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Pancreatic Differentiation of Sax 17 Knock-in Mouse Embryonic Stem Cells *in vitro*

Research Article

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ABSTRACT

The way to overcome current limitations in the generation of glucose-responsive insulin-producing cells is selective enrichment of the number of definitive endoderm (DE) progenitor cells. Sox17 is the marker of mesendoderm and definitive endoderm. The aim of the present research was to study the potential of Sox17 knock-in CGR8 mouse embryonic stem (ES) cells to differentiate into insulin producing cells in vitro by detection of specific markers at different stages of three-step protocol of differentiation. At day 5+9 cells strongly expressed Sox17 as well as CxCr4 and N-cadherine, while staining for Oct-4 (octamer-binding transcription factor 4) was faint. At day 5+16 the expression of Sox17 gradually disappeared and cells were positive for Neurogenin 3 (NGN3), Islet-1 and C-peptide. At last stage of differentiation protocol (day 5+23) Sox17 knock-in CGR8 ES derived cells showed insulin and C-peptide expression. We conclude that Sox17 knock-in mouse ES cells are equally suitable for differentiation into insulin producing cells as commonly used CGR8 ES cells. Genetically modified ES cells allow the selection of Sox17-expressing cells and the selective enrichment of DE progenitors.

KEY WORDS definitive endoderm, differentiation, embryonic stem cells, insulin, progenitors.

INTRODUCTION

A potential source of cells for the treatment of diabetes is embryonic stem cells, which have an almost unlimited proliferation capacity and retain the potential to differentiate *in vitro* into cells of the three germ layers (Wobus and Boheler, 2005).

The previous studies have successfully demonstrated the differentiation of ES cells via multi-lineage progenitors into pancreatic cells (Blyszczuk *et al*. 2003; Blysczuk *et al*. 2004; Schroeder *et al*. 2006). Recently, transcriptional profiling of undifferentiated ES cells, committed progenitors and islet-like clusters and their comparison with transcript profiles of the embryonic pancreas (Gu *et al*. 2004) revealed that the ES-derived pancreatic cells resemble an embryonic/fetal developmental stage (Rolletschek *et al*. 2010). The study suggested that additional factors/strategies are necessary to increase the amount of insulin-producing cells and their functional status.

Sox17 is expressed in mesendoderm and definitive endoderm (Tada *et al*. 2005) and Sox17-/- mutant mice showed complete depletion of gut definitive endoderm (Kanai-Azuma *et al*. 2002) giving rise to the digestive tract, pancreas and liver.

The most critical step to obtain high number of insulin producing cells is the selection, enrichment and cultivation of definitive endoderm progenitor cells. For this aim, it is necessary to characterize the DE cells in comparison to mesendoderm and extra-embryonic endoderm and to establish conditions for cultivation of DE progenitor cells. The

rationale behind this strategy is that only DE cells, but not the immature mesendoderm and extra-embryonic endoderm cells will give rise to pancreatic cells. Therefore, the aim of the present work was to study the potential of ES CGR8 cells expressing the Sox17+-DsRed-puromycin^r genes to differentiate into insulin producing cells in-vitro. The three-step protocol consists of: the formation of embryoid bodies (EBs), the spontaneous differentiation of embryoid bodies into progenitor cells of ecto-, meso- and endodermal lineages, and the induction of differentiation of early progenitors into the pancreatic lineage. Better characterization of Sox17 knock-in cells will enable the selection of Sox17-positive cells by FACS sorting and/or puromycin selection and finally yield higher efficiency of pancreatic differentiation protocol.

MATERIALS AND METHODS

Sox17 knock-in CGR8 mouse embryonic stem cells (CGR8 transfected by the vector "pSox17DsRedExpress NLS IRES2 Puro3 Neo", provided by H. Himmelbauer, T. Nolden, MPI Berlin) were differentiated into pancreatic cells applying protocol commonly used for embryonic stem cells differentiation (Blyszczuk *et al*. 2003). The cells were differentiated by the hanging drop technique (Schroeder *et al*. 2006). Briefly: Sox17 knock-in CGR8 ES cells were cultured on 60 mm tissue-culture dishes (Nunc) in embryonic stem cell culture medium. The culture medium was changed 1-2 h before passage. For subculture, the medium was aspirated and the culture dish was quickly rinsed with 2 mL of trypsin-EDTA (1:1; Sigma) to remove remaining serum containing medium. 0.5 mL of trypsin-EDTA was added and then incubated at room temperature for 30-60 seconds. The trypsin-EDTA (1:1) solution was removed and 2 mL of fresh culture medium was added. The cell population was resuspended with a 2 mL glass pipette into a single cell suspension and split 1:3 into new culture dishes (Nunc).

