Development of MAPC derived induced endodermal progenitors
Sambathkumar, Rangarajan

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Chapter 5

Epigenetic induction of definitive and pancreatic endoderm cell fate in human fibroblasts

Rangarajan Sambathkumar*, Eric Kalo*, Rob Van Rossom, Marijke M. Faas, Paul de Vos, Catherine M Verfaillie.

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* both first author
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Epigenetic induction of definitive and pancreatic endoderm cell fate in human fibroblasts

5.1 Abstract

Reprogramming can occur by the introduction of key transcription factors (TFs) as well as by epigenetic changes. We demonstrated that histone deacetylase inhibitor (HDACi) Trichostatin A (TSA) combined with a chromatin-remodeling medium (CRM) induced expression of a number of definitive endoderm and early and late pancreatic marker genes. When CRM was omitted, endoderm/pancreatic marker genes were not induced. Furthermore, treatment with DNA methyltransferase inhibitor (DNMTi) 5-azacytidine (5AZA) CRM did not affect gene expression changes, and when 5AZA was combined with TSA, no further increase in gene expression of endoderm, pancreatic endoderm, and endocrine markers was seen over levels induced with TSA alone. Interestingly, TSA-CRM did not affect expression of pluripotency and hepatocyte genes but induced some mesoderm transcripts. Upon removal of TSA-CRM, the endoderm/pancreatic gene expression profile returned to baseline. Our findings underscore the role epigenetic modification in transdifferentiation of one somatic cell into another. However, full reprogramming of fibroblasts to β-cells will require combination of this approach with TF overexpression and/or culture of the partially reprogrammed cells under β-cell specific conditions.

5.2 Introduction

Type-1 Diabetes mellitus (DM-I) is a severe metabolic disease that affects millions of people worldwide. It involves complete loss of functional insulin secreting β-cells in the pancreas due to autoimmune destruction. DM-1 leads to hyperglycemia, which can be treated with insulin injections. However in the long-term, DM-1 leads to micro and macro vascular, cardiovascular, neuronal, renal and ocular complications due to intermittent hyperglycemia. Replacement of the destroyed β-cells is the only
curative treatment. However, limited numbers of available donor organs and immunological issues restrict whole pancreas or islet transplantation [1, 2]. An alternative source for human cadaveric islets is generating insulin-producing β-cells from stem cells and/or somatic cells. However, the challenge in this area is to identify an adequate stem or progenitor cells and mechanisms to create a safe source of mature insulin-producing β-cells from such cells.

Over the last decade several studies have demonstrated that it is possible to generate functional β-cells from human embryonic stem cells (hESCs) by culturing the cells in conditions that mimic in vivo pancreatic development [3-6]. With the advent of induced Pluripotent Stem Cells (iPSCs) technology, developed by the Yamanaka team [7, 8], it has now also become possible to create Human Leukocyte Antigen (HLA)-identical β-cells to treat DM-I. However, the pluripotent nature of ESCs and iPSCs leaves the possibility for teratoma formation [6, 9] if full differentiation towards β-cells is not achieved. An alternative approach is to transdifferentiate somatic cells into insulin producing β-cells without passing through a pluripotent state, via ectopic expression of defined TFs and culture in supportive medium. A number of studies demonstrated in rodents that introduction of a single (PDX1) or group of TFs (PDX1, NGN3, MAFA) can transdifferentiate hepatocytes, intestinal cells, exocrine acinar or ductal pancreatic cells, or endocrine a-cells, thereby reprogramming these non-β-cell endodermal cells into β-cells [10-17]. In addition, Li et al., created endodermal progenitor cells by transiently overexpressing OCT4, SOX2, KLF4 and CMYC (OKSM) in mouse embryonic fibroblasts (MEFs) combined with small molecule epigenetic modifiers, which could subsequently be converted to β-cells [18]. Several recent studies have treated human or swine fibroblasts [19-21], mesenchymal stem cells [22], or rat liver stem cells [23], with epigenetic modifying molecules (DNA methyltransferase inhibitor and/or histone deacetylase inhibitor in CRM followed by culture under b-cell specifying conditions. This combined treatment resulted in the generation of endocrine pancreatic β-cells that reversed hyperglycemia in immunodeficient mice. Moreover, epigenetic modification also induced NGN3 expression and endocrine differentiation of the PNAC-1 human ductal cell line [24]. However many studies
reported that TSA itself could induce chromatin changes without the presence of chromatin remodeling medium (CRM). For instance, using fluorescence anisotropy imaging and fluorescence recovery after photo-bleaching (FRAP) and fluorescence correlation spectroscopy (FCS), it was demonstrated that TSA induced histone protein dynamics and expression in HeLa cells by increasing the euchromatin fraction and increasing core acetylation patterns, phosphorylation patterns, and nuclear volume [25]. Similarly TSA induces histone acetylation and reversible decondensation of interphase chromatin structure in HeLa cells, as demonstrated by image correlation spectroscopy (ICS) and spatially resolved scaling analysis (SRSA) methods [26]. In another study human hepatocellular carcinoma (HepG2) and NIH 3T3 cells, treatment with TSA and VPA (Valproic acid) increased the active chromatin marks, such as H3K9ac and H3K4me2 abundance, which might lead to chromatin decondensation [27, 28].

