Bachelor's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biological Sciences Ålesund

10003

Depigmentation - Possible causes of change in phenotype, potential genetic mechanisms, and methods for investigating the cause of depigmentation

Bachelor's thesis in Biotechnology
Supervisor: Ann-Kristin Tveten
May 2022

NTNU
Kunnskap for en bedre verden

10003

Depigmentation - Possible causes of change in phenotype, potential genetic mechanisms, and methods for investigating the cause of depigmentation

Bachelor's thesis in Biotechnology
Supervisor: Ann-Kristin Tveten
May 2022

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biological Sciences Ålesund

NTNU
Kunnskap for en bedre verden

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Ann-Kristin Tveten, Ph.D., Associate Professor and Program Manager of the Bachelor in Biotechnology at the Department of Biological Sciences in Aalesund. I am grateful for the advice and support that I have been given during this research project. The topic for this literary study was chosen based on a request from Dr. Lado Lako Loro from the hospital in Aalesund, with an aim of eventually conducting a case study. I have always had a fascination towards rare diseases. Understanding the cause of disorders is important to develop novel therapeutics that could make a difference and improve patients' quality of life. I would like to thank Dr. Lado Lako Loro for suggesting the topic matter for this project. It has been a privilege.

I would also like to extend my gratitude to my Godmother and Medical Doctor, Tove Haugan, and her husband, Kim Kise, for the hospitality and support they have given me, as well as many insightful and intriguing conversations on topics related to my degree.

Abstract

Defects in pigmentation are either classified as hypo-, hyper-, or depigmentation. As opposed to hypo- and hyperpigmentation, where the macules contain a decreased or increased amount of pigments respectively, the depigmented macules do not contain pigments. Patients with depigmented macules may face social stigmatization, reduced self-esteem, and an impaired quality of life. Dermatologists often encounter cases of depigmentation disorders that present a broad specter of differential diagnosis. It may be challenging to diagnose due to diversity, rarity, and progression over time. The challenge in setting diagnosis can also be explained by clinical findings, contraindications or the patient is not responsive to conventional treatment. Studying key enzymes involved in pigment production and signaling pathways regulating it, makes it possible to identify genes of interest. It is pivotal to study the cause of depigmentation by analyzing the patient's genetic makeup or altered gene expression to further understand the implications of the condition. Identification of culprit genes would make it possible to design innovative techniques for diagnosing future cases of depigmentation. By understanding the molecular underpinnings of depigmentation disorders, it would make it possible to develop novel therapeutics that can improve the patient's quality of life.

Sammendrag

Defekter i pigmentering klassifiseres som enten hypo-, hyper- eller depigmentering. Hypo- og hyperpigmentering er tilstander med henholdsvis redusert og økt mengde pigment. Depigmentering er en tilstand hvor områder av huden mangler pigment. Pasienter med depigmentering kan føle på lav selvtillit, sosial stigmatisering og redusert livskvalitet. Tilstander med depigmentering kan være vanskelig å diagnostisere grunnet et stort mangfold av ulike tilstander, sjeldenhet, samt progresjon av tilstanden over tid. Utfordringen med å stille rett diagnose kan også skyldes kliniske funn og kontraindikasjoner, eller at pasienten ikke responderer på konvensjonell behandling. Identifisering av mulige målgen kan oppnås ved å studere sentrale enzymer involvert i pigmenteringsprosessen og reguleringen av disse. Det er viktig å studere årsaken til depigmentering ved genomsekvensering eller genekspresjonsanalyser for å forstå tilstanden. Identifisering av målgen vil kunne gjøre det mulig å designe metoder for å diagnostisere fremtidige tilfeller av depigmentering, samt utvikling av behandlingsmetoder for å forbedre livskvaliteten til pasienter.

Abbreviations

UVR – Ultraviolet Radiation

NCCs – Neural Crest Cells

ROS – Reactive Oxygen Species

DAG – Diacylglycerol

DAMPs – Damaged Associated Molecular Patterns

UTR – Untranslated region

GOI – Genes of Interest

WGS – Whole Genome Sequencing

WES – Whole Exome Sequencing

RNA-seq – RNA sequencing

NGS – Next Generation Sequencing

TGS – Third Generation Sequencing

ONT – Oxford Nanopore Technology

PacBio – Pacific Biosciences

SNPs – Single Nucleotide Polymorphisms

SV – Structural Variation

GWAS – Genome-Wide Association Studies

RT-qPCR – Reverse Transcription Quantitative Polymerase Chain Reaction

FFPE – Formalin Fixed Paraffin Embedded tissue

FISH – Fluorescence *in situ* Hybridization

IHC – Immunohistochemistry

SDS-PAGE – Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

HKPs – Housekeeping Proteins

Table of Contents

1 Introduction

This literary study is a part of a greater project with an aim of conducting a case study on a patient with an unknown cause of depigmentation. Although there are three categories of pigmentation-related dermatoses, hypo-, hyper- and depigmentation, this study is restricted to studying depigmentation disorders that develop over time. Depigmentation disorders are characterized by defined macules or patches in the skin that lack pigmentation and occurs at some point in life. Depigmentation disorders constitute several genetic defects affecting mechanisms in melanin synthesis, melanosome maturation, translocation of melanosomes, dendritic projections, signaling from keratinocytes and dermal fibroblasts (1, 2). Dermatoses associated with defects in pigmentation can be acquired, congenital, skin-restricted, systemic, temporary, or permanent (3, 4). It is important to understand the biology of melanocytes, the biochemistry of melanogenesis, and signaling pathways between melanocytes, keratinocytes, and dermal fibroblasts to understand the implications of genetic variants and altered gene expression. There are more than 125 genes that are known to regulate pigmentation (5). Suggesting causal genes for the progression of depigmentation may be difficult. The aim of this study is to describe different techniques to search for culprit genes and altered expression to find the potential cause of rare conditions of depigmentation that are not vitiligo. Sequencing technologies for determining genotype, gene expression analysis and protein expression analysis will be discussed as methods for investigating the cause of depigmentation. Establishing a cell line from the patient's own skin cells is not going to be described in this literary study, considering that gene- and protein expression may be altered due to the different environment *ex vivo.* Cultivation of the patient's own skin cells may be done to evaluate the effect of prospective treatments (6). Skin biopsies from the patient will be recommended for studying gene- and protein expression.

2 Background

2.1 Epidermal melanocytes

Epidermal melanocytes are the pigment producing cells of the skin. Melanocytes and keratinocytes reside in the basal layer of the epidermis in a ratio of 1:10. Each melanocyte is in contact with approximately 36 keratinocytes through many protruding filopodia (2, 7). The keratinocytes that reside in the basal layer are constantly proliferating, while melanocytes are considered to be a stable population. The difference between light and dark phenotypes is not due to different numbers of melanocytes, but rather differences in the molecular mechanisms governing pigment production. Variance in skin color is manifested in the intensity of melanogenesis and the arrangement of melanocytic dendrites (2). E- and P-cadherins are important cell-cell junctions between melanocytes and keratinocytes (8). Filopodia are necessary in the transfer of melanin into keratinocytes where the melanin accumulate on the surface of the nucleus to protect it from ultraviolet radiation (UVR) and subsequent DNA damage (7). Dermal fibroblasts, melanocytes and keratinocytes communicate by the secretion of molecular cues and cell-cell junctions (2).

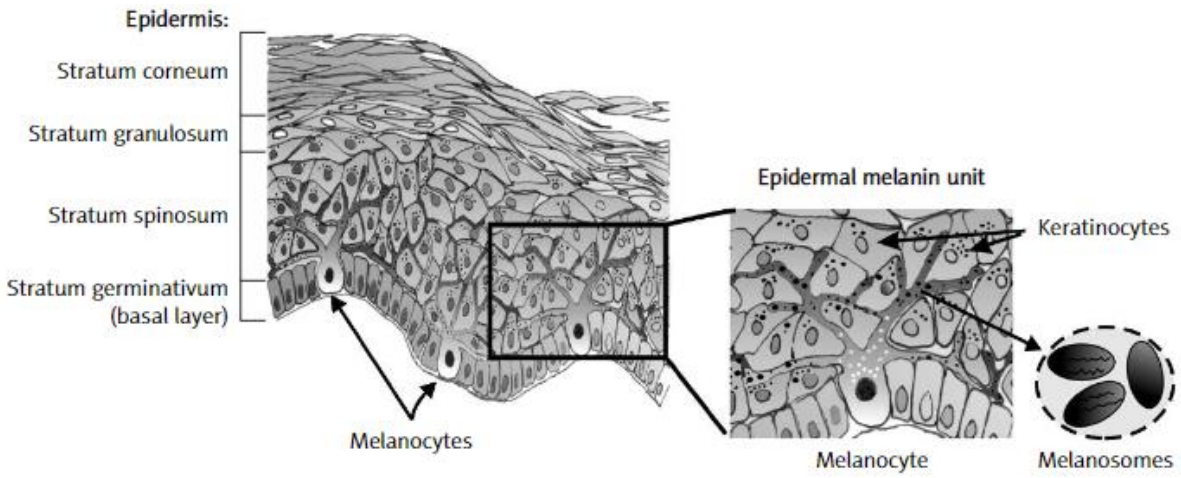


Figure 1. *Illustrating the structural components of the epidermis. The dermis is located below the basal layer and is not shown in this figure. Melanocytes with protruding filopodia and proliferating keratinocytes are adhered to the basal layer. As keratinocytes proliferate, the differentiated daughter cells are gradually pushed upwards. The outermost layer, stratum corneum, consists of dead keratinocytes. Melanocytes transport melanin containing organelles, melanosomes, to surrounding keratinocytes in the stratum spinosum. The melanin shields their nucleus from the harmful effect of UVR (2). Figure 1 by M. Cichorek et al. is licensed under creative commons.*

Melanocytes originate from several populations of neural crest cells (NCCs) in the embryonic phase (9, 10). *In vitro* experiments show that melanocyte proliferation requires many signaling pathways, but under normal circumstances epidermal melanocytes proliferate rarely and are hence thought of as a stable population (11). Melanocytes that are terminally differentiated have a limited potential of proliferation due to changes in cell cycle control elements (2, 12). Melanocytes can be identified molecularly by specific markers, such as TYRO, TYRP1, TYRP2, PMEL, MAR1 and MITF (13).