Generation of embryoid bodies (EBs) and early progenitor cells

The cells were trypsinized as described above and the single-cell solution was resuspended in differentiation medium I (composition details below). A cell suspension was prepared containing 600 cells in 20 µL of differentiation medium I. Single drops (n=50) of 20 µL of the cell suspension (containing 600 cells) were placed onto the lids of 100-mm bacteriological dishes (Greiner) containing 10 mL PBS and the lids were put upside down on the plates.

The cells in hanging drops were cultivated for 2 d. The cells aggregated and formed one EB per drop. Using light microscopy EBs were checked. Irregularly shaped strutures were not suitable for pancreatic differentiation.

The aggregates were carefully rinsed from the lids with 2 mL of differentiation medium I, transferred into 60-mm bacteriological Petri dishes with 5 mL of differentiation medium I, and cultivation in suspension was continued for next 3 days.

Gelatin-coated tissue-culture plates were prepared by adding a sufficient amount of 0.1% gelatin to cover the whole surface of the dish and incubated overnight at 4 ºC. For immunofluorescence analysis, cover slips (Schütt Labortechnik) were placed on the bottom of the dish before gelatin coating. Gelatin solution was removed before use. A sufficient amount of differentiation medium I was added and EBs were transferred onto the gelatin-coated culture plates. EBs (25-30) were placed on 60-mm tissue culture dishes (for immunofluorescence) and 5-10 EBs on 35-mm tissue culture dishes (for RT-PCR analysis), respectively. The medium was changed every second or third day until 9 day after EBs plating (=5-9 d).

Induction of pancreatic differentiation

Laminin is a crucial factor for pancreatic differentiation. In order to prepare poly-L-ornithine-laminin-coated dishes sterile poly-L-ornithine solution was added to the culture dishes and incubated for 3 h at 37 ºC. For immunofluorescence analysis, cover slips were placed on the bottom of the dish before adding the poly-L-ornithine solution. Poly-L-ornithine solution was aspirated, washed three times with distilled water and incubated with 5 mL distilled water at room temperature for 12 h. The dishes were rinsed three times with distilled water and dried at 40 ºC.

The medium from 60-mm tissue culture plates containing differentiating EBs derivatives was aspirated. The cultures were rinsed quickly with 2 mL of PBS. Sufficient amount of trypsin-EDTA (1:1) was added to cover the whole surface and incubated at room temperature for 30-60 s. The trypsin-EDTA solution was carefully removed and cells were gently detached with the cell scraper. Freshly prepared differentiation medium II (4 mL) supplemented with 10% FCS was added, cells were gently resuspended with a 5-mL glass pipette to obtain a suspension containing single cells and small clusters, and 1 mL suspension was plated onto four freshly prepared poly-L-ornithine-laminin (or collagen I)-coated 60-mm dishes. The differentiation medium II (3 mL) supplemented with 10% FCS was added and incubated overnight. FCS is needed for cell attachment. However, pancreatic differentiation requires serum-free conditions, therefore, FCS was removed after cell attachment. The next day (5+10 d), medium was aspirated and differentiation medium II (composition details below) without FCS was added. The medium was changed every second day. At days 5+16 and 5+23, RT-PCR and immunofluorescence for markers typical for pancreatic cells were performed.

Compositions of media used in pancreatic differenttiation procedure

Differentiation medium I: Iscove's modification of DMEM (Invitrogen) was supplemented with the following additives: 20% FCS, 2 mM L-glutamine, non-essential amino acids, 450 μMm 3-mercapto-1,2-propanediol (Sigma), 0.05 mg/mL streptomycin and 0.03 mg/mL penicillin.

