells (Figure 3). Each of the four shorter (S1-S4) constructs drove EGFP expression in pluripotent ES cells similarly to the original GOF18ΔPE-EGFP. Only a subset of cells expressed EGFP. This was expected, because the efficiency of transient transfection is never 100%.

In addition, we found that the shortest form, GOF18ΔPE-EGFP S4, carried the signal for repression in response to differentiation. We transfected the ES cells with the GOF18ΔPE-EGFP, GOF18ΔPE-EGFP S4 and positive control Pgk promoter-EGFP plasmids in triplicates. 24 hours later we trypsinized the transfected plates and plated the ES cells on two culture dishes each. One contained ES-conditioned medium whereas the other one contained regular medium. This latter plate, therefore, had no lymphocyte inhibitory factor (LIF) to suppress the differentiation of ES cells. We trypsinized the plates three days later and subjected the cells to FACS analysis (Table 2). We found that the percent of GFP positive cells was greatly and significantly reduced (−42%, p=0.00348) in the absence of LIF in the plates transfected with the GOF18ΔPE-EGFP S4 construct, similarly to the plate transfected with the parental construct (−57%, p=0.01592). This suggested that the GOF constructs have started to shut down in the absence of LIF. As cell division times may also be affected in the different culture conditions, a decrease in EGFP protein or RNA levels may simply indicate the differential loss of plasmid content or episome inactivation upon cell division in cell culture and not necessarily the downregulation of the *Pou5f1* promoter upon differentiation. This may also occur. However, this doesn't account for the great level of reduction observed for the GOF constructs, because the control, Pgk promoter-EGFP, construct showed only a modest (-11%, p=0.01847) reduction in percent GFP positive cells.

Four shorter forms of GOF18ΔPE-EGFP were not expressed in Hep3B cells

To test if these shorter forms could be correctly silenced in differentiated cells, we transfected them into Hep3B cells. We found that similarly to the parent GOF18ΔPE-EGFP transgene, none of the shorter (S1-S4) forms drove EGFP expression in Hep3B cells (Figure 4). The transfection control, red fluorescent protein (RFP), driven by the CMV promoter was expressed in each sample. Taken together, these results suggested that these four (S1-S4) shorter forms retained their restricted expression pattern specific to undifferentiated ES cells.

The shortest vector gives EGFP expression in 14.5 dpc mouse testis

We next investigated if the DE-PP regulatory sequences of *Pou5f1* could drive the EGFP expression in fetal mouse cells. We employed a simple approach to accomplish this goal through electroporation of this construct into cultured mouse embryonic gonads (Nakamura *et al.*, 2002). We injected and electroporated the GOF18ΔPE-EGFP S4 into 14.5 dpc male gonads. Green fluorescence signal was found in the testicular cords where the germ cells reside. Based on this EGFP pattern it is most likely that EGFP was expressed in the fetal germ cells (Figure 5).

DISCUSSION

Pou5f1 expression is tightly controlled during embryogenesis. Even though the *Pou5f1* gene's transcriptional regulation is not yet completely understood, the *Pou5f1* transgene has been a very useful tool to drive reporter gene expression in pluripotent embryonic cells and allowed the isolation of primordial germ cells from mixed cell population (Szabó *et al.*, 2002, Yoshimizu *et al.*, 1999). To find the minimal regulatory regions, essential and sufficient for specifying *Pou5f1* expression, we generated four shorter forms of GOF18ΔPE-EGFP and analyzed their expression patterns in pluripotent mouse ES cells and a differentiated cell line, Hep3B cells. We found that all four shorter forms were expressed in ES cell but not in Hep3B cells, similarly to the original GOF18ΔPE-EGFP form. The shortest vector contained the DE and the PP regions in front of the EGFP. This suggested that the shortest form harbors the essential regulatory elements in the DE that activate the promoter in pluripotent ES cells and also the essential elements that repress the promoter in differentiated Hep3B cells. We then used microinjection and gonad electroporation to introduce the shortest form into cultured fetal gonads and found that EGFP expression was localized in small foci in the testicular cords where germ cells reside. This suggests that the distal enhancer is sufficient to specify *Pou5f1* expression in fetal germ cells at least in cultured gonads. There may be differences in the level of expression between the constructs, but these may not hinder visualizing pluripotent cells as long as the EGFP signal is detectable in the right place and the right time. It remains to be proven that the GFP-positive cells within the gonad are genuine gonocytes. It would be tempting to differentiate the transfected ES cells to embryoid bodies and ask whether the EGFP transgene expression becomes completely downregulated upon differentiation. One could transplant S4-transfected ES cells to blastocysts, and then perform embryo transfers to assess the EGFP expression pattern in the embryo at 6.5-7.5 dpc and in the gonad at different time points. However, again, the suboptimal efficiency of transient transfection of ES cells and the normal downregulation of episomes in culture would pose technical limitations to these experiments and would make the interpretation difficult. Previous experiments in other species have shown that the entire *Pou5f1*genomic sequences are needed for specifying the correct expression pattern of the GOF18ΔPE-EGFP transgene (Kirchhof *et al.*, 2000). Our results suggest that the sequences that are essential and sufficient for proper *Pou5f1* gene regulation in the mouse may be concentrated on a shorter genomic region. To fully answer these questions one will need to develop a transgenic mouse line and test EGFP expression in embryos during development at different stages. Generation of a transgenic mouse with the shortest transgenic vector, GOF18ΔPE-EGFP S4, is needed to confirm whether the distal enhancer is sufficient to drive *Pou5f1* expression exclusively in pluripotent cells in the early embryo and in primordial germ cells after gastrulation. Nevertheless, we found that the most widely used *Pou5f1* reporter construct can be shortened to its one third without losing its specific expression pattern, at least in cultured cell types and in cultured gonad. We feel that this is useful information for future studies where visualization or genetic manipulation of pluripotent cells is desired.

