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MicroRNA Target Sites as Genetic Tools to Enhance Promoter-Reporter Specificity for the Purification of Pancreatic Progenitor Cells from Differentiated Embryonic Stem Cells

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Abstract Pluripotent cells hold great promise for cell replacement therapies in regenerative medicine. All known protocols for directed in vitro differentiation of pluripotent cells did not yield pure populations complicating the characterization of the derived cells. In addition, the risk of tumor formation due to residual undifferentiated cells is a serious unresolved problem. In the present study the tissue-specific mouse Pdx1 promoter was used to control the expression of the reporter gene GFP2 in mouse ES cells in order to purify them via FACS during in vitro differentiation. The background fluorescence of transduced ES cells hampered the purification of Pdx1-positive cells due to a contaminating population of partially undifferentiated cells. MicroRNAs (mir) are important regulators of gene expression and were used to enhance promoter specificity during differentiation towards pancreatic progenitor cells. The mouse mmu-mir-294 was found to be mainly expressed during pluripotency, whereas the expression of the mir-302 cluster was increased during early differentiation. Integration of a microRNA target site for the mmu-mir-294 into the lentiviral vector reduced the background fluorescence specifically during pluripotency and permitted re-occurrence of GFP2 expression upon differentiation. A combination of the microRNA target site with the Pdx1 promoter fragment allowed the purification of pancreatic progenitors from differentiated ES cells. This population reflected an early pancreatic progenitor population without other contaminating cell lineages. In conclusion, microRNA target sites are efficient regulatory elements to control transgene expression and to enhance tissue specificity as presented in this study facilitating the sorting and purification of Pdx1-positive pancreatic progenitor cells.

Keywords Embryonic stem cells · Differentiation · microRNA · Pancreatic progenitors · Pdx1 · Purification · Diabetes mellitus

Introduction

Pluripotent stem cells like embryonic stem (ES) cells hold great promise for cell-replacement therapies of degenerative diseases such as diabetes mellitus. Pluripotent stem cells can theoretically be differentiated into all somatic cell types of the human body and their self-renewal potential permits propagation of large cell numbers as a virtually unlimited source for in vitro differentiation. The in vitro differentiation into insulin-producing cells has been shown for pluripotent stem cells from different species [1–3]. Typically, differentiation protocols rely on the manipulation of signaling pathways either by recombinant cytokines/growth factors [1] or small molecules [4–7]. However, neither growth factor nor small molecule based differentiation protocols yielded so far pure and homogenous cell populations of the pancreatic lineage. The heterogeneous composition of differentiated stem cell populations severely

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complicates the detailed biochemical and functional characterization of pancreatic cells derived from in vitro differentiation cultures. Additionally, the mixed nature of the differentiation cultures harbors the risk of malignancies since the culture could be contaminated with ES cells that have resisted differentiation [8, 9]. A selection strategy would be conductive to enrich cell populations committed to the pancreatic lineage. Several publications have addressed these issues and the techniques used in these studies were either sorting techniques based on fluorescent reporter proteins [2, 10–13] or based on positive selection utilizing antibiotic resistance genes [14].

During pancreas development the transcription factor Pdx1 marks a pan-pancreatic progenitor population, which later gives rise to all adult pancreatic lineages [15]. Pdx1-positive pancreatic progenitor cells derived from human ES cells reversed upon implantation a chemically induced diabetes in mice [8, 16] demonstrating that these cells are capable of producing functional insulin-producing cells in vivo. Recently the derivation of mouse and human ES cells harboring a GFP knock-in into the Pdx1-locus [12, 17] has already proven the feasibility of reporter ES cell lines. MicroRNAs (miRNAs) are small-non coding single-stranded RNAs of approximately 22 nucleotides, which are an important class of negative regulators for mRNA translation. It has been shown that miRNAs are essential for proper development, differentiation, specification of cell fate, and can act as specific repressors during pathogenesis (reviewed in [18, 19]). In addition, the endogenous miRNA expression can be used to regulate the expression from transgenes via miRNA specific target sites (mirT) [20]. Recently, this system was utilized to trace differentiating neuronal progenitors by depleting the embryonic lineage from the differentiated ES cell cultures [21].

Here we report a different approach and constructed a lentiviral vector in which a tissue-specific promoter fragment of the mouse Pdx1 gene controls the expression of the fluorescent reporter gene GFP2 thereby permitting the purification of GFP2/Pdx1-positive cells via fluorescence activated cell sorting (FACS). Additionally, this new lentiviral vector comprises a genetic element, a mirT site for the ES cell specific mmu-mir-294 (mirT294) downstream of the GFP2 open reading frame. The mirT294 site reduced the expression of GFP2 and the background fluorescence of the lentiviral vector specifically in undifferentiated mouse ES cells. Upon differentiation the stage specific knock-down of the GFP2 expression was nullified. Combining the mirT technology with the tissue-specific promoter-reporter constructs can increase the specificity for the purification of pancreatic progenitor cells and potentially of any desired cell population from differentiated ES cell populations.

Materials & Methods

Materials

Knockout-DMEM, DMEM and RPMI1640 advanced culture media, glutamax, B27 media supplement, and non-essential amino acids were obtained from Life Technologies (Darmstadt, Germany). ES cell grade fetal calf serum (FCS) was purchased from PAA (Vienna, Austria), fetal bovine serum (FBS) from BioWest (Nuallié, France) and leukemia inhibitory factor (LIF) from Millipore (Schwalbach, Germany). IDE-1 was from Miltenyi Biotec (Bergisch Gladbach, Germany), (-)-Indolactam V from Sigma-Aldrich (Taufkirchen, Germany), and recombinant human FGF-10 from ReliaTech (Wolfenbüttel, Germany). All primer pairs, including random hexamer primers, were synthesized by Life Technologies. The RevertAid™ H Minus M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). The GoTaq® Taq polymerase was from Promega (Mannheim, Germany) and dNTPs were purchased from Genecraft (Münster, Germany). The restriction enzymes were delivered by Fermentas (St. Leon-Rot, Germany). Analytical grade chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Cloning of Lentiviral Vectors

A synthetic multiple cloning site was integrated into the pLenti6.3/V5 vector (Life Technologies) by the TOPO TA reaction. The CMV-promoter was removed by digestion with *Bsu*15I and *Bam*HI, followed by treatment with the Klenow-fragment and ligation to generate promoterless (PRL) constructs. The cDNA of the fluorescence proteins GFP2 and mCherry were subcloned into these vectors by PCR-amplification with specific primers containing the restriction sites for *Xho*I and *Bsp*119I (fw_*Xho*I_pTagGFP2: 5′-caactcgagatgagcgggggcgaggag-′3 and rev_pTagGFP2_Bsp119I: 5′-gcttcgaacctgtacagctcgtccatg-′3 for GFP2 from pTagGFP2 (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France); for mCherry (Takara Bio Europe/Clontech) fw_*Xho*I_pLenti6.3_mCherry: 5′-caactcgagatggtgagcaagggcgag-′3 and rev_pLenti6.3_mCherry_*Bsp*119I: 5′-gcttcgaacttgtacagctcgtccatgc-′3). All fluorescent reporter genes were cloned in-frame to the V5-Tag of the vector. The tissue-specific murine Pdx1 promoter was subcloned from the pBIpf1polA vector (a kindly gift from H. Edlund [22]) by *Sca*I/*Eco*RI digestion into the *Afe*I/*Eco*RI opened pLenti6.3 PRL vector containing a fluorescence reporter. The microRNA target site 294 (mirT294), synthesized by Geneart (Life Technologies, Germany), comprised 4 complementary repeats of the miRNA-294 and was integrated into the pLenti6.3 PRL vector utilizing *Pfo*I and *Sex*AI. Cloning of the CMV-early enhancer (CE) (described by

[23]) was carried out by PCR amplification of the enhancer element from the pcDNA3 vector (Life Technologies, Germany) with the following primer: CE-MfeI_fw: 5′-cagcaattggacattgattattgactagttattaatag-3′ and CE-MfeI_rev: 5′-gctcaattgatggggcggagttgttac-′3 and cloned by using the restriction enzyme *Mfe*I. All derived plasmids were verified by sequencing.