Differentiation medium II: DMEM/F12 (4.5 g/L glucose; Invitrogen) was supplemented with sterile additives: 20 nM progesterone (Sigma), 100 μM putrescine (Sigma), 1 μg/mL laminin, 10 mM nicotinamide (Sigma), 25 μg/mL insulin, 30 nM Na2SeO3 (Sigma), 50μg/mL transferring (Sigma), B27 media supplement (Invitrogen), 0.05 mg/mL streptomycin and 0.03 mg/mL penicillin. This medium was prepared with or without 10% FCS.

RT-PCR analysis

Preparation of the sample

Culture medium was removed and cells were washed twice with PBS. 100 μL of RNA lysis buffer per culture dish was added. The lysis buffer was allowed to spread across the surface of the dish and the lysate was transferred into a 1.5-mL micro tube.

Isolation of total RNA

All steps were performed on ice to avoid RNA degradation. Lysate (100 μL) was thawed and vortexed for 15 s., 10 μL (1/10 vol) of 2 M sodium acetate, pH 4.0 was added. 100 μL of acidic phenol was added and the mixture was vortexed vigorously. 20 μL of chloroform/isoamyl alcohol (24:1) was added and the mixture was vortexed again. Then it was incubated on ice for 15 min. The organic and aqueous phases were separated by centrifugation at 16,000 g for 10 min at 4 ºC. The upper aqueous phase was transferred carefully to a fresh tube; equal volume of isopropanol was added and mixed well. Incubation for 30 min at –80 ºC took place followed by centrifugation at 16,000 g for 10 min at 4 ºC. The supernatant was carefully discarded. The pellet was dissolved in 80 μL of proteinase K solution (Merck) and incubated for 1 h at 56 ºC. The mixture was cooled down; equal volume of isopropanol was added and mixed well. Then incubation at –80 ºC for 30 min took place. Mixture was centrifuged at 16,000 g for 10 min at 4 ºC. Supernatant was carefully discarded. The pellet was washed with 300 μL of 75% ice-cold ethanol, vortexed briefly and recentrifuged at 16,000 g for 10 min at 4 ºC. The supernatant was discarded and the pellet of nucleic acid was air dried. The RNA pellet was dissolved in 20 μL of H2O. 1 μL of RNA was diluted with 100 μL of H2O, the OD260 and the concentration of RNA was measured using a spectrophotometer (Bio-Rad), all samples were adjusted to the same RNA concentration (e.g. 0.2-0.3 μg/μL) with H2O, and measured again to confirm that the RNA concentration of all samples was equal.

Reverse transcription (RT) reaction

One PCR-reaction tube was labeled for each sample. The same amount of RNA (1.0 μg in 3 μL) was added to each tube. RT master mix for 1 reaction was prepared as follows: 10,45 μL of H2O, 4 μL of reaction buffer, 0,8 μL of 10 mM dNTPs mix, 0,48 μL of RNase inhibitor, 1 μL of oligo d(T)18 and 0,25 μL of Revert Aid M-MuLV reverse transcriptase, to a total volume of 17 μL. 17 μL of RT master mix was added to each tube, mixed carefully and centrifuged briefly at 2,000 g for 10 s at 4 ºC. Tubes were transferred to thermal cycler (Eppendorf) and reverse transcription reactions was performed for 1 h at 42 ºC, then heated to 99 ºC for 5 min. After that the samples were cooled to 4 ºC.

Polymerase chain reaction (PCR)

PCR master mix was prepared for 25 reactions containing 385 μL of H2O, 62.5 μL of 10×PCR buffer, 45 μL of 25 mM MgCl2, 40 μL of 10 mM dNTPs mix, 25 μL of 10 μM 5′ sense primer of target gene, 25 μL of 10 μM 3′ antisense primer of target gene and 6.25 μL of Taq DNA polymerase, to a total volume of 588.75 μL. The primer sequences of detected genes are presented in Table 1. New PCR-reaction tubes were labeled and 1.5 μL of RT reaction product was added to each tube as template DNA. 23.5 μL of PCR master mix was added to each tube, mixed by vortexing and centrifuged briefly at 2,000 g for 10 s at 4 ºC. Tubes were transferred to the thermal cycler. The cDNA was amplified through 25-40 thermal cycles. Standard conditions were: denaturation at 95 ºC for 40 s, annealing at 55-68 ºC for 40 s, and extension at 72 ºC for 40 s. The specific conditions were dependent on the primers and thermal cycler used. The samples were cooled to 4 ºC and stored at –20 ºC. GAPDH was used as a reference gene.

