Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Effect of oocyte factors on epigenetic memory in induced pluripotent stem cells for reduction of cell of origin dependency.

B.1.2 Abstract

The derivation of induced pluripotent stem cells (iPSCs) has opened doors for numerous applications for which limited human cell sources were available. However, their use has limitations due to epigenetic memory; histone and DNA modifications that iPSCs retain from their cells of origin. This affects chromatin structure and therefore activation or inhibition of specific genes. Some inhibitors against these modifications have been assessed to enhance reprogramming efficiency, but little has been reported about their influence on differentiation potential. What has been discovered, however, is that somatic cell nuclear transfer (SCNT) creates stem cells with DNA methylation levels more similar to embryonic stem cells (ESCs) compared to iPSCs, likely due to involvement of oocyte factors. Therefore, the aim of this proposed research is to determine the effect of a selection of these factors on epigenetic memory and differentiation potential of iPSCs generated from human dermal fibroblasts and neutrophils. Expression of TH2A, TH2B, ASF1A, GDF9, GLIS1, H3.3B, Wave1 and Gata3 will be transiently induced during reprogramming, while BMP7 will be supplemented to the culture medium and DJ-1 will be knocked down. Additionally, they will be investigated in combination of aforementioned inhibitors. Consequently, changes in modifications and differentiation potential of resulting iPSCs will be analysed and compared. This will provide the possibility of utilizing non-invasively acquired cells for creating a neutral iPSC line without limitations in differential potential, allowing for non-confounding comparisons across studies and between *in vitro* and *in vivo* circumstances due to no dependence on cells of origin.

B.1.3 Layman's summary

In the fight against diseases, there is a need for a supply of human cells and tissues that is representative of the specific disease or patient. While skin or blood cells can be quite non-invasively obtained for research and medical intervention purposes, this is not the case for cells from most organs, meaning that the supply is often limited. One way to obtain adult cells is to create them with stem cells present in embryos or the adult

human body. That being said, due to ethical concerns and low availability, these stem cells do not provide a solution. That is why researchers developed a way to create stem cells without these restrictions by reverting cells, like easily obtainable skin or blood cells, back to a stem cell-like state. Adult cells that are difficult to obtain from the human body can then be created from these stem cell-like cells, called induced pluripotent stem cells (iPSCs). However, these stem cell-like cells are not completely the same as natural stem cells due to retainment of specific DNA modifications from before they were reverted to a stem cell-like state. These modifications, collectively referred to as a memory, influence the structure of the cell's DNA and therefore cause specific genes to be turned on or off that normally would not be on or off. Because of this, iPSCs have a tendency to be more easily turned into cells that are related to the type of cells that were used to create the iPSCs. To ensure that iPSCs created from easily obtainable human cells can be used to create any type of human cell and there are no unwanted differences in results in research or medical interventions based on this dependence on cell of origin, this research is proposed to identify factors that can decrease the dependence.

The factors that will be investigated are proteins present in embryos and associated with a different way of creating stem cells, called somatic cell nuclear transfer (SCNT). This method has been found to reduce the memory in animal cells more than the method of generating iPSCs. While SCNT is not available for human cells for ethical reasons, this knowledge about the difference in memory can potentially be used to improve the method of creating iPSCs. Therefore, in this proposed research, the proteins, GLIS1, H3.3B, TH2A, TH2B, ASF1A, GDF9, Wave1, Gata3, BMP7 and DJ-1, will be induced to be produced or blocked in human skin or blood cells during the process of turning them into iPSCs. Consequently, changes in the memory modifications in the resulting iPSCs and the ability to create specific cells from these iPSCs will be analysed. This way more insight can be gathered into methods and mechanisms needed for removing iPSC memory and dependence on their cell type of origin to expand their general usability in the search for population-wide or patient-specific treatments in numerous diseases.

B.1.4 Keywords
Induced pluripotent stem cells, epigenetic memory, methylation, acetylation, differentiation potential, oocytes.

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B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

Since their derivation by Takahashi and Yamanaka in 2006 (1), induced pluripotent stem cells (iPSCs) have been a very promising tool for regenerative medicine and various other biomedical fields. Their advantage over embryonic stem cells with no ethical concerns (1) and the ability to generate autologous pluripotent stem cells, and advantage over adult stem cells because of their pluripotency (2), make iPSCs valuable for tissue repair and engineering. With the transcription factors introduced by Takahashi and Yamanaka, Oct4, Sox2, Klf4, and c-Myc (OSKM), somatic cells can be reprogrammed to a pluripotent stem cell state (1,3). Due to their pluripotency, they can subsequently be differentiated into various different cell types and lineages (2). However, it has become clear that this ability is not completely independent from their somatic cell origin; correlations have been identified between somatic cell origin and tendencies of their respective iPSCs to differentiate into specific cell types (4–6). For instance, iPSCs derived from mouse fibroblasts were discovered to have a higher tendency to differentiate into osteoblasts than iPSCs derived from mouse bone marrow cells (5). Moreover, in human umbilical blood-derived iPSCs, a keratinocyte differentiation marker was shown to be expressed 9.4 times lower than in keratinocyte-derived iPSCs (7,8).

Underlying this phenomenon are specific epigenetic modifications in iPSCs that they retained from their cells of origin (4,5,9,10). In other words, these cell-specific modifications, which are not removed during reprogramming, cause iPSCs to retain an epigenetic memory from their cells of origin (4,10). Modifications include DNA and histone methylation, and histone acetylation which can either prevent reactivation of genes during reprogramming or prevent inactivation of genes (10). As a result, the iPSCs showcase expression patterns of genes that are not susceptible to reprogramming and therefore differ from embryonic stem cells (10,11).