We here optimized the exposure of human fibroblasts to epigenetic modifiers to convert them to endoderm and pancreatic endocrine progenitors. We demonstrated that culture of adult human and foreskin fibroblasts with TSA combined with a chromatin remodeling medium (CRM), induces expression of endoderm and pancreatic endoderm genes, but that this is a transient phenomenon. Hence, further maintenance in β-cell-specifying conditions with or without forced expression of exogenous TFs will be needed to permanently convert fibroblasts to β-cells.
5.3 Materials and methods

5.3.1 Culture of BJ1 human fibroblasts

Human BJ1 adult fibroblasts were cultured in 90% DMEM-F12 + HEPES (Cat. No. 11330032, Gibco, Grand Island, NY - USA), 10% Fetal Bovine Serum (FBS)(Cat.No. F6178, Sigma-Aldrich, Saint Louis, MO, USA) and 1% Penicillin/Streptomycin (Cat.No. 15140122, Gibco, Carlsbad, CA, USA). Cells were maintained under normoxia conditions, 37°C temperature and 5% CO2.

5.3.2 Isolation and culture of primary human fibroblasts

A skin biopsy from healthy volunteer/ non-diabetic donor was obtained following informed consent and with approval from the ethical committee of University of KU Leuven. Fibroblasts were isolated in minimum DMEM (Cat.No.31885049, Gibco, Grand Island, NY, USA) supplemented with 20% FBS. After four passages, fibroblasts were frozen in liquid nitrogen in several aliquots. After thawing cells were grown in 90% DMEM HG + Glutamax™, pyruvate (Cat.No. 31966047, Gibco, Grand Island, NY - USA), supplemented with 10% FBS (Cat.No. F6178, Sigma-Aldrich, Saint Louis, MO, USA), and 1% Penicillin/ Streptomycin (Cat.No. 15140122, Gibco, Carlsbad, CA, USA). Cells were maintained under normoxia conditions, 37°C temperature and 5% CO2.

5.3.3 Reprogramming conditions

Chromatin remodeling medium (CRM) consisted of 10% knockout™ (KO) serum (Cat.No. 10828028,Gibco, Grand Island, NY – USA), Knockout™- DMEM (Cat.No. 10829018, Gibco, Grand Island, NY – USA), 50 μM, β-mercaptoethanol (50mM) (Cat.No. 31350010, Gibco, Grand Island, NY, USA), 1% MEM-Non-Essential Amino Acids solution (100x) (Cat.No. 11140050, Gibco, Grand Island, NY – USA), 1% B27 ® supplement (Cat.No. 17504044, Gibco, Grand Island, NY – USA), 2 mM L-glutamine (200mM) (Cat.No. 25030081, Paisley, Scotland, UK), 2% N2-supplement (100x)(Cat.No. 17502001, Gibco, Grand Island, NY – USA) all from Gibco, Life Technologies(Thermo Scientific), and 20 ng/ml recombinant human basic fibroblast growth factor (Cat.No. 100-18C, Peprotech, USA), 20 ng/ml epidermal growth factor (Cat.No.236-EG, R&D Systems, MN-USA), 1000 U/ml penicillin and Streptomycin (10,000U/ml) (Cat.No. 15140122, Gibco, Carlsbad, CA, USA), and 100nM L-Ascorbic Acid (Cat.No. A4403, Sigma-aldrich, Saint Louis, MO, USA). Cells were plated in 6 well
plate (Cat.No. 3516, Corning-Costar®, MA, USA) and exposed to 3 µM or 5 µM 5-AZA (Cat.No. A2385, Sigma Aldrich, Saint Louis, MO, USA) dissolved in DMSO (Cat.No.D2650, Sigma-Aldrich, Saint Louis, MO, USA) in CRM medium for 48 hours, with medium change at 24h. For treatment with Trichostatin A (TSA) - ready made solution 5mM (Cat.No. T1952, Sigma Aldrich, Saint Louis, MO, USA), cells were exposed to various concentrations between 100 nM to 100 µM for 24 hours. Untreated fibroblasts and DMSO treated fibroblasts cultured in CRM were used as controls.