Electrophoresis

5 μL of 6x loading buffer was added to 25 μL of PCR reaction. Half of each PCR reaction (15 μL) and 10 μL of DNA ladder (indicator of product size) were separated by electrophoresis on a 2% agarose (Invitrogen) gel in 1×TBE supplemented with 0.35 μg/mL of ethidium bromide at 5-10 V cm-1 for 70-100 min. The gel was then illuminated by UV light and digital image was obtained.

Immunofluorescence analysis

The cover slips containing cells were rinsed twice with PBS. Cells on cover slips were fixed with 4% PFA (Fluka) in PBS for 20 min. Cover slips were rinsed twice with PBS room temperature for 5 min.

The cells were incubated with 10% goat serum in a humidified chamber at room temperature for 30 min to prevent unspecific immunostaining. The cover slips were incubated with the primary antibody at 37 ºC for 60 min. The dilutions of antibodies for specific markers were found experimentally (Tables 2 and 3). The cover slips were rinsed three times with PBS at room temperature for 5 min. The cover slips were incubated with fluorescence-labeled specific secondary antibody diluted in PBS with 0.5% BSA in a humidified chamber at 37 ºC for 40 min.

Specimen was incubated with 5 µg/mL Hoechst 33342 in PBS at room temperature for 10 min (to label the cell nuclei). The cover slips were rinsed twice with PBS at room temperature for 5 min. Then they were quickly rinsed with distilled water at room temperature. The cover slips were embedded in mounting medium and immunolabeled cells were analyzed with inverted confocal laser-scanning microscope (CLSM) LSM 510 META (Carl Zeiss).

RESULTS AND DISCUSSION

In the present work we investigated the potential of Sox17 knock-in ES cells to differentiate into insulin producing cells *in vitro*. For reaching this goal we applied three-step protocol commonly used for pancreatic differentiation (Blyszczuk *et al*. 2004) and screened the cells for the presence of defined markers at all stages of the protocol. At the first stage (day 5+9) high expression of Sox17 was observed on protein as well as on mRNA level (Figure 1A2-D2 and Figure 4A, respectively). Sox17 is expressed in definitive endoderm (Tada *et al*. 2005). Therefore, it is a suitable cell marker that can be used to increase the efficiency of pancreatic differentiation. The rationale to use this strategy is that only DE cells will give rise to pancreatic precursors. At the first step of the protocol, ES-derived cells showed high expression level of N-cadherine and CxCr4 (Figure 1B3 and D3, respectively). High protein level of N-

Table 1 RT-PCR primer sequences and NCBI accessory numbers for detected genes

Sox 17	5'-CCA TAG CAG AGC TCG GGG TC-3' 5'-GTG CGG AGA CAT CAG CGG AG-3'	NM_011441.4
Isl-1	5'-GTT TGT ACG GGA TCA AAT GC-3' 5'-ATG CTG CGT TTC TTG TCC TT-3'	NM_021459.4
NGN3	5'-TGG CGC CTC ATC CCT TGG ATG-3' 5'-AGT CAC CCA CTT CTG CTT CG-3'	NM_009719.6
Insulin	5'-CCC TGC TGG CCC TGC TCT T-3' 5'-AGG TCT GAA GGT CAC CTG CT-3'	NM_008386.3
Glucagon	5'- CAT TCA CAG GGC ACA TTC ACC-3' 5'- CCA GCC CAA GCA ATG AAT TCC-3'	NM_008100.3
Somatostatin	5'-TCG CTG CTG CCT GAG GAC CT-3' 5'-GCC AAG AAG TAC TTG GCC AGT TC-3'	NM_009215.1
Brachyury (TG)	5'-CTG CAG ATG ACT TCA GAA CC-3' 5'-GGC CAT GAT CTC ATA CTG AC-3'	NM_001161832.1
N-cadherine	5'-TCA CAG CGG AGT GAA TCA G-3' 5'-CCC AAG CAA AGA TGG AAG-3'	NM_009868.4
Pax4	5'-ACC AGA GCT TGC ACT GGA CT-3' 5'-CCC ATT TCA GCT TCT CTT GC-3'	AY965054.1