Epigenetic memory modifications discovered to prevent gene reactivation by reprogramming factors due to enabling heterochromatin formation, are H2AK119ub (mono-ubiquitination of lysine 119 of histone H2A) (10,12), DNA methylation, H3K9me3 (lysine 9 trimethylation on histone H3) (10,13), and H3K27me3 (10). Reducing these modifications, results in increased gene expression in somatic cell nuclear transfer (SCNT) and should therefore enhance cell-fate reprogramming (10). For instance, H3K9me3 is present at higher levels in differentiated cells compared to embryonic stem cells (ESCs) (14), suggesting that to reduce epigenetic memory, its levels should be decreased during reprogramming. Removal of DNA methylation occurs during SCNT and iPSC generation. However, when insufficiently removed from genes regulating pluripotency in iPSCs, it results in a less efficient and incomplete conversion to iPSC (5,10). H3K27me3 has not specifically been identified as a modification contributing to memory in iPSC generation, but it has been in SCNT and transdifferentiation. Additionally, this modification induces inactivation of genes in iPSCs relating to their cell of origin (10). Furthermore, H3K9me2 (lysine 9 dimethylation on histone H3) is involved in inactivating Oct4 and other embryonic genes in differentiated cells (15,16), suggesting this should be reversed during reprogramming.

Opposite to this, H3K79me3, and H3K4me3 prevent inactivation of genes during reprogramming due to inducing euchromatin formation. Reduction of their levels during reprogramming results in inactivation of genes involved in differentiated cells and thereby enhance their reprogramming into iPSCs (10). There are however indications that H3K4me3 reduction causes inactivation of pluripotency genes, and thus consequently hinders iPSC generation (10,17). This corresponds with findings that H3K4me3 is globally less present in mouse somatic cells and incompletely reprogrammed iPSCs compared to in ESCs and fully reprogrammed iPSCs, suggesting that retained lower levels would interfere with pluripotency. The same was discovered for H3K36me2, H3K27ac (lysine 27 acetylation on histone H3), H3K9ac, H3ac (acetylation of histone H3), H4ac (acetylation of histone H4), and H4K5ac (18). Desired effects on gene expression thus depend on which specific genes are controlled by the modifications (pluripotent or differentiation-specific).

Differentiation tendencies caused by epigenetic memory could influence applications of iPSCs and the potential use of easily and non-invasively acquirable donor cells in generating difficult to obtain somatic cells. If donor cells from specific cell lineages are required for differentiation into specific target cells due to these tendencies, it can highly limit the source of non-invasively acquirable human cells for certain applications. Additionally, it creates a disadvantage in drug screening and disease modelling in regard of iPSC-derived cells potentially not representing *in vivo* cells due to epigenetic memory (6). Furthermore, it may affect personalized medicine or comparisons across different studies, due to donor cell- or tissue-specific variabilities in iPSCs and potentially their resulting differentiated cells. In organoid research, another major component of regenerative medicine, memory could influence proportions and presence of different cell types within these tissue structures (10,19).

Considering the involvement of the aforementioned epigenetic modifications in SCNT or iPSC generation, previous studies have utilized inhibitors to investigate effects on reprogramming efficiency (10,20). Histone acetylation has been increased with the use of Trichostatin A (TSA), valproic acid (VPA), and m-carboxycinnamic acid bishydroxamide (CBHA), inhibitors of histone deacetylases (10,11,21,22). Specifically, CBHA increases H3K9ac (11). Furthermore, H3K79me3 has been reduced with the inhibitor EPZ004777 (10,23). The inhibitor BIX-01294 has been used to decrease levels of H3K9me2 to improve iPSC generation (15). Additionally, improvement of iPSC generation has been shown as a result of reduction of DNA methylation with 5-aza-cytidine (AZA), an inhibitor of DNA methyltransferases (24). Epigenetic memory reduction has however not been a main focus of the use of these inhibitors, and their effect on fully removing memory remains elusive.

However, there is one technique that can more provide insight into a methodology to reduce epigenetic memory: SCNT. This is because, interestingly, epigenetic memory, based on DNA methylation, is retained less in stem cells created with SCNT. These stem cells are more similar to embryonic stem cells than iPSCs based on epigenetic memory (4,5). A possible explanation for this difference in memory reduction compared to iPSC generation, is the absence of factors in adult somatic cells that are present in oocytes or early embryos (25,26). If SCNT can reduce epigenetic memory to a level more similar to ESCs, knowledge from this technique could be potentially transferred to iPSC generation to achieve the same.

Although, SCNT is not fully efficient yet and epigenetic memory can still interfere with proper outgrowth of the embryos (10), a number of oocyte factors with a connection to reprogramming have been discovered: GLIS1, H3.3B, TH2A, TH2B, ASF1A, GDF9, Wave1, Gata3, BMP7, DJ-1 (25). Glis1, H3.3B, the collaboration of TH2A and TH2B, the collaboration of ASF1A and GDF9, Wave1, Gata3, and BMP7 improve SCNT or iPSC generation (25–33), while inhibition of DJ-1 causes activation of the p53 pathway (25,34), which is a pathway involved in iPSC generation (34).