5.3.4 RNA isolation, cDNA synthesis and quantitative real time- polymerase chain reaction (qRT-PCR)

Total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Cat.No. RTN350, Sigma-Aldrich, Saint Louis, MO, USA), for sample size <10⁵ cells, ZR RNA MicroPrep™ CA-USA (Cat.No. R1061, Zymo Research, CA, USA), was used and cDNA was synthesized from 500ng-1 µg total RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (Cat.No. 11752050, Invitrogen, CA, USA), according to manufacturer’s protocol. For qPCR: the cDNA underwent 40 rounds of amplification cycles on a ViiA™ 7 Real-Time PCR System with 384-well plate (Cat.No. 4453536, Applied Biosystems, Carlsbad, CA, USA) as follows: 40 cycles of a 2-step PCR (95°C for 15 sec; 60 °C for 45 sec) after initial denaturation (50 °C for 2 min, 95°C for 2 min) using specific primers, Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Cat.No. 11744500, Invitrogen, CA, USA) and 2 µl cDNA. For normalization purposes, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were used as a housekeeping control and results are shown in relative expression to GAPDH. All Primers were synthesized at (IDT technologies, Leuven, Belgium). The cycle threshold value >40 are considered undetectable and calculated as Ct of 40. A list of the primers can be found below (Supplementary table-1).
5.3.5 **Statistical analysis:** Parametric distribution of data points was confirmed using the Kolmogorov-Smirnov test. Comparisons between two groups were analyzed using an unpaired 2-tailed Student’s t-test. P-values < 0.05(*), < 0.01(**), <0.001(***) were considered significant. Data are shown as mean and error bars represent standard error of mean (SEM) of minimum three independent experiments. All results were analyzed using Graph Pad prism 6 software.
5.4 Results

5.4.1 Treatment of human primary adult and foreskin BJ fibroblasts with TSA induced definitive endoderm and pancreatic endocrine genes, but only transiently