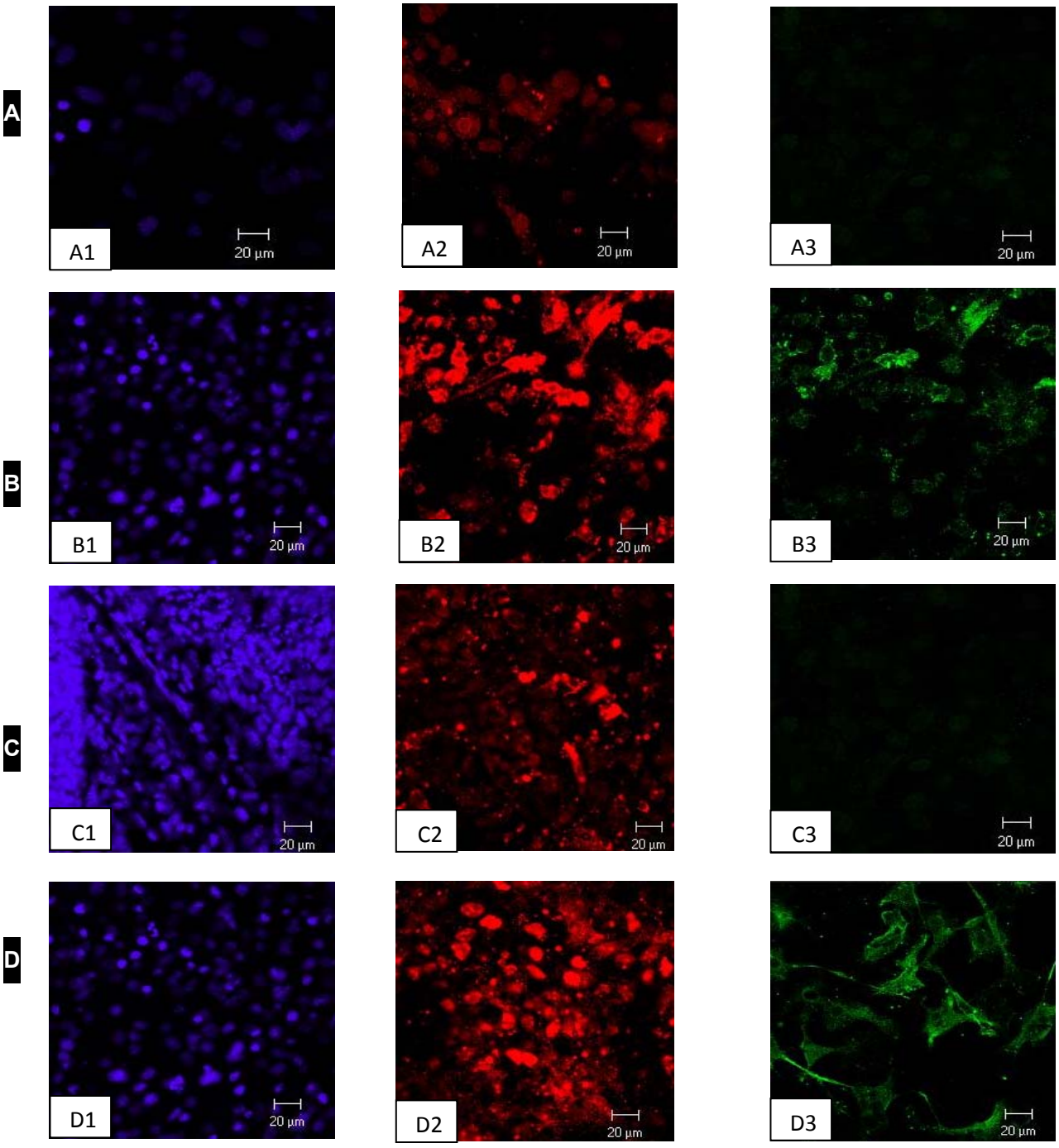

Figure 1 Immunofluorescence analysis of Sox17 knock-in ES derived cells at the first step of differentiation protocol (day 5+9). **A.** Oct-4 **(A3) B.** N-cadherine **(B3) C.** Brachyury **(C3) D.** CxCr4 **(D3). A1, B1, C1, D1** represent cell nuclei stained with Hoechst 33342. **A2, B2, C2, D2** represent Sox 17 expression