As incomplete processing of epigenetic modifications during OSKM-mediated reprogramming leads to retained epigenetic memory (10), experimentally reducing or inducing the modifications will most likely enable the creation of a fully reprogrammed and neutral iPSC cell line that can differentiate into any cell type for *in vitro* and *in vivo* applications. Additionally, as the main focus of prior studies was improving reprogramming efficiency, a clear overview or answer about the inhibitors' and oocyte factors' effect on removing epigenetic memory is lacking. Therefore, utilizing priorly acquired knowledge from SCNT, the aim of this research proposal is to determine the ability of specific oocyte factors to reduce epigenetic memory during iPSC generation by OSKM-mediated reprogramming. To investigate this, human neutrophils and dermal fibroblasts will be reprogrammed with OSKM-transduction and compared to the same procedure in combination with expression or inhibition of the aforementioned oocyte factors. Additionally, the factors' combinational effect with previously mentioned inhibitors will be analysed to provide insight into mechanisms underlying the factors' effect on epigenetic modifications in iPSC generation and their potential ability to enhance the inhibitors' effects. To confirm generation of iPSCs, pluripotency will be assessed based on embryonic bodies and expression markers. Finally, effects on epigenetic memory will be based on donor cell-specific expression, acetylation and methylation patterns, and the iPSCs' tendency to differentiate into osteoblasts. This will provide a first ever look into the usability of these SCNT-relating factors in OSKM-

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reprogramming regarding global changes in methylation and acetylation memory marks, and a start in the search for an optimal procedure to generate iPSCs independent of their cells or origin.

B.2.2 Approach

B.2.2.1 Cell isolation

Neutrophils will be isolated from human whole blood (obtained from University Medical Centre Utrecht with informed consent) according to the protocol previously reported (35). In short, whole blood will be added on isolation medium (sodium metrizoate and Dextran 500) after which centrifugation (35 min, 500 RCF) will be performed to separate the different cells into visible layers. The three layers on top will be discarded and the bottom layer will be collected. This layer contains neutrophils (and leftover red blood cells) which will be diluted with Hank's Balanced Salt Solution (HBSS) (minus magnesium and calcium). Centrifugation (10 minutes, 350 RCF) will be performed again. Red Cell Lysis Buffer will be added and the cells will be centrifuged (5 minutes, 250 RCF). Next, the cells will be washed with HBSS (minus magnesium and calcium) and lastly, HBSS / 2% HSA will be added (35).

Dermal fibroblasts will be isolated, as described with some adjustments (36), from human skin biopsies obtained from patients at University Medical Centre Utrecht with informed consent. Pieces will be cut and incubated in dispase (4 °C, overnight). Next, the dermis will be removed from the epidermis and added to collagenase (37 °C, 5% CO_2, 1 hour). DMEM (HEPES, pen/strep), with serum-free KnockOut Serum Replacement (Gibco, ThermoFisher Scientific, cat. 10828028) as replacement of fetal calf serum (FCS), is added and the cells will be filtered using a strainer. Finally, the cells are spun down (200 RCF, 5 minutes) and added to DMEM (HEPES, pen/strep), with serum-free KnockOut Serum Replacement (Gibco, ThermoFisher Scientific, cat. 10828028), for culturing (36).

B.2.2.2 iPSC generation

Human iPSCs will be generated from primary dermal fibroblasts and neutrophils following a previously reported procedure, with necessary adjustments, using oriP/EBNA1 episomal plasmids (37). These plasmids are viral packaging-free and non-integrating, which allows for factors to be transiently expressed (37,38). Additionally, they enable longer factor expression compared to original plasmid vectors, which makes them suitable for reprogramming purposes (38). Transient expression will prevent potential interference of OSKM and oocyte factors during differentiation of the iPSCs.

A pEP4 E02S EM2K episomal plasmid (Addgene plasmid # 20923 ; http://n2t.net/addgene:20923 ; RRID:Addgene_20923) (37) will be used for expression of OSKM factors. The dermal fibroblasts will be transfected via nucleofection with the use of the Human Dermal Fibroblast Nucleofector kit (Lonza, cat. VPD-1001). For the neutrophils, optimal conditions for transfection will be determined with the Primary Cell Optimization 4D-Nucleofector X kit (Lonza, cat. V4XP-9096) and consequently nucleofection will be performed with the 4D-Nucleofector X Unit (Lonza, cat. AAF-1003X). After, the cells will be seeded on mouse embryonic fibroblasts (MEF) feeder cells (Gibco, ThermoFisher Scientific, cat. A34181) and incubated in cell type-specific culture medium (refreshed on the second day). After four days, culturing is continued with human ES culture medium and after 18 to 20 days, iPSC colonies will be identified via alkaline phosphatase staining with an Alkaline Phosphatase Detection kit (Sigma-Aldrich, Merck, cat. SCR004). The cells will be in culture for a total of around four weeks before being processed for analysis (37).

B.2.2.2.1 Adapted iPSC generation

Expression or inhibition of a number of factors known to be present/relevant in oocytes and early embryos (GLIS1, H3.3B, TH2A, TH2B, ASF1A, GDF9, Wave1, Gata3, BMP7, and DJ-1) (25) will be induced in human dermal fibroblasts and neutrophils during reprogramming to investigate their effect on epigenetic memory. For this purpose, non-integrating episomal plasmids will be created and co-transfected with OSKM by nucleofection according to the protocol described in "iPSC generation" (37).

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TH2A and TH2B will be co-transfected together as they have been shown to have a combined effect on reprogramming of mouse somatic cells (28). The same goes for ASF1A and GDF9 regarding human somatic cells (26). Expression of GLIS1, H3.3B, Wave1 and Gata3 will be manipulated separately to analyse their individual effect. To create cDNA for all factors, reverse transcription PCR will be performed with the High-Capacity cDNA Reverse Transcription kit (Cat. 4368814, ThermoFisher Scientific). Next, an insert will be designed and incorporated into an pCEP4 Mammalian Expression Vector (ThermoFisher Scientific, cat. V04450) following the manufacturer's instructions.

Recombinant human BMP7 (PeproTech, cat. 120-03P) will be supplemented to the culture medium.

Transient knockdown of DJ-1 will be accomplished with siRNA transfection to investigate effects of their inhibition on epigenetic modifications, potentially regulated by p53 activation (25,34). siRNA targeting human DJ-1 (Santa Cruz Biotechnology, cat. Sc-37080) will be used for this purpose and co-transfected with OSKM plasmids mentioned in "Generating iPSCs from somatic cells" with the Lipofectamine 2000 Transfection Reagent (ThermoFisher Scientific, cat. 11668019) according to their siRNA plasmid co-transfection protocol.