MATERIALS & METHODS

Construction of shorter forms of GOF18ΔPE-EGFP

Four shorter forms of GOF18ΔPE-EGFP were generated by restriction endonuclease digestion (Figure 2). GOF18ΔPE-EGFP S1 was made by ClaI digestion, removing 7 kb of the *Pou5f1* gene sequence from the 3’ end. GOF18ΔPE-EGFP S2 was made by further digestion of S1 with EcoRV to remove 2 kb from the 5’end. GOF18ΔPE-EGFP S3 was made by subcloning a 14 kb long SpeI/NotI fragment (lacking 3 kb from the 5’end) into pBlueScript vector. GOF18ΔPE-EGFP S4 was made by subcloning a 4.7 kb AflII fragment, in which only *Pou5f1* distal enhancer and the promoter was retained, into pSL1180 vector. Pgk-EGFP plasmid was generated by replacing the CMV promoter of pEGFP-N1 (Clontech, USA) with 0.5 kb of Pgk promoter.

Cell lines and transient transfection

Mouse A2 ES cells (129S1), provided by Jeffrey Mann, were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL, USA) supplemented with 12% fetal bovine serum, 10^{-4} M β-mercaptoethanol, nonessential amino acids, L-glutamine, and antibiotics at standard concentration on a layer of mitomycin-inactivated LIF-producing STOC feeder cells. One day before transfection, coverslips were coated with 0.1% gelatin for 1 hour at 37 °C in 12-well plates. ES cells were trypsinized and plated on a 10 cm plate for 30 minutes twice to remove feeder cells by differential attachment. Suspended cells were counted and plated into 12-well plate at the density of 5×10^5 per well and were subsequently grown in ES-conditioned medium but without feeders. The following day, 2μg of plasmid DNA was used for transfection with 5μl of LipoFectamine 2000 according to the standard procedure provided by the manufacturer (Invitrogene, USA). Microscopy was done 48 hours after transfection when cells were washed with phosphate-buffered saline (PBS), and fixed with 4% formaldehyde for 10 minutes at room temperature. After three time of PBS wash, cells were mounted with Prolong Gold antifade regent with DAPI (Invitrogene, USA) overnight. Slides were sealed and kept at 4 °C before microscopy examination using an inverted fluorescence microscope. Alternatively, cells were trypsinized 24 hours after transfection and plated on duplicate 35 mm dishes with LIF (as supplied by freshly prepared conditioned medium) or without LIF (using regular medium). Three days later, cells were trypsinized, washed in PBS and fixed using 4% formaldehyde and analyzed by FACS for GFP positive cell content using a FACScalibur (BD Biosciences, USA) sorter.

Hep3B cells (ATCC) were grown in DMEM supplemented with 6% fetal bovine serum, nonessential amino acids, L-glutamine, and antibiotics, at standard concentrations. The transfection procedure was the same as for ES cells except the cell density was 3×10^5 in 12-

well plate, and DNA:LipoFectamine 2000 ratio used was 1μg:2.5μl. For internal control, one tenth of CMV-dsRed plasmid DNA (0. 1μg) was cotransfected with each shorter construct DNA.

