

ABSTRACT

Title of Dissertation: **INVESTIGATION OF ACCELERATED SKIN AGING AND PEROXISOMAL ABNORMALITIES IN HUTCHINSON-GILFORD PROGERIA SYNDROME**

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Hutchinson-Gilford progeria syndrome (HGPS) is a detrimental premature aging disease caused by a point mutation in human *LMNA* gene. This mutation results in the abnormal accumulation of a truncated pre-lamin A protein called progerin. Among the drastically accelerated signs of aging in HGPS patients, severe skin phenotypes such as alopecia and sclerotic skins always develop with the disease progression. In this dissertation, I study the HGPS molecular mechanisms focusing on early skin development by differentiating patient-derived induced pluripotent stem cells (iPSCs) to a keratinocyte lineage. Interestingly, HGPS iPSCs showed an accelerated commitment to the keratinocyte lineage than the normal control. To study potential signaling pathways that accelerated skin development in HGPS, I investigated the WNT pathway components during HGPS iPSCs-keratinocytes induction. Surprisingly, despite the unaffected β -

catenin activity, the expression of a critical WNT transcription factor LEF1 was diminished from an early stage in HGPS iPSCs-keratinocytes differentiation. Chromatin immunoprecipitation (ChIP) experiment further revealed strong bindings of LEF1 to early-stage epithelial development markers K8 and K18 and that the LEF1 silencing by siRNA down-regulates the K8/K18 transcription. During the iPSCs-keratinocytes differentiation, correction of HGPS mutation by Adenine base editing (ABE), while in a partial level, rescued the phenotypes for accelerated keratinocyte lineage-commitment. ABE also reduced the cell death in HGPS iPSCs-derived keratinocytes. These findings brought new insight into the molecular basis and therapeutic application for the skin abnormalities in HGPS.

One important feature in both HGPS and normal aging is the elevated levels of Reactive Oxygen Species (ROS), which are generated from metabolic pathways to cause oxidative damage to macromolecules within the cells. Although peroxisomal bioreactions can generate free radicals as their byproducts, many metabolic enzymes within the peroxisomes play critical roles as ROS scavengers, particularly catalase. In this dissertation, I observed impaired peroxisomes-targeting protein trafficking, which suggested that the poorly assembled peroxisomes might cause high oxidative stress, contributing to the premature senescent phenotype in HGPS. I also investigated the ROS clearance efficiency by peroxisomal enzymes and found a significantly decreased catalase expression in HGPS. Furthermore, I evaluated the effects of two promising HGPS-treatment drugs Methylene Blue and RAD001 (Everolimus, a rapamycin analog), on catalase in HGPS fibroblasts. I found that both drugs effectively reduced cellular ROS levels. As a well-known antioxidant, MB did not affect catalase expression or activity. Interestingly, the RAD001 treatment significantly upregulated catalase activity in HGPS cells. This is the first

characterization of peroxisomal function in HGPS and provides new insights into the cellular aspects of HGPS and the ongoing clinical trial.

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by

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Dedication

I dedicate this dissertation to my parents, Professors Duobin Mao and Chunxiao Jia,
for supporting every decision I made over these years.

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I would like to express my sincere gratitude to everyone who has helped me during my graduate education. Thank you so much for making me see this adventure through the end.

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List of Abbreviations

AAV9	Adeno-associated virus serotype 9
ABE	Adenine base editor
AON	Antisense Oligonucleotides
ATP	Adenosine triphosphate
BMP-4	Bone Morphogenetic Protein-4
BrdU	Bromodeoxyuridine
C/EBP α	CCAAT enhancer-binding protein alpha
CAT	Catalase
ChIP	Chromatin immunoprecipitation
COX	Cytochrome c oxidase
CRISPR	Clustered regularly interspaced short palindromic repeats
DCFDA	2', 7' - dichlorofluorescein diacetate
EC	Endothelial cells
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
ER	Endoplasmic reticulum
FTI	Farnesyltransferase inhibitors
GPx	Glutathione peroxidase
HGPS	Hutchinson-Gilford Progeria Syndrome
HUES64	human embryonic stem cells 64

ICMT	Isoprenylcysteine CarboxyMethyl Transferase
IFE	interfollicular epidermis
iPSC	induced pluripotent stem cells
K1	keratin 1
K14	keratin 14
K18	keratin 18
K5	keratin 5
K8	keratin 8
LEF1	Lymphoid Enhancer Binding Factor 1
LINC	Linker of Nucleoskeleton and Cytoskeleton
MB	Methylene blue
MMP	metalloproteinase
mTOR	Mammalian target of rapamycin
NAC	N-Acetyl-Cysteine
NHEJ	nonhomologous end joining
NLS	nuclear localization sequence
NO	nitric oxide
NPC	nuclear pore complex
Nrf2	nuclear factor erythroid 2-related factor 2
P-SMAD	phospho-smad
PARP1	poly(ADP-ribose) polymerase 1
Pex5	Peroxisomal Biogenesis Factor 5
Pex7	Peroxisomal Biogenesis Factor 7

PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI	propidium iodide
PMP70	70-kDa peroxisomal membrane protein
PPAR γ 2	Peroxisome proliferator-activated receptor gamma 2
PTS1	peroxisomal targeting sequence 1
RA	retinoid acid
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SCP2	sterol carrier protein 2
Sln	Sarcolipin
SMC	smooth muscle cells
SR	sarcoplasmic reticulum
SUN1	Sad1 And UNC84 Domain Containing 1
TCF7	Transcription Factor 7
TCF7L1	Transcription Factor 7 Like 1
TCF7L2	Transcription Factor 7 Like 2
UPR	unfolded protein response

Chapter 1: Introduction

1.1 Hutchinson-Gilford Progeria syndrome (HGPS)

1.1.1 General description and clinical features of HGPS

Hutchinson-Gilford progeria syndrome (HGPS) is a severe congenital genetic disorder that was first characterized by Jonathan Hutchinson and Hastings Gilford in 1886 and 1887, respectively (Gilford, 1904; Hutchinson, 1886). The progeria patients appear normal at birth, but start to develop premature aging symptoms from early childhood. The hallmarks of the disease include sclerotic skin, alopecia, bone, and joint abnormalities, cardiovascular system defects, etc. At an average age of 14.5, more than 90% of the patients die of aging-related heart failures (Merideth et al., 2008). HGPS occurs in all demographic groups. Its prevalence is 1 in 4-8 million births and approximately 400 children are living with HGPS all over the world (<https://www.progeriaresearch.org>). Currently, there is no cure for HGPS, but with many clinical treatments (Gordon, Kleinman, et al., 2012; Gordon et al., 2018; Harhour et al., 2018), the symptoms can be alleviated.

1.1.2 Molecular basis of HGPS

The genetic basis of the classical HGPS is a *de novo* point mutation at exon 11 of the human *LMNA* gene, which encodes A-type lamins that form the fibrillar network underneath the nuclear envelope named nuclear lamina (Karoutas & Akhtar, 2021). Although the nucleotide substitution in HGPS (c.1824C>T) does not affect the amino acid production (glycine, p.G608G), it may activate a cryptic splice site for a

truncated prelamin A isoform with 50 amino acids deletion named progerin (Eriksson et al., 2003) (Figure 1-1A).

In normal lamin A post-translational modifications, a cysteine from the CAAX box at the C-terminus of the prelamin A is farnesylated. This hydrophobic modification helps prelamin A targets the nuclear membrane with high affinity. After prelamin A reaches the nuclear membrane, the last three amino acids (-AAX) will be cleaved by Zmpste24, an endoprotease responsible for lamin A post-translational proteolytic modifications. The remaining cysteine at the C-terminus then undergoes carboxymethylation mediated by isoprenylcysteine carboxyl methyltransferase (ICMT). In the last step, 15 amino acids at the C-terminus which includes the farnesyl tail are removed by Zmpste24. After final processing, the lamin A protein builds up underneath the nuclear envelope and forms the nuclear lamina meshwork (Davies, Fong, Yang, Coffinier, & Young, 2009).

Because the cleavage site for Zmpste24 is within the deleted region caused by improper *LMNA* splicing in HGPS, the hydrophobic farnesyl tail was retained in progerin and built up an unstable nuclear lamina (Thomas Dechat, Adam, Taimen, Shimi, & Goldman, 2010) (Figure 1-1B). The aberrant accumulation of progerin at the nuclear envelope will cause nuclear blebbing and misshapen nuclei in progeria cells (T. Dechat et al., 2007).

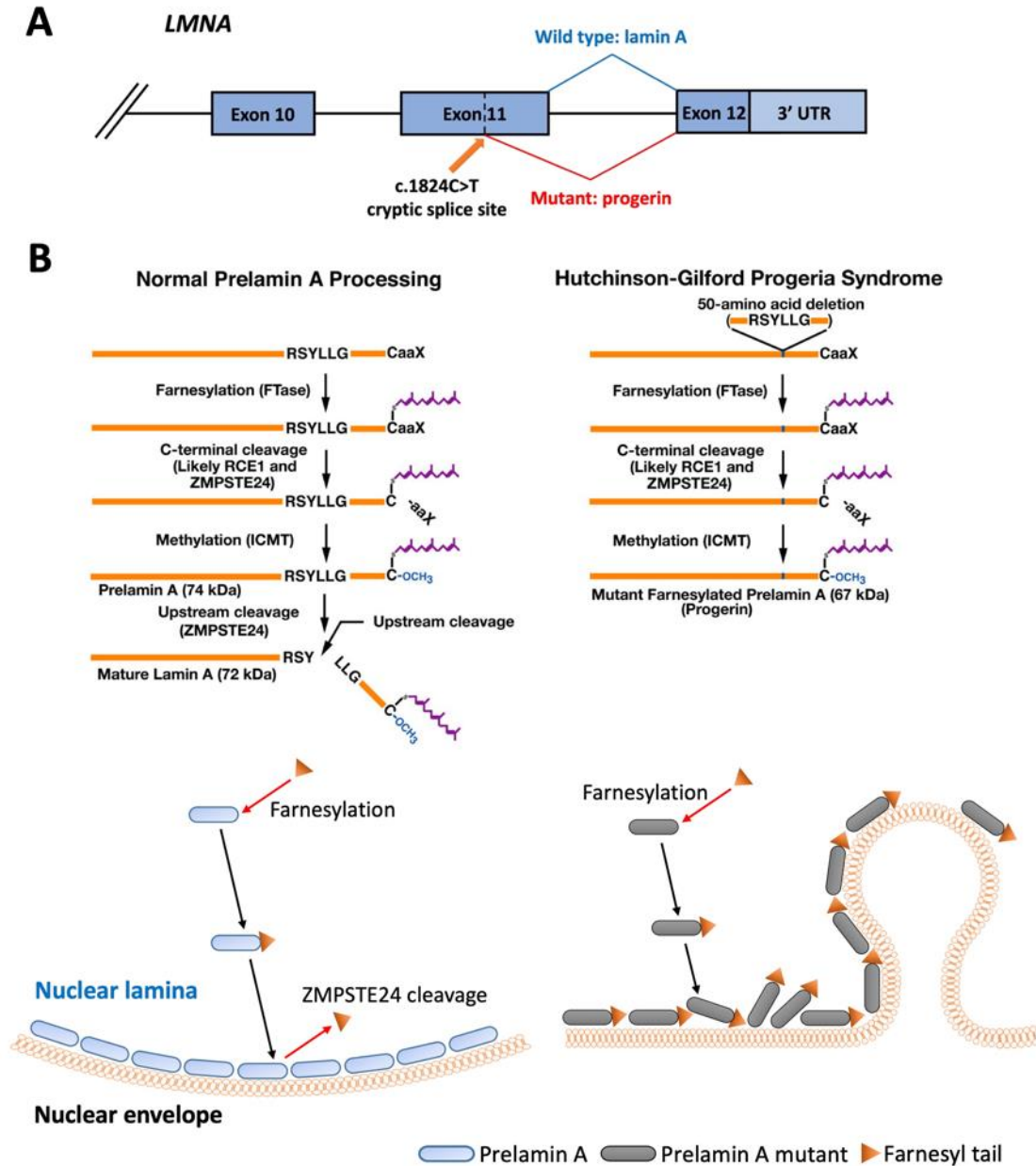


Figure 1-1 Molecular basis of HGPS. (A) Schematic of classical HGPS mutation in the human *LMNA* gene. (B) Post-translational modification of lamin A and progerin. Figure 1-1B is adapted from (Young, Meta, Yang, & Fong, 2006). Permission to republish this material was acquired. Illustrations were partially generated using templates from www.motifolio.com.

1.1.3 Cellular and molecular defects in HGPS

The accumulation of progerin in cells causes abnormal nuclear morphology and is usually accompanied by a disrupted spatial distribution of the nuclear pore complex (NPC) (Eriksson et al., 2003; Goldman et al., 2004). The farnesyl tail of progerin enhances its affinity with SUN1, a component of the LINC complex, which makes SUN1 aggregates at the nuclear envelope and disrupts the nuclear structure (Chen et al., 2014). Moreover, disruption of NPC organization by progerin impedes a series of cellular processes including the nuclear transportation of proteins, RNP assembly, mRNA quality control and SUMOylation (Kelley et al., 2011).

Progerin accumulation at the cell nucleus will also cause altered gene expression as well as chromatin structure and epigenetic changes (Goldman et al., 2004; McCord et al., 2013a). In HGPS cells, progressive loss of peripheral heterochromatin was found with abnormal histone modifications, including down-regulated H3K27me3 and H3K9me3 and upregulated H4K20me3 (McCord et al., 2013b; Scaffidi & Misteli, 2006; Shumaker et al., 2006). The loss of H3K27me3 in HGPS cells is likely due to the reduction of enhancer of zeste homolog 2 (EZH2), a methyltransferase that maintains its abundance (McCord et al., 2013a). Evidence showed that heterochromatin protein 1 α (HP1 α), an H3K9me3 binding protein, was significantly down-regulated in HGPS cells (T. Dechat et al., 2007; Z.-M. Xiong et al., 2016). Silencing experiments further suggested a critical role of HP1 α in H3K9me3 maintenance. DNA damage repair malfunctioning and genome instability were also reported in the HGPS cells (Y. Liu, Rusinol, Sinensky, Wang, & Zou, 2006).

Interestingly, progerin is also expressed in normal aging cells possibly due to the spontaneous activation of *LMNA* cryptic splice sites (Kan Cao, Blair, Faddah, & Al, 2011; Scaffidi & Misteli, 2006). This implies an innovative role of lamin A in the normal aging process.

1.2 Induced Pluripotent Stem Cells (iPSCs) as a model to study tissue-specific abnormalities in HGPS

1.2.1 Patient-derived iPSCs as a model to study cardiovascular failures in HGPS

Given its extremely-low prevalence as well as devastating symptoms, obtaining primary cell lines directly from HGPS patients for the purpose of research can be a fundamental challenge (Gordon, Rothman, López-Otín, & Misteli, 2014). To address that, *In vitro* differentiation of patient-derived iPSCs into various cell types can be utilized to circumvent this challenge in order to study tissue-specific abnormalities in HGPS.

The HGPS-specific iPSCs were first reprogrammed using dermal fibroblasts obtained from patients carrying the classic HGPS G608G mutation (G. H. Liu et al., 2011; J. Zhang et al., 2011). These iPSCs show no progerin expression, and exhibited normal nuclear shape and epigenetic features as in normal iPSCs. Their differentiation potential into fibroblasts, neural progenitors, vascular smooth muscle cells (SMCs), endothelial cells (ECs) and mesenchymal stem cells (MSCs) has been evaluated (J.

Zhang et al., 2011). Upon differentiation, many senescent phenotypes in HGPS are reestablished along with the accumulation of progerin (G. H. Liu et al., 2011).

To investigate the molecular mechanisms underlying the early-onset cardiovascular diseases, which are the major cause of deaths in HGPS patients, many cell types from cardiovascular system were generated using iPSCs model. According to Liu *et al*, directed differentiation of HGPS iPSCs to vascular SMCs leads to premature senescent phenotypes including misshapen nuclei, loss of heterochromatin mark H3K9me3, reduced telomere length and compromised cell proliferation (G. H. Liu et al., 2011). DNA-dependent protein kinase catalytic subunit (DNAPKcs, or PRKDC) was identified as a downstream target of progerin which may be responsible for numerous premature and physiological aging signs (G. H. Liu et al., 2011). In 2014, Zhang *et al*. further investigated the role of progerin in vascular SMCs loss in by differentiating the HGPS patient-derived iPSCs into SMCs (H. Zhang, Xiong, & Cao, 2014). Their work showed proliferative defects in HGPS iSMCs primarily due to caspase-independent cell death. Their work also revealed that the nuclear accumulation of progerin in HGPS iSMCs down-regulated poly (ADP-ribose) polymerase 1 (PARP1), and as a result, the nonhomologous end joining (NHEJ) response was activated. This error-prone DNA damage response pathway may further lead to prolonged mitosis and mitotic catastrophe observed in HGPS iSMCs (H. Zhang et al., 2014). By differentiating HGPS patient-derived iPSCs into SMCs, this study revealed a novel pathway to elucidate the progressive SMC loss in HGPS patients.

To monitor the mechanical stress endured by vascular SMCs *in vivo* due to pulsatile circulation, repeated contraction of SMCs was induced by electrical pulses (J. Zhang et al., 2011). Increased nuclear dysmorphology as well as accelerated senescence were detected in both normal and HGPS SMCs, while the effect was significantly greater in HGPS iSMCs. These iSMCs derived from HGPS iPSCs were also more sensitive to hypoxic conditions than the normal control (J. Zhang et al., 2011).

Endothelial dysfunction is a critical early event for atherosclerosis and may also plays an important role in HGPS disease progression. In 2019, Matrone *et al.* characterized the endothelial cells (ECs) differentiated from HGPS iPSCs. The tissue-specific phenotypes include abnormal nuclear morphology, shortened telomeres, reduced replicative capacity and impaired functions *in vitro* and in immunodeficient mice injected with HGPS iPSC-ECs (Matrone et al., 2019). To further investigate the angiogenic incompetence driven by endothelial dysfunction in HGPS, Gete *et al.* differentiated the patient-derived iPSCs into a endothelial cell lineage (Gete et al., 2021). They found that under both static and fluidic culture conditions, down-regulated endothelial nitric oxide synthase (eNOS) expression and activity as well as reduced intracellular nitric oxide (NO) level were detected in HGPS compared to normal control, resulting in defected capillary-like microvascular network formation. The expression of matrix metalloproteinase 9 (MMP-9) was also down-regulated in ECs generated from HGPS iPSCs (Gete et al., 2021). By differentiating HGPS

patient-derived iPSCs into ECs, these studies revealed new molecular mechanisms to elucidate the endothelial defects in HGPS.

To evaluate the functional defects of HGPS iPSCs-derived SMCs and ECs, in 2017, Atchison *et al.* developed a three-dimensional model of tissue engineered blood vessel (TEBV) consists of HGPS iPSCs-induced SMCs and normal human cord blood-derived endothelial progenitor cells (hCB-EPCs) (Atchison, Zhang, Cao, & Truskey, 2017). These TEBVs show reduced vasoactivity, increased medial wall thickness, increased calcification and apoptosis compared to TEBVs fabricated with normal iSMCs or primary MSCs (Atchison et al., 2017). Later, TEBVs fabricated with both HGPS iPSCs-derived SMCs and ECs were generated in 2020 (Atchison et al., 2020). Comparing to TEBVs fabricated with healthy cells, HGPS TEBVs showed impaired functions and expressed cardiovascular disease-related markers in endothelium. Moreover, HGPS TEBVs exhibited decreased vasoconstriction as well as vasodilation. Functional test for TEBVs consists of HGPS ECs and healthy SMCs was also performed to evaluate the EC-specific dysfunctions in this model (Atchison et al., 2020). Their work demonstrated great potentials of this iPSC-derived TEBV model to recapitulate HGPS disease phenotypes and perform drug tests.

Another cell type that bears the cardiovascular dysfunctions in HGPS are the cardiomyocytes, which have also been differentiated from HGPS iPSCs (Asensi, Kasai-Brunswick, De Figueiredo, & Campos-de-Carvalho, 2021; Monnerat et al., 2018). HGPS iPSCs-derived cardiomyocytes show normal electrophysiology

compared to control cells. However, alterations in cellular respiration suggested by significantly decreased oxygen consumption and mitochondrial abnormalities were detected in those cells. Proteomics analysis also revealed important differences in protein expression patterns in HGPS iPSCs-derived cardiomyocytes (Monnerat et al., 2018).

1.2.2 Patient-derived iPSCs as a model to study defects in HGPS mesenchymal lineage cells

Mesenchymal stem cells (MSCs) maintain the multipotency to differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes (Ding, Shyu, & Lin, 2011). Most severely affected tissues in HGPS are of mesenchymal origins. In HGPS iPSCs-derived MSCs, progerin-induced nuclear lobulation accompanied by mislocalized LAP2 was observed. Increased DNA damage was also detected in HGPS-MSCs (J. Zhang et al., 2011). Lineage-specific differentiation from MSCs under different *in vitro* culture conditions was often used in HGPS research.