2.2 Melanogenesis

The process of melanin synthesis is referred to as melanogenesis. Melanin is synthetized in a cascade of spontaneous and enzymatic reactions known as the Raper-Mason pathway (14). There are two types of melanin: eumelanin (dark brown) and pheomelanin (yellow reddish). Melanin has beneficial properties, such as absorption and scattering of UVR, ion storage, coupled redox reactions and scavenging of free radicals (2). The types of melanin produced is determined by the function of melanogenic enzymes and the availability of substrates. Cytotoxic intermediates are synthetized during melanogenesis (hydrogen peroxide and quinones), which makes it favorable that the melanogenesis takes place in separate compartments that are called melanosomes (2, 7). The eumelanin to total melanin ratio determines an individual's complexion. The amount of pheomelanin does not correlate with skin pigmentation, and a similar amount of pheomelanin is found in people with different complexions (2, 15). The number and size of melanosomes, the amount of eumelanin, transfer of melanosomes, distribution, and rate of degradation in surrounding keratinocytes are factors that make up the phenotypic diversity (16, 17). Eumelanin possesses a greater photoprotection with the ability to neutralize reactive oxygen species (ROS) and a higher resistance to degradation than its pheomelanin counterpart (2).

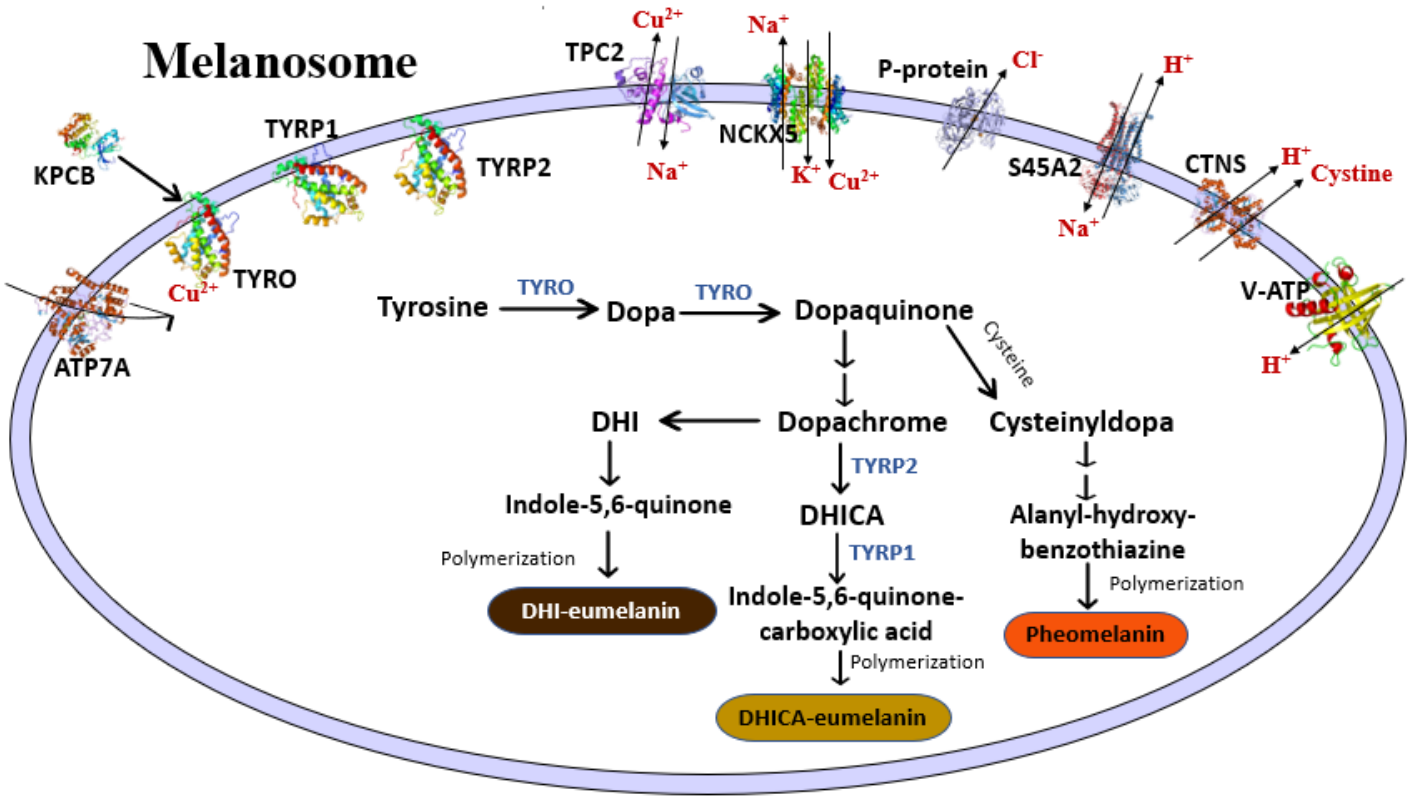


Figure 2. *Illustration of important enzymes and ion transport proteins in melanosomes. Melanogenesis takes place in the lumen of melanosomes. Tyrosinase (TYRO) is a membrane bound enzyme that catalyze the oxidation of tyrosine to form the intermediate 3,4-dihydroxyphenylalanine (dopa), which is further oxidized by TYRO to form dopaquinone. Dopaquinone conjugates with cysteine when present, which results in the formation of yellow reddish pheomelanin. When cysteine is depleted, dopaquinone undergoes an addition reaction to form dopachrome. Dopachrome can either spontaneously form 5,6-dihydroxyindole (DHI) or it can be converted to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by the action of TYRP2. TYRP1 is involved in the formation of DHICA-eumelanin, which has a lighter brown color compared to the dark brown DHI-eumelanin. TYRO, TYRP1 and TYRP2 are all membrane bound enzymes. KPCB phosphorylates and activates TYRO. The transport of substates and ions are regulated by a set of different channels in the melanosomal membrane. ATP7A is an ion channel that provides TYRO with its cofactor Cu^{2+}. The activity of TYRO is pH dependent. The transport of ions and amino acids through CTNS, p-protein, S45A2, TPC2, vacuolar ATP (V-ATP) complex, and NCKX5 is controlled by an intricate interconnected network of regulators. This figure is illustrated by the author and is based on information retrieved from a selection of articles (18, 19).*

The glycoprotein tyrosinase (TYRO) has a cytoplasmic-, transmembrane- and internal domain. The internal domain makes up 90% of the protein. TYRO is dependent on copper, which binds to histidine residuals in the catalytic internal domain (20). The cytoplasmic domain of protein kinase C beta (KPCB) activates TYRO by phosphorylating two of its serine residuals (4). Phenylalanine hydroxylase (PAH) with cofactor 6BH4 is located in the cytosol and converts phenylalanine to tyrosine, thus maintaining the tyrosine supply and promoting TYRO activation. TYRO is inactivated when copper is oxidized, but electron donors such as dopa, superoxide anion, and ascorbic acid can activate it (21). The amino acid, tyrosine, is hydroxylated to L-3,4-dehydroxyphenylalanine (dopa) and subsequently oxidized to dopaquinone, both reactions catalyzed by TYRO (2). Two additional enzymes present in the melanosomal membrane are important in the biogenesis of eumelanin, tyrosinase related protein 1 (TYRP1) and dopachrome tautomerase (TYRP2). TYRP2 requires zinc for its function (20, 21). If cysteine is present, it reacts spontaneously with dopaquinone in a condensation reaction, which yields either 3- or 5-cysteinyldopas. The cysteinyldopas oxidize and polymerize to form soluble yellow-reddish pheomelanin (22, 23). The synthesis of pheomelanin is favored until the melanosome is exhausted from cysteine, which results in a switch to synthesizing eumelanin. DHICA- and DHI-eumelanin is deposited on a pheomelanin granular core (18, 19). Eumelanin is produced when thiols like glutathione, cysteine and thioredoxin are absent. Then dopachrome is formed by a spontaneous cyclization of dopaquinone (2). Dopachrome forms 5,6-dihydroxyindole (DHI) by the spontaneous loss of carboxylic acid, which subsequently oxidize and polymerize to generate dark brown DHI-melanin. In the presence of dopachrome tautomerase (TYRP2), dopachrome is converted to DHI-carboxylic acid (DHICA) (24). Further conversions of DHICA are catalyzed by TYRO and TYRP1 to form a lighter brown DHICA-melanin (15, 23).