We initially tested the effect of addition of the HDAC inhibitor, TSA for 24h, on human primary adult and BJ foreskin fibroblasts, cultured in chromatin remodeling medium (CRM). We then assessed levels of transcripts of definitive endoderm, early and late pancreatic endocrine markers, as well as hepatocyte, skeletal muscle, endothelium, and pluripotency marker genes. TSA/CRM significantly induced the expression of the early endoderm marker genes, GATA4, EOMES, E-CADHERIN, SOX17, FOXA2, and CXCR4, in both cell populations while induction of SOX7 and MIXL1 was only seen in primary human fibroblasts (Fig. 1A, Fig. 2A). In addition, expression of the early and late pancreatic progenitor and endocrine marker genes, PTF1A, HLBX9, NKX6.1, ISL1, ARX, and MAFB was observed in both cell populations, while PDX1 expression was only detected in primary human fibroblasts treated cells (Fig. 1B-C, Fig. 2B-C). Transcripts for mature endocrine pancreatic cells including PAX4, NGN3, INS, GCG, and SST were however not increased following TSA/CRM culture (Fig. 1C, Fig. 2C). Transcripts for the hepatocyte marker genes, ALB, AFP, and HNF4A (Fig. 1D, Fig. 2D) were not expressed. We also assessed the effect of TSA/CRM on expression of mesodermal lineage transcripts and found an increase in MYOD1 and FLK1 expression but not the endothelium marker genes TIE2, VECADHERIN (Fig. 1E, Fig. 2E). Pluripotency marker genes OCT4, SOX2, and NANOG (Fig. 1F, Fig. 2F) were not induced. When the remodeling medium was removed, expression of the pancreatic endodermal genes was not maintained.
Figure-1: Trichostatin A (TSA) treatment of primary adult fibroblasts induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (GATA4, SOX7, MIXL1, EOMES, E-CADHERIN, SOX17, FOXA2, CXCR4, but not GSC) in TSA-CRM treated primary human fibroblast. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (PFT1A, PDX1, HIXB9, NNX6.1, but not SOX9, PAX6, NEUROD1) in TSA-CRM treated primary human fibroblast. C) qRT-PCR analysis demonstrated induction of pancreatic endocrine genes (ISL1, ARX, MAFB, but not PAX4, NGN3, MAFA, INS, SST, GCG) in TSA-CRM treated primary human fibroblast. D) qRT-PCR analysis demonstrated hepatocyte genes (ALB, GCG) were not induced except for AFP. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (MYOD1 and FLK1 but not Tie-2 and VE-CADHERIN) in TSA-CRM treated primary human fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) expression in TSA-CRM treated primary human fibroblast. Black bar – CRM treated primary human fibroblast cells; Light grey bar-DMSO-CRM primary human fibroblast treated cells; Dark grey bar-100µM TSA-CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed between TSA treated versus untreated fibroblast, and TSA treated versus DMSO treated fibroblast. *p<0.05, **p<0.01 and ***p<0.001 by unpaired 2-tailed Student’s t-test. NS- not significant.
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Figure 2: Trichostatin-A (TSA) treatment of BJ foreskin fibroblast induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (GATA4, GSC, EOMES, E-CADHERIN, SOX17, FOXA2) but not SOX7, MixL1, CXCR4) in TSA-CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (HNF4A) in TSA-CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated induction of pancreatic endocrine genes ARX, MAFB, but not ISL1, PAX4, NGN3, MAFA, INS, SST, GCG) in TSA-CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no induction of hepatocyte marker genes (ALB, AFP, HNF4A) in TSA-CRM treated BJ foreskin fibroblast. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (MYOD1 and FLK1 but not TIE-2 and VE-CADHERIN) in TSA-CRM treated BJ foreskin fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) in TSA-CRM treated BJ foreskin fibroblast. Black bar – CRM treated BJ foreskin fibroblast cells; Light grey bar-DMSO-CRM treated BJ foreskin fibroblast cells; Dark grey bar-100µM TSA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between TSA treated versus untreated and TSA treated versus DMSO treated. *p<0.05, **p<0.01 and ***p<0.001 by unpaired 2-tailed Student’s t-test. NS- not significant.
5.4.2 Combined treatment of fibroblasts with 5AZA and TSA did not result in further increased expression of definitive endoderm and pancreatic endocrine genes.

To test if combination of DNA methylation modification and inhibition of HDAC would induce a significant transdifferentiation of fibroblasts to endocrine pancreas, we next cultured primary adult fibroblasts and BJ fibroblast cells in CRM supplemented for 2 days with 3 or 5 µM 5AZA for 48 hours, followed by TSA for 24 hours. However, as significant cell death was seen with 5AZA at 5µM or more, studies were done using 3 µM 5AZA. Treatment for 2 days with 5AZA induced subtle morphological changes. In comparison with the typical elongated morphology of untreated fibroblasts 5AZA treated cells had a more rounded shape without long processes. 5AZA also decreased cell proliferation, which was analyzed by cell counting (data not shown). Following treatment with TSA on day 3, the cells appeared more flattened with granular cytoplasm and larger nuclei possibly due to more relaxed chromatin structure. Cell proliferation remained stable upon exposure to TSA.