In HGPS patients, a series of defects in skeletal development including low bone mineral density and high fracture risks were described previously (Merideth et al., 2008). Two studies examined the osteoblast lineage differentiated from HGPS MSCs and found an unexpected enhancement in osteogenic differentiation, despite the low bone mass observed in HGPS patients and animal models (Blondel et al., 2014; Lo Cicero et al., 2016). In a more recent study, Choi *et al.* initially observed this

phenotype transiently at the very early stage of osteogenic differentiation, but by comparing HGPS and control cells at the same cell densities, they found that the HGPS cells failed to gain the mineralization capacity. Reduced alkaline phosphatase activity and mineralization capacity were observed in HGPS osteoblasts (Choi et al., 2018). They further identified that the nuclear import of β -catenin, a key component in canonical WNT signaling pathway, was disrupted by progerin accumulated at the nuclear envelope. As a consequence, reduced active β -catenin in the cell nucleus causes insufficient activation of the WNT target genes transcription and thus impairs the osteogenesis in HGPS. Diminished nuclear β -catenin in HGPS osteoblasts can be restored by blocking the farnesylation of progerin, which eventually rescues the osteogenesis defects. By studying osteoblast differentiation using HGPS patient-derived iPSCs, this work provided molecular insights in understanding the impact of progerin on major signaling cascades in bone development in HGPS.

In HGPS iPSCs or MSCs-derived adipocytes, a severe lipid storage defect was observed at the late differentiation stage, recapitulating the lipodystrophies in HGPS patients. Expression analysis further suggested that progerin inhibited the transcription activation of $PPAR\gamma 2$ and $C/EBP\alpha$, but had little effects on the early adipogenic regulators. This work demonstrated a regulatory role of lamin A in the terminal differentiation phase of adipogenesis (Z.-M. Xiong, LaDana, Wu, & Cao, 2013).

In order to investigate the defects in HGPS cartilage formation, Mateos *et al.* utilized MSCs generated from human umbilical cord (UC) stroma with Lamin A (LA) and progerin (PG) overexpression for chondrogenesis experiments (Mateos *et al.*, 2013). The chondrogenic potential is defective in PG-MSCs indicated by increased hypertrophy markers expression during chondrogenic differentiation. Moreover, both PG-MSCs and LA-MSCs showed reduced manganese superoxide dismutase (MnSOD), elevated mitochondrial reactive oxygen species (ROS), and impaired migration capacity. These defects in HGPS chondrogenesis can be partially reversed by ROS-scavenger treatment (Mateos *et al.*, 2013). This study demonstrated that progerin induces chondrogenic defects possibly due to an imbalance in oxidative stress.

1.2.3 Patient-derived iPSCs as a model to study skin abnormalities in HGPS

Skin is the largest organ of human body. It consists of epidermis, dermis and subcutaneous tissue (Figure 1-2). The epidermis is the outermost layer of skin, which consists mostly of different types of keratinocytes that forms a stratified structure. Moving outward from the basement membrane, the basal layer (Stratum basale) keratinocytes undergo a sequential differentiation process to form the spinous layer (Stratum spinosum), granular layer (Stratum granulosum), and cornified layer (Stratum corneum) above (Figure 1-2, Fuchs & Raghavan, 2002). Other cell types including melanocytes that produce the pigment melanin as well as Langerhans cells also reside in the epidermis layer (Honari & Maibach, 2014). The dermis contains connective tissue, hair follicles and sweat glands. Fibroblasts that secrete various

extracellular matrix proteins (ECM) were also located in the dermis layer (Brown & Krishnamurthy, 2021). As the deepest layer of the skin, the subcutaneous tissue (or hypodermis) is made up of fat and connective tissue (Stecco, Hammer, Vleeming, & De Caro, 2015).

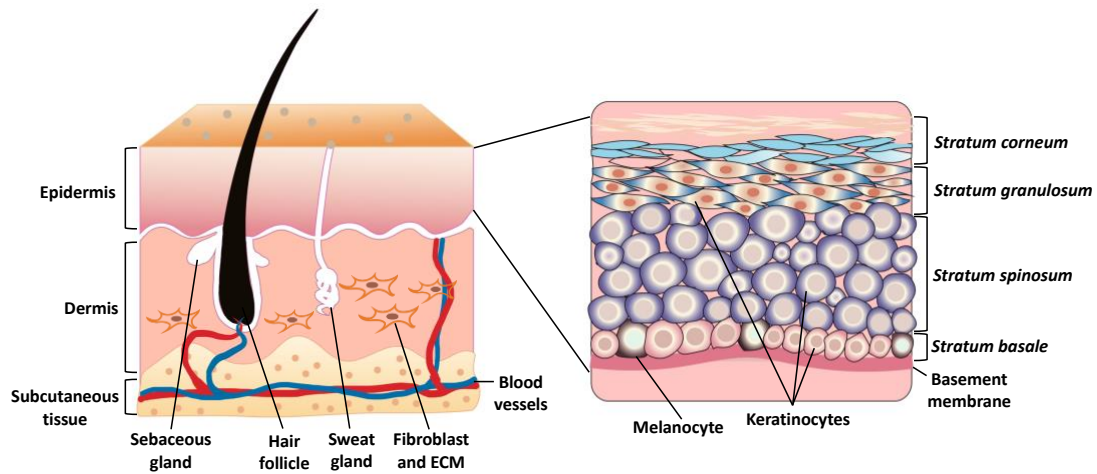


Figure 1-2 Structure of human skin (left) and stratified epidermis (right). The skin tissue consists of three major components: epidermis, dermis and subcutaneous tissue. Different types keratinocytes forms the stratified epidermis including basal layer (*Stratum basale*), spinous layer (*Stratum spinosum*), granular layer (*Stratum granulosum*) and cornified layer (*Stratum corneum*).

To study the skin abnormalities in HGPS that affects all skin components, including reduced epidermis thickness, alopecia, dimpling and wrinkling, hypopigmentation and decreased subcutaneous fat (Merideth et al., 2008), two types of cell lineages from human skins has been differentiated using HGPS patient-derived iPSCs to date, namely melanocytes and adipocytes (described in 1.2.2) (Lo Cicero et al., 2018; Z.-M. Xiong et al., 2013).

In HGPS iPSCs-induced melanocytes, measurements of melanin content revealed a lower melanin synthesis compared to normal cells. Analysis of the melanosome maturation process by electron microscopy revealed a lower percentage of mature, fully pigmented melanosomes. Functional rescue experiments uncovered a role of progerin in regulating pigment melanin production. Overall, these results suggested a new dysregulated pathway in HGPS and provided novel perspectives in the study of pigmentation phenotypes associated with progeria and normal aging (Lo Cicero et al., 2018).

As the primary cell type in the stratified epidermis, disease-specific iPSCs-induced keratinocytes has been generated in recessive dystrophic epidermolysis bullosa (RDEB) and Gorlin syndrome (GS) for mechanism studies (Itoh, Kiuru, Cairo, & Christiano, 2011; Morita et al., 2021). So far, there is no research conducted on the iPSCs-derived keratinocytes in HGPS. However, it can serve as a promising new model to investigate the skin abnormalities in progeria.

1.3 Skin abnormalities in HGPS

1.3.1 Skin abnormalities in HGPS and normal aging

As the outermost surface of human body, the skin is susceptible to external stimuli and develops signs of aging. The environmental factors, including air pollution, nutrition deficiency, smoking, and ultraviolet (UV) radiation, result in skin wrinkles,

loss of elasticity, laxity, and pigmentary changes (Krutmann, Bouloc, Sore, Bernard, & Passeron, 2017; Z.-M. Xiong et al., 2021; Z. M. Xiong et al., 2017). Skin aging can also be induced by intrinsic factors such as reactive oxygen species (ROS) generated from physiological process (Rinnerthaler, Bischof, Streubel, Trost, & Richter, 2015). HGPS skin abnormalities, including reduced epidermis thickness, alopecia, dimpling, wrinkling, altered pigmentation, and decreased subcutaneous fat, overlap significantly with signs of normal aging (Gordon, Brown, & Collins, 2019; Merideth et al., 2008). Thus, skin tissues with HGPS mutation may serve as a good model to study skin aging in normal population.

1.3.2 Skin abnormalities in HGPS transgenic mouse models

HGPS skin abnormalities have been studied in transgenic mice with inducible epidermal expression of progerin under the control of Keratin 5 (K5) or Keratin 14 (K14) promoters (Sagelius et al., 2008; Y. Wang et al., 2008). Postnatal expression of progerin under K5 resulted in premature exhaustion of adult stem cells in mouse skins (Rosengardten, Mckenna, Grochová, & Eriksson, 2011). Epidermal keratinocytes with aberrant proliferation capacity as well as altered gene expression have also been found in these animals (Rosengardten et al., 2011; Sagelius et al., 2008). In the same animal model with embryonic induction of progerin expression, keratinocytes from the interfollicular epidermis (IFE) presented an abnormal tendency to divide symmetrically, generating excessive basal keratinocytes at the basement membrane. As a result, basal cells were often spotted in suprabasal layers of the epidermis in HGPS transgenic mice and were likely to induce epidermal hyperplasia (Mckenna et

al., 2014; Sagelius et al., 2008; Sola-Carvajal et al., 2019). Pathway analysis indicated that impaired WNT/ β -catenin signaling may be responsible for this phenomenon, as progerin disrupted the nuclear localization of emerin and nesperin-2, impeding the nuclear transportation of β -catenin (Choi et al., 2018; Sola-Carvajal et al., 2019). While these transgenic animals with lineage-specific progerin expression served as great models to study skin abnormalities in HGPS, they may not fully represent the symptoms in HGPS patients due to genetic divergence between humans and mice. Moreover, these studies focused on keratinocytes from fully developed mouse skins and thus only those phenotypes at late developmental stages were characterized.

In this dissertation, I first characterized the phenotypes in HGPS epidermal development by differentiating the patient-derived iPSCs into a keratinocyte lineage. I also investigated the molecular mechanisms that contribute to the early disease progression in HGPS epidermal development.

1.4 Organelle abnormalities in HGPS

1.4.1 Mitochondrial defects in HGPS

Mitochondria are one of the most affected organelles in HGPS cells. Severe morphological defects including fragmentation and swollenness were found in a large portion of mitochondria in HGPS fibroblasts (Z.-M. Xiong et al., 2016), indicating that there might be an imbalanced mitochondrial fusion and fission process. Similar morphological defects were also seen in normal aging cells, including fragmented

mitochondria generated under elevated fission activities and enlarged mitochondria driven by excessive fusion (Seo et al., 2010). The accumulation of these abnormal mitochondria with compromised functions may cause the mitochondrial crisis, which may eventually lead to cell death (Seo et al., 2010). Elevated mitochondrial superoxide was detected in HGPS fibroblasts, along with MMP disruption, apoptosis and reduced ATP production (Z.-M. Xiong et al., 2016). Moreover, reduced expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), a master regulator of mitochondrial biogenesis and ROS detoxification (Austin & St-Pierre, 2012), was detected in HGPS adipocytes and fibroblasts, and its expression was suppressed by progerin (Z.-M. Xiong et al., 2016, 2013). PGC-1 α downregulation was also detected in aged mice, which disrupts mitochondrial biogenesis and causes ROS oxidative damages.

In HGPS skin fibroblast, the proteomic analysis indicated that there is a switch from oxidative to glycolytic metabolism in mitochondria. The lower expression of key components from the mitochondrial respiratory chain suggests that the switched metabolism was due to impaired oxidative phosphorylation. Among these down-regulated components, COX dysfunction is associated with increased mitochondria ROS generation and mitochondria membrane potential disruption. This metabolic transition also resulted in reduced mitochondrial ATP production (Rivera-Torres et al., 2013).

Since the majority of mitochondrial defects in HGPS are downstream effects caused by progerin expression, drugs that help progerin clearance may have a positive effect on improving mitochondria functions. For example, the farnesyl transferase inhibitor FTI-277 increased ATP synthesis and COX expression in *Zmpste24^{-/-}* and *LMNA^{G609G/G609G}* mouse fibroblasts (Rivera-Torres et al., 2013). Moreover, given that mitochondrial abnormalities are hallmarks in HGPS and normal aging, many treatment strategies are aimed at promoting mitochondrial functions and shows promising outcomes. For example, Methylene blue (MB), an antioxidant compound, improved the overall mitochondrial health and stimulated PGC-1 α expression in HGPS cells (Z.-M. Xiong et al., 2016). It has been further demonstrated that MB can also promote the longevity in normal aged skins (Z. M. Xiong et al., 2017).

1.4.2 Endoplasmic reticulum (ER) defects in HGPS

Endoplasmic reticulum, or ER, is an extension of the outer nuclear membrane that forms interconnected networks throughout the cytoplasm. Interestingly, nuclear localization sequence (NLS)-deleted Lamin A and progerin will both relocate to the ER membranes at the nuclear periphery, and recruit emerin to this new location (Wu, Flannery, Cai, Ko, & Cao, 2014). Disrupted ER network was found in HGPS fibroblasts possibly due to dysregulated interactions between Lamin A/progerin and SUN1 protein, and that farnesyltransferase inhibitor treatment weakens the binding between progerin and SUN1 (Chen et al., 2014). In HGPS animal tissues, up-regulated ER stress always activates unfolded protein response (UPR) to combat the

stress especially in blood vessels (Hamczyk et al., 2019). ER stress can also induce cellular senescence through the generation of excessive oxidative stress.

In myocytes from tissues such as skeletal muscle and heart, the specialized ER, sarcoplasmic reticulum (SR) harbors a different set of housekeeping proteins that enhance the Ca^{2+} storage and pumps them into the sarcoplasm to facilitate the excitation-contraction coupling in these cells. In HGPS, progerin-induced Sln upregulation impairs the calcium homeostasis and lead to thermogenic and metabolic defects (W. P. Wang et al., 2020).

1.4.3 Implications of abnormalities in other organelles in HGPS

Less is known about the role of progerin in disrupting other organelles in HGPS cells. Notably, from the same study on HGPS ER abnormalities, Golgi cisternae was found to be dispersed, particularly in cells expressing high levels of SUN1 (Chen et al., 2014).

In this dissertation, I first characterized the peroxisomal abnormalities in HGPS fibroblasts and investigated the interplay between progerin, oxidative stress and peroxisomes.

1.5 Therapeutic strategies for HGPS

1.5.1 CRISPR-based gene therapy for HGPS

Since HGPS is caused by a *de novo* point mutation in human genome, CRISPR-based gene editing technique seems to be a promising therapeutic approach specifically targeting the cause of disease. However, possibly due to impaired homologous recombination in progeria, currently, no original CRISPR technique-based correction of HGPS mutation was reported in cells from HGPS patients. In HGPS mouse model, knocking out of lamin A/progerin by a single dose administration of AAV-delivered CRISPR/Cas9 vector decelerated the HGPS disease progression and extended the lifespan of these animals (Beyret et al., 2019). However, this strategy diminished the expression of lamin A alongside progerin, which may only be applicable in mice due to their viability without lamin A (Osorio et al., 2011).

In 2021, Koblan *et al.* introduced the Adenine base editor for direct correction of the pathogenic HGPS mutation in cultured fibroblasts derived from progeria patients and in a mouse model (Koblan et al., 2021). Adenine base editor (ABE) linked a deoxyadenosine deaminase to a deactivated CRISPR vector, which conducts A•T to G•C base pairs conversion at the targeted genome region (Gaudelli et al., 2017). In HGPS, with the precise-targeting single-guide RNA (sgRNA), these base editors directly corrected the *LMNA* c.1824C>T mutation with high efficiency. *In vivo* base editing rescued the vascular pathology of the mice, preserving vascular smooth muscle cell counts and preventing adventitial fibrosis. The lifespan of progeria animals was also greatly extended (Koblan et al., 2021).

Most recently, Gete *et al.* reported ABE correction of the pathogenic c.1824C>T allele in HGPS iPSC-ECs (Gete et al., 2021). In their system, the clearance of progerin expression was highly efficient, and thus rescued pathogenic phenotypes as well as restored angiogenic competence in HGPS iPSC-ECs. Together, these results suggest that ABE could be a promising therapeutic approach to rescue HGPS-related phenotypes during iPSCs differentiation as well as treating HGPS patients.

1.5.2 Other treatment strategies for HGPS

In 2018, Harhour *et al.* thoroughly reviewed the current treatment strategies for HGPS targeting different components of the disease pathway (Harhour et al., 2018). Besides CRISPR-based therapeutic strategies that aims to correct the HGPS G608G mutation directly, multiple downstream process in the aberrant prelamin A production can be blocked by different treatment strategies.

To inhibit the aberrant splicing of progerin, Antisense OligoNucleotides (AONs) have been designed to block the *LMNA* splicing site for progerin production. HGPS fibroblasts treated with AONs displayed improved nuclear morphology and fixed gene expression profile (Scaffidi and Misteli, 2005). *In vivo* study by Osorio *et al.* also showed promising results after morpholino treatment in alleviating progeria phenotypes in *LMNA*^{G609G} mouse model (Osorio et al., 2011). Besides, an antidiabetic drug, Metformin, was shown to regulate the expression of HGPS cryptic splice site splicing factor SRSF-1. The down-regulation of progerin splicing by

Metformin treatment have been proved in HGPS cells (Egesipe et al., 2016; Park & Shin, 2017).

Next, to reduce the toxic post-translational modifications for progerin, prelamins A isoprenylation and methylation inhibitors including Lonafarnib, Zoledronate/Pravastatin, monoaminopyrimidines and isoprenylcysteine carboxyl methyltransferase (ICMT) inhibitor are suitable candidates. Notably, one farnesyltransferase inhibitor (FTI), Lonafarnib (branded as ‘Zokinvy’), is currently used in clinical trials for HGPS patients (Gordon, Cao, & Collins, 2012). To reduce the progerin accumulation in the nuclear envelope, Lee *et al.* screened a small molecule named JH4 that blocks the interaction between progerin and lamin A/C through direct interaction with progerin (Lee et al., 2016).

Moreover, the stimulation of progerin clearance by autophagy-activating drugs including rapamycin, sulforaphane and retinoids can also be applied in HGPS treatment. In 2011, Cao *et al.* showed that the formation of insoluble progerin aggregates was reduced upon rapamycin treatment, and found the progerin degradation activated by an autophagic mechanism in HGPS cells. This study also revealed that treatment with rapamycin improves HGPS nuclear morphology, delays cellular senescence and increases cell proliferation activity (Kan Cao, Graziotto, et al., 2011).

Additionally, various treatment were applied to alleviate damages caused by progerin in the cell, including a variety of antioxidants. Previous work in our group repurposed methylene blue (MB), a mitochondria-targeting antioxidant for alleviating cellular senescence in HGPS fibroblasts. Our study showed that MB treatment not only rescued the severe mitochondrial defects in HGPS, but also significantly delayed premature senescence, improved cell proliferation and restored nuclear morphology as well as histone modifications (Z.-M. Xiong et al., 2016). These results indicated MB as a promising therapeutic approach in HGPS treatment. Other potential targets for HGPS treatment include ROS generating pathways, NF- κ B activation, mitochondria abnormalities, etc. (Harhouri et al., 2018)

1.6 Significance of this dissertation

Skin is the largest organ of the body and the skin abnormalities in HGPS are severe and overlap greatly with normal aging. While previously established transgenic animals with lineage-specific progerin expression served as great models to study skin abnormalities in HGPS, they may not fully represent the symptoms in HGPS patients due to genetic divergence between humans and mice. As discussed in 1.2.1, obtaining primary cell lines directly from HGPS patients for the purpose of research may be challenging due to the extremely-low prevalence as well as devastating symptoms. Moreover, previous studies focused on keratinocytes from fully developed mouse skins and thus only those phenotypes at late developmental stages were characterized. In order to investigate the HGPS disease progression in early epidermal development with a humanized model, we took advantage of the patient-derived

iPSCs and monitored the full differentiation process towards a keratinocytes lineage. This is the first approach to differentiate HGPS patient-derived iPSCs into the skin keratinocytes.

In both progeria and normal skin aging, one crucial feature is elevated reactive oxygen species (ROS) level that causes oxidative damage to the cells. Usually, ROS can be generated from various metabolic pathways taken place in three major organelles: mitochondria, endoplasmic reticulum (ER) and peroxisomes. Both mitochondrial and ER defects have been reported in progeria cells, however, the role of peroxisomes have always been unrecognized in those studies (Hamczyk et al., 2019; Rivera-Torres et al., 2013). Our study presents the first characterization of peroxisomal function in HGPS and provides new insights into the cellular aspects of HGPS and the ongoing clinical trial.

Currently, there is no cure for HGPS, yet the recent application of Adenine Base Editing (ABE) in HGPS primary cells and an animal model offered very promising results (Koblan et al., 2021). Our study first applied ABE in HGPS iPSCs-keratinocytes differentiation to study the progerin-induced tissue-specific defects.

Chapter 2: Characterization of the epidermal differentiation from HGPS patient-derived iPSCs

2.1 Introduction

Conventionally, a succession of keratin genes expressed in keratinocytes has been identified during mammalian epidermal development: in the early embryonic stage, the expression of keratin pair K8-K18 peaks in single-layered epithelial progenitors that give rise to epidermis, and declines rapidly towards the keratinocyte commitment (Jackson, Grund, Winter, Franke, & Illmensee, 1981). Afterward, with the induction of p63, these progenitor cells are committed to the epidermal fate by turning on the K5-K14 expression (Nagarajan, Romano, & Sinha, 2008; Romano et al., 2012). These p63+, K5/K14+ cells are adult stem cells that can proliferate as well as differentiate into spinous cells, granular cells, and squames by upward stratification (Fuchs, 2007). One keratin type that marks this terminal differentiation is K1 (Fuchs & Green, 1980; L. Zhang, 2018).