Tissue Culture

The mouse embryonic stem cell lines ES-CCE and ES-D3 were maintained as described elsewhere [9]. Briefly, the cells were cultured on gelatine-coated dishes in KO-DMEM medium supplemented with 15 % (v/v) FCS (ES cell grade), penicillin/streptomycin, 2 mML-glutamin, 100 μM non-essential amino acids, 0.1 μM 2-mercaptoethanol, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 1,000 U/ml LIF in a humidified atmosphere at 37 °C with 5 % CO_2. The glucose-responsive insulin-producing MIN6 cell line [24, 25] and the 3T3 fibroblast cell line were maintained in DMEM supplemented with 10 % (v/v) FBS, penicillin/streptomycin and 2 mM glutamine in a humidified atmosphere at 37 °C with 5 % CO_2.

Lentiviral Production and Transfection

Lentiviral particles were produced as described elsewhere [26] with minor changes. Briefly, 1×10^7 HEK293T cells were transfected with the packaging plasmid pPAX2 (28.125 μg), the envelope plasmid pCDNA-MDG (9.375 μg) and different transfer plasmids (37.5 μg) by calcium phosphate precipitation. The viral particles were harvested from the culture medium 48 h after transfection and were purified at 4 °C and 3,000 g by Amicon Ultra Ultracel-100K ultrafiltration columns (Millipore, Schwalbach, Germany).

For the transduction of the MIN6 and 3T3 cell lines 25,000 cells per cm^2 were seeded, allowed to attach to the surface, and transfected with the purified lentiviral particles. In case of mouse ES cells 5,000 cells per cm^2 were used. The following day the medium was changed and the cells were washed once. Selection of positive cell clones was performed with 5 μg/ml blasticidin in the culture medium.

Differentiation of ES Cells

For differentiation experiments mouse ES cells were seeded with a density of approximately 2,500 cells per cm^2 and cultured overnight before starting the differentiation procedure. To induce the formation of the definitive endoderm germ layer, the cells were cultured for 6 days in RPMI advanced medium containing 1 μM IDE-1 and 0.2 % FCS (adopted from [5, 6]) followed by 4 days of cultivation in DMEM supplemented with 0.33 μM (-)-indolactam V, 50 ng/ml FGF-10, and 2 % B27 supplement to induce the differentiation of pancreatic progenitors. All cell culture media were additionally supplemented with 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Flow Cytometry and Cell Sorting

For analysis via flow cytometry of in vitro differentiated cells, they were washed with PBS, dissociated using trypsin/EDTA, and resuspended in PBS plus 2 % FCS. The antibody staining was performed following standard protocols. 250,000 cells were washed, incubated for 45–60 min with primary conjugated antibodies, washed three times, and analyzed by flow cytometry using the CyFlow ML flow cytometer (Partec, Münster, Germany). For every sample at least 20,000 events were counted for analysis and the data analysis was performed with FlowJo (Ashland, OR, USA). The following conjugated antibodies were used: anti-mouse CXCR4-PE (Clone 2B11, eBioscience, Frankfurt, Germany), anti-mouse SSEA1-APC (Life Technologies), and anti-mouse CXCR4-PerCP-eFluorophor 710 (Clone 2B11, eBioscience). Fluorescence activated cell sorting (FACS) was performed at the central facility of the Medical School Hannover on a MoFlo (Beckman-Coulter, Krefeld, Germany), XDP (Beckman-Coulter, Krefeld, Germany) or a FACS Aria (Becton Dickinson, Heidelberg, Germany) sorter.

MicroRNA Expression Analysis

Prior to isolation, the cells were stabilized and removed from the cell culture dish with RNAprotect (Qiagen). Total RNA was isolated with the miRNeasy kit (Qiagen, Hilden, Germany) and miRNAs were reverse transcribed with sequence-specific primers (Life Technologies) using TaqMan MicroRNA Reverse Transcription kit (Life Technologies). The expression of the analyzed miRNAs (mmu-mir294, mmu-mir302a, mmu-mir302d and snoRNA202) was performed by TaqMan miRNA Assays (Life Technologies) on a CFX96 Touch real time cycler (BioRad, München, Germany) or on a ViiA 7 real-time PCR system (Life Technologies). A dilution series was used for defining the arbitrary units and normalization was carried out against the snoRNA202.

Gene Expression Analysis

Total RNA from FACS-sorted cells was isolated with the RNeasy micro kit (Qiagen) and RNA quality was determined with an Experion automated electrophoresis station (BioRad). For cDNA synthesis, RevertAid™ H Minus M-MuLV Reverse Transcriptase was used with random hexamer primers. All samples were diluted to a final concentration of 2–5 ng/μl total RNA before analysis. The expression of the genes Pdx1, Nkx6.1, Lin-28, and Rbl2 was measured with commercial TaqMan assays (Life Technologies), whereas all other genes were analyzed with the

QuantiTect SYBR Green™ technology (Qiagen). Samples were incubated for 2 min at 50 °C and primarily denatured at 95 °C for 10 min followed by 40 cycles. Each cycle comprised a melting step at 95 °C for 15 s and the annealing/elongation step at 60 °C for 60 s. Each sample was measured as a triplicate using specific TaqMan assays or specific primer pairs (Supplementary Table 1). Data normalization was performed with qbasePLUS (Biogazelle, Zulte, Belgium) against the geometric mean of the housekeeping genes glucose-6-phosphate dehydrogenase (X-linked, G6PDH) and Tata box binding protein (TBP).

Immunocytochemistry

Immunocytochemistry was performed according to standard procedures. Briefly, FACS-sorted mouse ES cells were seeded on matrigel-coated glass slides (Zellkontakt, Nörten-Hardenberg, Germany) and fixed in 4 % (w/v) paraformaldehyde for 30–60 min at 4 °C. Subsequently, the cells were blocked for 20 min in PBS plus 0.2 % Triton X-100 and 5 % donkey serum (Dianova, Germany). Primary and secondary antibodies were diluted in PBS with 0.1 % Triton X-100 and 0.1 % BSA. Primary antibodies were incubated on the slides for 1–3 h at room temperature or overnight at 4 °C. Secondary antibodies were diluted 1:200–500 and incubated on the slides for 45–60 min at room temperature. The following primary antibodies were used: rabbit-anti-PDX1 (Abcam, ab47267) and mouse-anti-GFP (Clontech, 632375). Secondary antibodies were obtained from Dianova (Hamburg, Germany) and conjugated with Alexa® DyLight dyes, FITC or Cy fluorophores. Counterstaining of nuclei was performed with 1 μM DAPI for 5 min. Finally, the slides were mounted with Mowiol/DABCO anti photobleaching mounting media. The stained cells were examined using an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany).