We next assessed the effect of 5AZA combined with TSA on transcript expression as described for TSA only studies above. By contrast, following treatment with 5AZA followed by TSA, endodermal early and late pancreatic endocrine progenitor marker genes were induced in both primary and BJ foreskin fibroblasts (Fig. 3A-C, Fig. 4A-C). However, as with TSA only in this case also, mature pancreatic endocrine marker genes were not induced (Fig. 3C, Fig. 4C). As was seen for cultures treated with TSA alone, hepatocyte marker genes were not induced (Fig. 3D, Fig. 4D) but the mesodermal markers, MYOD1 and FLK1 were induced (Fig. 3E, Fig. 4E). Other mesodermal (Fig. 3E, Fig. 4E) and pluripotency marker genes (Fig. 3F, Fig. 4F) were also not induced. Again, when the epigenetic modifiers were removed, expression of the pancreatic endodermal genes was not maintained. Finally, we also demonstrated that aside from TSA, CRM was essential for induction of endodermal and pancreatic endodermal transcripts and that induction of the pancreatic endoderm genes was more efficient when early passage cells were used. We next assessed if combining 5AZA with TSA-CRM increases the expression level of definitive endoderm, early and later pancreatic endocrine progenitor transcripts. In both cell lines induction of
most, albeit not all, endodermal and early and late pancreatic marker genes were not improved by pretreatment of the cells with 5AZA before addition of TSA-CRM (Fig. 5-6A-C). 5AZA pretreatment enhanced expression of the skeletal muscle marker MYOD1 (Fig. 5E), but not hepatocyte and pluripotency marker genes (Fig. 5-6D and F). Despite the morphological changes observed following treatment with 5AZA-CRM for 2 days, no induction of pancreatic or other lineage specific markers was observed (Fig. 7-8).
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Figure 3: 5-azacytidine (5AZA) and Trichostatin A (TSA) treatment of primary adult fibroblast induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (GATA4, MIXL1, EOMES, ECADHERIN, SOX17, CXC4, but not SOX7, GSC, FOXA2) in 5AZA+TSA CRM treated primary human fibroblast cells. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (HNF1B, PDX1, HLXB9, PAX6, NKX6.1, but not SOX9, PTF1A, NEUROD1) in 5AZA+TSA CRM treated primary human fibroblast cells. C) qRT-PCR analysis demonstrated induction of the pancreatic endocrine genes (ISL1, ARX, MAFB, MAFA but not PAX4, NGN3, INS, SST, GCG) in 5AZA+TSA CRM treated primary human fibroblast cells. D) qRT-PCR analysis demonstrated no induction of hepatocyte genes (ALB, AFP, HNF4A) in 5AZA+TSA CRM treated primary human fibroblast cells. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (MYOD1 and FLK1 but not TIE-2 and VE-CADHERIN) in 5AZA+TSA CRM treated primary human fibroblast cells. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) in 5AZA+TSA CRM treated primary human fibroblast cells. Black bar – CRM treated primary human fibroblast cells; Light grey bar- DMSO-CRM treated primary human fibroblast cells; Dark grey bar- 3 µM 5AZA-CRM followed by 100µM TSA CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA+TSA treated versus untreated and 5AZA+TSA treated versus DMSO treated. *p<0.05, **p<0.01 and ***p<0.001 by unpaired 2-tailed Student's t-test. NS- not significant.
unpaired experiments. Statistical significance tests were performed in between 5AZA+TSA treated versus DMSO treated BJ foreskin fibroblast cells. Black bar analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) in 5AZA+TSA CRM treated BJ foreskin fibroblast cells. Black bar – CRM treated BJ foreskin fibroblast cells; Light grey bar - DMSO-CRM treated BJ foreskin fibroblast cells; Dark grey bar - 3µM-5AZA-CRM followed by 100µM TSA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA+TSA treated versus untreated and 5AZA+TSA treated versus DMSO treated. *p<0.05, **p<0.01 and ***p<0.001 by unpaired 2-tailed Student’s t-test. NS- not significant

Figure 4: 5-azacytidine (5AZA) and Trichostatin-A (TSA) treatment of BJ foreskin fibroblast induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (GATA4, EOMES, ECADHERIN, SOX17, CXCR4, but not SOX7, MIXL1, GSC, FOXA2) in 5AZA+TSA CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (HNF1B, PDX1, HLB9, PAX6, NKX6.1, NEUROD1, but not SOX9, PTF1A) in 5AZA+TSA CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated induction of pancreatic endocrine genes (ISL1, ARX, MAFB, but not PAX4, NGN3, MAFA, INS, SST, GCG) in 5AZA+TSA CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no induction of hepatocyte marker genes (ALB, AFP, HNF4A) in 5AZA+TSA CRM treated BJ foreskin fibroblast cells. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (FLK2 but not MYOD1, TIE-2 and VE-CADHERIN) in 5AZA+TSA CRM treated BJ foreskin fibroblast cells. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) in 5AZA+TSA CRM treated BJ foreskin fibroblast cells.
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2 and TSA treated versus 5AZA and followed by TSA treated fibroblasts.