Previous studies indicated that the accumulation of progerin at the nuclear envelope disrupts the separation of chromosomes at the anaphase of mitosis, resulting in an increased portion of mitotic-arrested or binucleated cells in progeria (Kan Cao, Capell, Erdos, Djabali, & Collins, 2007; T. Dechat et al., 2007). In HGPS iPSCs-derived smooth muscle cells (iSMCs), cell cycle abnormalities, including prolonged G2/M phase and mitotic catastrophe, were reported (H. Zhang et al., 2014). A higher frequency of mitotic keratinocytes marked by PHH3 was also observed in transgenic mice with epidermal-specific progerin expression (Sola-Carvajal et al., 2019). In this chapter, we characterized the keratinocytes induction from normal and HGPS patient-

derived iPSCs by detecting a series of keratinocytes-related markers expression and examined the cell proliferation activities using cell cycle analysis and apoptosis assay.

2.2 Results

2.2.1 Differentiating normal and HGPS-derived iPSCs into keratinocytes

To differentiate normal and HGPS patient-derived iPSCs into the keratinocyte lineage in vitro, we adapted a four-week differentiation protocol including two inductions with retinoid acid (RA) and BMP-4 at the initial stage, followed by keratinocyte growth medium changes every other day for up to 28 days (Itoh et al., 2011; Kogut, Roop, & Bilousova, 2014). At the end of differentiation, a subpopulation of basal keratinocyte-like cells with cobblestone morphology was observed (Figure 2-1A). To evaluate the efficacy of differentiation, we first detected the expression of lamin A/C, progerin, and basal keratinocyte markers Δ Np63 and K14 by immunofluorescence staining (Figure 2-1B&C). As expected, all these iPSCs-derived cells showed Δ Np63 and K14 expression with different fluorescence intensities. Their cellular distribution was comparable to those in primary keratinocytes (Figure 2-1C). Progerin expression was only detected in cells differentiated from HGPS iPSCs (Figure 2-1B).

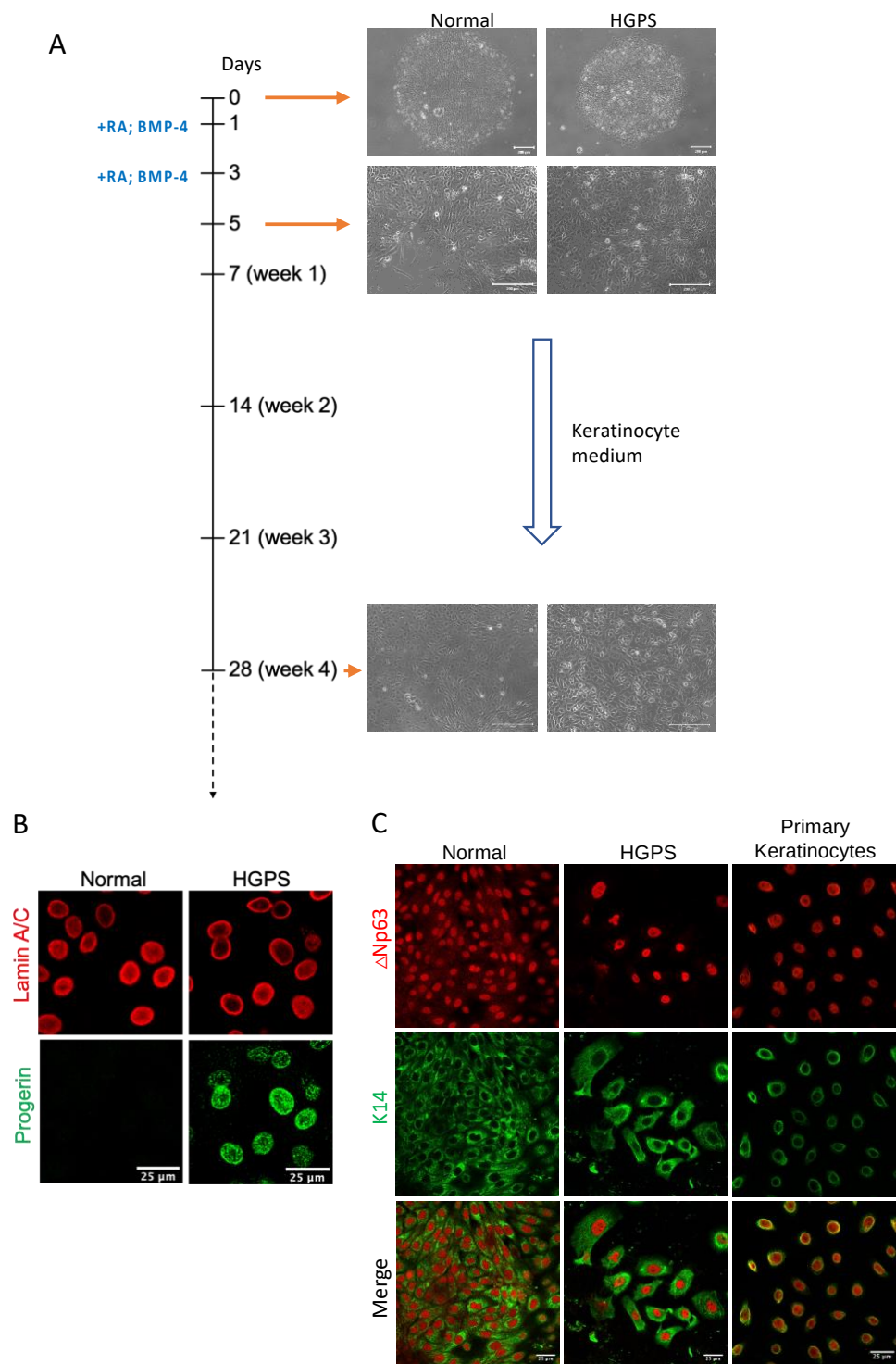


Figure 2-1 Differentiating normal and HGPS-derived iPSCs into keratinocytes. (A) Schematic representation of the protocol for differentiating normal and HGPS patient-derived iPSCs into a

keratinocyte lineage. RA and BMP-4 were added at day 1 and day 3. Cells were maintained in keratinocyte growth medium for more than 28 days (four weeks). Representative phase-contrast images of normal and HGPS iPSCs at a different time point during keratinocyte induction were shown next to the timeline (scale bar = 200 μ m). (B) Lamin A/C and progerin expression in normal and HGPS iPSCs differentiated cells at week four indicated by immunofluorescence staining (scale bar = 25 μ m). (C) Basal layer keratinocyte markers Δ Np63 and K14 expression in normal and HGPS iPSCs differentiated cells at week four as well as in primary keratinocytes indicated by immunofluorescence staining (scale bar = 25 μ m).

To characterize the normal and HGPS iPSCs-keratinocytes differentiation at different stages, the expression of these markers was quantified weekly for four weeks (Figure 2-2A&B) with quantitative RT-PCR and Western Blotting analysis. We found that Lamin A's expression was gradually upregulated during the four-week differentiation and detected progerin expression only in HGPS samples (Figure 2-2A). The early marker K8 was first detected upon the keratinocytes induction, and interestingly, its expression declined more rapidly in HGPS iPSCs-keratinocytes differentiation compared to the normal situation. Accordingly, basal layer keratinocyte markers Δ Np63 and K14 expression increased through normal and HGPS iPSCs differentiation. At each time point detected, Δ Np63 and K14 levels were consistently higher in HGPS than normal control. The terminal differentiation marker K1 was only seen in HGPS iPSCs-derived keratinocytes at week four but absent in normal iPSCs differentiation, suggesting that only the 4-week differentiated population in HGPS contained the fully matured keratinocytes to initiate the terminal differentiation (Figure 2-2A&B).

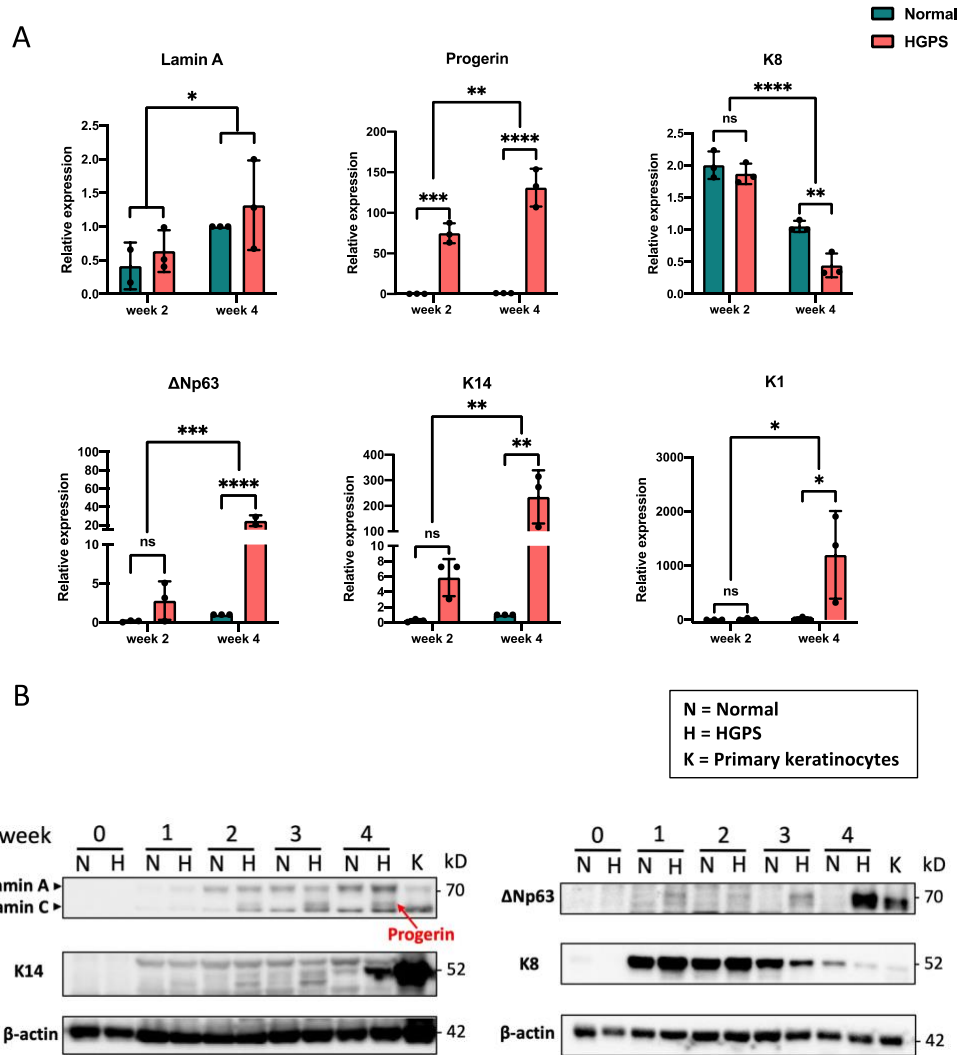


Figure 2-2 The expression of keratinocyte markers during iPSCs-keratinocytes differentiation. (A) Quantitative RT-PCR analysis of the relative expression of Lamin A, progerin, Δ Np63, K14, K8 and K1 during normal and HGPS iPSCs-keratinocytes induction. Data were normalized to endogenous ACTB mRNA and to the average of Normal week 4. Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, not significant, Two-way ANOVA followed by Tukey's multiple comparisons test. (B) Western blot analysis of Lamin A/C (including progerin in HGPS differentiated cells indicated by the red arrow), Δ Np63, K14, and K8 expression during normal and HGPS iPSCs-keratinocytes induction. Primary keratinocytes were used as a positive control. All experiments were repeated at least three times, and representative data were shown as indicated.

2.2.2 Dysregulated cell cycle and increased cell death in HGPS iPSCs-

keratinocytes differentiation

Progerin disrupts the cell cycle progression in mammalian cells (Kan Cao et al., 2007; T. Dechat et al., 2007; H. Zhang et al., 2014). To investigate the cell cycle partitioning in HGPS epidermal development, we first labeled the normal and HGPS cells at the intermediate (week 2) and late (week 4) iPSCs-keratinocytes differentiation stages using Bromodeoxyuridine (BrdU) as an indication for the DNA synthesis at the S phase. These cells were then stained with fluorescent BrdU antibody and propidium iodide (PI) and quantified for frequencies at each cell cycle stage by a flow cytometry analysis (Figure 2-4A&B, Figure 2-3A). Notably, we observed a more significant fraction of HGPS cells at the G2/M phase than normal differentiation at the intermediate stage. This disparity became more significant at the late stage of differentiation (Figure 2-3A).

Because the cell cycle abnormalities in differentiating HGPS epidermal cells resembles our previous findings in HGPS iPSCs derived smooth muscle cells (iSMCs), which is likely linked with mitotic catastrophe induced by progerin (H. Zhang et al., 2014), we next asked if there were increases in cell death during the HGPS iPSCs-keratinocytes induction. To address this question, we performed an Annexin V-PI assay using cells at the intermediate and late stages of differentiation. The population sorted as Annexin V-positive, and PI-negative (early apoptotic cells) was quantified as an indicator for cell death (Figure 2-3B). We observed that cell

death in both normal and HGPS differentiation increased from the intermediate stage to the late stage. There was a slightly higher percentage of dying cells in HGPS iPSCs differentiation at each stage than normal control (Figure 2-3C).

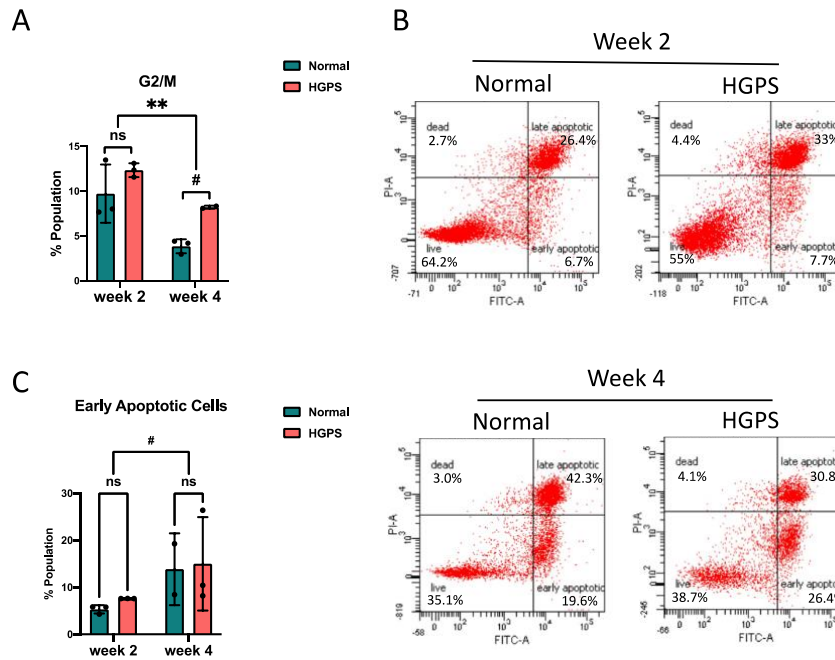
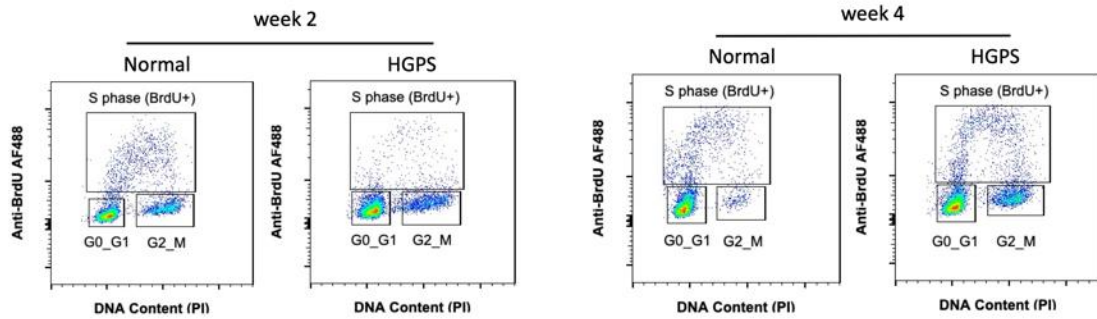


Figure 2-3 Cell cycle and apoptosis defects during iPSCs-keratinocytes differentiation. (A) Quantification of the G2/M phase cell cycle partitioning during normal and HGPS iPSCs-keratinocytes induction. Data were presented as mean $\pm$ SD ($n = 3$). # $P < 0.1$, ** $P < 0.01$, ns, not significant, Two-way ANOVA followed by Tukey's multiple comparisons test. (B) Representative flow cytometry plots showing PI-annexin V apoptosis assay during normal and HGPS iPSCs-keratinocytes induction. The gates were set according to the positive and negative controls as per the manufacturer's instructions. The cells in the lower right quadrant were quantified as early apoptotic populations. (C) Percentage of early apoptotic cells by PI-annexin V flow cytometry analysis during normal and HGPS iPSCs-keratinocytes induction. Data were presented as mean $\pm$ SD ($n = 3$). # $P < 0.1$, ns, not significant, Two-way ANOVA followed by Tukey's multiple comparisons test.

A



B

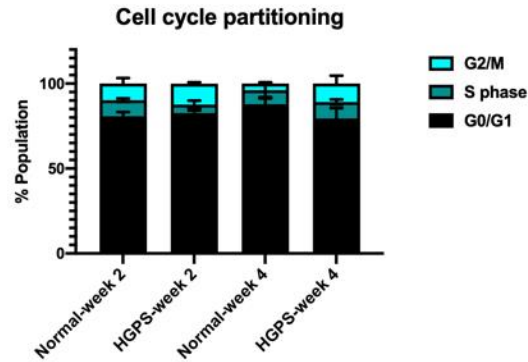


Figure 2-4 Cell cycle partitioning in normal and HGPS iPSCs-keratinocytes differentiation. (A) Representative flow cytometry plots showing BrdU-Pi cell cycle analysis during normal and HGPS iPSCs-keratinocytes induction. The distribution of cells at G0/G1, S, and G2/M phases were indicated within each plot. (B) Quantification of the cell cycle partitioning during normal and HGPS iPSCs-keratinocytes induction. Data were presented as mean $\pm$ SD (n = 3).

2.3 Discussion

2.3.1 Stem cell depletion may contribute to the accelerated keratinocyte-commitment in HGPS iPSCs differentiation

In 2007, Halascheck-Wiener *et al.* promoted a stem cell exhaustion theory to explain the segmental aging phenotypes that appears to be more severe in tissues under continuous strong mechanical stress or endure high turnover rate in HGPS (Halaschek-Wiener & Brooks-Wilson, 2007). For instance, the nuclear accumulation of progerin in HGPS endothelial cells and smooth muscle cells induces genome instability and increases cell death and turnover rate, resulting in a depletion of endothelial progenitor cells (EPCs). The continuing loss of EPCs will impair vascular maintenance and regeneration, causing severe cardiovascular conditions (Caplice & Doyle, 2005; Roberts, Jahangiri, & Xu, 2005). The stem cell exhaustion theory may also explain the hair loss and lipodystrophy in HGPS (Halaschek-Wiener & Brooks-Wilson, 2007). Moreover, epidermal hyperplasia and premature depletion of adult stem cells were reported in mouse skins with tissue-specific progerin expression (Rosengardten et al., 2011). This study observed an accelerated commitment of HGPS iPSCs to keratinocyte lineage, based on the keratinocyte development markers at early, middle and late stages. Consistent with studies in other HGPS cell types, these differentiating HGPS keratinocytes also showed a higher percentage of G2/M cells and an increased apoptosis rate. Together, these results suggest that HGPS keratinocytes differentiation is accelerated, resulting in undesired, hastened cell turnover and eventual depletion of epithelial stem cells.

Chapter 3: Investigation of the role of LEF1 in early epidermal differentiation from HGPS iPSCs

3.1 Introduction

Previous studies identified WNT signaling as one of the most affected pathways in mouse skin keratinocytes with epidermal-specific progerin expression (Sola-Carvajal et al., 2019). WNT signaling is an early morphogenetic pathway that has been activated upon gastrulation and orchestrates multiple processes in the skin development (Fuchs, 2007; Lim & Nusse, 2013). Impaired canonical WNT pathway with diminished nuclear β -catenin localization has been reported in progeroid mouse skins and HGPS osteoblast differentiation (Choi et al., 2018; Sola-Carvajal et al., 2019). In this chapter, I examined the activity of major WNT pathway components in normal and HGPS iPSCs-keratinocytes differentiation.