Gonad injection

Plasmid DNA was prepared using Qiagen endotoxin free maxiprep kit (Qiagen, USA), precipitated by ethanol, and dissolved in TE buffer at a concentration of 5μg/μl. As described by Nakamura et al (Nakamura *et al.*, 2002), gonads were isolated from 14.5 dpc CF1 mouse embryos and kept in cold DMEM supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine, and antibiotics at standard concentrations. A single gonad was washed with PBS and placed between a pair of electrodes (0.2 mm diameter, 15 mm length, 1 mm distance between electrodes; Nepa Gene Co., Ltd, Chiba, Japan) on glass dish with a small volume of PBS. The anterior-posterior axis of the gonad was parallel to the electrodes. Under microscope, approximately 0.3μl of DNA solution was hand injected by a glass capillary with mouthpiece. Right after injection, a set of electric pulses (50V, 50-ms, 100ms intervals, 10 times) was given by an electroporator (CUY21; Nepa Gene Co., Ltd, Japan) to the injected gonad to induce uptake of DNA by gonadal cells. Injected and electroporated gonads were cultured at 37 °C with 5% CO_2 for 48 hours on a culture insert membrane (Falcon, USA) in a 24-well dish with culture medium and examined using a Zeiss fluorescent dissecting microscope.

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Abbreviations

Pou5f1	(Pic-1, Oct1,2, Unc-86 transcription factor 1)
EGFP	(enhanced green fluorescent protein)
PGC	(primordial germ cell)
PP	(proximal promoter)
DE	(distal enhancer)
RFP	(red fluorescent protein) RFP

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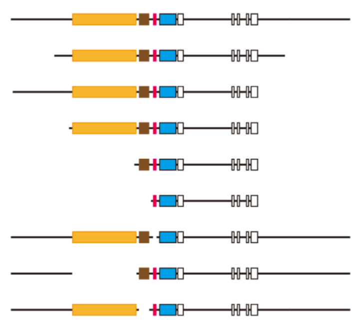

	Preimplantation	ES	Epiblast	EC	PGC	EGC
GOF18	+++	+++	+++	+++	+++	+++
GOF12	++		++			
GOF13	+++	+++		+++		
GOF9	+++	+++		++		
GOF6	-	-	++	++	-	
GOF5	-	-	-	-		
GOF18△PP		-		-		
GOF18△DE		-		+++		+
GOF18△PE	+++	+++	-	+	+++	+++

Fig.1. Considerations for shortening the GOF18ΔPE-EGFP construct

Summary of transgenic experiments done by the Schöler group (Yeom *et al.*, 1996). The different constructs are depicted with their components: LacZ reporter with SV40 polyA termination, blue box; distal enhancer (DE), orange box; proximal enhancer (PE), brown box; *Pou5f1* proximal promoter (PP), red box; *Pou5f1* exons, open boxes. Expression of the reporter gene is tabulated to the right: +++, high expression; ++, reduced expression; +, greatly reduced expression, compared to GF18; -, lack of expression at the different developmental stages: during preimplantation, in the epiblast, in primordial germ cells (PGC) and the cultured cell types: ES (embryonic stem) EC (embryonic carcinoma) EG (embryonic germ) cells.

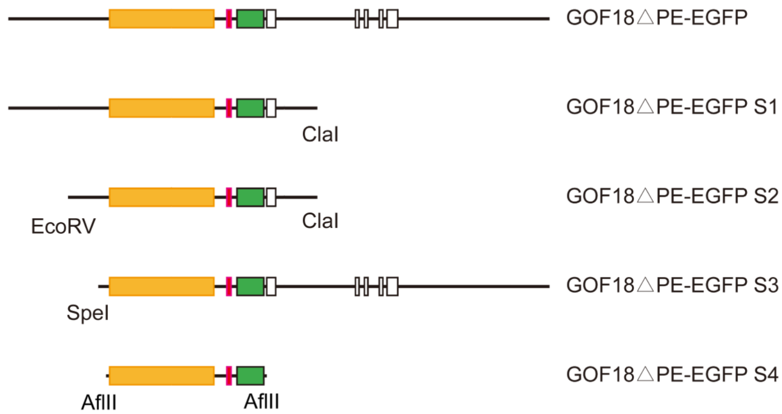

Fig. 2. Shortening of GOF18ΔPE-EGFP

Four shortened forms (S1-S4) of the 17 kb long GOF18ΔPE-EGFP were generated by restriction endonuclease digestions: EGFP reporter with SV40 polyA termination, green box; distal enhancer (DE), orange box; *Pou5f1* proximal promoter (PP), red box; *Pou5f1* exons, open boxes.