Electrochemical gradients are important for the transport of ions into and out of the melanosome. Ion homeostasis is maintained by the function of many transport proteins. Ions are indispensable factors that contribute to redox reactions, maintaining pH, enzymatic reactions, and electrical signaling (19). TYRO function relies on Cu^{2+} and pH. Na^{+}, K^{+}, Ca^{2+} and Cl^{-} may be important for maintaining membrane potential and internal pH. Melanosomal pH can determine the eumelanin to pheomelanin ratio and the rate of melanogenesis. TYRO, TYRP1 and TYRP2 have a diminished activity under acidic conditions but are active when the pH is neutral (1, 4).

Melanosomes has four maturation stages (20). Maturation of melanosomes depends on the proton pump (p-protein) in the melanosomal membrane, which increases the pH from 5 to 6.8 (21). Melanosome pH relies on the activity of a combination of ion transport proteins, e.g., S45A2, p-protein and TPC2. S45A2 and p-protein are positive regulators for pH neutralization, while TPC2 serves as a negative regulator (6, 19). Individuals with light skin tend to have melanosomes with more acidic conditions and subsequently a lower activity of melanogenic enzymes. Individuals with dark skin tend to have melanosomes with neutral pH. Experiments aiming to increase melanosomal pH using NH_4Cl showed an increased activity of TYRO in melanocytes from Caucasians (6). Mature melanosomes migrate bi-directionally towards the dendrites. Kinesin facilitates transport of melanosomes from the perinuclear area towards the plus ends of microtubules in the periphery. When the melanosomes reach the cell cortex, they bind actin filaments through a complex of MYO5A, MELPH, and RAB27A (20). The translocation of melanosomes happens through phagocytosis by keratinocytes via PAR2, as well as unidentified glycoproteins and lectins (1). Melanosomes entering the cytoplasm of keratinocytes disperse and covers the cytoskeleton surrounding the nuclei, protecting it from the harmful effect of UVR (25).

2.3 Signaling and regulation

Signal transduction regulates gene expression, resulting in up- or down regulation depending on the molecular mechanisms that are transduced. It is important to regulate melanogenesis to maintain homeostasis and adequate protection against UVR (1). Melanin absorbs ultraviolet and visible light, which leads to thermoregulation, photoreceptor shielding and photoprotection. Dermal fibroblasts, melanocytes and keratinocytes communicate by the secretion of molecular cues and cell-cell junctions (2). Extrinsic factors affecting melanogenesis is certain chemicals and UVR. Intrinsic factors are molecules secreted by keratinocytes, endocrine cells, fibroblasts, neural cells, inflammatory cells or by the melanocytes themselves (4). Melanocyte activity and growth is controlled by cell adhesion molecules and paracrine growth factors secreted from keratinocytes and dermal fibroblasts (8). Melanocytes also produce autocrine factors that influence its own activities, e.g., α-MSH and eicosanoids that elevates melanin synthesis, while the cytokines IL1A, IL6 and TNFA inhibit melanogenesis (2). Cytokines affect growth, pigmentation, morphology, mobility, dendricity as well as adhesive properties of melanocytes (26, 27) The activity of TYRO is enhanced in the presence of its substrates: dopa and tyrosine.

Melanocytes regulate melanogenic homeostasis by controlling the levels of tyrosine and dopa (1, 28). Phenylalanine hydroxylase (PAH) converts phenylalanine to tyrosine in the cytosol to serve further melanogenesis, activating TYRO (4). The membrane receptors, MSHR and KIT, are central in regulating melanogenesis by the binding of extracellular factors inducing intracellular signal transduction (1).

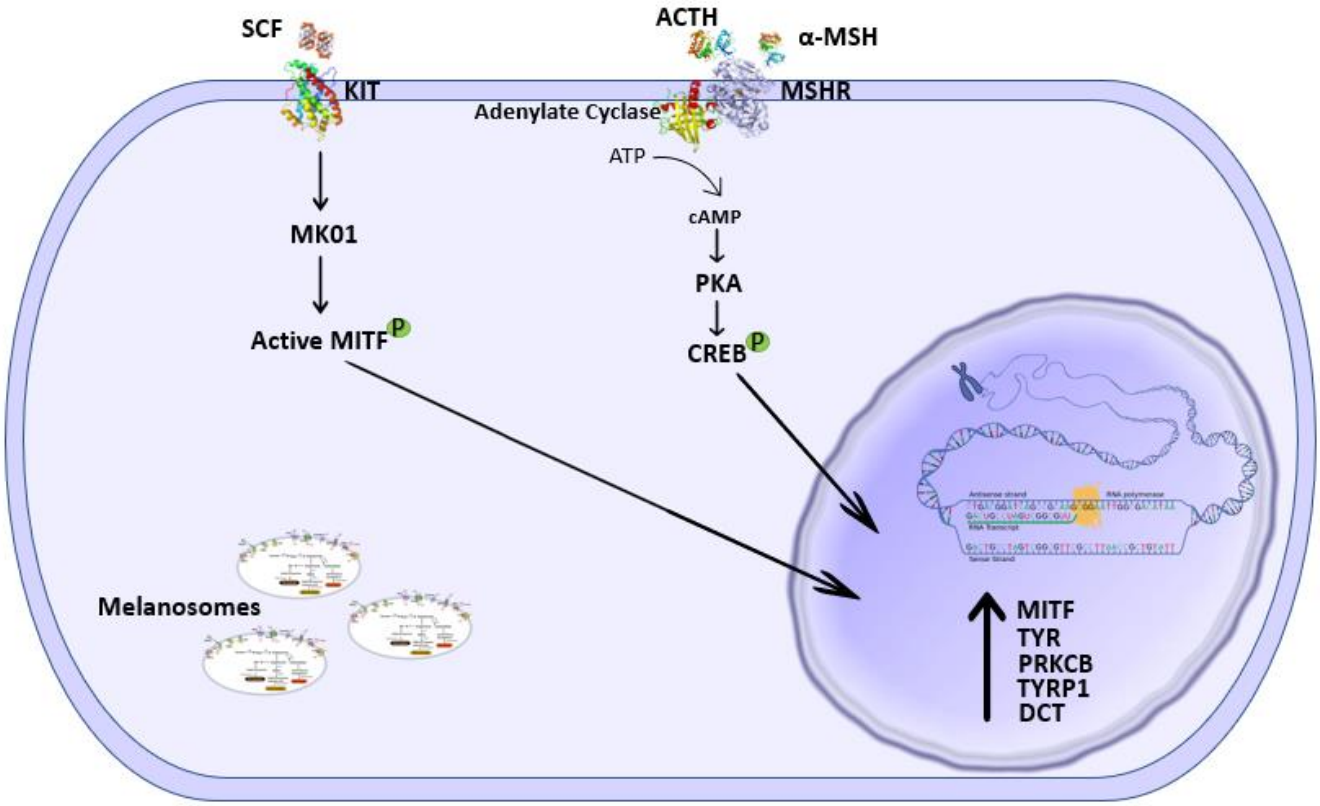


Figure 3. *Illustration of two central signaling pathways in melanocytes. SCF binds KIT in the melanocytic membrane. This results in an activation of MK01, which then phosphorylates and activates MITF. This results in an increased expression of melanogenic enzymes. The COLI peptides ACTH and α-MSH bind to MSHR in the melanocytic membrane. Adenylate cyclase then converts ATP to cAMP, which activates protein kinase A (PKA). PKA phosphorylates and activates CREB. This also results in an increased expression of enzymes involved in the biogenesis of melanin. Melanocytes have many protruding filopodia, which is not displayed here (1, 4). This figure is illustrated by the author.*

An upregulated synthesis of melanin is a natural response to UVR induced DNA damage, which results in acquired pigmentation. DNA damage, e.g., thymine dimers and pyrimidine derivatives promote the expression of the tumor suppressor gene *TP53* in keratinocytes. P53 is a transcription factor, which binds to a P53 consensus sequence in the promotor of the *POMC* gene, increasing

the expression of *POMC* (4). COLI is cleaved to produce the peptide hormones: α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH). When α-MSH and ACTH bind melanocortin 1 receptor (MSHR) in the melanocyte membrane it induces the expression of melanogenic enzymes. (17, 29). In melanocytes, P53 also increase the expression of the transcription factor, *HNF1A*, which in turn upregulates the expression of TYRO (4, 20). P53 mediates the adaptive response caused by exposure to UVR and DNA damage, which results in acquired pigmentation (20). Eller et al. (30) showed that small DNA fragments increased the expression and activity of TYRO and P53. UVR affects plasma membrane lipids to release diacylglycerol (DAG), resulting in a cascade reaction that ultimately activates TYRO (21). The number of melanocytic filopodia is also influenced by UVR (4).