Statistical significance tests were performed in between SAZA and followed by TSA treated versus TSA treated fibroblasts. *p<0.05, **p<0.01 and ***p<0.001 and TSA treated versus 5AZA and followed by TSA treated fibroblasts. #p<0.05, ##p<0.01 by unpaired 2-tailed Student’s t-test. NS- not significant.

Figure 5: Comparison of gene expression changes in primary adult fibroblasts treated with TSA-CRM vs. 5AZA-TSA-CRM. A) Comparative qRT-PCR analysis demonstrated induction of endodermal genes (GATA4, EOMES) (*significant indicates) were more highly expressed in SAZA+TSA CRM treated primary human fibroblast cells. However (SOX7 and MIXL1) (# significant indicates) were induced more in TSA-CRM treated primary human fibroblast. No significant differences in (GSC, E-CADHERIN, SOX17, FOXA2 and CXCR4) expression were found in 5AZA+ TSA CRM treated primary human fibroblast. B) Comparative qRT-PCR analysis demonstrated a greater induction of pancreatic endoderm genes (HNF6, PAX6, NKX6.1) (*significant indicates) in 5AZA+ TSA CRM treated primary human fibroblast cells. However SOX9 (# significant indicates) was induced more in TSA-CRM treated primary human fibroblast. No significant differences in (HNF1B, PTF1A, PDX1 and NEUROD1) expression were found in 5AZA+TSA CRM treated primary human fibroblast. C) Comparative qRT-PCR analysis demonstrated a greater induction of the pancreatic endocrine genes (ISL1, ARX, MAFB) (*significant indicates) in SAZA+TSA CRM treated primary human fibroblast cells. However (PAX4, NGN3, INS and SST) (# significant indicates) were induced more in TSA-CRM treated primary human fibroblast. No significant differences in (MAFB, GCG) expression were found in 5AZA+TSA CRM treated primary human fibroblast. D) Comparative qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (ALB, AFP, HNF4A) in 5AZA+TSA CRM treated primary human fibroblast. E) Comparative qRT-PCR analysis demonstrated a greater induction of the mesoderm lineage genes MYOD1 (*Significant indicates) in SAZA+ TASA CRM treated primary human fibroblast cells. However TIE-2 (# significant indicates) was induced more in TSA-CRM treated primary human fibroblast. No significant differences in (FLK1 and VE-CADHERIN) levels were found in SAZA+ TASA CRM treated primary human fibroblast. F) Comparative qRT-PCR analysis demonstrated that no differences in induction of pluripotency genes (OCT4, SOX2, and NANOG) in 5AZA+ TASA CRM treated primary human fibroblast. Black bar – TSA-CRM treated primary human fibroblast cells. CRM; Dark grey bar SAZA-CRM followed by 100μM TSA-CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between SAZA and followed by TSA treated versus TSA treated fibroblasts. *p<0.05, **p<0.01 and ***p<0.001 and TSA treated versus 5AZA and followed by TSA treated fibroblasts. #p<0.05, ##p<0.01 by unpaired 2-tailed Student’s t-test. NS- not significant.
performed in between 5AZA and followed by TSA versus TSA treated fibroblasts. *p<0.05, **p<0.01 (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA and followed by TSA versus TSA treated fibroblasts. *p<0.05, **p<0.01 and ***p<0.001 by unpaired 2-tailed Student’s t-test. NS- not significant.

