From the department of Clinical Science,
Intervention and Technology
The division of Obstetrics and Gynecology
Karolinska Institutet, Stockholm, Sweden

EXPLORING EARLY DEVELOPMENT AND REGENERATIVE MEDICINE USING CRISPR/CAS9

Nerges Winblad

Exploring Early Development and Regenerative Medicine using CRISPR/Cas9
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Nerges Winblad

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Principal Supervisor:
Assistant professor Fredrik Lanner, Ph.D.
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology
Division of Obstetrics and gynecology

Co-supervisor(s):
Associate professor Evren Alici, M.D., Ph.D.
Karolinska Institutet
Department of Medicine, Huddinge
Division of Hematology and Regenerative Medicine

Associate professor Björn Reinius, Ph.D.
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Biomaterials

Professor Juha Kere, M.D., Ph.D.
Karolinska Institutet
Department of Biosciences and Nutrition
Division of Functional Genomics

Opponent:
Professor Kathrin Plath, Ph.D.
University of California, Los Angeles
Department of Biological Chemistry

Examination Board:
Associate professor Fredrik Wermeling. Ph.D.
Karolinska institutet
Department of Medicine
Division of Rheumatology

Professor Gonçalo Castelo-Branco, Ph.D.
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Molecular Neurobiology

Professor Anders Lindahl, M.D., Ph.D.
Sahlgrenska Universitetssjukhuset
Department of Biomedicine
Division of Laboratory Medicine

Till mamma, för att du alltid sagt att jag kan bli vad jag vill.

To my family away from home, without you I wouldn't be where I am today.

POPULAR SCIENCE SUMMARY OF THE THESIS

Embryonic stem cells are derived from an embryo and exist in two different states: naive (before implantation) and primed (after implantation). The embryonic stem cells represent an early stage of embryo development and have the capacity to specialize into every cell that will make up the body.

By taking advantage of this characteristic, we can create protocols of how to guide the stem cells into becoming specific cells at the laboratory. These specialized cells can later be used to develop different types of cell therapies that can treat diseases such as age-related macular degeneration, diabetes type 1 or heart infarct. Embryonic stem cells can also be used to study molecular processes occurring in the embryo. That means that we can gain knowledge of what happens during the first week after egg and sperm have fused.

CRISPR/Cas9 is a molecular scissor that precisely can cut through the DNA strand at a region of interest. The cut results in a broken DNA molecule that needs to be repaired, which can be done in several different ways. If the cell with the broken DNA strand gets a template detailing what the repair should look like, it can fix the damaged DNA using that template. In this way it is possible to introduce special features by designing the template. If there isn't any added template, the two strands are glued back together, which results in scars at the joint. These scars are what we call mutations. Some types of mutations that are caused by CRISPR/cas9 results in a malfunctioning gene. Thus, we can study what happens when the gene and the protein it produces are missing in the cell.

Three papers are included in this thesis, in which we have used CRISPR/Cas9 to mutate genes, thus preventing them from carrying out their normal function in embryonic stem cells.

In **paper I**, we wanted to create a universal stem cell line, displaying reduced immunogenicity, that can be used for specialization into different cell types for cell therapy. The idea was that those cells would not need to be matched before transplantation, thus benefiting more patients. We mutated two genes that affect the presentation of HLA antigens. These antigens sit on the outside of the cells and work as their ID cards. Without them they cannot identify themselves to the immune system. The mutation was introduced in stem cells, after which these were specialized into a type of retina cell. Next, we wanted to investigate whether the mutated retina cells could avoid activating the immune system. Therefore, they were tested against two different immune cells: T cells and NK cells.

T cells distinguish the body's own cells from foreign ones by binding to and confirming that the HLA antigen that a cell is presenting is the same as that of the body. If there is a difference in HLA between the immune cell and the cell it is interacting with, the T cell becomes activated and destroys the cell. Since the mutated retina cells did not present HLA at all, they avoided activating the T cells. NK cells also recognize HLA, however, in contrast to T cells, they are also activated when the HLA antigens are missing. Therefore, the mutated retina cells did not avoid triggering a response from the NK cells. However, the NK cell response was lower than

anticipated. One explanation could be that these particular retina cells present other types of proteins on the cell surface, which act reduces the reaction from the NK cells. Future studies could explore ways to counteract the NK cell-mediated killing of the cells. Lastly, the mutated retina cells were transplanted into rabbit eyes. The result was a delay in rejection, which means that it took longer for the immune system to become activated and eliminate the cells.

In **paper II**, we wanted to study processes that occur during the first days of embryo development. To be able to perform such a study without using embryos, we used naive stem cells. They share characteristics with the stem cells residing in the preimplantation embryo, i.e. the embryo before it attaches and implants into the uterine wall. More specifically, we wanted to investigate if a particular protein complex (PRC2) is involved in the specialization that occurs during the early stages of embryo development. By using CRISPR/Cas9 or different inhibitors against the protein complex it was prevented from carrying out its normal function in the naive stem cells. Over a seven-day period we observed the naive cells activating non-naive gene networks, thus increasing their potential to become trophectoderm (which forms the outer layer of the embryo) or mesoderm (which is one of the three germ layers that are formed within the embryo). To summarize, we identified a specific role for the protein complex and concluded that it is an adaptive mechanism through which the specialization potential of the naive stem cells is limited during embryo development.

In **paper III**, we wanted to study another process that starts during the first week of embryo development, namely X chromosome inactivation. It is a mechanism with which one of the two X chromosomes in the female XX-embryo is shut down to balance the gene dosage against the male XY-embryos that only have one X chromosome. Through previous studies in mice, a protein (SPEN) important for this process had been identified. Thus, we mutated the *SPEN* gene in human primed stem cells. These particular stem cells have already undergone X chromosome inactivation, however, to study this process both X chromosomes need to be active. This can be achieved by converting primed stem cells to the naive state. When the mutated primed stem cells were transformed into naive stem cells, we observed that they could not be sustained in the naive state. This was surprising since the effect in mice was seen only after X chromosome inactivation had begun.

The results also suggested that more genes had been affected by the loss of the one that was mutated. This was particularly clear in the mutated cells, since a gene (*XIST*) that is normally highly expressed in the naive state, was barely detectable. Further exploration of these cells in the future will provide a better understanding of how X chromosome inactivation would ensue. However, based on the large effects that we have seen in the stem cells we can assume that the inactivation process will be affected as well.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Embryonala stamceller kommer som namnet antyder från ett embryo och finns i två stadier: naive (före implantering) och primed (efter implantering). Embryonala stamceller representerar ett tidigt stadie av embryoutvecklingen och har förmågan att specialisera sig till i princip vilken cell som helst. Det är genom specialiseringen av stamcellerna som cellerna som bildar vår kropp skapas. Om vi utnyttjar stamcellernas förmåga att specialisera sig så kan vi skapa protokoll för hur man gör celler på labb. Dessa kan senare användas för att utveckla olika typer av cellterapier som kan behandla, till exempel, åldersförändringar i gula fläcken, diabetes typ 1 eller hjärtinfarkt. Embryonala stamceller kan också användas för att studera molekylära processer som sker i embryot. Det i sin tur innebär att vi kan öka vår förståelse om vad som händer den första veckan efter att ägg och spermie smält samman.

CRISPR/Cas9 är en molekylär gensax som på ett exakt sätt kan klippa av DNA-strängen. Det resulterar i en trasig DNA-sträng som måste lagas, vilket kan ske på lite olika sätt. Om cellen vars DNA har klippts itu får en mall på hur det ska se ut där skadan uppstått så kan den laga brottet i strängen med hjälp av den. Då kan man skapa celler med specialdesignade egenskaper som introducerats genom mallen. Om det däremot inte bifogas någon mall så klistras de två ändarna ihop och då bildas små ärr i fogen. Dessa ärr kallar vi för mutationer. Vissa typer av mutationer som bildas som följd av CRISPR/Cas9 leder till att genen som muterats inte längre kan utföra sin funktion. Det betyder att vi kan studera vad som händer när genen och det protein den bildar saknas i cellen.

I den här avhandlingen ingår tre studier där vi har använt CRISPR/Cas9 för att mutera gener och på så vis förhindra att de kan utföra sina normala funktioner i embryonala stamceller.

I **den första studien** ville vi skapa en universell stamcellslinje som sedan kan specialiseras till diverse celler för cellterapi och som inte behöver matchas vid en transplantation. Vi muterade två gener som påverkar presentationen av så kallade HLA-antigener. De sitter på utsidan av cellen och fungerar som våra cellers ID-kort. Utan dem kan cellerna inte identifiera sig för immunförsvaret. Mutationerna introducerades i stamcellerna innan dessa specialiserades till en typ av näthinnecell. Vi utnyttjade detta för att undersöka om dessa muterade näthinneceller kan undvika att aktivera immunförsvaret. De testades mot två olika immunceller: T- och NK-celler.

T-celler känner igen kroppens andra celler med hjälp av HLA och aktiveras när HLA-antigenerna skiljer sig från kroppens egna version. Eftersom HLA-antigenerna inte finns på de muterade näthinnecellerna så aktiverades inte T-cellerna. NK-celler känner också igen HLA, men till skillnad från T-celler så aktiveras de även när HLA inte finns. De muterade näthinnecellerna aktiverade således NK-cellerna, men inte alls lika stark som förväntat. Det beror antagligen på att just dessa celler även presenterat andra protein på cellytan som motverkar aktiveringen av NK-cellerna. För framtida studier innebär det att man bör undersöka sätt att undvika reaktion från NK-cellerna. De muterade näthinnecellerna transplanterades också in i ögonen på kaniner. Det resulterade i längre tid till dess att cellerna avstöttes jämfört

med icke modifierade celler, vilket innebär att det tog längre tid för immunförsvaret att aktiveras och eliminera cellerna.

I **den andra studien** ville vi studera processer som sker under embryots första dagar. För att kunna göra det utan att använda embryon använde vi naive stamceller. Dessa delar egenskaper med stamcellerna som finns i embryot innan det implanterar i livmoderväggen. Mer specifikt så ville vi ta reda på om ett särskilt proteinkomplex (PRC2) spelade en roll i specialiseringen som sker under embryoutvecklingens tidiga skede. Genom att använda CRISPR/Cas9 eller olika inhibitorer mot proteinkomplexet förhindrades det att utföra sin funktion i de naive stamcellerna. Det resulterade i att cellerna över en sjudagarsperiod gick från att vara naive stamceller till att aktivera andra nätverk av gener och på så vis ökade sin potential mer och mer för att bli trophectoderm (vilket utgör det yttre höljet på embryot) eller mesoderm (ett av de tre groddlager som bildas i embryot). Sammanfattningsvis identifierade vi en specifik roll för det undersökta proteinkomplexet och konstaterar att det är en adaptiv mekanism som begränsar stamcellers potential att specialisera sig under embryoutvecklingen.

I **den tredje studien** ville vi än en gång studera en process som påbörjas under embryots första vecka, nämligen X-kromosominaktivering. Det är en mekanism genom vilken en av de två X-kromosomerna i XX-embryot stängs av för att balansera gendosen mot XY-embryon som bara har en X-kromosom. Ett protein (SPEN) som har visat sig viktig för den här processen i möss, muterades i de primed stamcellerna från människa. Primed celler har redan genomgått X-kromosominaktivering, så för att kunna studera det måste båda X-kromosomerna åter bli aktiva. Det går att åstadkomma genom att ta primed celler till det naive stadiet. Så när stamcellerna sedan genomgick konverteringen till det naive stadiet såg vi att de celler med den muterade varianten av genen inte kunde uppehålla sig som naive stamceller. Detta var förvånande eftersom effekten i möss synts först efter att X-kromosominaktiveringen påbörjats.

Resultatet antydde också att flera andra gener påverkats i de muterade cellerna. Särskilt tydligt syntes det på en gen (*XIST*) som uttrycks starkt i det naive stadiet, men som i cellerna med den muterade *SPEN*-genen knappt uttrycktes alls. Vi hann inte komma längre i vår undersökning av dessa celler den här gången, så vi vet inte hur X-kromosominaktivering har påverkats. Däremot kan vi baserat på de effekter vi redan sett i stamcellerna anta att inaktiveringsprocessen kommer att vara påverkad. Den cellmodell vi nu har kan vi använda för fortsatta studier av den muterade genen och dess effekt på X-kromosominaktivering.

ABSTRACT

With their intrinsic capacity to self-renew and their potential to differentiate to specialized tissues, human embryonic stem cells (hESCs) have many applications in the fields of cell therapy and developmental biology. By applying clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) genome engineering on selected genes in hESCs, we are able to create cells with desired characteristics.

In **paper I**, we wanted to explore the possibility of creating hypoimmunogenic stem cell lines for cell therapy. Therefore, the genes beta-2-microglobulin (*B2M*) and class II major histocompatibility complex transactivator (*CIITA*) were targeted to obtain hESCs that would not be able to present and express human leukocyte antigen (HLA) class I and class II, respectively. We derived three hESC lines, for which the expression of HLA class I, HLA class II or both were prevented. Whole genome sequencing (WGS) analysis identified few introduced off-target mutations, all of which were in non-coding regions and had not been attributed as pathogenic. These edited hESC lines were differentiated into retinal pigment epithelium (RPE) cells and tested *in vitro* against T cells and natural killer (NK) cells. Failure to present HLA class I and class II led to reduced $CD8^+$ and $CD4^+$ T cell responses, while NK cell degranulation increased and NK cell cytotoxicity remained unchanged. Finally, when the modified hESC-derived RPE cells were transplanted into a preclinical animal model, rejection was delayed regardless of the modification that had been introduced into the parental hESC lines.

In the following two studies, we used hESCs to model key molecular events during early human embryogenesis.

In **paper II**, we investigate lineage commitment during embryo development using naive and primed hESCs as models for the pre- and post-implantation epiblast, respectively. Inhibition of polycomb repressive complex 2 (PRC2) with pharmacologic inhibitors or through genetic ablation, led to the discovery that this protein complex maintains naive pluripotency and acts as a barrier, preventing naive hESCs from differentiating into trophectoderm or mesoderm lineages. New shared and naive-specific bivalent promoters were defined, identifying promoters of key trophectoderm and mesoderm genes as kept in a transcriptionally balanced state. Inhibition of PRC2 rendered naive hESCs in an activated state where both pluripotency and lineage-specific genes were expressed. Furthermore, transitioning through the activated state led to commitment to either trophectoderm or mesoderm lineages.