Western Blot

Cellular proteins were extracted using RIPA buffer (Sigma-Aldrich) and the protein concentration was determined by Bradford assays. 20 μg of total protein was separated using 12.5 % SDS-PAGE followed by electroblotting onto a PVDF membrane. To minimize non-specific binding the membrane was blocked by incubation with non-fat dry milk for 60 min at room temperature. Blots were then incubated with a specific primary antibody for 1–3 h at room temperature or overnight at 4 °C. The blots were subsequently incubated with a peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) at a dilution of 1:15,000. The following primary antibodies were used: mouse-anti-V5 1:5,000 (R960-25, Life Technologies) and goat-anti-actin (sc-1615, Santa Cruz). Protein bands were visualized by chemiluminescence using the ECL Plus detection system (Pierce, Bonn, Germany). Protein loading was quantified by densitometry and normalized against beta actin using the GelPro Analyzer 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistics

Unless stated otherwise the data values are expressed as mean ± SEM. Statistical analyses were performed using the GraphPad Prism analysis software (Graphpad, San Diego, CA, USA) applying *Student's t-test* or *ANOVA* followed by *Bonferroni's tes*t for multiple comparisons.

Results

Generation of ES Cell Clones Harboring the Pdx1-GFP2 Gene Cassette

The lentiviral vector system used in this study was based on the pLenti6.3 vector and contained a cPPT element and a WPRE element (Fig. 1a). A synthetic multiple cloning site was integrated into this vector. For the generation of tissue-specific reporter cassettes the constitutive CMV promoter was removed. Afterwards a tissue-specific promoter-reporter cassette comprising a 3.4 kb 5' promoter fragment of the mouse Pdx1 gene and the fluorescent reporter GFP2 was integrated into the multiple cloning site (Fig. 1a).

The functionality of the cloned mPdx1-promoter fragment was assessed in the insulin-producing MIN6 cell line, which showed a strong Pdx1 gene expression (data not shown) and in 3T3 fibroblasts as negative controls. Upon transduction with lentiviral particles the green fluorescence of MIN6 cells was increased, whereas 3T3 fibroblasts showed only a weak fluorescence (Supplementary Fig. 1A). ES cells transduced with lentiviral particles showed after clonal expansion the same morphology as wildtype ES cells. The staining for alkaline phosphatase (AP) was positive and the intensity comparable to wildtype ES cells (Fig. 1b). In addition the gene expression of the embryonic transcription factor Oct3/4 was not significantly changed between modified and wildtype ES cells (Supplementary Fig. 1C). This demonstrated that modified ES cells retained their pluripotency upon lentiviral transduction and clonal selection.

Clonally derived ES cells harboring the mPdx-1-promoter GFP2 (mPdx1-GFP2) construct exhibited an increased fluorescence compared to wildtype ES cells (Fig. 1c), as already observed for 3T3 fibroblasts. The increased background fluorescence was not correlated with the amount of lentiviral integrations per genome (Table 1). During differentiation with the small molecule based protocol the fluorescence gradually decreased and only a minor part of the population remained positive for GFP2 (Fig. 1d).

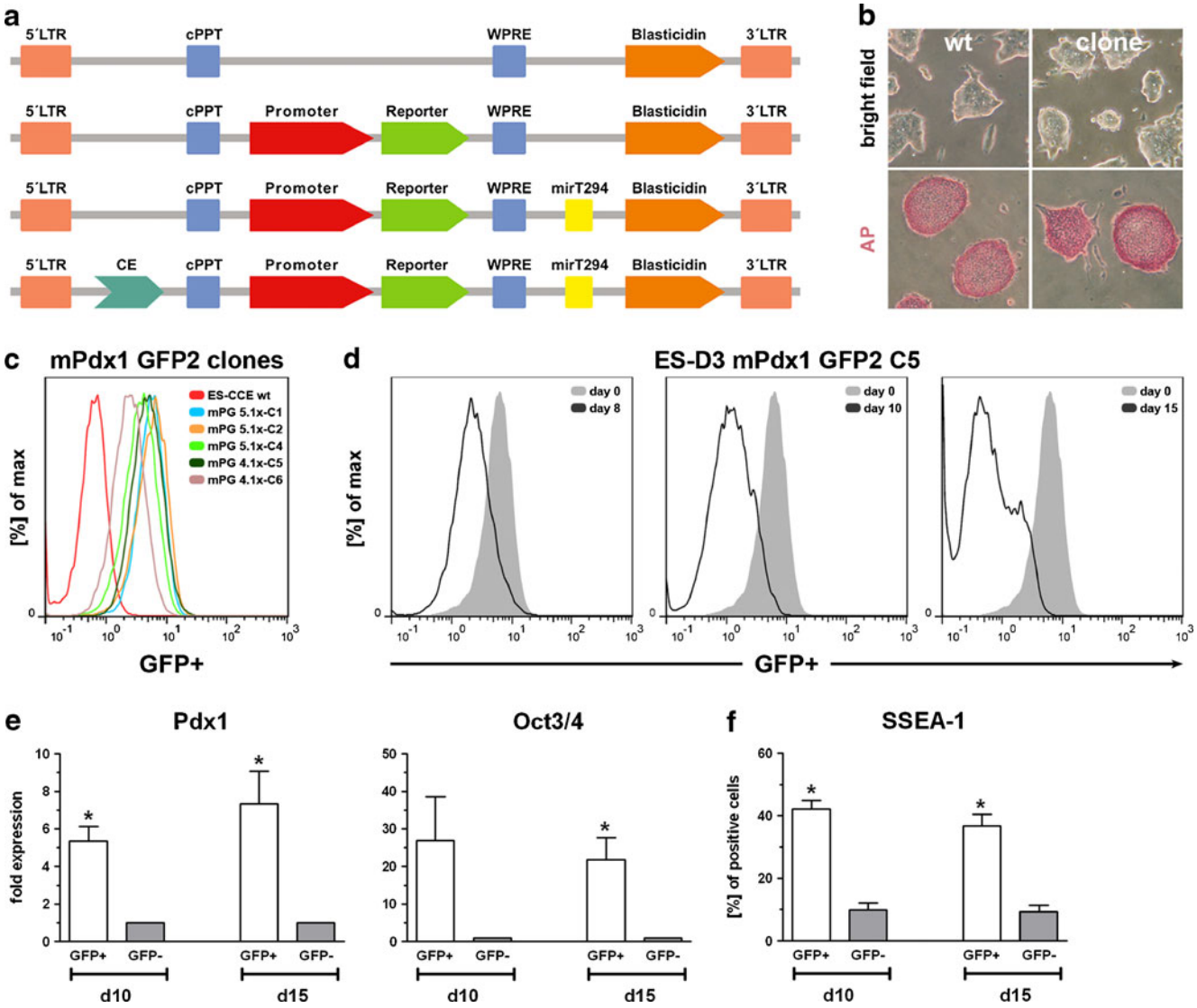


Fig. 1 Generation of ES cell clones harboring the Pdx1-GFP2 gene cassette. **a** Layout of the modified pLenti6.3 backbone vector, the integrated promoter-reporter gene cassette, the microRNA targest site (mirT) and a CMV early enhancer (CE) element. **b** ES cell clones selected by blasticidin treatment after lentiviral transduction showed the same positive staining for alkaline phosphatase (AP) as wildtype (wt) ES cells. **c** Flow cytometric analysis of the green fluorescence of typical mPdx1-GFP2 cell clones compared to wild type cells. **d** The green fluorescence of the cell clone ES-D3 mPdx1-GFP2 C5 was measured by flow cytometry during a small molecule based differentiation process at day 8, 10 and 15 of differentiation in comparison to the undifferentiated state. **e** Relative gene expression of the pancreatic transcription factor Pdx1 and the pluripotency marker Oct3/4 in sorted GFP-positive and GFP-negative cells normalized to the housekeeping genes G6PDH and TBP. Values are means ± SEM, n=4. *$p \leq$ 0.05, *Student's t-test*. **f** Distribution of cells positive for the embryonic surface marker SSEA1 in the GFP-positive population compared to the GFP-negative population at day 10 and 15 of differentiation as measured by flow cytometry. Values are means ± SEM, n=4. * $p \leq 0.05$, *Student's t-test*