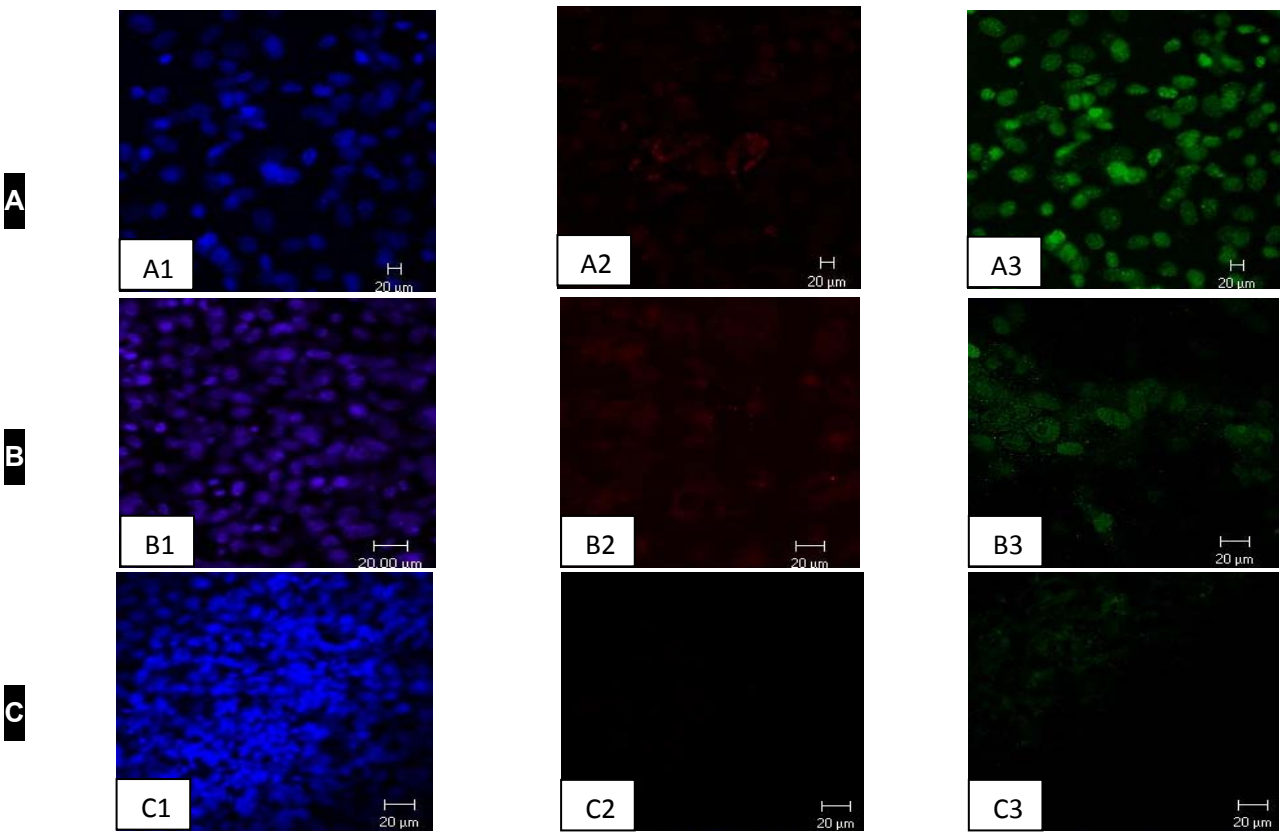

Figure 2 Immunofluorescence analysis of Sox17 knock-in ES derived cells at the intermediate step of differentiation protocol (day 5+16). **A.** Neurogenin3 **(A3)**. **B.** Islet-1 **(B3)**. **C.** C-peptide **(C3)**. **A1, B1, C1** represent cell nuclei stained with Hoechst 33342. **A2, B2, C2** represent Sox 17 expression

