Epigenetic memory in human induced pluripotent stem cells
Abstract

Human embryonic stem cells (HESCs), derived from the inner cell mass of blastocyst stage embryos, are capable of indefinite in-vitro culture while maintaining their potential to differentiate towards the three embryonic germ layers. These unique pluripotent stem cells hold a great promise in various research fields such as cell replacement therapy and disease modeling. Human induced pluripotent stem cells (hiPSCs) were recently generated from human somatic fibroblasts by introduction of defined factors. These pluripotent stem cells, deemed indistinguishable from human embryonic stem cells, may offer a possible solution to the problem of immune rejection in cell replacement therapy, and broaden the spectrum of genetic diseases that can be modeled in-vitro. Although shown to be very similar in nature, whether any differences do exist between hiPSCs and HESCs has been poorly addressed so far (i.e. epigenetic memory of tissue specific genes and in models of human diseases). In my work, I aim to generate and compare hiPSCs from fragile-X patients to HESCs that carry the same mutation. I would like to observe if hiPSCs faithfully recapitulate the developmental phenotype that was already observed in hESCs, or that an epigenetic memory prevents true modeling of this disease by iPSCs. In addition, I aim to generate iPSC cells from human pancreatic beta cells and observe if an epigenetic memory of the parental beta cell exists in the iPSCs and examine their potential to differentiate back to pancreatic progenitors. Epigenetic memory and skewed differentiation of beta-iPSCs may provide us with means to simplify the ways to generate beta cells, which are currently missing. Whether iPSCs faithfully recapitulate HESCs have broad implications to their ability to model genetic diseases and their use as an autologous source of cells in the regenerative medicine field.
**Scientific background:**

In 1957, Gurdon has shown in a milestone experiment that by enucleating an oocyte and transplanting a donor somatic nucleus instead, a clone of the donor can be generated (1). In this process, termed somatic cell nuclear transfer (SCNT), various factors in the oocyte’s cytoplasm reprogram the nucleus from a somatic identity to a pluripotent identity that can generate in principal the organism anew. In 1998, Wilmut was the first to repeat this feat in mammals (2). SCNT may provide a solution to the problem of immune rejection when using embryonic stem cells in cell replacement therapy, however generating human cloned embryonic stem cells has not been achievable so far. A different methodology to reprogram somatic cells to pluripotency is by fusing them with embryonic stem cells (3). However, this fusion creates tetraploid cells that cannot be used in therapy. Recently, Yamanaka has shown, first in mouse somatic cells (4) and then with human somatic cells (5-6) that by ectopically expressing defined transcription factors, these cells acquire a pluripotent identity that deems them indistinguishable from embryonic stem cells.

However, the question of differences between hiPSCs and HESCs has only been partially addressed so far (7-8). In the SCNT methodology, the reprogramming factors of the oocyte do not often completely erase the somatic cell identity, leaving memory of gene expression of the parental cell line or of an epigenetic memory in the level of chromatin on various loci (9-10). We thus sought to look at an epigenetic memory that may prevail in iPSCs because of incomplete erasure of the somatic cell identity by the reprogramming factors. It is common for cells to behave differently to the reprogramming factors, sometimes generating partial iPSCs that do not truly mimic ESCs. Because I aim to look at the differences between ESC and iPSCs, it is imperative to apply the most stringent criteria of characterization to the iPSCs I will generate to show that they are truly iPSCs and not partial iPSCs. These examinations include teratoma formation and embryoid bodies (EBs) generation, silencing of the retroviral transgenes and reactivation of many pluripotent-related genes by microarray (11-12). Only after a complete and thorough characterization, I will be able to faithfully compare the two types of pluripotent stem cells.
Fragile-X syndrome, the most common form of mental impairment in males is caused by expansion of CGG repeats in the 5 UTR of the FMR1 gene, leading to hetrochromatinization of the locus and its methylation (13). By using HESCs derived from PGD embryos that were shown to carry the fragile-X mutation, it was shown that in the pluripotent stage FMR1 is expressed, unmethylated and euchromatinized, however upon differentiation it is silenced, hetrochromatinized and methylated (14). Whether iPS cells from Fragile-X patients will reprogram the FMR1 locus and recapitulate the FMR1 phenotype observed in the hESCs model, or whether an epigenetic memory will persist in the iPSCs is the first goal I have addressed.