3.2 Results

3.2.1 LEF1 is down-regulated in early HGPS iPSCs-keratinocytes differentiation

We first checked the β -catenin level by Western blot analysis in iPSCs-keratinocytes differentiation. We detected the weekly expression of the total and active form of β -catenin throughout the differentiation process (Figure 3-1A). Our results indicated that the β -catenin expression was activated from a very early stage upon the iPSCs-keratinocytes induction and was decreased overtime under both normal and HGPS conditions. At each week, the β -catenin level in HGPS was quite comparable to normal conditions (Fig 3-1A and 3-2A). Because progerin was reported to disrupt the nuclear translocation of β -catenin (Choi et al., 2018; Sola-Carvajal et al., 2019), we then performed nuclear extraction for the early-stage (week 1) differentiating cells

with high β -catenin expression. Still, no significant difference in active β -catenin expression was detected in the HGPS nucleus (Fig 3-1B and 3-2B). We then asked if the transcription of those essential WNT pathway genes were activated upon the early iPSCs-keratinocytes induction. Indeed, despite the unchanged TCF7 mRNA level, significant upregulations of LEF1, TCF7L1, and TCF7L2 transcription were detected at differentiation day 5, following the second stimuli with BMP-4 and RA. Interestingly, the LEF1 mRNA level in HGPS did not catch up with normal conditions at differentiation day 5, while no other gene expression showed the same trend (Fig 3-1C). We further detected the weekly LEF1 expression throughout the differentiation process. Again, diminished LEF1 expression was observed in HGPS cells at all differentiation stages (Fig 3-1D&E).

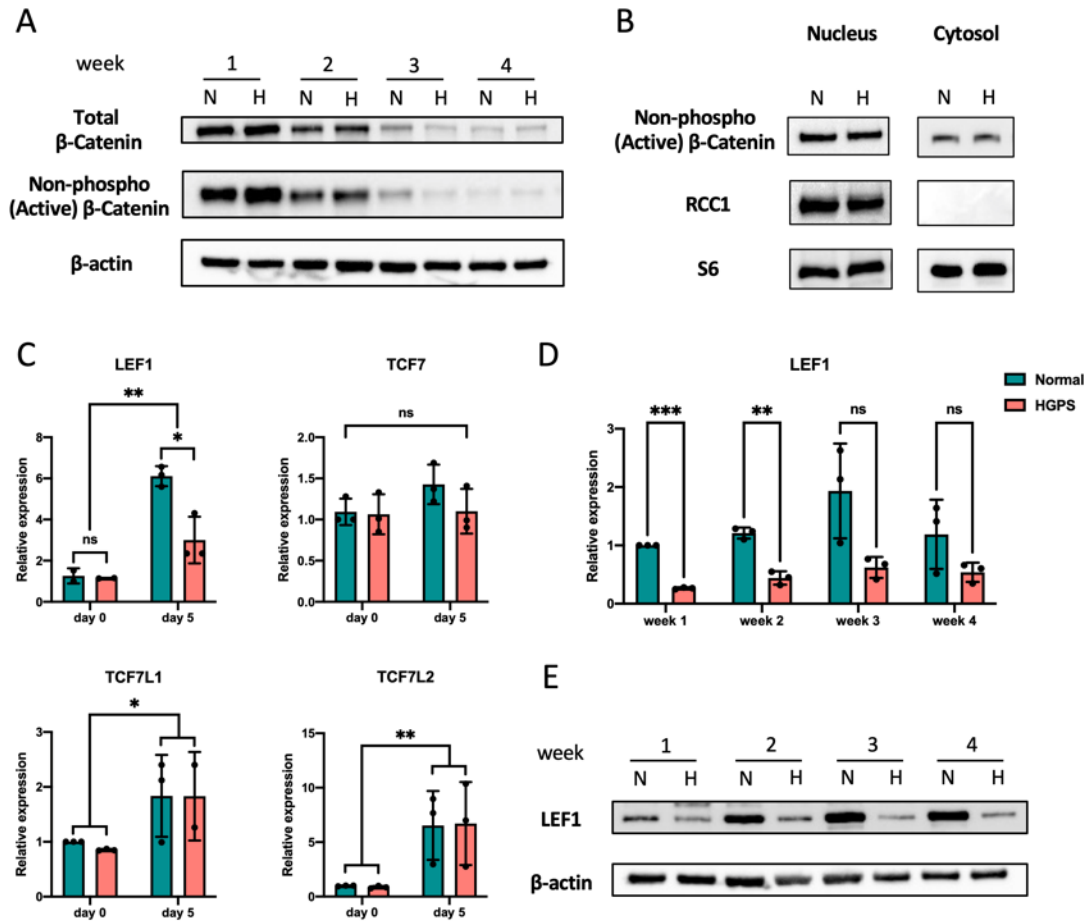


Figure 3-1 LEF1 down-regulation in early HGPS iPSCs-keratinocytes differentiation. (A) Western blot analysis of total and non-phospho (active) β-catenin expression during normal and HGPS iPSCs-keratinocytes induction. Experiments were repeated at least three times, and representative data were shown as indicated. (B) Western blot analysis of nuclear and cytosolic non-phospho (active) β-catenin level in early-differentiating (week 1) normal and HGPS iPSCs. RCC1 and S6 were used as a nuclear marker and overall loading control, respectively. Experiments were repeated at least three times, and representative data were shown as indicated. (C) Quantitative RT-PCR analysis of the relative expression of WNT transcription factors LEF1, TCF7, TCF7L1, and TCF7L2 before (day 0) and after induction with RA and BMP-4 (day 5) in normal and HGPS iPSCs differentiation. Data were normalized to endogenous ACTB mRNA and to the average of Normal day 0. Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01, ns, not significant, Two-way ANOVA followed by

Tukey's multiple comparisons test. (D) Quantitative RT-PCR analysis of the relative expression of LEF1 during normal and HGPS iPSCs-keratinocytes induction. Data were normalized to endogenous ACTB mRNA and the average of Normal week 1. Data were presented as mean $\pm$ SD (n = 3). **P < 0.01, ***P < 0.001, ns, not significant, Two-way ANOVA followed by Sidak's multiple comparisons test. (E) Western blot analysis of LEF1 expression during normal and HGPS iPSCs-keratinocytes induction. Experiments were repeated at least three times, and representative data were shown as indicated.

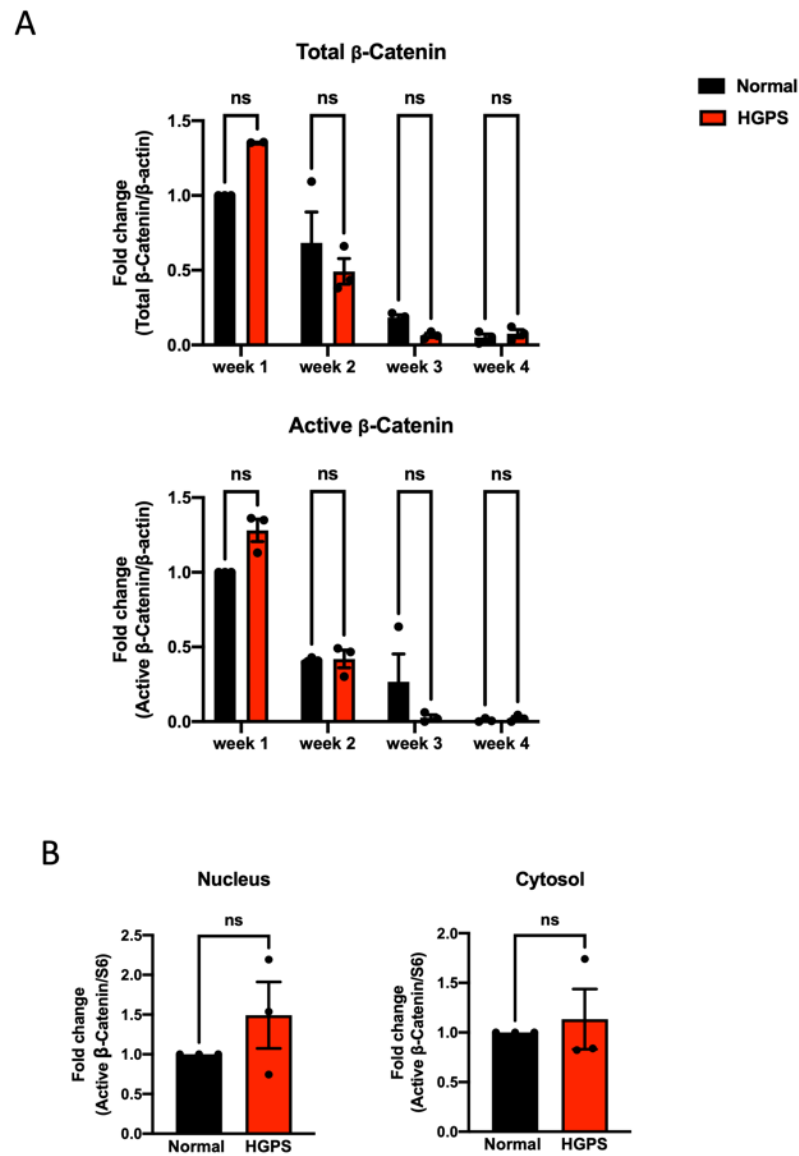


Figure 3-2 Quantification of β -catenin Western blotting analysis. (A) Quantification of total and active β -catenin expression during normal and HGPS iPSCs-keratinocytes induction. Representative Western blot result is shown in Figure 3-1A. Data were presented as mean $\pm$ SD (n = 3). ns, not significant, Two-way ANOVA followed by Sidak's multiple comparisons test. (B) Quantification of nuclear and cytosolic active β -catenin expression in early-differentiating (week 1) normal and HGPS iPSCs. Representative Western blot result is shown in Figure 3-1B. Data were presented as mean $\pm$ SD (n = 3). ns, not significant, unpaired two-tailed t-test.

3.2.2 LEF1 regulates keratinocytes differentiation through K8/K18

To investigate if LEF1 is an upstream regulator that determines the iPSCs' fate towards keratinocyte lineage upon RA and BMP-4 stimuli, we performed a genome-wide search for potential LEF1 binding targets among genes highly expressed in early epidermal development. At the gene locus of epidermal progenitor markers K8 and K18, which are adjacent to each other at chromosome 12, we identified seven putative LEF1 binding sites (Fig 3-3A). We then performed a Chromatin immunoprecipitation (ChIP) assay using LEF1 antibody and quantified the percent input of these binding sites by qPCR. Our results indicated strong LEF1 recruitment at binding sites K8-1, K8-5, and K8-6, which correlates with LEF1 ChIP-seq peaks in HEK293T and human embryonic stem cell (HUES64) derived mesoderm (Fig 3-3B and 3-4A). LEF1 binding at K8-1 was the strongest among them compared to a positive control at the *MYC* gene (Fig 3-3B).

To further test the impact of LEF1 on K8/K18 expression, we down-regulated LEF1 expression at differentiation day 5 using a combination of three LEF1-targeting

siRNAs. Our results showed that with a partial knockdown of LEF1 (Fig 3-3C&D), both K8 and K18 mRNA transcription decreased significantly (Fig 3-3E). Changes in their protein expression were not detected at this time point based on the Western blot analysis (Figure 3-4B&C).

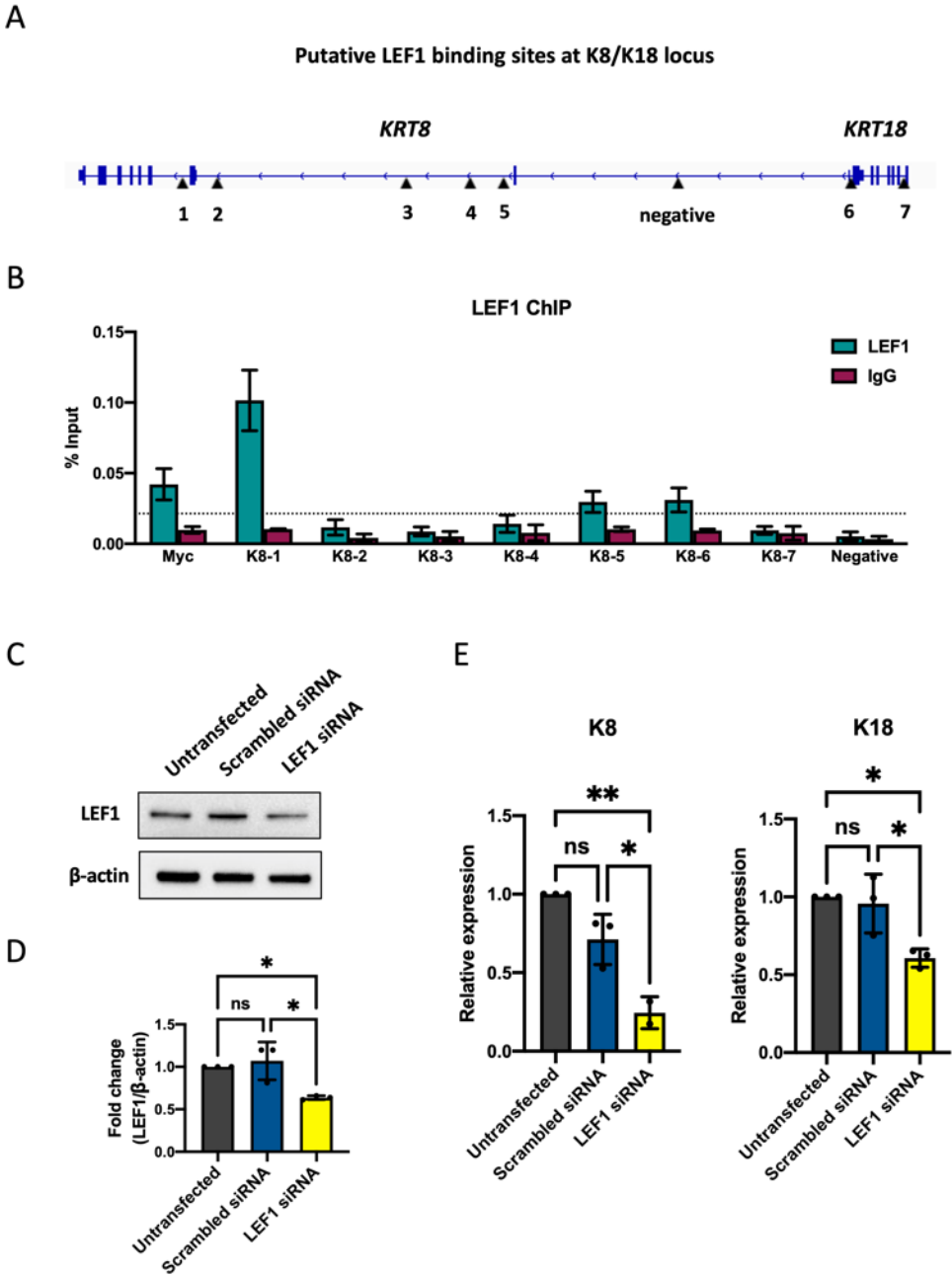


Figure 3-3 LEF1 regulates keratinocytes differentiation through K8/K18. (A) Schematic representation showing putative LEF1 binding sites at K8/K18 gene locus. The position of seven putative LEF1 binding sites and a non-specific locus (negative) were indicated by black arrowheads. (B) Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) analysis of the DNA binding activity of LEF1 at K8/K18 locus. A known LEF1 binding site at the *MYC* gene was used as a positive control. The binding sites with LEF1 enrichment 4 fold greater than the non-specific locus (above the dashed line) were considered to have strong LEF1 binding. Data were presented as mean $\pm$ SD (n = 3). (C) Representative Western blot result showing LEF1 protein expression 48 hours after LEF1 siRNA knockdown in normal iPSCs differentiation. (D) Quantification of LEF1 siRNA knockdown Western blot analysis in (C). Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, ns, not significant, One-way ANOVA followed by Tukey's multiple comparisons test. (E) Quantitative RT-PCR analysis of the relative expression of K8 and K18 48 hours after LEF1 siRNA knockdown in normal iPSCs differentiation. Data were normalized to endogenous ACTB mRNA and to the average of untransfected samples. Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01, ns, not significant, One-way ANOVA followed by Tukey's multiple comparisons test.

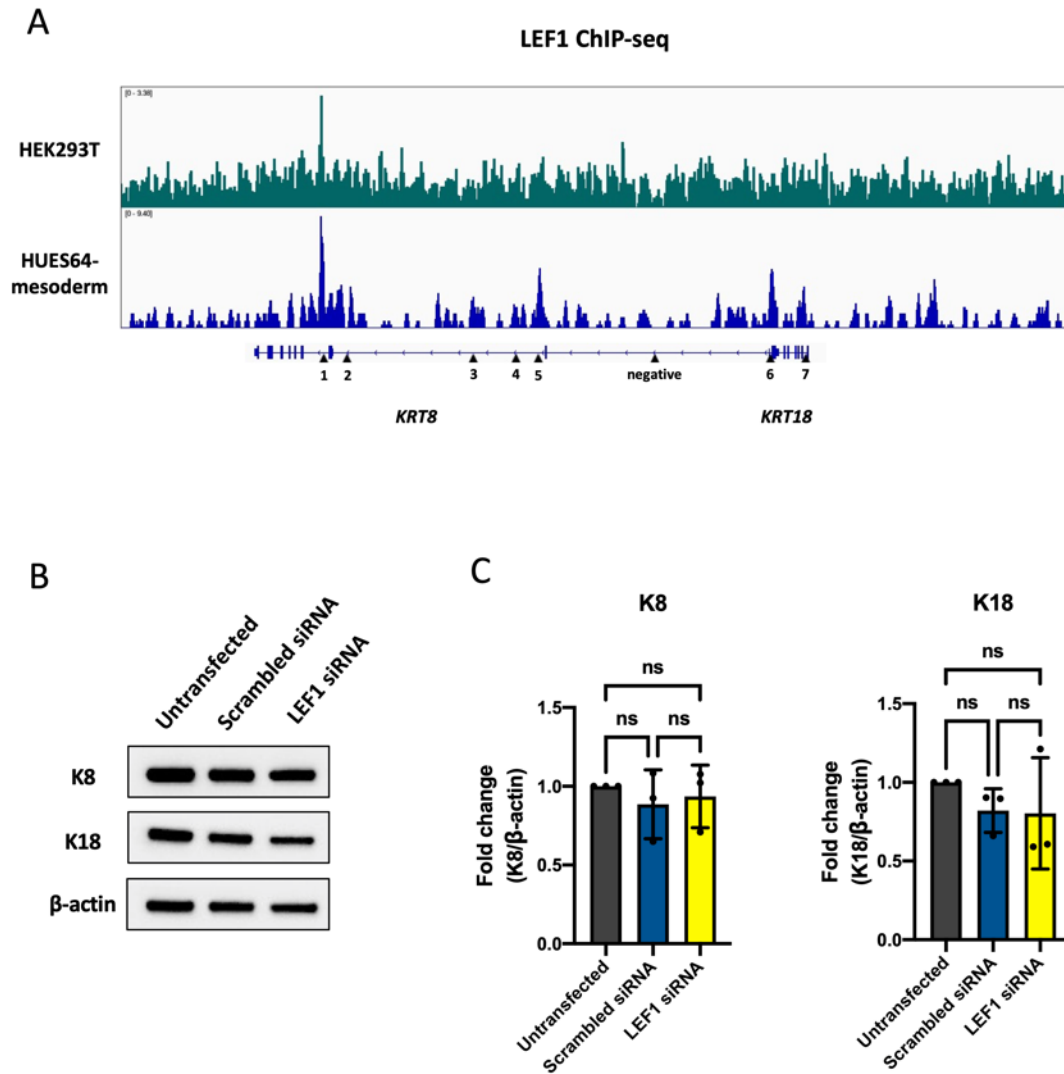


Figure 3-4 LEF1 ChIP-seq peaks at K8/K18 gene locus and Western blotting analysis for LEF1 siRNA knockdown experiments. (A) Genomic tracks display public available LEF1 ChIP-seq data. Shown are LEF1 enrichment profiles at K8/K18 locus in HEK293T (green) and human embryonic stem cell (HUES64) derived mesoderm (blue). The K8/K18 gene track with putative LEF1 binding sites was shown below the profiles. (B) Representative Western blot result showing K8 and K18 protein expression 48 hours after LEF1 siRNA knockdown in normal iPSCs differentiation. (C) Quantification of K8 and K18 protein expression after LEF1 siRNA knockdown in (B). Data were

presented as mean $\pm$ SD (n = 3). ns, not significant, One-way ANOVA followed by Tukey's multiple comparisons test.

3.3 Discussion

3.3.1 LEF1 is an early regulator in epidermal development, and its down-regulation accelerates the keratinocyte lineage commitment in HGPS

WNT signaling has been identified as one of the most affected pathways in the HGPS mouse keratinocytes (Sola-Carvajal et al., 2019). In iPSCs-keratinocytes differentiation, many WNT pathway components were highly expressed upon the induction of RA and BMP-4. Interestingly, we found that the expression of a critical WNT transcription factor LEF1 was diminished at an early stage in HGPS iPSCs differentiation. Moreover, the HGPS iPSCs-derived cells maintained a lower LEF1 expression level throughout the epidermal development than the normal control. We further investigated the impact of LEF1 on the expression of epidermal progenitor genes K8 and K18. In iPSCs-keratinocytes differentiation, K8/K18 expression was induced upon RA treatment and decreased over time significantly after p63 was activated (L. Zhang, 2018). In HGPS iPSCs-keratinocytes induction, the expression of K8/K18 decreases more rapidly, possibly due to insufficient LEF1 level, thus resulting in an accelerated commitment towards the p63 and K5/K14-positive keratinocyte lineage.

