T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

OPTIMIZATION AND RISK ASSESSMENT OF RAB27A-/- GENE THERAPY ALTERNATIVES USING CURRENT MOLECULAR TECHNIQUES AND TECHNOLOGIES

Özgür Doğuş EROL DUYAR (DVM)

Stem Cell Program

DOCTOR OF PHILOSOPHY THESIS

ANKARA

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Assoc. Prof. Fatima S. F. AERTS KAYA

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APPROVAL PAGE

Optimization and Risk Assessment of RAB27A-/- Gene Therapy Alternatives Using Current Molecular Techniques and Technologies

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In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in the data set. In the case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except for cited references. It was produced by myself in consultation with my supervisor Assoc. Prof. Dr. Fatima S. F. AERTS KAYA and written according to the rules of thesis writing at Hacettepe University Graduate School of Health Sciences.

Özgür Doğuş EROL DUYAR

ACKNOWLEDGMENT

I would like to express my sincere gratitude to my most amazing advisor, Assoc. Prof. Fatima Aerts Kaya, who enlightened me with her knowledge and experience throughout my doctoral education, motivated me and lifted me up every time I fell in the difficulties of scientific life.

I would like to thank Prof. Dr. Ayşen Özcan, Prof. Dr. Betül Çelebi Saltik, Prof. Dr. F. Duygu Çetinkaya, Prof. Dr. Fatma Visal Okur, who prepared me for the scientific life during my doctoral education. I would also like to express my respect to Prof. Dr. Emin Kansu, who showed me the importance of following the current literature through my education. Also, I am glad to have you all, my colleagues with whom I work in the department. I would like to express my gratitude to Prof. Dr. Petek Korkusuz and Prof. Dr. Y. Çetin Kocaefe and their students Merve Gizer and Hasan Basri Kılıç for their contributions to my doctoral thesis project. I am grateful to Prof. Dr. Gerard Wagemaker, who helped me understand the meaning of the PhD, and to Assist. Prof. Dr. Niek van Til and Trudi P. Visser, who broadened my horizon with respect to scientific research during my short visit to the Netherlands. I would like to thank my dear roommate Dr. H. Merve Tuna, who listened to my complaining at home for a long time. My dear teammates, everything would be incomplete without you, I'm glad to have you all. Burcu Pervin and Mehmet Emin Şeker, we are 1 in 3 together and I am glad we are. I would like to thank Şimal Şenocak, Ece Polat, Sema Nur Gür and Necef Özbek as well. I would like to express my gratitude to Dr. Burcu Özçimen, who is very valuable to me with her contributions in scientific life and who also never gets tired of being friends with me.

I would like to express my love to my dear dad and mom Ali Mehmet Erol and Zübeyde Erol who supported me during my educational life. Last but not least, my husband Arif Duyar, who made me smile during these stressful days and always made me feel his love and support.

I would like to thank the YÖK 100/2000 program and TÜBİTAK BİDEB-2211 Domestic Graduate Scholarship Program for providing me with financial support during my doctoral journey.

ABSTRACT

EROL DUYAR, Ö.D. Optimization and Risk Assessment of RAB27A-/- Gene Therapy Alternatives using Current Molecular Techniques and Technologies. Hacettepe University Graduate School of Health Sciences Stem Cell Doctor of Philosophy Thesis, Ankara, 2024. Griscelli Syndrome Type 2 (GS-2) is caused by a mutation in RAB27A which plays a role in exocytosis and membrane trafficking, resulting in immune deficiency. Here, we investigated 1) the efficacy of CRISPR/Cas9 gene editing of RAB27A using GS-2 MSCs and iPSC lines; 2) the efficacy of LV vectors to transduce MSCs and express RAB27A under the control of the SFFV, PGK and UCOE promoters, and 3) potential risks related to overexpression of RAB27A in immune deficient mice. We designed CRISPR/Cas9 constructs that target mutations in *RAB27A* and tested the gene correction efficacy on GS-2 MSC and iPSC lines. We generated a new GS-2 iPSC line and optimized cell culture/cryopreservation. Although editing of *RAB27A* using CRISPR/Cas9 is possible, survival of the stem cells was low. Transduction of GS-2 MSCs with LV vectors showed the highest RAB27A expression with SFFV, followed by PGK and UCOE. RAB27A+ MSCs and HSCs were transplanted into immune deficient mice but did not cause tumor formation. However, RAB27A overexpression may affect stem cell function. In conclusion, we compared the efficiency of gene editing using CRISPR/Cas9 with gene addition using LV vectors for the development of gene therapy for GS-2. Although gene editing results in acceptable levels of repair, the technology results in low cell viability. LV gene transfer was easy and robust, but high expression of RAB27A affected stem cell function. Thus, both methods can be developed into gene therapy for GS-2, but optimization of the procedures, increasing cell viability and fine-tuning of expression levels may be necessary before these therapies are ready for clinical use.

Keywords: Griscelli Syndrome Type 2, CRISPR/Cas9, lentivirus, stem cells.

(*) This study was supported by grants from the Scientific and Technological Research Council of Turkish Government TÜBİTAK no: 219S675 and Hacettepe University Scientific Research Projects Coordination Unit grant no: TUK-2019-17760 and THD-2022-19940.

ÖZET

EROL DUYAR, Ö.D. Güncel Moleküler Teknikler ve Teknolojiler Kullanılarak RAB27A-/- Gen Tedavi Alternatiflerinin Optimizasyonu ve Risk Değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Kök Hücre Doktora Tezi, Ankara, 2024. Griscelli Sendromu Tip 2 (GS-2), ekzositoz ve membran trafiğinde rol oynayan RAB27A geninde meydana gelen mutasyonların neden olduğu bir immün yetersizliktir. Bu tez kapsamında 1) GS-2 MKH'leri ve uPKH hatlarını kullanarak RAB27A'nın CRISPR/Cas9 gen düzenlemesinin etkinliğini; 2) LV vektörlerinin SFFV, PGK ve UCOE promoterlerinin kontrolünde MKH'lere aktarım ve RAB27A ekspresyon etkinliğini ve 3) immun yetmezlikli farelerde RAB27A'nın aşırı ifadesine bağlı potansiyel riskleri araştırılmıştır. RAB27A'daki mutasyonları hedef alan CRISPR/Cas9 elemanları tasarlanmış ve GS-2 MKH ve uPKH hatlarında gen düzeltme etkinliği test edilmiştir. Yeni bir GS-2 uPKH hattı oluşturulmuştur ve hücre kültürü/dondurma yöntemi optimize edilmiştir. RAB27A'nın CRISPR/Cas9 yöntemi ile düzeltilmesi mümkün olmasına rağmen kök hücrelerin hayatta kalım oranı düşük olarak bulunmuştur. GS-2 MKH'lere LV transdüksiyonu yapıldığında en yüksek ifadeyi SFFV'nin sağladığı ardından PGK ve UCOE'nun takip ettiği gösterilmiştir. RAB27A+ MKH'ler ve HKH'ler, immun yetmezlikli farelere nakledilmiştir ancak tümör oluşumuna neden olmamıştır. Ancak RAB27A'nın aşırı ifadesinin kök hücre fonksiyonunu etkilediği gösterilmiştir. Sonuç olarak, GS-2 gen tedavisinin geliştirilmesinde CRISPR/Cas9 ile gen düzenleme ve LV vektörü ile gen ekleme karşılaştırılmıştır. Gen düzenleme yeterli düzeyde onarım yapmasına rağmen CRISPR aktarım teknolojisi hücre canlılığının düşürmektedir. LV gen aktarımı kolay ve etkindir ancak RAB27A'nın yüksek ifadesi kök hücre fonksiyonu etkilemektedir. Bu nedenle, her iki yöntem de GS-2 için gen tedavisine yönelik olarak geliştirilebilir, ancak bu tedaviler klinik kullanıma hazır olmadan önce yöntemlerin optimizasyonu, hücre canlılığının arttırılması ve ifade seviyelerinin tam olarak ayarlanması gerekli olabilir.

Anahtar Kelimeler: Griscelli Sendromu Tip 2, CRISPR/Cas9, lentivirüs, kök hücreler

(*) Bu çalışma, Türkiye Cumhuriyeti Bilimsel ve Teknolojik Araştırma Kurumu TÜBİTAK no: 219S675 ve Hacettepe Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi hibe no: TUK-2019-17760 ve THD-2022-19940 ile desteklenmiştir.

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SYMBOLS and ABBREVIATIONS

ADA	Adenosine Deaminase
AML	Acute Myeloid Leukemia
ARS	Alizarin Red S
BD	Becton Dickinson
BM	Bone Marrow
bp	Base pair
cDNA	Complementary Deoxyribonucleic Acid
CGD	Chronic Granulomatous Disease
CHM	Inherited Choroideremia
CM	Complete Medium
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocytes
DAPI	Diamidino-2-Phenylindole Dihydrochloride
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DMEM-LG	Dulbecco's Modified Eagle Medium-Low Glucose
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic Acid
EF1α	Elongation Factor 1 Alpha
EPO	Erythropoietin
EVs	Extracellular Vesicles
FBS	Fetal Bovine Serum
GAP	GTPase Activating Proteins
GDI	GDP Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factors
GM-CSF	Granulocyte/Macrophage-Colony Stimulating Factor
GS	Griscelli Syndrome
GS2	Griscelli Syndrome Type 2
GTP	Guanosine Triphosphate
HDR	Homology Directed Repair
HEK293T	Human Embryonic Kidney 293T
HI	Heat Inactivation
HIV-1	Human Immunodeficiency Virus Type 1
HLH	Hemophagocytic Lymphohistiocytosis
HSC	Hematopoietic Stem Cell
HSV-1	Herpes Simplex Virus Type 1

IBMX	Isobutylmethylxanthine
IL-3	Interleukin 3
In	Leaden
iPSC	Induced Pluripotent Stem Cells
LB	Luria-Bertani
LCMV	Lymphocytic Choriomeningitis Virus
LV	Lentiviral
Mlph	Melanophilin
MMPs	Matrix Metalloproteinases
MNC	Mononuclear Cells
MΘ	Macrophage
NHEJ	Non-Homologous End Joining
NK	Natural Killer
O/N	Overnight
ORO	Oil Red O
OSKM	OCT4, SOX2, KLF4 And C-MYC
P/S	Penicillin/Streptomycin
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PDK	Phosphoglycerate Kinase
PI4K2α	Phosphatidylinositol 4-Kinase Type 2α
RAB27A	Ras-Related Protein 27A
RAG	Recombination Activating-Gene
RCL	Replication-Competent Lentiviral Vector
REP	RAB Escort Protein
RES	Resveratrol
Ri	ROCK Inhibitor
rmTPO	Recombinant Murine Thrombopoietin
RNP	Ribonucleoprotein
RPE	Retinal Pigment Epithelium
RRE	Rev Response Element
RT	Reverse Transcriptase
RV	Retroviral
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency Disease
SFFV	Spleen Focus Forming Virus
sgRNA	Single Guide Ribonucleic Acid
SIN	Self-Inactivating
SPL	Spleen

STF	SCF, TPO And Flt3-Ligand
ssODN	Single strand oligodeoxynucleotide
Th	Helper T Cell
TNC	Total Nucleated Cells
UCB	Umbilical Cord Blood
UCOE	Ubiquitous Chromatin-Opening Element
WAS	Wiskott–Aldrich Syndrome
X-SCID	X-Linked Severe Combined Immunodeficiency

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1. INTRODUCTION

Griscelli Syndrome Type 2 (GS-2) is an immune deficiency caused by a mutation in the *RAB27A* gene. The Rab GTPase encoded by this gene, plays an important role in exocytosis and intracellular membrane trafficking and cytoplasmic expression is found widely throughout the body, including in cells of the hematopoietic and immune systems. Mutations affecting interactions of the GTPase with its effectors have been linked to immune dysfunction, affecting both cells of the innate and the adaptive immune system, such as cytotoxic T cells. In addition, overactivation of the immune system may cause hemophagocytic lymphohistiocytosis (HLH) in some patients. Currently, the only curative treatment for GS-2 is Hematopoietic Stem Cell (HSC) transplantation. Induction of physiologic RAB27A expression or overexpression in HSCs through gene editing or the use of lentiviral vectors could be a potential approach to treat RAB27A deficiency. However, for rare genetically inherited diseases, such as GS-2, it is very difficult to obtain sufficient HSCs to design and test the efficacy of gene editing tools or viral vectors. Therefore, we used GS-2 mesenchymal stem cell (MSC) and induced pluripotent stem cell (iPSC) lines that we created previously and novel cell lines that we generated in the framework of this thesis. We then designed mutation-specific CRISPR/Cas9 constructs and cloned a codon-optimized RAB27A transgene (RAB27Aco) into three lentiviral vector constructs with different promoters that we then tested on these cell lines. Using these cell lines and constructs, we then aimed to assess 1) the efficacy of CRISPR/Cas9 gene editing of mutations in exon 3 and 7 of the *RAB27A* gene using GS-2 patient-derived MSC and iPSC lines and by measuring rates of homology directed repair (HDR), non-homologous end joining (NHEJ) and the occurrence of deletions; 2) the efficacy of third generation self-inactivating (SIN) lentiviral (LV) vectors to transduce MSCs and express RAB27A under the control of the constitutively active SFFV, the physiological PGK and the methylation-resistant UCOE promoters, and 3) potential (tumorigenic) risks related to overexpression of RAB27A in otherwise healthy stem cells in an immune deficient RAG2 mouse model.

2. GENERAL INTRODUCTION

2.1 RAB27A structure and function

RAB27A, also known as RAM or GS2, is a member of the small GTPases superfamily (1). The RAB27A gene is located on chromosome 15 (NC_000015.10) at the reverse strand of the 15q21.3 locus (Figure 2.1) and consists of approximately 87 base pairs (bp) (NG_009103). The *RAB27A* gene can be subject to alternative splicing and four transcript variants encoding the same protein have been described (Table 2.1) (2). The *RAB27A* gene has 7 exons of approximately 1 kb in size. However, the RAB27A coding sequence is only 666 bp and translates into a 25 kDa protein consisting of 221 amino acids (AA).

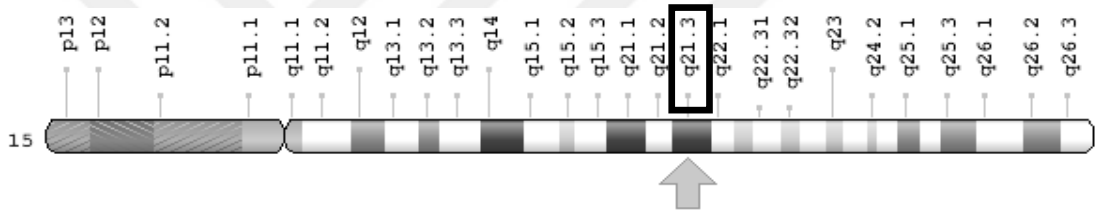


Figure 2.1. Chromosome localization of RAB27A. RAB27A is located at the q21.3 locus of chromosome 15.

The RAB27A protein has been shown to regulate multiple critical functions within the cell, including recycling of cell surface receptors, exocytosis, membrane trafficking, cell transduction, protein transport and degranulation of cells (3-7). RAB genes in the human genome have most likely arisen through gene duplications and alternative splicing results in the production of various isoforms with overlapping or similar functions, although some may show different abilities through binding to distinct effectors (Figure 2.2) (8). Currently, two different RAB27A isoforms are known that lack the 146th-153rd AA from the canonical protein that is produced by alternative splicing (9).

Table 2.1. mRNA transcripts of RAB27A.

Gene ID & name	Transcript accession	Transcript length	Protein transcript accession	Protein transcript-length
5873, RAB27A	NM_183235.3	3447	NP_899058.1	221
	NM_183236.3	3413	NP_899059.1	221
	NM_004580.5	3464	NP_004571.2	221
	NM_183234.2	3455	NP_899057.1	221

2.2 RAB27A mechanism of action

The RAB27A protein acts as a molecular switch that cycles between an active (GTP-bound) and an inactive (GDP-bound) form and its activity is modulated by a variety of guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and effectors (10).

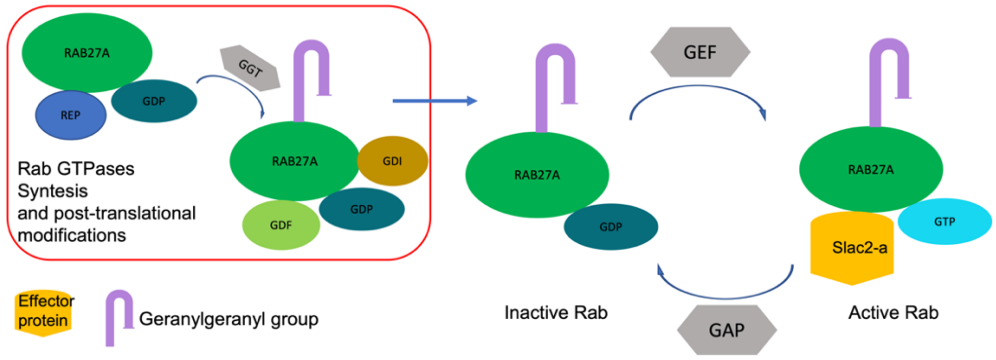


Figure 2.2. Switch mechanism of RAB27A. RAB27A switches that are ON in the GTP-bound (active) form and OFF in the GDP-bound (inactive) form like other GTPases. RAB27A has several effector proteins to show its function. REP: Rab escort protein; GDI: GDP Dissociation Inhibitor; GEF: Guanine Nucleotide Exchange Factors; GAP: GTPase Activating Proteins (11).

Conformational changes in the switch regions of RAB27A determine whether the molecule is in an active or inactive state. Similar to the other GTPases, the GDP-bound form of the RAB27A is inactive and becomes activated by GEFs, which recognize specific residues in the switch region that release GDP. In order to stimulate a cellular response, the GTP-bound form of RAB27A needs to bind with its effector proteins, such as Slp1, Slp2-a and Melanophilin (Figure 2.2). Thus, RAB27A function is activated (ON), when it is bound to GTP and inactive (OFF) when GTP is hydrolyzed

to GDP by GAPs (10). The G proteins contain a C-terminal prenylation motif which anchors them onto the cellular membrane (12). GDP Dissociation Inhibitor (GDI) can bind to the prenylated lipids, inhibiting GDP release from RAB and stabilizing the inactive form (12).

2.3. RAB27A function in healthy tissues and cells

RAB27A exerts different functions depending on the tissue and the presence of or binding to its effectors, but its main role is regulation of intracellular membrane trafficking, including fusion, cargo transport, lysosomal exocytosis and its function as a crucial element of secretory granules (13). Until now, three different types of Rab27a effectors have been found, including synaptotagmin-like protein (Slp), Slp homolog lacking C2 domains (Slac2/Melanophilin), and Munc13-4 (14). Among these, interaction with Slp2-a has been shown to promote the transport of Rab27a-containing vesicles to the membrane of secretory cells (15), Slac2a (Melanophilin, Mlph) is responsible for the transport of melanosomes (16), whereas Munc13-4 is the GTP-Rab27a specific-binding protein responsible for granule secretion in platelets (17). The function of RAB27A and its interaction with its effector proteins in physiological and pathological conditions is described in more detail below (Table 2.2). It has further been suggested that RAB27A may regulate two sequential phases of the secretory pathway i.e., a transport phase by interaction with a motor molecule, such as kinesin or myosin, and a docking phase, where RAB27A interacts through its effector molecules with a protein or lipid bound to the plasma membrane, resulting in docking/tethering of the vesicle or organelle (18).

Table 2.2. Rab27a effectors and functions in different tissues.

Effectors	Other names	Binding Partners	RAB27A affinity (19)	Functions
Slp1/JFC1	Exophilin-7	PIP3, Kinesin-1	High affinity	Promotes fusion of secretory granules not docked to the plasma membrane (20) Regulates exocytosis of neutrophilic granules (21) Regulates secretory lysosome exocytosis from CTLs (22) Regulates anterograde transport of TrkB in axons (23)
Slp2-a	Exophilin-4	Phosphatidyl-serine in the plasma membrane (24)	High affinity	Promotes transport of Rab27a-containing vesicles to the membrane of secretory cells (15) Regulates glucagon secretion in pancreatic endocrine cells (25) Regulates secretory lysosome exocytosis from CTLs (22) Regulates trafficking of myelin and myelination of Schwann cells (26) Regulates exocytosis of perforin-containing cytotoxic granule release from CTLs (27)
Slp3-a	Exophilin-6	Kinesin-1	Low affinity	Regulates transport of lytic granules in CTLs (28)
Slp4-a	Granuphilin, Exophilin-2	Munc18-1/ Syntaxin	High affinity	Regulates the exocytosis of insulin-containing dense-core granules in pancreatic beta cells (29) Regulates dense-core vesicle exocytosis in PC12 cells (30)
Slp5	Exophilin-9		High affinity	Induces hormone secretion in PC12 cells (31)
Slac2-a	Melanophilin, Exophilin-3	Myosin Va	Low affinity	Responsible for the transport of melanosomes (16)
Slac2-b	Exophilin-5		High affinity	Involved in exosome production (32)
Slac2-c	MyRIP Exophilin-8		Low affinity	Links secretory granules to actin and controls their release (33) Regulates insulin exocytosis (34) Cooperate with calcium channels for rapid activation of the photoreceptors (35, 36)
Rabphilin-3	Exophilin-1	Noc2	High affinity	Regulates dense-core vesicle exocytosis in PC12 cells (37)
Noc2a		Rabphilin-3	High affinity	Regulates dense-core vesicle exocytosis in PC12 cells (37)
Munc13-4	Unc13d			Responsible for granule secretion in platelets (17) and neutrophils (21) and CTLs Tethering of secretory lysosomes at the plasma membrane (38)
Coronin 3		actin		Controls endocytosis of secretory membrane in insulin-secreting cell lines (39)

Overall, Rab27a expression is remarkably widespread and found throughout different types of specialized secretory cells (both endocrine and exocrine), where it operates in unison with Rab3 molecules (13). Rab27a expression can be found in cells from the hematopoietic and gastrointestinal system, as well as in spleen and lung tissue, but not in other tissues, such as liver, heart, muscle, testis, or brain (40, 41).

2.3.1. Role of RAB27A in secretory cells

Throughout the gastrointestinal system, Rab27a expression is found in gastric glands and pits, as well as in mucus-secreting granules in the pylorus region and acid-secreting parietal cells of the stomach, Goblet cells of the small intestine and Paneth cells in the bottom of crypts. Exocrine salivary glands and exocrine pancreas cells also stained brightly positive for Rab27a expression. In addition, Rab27a expression co-localized with expression of insulin in the β-cells of the endocrine pancreatic islets of Langerhans and was also found to be present in the noradrenaline- and adrenalin-secreting cells of the adrenal glands. Outside of the gastrointestinal tract, mucus-secreting cells in the genital system, ovaries, oocytes and uterus endometrium of the reproductive system, Clara cells and ciliated cells in the bronchioles and epithelium type II cells in the alveolar region of the respiratory system have also been found to display high Rab27a expression (13, 42).

2.3.2. Role of RAB27A in melanosome distribution

RAB27A plays an important function in the distribution of melanosomes in both skin melanocytes and retinal pigment epithelium (RPE). Melanosomes in skin melanocytes travel along microtubules in both directions and are evenly distributed throughout the cell periphery, through interactions with the cortical actin cytoskeleton. These interactions are mediated by binding to a complex consisting of Rab27a/Mlph(Slac2-a)/Myosin Va (43, 44).

Melanosomes captured in the periphery are subsequently transported in granules to adjacent keratinocytes (Figure 2.3) (45-47). In contrast, melanosome motility in RPE cells is mediated through interactions of RAB27A with MyRIP and Myosin VIIa. Whereas MyRIP helps RAB27A to cooperate with calcium channels for rapid activation of the photoreceptors (35, 36), the interaction between RAB27A and Myosin VIIa regulates motility and fusion of melanosomes and phagosomes in the RPE (45). In mice melanosomes, the Rab27a-binding protein Slp2-is implicated in the regulation of Rab27a-mediated membrane transport. Slp-2 co-localizes with Rab27a and regulates melanosome distribution to the cell periphery (48).

2.3.3. Role of RAB27A in the nervous system

Rab27a has also been shown to play a role in the regulation of myelination in the nervous system. Through interaction with its effector protein Slp2-a and Rab27a form a complex that participates in the trafficking of the myelin protein and as such is involved in Schwann cell myelination and myelin-like membrane formation (26). Furthermore, Rab27a expression has been shown to increase during myelination, but decrease during neuron maturation (49) Furthermore, Rab27a has been shown to regulate the development of synaptic signaling through the release of extracellular vesicles and exosomes from immature neurons in the developing brain, as well as during repair after brain injury (50) revealing an important role for Rab27a-mediated paracrine communication.

2.3.4. Role of RAB27A in the hematopoietic system and immune cells

RAB27A is highly expressed in hematopoietic cells, particularly those with granules or secretomes that require the export of endosomes to the bloodstream. Additionally, RAB27A plays a vital role in immune system cells such as natural killer (NK) cells, cytotoxic T lymphocytes (CTL), macrophages (MΘ), mast cells and polymorphonuclear cells (neutrophil, basophil, eosinophil) which possess lytic

secretory granules to protect the body (5, 51-53). In the hematopoietic system, Rab27a has been shown to regulate the transport of cell surface receptors in hematopoietic cells, transport of lysosome-related organelles, release of secretory granules, as well as differentiation of bone marrow macrophages into functional, multinucleated osteoclasts (6).

2.3.5. Role of RAB27A in susceptibility to viral infections

Recent studies have revealed that Rab27a is involved in Human immunodeficiency virus type 1 (HIV-1) assembly and entrance to the cells via the phosphatidylinositol 4-kinase type 2α (PI4K2α) signaling pathway, which is activated by Rab27a effector Slp2a. Through control of PI4K2α levels in the plasma membrane Rab27a further affects HIV-1 replication in helper T cells (Th) and macrophages. These findings show that Rab27a is important for both viral assembly and replication (54). Similarly, Rab27a is implicated in the Herpes Simplex Virus type 1 (HSV-1) assembly through colocalization with glycoproteins of HSV-1 in the trans-Golgi network and viral assembly has been shown to be significantly decreased after Rab27a silencing, suggesting that Rab27a depletion/suppression may be used to decrease viral shedding (55).

2.4. The role of RAB27A in the development of diseases

2.4.1. Griscelli Syndrome subtypes

Griscelli Syndrome (GS) is an autosomal recessive disease caused by mutations in the *MYOVA, RAB27A or MLPH* genes, all of which present with different manifestations. Whereas the *MYOVA* mutations cause GS type 1 (GS-1), *RAB27A* mutations cause type 2 (GS-2) and *MLPH* mutations cause type 3 (GS-3).

GS-1 (or Elejalde Syndrome) is characterized by pigment dilution and severe neurological disabilities resulting from defects in actin-depended transport in secretory cells (56, 57). The often fatal neurological abnormalities are caused by

MyoVa, which plays an important role in axonal transport, development of dendritic spine structure, and synaptic plasticity of the brain (58). RAB27A is involved in the regulation of specialized secretory granules, such as melanosomes, lytic granules and platelet-dense granules in various cell types. Mutations in *RAB27A* and its effectors may cause the development of Griscelli Syndrome Type 2 (GS-2) (59, 60). GS-2 presents as a severe combined immunodeficiency disease (SCID), despite the presence of immune cells and is caused by a dysfunction of intracellular vesicle trafficking. This results in hypopigmentation and silver-gray hair due to the dysfunction of melanocytes, as shown in Figure 2.3 (51, 61), in combination with dysregulation of both innate and effector immune cells, including neutrophils, macrophages and T cells (62-64), as shown in Figure 2.4. Cells from the hematopoietic system affected by RAB27A mutations are shown in Figure 2.5. Dysregulation of immune cells in these children not only causes increased susceptibility to infections, but it may also cause hemophagocytic lymphohistiocytosis (HLH) in some of the patients (59, 60). Most of the *RAB27A* mutations cause either a null mutation or a frameshift mutation, but several missense mutant variants, such as Trp73Gly (W>G), Leu130Pro (L>P) and Ala152Val (A>P) are relatively common and have been reported by several centers (65). Since actions of RAB27A are mediated through interactions with a wide range of effector molecules, mutations affecting interaction with different effector molecules may have different molecular consequences (Supplement 7). For example, the L130P mutation affects GTP binding and, as a result, interferes with melanophilin binding, thus blocking melanosome transportation, whereas the W73G mutation does not inhibit binding to GTP, but prevents interaction with melanophilin (66). The W73G mutation of RAB27A also affects binding to its effector protein Munc13-4, causing the lack of activation of CTL and mast cells (67). In contrast, the A152P mutation neither affects GTP binding nor melanophilin interaction, but it acts as a mutant due to its overexpression (51, 66).

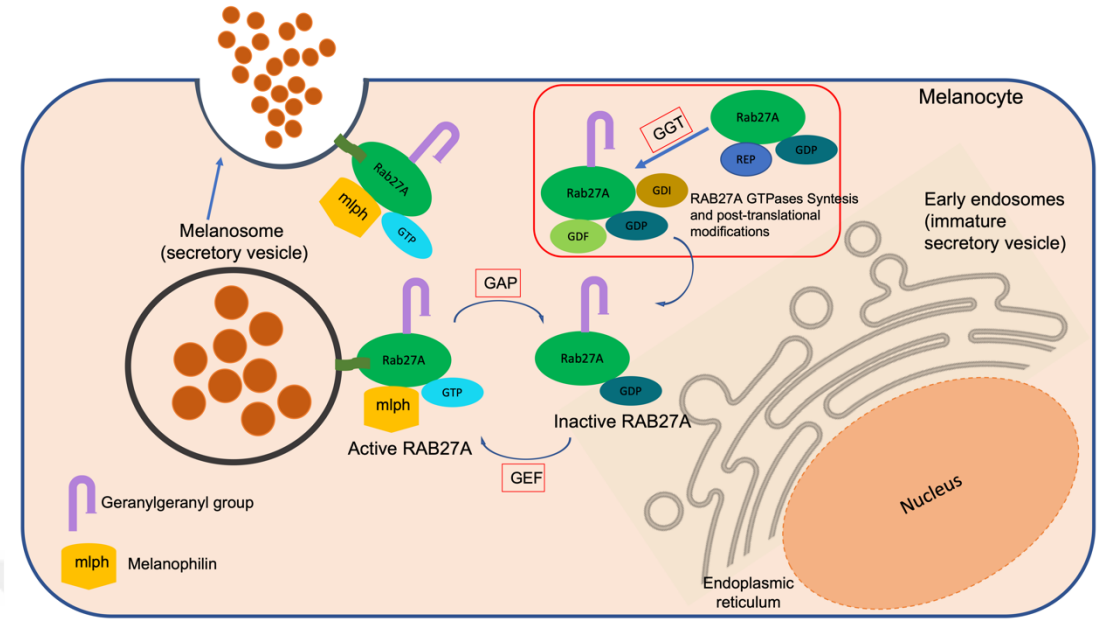


Figure 2.3. RAB27A protein and its effector melanophilin. The RAB27A protein is synthesized and maintained in an inactive state in the cell cytoplasm. When the cell gets the signal for granule secretion, RAB27A is switched on to its active state and works together with melanophilin to affect the exocytosis of granules in melanocytes. Mlph: melanophilin.

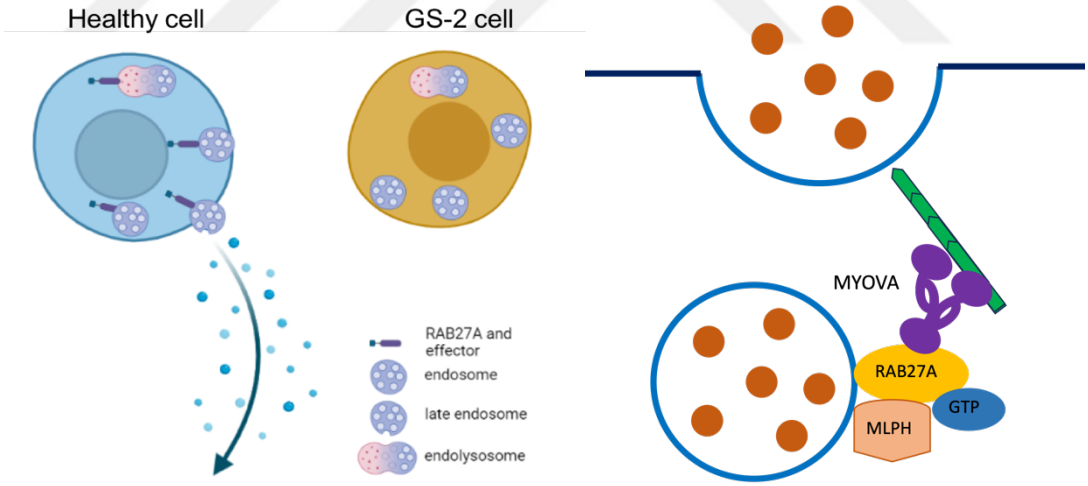


Figure 2.4. Functional consequences of RAB27A, MLPH and MYOVA mutations and interactions on cell function. Left: Healthy cells are able to secrete their endosomal content, whereas GS-2 cells are defective and cannot produce lytic granules or secrete endosomes. Right: Whereas MYOVA binds to the C-terminal of the RAB27A protein, MLPH binds its N terminal domain. Disturbance of interactions between MYOVA, RAB27A and MLPH results in respectively GS-1, GS-2 and GS-3.

Although mutations in the *RAB27A* gene lead to dysfunction of the immune system, not all patients develop HLH and some patients manifest with a late onset of HLH. Conversely, some patients diagnosed with GS-2 may have HLH as the presenting symptom. In contrast to GS-1, neurological involvement is not standard in GS-2 patients and exhibition of neurological manifestations are often secondary, resulting from the infiltration of lymphocytes into the central nervous system (CNS) (28).

GS-3, which is caused by a mutation in the *MLPH* gene generally has a milder clinical picture than GS-1 and GS-2, and mainly presents with disrupted melanosome transport in the skin and hair, resulting in hypopigmentation (63, 68). Whereas the Rab27a binding domain of Mlph is found at its N terminal, MyoVa has been shown to bind its C terminal domain (47). Thus, melanosome transport requires the functionality of all three *MLPH*, *RAB27A* and *MYOVA* genes, which explains the commonly shared feature of hypopigmentation among all GS types (Figure 2.4) (56, 68).

2.4.2. Diabetes

Diabetes mellitus (DM) is one of the most common metabolic disorders in the world. Recent studies have suggested that RAB27A may also play a role in the pathogenesis of diabetes. For example, RAB27A has been shown to regulate insulin secretion from pancreatic beta cells (29), through interaction with Slp2-a and Coronin 3 (39, 69), and glucagon secretion from alpha cells through interactions with Slp4-a (24, 25). Moreover, mutations in the RAB27A gene have been linked to an increased risk of developing Type 2 DM in some populations (70).

2.4.3. Kidney disease

Diabetic kidney disease has been shown to be exacerbated by an excessive inflammatory response in proximal tubular epithelial cells and increased secretion of exosomes (71, 72). Rab27a is involved in the regulation of secretion of extracellular vesicles (EVs) that exert an autocrine and paracrine effect in the Diabetic Kidney Disease (DKD) (73). Rab27a silencing has been suggested as a target to prevent the disease progression of different kidney diseases (73, 74).

2.4. The role of RAB27A in cancer and precancerous tissues

Aberrant regulation of RAB proteins has been implicated in the development and progression of many types of cancer. In particular, several studies have suggested that RAB27A may play a role in the invasion and metastasis of cancer cells. For example, RAB27A has been shown to promote the secretion of matrix metalloproteinases (MMPs), which degrade the extracellular matrix and facilitate cancer cell invasion. In addition, RAB27A has been shown to play a role in the control of intracellular trafficking and the regulation of exosome secretion, which can promote cancer cell survival and metastasis (32, 75) and have been associated with the facilitation of cancer development (11, 76).

In addition, RAB27A has been identified as a regulator of cancer progression and may through disruption of the extracellular matrix promote cancer cell invasion (77-80). Accordingly, RAB27A has been shown to promote the invasiveness of breast cancer cells through upregulation of metalloproteinases (80). However, although it is unknown whether increased expression of RAB27A in cancers is causative or secondary, RAB27A upregulation has been observed in many types of cancers and precancerous cells (80, 81). Forcefully induced RAB27A overexpression has been shown to promote cancer cell proliferation, invasion, metastasis and chemoresistance (82). Despite indications that RAB27A is involved in the progression of cancer development, there are currently not enough data that indicate a direct effect of RAB27A on neoplastic transformation of healthy (stem) cells.

2.5. Modelling and development of new treatment modalities for GS-2

2.5.1. *In vitro* models of GS-2

Even though the function of RAB27A and most of its known effectors have been largely unveiled, we still lack sufficient knowledge to treat the (symptoms of the) disease with any other treatment modality than with HSC transplantation. In addition, the cellular and molecular mechanisms underlying GS-2 and the reasons why some patients develop HLH and/or neurological sequelae, whereas others are barely affected or develop late-onset versions of the disease, remain poorly understood. A better understanding of the interactions between Rab27a and its effectors is therefore crucial to determine new treatment targets and to develop novel therapeutic strategies for GS-2. The rareness of GS-2 makes it difficult to obtain enough (stem) cells to study disease mechanisms or to develop and test novel treatment strategies. Recently, new technologies have been developed that allow the generation of induced Pluripotent Stem Cells (iPSCs) through ectopic overexpression of combinations of transcription factors, as described below. This has allowed the reprogramming of somatic cells and unlimited culture of disease-specific cells obtained from patients with rare genetically inherited syndromes and the establishment of fundamental biobanks, as sources of these cells (83).

We have previously developed and characterized several iPSC lines from a healthy donor (SK) and three different GS-2 patients (YF, YKÇ, İK) that we generated from donor and patient-derived mesenchymal stem cells. The donor and GS-2 iPSCs were differentiated using a two-step model, consisting of a 12-15 day period where 1) iPSCs are differentiated into primitive HSCs, 2) followed by a 10-14 day period used for terminal differentiation towards myeloid lineage (84). This model can now also be used for terminal differentiation into cells of the lymphoid lineage, to assess the effects and molecular mechanisms of RAB27A pathology during differentiation and activation of cells. In addition, we can now use these cells to develop and test treatment modalities, including gene therapeutics based on CRISPR/Cas9 and lentiviral vectors (discussed below).

2.5.2. Mouse models for GS-2 manifestations

Mouse models for GS are, similar to the disease in humans, classified into three types, with the *MyoVa* mutated *dilute (d)* mouse model representing GS-1, the *ashen (ash)* mouse model with mutations in *Rab27a* representing GS-2 (C3H/HeSn-ash/ash) (85) and the *leaden (ln)* mouse model (C57L/J-ln/ln) with a mutation in *Mlph* representing GS-3 (86). The naturally occurring *ashen* mouse model of GS-2, is quite similar to the disease in humans and is characterized by hypopigmentation due to altered expression of the *Rab27a* gene caused by splice site mutations in melanocytes and immune cells (85). However, in contrast to GS-2 in humans, the *ashen* mouse model does not develop HLH spontaneously and requires induction through exposure to the lymphocytic choriomeningitis virus (LCMV) strain in order to develop symptomatology resembling the HLH component of GS-2 (87). Other transgenic mouse models of Rab27a effectors, such as Rab escort protein 1 (Rep1), have been used to study inherited Choroideremia (CHM) and showed that ashen mouse retinas have a normal appearance whereas Rep1 knockout mice display retinal defects (88). In addition, the *ashen* mouse model has been used to study the effect of Rab27a in the regulation of dense granules and platelet function (89).

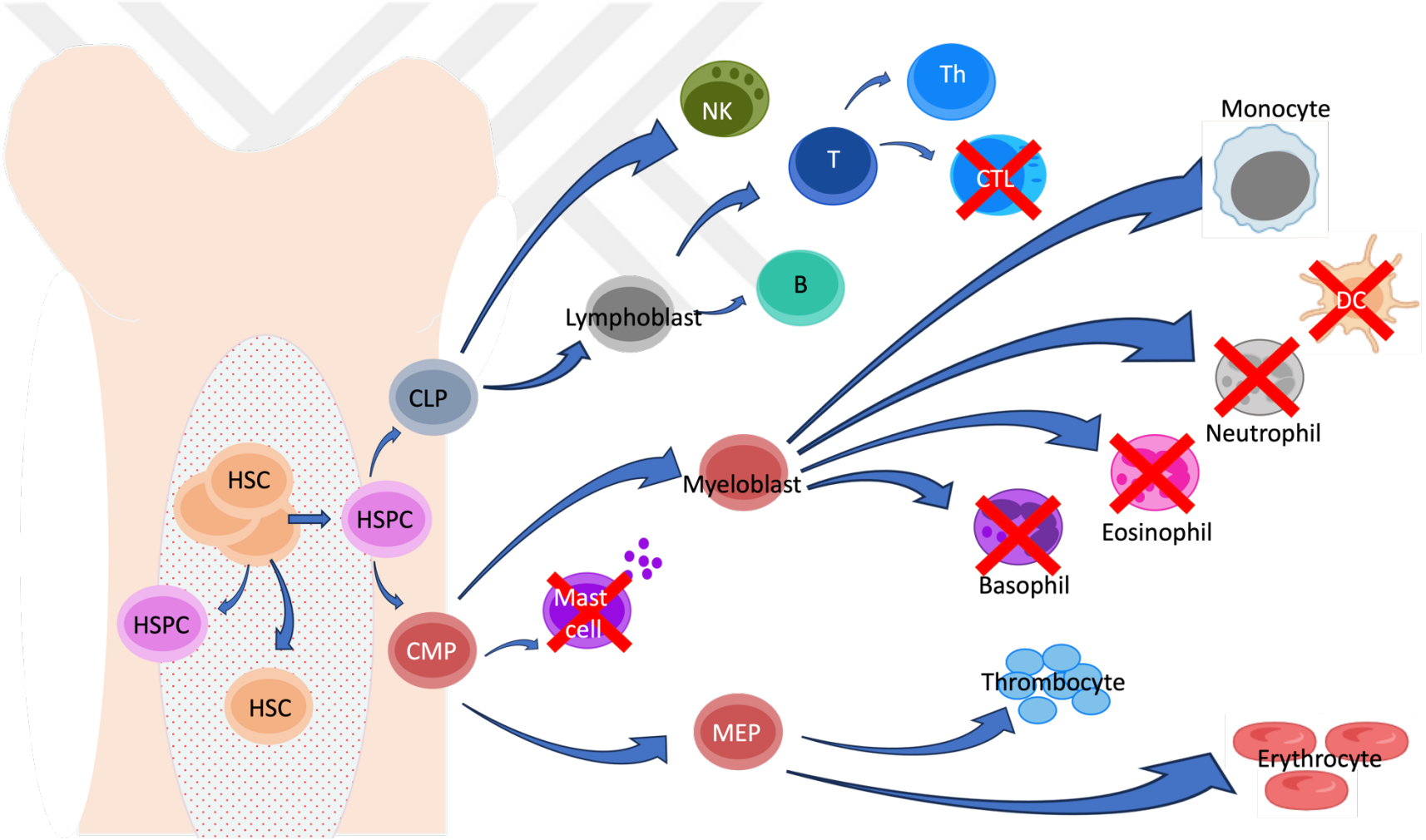


Figure 2.5. Hematopoietic cells are affected in GS-2. RAB27A is crucial for immune cell functions, and affects NK cells, CTL, MΦ, mast cells, neutrophils, basophils and eosinophils which all require functional lytic secretory granules.

2.6. Stem cells and their properties

2.6.1. Multipotent Mesenchymal Stromal Cells (MSCs)

MSCs are mesodermal derived multipotent stromal cells which can differentiate into adipogenic, osteogenic and chondrogenic tissues. They have a fibroblast-like shape and are positive for CD73, CD90 and CD105 surface markers and negative for hematopoietic markers, such as HLA-DR, CD45 and endothelial markers, such as CD31 and vWF (90). Among others, MSCs can be rapidly isolated from bone marrow, umbilical cord or adipose tissue (91).

MSCs are among the most commonly used stem cell types in regenerative medicine applications and have been tested for treatment of bone and cartilage diseases, autoimmune and acute graft versus host disease as well as skin disorders due to their immune modulatory and anti-inflammatory effects (92, 93). Additionally, MSCs migrate directly towards sites of inflammation or injury in response to a gradient of chemotactic cytokines, where they promote cell survival and angiogenesis (94). Although transplantation of MSCs in clinical trials has been shown to be overall safe, transplantation studies in mice have shown that prolonged culture of murine MSCs can cause the formation of sarcomas *in vivo* (95). In addition, the ability of MSCs to suppress the immune system, may cause additional risk in cancer-prone patients, since MSCs can thus promote the development or progression of tumors. Furthermore, MSCs can promote tumor development and progression due to remodeling of the extracellular matrix, fibroblast activation and neovascularization (96, 97).

2.6.2. Induced Pluripotent Stem Cells (iPSCs)

Current technologies allow the generation of iPSCs from somatic cells through ectopic overexpression of combinations of transcription factors, such as OCT4, SOX2, KLF4 and c-MYC (OSKM) MSCs (98) or OCT4, SOX2, NANOG and LIN28 (99). iPSCs can be used for disease modeling, drug development and studying of developmental

biology. The use of disease-specific or patient-derived iPSCs are especially important, since current mouse models that are being used in drug development and disease modeling, not always faithfully recapitulate the disease or disease pathophysiology in humans (100). Thus far, iPSCs have been shown to be play an important role in the modeling of diseases, such as immune deficiencies and lysosomal storage diseases (42, 84, 101-103).

In order to enhance reproducibility of results, especially studies using stem cells as source material require the use of optimized cell culture maintenance protocols, with special emphasis on stability of medium formulations, and the use of different types/sourced of extracellular matrix molecules (ECM). Most research labs use Matrigel as a source of ECM for the culture of iPSCs. The development of Matrigel has been shown to be of great value since its use makes the use of murine embryonic fibroblast (MEF) feeder layers unnecessary, thus allowing long-term cell culture in the absence of risks of murine cell contamination or potential negative effects due to the presence of xenogenic based components (104). Matrigel is extracted from Engelbreth–Holm–Swarm (EHS) mouse sarcomas, contains all known major components of tissue basement membranes and has been used for many decades for a variety of cell culture applications (105). Where the growth factor-reduced (GFR) molecule formulation is popular for its use in culture of ESCs and iPSCs, as well as tissue organoids (106), in different formulations, Matrigel has been used for culture of testis cells, angiogenesis assays, tumor invasion assays and other applications, both *in vitro* and *in vivo* (105). However, major issues associated with the use of Matrigel are that its suitability for studies of cellular biology and its potential for clinical use and/or drug testing are limited due to its heterogenous, indistinct and batch-to-batch variable composition. These lot-to-lot inconsistencies further contribute to unforeseen alterations in the physical and biochemical properties of Matrigel batches and may cause differences between experiments and decreased reproducibility (107).

2.6.3. Hematopoietic Stem Cells (HSCs)

HSCs are responsible for the daily production of myeloid and lymphoid cell lineages and can be isolated from bone marrow, umbilical cord blood or peripheral blood after stimulation with Granulocyte-Colony Stimulating Factor (G-CSF). Regulation of the HSC pool occurs through communication with osteoblasts, mesenchymal cells, ECM and cell signals (108). HSC transplantation has been used to treat hereditary diseases that affect the hematopoietic and immunologic systems, as well as for a selection of enzyme-deficiencies that can be treated by secretion of the missing enzyme (109, 110).

However, when the number of HSCs in the transplant is not sufficient or in the absence of a human leukocyte antigen (HLA) compatible donor, novel treatment modalities, such as gene addition allowing production of the missing enzyme or protein through vectors that provide permanent integration into HSCs or direct gene editing of the HSCs should be developed. For a number of immune deficiencies, retroviral and lentiviral vector-based systems have now been developed and are being used in the clinic (111-117). Gene editing has proven more difficult to achieve, but has recently also found its way into clinical practice (118, 119).

2.7. Gene therapy tools for treatment of inherited diseases

Currently, the only curative treatment for GS-2 patients is HSC transplantation (59, 60). But similar to other SCIDs, the development of lentiviral (LV) gene therapy for patients who do not have a suitable donor may be another option. For some of the SCIDs, including Adenosine Deaminase (ADA) SCID, X-linked SCID (X-SCID), Recombination activating-gene (RAG) 1 and 2 deficiency, Chronic Granulomatous Disease (CGD) and Wiskott–Aldrich Syndrome (WAS) gene therapy, using either retroviral and/or lentiviral vectors, has already been developed or is being tested in clinical trials (120-125). However, since the use of retroviral vectors has been associated with insertional mutagenesis due to integration in or near specific oncogenes (i.e. *MDS1-EVI1, LMO2, CCND2* and *SETBP1*) (126-129), most researchers

have switched to the development and use of lentiviral vectors because of their more suitable safety profile (122, 130). Although the currently used lentiviral vector systems have been shown to exhibit a decreased the risk for development of leukemia after HSC gene therapy, some of the promoters or transgenes used may still carry an intrinsic mutagenic risk (131-133). Therefore, in addition to the lentiviral vectors systems, novel gene editing tools, such as Zink fingers, TALENS and the CRISPR/Cas9 system have been developed. Below, we will discuss the development of lentiviral vectors and the CRISPR/Cas systems and their potential use for the treatment of inherited diseases.

2.7.1. Development of lentiviral vectors

Lentiviruses are members of the *Retroviridae* family consisting of identical ssRNA and are approximately 9 kb in length. The lentiviral vectors were developed, because the previously used Retroviral (RV) vectors were shown to have a preference for integration near specific protooncogenes (*LMO2, CCND2, MSD1-EVI1, PRDM16, SETBP1*) and required cell division for optimal gene transfer. In contrast, LVs can transduce both dividing and non-dividing cells and have been shown to integrate more randomly into the genome (134, 135). Lentiviral vectors (LVs) were developed using the genome of human immunodeficiency virus 1 (HIV-1). The lentiviral vector ssRNA genome consists of *gag, pol* and *env* genes and accessory genes. They have their own reverse transcriptase enzyme (RT) that transcribes the RNA into complementary DNA (cDNA), as well as an integrase enzyme encoded by *pol* which is required for the integration of cDNA into the host genome.

The first-generation (1st) LVs still contained HIV pathogenicity genes and was developed as a three-plasmid system: The packaging plasmid contained the accessory genes *vif, vpr, vpu, nef*, *rev* and *tat*. The second plasmid encoded the env gene and the third plasmid carried the original wild-type HIV 5’ and 3’ LTRs, the packaging sequence ψ, and the rev response element (RRE), as well as the transgene and its internal promoter (136). To develop a safer vector system, *vif, vpr, vpu* and nef were

eliminated in the second-generation (2nd) LV vector systems from the packaging plasmid. However, the *tat* gene could not be deleted from the genome due to its fundamental role in viral production and titration (137). In the third-generation (3rd) LV vector systems, the *rev* gene was separated from the packaging plasmid and placed *in trans* onto a fourth plasmid to prevent interaction with *gag* and *pol* (138). This way the generation of replication-competent LVs (RCL) requires at least two recombination events. Additionally, to increase the safety, the enhancer sequences in the 5'LTR-U3 region were deleted, resulting in self-inactivating (SIN) lentiviral vectors with a decreased risk for the generation of RCL (138) (Figure 2.6).