Furthermore, the effect of these factors will be screened with addition of inhibitors against epigenetic marks known to have an effect on SCNT or iPSC reprogramming: VPA (STEMCELL Technologies, cat. 100-1053), TSA (STEMCELL Technologies, cat. 72284), AZA (STEMCELL Technologies, cat. 72014), EPZ004777 (Tocris, Bio-Techne, cat. 5567), CBHA (BPS Bioscience, cat. 27204) and BIX-01294 (STEMCELL Technologies, cat. 72044) (10,11,15,21–24). The inhibitors will be added to the culture medium. The factors' potential to enhance the effect of the inhibitors will be analysed with experiments described below, providing insight into their potential mechanisms of action in removing epigenetic memory marks. As a control, the inhibitors will also be added to the OSKM reprogramming procedure without addition of the oocyte factors.

The following experimental procedures will be performed on iPSCs generated with OSKM, and iPSCs generated with the adapted procedures. When mentioned, their cells of origin, human dermal fibroblasts and neutrophils, will be included for comparison.

B.2.2.3 Reprogramming validation

B.2.2.3.1 Pluripotency

The pluripotency of the iPSCs will be determined by creating embryonic bodies and subsequently quantifying expression of ectoderm, mesoderm and endoderm markers with qPCR as described (39). Following reprogramming, cells are replated on non-adherent plates without feeder cells in human ESC medium. After 7 to 9 days, qPCR will be performed with the resulting embryonic bodies to analyse expression of PAX6 and Sox1 for ectoderm, TBX1 for mesoderm, and AFP, GATA4 and Sox17 for endoderm (39). Furthermore, expression levels of pluripotency markers Nanog, Sox2, Lin28, Oct4 (40), and alkaline phosphatase will be analysed (41). qPCR will be performed following a previously reported method (42). The RNeasy Mini kit (Qiagen, cat. 74104) will be used to collect RNA from the samples from which cDNA will be created with the SuperScript II Reverse Transcriptase (ThermoFisher Scientific, cat. 18064014). For the qPCR reactions, the Power SYBR Green PCR Master Mix (ThermoFisher Scientific, cat. 4368577) will be used and reactions are performed in triplicate (42). If available, previously designed primers will be used or they will be designed.

B.2.2.4 Epigenetic memory

B.2.2.4.1 iPSC gene expression profiles retained from donor cell

To identify memory in gene expression, expression levels of cell type specific markers will be quantified with qPCR in human dermal fibroblasts, neutrophils and their iPSCs, as described in "Pluripotency". The markers to identify granulocyte-specific gene expression, are Lyz1, Lyz2 and Ly6g (6). Decreased levels of expression are to be expected in iPSCs derived from neutrophil donor cells compared to donor cell levels due to reprogramming. On the other hand, higher levels are expected compared to iPSCs derived from dermal fibroblasts due to epigenetic memory.

B.2.2.4.2 iPSC histone acetylation and methylation marks retained from donor cell

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Acetylation or methylation marks known to play a role in maintaining epigenetic memory can be divided based on their function of either inducing heterochromatin formation (H2AK119ub, H3K27me3, H3K9me3, H3K9me2, and DNA methylation) (10,13,15,16) or inducing euchromatin formation (H3K4me3, H3K79me3, H3ac, H3K9ac, H3K27ac, H4ac, H4K5ac, H3K36me2) (10,18). To quantify global levels of these marks in iPSCs, chromatin immunoprecipitation sequencing will be performed. For comparison, levels in the dermal fibroblasts and neutrophils or origin will be analysed as well.

Chromatin immunoprecipitation sequencing will be executed as previously reported with some adjustments (23). iPSCs, human dermal fibroblasts and neutrophils will be fixed and processed in a Biorupter Pico sonication device (Diagenode, cat. B01080010) in SDS lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.1). This way the chromatin is sheared. Next, the chromatin will be diluted in a buffer (16.7 mM Tris-HCl, 1.2 mM EDTA, 0.01% SDS, 167 mM NaCl, 1.1% Triton-X100, pH 8.1) and antibodies to capture DNA that contains the memory marks, will be added (23). The following polyclonal antibodies will be used: anti-H2AK119ub (Invitrogen, ThermoFisher Scientific, cat. 720148), anti-H3K27me3 (Sigma-Aldrich, Merck, cat. 07-449), anti-H3K9me3 (Invitrogen, ThermoFisher, cat. PA5-31910), anti-H3K9me2 (Invitrogen, ThermoFisher Scientific, cat. PA5-120810), anti-H3K9ac (Invitrogen, ThermoFisher Scientific, cat. PA5-117092), anti-H3K4me3 (Bioss, ThermoFisher Scientific, cat. BS-53103R), anti-H3K79me3 (Invitrogen, ThermoFisher Scientific, cat. 49-1020), anti-H3ac (Invitrogen, ThermoFisher, cat. PA5-114693, against lysine 9, 14, 18, 23 and 27), anti-H3K27ac (Invitrogen, ThermoFisher Scientific, cat. PA5-96618), anti-H4ac (Invitrogen, ThermoFisher Scientific, cat. PA1-84526, against lysine 5, 8, 12 and 16), anti-H4K5ac (Invitrogen, ThermoFisher Scientific, cat. PA5-90308) and anti-H3K36me2 (Invitrogen, ThermoFisher Scientific, cat. PA5-96117).