In **paper III**, the relationship between long non-coding RNA (lncRNA) X-inactive-specific transcript (*XIST*) and the transcriptional repressor spen family transcriptional repressor (*SPEN*) was studied. Previous studies using mouse embryonic stem cells had identified SPEN as an *Xist*-interacting protein. Specifically, SPEN was described to bind to *Xist* RNA during initiation of X chromosome inactivation (XCI), to facilitate *Xist* RNA stability and to inhibit the negative regulator of *Xist*, namely *Tsix*, by recruiting chromatin remodelers to its promoter. We hypothesized that naive hESCs could be used to study the effect of loss of SPEN on *XIST* RNA,

since *XIST* is induced during conversion and X chromosome expression becomes biallelic, similar to the preimplantation epiblast. Thus, we targeted the *SPEN* locus in primed hESCs and converted single-cell derived *SPEN* knockout clones to the naive stem cell state. Surprisingly, the impact of SPEN depletion on *XIST* was apparent already in the naive state and manifested as low detectable levels of *XIST* RNA at naive passage 8, which remained low at naive passage 13.

LIST OF SCIENTIFIC PAPERS

I. Sandra Petrus-Reurer[1], **Nerges Winblad**[1], Pankaj Kumar, Laia Gorchs, Michael Chrobok, Arnika Kathleen Wagner, Hammurabi Bartuma, Emma Lardner, Monica Aronsson, Àlvaro Plaza Reyes, Helder André, Evren Alici, Helen Kaipe, Anders Kvanta, Fredrik Lanner[2].

Generation of Retinal Pigment Epithelial Cells Derived from Human Embryonic Stem Cells Lacking Human Leukocyte Antigen Class I and II.

Stem Cell Reports 2020, vol. 4, p. 648–662
https://doi.org/10.1016/j.stemcr.2020.02.006

II. Banushree Kumar[1], Carmen Navarro[1], **Nerges Winblad**[1], John P Schell, Cheng Zhao, Jere Weltner, Laura Baqué-Vidal, Angelo Salazar Mantero, Sophie Petropoulos, Fredrik Lanner[2], Simon J Elsässer[2].

Polycomb Repressive Complex 2 shields naïve human pluripotent cells from trophectoderm differentiation.

Nature Cell Biology 2022 (in press)

III. **Nerges Winblad**[2], Angelo Salazar Mantero, Simon J Elsässer, Fredrik Lanner[2]

SPEN is indispensable for XIST expression in naïve human embryonic stem cells

Manuscript

[1]Authors contributed equally
[2]Corresponding authors

CONTENTS

LIST OF ABBREVIATIONS

ABCG2	ATP Binding Cassette Subfamily G Member 2
AMD	Age-Related Macular Degeneration
ATRX	ATRX Chromatin Remodeler
B2M	Beta-2-Microglobulin
BMP4	Bone Morphogenic Protein 4
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
CDX2	Caudal Type Homeobox 2
CIITA	Class II Major Histocompatibility Complex Transactivator
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
cDNA	Complementary DNA
crRNA	CRISPR RNA
Cas9	CRISPR-Associated protein 9
DNA	Deoxyribonucleic Acid
DPPA3	Developmental Pluripotency Associated 3
DNMT3L	DNA Methyltransferase 3 Like
DSB	Double-Strand break
DMEM	Dulbecco's Modified Eagle Medium
E5	Embryonic Day 5
EED	Embryonic Ectoderm Development
ESCs	Embryonic Stem Cells
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
ELISA	Enzyme-linked immunosorbent assay
EPI	Epiblast
ERK	Extracellular-Signal-Regulated Kinase
FA	Fanconi Anemia

FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FACS	Fluorescence activated cell sorting
FISH	Fluorescent In Situ Hybridization
FRZB	Frizzled Related Protein
GATA2-6	GATA Binding Protein 2-6
ENPEP	Glutamyl Aminopeptidase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HnrnpK	Heterogenous Nuclear Ribonucleoprotein K
HnrnpU	Heterogenous Nuclear Ribonucleoprotein U
H2AK119ub	Histone 2A Lysine 119 ubiquitination
H3K27me3	Histone 3 Lysine 27 trimethylation
H3K4me3	Histone 3 Lysine 4 trimethylation
HDAC3	Histone Deacetylase 3
HDR	Homology-Directed Repair
hESCs	Human Embryonic Stem Cells
HLA	Human Leukocyte Antigen
IGF2	Insulin Growth Factor 2
IFN γ	Interferon-Gamma
IDR	Intrinsically Disordered Region
JAK	Janus Kinase
KO	Knockout
KSR	Knockout Serum Replacement
KLF17	Kruppel Like Factor 17
LIF	Leukemia Inhibitory Factor
lncRNA	Long Non-Coding RNA
$\mu Ci^{51}Cr$	Micro-Curie Chromium 51

MEFs	Mouse Embryonic Fibroblasts
MSX2	Msh Homeobox 2
MINUTE-ChIP	Multiplexed Indexed Unique Molecule T7 Amplification End-to-End Sequencing-Chromatin Immunoprecipitation
NANOG	Nanog Homeobox
NK cell	Natural Killer Cell
NODAL	Nodal Growth Differentiation Factor
NEAA	Non-Essential Amino Acids
NHEJ	Non-Homologous End-Joining
NCoR	Nuclear Receptor Corepressor
NR2F2	Nuclear Receptor Subfamily 2 Group F Member 2
NuRD	Nucleosome Remodeling and deacetylase
F12	Nutrient Mix F12
O_2	Oxygen
PFA	Paraformaldehyde
PBS	Phosphate-Buffered Saline
PEDF	Pigment Epithelium-Derived Factor
PRC1	Polycomb Repressor Complex 1
PRC2	Polycomb Repressor Complex 2
OCT4	POU Class 5 Homeobox 1
PE	Primitive Endoderm
PAM	Protospacer Adjacent Motif
RPE	Retinal Pigment Epithelium
RT-PCR	Reverse Transcription Polymerase Chain Reaction
ROCKi	Rho-Kinase Inhibitor
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein

RYBP	RING1 And YY1 Binding Protein
RRM	RNA-Recognition Motif
SMRT	Silencing Mediator for Retinoid or Thyroid-Hormone Receptor
sgRNA	Single-Guide RNA
SPEN	Spen Family Transcriptional Repressor
SPOC	SPEN paralogue/orthologue C-terminal
SOX2	SRY-Box Transcription Factor 2
SSEA3	Stage-Specific Embryonic Antigen 3
SSEA4	Stage-Specific Embryonic Antigen 4
SMCHD1	Structural Maintenance of Chromosomes Flexible Hinge Domain Containing 1
SUZ12	SUZ12 Polycomb Repressive Complex 2 Subunit
TIGIT	T Cell Immunoreceptor With Ig And ITIM Domains
tracrRNA	Trans-Activating CRISPR RNA
TFAP2C	Transcription Factor AP-2 Gamma
TFCP2L1	Transcription Factor CP2 Like 1
TGF-β	Transforming Growth Factor Beta
TE	Trophectoderm
Tsix	TSIX Transcript, XIST Antisense RNA
TP63	Tumor Protein P63
VEGF	Vascular Endothelial Growth Factor
WGS	Whole-Genome Sequencing
XCI	X Chromosome Inactivation
XCU	X Chromosome Upregulation
XIST	X-inactive Specific Transcript

1 INTRODUCTION

The first hESC lines were derived from human preimplantation embryos 24 years ago (1998)[1]. Since then, distinct stem cell states have been discovered, one of which representing the post-implantation epiblast cells and was named primed hESCs. With characteristics such as self-renewal and the potential to differentiate into to most, if not all, cell types that make up the human body, hESCs are of great interest and show a lot of potential for regenerative medicine and developmental biology alike[2]. Therefore, differentiation protocols have been established for a multitude of cell types, e.g. RPE cells for treatment of age-related macular degeneration (AMD)[3–6], dopaminergic neurons for treatment of Parkinson's disease[7–9], and beta cells for treatment of diabetes[10–12].

Simultaneously, hESCs culture conditions were further explored and improved[13], leading to feeder-free culture methods and development of naive hESC culture[14–17], which in contrast to primed hESCs represents the preimplantation epiblast. These naive hESCs are characterized by a domed morphology, specific transcriptional profile, epigenetic signature and metabolism, as well as biallelic X chromosome expression[14–16,18,19]. Furthermore, naive hESCs have the capacity to differentiate to the trophectoderm lineage[20–22]. Evidently, these preimplantation epiblast-like cells are advantageous for research on processes occurring during embryo development, such as lineage commitment events and X chromosome inactivation in females to ensure dosage compensation between the sexes.

Gene editing was revolutionized by the discovery and development of CRISPR/Cas9[23–28]. In 2012, the technology was shown to be programmable, which meant that it was possible to cut DNA at designated positions using dual RNAs together with the catalytic Cas9 protein[26]. It was early on proposed that this system could have the capacity to edit human DNA[26], which was soon thereafter demonstrated in 2013[27]. Since then, the gene editing technology has developed from being mainly plasmid-based to now include ribonucleoprotein complexes using improved variants of Cas9 protein and modified single-guide RNAs (sgRNA)[29,30].

In this thesis, I have described how we genetically modified primed hESCs to avoid recognition by immune cells. The modified hESCs were differentiated to RPE cells, which were tested *in vitro* against human T cells and NK cells as well as *in vivo* in an animal model. Through this study we learnt about potential risks of off-targets after gene editing and evaluated the potential of modified RPE cells as a cell therapy. Next, I detail how we inhibited chromatin remodelers PRC2 and SPEN, in two separate studies. When PRC2 was inhibited through pharmacologic or genetic ablation in naive hESCs, these cells drastically changed transcriptional and epigenetic profiles over the course of seven days. We learnt that PRC2 shields naive hESCs from differentiation into trophectoderm and mesoderm lineages. In the study where *SPEN* was knocked out, we again saw a dramatic effect in naive hESCs with the lncRNA *XIST* being severely impacted. Thus, we learnt that there is a relationship between the transcriptional repressor SPEN and the lncRNA *XIST*, and that loss of these factors led to failure to support these cells in the naive state.

In summary, in this thesis, hESCs have been combined with gene editing to try to answer questions about the suitability of gene edited cells for cell therapy and how inhibition of chromatin remodelers affects the primed and naive stem cell states, which in turn provides insight into the processes they govern in the human embryo. The findings presented in this thesis will hopefully provide a platform upon which new studies can be designed, so that we can continue to develop new models with which we can describe and understand human development.

2 LITERATURE REVIEW

2.1 EMBRYOGENESIS

During the first days of development the human embryo goes from being unicellular to multicellular while simultaneously transitioning from relying on maternally loaded transcripts to activating its own genome. This maternal to zygotic transition occurs between the four- and eight-cell stage in human, after which compaction begins and the morula is formed[31]. Around embryonic day 5 (E5) after fertilization a blastocoel, or cavity, forms inside the embryo[31], which coincides with lineage specification[32] (Figure 1). Trophectoderm (TE) forms the outer layer, which later will give rise to the placenta while the yolk sac will be formed by the primitive endoderm (PE) and the embryo proper is formed by the epiblast (EPI)[31]. These lineages segregate before the blastocyst matures and expands to prepare for implantation[32].
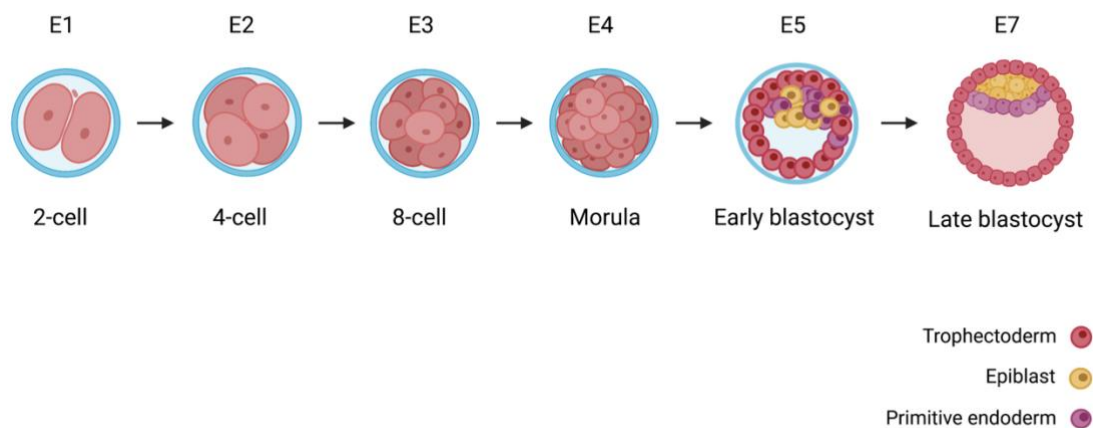

Figure 1| Human embryo preimplantation development. The embryo undergoes several cell divisions and when reaching E5, lineage specification takes place resulting in three emerging cell types: epiblast, primitive endoderm and trophectoderm. Afterwards, the embryo starts expanding and hatches from the zona pellucida (blue circle enclosing the embryonic cells). Created with BioRender.com.

Upon implantation, which takes place around E8, the embryo is imbedded in the uterine wall and TE cells start to migrate and differentiate, initiating the formation of what will become the placenta[33]. Pro-amniotic cavity formation and polarization of the EPI cells occurs around E8-9, while the bilaminar disc and yolk sac emerges around E11[33]. Through the process of gastrulation, migrating EPI cells form the primitive streak, which is as far as scientists are permitted to culture human embryos in accordance with Swedish law (SFS 2006:351) and international ethical regulations (14-day rule)[34].

To decipher what factors dictate successful development, more studies on key processes such as lineage commitment, X chromosome inactivation and implantation are necessary. Not unlikely, these studies will first be performed using alternative embryo-like models, such as hESCs, after which follow-up validation can be performed using actual human embryos.

2.1.1 Human embryonic stem cells

The inner cell mass of the pre-implantation embryo is used to derive hESCs[1]. Characteristic morphology of hESCs include high nucleus to cytoplasmic ratio, pronounced nucleoli and compact 2D colonies[1]. In addition, these cells have long telomeres, can self-renew unlimitedly and have the ability to differentiate into essentially any cell type of the body[1]. Pluripotency status of hESCs is assessed by three criteria;

1. cell surface marker genes such as *SSEA3*, *SSEA4* and *TRA-1-60*,
2. transcription factors *OCT4*, *SOX2* and *NANOG*, and,
3. their ability to differentiate *in vitro* and *in vivo* into the three germ layers; endoderm, ectoderm and mesoderm[35].

Initially, hESCs were derived on mouse embryonic fibroblasts (MEFs) as a support and feeder layer, however, advances in derivation procedures and culture techniques have rendered the use of xeno-derived products unnecessary in most protocols, making the hESCs culture models clinically relevant[13]. With these characteristics, hESCs have made their way into regenerative medicine research where they are used to develop cell differentiation protocols[36], several of which have entered clinical trials[37]. Additionally, hESCs can be classified as naive and primed hESC, representing pre- and postimplantation epiblast cells, respectively.