Sorting of these GFP-positive cells at day 10 of differentiation revealed a significantly 5.4±0.8 fold (p=0.01, n=4) increased gene expression of Pdx1 compared to the GFP-negative population (Fig. 1e). The increased Pdx1 gene expression was slightly higher at day 15 of differentiation with an 8.8±2.1 fold increase compared to the GFP-negative population (p=0.05, n=4). However, GFP-positive cells showed at day 10 a 26.9±11.6 fold (n.s.) and at day 15 and 16.3±0.5 fold (p=0.05, n=4) increased expression of the pluripotency gene Oct3/4 compared to the GFP-negative fraction (Fig. 1e). Moreover, flow cytometric analysis revealed that 42.1±2.8 % at day 10 ($p<$ 0.001) or 36.7±3.7 % at day 15 ($p<0.001$) of the GFP-positive cells remained positive for SSEA-1, a cell surface marker for embryonic stem cells (Fig. 1f). The GFP-negative population contained a minor part of cells (9.9± 2.3 % at day 10 or 9.4±1.9 % at day 15, respectively) remaining positive for SSEA-1. These results indicated that

Table 1 Comparison of lentiviral integrations and the fluorescence intensity of clones with the mPdx-1 promoter GFP2 (mPG) construct

Clone designation	Lentiviral integrations	Mean fluorescence compared to wt ES cells (x fold increase)
ES-CCE mPG 5.1x-C1	1.04	6.50
ES-CCE mPG 5.1x-C2	0.94	7.02
ES-CCE mPG 5.1x-C4	2.14	4.62
ES-CCE mPG 4.1x-C5	4.89	6.38
ES-CCE mPG 4.1x-C6	2.08	3.24

Lentiviral integrations were measured according to Sastry et al [48]. A standard containing the sequences of the lentiviral backbone and beta actin was used. All fluorescence intensities were normalized to wildtype (wt) ES cells and are presented as fold increase above background fluorescence

a mixed population of pancreatic progenitors and partially or fully undifferentiated cells were sorted from the GFP-positive population.

MicroRNA Expression in Mouse ES Cells During Directed In Vitro Differentiation into Pancreatic Progenitors

The miRNA expression of mmu-mir-294, -302a and -302d was assessed during the directed in vitro differentiation in two mouse ES cell lines, namely ES-CCE and ES-D3, to evaluate their potential to minimize the background fluorescence of the lentiviral constructs. We detected a prominent expression of mmu-mir-294 in pluripotent cells. Upon differentiation the expression of mmu-mir-294 decreased continuously (Fig. 2a). One-way ANOVA analysis revealed a statistically significant reduced expression of mmu-mir-294 at all analyzed time points compared to undifferentiated ES-CCE cells. After 6 days of differentiation the expression of mmu-mir294 was 4.9 fold decreased (0.35±0.03 AU, $p<0.001$) and decreased after 10 days by 29.0 fold (0.06±0.01 AU, $p<0.001$) compared to day 0. A similar result was found for the ES-D3 cell line with a 2.3 fold decrease at day 6 and a 5.5 fold decrease at day 10 of differentiation compared to day 0 (Fig. 2a). Overall the mmu-mir-294 expression was higher in ES-D3 cells and remained at a higher level compared to the ES-CCE line. Interestingly, the mmu-mir-302a and -302d showed nearly no detectable expression in undifferentiated mouse ES cells for both lines (Fig. 2b and c). The expression of these miRNAs in ES-CCE cells increased dramatically and statistically significantly at day 6 of differentiation (mir-302a: 4.43±0.65 AU; mir-302d: 5.56±1.04 AU, $p<0.001$) compared to day 0. However, after 10 days of differentiation both

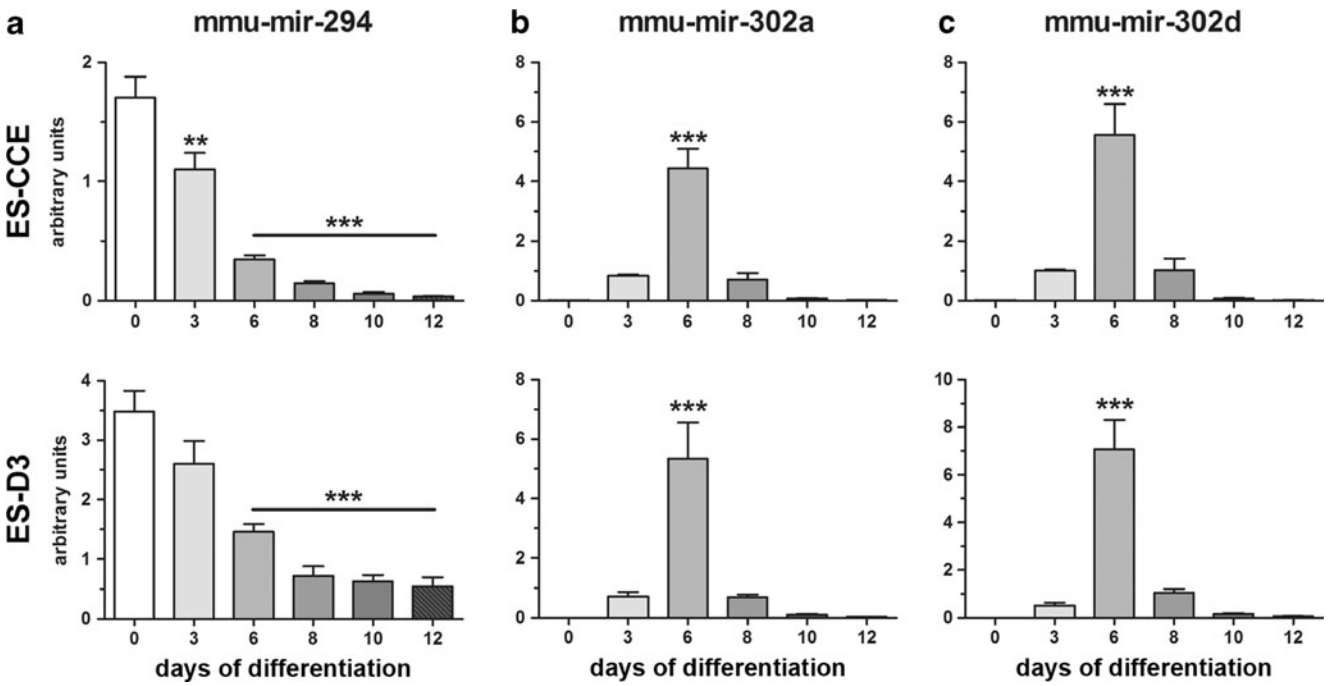


Fig. 2 MicroRNA expression in mouse ES cells during directed in vitro differentiation into pancreatic progenitors. MicroRNA expression of mmu-mir-294 (**a**), -302a (**b**) and -302d (**c**) in the cell lines ES-CCE and ES-D3 during the differentiation into pancreatic progenitors using the small molecule based protocol. MicroRNA expression was measured by TaqMan qPCR assays and normalized to the sno-RNA202. Values are mean ± SEM, $n=4–7$. *** $p\leq0.001$, ** $p\leq0.01$ compared to day 0. *ANOVA Bonferroni with Dunnett's post test*