Human beta cells are insulin producing cells that are damaged in type 1 Diabetes. HESCs may provide a possible source of beta cells for the treatment of type 1 Diabetes; however current protocols towards efficient beta cell generation are not very efficient (18). Human beta-cells undergo spontaneous de-differentiation in-vitro, losing the ability to express insulin; however they maintain an epigenetic memory on the insulin locus and retain the ability to re-differentiate towards insulin producing cells (15). Previously, iPS cells were generated from mouse beta cells using a lineage tracing systems (16). In human cells, reprogramming using a lineage tracing system was never reported, nor was it reported that cells from the endodermal lineage are capable of reprogramming. We used a lineage tracing system (17) to trace human beta cells and reprogrammed their de-differentiated progeny. We aim to examine epigenetic memory in the promoters of beta-cell related genes and see if we can harness this putative memory for a better and more efficient differentiation towards the generation of beta-cells in comparison to the limited ability of HESCs or fibroblast derived hiPSCs.
Research aims:

I. Generation of mouse and human induced pluripotent stem cells:

1) Generation of mouse and human iPSCs by the introduction of 4 transcription factors – OCT4, SOX2, KLF4 and cMYC.

2) Characterization of pluripotent markers in the iPSCs.

3) Characterization of in-vivo and in-vitro differentiation of the iPSCs.

II. Epigenetic memory in iPSCs that carry the Fragile-X mutation:

1) Generation of iPSCs from Fragile-X patients' fibroblasts and a teratoma cell line (Ter) derived from HESCs that carry the Fragile-X mutation (FX-ES).

2) Characterization of the Fragile-X (FX) iPSCs for pluripotent markers and in-vivo and in-vitro differentiation potential.

3) Molecular analysis of FX-ES, FX-iPSCs and FX-Ter-iPSCs for FMR1 gene expression, methylation promoter status and chromatin modifications in the FMR1 locus.

III. Epigenetic memory and skewed differentiation in human beta-cell derived iPSCs:

1) Generation of iPSCs from lineage-traced de-differentiated human beta cells (beta-iPSCs).

2) Characterization of the beta-iPSCs for pluripotent markers and in-vivo and in-vitro differentiation potential.

3) Analysis of the differentiation potential of beta-iPSCs, fibroblast-iPSCs and HESCs cells in-vitro and in-vivo towards insulin producing cells, by spontaneous differentiation and by defined factors differentiation protocols.

4) Analysis of the methylation profile and chromatin modifications occupancy in the promoters of beta cell related genes in beta-iPSCs vs. fibroblast derived iPSCs and HESCs.
IV. Global analysis of methylation profile and chromatin modifications in beta-iPSCs, fibroblast-iPSCs, keratinocyte-iPSCs and ES cells:

1. Analysis of global gene expression of beta-iPSCs, fibroblasts-iPSCs, keratinocyte-iPSCs and hESCs.

2. Analysis of global methylation of CpG sites in tissue specific promoters in beta-iPSCs, fibroblasts-iPSCs, keratinocyte-iPSCs and ES cells.

3. Analysis of global histone modifications occupancy in tissue specific promoters in beta-iPSCs, fibroblasts-iPSCs, keratinocyte-iPSCs and ES cells.

**Preliminary results:**

A. Generation and characterization of mouse and human iPSCs from fibroblasts:

1) Reprogramming of mouse and human fibroblasts to pluripotency and isolation of iPSC cell clones: Mouse embryonic fibroblasts (MEFs) or human foreskin fibroblasts (BJ) were infected with retroviruses carrying OCT4, SOX2, KLF4 and cMYC according to previous published protocols (4-5). Between three to four weeks after the infection, mES-like-colonies and human ES-like colonies appeared in the dishes. Five mouse iPSCs (miPSCs) and five human IPS cell lines (hiPSCs) were picked, propagated and characterized (See figure 1A and 2A-1).

2) Characterization of pluripotency markers in iPSCs: hiPSCs were positively stained for the pluripotent markers alkaline phosphatase (AP), OCT4, SOX2, NANOG and Tra-1-60 RT-PCR confirmed that while the parental fibroblast cell line did not express the endogenous OCT4 and SCX2, hiPSCs derived clones did express them, as well as NANOG and REX1. RT-PCR for the expression of the retroviral transgenes showed complete silencing of the viral promoter in all iPSCs, as expected in faithfully reprogrammed clones. Finally, iPSCs and their parental fibroblasts showed a normal 46XY karyotype. Gene expression arrays showed reprogramming and re-activation
of many pluripotent genes, similar to the gene expression levels of hESCs (See figure 1 and figure 2 and data not shown).

3) Characterization of in-vivo and in-vitro differentiation of iPSCs: Differentiation potential of miPSCs and hiPSCs was assessed in vitro by generating embryoid bodies (EBs) and in vivo by generating teratomas. For EBs formation, a 10 cm dish was trypsinized and resuspended in EBs media. To verify differentiation to the three germ layers, cell clumps were seeded after 10 days as floating EBs onto adherent culture dishes, allowed to propagate for additional 10 days and stained with antibodies directed against various markers of the three embryonic germ layers: FOXA2 and AFP (endoderm), Desmin and cardiac fetal actin (mesoderm) and NCAM1 (ectoderm). To verify differentiation to the three germ layers in teratomas, iPSCs were injected under the kidney capsule of SCID-beige mice. A month later, tumors were cryo-preserved, sectioned and stained with Hematoxylin and Eosin (H&E) and examined for various structures representing the three embryonic germ layers (See figure 1 and 2 and data not shown).