2.1.2 Primed and naive pluripotency states

The concept of naive and primed pluripotency states were first described in mouse, where naive cells were derived from the preimplantation blastocyst while primed cells were derived from the post-implantation embryo[38]. Primed hESCs are similar to the epiblast stem cells derived from the post-implantation mouse embryo[39,40]. They share features such as expression of *OCT4*, *NANOG* and *SOX2*, flattened morphology and self-renewal dependency on FGF and ACTIVIN[41]. More recently, naive pluripotency was established for human pluripotent cells and it differs considerably from human primed pluripotency[14–17,42].
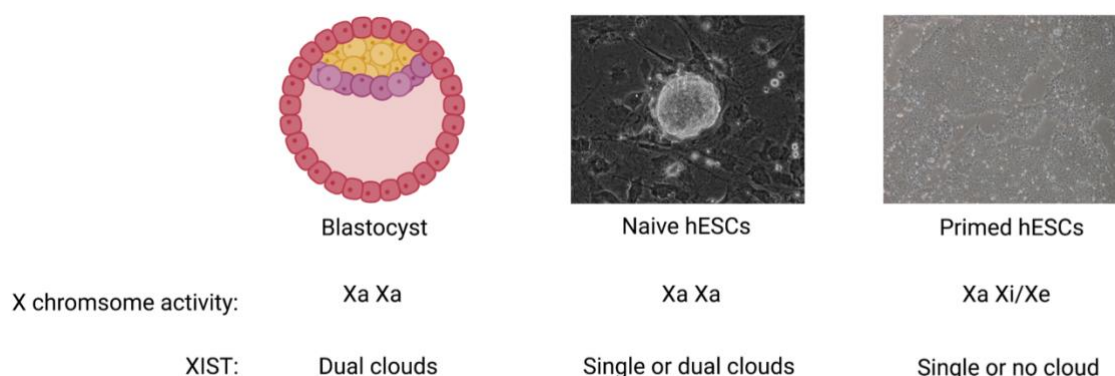
Figure 2| X chromosome activity and presence of *XIST* clouds on them. Naive hESCs share features with the preimplantation epiblast (yellow cells in blastocyst), such as both X chromosomes being actively transcribed (XaXa) and coated with *XIST* RNA. In contrast, primed hESCs have undergone classical XCI, thus only one X chromosome is active whereas the other is inactivated (XaXi). Time in culture often leads to partial reactivation of the inactive X chromosome, generating an eroded X chromosome (XaXe). Created with BioRender.com

The two stem cell states display differences in morphology, metabolism, epigenetic signatures and allelic expression state of X chromosome genes[14–17,19]. In female naive hESCs, *XIST* is upregulated and coats one or both X chromosomes (Figure 2). Upon differentiation to the primed pluripotency state, X chromosome expression becomes monoallelic as a result of X chromosome inactivation and only the inactive X chromosome is coated by *XIST*[18,43,44] (Figure 2). It is common among primed hESCs to lose *XIST* expression with time in culture and end up in an eroded state, where some genes, other than the classical escapees, are re-expressed from the previously inactive X chromosome[18,43,44]. Reverting primed hESCs into a naive state leads to reactivation of the inactive X chromosome and expression of *XIST* is restored[14–18]. Although the naive state models the preimplantation epiblast, differentiation back to the primed state uncovered preferential inactivation of the previously inactive X chromosome[18]. Efforts put into studying the naive culture conditions, further showed that it is possible to derive naive hESCs that will undergo random XCI upon differentiation[42,45].

2.2 CRISPR/CAS9 TECHNOLOGY

Clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas) is a bacterial immune system evolved to recognize recurrent viral infections[23–25]. The CRISPR/Cas system's ability to identify invading nucleic acids, bind and incapacitate them, was harnessed into a gene editing system with the capacity to specifically target and modify genes in eukaryotic cells[26,27]. As a gene editing technology, the Cas protein, with Cas9 being the most commonly used, is guided to its target site by a short RNA oligonucleotide designed specifically for the gene of interest[26,27]. The RNA/protein complex binds to the targeted genomic DNA and cleaves it, causing a double strand break[26,27]. The break is not always repaired flawlessly and insertion/deletion (indel) mutations are frequently generated at the cut site, which is exploited to create knockout models[27]. Although CRISPR/Cas is an exceptional gene editing tool, each guide RNA needs to be evaluated for its target specificity[46]. Promiscuous guide RNAs can bind to genomic sequences without exact homology, potentially leading to DNA breaks at unknown sites with various side-effects[46]. As a result, several *in silico* tools assessing sgRNA on-target efficiency and specificity, as well as predicting potential off-target sites have arisen alongside the gene-editing technology[47,48(p),49–52].

2.2.1 Gene editing

CRISPR/Cas was discovered in bacteria as short viral sequences integrated in the bacterial DNA[23,24]. Once the bacteria were infected by a virus, these short sequences were transcribed along with a Cas protein[23,25]. When complexed, the transcribed RNA guides the Cas protein to its target virus DNA and cuts it, thus leading to its degradation and the survival of the bacteria[23,25]. Engineering the CRISPR/Cas system to become a genome editing tool was done by generating a single-guide RNA (sgRNA) by combining the CRISPR RNA (crRNA, which contains the spacer), and the trans-activating (tracr) RNA[26,46]. The spacer sequence of the sgRNA is customizable to any genomic target, with the requirement that there is a protospacer adjacent motif (PAM) at the site[26,46]. The PAM sequence function as a DNA binding signal for the Cas protein, and depending on the Cas protein this sequence varies in length and motif[53].

The Cas protein forms a complex with the sgRNA that binds to the complementary genomic sequence, where it undergoes a conformational change aligning the catalytic sites with the DNA, allowing for it to be cut[26,27]. For Cas9, the cut occurs three base pairs upstream to the PAM.

2.2.2 DNA damage-repair pathways

Cuts generated by Cas9 are double strand breaks (DSBs), which are severe lesions in need of repair for normal cell function and survival[54]. Non-homologous end joining (NHEJ) and homology-directed repair (HDR) are the two main repair-pathways following DSBs in eukaryotic cells[54,55]. NHEJ can result in accurate repair or lead to the introduction of mutations at the cut site that is represented by insertion or deletion of few or many bases (Figure 3) [55,56]. In contrast, HDR can utilize homologous sequences that are either found endogenously or that have been deliberately delivered to the cell, thus leaving the repair site flawless (Figure 3)[55]. The Fanconi anemia (FA) pathway has been shown to be implicated in single-strand template repair (exogenous DNA)[57]. Knockout of FA genes revealed that the pathway is involved in balancing the repair outcomes between single-strand template repair and NHEJ, keeping editing efficiencies relatively similar[57].
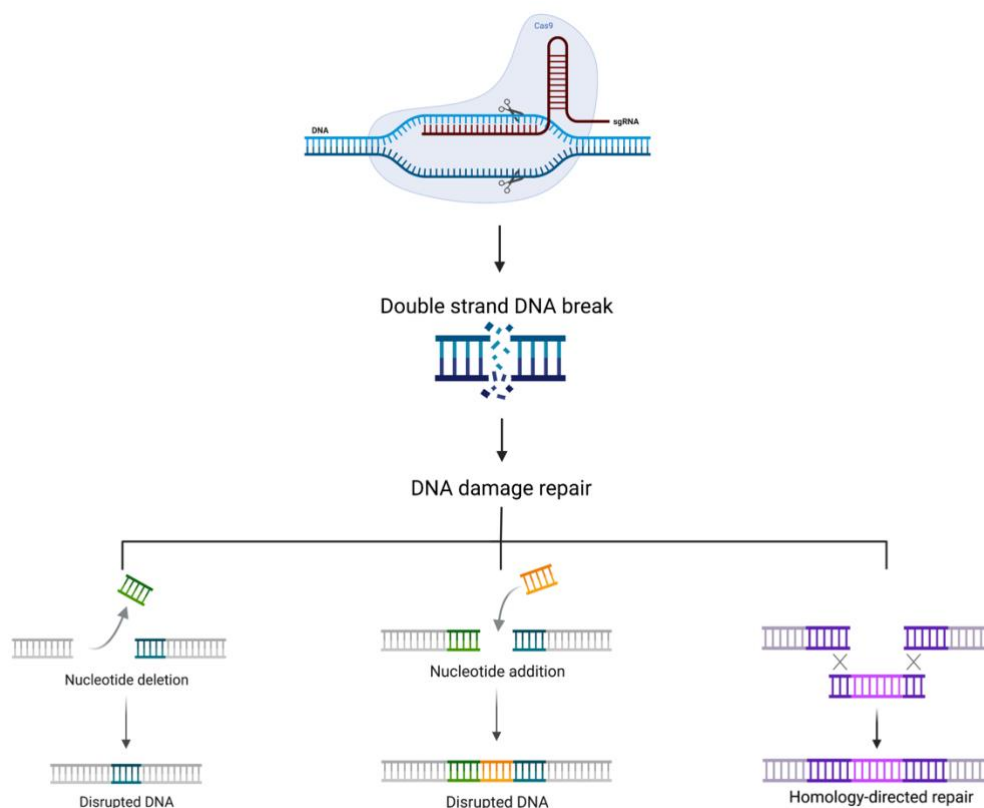
Figure 3| CRISPR/Cas9 gene editing and downstream repair pathways. Cas9 is guided to its genomic target by a sgRNA. Once bound to the DNA, Cas9 performs a double-strand cut leaving the DNA strands in need of repair. The two most common pathways for repair are NHEJ, which generates deletions or insertions at the cut site (left and middle), and, HDR, which uses a template to restore the DNA to what it was before or to introduce specifically designed features where the break occurred (right). Created with BioRender.com.

2.2.3 Off-targets: causes and solutions

Even though the CRISPR/Cas system is highly specific, there are still risks with off-target events due to sequence similarity between the twenty-nucleotide spacer and the three billion bases in the human genome. Studies of the mechanisms controlling Cas9 activity uncovered that the PAM sequence was required for DNA unwinding and that the specificity of Cas9 was strongly dictated by the 7-12 base pairs proximal to the PAM[58–60]. Mismatches further along the spacer are tolerated[46]. DNA breaks at unknown positions in the genome pose a risk, particularly if these breaks lead to gene knockouts and chromosomal truncations. As a result, several different *in silico* prediction tools have been developed to estimate the on- to off-target efficiency and identify potential off-target locations while designing the sgRNA[47–49,51]. It has also become more common to apply sequencing-based approaches to determine the off-target load in the modified cells[61–63], where the most powerful analyses study the whole genome and not only predicted sites. Furthermore, deriving single-cell clones is necessary to reduce heterogeneity since different genotypes may arise following CRISPR/Cas editing[64], leading to a diverse population with differences in off-target load and position.

2.3 REGENERATIVE MEDICINE AND CELL THERAPY

The differentiation potential that hESCs possess make them a highly relevant cell source for cell therapy. This exciting development is also shedding light on availability, specifically on the HLA matching[65] that is required before transplantation. Thus far, four strategies have been developed:

1. disrupting the function of all major HLA class I and class II genes[66–69],
2. generating homozygous super donors by knocking out specific HLA alleles[66,70–72],
3. overexpressing HLA-E/G to reduce the innate immune response[67,73], and,
4. creating haplobanks with human pluripotent stem cells[74,75].

All of these strategies significantly reduce the number of stem cell lines required to produce cell therapies for all patients in need.

2.3.1 Presentation of HLA class I and HLA class II

There are two classes of major histocompatibility complexes in humans, each represented by a set of highly polymorphic genes that help confer immunity[76]. The HLA class I locus consist of three major genes: *A*, *B* and *C*, whereas the HLA class II locus is made up by *DP*, *DQ* and *DR*[76,77] (Figure 4).

HLA Class I
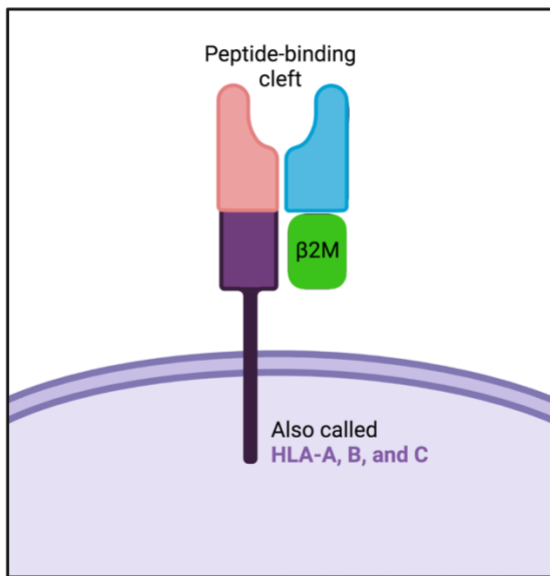
HLA Class II

Figure 4| Presentation of HLA class I and HLA class II molecules on the cell surface. In the peptide-binding cleft, antigens are presented to the immune cells with which the cells are interacting. Adapted from "MHC class I and MHC class II", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.
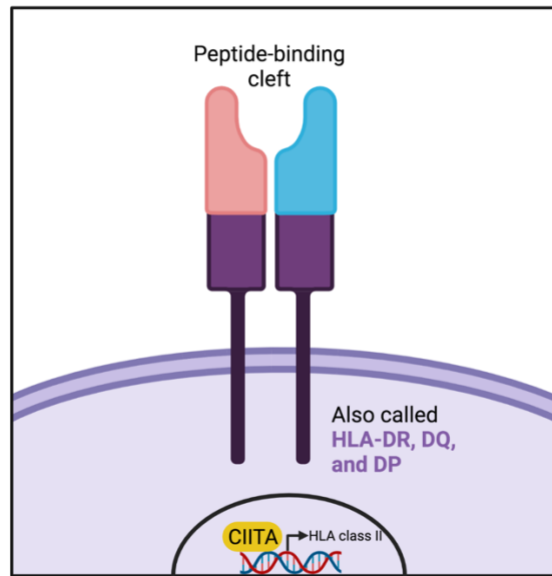
These genes are inherited co-dominantly, thus both maternal and paternal alleles are expressed equally[76]. Nucleated cells express HLA class I molecules, whereas HLA class II genes are expressed mainly by antigen presenting cells, however, under inflammatory conditions other, non-immune cells can upregulate HLA class II as well[76,77].

2.3.2 Transplantation rejection

There are two pathways of communication between allogenic cells and T lymphocytes; the direct and the indirect. The direct pathway signals through the interaction of the HLA class I molecule on the graft cell and the receptor on cytotoxic T cells[76]. The T cells recognize the structure formed by the allogenic HLA molecule and the bound peptide, which resembles a self HLA-foreign peptide complex[76]. The indirect pathway allows antigen-presenting immune cells to communicate with helper T cells through HLA class II molecules[76]. Usually, these immune cells have encountered a microorganism in the extracellular space, or in the case of transplantation, grafted cells. Activated helper T cells produce cytokines, which in turn activates the antigen presenting cell[76]. According to European standards, a 10/10 match is desirable for *HLA-A*, *-B*, *-C*, *-DRB1* and *-DQB1* loci because mismatches among these genes between donor and patient are associated with complications following transplantation[78].