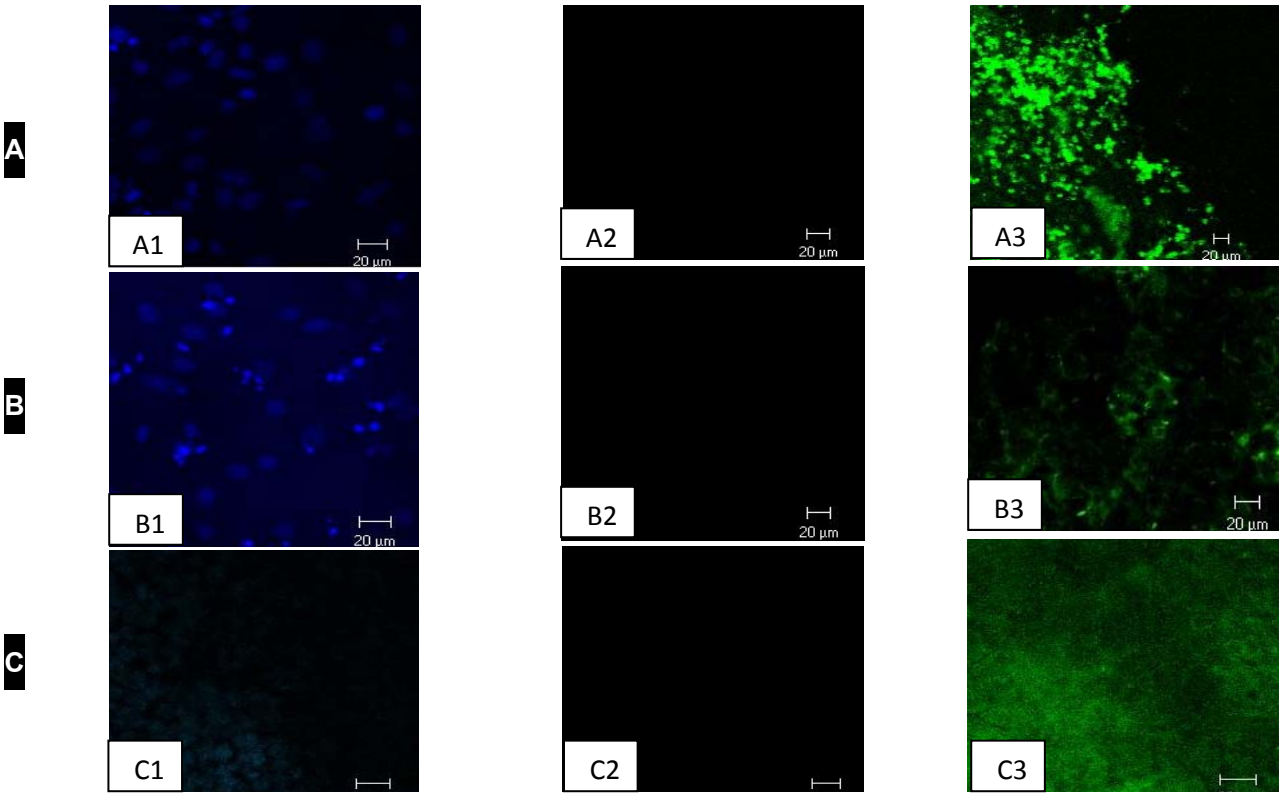

Figure 3 Immunofluorescence analysis of Sox17 knock-in ES derived cells at the terminal stage of differentiation (day 5+23). **A.** Neurogenin3 **(A3)**. **B.** C-peptide **(B3)**. **C.** Insulin **(C3)**. **A1, B1, C1** represent cell nuclei stained with Hoechst 33342**. A2, B2, C2** represent Sox 17 expression