miRNAs were more than 50 fold decreased compared to day 6 and only detectable at low levels. Compared to the ES-CCE line the ES-D3 cells showed a similar statistically significant peak after 6 days of differentiation (mir-302a: 5.34±1.21 AU; mir-302d: 7.07±1.22 AU, $p<0.001$) with a more than 40 fold decrease after 10 days of differentiation compared to day 6 (Fig. 2b and c). Day 6, which showed the highest expression of mmu-mir-302a and -302d, correlates with the generation of the definitive endoderm (DE) in this differentiation protocol. Hence, the mmu-mir-294 is an interesting miRNA to modify the reporter gene expression strength in mouse ES cell lines due to the high expression in the pluripotent state and a continuous decrease upon differentiation.

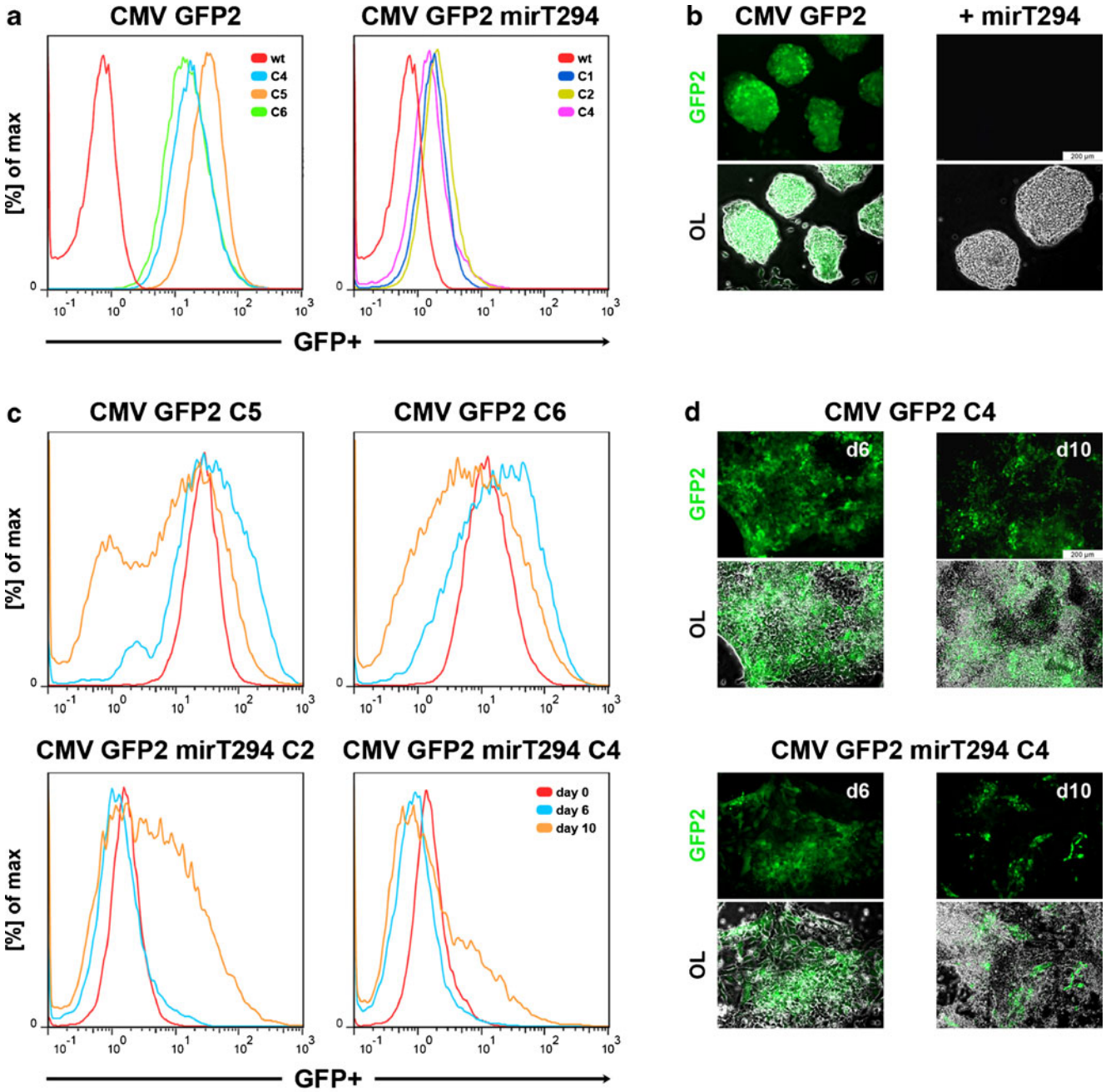
MirT294 Mediated Regulation of Transgene Expression in ES Cells

Efficient knock-down of the reporter gene expression via miRNA target sites in undifferentiated ES cells was tested using the strong constitutive CMV viral promoter (Supplementary Fig. 1B and Fig. 3a). The miRNA mmu-mir-294 was selected because this miRNA was highly expressed in ES cells and decreased upon differentiation. This should allow efficient knock-down of the CMV promoter controlled GFP2 expression specifically in the pluripotent state (Fig. 3a). Transduction of 3T3 fibroblasts with the CMV-GFP2 or CMV-GFP2 mirT294 construct showed no differences in the expression strength of the fluorescent reporter GFP2. Upon transduction of mouse ES cells without clonal selection, the CMV-GFP2 construct produced more than 50 % GFP-positive cells, whereas the construct containing the mirT294 showed only a marginal amount (≤5.5 %) of GFP-positive cells with a highly decreased mean fluorescence compared to the CMV-GFP2 (Supplementary Fig. 1B). To further characterize this system mouse ES cell clones were expanded after blasticidin selection. Cloning of the mirT294 into the pLenti6.3 vector decreased the mean fluorescence more than 10 fold compared to the CMV-GFP2 construct (mean fluorescence of three different clones CMV-GFP2 27.67±5.4 or CMV-GFP2 mirT294 2.2±0.2, Fig. 3a). Only clones generated with the CMV-GPF2 construct showed a detectable fluorescence via microscopy, whereas clones harboring the CMV-GFP2 mirT294 construct had no reliable fluorescence signal above background as illustrated by Fig. 3b. To exclude the possibility that different numbers of lentiviral integrations have caused this divergence, we measured the integrations via qPCR for the CMV-GFP2 and the CMV-GFP2 mirT294 construct. We found comparable transduction efficiencies with 3–4 integrations per clone (Table 2). To further validate this system, a potential effect of the integrated mirT294 site on mmu-mir-294 regulated genes was assessed by qPCR. For this purpose the positively regulated gene Lin-28 and the negatively regulated Rbl2 gene were selected [27–29]. No differences in the expression of both genes were detectable in clones containing a vector with the mirT294 site compared to clones without a mirT site or unmodified ES cells (Supplementary Fig. 1C). Hence, the mirT294 site did not alter the endogenous effect of the mmu-mir-294.