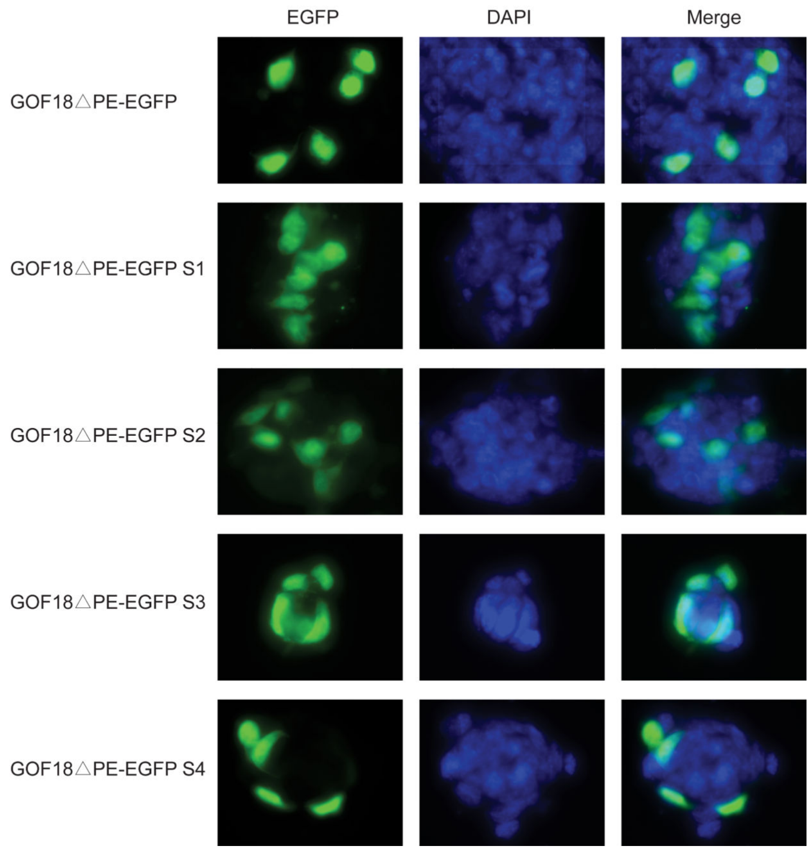

Fig. 3. Four shorter forms of GOF18ΔPE-EGFP were expressed in mouse ES cells

Each plasmid DNA was transfected into mouse ES cells. Microscopy showed that all four shorter forms of GOF18ΔPE-EGFP expressed green fluorescent protein in ES cells, similarly to the original GOF18ΔPE-EGFP.