2.3.3 Universal stem cells for off-the-shelf use

Highly diverse HLA class I and II alleles increase the chance of the body successfully developing an effective immune response against a large range of antigens[76]. A resulting medical and technical challenge is that great variance contributes to the difficulties in finding

matches for transplantations[78]. Creating haplobanks of human pluripotent stem cells lines that match the general population is an enticing proposal[74,75]. Studies of the HLA haplotypes among the populations of Japan and the United Kingdom detail the methods with which they have concluded that 50 induced pluripotent cell lines, would be necessary to cover 90,7% and 79% of the populations, respectively, only at loci *HLA-A*, *-B*, and *-DR*[74,79]. With the advances in gene editing, several efforts have been made to create so called universal (hypoimmunogenic) stem cells that can be utilized for downstream cell therapeutic applications[66–69,80,81]. Most of the strategies have involved modifying the *B2M* gene to knockout HLA class I[66–69]. B2M dimerizes with each of the HLA class I molecules thus allowing for their proper presentation on the cell surface (Figure 4)[76]. Consequently, loss of B2M led to internalization of HLA class I proteins. HLA class II genes have been targeted through the transcriptional master regulator *CIITA* (Figure 4)[82–84]. In summary, the combination of *B2M* and *CIITA* knockouts creates a cell type that cannot upregulate any major HLA genes.

2.3.4 Age-related macular degeneration and the retinal pigment epithelium

Age-related macular degeneration (AMD) is the leading cause of vision deterioration in the elderly population in industrialized countries[85,86]. It is caused by degeneration of the cells within the macula, gradually resulting in complete vision loss in the center field of vision[85]. In advanced cases of the disease, geographic atrophy or neovascularization is evident[85]. The former is a result of loss of the RPE and eventually the rest of the retinal cells, and the latter is a result of new vasculature growing beneath the retina which leads to detachment of the RPE and retinal layers[85].

The cells of the RPE are situated between the photoreceptors and Bruch's membrane, which in turn sits on top of the choriocapillaris[87]. RPE cells are pigmented, polarized, and have a hexagonal shape, creating a cobblestone-like monolayer of terminally differentiated cells that are important for maintaining homeostasis of the retina[87]. Specifically, the RPE functions as a barrier between the blood and the photoreceptors, also referred to as the neural retina[87]. The outer segments of the photoreceptors interact with the apical membrane of the RPE, where the RPE aid in the exchange of the outer segment disks by phagocytosing and degrading them[87,88]. The turnover of the outer segments is necessary to maintain the excitability of the photoreceptors[87]. Nutrients, ions and water are transported across the basal membrane[87]. In addition, RPE cells secret growth factors required for the photoreceptors, such as PEDF[89], and for the choroid, such as VEGF[88]. In diseases such as AMD, where the RPE degenerate, retinal homeostasis is severely affected, eventually leading to photoreceptor death and vision loss[86,87].

Since visual impairment restricts an individual's everyday life, developing therapies that prevent, delay or ultimately cure vision loss would greatly impact quality of life. Several studies have developed efficient protocols for generating RPE cells from human pluripotent stem cells[3–6,90–92], which is the first step towards developing a cell-based therapy against AMD.

2.4 LINEAGE COMMITMENT POTENTIAL IN NAIVE hESCs

Primed hESCs are derived from the postimplantation epiblast, which is why their differentiation potential is thought to be restricted to lineages arising after implantation[93]. In contrast, naive hESCs have been shown to retain the capacity to differentiate into trophectoderm, a lineage choice that is made before the blastocyst is formed[21,22,94,95]. Inhibition of NODAL and ERK rapidly induced trophectoderm differentiation of naive hESCs, which acquired cytotrophoblast, syncytiotrophoblast and trophoblast stem cell fates with time in culture[96]. Interestingly, outgrowths of the human epiblast uncovered that the pluripotent cells can regenerate the trophectoderm, suggesting there is plasticity among the inner cell mass cells even at the blastocyst-stage[22]. Another study used previously published single-cell RNAseq data from E5 and E7 preimplantation embryos to find cell surface markers of TE, which could be used to identify gene targets that would induce trophectoderm lineage commitment[95]. Again, inhibition of NODAL and ERK signaling pathways along with inhibition of JAK and stimulation BMP4 were described to robustly drive trophectoderm differentiation of the naive hESCs[95].

Development of blastocyst-like structures (blastoids) further strengthened the unrestricted potential of naive hESCs[94,97]. Blastoids mimicking the transcriptional signature and tissue architecture of the E6-7 blastocyst, were developed by inhibiting ERK and NODAL signaling pathways in 3D-cultured naive hESCs[94]. Lineage segregation into epiblast, hypoblast and trophectoderm was confirmed by comparison with *in vitro* cultured preimplantation embryos[94].

2.4.1 Chromatin remodeling and promoter bivalency

Histone modifications are associated with active or repressed regions of DNA, thus influencing gene expression[98]. Their enrichment at different genes varies depending on the gene and on the cell type[98]. Interestingly, two such histone modifications, H3K4me3 (activating) and H3K27me3 (repressive, catalyzed by PRC2), are found simultaneously enriched at certain gene promoters[98]. These bivalent domains have been shown to be particularly important for genes that change their status during development and are therefore kept in a poised state to facilitate a fast and accurate switch from active to repressed, or vice versa[98].

Murine ESCs lacking the PRC2 subunit EED were shown to increase transcription levels of *Gata3*, *Gata4* and *Gata6*, all of which are associated with differentiation[99]. Further examination of polycomb group target genes, were found to be similarly de-repressed in the absence of EED, supporting a role for PRC2 in maintaining pluripotency during embryonic development[99]. Similarly, the SUZ12 subunit of PRC2 was found at 8% of genes in hESCs, out of which 50% were involved in developmental processes[100]. Furthermore, the pluripotency factors OCT4, SOX2 and NANOG were found to bind a subset of these developmental genes that were occupied by PRC2, indicating that there is a connection between pluripotency and repression of developmental regulators[100].

2.5 X CHROMOSOME INACTIVATION

During preimplantation development, the embryo needs to undergo dosage compensation to compensate for having two X chromosomes in female cells while only having one copy in males. Different species have developed various strategies to resolve this challenge; *D. melanogaster* males upregulate the single X chromosome two-fold while *C. elegans* females downregulate the expression from both X chromosome to half and mammalian females inactivate one of the X chromosomes whereas the other remains active[101]. Recent advances in mouse X chromosome dynamics have led to a new model for XCI, where the inner cell mass cells experience two waves of X chromosome upregulation (XCU) concomitant with imprinted (first wave) and random (second wave) XCI[102]. Interestingly, rather than having distinct active and inactive X chromosome states throughout XCI, the model identifies XCU as a tuning mechanism that is used to balance the expression from the active X chromosome in relation to the future inactive X chromosome[102]. Studies of human preimplantation embryos have uncovered that both X chromosomes are active and coated with XIST RNA, and that XCI has started but is not completed by day 12[32,103–105]. Due to an increased awareness of species-dependent differences in dosage compensation, together with the shortage of human embryos available for research, naive hESCs have been used as a model system for the study of the human X chromosome inactivation process[18].

2.5.1 XIST

The lncRNA gene *XIST* is conserved in many mammalian species, such as cow, mouse and human[106]. The human *XIST* transcript is up to 19kb long, depending on the splice variant[107,108]. It contains several repetitive elements (A-F), serving different functions in the silencing process[109]. Early studies using mouse cells focused on the repeat A, which was found to be crucial for *Xist*-mediated silencing to occur[106,109]. This repeat region consists of 7.5 and 8.5 stem loops in mouse and human, respectively, that create a specific secondary structure found to confer silencing by recruiting repressor complexes[109–111]. Furthermore, cells lacking repeat A failed to silence the X chromosome even though it was coated by the *Xist* RNA, suggesting impaired recruitment of chromatin remodelers[109]. In contrast, human K562 cells lacking repeat A only moderately affected the *XIST* cloud formation and no global effect was observed on X-linked gene expression on the inactive X chromosome[112]. Therefore, it was suggested that repeat A may function during human embryogenesis when silencing of the X chromosome is initiated rather than in the maintenance of the already established landscape of the inactive X chromosome in differentiated cells[112]. Interestingly, erosion, or partial reactivation, of the inactive X chromosome, actually precedes loss of *XIST*, suggesting that epigenetic changes, rather than *XIST* per se, drive the alterations in allelic gene expression[43].

Other repeat regions of *Xist* have been implicated in recruitment of chromatin remodelers, spreading of heterochromatin and localization of *Xist* RNA on the future inactive X chromosome[113]. Repeat B and C were shown to be required to recruit non-canonical PRC1 by anchoring *Xist* to HNRNPK[114–116]. This Xist-HNRNPK-PRC1 pathway was also demonstrated to recruit SMCHD1, a protein that is required for *Xist* localization to the inactive X

chromosome[115,117]. Loss of the B and C repeats have been shown to lead to dispersed *Xist* clouds and depletion of H3K27me3 and H2AK119ub, which are laid down by PRC2 and PRC1, respectively[113,118,119]. Particularly, deletion of the B repeat led to less compaction of the X chromosome, which in turn affected PRC1 and its downstream effectors causing impairment of inactivation of genes that are slowly silenced during XCI[120]. Further deletional studies of *Xist* revealed that loss of repeat F caused decreased cloud intensity in combination with a reduction in H2K27me3 and H2AK119b, while loss of repeat E led to widespread dispersion of *Xist* RNA after four days of differentiation and aberrant regulation of genes that are known to escape XCI[113,121,122].

2.5.2 X chromosome inactivation during preimplantation development

Mammals achieve dosage compensation between the sexes by inactivating one of the X chromosomes in females. In mouse, this occurs in two waves during preimplantation; the first being imprinted and the second being random in the epiblast[105,123]. Imprinted XCI in mouse leads to inactivation of the paternally inherited X chromosome in all the embryonic and extraembryonic tissues[123–127]. Once the blastocyst has formed around E4.5-5.5, the paternal X chromosome is reactivated in the inner cell mass, which gives rise to the embryo proper[123]. Upon implantation, X chromosome inactivation is random in these cells while the paternal X chromosome remains inactive in the extraembryonic tissues[123,126,127]. In contrast, human preimplantation embryos do not undergo imprinted XCI, and it is not yet known how *XIST* mediates dosage compensation through random XCI. Two studies using single-cell RNA sequencing to analyze human preimplantation embryos found that the transcriptional output from both of the X chromosomes were balanced until E6-7[32,103]. Initiation of dampening of the transcriptional output from the X chromosomes was observed at E4 and maintained until E7, when the embryo is preparing for implantation[32]. Furthermore, although random XCI has begun it is not considered fully completed even at E12[103].

2.5.3 Protein interactome of XIST

In mouse, *Xist* interacts with a multitude of proteins during the different phases of XCI. Mass spectrometry of *Xist*-binding proteins in mouse ESCs and epiblast stem cells, captured 81 proteins in total including those encoded by the genes *HnrnpK*, *HnrnpU* and *Spen*[111]. HNRNPK interacts with *Xist* RNA repeats B and C, and through this connection non-canonical PRC1 is recruited, which lays down the repressive H2AK119ub mark on the future inactive X chromosome[111,119,128]. Although loss of HNRNPK had no effect on *Xist* localization, ablating HNRNPU led to dispersal of *Xist* in the nucleus[111].

Another protein interacting with *Xist*, specifically through repeat A, is SPEN[110,111,129–131]. It was demonstrated not to be needed for localization of *Xist* RNA[111], instead it was shown to be crucial for initiation of XCI. *Spen*-knockout cells displayed abrogated *Xist* gene silencing function, which, in addition, resulted in impaired recruitment of chromatin modifiers[110,129,132,133].

Specifically, the SPEN paralogue/orthologue C-terminal (SPOC) domain interacted with corepressors NCoR and SMRT, which are known interactors of HDAC3[133]. Another study proposed that HDAC3 was pre-loaded on the future inactive X chromosome, and that the protein was activated upon differentiation leading to deacetylation[134]. The epigenetic landscape is then re-shaped by rapid accumulation of H2AK119ub mark, which is later followed by the addition of the PRC2 mark H3K27me3[134]. Furthermore, the m^6A methyltransferase complex as well as the NuRD complex were also identified to bind to the SPOC domain of SPEN, leading to the conclusion that SPEN binds to *Xist* RNA and then connects it with a variety of transcriptional regulators and chromatin modifiers[133]. The RNA recognition motifs (RRM) were shown to be crucial for linking SPEN to *Xist* RNA, where *Spen* mutants lacking RRM2-4 failed to accumulate on the future inactive X chromosome[133]. Additionally, SPEN ensures that *Xist* is upregulated and that the RNA remains stable in the cells once differentiation has begun[132]. RNA stability was achieved by SPEN binding to the promoter of the *Xist* antagonist *Tsix*, after which HDAC3 were either recruited to or activated at the site, thus removing acetyl groups and weakening the *Tsix* promoter[132].

The number of *Xist* foci on the future inactive X chromosome within a nucleus was determined to be approximately 50, each one containing ~2 *Xist* RNA molecules[120]. These foci create compartments into which protein interactors are recruited at concentrations far surpassing that of *Xist*[120]. Among the proteins found in these compartments were SPEN, RYBP and EZH2, respectively, as well as HNRNPK[120]. Here, SPEN was shown to increase in concentration upon differentiation with around 35 molecules of SPEN being detected within one compartment (to the 2 *Xist* molecules in the same space)[120]. Interestingly, the intrinsically disordered regions (IDRs) located within the gene body of SPEN, were found to be essential for accumulation of SPEN in these compartments[120]. In addition, silencing was severely affected by the decrease in SPEN concentration in SPEN IDR-mutants[120].

3 RESEARCH AIMS

The overall aim of my research projects have been to use CRISPR/Cas9 to create models for studies in regenerative medicine and developmental biology. Specifically, the aims for the three projects are the following:

I. The first aim of paper I, was to knockout HLA-associated genes *B2M* and *CIITA* in primed hESCs to create hypoimmunogenic stem cell lines while retaining the potential to differentiate into RPE cells. The second aim of paper I, was to test the modified hESCs and their RPE derivatives against T cells and NK cells to evaluate their immune-activating capacities as well as studying the immune response after transplantation into the rabbit xeno-model.

II. In paper II, the first aim was to study bivalent genes in naive and primed hESCs, respectively, to uncover differences between the two stem cell states. The second aim was to study the effect of loss of the PRC2 complex in both stem cell states through pharmacologic and gene editing approaches targeting different subunits of the PRC2 complex.