Figure 6: Comparison of gene expression changes in BJ1 foreskin fibroblasts treated with TSA-CRM vs. 5AZA-TSA-CRM. A) Comparative qRT-PCR analysis demonstrated induction of endodermal genes for (EOMES, E-CADHERIN) (* significant indicates) were more highly expressed in 5AZA+ TSA CRM treated BJ foreskin fibroblast. No significant differences in (GATA4, SOX7, MIXL1, GSC, SOX17, FOXA2 and CXCR4) expression were found in 5AZA+TSA CRM treated BJ foreskin fibroblast. B) Comparative qRT-PCR analysis demonstrated significantly greater induction of pancreatic endoderm genes, (HNF1B, SOX9, PTF1A, PDX1 and NEUROD1) expression were found in 5AZA+ TSA CRM treated BJ foreskin fibroblast. C) Comparative qRT-PCR analysis demonstrated greater induction of pancreatic endocrine genes (ISL1, ARX) (* significant indicates) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. No significant differences in (PAX4, NGN3, MAFB, MAFA, INS, SST and GCG) expression were found in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. D) Comparative qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (ALB, AFP, and HNF4A) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. E) Comparative qRT-PCR analysis demonstrated no differences in induction of mesodermal lineage genes (MYOD1, TIE-2, FLK1, and VE-CADHERIN) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. F) Comparative qRT-PCR analysis demonstrated no differences in induction of pluripotency genes (OCT4, SOX2, NANOG) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. Black bar – TSA-CRM treated BJ foreskin fibroblast cells; Dark grey bar: 5AZA-CRM followed by 100µM TSA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments.
5-azacytidine (5AZA) treatment of primary adult fibroblasts does not induce marker expression. A) qRT-PCR analysis demonstrated no differences in induction of endodermal genes for (GATA4, SOX7, MIXL1, GSC, EOMES, E-CADHERIN, SOX17, FOXA2, and CXCR4) in 5AZA CRM treated primary human fibroblast. B) qRT-PCR analysis demonstrated no differences in induction of pancreatic endoderm genes (HNF1B, SOX9, PTF1A, PDX1, HLB9, PAX6, NKX6.1, and NEUROD1) in 5AZA CRM treated primary human fibroblast. C) qRT-PCR analysis demonstrated no differences in induction of pancreatic endocrine genes (ISL1, ARX, PAX4, NGN3, MAFB, MAFA, INS, SST, and GCG) in 5AZA CRM treated primary human fibroblast. D) qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (ALB, AFP, HNF4A) in 5AZA CRM treated primary human fibroblast. E) qRT-PCR analysis demonstrated no differences in induction of mesoderm lineage genes (MYOD1, TIE2, FLK1, and VE-CADHERIN) in 5AZA CRM treated primary human fibroblast. F) qRT-PCR analysis demonstrated no differences in induction of pluripotency genes (OCT4, SOX2, NANOG) in 5AZA CRM treated primary human fibroblast. Black bar – CRM treated primary human fibroblast cells; Dark grey bar-3µM 5AZA–CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA-CRM treated primary human fibroblast versus untreated primary human fibroblasts in CRM by unpaired 2-tailed Student’s t-test. NS- not significant.
untreated BJ foreskin fibroblasts in CRM by unpaired significance tests were performed in between 5AZA represent the mean BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene CADHERIN.) demonstrated no differences in induction of endodermal genes for (GATA4, SOX7, MIXL1, GSC, EOMES, E-CADHERIN, SOX17, FOXA2, and CXCR4) in 5'AZA CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated no differences in induction of pancreatic endoderm genes (HNF1B, SOX9, PTF1A, PDX1, HXB9, PAX6, NKX6.1, and NEUROD1) in 5'AZA CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated no differences in induction of pancreatic endocrine genes (ISL1, ARX, PAX4, NGN3, MAFA, INS, SST, and GCG) in 5'AZA CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (ALB, AFP, HNF4A) in 5'AZA CRM treated BJ foreskin fibroblast. E) qRT-PCR analysis demonstrated no differences in induction of mesoderm lineage genes (MYOD1, TIE2, FLK1, and VE-CADHERIN) in 5'AZA CRM treated BJ foreskin fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) expression in 5'AZA CRM treated BJ foreskin fibroblast. Black bar – CRM treated BJ foreskin fibroblast cells; Dark grey bar-3µM 5AZA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between SAZA-CRM treated BJ foreskin fibroblast versus untreated BJ foreskin fibroblasts in CRM by unpaired 2-tailed Student’s t-test. NS- not significant.

Results

Figure-8: 5-azacytidine (5AZA) treatment of BJ foreskin adult fibroblast does not induces marker expression. A) qRT-PCR analysis demonstrated no differences in induction of endodermal genes for (GATA4, SOX7, MIXL1, GSC, EOMES, E-CADHERIN, SOX17, FOXA2, and CXCR4) in 5'AZA CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated no differences in induction of pancreatic endoderm genes (HNF1B, SOX9, PTF1A, PDX1, HXB9, PAX6, NKX6.1, and NEUROD1) in 5'AZA CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated no differences in induction of pancreatic endocrine genes (ISL1, ARX, PAX4, NGN3, MAFA, INS, SST, and GCG) in 5'AZA CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (ALB, AFP, HNF4A) in 5'AZA CRM treated BJ foreskin fibroblast. E) qRT-PCR analysis demonstrated no differences in induction of mesoderm lineage genes (MYOD1, TIE2, FLK1, and VE-CADHERIN) in 5'AZA CRM treated BJ foreskin fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (OCT4, SOX2, NANOG) expression in 5'AZA CRM treated BJ foreskin fibroblast. Black bar – CRM treated BJ foreskin fibroblast cells; Dark grey bar-3µM 5AZA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene GAPDH. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between SAZA-CRM treated BJ foreskin fibroblast versus untreated BJ foreskin fibroblasts in CRM by unpaired 2-tailed Student’s t-test. NS- not significant.
5.5 Discussion