In order to allow re-occurrence of GFP2 transgene expression under the control of any tissue-specific promoter the mirT site must permit expression upon differentiation of mouse ES cells. For the generation of pancreatic progenitors a differentiation procedure based on small molecules was used. The expression of the key transcription factor Pdx1 is usually expected between 8 and 12 days of in vitro differentiation with this protocol. Thus, we analyzed the re-occurrence of GFP2 expression via flow cytometry after differentiation of the CMV-GFP2 mirT294 clones. Upon differentiation for 10 days GFP2 re-appeared in CMV-GFP2 mirT294 clones as illustrated in Fig. 3c. The amount of GFP-positive cells ranged from 16 to 50 % depending on the differentiation experiment and the analyzed clone. The first GFP-positive cells were observed after 3 to 6 days of differentiation but the main peak of positive cells was detected after 10 days (Fig. 3c and d). This re-occurrence of GFP2-fluorescence correlated with the decreased expression of mmu-mir-294 in mouse ES cells during differentiation. In contrast, the clones harboring the CMV-GFP2 construct showed a decrease of the fluorescence intensity upon differentiation. A population of the cells, ranging from 15 to 62 %, showed a complete loss of the GFP2 signal after 10 days of differentiation. This effect was observed by flow cytometric analysis and fluorescence microscopy (Fig. 3c and d) and is most likely attributed to gene silencing. The protein expression of GFP2 was analyzed by Western blot (Fig. 3e). Clones generated with the CMV-GFP2 construct showed at day 0 and day 10 a strong GFP2 expression, whereas the integration of the mirT294 site decreased the GFP2 expression significantly in the pluripotent state. Upon differentiation CMV-GFP2 mirT294 clones showed a strong band for GFP2 at day 10 indicating the re-expression of GFP2 under the control of the constitutive CMV-promoter upon decreased mmu-mir-294 expression. Consequently, the mirT294 site was able to knock down the expression of the fluorescence marker gene specifically in mouse ES cells and the expression of the promoter could be re-activated upon differentiation.

The Background Fluorescence of Tissue-Specific Promoter-Reporter Constructs was Reduced by Integration of mirT294

Integration of the mirT294 site into the mPdx1-GFP2 construct completely decreased the background fluorescence compared to clones without a mirT294 site. Furthermore, integration of a viral enhancer element (CMV early enhancer, CE) in addition to the mirT294, to increase the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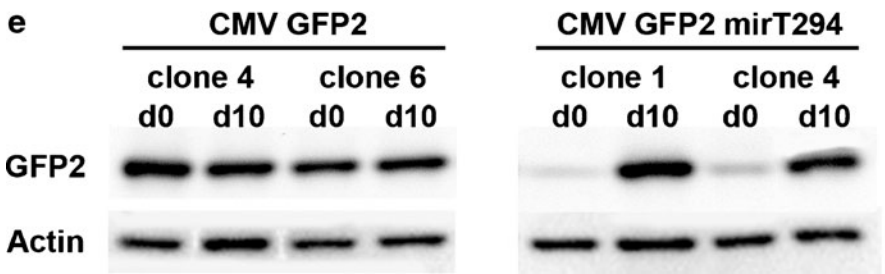
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clone 6
clone 1
clone 4
d0
d10
GFP2
Actin

◀ **Fig. 3** Reduction of CMV promoter controlled GFP2 expression in mouse ES cells by mirT294. **a** Flow cytometry analysis of different ES cell clones with a stable integration of either the CMV-GFP2 or the CMV-GFP2 mirT294 construct. **b** Fluorescence microscopy of different clones in the pluripotent ES cell state. **c** Expression of the reporter gene GFP2 of different clones during the pancreatic in vitro differentiation determined by flow cytometry. **d** Representative pictures of different clones during the pancreatic differentiation protocol at day 6 and 10. **e** Protein expression of GFP2 was determined by Western Blot at day 0 and day 10 of the pancreatic differentiation protocol. Shown are two representative clones harboring either the CMV-GFP2 or the CMV-GFP2 mirT294. As internal control beta actin was used

expression of weak endogenous promoters, did not alter this effect (Fig. 4a). All clones showed slightly lower fluorescence intensities compared to wildtype ES cells and the amount of lentiviral integrations had no significant influence (Table 3). Upon differentiation for at least 10 days, a small population ranging from 1 to 6 % of the cells started to express the reporter gene GFP2 (Fig. 4b). This population was purified by FACS and subjected to gene expression analysis and immunofluorescence. After 10 days of differentiation the expression of Pdx1 was significantly increased by 13.7±2.6 fold ($p=0.01$, $n=5$) in GFP-positive cells compared to the negative fraction (Fig. 4c). Furthermore, both populations expressed the embryonic marker gene Oct3/4 only at marginal levels compared to pluripotent ES cells (GFP-positive: > 200 fold decrease and GFP-negative: 14.6 fold decrease). A significant reduction of the Oct3/4 expression ($p<0.001$, $n=8$, 14.0 fold) was as well determined comparing the GFP-positive population (0.04±0.01) to GFP-negative cells. Interestingly, Hnf6 was significantly lower transcribed in GFP-positive cells (0.35±0.08, $p<0.001$, $n=8$) compared to the negative population. Another important marker for further maturation of Pdx1-positve cells is Nkx6.1, which was not significantly but slightly increased (2.21±0.62, n.s., $n=6$) in GFP-positive cells compared to the negative population. To further characterize the sorted populations, immunofluorescence analysis was performed. Cells of the GFP-positive population were double-positive for PDX1 and GFP2, whereas the negative population contained only a minor part of cells positive for PDX1 (Fig. 4d). Interestingly, nearly all cells in both populations positive for PDX1 were also positive for GFP2 and only a very low amount of PDX1 single-positive cells was detectable (data not shown). Moreover, some GFP-positive cells showed PDX1-positivity in the nucleus and in addition in the cytoplasm (Fig. 4d).