III. In paper III, the aim was to generate single-cell derived *SPEN*-KO hESC lines and revert them to the naive state to investigate the relationship between SPEN and *XIST*. Thus, we wanted to uncover if there is a connection between the human genes, which correlate with that recently described in mouse.

4 MATERIALS AND METHODS

Samples, materials and methods (**Table 1**) are thoroughly described in the methods section in each paper included in this thesis. Additionally, a selection of methods and their rationale will be discussed here.

Table 1| Methods and samples that have been used to acquire the data presented in this thesis.

Method	Samples	Paper
Ethical statements	Human embryonic stem cells, rabbits	I, II, III
Cell culture	Primed and naive hESCs, HEK293T cells	I, II, III
Co-culture with immune cells	Primed hESCs, RPE cells, T cells, NK cells	I
Gene editing, molecular cloning, electroporation	Primed and naive hESCs, HEK293T cells	I, II, III
PCR and Sanger sequencing	Primed hESCs	I, III
Pharmacologic inhibition	Primed and naive hESCs	II
Immunofluorescence	Primed and naive hESCs	I, II, III
Histology	Rabbit retina	I
RNA extraction, RT-PCR	Primed and naive hESCs, RPE cells	I, III
RNA FISH	Naive hESCs	II, III
Immunoblotting	Primed and naive hESCs, RPE cells	I, II
Flow cytometry	Primed hESCs, RPE cells, NK cells	I
Fluorescence activated cell sorting (FACS)	Primed hESCs	I
Enzyme-linked immunosorbent assay (ELISA)	RPE cells, T cells	I
Subretinal transplantation of RPE cells	RPE cells, rabbits	I
Multimodal retinal imaging, retinal measurements	Rabbits	I
Whole-genome sequencing (WGS) analysis	Primed hESCs, RPE cells	I
Minute-ChIP sequencing and analysis	Primed and naive hESCs	II
Bulk RNA sequencing and analysis	Primed and naive hESCs	II
Single-cell RNA sequencing and analysis	Primed and naive hESCs	II
Image analysis	Naive hESCs	II, III
Statistics		I, II

4.1 ETHICS

4.1.1 Human embryonic stem cells

Human embryonic stem cell lines HS975 and HS980 were derived in house from donated surplus embryos after obtaining informed consent from couples undergoing *in vitro*-fertilization and with approval from the Swedish Ethics Review Authority (Etikprövningsnämnden; dnr 2011/745-31 with amendment 2016/1528-32 and 2012/1765-31/1 with amendment 2016/2045-32).

4.1.2 Animal work

In **paper I**, New Zealand white albino rabbits were used during the subretinal transplantation of wildtype, B2M-single KO, CIITA-single KO, and B2M- and CIITA- double KO RPE cells, respectively. The animals were provided by Lidköping's rabbit farm (Lidköping, Sweden). Experiments were carried out at St Eriks Ögonsjukhus after approval from the Northern Stockholm Animal Experimental Ethics Committee (Dnr N25/14) and in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research.

4.2 CELL CULTURE

Human embryonic stem cells retain the capacity to divide, differentiate and have long, healthy telomers. In the studies that are included in this thesis, we take advantage of these characteristics by differentiating primed hESCs into RPE cells and by converting primed hESCs to naive hESCs, the latter mimicking the pre-implantation inner cell mass cells.

In **paper I**, we genetically modified primed hESCs, expanded them to obtain single cell-derived clones and then differentiated them into RPE cells. As RPE cells constitute a future cell therapy product and genetic engineering may one day become part of the solution to offer such a cell therapy product to a wider population, we wanted to test these cells in different *in vitro* co-cultures with donor-derived T- and NK cells, respectively. This allowed us to understand how the hESC-derived RPE cells would perform against human immune cells and complemented the *in vivo* data we obtained from the subretinal transplantation in the rabbit animal model.

In **paper II**, we used naive and primed hESCs to study how inhibition of PRC2 affected the two cell types, which represent the pre- and post-implantation epiblast, respectively, thus allowing us to appreciate the effects it could have in the human embryo. Not only were pharmacologic inhibitors used against PRC2 subunits EZH2 and EED, but also CRISPR/Cas9-mediated gene editing of *EED* to demonstrate that the effect observed after inhibitor treatment was retained by gene editing. Here, in contrast to **paper III**, gene editing was done in naive hESCs.

Again, in **paper III**, primed and naive hESCs were used to create a model with which it would be possible to study the relationship between human SPEN and *XIST*, which are known players in mouse XCI. Since XCI is a process that occurs in the early phase of embryogenesis, it is

hard to study, and models are necessary. Gene editing of *SPEN* was done in primed hESCs, which were expanded as single cell-derived clones and then converted to naive hESCs, allowing reactivation of the inactive X chromosome and upregulation of *XIST*.

4.2.1 Primed hESCs

Primed hESCs were cultured on 10µg/ml laminin 521 ((Biolamina; LN521-02) using Nutristem hESC XF medium (Biological Industries; 05-100-1A) at 37°C, 5% O_2 and 5% CO_2. hESCs were passaged when reaching 70-80% confluency. Primed cells used in **papers I and II** are HS980 and HS975 (derived in-house; ethical permit 2011/745-31 with amendment 2016/1528-32), respectively. In **papers II and III**, commercial hESC line H9 (Wicell; WA09) was used for the majority of experiments. These cells had been adapted to laminin 521 prior to use in these studies.

4.2.2 Naive hESCs

4.2.2.1 *Mouse embryonic fibroblast culture as matrix for naive cells*

Mouse (ICR) Inactivated Embryonic Fibroblasts (Gibco; A24903) were cultured on plates that had been pre-coated with 0.1% gelatin (Sigma-Aldrich; G1890) at 37°C, 5% O_2 and 5% CO_2 for at least 30 min. MEF media consisted of DMEM (Gibco; 11995-065) supplemented with 10% FBS (Gibco; 16140-071), 1% NEEA (Gibco; 11140050) 1mM Glutamine (Gibco; 25030-149) and 0.1mM beta-2-mercaptoethanol (Gibco; 21985-023). MEFs were plated at densities of around 333.000 cells per 6-well.

4.2.2.2 *Naive hESC culture*

Primed H9 cells were plated onto wells containing a high-density layer of MEFs and kept in Nutristem hESC XF medium (Biological Industries; 05-100-1A) supplemented with 10µM ROCKi (Merck; Y-27632) for 24h at 37°C, 5% O_2 and 5% CO_2. To establish naive hESCs, the manufacturer's protocol for NaïveCult™ Induction Kit (Stemcell Technologies; 05580) was followed. Briefly, 24h after plating the primed cells, media was changed to Induction Medium I supplemented with 0.75 mM Sodium Butyrate (Sigma Aldrich; B5887-1G). Daily changes using this media was carried out for a total of 3 days before changing to Induction Medium II on day 4. On the eleventh day, cells were passaged for the first time since starting the conversion protocol using TrypLE Select (Gibco; 12563011) and incubating at 37°C, 5% O_2 and 5% CO_2 for 4 min. The converting cells were pelleted, resuspended in Induction Medium 2 supplemented with 10µM ROCKi and plated in freshly-coated MEF wells. During the early passages, cells were split at a 1:1 ratio. This was repeated during passage 2, and at passage 3 medium was changed to Induction Medium 3, which the converting cells were kept in until passage 8. From passage 8 onwards, NaïveCult™ expansion medium was used to culture the now naive cells. During each passage, medium was supplemented with 10µM ROCKi and a 40µm cell strainer was used to remove larger sheets of MEFs. Between passages naive cells were cultured without the addition of ROCKi, and passaging was done every 4-5 days. In the

instances of the *SPEN* knockout cells in **paper III**, this time was increased to every 5-6 days due to slower growth rates/collapse of the $SPEN^{-/-}$ mutants.

In **paper II**, naive HS975 cells were cultured using the 5iLAF protocol[14]. In brief, the 5iLAF medium is composed of 240ml DMEM/F12 (Invitrogen; 11320) and 240ml Neurobasal (Invitrogen; 21103) supplemented with 5ml N2 supplement (Invitrogen; 17502048), 10ml B27 supplement (Invitrogen; 17504044), 1mM glutamine (Gibco; 25030081), 1% NEAA (Gibco; 11140050), 0.1mM beta-mercaptoethanol (Gibco; 21985023), 50mg/ml BSA (Sigma; A9418), 0,5% KSR (Gibco; 10828028), 1µM PD0325901 (Tocris; 4192), 0.5µM SB590885 (Sigma; SML0501), 1µM WH-4-023 (Sigma; SML1334), 1µM IM-12 (ENZO; BMLWN1020025), 20ng/ml Activin A (Biotechne; 338-AC-050), 8ng/ml FGF2 (Biotechne; 234-FSE-025), 10µM ROCKi (Merck; Y-27632) and 20ng/ml rhLIF (Merck; LIF1050). Previously converted naive HS975 were plated onto pre-coated plates containing 333.000 MEFs. Media was changed daily.

4.2.3 Pharmacologic inhibition of EZH2 and EED

In **paper II**, pharmacologic inhibition of PRC2 subunits EZH2 (EZH2i; 10µM EZSolution™ EPZ-6438, BioVision; 2428-5) and EED (EEDi; 10µM EED226, Cayman Chemical; CAYM22031-10) was performed in both primed and naive H9 hESCs as well as in naive HS975 hESCs. The inhibitors were simple supplemented to the respective culture media and kept in culture throughout the experiment, including during passaging, which was generally done on day 3-4 of treatment.

4.2.4 *In vitro* differentiation of hESCs to RPE

In **paper I**, 2.4×10^4 cells/cm^2 HS980 hESCs were plated in wells pre-coated with 20µg/ml laminin 111 (Biolamina; LN111-02) using Nutristem hESC XF medium (Biological Industries; 05-100-1A) supplemented with 10µM ROCKi (Millipore; Y-27632) at 37°C, 5% O$_2$ and 5% CO$_2$. After 24h, the stem cell culture medium was replaced with differentiation medium Nutristem hESC XF without bFGF and TGFβ (Biological Industries; 06-5100-01-1A) and cultured at 37°C, 5% CO$_2$. At day 6 after plating, 100ng/ml Activin A (R&D systems; 338-AC-050) was added to the medium. Media changes occurred three times per week and the cells were maintained without passaging for 5 weeks. The monolayers were then passaged by incubation with TrypLE Select (Gibco; 12563011) for 10 min at 37°C, 5% CO$_2$. The dissociation reagent was carefully removed and the cells were resuspended in differentiation medium by pipetting to obtain a single-cell suspension, which was centrifuged for 4 min at 300 x g. The resulting pellet was resuspended in fresh, pre-heated differentiation media and passed through a 40µm cell strainer before plating 7×10^4 cells/cm^2 in laminin 111 pre-coated wells. Media changes were done three times per week and these re-plated cells were kept in culture for another four weeks, at which point they had adopted the morphology and characteristics of RPE cells.

4.2.5 Co-culture with T cells (T cell proliferation assay)

To assess whether T (cytotoxic and helper) cells were activated by the genetically modified RPE cells, co-cultures were performed. Cytotoxic T cells interact with cells via HLA-I and would become activated upon recognition of a non-self HLA-type. Helper T cells on the other hand, interact with cells through HLA-II molecules and when they become activated they produce cytokines.

Day 30 after re-plating, unstimulated or 2 days IFNγ (100ng/ml, Peprotech; 300-02) pre-stimulated RPE cells were dissociated, irradiated (30 Gy) and plated at a cell density in the range $1x10^3$ to $5.5x10^3$ cells/cm^2 on 20 µg/ml laminin 521 pre-coated plates using RPMI medium (Hyclone; SH3025501) supplemented with 10% AB serum (Sigma; H3667), 100 units/ml penicillin and 100 µg/ml streptomycin (Hyclone; SV30010). RPE cells were allowed to attach for 3h. Meanwhile, human peripheral blood mononuclear cells that had been isolated from buffy coats of healthy donors through Lymphoprep density gradient centrifugation (Axis-Shield PoC AS; 1114547), were stained with 2.5µg/ml CellTrace CFSE Cell Proliferation Kit (ThermoFisher; C34554) or were divided for CD4$^+$ and CD8$^+$ isolation, respectively, using CD4- and CD8-negative selection beads (MACS Milteny Biotec; CD4: 130-096-533 and CD8: 130-096-495). One million of labeled or unlabeled PBMCs, or isolated CD4$^+$ or CD8$^+$ T cells, were plated per well on top of the previously plated unstimulated or IFNγ pre-stimulated RPE cells. 1 ng or 100U IL-2 (BD Biosciences; 554603), 1.25µg/ml CD28 (Biolegend; 302902[CD28.2]) or 25ng/ml OKT-3 (Biolegend; 317315) were added to the wells where they were required. Co-cultures were maintained for 5 days at 37°C before analysis.

4.2.6 Co-culture with NK cells

4.2.6.1 Cytotoxicity assay

NK cell-mediated cytotoxicity was measured in a chromium (^{51}Cr) release assay, which means that the amount of released chromium corresponded to the extent to which the RPE cells had been killed by the NK cells.

NK cells had been pre-stimulated with IL-2 overnight and RPE cells had been stimulated with IFNγ for 2 days prior to the start of the experiment and control RPE cells were kept alongside. RPE cells were labeled with 70 µCi ^{51}Cr (PerkinElmer, Waltham, MA) for 1h at 37°C after which NK cells were mixed with the labeled RPE cells at the following ratios: 10:1, 3:1, 1:1, 0.3:1 (NK:RPE) and incubated at 37°C for 4 h. Supernatants were then analyzed using a MicroBeta LumiJET Microplate counter (PerkinElmer, Waltham, MA). The percentage of lysis per sample was calculated as follows:

$$Percentage\ of\ lysis = \frac{(Experimental\ release - Spontaneous\ release)}{(Maximum\ load - Spontaneous\ release)} \times 100$$

4.2.6.2 Degranulation assay

The second NK cell activity measurement performed against the RPE cells was the degranulation assay. Release of lytic granules onto the target cells, in this case the RPE cells, was measured using an antibody against CD107 (lysosome-associated membrane protein-1 (LAMP-1)).

Once again, NK cells had been pre-stimulated with IL-2 overnight and RPE cells had either been stimulated with IFNγ for 2 days prior to the experiment or remained untreated. NK cells were mixed with RPE cells at a ratio of 3:1 and incubated with an anti-CD107 antibody at 37°C for 1h. Protein transport was blocked using monesin (GolgiStop, BD Bioscences; 554724) and the samples were incubated at 37°C for an additional 3h. Next, the cells were stained for intracellular IFNγ using BD Cytofix/Cytoperm kit (BD Biosciences; 554714). The cells were then analyzed by flow cytometry.