After overnight incubation (4 °C), Pierce^{TM} Protein A/G Agarose beads (ThermoFisher Scientific, cat. 20421) will be added. After an hour of incubation (4 °C), the precipitate will be washed (5 minutes, 4 °C) in a low-salt solution (20 mM Tris-HCl, 2 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% Triton-X100, pH 8.1), a high-salt solution with 500 mM NaCl, a LiCl solution (10 mM Tris-HCl, 1 mM EDTA, 1% IGEPAL CA-630, 1% deoxycholic acid, 0.25 M LiCl, pH 8.1), and a TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.1) in this order. Next, elution buffer (0.1 M NaHCO3, 1% SDS) will be added twice for 15 minutes (room temperature) and the solution will be incubated (65 °C) with addition of sodium chloride. The following day, the DNA will be processed with the QIAquick PCR Purification kit (Qiagen, cat. 28104) for purification (23).

With the TruSeq ChIP Library Preparation kit (Illumina, cat. IP-202-1012) libraries will be generated from the purified DNA, which will be sequenced with the NextSeq 2000 sequencer (Illumina). With the latest version of the open-source program Bowtie (43), Bowtie 2, sequenced reads will be aligned to the latest version of the human genome assembly, hg38. Memory mark levels will be determined as amount of reads per million per kilobase. In case of histone methylation and acetylation marks, their transcription start site will be used to determine what window will be analysed. This will not be applied to DNA methylation marks. Reads found to align to more than one location of the genome will be excluded (23).

B.2.2.4.3 iPSC global DNA methylation pattern

To analyse global DNA methylation, methylated DNA immunoprecipitation (next-generation) sequencing (MeDIP-seq) will be performed on iPSCs, human dermal fibroblasts and neutrophils of origin as previously reported (44), with some adjustments. With the DNeasy kit (Qiagen, cat. 69504) DNA will be collected according to the manufacturer's protocol. Next, the MagMeDIP kit (Diagenode, cat. C02010021) and an antibody against 5-methylcytidine (Invitrogen, ThermoFisher Scientific, cat. MA1-80331) will be used to acquire methylated DNA. With the NEBNext Ultra II DNA Library Prep kit (New England Biolabs, cat. E7645L) libraries will be generated for sequencing with the NextSeq 2000 sequencer (Illumina). Same as described in "iPSC histone acetylation and methylation marks retained from donor cells", Bowtie 2 will be used to align sequenced reads to the human genome assembly hg38 (44).

B.2.2.4.4 iPSC differentiation

The iPSCs will be differentiated into osteoblasts to analyse their inclination to differentiate into a specific cell type based on their respective somatic donor cells. Differentiation will be performed as previously described

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(41) with an intermediary differentiation step into mesenchymal stem cells (MSCs). iPSCs will be dissociated and reseeded in new flasks with 0.1% gelatine and MSC medium (α-MEM, HEPES, L-glutamine, sodium pyruvate, L-ascorbate-2-phosphate, non-essential amino acids, pen/strep). The FCS in this medium will be replaced by the serum-free KnockOut Serum Replacement (Gibco, ThermoFisher Scientific, cat. 10828028). Two weeks later, the cells will be passaged (1:3) on gelatine twice and then without gelatine till visible features of MSCs are observed. To validate successful differentiation, MSCs will be selected with flow cytometry based on a primary and subsequent more extensive secondary selection of presence of MSC markers (CD166, CD105, CD146, CD73, CD90) and lack of pluripotency markers (TRA181 and TRA160). In the secondary selection, the lack of hematopoietic markers (CD34, CD14, CD45) is included (41).

Next, the selected MSCs will be differentiated into osteogenic cells by culturing them for around four weeks in osteoinductive medium (α-MEM, L-glutamine, sodium pyruvate, L-ascorbate-2-phosphate, pen/strep, inorganic phosphate, and dexamethasone) (41) with serum-free KnockOut Serum Replacement (Gibco, ThermoFisher Scientific, cat. 10828028) replacing FCS.

Based on prior observations of epigenetic memory in mouse iPSC differentiation (5,7), the fibroblast derived iPSCs are expected to exhibit a higher tendency to differentiate into osteoblasts than the neutrophil iPSCs. Alizarin red staining to analyse osteoblast morphology, and qPCR to quantify expression levels of osteogenic markers, Runx2, Bglap and Sp7 will be conducted to confirm and compare osteoblast differentiation (5). qPCR will be performed as described in “Pluripotency”. Differentiated cells will be stained with alizarin red as previously reported (5). The cells will be fixed and incubated with Alizarin-Red Staining Solution (Sigma-Aldrich, Merck, cat. TMS-008). After 15 minutes, they will be washed using Tris-HCl (pH 4.0) (5). Images will be analysed using ImageJ.

B.2.2.5 Timeline

Table 1: Timeline of the proposed research over the course of four years.

Tasks	Months							
	1-6	7-12	13-18	19-24	25-30	31-36	37-42	43-48
Designing and optimizing vectors	▨	▨						
Optimizing OSKM reprogramming	▨	▨						
Reprogramming assays			▨	▨				
Epigenetic memory analysis				▨	▨	▨		
Differentiation analysis						▨	▨	▨

The proposed research will be conducted over the course of four years (Table 1) using facilities and equipment available in the University Medical Center Utrecht. The project will start with designing and optimizing the episomal plasmid vectors for OSKM and adapted reprogramming procedures. At the same time, dermal fibroblasts and neutrophils will be isolated, and the OSKM procedure will be tested and optimized if needed. This will be done to ensure effective reprogramming before commencing the OSKM and adapted reprogramming assays (including pluripotency validation), and epigenetic memory analysis. The epigenetic memory analysis involves assessing retained gene expression profiles, histone methylation and acetylation marks, and DNA methylation patterns. Towards the end of the four-year project, analysis of differentiation potential will be performed.