Discussion

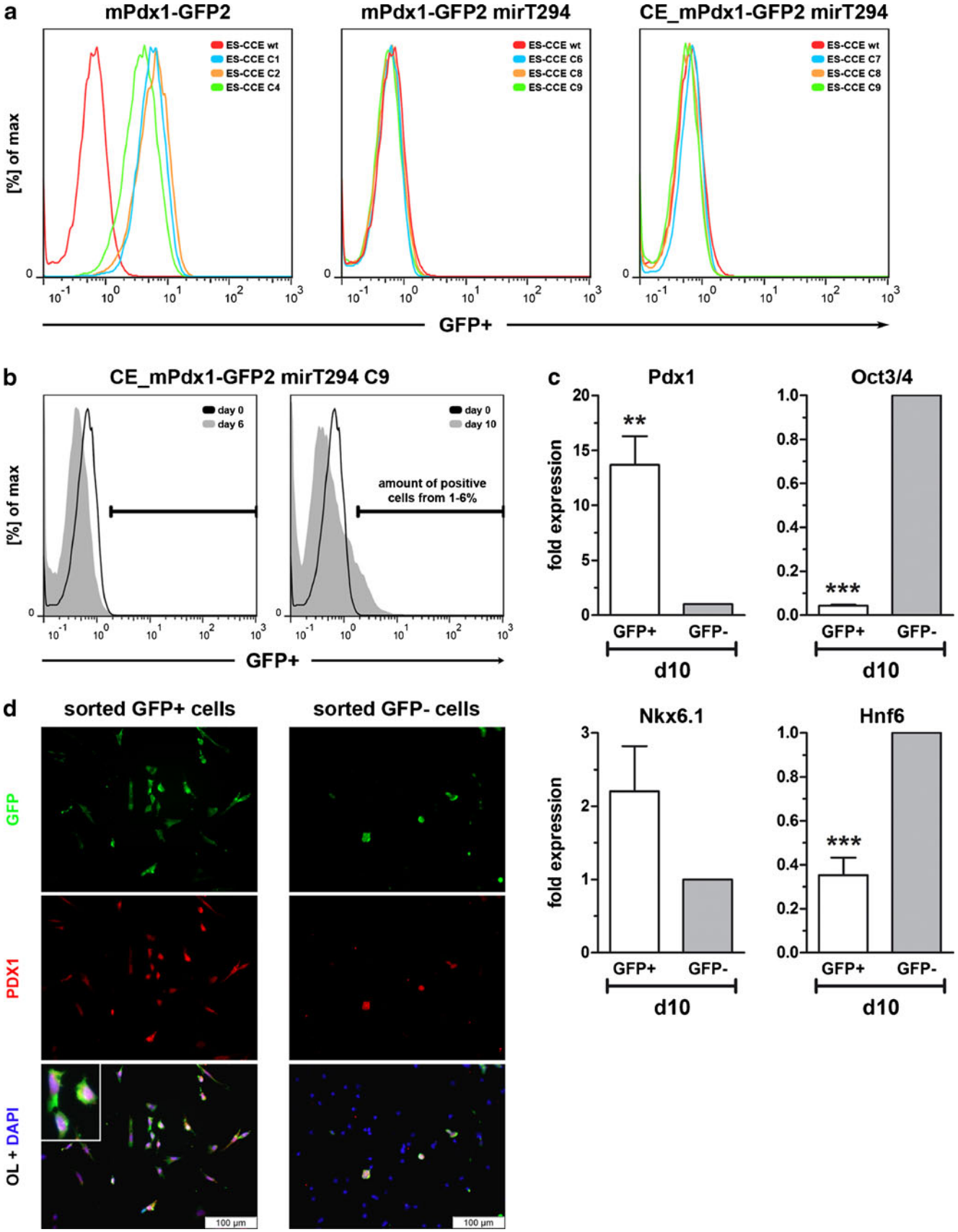
The differentiation of ES cells into insulin-producing cells for cell replacement therapy is a promising and feasible technique to generate insulin-producing cells in vitro [30]. However, to date all known protocols have been able to yield heterogeneous cell populations only with a limited extent of pancreatic endocrine cell enrichment [11]. The pancreatic cells were contaminated with various undefined cell types that did resist the directed differentiation procedure towards the pancreatic endocrine lineage. Other cell types that can potentially arise during differentiation are cells, which are not sufficiently committed to the definitive endoderm germ layer and may instead be routed into ectodermal or mesodermal derivatives, cells that were routed to other endoderm lineages with hepatic, intestinal, or pulmonal character, and most undesirably cells that still harbour embryonic potential. These unwanted cells harbor a residual risk of malignancy that can lead to tumor formation [8]. Moreover, any biochemical analysis of mixed cell populations is prone to misinterpretation of the data. To tackle these drawbacks and to enable the characterization of defined and homogenous cell populations, all unwanted lineages need to be depleted.

For this purpose tissue-specific promoter-reporter constructs have been developed to purify various cell types after in vitro differentiation [2, 14, 31–33]. In the present study a fragment of the mouse Pdx1-promoter controlled the expression of the fluorescent reporter gene GFP2 and allowed the purification of Pdx1-positive pancreatic progenitor cells from differentiated ES cell cultures. However,

Table 2 Lentiviral integrations and the fluorescence intensity of clones harboring the CMV-GFP2 or the CMV-GFP2 mirT294 construct

Clone designation	Lentiviral integrations	Mean fluorescence compared to wt ES cells (x fold increase)
ES-CCE CMV-GFP2 C4	3.98	23.94
ES-CCE CMV-GFP2 C5	3.29	38.74
ES-CCE CMV-GFP2 C6	2.15	21.20
ES-CCE CMV-GFP2 mirT294 C1	3.07	2.03
ES-CCE CMV-GFP2 mirT294 C2	4.09	2.53
ES-CCE CMV-GFP2 mirT294 C4	3.03	2.02

Lentiviral integrations were measured according to Sastry et al [48]. A standard containing the sequences of the lentiviral backbone and beta actin was used. All fluorescence intensities were normalized to wildtype (wt) ES cells and are presented as fold increase above background fluorescence


a
mPdx1-GFP2
mPdx1-GFP2 mirT294
CE_mPdx1-GFP2 mirT294
ES-CCE wt
ES-CCE C1
ES-CCE C2
ES-CCE C4
ES-CCE C6
ES-CCE C8
ES-CCE C9
ES-CCE C7
[%] of max
GFP+
b
CE_mPdx1-GFP2 mirT294 C9
day 0
day 6
day 10
amount of positive cells from 1-6%
c
Pdx1
Oct3/4
Nkx6.1
Hnf6
fold expression
GFP+
GFP-
d10
d
sorted GFP+ cells
sorted GFP- cells
GFP
PDX1
OL + DAPI
100 µm

◀ **Fig. 4** Reduction of the background fluorescence from tissue-specific promoter-reporter constructs by integration of mirT294 allowed the purification of a pancreatic progenitor population. **a** Measured fluorescence by flow cytometry of clones harboring the tissue-specific promoter constructs mPdx1-GFP2, mPdx1-GFP2 mirT294 or CE_mPdx1-GFP2 mirT294 construct. **b** Expression of the reporter GFP2 from the mPdx1-promoter during the pancreatic in vitro differentiation in comparison to the undifferentiated state was determined by flow cytometry for the clone CE_mPG mirT C9. **c** Relative gene expression of sorted GFP-positive cells compared to the negative fraction of the CE_mPG mirT C9 clone. Gene expression was normalized to the housekeeping genes G6PDH and TBP and GFP-negative cells were set to one. Values are means ± SEM, n=5–8. *** $p \leq 0.0001$, ** $p \leq 0.001$, *Student's t-test*. **d** Immunofluorescence staining of the two sorted populations for GFP2 (*green*) and PDX1 (*red*). The nuclei were counterstained with DAPI (*blue*). The inlet in the overlay of GFP+ sorted cells shows a higher magnification

sorted Pdx1-positive cells were mixed with partially undifferentiated cells expressing the embryonic transcription factor Oct3/4. The background fluorescence of all tested lentiviral constructs, which was specifically detected during pluripotency, hampered the purification to obtain a homogenous Pdx1-positive population. Pdx1, which is slightly expressed in undifferentiated mouse ES cells, can be one possible complication because the slight fluorescence masks Pdx1-positive cells arising during differentiation. However, all tested constructs as well as other promoter fragments (data not shown) showed this slight background fluorescence indicating an unspecific activation of ectopic promoters in mouse ES cells. Other possible explanations for the observed GFP2 expression are weakly active viral promoter sequences within the remaining LTRs or in the vector, the open chromatin structure of ES cells allowing a simplified access to the promoter, and positional effects of flanking chromatin at the random integration site of the genome [34].