B. Epigenetic memory in iPSCs that carry the Fragile-X mutation:

1. Fibroblasts from three Fragile-X patients (GM05848, GM07072 and GM09497) and a teratoma cell line derived from human embryonic stem cells carrying the fragile-X mutation were infected with retroviruses encoding for the 4 factors. Approximately 3 weeks after the infection ES-like colonies emerged (termed FX-iPS cells). Seven colonies from patient GM05848, two from patient GM07072 and two from patient GM09497 were established. From the teratoma cell line, 6 cell lines were established (termed FX-Ter-iPS) (See figure 3).

2. Characterization of FX-iPSCs:

FX-iPSCs cells were positively stained for pluripotent markers such as alkaline phophatase, OCT4, SOX2, NANOG, Tra-1-60 and SSEA3. RT-PCR for pluripotent markers confirmed that while the parental fibroblasts cells did not express OCT4, SOX2, NANOG and REX1 transcripts, the FX-iPS did express them. FX-iPS cell clones silenced the retroviral transgenes as was tested by RT-PCR, showing faithful
reprogramming. In addition, Bisulfite sequencing of the NANOG and OCT4 promoters showed complete demethylation in the iPS cells lines in contrast to extensive methylation in the parental FX-fibroblasts. FX-iPS cell lines maintained a normal karyotype at passage 10. Gene expression arrays of FX-iPSCs showed reprogramming and re-activation of many pluripotent genes with level similar to ES and WT-iPS cells. To examine the differentiation potential of FX-iPSCs in-vitro, FX-iPSCs were trypsinized and allowed to aggregate as cell clumps (EBs) for 10 days, after which they were seeded onto adherent culture dishes for additional 10 days and stained for markers of the three embryonic germ layers: FOXA2 and AFP (endoderm), Desmin and cardiac fetal actin (mesoderm) and NCAM1 (ectoderm). For in-vivo differentiation, FX-iPSCs were injected under the kidney-capsule of SCID-Beige mice. After 4 weeks tumors were dissected and stained with H&E and various differentiated structures from the three embryonic germ layers were observed (See Figure 4).

3. Molecular analysis of the FMR1 gene and locus:

Real-time PCR for FMR1 transcript level was performed on FX- fibroblasts, FX-iPSCs, FX-ES cells, WT-ES cells, WT-fibroblasts and WT-iPSCs. While FX-ES cells, WT-ES cells, WT-fibroblasts and WT-iPSCs expressed the FMR1 gene, FX-fibroblasts and FX-iPSCs did not, conferring that the FMR1 locus was refractory to reprogramming by defined factors and that FX-iPSCs do not recapitulate the phenotype observed in FX-ES cells. FX-Ter-iPSCs also failed to reactivate FMR1 expression to the FX-ES level following reprogramming. Histone modifications at the FMR1 locus were assessed using chromatin Immunopercipitation assays. In contrast to FX-ES cells, WT-ES cells, WT-fibroblasts and WT-iPSCs that showed high levels of H3 acetylation, H3K4 methylation and low level of H3K9 methylation, FX-Fibroblasts and FX-iPSCs showed heterochromatinization of the FMR1 locus as can be determined by low levels of H3 acetylation, H3K4 methylation and high levels of H3K9 methylation. These results show epigenetic memory in the chromatin level in the FMR1 locus. Finally, while WT fibroblasts and WT-iPSCs did not show any methylation on the FMR1 promoter as was detected by bisulfite sequencing assay, the FX fibroblasts showed extensive
methylation that was maintained in the FX-iPS cell lines, showing an epigenetic imprint on the DNA level as well (See figures 5, 6).

C. Epigenetic memory and skewed differentiation in beta-cell derived iPSCs:

1) Lineage tracing of human beta-cells and their reprogramming to pluripotency:

Human pancreases were dissociated and infected with two lenti viruses. The first encodes for Cre under insulin promoter and the second encodes for RFP-GFP under CMV promoter. The RFP gene is flanked by two loxP sites, so that upon Cre activity it is excised, bringing the downstream GFP gene under the CMV promoter. Only the insulin expressing cells in the pancreas are labeled with GFP. These lineage traced beta cells undergo spontaneous de-differentiation in-vitro, while maintaining the ability to re-differentiate towards insulin producing cells and maintaining an open chromatin histone marks on the insulin locus (15). Lineage-traced cells from two different lineage-tracing systems and from three different donors were infected with retroviruses carrying the 4 factors. Five weeks after infection numerous colonies had emerged, however with a marked lower efficiency than fibroblasts. Several colonies were picked and propagated for further characterization, however only three were shown to be indeed beta-cell derived by PCR for the Cre-mediated recombination (Figure 7 and 8).