2.7.2. Development of new promoter systems

Long term stable transgene expression is one of the challenges in the gene therapy field and is mostly manipulated using a variety of virally-derived promoters. The most commonly used promoters are the constitutively active promoters PGK (phosphoglycerate kinase), EF1α (Elongation Factor 1 alpha) and SFFV (Spleen Focus Forming Virus). In addition to the tissue/cell-specific promoters, recently, a novel class of promoters, such as the UCOE (Ubiquitous Chromatin-Opening Element) promoter has been developed. We have previously discussed the design and function of the LV plasmids and the use of these vectors for the treatment of genetically inherited diseases in detail (110). PGK is a constitutively active physiological promoter, which is preferred in many gene therapy constructs. In contrast, the SFFV promoter is a strong constitutively active promoter that may activate nearby genes and may cause clonal expansion and tumor progression (132, 139). Therefore, PGK is often preferred to reduce genotoxicity in both LV and RV vectors (139, 140). However, due to their constitutively active nature, both of these plasmids are prone to silencing due to methylation of CpG islands (141).

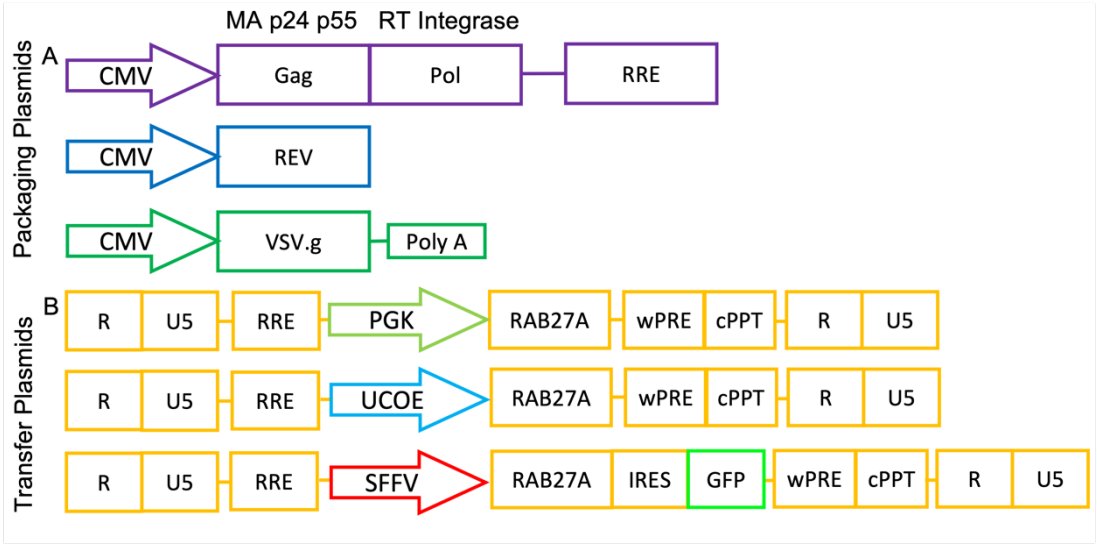


Figure 2.6. Generation of third generation self inactivating (SIN) LVs. A) the packaging plasmids encoding Gag (matrix, capsid and nucleocapsid proteins) and pol gene (reverse transcriptase). The rev response element (RRE) containing plasmid assists Rev with the posttranscriptional export of viral mRNAs and the envelop plasmid, pseudotyped with VSV.g for broad tissue tropism. B) Transfer plasmid components can be used for cloning of different promoters (PGK, UCOE, SFFV) and transgene of choice (in our case RAB27Aco).

The newly developed UCOE promoter, is specifically designed to increase resistance to epigenetic silencing in several cell types, especially multipotent and pluripotent stem cells by counteracting epigenetic silencing and preventing position effect variegation (142, 143).

2.7.3. Development of CRISPR/Cas9 gene editing tools

The CRISPR/Cas9 system was discovered as a defence system in prokaryotes against bacteriophages (144) and has since then been developed as a powerful, programmable, RNA guided gene editing tool (Figure 2.7) (145). Initially, the CRISPR/Cas system was used as a gene editing tool in combination with the use of the SpCas9 (*Streptococcus pyogenes* CRISPR associated protein 9) enzyme (146, 147). This Cas9 enzyme recognizes the PAM (protospacer adjacent motifs) and creates double strand breaks in the DNA with the guidance of two RNA subunits i.e., crRNA (crispr RNA) and tracrRNA (trans activating RNA) that are together called the guide RNA

(gRNA) when they are hybridized (146, 148). Since its discovery, the CRISPR/Cas9 system has been improved and optimized and new Cas enzymes and a new hybridized guide RNA system consisting of a single guide RNAs (sgRNA) have been developed.

Currently, different bacterial Cas enzymes, such as Cas12a (cpf1), dead SpCas9 (dCas9) and Cas13 can be used to target different PAM sequences in the genome for genetic editing of dsDNA and ssRNA, respectively (146). This feature provides more precise editing of the human genome with increased efficiency and reduced insertion/deletion off-target effects due to non-homologous end joining (NHEJ). Although Cas9 is the most preferred CRISPR enzyme the newly developed Cas12 has been favored for gene therapy because it targets AT-rich sequences, reducing off-target effects in the genome. However, the use of the Cas12 enzyme has been challenging due to its restriction site precision. Whereas the Cas12a enzyme cuts the DNA at the 12th-13th base after the PAM sequences (TTTV) with a sticky end, the Cas9 enzyme cuts at the 3rd base after the PAM sequences (NGG) with a blunt end. Because of this feature, as well as its high efficiency and relatively low off-target effects, Cas9 is still the most preferred enzyme for genetic correction of mammalian genomes (149, 150). The simplicity of the CRISPR/Cas system, in combination with its exceptionally effective DNA targeting and cleaving efficiency, as well as the possibility to choose from many naturally occurring type II (single Cas) system variants, has allowed researchers to precisely and efficiently target and edit specific genomic sequences (151).

2.8. Hypothesis and aim

In the framework of this PhD thesis, we wanted to develop and test different gene therapy tools to test their efficacy and feasibility for the treatment of GS-2. Our “0” hypothesis is therefore, “gene editing with CRISPR/Cas9 is as efficient as gene addition using a lentiviral vector with a codon-optimized RAB27A transgene for the treatment of GS-2”.

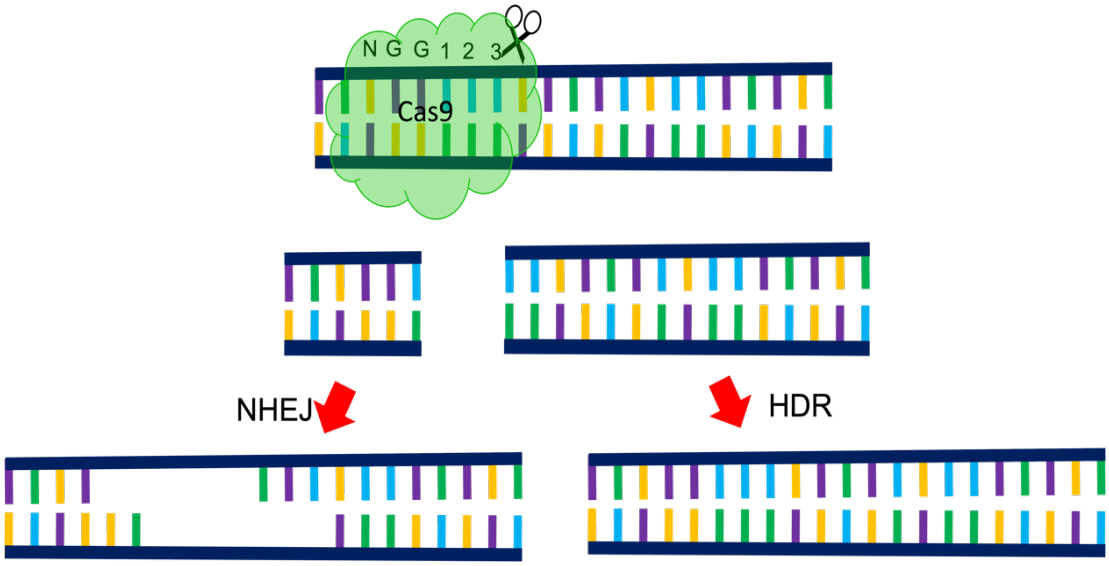


Figure 2.7. Working mechanism of the CRISPR/Cas9 gene editing tool. CRISPR/Cas9 induces double strand breaks and repair through non-homologous end joining (NHEJ) or homology directed repair (HDR) using the gRNA as a template.

We therefore designed different CRISPR/Cas9 constructs to specifically edit and repair mutations in the exon 3 and exon 7 of the RAB27A gene using MSCs and iPSCs from two GS-2 patients and assessed efficiency, cell viability and the frequency of homology directed repair (HDR). We then designed and cloned several lentiviral constructs carrying a codon-optimized version of the RAB27A gene under the control of PGK, SFFV and UCOE promoters and tested their efficacy *in vitro* using MSCs and iPSCs from GS-2 patients and we tested the presence of any tumorigenic potential of our lentiviral constructs by overexpressing RAB27A in healthy MSCs and HSCs, using the strongest promoter and the highest MOI and by transplanting these cells in immune deficient animals. Although our main goal was to “functionally repair” GS-2 HSCs, for construct optimization we used GS-2 MSCs, due to their availability, rapid culture and the presence of the same RAB27A mutations.

This study aimed further to increase our knowledge and experience using current molecular gene therapy tools and to develop an alternative treatment for patients with GS-2.

3. MATERIALS AND METHODS

3.1. Stem Cell Cultures

3.1.1. Mesenchymal Stem Cells (MSCs)

The MSCs used in this thesis were initially obtained from healthy donors (n=4) and GS-2 patients (n=4) after approval by the Hacettepe University Ethical Committee for Non-Interventional Clinical Research (GO14/424) (84) and re-used after approval by the Hacettepe University Ethical Committee for Non-interventional Clinical Research (GO20/316). MSCs were cultured in presence of DMF10, consisting of a mixture 60% DMEM-LG (Dulbecco's Modified Eagle Medium-Low Glucose, Thermo Fisher Scientific, #31885), 40% MCDB-201 (Sigma, #M6770) medium, supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Life Technologies, #10270), 1% penicillin/streptomycin (P/S, Gibco, #15140), and 2 mM L-glutamine (Sigma, #G3126). The FBS used in this study was batch tested by confirming support of MSC proliferation and differentiation before purchase and lot#45F5081K was used in all experiments. Heat inactivation (HI) was obtained by thawing the frozen FBS in 37^{o}C and then heating the FBS for 45 minutes at 56^{o}C. FBS-HI batches were spun down to remove complement debris and sterile aliquoted and stored at -20^{o}C until use. MSC culture media were changed twice a week. When cells reached 70-80% confluency, cells were passaged using 0,25% Trypsin/1 mM Ethylenediaminetetraacetic acid (EDTA). Briefly, cells were washed with 1X Phosphate Buffered Saline (PBS, Advanta, #R01038) and then incubated for 5 min at 37^{o}C 5% CO_2. After detachment cells were collected with 10 mL DMF10 and centrifuged at 450 x*g* for 5 min in a tabletop centrifuge (Eppendorf, #5810R). Cells were counted with a Bürker-Türker hemocytometer using 0,4% Trypan Blue staining and 3x10^5 cells were seeded per T75 flask (4000 c/cm^2). For optimization of freezing conditions, cells were frozen in presence of 10% DMSO or 5% DMSO with or without the addition of the anti-oxidants, SUL-109 or Resveratrol (RES).

3.1.2. Hematopoietic Stem and Progenitor Cells (HSPCs)

Umbilical cord blood (UCB) was collected from the healthy term newborns born by Cesarean section at the Department of Gynecology and Obstetrics after approval by the Hacettepe University Ethical Committee on Non-Interventional Clinical Research (GO20/316) and obtaining of informed consent of the mothers. Up to 60 mL UCB was collected in 20 mL PBS containing 20 μL Heparin (5.000 IU/mL, Poliparin). Collected UCBs were inspected for the presence of clots. If the collected volume exceeded >40 mL, cells were processed for use or cryopreservation. Viable nucleated cell counts were assessed before (total nucleated cells, TNC) and after (mononuclear cells, MNC) density centrifugation using Lymphocyte Separation Medium (Cegrogen, #J0500). Cells were counted using Turk's solution (0,01% Crystal violet/1% glacial acetic acid) to lyse red blood cells. Recovery was calculated for quality control purposes and MNCs were used if the recovery was between 20-40% of the original TNC number. CD34+ HSPCs were isolated using magnetic activated cell sorting (MACS). Briefly, MNCs were centrifuged at 300 x*g* for 5 min twice and then resuspended in 300 μL of MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA [Miltenyi Biotech, #130-091-376]) for up to 10^8 total cells. Cells were then incubated with 100 μL FcR Blocking Reagent and 100 μL CD34 MicroBeads (Miltenyi Biotech, #130-046-703) and incubated at 4^{o}C for 30 min at a MACS rotator. After incubation, cells were washed with 4 mL MACS buffer and centrifuged at 300 x*g* for 5 min twice. Supernatant was discarded and cells were resuspended with 3 mL MACS buffer. Cells were layered onto pre-wetted LS columns (Miltenyi Biotech, #130-042-401) containing a pre-separator 40 um cell sieve (Miltenyi Biotech, #130-041-407) and placed into a magnetic field (MidiMACS). Columns were rinsed with 4x3 mL ice cold MACS buffer and the negative flow through was collected in a 15 mL tube (CD34- cell fraction). The CD34+ cell fraction was collected after removal of the column from the magnetic field and rinsing of the columns using 2 x 6 mL of MACS buffer.

Healthy male Balb/c bone marrow (BM) cells were collected from femurs and tibia and CD117+ BM cells were selected using a Lineage depletion kit (Miltenyi, #130-090-858), followed by positive selection for CD117 (Miltenyi, #130-091-224), according to the manufacturer's instructions. Briefly, BM cells were washed with 1X PBS twice and resuspended in MACS buffer. Resuspended cells were counted and centrifuged at 300 x*g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in 40 µL MACS buffer and 10 µL biotin-antibody against lineage positive cells per 10^7 total cells and incubated at 4°C for 10 min. After the addition of 30 µL MACS buffer and 20 µL of anti-Biotin MicroBeads per 10^7 total cells were added and incubated for 15 min at 4°C. Cells were washed with 2 mL MACS buffer twice and centrifuged at 300 x*g* for 5 min. Cells were resuspended in 2 mL MACS buffer and then passed through a pre-separation filter (Miltenyi, #130-041-407) to prevent clogging of the column. The column was placed on the magnet and pre-wetted with MACS buffer before use. While the lineage positive cells, bound to biotin-antibodies were captured onto the magnet, the flow-through contained the lineage-negative stem cell enriched fraction. The flow-through was collected by washing the column twice with 6 mL MACS buffer. The lineage positive cells were collected by washing with MACS buffer after the removal of the column from the magnetized field. Both cell fractions were counted and recovery of the cell fractions was calculated.

For the selection of CD117+ cells, the lineage negative fraction was resuspended in MACS buffer and centrifuged at 300 x*g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in 80 µL of MACS buffer per 10^7 total cells. Per 10^7 total cells 20 µL of anti-CD117-microBeads were added and cells were incubated at 4°C for 30 min. After incubation, the cells were washed with 2 mL MACS buffer per 10^7 cells and centrifuged at 300×*g* for 10 minutes. The supernatant was discarded and the cells were resuspended in 2 mL MACS buffer and counted.

CD117+ BM cells were cultured in HSC expansion medium (Miltenyi, #130-100-463) supplemented with 30 ng/mL recombinant murine TPO (rmTPO, Peprotech, #315-14) (152).

3.1.3. Induced Pluripotent Stem Cells (iPSCs)

Some of the GS-2 iPSC clones used within the framework of this thesis were previously generated and characterized (84). Other new GS-2 iPSC clones were generated as part of this thesis after approval by the Hacettepe University Ethical Committee on Non-Interventional Clinical Research (GO20/316). Characteristics of all samples used are shown in Table 3.1.

Table 3.1. Confirmed RAB27A mutations in healthy donor and GS-2 MSCs and iPSCs.

clone	Patient	Mutation	Exon	characterization
YF #A2A3	GS-2	c.148-149delinsC	3	(84)
İK #5	GS-2	c.514-518delCAAGC	7	(84)
GP #1	--	ok	--	(84)
AB #2	GS-2	c.T217Ghom*	5	This thesis
AB #4	GS-2	c.T217G hom	5	This thesis
ARB	carrier	c.T217G het*	5	This thesis
ABM	--	ok	--	This thesis

*hom: homozygote, het: heterozygote

Generation of novel GS-2 iPSC clones

GS-2 patient AB-derived MSCs were seeded at different concentrations (12.500 and 25.000 cell/well) into wells of a 12-well plate in 2 mL DMF10 medium and incubated overnight (O/N). The following day 1 mL of unconcentrated LV-SF-OSKM (Figure 3.1) lentiviral vector was added onto cells and cells were incubated for 1 day. The LV-SF-OSKM construct was kindly provided by Prof. Dr. Axel Schambach (MH Hannover) and production and testing of the viral vector supernatants have been described before (84). Briefly, HEK293T were used as packaging cells. For transfection of the HEK293T cells a mixture of 20 µg transfer vector (SFFV-OSKM), 7 µg envelope (pMD2-VSV.g), 13 µg p.GAG.POL.RRE (pMDLg/p-RRE) and 5 µg p.REV (pRSV-REV) plasmids was used (153). Transfections were done using a calcium phosphate kit (CAPHOS, Takara bio, #631312). 18 hours after transfection, the medium was changed, and after 24 hours, the supernatants containing the lentiviral vector were collected and passed through a 45 µm filter (Millipore, #SLHP033RS). The filtered viral vector supernatants were concentrated using a Beckman Coulter (#326823) with a

SW32 rotor at 20,000 rpm for 2 hours at 4°C. MSCs were transduced overnight with LV-OSKM, followed by culture with DMF10 for 7 days (or until confluent) and media were changed every 2 days. 6-well plates were coated with Matrigel (Corning, #354277, Lot #3025004), according to the manufacturer's instructions.

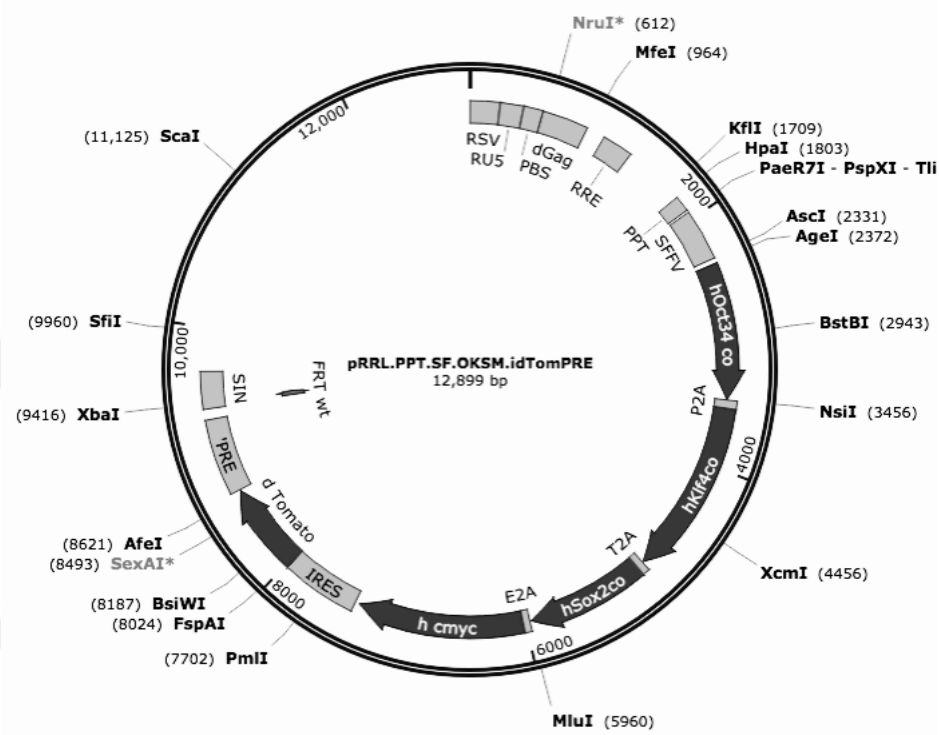


Figure 3.1. The LV-OSKM transfer plasmid backbone. OSKM contains the SFFV promoter and human OCT3/4, SOX2, KLF4 and c-MYC transgenes with idTom as a tag.

24 hours before cell culture, an aliquoted Matrigel sample was thawed at 4°C O/N and carefully diluted in 25 mL DMEM:F12 media (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12, Thermo Fisher, #11330). Using pre-cooled pipettes, 1 mL Matrigel was layered onto each well of a 6-well plate and either incubated at 37°C for 30 min or at room temperature for 60 min. LV-OSKM-transduced MSCs were collected with 0,25% Trypsin/1 mM EDTA, as described above, and seeded onto the Matrigel coated 6-well plates in presence of DMF10. The next day, wells were checked for cell adherence and medium was replaced with 1,5 mL iPS Brew (Miltenyi, #130-104-368), TeSR-E8 (Stem Cell Technologies, #05990) or mTeSR Plus (Stem Cell Technologies, #100-0276) media to test which cell culture medium supported

reprogramming of somatic cells best. Media were changed daily and cell culture was continued until the colony formation was observed. When cell colonies were sufficient in number and size, several clones were picked. Colonies that were sufficiently large for selection, were divided into small pieces using a 10 μL pipette tip or an insulin injection needle. Colonies were then carefully selected and transferred onto fresh Matrigel-coated 12-well plates, prefilled with TeSR-E8 or iPS Brew XF and 10 μM Rock inhibitor (Ri, Y27632, Tocris, #1254).

Maintenance and cryopreservation of iPSCs

Since we noticed spontaneous differentiation in some of the iPSC cultures, we decided to test different conditions for the maintenance and cryopreservation of the cells. In similar the reprogramming, we tested the three media described above for long-term culture maintenance and optimized cryopreservation of the iPSCs using 10% DMSO or DMSO-reduced cryopreservation medium formulations, supplemented with the antioxidants SUL-109 (Sulfateq, #FD01) at 1:25 (154) or Resveratrol (RES, Enzo, #BML-FR104-0100) at a concentration of 1 μM and 10 μM. Cryovials were placed in a Mr. Frosty (Nalgene) and stored at -80°C for at least 24 hours before being transferred to a liquid Nitrogen tank.

Passaging of iPSCs

iPSCs were passaged using ReLeSR (Stem Cell Technologies, #100-0484). Briefly, 30 min before passaging, fresh Matrigel-coated 6 well plates were prepared. The culture medium was brought to room temperature. Cells were washed with DMEM:F12 or DPBS (Gibco, #14190) and the medium was aspirated. 1 mL ReLeSR was added into the wells and cells were incubated for 1 min. After the ReLeSR was discarded, the plate was placed into an incubator to incubate the cells for 5 more minutes at 37°C. After incubation, colonies were collected carefully in order to prevent breaking up of the colonies into single cells and transferred as clumps to fresh Matrigel-coated wells in the presence of media and 10 μM Ri. For thawing, frozen iPSCs were placed in a water bath at 37°C for 3-4 min and then immediately

transferred to thawing media, consisting of E8 medium (Stem Cell Technologies, #05990) supplemented with 1% P/S with or w/o 10 μM Ri, SUL-109 or RES. Cells were centrifuged at 200 x*g* for 2 min. After the supernatant was discarded, the pellet was gently resuspended in culture media without breaking up the colonies into single cells and directly seeded on Matrigel-coated plates. The first 24 hours after thawing, cells were cultured for 48 hours in the presence of Ri with or w/o the addition of SUL-109 and RES to prevent cryopreservation-related delayed cell death. Thereafter, cell media were daily replaced with maintenance media, consisting of E8 medium with 1% P/S only.

3.2. Characterization of stem cells

3.2.1. Immunophenotyping by flow cytometry

Immunophenotyping of MSCs

MSCs were trypsinized and centrifuged at 300 *xg* for 5 min. The supernatant was discarded and the pellet was resuspended in 1 mL PBS. Cells were counted and $1x10^5$ cells were incubated with a panel of directly labeled fluorescent antibodies (shown in Table 3.2). Antibody concentrations were used according to the manufacturer's instructions or adapted after careful testing. All incubations were done in PBN (PBS; 5% BSA; 0,5% NaN_3) with 2% human AB serum to block non-specific protein binding for 15 minutes at RT, in the dark. After incubation, the cells were washed with PBN three times and resuspended in 200 μL PBN. Before acquisition, cellular gates were determined and data of 10.000 viable cells (based on the SSC vs FSC gating strategy) were recorded using a BD Accuri C6 plus flow cytometer (Becton Dickinson) and analyzed using the BD CSampler software.

For intracellular staining of RAB27A, MSCs were collected with trypsinization and centrifuged at 300 *xg* for 5 min. The supernatant was discarded and the pellet was resuspended in 1 mL PBS. Cells were counted and were divided at $1x10^5$ cells/tube. Cells were fixed in 250 μL Fixation buffer (Becton Dickinson, #554655) and incubated for 30 min at 4°C. The cells were washed once with 1 mL PBN (PBS/5%

BSA/0,5% NaN_3) and then permeabilized with 500 μL Perm Buffer III (Becton Dickinson, #558050). Cells were washed with 1 mL PBN twice and centrifuged at 300 *xg* for 5 min. The pellet was resuspended and incubated with 1 μL RAB27A primary antibody (Thermo Scientific, #4D3F11) in 25 μL PBN for 45 min at RT. After incubation cells were washed 3 times with 1 mL PBN. For secondary antibody staining, rat anti-mouse IgG2b-FITC (eBioscience, #11-4220-82) was used. Cells were washed 3 times and for the final step, cells were resuspended with 200 μL PBN. Cells were assessed using a BD Accuri C6 plus flow cytometer (Becton Dickinson) and analyzed using the BD CSampler software.

Table 3.2. Antibodies used for characterization of MSCs.

Antibody and conjugate	**Brand**	**Catalog number**
CD29-APC	BD Pharmingen	559883
CD44-APC	eBioscience	17-0441-82
CD73-FITC	BioLegend	344016
CD90-FITC	BioLegend	328108
CD105-PE	eBioscience	12-1057-42
CD166-PE	BioLegend	343903
RAB27A	Thermo Fisher	4D3F11
RAB27A	Invitrogen	PA5-79904
IgG2b-FITC	eBioscience	11-4220-82
Goat anti-mouse IgG-AF568	Abcam	ab175473

Immunophenotyping of HSPCs

Human and murine HSPCs were immunophenotyped before and after selection. Cells were centrifuged at 300 *xg* for 5 min. The supernatant was discarded and the pellet was resuspended in 1 mL PBS. Cells were counted and $1x10^5$ cells were incubated with a panel of directly labeled fluorescent antibodies (shown in Table 3.3). Antibody concentrations were used according to the manufacturer's instructions or adapted after careful testing. All incubations were done in PBN with 2% normal mouse serum to block non-specific protein binding for 30 minutes at +4°C, in the dark. After incubation, the cells were washed with PBN three times and resuspended in 200 μL PBN. Before acquisition, cellular gates were determined and data of 10.000 viable cells were recorded using flow cytometry.

Table 3.3. Antibodies used for characterization of human and mouse HSPCs.

Antibody and conjugate	Brand	Catalog number
human CD34-APC	BioLegend	343510
human CD38-PE	BioLegend	303506
human CD45-FITC	BD Bioscience	345808
human CD45-APC	BD Pharmingen	555485
mouse Sca-1 (Ly6A/E)-PE	BD Pharmingen	553108
mouse c-kit (CD117)-APC	BioLegend	135108
mouse CD45-FITC	BioLegend	103108

Immunophenotyping of iPSCs

iPSC clones were broken up into single cells using ReLeSR and centrifuged at 200 x*g* for 3 min. The supernatant was discarded and the pellet was resuspended in PBN+2% human AB serum. Antibodies used are shown in Table 3.4. Concentrations and staining protocols were used as per the manufacturer's instructions. Briefly, cells were incubated at RT for 15 min in the presence of the specified antibodies. After incubation cells were washed with PBN three times and resuspended with 200 μL PBN. Cells were assessed using the BD Accuri C6 plus flow cytometer (Becton Dickinson) and analyzed using the BD CSampler software.

Table 3.4. Antibodies used for characterization of iPSCs.

Antibody and conjugates	Brand	Catalog number	Dilution
OCT3/4-APC	Miltenyi	130-105-555	1:10
TRA-1-60-PE	Miltenyi	130-100-347	1:10
TRA-1-81-PE	Miltenyi	130-101-410	1:10
SSEA1-APC	Miltenyi	130-104-937	1:10
SSEA4-APC	Miltenyi	130-098-347	1:10
CD34-APC	Biolegend	343510	2 μL/test
CD43-PE	Biolegend	303506	2 μL/test
CD45-FITC	BD Bioscience	345808	2 μL/test

3.2.2. Immunofluorescence Staining (IF)

MSCs and iPSCs were seeded into 8-well chamber slides (Nalgene Nunc International, #154534) and were grown until they reached 70-80% confluency. The culture medium was discarded and cells were washed with PBS. Cells were fixed with

4% Formaldehyde solution (J.T. Baker, #7041) and incubated at 4°C for 10 min. Formaldehyde solution was discarded and cells were permeabilized with 500 µL 0.2% Triton-X-100 (Sigma-Aldrich, #9036-19-5) in PBS at RT for 10 min. After incubation, 100 µL blocking buffer consisting of 0.1% Tween-20 (Merck, #822184), 5% BSA (Miltenyi Biotech, #130-091-376) and 5% antibody's host animal serum was added to each well and incubated for 1h at 4°C, while the chamber slides were covered with parafilm (Sigma-Aldrich, # P7793). Antibodies were diluted in blocking buffer according to the manufacturer's instructions and added to each well and incubated at 4°C O/N. The next day, the primary antibody was discarded and washed with PBS three times. The secondary antibody was diluted with blocking buffer and 100 µL was added to each well, after which the cells were incubated at RT for 1h. DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma, #D8417) stock solution (5 mg/mL) was diluted to 5 µg/mL and added to the wells followed by incubation for 5 min at RT. Then cells were washed with PBS three times and mounting media was added onto the cells. The chamber slide was closed with coverslip glass and the samples were ready to image with Olympus fluorescence microscope (IX73). Images were analyzed with the free online ImageJ software (NIH).

3.2.3. Assessment of RAB27A expression by Western Blotting (WB)

MSCs and iPSCs were lysed with RIPA buffer containing protease inhibitors (Roche, #5892970001). Proteins (20 ug per lane) were separated using a 15% polyacrylamide gel and transferred to the PVDF membrane with a Trans-Blot system (Biorad, #1704150). Membranes were blocked with 5% non-fat dry milk (Bio-Rad, #1706404) in 0,1% Tween-20 in TBS (TBS-T) and incubated with anti-RAB27A antibody (1:1000, Saint John's, #STJ25258) overnight. Membranes were then stained with a secondary rabbit antibody (1:10000, Advansta, #R-05072-500) at room temperature for 2 hours. GAPDH (1:10000, Invitrogen, #MA5-15738) was used as a housekeeping protein. Proteins were detected using ECL (Thermo Scientific, #32132) and imaged using a FluorChem FC3 (R&D Systems).

3.2.4. RNA Isolation

MSCs and iPSCs were collected with Qiazol reagent (Qiagen, #79306) to solubilize the DNA and RNA and denature the proteins. RNA isolation was performed using the Direct-zol RNA isolation kit (Zymo, #R2062), according to the manufacturer's protocol. Briefly, absolute ethanol (Sigma-Aldrich, E7023) was added in an equal volume to TRIzol and vortexed. The mixture was transferred to a column placed in a collection tube and centrifuged at 16.000 x*g* for 30 sec. The flow-through was collected in a 15 mL polypropylene tube for protein isolation later. The column was then washed with RNA wash buffer and centrifuged at 16.000 x*g* for 30 sec. The DNase I solution was prepared by diluting 5 µL DNase I (6 IU/µL) in 35 µL DNA Digestion Buffer and directly added onto the column and incubated for 20 min at RT. After incubation the column was washed with 400 µL RNA PreWash buffer and centrifuged at 16.000 x*g* for 30 sec. The column was washed with 700 µL RNA Wash Buffer and centrifuged at 16.000 x*g* for 60 sec. Next, the column was transferred to an RNase-free tube. To elute the RNA, 15 µL of pre-warmed (37^{o}C) DNase/RNase-Free water was added directly onto the column and centrifuged at 16.000 x*g* for 60 sec. Eluted RNA was measured using a Nanodrop 1000 (Thermo Fisher).

3.2.5. Quantitative Real-Time PCR (qRT-PCR)

RNA was reverse transcribed to cDNA (Table 3.5) and the thermal cycler was set for annealing at 25°C for 5 minutes, extension at 42°C for 60 minutes and inactivation at 70°C for 15 minutes. At the end of the protocol, cDNA was diluted 5 times in DNase/RNase-free water and stored at -20^{o}C. The qRT-PCR was performed using the GoTaq 2-Step RT-qPCR kit (Promega, #A6010) and LightCycler 480 Probes Master mix (Roche, #04707494001) and assessed using a Light Cycler 480 II (Roche, USA). For details see Table 3.6. The SYBR protocol was set for 40 cycles of denaturation at 95^{o}C for 15 sec and annealing/extension at 60^{o}C for 1 minute. The primer sequences used are shown in Table 3.7. *GAPDH* and *B2M* were used as a housekeeping gene for normalization and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression (155).

Table 3.5. cDNA synthesis protocol and required volume of components.

Components	**Volume (μL)**
RNA template	Depends on concentration
Oligo dT primer (0.5μg/μl)	1
5X GoScript Buffer	4
$MgCl_2$ (25 mM)	2
dNTP mix	1
Ribonuclease Inhibitor	0,5
Reverse Transcriptase	1
Nuclease free water	Up to 20 μL

Table 3.6. Required volume of components for two different qRT-PCR kit.

Components	**Promega SYBR Mix**	**Roche Probe Master mix**
SYBR master mix (2X)	5 μL	5 μL
Primer F	0,5 μL	1 μL
Primer R	0,5 μL	
dH_2O	2 μL	2 μL

Table 3.7. Forward and reverse primer sequences.

Gene	**Forward**	**Reverse**
Primer/Probe sets		
RAB27A	CTGAAGAGGACATGTGATTGGA	GTCTTTGAGCCTTAGATTTCCAG
B2M	CCGTGTGAACCATGTGACTTT	CCTCCATGATGCTGCTTACA
OCT4	GCAAAACCCGGAGGAGTC	TCCCAGGGTGATCCTCTTCT
SOX2	ATGGGTTCGGTGGTCAAGT	GGAGGAAGAGGTAACCACAGG
NANOG	ATGCCTCACACGGAGACTGT	CTGCAGAAGTGGGTTGTTTG
PPARG	GACAGGAAAGACAACAGACAAAT	GGGGTGATGTGTTTGAACTTG
SCD	CCGGGAGAATATCCTGGTTT	AGGAGTGGTGGTAGTTGTGGA
ALPL	AGAACCCCAAAGGCTTCTTC	CTTGGCTTTTCCTTCATGGT
SP7	GACTGCAGAGCAGGTTCCTC	TAACCTGATGGGGTCATGGT
Gene	**Forward**	**Reverse**
Primers (w/o probe)		
GAPDH human/mouse	CATCACTGCCACCCAGAAGAC	TGACCTTGCCCACAGCCTTG
RAB27A human	CTGAAGAGGACATGTGATTGGA	GTCTTTGAGCCTTAGATTTCCAG
RAB27Aco	AGTACGGCATCCCTTATTTCG	ATCCAAGACTTGTCCACGCA
Sry mouse	CATCGGAGGGCTAAAGTGTCAC	TGGCATGTGGGTTCCTGTCC

3.3. Stem Cell Differentiation assays

3.3.1. Adipogenic and Osteogenic differentiation of MSCs

For adipogenic differentiation, healthy donor and GS-2 MSCs were maintained in DMEM-LG with 10% FBS-HI, 1 µM dexamethasone (Invitrogen, #D1756), 60 µM indomethacin, (Sigma, #I7378), 500 µM isobutylmethylxanthine (IBMX, Sigma, #I5879), and 5 µg/mL Insulin (Sigma, #I6634) for 7 days. After this period, cells were collected to assess changes in gene expression of the adipogenic markers *SCD* and *PPARG* using RT-PCR, as described before (156).

For osteogenic differentiation, healthy donor and GS-2 MSCs were grown in DMEM-LG with 10% FBS-HI, 100 nM dexamethasone, 10 mM β-glycerophosphate (Applichem, #2250), and 0.2 mM L-ascorbic acid (Sigma, #A4034) for 7 days. Cells were collected and assessed for changes in gene expression of the osteogenic markers *ALPL* and *RUNX2*, as described before (84, 156).

3.3.2. Hematopoietic differentiation of iPSCs

For optimization of hematopoietic differentiation of healthy donor and GS-2 iPSCs two different protocols were used. The first differentiation protocol was feeder-free, using a Matrigel-coated 10 cm tissue culture-treated dishes, whereas the second protocol makes use of the Op9 feeder layer system (157). The Op9 cell line was kindly provided by Prof. Dr. Juan Carlos Zúñiga-Pflücker (Sunny Brook Research Institute, Canada) (158) and maintained in αMEM (Minimum Essential Medium Eagle, Thermo Fisher, #11095) containing 20% FBS (Gibco, #10270) and 1% P/S. The Op9 cells were seeded onto a 10 cm petri dishes and used as feeder layers when the layers reached approximately 70-80% confluency. Then the iPSCs were seeded onto either the Matrigel-coated plates or the Op9 feeder layers in mTeSR Plus medium and observed for 10-15 days until hematopoietic island formation was seen. When clear islands were observed, induction of hematopoietic differentiation was initiated using the hematopoietic differentiation kit (Stem Cell Technologies, #05310). The culture

medium was replaced with medium A to induce mesodermal differentiation (first stage). After 3 days, the medium was changed to medium B for the second stage of differentiation, hematopoietic induction (Figure 3.2). At the end of this stage, cells should have differentiated up till the early hematopoietic stem cell (CD43+/CD34+). To assess hematopoietic differentiation, cells were collected and used for interim analyses of CD34, CD38, CD43 and CD45 expression (and loss of expression of iPSC markers) by flow cytometry and colony assays (Stem Cell Technologies, #H4434) to induce terminal hematopoietic differentiation. Briefly, 1:10 of the cells were collected from the cultures in 0,3 mL IMDM and directly mixed with 2,7 mL of methylcellulose containing Stem Cell Factor (SCF), Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF), Interleukin 3 (IL-3), Erythropoietin (EPO). Cell mixtures were plated in non-tissue culture treated 35 mm wells (BD Falcon 35-1008) at 1 mL/well and maintained in a humidified atmosphere for 10-14 days. After visible colonies appeared, colonies were counted and collected in 2 mL PBS for staining. Cells were centrifuged at 300 x*g* for 5 minutes and stained for expression of CD16, CD33, CD34, CD38, CD43 and CD45, as described above.

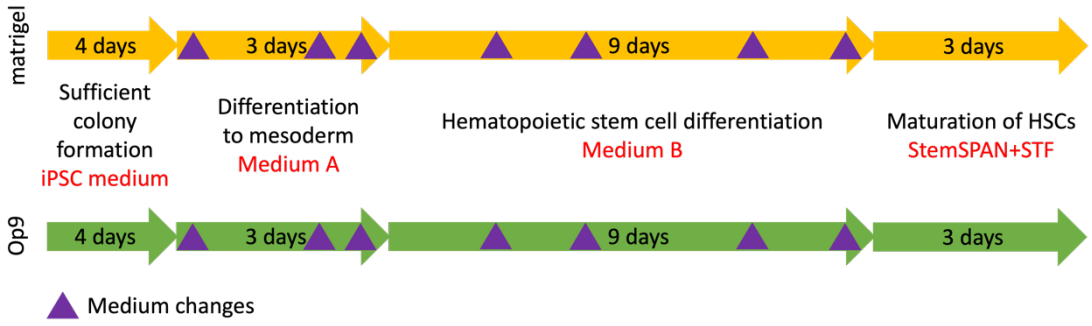


Figure 3.2. Hematopoietic differentiation of iPSCs. iPSCs were differentiated towards mesodermal and hematopoietic lineage. Plates were coated with Matrigel (upper panel) or Op9 cells (lower panel). iPSCs were seeded onto the plates. Mesodermal differentiation was induced using Medium A for 3 days, followed by HSC induction for 9 days using Medium B. Final cell expansion and maturation were done for 3 additional days using StemSPAN medium with SCF, TPO and Flt3-ligand.

3.4. Design, preparation and testing of RAB27A CRISPR/Cas9 constructs

3.4.1. gRNA design and T7 Endonuclease Assay

The guide RNA (gRNA) sequences targeting the RAB27A gene exon 3 and exon 7 mutations specific to the patient cells, were designed using the CHOPCHOP website (https://chopchop.cbu.uib.no) (159). The GS-2 mutations were annotated on the RAB27A DNA coding sequence using SnapGene software. For each mutation 3 mutation-specific gRNAs were designed and tested on GS-2 MSCs (Table 3.8).

Table 3.8. Selected gRNAs from CHOPCHOP for design of CRISPR constructs.

gRNA name	gRNA sequence	PAM
gRNA3.2	TTGATTTCAGGGAAAAACAG	TGG
gRNA3.3	CTCCAAATTTATCACAACAG	TGG
gRNA3.5	TGTATATTGGTAAAGTACAC	TGG
gRNA7.1	ATAAGCAATTGAGATGCTTC	TGG
gRNA7.2	ATTGCTTATGTTTGTCCCAT	TGG
gRNA7.3	GGACCTGATAATGAAGCGAA	TGG

Transfections were performed using the Neon Transfection System Kit (ThermoFisher Scientific, #MPK10096). Briefly, gRNAs were diluted in IDTE buffer in the presence of 200 uM crRNA and 200 μM tracrRNA to form the sgRNA complex. The sgRNA complexes were then incubated with Cas9 enzyme (Integrated DNA Technologies, IDT, #1081059) for 15 minutes at room temperature to allow formation of the ribonucleoprotein (RNP) complex. After transfection, cells were incubated at 37°C with 5% CO_2 for 72 hours. Cells from a single well were pooled and used to extract genomic DNA (gDNA). Briefly, samples were incubated with nuclear lysis buffer (50 mM Tris-Cl, 10 mM EDTA, 0.8% SDS) O/N and then digested with phenol/chloroform/isoamyl alcohol (25:24:1) for 2 min. After centrifugation, samples were transferred to 100% ethanol to precipitate the DNA. gDNA was used for PCR and loaded onto agarose gels. When PCR bands were visible on the gel, we performed the T7 Endonuclease I (T7E1) assay (New England Biolabs, #M0302) to assess the genome targeting efficiency of the designed gRNAs. Amplicons were tested using gDNA from the target GS-2 MSCs and from negative control healthy donor MSC DNA.

After the assay, NHEJ events were calculated from the agarose gel images using Gel Analyzer software (http://www.gelanalyzer.com/) followed by mismatch detection with the T7EI calculator (https://horizondiscovery.com/en/ordering-and-calculation-tools/t7ei-calculator). The estimated gene modification rate was calculated using the following formula: % gene modification = 100 x (1-(1-fraction cleaved)) (160). Single strand oligodeoxynuclotide (ssODN) repair templates were designed with homologous genomic sequences that were obtained from NCBI (Gene ID: 5873) for selected gRNAs (Table 3.9).

Table 3.9. Donor DNA sequences for to test HDR.

Strand	ssODN Sequence
E3 +	CAATATACAGATGGTAAATTTAACTCCAAATTTATCACAACAGTGGGCATTGATTTCAGGGAAAAAAGAGTGGTAAGTTCTATATCCTTCTATGTAAAAATGTAATCG
E3 -	CGATTACATTTTTACATAGAAGGATATAGAACTTACCACTCTTTTTTCCCTGAAATCAATGCCCACTGTTGTGATAAATTTGGAGTTAAATTTACCATCTGTATATTG
E7 +	CTACTTTGAAACTAGTGCTGCCAATGGGACAAACATAAGCCAAGCAATTGAGATGCTTCTGGACCTGATAATGAAGCGAATGGAACGGTGTGTGGACAAGTCCTGGTTCCTGAAGG
E7 -	CCTTCAGGAATCCAGGACTTGTCCACACACCGTTCCATTCGCTTCATTATCAGGTCCAGAAGCATCTCAATTGCTTGGCTTATGTTTGTCCCATTGGCAGCACTAGTTTCAAAGTAG

3.4.2. Correction of RAB27A mutations using CRISPR/Cas9

For correction of GS-2 MSCs, cells were collected with Trypsin/EDTA and single cells were resuspended in R-solution (Thermo, #1096B). For correction of GS-2 iPSCs, iPSCs from a single well were collected with ReLeSR solution and cell aggregates were resuspended in R-solution. The RNP complex was prepared from a mixture of gRNA (50 μM), Cas9 (61 μM) and PBS. The cell suspension was mixed with the RNP complex, homology-directed repair (HDR) donor DNA at a concentration of 100 μM, electroporation enhancer (Integrated DNA Technologies, #1075916) at 20 μM and pipetted onto the Neon Transfection System (Thermo, #1096). For negative controls DMSO was used instead of donor DNA. Transfections were performed at 1400 V, 10 ms and 3 pulses.

Transfected MSCs were cultured in 6-well plates in DMF10 without P/S for 2 days. After transfection of iPSCs, cells were placed onto fresh Matrigel-coated dishes and cultured in TeSR-E8 with 10 μM Ri for 2 days.

3.4.3. Mutation repair analysis using Sanger or NGS

After 2 days of culture, transfected and control GS-2 MSCs and iPSCs were collected and gDNA was isolated using phenol:chloroform:isoamyl alcohol (25:24:1) extraction, as described above. Sequencing was done using gDNA via Sanger or MiSeq (Illumina) by Intergen. Aligned ".bam" files were analyzed with the IGV 2.3 (Broad Institute) software.

3.5. Design and preparation of different RAB27Aco expressing lentiviral vectors

3.5.1. Subcloning of the RAB27Aco Gene and Preparation of Bacterial Stock

The transgene encoding codon-optimized human *RAB27Aco*, was modified with a Kozak sequence to improve translation, and cloned into a lentiviral backbone plasmid. We used the PGK-RAB27Aco (GenScript), UCOE-RAG2 (generously provided by Prof. Dr. Axel Schambach, MH Hannover) (125) and LeGo-SF-Angptl3-iG2 plasmids (161) to make three lentiviral RAB27Aco constructs under the control of the UCOE, PGK and SF promoters (162-164). The PGK-RAB27Aco transgene was cloned under the control of an UCOE promoter by using 1 μL AgeI-HF (R3552S, New England Biolabs) and 1 μL SbfI-HF (R3642S, New England Biolabs) restriction enzymes. Details of the protocol used are given in Table 3.10. Plasmid maps of the original constructs are shown in Figure 3.3.

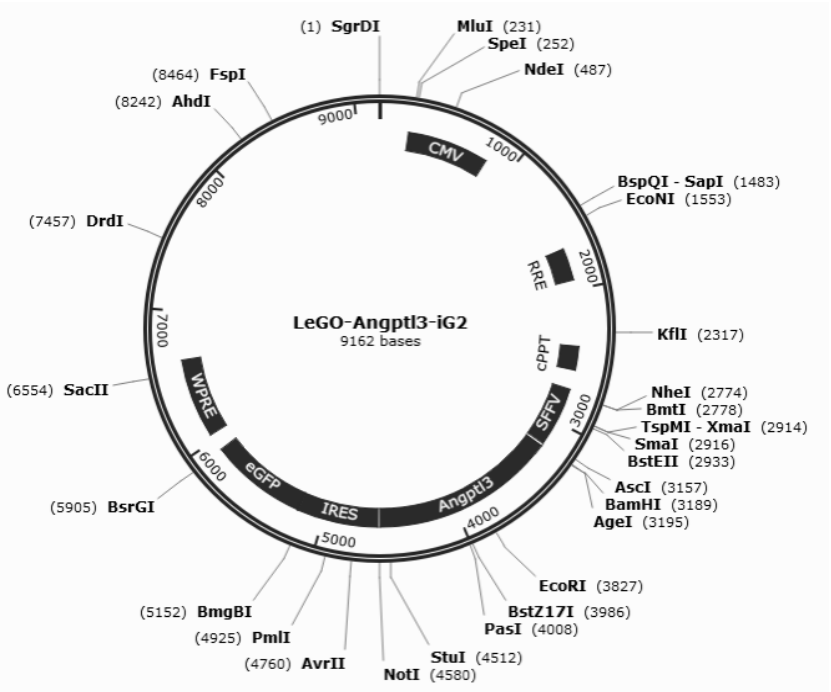
For RAB27Aco gene transfection 0,5 μL transgene plasmid was used for transformation of 12,5 μL Stbl3 competent bacteria (Invitrogen, #C737303) and incubated for 30 min on ice. Bacteria were then exposed to heat shock at 42^{o}C in a water bath for 1 min and immediately put into ice.

350 µL SOC medium (Invitrogen, #15544034) was added and the mixture was shaken for 30 min at 37°C. The SF-RAB27Aco-IRES-GFP plasmid was expressed in XL1 Blue competent bacterial cells using the same basic heat shock protocol, as described above.

Table 3.10. Cloning of RAB27Aco under the control of the UCOE and SFFV promoters.

Backbone	**PGK-RAB27A insert**	**UCOE-RAG2**	**LeGo-A3-iG2**
Plasmid	3 µg (1.75 µL)	0.5 µg (0.45 µL)	0.5 µg (0,55 µL)
CutSmart (Buffer)	2 µL	2 µL	2 µL
AgeI-HF	1 µL	1 µL	1 µL
SbfI-HF	0.5 µL	0.5 µL	0.5 µL
H_2O	14.75 µL	16.18 µL	16.45 µL
Total Volume	**20 µL**	**20 µL**	**20 µL**

After heat shock, the bacteria were transferred to Luria-Bertani (LB) agar plates and maintained at 37°C O/N. To prepare LB agar, we used 7,5 g bacto agar (BD Thermo Fisher, #DF0140-01-0), 5 g tryptone, 5 g NaCl, 2,5 g yeast extract and up to 500 mL dH_2O. After sterilization and cooling down of the LB agar, we added 500 µL ampicillin (100 ug/mL).