Previous studies revealed a β -catenin loss in the HGPS cell nucleus due to disrupted nuclear transportation caused by the progerin (Choi et al., 2018; Sola-Carvajal et al.,

2019). In our iPSCs-keratinocytes differentiation process, the nuclear β -catenin level was unchanged at the early stage of iPSCs-keratinocytes differentiation, when progerin aggregates were not yet formed at the nuclear envelope. Although it is unclear how LEF1 transcription was inhibited at this stage of differentiation, other potential pathways regulate LEF1 expression in a β -catenin-independent manner, including the BMP pathway, which activates the LEF1 transcription via P-SMAD in neural stem cells differentiation (Armenteros, Andreu, Hortigüela, Lie, & Mira, 2018).

Chapter 4: Adenine base editing in HGPS iPSCs-keratinocytes differentiation

4.1 Introduction

Currently, there is no cure for HGPS. Yet, the recent application of Adenine Base Editing (ABE) in HGPS primary cells and an animal model offered up-and-coming results (Koblan et al., 2021). This novel gene-editing tool linked a deoxyadenosine deaminase to a deactivated CRISPR vector, which conducts A•T to G•C base pairs conversion at the targeted genome region (Gaudelli et al., 2017). In HGPS, with the precise-targeting single-guide RNA (sgRNA), these base editors directly corrected the *LMNA* c.1824C>T mutation with high efficiency. *In vitro* base editing in HGPS primary fibroblasts and endothelial cells showed complete progerin clearance, while the embryonic ABE-AAV9 injection greatly improved the health of HGPS mice and significantly extended their lifespans (Gete et al., 2021; Koblan et al., 2021).

In this chapter, we described our approaches in correcting the HGPS mutation during iPSCs-keratinocytes differentiation by ABE and characterized the phenotypes in HGPS-corrected iPSCs-derived keratinocytes.

4.2 Results

4.2.1 Correcting the HGPS mutation in iPSCs-keratinocytes differentiation with ABE

To revert the *LMNA* c.1824 C>T mutation and curb its impact on HGPS iPSCs-derived keratinocytes from an early developmental stage, we conducted Adenine base editing (ABE) using the c.1824 C>T targeting ABE7.10max lentiviral vector at

differentiation day 5, followed by three days' puromycin selection. ABE7.10max vector with human non-targeting sgRNA was included (mock-corrected) (Figure 4-1A). At the end of iPSCs-keratinocytes differentiation (week 4), about 50% of *LMNA* c.1824 T (pathogenic) was corrected to C (wild-type) (Figure 4-1B). Although progerin was still detectable in HGPS-corrected iPSCs-keratinocytes, immuno-stained cells with very bright nuclei consisting of highly expressed progerin were rarely seen after ABE correction (Figure 4-1C). Our RT-qPCR results showed a 50% reduction of progerin mRNA transcription in HGPS-corrected iPSCs-derived keratinocytes (Figure 4-1D) and that the ABE down-regulation in progerin expression was also prominent in Western blotting analysis (Figure 4-1E). Interestingly, despite the moderate correction efficiency, the keratinocyte markers expression in HGPS-corrected cells was significantly reversed. Down-regulated p63, K14 and K1 and up-regulated K8 and K18 expression were detected after ABE (Figure 4-1F and Figure 4-2A). At differentiation week 4, the mRNA transcription of LEF1 was slightly restored in HGPS-corrected iPSCs-keratinocytes (Figure 4-1F). Moreover, although our ABE procedure did not change the cell cycle partitioning in HGPS iPSCs-derived keratinocytes (Figure 4-2B), the phenotype of massive cell death was alleviated (Figure 4-1G).

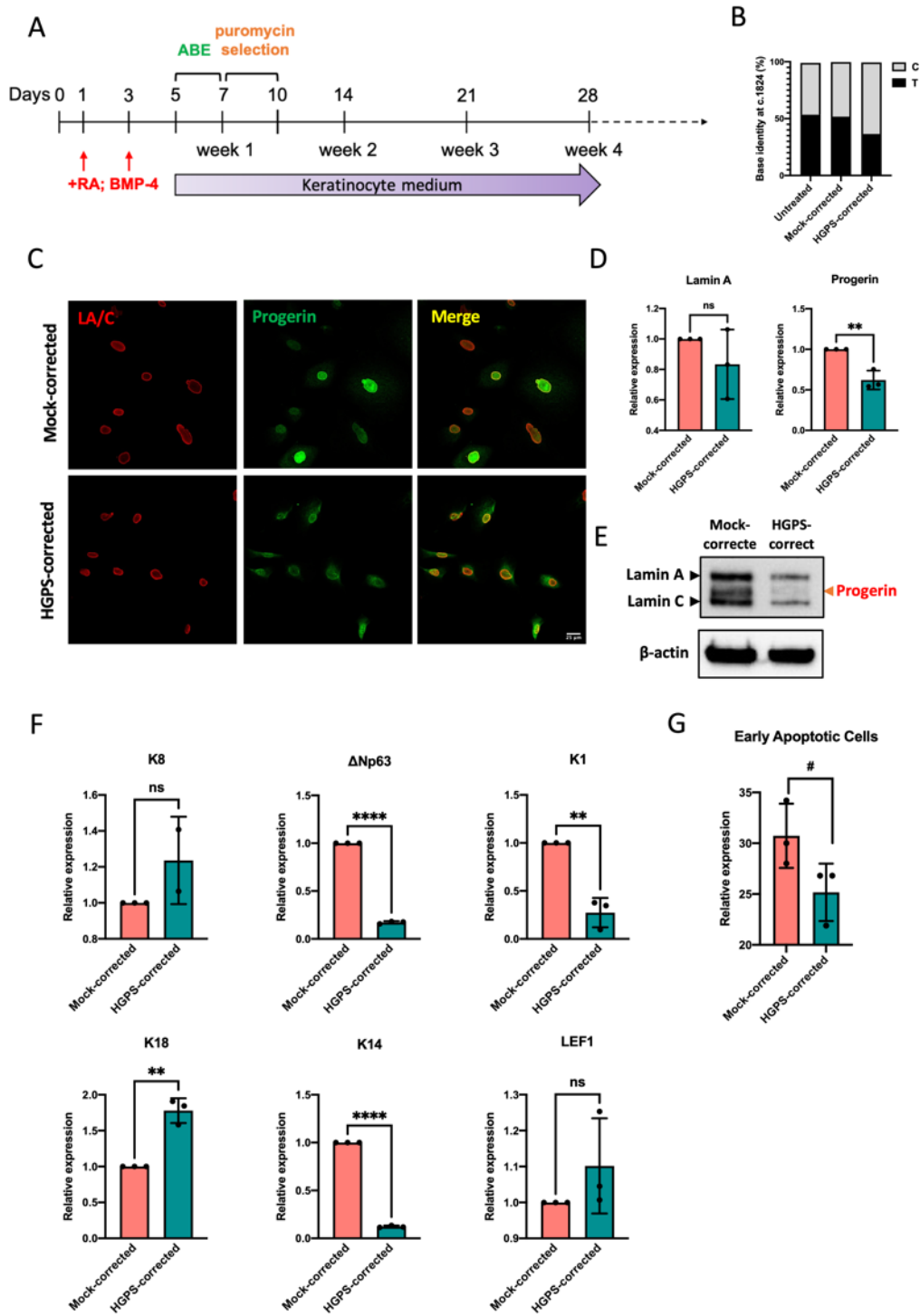


Figure 4-1 ABE corrects the HGPS mutation in iPSCs derived keratinocytes. (A) Schematic representation of the Adenine base editing in HGPS patient-derived iPSCs-keratinocytes induction. (B)

LMNA c.1824 nucleotide identity in HGPS iPSCs derived-keratinocytes untreated or treated with ABE7.10max-VRQR lentivirus (mock and HGPS mutation-targeting) at differentiation week 4. (C) Lamin A/C and progerin expression in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week four indicated by immunofluorescence staining (scale bar = 25µm). (D) Quantitative RT-PCR analysis of the relative expression of Lamin A and progerin in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week 4. Data were normalized to endogenous *ACTB* mRNA and to the average of mock-corrected cells. Data were presented as mean $\pm$ SD (n = 3). **P < 0.01, ns, not significant, unpaired two-tailed t-test. (E) Western blot analysis of Lamin A/C and progerin expression in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week 4. Experiments were repeated at least three times, and representative data were shown as indicated. (F) Quantitative RT-PCR analysis of the relative expression of Δ Np63, K14, K8, K18, K1, and LEF1 in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week 4. Data were normalized to endogenous *ACTB* mRNA and to the average of mock-corrected cells. Data were presented as mean $\pm$ SD (n = 3). **P < 0.01, ****P < 0.0001, ns, not significant, unpaired two-tailed t-test. (G) Percentage of early apoptotic cells by PI-annexin V flow cytometry analysis in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week 4. Data were presented as mean $\pm$ SD (n = 3). #P < 0.1, unpaired two-tailed t-test.

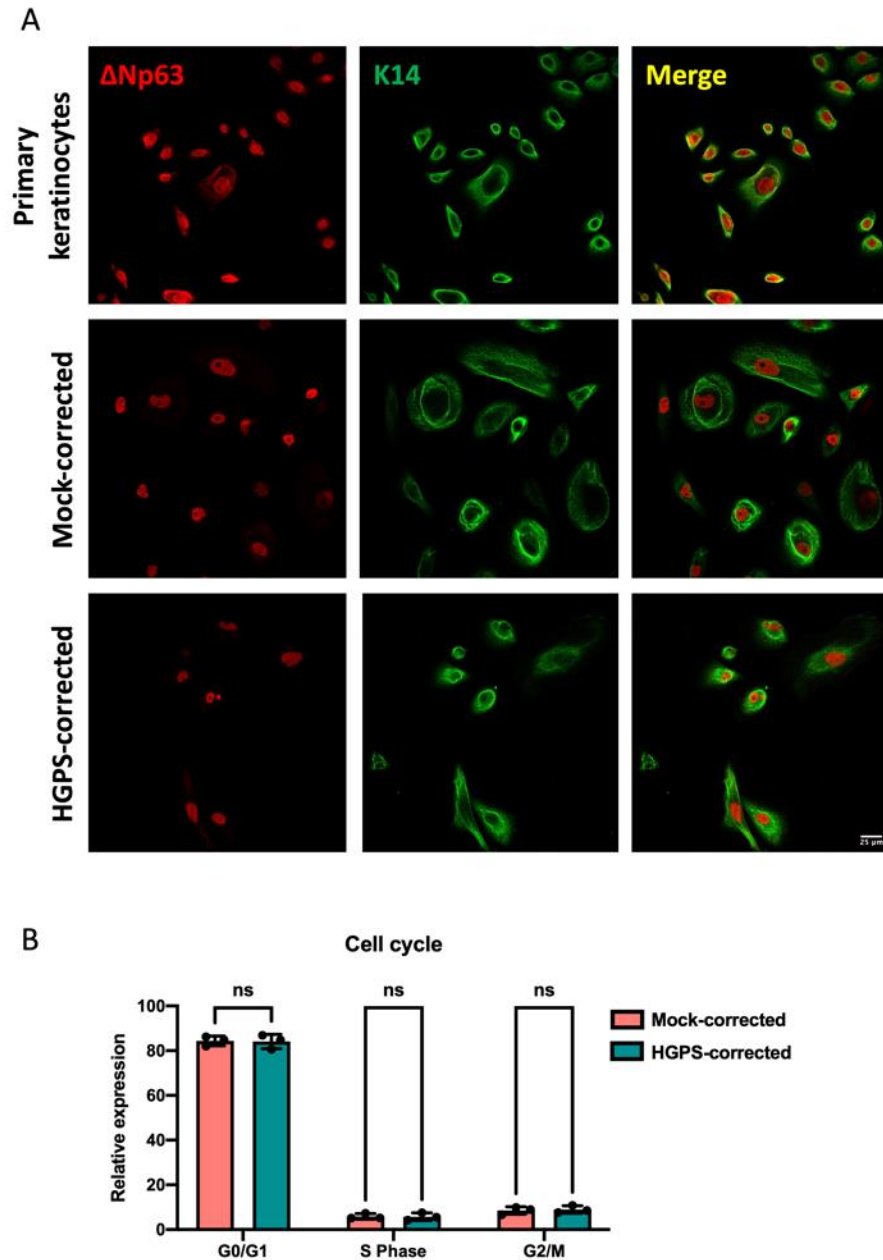


Figure 4-2 Keratinocytes markers expression and cell cycle analysis in HGPS iPSCs-keratinocytes after ABE. (A) Basal layer keratinocyte markers Δ Np63 and K14 expression in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week four as well as in primary keratinocytes indicated by immunofluorescence staining (scale bar = 25 μ m). (B) Quantification of the cell cycle partitioning in mock-corrected and HGPS-corrected iPSCs-derived keratinocytes at week 4. Data were

presented as mean $\pm$ SD (n = 3). ns, not significant, Two-way ANOVA followed by Sidak's multiple comparisons test.

4.3 Discussion

4.3.1 ABE in HGPS iPSCs-keratinocytes differentiation

Since the developmental defects in HGPS iPSCs-keratinocytes have an early onset, we strived to correct the c.1824C>T mutation at an early developmental stage without disturbing the initial inductions with RA and BMP-4. Our ABE in HGPS iPSCs-derived keratinocytes did not reach the high correction efficiency of primary fibroblasts or endothelial cells. As a result, approximately 30% of the cells still carry the HGPS mutation at differentiation day 28. Notably, in HGPS animals with embryonic ABE-AAV9 virus injection, the base editing efficiency in skins was also lower than in other tissues such as the liver and heart (Koblan et al., 2021). One possible explanation lies in the cell cycle abnormalities in HGPS epidermal development. Because progerin was relatively stable, they may persist in the HGPS-corrected cells long after the mutation has been corrected. Thus it may take a long period for complete progerin clearance. However, as basal layer keratinocytes have limited self-renewal potential and start spontaneous terminal differentiation under the *in vitro* culture condition, there was only a short window to evaluate the properties of HGPS-corrected cells. Based on our cell cycle analysis, progerin left a constant impact on the HGPS-corrected cell division. According to the cell cycle partitioning in normal and HGPS cells, at differentiation week 2, when ABE had its optimal effects, reducing the S phase population made it difficult for genome editing to occur.

Another unique feature in epidermal development may also reduce the base editing efficiency: the high cell death and turn-over rate in normal and HGPS differentiation. ABE only slightly reduced the cell death in HGPS-corrected cells. Interestingly, our results indicated that even a tiny portion of correction makes a big difference in decelerating the commitment towards keratinocytes in HGPS. A similar phenomenon was seen in ABE-corrected HGPS animals. Despite the small percentage of correction in many tissues, their health condition improved greatly with significantly extended lifespan (Koblan et al., 2021). At the end of differentiation, only a slightly higher LEF1 expression was detected, indicating a less critical role of LEF1 at the late stage of epidermal development. Future experiments such as ABE in HGPS iPSCs may increase the correction efficiency in HGPS iPSCs-derived keratinocytes.

Chapter 5: Investigation of peroxisomal abnormalities in HGPS fibroblasts

5.1 Introduction

One defining feature in both HGPS and normal aging is the accumulated cellular oxidative stress, which occurs when there is an imbalance between the production of reactive oxygen species (ROS) and antioxidants that curb ROS initiation and propagation (Liguori et al., 2018). The mitochondria act as a significant source of ROS through oxidative phosphorylation and other metabolic processes (Rivera-Torres et al., 2013). In HGPS, ROS levels become elevated due to mitochondrial dysfunction, as there is a distinct downregulation of mitochondrial oxidative phosphorylation proteins, morphological abnormalities, and lessened movement (Rivera-Torres et al., 2013; Z.-M. Xiong et al., 2016). Increased levels of ROS can lead to cellular and molecular damage in the body, which is why ROS scavengers in peroxisomes play a vital role in the management of the oxidative stress (Moldovan & Moldovan, 2004).

Peroxisomes are small, membrane-bound eukaryotic organelles that contain various enzymes to carry out metabolic reactions that convert ROS into hydrogen peroxide, which is then decomposed by ROS scavenging enzymes such as catalase or glutathione peroxidase (GPx) into water and oxygen (Bonekamp, Völkl, Fahimi, & Schrader, n.d.; Schrader & Fahimi, 2006). Peroxisomes biogenesis disorders, peroxisomal matrix proteins mislocalization and catalase deficiency have been reported in standard aging cells (Terlecky, Koepke, & Walton, 2006).

Many treatment strategies for HGPS have been proved to help reduce ROS production in the organism, including RAD001 and Methylene Blue (MB) (Harhour et al., 2018). RAD001 (or Everolimus) is a rapamycin analog that promotes the progerin clearance in HGPS cells by activating the autophagy machinery (K. Cao et al., 2011; Graziotto, Cao, Collins, & Krainc, 2012). It rescues many cellular defects in HGPS cells and is currently being used in clinical trials for HGPS patients (DuBose et al., 2018). Methylene Blue (MB) is a mitochondrial-targeting antioxidant whose redox property allows it to cycle between reduced and oxidized forms, thus facilitating electron transfer in the mitochondria. This reduces ROS production by increasing mitochondrial oxidative phosphorylation and the reduction of electron leakage (Atamna & Kumar, 2010). Our recent work with MB in HGPS demonstrates that the antioxidant rescues many aging signs of HGPS, such as nuclear blebbing and heterochromatin loss, and improves mitochondrial health (Z.-M. Xiong et al., 2016).

In this chapter, we investigated the interplay between progerin, oxidative stress, and peroxisomes in HGPS affected cells. We characterized the peroxisomes in HGPS fibroblasts by examining their assembly, protein import, and ROS scavenging enzyme activity. We also evaluated the effect of two promising HGPS- treatment drugs, Methylene Blue and RAD001, on peroxisomes in HGPS cells.

5.2 Results

5.2.1 Peroxisomal biogenesis in HGPS fibroblasts

To determine whether the extreme cellular environment in HGPS fibroblasts causes peroxisomal biogenesis disorder, we first detected the peroxisomal distribution by confocal fluorescence microscopy. Within the cytosol of each cell, the puncta co-stained with both 70-kDa peroxisomal membrane protein (PMP70) and catalase antibodies are identified as mature and functional peroxisomes (Figure 5-1A). We found that most peroxisomes stained are mature and that there is no significant difference between the PMP70-catalase colocalizations in normal and HGPS fibroblasts (Figure 5-2A). We then quantified the peroxisomes abundance in more than 200 fibroblast cells from normal and HGPS skins using PMP70 as a marker (Figure 5-1B) and found that compared to normal cells, there was no significant change in peroxisome density in HGPS cells.

In cells with peroxisomal biogenesis defects, aberrant peroxisomes lacking some of the matrix proteins were often observed, referred to as “peroxisome ghosts” (Yamasaki et al., 1999). To investigate the peroxisomal matrix proteins import machinery in HGPS fibroblasts, cells were transfected by vectors encoding GFP-tagged sterol carrier protein 2 (GFP-SCP2). SCP2 is a peroxisomal targeting protein with comparable expression levels between normal and HGPS fibroblasts (Figure 5-2B&C). At 48 hours post-transfection, these cells were fixed and immunostained with an antibody against the peroxisomal membrane protein PMP70. The cellular distribution of GFP-SCP2 and PMP70 was detected by confocal fluorescence

microscopy. We identified the SCP2-deficient “peroxisome ghosts” by a customized pipeline in CellProfiler (McQuin et al., 2018), which quantified the portion of PMP70 puncta not co-localized with GFP-SCP2 in each cell (Figures 5-1C). The results acquired from more than 100 cells indicated that compared to normal fibroblasts, there was a more significant portion of HGPS cells with higher SCP2-deficient “peroxisome ghosts” index (Figure 5-1C&D).

In mammalian cells, the recognition and transportation of peroxisomal-targeting proteins were carried out by two cytosolic transporters, Pex5 and Pex7 (Rachubinski & Subramani, 1995). Quantitative RT-PCR revealed the relative expression of Pex5, the receptor for peroxisomal targeting sequence 1 (PTS1)-containing proteins including catalase (Purdue & Lazarow, 1996) and SCP2 (Stanley et al., 2006), decreased slightly in HGPS fibroblasts (Figure 5-1E). However, this reduction was not detected in the Western blot assay (Figure 5-1G). Meanwhile, the expression of Pex7 remained unchanged in HGPS fibroblasts (Figure 5-1F&G).