2) Characterization of the beta-iPSCs for pluripotent markers and in-vivo and in-vitro differentiation potential:

Beta-iPSCs were positively stained for pluripotent markers such as alkaline phosphatase, OCT4, SOX2, NANOG and Tra-1-60. RT-PCR for pluripotent markers confirmed that while the parental cells did not express OCT4, SOX2, NANOG and REX1 transcripts, the beta-iPSCs did express them. Beta-iPSCs clones silenced the retroviral transgenes as was tested by RT-PCR, showing faithful reprogramming. Beta-iPSCs maintained normal karyotype at passage 10. Gene expression arrays of beta-iPSCs showed reprogramming and re-activation of many pluripotent genes with level similar to ES and WT-iPS cells. To examine the differentiation potential of beta-iPSCs in-vitro, beta-iPSCs were trypsinized and allowed to aggregate as cell clumps
(EBs) for 10 days after which they were seeded onto adherent culture dishes for additional 10 days and stained for markers of the three embryonic germ layers- FOXA2 and AFP (endoderm), Desmin and cardiac fetal actin (mesoderm) and NCAM1 (ectoderm). For in-vivo differentiation, beta-iPSCs were injected under the kidney-capsule of SCID-Beige mice. After 4 weeks tumors were dissected and stained with H&E and various differentiated structures from the three embryonic germ layers were observed (See figures 9 and 10).

3) Analysis of the differentiation potential of beta-iPSCs, fibroblast-iPSCs and ES cells in-vitro and in-vivo towards insulin producing cells:

Three beta-cell derived iPSCs, 5 fibroblasts derived iPSCs and 3 human ES cells were trypsinized and allowed to aggregate as floating EBs for 20 days after which RNA was isolated. Real-time PCR for beta-cell markers was performed as well as for embryonic mesoderm and ectoderm. While the differentiation potential for mesoderm and ectoderm was overall the same, the three iPSCs showed a marked skewed differentiation toward Insulin producing cells as was determined by Real-time PCR showing high level of insulin and PDX1 mRNA expression. Moreover, C-Peptide staining could have been detected only in EBs sections from beta-Cell derived iPSCs. The differentiation potential was observed also in beta-iPSCs derived teratomas, showing the highest level of insulin mRNA expression and PDX1 mRNA expression (See figure 11).
Research plan:

a. Generation of mouse and human iPSCs:

1) Reprogramming of mouse and human fibroblasts to pluripotency and establishment of pluripotent cell lines. This part was completed.

2) Complete molecular characterization of the iPSCs and their potential to differentiate in-vitro and in-vivo. This part was completed.

b. Epigenetic memory in a disease model:

1) Reprogramming of 3 fragile-X patients derived fibroblasts and a teratoma cell line and complete characterization of cell lines. This part was completed.

2) Real-time PCR for FMR1 expression, chromatin immunoprecipitation for H3 acetylation, H3K4 and H3K9 methylation, bisulfite sequencing of the FMR1 promoter. This part was completed.

c. Epigenetic memory and skewed differentiation in beta-cell derived iPSCs:

1) Lineage tracing and reprogramming of beta cells to pluripotency and beta-iPSCs characterization. This part was completed.

2) Assessment of the differentiation potential of fibroblast-iPSCs, ES cells and beta-iPSCs to differentiate towards beta-like cells in-vitro and in-vivo. This part was completed.

3) Chromatin Immunoprecipitation for H3 acetylation and analysis of its enrichment in PDX1 and Insulin promoters. Chromatin Immunoprecipitation will be performed on de-differentiated beta cells, beta-iPSCs, fibroblast-iPSCs and hESCs cells using an anti-H3 acetylation antibody. By Real-time PCR I intend to look at the enrichment of this modification, associated with open chromatin, at the promoters of tissue specific genes like PDX1, Insulin and MAFA1 in all cell types.

4) Bisulfite sequencing of PDX1 and Insulin promoters. Here I intend to perform bisulfite sequencing for PDX1 and Insulin promoters. HEla cells and fibroblast-iPSCs
will be used as a negative control for the level of methylation in beta cells, de-differentiated beta cells and beta-iPSCs.

5) Assessment of the differentiation potential of fibroblast-iPSCs, hESCs and beta-iPSCs to differentiate towards beta-like cells using directed differentiation protocols (18). Using defined factors that mimic the normal pancreatic development I intend to examine the differentiation potential of beta-iPSCs to differentiate towards beta-like cells in comparison to fibroblast-iPSCs and hESCs. After 14 days of the differentiation protocol of monolayer cultures, I will isolate RNA and perform Real-time PCR for PDX1 expression levels. Should I see again skewed differentiation towards PDX1 expression in beta-iPSCs, I will perform the same protocol over a time period of 20 days and examine Insulin and c-Peptide mRNA expression.

d. Global analysis of methylation profile and chromatin modifications occupancy in beta-iPSCs, fibroblasts-iPSCs and ES cells using various global arrays:

In this final part of my work I intend to look globally at the differences between iPSCs and ES cells and between iPSCs generated from different parental cell lines (i.e. fibroblasts vs. beta cells). My goal is to search for epigenetic memory in iPSCs globally.