4.3 GENE EDITING USING CRISPR/CAS9

To be able to make the models necessary to answer the research questions presented in **papers I, II and III**, we employed CRISPR/Cas9-mediated gene editing in primed and naive hESCs.

In **paper I**, we used a validated sgRNA sequence for *B2M* and designed several sgRNAs targeting *CIITA* to create the B2M single-KO, CIITA single-KO and the double-KO (B2M and CIITA) cell lines. The sgRNAs were cloned into the Cas9-containing plasmid pX459 and were expressed under the U6-promoter. The efficiency was initially evaluated through a nuclease assay, which we moved on from in favor of Sanger sequencing of the PCR amplified region of interest.

After having used two different pharmacological inhibitors against the PRC2 subunits EZH2 and EED, respectively, we wanted to prevent PRC2 function through genetic intervention. Thus, in **paper II**, we used pre-made sgRNAs against *EED* (Synthego) that were complexed with Cas9 protein before being electroporated into naive hESCs. Editing was evaluated on protein level through immunofluorescence staining of EED and H3K27me3.

Lastly, in **paper III**, we wanted to study the transcriptional repressor SPEN and its relationship to *XIST* using hESCs. This time, sgRNA targets were designed using Benchling's CRISPR tool and ordered as oligonucleotides that were assembled into functional sgRNAs in-house. Primed hESCs were electroporated with complexed sgRNA and Cas9 protein, allowed to recover before being expanded as single-cell clones. To be able to study how loss of function of *SPEN* affected *XIST*, the resulting clones needed to be converted to the naive state where *XIST* is upregulated and both X chromosomes are active.

4.3.1 Editing in primed hESCs

Gene editing was carried out using plasmid pX459 and complexed sgRNA and Cas9, respectively. Primed hESCs were grown until they reached 70-80% confluency at which point they were ready for electroporation using the NEON Transfection System (ThermoFisher

Scientific; MPK5000). First, the cells were dissociated with TrypLE Select at 37°C, 5% O_2 and 5% CO_2 for 4 min. Then, collected in fresh media and counted using MOXI Z Mini Automated Cell Counter (Orflo; MXZ001). After centrifugation at 300 x g for 4 min, cells were resuspended in PBS$^{-/-}$ (Gibco; 14190144) and spun down again using the same settings. PBS$^{-/-}$ was carefully removed and cells were resuspended in Buffer R and 200.000 cells was administered into separate tubes containing either 2 µg pX459, pre-complexed 0.5µl Cas9 (Invitrogen; A36496) and 500ng sgRNA, GFP mRNA or nothing. Each tube was vortexed to mix the cells with the contents, and 10µl suspension was electroporated using the following NEON settings: 1050 V, 30 ms and 2 pulses. Electroporated cells were plated onto laminin 521 pre-coated wells in fresh Nutristem supplemented with 10µM ROCKi and allowed to recover. Primed hESCs electroporated with pX459 were exposed to 0.5µg/ml puromycin (ThermoFisher Scientific; A1113803) selection 24h post-transfection to ensure that only cells that had taken up the plasmid would grow. Clonal expansion was done by serial dilution, plating 2 cells/well in a 96-well plate pre-coated with 15µg/ml laminin 521 and 1.7µg/ml E-cadherin (R&D systems; 8505-EC050).

4.3.2 Editing in naive hESCs

In naive hESCs, the method differed slightly as we needed to take the MEF cells into account while preparing for electroporation. The day before electroporation, fresh MEF cells were plated at high density in 6-well plates and allowed to attached overnight. The next day, MEF medium was removed and the MEF cells were rinsed with PBS$^{-/-}$ before fresh naivecult expansion medium supplemented with 10µM ROCKi was added to each well. Plates were kept at 37°C, 5% O_2 and 5% CO_2 until the electroporation started. Note to those who want to perform this experiment themselves: 1 x 6-well plate of naive hESCs was used to achieve the cell numbers required to perform this experiment and using fewer cells is not recommended.

Dissociation of naive hESCs was done using TrypLE select at 37°C, 5% O_2 and 5% CO_2 for 4 min after which the cells were collected by pipetting and passed through a 40µm cell strainer to remove any MEF sheets. After diluting the TrypLE cell suspension in fresh naivecult expansion medium, naive cells were counted using the small particle setting (<8µm) on MOXI Z Mini Automated Cell Counter. At this point the cells were centrifuged at 300x g for 4 min, the pellet was resuspended in PBS$^{-/-}$ and centrifuged again. This time, 100.000 cells were distributed into tubes containing pre-complexed 0.5µl Cas9 (Invitrogen; A36496) and 1µl 100µM EED sgRNAs or 1µl 100µM non-targeting control sgRNA. Electroporation was done using the NEON electroporator with settings 1400 V, 20 ms, and 1 pulse. Media were changed daily until the naive hESCs had grown for 7 days, at which time they were fixed in 4% paraformaldehyde (PFA) and used for immunofluorescence staining. Since we were investigating PRC2 and how it may in fact contribute to shielding the naive hESCs from differentiation, mutating its EED subunit may result in differentiating cells. Therefore, we did not passage these cells after 4 days as per usual, since passaging may select against these differentiating cells in the naivecult medium.

4.3.3 Mutation detection and evaluation

Initially, surveyor nuclease assay was used to determine if there was a mutation in the edited sample. Briefly, this method relies on the amplification of mutant and control gDNA from the bulk targeted sample. These fragments are then annealed by increasing the temperature, which denatures the DNA, and are then allowed to slowly renature. Once these samples are incubated with the nuclease, any mismatch between the annealed fragments will be cleaved and can then be separated using gel electrophoresis. Quantification was done by comparing the intensity of the cleaved fragments to that of the intact control fragment.

Once we could PCR amplify the region of interest in gene edited and control cells, respectively, and send these for Sanger sequencing we were able to analyze many more samples in parallel. The online tool, ICE analysis (Synthego; https://ice.synthego.com/#/), allowed us to upload the sgRNA sequence along with the Sanger sequencing files containing edited and control sample chromatograms.

4.4 APPROACHES TO STUDY RNA EXPRESSION

Throughout the studies presented in this thesis, RNA expression has been assessed at using various techniques: bulk RNA sequencing, single-cell RNA sequencing, RNA FISH and qPCR.

The master regulator of transcription of HLA-II genes, *CIITA*, was knocked out in **paper I**. To evaluate if the RPE cells derived from the CIITA-KO hESC line had lost HLA-II gene expression, RPE cells were cultured with IFNγ for 5 days before RNA was extracted. The IFNγ stimulation is necessary for the RPE cells to upregulate HLA-II genes, which they otherwise would not express. Real-time RT-PCR using TaqMan^{TM} probes was employed to measure the gene expression of *CIITA* and the HLA-II genes under its control, namely, *HLA-DR*, *HLA-DP* and *HLA-DQ*.

In **paper II**, several methods were employed to study the impact of PRC2 inhibition on the transcriptional profiles of naive and primed hESCs. First, naive and primed hESCs were cultured with EZH2i (EPZ-6438, BioVision; 2428-5) and untreated cells were kept as controls. After seven days of inhibitor culture, treated and untreated primed hESCs were collected in RLT buffer (QIAGEN), while treated and untreated naive hESCs went through a MEF removal step using mouse-specific beads before resuspending the resulting pellets in RLT buffer. The samples were then sent for bulk RNA sequencing. This allowed us to understand the general trends among the treated cells when compared to untreated control cells within each sample type using bioinformatic analysis. Furthermore, it was also possible to compare the effect of PRC2 inhibition in primed hESCs compared to naive hESCs. Based on these results, an inhibitor treatment time course using naive hESCs treated for 2, 4 or 7 days along with untreated control as well as primed hESCs treated for 7 days along with untreated control cells was set up. Here, single-cell RNA sequencing, specifically the Chromium next GEM single cell 3' Reagents Kits 3.1 (10X Genomics) protocol together with the accompanying

bioinformatic analysis, was used to dissect the transcriptional profiles emerging at different time points during the inhibitor treatment.

To investigate if the SPEN protein had any association with the *XIST* RNA, RNA FISH was used to study presence of *XIST* in gene edited and control naive hESCs, respectively, in **paper III**. RNA FISH makes use of gene-specific probe sets that bind to the transcripts originating from the gene of interest. A set of fluorescently-labeled hairpins are then applied to the samples, which bind to the probe sets thus triggering growth of the amplification polymer. Samples were mounted using SlowFade™ Gold Antifade Mountant (ThermoFisher Scientific; S36940). Images were acquired on a Nikon Eclipse Ti spinning disk confocal microscope with a 60X oil immersion objective, and the resulting Z-stack images were analyzed using CellProfiler and ImageJ. Next, gene edited and control primed and naive hESCs were collected and RNA extracted. 55ng total RNA was used to synthesize cDNA. Of the resulting cDNA, 1µl was used in each reaction containing a target probe and a *GAPDH* control probe. The C_T values generated in the RT-PCR were analyzed according to the $\Delta\Delta C_T$ method[135].

4.5 APPROACHES TO STUDY PROTEIN EXPRESSION

Confirming loss of protein, as in the case of CRISPR/Cas9 gene editing, or to evaluate the changes caused by inhibition of proteins, as in the case of the EZH2i and EEDi treatments, there are a multitude of methods that can be used.

In **paper I**, flow cytometry, immunoblotting and immunofluorescence were used to determine if the proteins B2M, CIITA, HLA-I and HLA-II were present in the genetically modified primed hESCs. Initially, immunoblotting and immunofluorescence of B2M and CIITA was performed to confirm their absence in the gene edited hESCs. This allowed us to look at total protein as well as localization of said proteins in the cells. Thereafter, since HLA-I, B2M and HLA-II are all surface proteins, flow cytometry allowed clear confirmation of whether these proteins persisted on the cell surface.

In **paper II**, immunofluorescence and MINUTE-ChIP were employed offering both visual and sequencing information, respectively. Since PRC2 is responsible for laying down the H3K27me3 mark thus affecting the epigenetic landscape, assessing what regions of DNA are bound by different chromatin marks and how this changes when PRC2 is prevented from performing its function was of great interest. Briefly, cell pellets were lysed and MNase treated rendering mono- to trinucleosome fragments[136]. These were then ligated with dsDNA adaptors consisting of a T7 promoter, 8bp sample barcode and a 6bp unique molecular identifier[136]. Once barcoded, samples were pooled and distributed into separate ChIP reactions containing protein A/G magnetic beads (BioRad; 161-4013/23) that had been coupled with H3K27me3 (Millipore; 07-449), H3K4me3 (Millipore; 04-745) and H2AUb (Cell Signaling; 8240S), respectively[136]. ChIP reactions were incubated at 4°C for 4h, rotating, after which the ChIP-DNA was isolated[136]. The isolated DNA was used for *in vitro* transcription, followed by RNA 3'adapter ligation, reverse transcription and PCR amplification before the libraries were created and ready for sequencing[136].

Although there are not many validated antibodies against SPEN that are suitable for immunofluorescence, the antibody (dilution 1:200, Abcam; ab72266) used in **paper III** gave signal in naive hESCs. Here, we used antibodies against various proteins that had been previously proposed or identified as impacted by loss of SPEN. In brief, samples were fixed in 4% PFA for 10 min, washed using PBS$^{-/-}$, permeabilized in 0.3% Triton X-100 for 10 min, washed using PBS$^{-/-}$ and blocked in 0.1% Tween-20 and 4% FBS (Gibco; 16140-071) for 2h before primary antibody solution was added to the samples. 16h later primary antibody solution was washed away using blocking buffer. Thereafter, samples were incubated with secondary antibody solution for 2h, then washed and mounted using DAKO fluorescent mounting media (DAKO; S3023).

5 RESULTS

5.1 GENERATION OF RETINAL PIGMENT EPITHELIAL CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS LACKING HUMAN LEUKOCYTE ANTIGEN CLASS I AND II (PAPER I)

Here, the aim was to generate hESCs lacking HLA class I and II expression and evaluate their immunological properties after differentiation to RPE cells through *in vitro* and *in vivo* studies.

First, targets for HLA class I and HLA class II were set to *B2M* and *CIITA*, respectively. Gene editing was done sequentially in hESCs; first single-knockout of *B2M* (B2M-SKO) and single-knockout of *CIITA* (CIITA-SKO) cell lines were derived separately. Next, the *CIITA* locus was edited in the B2M-KO cell line, thus generating the double-knockout (DKO) cell line. Validation of the resulting cell lines demonstrated that:

1) B2M-SKO led to displacement of HLA class I proteins, i.e. they were expressed but not presented on the cell surface,
2) CIITA-SKO completely abolished HLA class II gene expression, thus their protein products were not found on the surface of or intracellularly in the cells, and,
3) DKO cells lacked surface expression of both HLA class I and class II proteins.

Since this was a preclinical study, where the hESCs were genetically engineered before being differentiated into RPE cells, WGS was employed to evaluate any off-target mutations that were introduced by CRISPR/Cas9 or through time in culture. HS980 control, hESC-B2M-SKO and hESC-DKO cells were sequenced. Off-target lists were generated using the online tools E-CRISP[137] and Cas-OFFinder[52], evaluating 19 277 and 22 618 potential targets for *B2M* and *CIITA* sgRNAs, respectively. Apart from the on-target mutations at *B2M* and *CIITA* loci, which were found at allele frequency 1, the genome analysis revealed another three indels at lower allele frequencies indicating that these had arisen over time in culture rather than through gene editing. Furthermore, when comparing hESC-B2M-SKO to HS980 13 single nucleotide variants were identified within non-redundant exonic boundaries whereas 16 were identified when comparing hESC-DKO to hESC-B2M-SKO. After filtering, three heterozygote SNPs remained and these were either located within the 3' UTR, a nonsense mutation or a silent mutation, and importantly, none of these had been described as pathogenic.

The hESC-B2M-SKO and hESC-DKO as well as HS980 control cells were differentiated to RPE cells and were challenged against T cells. To mimic the inflammatory environment that is associated with transplantation, all RPE cells were pre-stimulated with IFNγ before being co-cultured with CD8$^+$ and CD4$^+$ T cells, respectively. The amount of IFNγ produced during the co-cultures directly correlates to the activation level of the T cells. In the presence of RPE-B2M-SKO, CD8$^+$ T cells but not CD4$^+$ T cells, showed reduced IFNγ secretion. RPE-DKO activated neither CD8$^+$ nor CD4$^+$ T cells, which was demonstrated by the low levels of IFNγ secretion.