The production of ACTH from the pituitary gland is insufficient to activate melanogenesis, instead melanocytes and keratinocytes are responsible for producing enough COLI peptides. ACTH and α-MSH has a tetrapeptide (His-Phe-Arg-Trp) in common, which is essential for activating MSHR (17, 29). Polymorphisms of the *MC1R* gene cause different responses to UVR (20, 29). COLI peptides stimulate a chain reaction that ultimately results in an elevated transcription of *MITF*, *PRKCB*, *TYR*, as well as *TYRP1* and *DCT*. Mutations affecting the function of MSHR will not activate the transcription of *TYRP1* and *DCT*, which in turn reduces the production of eumelanin (4). When α-MSH or ACTH binds to MSHR, adenylate cyclase is activated and increases the cAMP concentration intracellularly. cAMP activates protein kinase A (PKA). Activated PKA phosphorylates and activates CREB, which in turn increases the expression of *MITF*. Phosphorylated MITF is active and promotes the expression of enzymes involved in the biogenesis of eumelanin (20, 29). MK01 activation is induced when KIT binds to SCF. MK01 is responsible for phosphorylating and activating MITF (17). Besides upregulating the expression of melanocytic enzymes, MITF also regulates the expression of RAB27A, BCL2 and, PMEL (4, 20).

2.4 Vitiligo and vitiliginoid disorders

The most common depigmentation disorder is non-segmental vitiligo, which has a prevalence between 0.5-1% of the population. Vitiliginoid conditions resemble vitiligo in phenotype but are caused by something other than an autoimmune loss of pigment cells (31). Examples of vitiliginoid disorders are chemical leukoderma, scleroderma leukoderma, melanoma-associated

leukoderma and Vogt-Koyanagi-Harada disease. Vitiliginoid conditions are diagnosed clinically, and in most cases, it is unnecessary with histological confirmation. Obtaining a biopsy specimen of the skin is rarely conducted but would be recommended for patients that are refractory to conventional treatment, sensory changes, other epidermal changes, alopecia, or induration (3, 31). It might be hard to determine whether a condition of depigmentation is vitiligo or not. The clinical distribution between depigmentation and hypopigmentation can be difficult. Dermatologists use a handheld black light or a Wood's lamp to accentuate depigmented skin in order to differentiate between the two. Underlying compounds of the skin absorbs UV light and autofluoresce a white-blue light when examined as mentioned. This does not occur if the macules are hypopigmented (3).

Melanocytes from vitiligo patients are more susceptible to damage from oxidative stress, which ultimately activates cascade of events leading to an activation of the immune system and subsequent loss of melanocytes. DNA and HMGB1 are damaged associated molecular patterns (DAMPs) released by melanocytes (32). IFNG is the key drive to vitiligo pathogenesis. Keratinocytes produce IFNG-induced chemokines CXCL9 and CXL10, which recruits $CD8^+$ T cells to the epidermis (33). A reported study of mRNA levels show that keratinocytes had a decreased expression of SCF and an increased level of IL6 and TNFA, all of which have a negative influence on the synthesis of melanin. The former being especially important in melanocyte survival (31). The keratinocytes had abnormal levels of NEF2, but also the downstream genes *glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H quinone dehydrogenase 1 (NQO1)* and *glutamate-cysteine ligase modifier subunit (GCLM*), all important in ROS regulation. Whether these alterations were present prior to melanocyte death or if it was consequential is not clarified. Melanocytes also show a deficit antioxidant mechanism and increased generation of ROS. (31, 34). The keratinocytes have features of reduced antioxidant capacity, increased ROS production, mitochondrial dysfunctions, activation of caspase 3, but also activation of RELA NF-kB subunit, MK14, P53 as well as DBLOH proteins (35). CXL10 in combination with IL6 was shown to be a reliable marker of vitiligo (36). Stimulation of IFNG and CXL10 increased the expression of *CD80, CD40, ICAM1* and *CIITA* on the surface of melanocytes where they presented their own antigens. This triggers T cells with specificity towards melanocyte related antigens (37). miRNAs regulate expression post-transcriptionally by interfering with the translation of mRNA when binding to 5'-UTR or 3'-UTR (31). 13 miRNAs

were shown to be differentially expressed in skin of patients with non-segmental vitiligo by Mansuri et al. (38).

2.5 Analyzing depigmentation

Analyzing the cause of depigmentation disorders can be conducted with genotyping, gene- and protein expression analysis. DNA sequencing can be useful to identify and study the implications of specific mutations. There are several approaches for DNA sequencing, ranging from traditional Sanger sequencing to next generation sequencing (NGS) and third generation sequencing (TGS) (39). There have been made many advancements in sequencing technologies, from manual, labor intensive and time-consuming approaches to automated, efficient and accurate applications (40). NGS was designed to make sequencing of whole genomes more applicable, making it possible to sequence millions of bases in a fast and highly accurate manner (39). Whole genome sequencing (WGS) applies NGS technology and can be used to screen for variations and culprit genes. Whole exome sequencing (WES) can be used to search for mutations in the protein-coding regions of the genome, creating less data than WGS. RNA sequencing (RNA-seq), however, can both give information about the transcript's sequence, splicing events, and gene expression simultaneously (41).

Evaluation of differentially expressed genes allows the understanding of cellular responses to induced signals, changes in phenotype, biological functions and diversity (42). Gene expression can be analyzed *in vivo* with Fluorescence *in situ* Hybridization (FISH) on fixed tissue sections by using fluorescently labeled probes specific to mRNAs of interest. When studying gene expression of more than one gene, it is important that the different probes are labeled with different fluorophores. FISH allows for detection of gene expression *in vivo* with fluorescence microscopy (39, 43). Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) allows the detection of gene expression in real time. PCR is a powerful technique allowing the detection of minute amounts of target genes. It is not labor intensive and produces a great amount of data in a short period (44). PCR is a great choice for studying gene expression where the amount of available tissue is limited. RNA must be converted to complementary DNA (cDNA) before it can be amplified in the following PCR cycles. This process is referred to as reverse transcription PCR (RT-PCR) (39). Microarray analysis is used to study the transcriptome and the method has widespread use in fields like experimental biology, identification of drug targets, and

pharmacogenetics. Microarray technology has revolutionized the understanding of regulated transcription by integrating analysis of comparative genomic hybridization, exon-based arrays, tiling and single nucleotide polymorphisms (SNPs) (42). The gene chip contains thousands of spots, where each spot on the microarray represents a specific gene, and the brighter the spot fluoresce the greater the expression of the given gene (39).

Protein expression can be interesting to analyze, since gene- and protein expression does not always correlate (45). Immunohistochemistry (IHC) is a method for analyzing protein expression *in vivo* using fluorescently labeled antibodies with specificity for proteins of interest. This method also shows the proteins localization within the tissue section (13). Western blotting is another method for analyzing protein expression. Proteins from the sample is separated on SDS-PAGE. Protein bands are then transferred to a nitrocellulose membrane. Western blot uses primary antibodies with specificity for proteins of interest and secondary conjugated antibodies that bind the primary antibodies to detect the amount of proteins of interest (46).

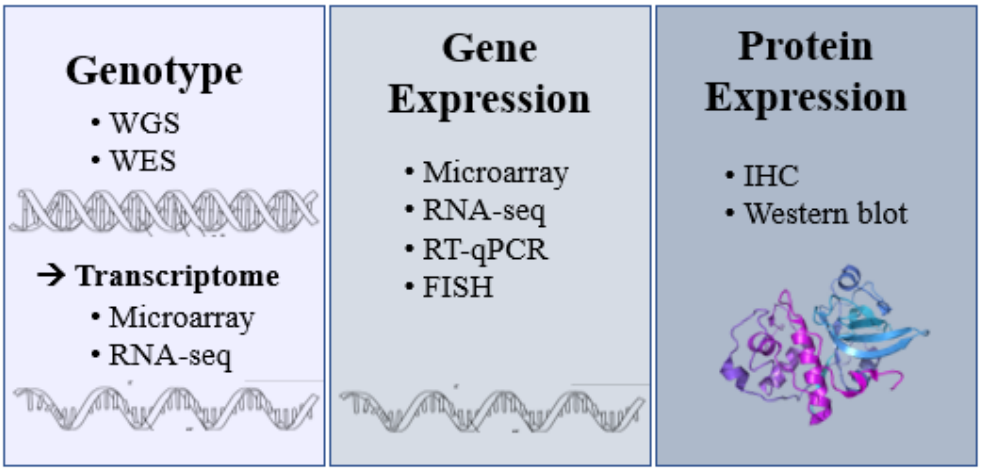


Figure 4. *Illustrates the different methods for analyzing depigmentation. The transcriptome is placed in the same box as genotype, because versions of genes can be detected using microarray and RNA-seq. Microarray and RNA-seq detects gene expression of transcripts. RT-qPCR and FISH can be used to detect gene expression of target genes. Quantification of target proteins can be conducted with IHC or Western blot (13, 40-42, 47). This figure is illustrated by the author.*

3 Methods

This literary study is based on articles from PubMed. Searching for “Skin depigmentation” on PubMed gave close to 2900 results from the last 10 years. Other keywords that were used to find articles were “depigmentation disorders, melanogenesis, genetics of pigmentation, signaling pathways in melanocytes, and vitiligo”. Studies on pigmentation and pigmentation defects have been going on since the 50s, but most of the advances have been made the last 20 years. Literature from early 2000 until today was primarily chosen for this study. Technology is constantly evolving with improved protocols, automation of procedures, and higher precision make research articles from recent years more reliable and updated. However, there have been some interesting findings in research articles and reviews that are older than 20 years. Articles from the 50s up to the late 90s are therefore not excluded entirely from this study. The book “*Introduction to Biotechnology*” by William J. Thieman and Michael A. Palladino describes laboratory techniques for analyzing gene and protein expression and has been an asset to this study. Additional research articles on different methods for analyzing gene and protein expression has also been found on PubMed.