1. Analysis of global gene expression of beta-iPSCs, fibroblast-iPSCs and ES cells: In this part of my work, I intend to look at the gene expression profiles of ES cells and iPSCs that were generated from beta cells or fibroblasts. I will also take advantage of expression arrays of iPSCs that were generated from human blood, neurons and keratinocytes that are available at the GEO site. I would like to see how they will cluster together and whether reminiscent gene expression of tissue specific genes persists in the iPSCs from the original cell line. More specifically I intend to look at the decrease in somatic cell markers and tissue specific cell markers and see if somatic cell markers are down regulated in iPSCs from different cell types while the down regulation of tissue specific genes is different. That an epigenetic memory exists in iPSCs I am beginning to see; however gene expression memory as a result of incomplete reprogramming may also prevail in iPSCs from the original cell line, as was shown before in SCNT. Previous work has shown different gene expression
between hESCs and hiPSCs (7). In this work, it was suggested that reminiscent gene expression of fibroblast related gene persist in the hiPSCs that is not found in hESCs. I intend to look at similar patterns in other cell types and observe if this phenomenon is wide and global.

2. Analysis of global methylation of CpG promoter sites of beta-iPSCs, fibroblast-iPSCs and HESCs: Here I intend to perform a global analysis of CpG sites in the promoters of tissue specific genes. I will look after epigenetic memory in other loci and specifically CpG sites of tissue specific genes. The CpG array recognizes ~28,000 CpG of about ~14,000 genes. Previous work (19) looked at CpG patterns in hiPSCs and hESCs, seeing both similarities and differences between the two, however all iPSCs were originated from fibroblasts. In my work I intend to widen the scope and look at the CpG pattern in iPSCs derived from different cell types and compare their pattern.

3. Analysis of global histone modifications at the promoters of beta-iPSCs, fibroblast-iPSCs and HESCs: Here I would like to perform ChiP on ChiP assays for H3K4 and H3K27 histone modifications. These two modifications are associated with bivalency—a unique property of ES cells. I would like to see whether beta cells are poorly enriched for H3K27 and highly enriched for H3K4—which may persist in beta-iPSCs. This may shed light on the skewed differentiation that I observe—this "skewed bivalency" will exist to a lesser degree in ES cells and fibroblast-iPSCs. In fibroblasts I will look for H3K4 enrichment and low levels of H3K27 in fibroblasts transcription factors, having again ES cells as my reference and control. Previous work (20) have generated chromatin maps that examined changes of chromatin modifications following reprogramming, highlighting mainly similarities between hESCs and hiPSCs. I will search for those genes whose chromatin modification are not the same, and see whether they are related to tissue specific genes and whether these changes are associated with the cell type that has been reprogrammed.
References:


Figure 1: Induced pluripotent stem cells from BJ fibroblasts

A) Bright field image of a typical iPS culture (bar = 500 μm), B) Alkaline phosphatase (bar = 500 μm), C) Tra-1-60 (bar = 100 μm), D) OCT3/4 (bar = 100 μm), E-F) Hematoxylin and eosin staining of slides showing various differentiate structures, E) (bar = 25 μm), F) bar = 50 μm and G) bar = 25 μm, H) RT-PCR showing silencing of transgenes in the three lines, +Tg = Samples day 7 post infection still expressing transgenes, iPS = induced pluripotent stem cells.
Figure 2: Generation and characterization of miPSCs

(A) Morphology of miPS. (B) miPS are positive for alkaline phosphatase staining. (C-D): Immunocytochemical staining shows that miPS express pluripotency-related markers such as Oct4 (C), Sox2 (D). Nuclei were stained with Hoechst 33258 (blue). (E) miPS differentiate towards the three germ layers in-vivo and in-vitro showing representative of all three germ layres, miPS maintain a normal (40 XY) karyotype.
Figure 3: Characterization of the undifferentiated FX-IPS cells

A: Fragile-X (FX) iPS cells have a similar morphology to human ES cells and express markers of undifferentiated cells. I: FX-iPS cell line, II: FX-iPS cells are positive for alkaline phosphatase staining, III-VI: Immunocytochemical staining shows that FX-iPS cells express pluripotency-related markers such as Tra-1-60 (III), Oct4 (IV), Sox2 (V) and Nanog (VI). Nuclei were stained with Hoechst 33258 (blue).