Next, we challenged RPE-B2M-SKO, RPE-DKO and RPE control against NK cells in both degranulation and cytotoxicity assays. In the degranulation assay, NK cell activity is measured through CD107 expression after co-culturing the immune cells with IFNγ pre-stimulated RPE cells. There was an evident donor-dependent response; stronger in NK cells from donor 1 compared with NK cells from donor 2 and 3, respectively. The RPE control cells contributed to the least amount of NK cell activation whereas RPE-B2M-SKO and RPE-DKO showed a significant increase in NK cell activation instead. Moreover, evaluating NK cell cytotoxicity did not reveal the same striking difference between the RPE control and edited cell lines. Therefore, NK cell ligands expressed by the RPE cells were examined through flow cytometry. CD112, a ligand binding both NK activating receptor DNAM-1 and NK inhibitory receptor TIGIT, was highly expressed in all cell lines, whereas CD155, a ligand with both activating and inhibitory potential, which also binds DNAM-I and TIGIT in addition to CD96, was expressed at lower levels in RPE-B2M-SKO and RPE-DKO compared to RPE control under inflammatory conditions. Furthermore, RPE control cells also expressed CD47, CD55, and CD59, which have all been previously assigned inhibitory roles[66,138,139].

Assessing the immune response *in vivo* was done after subretinal transplantation of RPE-B2M-SKO, RPE-DKO and RPE control into a xenogeneic model without immunosuppression. Signs of early rejection (within one week) were lower in both of the gene edited cell lines compared to RPE control, however, RPE-DKO did not have increased survival rate compared to RPE-B2M-SKO. To understand the contribution of an HLA class II knockout, hESC-CIITA-SKO were differentiated to RPE after which they were subjected to subretinal transplantation. Interestingly, RPE-CIITA-SKO also showed a reduced rejection rate, similar to RPE-B2M-SKO and RPE-DKO.

5.2 POLYCOMB REPRESSIVE COMPLEX 2 SHIELDS NAIVE HUMAN PLURIPOTENT CELLS FROM TROPHECTODERM DIFFERENTIATION (PAPER II)

In this study, we sought to elucidate the function of PRC2 in naive and primed hESCs and evaluate how loss of PRC2 affected the epigenetic and transcriptional profiles of those cells.

Naive and primed hESCs were maintained in culture with and without an EZH2 inhibitor. Using MINUTE-ChIP, three histone modifications associated with bivalent promoters, namely H3K27me3, H3K4me3 and H2Aub, were outlined in treated and untreated cells, respectively. Notably, H3K27me3 levels were significantly higher in naive than in primed hESCs and so were H2Aub levels too, whereas H3K4me3 levels only were marginally increased at active promoters. In the EZH2 inhibitor treated cells, H3K27me3 levels were reduced 97% in naive hESCs and 92% in primed hESCs. Furthermore, time course analysis using immunofluorescence on EZH2 inhibitor treated naive hESCs revealed that H3K27me3 was broadly depleted between days 2 and 4 and then lost on day 7. Surprisingly, H3K27me3 accumulated on the naive X chromosomes to a greater extent than on naive autosomes. We estimated that 10% of H3K27me3 marks in naive hESCs originated from the X chromosomes. In spite of the increase in H3K27me3, there was no change in overall transcriptional output

from the X chromosomes compared to autosomes. When PRC2 was inhibited, thus H3K27me3 marks were lost, there was no global upregulation of X-linked genes.

After defining promoter bivalency based on H3K4me3/H3K27me3 enrichment in naive and/or primed hESCs, we used RNA-seq to understand if H3K27me3 participated in regulation of these promoters. Naive-bivalent genes, which were designated as H3K4me3-positive with higher H3K27me3 levels in the naive state, had lower expression in naive hESCs. Consequently, after EZH2 inhibitor treatment, these same genes were de-repressed. Primed bivalent genes, designated as H3K4me3-positive with higher H3K27me3 levels in the primed state, followed the same trend and expression increased after inhibitor treatment.

Several genes, many were known markers of naive and primed pluripotency, changed bivalency status between naive and primed stem cell states. One such gene, *TFAP2C*, was described to be involved in setting up the naive transcriptional profile. Its promoter was enriched in H3K4me3 and lacked H3K27me3 in the naive state, whereas it was bivalent in the primed state. EZH2 inhibitor treatment in primed hESCs led to loss of H3K27me3, which activated *TFAP2C* although not to the same extent as in naive hESCs. A comparable response was also seen for primed markers that were enriched for H3K4me3 in the primed state and devoid of H3K27me3, which acquired a bivalent status in the naive state with promoters high in H3K27me3 and low in H3K4me3. Depleting H3K27me3 in the naive state led to activation of these primed genes but again not reaching the transcriptional levels observed in the primed state.

Investigating differential gene expression in naive hESCs uncovered a set of known developmental genes, *GATA2*, *FRZB*, and *IGF2* among others, within the group of genes that were upregulated following EZH2 inhibitor treatment. In fact, trophectoderm progenitor marker *ENPEP*, plasma membrane transporter *ABCG2* found in both trophectoderm and placental tissues, as well as *TP63*, which defines the stem cell division of the cytotrophoblast cells, were amidst the most highly upregulated genes. In addition, other known genes involved in establishing the trophectoderm lineage, such as *GATA3*, *CDX2*, *MSX2* and *NR2F2*[140–145], were expressed in EZH2 inhibitor treated naive hESCs. Notably, these transcription factors were all found to have bivalent promoters. Furthermore, taking advantage of previously published ChIP-seq data from trophoblast progenitors and comparing the naive hESC treated with EZH2 inhibitor, we found that 20% of the upregulated genes were bound by GATA3. One of these genes was *ENPEP*, which did not carry H3K4me3 in the naive state, however, GATA3 binding sites were found within its gene body.

Next, we wanted to explore whether GATA3 protein was induced upon loss of H3K27me3. Naive hESCs were cultured with another inhibitor in addition to EZH2, namely EED226, which binds to the H3K27me3 binding site of the EED subunit of PRC2. The 7-day treatment reduced H3K27me3 levels regardless of which inhibitor was used, and a subset of cells gained GATA3 protein expression while simultaneously losing pluripotency. Furthermore, CRISPR/Cas9-mediated gene editing of EED resulted in loss of EED and thus also H3K27me3,

supporting the results from the inhibitor studies. A fraction of the EED-negative cells gained GATA3 protein expression.

Clearly, inhibitor treatments and gene editing alike produce heterogenous naive hESCs. To resolve the heterogeneity, we performed single-cell RNA-seq on naive hESCs treated with EZH2 inhibitor for 2, 4 or 7 days together with untreated cells as well as primed hESCs treated with EZH2 inhibitor for 7 days along with untreated control. Among the untreated cells, a small subset (1.8%) clustered within the trophectoderm reference datasets and another fraction (1%) clustered within the mesoderm reference datasets, however, the majority clustered within the preimplantation epiblast cell reference datasets. EZH2 inhibitor-treated naive hESCs progressively clustered within the differentiated reference datasets, culminating in 9.1% trophectoderm-like cells and 11.7% mesoderm-like cells after 7 days. Furthermore, a large portion of cells displayed a shift in their transcriptional profiles after 4 and 7 days of inhibitor treatment even though they remain within the epiblast-like cell cluster. In contrast, the majority of primed hESCs treated with EZH2 inhibitor (99.96%) clustered with the epiblast-like cells and hardly produced any differentiated cells.

Finally, we were able to describe the exit from pluripotency among the naive hESCs by performing a trajectory analysis using differentially expressed genes from the identified subpopulations and the EZH2 inhibitor time course. Interestingly, this resulted in a bifurcated trajectory consisting of naive hESCs that transition towards the bifurcation as they acquire differentiation potential, and upon reaching the bifurcation point can access trophectoderm and mesoderm lineages.

5.3 SPEN IS INDISPENSABLE FOR XIST EXPRESSION IN NAIVE HUMAN EMBRYONIC STEM CELLS (PAPER III)

Here, we sought to explore the relationship between SPEN and *XIST* by knocking out *SPEN* through gene editing, deriving single-cell clones and converting them to naive hESCs.

First, the *SPEN* loci was edited using sgRNA/Cas9 ribonucleic complex in primed hESCs. To reduce heterogeneity in future experiments, single-cell clones were derived and analyzed for indels at the Cas9 cute site. Out of 23 edited clones, three were chosen based on their indel status. *SPEN$^{-/-}$* clone 6 had a homozygote mutation introducing 1bp, *SPEN$^{-/-}$* clone 15 had a compound heterozygote mutation, which introduced +1bp and +2bp on each allele, and lastly, *SPEN$^{+/-}$* clone 20 had a heterozygote mutation introducing +8bp on one allele, while the other was intact.

The initial phase of the naive conversion progressed similarly for all clones and control cells. After approximately 5 passages in naive conditions, *SPEN$^{-/-}$* clone 6 cells collapsed. Thus, we did not acquire enough material to include it in the downstream experiments. After yet another few passages *SPEN$^{-/-}$* clone 15 followed suit. In contrast, the heterozygote *SPEN$^{+/-}$* clone 20 converted similarly to control hESCs. Interrogating gene expression of naive and primed pluripotency markers, respectively, showed that even though *SPEN$^{-/-}$* clone 15 was not expanding in culture at later passages, it did upregulate naive marker genes and reduced primed

marker genes albeit not at the same levels as seen in *SPEN*^{+/-} clone 20 and control hESCs. An outlier among the gene expression in primed hESCs was the naive pluripotency gene *DPPA3*, which was undetected in *SPEN*^{+/-} clone 20 and control but highly increased in SPEN^{-/-} clone 15. In the naive state, *SPEN*^{+/-} clone 20 and control increase *DPPA3* expression while naive *SPEN*^{-/-} clone 15 presents it at similar levels as in the primed state.

Using the naive hESCs, we wanted to examine the *XIST* status in each of the two remaining clones and compare with control. Employing RT-PCR and RNA FISH, uncovered a much reduced induction of *XIST* at passage 8 in *SPEN*^{-/-} clone 15, which, at passage 13 culminated in absence of *XIST* RNA. Meanwhile, *SPEN*^{+/-} clone 20 and control had comparable *XIST* gene expression levels and intensity of *XIST* RNA clouds at passage 13, however, *ATRX* intensity levels were reduced in the former.

Upon initiation of differentiation in mouse ESCs, SPEN binds to *Xist* causing the recruitment of chromatin remodelers to change the epigenetic landscape of the future inactive X chromosome[111,120,132,133]. Therefore, we wanted to evaluate the H3K27me3 mark to assess PRC2 functionality. Surprisingly, neither H3K27me3 nor EED, a subunit of PRC2, were observed in *SPEN*^{-/-} clone 15. In contrast, both *SPEN*^{+/-} clone 20 and control naive hESCs displayed a distinct H3K27me3 accumulation in the nuclei.

We then hypothesized that the *SPEN*^{-/-} clone 15 cells may be differentiating, since inhibition of PRC2 has been shown to induce GATA3 and GATA6-positive cells. Surprisingly, *SPEN*^{-/-} clone 15 did not express more *GATA3* or *GATA6* than control and was negative for both markers in immunofluorescence. Moreover, in its primed state, *SPEN*^{-/-} clone 15 expressed GATA3 at similar levels seen in its naive derivative. A comparable trend was observed in *SPEN*^{+/-} clone 20, while control naive hESCs expressed *GATA3* at higher levels compared to primed hESCs.

6 DISCUSSION

6.1 GENERATION OF RETINAL PIGMENT EPITHELIAL CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS LACKING HUMAN LEUKOCYTE ANTIGEN CLASS I AND II (PAPER I)

The efforts put into this study demonstrate that it is possible to genetically engineer hESCs using CRISPR/Cas9 and create stem cell lines that are interesting for future cell therapy endeavors. Mutating *B2M* and *CIITA*, allowed us to remove the protein products of a total of 8 genes. Minimizing the amount of genomic targets may be beneficial in terms of reducing CRISPR-mediated off-targets. WGS of the resulting hESC-B2M-SKO and hESC-DKO lines, respectively, identified several heterozygous mutations when compared with the control HS980 cell line. Even though we had lists of ~20 000 potential off-targets per sgRNA, only 3 of these loci had been edited and they were not within known genes. Another 4 locations in the genome had acquired mutations and due to their low allele frequencies were assigned as more likely caused by time in culture than by CRISPR/Cas9. Of note, none of these mutations has been described as pathogenic. Furthermore, the gene editing had not affected the differentiation capacity of the cells and the resulting RPE cell lines were not able to upregulate either HLA class I or class II genes.

Although mostly immune cells interact via HLA class II genes, our experiments clearly show that other cell types may upregulate these genes under inflammatory conditions. Our HLA class I negative cell line, RPE-B2M-SKO, did not trigger CD8^{+} T cell activation, however, CD4^{+} T cells were activated since they were able to interact with the cells through the intact HLA class II surface proteins. Supporting these data, RPE-DKO, which lacked both HLA class I and HLA class II surface antigen presentation, did not activate any of the T cells. Since it is often perceived as a "rule" that immune cells are the "only" cells that present HLA class II antigens, our study also serves as a reminder that it may not always be the case.

In contrast, the "missing-self" in RPE-B2M-SKO and RPE-DKO gave stronger activation than the mismatch of HLA class I in the co-cultures of RPE control and NK cells. NK cell-mediated killing may be attributed to the various stimulatory ligands presented on the RPE cell surfaces, such as the DNAM-1 activating ligand CD112. Under inflammatory settings, produced by INFγ stimulation, the overall NK cell activation was reduced in all cell lines. This reduction could be a result of changes in what ligands are presented by the RPE cells during inflammation. CD155, a ligand that has been attributed both inhibitory and activating roles depending on what receptor it binds to, offers one explanation as to why RPE-SKO-B2M and RPE-DKO were killed to a larger extent than RPE control since their expression levels were lower. Another NK inhibitor candidate is *HLA-E*, which has been introduced into hPSCs through genetic engineering to circumvent NK-mediated killing[67,146]. Although we did not evaluate the potential of an *HLA-E* knock-in in this study, it does offer an approach to making hypoimmunogenic stem cells that are not only protected from T cells but also from NK cells.

Finally, the gene edited RPE cells along with RPE control cells were transplanted into rabbit eyes to allow us to assess rejection *in vivo*. Regardless of whether the RPE cells lacked HLA class I, HLA class II or both, they all delayed onset of rejection.

6.2 POLYCOMB REPRESSIVE COMPLEX 2 SHIELDS NAIVE HUMAN PLURIPOTENT CELLS FROM TROPHECTODERM DIFFERENTIATION (PAPER II)

This study started out exploring the epigenetic landscape of naive and primed hESCs and how it changed after PRC2 inhibition using quantitative ChIP-seq. We found a discernable difference in H3K27me3 enrichment in naive compared to primed hESCs. Specifically, the naive X chromosomes had accumulated a significant portion of these chromatin marks but they appeared to not affect the X chromosome dosage compensation. Similarly, in mouse ESCs lacking *Xist* B/C repeats, which led to loss of H3K27me3 through impaired recruitment of PRC2, XCI can still be initiated and it was only at later time points that it was possible to discern deregulation of XCI[113,116,147]. This suggests that if there is such a correlation in hESCs we would not have been able to detect it in our study as we limited PRC2 inhibition to 7 days.