There are many different names associated with genes and proteins. The HGNC database was used to search for genes to find the approved names and symbols, but also chromosomal locations. The *InterPro* site on HGNC was used to find the correct short names for proteins. To find clinical variants of genes, the *ClinVar* site by NCBI was used. *ClinVar* is a database with submitted clinical variants of genes, making it possible to search for different variants and to find information about these. The column to the left on *ClinVar* displays the number of variants that fall into different categories, such as clinical significance, variation type, molecular consequence, variant length, and review status. The protein resources on the HGNC site were also frequently used to find information about protein’s molecular effects. Attached to this document is a table with the approved names of genes and proteins mentioned in this literary study. Many research articles use alias symbols of genes and proteins. These have been changed in accordance with the approved names on HGNC and *InterPro*.

4 Results

The result from searching for articles on PubMed was 8 articles that were of strong relevance for this literary study covering subjects on melanocyte biology, signaling pathways, genetics, and different methods for analyzing gene- and protein expression.

Table 1: Central articles used in this literary study.

Title	Authors	Published	Year	Relevance
Skin melanocytes: biology and development	M. Cichorek et al	PubMed	2013	Describes the biology of melanocytes, melanogenesis and maturation of melanosomes
Signaling Pathways in Melanogenesis	S. D'Mello et al	PubMed	2016	Describes central molecular pathways that influence melanogenesis
The Genetics of Human Skin and Hair Pigmentation	W. Pavan et al	PubMed	2019	Describes genes involved in the pigmentation process, normal phenotypic variation, and diseases
Acquired disorders with depigmentation: A systematic approach to vitiliginoid conditions	M. Saleem et al	PubMed	2019	Describes different vitiliginoid conditions and their clinical features
Immunohistochemistry and in situ hybridization in the study of human skin melanocytes	T. Passeron et al	PubMed	2007	Experiment of detecting gene and protein expression of human melanocytes
Real-time PCR (qPCR) primer design using free online software	B. Thornton and C. Basu	PubMed	2011	Describes the experimental design of qPCR.
Microarray analysis sheds light on the dedifferentiating role of agouti signal protein in murine melanocytes via the Mc1r	E. Le Pape et al	PubMed	2009	Experiment where melanocytes were treated with ASIP and α-MSH, and gene expression was assessed with microarray
Protein purification and analysis: next generation Western blotting techniques	M. Mishra et al	PubMed	2017	Describes Western blotting and its different applications

5 Discussion

5.1 Possible genetic mechanisms

There are many possible reasons for depigmentation disorders that progress over time. Numerous genes are involved in the pigmentation process and suggesting which mechanism is altered may be difficult. Melanin acts as a photosensitizer and has a potential of generating superoxide radicals that in turn could cause cellular damage and altered functions (15). The focus here is depigmentation disorders that develop over time and is not present at birth. Epidermal melanocytes are considered to be a stable population that are seldomly replaced (2, 48). The probability that a spontaneous somatic mutation of any of the pigmentation related genes of an epidermal melanocyte resulting in the progression of depigmented macules is low (2, 7). Keratinocytes residing in the basal layer are proliferating regularly, and for each division cycle resulting in two daughter cells, one differentiated and one undifferentiated keratinocyte. The undifferentiated keratinocyte resides in the same location as the parent cell, while the differentiated keratinocyte is pushed upwards replenishing the pool of cells in the epidermis (2). Alterations in keratinocytes ability to communicate with melanocytes via peptide hormones, may result in an altered pigment production of nearby melanocytes. If a spontaneous mutation occurs in one of the proliferating keratinocytes in the basal layer, it only affects a very small number of neighboring cells (1, 4). It is unlikely to have a significant effect unless some of the key cell cycle regulators are mutated as well, making them proliferate faster than normal. This would not necessarily explain the onset of more than one depigmented macule in different parts of the body. The probability that a depigmentation disorder is a result of spontaneous mutations in proliferating keratinocytes is considered to be low.

Depigmentation could be caused by a downregulation in gene expression. Down regulated expression of vital hormones, enzymes, transcription factors, kinases, phosphatases, or transport proteins may affect the pigmentation process leading to the formation of depigmented macules. The pigmentation process relies on a multitude of different molecular processes and the fine balanced relationship between them (1). If the transcription of *TYR* was halted, there would be less or no production of both eumelanin and pheomelanin. If the transcription of *TYRP1* and *DCT* were reduced, it would result in a reduced production of eumelanin. The transcription of *TYR*, *TYRP1* and *DCT* is controlled by MITF. If any of the important factors involved in the signal

transduction of inducing the transcription of MITF is affected, the potential cause could be a lack of melanin synthesis (20). A reduction in the number of MSHR in the melanocyte membrane may cause a reduced signal transduction and subsequently a more seldom activation of CREB, which is responsible for enhancing the transcription of *MITF*. An upregulated transcription of *ASIP* leads indirectly to a downregulation in the transcription of eumelanin by preventing the intracellular cascade of reactions when MSHR is activated (1). An imbalance in the ion transporters of melanosomes may also affect the melanogenic enzymes negatively (19). It is difficult to pinpoint which mechanism is most likely to be affected, considering that there could be several affected mechanisms and even combinations of different versions of genes giving a genetic predisposition for developing depigmented macules later in life (1, 18). A down regulation in expression will likely result in hypopigmented macules where there is some pigment present, but not completely absent. This downregulation in expression could possibly progress over time to be completely lost, resulting in no expression of key proteins in melanin production and subsequent depigmentation (18, 49). Post-transcriptional modifications, e.g., alternative splicing may change the function of the translated proteins and could result in decreased pigmentation. Post-translational modifications may affect the function of proteins as well (41). miRNAs that interfere with the translation of mRNAs can also explain changes in phenotype (38).

Old age can result in a gradual reduction in pigmentation over time. After the age of 30, it is estimated that between 10-20% of melanocytes in the epidermis are lost for each decade. Melanocyte morphology is also altered in elderly individuals, being larger and more dendritic. This could be an adaptation to fewer melanocytes, but the activity of TYRO is reduced (2, 50). ROS accumulate because of reduced catalase amount and activity, as well as a downregulation of *BCL2*. Apoptosis of terminally differentiated melanocytes explains the decreased number of melanocytes due to senescence (12, 50). The age of onset for a depigmentation disorder should be taken into account when investigating the cause of the condition.

Table 2: Genes put into categories of their respective molecular functions

Molecular functions	Genes
Enzymes in melanogenesis	*TYR, TYRP1* and *DCT*
Proteins within the melanosome that are important for melanogenesis	*OCA2, SLC45A2, SLC24A5, TPCN2* and *PMEL*
Signal transduction	*MC1R, POMC, ASIP* and *ATRN*
Transcription factors regulating melanin synthesis	*MITF, PAX3, SOX9* and *SOX10*
Melanosome transport	*RAB27A, MYO5A* and *MYO7A*
Ligands and receptors important developmentally in migration and differentiation of melanoblasts	*SCF + KIT* and *EDN3 + ENDRB*
Differentiation of melanocytes	*LRMDA, WNT1* and *WNT3A*

5.2 Genes of interest

There are more than 125 genes that are known to regulate pigmentation (5). Some genes have shown a stronger association with phenotypic diversity then others, and some of those genes will be described here. This includes *MC1R, POMC, ASIP, TYR, TYRP1, DCT, LRMDA, OCA2, SLC45A2, SLC24A5, TPCN2, SCF, KIT, MITF, WNT1, WNT3A, PRKCB, SOX9, SOX10* and *PAX3*. Most mechanisms governing transcriptional and post-transcriptional processes of pigmentation related genes remain uncertain (18, 49).