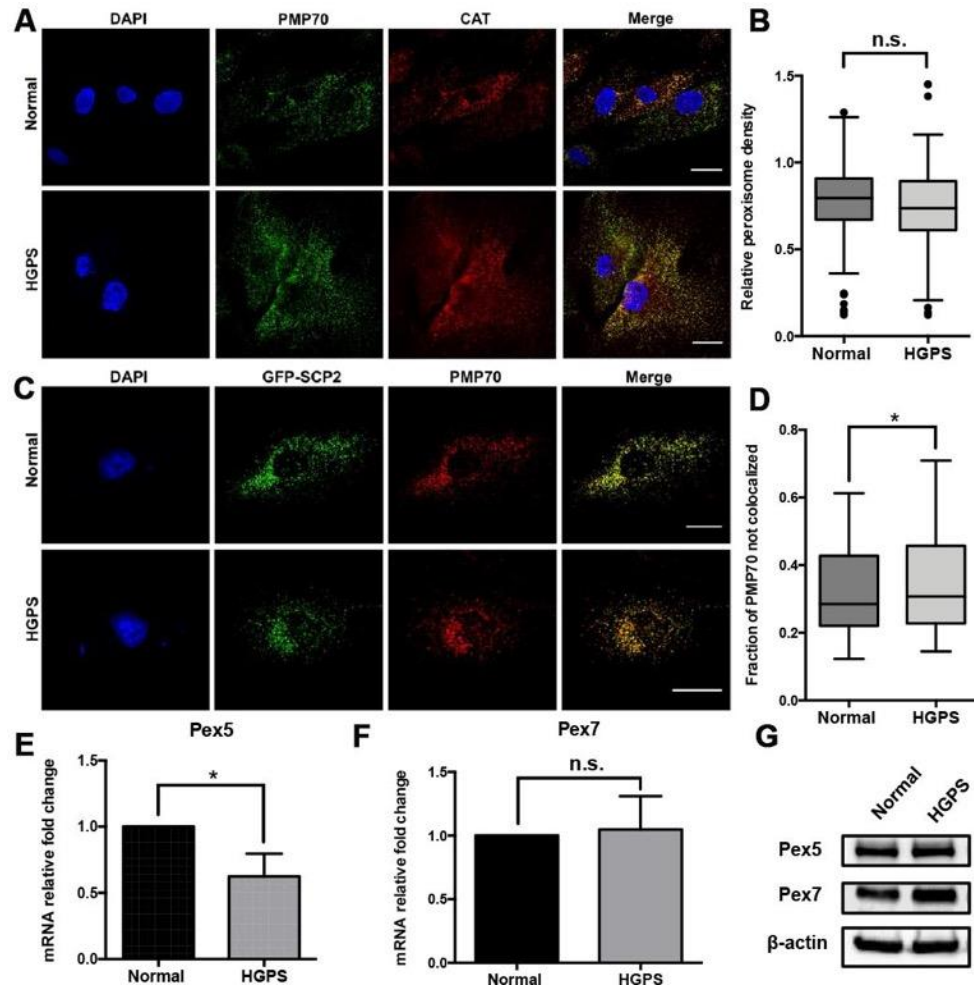


Figure 5-1 Characterization of the peroxisomes in HGPS fibroblasts. (A) Peroxisomes localization indicated by PMP70 and catalase immunofluorescence staining in HGPS patient-derived dermal fibroblasts and their normal counterpart. Bar = 25μm. (B) The peroxisomes density per cell was indicated by the quantification of PMP70 puncta per square unit in normal and HGPS fibroblast cells. More than 100 cells from 3 independent experiments were analyzed, and the data was represented in a Tukey box plot. Boxes show the 25th, 50th, and 75th percentiles, and the dots indicate the outliers. (C) Confocal fluorescence microscopy analysis of normal and HGPS fibroblasts transfected with GFP-SCP2 and probed with PMP70 antibody. Bar = 25μm. (D) Box plot of the portion of PMP70 puncta not colocalized with SCP2 (peroxisomal ghosts) in each cell. More than 100 cells from 3 independent experiments were analyzed. Boxes show the 25th, 50th, and 75th percentiles. Kolmogorov-Smirnov test was used to compare the distributions of these two samples. (E, F) Quantitative RT-PCR analysis

of the relative expression of Pex5 and Pex7 in normal and HGPS fibroblasts. (G) Western blot analysis of Pex5 and Pex7 in normal and HGPS fibroblasts at passage 21. All experiments were performed using mid-passage cells between p15 to p25. All experiments were repeated at least three times, and representative data were shown as indicated. *, $p < 0.05$, n.s., not significant.

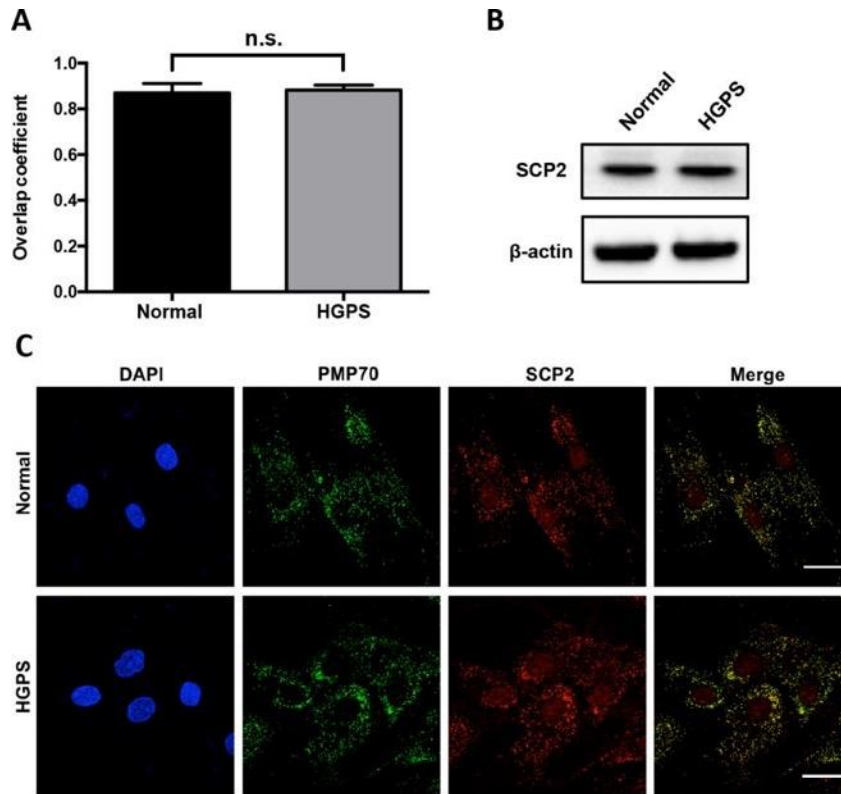


Figure 5-2 Peroxisomal proteins colocalization and endogenous SCP2 in HGPS fibroblasts. (A) Colocalization of PMP70 and catalase fluorescence in normal and HGPS fibroblasts was measured by a customized colocalization pipeline in CellProfiler. More than 100 cells from 3 independent experiments were analyzed. (B) Western blot analysis of endogenous SCP2 expression in normal and HGPS fibroblasts (cell passage number = 18). (C) Immunofluorescence staining of endogenous SCP2 in normal and HGPS fibroblasts. PMP70 antibody was used to indicate the peroxisomes localization. Bar = 25μm, n.s., not significant. All experiments were repeated at least three times, and representative data were shown as indicated.

Based on these findings, we conclude that the peroxisomes proteins import was affected in HGPS fibroblasts, further contributing to their premature senescent phenotype.

5.2.2 Catalase deficiency in HGPS fibroblasts

Since elevated oxidative stress was a hallmark in HGPS cells (Z.-M. Xiong et al., 2016), we then investigated the behaviors of two major ROS scavenging enzymes in peroxisomes, namely catalase (CAT) and glutathione peroxidase (GPx) (Fransen, Nordgren, Wang, & Apanasets, 2012). We detected a significant reduction in catalase mRNA transcription and protein expression in HGPS fibroblasts (Figure 5-3A&B). The overall catalase activity showed a significant drop but turned back to a similar level as normal cells after normalizing with its protein abundance (Figure 5-3C&D). Contrarily, the relative expression and enzymatic activity of the GPx family were not affected in HGPS cells (Figure 5-3E&F). In conclusion, our findings suggested that the inefficient ROS clearance in HGPS fibroblasts was at least partially due to a massive catalase deficiency.

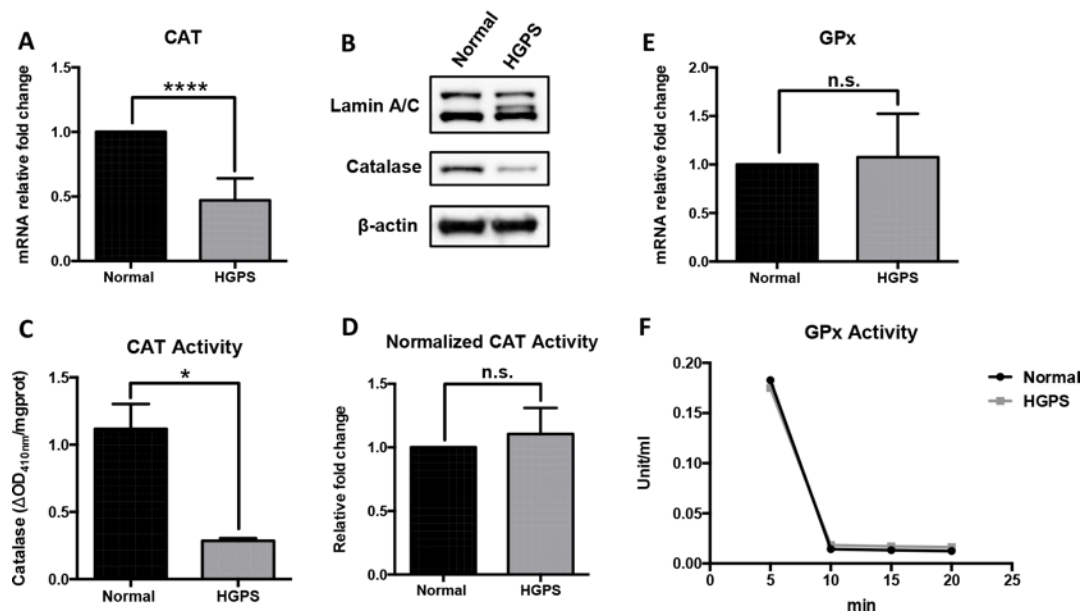


Figure 5-3 Characterization of the ROS-scavenging enzymes in HGPS peroxisomes. (A) Quantitative RT-PCR analysis of the relative expression of catalase in normal and HGPS fibroblasts. ****, $p < 0.0001$. (B) Western blot analysis of Lamin A/C, progerin, and catalase expression in normal and HGPS fibroblasts at passage 25. (C) Catalase activity assay measured by the decomposed H_2O_2 per mg total protein from normal and HGPS fibroblasts. *, $p < 0.05$. (D) Catalase activity normalized with the protein expression level in normal and HGPS fibroblasts. n.s., not significant. (E) Quantitative RT-PCR analysis of the relative expression of Glutathione peroxidase in normal and HGPS fibroblasts. n.s., not significant. (F) Glutathione peroxidase activity in normal and HGPS fibroblasts. All experiments were performed using mid-passage cells between p15 to p25. All experiments were repeated at least three times, and representative data were shown as indicated.

5.2.3 Exogenous progerin induced ROS production while down-regulating catalase expression in fibroblast cells

We further questioned if the catalase deficiency in HGPS fibroblasts was a downstream effect of progerin expression. To address that, human dermal fibroblasts (HGFDFN168) were transduced with lentiviral vectors expressing DsRed-tagged lamin A (DsRed-LA) and progerin (DsRed-Prg). We then identified the DsRed-positive cells and detected the peroxisomes distribution within these cells by confocal fluorescence microscopy (Figure 5-4A). The peroxisome density in DsRed-Prg positive cells turned out to be similar to the DsRed-LA positive control (Figure 5-4B). Consistent with Figure 5-1, no significant difference in GPx, Pex5, and Pex7 expression was detected (Figure 5-5A&B). At three weeks post-viral infection, we saw higher ROS levels in cells with DsRed-Prg expression by 2', 7'-dichlorofluorescein diacetate (DCFDA) flow cytometry assay (Figure 5-4C). However, there was a remarkable reduction of catalase transcription in DsRed-Prg expressing cells revealed by quantitative RT-PCR (Figure 5-4D). We did not detect a noticeable reduction in catalase protein level in this experiment (Figure 5-5A), likely due to the small portion of DsRed positive cells remaining in the population three weeks after virus transduction. The normalized catalase activity was consistently unaffected in cells with exogenous progerin expression (Figure 5-4E). These findings suggested that the accumulation of exogenous progerin in the cell could cause excessive ROS production and reduced catalase expression.

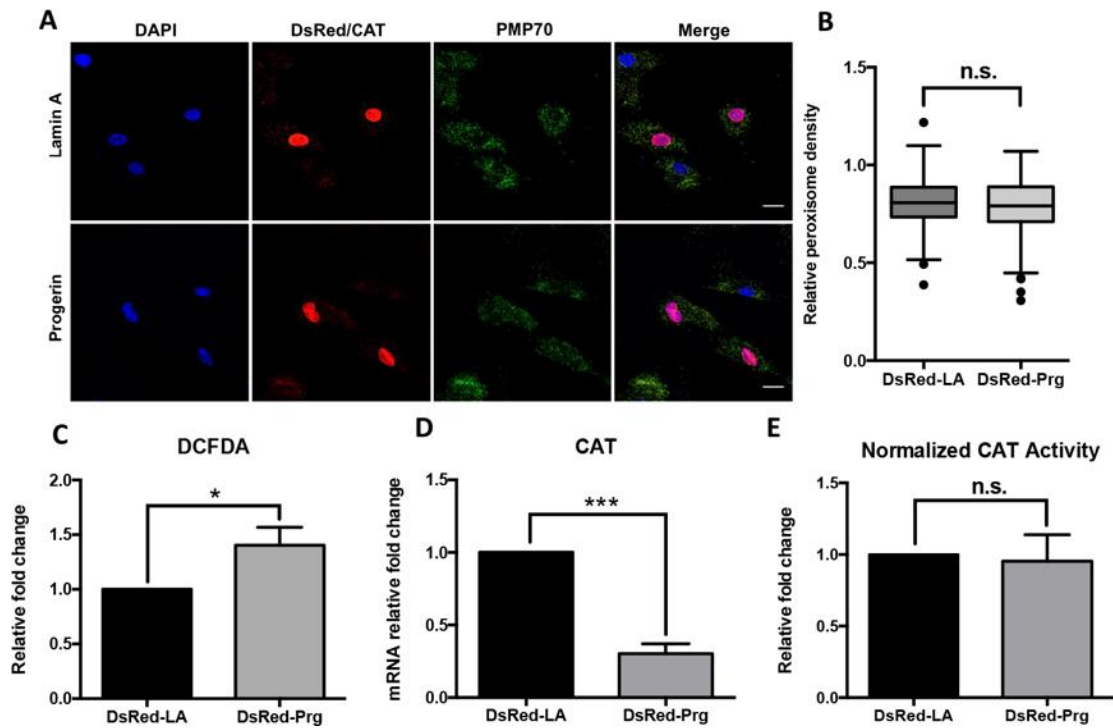


Figure 5-4 Peroxisomal defects in normal fibroblasts expressing progerin. (A) Normal human dermal fibroblast cells were infected by DsRed-LA and DsRed-Pg lentiviruses. The exogenous Lamin A and progerin expression were detected by DsRed fluorescence in the nuclei. Peroxisomes localization was indicated by PMP70 and catalase immunofluorescence staining. Bar = 25 μ m. (B) Quantification of PMP70 puncta per square unit in fibroblasts overexpressing lamin A and progerin. More than 200 cells from 3 independent experiments were analyzed, and the data was represented in a Tukey box plot. Boxes show the 25th, 50th, and 75th percentiles, and the dots indicate the outliers. (C) Relative fold change of ROS activity measured by DCFDA flow cytometry analysis in fibroblasts overexpressing lamin A and progerin. *, $p < 0.05$. (D) Quantitative RT-PCR analysis of the relative expression of catalase in fibroblasts overexpressing lamin A and progerin. ***, $p < 0.001$. (E) Normalized catalase activity in fibroblasts overexpressing lamin A and progerin. n.s., not significant. All experiments were performed using mid-passage cells between p15 to p25. All experiments were repeated at least three times, and representative data were shown as indicated.

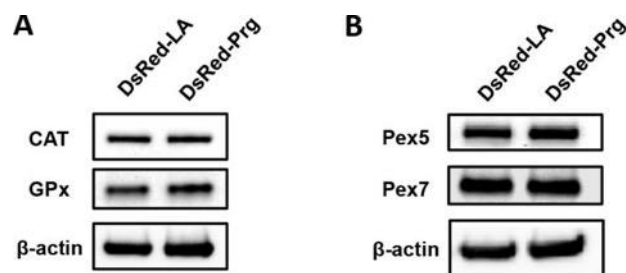


Figure 5-5 (A, B) Western blot analysis of catalase, Glutathione peroxidase (GPx), Pex5, and Pex7 expression in DsRed-lamin A and DsRed-progerin expressing fibroblasts (cell passage number = 21). All experiments were repeated at least three times, and representative data were shown as indicated.

5.2.4 Catalase overexpression alleviates the oxidative stress in HGPS fibroblasts

We then tested if catalase overexpression can reverse some aging hallmarks in normal and HGPS fibroblasts. We transduced the normal and HGPS fibroblast cells with recombinant human catalase-encoding retroviral vector (pBABE-CAT) and controlling pBABE-puro vector (vehicle). The catalase overexpression in pBABE-CAT infected cells was confirmed by quantitative RT-PCR (Figure 5-6A&B), Western blot (Figure 5-6E), and immunofluorescence staining (Figure 5-7A) in both normal and HGPS fibroblasts. The compensated catalase expression resulted in a significant reduction of the cellular ROS level (Figure 5-6C&D). Again, no significant difference in Pex5 expression was observed (Figure 5-7B&C).

We then detected the expression level of nuclear factor erythroid 2-related factor 2 (Nrf2) and p16 in fibroblasts with catalase overexpression. Nrf2 is a master regulator of the cellular antioxidant defense system (Ma, 2013). In HGPS cells, the Nrf2

pathway was suppressed, possibly due to the interference of the progerin (Kubben et al., 2016). p16 is a biomarker of cellular senescence and skin aging (A. S. Wang & Dreesen, 2018). We found that although in normal fibroblasts, these proteins expression levels remained unchanged, in HGPS cells overexpressing catalase, Nrf2 expression was restored moderately, and that the p16 expression was down-regulated compared to cells transduced by vehicle (Figure 5-6F, G&H). To conclude, our findings suggested that catalase overexpression could alleviate the oxidative stress in HGPS fibroblasts.

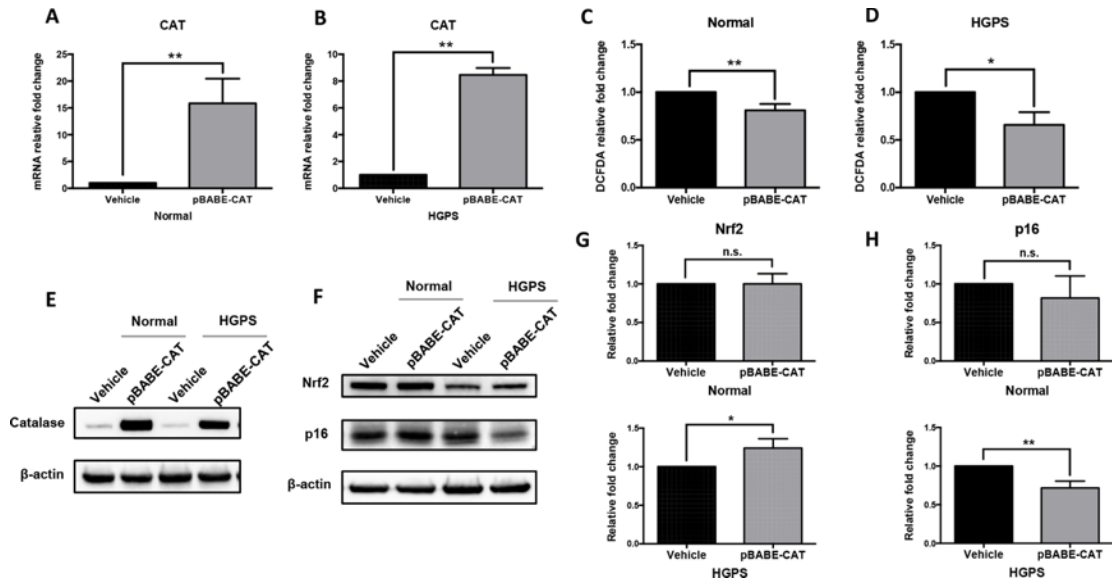


Figure 5-6 Catalase overexpression alleviates oxidative stress in normal and HGPS fibroblasts. (A, B) Quantitative RT-PCR analysis showed the relative expression of catalase in normal and HGPS fibroblasts infected by pBABE-CAT and control pBABE-puro retroviral vectors (Vehicle), respectively. **, $p < 0.01$. (C, D) Relative fold change of ROS activity measured by DCFDA flow cytometry analysis in normal and HGPS fibroblasts overexpressing catalase. *, $p < 0.05$, **, $p < 0.01$.

(E) Western blot analysis showed catalase expression in normal and HGPS fibroblasts infected with pBABE-CAT and control vectors (cell passage number = 18). (F) Western blot analysis showed Nrf2 and p16 expression in normal and HGPS fibroblasts infected with pBABE-CAT and control vectors (cell passage number = 18). (G, H) Quantification of Nrf2 and p16 relative expression from (F). *, $p < 0.05$, **, $p < 0.01$, n.s., not significant. All experiments were performed using mid-passage cells between p15 to p25. All experiments were repeated at least three times, and representative data were shown as indicated.