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B.2.3 Feasibility / Risk assessment

Utilizing factors that have been shown to be connected to somatic cell reprogramming by OSKM reprogramming and/or nuclear transfer, will potentially reduce epigenetic memory. That being said, the selected factors have not all been identified in human oocytes or human iPSCs. This means that the translation from oocytes and cells from other species to human species is not guaranteed and should become clear from the proposed research.

Additionally, the chosen factors are a selection from a number of factors known to be present in oocytes. To ensure feasibility, not all known factors will be tested which provides for opportunities to expand the proposed research with other factors depending on the data from this proposal. Furthermore, a conscious decision was made to assess the factor's individual effects to ensure feasibility, which allows for further research on combined effects.

Due to the use of commercially available expression vectors for some of the oocyte factors, it is possible to investigate a selection of factors in the intended timeframe of this research. However, for some factors a vector will have to be designed. This requires time and expertise which has been taken into account in the outline of this proposal. If proven to be too challenging, changes can be made to the selected list of oocyte factors, replacing or omitting some.

Furthermore, due to limited non-invasive human cell sources, epigenetic memory has not priorly been investigated in the exact human somatic cell setup as proposed here. However, presence of epigenetic memory is expected based on literature (5,7).

B.2.4 Scientific and societal impact

In the short-term, the proposed research will provide more insight into somatic cell reprogramming and underlying (epigenetic) mechanisms. Based on its results, an overview can be made of factors that can or cannot be used to reduce epigenetic memory, which creates the basis for the search towards optimal combinations of factors and/or inhibitors for a general procedure of generating neutral iPSCs. For this purpose, future research can make use of vectors generated in this proposed research for the expression of the different oocyte factors. Furthermore, factors that show an effect on memory can be further analysed regarding specific pluripotency or differentiation-specific genes they affect.

Considering there is a correlation between epigenetic modifications and reprogramming efficiency (7,10,22), potential improvement in removing memory, will be useful for future research into enhancing this efficiency. Since the effects of some of the proposed oocyte factors and epigenetic inhibitors on reprogramming efficiency of mostly mice cells and oocytes have been shown previously, it is possible that their addition to the reprogramming protocol could also result in an increased reprogramming efficiency. Because epigenetic memory is a component potentially influencing reprogramming efficiency (7,10,22), future research can be executed to explore the factors' effects on efficiency based on their effect on epigenetic memory that might become apparent from the proposed research. Also, insight into factors involved in (reducing) epigenetic memory provides opportunities to more precisely and timely regulate epigenetic changes during reprogramming.

Apart from reprogramming efficiency, the applicability of iPSCs can also be challenged by low differentiation efficiencies into specific cell types. For instance, differentiation into neuroepithelia has been found to be more inefficient when using iPSCs than ESCs (45,46). Considering chromatin accessibility of specific genes is regulated in cells in order for differentiation to take place (13), and retained chromatin modifications influence differentiation potential of iPSCs (4), epigenetic memory may also influence differentiation efficiency of iPSCs into specific somatic cell types. For instance, differentiation of progenitors into oligodendrocytes is negatively affected when H3K9me3 is reduced (13,47). With this in mind, insight gained

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from the proposed research might be beneficial for future research for enhancement of iPSC differentiation procedures.

Additionally, in the long-term, improving differentiation potential of iPSCs by removing epigenetic memory, will benefit future research by allowing the use of any type of non-invasively acquired cell type to be used for the generation of iPSCs for any application. At the same time, removing donor cell dependence could provide more control over proportions of cell types within organoid cultures (10,19). Supporting these scientific benefits, will be the possibility of creating biobanks with these versatile, neutral iPSCs.

Apart from broadening research opportunities, the aforementioned advantages will be beneficial on a patient and societal level. iPSCs form an important source of human somatic cells for allogenic or autologous applications in numerous scientific fields including regenerative medicine. Considering, epigenetic changes play a fundamental role in differentiation, retained epigenetic memory marks, can influence differentiation of iPSCs, as seen before in differences of differentiation potential (4–6). This could restrict their range of differentiation opportunities depending on cell of origin (6,8,10). Thus, as epigenetic memory influences differential potential of these stem cells, there is a need for the generation of a fully reprogrammed and neutral iPS cell line. This will allow for less genetic heterogeneity in cells, tissues or organoids derived from iPSCs for personalized medicine and comparisons across studies with different used cells of origin. With a neutral iPS cell line, the cell of origin should not matter, allowing researchers to utilize any cell type available without the risk of confounding results. This is especially important in disease modelling and drug screening since epigenetic memory might affect how well the differentiated cells can replicate *in vivo* disease mechanisms (6). For instance, a previous study has revealed differences in response levels between cardiomyocytes derived from cardiomyocyte-derived iPSCs or derived from fibroblast-derived iPSCs when exposed to caffeine (48).

Looking beyond *in vitro* applications, iPSCs are promising tools for *in vivo* interventions against diseases or tissue damage (49). In the long-term, when general obstacles for *in vivo* use of iPSCs, including mutagenesis, immune rejection of allogenic cells, and tumorigenicity (49), are resolved, the aforementioned advantageous of the proposed research can be extended to *in vivo* use of a neutral iPS cell line. The use of non-integrating, viral package-free episomal vectors for gene expression during reprogramming in this proposed research is already a start in this direction as its transient expression prevents tumorigenesis (50).

Due to the lack of pluripotency of adult stem cells, the invasiveness of obtaining said cells, and ethical concerns for the use of ESCs, the ability to generate iPSCs provides numerous new opportunities for research and treatment of diseases (1,2). It has especially opened the door in personalized medicine for developing patient-specific treatments by allowing researchers to utilize an autologous cell or tissue source (2). However, if epigenetic memory possibly interferes with representative results or limits the range of cells usable for specific applications, the full potential of iPSCs has not yet been reached. Therefore, to improve the usability of iPSCs against many diseases, it is essential to remove their dependence on their cell of origin and thereby the epigenetic limit in differentiation potential.