Melanocortin 1 receptor

Melanocortin 1 receptor (MC1R) encodes the protein MSHR, which resides in the melanocyte cell membrane. Genetic, pharmacological, and biochemical evidence suggest that signal transduction from MSHR is the main factor inducing melanogenesis. This receptor is a subgroup of the G-coupled receptors class A (14). Its agonists ACTH and α-MSH stimulates eumelanin synthesis, whereas the antagonist ASIP stimulates pheomelanin synthesis (4, 51). Variants of *MC1R* has been associated with fair skin and red hair. These variants show reduced affinity for α-MSH or a reduced ability to activate adenylyl cyclase (49). *MC1R* has the chromosomal location 16q24.3. There are 7 likely pathogenic and 32 pathogenic variants of *MC1R* submitted to *ClinVar*, most of which are associated with hypopigmentation and Tyrosinase-positive oculocutaneous albinism or Falconi anemia. In most cases the clinical condition associated with the variants are not provided (52).

Proopiomelanocortin

Proopiomelanocortin (POMC) encodes the protein COLI, which is cleaved to produce a set of different peptide hormones, such as ACTH and α-MSH (14). The expression of *POMC* is stimulated by UVR and UVR-derived ROS. α-MSH and ACTH stimulates TYRO activity through the cAMP pathway. When α-MSH or ACTH binds to MSHR on the melanocyte surface, adenylyl cyclase is activated intracellularly leading to an elevated level of cAMP. The cAMP-dependent pathway results in an elevated expression of melanogenic enzymes (1). The chromosomal location of *POMC* is 2p23.3. There are 27 pathogenic variants of *POMC* submitted to *ClinVar*. Many of these result in proopiomelanocortin deficiency characterized by an early onset of obesity and a tendency to have pale skin and red hair (52).

Tyrosinase, tyrosinase related protein 1 and *dopachrome tautomerase*

TYR encodes the melanogenic enzyme tyrosinase (TYRO). Mutations affecting *TYR* causes the most severe form of oculocutaneous albinism (OCA1), which is an autosomal recessive disease. (53). OCA1A is a condition when there is a lack of TYRO activity. This results in an absence of pigment in eyes, skin and hair, as well as visual anomalies. OCA1B is when there is a partial lack of TYRO activity, causing an impaired melanogenesis and reduced pigment in eyes, skin, and hair. Some phenotypes of OCA1B shows a reduced activity of TYRO when temperatures rise above 35-37 ℃ (18). The cytogenetic location of *TYR* is 11q14.3. There are 113 submitted cases of pathogenic variants of *TYR* on *ClinVar.* Many of these variants cause tyrosinase-negative oculocutaneous albinism (52). *Tyrosinase related protein 1 (TYRP1)* encodes the protein TYRP1. Loss of function of *TYRP1* leads to albinism type OCA3, which is characterized by red hair and a light brown complexion (18). *TYRP1* has the chromosomal location 9p23. There are 116 pathogenic variants of *TYRP1* that are registered on *ClinVar,* where many are linked to polygenic conditions and some variants of *TYRP1* causing oculocutaneous albinism type 3 (52). *Dopachrome tautomerase (DCT)* encodes the protein TYRP2 (14). TYRP1 and TYRP2 are important in the biogenesis of eumelanin (1). *DCT* is located on chromosome 13 (13q32.1). There are 69 pathogenic variants of *DCT* that are submitted to *ClinVar*. Some of these variants cause oculocutaneous albinism type 8 (52).

Agouti signaling protein

Agouti signaling protein (ASIP) encodes the 132 amino acids long protein ASIP (52). ASIP is the antagonist of MSHR. When ASIP binds to MSHR in the melanocyte cell membrane, it promotes the biogenesis of pheomelanin. Decreased expression of *ASIP* would result in decreased antagonism of α-MSH, which would ultimately leave α-MSH free to bind MSHR, inducing eumelanin synthesis (14, 49). Unfunctional variants of ASIP or significantly reduced expression of *ASIP* would lead to an increased biogenesis of eumelanin resulting in a darker complexion. In the opposite case, with a gain of function variant of ASIP or an upregulated expression of *ASIP* would lead to an increased synthesis of pheomelanin as opposed to eumelanin resulting in a lighter skin complexion (49). *ASIP* has the chromosomal location 20q11.22. There are 12 pathogenic variants of *ASIP* submitted to *ClinVar*, where most of the reported variants includes several other genes (52).

Solute carrier family 45 member 2 and *solute carrier family 24 member 5*

The transport protein S45A2 is encoded by the gene *solute carrier family 45 member 2 (SLC45A2). Solute carrier family 24 A member 5 (SLC24A5)* encodes the transport protein NCKX5. Both *SLC45A2* and *SLC24A5* are members of the solute carrier family (SLC), which share homology with symporters of sucrose and protons. Melanosomes acquire enzymes involved in melanogenesis through S45A2. Mutations affecting *SLC45A2* cause oculocutaneous albinism type 4 (16, 53). Nonsense mutations of *SLC45A2* causes misrouting of TYRO, giving a similar phenotype as oculocutaneous albinism type 2, with a reduced or a lack of pigment in eyes, skin, hair and ocular abnormalities (18). *SLC45A2* has the chromosomal location 5p13.2. There are 49 pathogenic variants of *SLC45A2* registered on *ClinVar*, where many are linked to oculocutaneous albinism type 4. (52). NCKX5 is a Na^+/Ca^{2+} exchanger located in the melanosomal membrane that is K^+ dependent. NCKX5 transports Ca^{2+} into the melanosome lumen, mediating Ca^{2+} signaling (18). *SLC24A5* has the cytogenetic location 15q21.2. There are registered 30 pathogenic variants of *SLC24A5* on *ClinVar*. Some of these pathogenic variants cause the disease oculocutaneous albinism type 6 in combination with variants of *MYEF2* (52).

Leucine rich melanocyte differentiation associated

Leucine rich melanocyte differentiation associated (LRMDA) encodes a leucine rich protein containing 198 amino acids, which is associated with the differentiation of melanocytes. Mutation of the *LRMDA* gene results in oculocutaneous albinism type 7 (OCA7), which is a rare form of albinism (18). Experiments with zebrafish, where the ortholog of *LRMDA* was knocked down demonstrated a lower amount of pigment per cell, as well as a reduction in the number of pigment cells. This experiment supports that LRMDA plays a pivotal role in the differentiation of melanocytes (18, 54). *LRMDA* has the chromosomal location 10q22.2-q22.3. There are 15 variants of *LRMDA* on *ClinVar* that are pathogenic (52).

OCA2 melanosomal transmembrane protein

OCA2 melanosomal transmembrane protein (OCA2) encodes the p-protein, which spans over 345 kb, divided into 24 exons, 23 of which make up the coding region and exon 1 represents a 5' UTR. The p-protein is an 836-amino acid long protein consisting of 12 transmembrane domains (18, 52). The p-protein assists the processing and trafficking of TYRO, metabolism of gluthathione and regulation of the melanosomal pH, as well as increasing the Cl^- conductance from melanosomes. Oculocutaneous albinism 2 (OCA2) is a common form of albinism, caused by a deletion in *OCA2*. Nonsynonymous polymorphisms in the coding regions of *OCA2* are associated with phenotypic diversity (18). The p-protein regulates melanosomal pH and maturation. *OCA2* has the chromosomal location 15q12-q13.1 (52). The transcription of *OCA2* is controlled by an enhancer element that is encoded in a nearby intron of HERC2. The transcription factors MITF, HLTF and LEF1 binds to the enhancer element, which results in an increased transcription of *OCA2*. C-allele carriers of polymorphism rs12913832 have a reduced binding of transcription factors to the enhancer element, which results in a reduced expression of *OCA2*. Homozygous individuals of the C-allele have light blue eyes (18). There are more than 340 variants of *OCA2* on *ClinVar* that are pathogenic, most causing albinism (52).

Two pore segment channel 2

Two pore segment channel 2 (TPCN2) encodes the protein TPC2, which is associated with skin and hair color. A. Ambrosio et al. (55) suggests that TPC2 regulates the acidity and size of melanosomes by mediating the release of Ca^{2+}. Knockout of *TPCN2* caused a less acidic melanosomal lumen and an increase in melanin content (55). Phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$) has been implicated in modulating the function of TPC2 in controlling the pH. $PI(3,5)P_2$ modulated TPC2 in such a way that the melanosome becomes more acidic with a subsequent reduction in pigmentation (18). *TPCN2* has the chromosomal location 11q13.3. There are registered 5 pathogenic variants of *TPCN2* on *ClinVar*. All of these include several genes as well as *TPCN2* (52).

SRY-box transcription factor 9 and *SRY-box transcription factor 10*

The SOX family contains around 20 transcription factors with SRY high mobility box (HM-box). Sequence-specific binding of DNA is mediated by the HM-box domain. There's a great diversity of mechanisms that SOX proteins regulate transcription. *SOX9* and *SOX10* (SOXE) are essential in the development and regulation of melanogenesis. SOX10 is attributed unique functions in the development of melanocytes from NCCs (1). SOX10 controls the MITF transcription, and in the absence of SOX10 there would be no MITF induced expression of *TYR*. Both SOX10 and MITF is needed to activate TYRP2 (56). SOX9 has an ability to induce the expression of *SOX10* (1, 57). During melanocyte differentiation, *SOX10* is downregulated, while *SOX9* is upregulated. An ectopic expression of *SOX9* is enough to promote differentiation into melanocytes (57). Irradiation of UVB upregulates the expression of melanogenic enzymes by SOX9. The SOX9 activity depends on the cAMP pathway (1). *SOX9* has the cytogenic location 17q24.3. There are registered 75 pathogenic variants of *SOX9* on *ClinVar*, many resulting in the condition Campomelic dysplasia. *SOX10* has the chromosomal location 22q13.1. 103 pathogenic variants of *SOX10* are submitted to *ClinVar*, where many of these cause the condition Waardenburg syndrome type 2E (52).