The decrease of the background fluorescence in pluripotent ES cells without effects on tissue-specificity of the promoter would permit the mandatory depletion of the contaminating embryonic population as well as the other cell lineages and would in turn facilitate the purification of bona fide Pdx1-positive cells. The Pdx1-positive cell is the earliest cell type defining the pancreatic endoderm and thus presents a cell that is not yet primed towards exocrine, ductal, or endocrine progeny [15]. Thus, Pdx1-positive cells do not represent optimal transplant material for a potential transplantation therapy of diabetes mellitus in humans. This would ideally require pure insulin-producing cells or alternative cells that are exclusively committed to the endocrine lineage such as Ngn3-expressing cells. However, purified Pdx1-positive populations can be utilized to screen for new compounds and growth factors that can specifically control differentiation from early pancreatic endoderm into later pancreatic cell types such as the aforementioned Ngn3-positive endocrine cell. Furthermore, this purified cell population could be used to analyze the cell surface proteome to find specific surface markers, which would allow the purification of genetically unaltered Pdx1-positive cells.

Recently it has been shown that miRNAs are important post-transcriptional regulators of gene expression in all cell types and that these small non-coding RNAs can be used to regulate the expression of proteins from viral vector systems via mirT sites [20]. The integration of a mirT site can be used as a genetic tool to enhance the specificity of gene expression in a certain cell type [35–37]. Alternatively, a constitutive promoter has been utilized to deplete undifferentiated cells from a heterogeneous population enriched of neuronal progenitor cells [21]. Especially those ES cell specific miRNA clusters, which are important for cell cycle regulation, the expression of necessary transcription factors, and the regulation of the pro-differentiation let7-miRNA family [29, 38, 39], are interesting candidates for the down-regulation of the background fluorescence of lentiviral vectors. Consistent with the literature, the expression of mmu-mir294 from the mouse ES cell specific mmu-mir-290-295 cluster showed a gradual decrease during differentiation pointing to a major role of this miRNA during the maintenance of the pluripotent state of mouse ES cells [40, 41]. This characteristic decrease of the mmu-mir-294 expression renders this miRNA as an interesting candidate for the mirT design to reduce the background fluorescence in the ES cells. Interestingly, the mmu-mir-302a and the mmu-mir-302d,

Table 3 Lentiviral integrations and the fluorescence intensity of clones harboring the mPdx-1 promoter GFP2 (mPG) mirT294 or CE_mPdx-1 promoter GFP2 (CE_mPG) mirT294 construct

Clone designation	Lentiviral integrations	Fluorescence compared to wt ES cells (x fold increase)
CCE mPG mirT294 5.1x-C6	1.06	0.82
CCE mPG mirT294 5.1x-C8	1.11	0.81
CCE mPG mirT294 5.1x-C9	2.85	0.78
CCE CE_mPG mirT294 5.1x-C7	0.99	0.95
CCE CE_mPG mirT294 5.1x-C8	1.99	0.82
CCE CE_mPG mirT294 5.1x-C9	1.07	0.78

Lentiviral integrations were measured according to Sastry et al [48]. A standard containing the sequences of the lentiviral backbone and beta actin was used. All fluorescence intensities were normalized to wildtype (wt) ES cells and are presented as fold increase above background fluorescence

which are highly conserved between different species, were not expressed at detectable levels in undifferentiated mouse ES cells. Their expression rapidly increased during the first differentiation step towards the definitive endoderm indicating a major role during the early differentiation events. For human ES cells a role of the mir-302-cluster for the pluripotent state and for the mesendo-/endodermal differentiation has been previously described [42, 43]. Mouse and human ES cells have been characterized as different in terms of their developmental stage and thus human ES cells are more similar to mouse epiblast stem cells [44]. Stadler and collaborators showed that mouse epiblast stem cells had an increased expression of the mmu-mir-302b compared to mouse ES cells [45]. Recently it has been shown that the mir-302 cluster has a regulatory effect on inhibitor proteins of the Tgf-ß and BMP signaling pathways [43, 46]. These signaling pathways play important roles during early lineage commitment towards mesendo-/endoderm (mainly Tgf-ß signaling) or trophoectoderm (mainly BMP signaling) [46]. During the first patterning of the endoderm a high ActivinA/Nodal concentration is used for activation of Tgf-ß signaling by SMAD phosphorylation (reviewed in [47]). Moreover, down-regulation of the mir-302 cluster reduces the expression of marker genes for the endoderm (Sox17/FoxA2) and mesoderm (Brachyury) linages upon differentiation [43, 46]. The high expression of the mir-302 cluster eases Tgf-ß signaling by repressing the inhibitory proteins Lefty1/2. Thus, the mir-302 cluster, shown here for the mmu-mir-302a and -302d, seems to have a conserved role within the different species during early differentiation events and during maintenance of these states as it was described for human ES cells by "simplifying" the activation of important signaling pathways [43, 46].

The integration of the mirT294 site into the lentiviral vector reduced the background fluorescence of the reporter gene GFP2 virtually completely even when the strong viral CMV promoter was used. During differentiation the mmu-mir294 expression decreased, which allowed the re-occurrence of the reporter gene expression. As described in the literature, integration of a mirT site, here the mirT294, did not affect the expression of genes regulated by the miRNA complementary to the mirT site as shown here for Lin28 and Rbl2 [27]. One of the positively regulated genes of the mmu-mir-294 is Lin-28, which is known to block the let-7 maturation, thus ensuring the expression of let-7 targets like c-myc and maintaining pluripotency of the cell [29, 38, 39]. The specific down-regulation of the reporter gene expression in the ES cell state can be used to erase the background fluorescence of ectopic cellular promoters without a significant effect on the cellular function of mmu-mir-294. The combination of a tissue-specific promoter fragment, here from the Pdx1 gene, and the mirT site allowed the purification of Pdx1-positive pancreatic progenitors from differentiated mouse ES cell cultures without the contaminating embryonic lineage. The GFP2-positive population showed a significantly decreased Oct3/4 expression, which was close to the detection limit and an increased Pdx1 and Nkx6.1 expression thereby indicating an early pancreatic progenitor population. The very low Oct3/4 gene expression in sorted GFP2-positive cells might be caused by minor cell contaminations of GFP2-negative cells due to the technical limitations of the sorting process. However, whether GFP2+/Pdx1+ cells represent complete homogeneity without any risk of residual tumour formation potential has now to be verified by implantation in immunodeficient animals.

The present lentiviral vector system permits the purification of pancreatic progenitors via cell sorting and allows a reliable characterization of pure cell populations. Moreover, transgene expression could be specifically down-regulated in a time- or differentiation-dependent manner by using stage specific miRNAs opening other potential applications. (1) Combined with other cellular promoters, other cell types might be purified from stem cell differentiation cultures. (2) Another potentially useful application is the purification of specific progenitor populations that can be subsequently screened by drug/compound testing for new mechanisms controlling ES cells differentiation into certain somatic cell types. (3) Cancer cells show a miRNA profile different from normal cells. These might be selectively destroyed by utilizing transcript knock-down via mirT site of a suicide gene in healthy cells by an abundant microRNA, which is decreased or ideally absent in cancer cells. This would render degenerated cells prone to cell death.

In summary, the depletion of the background fluorescence of this lentiviral vector system via mirT sites erased the embryonic population from sorted cells and in combination with a tissue-specific promoter a pancreatic progenitor population could be purified.

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Conflicts of interest The authors declare no potential conflicts of interest.

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