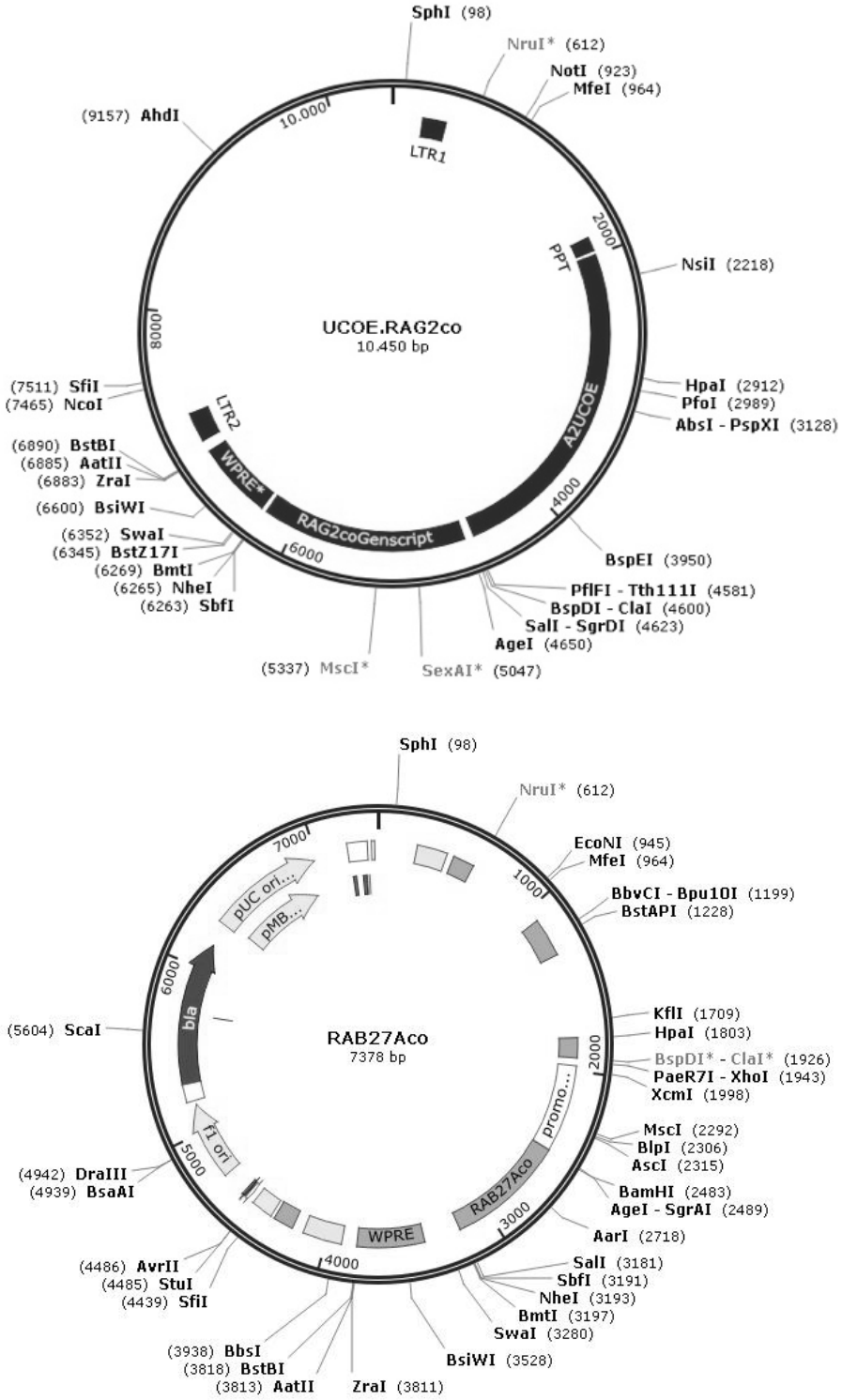


Figure 3.3. Maps of the backbone plasmids used for cloning. RAB27Aco was cloned into 3 lentiviral backbone constructs with different promoter sequences (PGK, UCOE and SFFV) for comparison of promoter efficiency.

After 18-24 hrs, selected bacterial colonies were transferred to 5 mL LB broth. For LB broth preparation we used 10 g tryptone, 10 g NaCl, 5 g yeast extract and up to 1 L of dH_2O. After 12 hrs the OD600 value was measured. When the OD value reached levels between 0.6-0.8, 2.5 mL of the bacterial suspension was added to 250 mL LB broth containing 250 µL ampicillin. The bacterial suspension was then incubated at 37°C O/N on a shaker. The next day, the bacterial suspension was centrifuged at 5000 *xg* for 30 min at 4°C in a Thermo Fisher Sorvall RCG+. After centrifugation, the supernatant was discarded and the pellets were suspended with the buffer supplied by the kit. For plasmid isolation we followed the Maxiprep (Macherey-Nagel, #740414) kit's instructions. After isolation, the plasmid pellet was resuspended in dH_2O and the DNA concentration was measured using a Nanodrop 1000.

3.5.2. Lentiviral Vector Production

For production of viral vector particles, we used the HEK293T packaging cell line (kindly provided by Dr. Niek van Til, Utrecht University Medical Center, The Netherlands). Cells were cultured in IMDM (Gibco, #12440) with 10% FBS and 1% P/S. For vector production, we used the PGK-RAB27Aco, UCOE-RAB27Aco and SF-RAB27Aco plasmids and a calcium transfection kit (Takara bio, #631312). On day 1, HEK293T cells were grown with DMEM/Glutamax, 10% FCS and 1% P/S medium. Confluent T-175 flasks of HEK293T cells were split into 5 separate 145 mm dishes, each containing 18 mL of medium. On day 2, the plasmid mix was prepared (Table 3.11) and stored at -80°C for 4-5 hrs. Four hours before the transfection the HEK293T culture media were changed. After that, the plasmid mixture was spun down at 4°C at maximum x*g* for 30 min.

The supernatant was discarded and the pellet was washed with 500 µL 70% ethanol and then spun down at 4°C at maximum *xg* for 5 min. The pellet was dissolved in 875 µL water and 125 µL calcium chloride solution per plate. The mixture was vortexed and incubated for 10 min.

Lastly, 1 mL of 2x HEPES was added while bubbling the pellet mixture. The mixture was then transferred to the HEK293T culture plates and incubated at 37ºC, 5% CO_2.

Table 3.11. Plasmid mixture components.

Vectors	**plasmid (μg)**
Transfer vector with RAB27A	20 μg
pMD2-VSV.g (invitrogen #PF532)	7 μg
pMDLg/p-RRE (invitrogen #PF531)	13 μg
pRSV-REV (invitrogen #PF530)	5 μg
Sodium acetate	10% of the total volume
100% Ethanol	x2,5 of the total volume

After 16 hrs of transfection, media were refreshed. Twenty four hours later the viral vector-containing culture supernatants were collected and filtered using 0,45 μm filters to prevent the inadvertent presence of vector-producing HEK293T cells in the supernatant. After filtration, (for reference purposes) some of the unconcentrated virus was aliquoted, frozen and stored at -80ºC, whereas the remaining volume was concentrated using an ultracentrifuge (Beckman Coulter, #326823) with SW32 rotors at 20.000 rpm for 2 h at 4ºC. The viral pellet was resuspended with 250 μL PBS and aliquoted at 25 μL/vial. Vials were frozen at -80ºC. The medium of the transfected HEK293T cells was refreshed and the next day the viral supernatant was collected and processed, as described above. After collection of day 2 viral supernatants, the transfected HEK293T cells were inactivated and discarded.

3.5.3. Virus Titration and RAB27A Staining

Viral vectors were titrated using HeLa cell lines (kindly provided by Prof. Dr. Gerard Wagemaker, Erasmus University Rotterdam, The Netherlands). HeLa cells were cultured in complete medium (CM), consisting of DMEM-LG (DMEM+Glutamax, Life Technologies, #31966), 10% FBS-HI (Life Technologies, #10270) and %1 P/S.

HeLa cells were plated at 200.000 cells/well in 6 well plates and 5 µL, 50 µL and 500 µL of 1:100 diluted concentrated viral vectors were added to the cells. After 4 days, cells were collected and stained with anti-RAB27A antibodies.

3.6. Transplantation assays

3.6.1. Animals

Mouse studies were carried out at the Hacettepe University Laboratory Animals Research and Application Center after approval of the experimental procedures by the Hacettepe University Animal Experiments Ethical Committee (2020/02-03). BALB/c-Rag2$^{-/-}$ (Rag2) mice were kindly provided by Prof. Dr. Gerard Wagemaker (Erasmus University Medical Center, Rotterdam, The Netherlands) (125). Healthy Balb/c mice were purchased from the Ankara University Experimental Animal Research Laboratory. Animals were allowed free access to irradiated chow. All animals were euthanized at the termination of the experiment by light sedation followed by cervical dislocation.

3.6.2. Transplantation of RAB27A+ MSCs in Rag2 mice

Healthy donor MSCs were transduced with LV-SF-RAB27A-IRES-GFP viral vectors for 24 hours at and MOI of 20. RAB27A expression was measured in MSCs and remaining transduced cells were cultured and 1x10^6 cells were injected with Matrigel (Corning, #354234) into Rag2 mice subcutaneously to intrascapular region (n=6). 1x10^6 healthy control MSCs were injected into the control mice (n=3). Mice were followed up to 3 months after injection. Upon sacrifice, tissues surrounding the injection site and lungs (which are the primary site for metastatic behavior after transplantation of tumorigenic cells in immune deficient mice) (165) were collected and assessed for any signs of tumor formation. PB cells were used for FACS analysis.

3.6.3. Transplantation of RAB27A+ human and murine HSCs in Rag2 mice

Overexpression of RAB27A was obtained by overnight lentiviral transduction of CD34+ and CD117+ cells with LV-SF-RAB27A-IRES-GFP at an MOI of 30. RAB27A overexpressing CD34+ HSPCs (n=3) and CD117+ (n=3) were injected intravenously at a concentration of $1,2x10^5$ CD34+ HSPCs or $3x10^4$ CD117+ cells, respectively, into Rag2 mice pretreated with 25 mg/kg Busulfan intraperitoneal (i.p.) 24 hours prior to transplantation. Non-transduced human CD34+ (n=3) and murine CD117+ (n=3) cells were injected in control mice. Mice were observed for 6 months after transplantation. Upon sacrifice, spleen (SPL), peripheral blood (PB) and bone marrow (BM) cells were collected and assessed for the presence of RAB27A expressing cells, overall engraftment of hematopoietic cells and signs of leukemia (Table 3.12).

3.6.4. Histological analysis

For histological analysis, tissue samples surrounding the injection sites were obtained upon sacrifice of transplanted and healthy control mice. In addition, whole lungs were collected, since these are often the primary metastatic sites of malignant cells after transplantation in the intrascapular subcutaneous region (165).

Table 3.12. Antibodies used for assessment of immune reconstitution

Antibody and conjugate	**Brand (catalog nr.)**
human CD34-APC	Biolegend (343510)
human CD38-PE	Biolegend (303506)
human CD3-APC	BioLegend (344812)
human CD19-PE	BD Pharmingen (555413)
human CD45-APC	BD Pharmingen (555485)
human RAB27A	Invitrogen (PA5-79904)
mouse c-kit-APC	BioLegend (135108)
mouse Sca-1-PE	BD Bioscience (553108)
mouse CD3-APC	BioLegend (100236)
mouse CD19-PE	BioLegend (115508)
mouse CD45-FITC	BioLegend (103108)

All samples were fixed in 10% phosphate-buffered formaldehyde solution dehydrated through a graded series of ethanol and cleared in Xylene in an automated tissue processor (TP1020, Leica, Germany). Paraffin-embedded samples (LG1150H-C, Leica, Germany) were cut into 3 µm thick microtome (SM2000R, Leica, Germany) sections, deparaffinized at 60^{o}C overnight and stained with Hematoxylin/Eosin (H&E). Samples were assessed for the presence of MSCs or any pathological signs related to the transplantation (i.e., infections, signs of inflammation, tumor growth, aberrant cell differentiation, etc.) using a bright field microscope (DM6B, Leica, Germany) and analyzed using the attached image analysis program (LASX, Leica, Germany).

3.7. Statistics

Student T-test analysis was performed to determine statistical significance (p-value <0,05) for differences between two groups. qRT-PCR analysis was done as described above. All calculations were performed using the Microsoft Excel spreadsheet program.

3.8. Summary of methods

A schematic overview of the methods used is given below (Figure 3.4).

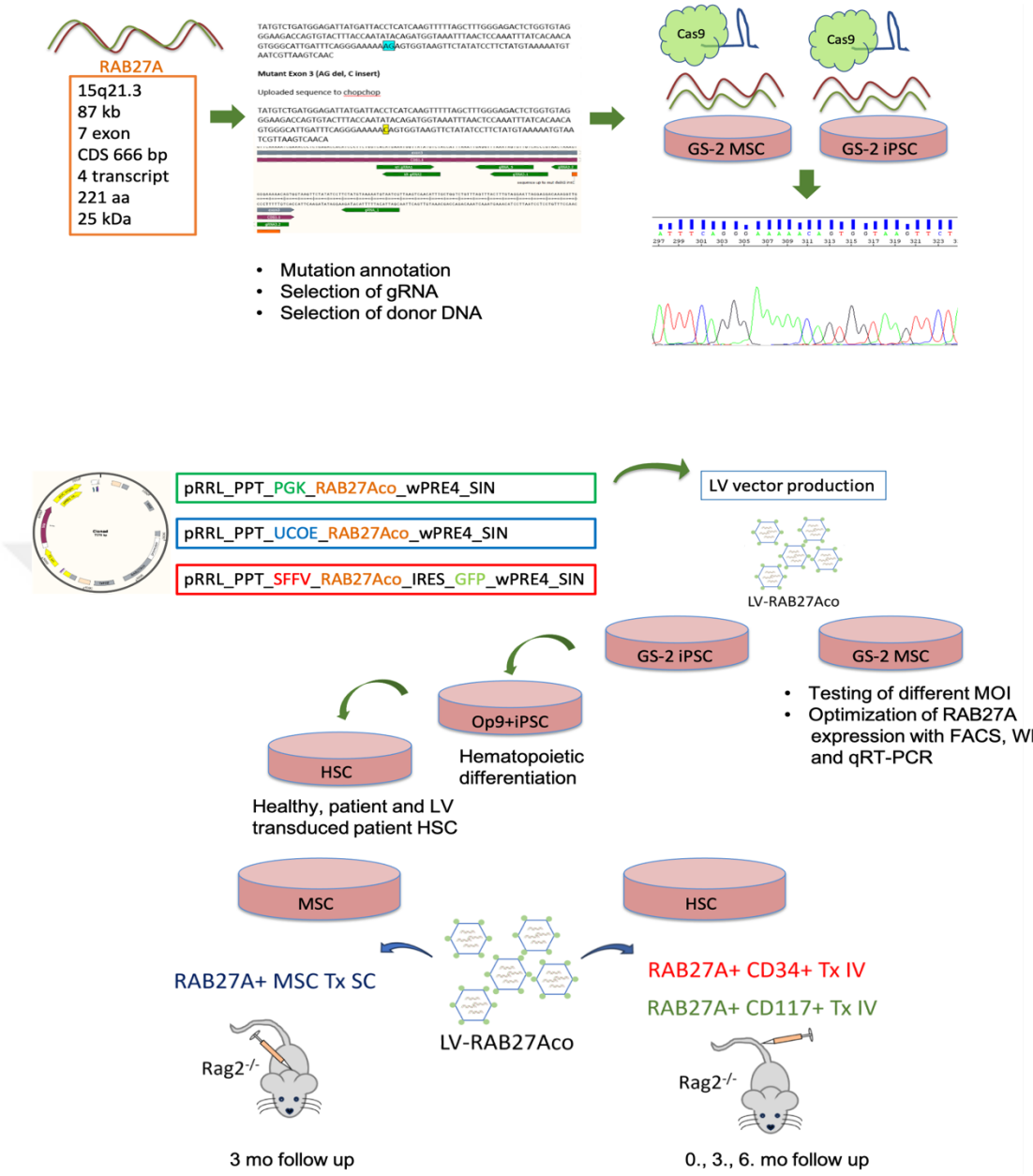


Figure 3.4. Overview of methods used in this thesis. GS-2 patient derived MSCs and iPSCs were subjected to gene editing using CRISPR/Cas and gene correction and editing efficiency were assessed using sequencing techniques. Three different lentiviral constructs carrying the codon-optimized RAB27Aco transgene under the control of a PGK, UCOE and SF promoter were subcloned into existing backbones and tested for efficacy of RAB27A gene expression in GS-2 MSCs and iPSCs. Healthy donor MSCs and HSCs were transduced with LV-SF-RAB27Aco and tumorigenic activity was assessed after transplantation in immune deficient RAG2 KO mice.

4. RESULTS

4.1. Optimization of stem cell culture and cryopreservation conditions

4.1.1. Maintenance of GS-2 MSC cultures

Here we characterized and reprogrammed MSCs from a GS-2 patient (AB) and his related donor (ARB). AB and ARB were sequenced to define their mutations (shown in the material method) and were shown to have a homozygous and heterozygous mutation in exon 5 of the RAB27A gene, respectively. Although MSCs from all patients/donors were easily cultured and overall morphology appeared to be the same (Figure 4.1). No significant differences were observed in adipogenic and osteogenic differentiation capacities of the GS-2 patients AB and the carrier ARB (Figure 4.2), since the sample number of the groups was too low. However, especially GS-2 MSCs with the homozygous RAB27A mutation appeared to display lower than average osteogenic differentiation.

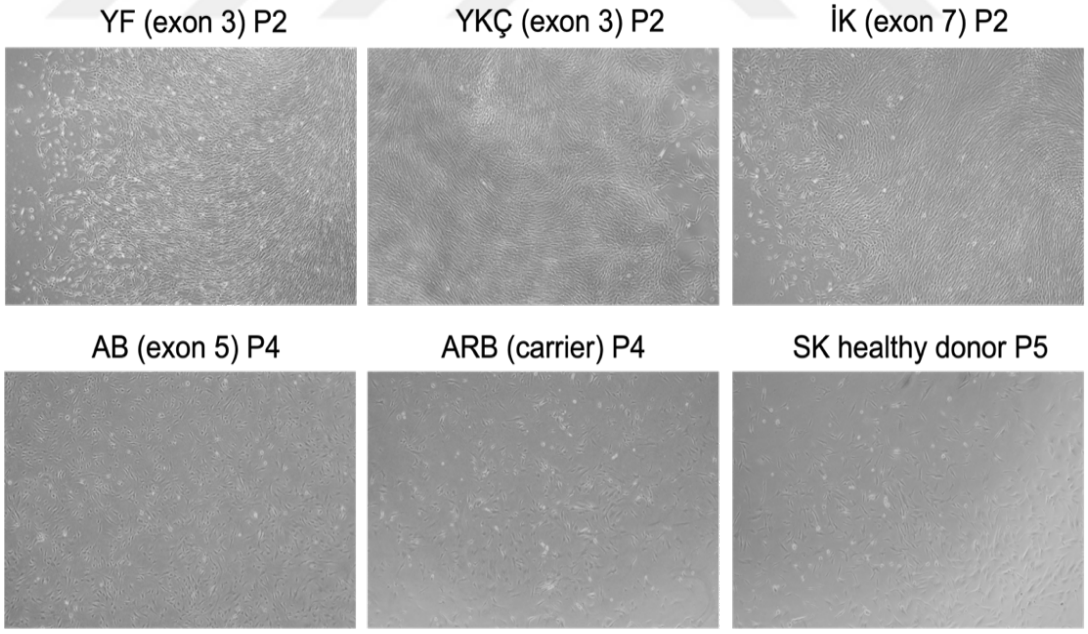


Figure 4.1. GS-2 patient and healthy donor-derived MSC morphology. MSCs from the RAB27A-/- GS-2 patients (YF, YKÇ, İK, AB), a heterozygous RAB27A mutation carrier (RAB27A+/-) (ARB) and healthy RAB27A+/+ donor (SK) showed overall similar morphology (4x).

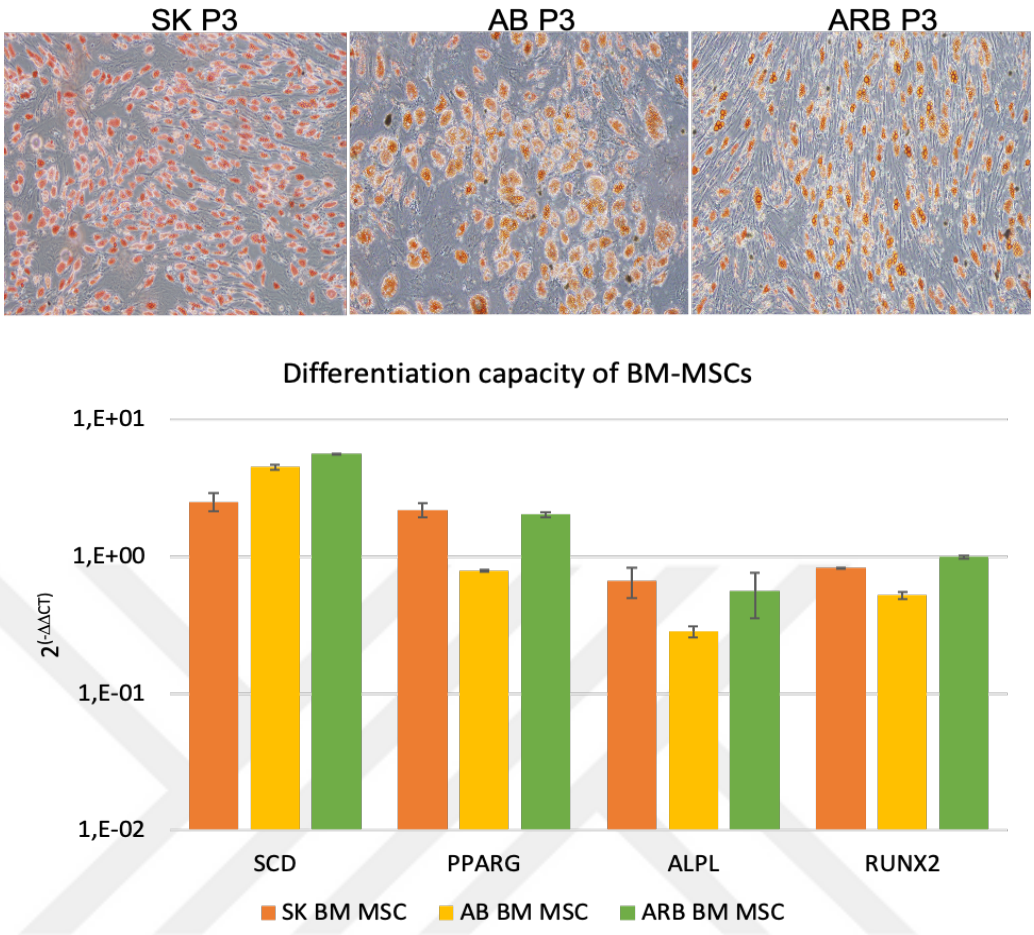


Figure 4.2. Differentiation of healthy donor, GS-2 and carrier MSCs. Upper panel: Adipogenic differentiation of healthy donor (SK), GS-2 patient (AB) and his carrier donor (ARB) derived MSCs after 21 days. Lower panel: SK, AB and ARB BM-MSCs were exposed to adipogenic or osteogenic differentiation media for 7 days and gene expression of *SCD* (early adipogenic marker), *PPARG* (late adipogenic marker), *ALPL* (early osteogenic marker) and *RUNX2* (late osteogenic marker) was measured using qRT-PCR.

4.1.1. Optimization of iPSC cultures

GS-2 iPSCs were thawed from -80°C and plated in 6-well culture dishes coated with Matrigel. During the first 24 hours of culture, ROCK inhibitor (Ri, Y27632) was added to commercially available iPS Brew XF+supplement medium (Miltenyi) and cells were cultured at 37°C with 5% CO_2. In contrast to MSC cultures, daily medium changes are required for the survival and maintenance of iPSCs to sustain their undifferentiated state.

In addition, storage of iPSCs for prolonged times at -80°C resulted in the loss of several of the iPSC lines that we created previously. We therefore sought out alternative culture and freezing conditions to support iPSC growth and survival in the absence of uncontrolled differentiation. We found that different cell freezing and thawing procedures directly affect the cells' colony formation, proliferative and differentiation capacities. Especially, when iPSCs were stored for prolonged periods of time at -80°C, these cells showed increased cell death after thawing, visible as centers of necrosis in the middle of the iPSC colonies, as well as uncontrollable (irrepressible) spontaneous differentiation (Figure 4.3).

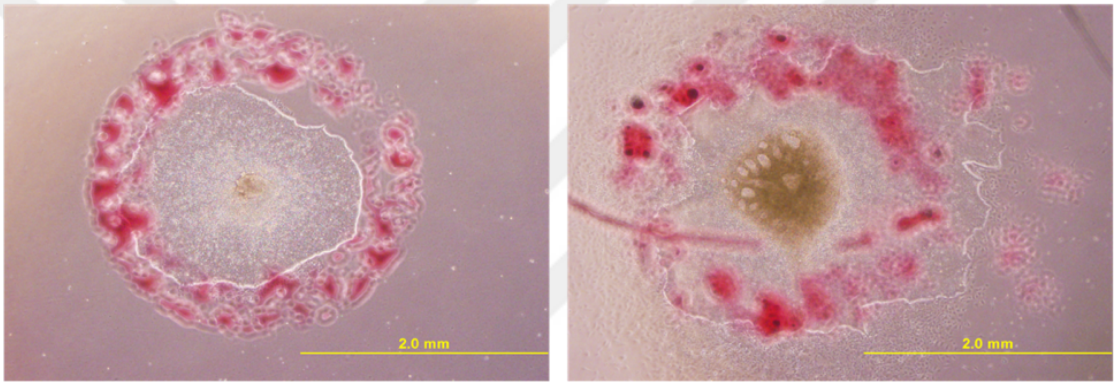


Figure 4.3. GS-2 (YF/A1A3/P11) iPSC culture. After thawing iPSCs stored at -80°C displayed increased cell death and adherent colonies showed spontaneous differentiation.

To prevent proliferation and uncontrollable differentiation of the GS-2 iPSC lines, cells were cultured in presence of iPS-Brew XF from Miltenyi, TeSR-E8 or mTeSR Plus media, both from Stem Cell Technologies (Figure 4.4). The mTeSR Plus medium, which was modified from mTESR-1 medium by using more stable essential medium components, such as bFGF, and improved pH buffering, was designed to maintain optimal cell quality and increase cell expansion rates with restricted feeding protocols, allowing every other day medium changes instead of daily medium changes (166). When we compared this culture medium with iPS-Brew XF and TeSR-E8, we found that TeSR Plus provides the best maintenance of GS-2 iPSCs in terms of survival and proliferation, while preventing spontaneous and uncontrollable differentiation and therefore this medium was used in following cultures.

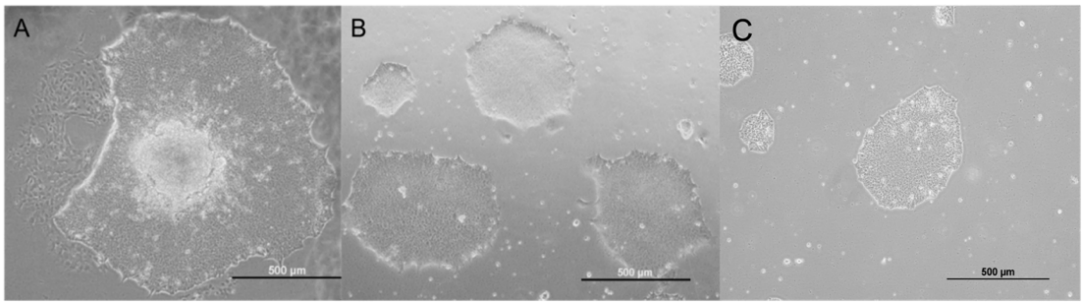


Figure 4.4. Effect of different culture media on iPSC culture. A) Extensive spontaneous differentiation is observed in cells cultured with iPS Brew; B) Cells cultured with TeSR-E8 support short-lived cultures, but differentiation is seen in long-term cultures; C) mTeSR-Plus medium supports long-term culture without differentiation.

4.1.2. Assessment of iPSC medium efficiency during reprogramming

To compare the effects of different iPSC media and cell concentration on reprogramming efficiency, we reprogrammed GS-2 patient AB-derived MSCs in the presence of different culture media. In contrast to the results above, we found that under these conditions iPS Brew XF+supplement showed the support of reprogramming with the highest efficiency: where no iPSC colonies were formed in wells maintained in E8, robust colony formation was seen in presence of iPS Brew XF + supplement. Both conditions using 12.500 cells and 25.000 cells showed a similar efficiency of reprogramming (Figure 4.5).

After initial colonies were seen in wells from AB cultures with iPS Brew using 12.500 cells for reprogramming cells, the morphologically best colonies were picked (Figure 4.6) and transferred to a Matrigel-coated 12-well plate for expansion. Using the continued colony picking method, colonies were cleared from differentiating and non-iPS cells until pure non-differentiated iPSC clones were formed. Among these clones, AB/#2 and AB/#4 iPSCs were chosen for further experiments and characterized with qRT-PCR (Figure 4.7). Both clones showed increased expression of the pluripotency markers *OCT3/4, SOX2* and *NANOG*.

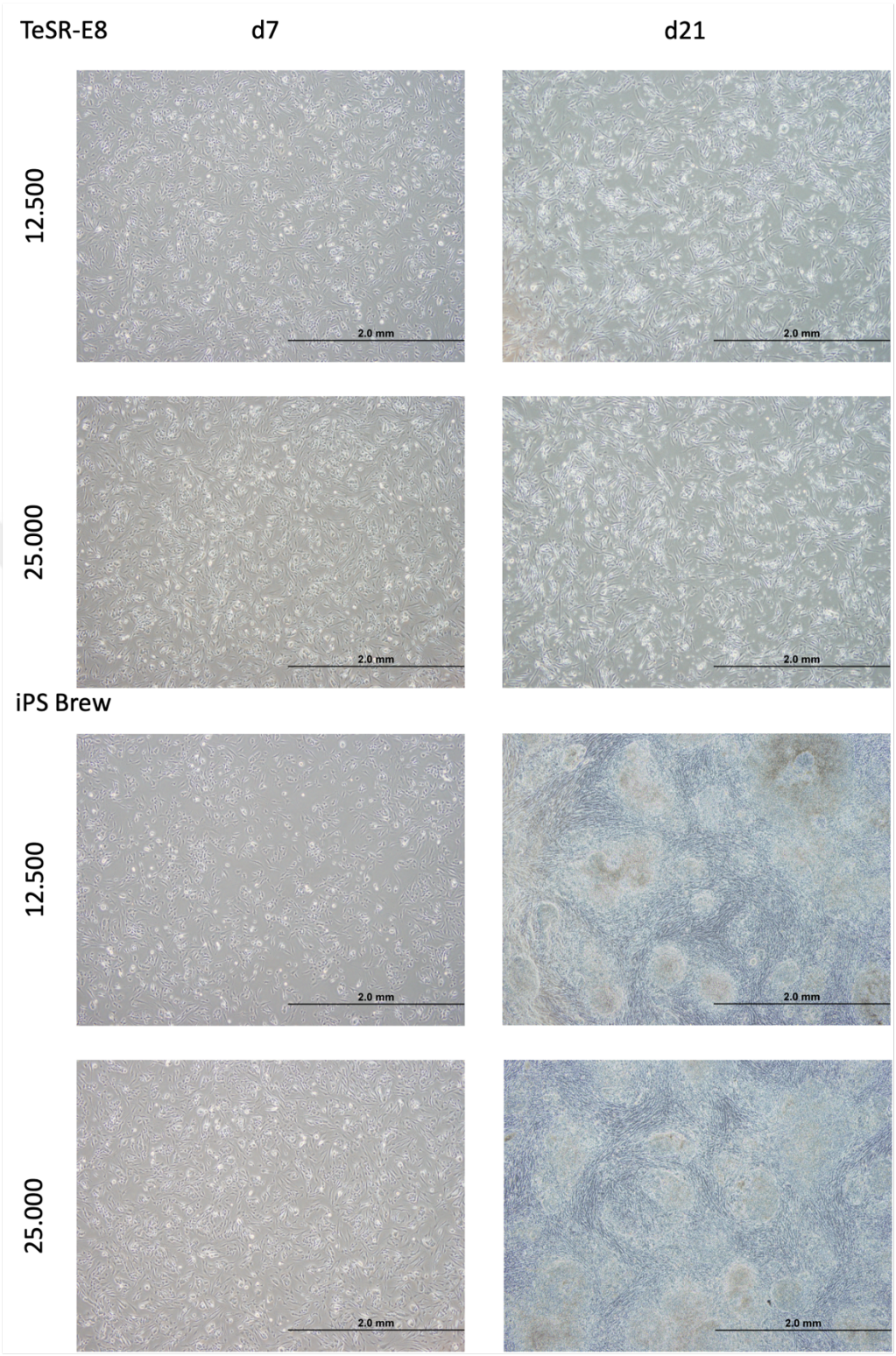


Figure 4.5. Effects of different culture media on reprogramming efficiency. AB/P1/GS-2 MSCs were seeded at different concentrations (12.500 and 25.000) onto 12-well plates and reprogrammed using LV-OSKM viral vectors. After a week, cells were transferred to a fresh Matrigel-coated 6 well plate and reprogramming of the cells were observed for 21 days. Upper panel: TeSR-E8, Lower panel: iPS Brew XF

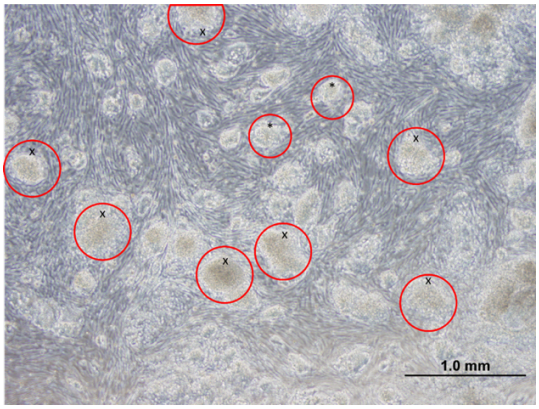


Figure 4.6. Colony growth after reprogramming. X: proper colony size, *: small colony size.

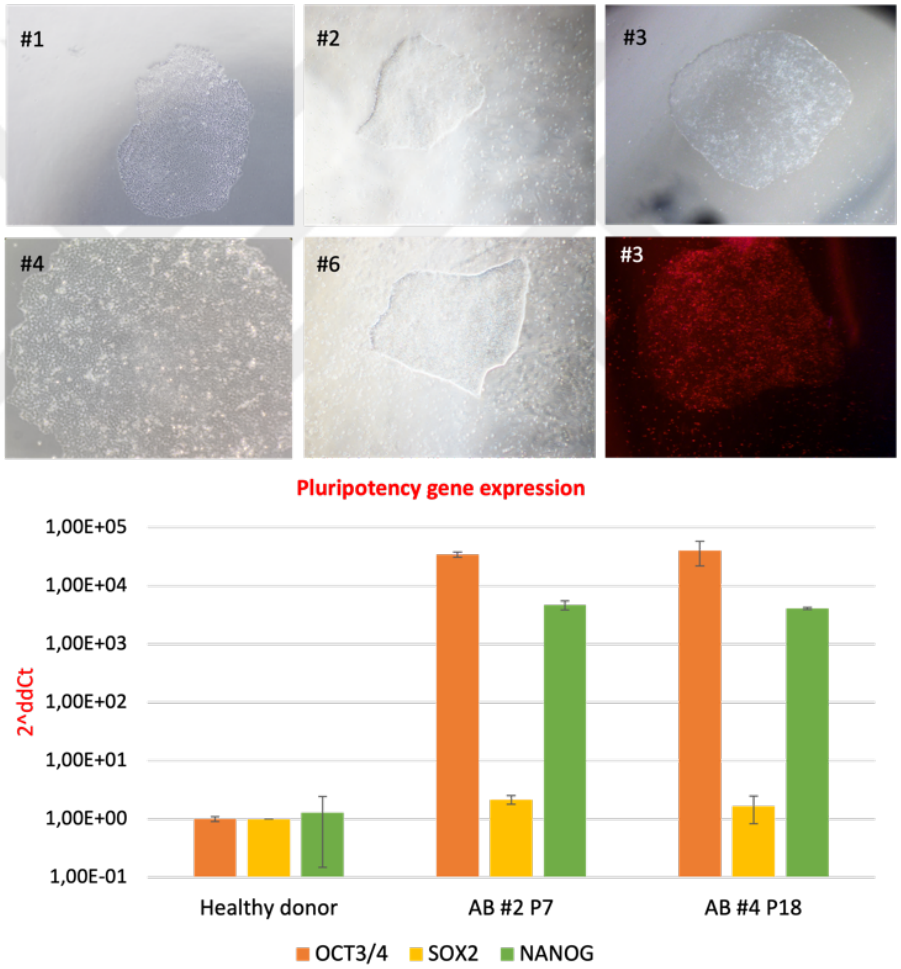


Figure 4.7. GS-2 iPSCs (AB) after reprogramming with LV-OSKM. Upper panel: AB iPSC clones (#1-4 and #6) light microscopy photographs (4X) and fluorescence microscopy for expression of dTom (#3). Lower panel: Clones #2 and #4 were assessed for expression of the pluripotency genes *OCT3/4, SOX2* and *NANOG* and compared with healthy donor MSCs. Data are shown as mean ± standard deviation of technical replicates.

4.1.3. Optimization of iPSC cryopreservation

Breaking up of the iPSC colonies into single cells resulted in decreased cellular viability after thawing with lower plating efficiency in comparison to iPSCs frozen as aggregates (Figure 4.8).

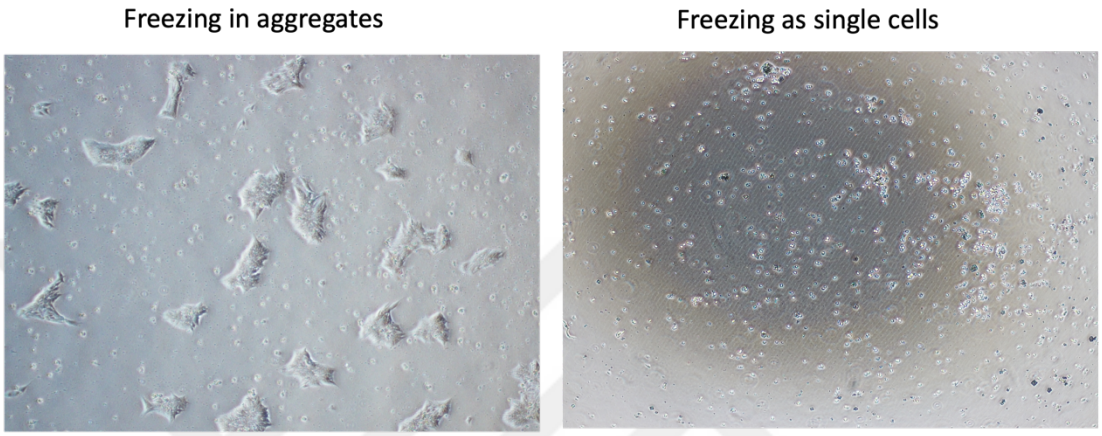


Figure 4.8. Morphology of iPSCs after freezing and thawing as aggregates or as single cells. iPSCs were thawed and plated in TeSR-E8 medium. Left: Cells frozen as aggregates show a healthier morphology and rapidly form colonies in culture. Right: Cells frozen as single cells do not recover from the freezing/thawing procedure.

Our results show that decreasing the concentration of DMSO from 10% to 5% is not associated with significant cell death or decreased colony formation and could therefore be used in subsequent studies. We found in our experiments that addition of Ri always supported better survival after thawing and that DMSO levels could be easily reduced to 5%, but extra additives, such as the antioxidants SUL-109 or RES in addition to Ri did not further improve colony formation or cell survival of iPSCs. Based on these data we recommend the use of 5% DMSO with Ri and freezing in aggregates as the best protocol to cryopreserve iPSCs.

4.2. Use of CRISPR/Cas9 to correct RAB27A mutations in MSCs and iPSCs

4.2.1 Assessment of RAB27A expression by GS-2 MSCs and iPSCs

The healthy donor and GS-2 MSCs and iPSCs used in this study were characterized in detail before and the presence of RAB27A mutations were confirmed using sequencing of the gene (84). iPSC clones generated from the GS-2 MSCs showed the same RAB27A mutations and were thus confirmed to be representative cell lines of GS-2. We did not observe differences in morphology or proliferation of GS-2 MSCs and iPSCs in comparison to healthy donor MSCs and iPSCs (Figure 4.9).

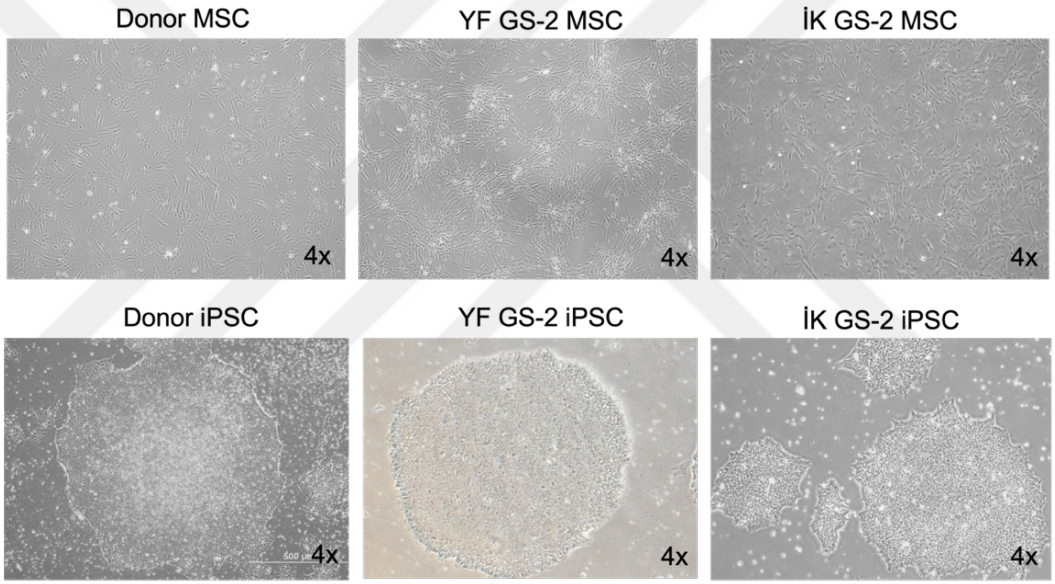


Figure 4.9. Morphology of healthy donor and GS-2 patient-derived MSCs and iPSCs. Light microscope pictures of donor and GS-2 MSC cultures (upper lane) and their iPSCs (lower lane). MSCs show typical fibroblast shapes, whereas iPSCs grow in distinct colonies.

Since RAB27A protein and gene expression levels in these cells were not previously assessed, we measured baseline protein expression levels by Western Blot and immune fluorescence (Figure 4.10.A) and gene expression levels of *RAB27A* in healthy donor and GS-2 MSCs (Figure 4.11.B). RAB27A protein expression in healthy donor MSCs was found to be relatively low in comparison to the positive control cell line K562, but expression was completely absent in MSCs samples of the two GS-2 patients (İK and YF).

After confirmation of expression of the *OCT4*, *SOX2* and *NANOG* pluripotency genes in our GS-2 iPSCs, we then assessed gene expression of *RAB27A* and found complete absence of *RAB27A* expression in both GS-2 patient-derived iPSC clones (Figure 4.11).

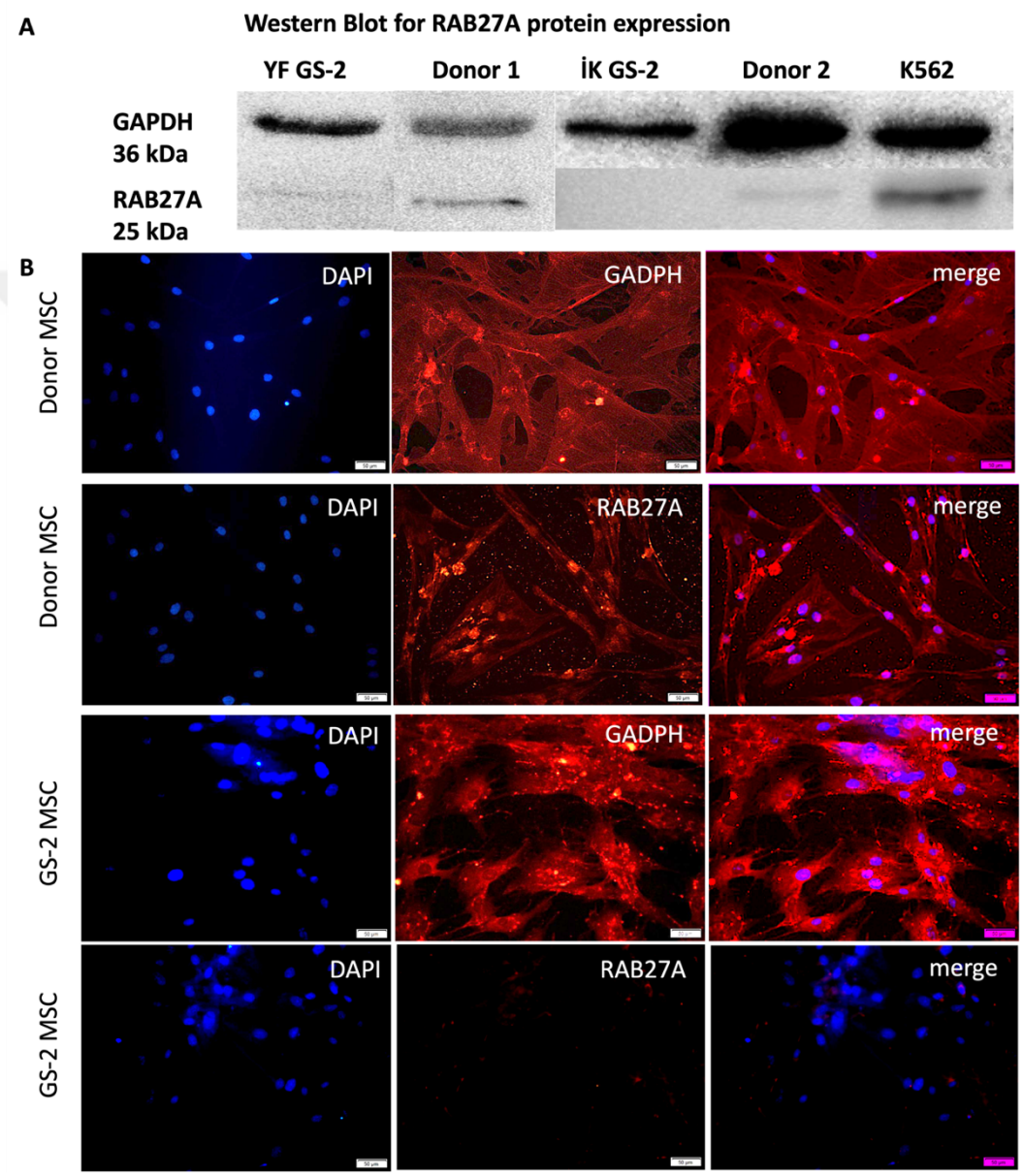


Figure 4.10. RAB27A expression in healthy donor and GS-2 MSCs. A) RAB27A protein expression in healthy and GS-2 donor MSCs by Western Blots. Positive control: K562. B) Healthy donor and GS-2 MSCs were stained with anti-GAPDH or anti-RAB27A. Nuclei were counterstained with DAPI. Photographs were taken with an inverted microscope (Olympus LS, IX73) and analyzed using ImageJ software (NIH, Java, 2022).

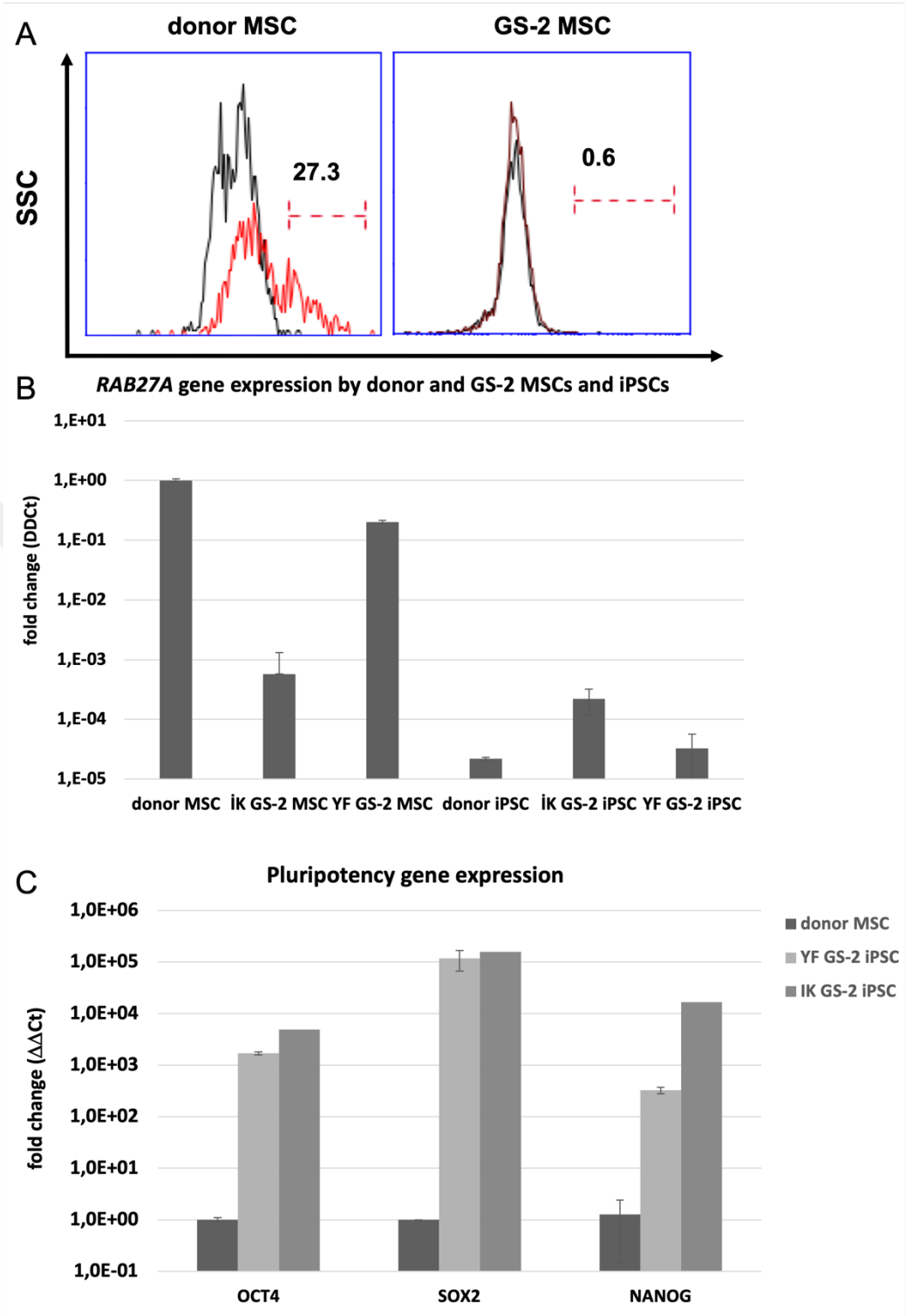


Figure 4.11. RAB27A expression by donor or GS-2 MSCs and iPSCs. A) Donor and GS-2 patient-derived MSCs were stained for RAB27A expression using flow cytometry. B) RAB27A gene expression in GS-2 MSCs from two donors (İK and YF) and GS-2 iPSCs were calculated relative to RAB27A expression in healthy donor MSCs. C) GS-2 iPSC expression of pluripotency genes was confirmed for *OCT4, SOX2* and *NANOG*.