KIT proto-oncogene, receptor tyrosine kinase and *KIT ligand*

KIT ligand (KITLG) encodes SCF. *KIT proto-oncogene, receptor tyrosine kinase (KIT)* encodes the protein KIT. Mutations affecting *KIT* has been associated with the autosomal dominant disorder piebaldism, causing an altered migration and proliferation of melanocyte precursors, resulting in white patches of both skin and hair (18, 52). KIT and SCF regulates the distribution of melanin in the skin and the number of melanocytes during development. Mutations affecting *KITLG* results in a range of disorders with either hypo- or hyperpigmentation. The mutations are referred to as loss of function or gain of function defects respectively (18). *KITLG* has the chromosomal location 12q21.32 and *KIT* has the location 4q12. There are 89 pathogenic variants of *KIT* submitted to *ClinVar*. Many of these were associated with diseases like gastrointestinal stroma tumor, partial albinism, mastocytosis, melanoma and piebaldism. There were registered 16 pathogenic variants of *KITLG* on *ClinVar*, some of which were associated with inherited progressive hyperpigmentation with or without hypopigmentation (52).

Melanocyte inducing transcription factor

Melanocyte inducing transcription factor (MITF) contains several promoter regions. It has at least nine promoter-exons. The M promoter is used selectively in melanocytes and many transcription factors bind to it, e.g., SOX9, SOX10, CREB, PAX3, ONEC2, LEF1 as well as MITF itself (1, 58). Post-translational modifications (sumoylation) are mediated by protein inhibitor of activated STAT 3 (PIAS3), which affects the transcriptional activity of MITF. The unsumoylated version appears to be more active in binding multiple sites in the promoter. It is evident that post-translational modifications regulate MITF activity (58). The phosphorylation pattern of MITF dictate the interaction between PIAS3 and MITF. The MITF-PIAS3 interaction increases when Ser^{73} is phosphorylated and decreases when Ser^{409} is phosphorylated (59). MITF is an important regulator of melanogenesis (58). *MITF* has the chromosomal location 3p13. 55 pathogenic variants of *MITF* are registered on *ClinVa*r, where most are associated with Wardenberg syndrome type 2A (52).

Wnt family member 1 and *Wnt family member 3A*

Wnt family member 1- and 3A (WNT1 and WNT3A) are genes that promote the differentiation of NCCs into pigment producing melanocytes. WNT1 signals to melanoblasts to proliferate and produce more melanocytes, whereas β-catenin and WNT3A promote differentiation. WNT3A maintains the expression of *MITF* (1, 60). The WNT pathway can also increase the expression of MITF (1). LEF1 and β-catenin regulate the *MITF* expression synergistically through LEF1 binding sites in the MITF-M promoter (60). *WNT1* has the cytogenic location 12q13.12. 22 pathogenic variants of *WNT1* have been submitted to *ClinVar*. *WNT3A* has the chromosomal location 1q42.13. 28 pathogenic variants of *WNT3A* have been submitted to *ClinVar*, where all the reported cases included changes in several other genes (52).

Protein kinase C beta

Protein kinase C beta (PRKCB) encodes the protein KPCB, which phosphorylates and activates TYRO. There are nine genes that encode protein kinase C (PKC). PKCs have different requirements for activation and are therefore divided into three subclasses. The pattern of alternative splicing leads to a greater heterogeneity of PKCs (1). KPCB is important because it activates TYRO. It is the activity of TYRO that determines total melanin production rather than the amount of TYRO present. Its activity depends on phosphorylation of Ser^{505} and Ser^{509} of the cytoplasmic domain. UV irradiation upregulates the expression of *PRKCB*, which is activated by DAG obtained from UVR exposed cell membranes. Activated KPCB is then translocated from the cytoplasm to the melanosomal membrane where it phosphorylates and activates TYRO (61). The translocation of KPCB is carried out by RACK1 (62). *PRKCB* has the chromosomal location 16p12.2-p12.1. There are submitted 27 pathogenic conditions where *PRKCB* is affected on *ClinVar*, but all the reported pathogenic conditions include several other genes (52).

Paired box 3

Paired box 3 (PAX3) is a member of paired box family and plays a central role in the development of melanocyte progenitors. PAX3 is an important factor in the transition from early melanoblasts derived from NCCs to mature melanocytes, and evidence suggest that PAX3 contributes to growth and survival of the melanocytic lineage (1). The promoter sequence of *MITF* contains a binding site for PAX3 located between position -40 and -26, a region known to

be critical for activation of MITF transcription (63). *PAX3* has the chromosomal location 2q36.1. There are 91 submitted cases of pathogenic variants of *PAX3* on *ClinVar*, where many cause the condition Waardenburg syndrome type 1 (52).

5.3 Investigating the cause of depigmentation

Screening for abnormal pigmentation related genes through WGS, WES or RNA-seq may be insightful for diagnostic purposes. However, sequencing a patient's genome will not necessarily show any clear context as to why a patient has developed depigmentation (41). Many conditions are polygenic, making it hard to determine all the variants involved in the progression of a given disease. If the cause of change in phenotype is a gradual reduction in depigmentation over time, it may be more suitable to analyze gene expression and protein expression. Melanocytes, keratinocytes and dermal fibroblasts are located superficially, which makes biopsy relatively easy (13). There are numerous methods available to study gene expression, involving northern blotting, RT-qPCR, microarray and FISH. Protein expression can be analyzed with IHC or western blotting.

5.3.1 Nucleic acid sequencing

Genotyping can be conducted by sequencing the whole genome, the exome, or the actual expressed mRNAs. Sequencing technologies have evolved considerably over the past decades, from traditional Sanger sequencing to NGS and TGS (39). There are in general three strategies for WGS, 1) short-read sequencing with Illumina technology, 2) long-read sequencing with Oxford Nanopore Technologies (ONT) or Pacific Biosciences (PacBio), and 3) linked-read sequencing with 10X Genomics (39, 64). Illumina sequencing technologies yield paired-end reads of around 150 bp with error rates of 0.1-0.5%. TGS technology, such as ONT and PacBio yield between 10-100 kb reads with error rates up to 15%. 10X Genomics produce short reads from longer DNA molecules. Most WGS is performed with NGS platforms, such as Illumina NovaSeq or HiSeq due to high accuracy, efficiency and low cost (64). NGS applies capillary electrophoresis, parallel formats, and fluorescens imaging. Specific software programs compare the sequence data of many simultaneously sequenced strands. NGS produces overlapping short read lengths, which are put together like a puzzle by a software to produce a complete and coherent sequence. The Illumina HiSeq approach can generate 600 Gb of data in 10 days (39). In

rare disease studies (n = 1), the sequencing method targets deeper coverage by sequencing >30x for improved and robust accuracy of rare heterozygous variants. Studies of complex traits in a larger scale population, e.g., GWAS, may target a reduced coverage of >20x to stay within a specific budget due to increased sample size. This will still allow accurate detection of rare variants (39, 64). Detection of genetic variants can also be done with WES, which produces less data by only sequencing the protein-coding regions (the exome). In some cases where mutations are detected with WGS or WES, it might be hard to interpret what the final transcript looks like and the molecular functions of the variants. RNA-seq makes it possible to determine the actual sequence of transcripts, splicing events and gene expression simultaneously (41).

For diagnostic purposes where the genes of interest are not known, WGS can be used to screen for a broad range of mutations, e.g., SNPs and large structural variation (SV) affecting pigmentation related genes (64). The reduced amount of sequencing and data storage makes WES a more affordable method when compared to WGS. However, WES does not detect large structural variants (SV), such as inversions, translocations, and copy number variations (duplications). It also does not detect perturbed splicing events, variations in non-coding regions or gene expression. Splicing defects caused by mutations located in introns or silent mutations in exons, will not be detected by WES (40, 41). Some studies indicate that the diagnostic rate for monogenic disorders by WES is between 20-40%, which can be explained by the lack of detecting intronic variants and silent exonic variants resulting in aberrant splicing events (41).

Sequencing the genome will not detect changes in transcription, unless the sequence of pigmentation related transcription factors, e.g., MITF, SOX9, SOX10 and PAX3 have a loss of function mutation. Mutations in the promotor sites of pigmentation related genes may reduce the affinity for specific transcription factors. Whether the affinity is affected or not can be hard to tell. Either way leads to a potential reduction in gene expression of pigmentation related genes (1, 41). RNA-seq could help determine this by measuring the amount of gene expression. RNA-seq is a multifaceted method for sequencing the whole transcriptome, making it possible to screen for different variants, splicing defects, and aberrant expression. cDNA library is synthesized in a manner dedicated to RNA-seq, which differs from conventional cDNA synthesis. RNA-seq may be a powerful technique to facilitate the diagnostics of rare genodermatoses by screening for causative variants, as well as differential expression (41).