B: Expression of endogenous pluripotent and exogenous genes. I: RT-PCR for markers of pluripotency in FX (Fragile-X) fibroblasts compared to FX derived iPS cell clones and human ES cell (HES13) (Cowan et al., 2004). Primers recognizing the endogenous Oct4 and Sox2 transcripts were used. GAPDH was used as a loading control for each lane. II: PCR analysis of expression of retroviral transgenes. FX-iPS cell clones and fibroblasts 7 days after transduction with the 4 retroviruses were analyzed for expression of the PMXs retroviral transgenes.

C: Reprogramming of expression of pluripotent markers. Gene expression of multiple markers of pluripotent cells analyzed by DNA microarray is shown for FX-fibroblasts (FX-Fib), FX-iPS cells (FX-ips), FX-ES cells (FX-ES) and normal ES cells (WT-ES). D: Karyotype analysis of the iPS cells. Shown is a karyotype of the FX fibroblasts and the iPS cells that were derived from them. Both cell types have normal diploid 46XY chromosomes.
Figure 4: Characterization of the pluripotency of FX-iPS cells
A: *in-vitro* differentiation of FX-iPS cells. I: Cystic embryoid bodies (EBs) at day 7. I-VI: Images of differentiated FX-iPS cells from 8 days old EBs plated onto adherent culture tissue dishes, and stained at day 16 for markers of the three embryonic germ layers. II: FoxA2 (endoderm), III: alpha-fetoprotein (endoderm), IV: Cardiac-fetal actin (mesoderm), V: Desmin (mesoderm), VI: NCAM1 (ectoderm). Nuclei were stained with Hoechst-33342 (blue).
B: *in-vivo* differentiation of FX-iPS cells. Hematoxylin and eosin staining of iPS cell derived teratoma sections, showing I: neural rosette and pigmented retinal epithelium (ectoderm), II: adipose tissue (mesoderm), III respiratory epithelium (endoderm), IV: neuronal tissue (ectoderm), V: muscle and cartilage (mesoderm), and VI: gut-like epithelium (endoderm).
Figure 5: FMR1 expression in FX-IPS cells
A: FMR1 transcript expression as detected by real-time PCR in FX-fibroblasts, 11 FX-IPS cell clones, normal fibroblasts, two normal iPS cell clones, FX-ES (human embryonic stem cell line established from fragile-X affected embryo) and normal ES cells. B: Comparison of FMR1 expression levels in low passage (p5) and high passage (p17 and p18) FX-IPS cells. C: Immunostaining for FMRP in ES, iPS, FX-ES and FX-IPS cells using goat anti human FMRP antibody and Hoechst 33258 for nuclear staining. D: FMR1 and NANOG expression levels in FX-ES cells, differentiated FX-ES cells, and reprogrammed differentiated FX-ES cells.
Figure 6: Epigenetic memory in FX-iPS cells
Bisulphite sequencing analysis of the FMR1 promoter in: A. Normal fibroblast and two iPS cell lines derived from these fibroblasts (IPS #28, #94); B. FX-fibroblasts and seven iPS cell lines derived from these fibroblasts (FX-iPS A-12, A-17, A-47, A-50, A-52, A-55, A-89). C. Bisulphite sequencing analysis of the OCT4 promoter in FX-fibroblasts and FX-iPS cell clones. Open circles represent unmethylated CpGs; Black circles represent methylated CpGs. D. Histone modifications at the FMR1 locus in FX-iPS cells: ChIP analysis of histone H3-tail acetylation and H3K4 and H3K9 methylation in FX-iPS cells. Real-time PCR was performed on bound and input sonicated DNA fragments using primers for the FMR1 promoter. Adenine phosphoribosyl transferase (APRT) and Crystalline (CRYST) served as either positive or negative controls. Values were normalized relative to the appropriate positive control.
Figure 7: Lineage tracing of human beta cells

(A) Shown are lineage traced human beta cells at passage 3 following their spontaneous de differentiation. In red: none traced cells, green: traced human beta cells. (B) Two lineage tracing systems were used to trace human beta cells using lentivirus. From both tracing systems, beta-iPSCs were generated. In both systems, the human insulin promoter was followed by CRE that excises loxP sites in beta cells only, tracing them with GFP.
Figure 8: Reprogramming of de-differentiated human Beta cells to pluripotency:

(A) Morphology changes in lineage-traced de-differentiated human beta cells, acquiring morphology of hESCs in day 21. (B) PCR for the excised (recombined) transgene. (4, 8, 9) show a recombined band in 3 beta-derived iPSCs clones. 1: Sorted Beta Cell Derived DsRED+ GFP+, 2: Sorted eGFP+, 3: Lineage traced cells (system 1), 4: Beta-iPS #1, 5: neg control, 6+12: Marker, 7: Plasmid, 8: S26#2 Beta-iPS #2, 9: F15#3 Beta-iPS #3, 10: Lineage traced cells (system 2), 11: neg control, 13: F15#4 islet cell derived iPS (none beta cell derived).
Figure 9: Characterization of the undifferentiated IPS cells:

I: Markers of undifferentiated cells in beta-iPSCs. A-E: Immunocytochemical staining shows that Beta-iPS cells express pluripotency-related markers such as Oct4 (A), Sox2 (B) NANOG (C), Tra-1-60 (D). E: Beta-iPS cells are positive for alkaline phosphatase staining. Nuclei were stained with Hoechst 33258 (blue). II: PCR analysis of expression of retroviral transgenes. Beta-iPS cell clones and fibroblasts 7 days after transduction with the 4 retroviruses were analyzed for expression of the PMXs retroviral transgenes. III: Reprogramming of expression of pluripotent markers. Gene expression of multiple markers of pluripotent cells analyzed by DNA microarray is shown for De-differentiated beta cells and Beta-iPS cells. IV: Karyotype analysis of the beta-iPS cells, showing normal diploid 46XY chromosomes.
Figure 10: Characterization of the pluripotency of the beta-iPS cells:

Figure 11: Skewed differentiation in EBs towards insulin producing cells in beta-cell derived iPSCs:

Three beta-cell derived iPSCs, 5 fibroblasts derived iPSCs and 3 human hESCs were trypsinized and allowed to aggregate as floating EBs for 20 days after which RNA was isolated. Real-time PCR for beta-cell markers was performed as well as for embryonic mesoderm and ectoderm. While the differentiation potential for mesoderm and ectoderm was overall the same (data not shown), the three beta-iPSCs showed a marked skewed differentiation toward Insulin producing cells as was determined by Real-time PCR showing high level of insulin and PDX1 mRNA expression. The graph shows 8 biological reproductions of 3 diff beta-iPSCs averaged, 5 diff WT-iPSCs averaged and 3 differentiated hESCs averaged + Standard Error (two tailed students t-test). Moreover, C-Peptide staining could only be detected in EBs from beta cell derived iPSCs. The skewed differentiation was observed also in beta-iPSCs derived teratomas, showing the highest level of insulin mRNA expression and PDX1 mRNA expression (data not shown).
Materials and methods:

Cell culture

ES and iPS cells were cultured in standard ES cell culture media containing KnockOut DMEM (Gibco-Invitrogen) supplemented with 15% Knockout serum replacement (Gibco-Invitrogen), 2mM L-glutamine (Sigma-Aldrich), 1:100 dilution of non-essential amino acids (Gibco-Invitrogen), 1:100 dilution of ITS (insulin, transferrin and selenium) (Gibco-Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 8 ng/ml basic fibroblast growth factor (bFGF) (PeproTech), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco-Invitrogen).

GM05848 fibroblasts from a 4 year old fragile-X patient (FX-fibroblast A) and GM07072 fibroblasts from a 22 week old fetus with a fragile X mutation (FX-fibroblast B) were purchased from Coriell Institute for Medical Research and were grown in Eagle's Minimum Essential Medium (Sigma) supplemented with 15% fetal calf serum (Biological Industries), 2mM L-glutamine (Sigma-Aldrich), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco-Invitrogen). Differentiated teratoma derived FX-ES cells (3) were cultured on gelatin-coated dishes in DMEM (Gibco-Invitrogen) supplemented with 10% FCS (Biological Industries), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco-Invitrogen).

Retroviral production and iPS cell generation

For iPS cell generation, approximately 2x10^6 293T cells were transfected in the presence of Eugene 6 (Roche) with 4.5 µg of pMXs retroviral vectors containing either human Oct4, Sox2, KLF4 or cMyc (obtained from Addgene), and 4.5 µg of PCL-Ampho plasmid. Twenty-four hours after transfection the culture was replaced with fresh medium, and forty-eight hours after transfection the supernatant was collected, filtered through a 0.45 uM cellulose acetate filter (Whatman) and supplemented with 4µg/ml of Polybrene (Sigma-Aldrich). Virus containing medium was then transferred to a 10 cm dish containing 2x10^5 FX-fibroblast A, FX-fibroblast B or differentiated FX-ES cells at passage number 8, 5 and 5, respectively. Seventy two hours post transfection a second round of infection was performed. Four days post
infection, ES cell medium was added to the infected fibroblasts and mitomycin treated mouse embryonic fibroblasts (MEFs) were seeded onto the infected fibroblasts dish. Between 21 to 30 days post infection, ES like colonies were picked and allowed to propagate in a 12 wells plate. At this stage the culture was defined as passage 0 and passaged mechanically until passage 5, thereafter they were passaged using 0.25% Trypsin (Biological Industries).