4.2.2. gRNA and donor DNA design and assessment of genome targeting efficiency

For each patient (YF: exon 3; c.148-149delAGinsC and IK: exon 7; c.514-518delCAAGC), we designed 3 different mutation-specific gRNAs (Table 3.8 in the material & methods section). We used the CHOPCHOP online tool to design all gRNAs using annotation of the GS-2 mutations on the RAB27A DNA coding sequence (Figure 4.12). We then tested the genome targeting efficiency of the 3 selected gRNAs for each exon using the T7 endonuclease assay. Using the gel analyzer software, we found the highest efficiency with gRNA 3.3 (10% efficiency) and gRNA 7.3 (27% efficiency). Based on these data we used the HDR donor DNA sequences as shown in Table 3.9 above in materials and methods.

4.2.3. Transfection of the RNP complex and donor DNA into MSCs and iPSCs

We then proceeded to test the efficiency of the homology-directed repair (HDR) on GS-2 patient-derived MSCs using the designed RNP complexes or DMSO controls. Cells were allowed to recover for two days after transfection and then used for DNA mutation analysis. Despite good results with the gRNAs designed to correct exon 3 in the T7 endonuclease assay, we found no correction of exon 3 after HDR (Figure 4.13.A). Although HDR was observed in some of the cells with mutations in exon 7, a considerable fraction of cells obtained deletions in the gene (Figure 4.13.B). After optimization of the procedures with different donor DNA concentrations, we then decided to test HDR efficacy on GS-2 iPSCs. Sequencing analysis revealed the presence of HDR in a minority of the transfected cells only (Figure 4.13.C). After transfection, the iPSCs were cultured for 2 days, but despite the addition of ROCK inhibitor (Ri), massive iPSC cell death, decreased colony formation and spontaneous differentiation were observed (Figure 4.13.D).

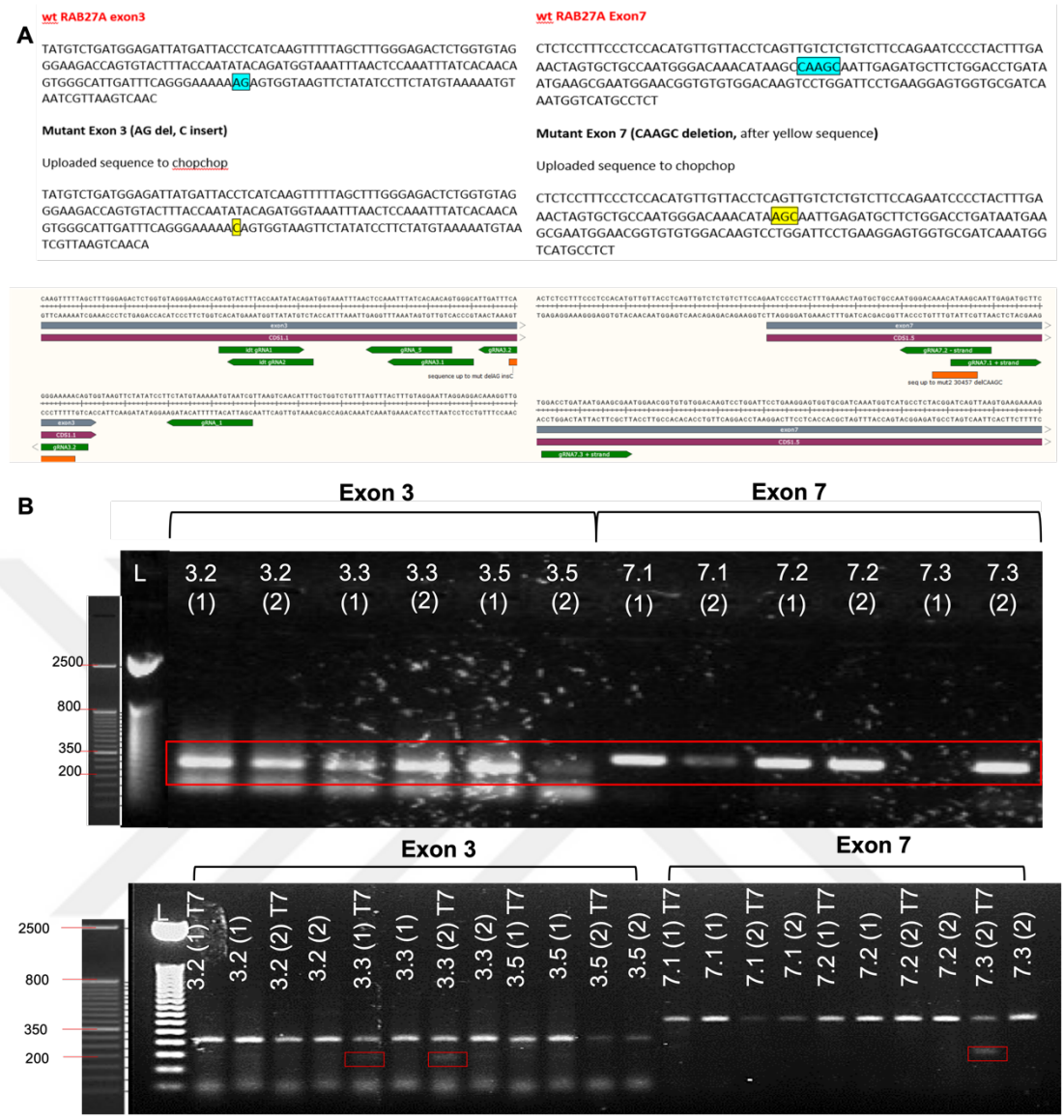


Figure 4.12. Design of gRNAs and testing of genome targeting efficiency. A) To design gRNAs, the coding sequence of RAB27A was annotated with patient mutations and then uploaded to the CHOPCHOP online tool (167). The best-ranking sequences near or on the targeted mutation sites for RAB27A exon 3 (left) and RAB27A exon 7 (right) were selected. B) Genome targeting efficiency of different gRNAs. The genome targeting efficiency of the designed gRNAs (3.2, 3.3 and 3.5 for exon 3 and 7.1, 7.2 and 7.3 for exon 7) was tested using the T7 Endonuclease assay. Upper panel: DNA of gRNA-transfected cells was controlled with PCR; Lower panel: The PCR products were loaded onto a 2% agarose gel to detect gRNA efficiency after the addition of T7 Endonuclease. All tests were run in duplicate. Genome targeting efficiency was the highest with gRNA 3.3 and 7.3 (as shown by the red rectangles in the lower panel).

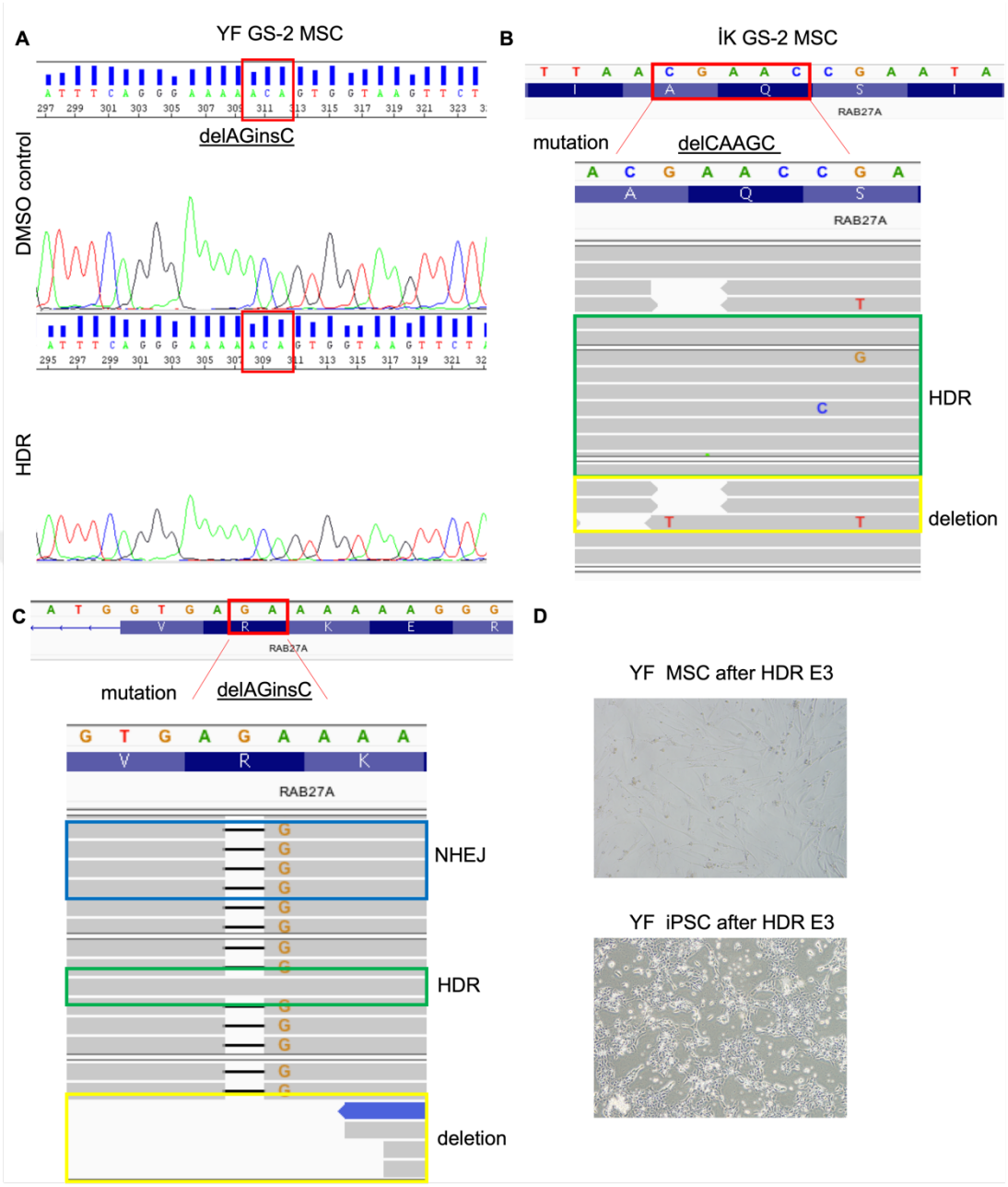


Figure 4.13. Mutation analysis of GS-2 MSCs after transfection. MSCs from two different GS-2 patients (YF: exon 3 delAGinsC; İK: exon 7 delCAAGC) were transfected and cultured for two days. Mixed cell populations from a single well were collected for DNA analysis. Sequencing analysis revealed the absence of HDR in exon 3 (A), but the presence of HDR in up to 50% of the cells with exon 7 mutations (B). Transfection of GS-2 iPSCs with the RAB27A exon 3 mutation results in low HDR efficacy (C) and loss of viability and spontaneous differentiation (D).

4.3. Design and testing of RAB27Aco expressing lentiviral vectors

4.3.1. Subcloning of RAB27Aco under the control of different promoters

The coding sequence of the RAB27A gene is 666 base pairs long. In order to allow optimal expression of the gene as well as a transgene size that is optimal for packaging into a lentiviral vector, we performed codon optimization. Codon optimization of the RAB27A gene (RAB27Aco) further enhances effective protein expression from the transgene plasmid. In addition, a Kozak nucleic acid motif consensus sequence, which serves as an initiation site for protein translation of mRNA transcripts, was added to the transgene 5' end, and the TGA stop codon was added to the 3' end (see Supplement 8). The RAB27Aco sequence was then cloned under the control of a PGK promoter, which allows constitutively active expressing at physiological levels in most tissues (Figure 4.14.A). RAB27Aco cloning process was performed using NotI and EcoRI restriction enzymes, which cut the plasmid with a sticky end, resulting in optimal ligation (Figure 4.14.B).

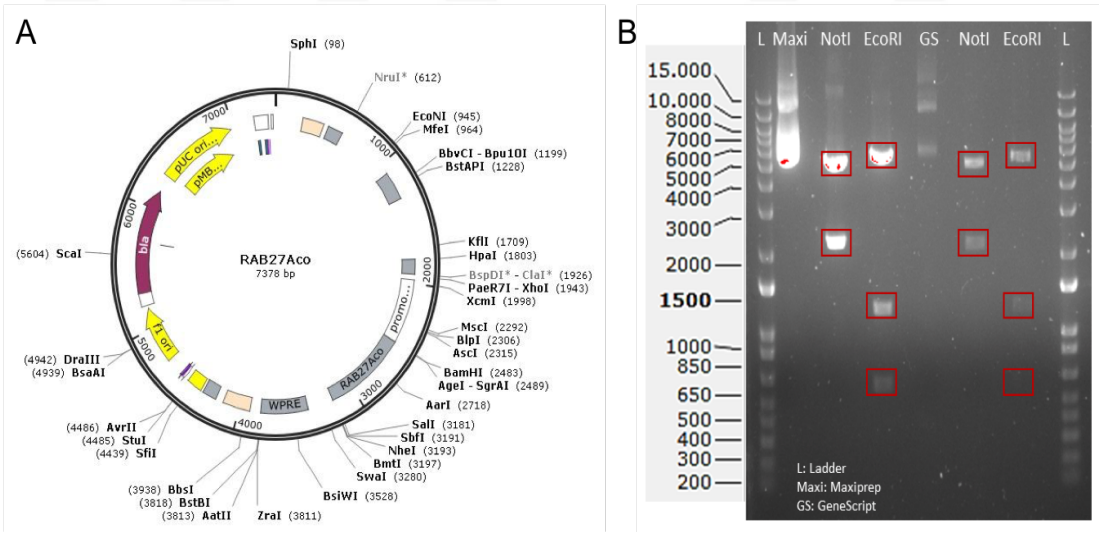


Figure 4.14. Plasmid map of PGK-RAB27co after cloning. A) PGK-RAB27Aco plasmid map using GenScript, **B)** DNA fragments after plasmid transformation of RAB27Aco and correction of the cloning process. EcoRI cuts the plasmid into 3 pieces, whereas NotI cuts it in 2 pieces.

RAB27Aco was also cloned under the control of a UCOE promoter using the UCOE-RAG2co plasmid, which was designed by Dr. Niek van TIL (125) (Figure 4.15.A-B). Both plasmids were cut with AgeI-HF and SbfI-HF restriction enzymes, which cut the plasmid at the one site with a sticky end. After cutting the plasmids, the size of the pieces was 6.7 kb for the PGK backbone, 0.7 kb for RAB27Aco, 8.8 kb for the UCOE backbone, and 1.6 kb for RAG2co. Based on fragment size, the UCOE backbone and RAB27Aco transgene were cut from the gel (Figure 4.15.C) and ligated (Figure 4.15.D). The ligated plasmid was mixed with 6X loading dye and was plasmid sizes were checked using electrophoresis on a 0.8% agarose gel. The original UCOE-RAG2co, PGK-RAB27Aco, and the ligated UCOE-RAB27Aco plasmids (old1, new1, new2) were analyzed using different restriction enzymes, using NsiI for a single cut into the UCOE promoter sequence and SbfI to cut a single site of the plasmid sequence (Figure 4.15.E), resulting into fragments of respectively 6.4 kb and 3.1 kb in length. The corrected plasmids were then expressed into transformed Stbl3-competent bacteria and isolated using mini/maxiprep.

Next, RAB27Aco was cloned under the control SFFV promoter using SFFV-ANGPTL3-IRES-iG2 as a backbone and the PGK-RAB27Aco as the insert plasmids (Figure 4.16.A-B). Both plasmids were cut with BamHI and NotI restriction enzymes to get a sticky end for the insert sequence. After cutting the plasmids the size of the pieces was 6.7 kb for the PGK backbone, 0.7 kb for RAB27Aco, 8.8 kb for the SFFV backbone, and 1.3 kb for ANGPTL3 (Figure 4.16.C-D). The ligated RAB27Aco-IRES-iG2 plasmid was transformed into the competent bacteria and plasmids were isolated.

4.3.3. RAB27Aco lentiviral (LV) vector production and titration

Several batches of PGK-RAB27Aco, UCOE-RAB27Aco, and SFFV-RAB27Aco-IRES-GFP LV vectors were produced after packaging into HEK293T cells, and titers were calculated using the PGK-GFP plasmid as a (Figure 4.17).

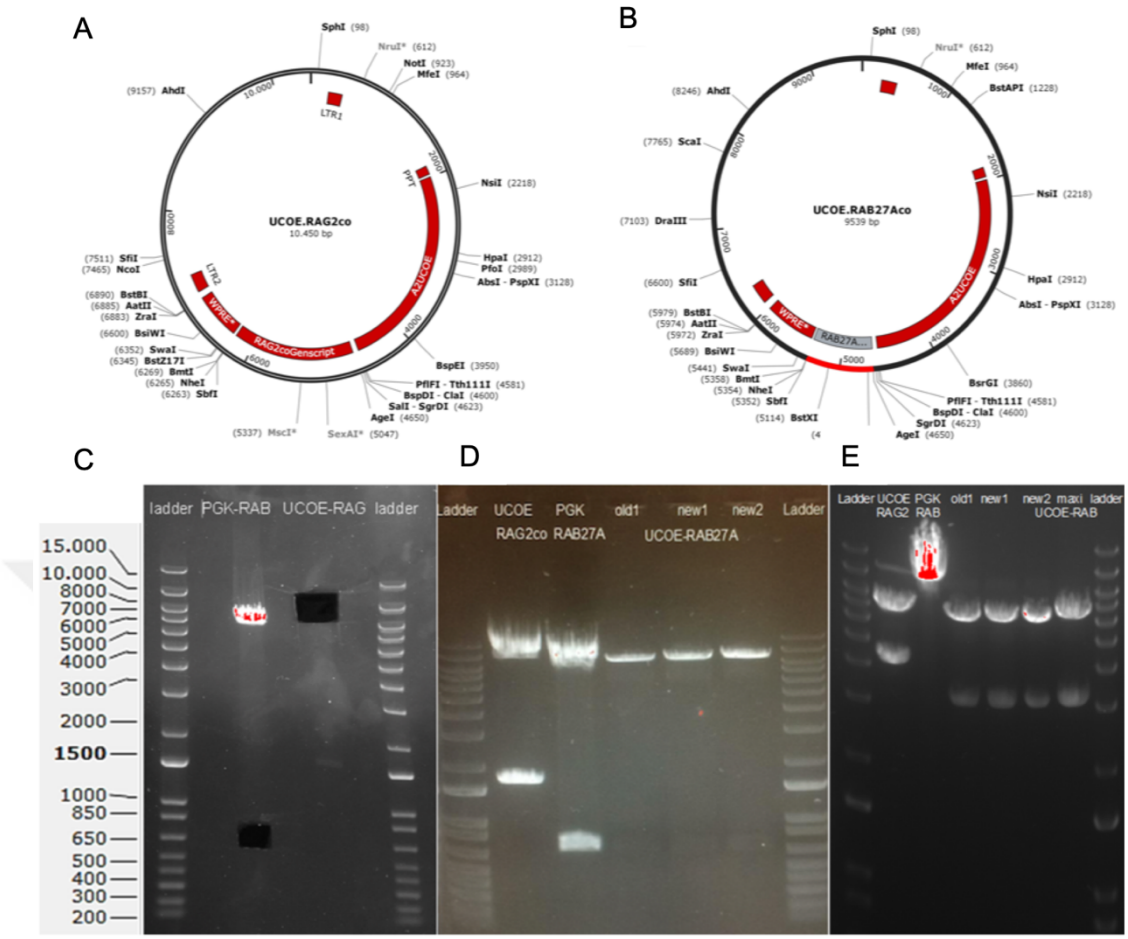


Figure 4.15. Cloning of RAB27Aco under the UCOE promoter and confirmation of the plasmids. A) The original UCOE-RAG2co plasmid map, B) RAB27Aco plasmid cloned under control of the UCOE promoter, C) PGK-RAB27Aco and UCOE-RAB27Aco plasmids after digestion with with AgeI and SbfI restriction enzymes (the image was taken after the bands were cut out of the agarose gel, as visible by the black holes), D) Confirmation of correct ligation of the UCOE backbone and RAB27Aco fragments (old1, new1 and new2), E) Control of the UCOE-RAB27Aco plasmid sequence with NsiI and SbfI restriction enzymes.

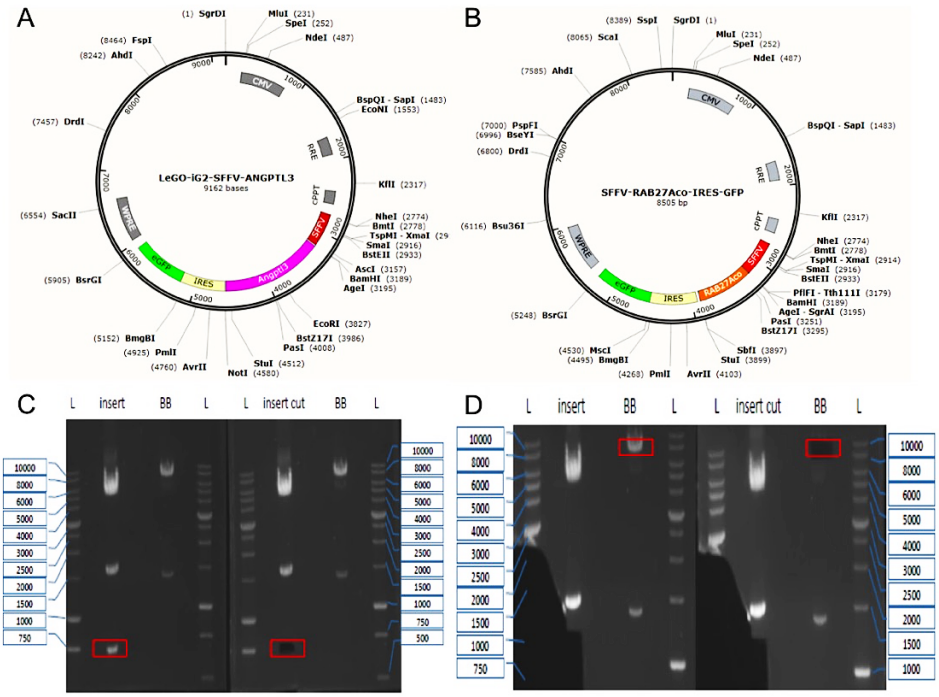
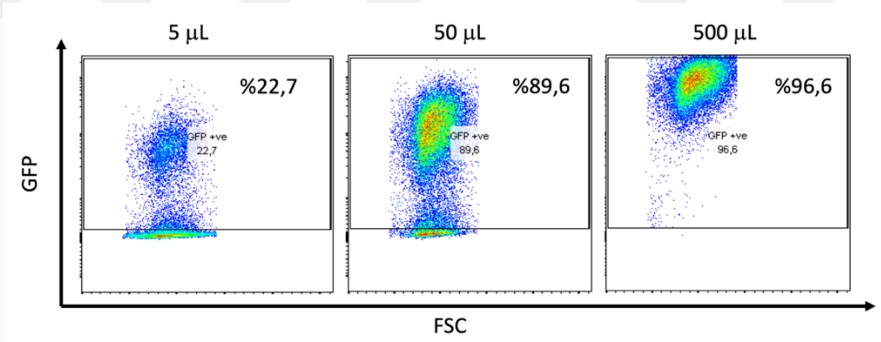


Figure 4.16. Cloning of SFFV-RAB27Aco-IRES-iG2. A) LeGO-ANGPTL3-IRES-iG2 backbone plasmid. B) Cloned SFFV-RAB27Aco-IRES-iG2 plasmid. C-D) Backbone and insert plasmids after digestion with BamHI and NotI restriction enzymes.



Construct	Virus dilution (µL)	GFP positive cell (%)	Total cell number	Positive cell number	Dilution factor	Titer (particle/ml)
PGK-GFP	5	22,7	200	45400	200	9,08E+08
	50	89,6	200	179200	20	3,5E+08
	500	96,6	200	193200	2	3,86E+07

Figure 4.17. Titration of the PGK-GFP transfer plasmid. PGK-GFP LV vector supernatant was concentrated and then titrated using the Jurkat cell line. Serial dilutions left: 5 µL, middle: 50 µL, right: 500 µL of the concentrated viral vector were added onto cells. After 4 days of transduction, GFP percentages were assessed using flow cytometry.

4.3.4. Lentiviral transduction of MSCs

Concentrated viral vector batches of PGK-RAB27Aco, UCOE-RAB27Aco, SFFV-RAB27Aco-GFP and PGK-GFP LV vectors were used for transduction of İK/GS-2 MSC, YF/GS-2 MSC and YKÇ/GS-2 MSC patient samples in a 6-well plate at different MOIs (10, 30, 100). Cells were transduced overnight and then collected for flow cytometric analysis. After flow cytometry analysis, dim and highly positive populations were identified in GS-2 patient cells transduced with RAB27A, most likely indicating the presence of cells with single or multiple copies of vector integrations per cell (Figure 4.18).

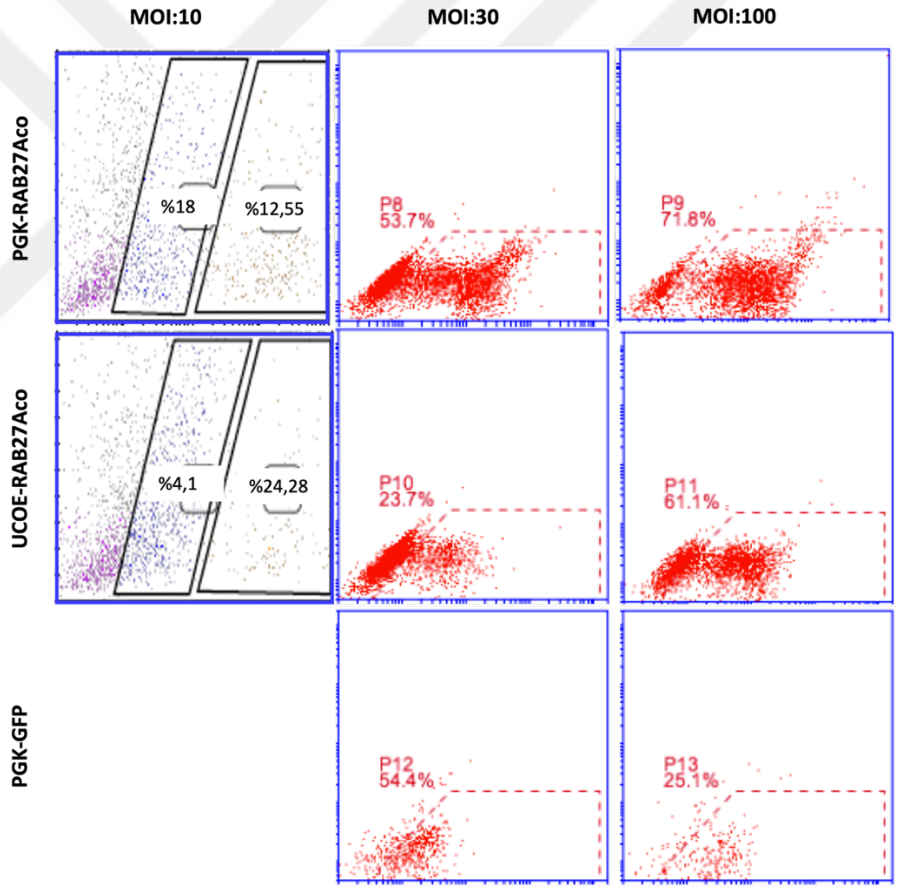


Figure 4.18. GFP expression after transduction with LV-PGK-GFP, PGK-RAB27Aco and UCOE-RAB27Aco of GS-2 MSCs. LV-PGK-RAB27Aco and LV-UCOE-RAB27Aco viral supernatants were added to GS-2 MSCs at an MOI of 10. RAB27A expression was assessed using flow cytometry. Expression of RAB27A increased with MOI resulting in more than 1 integration per cell, affecting overall RAB27A protein expression. The PGK promoter showed a 0.5 log higher transduction efficiency than the UCOE promoter.

After transduction we found a GFP positivity rate of 30% after use of the LV-PGK-RAB27Aco vector and 28% after use of the LV-UCOE-RAB27Aco vector. Higher MOI resulted in increased RAB27A expression with 53.7% RAB27A expression after transduction with PGK-RAB27A at MOI=30 and 71.8% at MOI=100; similarly, we found 23.7% positivity for RAB27A after transduction with UCOE-RAB27A at MOI=30 and 61.1% positivity at MOI=100. Control wells transduced with LV-PGK-GFP showed 54.4% GFP at MOI=30, but a decreased GFP expression (25.1%) at MOI=100. Western blot, immunofluorescence labeling, and qRT-PCR were used to confirm transduction (Figure 4.19). The PGK promoter provided higher RAB27A gene and protein expression compared to the UCOE promoter. Similarly, we found that RAB27A gene expression was higher when the PGK promoter was sure compared to UCOE promoter. Transduction efficiency of UCOE never reached PGK levels, despite a rise in transduction efficiency with increasing MOI.

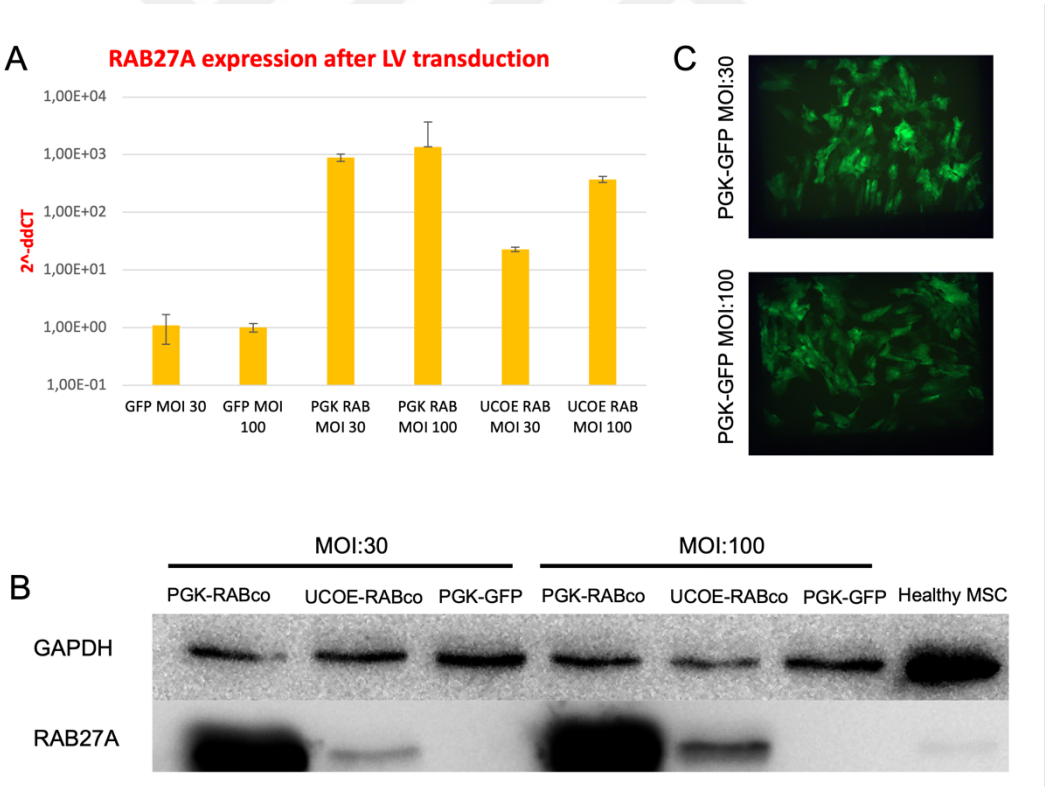


Figure 4.19. RAB27Aco expression after LV vector transduction in different promoters. LV-PGK-GFP, PGK-RAB27Aco and UCOE-RAB27Aco of GS-2 MSCs at an MOI:30 and MOI:100. GAPDH: 36 kDa, RAB27A: 25 kDa.

Overall, overexpression of RAB27A in GS-2 MSCs was achieved with both the UCOE and PGK promoters, while the effects of PGK were more pronounced. Using codon-optimized PGK-RAB27Aco and UCOE-RAB27Aco LV vector design, RAB27A gene and protein expression were successfully achieved in GS-2 MSCs.

4.3.5. Lentiviral transduction of iPSCs

GS-2 iPSCs were transduced with LV-PGK-RAB27Aco and the increase in *RAB27A* gene expression was confirmed with both qRT-PCR and flow cytometry (Figure 4.20). We found that PGK-RAB27Aco at MOI:30 provides a robust RAB27A expression in both MSCs and iPSCs.

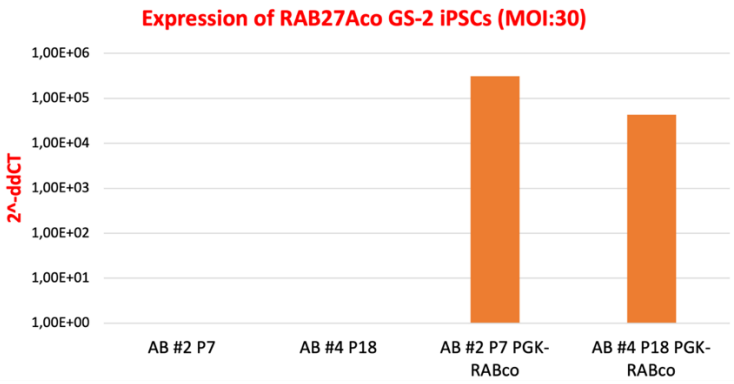
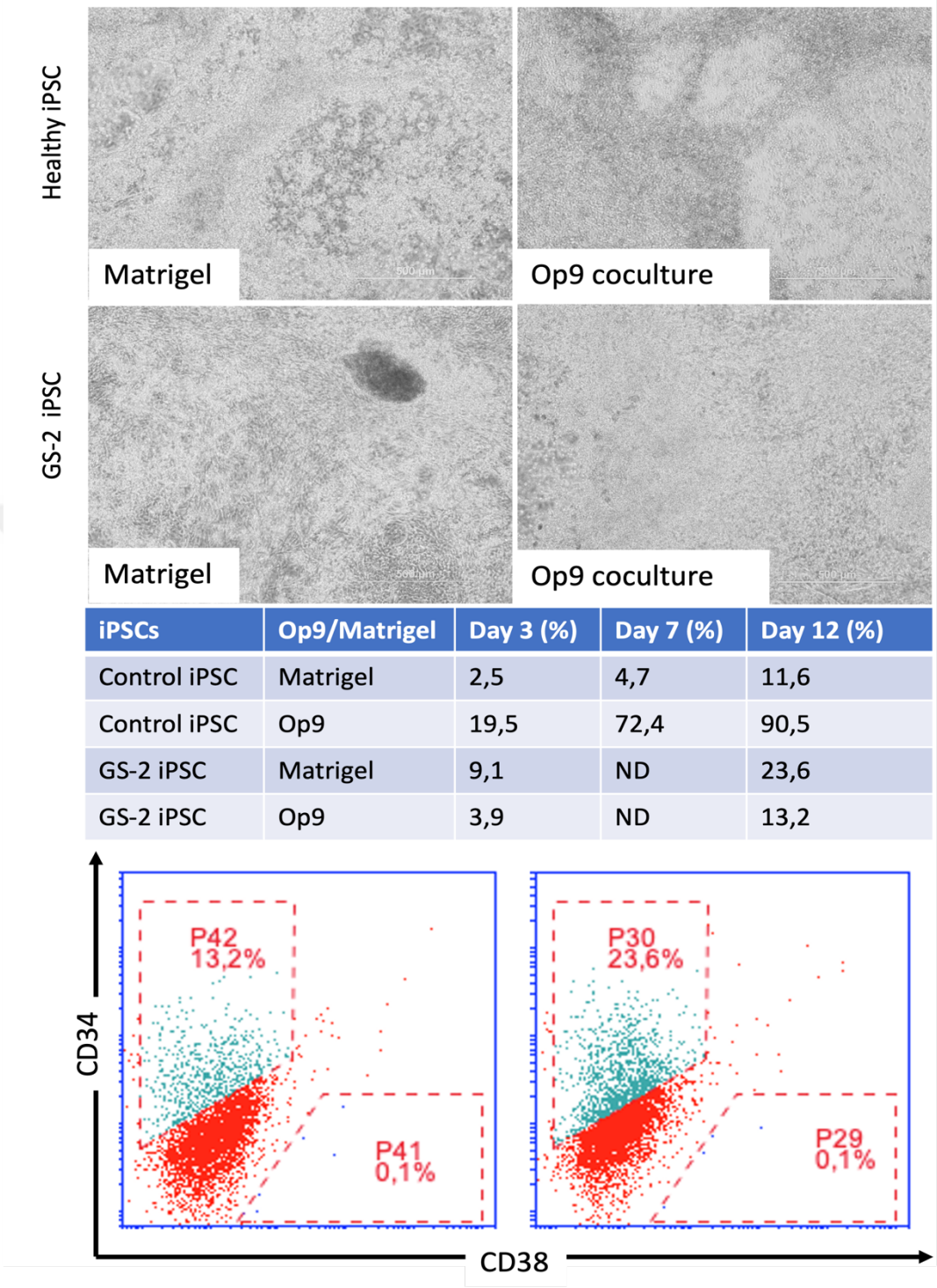


Figure 4.20. RAB27Aco expression in GS-2 iPSCs after LV vector transduction. Two different GS-2 iPSC clones were transduced at MOI:30 with LV-PGK-RAB27Aco. Expression of RAB27Aco was measured 24 hours after transduction.

4.3.6. Hematopoietic differentiation of iPSCs

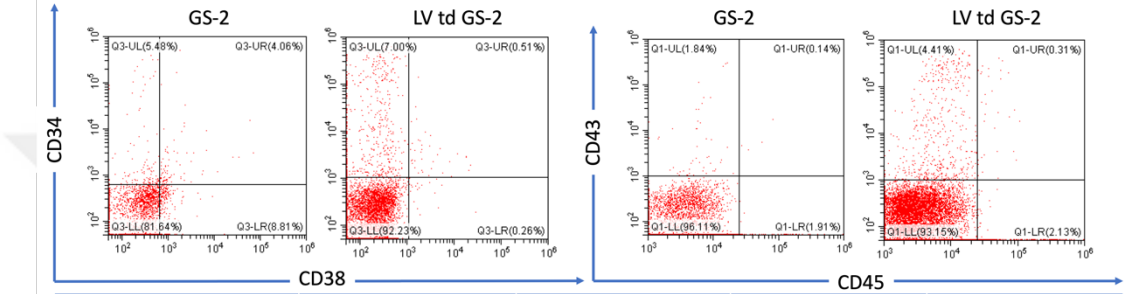
Firstly, we optimized hematopoietic differentiation from iPSCs using donor cells by comparing differentiation efficiency of the iPSCs after plating on Matrigel-coated plates or confluent Op9 cell layers. Since we found increased numbers of CD34+ cells in cultures in the presence of Op9, we used these conditions in subsequent experiments (Figure 4.21).



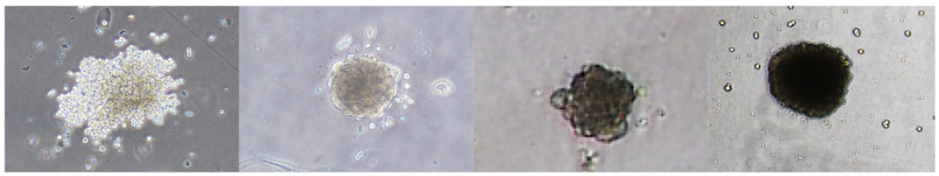
iPSCs	Op9/Matrigel	Day 3 (%)	Day 7 (%)	Day 12 (%)
Control iPSC	Matrigel	2,5	4,7	11,6
Control iPSC	Op9	19,5	72,4	90,5
GS-2 iPSC	Matrigel	9,1	ND	23,6
GS-2 iPSC	Op9	3,9	ND	13,2

Figure 4.21. Optimization of hematopoietic differentiation. Healthy donor and GS-2 iPSCs were differentiated towards hematopoietic lineage in the presence of Matrigel-coated plates or confluent Op9 cell layers. Upper panel: Hematopoietic islet formation on Matrigel or Op9 cell layers. Middle panel: Comparison of hematopoietic differentiation on Matrigel and Op9 cells on day 3, 7 and 12. Lower panel: Expression of CD34 HSC and CD38 mature cell markers after 12 days of differentiation.

After differentiation, we compared the differentiation capacity of the original GS-2 iPSCs with RAB27A transduced GS-2 iPSCs. Differentiation capacity of the transduced, RAB27A expressing and non-transduced RAB27A- iPSCs was comparable in terms of surface marker expression. However, hematopoietic colonies were larger in size and showed a healthier appearance before transduction than after transduction. Interestingly, although colonies were smaller in size after transduction, their total colony numbers increased (Figure 4.22).



Cells	CD34 (%)	CD38 (%)	CD43 (%)	CD45 (%)
GS-2 iPSC #2	9,54	12,87	1,98	2,05
GS-2 iPSC #4	7,65	0,47	1,90	1,04
LV td GS-2 iPSC #2	7,51	0,77	4,72	2,44
LV td GS-2 iPSC #4	6,33	0,47	1,05	1,17



Criteria	GS-2 iPSC #2	GS-2 iPSC #4	LV td GS-2 iPSC #2	LV td GS-2 iPSC #4
Colony number	88	140	225	218
Diameter (μm)	42,9 ± 41,7	16,4 ± 6,2	34,9 ± 8,6	55,1 ± 32,3

Figure 4.22. Hematopoietic differentiation of GS-2 iPSCs and PGK-RAB27Aco transduced GS-2 iPSCs. Upper panel: Analysis of expression of the hematopoietic markers CD34, CD38, CD43 and CD45 in GS-2 iPSCs before and after transduction with LV-PGK-RAB27Aco. Lower panel: After 12 days of hematopoietic differentiation, we assessed the colony formation of HSCs from the GS-2 and RAB27A transduced GS-2 iPSCs. Whereas the colony number after transduction was higher in both transduced GS-2 iPSC clones, colony size was larger in the GS-2 iPSC derived cells.

4.4. Assessment of potential risks related to overexpression of RAB27A

4.4.1. Assessment of RAB27A expression in cell lines and primary cells

To assess the effect of RAB27A overexpression, we first wanted to identify cells with a relatively low endogenous RAB27A expression level. We therefore measured basic (endogenous) RAB27A protein expression levels in different stem cell types and cell lines, including three different leukemic cell lines (Daudi, HL60 and Jurkat) and one non-tumorigenic human embryonic kidney HEK293T cell line (Figure 4.23). Jurkat's, HL-60 and Daudi cell lines were used to test whether the RAB27A antibody worked. Cells were intracellularly stained as described in the methods section above. $1x10^5$ cells were taken into the tube from each cell line. Cells were unstained to determine negative and were stained with primary antibodies to determine false positivity and with both primary and secondary antibodies to determine positivity. After staining, RAB27A expression in the cells was determined by reading on the flow cytometry device. To check whether the RAB27A antibody worked, Jurkat's with high positive RAB27A expression, medium positive HL-60 and low positive Daudi cell lines were used. Cells were intracellularly stained as described in the methods section above. $1x10^5$ cells were taken into the tube from each cell line. Cells were unstained to determine negative and were stained with primary antibody to determine false positivity and with both primary and secondary antibodies to determine positivity. After staining, RAB27A expression in the cells was determined by reading on the flow cytometry device (Figure 4.23). RAB27A protein expression was found to be the highest in the HEK293T cells, whereas expression of RAB27A in the leukemic cell lines was highly variable, ranging from almost 20 to 90% expression. In contrast, the basic endogenous expression of RAB27A in both MSCs and HSPCs samples was found to be considerably lower ($\leq$20%).

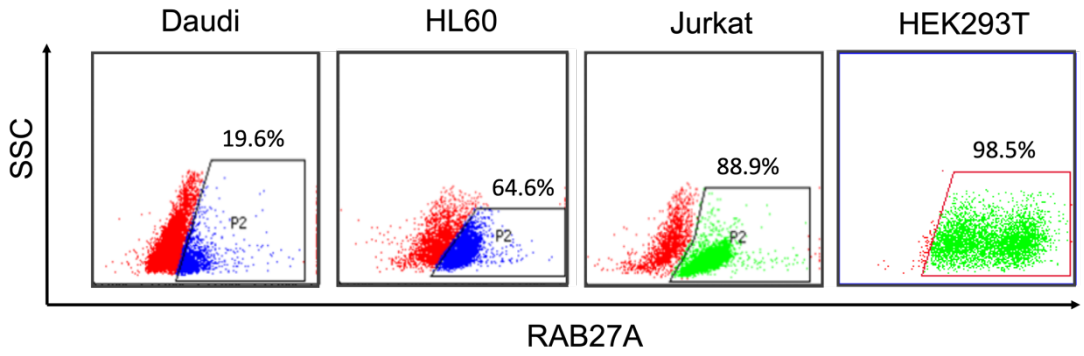


Figure 4.23. Endogenous RAB27A protein expression in different cell lines. RAB27A was determined using flow cytometry in three different leukemic cell lines (Daudi, HL60 and Jurkat) and the healthy human embryonic kidney cell line (HEK293T). All cell lines showed high but variable levels of RAB27A expression.

4.4.2. Overexpression of RAB27A in MSCs and HSPCs may interfere with stem cell function

Based on their relatively modest endogenous RAB27A expression levels (in comparison to cancer cell lines and other tissues), we decided to use these stem cells for the overexpression assays. We therefore used healthy donor BM-MSCs, human UCB CD34+ HSPCs and murine BM CD117+ HSPCs. Passage 3 MSCs expressed high levels of the MSC-specific markers CD29, CD44, CD73, CD90, CD105 and CD166 (all>95%) and showed differentiation into adipogenic and osteogenic lineage (Figure 4.24). RAB27A protein and gene expression levels, as well as differentiation capacity, were assessed for MSCs before and 24 hours after transduction. Overnight lentiviral transduction resulted in a rapid increase in RAB27A expression (Figure 4.25.A). However, overexpression of RAB27A resulted in an overall decrease in expression of typical MSC adherence markers, including CD29 (68,6%), CD44 (92,6%) and CD105 (18,4%) and complete loss of expression of the MSC stem cell markers CD73, CD90, CD166, but did not affect adipogenic and osteogenic differentiation capacity of RAB27A+ MSCs (Figure 4.25.B), indicating possible interference of RAB27A with MSC stemness/function, but not adherence or viability.

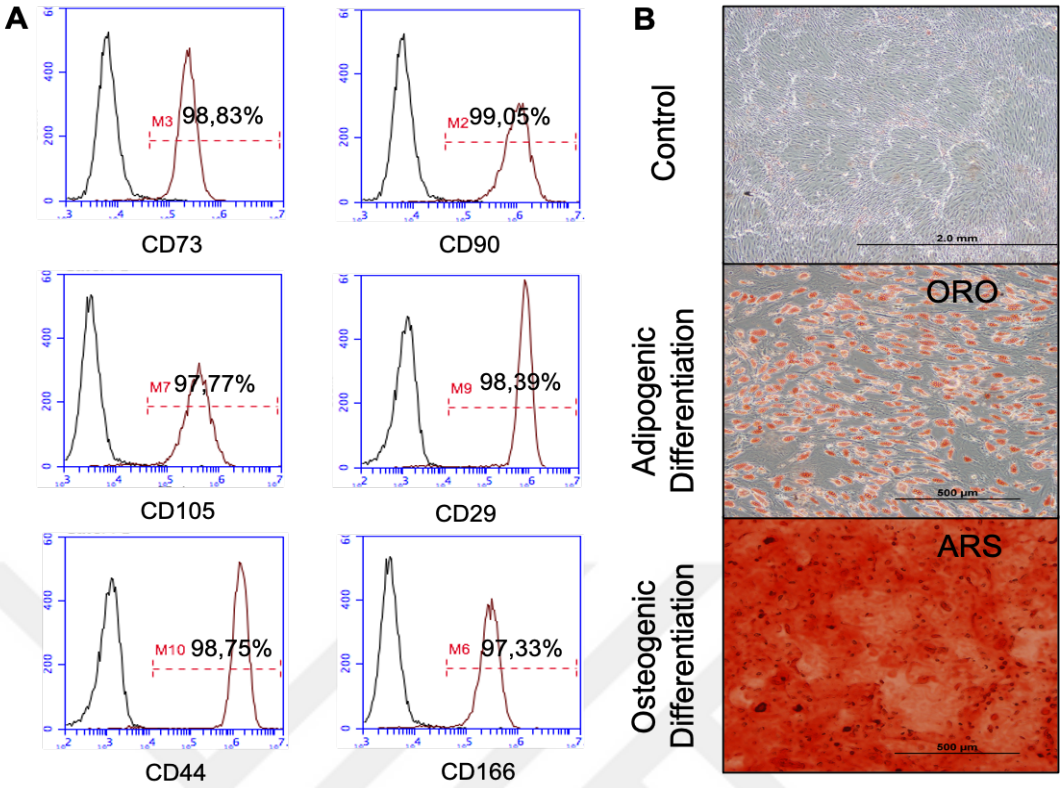


Figure 4.24. Characterization of healthy donor BM-MSCs. For transduction and transplantation experiments healthy donor bone marrow-derived mesenchymal stem cells (MSCs) were used. The MSCs were characterized at passage 3 and used for immunophenotyping (**A**) and differentiation assays (**B**). The cells displayed high expression of typical MSC markers, including CD29, CD44, CD73, CD90, CD105 and CD166. Differentiation assays were performed for adipogenic and osteogenic lineages. After 21 days of differentiation cells were stained with Oil Red O (ORO) or Alizarin Red S (ARS) and compared with unstained control wells. After confirmation of immunophenotype and differentiation potential, the MSCs from this donor were used in further experiments.

Next, we assessed RAB27A levels in human UCB CD34+ and murine BM CD117+ HSPCs. Similar to MSCs, we found low level endogenous expression of RAB27A, which rapidly increased after transduction (Figure 4.26).