Even though de-repression of naive-specific genes in primed hESCs and primed-specific genes in naive hESCs was accomplished through PRC2 inhibition, neither cell type expressed these genes to the same extent as in their native state. Furthermore, these genes were often H3K4me3-high and H3K27me3-low in their native states and acquired a bivalent status in the other. Thus, PRC2 appears to lay down an epigenetic barrier between the two stem cell states through deposition of H3K27me3.

Interestingly, GATA3 binding sites were identified in many of the genes that were upregulated in the EZH2 inhibitor-treated naive hESCs, which suggest that these genes may act downstream of *GATA3* induction. Moreover, inhibition of PRC2 through pharmacological substances targeting EZH2 or EED as well as gene editing of *EED*, resulted in induction of *GATA3* in a subset of cells after 4-7 days in culture. In conclusion, over time, a reduction of H3K27me3 levels in naive hESCs correlated with activation of a differentiation potential in these cells in the early phase of the inhibition, which was further solidified at the later stages when these activated cells reached the bifurcation point, thus also the lineage choice.

Since untreated naive hESCs also mapped onto all states of the trajectory, albeit mostly in the ground state, PRC2 inhibition appeared to accelerate the transition from ground state to activated state with more cells acquiring a lineage commitment. The H3K27me3 landscape and the absence of it following PRC2 inhibition, suggest that it has a dynamic role where it can maintain pluripotency in hESCs but also support and reinforce lineage commitment during development.

6.3 SPEN IS INDISPENSABLE FOR XIST EXPRESSION IN NAIVE HUMAN EMBRYONIC STEM CELLS (PAPER III)

This study was designed based on knowledge derived solely from mouse studies, since there had not been any published studies of SPEN function in human X chromosome regulation at

the start of the project. There are several established differences in XCI during mouse and human embryo development, however, in both species *XIST* RNA participate in dosage compensation[148].

Mouse SPEN protein has been attributed with at least three functions during XCI:

1) binding to *Xist* to increase its stability,
2) binding to the promoter of *Tsix* together with HDAC3 to inhibit *Tsix* expression from the future inactive X chromosome, and
3) to recruit chromatin remodelers to establish the heterochromatin landscape causing X chromosome silencing[133,132,120].

Unexpectedly, knocking out *SPEN* in hESCs resulted not only in loss of *SPEN* but also in failure to induce *XIST* at levels comparable to control. Morphologically the *SPEN*^{-/-} clones were similar to the *SPEN*^{+/-} clone and control hESCs during naive conversion, however, importantly, lack of SPEN severely affected the expansion potential in the naive environment even though naive pluripotency genes *KLF17*, *DNMT3L* and *TFCP2L1* were upregulated similarly to control. Thus, we hypothesize that it is the loss of *SPEN* expression and the downstream implications of it that leads to reduced naive competence in these clones. Clearly, having one functional allele of *SPEN* is enough to gain a phenotype similar to that of control.

Dual-color RNA FISH was used to evaluate *XIST* clouds and the allelic expression status of *ATRX*. Interestingly, when analyzed per colony *SPEN*^{+/-} clone 20 displayed reduced intensity of *ATRX* foci colocalizing with *XIST* clouds, however, the intensity of *XIST* clouds between *SPEN*^{+/-} clone 20 and control were not drastically different. *SPEN*^{-/-} clone 15 showed the same spread in intensity of *ATRX* foci, but displayed no evident *XIST* clouds that were comparable to control. It remains to be seen what the significance of reduced *ATRX* within the *XIST* cloud may be, and exploring global X-linked gene expression will be necessary to evaluate if it is a common trend among all X chromosome transcripts. Furthermore, since ATRX together with DAXX deposit histone 3.3 (H3.3) in heterochromatin and is important for silencing of repetitive elements[149], it does raise the question whether ATRX was affected by loss of SPEN or by the resulting change in the epigenetic landscape following loss of SPEN.

Another unexpected finding was that *SPEN*^{-/-} clone 15 and *SPEN*^{+/-} clone 20 had both upregulated *GATA3* in the primed state, however, since *SPEN*^{+/-} clone 20 naive conversion was analogous to control hESCs it does not appear to prevent the conversion provided that the cells had one functional allele of *SPEN*. In the naive state, both *SPEN* clones had downregulated *GATA3* to levels lower than control hESCs. Unfortunately, since *SPEN*^{-/-} clone 15 collapsed over time in culture during the establishment of the naive state, we had to use an earlier passage for the gene expression analysis compared to the RNA FISH and immunofluorescence. There is a clear discrepancy between these passages: in the earlier passage we detected *XIST*, albeit at lower levels than control, whereas at the later passage we did not. Overall, gene expression of naive pluripotency genes as well as *GATA6* and *GATA3* were observed at the earlier naive passage while no protein was detected at the later passage. Repeated naive conversions

combined with RNA FISH, epigenomic profiling and transcriptional analysis, at the same passages over a time course will be required to deduce when this change in expression occurs. Furthermore, evaluating the allelic expression of X-linked genes, such as *ATRX*, in the *SPEN*^{+/-} and *SPEN*^{-/-} clones could discern whether the X chromosome expression has gone from monoallelic to biallelic in the naive state. Additionally, following the allelic expression during differentiation, from initiation until establishment of XCI, we would be able to uncover whether XCI is random or skewed. Previous studies of XCI in hESCs have shown that differentiation following resetting using 5iLAF leads to skewed XCI[18], unless domey, TFCP2L1-high colonies are selected and propagated in the conversion media[42]. We used a commercial media based on the t2iLGö[15,17] media formulation, in which random XCI has not been evaluated. Thus, differentiation of the naive control hESCs may induce skewed XCI if there is an epigenetic memory that is not completely reset during the t2iLGö conversion. Interestingly, the *SPEN*^{+/-} and *SPEN*^{-/-} clones, which carry deleterious mutations in an epigenetic factor, may display aberrant XCI or failure to induce XCI. Particularly the *SPEN*^{+/-} clone will be interesting to follow during XCI, because it is possible that having only one functional allele of *SPEN* will have an impact on how well the epigenetic landscape is shaped thus influencing the completeness of the X chromosome silencing.

6.4   ETHICAL REFLECTIONS

This thesis is comprised of three studies that all used CRISPR/Cas9 to mutate selected genes in human embryonic stem cells to be able to study cellular responses in their absence.

Today, CRISPR/Cas9 is a widely used molecular tool in research. While revolutionizing genome engineering, its potential clinical applications have been a subject for debate ever since being attributed the possibility of editing the human genome. Changes introduced into cells that can give rise to the human embryo, thus being inherited by the next generation, are controversial, in many countries illegal, and strongly advised against by the scientific community worldwide due to the implications they would have on the resulting individuals and on society. Currently, in the case of heritable diseases there are available methods to ensure that the disease allele is not transmitted to the next generation, such as preimplantation genetic diagnosis. Although carrier-screening is not widely adopted, it is also a method with which it is possible to asses recessive mutations in the genome before conception. Furthermore, there is still much to learn about how to make CRISPR/Cas9 safe for clinical use. Specifically, design and delivery of CRISPR/Cas constructs to minimize, or ideally abolish, off-target edits need to be optimized and standardized. Similarly, how to perform the analysis the edited genome need to be developed alongside the technology.

In Sweden, research conducted using human embryos is subject to strict laws detailing the time embryos can be kept in culture (14 days) and forbidding research embryos to be transferred into a woman's uterus. There are two main categories of embryos that are donated to research:

1) those that carry genetic mutations making them unsuitable for embryo transfer, and,

2) those that have reached the end of the cryopreservation, which is set to maximum 10 years today[150].

In the studies presented in this thesis, the human embryonic stem cells we used had been derived from surplus embryos donated by couples undergoing assisted reproduction. Using these cells, we were able to:

I.   Generate hypoimmunogenic hESCs using CRISPR/Cas9. These cells were then differentiated into RPE, a cell type relevant for cell therapy of age-related macular degeneration. These engineered stem cells could be used in other differentiation efforts as well. However, only provided that the resulting cell type is one that will not contribute to forming germ cells or any derivative thereof. The use of the genetically engineered hESC-derived RPE cells for the treatment of e.g. age-related macular degeneration, will not affect the individual's germ cells thus are not considered to carry germline mutations.

II.   Confirm a phenotype seen using pharmacological inhibitors by targeting the gene, whose protein product was targeted by the inhibitors. This study focused on understanding how a chromatin remodeler and the loss of it changed the epigenetic landscape in two stem cell states, which represent the pre- and post-implantation epiblast, respectively. The use of hESCs allowed us to assess developmental processes without using embryos.

III.   Create a stem cell model with which we will be able study X chromosome inactivation, which occurs early in the embryo. Already, by knocking out one gene we were able to assess the impact on other genes and how this changed between stem cell states representing the pre- and post-implantation epiblast.

7 CONCLUSIONS

This thesis presented its overall aim as being the use of CRISPR/Cas9 to create stem cell models that could be used in studies of regenerative medicine and developmental biology, with individual aims for each paper. In **paper I**, we evaluated the genetically modified hESC-derived RPE cells' capacity of activating the immune system through *in vitro* and *in vivo* experiments, respectively. In **paper II**, we investigated the role of PRC2 in both naive and primed hESCs through employment of pharmacological inhibitors and gene editing. Finally, in **paper III**, we sought to understand the relationship between SPEN and *XIST* in naive hESCs through genetic ablation of *SPEN*.

The main conclusions from each paper are the following:

- Gene modified hESCs were able differentiate to RPE cells. WGS revealed that neither gene editing nor the differentiation process had introduced deleterious mutations, demonstrating that it is possible to use these protocols in preclinical studies. Furthermore, gene edited RPE cells reduced CD4$^+$ and CD8$^+$ T cell activation, while slightly increasing NK cell activation *in vitro*. After transplantation, the same cells reduced early rejection and delayed anti-human antibody production, which is associated with late rejection.

- Capturing the epigenomic profiles of naive hESCs, revealed that bivalency was broadly maintained and hundreds of other promoters had gained bivalency uniquely in these cells. Inhibition of PRC2, pharmacologically or genetically, in the naive hESCs led to loss of H3K27me3, which in turn resulted in specific gene sets being activated thus priming the cells for trophectoderm and mesoderm lineages.

- Loss of SPEN affected *XIST* expression in naive hESCs to the point where *XIST* RNA was detected at very low levels in cells where *SPEN* had been mutated on both alleles. Having only one functional allele of *SPEN* did not appear to have any effect on *XIST* expression nor on gene expression in general, however, it did impact the intensity of *ATRX* foci colocalizing with *XIST*.

8 POINTS OF PERSPECTIVE

This thesis has included studies in regenerative medicine and early development. What has unified them is the application of CRISPR/Cas9 in hESCs. Looking at the future of cell therapies, gene editing and embryo research, I will discuss implications our studies may have on these topics.

8.1 GENETIC ENGINEERING IN THE ERA OF CELL THERAPIES

There are many uses for human pluripotent stem cells (hPSCs) in cell therapy. Differentiating hPSCs to clinically relevant cell types that can be used for cell therapy is a field that has been growing and is entering its next phase: clinical trials[151,152]. This raises questions about how to be able to treat all patients in need and several strategies have been considered and can be summarized into three categories: autologous, allogeneic and allogeneic in combination with genome engineering. Autologous transplantation implies that the patient's own cells have been used to develop the necessary cell type while an allogeneic transplantation have used donor cells for the same process. The latter requires matching of HLA haplotypes and unless haplobanks[75] containing cell lines that match the majority of the population are established, the cell therapy will only be available to the lucky few. The third strategy is to use gene editing to create the cell lines required to cover the majority of the population. In our study, HLA class I and class II are removed by editing *B2M* and *CIITA*, respectively, and although this worked well against T cells it may not be enough to protect the cells from NK cell killing. Furthermore, in a clinical setting, retaining at least one of the HLA class I genes may be beneficial, since those cells would be able to "communicate" with the immune system of the patient. After all, our immune system clears infected cells as well as those that have acquired mutations and become cancerous from our bodies. Facilitating normal interaction between transplanted cells and the immune system must be regarded as beneficial for the patient.

Gene editing in general and CRISPR/Cas9-technology specifically, is currently in the early stages of clinical trials[153]. In our study, we used one of the earliest version of the technology, which was plasmid-based. The benefit of using ribonucleoprotein (RNP) complexes is that they are pre-assembled and ready to cut the DNA upon entering the cell. Consequently, the halftime of a RNP complex is also shorter than that of a plasmid, which could reduce the number of off-target edits. Additionally, streamlined genome-wide analysis protocols are needed to identify off-targets and evaluate their clinical and biological relevance.

Although cell therapies have great clinical potential, there are also risks associated with the use of stem cell-derived cell products. Time in culture, gene editing, and dedifferentiation can all affect the integrity of the genome[154]. Introduction of deleterious mutations through any of these processes can cause the cells to undergo tumorigenic transformation, which is very serious as this could lead to cancer development. Rigorous testing and good manufacturing protocols need to be developed to ensure safety of the product for the patients.

8.2 UNDERSTANDING HUMAN EMBRYO DEVELOPMENT USING STEM CELLS

Studying the processes regulating human embryo development is critical for understanding how we as a species evolve. Importantly, there are laws preventing the misuse of embryos in research in Sweden, but in other countries it is not only impossible to use human embryos for research, it is also considered controversial and unethical.

Human embryos represent a finite source of material, however, their derivatives, namely the hESCs are not. Over the past eight years[14–17], hESC culture protocols have developed to include naive and primed pluripotency states which can be described as representatives of pre- and postimplantation epiblast, respectively. Now, instead of using human embryos we can use naive or primed hESCs to create models of a particular developmental process. The naive hESCs are also being used together with other cell types, to create synthetic embryos. This could facilitate and improve knockout studies, as the edited cells could be established from the primed or naive hESCs and propagated as single-cell clones before being used to form blastoids[93,94]. Indeed, alternative strategies to using human embryos will continue to improve and develop, which will create a need for new ethical discussions and regulations.

In **papers II and III** we knocked out *EED* and *SPEN*, respectively, and creating a synthetic embryo using those cell lines would allow us to investigate how the blastoids develop and what impact the loss of function would have on growth, transcriptional signatures and the epigenetic landscape that are shaped during this period. Looking beyond the understanding how one of the X chromosomes become inactivated or how PRC2 act as a barrier regulating development, we can also learn about their deregulation, which is commonly seen is disease. Many times, understanding what establishes the "normal state" facilitates the understanding of the disease and ultimately how new drugs are designed.

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