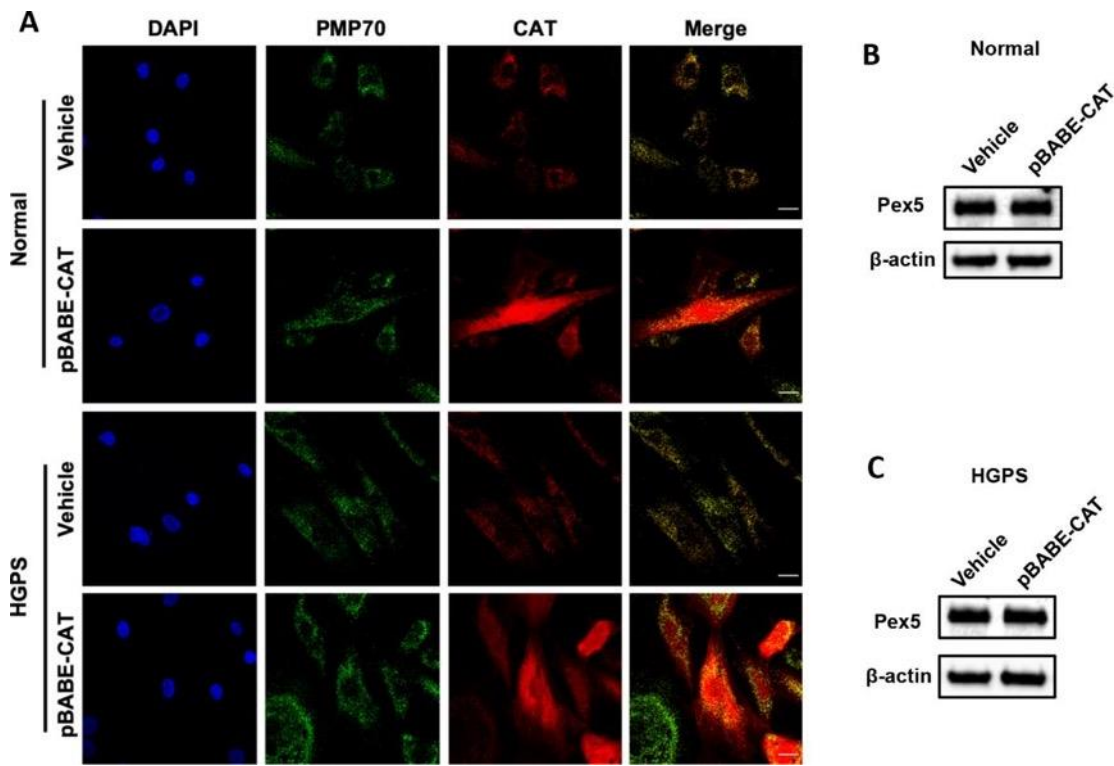


Figure 5-7 Catalase overexpression in normal and HGPS fibroblasts (A) Immunofluorescence staining of catalase in normal and HGPS fibroblasts infected by pBABE-CAT and control vectors (Vehicle). PMP70 antibody was used to indicate the peroxisomes localization. Bar = 25 μ m. (B, C) Western blot analysis showed Pex5 expression in normal and HGPS fibroblasts infected by pBABE-CAT and Vehicle (cell passage number = 21). All experiments were repeated at least three times, and representative data were shown as indicated.

5.2.5 RAD001 reduces cellular ROS and stimulates catalase activity in HGPS fibroblasts

We treated both normal and HGPS fibroblasts with 100 nM MB and RAD001 for two weeks, as described previously (DuBose et al., 2018; Z.-M. Xiong et al., 2016). We detected the ROS clearance effect of both treatments by DCFDA flow cytometry assay. We found that RAD001 treatment appears to be more effective than MB, a well-known mitochondrial-targeting antioxidant (Figure 5-8A&B). Furthermore, we analyzed the mRNA transcription, protein expression, and enzymatic activity of catalase after drug treatment. Our findings indicated that the administrated MB treatment did not affect catalase behaviors (Figure 5-8C&D). There was a reduction of catalase expression level after RAD001 treatment (Figure 5-9A). However, very interestingly, the unit catalase activity was significantly enhanced in RAD001-treated HGPS cells (Figure 5-8E and Figure 5-9B). Our findings suggested RAD001 alleviated ROS through stimulation of catalase activity.

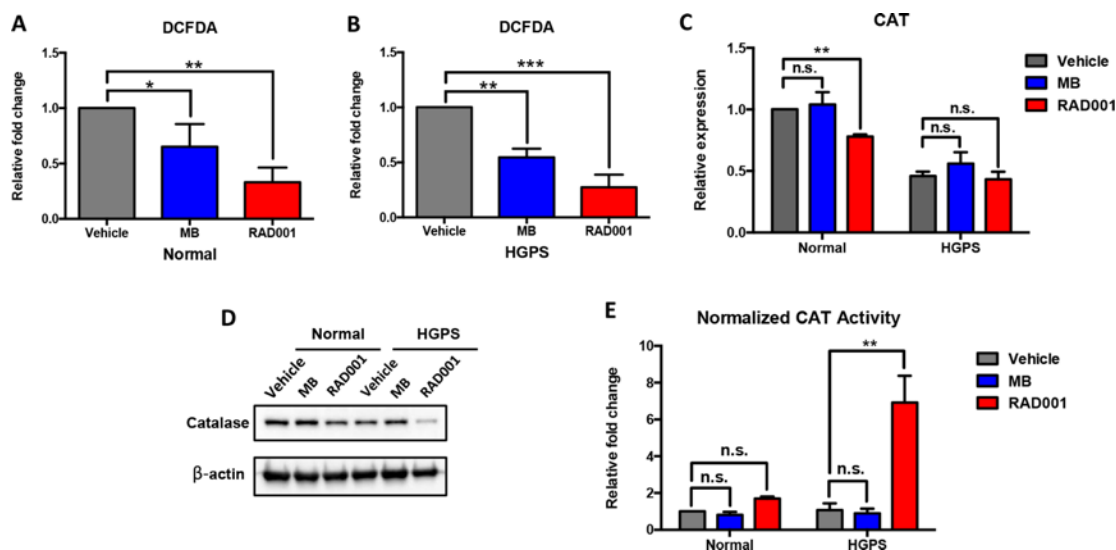


Figure 5-8 Methylene Blue (MB) and RAD001 reduce cellular ROS and RAD001 activated catalase activity in HGPS cells. Normal and HGPS fibroblasts were treated with 100nM Methylene Blue and RAD001 for two weeks. (A, B) The relative fold change of ROS activity was measured by DCFDA flow cytometry analysis. One-way ANOVA followed by Dunnett's multiple comparisons test was used to compare the effect of Methylene Blue and RAD001 treatment with the control group (Vehicle). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. (C) Relative mRNA expression of catalase was detected by quantitative RT-PCR analysis. Two-way ANOVA followed by Dunnett's multiple comparisons test was used to compare the mRNA expression of Methylene Blue, and RAD001 treated cells with the control group within each block (normal and HGPS). n.s., not significant, **, $p < 0.01$. (D) The catalase expression level was detected by Western blot analysis (cell passage number = 19). (E) Normalized catalase activity. Two-way ANOVA followed by Dunnett's multiple comparisons test was used to compare the catalase activity of Methylene Blue, and RAD001 treated cells with the control group within each block (normal and HGPS). n.s., not significant, **, $p < 0.01$. All experiments were repeated at least three times, and representative data were shown as indicated.

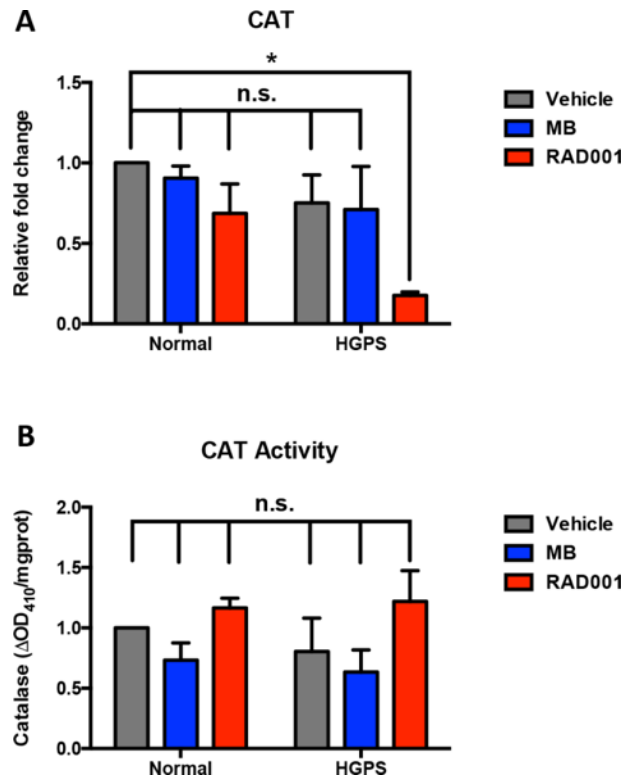


Figure 5-9 Quantification of catalase expression and activity in MB and RAD001-treated fibroblasts. (A) Quantification of catalase expression in normal and HGPS fibroblasts with MB and RAD001 treatment. (B) Raw catalase activity in normal and HGPS fibroblasts with MB and RAD001 treatment. *, $p < 0.05$, n.s., not significant. All experiments were repeated at least three times.

5.3 Discussion

5.3.1 Elevated oxidative stress may affect peroxisomal protein trafficking via Pex5

Peroxisomes targeting (especially PTS1 containing) proteins import have been found compromised in aging cells (Terlecky et al., 2006). A recent study revealed that the PTS1 transporter Pex5 is a redox-sensitive protein and that the ROS-inactivated

monoubiquitylation at Pex5 Cys-11 may affect its PTS1 binding affinity (Apanasets et al., 2014; Walton, Brees, Lismont, Apanasets, & Fransen, 2017). It is reasonable to infer that the interactions between Pex5 and PTS1-containing proteins weaken during normal aging due to the ROS accumulation in the cellular environment. Similarly, elevated oxidative stress may contribute to the moderately impaired peroxisomal protein trafficking we observed in HGPS fibroblasts.

5.3.2 Catalase deficiency and oxidative stress in HGPS

The mammalian catalase contains a noncanonical PTS1(-KANL), with a relatively low binding affinity to the Pex5 receptor (Purdue & Lazarow, 1996). Previous studies indicated that in cells under oxidative stress, an increasing portion of catalase was retained in the cytosol, which may serve as an antioxidative defense system (Murakami et al., 2013; Walton et al., 2017). In this study, we did not observe significantly mislocalized catalase in mid-passage HGPS fibroblasts and cells overexpressing progerin. Instead, we discovered a catalase deficiency that may be responsible for the ineffective ROS clearance in HGPS cells. Interestingly, humans with inherited catalase deficiency (or acatalasemia, hypocatalasemia) tend to have a high risk of developing age-related disorders, including diabetes, atherosclerosis, and cancer (Dasouki, 2017; Góth & Nagy, 2013). Thus, the down-regulated catalase expression in HGPS cells may be a downstream effect of progerin that further contributes to multiple premature aging phenotypes. Moreover, we found catalase overexpression in HGPS fibroblasts restored the Nrf2 antioxidant pathway suppressed

by progerin (Kubben et al., 2016) and significantly improved the cellular redox homeostasis.

5.3.3 Methylene Blue (MB) and RAD001 reduce cellular ROS and RAD001 activated catalase activity in HGPS fibroblasts

As a promising HGPS treatment, RAD001 promotes the progerin clearance by activating the autophagy machinery in the HGPS cells (K. Cao et al., 2011; DuBose et al., 2018). We observed a reduction of catalase expression in HGPS fibroblasts after RAD001 treatment, which may be regulated by the altered mTOR pathway. Surprisingly, our findings suggested that despite the overall reduction in catalase, RAD001 treatment enhances catalase unit activity in HGPS cells. On the contrary, MB, a mitochondrial-targeting antioxidant, significantly reduced cellular ROS but did not appear to affect either catalase activity or expression in HGPS. Our findings revealed a new role of RAD001 treatment in stimulating the catalase activity in HGPS.

Chapter 6: Summary and future directions

6.1 Summary

This dissertation mainly focuses on two studies conducted in different cell types from HGPS skin. My first goal was to investigate the potential abnormalities in HGPS epidermal development. To achieve that, this work was performed in normal and HGPS patient-derived iPSCs-keratinocytes induction system.

In Chapter 2, I characterized the normal and HGPS iPSCs-keratinocytes differentiation at different stages. The expression of epidermal developmental markers was quantified with quantitative RT-PCR and Western Blotting analysis. The early marker K8 was first detected upon the keratinocytes induction, and interestingly, its expression declined more rapidly in HGPS iPSCs-keratinocytes differentiation compared to the normal situation. Accordingly, basal layer keratinocyte markers Δ Np63 and K14 expression increased through normal and HGPS iPSCs differentiation. At each time point detected, Δ Np63 and K14 levels were consistently higher in HGPS than normal control. The terminal differentiation marker K1 was only seen in HGPS iPSCs-derived keratinocytes at week four but absent in normal iPSCs differentiation, suggesting that only the 4-week differentiated population in HGPS contained the fully matured keratinocytes to initiate the terminal differentiation (Figure 6-1A). These results indicated that HGPS iPSCs showed an accelerated commitment towards the keratinocyte lineage under the differentiation stimuli compared to normal cells.

Next, I detected the cell cycle partitioning during HGPS iPSCs-keratinocytes induction. I observed a more significant fraction of HGPS cells at the G2/M phase compared to normal differentiation at the intermediate stage. This disparity became more significant at the late stage of differentiation. Notably, a higher frequency of mitotic keratinocytes marked by PHH3 was also observed in transgenic mice with epidermal-specific progerin expression (Sola-Carvajal et al., 2019). This data showed progerin-induced mitotic defects in HGPS epidermal development.

To measure the cell death during HGPS iPSCs-keratinocytes induction, I performed an Annexin V-PI assay using cells at the intermediate and late stages of differentiation. I observed that cell death in both normal and HGPS differentiation increased from the intermediate stage to the late stage. At each stage, there was a slightly higher percentage of dying cells in HGPS iPSCs differentiation than normal control.

In Chapter 3, I investigated the WNT signaling pathway in normal and HGPS iPSCs-keratinocytes differentiation. Surprisingly, despite the unaffected β -catenin activity, the expression of a critical WNT transcription factor LEF1 was diminished from an early stage in HGPS iPSCs-keratinocytes differentiation. Chromatin immunoprecipitation (ChIP) experiment further revealed strong bindings of LEF1 to early-stage epithelial development markers K8 and K18 and that the LEF1 knockdown by siRNA down-regulates the K8/K18 transcription. In HGPS iPSCs-keratinocytes induction, the expression of K8/K18 decreases more rapidly, possibly

due to insufficient LEF1 level, thus resulting in an accelerated commitment towards the Δ Np63 and K14-positive keratinocyte lineage (Figure 6-1B).

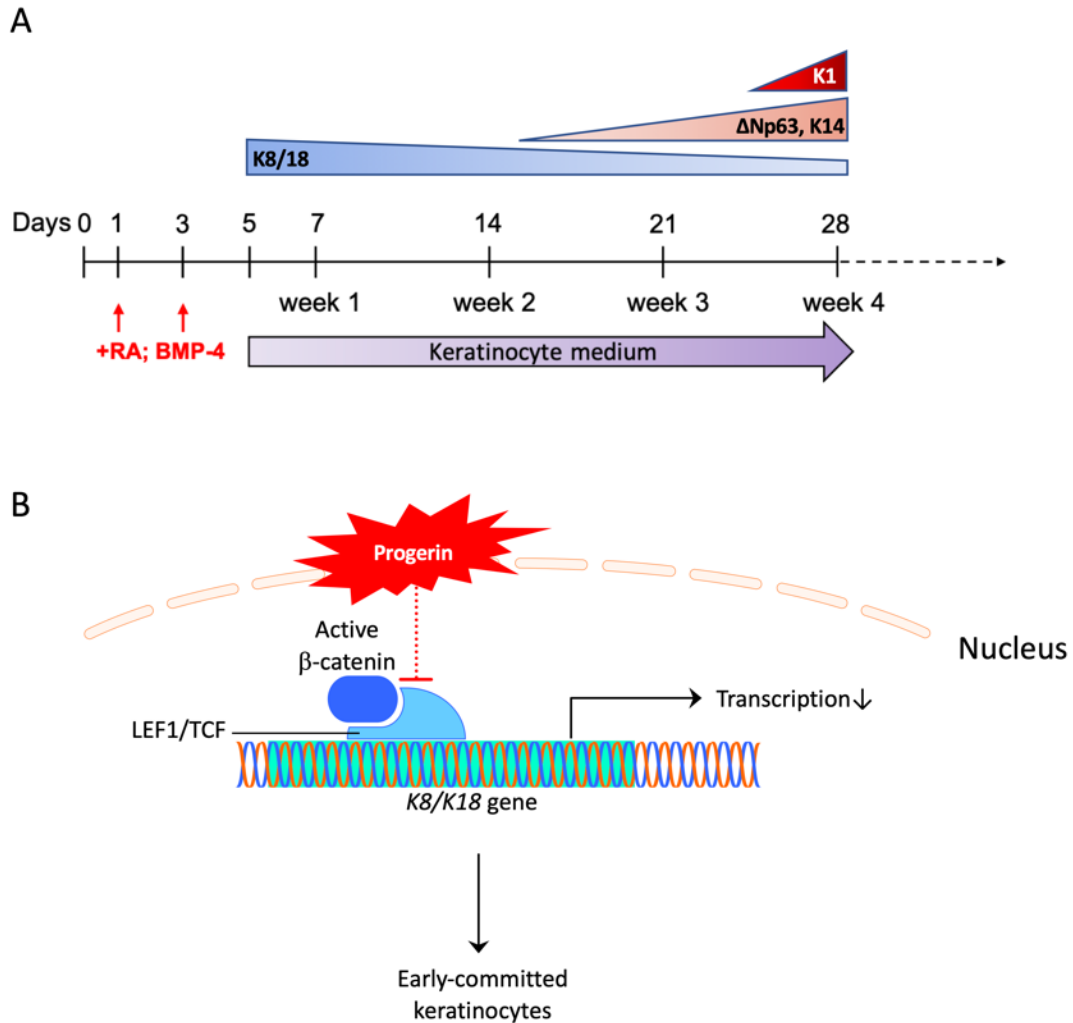


Figure 6-1 Working model of epidermal development in HGPS. (A) Schematic representation of the keratinocyte-related markers succession during iPSCs-keratinocytes induction. (B) Schematic diagram showing the molecular mechanism for the early commitment to the keratinocytes lineage in HGPS iPSCs differentiation. Illustrations were partially generated using templates from www.motifolio.com.

In Chapter 4, I endeavored to correct the HGPS mutation during the iPSCs-keratinocytes differentiation by Adenine base editing (ABE). While in a partial level, I found that this approach rescued the phenotypes for accelerated keratinocyte lineage-commitment in HGPS. ABE also reduced the cell death in HGPS iPSCs-derived keratinocytes. These findings brought new insight into the molecular basis and therapeutic application for the skin abnormalities in HGPS.

In Chapter 5, I investigated the peroxisomal abnormalities in HGPS skin fibroblasts. I observed impaired peroxisomes-targeting protein trafficking, which suggested that the poorly assembled peroxisomes might cause high oxidative stress, contributing to the premature senescent phenotype in HGPS. I then investigated the ROS clearance efficiency by peroxisomal enzymes and found a significantly decreased catalase expression in HGPS. In the end, I evaluated the effects of two promising HGPS-treatment drugs Methylene Blue and RAD001 (Everolimus, a rapamycin analog), on catalase in HGPS fibroblasts. I found that both drugs effectively reduced cellular ROS levels. As a well-known antioxidant, MB did not affect catalase expression or activity. Interestingly, the RAD001 treatment significantly upregulated catalase activity in HGPS cells. This study first characterized the peroxisomal abnormalities in HGPS and provided new insights into the cellular aspects of HGPS and the ongoing clinical trial.

6.2 Future directions

In HGPS iPSCs-keratinocytes differentiation, I identified an early onset LEF1 down-regulation in an β -catenin independent manner. However, the underlying mechanisms or signaling pathways controlling this LEF1 reduction remain unclear. Previous studies indicated BMP as a potential pathway that regulates LEF1 expression without β -catenin involvement, which has been achieved via P-SMAD in the neural stem cells differentiation (Armenteros et al., 2018). Moreover, the role of progerin at this differentiation stage is also unclear. Future work needs to be done to address these questions.

According to Chapter 4, our Adenine base editing in HGPS iPSCs differentiation was partial and could not achieve a complete progerin clearance at the end of differentiation. Thus, future work needs to be done to improve the ABE efficiency in HGPS iPSCs-keratinocytes. Two potential approaches could be tested: Reprogramming the HGPS-corrected fibroblasts to the iPSCs, or performing ABE directly in HGPS iPSCs. For future research, the HGPS-corrected iPSCs can then be differentiated into keratinocytes or other cell types of interest.