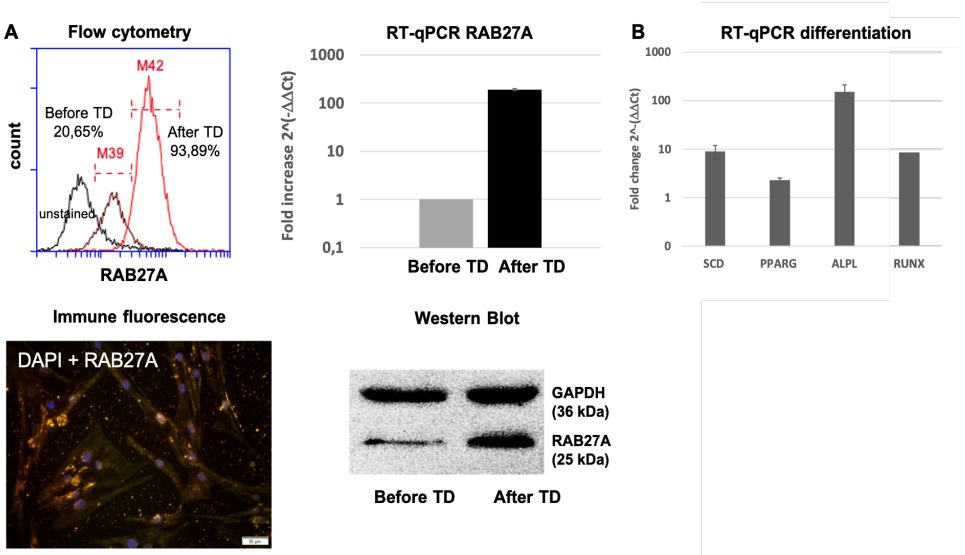


Figure 4.25. RAB27A expression in MSCs before and after transduction. Healthy donor BM-MSCs were transduced overnight with LV-RAB27Aco. (A) Expression of RAB27A in MSCs before and after transduction was confirmed using flow cytometry (upper left), RT-qPCR (upper right), immunofluorescence microscopy (lower left) and Western Blot (lower right). (B) RAB27A RAB27A+ MSCs were exposed to differentiation media for 7 days and gene expression of *SCD* (early adipogenic marker), *PPARG* (late adipogenic marker), *ALPL* (early osteogenic marker) and *RUNX2* (late osteogenic marker) was measured.

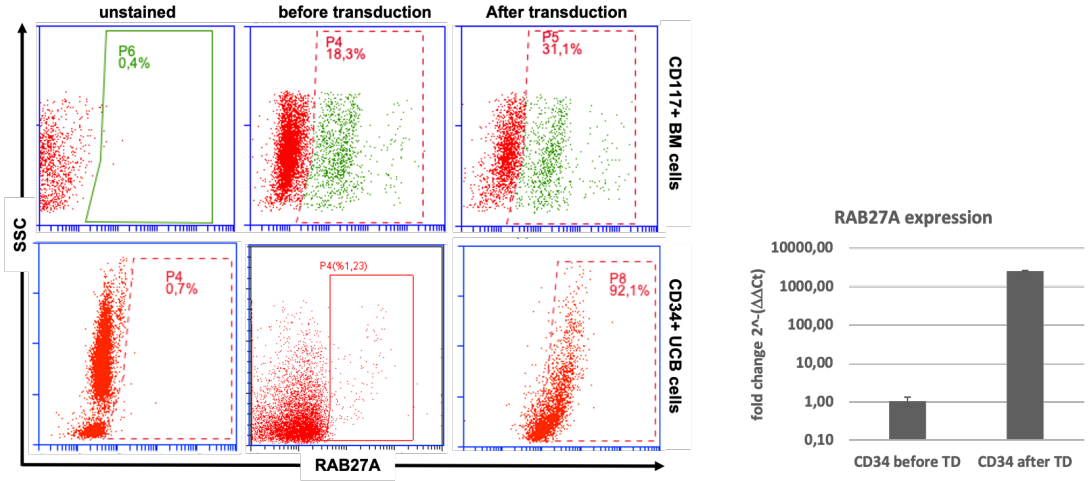
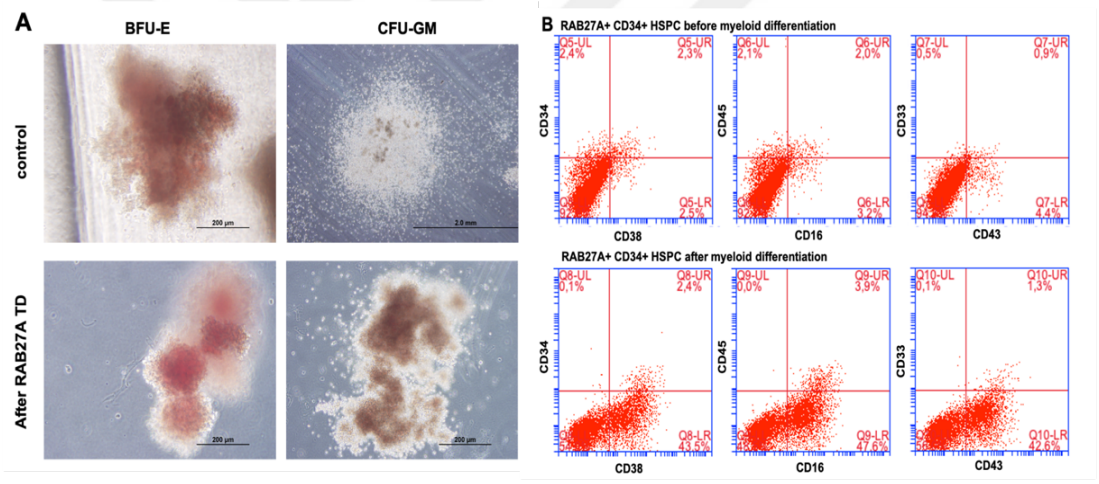


Figure 4.26. RAB27A expression in human and murine HSPCs before and after transduction. Healthy donor umbilical cord blood (UCB) derived CD34+ HSPCs and murine bone marrow (BM) CD117+ cells were transduced overnight with LV-RAB27Aco. Expression of RAB27A in the CD34+ and CD117+ cells before and after transduction was assessed using flow cytometry (left) and in CD34+ cells confirmed with RT-qPCR (right).

Effects of RAB27A overexpression on HSPC function were further assessed by enumeration and immunophenotyping of the cells after myeloid differentiation (Figure 4.27.A). Despite a noticeable decrease in colony forming numbers, overall CFU morphology remained unchanged after the transduction of HSPCs and overexpression of RAB27A. We observed no lineage bias towards either erythroid or granulocytic lineage. Flow cytometric assessment of RAB27A transduced HSPCs before and after myeloid differentiation revealed a normal increase in myeloid-specific markers (Figure 4.27.B). These data indicate that RAB27A overexpression is not directly associated with MSC or HSPC toxicity or viability. However, general stem cell function and/or stemness may be affected by continuous overexpression of the protein.



Colonies/10^3 cells	BFU-e	CFU-GM	CFU-GEMM
Before TD	7,3	33,6	6,4
After TD	3,6	22,7	1,8

Figure 4.27. RAB27A expression does not affect the differentiation potential of HSPCs. Myeloid differentiation of CD34+ HSPCs before and after transduction was assessed using colony forming unit assays. (A) Control and RAB27A transduced CD34+ HSPCs we assessed for Erythroid (BFU-E) and Granulocytic/Monocytic (CFU-GM) colony formation; (B) RAB27A transduced CD34+ HSPCs were assessed for expression of early HSPC (CD43 and CD34) and mature myeloid cell markers i.e., CD16, CD33, CD38 and CD45 before and after differentiation.

4.4.3. RAB27A+ MSCs do not cause development of mesenchymal tumors

We then proceeded with subcutaneous transplantation of control (non-transduced) MSCs or LV-RAB27A-transduced MSCs (RAB27A+ MSCs) in Rag2 mice. To prevent the cells from direct migration from the injection site, cells were injected in the presence of Matrigel, which polymerizes when exposed to temperatures of 37°C. Local inspection of the injection sites did not show any signs of tumorigenic or immune activity. However, one mouse (HU-R338) from the MSC control group was found to suffer from considerable dyspnea shortly before sacrifice at 3 months post-transplantation. Assessment of the peripheral blood smear of this mouse, revealed widespread blastic infiltration of peripheral blood, indicating the presence of acute myeloid leukemia (AML), whereas none of the other mice transplanted with MSCs, either transduced or non-transduced, showed any signs of leukemia (Figure 4.28).

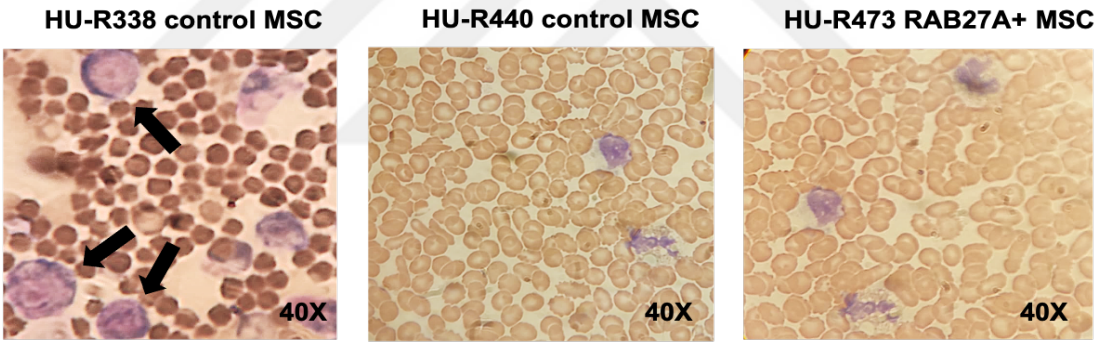


Figure 4.28. Rag2 mice were transplanted with non-transduced control MSCs (n=3) or transduced RAB27A+ MSCs (n=3) and followed for 3 months. A: mouse HU-R338 received control MSCs and showed clinical signs of leukemia (shortness of breath, failure to thrive), as well as the presence of myeloid blasts (black arrows) in the peripheral blood smear, highly indicative for acute myeloid leukemia (AML); B: mouse HU-R440 was transplanted with the same batch of control MSCs, but lived throughout the experiment without signs of leukemia or other sequelae; C: mouse HU-R473 received RAB27A transduced MSCs and showed no signs of any discomfort, nor abnormalities in its peripheral blood smear. The peripheral blood smears from HU-R440 and HU-R473 are representative for the other mice in their groups, no other adverse events were observed in any of the other mice.

Daily clinical inspection of the mice did not suggest the development of solid (mesenchymal) tumors and all mice, except for one mouse in the SF-RAB27A treated group (HU-R490), were sacrificed at the intended period. Although mouse HU-R490 was transplanted with the same batch of RAB27A+ MSCs as the other mice, this mouse was found dead shortly before the end of the transplantation study. Macroscopically, no visible signs of tumor tissue or infection were detected, but clinical inspection of the animal revealed signs of trauma and bite marks around the tail and back, indicating the mouse had been most likely killed by its litter mates. Tissues from all other mice were available for histological examination. Detailed inspection of the tissues excised from the area adjacent to the injection site showed no signs of aberrant growth or malignant cells, although mononuclear cell infiltrates were observed in the surrounding loose subcutaneous connective tissue and striated muscles in mice from both groups (Figure 4.29.A-L). Similarly, inspection of the lung tissue, as the primary tissue for metastasis in this area, showed the presence of widespread granulation tissue, characterized by dense cellular infiltrates in the peribranchial and bronchiolar lung stroma of both mice transplanted with MSCs and RAB27A+ MSCs, but no evidence of malignant cells (Figure 4.29.M-Y).

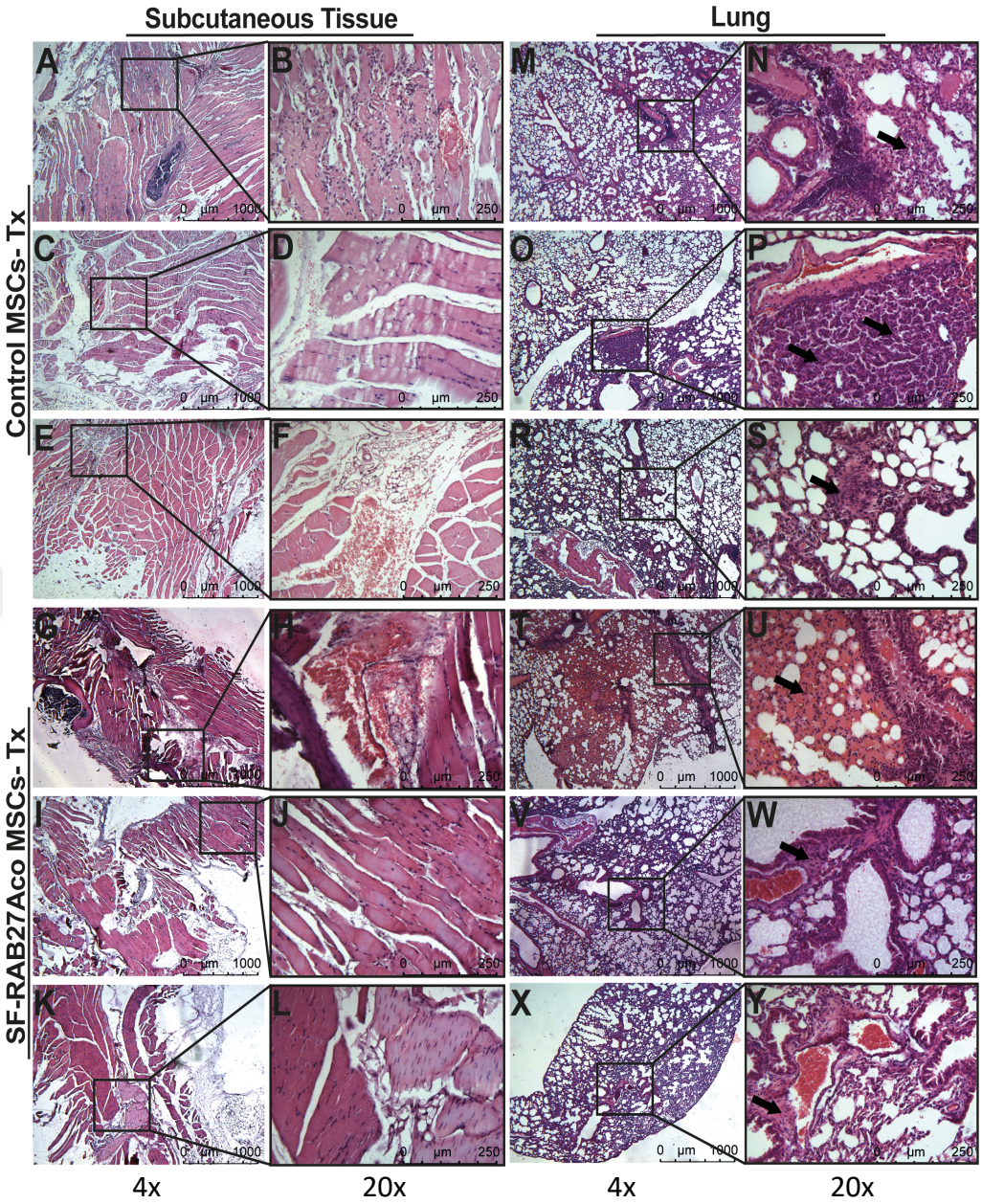


Figure 4.29. Histological assessment of MSC injection sites. Control and RAB27A+ MSCs were transplanted into Rag2 mice. Histological preparates stained with H/E from the injection site (A-F) control MSCs; (G-L) RAB27A+ MSCs) and lung tissue (M-S) control MSCs; (T-Y) RAB27A+ MSCs) were assessed for the presence of malignancy or inflammation. Granulation tissue with mononuclear cell infiltrates was detected in connective tissue and striated muscles of mice in both groups' injection sites (A-L). Lung parenchyma showed healthy alveoli, bronchi and bronchioles. Peribranchial and bronchiolar lung stroma exhibited cellular infiltrates (M-Y) without signs of malignant cells. Black arrows indicate the presence of cellular infiltrates.

4.4.4. RAB27A overexpression in HSPCs affects their long-term engraftment potential

We then proceeded with hematopoietic transplantations in Busulfan pre-treated Rag2 mice with control or RAB27A-transduced murine BM CD117+ or human UCB CD34+ HSPCs and followed the mice for 6 months post-transplantation. Engraftment kinetics in the Rag2 mouse model were followed by assessing circulating CD3+ T cell and CD19+ B cell numbers in the peripheral blood. Transplantation of murine CD117+ HSPCs resulted in robust immune recovery, whereas transplantation of human CD34+ HSPCs resulted in only marginal immune recovery (Figure 4.30.A). The latter is expected since the Rag2 mouse model is a leaky immune deficient mouse model with residual NK cell activity and may only allow for relatively low engraftment in a xenotransplant setting. We found no significant differences in c-kit+ or CD34+ cell numbers in mice transplanted with control or RAB27A+ HSPCs.

We observed the appearance of considerable numbers of CD3 and CD19 cells derived from the transplants, especially in the mice transplanted with murine RAB27A+ CD117+ cells. However, the level of intracellular RAB27A expression in the peripheral blood, bone marrow and spleen total nucleated cells of these mice was unexpectedly low at 6 months post-transplantation (Figure 4.31), despite very high transduction levels before transplantation. These data most likely indicate that the RAB27A transduced cells may have contributed only marginally to the total engraftment or alternatively that RAB27A expression has been lost in time. Because the detection capacity of low protein expression of flow cytometers is limited, we decided to assess *RAB27A* gene expression in the BM of mice transplanted with RAB27A+ CD117+ and CD34+ HSPCs using RT-qPCR (Figure 4.30.B).

Since we used primer sequences designed to detect our codon-optimized transgene, we cannot measure the presence of physiological *RAB27A* gene expression before transplantation. We therefore used the ΔCt method to assess relative expression in relation to *gapdh*, rather than the $\Delta\Delta$Ct method.

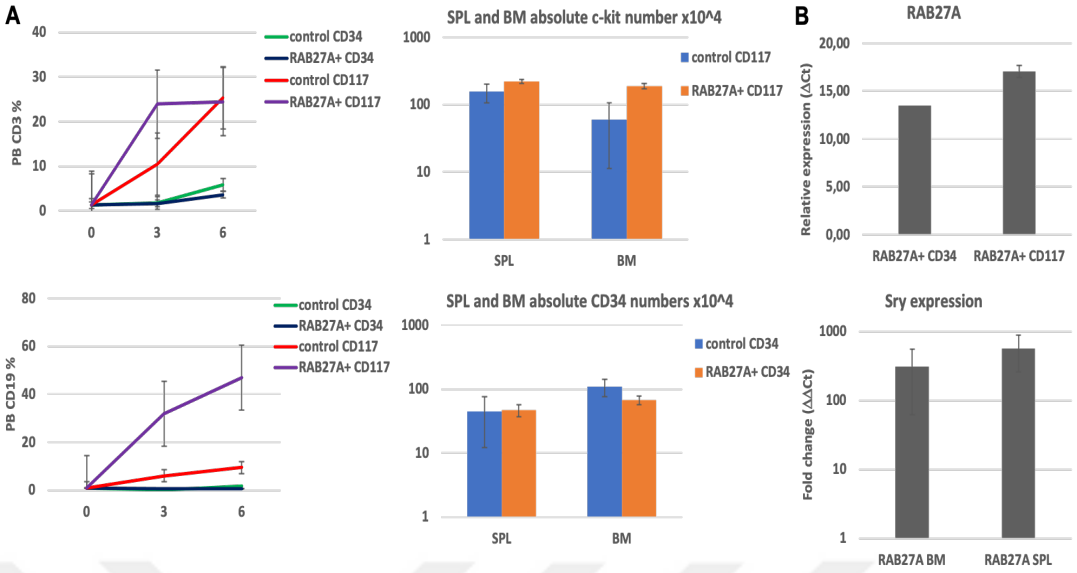


Figure 4.30. Assessment of engraftment potential of RAB27A transduced HSPCs. (A) Control and RAB27A+ human CD34+ and murine CD117+ HSPCs were transplanted in Rag2 mice. Engraftment was followed by assessing immune reconstitution in these mice for 6 months, as apparent from the appearance of murine or human CD3 T cells and CD19 B cells in the peripheral blood (left). After sacrifice changes in CD117+ and CD34+ cell numbers in bone marrow (BM) and spleen tissue (SPL) were assessed (middle). (B) Despite bone marrow (BM) and spleen (SPL) chimerism (as measured by *Sry*) *RAB27A* gene expression in BM of mice transplanted with RAB27A transduced cells was very low and not detectable in SPL (right).

Using this method we detected gene expression of codon-optimized human *RAB27A* in the bone marrow of the transplanted mice. However, in similar the results obtained with flow cytometry, the relative expression levels of the *RAB27A* transgene were very low. To assess the overall engraftment of male (Sry+) Balb/c derived control and transduced CD117+ cells in female (Sry-) mice, we measured *Sry* expression in BM samples of transplanted animals. In agreement with the immune reconstitution data, we found high expression of Sry in BM and spleen tissue.

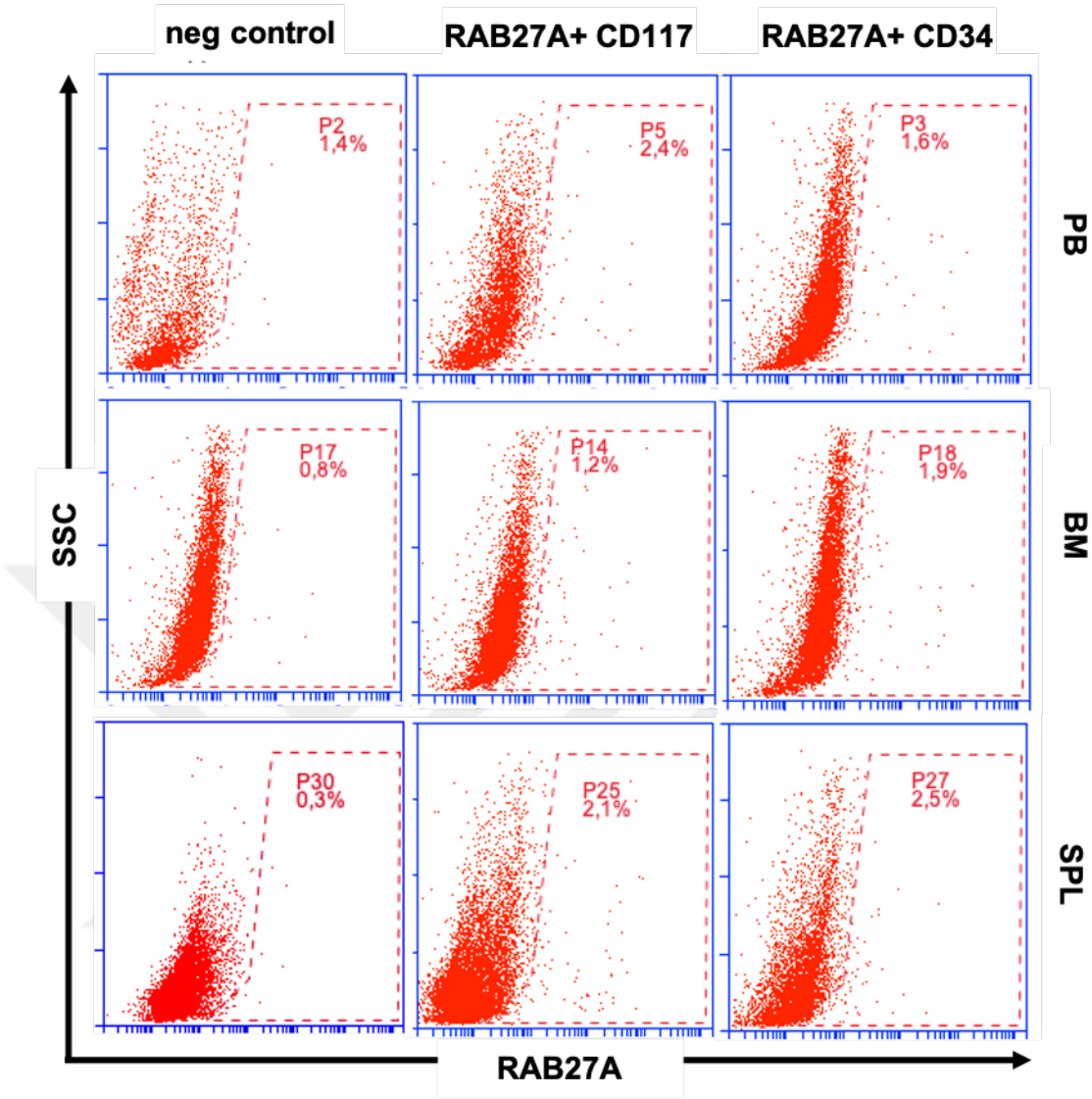


Figure 4.31. RAB27A expression 6 months after transplantation. Rag2 mice were transplanted with murine RAB27A+ CD117 cells (n=3) or human RAB27A+ CD34 cells control MSCs (n=3) and followed for 6 months. Upon sacrifice, peripheral blood (PB, upper panel), bone marrow (BM, middle panel) and spleen (SPL, lower panel) total nucleated cells were collected and assessed for intracellular RAB27A expression. Despite high levels of RAB27A expression in the transplanted cells, RAB27A expression was barely detectable using FACS analysis at 6 months post transplantation. Representative plots from each group are shown.

5. DISCUSSION

GS-2 is an immune deficiency caused by a mutation in the *RAB27A* gene. Although mutations in the *RAB27A* gene may affect other cell types, such as melanocytes, causing pigmentary dilution of skin and hairs resulting in the characteristic silver-gray hair complexion (168) and secretory cells (18, 44), the latter has not been reported to result in any specific functional problem, likely due to the compensatory effect of RAB27B and thus major clinical issues of mutations in *RAB27A* can be largely contributed to dysfunction of the immune system (52). Currently, the only curative treatment for children with GS-2 is HSC transplantation. In the absence of a suitable, matched donor, the reintroduction of physiologic RAB27A expression in the stem cells of the hematopoietic system through gene editing or overexpression of RAB27A in HSCs through use of lentiviral vectors could be a potential approach to treat RAB27A deficiency. However, for rare diseases, such as GS-2, it is very difficult to obtain sufficient numbers of (hematopoietic stem) cells to design and test the efficacy of gene editing tools or viral vectors. The development of induced pluripotent stem cells (iPSC) through overexpression of a several combinations of transcription factors (169) has allowed reprogramming and indefinite culture of patient-derived somatic cells and the creation of accessible, shared biobanks (83). Using this system, we aimed to assess within the framework of this thesis 1) the effects of correction of GS-2 RAB27A mutations through use of the CRISPR/Cas9 gene editing of patient-derived MSCs and iPSCs, 2) the efficacy of different lentiviral vectors to express a codon-optimized version of RAB27A (RAB27Aco) under the control of different physiologic and constitutive promoters, and 3) to assess potential risks related to overexpression of RAB27Aco *in vivo* in an immune deficient mouse model.

iPSC culture media may differ in constituents, but unfortunately many of these media have proprietary medium formulations, making a side-to-side comparison of medium supplements difficult. We found that iPS-Brew showed the best support of iPSC reprogramming, whereas TeSR E8 medium showed the best performance during maintenance culture. However, one downside of the use of TeSR E8 medium is that although it is one of the most commonly used media, its use requires daily medium

changes in order to keep the iPSCs undifferentiated. Further culture optimization showed that use of TeSR Plus medium was ideal, since it supported iPSC maintenance to a similar extent as the TeSR E8 medium, but at the same time allowed us to change medium once in two or even in three days.

In addition to the standard maintenance protocols, also the importance of storage (cryopreservation) protocols is often overlooked and discarded as if of lesser consequence. Similar to the above-mentioned maintenance protocols, storage conditions and freezing medium supplements have a major impact on cell function, preservation of stemness and differentiation potential of stem cells (170). Current protocols for cryopreservation of iPSCs have focused on the effects of freezing of the cells as small aggregates vs single cell freezing (171). Our results show that breaking up of the colonies resulted in suboptimal recovery after thawing with 1) induction of uncontrolled differentiation, initiating from the borders of the colonies, 2) increased necrosis in the center of the colonies, and 3) lower plating efficiency in comparison to iPSCs frozen in aggregates. In addition, other studies have assessed the effects of lowering the concentrations of DMSO in order to prevent DMSO-related cell death during thawing of the cells (171, 172). Our results show that decreasing the concentration of DMSO from 10% to 5% is not associated with significant cell death or decreased colony formation and could therefore be used in subsequent studies. Further decreasing of the DMSO concentrations however may require addition of the non-toxic cryoprotectant agent (CPA) glycerol to prevent cell death related to dehydration damage during freezing (172). The use of Knockout Serum Replacement (KSR) has also gained popularity as a serum replacement in the development of xenogenic-free cryopreservation formulations. In combination with 10% DMSO, KSR has been used at concentrations of 25%-90% to freeze effectively iPSCs, ESCs and iPSC-derived cells with high post-thaw viability, as reviewed by Erol et. al. (170). Lastly, ROCK inhibitors have been shown to promote plating and cloning efficiency of both ESCs and iPSCs by inhibition of Rho kinase activity (173). Other methods to increase cell survival after freeze/thawing may be by effectively inhibiting cellular stress, including oxidative stress by adding anti-oxidants, such as vitamin E (174) and its

derivative, SUL-109 (175) or polyphenols, such as Resveratrol (RES). We found in our experiments that addition of Ri always supported better survival after thawing and that DMSO levels could be easily reduced to 5%, but extra additives, such as SUL-109 or RES in addition to Ri did not further improve colony formation or cell survival of iPSCs.

After optimization of reprogramming and maintenance culture conditions of healthy donor MSCs and iPSCs and GS-2 iPSCs, we proceeded to use these cells to test the CRISPR/Cas9 and lentiviral vector constructs that we designed. Although a variety of genome editing tools have been developed, such as Zinc-finger nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs), none of these is remotely as effective as the recently described CRISPR/Cas9 system (146). In addition, although the ZFNs and TALENs systems can both be used to induce genome modification, their design is generally costly and time-consuming, limiting their large-scale use. In contrast, the CRISPR/Cas9 system enables researchers to directly modify DNA sequences and genes, allowing not only the study of biological systems, but also providing a simple, rapid and precise method for genetic manipulation.

In this thesis, we assessed the efficacy of the CRISPR/Cas9 gene editing tool to correct two different mutations in the *RAB27A* gene of GS-2 patient-derived MSCs and iPSCs. We designed 3 different gRNA constructs to target each exon (i.e., exon 3 and exon 7) and tested the gene correction efficacy through HDR. We used electroporation (nucleofection) to transfer the RNP complex and the donor DNA to GS-2 MSCs and iPSCs and found that gene correction using viral-free methods to correct GS-2 MSCs and iPSCs is feasible, with correction rates similar to or even exceeding those previously published for iPSCs (176, 177). However, survival of MSCs and iPSCs after nucleofection was very low, despite the presence of optimized cell culture conditions and the addition of Ri to prevent apoptosis (178). Furthermore, we also observed unwanted differentiation of iPSCs after nucleofection (179). Although the rates of HDR in our settings were moderate, performance of CRISPR/Cas9 in our hands was suboptimal due to low cell viability and therefore we aim to improve the method in future experiments. Repair through HDR has been shown to depend on

the cell cycle and take place in the S/G2 phase (180). Therefore, use of actively cycling cells (181), induction of cell cycle synchronization, temporarily arresting of cells in the S/G2 phase, as well as suppression of NHEJ through use of specific inhibitors may contribute to obtain increased rates of HDR (182-184). Alternative methods to increase HDR efficiency may further include usage of single-stranded oligo DNA nucleotides (ssODN) or the use of donor DNA designed with asymmetric homology strands (185). However, even if HDR rates can be enhanced using these methods, we would still have to improve cell viability/reduce transfer-related apoptosis of MSCs and iPSCs after nucleofection (179) in order to be able to expand the cells and use for future research purposes. The use of viral vector systems, including Adeno-associated viral (AAV) vectors (186) or lentiviral vectors (LV) (187) could help to overcome this problem. We show here that it is possible to use CRISPR/Cas9 to correct mutations in the *RAB27A* gene. However, in order to make this system useful for clinical implementation, we need to enhance the HDR efficiency, and improve cell survival after gene correction. Thus, although the currently available CRISPR/Cas techniques can be and have been used almost effortlessly to create gene knock-out cell lines and models, we show here that using the same system for site directed knock-in using primary (patient-derived) cells requires further development and optimization. Current gene editing tools require the design of different gRNAs for each unique mutation, followed by testing of the constructs on patient-derived material. Therefore, this system may be, although in theory the best way to cure genetically inherited diseases, not be the most (cost) effective method. Nevertheless, recently the first advanced therapy medicinal product (ATMP) based on the use of CRISPR/Cas9, Casgevy, has been approved by the FDA. Using CRISPR/Cas9 the researchers targeted a specific locus on the DNA that is involved in switching the fetal hemoglobin (HbF) gene off after birth. By deleting this region, expression of fetal hemoglobin is resumed alleviating the symptoms of a sickle-cell disease or β-thalassemia (188). Thus, using knock-out rather than knock-in, the CRISPR/Cas9 system can be used to treat a large group of patients, despite different mutations. In our system, we may use this feature to increase RAB27B expression.

RAB27A and RAB27B have been shown to be functionally redundant (40) and show partially overlapping functions. In fact, in secretory cells RAB27A and RAB27B are often co-expressed (189). Furthermore, RAB27B has been shown to bind many of the RAB27A effectors (190). The miRNA miR-599 has been shown to directly target *RAB27B* expression and expression levels of RAB27B have been shown to be inversely correlated with miR-599 levels (191). Therefore, instead of focusing on repairing of mutations in RAB27A with CRISPR/Cas9, it may be easier to, similar to Casgevy, knock-out expression of the suppressor miR-599 with CRISPR/Cas, thus increasing *RAB27B* expression. In conclusion, we believe that the use of custom designed CRISPR/Cas9 gene therapy for the treatment of Griscelli Syndrome Type 2 is feasible, but current low rates of HDR efficiency and cell viability after transfection require further optimization to make this system ready for clinical use.

We therefore sought out different methods in addition to CRISPR/Cas gene correction to reinstate ectopic RAB27A expression in GS-2 MSCs and iPSCs by designing different types of lentiviral vectors. An advantage of the lentiviral vector systems is that 1) most currently used vectors are amphotropic or even pantropic, being able to target almost any mammalian cell; 2) using different promoter systems, different levels of expression, reaching from near physiologic to high overexpression can be achieved; 3) high transduction efficiencies can be obtained by use of lentiviral vectors without a negative effect on cellular viability; and 4) the lentiviral vectors used are not mutation or patient-specific and can be developed to work with any patient. In the last decade, several gene therapeutic ATMPs for genetically inherited diseases have been developed and received marketing authorization from the FDA, including Zynteglo for the treatment of ß-thalassemia (109). Since expression of RAB27A into progeny of transduced HSCs requires permanent integration of the gene into the genome, gene therapy for GS-2 would be ideally done using lentiviral vector transfer systems (192). Although the lentiviral vector systems have been proven highly effective in the development of treatment for certain metabolic (e.g., CALD) and hematopoietic diseases (e.g., b-thalassemia/sickle cell disease), potential risks related to the lentiviral integrations sites, such as integrational mutagenesis (135) or risks

related to overexpression of the transgene (193) have led researchers to develop newer generations of 3rd generation self-inactivating (SIN) lentiviral vectors (194), and 4th generation lentiviral vectors, which lack HIV-1 packaging sequences in the LV vector proviruses (195). In addition, regulated expression of transgenic proteins by use of miRNA cassettes in the construct, as well as improved promoter systems, including tissue-/cell-specific promoters, physiologic promotors, and methylation-resistant promoters have been used to further improve protein expression. Here, we tested the efficacy of 3 different promoters on the expression of RAB27A in GS-2 MSCs and iPSCs. Using our lentiviral constructs, we were able to obtain high expression of the RAB27Aco transgene in MSCs after use of both the physiological promoter PGK and the strong SFFV promoter. However, in order to gain similar transduction percentages as with SFFV, we needed a 0,5 log higher MOI when using the PGK promoter. A similar effect was seen when the UCOE promoter was used, which showed a transduction efficiency of 0,5 log lower than PGK.

Many Rab GTPases, including RAB27A (and RAB27B), have been shown to play a role in the development, invasiveness and metastasis of numerous malignancies resulting in poor survival, reviewed by Erol et al. (196) and promote proliferation and invasiveness of cancer cell lines (81, 197, 198). In contrast, suppression of RAB27A resulted in a better prognosis of cancer *in vivo* and decreased aggressiveness of cancer cells *in vitro* (199, 200). However, it is not known whether RAB27A functions as an initiating factor or simply provides an optimal biochemical environment for cancer growth and metastasis via upregulation of cell signaling and cytokine secretion. In order to assess potential risks related to overexpression of RAB27A after LV gene therapy of GS-2, we wanted to overexpress RAB27A in healthy stem cells using a strong constitutive promoter, i.e., SF (the promoter of Spleen Focus Forming Virus, SFFV) (162) and assess the development of tumors/leukemia in mice. Use of the SFFV promoter was chosen for several reasons: firstly, we have shown that SFFV is the strongest promoter, thus the chances of revealing the presence of any negative side effects would be the highest with this promoter; and secondly, the SFFV promoter itself is known to cause tumor progression and use of this promoter would

therefore give information on the risks related to these types of constructs. In our *in vivo* transplantation studies, we did not find any evidence for direct tumorigenesis-initiating effects of RAB27A overexpression. Interestingly however, we noticed that both MSCs and HSPCs appeared to be functionally impaired after RAB27A overexpression, as evident from a decrease in expression of stem cell markers by MSCs and decreased colony formation by HSPCs. Since we found relatively low physiologic expression of RAB27A in healthy donor MSCs and HSPCs, we believe that after transduction the SF-regulated supraphysiological RAB27A levels may have affected stem cells by interference with normal cellular functions. For example, Rabs have been implicated in the development of neurodegenerative diseases and increased RAB27 expression in forebrain neurons has been correlated with accelerated cognitive decline in patients with Alzheimer's disease (201) possibly due to interference with neurotransmitter release and dysregulation of axonal transport (23).

To reveal any potential side effects related to RAB27A overexpression in stem cells, we lentivirally transduced MSCs and HSPCs with high MOIs using the SF promoter to forcefully induce RAB27A expression and then performed transplantation assays in immune deficient Rag2 mice. We did not find any signs of mutagenesis related to our transplantations, although we encountered a spontaneous leukemia in one of the control mice, transplanted with non-transduced MSCs. We have previously used this mouse model to develop gene therapy for RAG2-SCID and the occurrence of spontaneous leukemias or other tumors in this immunodeficient mouse model is not rare and has been observed in other SCID mouse models as well (202, 203). Besides this leukemia, we observed mononuclear cell infiltrates upon histological examination of the pulmonary tissue of mice transplanted with both control MSC and RAB27A+ MSCs, indicating that MSCs may have induced a tissue response, but that this is not related to RAB27A overexpression. Mice transplanted with non-transduced and RAB27A expressing human CD34+ HSPCs showed low engraftment of human cells in BM and spleen, and human *RAB27A* expression levels were very low, despite high levels of transduction. We did not find

signs of leukemia in any of the transplanted mice. Mice transplanted with murine HSPCs showed better engraftment with robust immune reconstitution, in particularly mice transplanted with RAB27A+ CD117 cells. However, despite confirmed expression of Sry, indicating engraftment from male into female mice, again RAB27A expression was barely detectable. We believe these data may indicate that 1) RAB27A may affect long-term engraftment potential of and stemness of HSPCs, 2) RAB27A+ HSPCs may have lost expression of RAB27A due to methylation of CpG islands in the SFFV promoter (141). The latter would be highly plausible, since transgene expression has been shown to be silenced regularly in constructs using SFFV due to methylation of the promoter or enhancer elements (141, 204). Therefore, use of silencing resistant UCOE promoter, which has been shown to provide stable gene expression both in multipotent and pluripotent stem cells, could be a good alternative. However, use of a UCOE promoter to drive transgene expression has been shown to result in decreased viral titers during vector production and relatively low gene expression due to its bidirectional expression. However, this *Sry* expression did not correlate with *RAB27A* expression, indicating that RAB27A transduced cells were not a major contributing cell fraction to the overall success of the transplantation. Thus, firstly our *in vivo* experiments using RAB27A overexpression in MSCs and HSPCs did not indicate an initiating role for RAB27A in the development of any type of malignancy, although upregulation of RAB27A may create a biochemical environment that is supportive for progression of cancer cell growth. Secondly, we believe overexpression of RAB27A may cause changes in cellular signaling and behavior that may affect the cells' stemness potential. However, since clinically effective treatment of GS-2 does not require high levels or supraphysiological expression of RAB27A, use of the PGK or methylation-resistant UCOE promoter may be sufficient to obtain clinical improvement (125).

In summary, we optimized iPSC reprogramming, culture and cryopreservation conditions, we then tested the efficacy of CRISPR/Cas9-mediated gene editing to repair mutations in the RAB27A gene of different patients using MSCs and iPSCs. Although we found a significant level of HDR, low viability affected subsequent testing

of the cells in terms of function and quality and RAB27A expression. However, we only tested the efficacy of CRISPR/Cas9 using electroporation, and we did not test transfer of Cas9 using lentiviral vectors. We then compared the efficacy of different lentiviral promoter systems in the ectopic overexpression of RAB27A, again on MSCs and iPSCs, but we omitted the use of promoter of RAB27A itself, which might have given a more appropriate expression pattern that may not affect stem cell function. In addition, when we tested the potential tumorigenic effects of RAB27A after overexpression in healthy MSCs and HSCs using a lentiviral construct with an SFFV promoter, we only followed these mice for a period of 6 months. We know from early RV gene transfer studies that this may not be sufficient to predict long term tumorigenic effects and that secondary transplants may be necessary to increase the follow-up period and enhance any intrinsic risks related to RAB27A expression. However, although we collected spleen and bone marrow stem cells from the transplanted mice, the absence of high levels of engraftment as well as the absence of a high level of RAB27A expression did not make these cells suitable for secondary transplantation. Therefore, in future studies we aim to repeat these experiments and perform secondary transplants if the animals show sufficient engraftment with RAB27A expressing cells.

6. CONCLUSIONS

Within the framework of this thesis, we aimed to develop gene therapy for the treatment of Griscelli Syndrome Type 2 using two GS-2 patient-derived MSC and iPSC lines with mutations in exon 3 and 7, that we generated previously and new cell lines from a GS-2 patient with a mutation in exon 5 of the RAB27A gene that we generated during this thesis. We designed and produced different mutation-specific CRISPR/Cas9 gene editing tools and several lentiviral vectors that express the codon-optimized RAB27Aco transgene under the control of the constitutively active SFFV promoter, the constitutively active physiological PGK promoter and the methylation-resistant UCOE promoter.

We optimized cell culture and cryopreservation conditions of iPSCs and found that iPS Brew medium is optimal for use during reprogramming, whereas use of TeSR Plus medium allows medium changes of iPSC cultures every two or even three days, instead of the daily changes required with standard TeSR E8 medium. We show that for optimal cryopreservation of iPSCs cells should be frozen as aggregates, rather than as single cells and levels as high as 10% DMSO are not per se necessary, since addition of Ri to 5% DMSO freezing media is sufficient to obtain high viability after thawing.

We designed 3 different gRNA constructs to target each exon (i.e., exon 3 and exon 7) and tested the gene correction efficacy through HDR. We used electroporation (nucleofection) to transfer the RNP complex and the donor DNA to GS-2 MSCs and iPSCs and found that gene correction using viral-free methods to correct GS-2 MSCs and iPSCs is feasible, with correction rates similar to or even exceeding those previously published for iPSCs. However, electroporation resulted in low cellular viability and uncontrolled differentiation of iPSC colonies.

Using three different promoters in a 3rd generation SIN LV vector system we show that the highest RAB27A transduction efficiencies are obtained with the SFFV promoter, followed by PGK and UCOE. However, too high expression levels of RAB27A resulted in loss of stemness as apparent from loss of stem cell markers by MSCs, loss of colony formation by HSCs and loss of long-term repopulation ability when cells were transplanted *in vivo*. However, we did not find any side effects, tumorigenic or

otherwise, related to overexpression of RAB27A, indicating that although RAB27A has been previously associated with a negative prognostic role in cancer and increased aggressiveness of tumors, overexpression of RAB27A by itself is not an initiating factor for the induction of malignancies.

In conclusion, we compared CRISPR/Cas9 and LV vectors for the development of gene therapy for GS-2. Although gene editing results in acceptable levels of repair, the technology results in low cell viability. LV gene transfer was easy and robust, but high expression of RAB27A affected stem cell function. Thus, both methods can be developed into gene therapy for GS-2, but optimization of the procedures, increasing cell viability and fine-tuning of expression levels may be necessary before these therapies are ready for clinical use.

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8. SUPPLEMENTS

Supplement 1. Non-interventional ethical Committee approval

T.C.
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Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557-933
Konu : ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 09 HAZİRAN 2020 SALI
Toplantı No : 2020/11
Proje No : GO 20/316(Değerlendirme Tarihi: 31.03.2020)
Karar No : 2020/11-25

Üniversitemiz Kök Hücre Araştırma ve Uygulama Merkezi öğretim üyelerinden Dr. Öğr. Üyesi Fatima Aerts KAYA'nın sorumlu araştırmacı olduğu, Doç. Dr. Özgür ÖZYÜNCÜ, Uzm. Bio Burcu PERVİN, Mehmet Emin ŞEKER ile birlikte çalışacakları ve Vet. Hek. Özgür Doğuş EROL'un doktora tezi olan, GO 20/316 kayıt numaralı, ***"RAB27A Mutasyonunun Düzeltilmesine Yönelik Gen Tedavisi Geliştirilmesi ve Risk Değerlendirilmesi"*** başlıklı proje önerisi araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup, RAB27A geninin klonlanması ve plazmit izolasyonu, LV-RAB27A transdüksiyon sürecinin optimizasyonu ve GS-2 uPKH'lerden farklılaşmış HKH'lerde gen düzeltilmesi alt iş paketlerini içeren bölümü HÜBAB Lisansüstü tez desteği kapsamında; optimizasyonu yapılan LV-RAB27A konstraktlarının transdüksiyonu ve risk değerlendirmesi alt iş paketlerini içeren bölümü ise TÜBİTAK Hızlı Destek kapsamında yürütülmek üzere 10 Haziran 2020–10 Aralık 2022 tarihleri arasında geçerli olmak kaydı ile etik açıdan **uygun bulunmuştur.** Çalışma tamamlandığında sonuçlarını içeren bir rapor örneğinin Etik Kurulumuza gönderilmesi gerekmektedir.

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Supplement 2. Animal Ethical Committee approval

T.C.
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HAYVAN DENEYLERİ ETİK KURUL KARARI

TOPLANTI TARİHİ	:	25.02.2020 (SALI)
TOPLANTI SAYISI	:	2020/02
DOSYA KAYIT NUMARASI	:	2020/07
KARAR NUMARASI	:	2020/02 03
ONAY BİTİŞ TARİHİ		25.02.2025
ARAŞTIRMA YÜRÜTÜCÜSÜ	:	Dr. Öğr. Üyesi Fatima Aerts KAYA
HAYVAN DENEYLERİNDE GÖREVLİ ARAŞTIRMACILAR	:	Dr. Öğr. Üyesi Fatima Aerts KAYA, Vet. Hek. Özgür Doğuş EROL(Doktora Tezi), Bio. Burcu PERVİN, Mol. Bio. Mehmet Emin ŞEKER
DİĞER YARDIMCI ARAŞTIRMACILAR	:	-
ONAYLANAN HAYVAN TÜRÜ ve SAYISI	:	***67 Adet Balb/c Rag2 Fare (1-12 Ay)***

Üniversitemiz Kök Hücre Araştırma ve Uygulama Merkezi öğretim üyelerinden Dr. Öğr. Üyesi Fatima Aerts KAYA'nın araştırma yürütücüsü olduğu 2020/07 kayıt numaralı ***"RAB27A Mutasyonunun Düzeltilmesine Yönelik Gen Tedavisi Geliştirilmesi ve Risk Değerlendirilmesi"*** isimli çalışma Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre uygun bulunarak oy birliği ile onaylanmasına karar verilmiştir.

Araştırma yürütücüsü en geç, onay bitiş tarihinden sonraki 1 ay içerisinde proje sonuç raporunu Kurulumuza teslim etmekle yükümlüdür.

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Etik Kurul Başkanı

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Ayrıntılı Bilgi için:

Supplement 3. Thesis related publications

1. **Özgür Doğuş Erol**, Burcu Pervin, Mehmet Emin Şeker, Fatima Aerts-Kaya. Effects of storage media, supplements and cryopreservation methods on quality of stem cells. Invited review. *World Journal of Stem Cells* 2021:13(9), 1197-1214. DOI: https://dx.doi.org/10.4252/wjsc.v13.i9.1197. SCIE indexed. Q2

2. **Özgür Doğuş Erol**, Mehmet Emin Şeker, Fatima Aerts-Kaya. Uyarılmış Pluripotent Kök Hücrelerle nadir hastalık modellemesi (Modelling of rare diseases with induced pluripotent stem cells). *Türkiye Klinikleri Tıbbi Genetik - Kök Hücre ve Rejeneratif Tıp - Special Topics*, 2021(1): 10-17. https://www.turkiyeklinikleri.com/journal/tibbi-genetik-ozel/547/tr-index.html Q3

3. **Özgür Doğuş Erol**, Şimal Şenocak, Fatima Aerts-Kaya. The Role of Rab GTPases in the development of genetic and malignant diseases and their potential as a treatment target for stem cell gene therapy. *Molecular and Cellular Biochemistry*, 2023. DOI: 10.1007/s11010-023-04727-x. SCIE indexed. Q2

4. **Özgür Doğuş Erol**, Mehmet Emin Şeker, Burcu Pervin, Merve Gizer, Petek Korkusuz, Özgür Özyüncü, Niek P. Van Til, Fatima Aerts-Kaya. Assessment of potential side effects related to RAB27A overexpression in mesenchymal and hematopoietic stem cells. Experimental Cell Research, 2023. Submitted. SCIE indexed. Q3

5. **Özgür Doğuş Erol**, Şimal Şenocak, Burcu Özçimen, Gülen Güney-Esken, Hasan Basri Kiliç, Çetin Kocaefe, Niek P. Van Til, Fatima Aerts-Kaya. Correction of Griscelli Syndrome Type 2 causing mutations in the RAB27A gene with CRISPR/Cas9. Turkish Journal of Biology, 2023. Submitted. SCIE indexed. Q2

Supplement 4. Thesis related abstracts

1. **Özgür Doğuş Erol**, Mehmet Emin Şeker, Burcu Pervin, Niek van Til, Ayşen Günel-Özcan, Çetin Kocaefe, Fatima Aerts-Kaya. Repair of Griscelli Type 2 mesenchymal stem cells with a lentiviral vector carrying RAB27Aco. İstinye University Stem Cell and Tissue Engineering Symposium, 27-28 feb İstanbul 2021. Abstract SB18, p47-48.

2. **Özgür Doğuş Erol**, Mehmet Emin Şeker, Fatima Aerts-Kaya. Correction of RAB27A from Griscelli Syndrome Type II-derived Mesenchymal Stem Cells. ESGCT 2021. Human Gene Therapy 2021:32(19-20)A135. DOI: 10.1089/hum.2021.29180.abstracts. Q1

3. **Özgür Doğuş Erol**, Mehmet Emin Şeker, Burcu Pervin, Burcu Özçimen, Şimal Şenocak, Sema Nur Gür, Hasan Basri Kılıç, Merve Gizer, Petek Korkusuz, Yusuf Çetin Kocaefe, Nico Peter van Til, Fatima Aerts-Kaya. Comparison of different methods for genetic correction of Griscelli Syndrome type II-derived stem cells. European Hematology Association (EHA), 2023. Hemasphere Q1

4. **Özgür Doğuş Erol**, Mehmet Emin Şeker, Burcu Pervin, Burcu Özçimen, Şimal Şenocak, Sema Nur Gür, Hasan Basri Kılıç, Merve Gizer, Petek Korkusuz, Yusuf Çetin Kocaefe, Fatima Aerts-Kaya. RAB27A Gen Tedavisi ve Olası Tümorijenik Etkilerinin İncelenmesi. 18. Tıbbi Biyoloji ve Genetik Kongresi, Ekim 2023.