Alkaline phosphatase staining and Immunocytochemistry

Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich) according to the manufacturer's instructions. For immunocytochemistry, cells were washed twice with PBS, crosslinked with 10% formalin solution for 10 min, washed twice with PBS and blocked for one hour at room temperature with PBS containing 2% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Triton-X-100. Primary antibody staining was performed for 1 hour in room temperature with antibodies diluted in blocking solution. The antibodies used were mouse anti human OCT3/4 (IgG, 1:200, Santa Cruz Biotechnology), goat anti human SOX2 (IgG, 1:100 Santa Cruz Biotechnology), goat anti human NANOG (IgG, 1:100, R&D Systems), mouse anti human Tra-1-60 (IgM, 1:500 Santa Cruz Biotechnology), mouse anti human FMRP (IgG, 1:100, Chemicon International), rabbit anti human alpha-fetoprotein (IgG, 1:200, Dako), rabbit anti human FOXA2 (IgG, 1:1000, Abcam), mouse anti human Desmin (1:200, IgG, Dako), mouse anti human cardiac fetal actin (1:200, IgG, Maine Biotechnology) and goat anti human NCAM1 (1:150, IgG, R&D Systems).

DNA microarray analysis

Total RNA was extracted according to the manufacturer's protocol (Affymetrix, Santa Clara, CA) from populations of FX fibroblasts, FX-iPSCs and normal and FX ESCs. Hybridization to the GeneChip Human Gene 1.0 ST Arrays, washing, and scanning were performed according to the manufacturer's protocol (Affymetrix), and expression patterns were compared between samples.

DNA and RNA isolation and reverse transcription
Total genomic DNA was extracted using genomic DNA extraction kit (RBC) and total RNA (DNase treated) was extracted using RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed with random hexamer primers using ImProm-II reverse transcriptase (Promega).

**Real-time PCR and PCR**

Real-time PCR was carried out in triplicates using SYBR Green ROX mix (Applied Biosystems) and 7300 Real-time PCR System (Applied Biosystems). PCR was performed using ReadyMix PCR Reaction Mix (Sigma-Aldrich).

**In vitro differentiation**

For EB formation, a semi-confluent 10 cm plate of the cells was harvested using trypsin, and cell clumps were resuspended in ES cell media without bFGF, allowed to aggregate and transferred to one well of a non-adherent six well plate. After 8 days as floating EBs, cell clumps were collected and seeded onto a 12 well plate adherent culture dish. After an additional 8 days of growth, adherent cells were stained for the presence of markers from the three embryonic germ layers.

**In vivo differentiation**

For teratoma formation, a confluent 10 cm plate was harvested using trypsin. Cells were centrifuged and cell pellets were resuspended in 50μL of IPS cell medium. The IPS cells were injected either subcutaneously into the dorsal flank, or into the kidney capsule of 4 weeks old SCID-Beige male mice (The Jackson Laboratory). Tumors were dissected after 4-6 weeks and cryopreserved in O.C.T. (Sakura) Cryosections were stained with haematoxylin and eosin for histological analysis. All animal experiments were conducted under the supervision of the Hebrew University Faculty of Sciences and Animal Care and Use Committee.

**Bisulphite genomic sequencing**

Bisulphite treatment of genomic DNA was carried out using EZ DNA Methylation™ Kit (Zymo Research Corporation) according to the manufacturer’s protocol. Bisulfite treated DNA was amplified by Faststart high fidelity taq polymerase (Roche) using
the following primers: OCT4: 5’ primer TTAGGAAAAATGGGTAGTAGGGATTT; 3’ primer TACCCAAAACAAAATAATTTAAACCT FMR1: 5’ primer AGTGTATTGTTGAGAAAATGG; 3’ primer TCTCTCCTCAAATAACCTAAAAAC. PCR products were cloned into bacteria using TOPO TA Cloning Kit (Invitrogen). Single colonies were analyzed for CpG methylation at all potential sites by direct sequencing. The methylation status of each single CpG site was found by comparison of the sequence result to the genomic sequence using QUMA software (http://quma.cdb.riken.jp/).

Chromatin immunoprecipitation (ChIP)

Approximately 1.5x10⁶ cells were crosslinked with formaldehyde solution, lysed and the chromatin was sonicated to 200bp-1000bp DNA fragments. Chromatin was pre-cleared using 30μL of salmon sperm agarose beads (Upstate Biotechnology) for 1 hour at 4⁰C. Immunoprecipitation of chromatin was performed overnight using anti-acetylated histone H3 antibody (Upstate Biotechnology), anti-methylated histone H3K4 (Upstate Biotechnology), or anti-methylated histone H3K9 antibody (Upstate Biotechnology). After the overnight incubation, crosslinking was reversed and DNA recovered using a PCR clean-up kit (Qiagen). Eluted DNA fragments were used for quantitative PCR analysis using primers for the FMR1 promoter region as well as appropriate positive and negative controls (APRT and Crystalline).
Publications:


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