B.2.5 Ethical considerations

Ethical dilemmas are avoided by investigating effects on OSKM reprogramming of only non-invasive human somatic cells with no need for embryonic cells or oocytes. Using comparisons of OSKM reprogramming, adapted reprogramming and wildtype somatic cells, allows for a complete insight into epigenetic memory without the use of ESCs. Furthermore, pluripotency will be determined without the use of an (chimera or teratoma) animal model. Additionally, in the experimental procedures, serum-free KnockOut Serum Replacement (Gibco, ThermoFisher Scientific, cat. 10828028) will be used as replacement of FCS to minimize the use of material originating from animals.

Material that does originate from animals used in the proposed research, is the MEF feeder cells (37). Considering these cells will be commercially acquired, no additional animals or primary animal cells are

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required for conducting the proposed research. All necessary ethical and animal welfare requirements will be ensured by the manufacturer.

Furthermore, informed donor consent with explanation of the use of the donors' somatic and resulting iPSCs, will be required of the whole blood and skin biopsy donors with the option of withdrawal at any given moment.

B.2.6 Literature/references

1. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 2006 Aug 25;126(4):663–76.
2. Zakrzewski W, Dobrzyński M, Szymonowicz M, Rybak Z. Stem cells: past, present, and future. Stem Cell Res Ther. 2019 Feb 26;10(1).
3. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. 2007 Nov 30;131(5):861–72.
4. Zwaka TP. Troublesome memories. Nature. 2010;467:280–1.
5. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, et al. Epigenetic memory in induced pluripotent stem cells. Nature. 2010;467:285–90.
6. Polo JM, Liu S, Eugenia Figueroa M, Kulalert W, Eminli S, Yong Tan K, et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat Biotechnol. 2010;28:848–55.
7. Noguchi H, Miyagi-Shiohira C, Nakashima Y. Induced tissue-specific stem cells and epigenetic memory in induced pluripotent stem cells. Int J Mol Sci. 2018 Apr 1;19(4).
8. Kim K, Zhao R, Doi A, Ng K, Unternaehrer J, Cahan P, et al. Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. Nat Biotechnol. 2011 Nov 27;29:1117–9.
9. Ji H, Ehrlich LIR, Seita J, Murakami P, Doi A, Lindau P, et al. Comprehensive methylome map of lineage commitment from haematopoietic progenitors. Nature. 2010;467:338–42.
10. Hörmanseder E. Epigenetic memory in reprogramming. Curr Opin Genet Dev. 2021 Oct 1;70:24–31.
11. Song Y, Hai T, Wang Y, Guo R, Li W, Wang L, et al. Epigenetic reprogramming, gene expression and in vitro development of porcine SCNT embryos are significantly improved by a histone deacetylase inhibitor—m-carboxycinnamic acid bishydroxamide (CBHA). Protein Cell. 2014 May 1;5(5):382–93.
12. Tamburri S, Lavarone E, Fernández-Pérez D, Conway E, Zanotti M, Manganaro D, et al. Histone H2AK119 Mono-Ubiquitination Is Essential for Polycomb-Mediated Transcriptional Repression. Mol Cell. 2020 Feb 20;77:840–56.
13. Nicetto D, Zaret KS. Role of H3K9me3 heterochromatin in cell identity establishment and maintenance. Curr Opin Genet Dev. 2019 Apr 1;55:1–10.
14. Favaedi R, Shahhoseini M, Pakzad M, Mollamohammadi S, Baharvand H. Comparative epigenetic evaluation of human embryonic stem and induced pluripotent cells. International Journal of Developmental Biology. 2016;60:103–10.
15. Shi Y, Desponts C, Do JT, Hahm HS, Schöler HR, Ding S. Induction of Pluripotent Stem Cells from Mouse Embryonic Fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds. Cell Stem Cell. 2008 Nov 6;3(5):568–74.
16. Feldman N, Gerson A, Fang J, Li E, Zhang Y, Shinkai Y, et al. G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. Nat Cell Biol. 2006 Feb;8:188–94.
17. Kidder BL, Hu G, Yu ZX, Liu C, Zhao K. Extended Self-Renewal and Accelerated Reprogramming in the Absence of Kdm5b. Mol Cell Biol. 2013;33(24):4793–810.
18. Mattout A, Biran A, Meshorer E. Global epigenetic changes during somatic cell reprogramming to iPS cells. J Mol Cell Biol [Internet]. 2011 [cited 2024 Mar 1];3(6):341–50. Available from: https://academic.oup.com/jmcb/article/3/6/341/882201
19. Adhya D, Annuario E, Lancaster MA, Price J, Baron-Cohen S, Srivastava DP. Understanding the role of steroids in typical and atypical brain development: Advantages of using a "brain in a dish" approach. J Neuroendocrinol. 2018 Feb 1;30(2):e12547.