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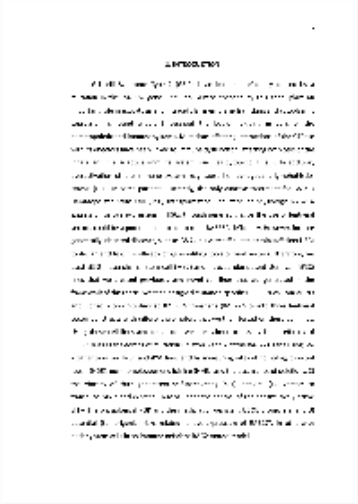
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Supplement 7. Pathogenic RAB27A mutations with different molecular consequences

Name	Consequence	Conditions
NM_183235.3(RAB27A):c.467+3_467+6del		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.240-2A>C		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.147_148del (p.Arg50fs)	R50fs	Griscelli syndrome type 2
NC_000015.10:g.55224013del		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.239+1G>T		Autoinflammatory syndrome|Griscelli syndrome type 2
NC_000015.9:g.55514530_55552423dup		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.467+5G>A		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.400A>G (p.Lys134Glu)	K134E	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.598C>T (p.Arg200Ter)	R200*	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.460A>T (p.Lys154Ter)	K154*	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.335del (p.Asn112fs)	N112fs	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.227C>T (p.Ala76Val)	A76V	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.137T>G (p.Phe46Cys)	F46C	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.439G>T (p.Glu147Ter)	E147*	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.400_401del (p.Lys134fs)	K134fs	Griscelli syndrome type 2|Autoinflammatory syndrome
NC_000015.9:g.(?_55497685)_(55527152_?)del		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.377del (p.Pro126fs)	P126fs	Griscelli syndrome type 2|Inborn genetic diseases
NM_183235.3(RAB27A):c.251dup (p.Leu84fs)	L84fs	Griscelli syndrome type 2
NC_000015.10:g.(?_55234762)_(55234954_?)del		Griscelli syndrome type 2
NC_000015.10:g.(?_55223869)_(55234954_?)del		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.423_424del (p.Arg141fs)	R141fs	Griscelli syndrome type 2

NM_183235.3(RAB27A):c.467+1G>C		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.149del (p.Arg50fs)	R50fs	Griscelli syndrome|Griscelli syndrome type 2
NM_183235.3(RAB27A):c.550C>T (p.Arg184Ter)	R184*	Autoinflammatory syndrome|Griscelli syndrome type 2|not provided
NM_183235.3(RAB27A):c.244C>T (p.Arg82Cys)	R82C	Griscelli syndrome type 2|not provided|Autoinflammatory syndrome
NM_183235.3(RAB27A):c.514_518del (p.Gln172fs)	Q172fs	not provided|Griscelli syndrome type 2
NM_183235.3(RAB27A):c.352C>T (p.Gln118Ter)	Q118*	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.53_54del (p.Ser18fs)	S18fs	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.454G>C (p.Ala152Pro)	A152P	Griscelli syndrome type 2
NM_183235.3(RAB27A):c.389T>C (p.Leu130Pro)	L130P	Griscelli syndrome type 2
NG_009103.1:g.(13486_?)_(?_81610)del		Griscelli syndrome type 2
NM_183235.3(RAB27A):c.217T>G (p.Trp73Gly)	W73G	Griscelli syndrome type 2

Supplement 8. Codon optimization DNA sequence

```
Optimized    22   ATGTCCGACGGCGATTACGACTATCTGATCAAGTTCCTGGCCCTGGGCGATAGCGGAGTG
Original     22   ATGTCTGATGGAGATTATGATTACCTCATCAAGTTTTTAGCTTTGGGAGACTCTGGTGTA

Optimized    82   GGCAAGACCTCCGTGCTGTACCAGTATACAGACGGCAAGTTCAACAGCAAGTTTATCACC
Original     82   GGGAAGACCAGTGTACTTTACCAATATACAGATGGTAAATTTAACTCCAAATTTATCACA

Optimized   142   ACAGTGGGCATCGACTTCCGGGAGAAGCGCGTGGTGTACCGGGCCTCCGGACCAGACGGA
Original    142   ACAGTGGGCATTGATTTCAGGGAAAAAAGAGTGGTGTACAGAGCCAGTGGGCCGGATGGA

Optimized   202   GCAACCGGCAGGGGACAGAGAATCCACCTGCAGCTGTGGGATACAGCCGGCCAGGAGCGG
Original    202   GCCACTGGCAGAGGCCAGAGAATCCACCTGCAGTTATGGGACACAGCAGGGCAGGAGAGG

Optimized   262   TTCAGATCTCTGACCACAGCCTTCTTTCGGGATGCCATGGGCTTCCTGCTGCTGTTTGAC
Original    262   TTTCGTAGCTTAACGACAGCGTTCTTCAGAGATGCTATGGGTTTTCTTCTACTTTTTGAT

Optimized   322   CTGACCAATGAGCAGTCCTTTCTGAACGTGAGAAATTGGATCTCTCAGCTGCAGATGCAC
Original    322   CTGACAAATGAGCAAAGTTTCCTCAATGTCAGAAACTGGATAAGCCAGCTACAGATGCAT

Optimized   382   GCCTATTGCGAGAACCCCGACATCGTGCTGTGCGGCAATAAGAGCGATCTGGAGGACCAG
Original    382   GCATATTGTGAAAACCCAGATATAGTGCTGTGTGGAAACAAGAGTGATCTGGAGGACCAG

Optimized   442   AGGGTGGTGAAGGAGGAGGAGGCCATCGCCCTGGCCGAGAAGTACGGCATCCCTTATTTC
Original    442   AGAGTAGTGAAAGAGGAGGAAGCCATAGCACTCGCAGAGAAATATGGAATCCCCTACTTT

Optimized   502   GAGACAAGCGCCGCCAACGGCACAAATATCAGCCAGGCCATCGAGATGCTGCTGGATCTG
Original    502   GAAACTAGTGCTGCCAATGGGACAAACATAAGCCAAGCAATTGAGATGCTTCTGGACCTG

Optimized   562   ATCATGAAGAGGATGGAGCGGTGCGTGGACAAGTCTTGGATTCCCGAGGGAGTGGTGCGC
Original    562   ATAATGAAGCGAATGGAACGGTGTGTGGACAAGTCCTGGATTCCTGAAGGAGTGGTGCGA

Optimized   622   AGCAACGGACACGCCTCCACAGATCAGCTGTCTGAGGAGAAGGAGAAGGGCGCCTGCGGC
Original    622   TCAAATGGTCATGCCTCTACGGATCAGTTAAGTGAAGAAAAGGAGAAAGGGGCATGTGGC

Optimized   682   TGT
Original    682   TGT
```

Supplement 9. Thesis related paper 1

WJSC World Journal of Stem Cells

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World J Stem Cells 2021 September 26; 13(9): 1197-1214

DOI: 10.4252/wjsc.v13.i9.1197

ISSN 1948-0210 (online)

REVIEW

Effects of storage media, supplements and cryopreservation methods on quality of stem cells

Ozgur Dogus Erol, Burcu Pervin, Mehmet Emin Seker, Fatima Aerts-Kaya

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Author contributions: Erol OD, Pervin B and Seker ME drafted the first version of the article and contributed equally to this work; Aerts-Kaya F designed the manuscript, reviewed the contents and wrote the final version of the article to be published; All authors have read and approved the final manuscript.

Supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK), No. 1188738 and No. 2198675.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Abstract

Despite a vast amount of different methods, protocols and cryoprotective agents (CPA), stem cells are often frozen using standard protocols that have been optimized for use with cell lines, rather than with stem cells. Relatively few comparative studies have been performed to assess the effects of cryopreservation methods on these stem cells. Dimethyl sulfoxide (DMSO) has been a key agent for the development of cryobiology and has been used universally for cryopreservation. However, the use of DMSO has been associated with *in vitro* and *in vivo* toxicity and has been shown to affect many cellular processes due to changes in DNA methylation and dysregulation of gene expression. Despite studies showing that DMSO may affect cell characteristics, DMSO remains the CPA of choice, both in a research setting and in the clinics. However, numerous alternatives to DMSO have been shown to hold promise for use as a CPA and include albumin, trehalose, sucrose, ethylene glycol, polyethylene glycol and many more. Here, we will discuss the use, advantages and disadvantages of these CPAs for cryopreservation of different types of stem cells, including hematopoietic stem cells, mesenchymal stromal/stem cells and induced pluripotent stem cells.

Key Words: Cryoprotective agents; Dimethyl sulfoxide; Hematopoietic stem cells; Mesenchymal stromal/stem cells; Induced pluripotent stem cells

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Core Tip: The manuscript is an overview of current cryopreservation protocols used for

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Manuscript source: Invited manuscript

Specialty type: Cell biology

Country/Territory of origin: Turkey

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 27, 2021
Peer-review started: February 27, 2021
First decision: April 20, 2021
Revised: April 21, 2021
Accepted: August 24, 2021
Article in press: August 24, 2021
Published online: September 26, 2021

P-Reviewer: Liu L, Schenke M
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Wang LYT

cold storage of hematopoietic stem cells, mesenchymal stem cells and induced pluripotent stem cells. Although dimethyl sulfoxide (DMSO) is commonly used in cryopreservation of cell lines, primary cells and stem cells, the use of DMSO has been associated with certain toxicity, both directly on the cells, as well as upon infusion with the stem cell product. As a result of this many groups have undertaken efforts to find suitable replacements for DMSO that are equally potent but less toxic. In this review, we summarize the current *status quo* of stem cell freezing protocols and we describe the most commonly used cryoprotective agents and their effects on stem cells and stem cell function.

Citation: Erol OD, Pervin B, Seker ME, Aerts-Kaya F. Effects of storage media, supplements and cryopreservation methods on quality of stem cells. *World J Stem Cells* 2021; 13(9): 1197-1214
URL: https://www.wjgnet.com/1948-0210/full/v13/i9/1197.htm
DOI: https://dx.doi.org/10.4252/wjsc.v13.i9.1197

INTRODUCTION

Although optimization of stem cell culture, expansion and differentiation methods has been the main focus of stem cell research, an equally important and largely ignored topic in stem cell research is long term storage and cryopreservation. No matter the quality of the stem cell cultures, without optimization and careful control of cryopreservation, reproducibility and clinical (side) effects may be difficult to interpret. Furthermore, effects may be unexpected and suboptimal if cells are not stored, frozen and thawed under the most favorable conditions. Cryopreservation of cells, tissues and embryos has been common practice since the 1950s and took flight with the development of *in vitro* fertilization practices and hematopoietic stem cell (HSC) transplantation.

Storage under low temperature conditions reduces the rates of intracellular enzymatic and chemical reactions that may be harmful and allows the cells to be stored long-term without damage. The basic principle underlying successful cell cryopreservation is prevention of the formation of intra- and extracellular ice crystals during freezing, since this is the primary cause of cell damage[1]. Cryopreservation methods can be classified into slow freezing and fast freezing (vitrification) procedures. Both methods are based on the freezing or solidification of the cells or tissues and may cause cell injury in the process. However, the mechanisms that cause cell damage are quite distinct. Whereas rapid cooling results in the formation of intracellular ice crystals causing physical stress to the cells and mechanical breakdown, slow cooling causes osmotic changes in the cells and mechanical stress due to the formation of extracellular ice[2]. During vitrification a liquid is transformed into a glass-like non-crystalline solid state due to overcooling without freezing. Its most important feature is the prevention of ice formation[3,4]. During vitrification, cells kept in cryoprotectant solutions are briefly exposed to nitrogen vapor and subsequently immersed in liquid nitrogen[5] and usually a permeable cryoprotectant [dimethyl sulfoxide (DMSO) or glycerol] and an impermeable cryoprotectant [hydroxyethyl starch (HES), polyvinyl alcohol, trehalose] are used together[6,7]. During slow freezing, extracellular ice crystals may cause an increase in cellular osmolality and dehydration, and therefore the cooling rate during freezing should be sufficiently slow to allow a suitable amount of water to leave the cell[8,9]. The optimal cooling rate depends on cell size, sample size, water permeability and the presence of nucleating agents, which initiate and catalyze the freezing process. In addition, the cryoprotectant used, the temperature and surface/volume ratio should also be taken into consideration to determine the optimal cooling rate[10]. A cooling rate of 1-3 °C/min during the initial freezing phase (+4 °C to -40 °C) is optimal for most mammalian cells when frozen in the presence of cryoprotective agents, such as glycerol or DMSO[11]. Automated freezing devices, such as KRYO 10 series III (Planer Products, Sunbury-on-Thames, United Kingdom)[12], CryoMed 1010 (Forma Scientific, Marjetta, OH, United States)[13] and Cryomed (New Baltimore, MD, United States)[14] provide a temperature decrease at a controlled rate. Differences between vitrification and cryopreservation are depicted schematically in Figure 1.

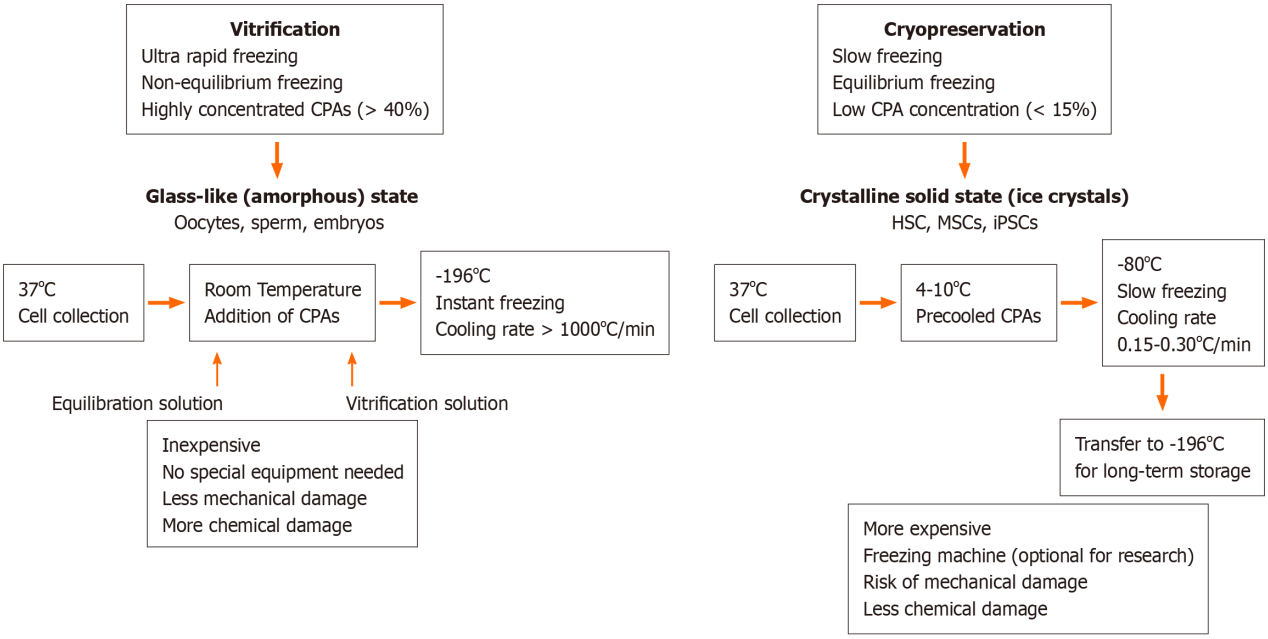


Figure 1 Comparison of vitrification and cryopreservation procedures. CPAs: Cryoprotective agents; HSCs: Hematopoietic stem cells; MSCs: Mesenchymal stem cells; iPSCs: İnduced pluripotent stem cells.

Despite a vast amount of different methods, protocols and cryoprotectants, stem cells are often frozen using protocols optimized for cell lines and relatively few comparative studies have been performed to assess the effects of cryopreservation methods and supplements on stem cell quality and viability. A list of commercially available cryopreservation media is provided as Supplement 1. Here, we summarize the use, advantages and disadvantages of cryopreservation methods used for different types of stem cells, including HSCs, mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC).

CRYOPROTECTIVE AGENTS, ADDITIVES AND SOLUTIONS

In order to serve as an effective cellular cryoprotective agent (CPA), the compound should have certain properties, including (1) High water solubility, even at low temperatures; (2) Free penetration of cell membranes; and (3) Low toxicity. Although many compounds may have these properties, including the most commonly used agents DMSO and glycerol, the choice of the compound may differ depending on the type of cell. CPAs are often used in combination with a carrier solution, which may provide different concentrations of (nutritional) salts, a variety of buffers, osmogens and/or apoptosis inhibitors. The contents of this carrier solution further help the cells maintain an isotonic concentration (300 milliosmoles) to prevent swelling or shrinking during the freezing process[15].

DMSO [Me_2SO, $(CH3)_2SO$]

DMSO has been a key agent for the development of cryobiology. For cryopreservation of HSCs, use of DMSO, in combination with a temperature-controlled freezing technique followed by a rapid thawing procedure of 1-2 °C/min, is considered the clinical standard[16]. The use of DMSO as a CPA to prevent freezing-related cell damage was first proposed by Lovelock and Bishop[17], who used it during slow cooling of bull sperm. Due to its low hydrophilicity and molecular weight, DMSO freely penetrates cell membranes. It can disrupt ice crystal nucleation by forming hydrogen bonds with intracellular water molecules and prevents dehydration by reducing the amount of water absorbed into ice crystals[18]. However, prolonged exposure to DMSO negatively affects cellular function and growth by interfering with metabolism, enzymatic activity, cell cycle and apoptosis[19]. DMSO is also thought to modulate intracellular calcium concentrations[19,20] and may induce or inhibit cell apoptosis and differentiation, depending on the cell type, the stage of cell growth and differentiation, the concentration of DMSO (typically 5%-10%), duration of exposure

and temperature[21,22]. Whereas high concentrations of DMSO may cause instant hemolysis, white cell stacking and fibrinogen precipitation, intravenous administration of DMSO has been associated with local irritation and necrosis[23]. Infusion of cell products that contain DMSO is associated with a wide range of gastrointestinal side effects (nausea, vomiting, abdominal pain, diarrhea)[24-26]; cardiovascular effects (hypertension, bradycardia, tachycardia)[25-27]; respiratory (dyspnea) and dermatological effects (urticaria, itching, and redness)[28,29]. Furthermore, even very low concentrations of DMSO can affect cellular processes by causing differential expression of thousands of genes, changing DNA methylation profiles and tissue-specific deregulation of miRNAs[30,31], and may affect stem cell fate by inducing unwanted differentiation[32].

Glycerol ($C_3H_8O_3$)

Glycerol is a simple polyol compound. Its cryoprotective effects have been known since the early 1950s, when glycerol was first tested on fowl spermatozoa, rabbit red blood cells and water amoeba[33,34]. Glycerol is a colligative CPA that prevents dehydration damage by increasing the total solute concentration, including sodium ions, thus preventing ice formation and reducing the amount of water absorbed by ice crystals[7,35]. Although glycerol at low concentrations (< 20%) is not sufficient to prevent crystallization completely, it does protect different cells from cell death. High concentrations (70%) of glycerol were used without significant toxicity and were shown to provide substantial protection[36].

Hydroxyethyl starch

Hydroxyethyl starch was synthesized by Ziese W in 1934. The hydroxyethyl starch molecule is a high molecular weight synthetic polymer and can be purified from corn or potatoes[37]. Since high molecular weight CPAs are generally unable to enter cells, HES accumulates in the extracellular space. Here, it regulates water flow during cooling and heating and provides cryoprotection by absorbing the water molecules and keeping them thermally inert. Although HES remains extracellulary, it can minimize intracellular ice crystal formation and provides membrane stabilization[38]. By increasing the extracellular viscosity it further prevents osmotic stress and damage, reducing the rate at which water is withdrawn from the cells during cooling[39,40].

Trehalose

Trehalose is a non-toxic disaccharide and helps maintaining the structural integrity of cells during freezing and thawing[41,42]. Trehalose has high water retaining properties and is found in a large number of organisms, such as nematodes and yeasts that can survive freezing and drying[43] and can be isolated from yeasts, plants and fungi[42,44]. However, trehalose does not display any significant cryoprotective potential by itself and should therefore be used in combination with other CPAs[45].

Albumin

The albumin protein consists of three homologous domains, each with specific structural and functional properties[46]. Human serum albumin (HSA) is present in serum at high quantities and serves as a buffer or depot for hormones, growth factors, fatty acids and metals. Due to its stabilizing function, albumin is an important component of common preservation and cell culture media. During freezing, albumin is used for its ability to coat surfaces, buffer function and binding capacity[47], but, similar to trehalose, albumin is only used as a supplementary cryoprotective agent during freezing of cells and tissues[48].

Dextran

Dextran is a branched polysaccharide with α-1.6 glycosidic links between glucose molecules[49]. Dextran can interact with lipoproteins, enzymes and cells, and has the ability to stabilize proteins[50]. Dextran is non-toxic, only weakly antigenic and usually used at a concentration of 10%[51,52]. Dextran has been used as a cryoprotect during freezing of HSCs and sperm[53,54]. Similar to albumin and trehalose, dextran is only used in combination with other CPAs, such as DMSO or glycerol.

CRYOPRESERVATION OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cell transplantation (HSCT) is used for the treatment of various

malignant and non-malignant diseases affecting the hematopoietic and immune system as well as for the treatment of a variety of inborn errors of metabolism[55]. HSC products derived from bone marrow (BM), peripheral blood or umbilical cord blood (UCB) are usually stored for a brief period that may range from a few days to months but may increase up to several years, depending on the disease state of the patient and treatment schedule[56]. Banking of HSC transplants is becoming increasingly important because of the possibility to use previously stored material even years after collection. In addition, storage of UCB for personal (private banking) or transplantation purposes (biobanks) is becoming increasingly popular and may require banking for up to several decades. For this reason, it is critically important that HSCs retain their potential during the freezing, banking and thawing[57]. HSCs can be stored unprocessed at +4 °C or room temperature for approximately 72 h after collection without massive apoptosis, cell death or loss of stem cell function. Within this time period, they can be transported and engrafted without any problems, but additional protocols may be required for longer storage[22,58,59]. Freezing the cells extends their shelf life greatly and increases the safety of HSC therapy by providing time to perform quality controls (microbiologically) and product testing (HSC content, colony assay, CD34+ enumeration). Despite these benefits, cryopreservation of HSCs poses several challenges, most notably a decrease in cell viability after thawing and side effects in patients due to the CPAs used[60]. An overview of current protocols used for cryopreservation of HSCs has been provided in Table 1.

Throughout the years, DMSO has been the CPA of choice in most studies. It has been tested at different concentrations, ranging from 2.5% to 10% with variable results. Since DMSO is highly hyperosmotic, rapid infusion of the cryopreserved cells into the isosmotic blood system may cause osmotic damage, excessive cell expansion and decreased cell viability. This in turn may cause immediate side effects but can also affect engraftment in the long term[14,22]. Generally, lower doses of DMSO provided less toxicity, but in some cases, this was accompanied by a decrease in cell viability. Nevertheless, observed effects and side-effects of DMSO may differ widely between the protocols used due to the addition of other supplements (HES, HSA, Trehalose), cell dose (ranging from 15 x 10^6 cells/mL-4000 x 10^6 cells/mL), cell source (peripheral blood/BM/UCB), use of controlled rate or uncontrolled rate freezing, duration of storage (< 1 wk to > 1 decade) and the temperature used for long-term storage (-80 °C to -196 °C). To reduce the toxic effects of DMSO-cryopreserved HSCs during transplantation, it has been opted to divide the infusions into multiple portions, given at intervals of several hours or days, or alternatively to concentrate further HSC grafts to reduce cryopreservation volume and DMSO content[61]. In addition, alternatives such as different CPAs to reduce or replace DMSO for cryopreservation[14,62] or complete removal of DMSO prior to infusion[63,64] are being investigated. Even though a concentration of 10% DMSO in HSC cryopreservation is widely accepted as the cryopreservation medium of choice[65,66], similar or even more successful results have been obtained using percentages of DMSO as low as 2.5%-5%, with or without the addition of HES. Using these protocols similar engraftment was observed but with less toxicity[14,67,68]. Use of trehalose in combination with DMSO in UCB-derived HSC freezing has been shown to increase survival and cell differentiation capacity of HSCs in comparison to HSCs frozen without trehalose[53]. Direct comparison of trehalose and DMSO for cryopreservation of BM-HSCs showed no differences on viability between both groups[45]. Similarly, in NOD-SCID mice, the use of low amounts of DMSO (5%) and trehalose (5%) to reduce the toxic effects of DMSO showed a positive effect on HSC survival and engraftment after transplantation[69]. When BM-derived HSCs were frozen using a combination of 7.5% DMSO and 4% HSA, cells displayed high viability and sustained engraftment[70]. Studies using combinations of DMSO with dextran-40 showed increased HSC viability and functionality in comparison to the DMSO only group[71]. In conclusion, a lower concentration of DMSO and addition of a non-toxic second CPA or supplement, such as HSA and trehalose, decreases toxicity related to DMSO, while maintaining high HSC viability and sustaining engraftment.

CRYOPRESERVATION OF MESENCHYMAL STEM/STROMAL CELLS

Multipotent mesenchymal stem/stromal cells (MSCs) can be isolated from many tissues, including the bone marrow (BM-MSC), adipose tissue (adipose tissue derived stem cell), umbilical cord Wharton Jelly (Wharton Jelly-MSC), placenta (placenta-MSC), tooth germ (tooth germ MSC) or dental pulp (dental pulp stem cell) and many

Table 1 Comparison of different protocols used during cryopreservation of hematopoietic stem cells

HSC source	Storage period and temperature	Cryopreservation	Viability post freezing	Engraftment in days	Results	Ref.
< 600 x 10^6 cells/mL autologous PBSC	5-15 yr, -150 °C	10% DMSO and 23.3% Plasma Lyte A	66.4%	12	Viable CD34+ cells or CFU-GM is a reliable predictor of rapid engraftment	[13]
< 300 x 10^6 cells/mL autologous PBSC	< 6 mo, -80 °C	3.5% DMSO, 1% HSA and 2.5% HES	72%	14	Low DMSO conc allows successful engraftment and reduces toxicity (8%); Similar engraftment after combination of DMSO with or without HES and HSA	[115]
< 100 x 10^6 cells/ mL autologous PBSC	< 6 mo, -80 °C	5% or 10% DMSO, autologous plasma, 5% ACD	85%	14	19.1% infusion-related toxicity in the 10% DMSO group *vs* 6.8% in the 5% DMSO group, lowering DMSO results in reduction in infusion toxicity and lower costs with a similar hematopoietic reconstitution	[116]
Autologous PBSC	< 11 yr, -80 °C	3.5% DMSO + 1% HSA and 2.5% HES *vs* 6% DMSO + 6% HES	no significant change	11-12	Uncontrolled-rate freezing and cryopreservation with 5% DMSO/HES at −80 °C supports hematopoietic reconstitution comparable to that of controlled-rate freezing and liquid nitrogen storage	[117]
< 4000 x 10^6 cells/mL autologous PBSC	1-98 wk, -80 °C	3.5% DMSO, 2.5% HES and 1% HSA	60.8%	11-20	Reduction in DMSO concentration decreases transfusion-related adverse events. PBPCs cryopreserved in low DMSO/HES/HSA at -80°C allow successful engraftment	[24]
50 x 10^6 cells/mL autologous PBSC and BM	PB: 35 mo (26-78); BM 16 mo (27-71), -90 C	5% DMSO, 6% HES and 4% HSA in RPMI1640	93%		DMSO-associated toxicity during infusion, storage of HSCs at -90°C in DMSO/HES/HSA did not cause loss of cell numbers, viability, and clonogenic activity	[118]
Autologous PBSC	Controlled rate freezing at -186 °C	5% or 10% DMSO and 6% HES		10-20	Two patients who received components cryopreserved with DMSO alone experienced serious neurological toxicity, none of the recipients who received components frozen in DMSO/HES experienced serious infusion-related toxicity, better hematopoietic recovery in presence of HES independent of DMSO concentration	[14]
100 x 10^6 cells/mL – 200 x 10^6 cells/mL autologous PBSC	5-6 yr, controlled rate freezing at -160 °C	2%-10% DMSO, 10% ACD	73% with 5% DMSO	10-14	Cryopreservation using 5% instead of 10% DMSO improves CD34 + cell and leukocyte viability, but has only minor effects on supernatant levels of leukocyte- and platelet-derived soluble mediators	[61]
75 x 10^6 cells/mL - 250 x 10^6 cells/mLautologous PBSC	32-180 d, controlled rate freezing, -196 °C	5% or 10% DMSO	84%-95%	10-14	The use of 5% instead of 10% DMSO was associated with a decrease in side effects, cryopreservation with 5% DMSO followed by storage in nitrogen is a simple, highly standardized, and safe procedure for cryopreservation of autologous stem cell graft	[119]
UCB	1-2 mo, uncontrolled *vs* controlled rate freezing at -90 °C	5% or 10% DMSO	Uncontrolled 84.2%; controlled 92.5%		Best recovery of UCB cells when controlled-rate freezing and 5% DMSO were combined	[120]
15 x 10^6 cells/mL UCB	> 2 wk, controlled rate freezing at -170 C	5%, 10% or 20% DMSO and 2% HSA or autologous plasma	89%		Optimal conditions for cryopreservation were 10% DMSO and 2% HSA with fast addition and removal of DMSO	[121]
800 x 10^6 cells/mL UCB	10 yr, controlled rate freezing at -196 °C	10% DMSO and 5% Dextran	83.7%		Long term storage of UCB units does not affect the quality of the HSCs	[122]
Autologous BM	4 mo, -80 °C	5% DMSO and 6% HES	82.2%	21	BM cells can be rapidly and inexpensively cryopreserved in DMSO/ HES, without need for rate-controlled freezing or storage	[123]

				in liquid nitrogen	
20 x 10^6 cells/mL BM or 17 x 10^6 cells/mL PBSC	Controlled rate freezing at -196 °C	10% DMSO or 0.25-1 mol/L TH with or without 0.25 IU/mL insulin (I)	DMSO: 33%TH: 32%; TH/I: 30%	DMSO-cryopreserved cells exhibited the best median viability-rate after thawing. Comparable results could be achieved with trehalose 0.5 mol/L with/without insulin	[45]
200 x 10^6 cells/mL autologous BM or PBSC	BM: 11.8 yr *vs* PB: 33 d controlled rate freezing at -196 °C	10% Medium 199 , 80% autologous plasma and 10% DMSO	BM: 81.5%; PBSC: 68.0%	BM can be cryopreserved for more than a decade without apparent loss of progenitor activity in comparison to short-term cryopreserved PBSC	[124]

HSC: Hematopoietic stem cell; DMSO: Dimethyl sulfoxide; CFU-GM: Colony Forming Unit-Granulocyte/Macrophage; ACD: Acid citrate dextrose; RPMI: Roswell Park Memorial Institute Medium; HES: Hydroxyethyl starch; HSA: Human serum albumin; BM: Bone marrow; UCB: Umbilical cord blood; TH: Trehalose.

other connective tissues[72,73]. MSCs can differentiate into cells from several mesenchymal lineages, including but not limited to osteoblasts, adipocytes and chondrocytes[74,75]. MSCs are highly positive for cell surface molecules like CD29, CD44, CD73, CD90 and CD105[76]. They hold great potential for clinical application due to their capacity for regeneration of damaged or injured tissues, migration to sites of injury and regulation (usually suppression) of local and generalized immune responses. In order to obtain a sufficient amount of MSCs for clinical application, cells are often profoundly expanded in culture. Since MSCs themselves do not express HLA-DR, the cells are considered immunologically inert and expanded MSCs from unrelated, third-party donors can be used for treatment of a variety of diseases, ranging from graft *vs* host disease to severe acute respiratory distress syndromes[77,78]. These characteristics make MSCs ideal for ready, off-the-shelf treatments but require significant expansion and long-term cryopreservation[79-81]. Similar to the protocols developed for freezing of HSCs, a variety of freezing solutions and protocols has been tested for cryopreservation of MSCs (Table 2). Similar to freezing protocols used for HSCs, MSC freezing media generally consists of a basic medium [alpha-modified minimal essential medium, Dulbecco's Modified Eagle's Medium (DMEM) or advanced DMEM], supplemented with 3%-10% DMSO. In most studies expression of MSC surface markers (CD29, CD44, CD73, CD90, CD105 and/or CD166) was assessed before and after cryopreservation, and in almost all cases, MSC phenotype was not affected by cryopreservation, with overall expression levels > 90%. Cell viability ranged from 60% to 95% when fetal bovine serum (FBS) was used in addition to DMSO. In the presence of 10% DMSO, viability was typically very high (80% to 100%) after thawing, regardless of the duration of the freezing period[81-84].

While there was no significant difference between 2% and 10% DMSO in terms of viability after a 1 mo freezing period, a significant portion of the cells frozen in presence of 2% DMSO died after long-term cryopreservation[81]. Therefore, in order to reduce the toxicity related to DMSO, either the percentage of DMSO was reduced or secondary CPAs (trehalose, sucrose, boron) were added to the freezing media[83-85]. Alternatively, high molecular weight macromolecules, such as FBS, polyethylene glycol (PEG) or polyvinylpyrrolidone were added as secondary CPAs to the freezing media[83,84,86]. However, since FBS contains animal components, cell products may contain remnants of FBS despite post-thaw washing that may trigger adverse (immune) reactions when used in a clinical setting[87]. Therefore, animal component free media, such as Cryostor, have been developed as an alternative to standard freezing medium formulations[81]. Studies using adipose tissue-derived MSCs frozen with 10% DMSO, 0.9% NaCl and human serum, HSA or knockout serum replacement (KSR)[88] revealed that all FBS replacements supported a similar multilineage differentiation potential, expression of cell surface markers and gene expression of stem cell markers, indicating that these may be good alternatives for clinical use. Carnevale *et al* [89] used 5% DMSO and human serum instead of FBS for cryopreservation of BM-MSCs and found no differences in terms of differentiation or phenotype. Cryopreservation of BM-MSCs using 7.5% DMSO, supplemented with 2.5% PEG and 2% BSA or even 5% DMSO, supplemented with 5% PEG and 2% BSA were shown to be almost as good as 10% DMSO in terms of viability and similar in terms of differentiation[84]. Comparison of mixed osmolyte solutions, consisting of sucrose/glycerol/creatine and sucrose/glycerol/isoleucine with standard DMSO containing freezing media further showed the potential of these type of cryopreservation solutions by improving post-thawing function of MSCs[31].

Table 2 Comparison of different protocols used during cryopreservation of mesenchymal stem/stromal cells

MSC source, passage	Culture medium	Storage period and temperature	Cryopreservation	Viability	Phenotype	Results	Ref.
BM-MSC/P3	MEM, 15% FBS, 1% P/S, 1% L-glutamin	7 wk at -196 °C	90% FBS and 10% DMSO		Osteogenic and adipogenic differentiation, high expression of CD44, CD73, CD90 and CD105	No effects of freezing on function, differentiation and phenotype of the cells	[125]
1 x 10^6BM-MSC/P3, P4, P8, P13, P18	MEM, 10% FBS, 1% P/S, 1% L-glutamin	12 mo, controlled rate freezing at -80 °C	30% FBS, 60% MEM and 10% DMSO	85%-100%	Chondrogenic, adipogenic, neurogenic differentiation, no difference in expression of cell surface markers between passages	No differences in phenotype or differentiation between different cryopreserved MSCs from different passages	[82]
0.5 x 10^6 /mL; BM-MSC	MSC growth medium, 10% FBS	1-5 mo, controlled rate freezing at -196 °C *vs* 4 d at 4 °C	Freezing medium (FM): 10% DMSO, 10% FBS, MSC growth medium, 30% BSA *vs* CryoStor (CS) animal component free freezing medium with 2%, 5% or 10% DMSO *vs* storage in HypoThermosol-FRS medium (HTS-FRS) at 4°C	FM 10% DMSO: 102.8%; CS 2% DMSO: 91.7%; CS 5% DMSO: 95.6%; CS 10% DMSO: 95.4%; HTS-FRS: 85.0% (rapid loss of viability after > 6 d)	Osteogenic differentiation, high expression of CD44, CD90, CD105, CD166, loss of expression of CD9 after hypothermic storage	No difference in differentiation or phenotype before and after freezing; HTS-FRS preserved MSC marker expression, proliferation and osteogenic differentiation after storage for at least 4 d	[81]
1 x 10^6 /mL; BM-MSC	MEM, 10% FBS, 1% P/S	7 wk at -196 °C	10% DMSO ± 10% or 90% FBS, 7.5% DMSO, 2.5% PEG ± 2% BSA, 5% DMSO, 5% PEG, 5% DMSO, 2% PEG, 3% Trehalose ± 2% BSA, 2.5% DMSO, 7.5% PEG ± 2% BSA, 10% Propanediol, 2%BSA, 7.5% Propanediol 2%BSA, 2.5% PEG	Highest viability with 7.5% DMSO, 2.5% PEG and 2% BSA: 82.9% ± 4.3% *vs* 10% DMSO: 82.7% ± 3.7%	Adipogenic, osteogenic and chondrogenic differentiation	In comparison to 10% DMSO, best results with 7.5% DMSO, 2.5% PEG and 2% BSA. In presence of and 2% BSA also good results with 5% DMSO, 5% PEG or 7.5% propanediol with 2.5% PEG	[84]
BM-MSC/P1-6	MEM, 10% Human Serum, 1% L-glutamine, 1% P/S	1 yr at -196 °C	MEM, 40% Human Serum, 5% DMSO		Osteogenic, adipogenic and myogenic differentiation, before and after thawing high expression of CD73, CD90 and CD105, no expression of CD16, CD34, CD45 and HLA-DR	Cryopreserved MSCs show slightly lower proliferation rate, no differences in differentiation, senescence markers, CFU-F or karyotype between frozen and fresh cells	[89]
5 x 10^5 /mL; BM-MSC/P1	MEM, 15% FBS, 1% P/S	< 6 mo *vs* 33-37 mo	CELLBANKER cryopreservation medium (contains serum and DMSO)	90%	Osteogenic differentiation, both fresh and cryopreserved MSCs were negative for CD14, CD34, CD45 and HLA-DR and positive for CD29 and CD105	No difference in osteogenic potential between fresh and cryopreserved cells. Long-term cryopreserved MSCs retained high osteogenic potential, no difference in phenotype	[86]
1 x 10^6 /mL; WJ-MSC	ADMEM, 10% FBS, 1% P/S, 1% L-glutamine	3 mo, controlled rate freezing at -196 °C	A: ADMEM, 10% PVP ± 10% FBS, B: ADMEM, 10% FBS, 0.05 mol/L glucose, 0.05 mol/L sucrose, 1.5 mol/L ethylene glycol ± 10% FBS, C: ADMEM, 10% DMSO ± 10% FBS	A: 62.9% ± 0.4%; A without FBS: 6.8% ± 0.2%; B: 72.2% ± 0.23%; C: 81.2% ± 0.6%	Adipogenic and osteogenic differentiation, both fresh and cryopreserved MSCs were negative for CD34 and CD45 and positive for CD73, CD90 and CD105	Complete elimination of FBS in cryoprotectants resulted in drastic reduction in cell viability. Cryopreservation did not alter basic stem cell characteristics, plasticity and multipotency, except for proliferation rate	[83]
1 x 10^6 /mL; tgMSC	DMEM, 10% FBS, 1% P/S/A	1 d or 6 mo, freezing at -196 °C	20 μg/mL NaB, 20% FBS, 1% P/S/A , 10%, 7%, 5%, 3% or 0% DMSO	First cycle: > 90%; Second cycle: > 70%; Third cycle: > 80%; Fourth cycle: > 80%	Osteogenic, chondrogenic, and adipogenic differentiation, high expression of CD29 and CD73, medium expression of CD90, CD105 and CD166, no	< 5% DMSO in freezing medium resulted in increased cell death, NaB improved cellular viability after freeze-thaw cycles, addition of NaB to the freezing medium did not affect	[85]

					expression of CD14, CD45, CD34	differentiation capacity of MSCs	
5 x 10^5/mL ADSC/P2	DMEM-LG, 10% FBS	2 wk, freezing at -196 °C	0.9% NaCl containing 10% DMSO HSA, HS, KSR or 90% FBS	DMSO + 9%; HSA: 78.0%; DMSO + 90%; HS: 72.4%; DMSO + 90%; KSR: 77.0%; DMSO + 90%; FBS: 78.5%; DMSO alone: 19.6%	No differences in adipogenic, osteogenic, and chondrogenic differentiation, gene expression of *CD73, CD90, CD105, CD106, CD166, SCF, REX1* and *NANOG*. All ADSCs were positive for surface expression of CD44, CD73, CD90, CD105, CD166 and HLA-ABC and negative for CD31, CD34 and HLA-DR	ADSCs frozen with HSA, HS, or KSR showed similar growth kinetics as cells frozen with FBS. Multilineage differentiation of ADSCs did not differ between groups	[88]
1 x 10^6/mL DPSC/P5-7	MEM, 15% FBS, 1% P/S/A, 100 uM L-ascorbic acid 2-phosphate	1 wk, freezing with Mr. Frosty (NMF) *vs* magnetic freezing (MF)	Serum-free cryopreservation medium (SFM) containing 3% DMSO, SFM + 10% DMSO, FBS + 3% DMSO, FBS + 10% DMSO	SFM + 3%; DMSO: 75%; SFM + 10%; DMSO: 78%; FBS + 3%; DMSO: 70%; FBS + 10%; DMSO: 73%	CD29, CD44 and STRO-1 expression did not differ between the NMF and the MF groups, whereas levels of CD73, CD90, CD146 and CD166 in the MF group increased compared to the NMF group.	DPSC viability using MF was significantly superior to that of the NMF using 2%–10% DMSO; Post-thaw MF-DPSCs expressed MSC markers and showed osteogenic and adipogenic differentiation similar to fresh DPSCs	[90]
ESC-derived MSC	MEM, 10% FBS, 1% NEAA	Controlled rate freezing at 196 C	Sucrose, glycerol, creatine (SGC) and sucrose/glycerol/isoleucine (SGI) solutions were incubated for 1h before freezing, Sucrose, mannitol, creatine (SMC) solutions were incubated for 2 h before freezing	SGI>SGC>SMC	Osteogenic and chondrogenic differentiation, all groups were positive for CD73, CD90 and CD105, and negative for CD45	Osmolyte-based cryopreservation formulations retain MSC post-thaw viability, cell surface markers expression, proliferation, and osteochondral differentiation potential	[31]

MSC: Mesenchymal stem/stromal cell; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; ESC: Embryonic stem cell; NEAA: Non essential aminoacids; MEM: minimal essential medium; KSR: Knockout serum replacement; BSA: Bovine serum albumin; P/S: Penicillin/Streptomycin; DPSC: Dental pulpa stem cells; ADSCs: Adipose derived stem cells.

For research purposes often non-controlled, simple isopropanol-jacketed freezing containers (such as the Mr. Frosty from NALGENE) are used. Using this system, temperature in cryovials decreases approximately 1 C/min[89,90]. In contrast, for clinical use, temperature controlled freezing devices are often preferred. Lee *et al*[90] used a programmed freezer with a magnetic field to freeze human dental pulp MSCs. Using the magnetic freezing procedure, the researchers were able to decrease the level of DMSO to 3% without a significant difference in cell viability. Using the magnetic field freezer "Cells Alive System" (CAS) rat BM-MSCs were frozen in serum-free freezing medium (10% DMSO, 5% Albumin, 0.2% D-Glucose, 0.6% NaCl, 0.03% glutamine, 0.2%$NaHCO_3$)[91]. After 3 years, viability and *in vivo* bone formation in the CAS group was significantly higher than that in cells stored in a non-programmed or non-magnetic freezer (87.7% and 48.5%, respectively). These data show the potential for use of alternative freezing systems for cryopreservation of MSCs as well as the use of secondary CPAs that decrease the need for DMSO. Most clinical trials use MSCs from related donors rather than off-the-shelf products. These MSCs are often directly after expansion infused into the patients. However, considering the increasing requirement for readily available MSC products, MSC culture and cryopreservation protocols under good manufacturing practice conditions will need to be revisited and low DMSO protocols that are optimized for clinical use and support MSC function in the absence of animal components remain to be developed.

CRYOPRESERVATION OF INDUCED PLURIPOTENT STEM CELLS

Whereas studies on HSCs have been the focus of stem cell research since the 1960s-70s, studies assessing the role and function of MSCs have intensified since the 1990s. Since 2006, a substantial portion of the focus within the stem cell field has moved steadily towards the use of the new kid on the block, *i.e.* induced pluripotent stem cells (iPSC).

iPSCs are stem cells with embryonic stem cell (ESC)-like properties, but lack the ethical issues involved with the use of ESCs. This is related to the fact that iPSCs are artificially generated from somatic cells by forced overexpression of the pluripotency transcription factors OCT4, SOX2, KLF4 and c-Myc[92,93]. New protocols using different combinations of transcription factors, including NANOG and LIN28[94] and others, devoid of oncogenic potential, as well as different methods for transfer (*e.g.*, integrating lentiviral vectors, non-integrating sendai based vectors, episomal vectors, direct mRNA transfer, *etc*.)[95] have not affected the characteristics of the derived iPSCs: iPSCs have unlimited self-renewal capacity and the ability to differentiate into cells from all three germ layers (endoderm, mesoderm, ectoderm). iPSCs thus provide the tools to study early developmental biology *in vitro* and can be used for disease modeling and drug discovery. In addition, patient-derived iPSCs offer the opportunity to study the pathophysiology of diseases that could not be studied previously and can be used for the development of personalized medicine. All these features further stimulated iPSCs to become an important source of stem cells, and biobanks for storage of healthy and patient-derived iPSCs have now been established in many countries. However, efficient banking requires cell production facilities where cells can be expanded, maintained and cryopreserved under optimal conditions to ensure protection of iPSC characteristics and properties for weeks to years. In contrast to the cryopreservation protocols developed for HSCs and MSCs, current protocols for cryopreservation of iPSCs have focused on different issues, including freezing of cells in small aggregates *vs* single cell freezing in the presence of absence of DMSO[96-99], cell freezing using vitrification or different combinations of CPAs[100-102], cell recovery after cryopreservation using small molecules, such as the Rho kinase (ROCK) inhibitor Y-27632[103-105] and development of animal-component free formulations of culture and cryopreservation media using KSR instead of serum[106-108] (Table 3).

Using Raman spectroscopy to assess intracellular ice formation in iPSCs during cooling, Li *et al*[96] showed that iPSC aggregates are more sensitive to supercooling than single iPSCs in suspension due to the decreased water permeability of iPSCs in aggregates *vs* single cells. They also showed a greater variation in DMSO concentration across the aggregates than in single cells, suggesting that the size of the aggregates may hinder equal diffusion of the cryoprotectant to the cells. They also found that iPSC aggregates frozen in an optimized solution consisting of non-essential amino acids, sucrose, glycerol, isoleucine and albumin dissolved in a buffer made of poloxamer 188 (P188) in Hank's Balanced Saline Solution, did not exhibit the same sensitivity to undercooling as those frozen in non-optimized solutions or those containing 7.5% DMSO[97]. In addition, cryopreservation of iPSCs in aggregates requires a significantly modified freezing technique, where iPSC aggregates are first incubated at room temperature for 30 min to 1 h before freezing to allow sufficient internalization of the CPAs[97], in contrast to freezing with DMSO, which usually requires working at low temperatures (4 °C) and rapid mixing of cells.

Miyamoto *et al*[100] compared the efficacy of a variety of different cryopreservation media on an established murine iPSC line. These media consisted of control 10% DMSO formulations to reduced DMSO solutions, glycerol-containing solutions, combinations of DMSO and glycerol and commercially available cryopreservation media (CELLBANKER 1, 1+, 2 and STEM-CELLBANKER) and were used to freeze mouse iPSCs in suspension. Comparison of viability, proliferation and multipotency after long-term freezing of iPSCs in these media showed optimal results with the serum-free formulations of CELLBANKER (CELLBANKER 2 and STEM-CELLBANKER)[100]. However, the precise formulations of these freezing media is proprietary, Hank's Balanced Saline Solution and the researchers did not mention whether the STEM-CELLBANKER formulation used contained DMSO. Katkov *et al*[98] compared freezing of iPSCs in aggregates and as single cells using different CPAs including DMSO, ethylene glycol (EG), propylene glycol and glycerol. After extensive comparison, they found that freezing in aggregates resulted in favorable iPSC recovery after thawing. In addition, toxicity tests revealed that EG was not only less toxic than DMSO, it also supported better maintenance of pluripotency than propylene glycol or glycerol[98].

The use of KSR as a serum replacement has shown promising results and is another step in the development of animal component-free cryopreservation solutions. In combination with 10% DMSO, KSR has been used at concentrations of 25%-90% to freeze effectively iPSCs, ESCs and iPSC-derived cells with high post-thaw viability [105,106,108,109]. Inhibition of Rho kinase activity with ROCK inhibitors has shown favorable outcomes after freezing of both ESCs and iPSCs, and although not added during cryopreservation itself, it promotes both plating and cloning efficiency[104,105,108,110,111] by preventing apoptosis of detached cells[112]. Since addition of ROCK

Table 3 Comparison of different protocols used during cryopreservation of induced pluripotent stem cells

Source of cell	Storage periode and temperature	Cryopreservation	Viability	Parameters	Results	Ref.
1.5 x 10^6-2 x 10^6 hiPSC line UMN PCBC16iPS	Controlled rate; -196 °C	NEAA, sucrose, glycerol, isoleucine and albumin in a P188 in HBSS *vs* 7.5% DMSO; Aggregates *vs* single cells		Viability, adherence and intracellular ice formation	P188 was found here to not only inhibit ice formation significantly but also soften the solid-liquid interface of ice and increase the distance between adjacent ice crystals; The cryoprotective effects of the DMSO- free CPA cocktail could be capitalized only with the optimized composition. Deviation from the optimum may result in less desirable outcomes	[96, 97]
H9 hESC and hiPSC	3-6 d,controlled rate; -80 °C	10% DMSO, 10% EG, 10% PG, 10% glycerol, clumps *vs* single cells; ROCK inhibitor after thawing	EG-DMSO> PG|||<***glycerol	Toxicity of CPAs, expression of NANOG by hiPSCs	Freezing single cell iPSCs in the presence of a ROCK inhibitor and EG and programmable freezing drastically improved the yield of iPSCs in comparison to standard freezing in clumps without ROCK inhibitor	[98]
1-2x10^6 hiPSC	-196 °C	A: 10% DMSO/90% FBS; B: 10% DMSO/90% KSR; C: 10% DMSO/ESC medium + 20%KSR + ROCK inhibitor; Single cells	A: 90%; B: 70%; C: 70%	Viability, karyotype, expression of pluripotency markers TRA-1-60, TRA-1-81, Oct4, SSEA-3, and SSEA-4, embryoid body formation, neuronal differentiation, colony formation	Addition of ROCK inhibitor to pre- and post-thaw culture media increased survival rate, hiPSCs retained typical morphology, stable karyotype, expression of pluripotency markers and the potential to differentiate into derivatives of all three germ layers after long-term culture	[103, 105, 108]
hiPSC	-196 °C	10% DMSO in KO DMEM, 20% KSR, 1% NEAA, 1% L-glutamine, 0.2% b-mercaptoethanol, 1% antibiotic/ antimycotic and 8 ng/mL bFGF; ROCK inhibitor after thawing; Single cells		Colony number and size	ROCK inhibitor Y-27632 significantly improves the recovery of cryopreserved human iPS cells and their growth upon subculture	[104]
hiPSC line 253G4 and 201B2	7 d, Vitrification in; -196 °C	VS2E vitrification solution (40% EG, 10% PEG in Euro-Collins medium), DAP213 vitrification solution (1.2% DMSO, 22% PG, 5.9% acetamide); Single cells	VS2E>DAP213	Proliferation, expression of pluripotency markers Oct3/4, SSEA4, ALP, pluripotency in teratoma assay	Higher recovery rate of hiPSCs with DMSO and serum-free VS2E vitrification medium, cells after vitrification expressed Oct-3/4 and SSEA-4 and alkaline phosphatase and retained their pluripotency	[114]

iPSC: Induced pluripotent stem cells; NEAA: Non-essential amino acids; DMSO: Dimethyl sulfoxide; CPA: Cryoprotective agents; ESC: Embryonic stem cell; bFGF: basic Fibroblast Growth Factor; ROCK: Rho Kinase; ALP: Alkaline phosphatase; KSR: Knockout Replacement; FBS: Fetal Bovine Serum; HBSS: Hank's Balanced Salt Solution.

inhibitors up to 5 d after thawing still promotes colony formation, and since the effects of ROCK inhibition appear to be reversible, it has been also been suggested that ROCK inhibitors may relieve cellular stress[104].