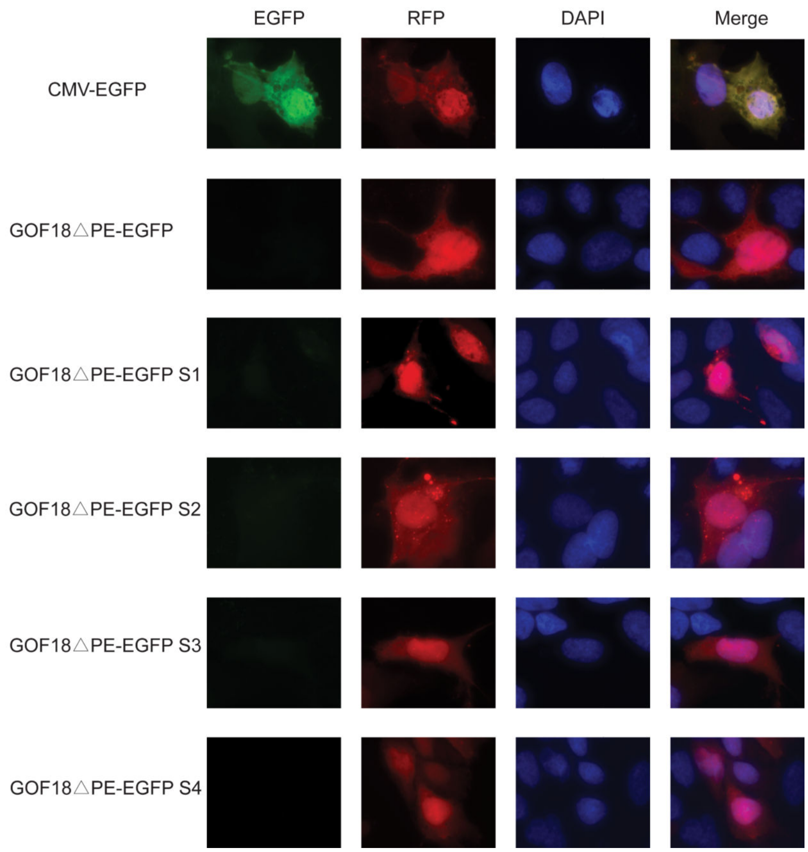

Fig. 4. Four shorter forms of GOF18ΔPE-EGFP were not expressed in Hep3B cells

he expression constructs indicated to the left were transfected into Hep3B cells together with the CMV-dsRed plasmid as transfection control. Microscopy examination showed that similarly to the original GOF18ΔPE-EGFP, none of the shorter forms drove EGFP expression in differentiated Hep3B cells. However, the CMV promoter drove the control dsRed expression in each sample.

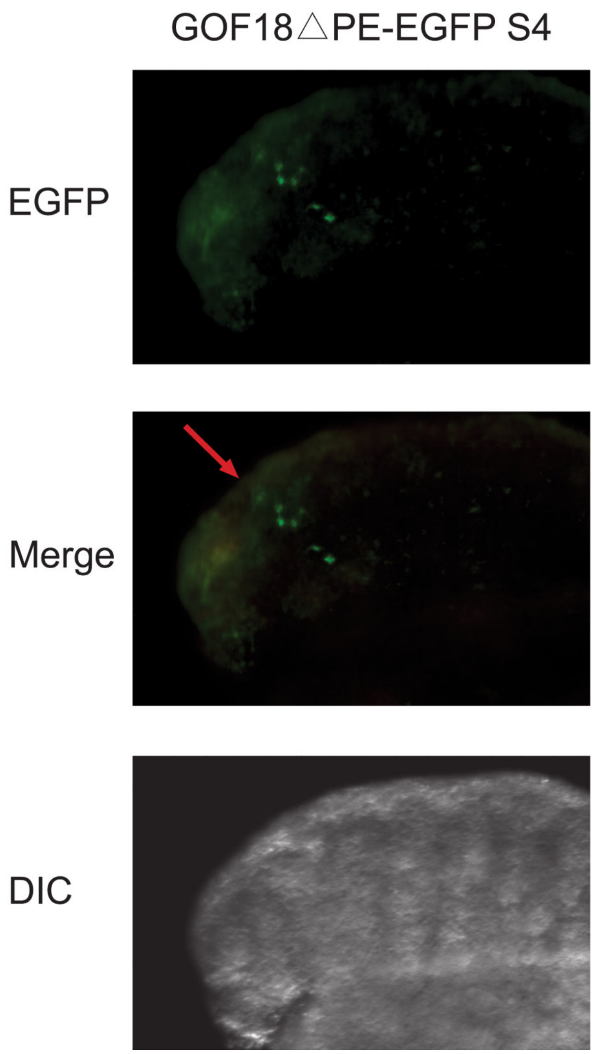

Fig. 5. Distal enhancer of *Pou5f1* drove EGFP expression in 14.5 dpc mouse fetal gonad

ale gonads from 14.5 dpc mouse fetuses were injected with GOF18ΔPE-EGFP S4 (red arrow) and cultured for 48 hours after electroporation. GFP positive cells were observed inside the testicular cords, where the germ cells reside.

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Table 1

Pou5f1 regulatory sequences used in the different constructs.

	Pou5f1 **regulatory sequence length (kb)**		
Construct	**Upstream**	**Downstream**	**Total**
GOF18ΔPE-EGFP	7.5	9	16.5
GOF18ΔPE-EGFP S1	7.5	2	9.5
GOF18ΔPE-EGFP S2	5.5	2	7.5
GOF18ΔPE-EGFP S3	4.5	9	13.5
GOF18ΔPE-EGFP S4	4	0	4

Table 2

Downregulation *of Pou5f1-EGFP* in the absence of conditioned medium

	Percent GFP+ cells in FACS						
	AVERAGE (n=3)		STDEV (n=3)		Change		
Construct	**LIF+**	**LIF−**	**LIF+**	**LIF−**	**Total**	**%**	**TTEST**
Pgk-EGFP control	28.51	25.15	2.01	0.93	−3.36	−11.77	0.01847
GOF18ΔPE-EGFP	1.06	0.46	0.16	0.04	−0.60	−56.78	0.01592
GOF18ΔPE-EGFP S4	3.25	1.88	0.20	0.36	−1.38	−42.32	0.00348