20. Stricker SH, Götz M. Epigenetic regulation of neural lineage elaboration: Implications for therapeutic reprogramming. Neurobiol Dis. 2021 Jan 1;148.
21. Yang G, Zhang L, Liu W, Qiao Z, Shen S, Zhu Q, et al. Dux-Mediated Corrections of Aberrant H3K9ac during 2-Cell Genome Activation Optimize Efficiency of Somatic Cell Nuclear Transfer. Cell Stem Cell. 2021 Jan 7;28(1):150-163.e5.
22. Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, et al. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol. 2008 Jul;26:795–7.
23. Onder TT, Kara N, Cherry A, Sinha AU, Zhu N, Bernt KM, et al. Chromatin-modifying enzymes as modulators of reprogramming. Nature. 2012 Mar 29;483:598–602.
24. Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, et al. Dissecting direct reprogramming through integrative genomic analysis. Nature. 2008;454:49–55.
25. Han F, Li X, Song D, Jiang S, Xu Q, Zhang Y. SCNT versus iPSCs: Proteins and small molecules in reprogramming. International Journal of Developmental Biology. 2015;59(4–6):179–86.
26. Gonzalez-Muñoz E, Arboleda-Estudillo Y, Otu HH, Cibelli JB. Histone chaperone ASF1A is required for maintenance of pluripotency and cellular reprogramming. Science (1979). 2014 Aug 15;345(6198):822–5.
27. Maekawa M, Yamaguchi K, Nakamura T, Shibukawa R, Kodanaka I, Ichisaka T, et al. Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. Nature. 2011 Jun 8;474:225–9.
28. Shinagawa T, Takagi T, Tsukamoto D, Tomaru C, Huynh LM, Sivaraman P, et al. Histone variants enriched in oocytes enhance reprogramming to induced pluripotent stem cells. Cell Stem Cell. 2014 Feb 6;14(2):217–27.
29. Miyamoto K, Teperek M, Yusa K, Allen GE, Bradshaw CR, Gurdon JB. Nuclear Wave1 Is Required for Reprogramming Transcription in Oocytes and for Normal Development. Science (1979). 2013 Aug 8;341(6149).
30. Wen D, Banaszynski LA, Liu Y, Geng F, Noh KM, Xiang J, et al. Histone variant H3.3 is an essential maternal factor for oocyte reprogramming. Proc Natl Acad Sci U S A. 2014 May 20;111(20):7325–30.
31. Samavarchi-Tehrani P, Golipour A, David L, Sung HK, Beyer TA, Datti A, et al. Functional genomics reveals a BMP-Driven mesenchymal-to-Epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell. 2010 Jul 2;7(1):64–77.
32. Chen J, Liu J, Yang J, Chen Y, Chen J, Ni S, et al. BMPs functionally replace Klf4 and support efficient reprogramming of mouse fibroblasts by Oct4 alone. Cell Res. 2011 Dec 7;21:205–12.
33. Shu J, Zhang K, Zhang M, Yao A, Shao S, Du F, et al. GATA family members as inducers for cellular reprogramming to pluripotency. Cell Res. 2015 Jan 16;25:169–80.
34. Miyamoto K, Nagai K, Kitamura N, Nishikawa T, Ikegami H, Binh NT, et al. Identification and characterization of an oocyte factor required for development of porcine nuclear transfer embryos. Proc Natl Acad Sci U S A. 2011 Apr 26;108(17):7040–5.
35. Oh H, Siano B, Diamond S. Neutrophil isolation protocol. Journal of Visualized Experiments. 2008;(17).
36. Kisiel MA, Klar AS. Isolation and culture of human dermal fibroblasts. Methods in Molecular Biology [Internet]. 2019 [cited 2024 Feb 28];1993:71–8. Available from: https://link-springer-com.proxy.library.uu.nl/protocol/10.1007/978-1-4939-9473-1_6
37. Junying Y, Kejin H, Kim SO, Shulan T, Stewart R, Slukvin II, et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science (1979). 2009 May 8;324(5928):797–801.
38. Malik N, Rao MS. A review of the methods for human iPSC derivation. Methods in Molecular Biology. 2013;997:23–33.
39. Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, et al. Vitamin C Enhances the Generation of Mouse and Human Induced Pluripotent Stem Cells. Cell Stem Cell. 2010 Jan 8;6(1):71–9.
40. Cevallos RR, Hossain ME, Zhang R, Hu K. Evaluating Reprogramming Efficiency and Pluripotency of the Established Human iPSCS by Pluripotency Markers. In: Methods in Molecular Biology. Humana Press Inc.; 2021. p. 235–49.

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41. Hynes K, Menicanin D, Mrozik K, Gronthos S, Bartold PM. Generation of functional mesenchymal stem cells from different induced pluripotent stem cell lines. Stem Cells Dev. 2014 May 15;23(10):1084–96.
42. Sun T, Ding CKC, Zhang Y, Zhang Y, Lin CC, Wu J, et al. MESH1 knockdown triggers proliferation arrest through TAZ repression. Cell Death Dis. 2022 Mar 1;13(3):221.
43. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009 Mar 4;10(R25).
44. Li W, Huang Y, Sargsyan D, Khor TO, Guo Y, Shu L, et al. Epigenetic alterations in TRAMP mice: epigenome DNA methylation profiling using MeDIP-seq. Cell Biosci. 2018 Jan 12;8:3.
45. Liu G, David BT, Trawczynski M, Fessler RG. Advances in Pluripotent Stem Cells: History, Mechanisms, Technologies, and Applications. Stem Cell Rev Rep. 2020 Feb 1;16(1):3–32.
46. Hu BY, Weick JP, Yu J, Ma LX, Zhang XQ, Thomson JA, et al. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proc Natl Acad Sci U S A. 2010 Mar 3;107(9):4335–40.
47. Liu J, Magri L, Zhang F, Marsh NO, Albrecht S, Huynh JL, et al. Chromatin Landscape Defined by Repressive Histone Methylation during Oligodendrocyte Differentiation. The Journal of Neuroscience. 2015 Jan 1;35(1):352–65.
48. Firas J, Liu X, Polo JM. Epigenetic memory in somatic cell nuclear transfer and induced pluripotency: Evidence and implications. Differentiation. 2014 Jul 1;88(1):29–32.
49. Yamanaka S. Pluripotent Stem Cell-Based Cell Therapy—Promise and Challenges. Cell Stem Cell. 2020 Oct 1;27(4):523–31.
50. de Lázaro I, Cossu G, Kostarelos K. Transient transcription factor (OSKM) expression is key towards clinical translation of in vivo cell reprogramming. EMBO Mol Med. 2017 Jun;9(6):733–6.