Similar to studies in MSCs, the effects of magnetic fields on iPSC recovery after freezing have been assessed. Using the CAS researchers showed improved survival after thawing of iPSCs, but no effect on proliferation, gene expression and multilineage differentiation[113]. Reubinoff *et al*[101] previously showed that vitrification of both ESCs and iPSCs is feasible, using precooled freezing medium consisting of 90% FBS and 10% DMSO and a cooling rate of 1 C/min. ESC aggregates were preincubated in 80% DMEM, 10% DMSO and 10% EG and then placed into small 1-2 mL droplets containing 60% DMEM, 20% DMSO, 20% EG and 0.5 mol/L sucrose. All vitrified ESC aggregates recovered upon thawing and gave rise to colonies after plating. However, vitrified colonies were significantly smaller and showed increased differentiation compared with control colonies. Nevertheless, colonies generally recovered within 1-2 d of cell culture. Using a similar method for iPSCs, but using a DMSO and serum-free medium based on 40% EG and 10% PEG, Nishigaki *et al*[114] obtained a higher recovery rate of iPSCs than with a vitrification solution containing DMSO and serum.

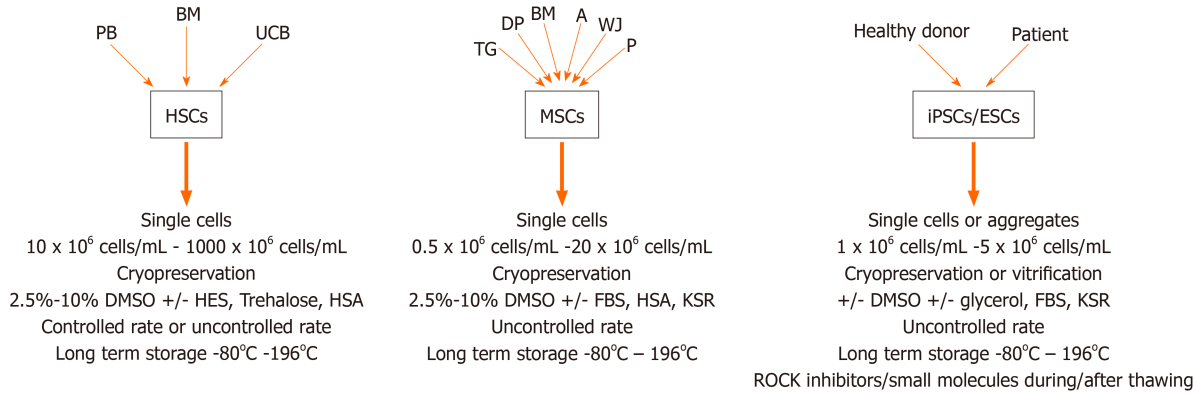


Figure 2 Preferred cryopreservation protocols for different types of stem cells. PB: Peripheral blood; BM: Bone marrow; UCB: Umbilical cord blood; HSCs: Hematopoietic stem cells; DMSO: Dimethyl sulfoxide; HES: Hydroxyethyl starch; HSA: Human serum albumin; TG: Tooth germ; DP: Dental pulp; A: Adipose tissue; WJ: Wharton Jelly; P: Placenta; MSCs: Mesenchymal stem/stromal cells; FBS: Fetal bovine serum; KSR: Knockout serum replacement; iPSCs: Induced pluripotent stem cells; ESCs: Embryonic stem cells; ROCK: Rho-associated protein kinase.

CONCLUSION

The universally used cryoprotectant DMSO has been associated with *in vitro* and *in vivo* toxicity and has been shown to affect many cellular processes through dysregulation of gene expression and changes in DNA methylation. Despite studies showing that DMSO affects cell characteristics including differentiation potential, DMSO remains to be the CPA of choice both in a research setting and in the clinics. Many different protocols have been developed for different types of stem cells and a broad range of alternatives to DMSO have been shown to hold promise for use as a CPA (Figure 2). These alternatives include such molecules as trehalose, sucrose, EG, PEG and many more. It is obvious that a single protocol that can be used for all types of stem cells is not feasible, but the enormous amount of available alternatives should make it possible to adapt and optimize DMSO-free and animal component and serum-free cryopreservation solutions adapted for different types of stem cells in the foreseeable future.

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Supplement 10. Thesis related paper 2

The Role of Rab GTPases in the development of genetic and malignant diseases

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Received: 16 March 2023 / Accepted: 1 April 2023
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Abstract
Small GTPases have been shown to play an important role in several cellular functions, including cytoskeletal remodeling, cell polarity, intracellular trafficking, cell-cycle, progression and lipid transformation. The Ras-associated binding (Rab) family of GTPases constitutes the largest family of GTPases and consists of almost 70 known members of small GTPases in humans, which are known to play an important role in the regulation of intracellular membrane trafficking, membrane identity, vesicle budding, uncoating, motility and fusion of membranes. Mutations in Rab genes can cause a wide range of inherited genetic diseases, ranging from neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) to immune dysregulation/deficiency syndromes, like Griscelli Syndrome Type II (GS-II) and hemophagocytic lymphohistiocytosis (HLH), as well as a variety of cancers. Here, we provide an extended overview of human Rabs, discussing their function and diseases related to Rabs and Rab effectors, as well as focusing on effects of (aberrant) Rab expression. We aim to underline their importance in health and the development of genetic and malignant diseases by assessing their role in cellular structure, regulation, function and biology and discuss the possible use of stem cell gene therapy, as well as targeting of Rabs in order to treat malignancies, but also to monitor recurrence of cancer and metastasis through the use of Rabs as biomarkers. Future research should shed further light on the roles of Rabs in the development of multifactorial diseases, such as diabetes and assess Rabs as a possible treatment target.

Keywords Rab GTPases · Inherited diseases · Cancer · Stem cell · Gene therapy · Biomarkers

Introduction

Small GTPases are single chain polypeptides, which serve as GTP binding proteins. The small GTPase superfamily consists of the Ras, Rho, Rab, Arf and Ran families [1] and play an important role in several cellular functions, including cytoskeletal remodeling, cell polarity, intracellular trafficking, cell-cycle progression, lipid transformation and so forth [1, 2]. The first Rab gene (*YPT1*) was identified in 1983 by D. Gallwitz et al. as an open reading frame between the actin and tubulin genes in the yeast *Saccharomyces cerevisiae*. The Ras-associated binding (Rab) GTPases constitute the largest family of the almost 70 known members of small GTPases in humans and are known to play an important role in the regulation of intracellular membrane trafficking [3]. Rab GTPase coding areas are divided over several locations in the human genome and seem to have arisen through a series of gene duplications, resulting in multiple Rab GTPase isoforms with high sequence similarity and partially overlapping functions. In addition to the vast array of different Rab GTPases, alternative splicing of some of the Rab genes may result in even more isoforms, with different abilities to bind their effectors [4]. The Rab GTPases are localized on different intracellular membranes in the cell, facing the cytosol in order to regulate the specificity and the direction of the membrane trafficking [5, 6]. By doing so, the Rab proteins have been shown to not only control intracellular membrane trafficking, but also serve to aid in membrane identity, vesicle budding, uncoating, motility and fusion of membranes [3, 6]. In addition, Rab GTPases have been shown to participate in the regulation of basic cellular functions, mediated through interactions between the Rab GTPases and their effectors. Furthermore, the Rab GTPases

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Published online: 15 April 2023

have also been shown to be involved in the regulation of cell signaling, cell proliferation and survival, as well as migration. These processes take place via recruitment of effector molecules and regulatory proteins, which affect the binding of Rab proteins to membranes and thus regulate their activity. Examples of effector molecules that are recruited by these Rab proteins include membrane (coat) proteins, tethering factors, molecular motor proteins, phospholipid modulators, kinases, phosphatases and others [7, 8].

Mutations in the genes that encode Rab GTPases and result in altered proteins and/or protein interactions are associated with a number of inherited diseases, such as immune deficiencies, hereditary blindness and mental retardation and may be a contributing factor in the development of multifactorial diseases, such as neurodegenerative diseases, malignancies, inflammatory diseases, obesity and diabetes [7, 9–11].

A better understanding of the interactions between the Rab GTPases and their effectors and their role in the development of Rab-related diseases is crucial to determine new treatment targets and to develop novel therapeutic strategies. Therefore, we aim to provide a comprehensive overview of the roles of the different Rabs in the development of genetic and acquired diseases by assessing their role in cellular structure, regulation, function and biology and we summarize preliminary data that may support their possible use as target for (stem cell) gene therapy.

Function, evolution and structure of Rab proteins

The number of Rab GTPases has been shown to vary widely among species, and in humans, currently 70 Rabs, belonging to 44 subfamilies have been identified. Among all the Rab GTPases, almost 75% is involved in endocytic trafficking, serving as *post office* molecules. They are generally found firmly located in the bilayer, where they specify the spatial and temporal identity, direction and function of vesicles and organelles. Although Rabs belonging to functional subgroups often cooperate, their functions are not always redundant. Evolutionary assessment of the Rab genomes supports the diversification of Rabs from their last eukaryotic common ancestor (LECA) into 6 functionally distinct subgroups that are involved in secretion (I), early endosomes (II), late endosomes (III), recycling of endosomes to the surface (IV), recycling of endosome to the Golgi complex (V) and trafficking associated with cilia/flagella (VI) [12]. Although the LECA must have had at least 20 different Rabs, most of these Rabs have been shown to be dispensable during evolution, with only five Rabs (i.e., Rab1, Rab5, Rab6, Rab7 and Rab11), each one belonging to a different group, being conserved throughout all eukaryotic genomes and being likely essential for proper functioning [12]. Of these Rab1 and Rab6 are located in the Golgi apparatus, whereas Rab5, Rab7 and Rab11 are involved in endo- and phagocytosis (Table 1, Fig. 1). Changes in the expression of the essential Rab genes directly affect physiological membrane trafficking and may cause dysregulation of multiple intracellular signaling pathways that control the balance between cell survival, cell proliferation and cell death.

All Rab proteins consist of conserved globular G motifs (guanine moiety binding motifs, Rab family specific motifs, Rab subfamily specific motif, C-terminal interacting motif, geranylgeranylation motif) and structural elements (consisting of switch regions, complementary determining regions and a hypervariable C-terminal domain) [5, 8, 13]. In addition to the small GTPases, a separate group of large Rab GTPases, consisting of RAB44, RAB45 and RAB46 has been described (reviewed by Tsukuba et al.) [14]. These large Rabs have a size of about 70–150 kDa (in contrast to the small GTPases with a MW of 20-30 kDa) and consist of several isoforms that arise as a result of splicing variants, with long (L) and short (S) versions of the same protein that may show a different distribution pattern, but share a similar function (Table 1). The group of large Rab GTPases is structurally characterized by the presence of a common structural domain, consisting of an amino-terminal EF-hand domain (EFD), a coiled-coil domain (CCD), and a carboxy-terminal Rab domain.

Rab GTPase activation and molecular switch

As described above, most Rabs are typical Ras-like small GTPases that function as membrane-bound molecular switches. All Rabs have a GTPase domain that contains a C-terminal prenylation motif, where hydrophobic molecules can be added onto the protein, and which supports Rab anchoring to cellular membranes [15]. In order to perform their function, all Rabs bind to the Rab escort protein (REP) first, after which they are prenylated at their C-terminal cysteine residues by Rab geranylgeranyl-transferase (RabGGTase). The Rabs are then transferred to their respective internal membranes. Here, the Rabs function as nucleotide-dependent switches that are activated (ON), when they are bound to GTP and inactive (OFF) when GTP is hydrolyzed to GDP [8] (Fig. 2). Usually, inactive GDP-bound Rabs are activated by Guanine Nucleotide Exchange Factors (GEFs), which recognize-specific residues in the switch region. To initiate the signaling cascade, the activated GTP-bound Rab needs to bind to an effector protein. The only exception to this rule is the effector protein protrudin, which binds to the inactive GDP-bound form of Rab11 to become functional [16]. Reversion from the active state to the non-active state is mediated by GTPase-Activating Proteins (GAPs), which display conserved arginine and glutamine

Table 1 Human Rabs, their functions, effectors, tissue expression and related genetic diseases

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Group I (secretion)						
Rab1 (essential) Rab1a, Rab1b	ER and early Golgi membranes	Ypt32p, Uso1p, COPII, TBC1D20, Golgin-84	Maintenance of the Golgi complex, anterograde protein trafficking from ER-to-Golgi, authophagy, conversion of proinsulin to insulin	Low tissue specificity	Oculocerebrorenal syndrome, cardiomyopathy, Parkinson's disease, bacterial and viral infections, diabetes	[10, 34–37]
Rab19 (Rab19a)	Recycling endosome, Golgi	ATG16L1, TBC1D4	Intracellular protein transport, ciliogenesis	Intestinal epithelial, duct and tubular cells, colon enterocytes, bone marrow, T-cell	Inflammatory bowel disease (IBD), Huntington's disease	[38]
Rab43 (Rab19b)	Golgi	USP6NL	ER-Golgi trafficking, virion assembly and replication	Liver tissue, epithelial cells	Disruption of Golgi apparatus	[35, 36, 39, 40]
Rab30	Golgi, early endosome, recycling endosomes	Golgin, GCC88	Retrograde trafficking and maintenance of endosome-Trans Golgi Network organization	Highly expressed in brain tissue	Syndromic X-Linked Intellectual Disability Shashi Type	[41, 42]
Rab33a	Golgi	Singar1	Anterograde vesicular transport for membrane exocytosis and axon outgrowth, regulation of immune response against infections	Highly expressed in brain tissue, lymphocytes, monocytes and melanocytes	Combined oxidative phosphorylation deficiency, deafness, X-linked 5, peripheral neuropathy	[43, 44]
Rab33b	Golgi apparatus, ER	UVRAG, CLN3, Atg16L	Cis-Golgi network to ER cycling of Golgi enzymes, intra-Golgi retrograde trafficking	Broad tissue expression	Dyggve-Melchior-Clausen syndrome and Smith-McCort dysplasia (autosomal-recessive spondylo-epimetaphyseal dysplasias)	[45]
Rab35 (Rab1c)	Endosome	ACAP2 (Arf6 GAP), RUSC2, MICAL-L1	Intracellular membrane trafficking, recycling of endosomes towards the plasma membrane, clathrin-mediated endocytosis, cell surface homeostasis, neurite outgrowth, cell migration	Highly expressed in neuronal tissue and myoblasts	Associated with Lowe oculocerebro-renal Syndrome, possible involvement in Parkinson's disease, bacterial infections	[26, 33, 46, 47]
RabX6*						

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab8 Rab8a, Rab8b	Golgi, Endosome, cytoskeleton	EHBP1L1, OCRL1, EHBP1, Sec15, TBC1D4	Vesicle formation, movement, tethering, fusion, neurotransmitter release, neuronal maturation and outgrowth and adipogenic differentiation	Broad tissue expression, including gastrointestinal tract,	Absence of Rab8 results in blockage of neuronal maturation and is associated with Lowe syndrome, Parkinson's disease, microvillus inclusion disease (MVID)	[28, 48–51]
Rab3a, Rab3b, Rab3c, Rab3d (Rab16)	Cytoplasm, intracellular vesicles and cell membrane	Rabphilin3a, RIM1a, RIM2a, Noc2, Munc18-1, Synapsin, PRA1, Calmodulin, granuphilin	Synaptic vesicle exocytosis, neural transmission, plasma membrane repair, acrosomal exocytosis, insulin secretion	Highly expressed in neurons and brain tissue	Rab3s are functionally redundant, low Rab3a is associated with Lewy body dementia, high Rab3b is a protective factor in Parkinson's disease, association with Diabetes Type II	[52–57]
Rab12	Golgi apparatus, Lysosome and endosome	RILP, RILP-L1 and RILP-L2	Protein transport, degradation of transmembrane protein, autophagy	Highly expressed in brain and retina, breast, muscle and skeletal tissue	Dystonia, Newfoundland Rod-Cone Dystrophy	[58]
Rab13 (Rab8c)	Golgi apparatus and endosome	DENND2B, PRKACA, JRAB/MICAL-L2	Endocytic recycling, neuronal regeneration and neurite outgrowth, glucose homeostasis, assembly of tight junctions, reorganization of the actin cytoskeleton	Broad tissue expression, including nervous system, gastrointestinal system	Crohn's disease	[30, 59, 60]
Rab10	Golgi, ER and endosome	EHBP1, EHBP1L1, SEC16A, MARCKS	Lysosome exocytosis, plasma membrane repair and intracellular membrane trafficking, axonal development, critical for normal neuronal function, regulates ciliogenesis	Expressed by all cell types in the brain, broad tissue distribution	Legionella pneumophila pathogenesis, association with Parkinson's and Alzheimer's disease	[29, 61, 62]
Rab15	centriolar satellite and vesicles	REP15	Regulation of synaptic vesicle membrane, endocytic recycling, early endocytic trafficking, anti-viral immunity	Highly expressed in the brain	Stiffness of Calcaneus	[63–66]
Rab26 (Rab26a)	Golgi apparatus and secretory vesicles	Atg16L1, CvpF	Protein transport (Golgi to plasma) and exocytosis, lysosome traffic, autophagy	Highly expressed in brain and breast tissue	Sensitivity to Coxiella infection	[67, 68]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab37 (Rab26b)	Recycling endosome	MUNC13-1, ATG5	Exocytosis of secreted glycoproteins, mast cell degranulation, exocytosis of insulin, autophagosome formation	Highly expressed in lympho-hematopoietic tissues (bone marrow, spleen, lymphnodes, macrophages and mast cells) and the gastrointestinal system	Inhibition of neovascularization, loss of insulin regulation	[69–72]
Rab27a	Lysosome and endosome	MUNC13-4, Myosin Va, Melanophilin (Slac2-a), Coronin3, Slp4-a, MyRIP, Noc2	Exocytosis of organelles, granule maturation, degranulation, nociception, pro-insulin conversion to insulin (Noc2-Rab2), insulin exocytosis (Noc2) and endocytosis of insulin granules (Coronin3)	Broad tissue expression, including immune cells	Griscelli Syndrome type II immune deficiency, Diabetes type II	[73–80]
Rab27b	Endosome	Slac2-b, MUNC13-4, melanophilin, MyRIP, Slp1–5, granulophilin	Endocytosis, exocytosis and secretion, co-localization with RAB27a, delivery of secretory granules near the plasma membrane	Broad tissue expression, including neurons, endocrine and exocrine cells, secretory cells, goblet cells, platelets	None	[81]
Rab34	Golgi apparatus	RILP, MUNC13-2	Maturation of phagosomes to engulf pathogens, lysosome/phagosome fusion	Broad tissue expression, including nervous system, digestive system and reproductive system	Bacterial and viral infections	[18, 19, 82, 83]
Rab36	Late endosome, Golgi apparatus and lysosome	RILP	Intracellular trafficking, spatial distribution of late endosomes and lysosomes, retrograde melanosome transport in melanocytes	Ubiquitous expression throughout all tissues	Deletion is associated with malignant rhabdoid tumors	[84–86]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab44*** Rab44L (long), Rab44S (short)	L: lysosome, S: early endosome, ER	VAMP-8	Retrograde melanosome transport in melanocytes, negative regulation of osteoclast differentiation, degranulation of mast cells	High expression in bone marrow, spleen and immune cells, marginal expression in the epididymis, lungs, skin, thymus, ovary, uterus, and liver	Allergies, atopic and auto-immune diseases mast cell-driven anaphylaxis	[87–92]
Rab45/RASEF*** Rab45-L (long), Rab45-S (short)	Perinuclear region	L: Dynein-dynactin, ERK1/2	Endocytic trafficking and apoptosis	Rab45-L: epithelial cells Rab45-S: testes	Regulation of anti-cancer responses	[93]
Rab46*** CRACR2A-L (long), CRACR2A-S (short)	Weibel-Palade bodies, Perinuclear region	Vav1, Dynein -dynactin	Endocytic trafficking, calcium transport and neutrophil degranulation	Rab46: endothelial cells, T-cells CRACR2-S: T-cells	Impaired T-cell differentiation, cardiovascular disease, non-alcoholic fatty liver disease, periodontal disease, cancer metastasis	[94]
RabX4*						
Rab18	ER and Golgi, lipid droplets	DFCP1, NRZ, syntaxin-18	Apical endocytosis and recycling, neuronal migration in the developing cerebral cortex, eye and brain development and neurodegeneration	Ubiquitous expression in all organs	Warburg Micro syndrome	[24, 95, 96]
Rab40a Rab40aL (RLGP), Rab40b, Rab40c	Rab40c: Lipid droplets	Rab40b: Cullin5, Rab40c: TIP47, Cullin5	Rab40b: Secretion of MMPs to facilitate ECM remodeling during cell migation, and actin cytoskeleton dynamics during cell migration Rab40c: biogenesis of lipid droplets, regulation of actin dynamics, cell migration, secretory membrane trafficking	Brain, kidney, lung, heart, liver and skeletal muscle	RAB40aL disruption causes Martin–Probst syndrome (MPS)	[97–100]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Group II (early endosomes)						
Rab5 (essential) Rab5a, Rab5b, -Rab5c	Cytosol Rab5a: early endosome Rab5b,c: late endosome, exosome, clathrin-coated vesicle membrane	Rab5ip, Rabip4, Rabenosyn-5, EEA1, Rabex-5, Rabaptin-5, FHF, Rabankyrin-5, APPL1, ZFYVE21	Vesicle formation, movement, tethering and fusion, filopodia formation, clathrin-mediated early endocytosis, viral cell entry/egress in a variety of cell types, clathrin-mediated endocytosis	Ubiquitous tissue expression	Pathological activation of Rab5 drives endocytic dysfunction in Alzheimer's disease and Down syndrome, PHEV entrance, Nephrotic syndrome,	[101–108]
Rab17	Endosome	ALS2, Rabex-5	Transcytosis, epithelial cell polarization, melanocytic filopodia formation and melanosome trafficking, filopodia formation, dendrite morphogenesis in neurons	Ubiquitous tissue expression with preference for epithelial cells	Increased expression in Amyotrophic Lateral Sclerosis	[31, 109–111]
Rab21	Endosome, ER and Golgi apparatus	APPL1, Rabex-5	Early endosome morphology and function, cell adhesion and migration, integrin trafficking, pro-inflammatory cytokines	Ubiquitous tissue expression	Alzheimer's disease, increased in acute pancreatitis	[25, 112–114]
Rab22 (Rab22a)	Early endosome	Rabex-5, RAD51, EEA1	Early endosome dynamics, internalization of MHC-I molecules, recycling of cargo trafficking through a clathrin-independent pathway, NGF signaling and neurite outgrowth, sorting of Transferrin	Ubiquitous tissue expression, high expression in nervous system and gastrointestinal system	Reduced recycling endosome dynamics and cargo accumulation in the sorting endosomes or lysosomes	[32, 115–122]
Rab31 (Rab22b)	Endosome, Golgi apparatus	FLOT1, FLOT2	post-Golgi trafficking, endosomal sorting complex required for transport (ESCRT)-independent exosome pathway, intraluminal vesicle formation and suppression of multivesicular endosome degradation, regulation of EGFR, early-stage phagosomes, regulation of innate immunity	Brain, female tissues, endocrine tissues, muscle and hematopoietic tissues	Kawasaki disease	[123–125]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab24	Cytosol, perinuclear area	RILP	Clearance of late autophagic lysosomes, endosomal degradation	Ubiquitous tissue expression	Possible involvement in hereditary ataxia, decreased in atherosclerosis and multiple sclerosis and elevated expression in tuberculosis	[126–129]
Rab20	Golgi apparatus	Rabex-5	Maturation and acidification of phagosomes, protection from fungal and bacterial microorganisms, hypoxia-induced apoptosis, inhibition of neurite outgrowth	Ubiquitous tissue expression	Increased susceptibility to infections, upregulation of Rab20 under hypoxic conditions	[20, 130–133]
RabX1*						
Group III (late endosomes)						
Rab7 (essential) (Rab7a)	Early to late endosomes, lysosome	PLEKHM1, RILP, ORP1L, FYCO1, WDR91, NRBF2, Rabring7	Endocytosis, endolysosomal trafficking, autophagy, mitophagy, growth-factor-mediated cell signaling, nutrient-transportor mediated nutrient uptake, neurotrophin transport in the axons of neurons, lipid metabolism, maturation of melanosomes, pathogen-induced phagosomes, phagosomal acidification, neurite outgrowth, participates in life cycle of viruses	Ubiquitous tissue expression, high expression found in skeletal muscle	Charcot-Marie-Tooth neuropathy type 2B (CMT2B), Alzheimer's and Down Syndrome, ARC Syndrome, Diabetes Type II	[134–137]
Rab7b	Late endosome, lysosome, TGN	Atg4B, Myosin II	Transport from late endosomes to the TGN, dendritic cell migration, autophagosome maturation, megakaryocytic differentiation, regulation of toll-like receptor signaling	monocytes (CD14 + cells), immature dendritic cells (DCs), hematopoietic stem cells	None	[23, 138, 139]
Rab9 (Rab9a)	Endosome, Golgi, ER	TIP47, RUTBC2, p40 (RABEPK)	Endosome and trans golgi network (TGN), antiviral activity	Ubiquitous tissue expression, highly expressed in monocytes	Rab9 overexpression is related with Niemann-Pick C1, cholesterol metabolism	[84, 140–143]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab9b (Rab9L)	Cell membrane and phagosome	Atg7	Late endosome/late endosome to TGN transport, alternative autophagy	Visceral in adipose tissue	None	[144]
Rab23	Endosome	unknown	Autophagosome assembly, cellular defense response, intracellular protein transport, plasma membrane, early endosome/ endocytosis, and sonic hedgehog signaling	Whole blood (monocyte, platelet), intestine	Carpenter Syndrome	[145]
Rab29 (Rab7L1)*	Golgi apparatus and cytoskeleton	unknown	Immune synapse assembly and ciliogenesis, membrane trafficking and neurite outgrowth, maintenance of the TGN, retrograde Trafficking	Ubiquitous tissue expression	Parkinson's disease	[146–149]
Rab32 Rab32a	Mitochondrion	VARP, reticulon 3L	Melanogenesis and pigment biosynthesis, phagosome maturation, autophagic degradation of mitochondria-associated membrane (MAM) proteins	Ubiquitous tissue expression	Parkinson's disease, Multiple sclerosis, Hermansky-Pudlak syndrome	[150–153]
Rab38 (Rab32b)	Lysosome related organelles	VARP	Vesicle trafficking (melanogenesis)	Alveolar lung cells, melanocytes, retinal pigmented epithelium	Oculocutaneous albinism, Hermansky-Pudlak syndrome	[151, 152, 154]
Group IV (recycling of endosomes to the surface)						
Rab2 Rab2a	ER and Golgi	ICA69, RIC19, golgin-45, GAPDH, Noc2	ER-to-Golgi traffic or in the formation of secretory granules, facilitates ER-associated degradation or secretion of (pro) insulin, pro-insulin to insulin processing, acrosome formation	Ubiquitous tissue expression with high expression in oral epithelium, adipocytes and Langerhans islets	Huntington's disease, diabetes, non-alcoholic fatty liver	[77, 155–159]
Rab2b	Golgi	GARI-L1 (FAM71F1), GARI-L4, GARI-L5	Regulates DNA-induced innate immune responses (limits replication of DNA viruses), regulation of Golgi morphology, acrosome formation	Kidney, prostate, lung, liver, thymus, colon, pancreas ductal cells, Langerhans islets and skeletal muscle	Male fertility	[159–162]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab39a	Late endosome, lysosome	UACA	Maturation/acidification of phagosome, phagosome-lysosome fusion, vesicular trafficking, autophagy, sphingolipids and phospholipids transport, basolateral exosome release, phagosome maturation into MHC-I antigen-presenting compartments	Brain frontal cortex and cerebellar hemisphere, antigen-presenting cells	Unknown, overexpression is associated with cancer stemness	[163–167]
Rab39b	ER and Golgi	PICK1, UACA	Intracellular protein transport and autophagy, synapse formation and maintenance, sphingolipids and phospholipids transport, basolateral exosome release	Brain frontal cortex, anterior cingulate cortex, retina	Early onset Parkinson's Disease, X-linked intellectual disability with autism, epilepsy, loss-of-function on synaptic activity, X-linked 72 (MRX72), Waisman syndrome (WSMN)	[153, 165, 166, 168–171]
Rab42 (Rab39c)	Cytoplasm	Unknown	Secretory membrane trafficking	Ubiquitous tissue expression	Unknown	[172]
Rab4 Rab4a, Rab4b	Endosome	Rabip4	early sorting endosome/endosomal recycling, Glut4 recycling, transport through early endosomes, antigen processing, maintaining synaptic balance	Ubiquitous tissue expression	Alzheimer's disease, Huntington's disease, Protection against Huntington	[57, 173–176]
Rab11 (essential) Rab11a, Rab11b	Endosome and cell membrane	FIP3, MYO5B, FIP2, SH3TC2	Endocytic recycling and membrane delivery during cytokinesis, with MYO5B and RAB8A become part of epithelial cell polarization	Ubiquitous tissue expression with high expression in the brain, bone, monocytes, T cells, skin, adipocyte, pancreas and kidney	Huntington's Disease, Cystic fibrosis, colorectal cancer	[177–184]
Rab25 (Rab11c)	Plasma membrane	MYO5B, RAB11FIP1, RAB11FIP2, RAB11FIP3, RAB11FIP5	Cell survival, autophagy, promotes invasive migration of cells, regulation of apical recycling endosomes	Skin, gastrointestinal system	Intestinal neoplasia	[185, 186]

Table 1 (continued)

Rab	Localization	Effectors	Cellular Function	Tissue expression	Disease	References
Rab14	Recycling endosome between the ER and Golgi	YUFY1/Rabip4, RCP (FIP effector)	Endocytic recycling, membrane trafficking between ER and Golgi, cell migration	Ubiquitous tissue expression	Unknown	[175, 187–190]
Group V (recycling of endosomes to the Golgi)						
Rab6 (essential) Rab6a, Rab6b, Rab6c	Golgi	Rab6IP, PIST, BicaudalD1, BicaudalD2, p150	Membrane trafficking from Golgi to ER, intra-Golgi transport, retrograde trafficking, Golgi homeostasis	Rab6b: expressed in brain Rab6c: brain, testis, prostate and breast	Rab6 knockout in mice is embryonically lethal, Alzheimer's disease	[22, 191–194]
Rab41 (Rab6d)	Cytoplasm	dynactin 6, syntaxin 8	ER-to-Golgi trafficking and cell growth, Golgi organization	Low tissue expression	Fragmented Golgi apparatus	[195, 196]
Group VI (traffic associated with cilia/flagella)**						
Rab28	Cytoplasm/ nucleus	unknown	Phagocytosis of cone outer segments	Ubiquitous tissue expression, high expression in photo-receptor cells, retinal pigment epithelium (RPE)	autosomal-recessive cone-rod dystrophy (CORD)	[197]
RabL4 (IFT27)					RabL4 is lost from the genomes of organisms that lack cilia	[12, 198]

*Lost in vertebrates; **Group VI Rabs are not typical Rab proteins and might be better classified as a separate Rab family subgroup; ***Large Rab GTPases with long *N*-terminal regions in addition to the *C*-terminal Rab-like GTPase domains

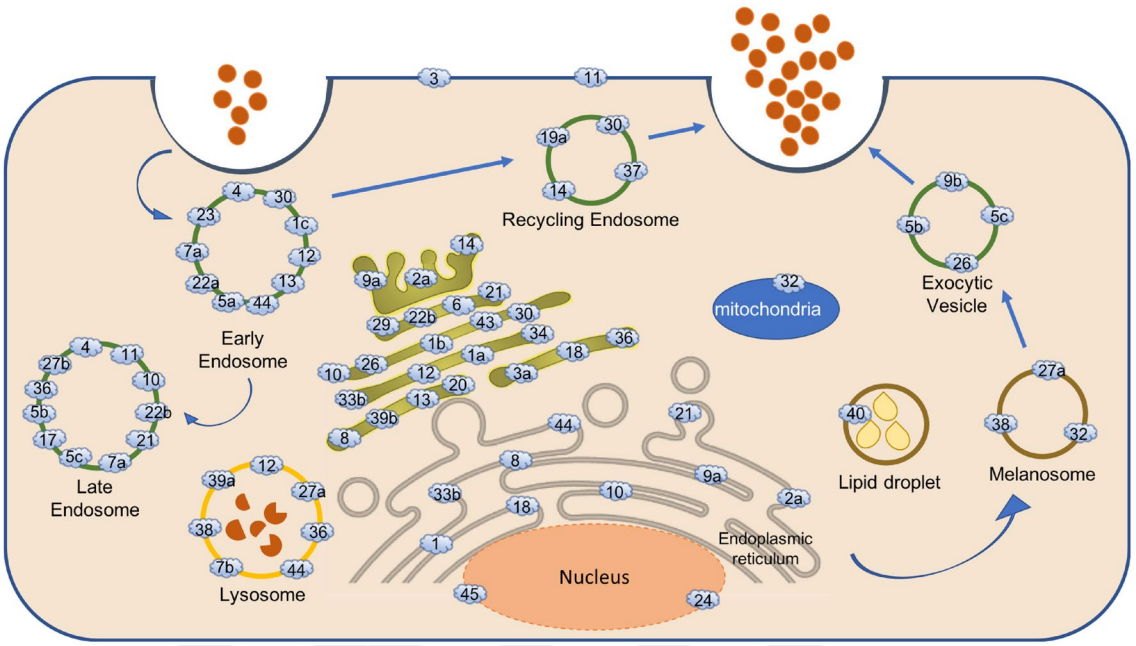


Fig. 1 Localization of Rab GTPases in the cell Each Rab GTPase has a specific role and localization within the cells where it regulates spatial and temporal identity, direction and function of vesicles and organelles

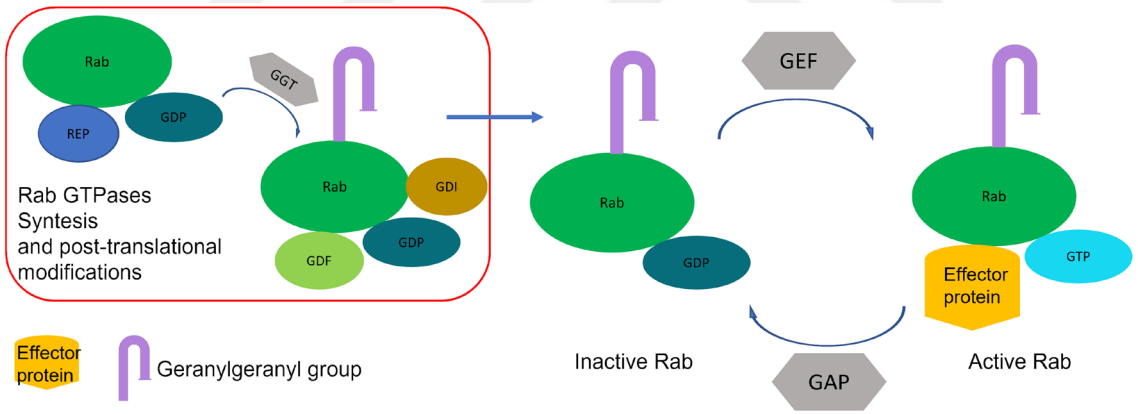


Fig. 2 Rab GTPases Ras related protein in brain (Rab) GTPases function as membrane-associated molecular switches that are ON in the GTP-bound (active) form and OFF in the GDP-bound (inactive) form. *REP* Rab escort protein; *GDI* GDP dissociation inhibitor; *GEF* guanine nucleotide exchange factors; *GAP* GTPase-activating proteins

residues that can accelerate the catalysis. GDP Dissociation Inhibitors (GDI) can bind to the prenylated lipids, inhibiting GDP release from the Rabs and stabilizing them in their inactive state [15].

The role of Rab proteins in healthy cells

Based on the presence of different combinations of Rabs within specific cells, the Rab GTPases regulate different physiological functions within distinct types of cells, including regulation of cytoskeletal remodeling, cell polarity, intracellular trafficking from and to the ER and Golgi apparatus, and cell-cycle progression. However, Rab GTPases are also responsible for cell–cell interactions. In order to perform all these different functions flawlessly, they have to recruit a specific effector to switch from an inactive, GDP-bound to an active, GTP-bound state. For example, active Rab proteins interact with motor proteins, such as actin filaments and microtubules, to regulate vesicle transport, cell maturation, cell proliferation and cell migration.

Whereas most Rab GTPases are characterized by a ubiquitous tissue expression, some Rabs are particularly highly expressed in the nervous system, where they play a critical role. These Rab have been shown to be involved in neurotransmitter release (Rab8), neurite outgrowth (Rab13, Rab22, Rab29, Rab35), neuronal maturation (Rab8), cell migration (Rab18, Rab21, Rab35, Rab40), differentiation (Rab8, Rab44), normal functioning/regeneration (Rab3, Rab5, Rab7, Rab10, Rab13, Rab17, Rab27) and, as a consequence, mutations in these Rabs have been associated with the development of specific neurological syndromes, deficits and/or neurodegenerative diseases, caused by aberrant protein folding, vesicle trafficking, and secretion (Table 1).

Similarly, defects in Rab GTPases involved in innate/adaptive immunity and inflammation, e.g., regulation of mast cell degranulation, neutrophil granule maturation, granule docking and priming, lytic granule exocytosis (Rab27a), fusion of early endosomes with phagosomes (Rab5, Rab14, Rab20, Rab22A), late phagosomes (Rab7 and Rab34), phagosome maturation (Rab7, Rab20, Rab22b, Rab32, Rab34, Rab38 and Rab43), macropinocytosis (Rab5, Rab7, Rab20, Rab34) [17], lysosome/phagosome fusion (Rab34) [18, 19], autophagy (Rab7, Rab12, Rab24, Rab25, Rab26, Rab33, Rab37, Rab39a), and modulation of viral/bacterial infections (Rab1, Rab6, Rab20, Rab26, Rab27a, Rab33a, Rab34, Rab35), may result in immune deficiencies, autoimmunity, increased susceptibility to infections and asthma/COPD (reviewed by Prashar et al.) [20]. In addition, responses to and severeness of infectious diseases caused by intracellular microorganisms are strongly dependent on the function of endocytic Rabs and altered Rab expression affects both effective host defenses and the pathogens life cycle and survival [21].

In addition to the above, Rabs also play a critical role during embryonic development and stem cell differentiation, starting from their role in germline stem cells (Rab6) [22] and cell migration (Rab7b, Rab18, Rab21, Rab35) [23–26] to their role in the development of the brain (Rab7a, Rab8, Rab10, Rab13, Rab17, Rab22, Rab35) [27–33] and adult stem cells (Rab5, Rab8, Rab44). Furthermore, Rab2 and Rab3 have been shown to play an important role in the development of functional sperm cells. Development of novel (stem cell-directed) therapies that target these Rabs or their respective effectors may be helpful in the treatment, prevention or diagnosis of Rab-associated diseases.

Role of Rabs in the development of genetic diseases

Due to the important role of the Rab proteins in healthy cell physiology and embryonic development, any interference with Rab function or activity due to mutations in the Rab proteins or its direct effectors may result in the development of genetic diseases. The effects of Rab mutations depend not only on Rab tissue distribution and expression, but also strongly on the presence of other Rabs that may have arisen evolutionarily through gene duplication and may display (partially) overlapping functions (eg Rab3) [52]. Although discussion of all Rabs is impossible, we will give a brief summary here of diseases related to the absence/malfunctioning of some specific Rabs. However, it should be kept in mind that although strong associations between changes in Rab expression and diseases can be found, in many diseases it remains unclear whether these changes in Rab expression are cause or result of the disease.

A selection of mutations in Rab genes that have been directly linked to the development of a specific genetic diseases will be discussed here in more detail. The RAB27a GTPase regulates secretion of cytolytic granules by binding to its effector MUNC13-4 [199]. Mutations in either *RAB27a* or *MUNC13-4* may result in the development of Griscelli Syndrome Type 2 (GS-2) with or without accompanying primary hemophagocytic lymphohistiocytosis (HLH) [200]. GS-2 is clinically characterized by the presence of a combined immune deficiency, caused by the loss of cytotoxic killing activity in T and NK cells [200] and a low neutrophil number and function [201]. If the *RAB27A* mutation interferes with Melanophilin (MLPH/SLAC2-A) binding as well, this immune deficiency may be further accompanied by the typical partial albinism that occurs as a result of a defect in melanosome transport and results in silver/gray hair [199, 202].

RAB7a is found in late endosomes and does not only play a role in the regulation of endocytosis, autophagy, and neurite outgrowth, it is also involved in the process of inducing an effective immune response against pathogens through its role in phagocytosis, microbial pathogen infection and the viral life cycle [136]. Mutations in the gene for RAB7a are directly associated with Charcot-Marie-Tooth type 2B (CMT2B) disease [203]. CMT2B is an autosomal dominantly inherited peripheral neuropathy, which develops in the teenage years and is characterized by chronic axonal degeneration with subsequent loss of nerve fibers, causing distal muscle weakness, atrophy, sensory loss, loss of deep-tendon reflexes, infections, and foot ulcers that may require (partial) amputation because of recurring infections [204].

Both Warburg Micro and Martsolf syndromes are autosomal-recessive developmental disorders and have been linked to mutations in RAB genes and/or RAB GTPase-activating proteins (GAP) [96, 205, 206]. Although both diseases show high similarity and are characterized by ocular and neurodevelopmental symptoms, including mental retardation and development of childhood cataracts, symptoms are generally more severe in Warburg Micro syndrome. The RAB3 proteins modulate secretion of neurotransmitters and hormones

and are regulated by RAB3 GTPase-activating proteins (RAB3GAP) [207, 208]. *RAB3GAP1* and *RAB3GAP2* encode, respectively, the catalytic and non-catalytic subunits of the RAB3 GTPase-activating protein and mutations in these genes have been directly linked to Warburg Micro syndrome. Linking studies in patients with similar symptoms but without these *RAB3GAP1/2* mutations revealed the involvement of another RAB, i.e., RAB18. RAB3GAP1 and RAB3GAP2 form a RAB3GAP complex, which in turn functions as a RAB18GEF [96]. Studies in mice have shown that Rab18 is a critical regulator of neuronal migration and morphogenesis of the developing cerebral cortex [24] and consequently, loss-of-function mutations in the *RAB18* have been associated with about 50% of Warburg Micro syndrome cases [96].

Other diseases related to loss-of-function mutations in Rabs that are not further discussed here are autosomal-recessive Carpenter syndrome (RAB23) [145] and X-linked mental retardation associated with autism, epilepsy, and macrocephaly, and early onset X-linked Parkinson's disease, which are both caused by mutations in RAB39b [169, 170] (Table 1).

Rab mutations can also (in)directly affect disease (progress) by independently contributing to the development of multifactorial neuronal diseases, such as Alzheimer's disease (Rab3a, Rab4, Rab5, Rab6, Rab7, Rab10, Rab21) [29, 62], Parkinson's disease (Rab10, Rab29, Rab32, Rab39B) [148, 149, 153, 170], Huntington's disease (Rab4, Rab5, Rab8, Rab11) [28, 176, 183, 184, 209] and metabolic diseases, such as diabetes (involvement of Rab1a, Rab2a, Rab3, Rab7, Rab8a, Rab11b, Rab26, Rab27a, Rab37, reviewed by Gendaszewska–Darmach et al.) [210].However, in these cases, disease progression and prognosis are not always related to loss-of-function mutations: e.g., overexpression of RAB5 and RAB7a has been reported in hippocampal neurons from patients with Alzheimer's disease (AD) [211, 212]. Furthermore, overexpression of other Rabs, such as RAB1 for example, has been shown to confer relative protection against the development of Amyotrophic Lateral Sclerosis (ALS) [213] and Parkinson's disease (PD) [214], RAB3b, which has been shown to protect dopaminergic neurons [215] or RAB4a, which is able to rescue synaptic defects and improve lifespan, and locomotor skills in Huntington's disease [176]. Similarly, increased Rab expression has been found to ameliorate the clinical phenotype in a range of disease models [13, 15, 16, 199].

In addition to mutations in the Rabs themselves, mutations in Rab-related proteins are also associated with (partial) dysfunction of Rab proteins or changes in Rab activity, e.g., mutations in Rab escort protein-1 (*REP1*) result in X-linked choroideremia blindness, due to the absence of functional RAB27A in the retinal pigment epithelium [216, 217], mutations in the geranylgeranyl-transferase (GGT) of Rab32/38 causes Hermansky–Pudlak syndrome [154] and malfunctioning of Rab GDP dissociation inhibitor-α (RabGDI-α) causes human X-linked mental retardation with epileptic seizures.

Together these data further underline the importance of intracellular membrane trafficking in the etiology of a wide range of diseases [57]. In addition, since altered Rab expression is often associated with disease progression and prognosis, better insight in the roles of Rabs, identification of new Rab effectors and other Rab interacting proteins will inevitably have an important impact on a better understanding of the diseases and the identification of novel treatment targets. Future therapies targeting these Rabs (through suppression or overexpression) and their downstream signaling partners could be helpful in the development of new treatment options for these specific diseases.

Rabs as treatment target for treatment of Rab-related inherited diseases

Most Rab GTPases display ubiquitous tissue expression and therefore diseases related to mutations in Rabs often affect multiple systems at the same time (Table 1). In most cases, treatment is therefore symptomatic, e.g., support of the immune system with IvIg infusions and antibiotics, or anti-epileptic medication for diseases with neurological involvement and epilepsy. None of these treatments however are curative. For Rab-related diseases, where the absence of Rab expression only affects a few tissues, such as is the case with GS-2 (RAB27a) or X-linked early onset Parkinson's disease (RAB39b), development of gene therapy could have therapeutic and clinical potential. The rarity of these type of mutations however, greatly impacts the possibilities for the development of novel (stem cell) gene therapy treatments. Recently, biobanks and biobanking projects have started to offer the use of patient-derived-induced pluripotent stem cells (iPSCs) that can be propagated indefinitely and can be used for human disease modeling and development of novel therapies. For both GS-2 [218, 219] and X-linked early onset Parkinson's disease [220] iPSC lines have recently been generated. However, a corrective gene therapy for these diseases is still under development [221].

Currently, the most effective gene therapy for Rab-related diseases is not actually targeting a Rab, but rather the Rab escort protein-1 (REP1). Choroideremia (CHM), an X-linked retinal degeneration of photoreceptors is caused by loss-of-function mutations in *REP1*. Using an AAV2 vector carrying the *REP1* gene, researchers showed the ability of the vector to effectively transduce human photoreceptors in vitro and CHM mouse retinas in vivo [222]. Using patient-derived lymphoblasts and iPSCs, the effectivity of the AAV2 constructs was confirmed [223], leading to the initiation of a

clinical trial [224]. An independent study using lentiviral vectors to transfer REP1 has also shown promise [225].

Primary HLH is characterized by uncontrolled inflammation and immune dysregulation and can be caused by a mutation in *RAB27a*. Previous success with the development of retroviral and lentiviral gene therapy for immune disorders and immune deficiencies has paved the way for the development of gene therapy for HLH/GS-2 [219, 226, 227].However, thus far pre-clinical gene therapy studies have focused on using retroviral vectors encoding the genes for Perforin and MUNC13-14, but not yet RAB27a. Development of gene therapy for CMT has only recently been initiated using AAV mediated delivery of neurotrophin-3 directly to muscle [228].However, vectors carrying the RAB7a gene have not been developed yet. Similarly, lentiviral gene therapy for Hermansky–Pudlak syndrome (HPS), directed at transfer of *BLOC-3* (a Rab32/38-GEF) to the lungs, bone marrow and the gastrointestinal system has recently gained some interest [229, 230] and shown some promise after efficient transduction of HPS patient-derived melanocytes. Thus, development of gene therapy for selected Rab-related monogenic diseases may be feasible in the future and may offer an alternative solution to otherwise untreatable diseases.

Role of Rabs in the development or progression of cancer

Mutations in Rab genes that result in aberrant Rab expression, alternative splicing variants, morphological changes in the Rab proteins affecting interaction with Rab effectors, or post-translational modifications of Rab proteins have been shown to cause functional impairments that may contribute to tumorigenesis and metastasis. Interestingly, different Rabs have been shown to be able to serve as oncogenes, as well as tumor-suppressor genes (reviewed by Krishnan et al. 2020) [231]. Because of the broad involvement of Rab proteins in the regulation of apoptosis, cell proliferation and survival of healthy cells, their role in the development and progression of cancer should not be underestimated. Below, we will give a brief overview of the most commonly observed cancers related to aberrant Rab signaling (also summarized in Table 2).

Brain cancer

RAB3a is involved in synaptic vesicle trafficking in the healthy brain. Elevated RAB3a expression in glioma cell lines and primary astrocytes is associated with increased cell proliferation and overexpression of RAB3a has been shown to increase the tumorigenicity of glioma cells, p53-/- astrocytes and the expression levels of various stem cell markers [235]. In addition, cells became resistant to irradiation and a variety of anti-cancer drugs. RAB3b expression was correlated with the severity of the gliomas, with high-grade gliomas expressing significantly higher levels of RAB3b than low-grade gliomas. Silencing of RAB3b was shown to inhibit glioma proliferation through cell-cycle arrest and induction of apoptosis [236]. RAB21 is also overexpressed in glioma cells and suppression of RAB21 has been shown to decrease glioma proliferation and induce expression of apoptosis-related proteins, such as Caspase7, BIM, and BAX [250]. RAB42 and RAB42 have been shown to be highly expressed in, respectively, glioblastoma and glioma cells and expression levels were directly correlated with a poor prognosis [265, 266].