To establish a human 3D skin model that resembles the stratified epidermis tissue, fibroblasts and epidermal keratinocytes can be tested for 3D bioprinting. With the basal layer keratinocytes differentiated from iPSCs, future work can be done to reconstruct the human 3D epidermis *in vitro* with keratinocytes carrying the HGPS mutation or the ABE edited isogenic controls. Structural and functional tests of the

engineered tissue and drug tests can be performed to evaluate potential defects in this 3D disease model.

In HGPS fibroblasts, Kubben and colleagues (Kubben et al., 2016) have shown that progerin impairs the transcriptional activation of NRF2-ARE regulated antioxidants through sequestration of NRF2 away from its transcriptional targets (NRF2 mislocalization), while the NRF2 expression level remained unchanged between normal and HGPS cells. In this dissertation, we have not looked into the localization of NRF2 nor the impact of overexpression of catalase on NRF2. Future experiments could be done to address this question. Furthermore, future studies need to characterize other organelles functions in HGPS cells.

Chapter 7: Materials and methods

7.1 Chapter 2-4

7.1.1 Keratinocytes differentiation from iPSCs

Normal and HGPS patient-derived iPSCs used in this study were reprogramed from skin fibroblasts of a male HGPS patient who carried the classic G608G HGPS mutation (HGADFN167) and his normal father (HGADFN168) and provided by Progeria Research Foundation Cell and Tissue Bank. *In vitro* differentiation protocol towards the keratinocyte, lineage was adapted from previous publications with minor modifications (Itoh et al., 2011; Kogut et al., 2014). In brief, feeder-free iPSCs were cultured in Defined keratinocytes serum-free medium (DKSFM, Gibco) containing 25 ng/ml bone morphogenetic protein-4 (BMP-4, R&D Systems) and 1 μ M all-trans Retinoid Acid (RA, Sigma-Aldrich) at the initial stage of differentiation (medium was changed once at differentiation day 3). Starting from day 5, DKSFM was changed every other day for up to 28 days. Fully differentiated keratinocytes can be passed 3-5 times on Collagen I (Sigma-Aldrich)-coated tissue culture dishes. Cells were maintained at 37°C and 5% CO² in a humidified incubator.

7.1.2 Adenine base editor lentiviral vectors production

The ABE7.10max-VRQR lentiviral vector targeting the HGPS G608G mutation was generated as described in Koblan *et al.*, 2021. Human non-targeting control vectors were generated as described in Gete *et al.*, 2021. In brief, oligos containing these non-targeting sgRNAs with 5' overhang BsmBI digestion sites were synthesized by

Integrated DNA Technologies, Inc. The oligos were first annealed and inserted into the lentiCRISPR V2 plasmids (a gift from Feng Zhang, Addgene Plasmid #52961) as described previously in Sanjana, Shalem, & Zhang, 2014. The fragments containing these non-targeting sgRNAs were digested from the recombinant lentiCRISPR V2 plasmids by restriction enzymes KpnI and NheI. These fragments were then ligated into the ABEmax7.10 backbone, which was extracted from the digests of KpnI and NheI. The sequences of the recombinant plasmids were confirmed by Sanger sequencing.

7.1.3 Immunocytochemistry

For immunocytochemistry, cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA) for 15 minutes, followed by permeabilization with 0.5% Triton in PBS for 5 minutes. Cells were then blocked with 4% BSA in PBS for one h. After that, cells were incubated with primary antibodies in 4% BSA in PBS overnight at 4°C. Next day, after five PBS washes, cells were incubated in secondary antibodies diluted in 4% BSA in PBS for 1 h. Cells were then washed five times with PBS, and fluorescence images were acquired with Zeiss LSM 710 confocal microscope (Zeiss International, Oberkochen, Germany). Fluorescence intensity was adjusted with ImageJ software (NIH). Primary antibodies used for immunocytochemistry were: lamin A/C (1:250, Millipore MAB3211), progerin (1:250, Cao et al., 2011), ΔNp63 (1:250, Biolegend #619001), K14 (1:500, Invitrogen MA5-11599). Secondary antibodies used were: Alexa Fluor 488 donkey anti-rabbit IgG (1:1000, Invitrogen), Alexa Fluor 594 donkey anti-rabbit IgG (1:1000, Invitrogen), Alexa Fluor 488

donkey anti-mouse IgG (1:1000, Invitrogen), and Alexa Fluor 594 donkey anti-mouse IgG (1:1000, Invitrogen).

7.1.4 Western blot

Whole-cell lysates for immunoblotting were prepared by dissolving cells in Laemmli Sample Buffer containing 5% 2-mercaptoethanol (Bio-Rad). Nuclear and cytosolic fractions of iPSCs-differentiated cells were acquired using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific #78833) as per the manufacturer's instructions. Protein samples were loaded on 10%-12% polyacrylamide gels and transferred onto 0.45 µm pore-size nitrocellulose membranes (Bio-Rad) using the Turboblot (BioRad). Blots were incubated overnight at 4°C with primary antibodies and then probed with secondary antibodies before ECL development and imaging (Bio-Rad). Primary antibodies used for immunoblotting are as follows: lamin A/C (1:500, Millipore MAB3211), progerin (1:500, Cao et al., 2011), ΔNp63 (1:500, Biolegend #619001), K14 (1:500, Invitrogen MA5-11599), K8 (1:500, Biolegend #904804), K18 (1:1000, Cell Signaling #4548), total β-Catenin (1:1000, Cell Signaling #9562), Non-phospho (Active) β-Catenin (1:1000, Cell Signaling #8814), RCC1 (1:1000, Cell Signaling #3589), S6 Ribosomal Protein (1:1000, Cell Signaling #2217), LEF1 (1:250, Santa Cruz sc-374412) and β-actin (1:1000, Sigma-Aldrich A3854).

7.1.5 RNA isolation and Real-Time quantitative PCR

Total genomic RNA was extracted with Trizol (Life Technologies) and purified using the RNeasy Mini kit (Qiagen) as per the manufacturer's instructions. The RNA yield was determined by the NanoDrop 2000 spectrophotometer (Thermo Fisher). 1 µg of total RNA was converted to cDNA using iScript Select cDNA Synthesis kit (Bio-Rad). Quantitative RT-PCR was performed in triplicate using SYBR Green Supermix (Bio-Rad) on CFX96 Real-Time PCR Detection System (C1000 Thermal Cycler, Bio-Rad). All primers used in this study are listed as follows:

Table 7-1 Primer sequences used for iPSCs-keratinocytes RT-qPCR experiments

ACTB	Forward	5'-CTGGAACGGTGAAGGTGACA-3'
	Reverse	5'-AAGGGACTTCCTGTAACAATGCA-3'
LMNA	Forward	5'-GCAACAAGTCCAATGAGGACCA-3'
	Reverse	5'-CATGATGCTGCAGTTCTGGGGGCTCTGGAT-3'
Progerin	Forward	5'-GCAACAAGTCCAATGAGGACCA-3'
	Reverse	5'-CATGATGCTGCAGTTCTGGGGGCTCTGGAC-3'
K8	Forward	5'-CAGAAGTCCTACAAGGTGTCCA-3'
	Reverse	5'-CTCTGGTTGACCGTAACTGCG-3'
K18	Forward	5'-GGCATCCAGAACGAGAAGGAG-3'
	Reverse	5'-ATTGTCCACAGTATTTGCGAAGA-3'
ΔNp63	Forward	5'-ACCTGGAAAACAATGCCCAGA-3'
	Reverse	5'-GGCAATCTGTCCCTCGTTGA-3'
K14	Forward	5'-TGAGCCGCATTCTGAACGAG-3'
	Reverse	5'-GATGACTGCGATCCAGAGGA-3'
K1	Forward	5'-GGTGCTTATATGACCAAGGTGG-3'
	Reverse	5'-ATGCTGTCCAGGTCGAGACT-3'
LEF1	Forward	5'-CGATGACGGAAAGCATCCAG-3'
	Reverse	5'-CCACCCGGAGACAAGGGATA-3'
TCF7	Forward	5'-AACATTTCAACAGCCCACATCC-3'
	Reverse	5'-GGAGTAGAAGCCAGAGAGGTC-3'
TCF7L1	Forward	5'-CCACAGTCAAGGACACGAGG-3'
	Reverse	5'-TCGATCTCTGGGAGAGGTG-3'
TCF7L2	Forward	5'-CCTCACGCCTCTTATCACGTA-3'
	Reverse	5'-AGGCGATAGTGGGTAATACGG-3'

7.1.6 Cell cycle analysis

Cell cycle analysis was performed at differentiation week 2 and week 4 in normal and HGPS iPSCs-keratinocytes induction. Before harvest, cells were incubated in 10 μ M BrdU (BD) labeling medium for 1 h. The collected cells were then washed once and resuspended in 100 μ L PBS. The cell suspension was fixed in 1 mL of 70% (vol/vol) ethanol for 1 h on ice, followed by a PBS wash and denatured in 2N HCl with 0.5% Triton X-100 for 30 min at RT. Cells were washed again with PBS, blocked and incubated in Alexa Fluor 488 anti-BrdU antibody solution (1:100, Invitrogen #B35130) at 4 °C overnight. On the next day, the cells were pelleted, rinsed with PBS, and resuspended in 50 μ g/mL propidium iodide (PI, Invitrogen) with 100 μ g/mL DNase-free RNase (Thermo Scientific) in PBS for 30 min at 37 °C. Flow cytometry was performed with FACS CantoII (BD) and the data were analyzed by FlowJo software.

7.1.7 Apoptosis assay

PI-annexin V apoptosis assay was performed at differentiation week 2 and week 4 in normal and HGPS iPSCs-keratinocytes induction according to the manufacturer's instruction (BD). In brief, cells were harvested and rinsed with PBS, then resuspended and stained with 100 μ L of 1x annexin V binding buffer containing 5 μ L of annexin V and 5 μ L of PI for 25 min in the dark at room temperature. Stained samples were analyzed by FACS CantoII (BD) and the data were processed by FlowJo software.

7.1.8 Chromatin immunoprecipitation

LEF1 ChIP was performed in early-differentiating (week 1) epidermal cells derived from normal iPSCs. Detailed experimental procedures were described in the protocol of SimpleChIP® Plus Sonication Chromatin IP Kit (Cell Signaling). Briefly, protein and DNA were cross-linked using 1% formaldehyde for 10 min. Nuclei were isolated and lysed, and chromatin was sheared to an average size of 400 bp by sonication. A small aliquot of the supernatant was used as input control, and the remaining sonicated chromatin was divided into two aliquots, which were incubated with an anti-LEF1 antibody (1:50, Cell Signaling #76010) and anti-rabbit IgG antibody (1:1000, or 1 µL for one immunoprecipitation, Cell Signaling #2729) as a negative control, respectively. The antibody-bound complex was precipitated with Dynabeads protein G (Invitrogen). The DNA fragments were released from the immunoprecipitated complexes by reversing the cross-linking at 65°C overnight. The precipitated DNA was isolated, purified and used as a template for PCR. Primers used for ChIP-qPCR were listed below.

Table 7-2 Primer sequences used for LEF1 ChIP-qPCR experiments

MYC	Forward	5'-CCCAAAAAAAGGCACGGAA-3'
	Reverse	5'-TATTGGAAATGCGGTCATGC-3'
K8-1	Forward	5'-TCCCAGGAGCCAGTCAGCGT-3'
	Reverse	5'-GCCCATTGAGCCCGTGTCCC-3'
K8-2	Forward	5'-GCTCCCAACGGGCCAGAGGA-3'
	Reverse	5'-GCCTGGGGTGCCTTGCTCAG-3'
K8-3	Forward	5'-ACCTTCAGACCACCCTCCCCC-3'
	Reverse	5'-ACCCTGGGAAGAGGCAGCAGA-3'
K8-4	Forward	5'-CGTCCTGTGAACTGACCCTCCC-3'
	Reverse	5'-TGTTTTCCTGCTACTGCACA-3'
K8-5	Forward	5'-CCCCTCCGTCATCCTGGCCT-3'
	Reverse	5'-CCTGGGGCTGGCTGGTAGGA-3'

K8-6	Forward	5'-CTGCGCCCGGTCAAGACTCC-3'
	Reverse	5'-CCGGGCATGGACACGGACAG-3'
K8-7	Forward	5'-CACAGACCCGGGCAGAGGGA-3'
	Reverse	5'-TCTTCCAGCAGGCGGCGGTA-3'
Negative	Forward	5'-GCCACTTGGGAGGCTGAGGC-3'
	Reverse	5'-TTGAGCCACTGCACCCTGCC-3'

7.1.9 Silencing experiments

The siRNA plasmids consist of a pool of three human LEF1-targeting sequences (Santa Cruz, sc-35804) and the control scrambled siRNA (Santa Cruz, sc-37007) were purchased from Santa Cruz. Transient transfections were carried out using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the manufacturer's protocol. After 48 h of transfection, cells were collected for RT-qPCR and Western blotting analysis.

7.1.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software. Data were analyzed using unpaired Student's *t*-test for two groups. One-way and two-way analysis of variance (ANOVA) followed by post hoc multiple comparisons were used to compare means of three or more groups. Analysis method adapted in each experiment was indicated in figure legends. All experiments were repeated at least three times and results were presented as the mean $\pm$ SD. *p* value < 0.05 or < 0.1 was considered significant.

7.2 Chapter 5

7.2.1 Cell culture and drug treatment

The normal and HGPS human dermal fibroblast lines HGFDFN168 and HGFDFN167 were obtained from Progeria Research Foundation (PRF). HGFDFN167 carries the canonical *LMNA* c.1824C>T mutation of HGPS. All fibroblast cell lines were cultured in EMEM (Lonza) supplemented with 15% FBS (VWR International) at 37 °C with 5% CO₂. Methylene Blue (MB; Acros Organics) and RAD001 (Everolimus; AdooQ BioScience) were dissolved in PBS and added to the growth medium at a final concentration of 100 nM. Fresh medium was provided twice a week, and the cells were passaged at ratio 1:3 when reaching 95% confluency. All the biomolecular detections in this work were performed using mid-passage cells between p15 to p25.

7.2.2 Immunocytochemistry and peroxisomal quantification

Immunostaining was carried out using the following antibodies: catalase (1:1000, Cell Signaling #12980), PMP70 (1:200, Santa Cruz sc-514728). DAPI (Vector Laboratories) was used to counterstain cell nuclei. Images were acquired with Zeiss LSM 710 confocal microscope (Zeiss International, Oberkochen, Germany). Fluorescence intensity was adjusted with ImageJ software (NIH). A quantitative measurement of peroxisomes number per square unit was performed using ImageJ. In each green-fluorescence channel image, cells of interest were outlined and their surface areas (in pixels) were measured. These outlined cells were then analyzed

using the particle analysis function for the number of particles greater than 4×4 pixels, which indicated the PMP70-positive peroxisomes. To account for the size difference of the cells, the peroxisomes counts were normalized with the cell surface areas.

7.2.3 GFP-SCP2/PMP70 colocalization analysis

Normal and HGPS fibroblasts were transfected with GFP-SCP2 plasmid (a kind gift from Dr. Ralf Erdmann, RUB, Germany) using Lipofectamine 2000 (Thermo Fisher). At 48 hours post transfection, cells were fixed and immunostained with PMP70 antibody (1:200, Santa Cruz sc-514728). Zeiss RGB fluorescence images were separated into respective channels in CellProfiler and named accordingly. The peroxisomes were identified and segmented by a primary object identification module, whereas cell borders were manually defined with the manual free drawing object module. Peroxisomes intensity was constrained using Otsu threshold and segmented by intensity automatic settings. A related object module was implemented to quantify the percentage of PMP70 puncta not colocalized with GFP-SCP2.

7.2.4 RNA isolation and Real-Time quantitative PCR

Total genomic RNA was extracted with Trizol (Life Technologies) and purified using the RNeasy Mini kit (Qiagen) as per the manufacturer's instructions. The RNA yield was determined by the NanoDrop 2000 spectrophotometer (Thermo Fisher). 1 μ g of total RNA was converted to cDNA using iScript Select cDNA Synthesis kit (Bio-

Rad). Quantitative RT-PCR was performed in triplicate using SYBR Green Supermix (Bio-Rad) on CFX96 Real-Time PCR Detection System (C1000 Thermal Cycler, Bio-Rad). All primers used in this study are listed as follows.

Table 7-3 Primer sequences used for fibroblasts RT-qPCR experiments

PEX5	Forward	5'-CTGAATTCCTGCAGGACCAGAATGCA-3'
	Reverse	5'-GTTCCCTCAGGTTCTCCCAGCCA-3'
PEX7	Forward	5'-TTGATGTGACTTGGAGTGAGAAC-3'
	Reverse	5'-CCCACAATTTGACAGTTTGATCC-3'
CAT	Forward	5'-TGGAGCTGGTAACCCAGTAGG-3'
	Reverse	5'-CCTTTGCCTTGGAGTATTTGGTA-3'
GPx	Forward	5'-AGATGAACGAGCTGCAGCGGCG-3'
	Reverse	5'-ACCGGAGACCAGGTGATGAGCTTG-3'

7.2.5 Western blot

Whole-cell lysates for immunoblotting were prepared by dissolving cells in Laemmli Sample Buffer containing 5% 2-mercaptoethanol (Bio-Rad). Antibodies used in this study are as follows: lamin A/C (1:500, Millipore MAB3211), catalase (1:1000, Cell Signaling #12980), Nrf2 (1:200, Santa Cruz sc-722), p16 (1:200, Santa Cruz sc-468), and β -actin (1:1000, Sigma-Aldrich A3854).

7.2.6 Catalase activity and glutathione peroxidase activity assay

Cells grown on 100-mm culture dishes were trypsinized at 90% confluency, pelleted, and analyzed using the OxiSelect™ Catalase Activity Assay Kit, Fluorometric (STA-339, Cell Biolabs), and the Glutathione Peroxidase Assay Kit (Abcam) as per the manufacturer's instructions. All "Normalized Catalase activity" data are normalized using Western blot quantification results.

7.2.7 ROS measurement by DCFDA assay

To measure cellular ROS activity, cells grown on 100 mm dishes were trypsinized at 90% confluency, rinsed with PBS, and then incubated in 1x dilution buffer containing 12.5 μ M DCFDA (Abcam) at 37 °C. After 30 min of incubation, the DCFDA intensity was detected by flow cytometry assay excited by a laser at 488 nm, and the data were recorded at 530 ± 15 nm. Flow cytometry was performed with FACS CantoII (BD Biosciences). The data were analyzed by FlowJo software.

7.2.8 Retroviral vectors production and virus transduction

Restriction enzymes SnaBI and SalI (New England Biolabs) were used to digest the human catalase sequence from the CAT-pC1 plasmid (He et al., 2006), which was then subcloned into the pBABE-puro retroviral vectors (a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg, Addgene plasmid # 1764). After verification by sequencing, these retroviral constructs were co-transfected into HEK293T cells with two packaging vectors VSV-G, and Gag-Pol using Fugene 6 (Promega). 48 h after transfection, the culture supernatant containing viruses was purified by filtration through 0.45 μ m filters and stored at -80°C. Puromycin selection was performed after virus transduction at 1 μ g/ml in culture media for three days.

7.2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. The data are shown as mean $\pm$ SEM. The student's *t*-test (two-tailed) was performed to compare two groups except for Figure 5-1D, where a Kolmogorov-Smirnov test was utilized. One-way ANOVA followed by Dunnett's multiple comparisons test was used to compare the effect of Methylene Blue and RAD001 treatment with a control group (Vehicle); Two-way ANOVA followed by Dunnett's multiple comparisons test was used to compare the mRNA expression of Methylene Blue, and RAD001 treated cells with the control group within each block (normal and HGPS); Two-way ANOVA followed by Dunnett's multiple comparisons test was used to compare the catalase activity of Methylene Blue, and RAD001 treated cells with the control group within each block (normal and HGPS). $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times, and representative data were shown as indicated.

Appendices

Besides the above studies, I was also involved in the following research:

Ultraviolet radiation protection potentials of Methylene Blue for human skin and coral reef health (Z.-M. Xiong et al., 2021)

Methylene blue (MB) is a century-old medicine, a laboratory dye, and recently shown as a premier antioxidant that combats ROS-induced cellular aging in human skins.

Given MB's molecular structure and light absorption properties, we hypothesize that MB can be considered sunscreen active for UV radiation protection. In this study, we tested the effects of MB on UVB ray-induced DNA double-strand breaks in primary human keratinocytes. We found that MB treatment reduced DNA damages caused by UVB irradiation and subsequent cell death. Next, we compared MB with Oxybenzone, the most commonly used chemical active ingredient in sunscreens but recently proven to be hazardous to aquatic ecosystems, particularly coral reefs. At the same concentrations, MB showed more effective UVB absorption ability than Oxybenzone and significantly outperformed Oxybenzone in preventing UVB-induced DNA damage and the clearance of UVA-induced cellular ROS. Furthermore, unlike Oxybenzone, MB-containing seawater did not affect the growth of the coral species *Xenia umbellata*. Altogether, our study suggests that MB has the potential to be a coral reef-friendly sunscreen active ingredient that can provide broad-spectrum protection against UVA and UVB.

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