We demonstrated that treatment of two different sources of human fibroblasts with TSA in CRM induced expression of endoderm lineage markers and pancreatic endoderm markers known to be important for β-cell differentiation, albeit at modest levels. By contrast, 5AZA did not induce expression of endoderm and pancreatic endoderm markers though morphological changes occurred. However, the induction resulting from treatment with TSA, or a combination of 5AZA followed by TSA was reversible and disappeared upon removal of the epigenetic modifiers and the chromatin remodeling medium. Interestingly, already in 1977, Taylor and Johns demonstrated that 5AZA can support transdifferentiation of mouse fibroblasts into skeletal myoblasts by the master regulator MyoD1 [29, 30] but did not recognize the involvement of epigenetics.

In line with our studies, Pennarossa et al., [19] described that culture of human fibroblasts with 5AZA resulted in minimal induction of endodermal and pancreatic marker genes. However, they demonstrated that such pretreatment allows cells subsequently to differentiate into β-cells when cultured in the presence of cocktails of growth factors to induce pancreatic endoderm and subsequently mature endocrine pancreatic cells. In a second study by the same group [20], similar results were reported for pig fibroblasts, where again they described that treatment with 5AZA alone does not cause robust changes in endoderm and pancreatic gene expression patterns, but may change the epigenetic state of fibroblasts such that they subsequently become susceptible to growth factors that support pancreatic endocrine and β-cell differentiation.

Another approach, described by Katz et al., combined the use of TF overexpression followed by treatment of the transduced cells on day 3 with a HDACi (Romidepsin) and 5AZA to transdifferentiate human dermal fibroblasts into β-cells [21]. They demonstrated that Romidepsin could activate some endocrine pancreas genes, whereas 5AZA alone did not. This corroborates our observations that the HDAC inhibitor TSA can induce endoderm and pancreatic marker genes. They further found
that PDX1 was only minimally induced despite the fact that the combination of Romidepsin and 5AZA could induce a number of markers of pancreatic endoderm and more mature pancreatic cells. Only when overexpression of PDX1 was combined with the epigenetic modifiers human fibroblasts could be reprogrammed to β-cells.

We speculate that TSA combined with CRM may induce a “versatile” epigenome surrounding endodermal and pancreatic genes, but cannot induce full reprogramming. Full reprogramming may require that we combine this approach with transduction of a limited number of key pancreatic TFs (like PDX1) or culture in growth factor cocktails known to allow differentiation of PSC to β-cells. Aside from the HDACi, TSA, CRM was also needed to induce the endodermal and pancreatic genes. Which factor from CRM has this added epigenetic effect is currently still unclear, but it might be L-Ascorbic acid, as this compound has been shown to enhance reprogramming via the induction of Ten-eleven translocation (TET) hydroxylase dependent DNA demethylation [31-33].

5.6 Conclusion

In conclusion, we described an optimized schedule for treatment of human fibroblasts with epigenetic modifiers to activate endocrine pancreatic marker genes. We demonstrated that culture of adult human fibroblasts with and TSA combined with CR medium, induces expression of endoderm and pancreatic endoderm genes, but that this is a reversible phenomenon. Treatment with TSA/CRM also induced some mesoderm lineage markers MYOD1 and FLK1 in both fibroblast cell lines. When treatment with 5AZA-CRM was added before TSA-CRM treatment, an increase in mesoderm lineage markers MYOD1 was seen beyond levels induced by TSA-CRM, but 5AZA/CRM itself did not induce any lineage marker expression. Hence, further maintenance in β-cell-specifying conditions with or without forced expression of exogenous TFs will be needed to eventually convert fibroblasts to β-cells.
References


### Supplementary table 1

Primer sets used for gene expression analysis by qRT-PCR

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