Lung cancer

Invasiveness and metastasis of lung carcinoma cells triggered by hypoxia depends on RAB5 expression [241]. In some cases downregulation of the tumor suppressive abilities of Rabs has been shown to result in a poorer prognosis. For example, tissue inhibitor of metalloproteinase 1 (TIMP1) is carried by RAB37 and inhibits extracellular matrix turnover. Dysfunction or low expression of RAB37 or TIMP1 has been observed to support metastasis and result in poor survival [264]. Similarly, downregulation of RAB17 has been shown to promote cell invasion and enhance tumorigenicity of non-small cell lung cancer [190].

Gastric cancer

Increased RAB13 expression in gastric cancer cells has been associated with poor overall survival. Functional deletion of RAB13 has been shown to inhibit proliferation of gastric cancer cells and induce apoptosis [245].

Colorectal cancer

RAB3c has been found to be overexpressed in colorectal cancer tissue and increased expression was associated with risk of metastasis [237]. Similarly, RAB3d expression in colorectal cancer cells was increased and expression levels correlated with tumor size, lymphatic metastases and distant metastases [239]. Overexpression of RAB17 in colorectal cancer cells was shown to directly accelerate cancer cell proliferation [249]. In contrast, RAB31 expression in late-stage colorectal cancer cells affects the cancer stem cell niche by increasing cancer-associated fibroblasts, thus promoting further growth of the cancer cells [262, 267].

Pancreatic cancer

Several Rabs have been shown to play a role in the development or progression of pancreatic cancer. RAB3a,

Table 2 Relation between expression of Rabs, their use as a biomarker and effects on the development/progression of cancer

Rab protein	Cancer Type	Biomarker	Expression/Metastasis	References
Rab1A	Prostate		Downregulation; metastasis	[232]
	Bladder cancer		Upregulation; metastasis	[233]
	Liver		Upregulation; malignant growth, invasion, migration, metastasis	[232]
Rab2A	Breast		Upregulation; poor prognosis and relapse	[234]
Rab3A	Brain (glioma)		Upregulation; anti-cancer drug resistance, tumorigenesis and cancer stemness	[235]
Rab3B	Brain (glioma)		Silencing; inhibits proliferation and induces apoptosis	[236]
Rab3C	Colon		Upregulation; distant metastasis and poor prognosis	[237]
Rab3D	Breast (Triple Negative)	Tumor progression, diagnosis		[238]
	Colorectal	guidance in cancer diagnosis and treatment	Upregulation; metastasis and invasion	[239]
Rab4	Breast		Cooperation with Rab5, responsible for the invadosome	[240]
Rab5	Breast (triple negative)	Lymph node marker	Upregulation; metastasis and tumor cell migration, poor prognosis	[240, 241]
	Lung		Upregulation; invasiveness and metastasis	[241]
Rab7	Pancreas	Prognosis	Upregulation; poor prognosis	[242]
Rab9	Breast (triple negative)	Potential inhibitor therapy	Promotes proliferation, migration	[243]
Rab11	Breast		FIP1C; poor prognosis, downregulation may cause metastatic behavior	[244]
Rab13	Gastric		Upregulation; poor survival. Rab13GEF promotes metastasis and tumorigenesis	[245, 246]
Rab14	Gastric		Upregulation; metastasis. Potential therapeutic target for treating metastases	[188, 247]
Rab17	Liver		Normal expression; Rab17 acts as a tumor-suppressor (reduced tumorigenic properties)	[248]
	Lung (NSCLC)		Downregulation; promotes cell invasion and enhances tumorigenicity	[190]
	Colon		Upregulation; cancer proliferation	[249]
Rab21	Brain		Upregulation; poor prognosis and tumorigenesis	[250]
Rab23	Breast		Upregulation; inhibits growth and proliferation	[251]
	Bladder		Upregulation; invasiveness and metastasis	[252]
Rab25	Breast		Regulates apoptosis	[253, 254]
	Ovarian		Regulates apoptosis	[255, 256]
Rab27A	Liver		Upregulation; metastasis and reduced survival rate	[257]
	Prostate		Downregulation; aggressiveness and relapse	[258, 259]
Rab27B	Breast		Upregulation; poor prognosis	[260]
	Liver		Upregulation; reduced overall survival	[257]
Rab31	Breast		Upregulation; poor prognosis	[261]
	Colon		Promote cancer growth	[262, 263]
Rab37	Lung		Downregulation; poor prognosis and metastasis	[264]
Rab42	Brain (glioma)		Upregulation; poor prognosis	[265]

RAB21, RAB22a, RAB25 and RAB26 were all shown to be overexpressed in pancreatic cancer cells [68, 235, 250, 268]. In addition, overexpression of both RAB27a and RAB27b has been shown to be correlated with poor overall survival in pancreatic cancer [269, 270]. Therefore, downregulation of RAB27a and RAB27b has been suggested as a way to induce apoptosis in pancreatic ductal carcinoma cells [271]. Similarly, downregulation of RAB38 (which is highly expressed in pancreatic adenocarcinoma cells and promotes cell proliferation) was shown to suppress pancreatic cell proliferation and metastasis [272]. Upregulation of RAB7A in pancreatic adenocarcinoma cells has been linked to a poor prognosis as well [242].

Bladder cancer

RAB1a has been found to be overexpressed in bladder cancer cells and its expression was positively correlated with tumor size and risk of metastasis [233]. Increased RAB23 and RAB27 expression has also been linked to bladder cancer by activation of the NF-kB signaling pathway and knockdown of these Rabs has been shown to result in inhibition of cell growth and invasion [252, 273].

Breast and ovarian cancer

Many Rabs have been shown to be involved in the development or progression of breast cancer. Some of these Rabs have been demonstrated to specifically promote breast cancer invasiveness by regulating endocytic recycling of membrane type 1 matrix metalloproteinase MT1-MMP, e.g., RAB2a [234], by increasing MT1-MMP activation (RAB4 and RAB5) [240], by promotion of epithelial-to-mesenchymal transition (RAB3d) [238] or by regulation of α6β4 integrin trafficking (RAB11) [274]. Other Rabs have been shown to modulate breast cancer cell proliferation and survival by inhibition of apoptosis (RAB9) [243]. RAB23 has been shown to inhibit growth and proliferation of breast cancer cells by inhibiting DNA synthesis and induction of apoptosis [251], whereas RAB25 regulates apoptosis in both breast and ovarian cancer [254, 275]. Overexpression of RAB15, RAB27b and RAB31 is associated with a poor prognosis in both breast and ovarian cancer [260, 261, 276].

Prostate cancer

Both RAB27 and RAB28 are downregulated in advanced prostate cancer and their expression levels are inversely correlated with the severity of the cancer [259]. In contrast, RAB3b expression was found to be increased in prostate cancer cells and supports relapse of prostate cancer. Mechanistically, it has been shown that the NKX3-1 homeobox gene, which is involved in prostate cancer progression, and androgen receptors (AR) regulate each other in a feed-forward regulatory loop and promote prostate cancer cell survival through overexpression of RAB3b [258].

Miscellaneous

As described above, due to their tissue-wide spread expression pattern, mutations in Rabs have been shown to affect many important cell systems. Upregulation of Rabs that function as oncogenes or downregulation of Rabs that function as tumor-suppressor genes both have been related to the development of many different types of cancer. Although the Rabs involved in the development of the most commonly occurring malignancies have been described above, some Rabs deserve separate mentioning. Gene expression analysis studies have shown increased expression of RAB45/RASEF in a variety of cancer cells, including myeloid leukemia, uveal malignant melanoma, colorectal cancer, lung cancer and breast cancer [14]. Detection of RAB39B expression in diffuse large B-Cell lymphoma has shown promise for its use as a chemosensitivity-related biomarker of progression [277]. Lastly, Rab35 has been linked to the development of several cancers, such as breast and lung cancer, due to its involvement in cell migration and activation of the Akt signaling pathway [278].

Conclusions

Tight regulation of Rab expression and its effectors is important to maintain cellular homeostasis. Genetic diseases related to loss-of-function mutations in Rabs affect multi-organ systems and may become apparent during embryonic development, affecting development of the brain and eye and the musculoskeletal system. Others may reveal themselves later and result in immune dysregulation or slowly progressive diseases and neurodegenerative syndromes. In addition, aberrant expression of multiple Rabs has been shown to be strongly linked to the development of many types of malignancies. Targeting the Rabs for the treatment of inherited genetic diseases using stem cell gene therapy shows great potential, but is still in its infancy. Monitoring of Rabs as tumor-specific biomarkers to measure tumor progression, distant metastases and the effects of treatments also shows great promise (Table 2). Lastly, targeting of cancer-related Rabs with miRNAs to control their expression levels will show its effects in the coming decade.

Acknowledgements This work was supported by grants from the Hacettepe University, Scientific Research Coordination Unit project nr. TUK-2019-17760 and THD-2022-19940 and the Scientific and Technological Research Council of Turkey (TÜBİTAK) project nr. 219S675.

Author contributions ODE and SS: drafted the first version of the article; FA-K: designed the manuscript, reviewed the contents and wrote the final version of the article to be published; all authors have read and approved the final manuscript.

Data availability Not applicable.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Not applicable.

Consent to participations Not applicable.

Consent to publications Not applicable.

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Supplement 11. Thesis related submitted paper 3

Human Cell

Assessment of potential side effects related to RAB27A overexpression in mesenchymal and hematopoietic stem cells.

–Manuscript Draft–

Manuscript Number:	HUCE-D-23-00861
Full Title:	Assessment of potential side effects related to RAB27A overexpression in mesenchymal and hematopoietic stem cells.
Article Type:	Research Article
Keywords:	Keywords: GTPases, RAB27A, Griscelli Syndrome Type 2, Lentivirus, Gene Therapy, Cancer
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Funding Information:	Hacettepe Üniversitesi (THD-2022-19940) — Assoc. Prof. Fatima S. F. AERTS KAYA
	Hacettepe Üniversitesi (TUK-2019-17760) — Assoc. Prof. Fatima S. F. AERTS KAYA
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Abstract:	RAB27A plays an important role in the regulation of exocytosis and intracellular vesicle trafficking. Loss-of-function mutations in the RAB27A gene cause dysfunctional immune cells and Griscelli Syndrome Type 2 (GS-2), whereas upregulation of RAB27A in cancer cells is associated with a worse prognosis and increased metastasis. Here, we wanted to assess the potential side effects of overexpression of RAB27A in different types of healthy stem cells, as preparation for the development of gene therapy for GS-2. Mesenchymal stem cells (MSCs) and hematopoietic stem/progenitor cells (HSPCs) were transduced with a lentiviral vector, carrying a co-don-optimized RAB27A transgene. Cells were used for in vitro functional assays and in vivo transplantation assays to assess the effect of RAB27A on stem cell function. Overexpression of RAB27A resulted in phenotypic changes in MSCs, and decreased colony forming capacity of HSPCs. Transplantation of RAB27A+ stem cells was not associated with any tumorigenesis. Despite high expression of RAB27A in HSPCs

Assessment of potential side effects related to *RAB27A* overexpression in mesenchymal and hematopoietic stem cells.

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Abstract

RAB27A plays an important role in the regulation of exocytosis and intracellular vesicle trafficking. Loss-of-function mutations in the RAB27A gene cause dysfunctional immune cells and Griscelli Syndrome Type 2 (GS-2), whereas upregulation of RAB27A in cancer cells is associated with a worse prognosis and increased metastasis. Here, we wanted to assess the potential side effects of overexpression of RAB27A in different types of healthy stem cells, as preparation for the development of gene therapy for GS-2. Mesenchymal stem cells (MSCs) and hematopoietic stem/progenitor cells (HSPCs) were transduced with a lentiviral vector, carrying a co-don-optimized RAB27A transgene. Cells were used for in vitro functional assays and in vivo transplantation assays to assess the effect of RAB27A on stem cell function. Overexpression of RAB27A resulted in phenotypic changes in MSCs, and decreased colony forming capacity of HSPCs. Transplantation of RAB27A+ stem cells was not associated with any tumorigenesis. Despite high expression of RAB27A in HSPCs before transplantation, RAB27A levels in peripheral blood, bone marrow and spleen cells remained low, indicating overexpression of RAB27A may have affected the long-term reconstitution potential. Development of gene therapy for GS-2 may require finetuning of RAB27A expression, but is not likely to be complicated by RAB27A-induced tumorigenesis.

Keywords: GTPases, RAB27A, Griscelli Syndrome Type 2, Lentivirus, Gene Therapy, Cancer

Introduction

Rab proteins are small GTPases that play a role in intracellular membrane trafficking [1]. They control membrane identity, migration to and fusion of membranes, regulating cell signaling, cell proliferation and survival. Many Rabs, including Rab3, Rab5, Rab7, Rab9, Rab14, and Rab27 have been associated (in)directly with the development of different types of cancers [2].

RAB27A is involved in the regulation of specialized secretory granules, such as melanosomes, lytic granules and platelet-dense granules. Mutations in *RAB27A* and its effectors, *MUNC14-4* and *MYO-VA*, may cause the development of Griscelli Syndrome Type 2 (GS-2) [3, 4], which presents as an immunodeficiency and is caused by a dysfunction of intracellular vesicle trafficking. Where dysfunction of melanocytes results in hypopigmentation, dysregulation of innate and effector immune cells, including neutrophils, macrophages and T cells, causes an increased susceptibility to infections and hemophagocytic lymphohistiocytosis (HLH) [3, 4].

Current treatment for GS-2 is hematopoietic stem cell (HSC) transplantation [3, 4], but in the absence of a donor lentiviral (LV) gene therapy may be another option. For some immune deficiencies, including Adenosine Deaminase (ADA) SCID, X-linked SCID (X-SCID), Recombination activating-gene (RAG) 1 and 2 deficiency, Chronic Granulomatous Disease (CGD) and Wiskott–Aldrich Syndrome (WAS) gene therapy has been developed [5-10]. However, since the use of retroviral vectors has been associated with insertional mutagenesis [11-14], lentiviral vectors with a more favorable safety profile are being preferred for clinical development [7, 15]. Although LV gene therapy has been linked with an increase in leukemia after HSC gene therapy, these events were linked to the promoter or the transgene, rather than to the vector [16-18].

The role of RAB GTPases in (the progression of) cancer has become an important topic in cancer research. Intracellular vesicular transport and secretion affects critical mechanisms, such as tumor cell metastasis and invasiveness [2]. RAB27A is an important regulator of these mechanisms and it may play a direct role in the invasion and metastasis of cancer [19]. For example, RAB27A is involved in the secretion of matrix metalloproteinases (MMPs) [20-22], which through degradation of extracellular matrix may promote cancer cell invasion. Furthermore, upregulation of RAB27-dependent recycling of MT1-MMP may promote invasiveness of breast cancer cells [22]. RAB27A has been implicated in the regulation of exosome secretion at the plasma membrane [23], facilitating cancer cell survival and metastasis [24]. However, upregulation of RAB27A may be secondary to other changes in precancerous cells [22, 25], although enforced overexpression of RAB27A has been shown to increase cell proliferation, invasion and chemoresistance of cancer cells [26].

Here, we wanted to assess the possible negative or neoplastic stimulating effects of overexpression of RAB27A in mesenchymal stem cells (MSCs) and hematopoietic stem and progenitor cells (HSPCs). We used a lentiviral vector with the constitutively active SF promoter to unveil any risks related to overexpression of RAB27A, as a preliminary safety study for the development of lentiviral gene therapy for GS-2. This study may enhance our understanding of the cancer biology of stem cells and identify RAB27A as a potential therapeutic target. In addition, it will allow the development of safe gene transfer methods for the treatment of GS-2.

Results

Overexpression of RAB27A in MSCs and HSPCs may interfere with stem cell function

First, we wanted to identify cells with low endogenous RAB27A expression. We measured RAB27A expression in three leukemic (Daudi, HL60 and Jurkat) and the HEK293T cell lines (Figure S1). RAB27A expression was highest in the HEK293T cells, whereas expression of RAB27A in the leukemic cell lines was highly variable (20-90%). Since endogenous expression of RAB27A in MSCs and HSPCs was considerably lower (≤20%), we decided to use these stem cells for the overexpression assays. We used human BM-MSCs, human UCB CD34+ and murine BM CD117+ HSPCs. BM-MSCs expressed high levels of the MSC-specific markers CD29, CD44, CD73, CD90, CD105 and CD166 (all>95%) and showed normal differentiation (Figure S2). Lentiviral transduction rapidly increased RAB27A expression (Figure 1A), but caused a decrease in MSC adherence markers, including CD29 (68,6%), CD44 (92,6%) and CD105 (18,4%) and complete loss of expression of the MSC stem cell markers CD73, CD90, CD166. However, it did not affect differentiation of RAB27A+ MSCs (Figure 1B), indicating possible interference of RAB27A with MSC stemness/function, but not adherence or viability.

Similar to MSCs, we found low endogenous expression of RAB27A in UCB CD34+ and BM CD117+ HSPCs, which rapidly increased after transduction (Figure 2). RAB27A overexpression in HSPCs resulted in a decrease in colony forming numbers, although overall CFU morphology remained unchanged (Figure 3A, Table S2).

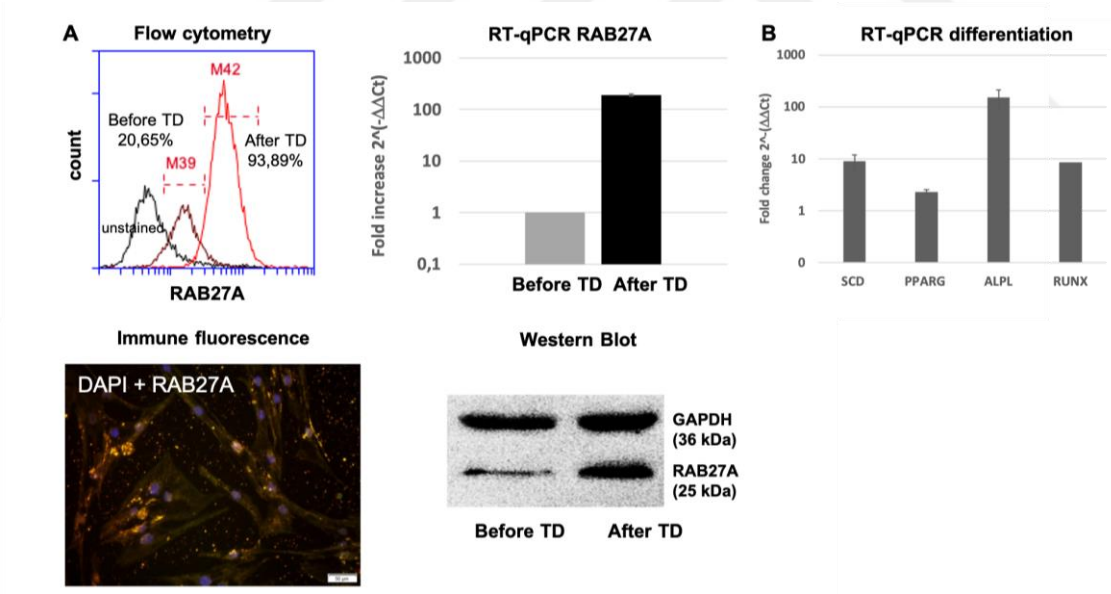


Figure 1. RAB27A expression in MSCs before and after transduction. Healthy BM-MSCs were transduced with LV-RAB27Aco. (A) Expression of RAB27A in MSCs before and after transduction was confirmed using flow cytometry (upper left), RT-qPCR (upper right), immunofluorescence microscopy (lower left) and Western Blot (lower right). (B) RAB27A+ MSCs were differentiated for 7 days and expression of *SCD* (early adipogenic marker), *PPARG* (late adipogenic marker), *ALPL* (early osteogenic marker) and *RUNX2* (late osteogenic marker) was measured.

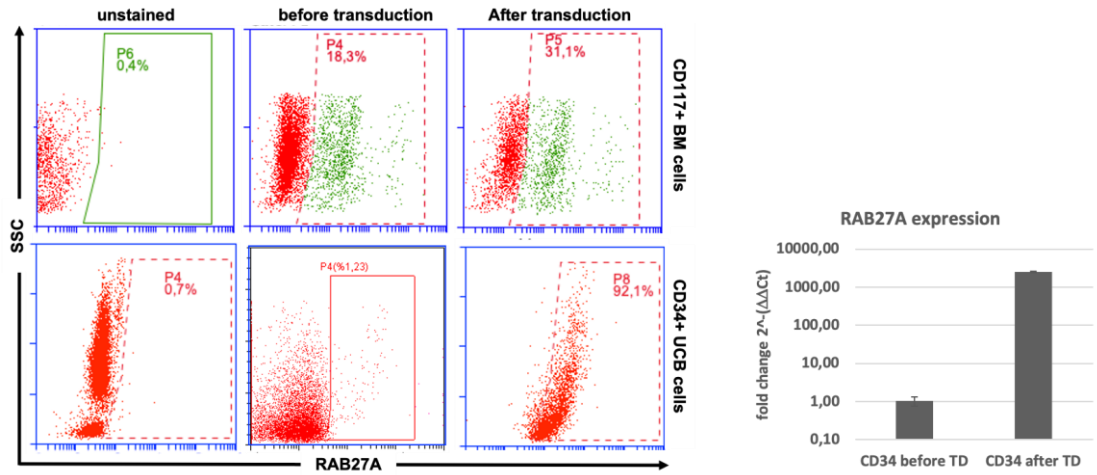


Figure 2. RAB27A expression in human and murine HSPCs before and after transduction. Healthy donor UCB CD34+ HSPCs and murine BM CD117+ cells were transduced with LV-RAB27Aco. Expression of RAB27A in the CD34+ and CD117+ cells before and after transduction was assessed using flow cytometry (left) and in CD34+ cells confirmed with RT-qPCR (right).

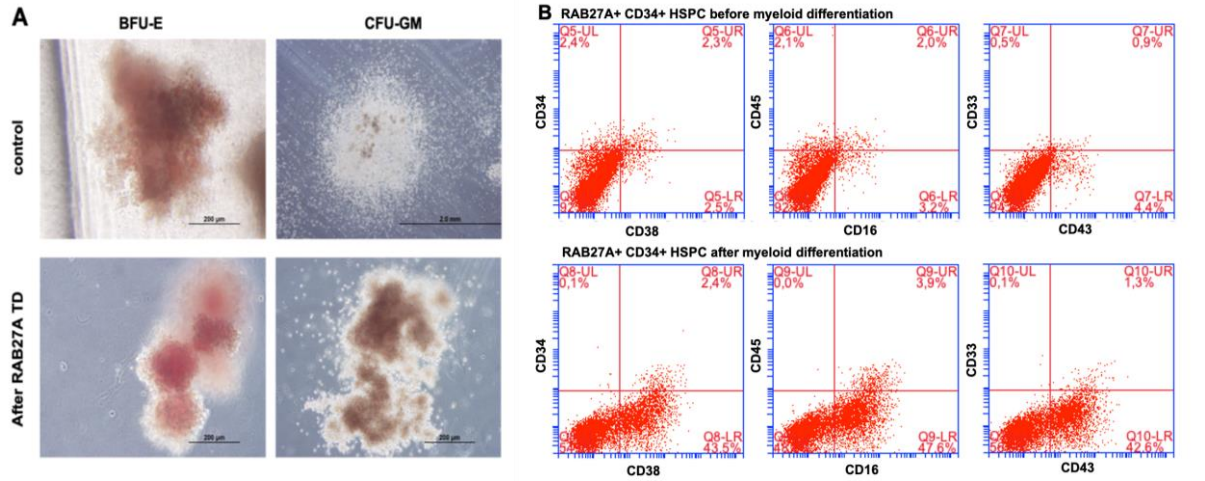


Figure 3. RAB27A expression does not affect the differentiation potential of HSPCs. Myeloid differentiation of CD34+ HSPCs before and after transduction. (A) Control and RAB27A transduced CD34+ HSPCs were assessed for Erythroid (BFU-E) and Granulocytic/Monocytic (CFU-GM) colony formation; (B) RAB27A transduced CD34+ HSPCs were assessed for expression of HSPC (CD43 and CD34) and mature myeloid markers i.e., CD16, CD33, CD38 and CD45 before and after differentiation.

We observed no lineage bias towards erythroid or granulocytic lineage. Assessment of RAB27A+ HSPCs before and after differentiation revealed a normal increase in myeloid-specific markers (Figure 3B). These data indicate that RAB27A overexpression is not directly associated with MSC or HSPC toxicity or viability. However, general stem cell function and/or stemness may be affected by continuous overexpression of the protein.

RAB27A+ MSCs do not cause development of mesenchymal tumors

After transplantation of LV-RAB27A-transduced or control MSCs in Rag2 mice we did not observe any signs of tumorigenic or immune activity. Although one mouse (HU-R338) from the MSC control group developed acute

myeloid leukemia (AML), none of the other mice transplanted with MSCs, either transduced or non-transduced, showed any signs of leukemia (Figure S3). Clinical inspection of the mice did not suggest the development of solid tumors and all mice, except for one mouse in the SF-RAB27A treated group (HU-R490), were sacrificed at the intended period. However, this mouse was killed by its litter mates and could not be assessed. Detailed inspection of the tissues adjacent to the injection site showed no signs of aberrant growth or malignant cells, although mononuclear cell infiltrates were observed in the surrounding loose subcutaneous connective tissue and striated muscles in mice from both groups (Figure 4A-L). Similarly, inspection of the lung tissue, as the primary tissue for metastasis in this area, showed the presence of widespread granulation tissue, characterized by dense cellular infiltrates in the peribranchial and bronchiolar lung stroma of both mice transplanted with MSCs and RAB27A+ MSCs, but no evidence of malignant cells (Figure 4M-Y).

RAB27A overexpression in HSPCs affects their long-term engraftment potential

We then transplantated control or RAB27A-transduced BM CD117+ or UCB CD34+ HSPCs into Rag2 mice. Engraftment was followed by assessing PB CD3+ T cell and CD19+ B cells. Whereas transplantation of murine CD117+ HSPCs resulted in robust immune recovery, transplantation of human CD34+ HSPCs resulted in only marginal engraftment (Figure 5A). The latter is expected since the Rag2 mouse model is leaky with residual NK cell activity, and may allow low engraftment in a xenotransplant setting. We found no differences in c-kit+ or CD34+ cell numbers in mice transplanted with control or RAB27A+ HSPCs. We observed the appearance of of CD3 and CD19 cells derived from the transplants, especially in the mice transplanted with murine RAB27A+ CD117+ cells. However, the level of intracellular RAB27A expression in the PB, BM and spleen total nucleated cells of these mice was unexpectedly low (Figure S4), despite high transduction levels before transplantation. Thus, RAB27A transduced cells may have contributed only marginally to the total engraftment or alternatively that RAB27A expression may have been lost in time. We therefore decided to assess *RAB27A* gene expression in the BM of mice transplanted with RAB27A+ CD117+ and CD34+ HSPCs using RT-qPCR (Figure 5B). Since we used primer sequences designed to detect our codon-optimized transgene, we are not able to measure the presence of physiological *RAB27A* gene expression before transplantation. We therefore used the ΔCt method, rather than the ΔΔCt method. Although we detected gene expression of *RAB27Aco* in the BM of transplanted mice, the relative expression levels *RAB27Aco* were very low. To assess overall engraftment of male (Sry+) Balb/c control and transduced CD117+ cells in female (Sry-) mice, we measured *Sry* expression in BM of transplanted animals. In agreement with the immune reconstitution data, we found high expression of Sry in BM and spleen tissue. However, since this *Sry* expression did not correlate with *RAB27A* expression, we conclude that RAB27A transduced cells were not a major contributing cell fraction to the overall success of the transplantation.

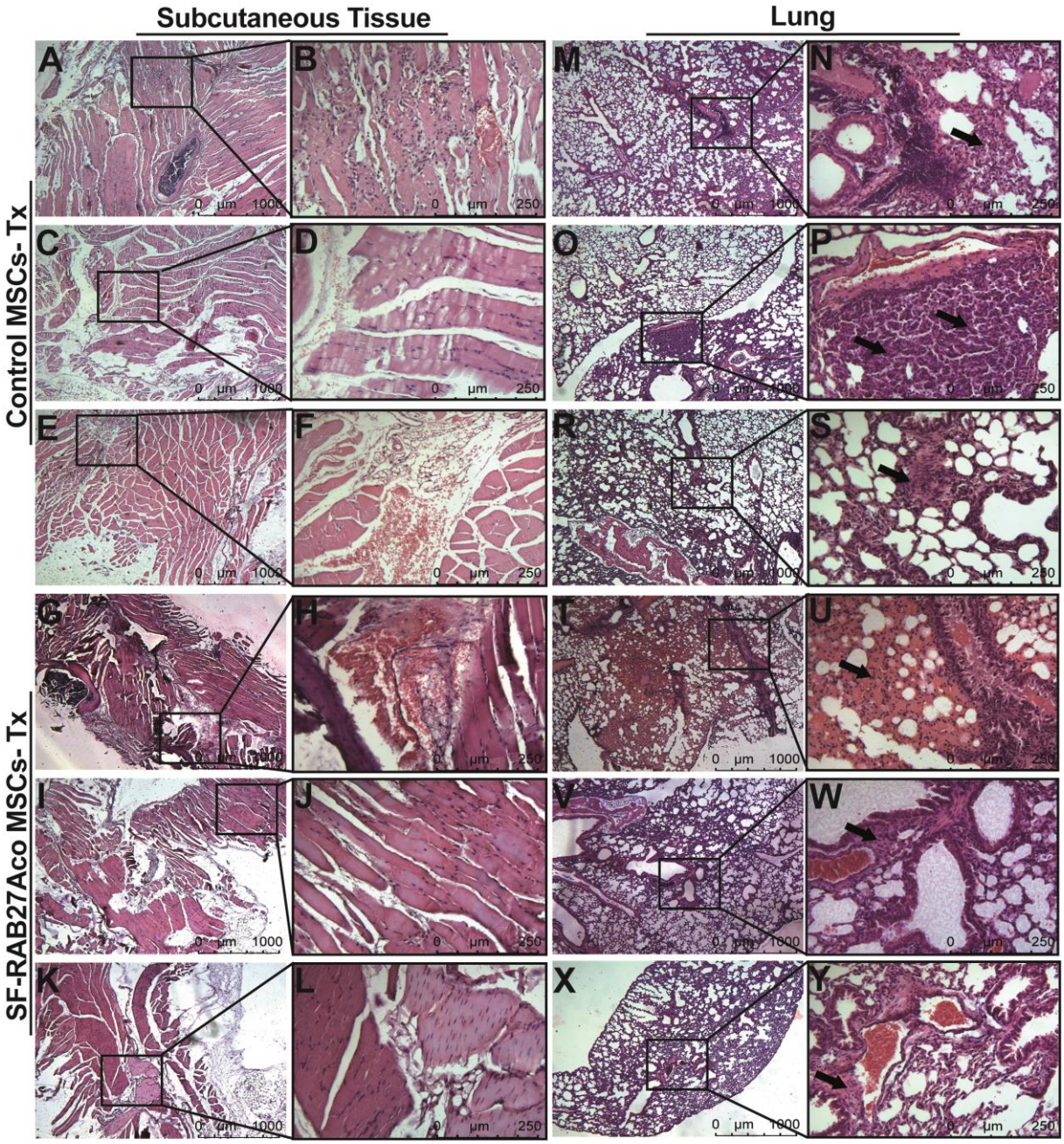


Figure 4. Histological assessment of MSC injection sites. Control and RAB27A+ MSCs were transplanted into Rag2 mice. Histological staining with H/E of injection sites (A-F) control MSCs; (G-L) RAB27A+ MSCs and lung tissue (M-S) control MSCs; (T-Y) RAB27A+ MSCs were assessed for the presence of malignancy or inflammation. Mononuclear cell infiltrates was detected in connective tissue and striated muscles of mice in both groups (A-L). Lung parenchyma showed healthy alveoli, bronchi and bronchioles. Peribranchial and bronchiolar lung stroma exhibited cellular infiltrates (M-Y) without signs of malignant cells (black arrows).

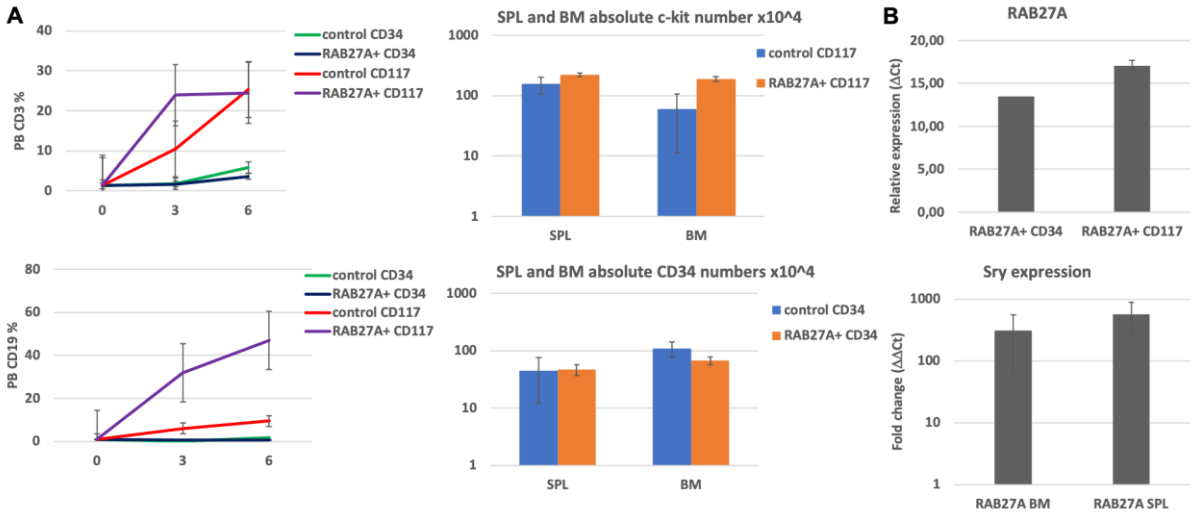


Figure 5. Assessment of engraftment potential of RAB27A transduced HSPCs. (A) Control and RAB27A+ human CD34+ and murine CD117+ HSPCs were transplanted in Rag2 mice. Engraftment was followed by assessing the appearance of CD3 T cells and CD19 B cells in the PB (left). After sacrifice changes in CD117+ and CD34+ cell numbers in bone marrow (BM) and spleen tissue (SPL) were assessed (middle). (B) Despite BM and SPL chimerism (as measured by *Sry*) *RAB27A* gene expression in BM of mice transplanted with RAB27A transduced cells was very low and not detectable in SPL (right).

Discussion

Many Rab GTPases are involved in the initiation, progression or metastasis of a wide range of malignancies [2]. RAB27A plays a role in intracellular membrane trafficking, exocytosis and regulation of cell-to-cell communication through extracellular vesicles. However, the potential role of RAB27A in the development of malignancies is unknown. RAB27A expression is upregulated in specific cancers [20, 25, 27] and promotes proliferation and invasiveness of cancer cell lines [27, 28]. In contrast, inhibition or suppression of RAB27A expression has been correlated with a better prognosis in certain cancers and decreased proliferation and invasiveness of cancer cells [29, 30]. Nevertheless, it remains unclear whether RAB27A functions as a trigger, merely facilitates cancer or metastasis or is actively involved in the development of the malignancy. Here, we assessed potential risks related to overexpression of RAB27A, as a preparation for the development of gene therapy for GS-2.

We did not find any evidence for tumorigenesis-initiating effects of RAB27A overexpression in MSCs or HSPCs. However, we did notice that after RAB27A overexpression both MSCs and HSPCs appear to be functionally impaired. Although lentiviral transduction was not associated with loss of viability, overexpression of RAB27A in MSCs resulted in an isolated decrease in expression of MSC stem cell, but not adhesion markers, indicating a possible effect on stemness. Conversely, ectopic overexpression of RAB27A in cancer stem cells has been shown to promote stemness of these cells via upregulation of cytokine secretion, resulting in promotion of proliferation and metastasis [25]. These contradictory findings suggest that RAB27A may suppress mesenchymal epithelial transition (MET) in healthy cells, but suppress epithelial mesenchymal transition (EMT) in cancer cells [31]. We did not observe any negative effects of RAB27A overexpression on differentiation. RAB27A overexpressing CD34+ HSPCs showed normal myeloid differentiation, although the CFU numbers appeared to be decreased.

Other mechanisms through which RAB27A overexpression may affect stem cells is by interference with normal physiological functions. Previous studies have shown a role for Rabs in the development of neurodegenerative diseases and upregulation of RAB27 in cholinergic neurons has been shown to be correlated with cognitive decline in individuals with cognitive impairment and Alzheimer's disease [32]. It has been suggested that RAB27A may mediate cognitive impairment through interference with neurotransmitter release and dysregulation of axonal transport [33].

To assess the effects of RAB27A overexpression *in vivo*, we transplanted Rag2 mice with RAB27A overexpressing MSC or HSPCs. We did not observe any tumors in the RAB27A+ MSC transplanted group, although we did encounter one case of AML in the MSC control group. However, since the spontaneous development of leukemias has been described for several immune deficient mouse models [34, 35], and since the origin of the leukemia is hematologic and not mesenchymal, we believe this leukemia to be a result of the weakened immune system. Other than this, we only observed mononuclear cell infiltrates around the MSC injection sites and in lung tissue [36] in both control MSC and RAB27A+ MSC transplanted groups.

Since chimerism after transplantation with human CD34+ HSPCs were low (<5%, based on PB T/B cell numbers), it was difficult to assess any effect of RAB27A overexpression. We found 4% CD34+ in BM and SPL tissue, indicating low, but persistent human cell engraftment, with no differences in engraftment between the control CD34 and RAB27A+ CD34 transplanted mice. We did not detect evidence of leukemia in any of the transplanted mice. Considering the high level of transduction efficiency (>90% in CD34+ HSPCs), we expected a significant amount of RAB27A expression in PB, BM and spleen. However, when we assessed these tissues, we found very low expression of *RAB27A*. Mice transplanted with CD117+ cells showed engraftment with high levels of T/B cells, especially in mice transplanted with RAB27A+ CD117 cells. Despite high levels of *Sry* expression, RAB27A expression in RAB27A+ CD117 mice was not higher than that of control CD117 mice, indicating that either 1) RAB27A transduced cells did not contain the HSPCs with long-term engraftment potential; 2) RAB27A+ HSPCs were functionally defect, resulting in a lower-than-expected contribution to overall engraftment; 3) RAB27A+ HSPCs may have lost expression of RAB27A due to methylation of CpG islands in the SF promoter, which has been shown to occur commonly during hematopoietic differentiation [37].

Thus, RAB27A overexpression in healthy stem cells does not appear to initiate a cascade of events that result in the formation of tumorigenic tissue, nor did it interfere with their viability or differentiation capacity. However, upregulation of RAB27A in many cancers indicates that RAB27A may create a favorable environment for cancer cell growth and progression. We found that stem cells overexpressing RAB27A may show changes in cellular behavior that may result in loss of stemness. Since effective treatment of GS-2 with LV-RAB27A does not require cross-correction of neighboring cells (as is the case with many metabolic diseases), localized RAB27A expression in specific immune cells only would be sufficient. For the development of gene therapy for GS-2 we should therefore aim at RAB27A expression levels that are closer to physiological expression levels, for example by using a PGK or methylation-resistant UCOE promoter [10]. Alternatively, RAB27A expression could be regulated by using the RAB27A promoter itself or miRNA to suppress excessive RAB27A expression in the stem cells.

Materials and methods

Animals

Mouse studies were carried out at the Hacettepe University Laboratory Animals Research and Application Center after approval of the experimental procedures by the Hacettepe University Animal Experiments Ethical Committee (2020/02-03). BALB/c-Rag2$^{-/-}$ (Rag2) mice were kindly provided by Prof. Dr. Gerard Wagemaker (Erasmus University Medical Center, Rotterdam, The Netherlands) [10]. Healthy Balb/c mice were purchased from Ankara University Experimental Animal Research Laboratory. Animals were allowed free access to irradiated chow.

Isolation of MSCs and HSPCs

BM-MSCs from healthy donors and GS-2 patients were obtained after approval by the Hacettepe University Ethical Committee for Non-Interventional Clinical Research (GO14/424) and informed consent [38]. MSCs were cultured with DMF10, consisting of DMEM-LG (Dulbecco's Modified Eagle Medium-Low Glucose, Thermo Fisher Scientific, #31885), MCDB-201 (Sigma, #M6770) medium, 10% Fetal Bovine Serum (FBS, Life Technologies, #10270), 1% penicillin/streptomycin (P/S, Gibco, #15140) and 2 mM L-glutamine (Sigma, #G3126). Adipogenic and osteogenic differentiation of MSCs was done, as described before [39].

Human umbilical cord blood (UCB) was collected from healthy newborns at the Hacettepe University of Obstetrics and Gynecology Department after approval by the Hacettepe University Non-Interventional Ethics Committee, GO20/316. UCB CD34+ HSPCs were selected, as previously described [40]. During lentiviral transductions CD34+ HSPCs were cultured overnight in HSC expansion medium (Miltenyi, #130-100-463) with 30 ng/mL human TPO (rhTPO, R&D Systems, #288-TP-025). For colony assays 10^3 cells were resuspended in Methocult Classic (Stem Cells Technologies, #H4434). Myeloid colonies were counted 10-14 days later.

Balb/c BM cells were collected from male mice and used for CD117+ selection using lineage depletion (Miltenyi, #130-090-858), followed by positive selection for CD117 (Miltenyi, #130-091-224). CD117+ BM cells were cultured with HSC expansion medium (Miltenyi, #130-100-463) with 30 ng/mL murine TPO (rmTPO, Peprotech, #315-14) [41].

Lentiviral vector constructs and transduction

The transgene encoding codon-optimized human *RAB27Aco*, was cloned into a lentiviral backbone plasmid with a constitutively active SFFV promoter (pRRL.PPT.SF.RAB27Aco.IRES.EGFP.wPRE4*.SIN, or briefly LV-RAB27Aco) [10, 42]. LV production and titration were done according to standard procedures [42]. Overexpression of RAB27A was obtained by overnight lentiviral transduction of MSCs (MOI:20), CD34+ and CD117+ cells (both MOI:30).

Immunophenotyping

MSCs were resuspended in PBN (PBS, 5% BSA, 0,5% NaN3) + 2% human AB serum. Cells were stained with antibodies against CD29, CD34, CD44, CD73, CD90, CD105 and CD166. CD34+ HSPCs were stained with anti-CD34, CD38 and hCD45, murine CD117+ cells were stained with anti-CD117, Sca-1 and mCD45. All antibodies were from BioLegend. For intracellular RAB27A staining, cells were fixed with Fixation buffer (Becton Dickinson, #554655) and permeabilized with Perm Buffer III (Becton Dickinson, #558050). Cells were incubated

with anti- RAB27A antibody (Thermo Scientific, #4D3F11), followed by incubation with anti-mouse-FITC (eBioscience, #11-4220-82) antibody. Cells were assessed using a BD Accuri C6 plus flow cytometer (Becton Dickinson).

Western Blotting

Cells were lysed with RIPA buffer containing protease inhibitors (Roche, #5892970001). Proteins (20 ug) were loaded onto a 15% polyacrylamide gel and transferred to the PVDF membrane with a Trans-Blot system (Biorad, #1704150). Membranes were blocked with 5% non-fat dry milk (Bio-Rad, #1706404) in 0,1% Tween-20 in TBS (TBS-T) and incubated with anti-RAB27A antibody (1:1000, Saint John's, #STJ25258). Membranes were then stained with a secondary antibody (1:10000, Advansta, #R-05072-500). GAPDH (1:10000, Invitrogen, #MA5-15738) was used as a housekeeping protein. Proteins were detected using ECL (Thermo Scientific, #32132) and imaged using a FluorChem FC3 (R&D Systems).

Immunofluorescence staining

LV-RAB27Aco transduced BM-MSCs were fixed with 4% paraformaldehyde (Sigma, #8187085000) and permeabilized with 0,1% Triton X-100 (Merck, CAS # 9036-19-5) in PBS. Cells were blocked with blocking buffer (BB) consisting of 5% BSA (Merck, #10735094001), 0,1% Tween-20 (Cellconic, CAS #9005-64-5) in PBS and stained with anti-GAPDH and anti-RAB27A antibodies followed by incubation with goat anti-rabbit IgG (#ab175471) and goat anti-mouse IgG (#ab175473), respectively. Nuclei were counterstained with 5 mg/mL DAPI (Sigma, #D8417-5MG). Photographs were taken with an inverted microscope (Olympus LS, IX73) and analyzed using ImageJ software (NIH, Java, 2022).

qRT-PCR

RNA isolation was performed with the Direct-zol RNA isolation kit (Zymo, #R2062), according to the manufacturer's protocol. RNA was reverse transcribed to cDNA and qRT-PCR was performed using the GoTaq 2-Step RT-qPCR kit (Promega, #A6010) and LightCycler 480 Probes Master mix (Roche, # 04707494001) assessed using a Light Cycler 480 II (Roche, USA). The primer sequences used are shown in Table S1. *GAPDH* and *B2M* were used as a housekeeping gene for normalization and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression.

Transplantation and follow-up

$1x10^6$ RAB27A+ or control MSCs were injected s.c. with 100 uL Matrigel (Corning, #354234) into Rag2 mice (n=3 per group). Mice were followed for 3 months. Upon sacrifice, tissues surrounding the injection site and lungs were collected. PB cells were used for FACS analysis. RAB27A+ CD34+ (n=3) and CD117+ (n=3) HSPCs were injected i.v. at a concentration of $1,2x10^5$ CD34+ or $3x10^4$ CD117+ cells, respectively, into Rag2 mice pretreated with 25 mg/kg Busulfan. Non-transduced human CD34+ (n=3) and murine CD117+ (n=3) cells were injected in control mice. Mice were observed for 6 months. Upon sacrifice, spleen (SPL), peripheral blood (PB) and bone marrow (BM) cells were collected and assessed for the presence of RAB27A.

Histological analysis

Tissue samples surrounding the injection sites and lung tissue were collected and assessed since these are often the primary metastatic sites of malignant cells after transplantation in the intrascapular subcutaneous region [36]. Samples were fixed in 10% phosphate-buffered formaldehyde solution, dehydrated through a graded series of ethanol and cleared in Xylene in an automated tissue processor (TP1020, Leica, Germany). Paraffin-embedded samples (LG1150H-C, Leica, Germany) were cut into 3 µm thick sections (SM2000R, Leica, Germany), deparaffinized at 60ºC overnight and stained with Hematoxylin/Eosin (H&E). Samples were assessed for the presence of MSCs or any pathology related to the transplantation (i.e., infections, signs of inflammation, tumor growth, aberrant cell differentiation, etc.) using a bright field microscope (DM6B, Leica, Germany) and analyzed using the attached image analysis program (LASX, Leica, Germany).

Statistical analysis

Student T-test analysis was performed to determine statistical significance (p-value <0,05) for differences between two groups. All calculations were performed using the Microsoft Excel spreadsheet program.

Author Contributions

Conceptualization: Ö.D.E., N.v.T. and F.A.K;
Methodology, Ö.D.E., M.E.Ş., B.P., M.G. and N.v.T.;
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Investigation, Ö.D.E. and F.A.K.;
Resources, Ö.Ö.;
Data curation, Ö.D.E., M.E.Ş., B.P. and M.G.;
Writing—original draft preparation, Ö.D.E.;
Writing—review and editing, F.A.K.;
Visualization, M.G. and P.K.;
Supervision, F.A.K.;
Funding acquisition, Ö.D.E. and F.A.K.
All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by grants from the Scientific and Technological Research Council of Turkey (TÜBİTAK) project nr. 219S675 and the Hacettepe University Scientific Project Coordination Unit (BAP), TUK-2019-17760 and THD-2022-19940.

Institutional Review Board Statement:

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Hacettepe University Animal Experiments Ethical Committee (2020/02-03).

Informed Consent Statement:

Informed consent was obtained from all subjects involved in the study for collection of cord blood.

Data Availability Statement:

Supporting data are found as supplementary material. Remaining data can be obtained from the corresponding author upon reasonable request.

Acknowledgments

We are grateful to Turkish Academy of Sciences (TUBA) for its partial support.

Conflict of interest statement

The authors declare no conflicts of interest.

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9. CURRİCULUM VITAE

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14. Burcu Pervin, Merve Gizer, İlke Sari, Sema Nur Gür, Ece Gizem Polat, Mehmet Emin Şeker, Özgür Doğuş Erol, Bahar Değirmenci Uzun, Petek Korkusuz, Fatima Aerts Kaya. Akut Greft Versus Host Hastalığının Rag2-/- Fare Kolonoidlerde İn Vitro Modellenmesi. 18. Tıbbi Biyoloji ve Genetik Kongresi, Ekim 2023.

Awards and Prizes:

1. 3. Kök Hücre ve Hücresel Tedaviler Kongresi 2019. "Gazi Nasuh TUĞLU Özel Öğrenci Proje Ödülü" 3rd place award for the abstract by Burcu Pervin, **Özgür Doğuş Erol**, Duygu Uçkan-Çetinkaya, Gerard Wagemaker & Fatima Aerts Kaya. RAG2$^{-/-}$ ağır kombine immün yetmezliği hematopoetik kök hücre nakli ve gen tedavi optimizasyonu. Abstract Kök Hücre ve Hücresel Tedaviler Kongresi, oral presentation, Istanbul 2019.

Projects:

1. TUK-2019-17760 HÜ-BAP, 2019-2021 (26/04/19 – 30/04/21) Preparation of CRISPR/Cas9 and lentiviral constructs for genetic modification of Griscelli Syndrome. Hacettepe University/Utrecht University cooperation project.

2. 219S675 TÜBİTAK 1001, 2019-2023 Development of gene therapy and genetic modification of Griscelli Syndrome induced pluripotent stem cells.

3. THD-2021-19634 HÜ-BAP, 2021-2022 (26/11/21 – 26/02/23) Assessment of the effects of CXCR4(+) Mesenchymal Stem Cells in a model of inflammatory bowel disease.

4. THD-2022-19940 HÜ-BAP, 2022-2023 (22/02/22 – 22/02/23) Development of gene therapy and genetic modification of Griscelli Syndrome induced pluripotent stem cells, 219S675 TÜBİTAK additional budget support.

5. TKA-2022-19508 HÜ-BAP, 2022-2024 (28/06/22 – 28/06/24) Assessment of the protective effects of SUL-109 and Resveratrol in the optimization of freeze/thaw procedures of mesenchymal, hematopoietic and induced pluripotent stem cells.

6. TAY-2022-20436 HÜ-BAP, 2022-2024 (15/12/2022-16/12/2024) Research of viral vector and genetic modification methods in basic sciences.

7. TÜBİTAK 2218 Yurt İçi Doktora Sonrası Araştırma Burs Programı, Lentiviral vektör plazmit tasarımlarının geliştirilmesi ve optimize edilmesi. (Submitted)