Table 2 Primary antibodies to characterize progenitor and pancreatic cells

Rabbit anti-Isl-1 IgG	1:50	Abcam, UK
Mouse anti-insulin IgG (clone K36AC10)	1:100	Sigma-Aldrich, Germany
Sheep anti-C-peptide IgG	1:100	Acris, Germany
Rabbit anti-neurogenin3 IgG	1:100	Abcam, UK
Rabbit anti-brachyury IgG	1:100	Abcam, UK
Rabbit anti-N-cadherin IgG	1:100	Santa Cruz Biotechnology Inc.
Rabbit anti-Oct-4 IgG	1:100	Abcam, UK

cadherine at the first stage of protocol indicated that a remarkable proportion of definitive endoderm cells were still present in the population of early progenitors.
Similarly, the signal for Oct-4 was faint on the protein and mRNA level (Figure 1A3 and Figure 4. A, respectively). Low expression level of Oct-4, the marker of pluripotent cells, indicates that population of ES derived cells consisted mainly of progenitor cells of defined lineages with very few pluripotent cells.

ES cells successfully passed through all steps of differentiation protocol and delivered insulin producing cells in-vitro.However, still further studies are needed to check the potential of these cells to regulate the glucose level following transplantation.

Until now, in spite of intensive research, no insulin-secreting cell population produced *in vitro* fulfils the requirements for potential therapeutic applications both with recent studies showed that Activin A and the sequential addition of pancreatic growth and differentiation factors activated endoderm- and pancreatic endocrine-specific pathways resulting in all cell types of the islet, including insulin-, glucagon-, somatostatin-, PP- and ghrelin-positive cells (D'Amour *et al*. 2006), but the insulin positive beta-like cells did not respond to glucose.

Table 3 Fluorescence-labeled secondary antibodies

ALEXA 488-conjugated donkey anti-sheep IgG	1:100	Molecular Probes, Germany
Cy3-conjugated goat anti-rabbit IgG	1:600	Jackson ImmunoResearch Laboratories, USA
ALEXA 488-conjugated goat anti-mouse IgG	1:100	Molecular Probes, Germany

The next step of differentiation protocol (day 5+16) was characterized by the enhanced expression of pancreatic progenitor markers such as neurogenin3 and Islet-1 as well as C-peptide- a by-product of insulin synthesis (Figure 2A3-C3). The same expression pattern was found on mRNA level (Figure 4B). On the other hand, the expression of Sox17 was very weak on protein and mRNA level (Figure 2A3-C3 and Figure 4B, respectively), which indicated that cells were much more advanced in the differentiation process in comparison to the first stage of the protocol of pancreatic differentiation and no (or very few) definitive endoderm cells were present in the population.

The last stage of cell differentiation (day 5+23) was characterized by enhanced expression of neurogenin3, C-peptide and insulin (Figure 3 A3-C3). Studies performed on mRNA level also revealed the presence of some other pancreatic genes such as glucagon, Pax4 and somatostatin (Figure 4C). This indicates that some of Sox17 ES knock-in respect to functional competence/glucose-responsiveness and the level of insulin-production.

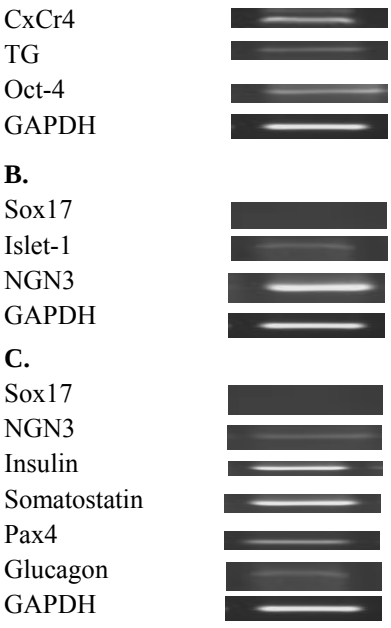

Figure 4 mRNA levels of marker genes and housekeeping gene (GAPDH) of Sox17 knock-in ES derived cells at three steps of differentiation protocol: **A.** Day 5+9 **B.** Day 5+16 **C.** Day 5+23

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