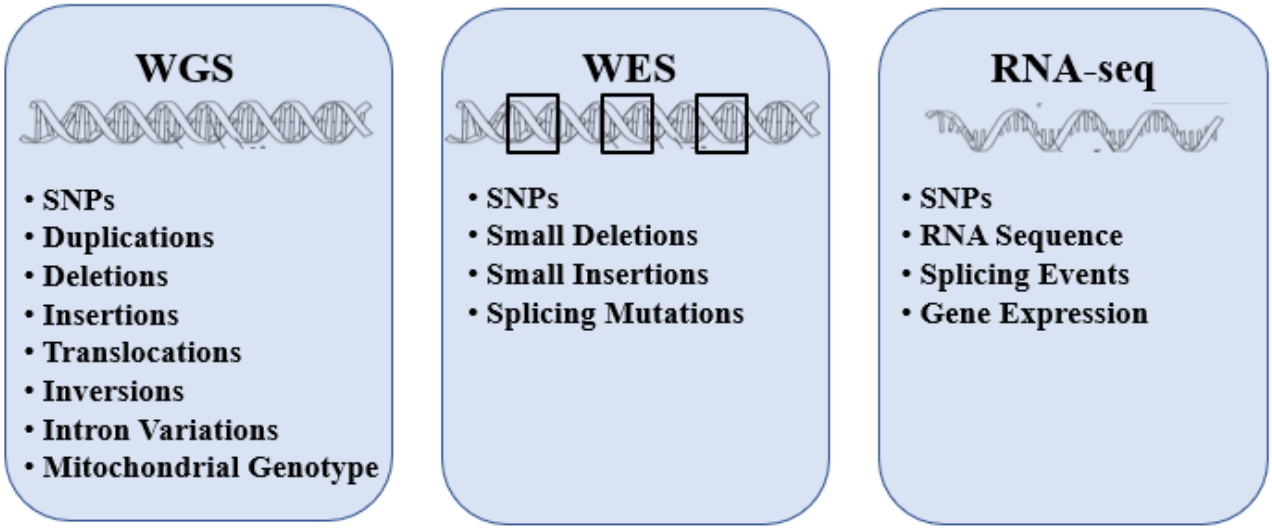


Figure 5. *Illustrates what kinds of mutations WGS, WES and RNA-seq can detect. All three can detect SNPs, but WES and RNA-seq can only detect SNPs that are in the protein-coding regions. Although WGS generates a substantial amount of data when compared to WES, it also detects a larger spectrum of mutations that are not possible to detect with WES. RNA-seq provides information on actual RNA sequence, splicing events and gene expression, but it does not detect large SV. The best method for genotyping should be evaluated on the basis of research aims, budget and availability. This figure was illustrated by the author based on information retrieved from a selection of articles (41, 64).*

Genome-wide functional genomics allows for interpreting molecular effects of different gene variants, from no significant change to reduced function, loss of function, or gain of function mutations (64). Data analysis is important for interpreting the obtained sequenced data. There are numerous software programs for bioinformatics analysis of obtained NGS data, each serving different purposes depending on the areas covered and the experimental aims. The software programs range from statistical data analysis to whole-genome association studies, and map-read to reference genome (41). The short reads generated when sequencing with Illumina NovaSeq or HiSeq are aligned to a reference genome by WGS analysis pipelines, which map the variants according to the reference. Modern WGS pipelines use BWA-MEM for the alignment of reads (64).

5.3.2 Gene expression analysis

Microarray analysis makes it possible to analyze the transcriptome of skin cells. Differential exon combinations, because of alternative splicing, can be assessed when using microarray technology. Thousands of specific genes can be assayed in parallel, even measuring rare events of alternative splicing and low abundance transcripts (39, 42). The procedure for obtaining a transcriptome profile of skin cells using microarray consists of 1) skin biopsy, 2) extraction of mRNA, 3) synthesis of fluorescently labeled cDNA, 4) overnight incubation on the microarray chip (hybridization), 5) washing step, 6) detection, and 7) data processing (39). cDNA is synthesized with reverse transcription, primers complimentary to the poly-A tail, and fluorescently labeled nucleotides (39, 42). cDNA is then applied to the microarray chip and incubated overnight. The complimentary cDNA strands hybridize to the ssDNA strands fixed to the microarray chip. There is a washing step before the microarray chip is scanned by a laser. The laser causes the hybridized cDNA to fluoresce, which is detected and interpreted by a software. The fluorescence intensity is integral to the relative amount of gene expression (39). E. Le Pape et al. (51) used competitive hybridizations of ASIP-treated versus untreated melanocytes, and α-MSH-treated versus untreated melanocytes, to assess changes in the transcriptome as a response to ASIP or α-MSH. They looked at the response after 3 hours, 1-, 2-, 3- and 4 days of treatment with the respective peptide hormones. This study confirmed that treatment with ASIP resulted in a downregulation of pigmentation related genes, while α-MSH upregulated the expression of these genes (51). In many cases it is interesting for researchers to compare the transcriptome of two different samples, e.g., lesioned versus non-lesioned areas of patients with depigmentation, or in other cases it could be cancer versus normal tissue. The findings of such studies can help researchers better understand phenotypical changes and culprit genes (39).

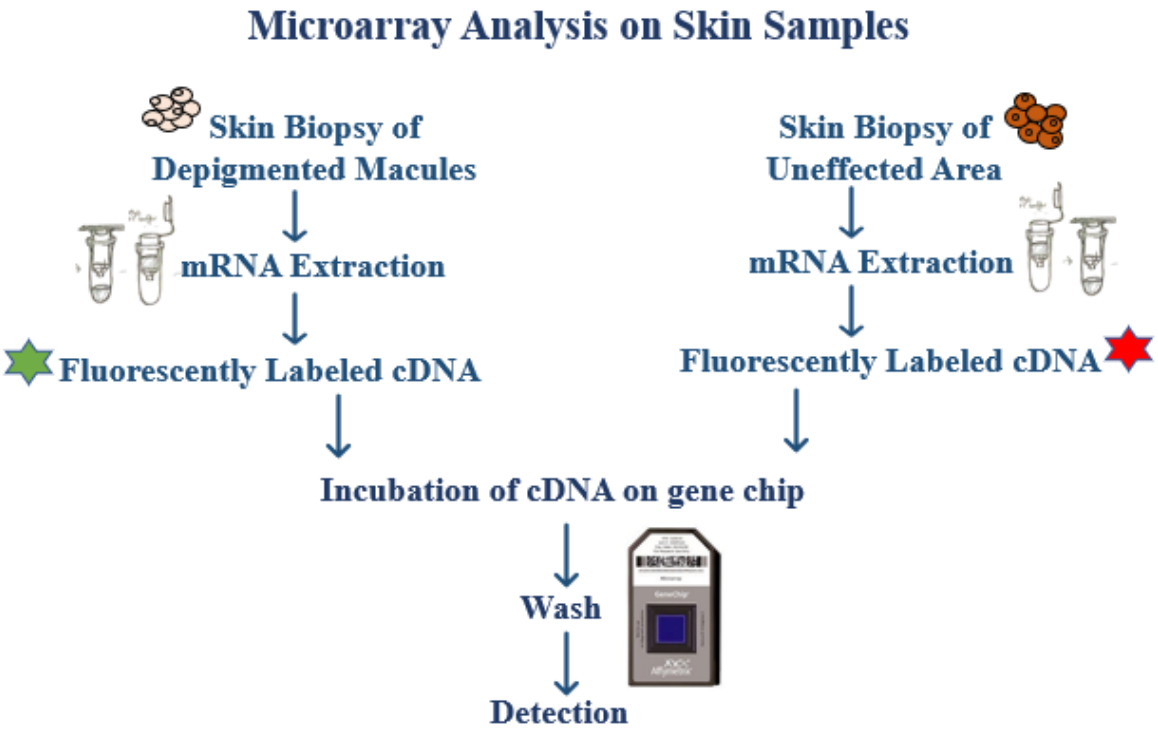


Figure 6. *Flow chart illustrating the sequential steps in creating a transcriptome profile with DNA microarray. A skin biopsy can be taken from depigmented areas and from unaffected areas. mRNAs from both samples is extracted. cDNA is synthesized by using reverse transcriptase and nucleotides labeled with a fluorescent dye. When comparing the two different skin samples, it is essential to use two different fluorophores in order to distinguish between them. The fluorescently labeled cDNA is then incubated on the gene chip overnight, so that it binds to complementary DNA sequences. After hybridization there is a washing step followed by detection. A laser scans the microarray causing the cDNA to fluoresce, which is visualized as bright spots. The brighter the spots are, the more of the given genes have been expressed in the skin samples. The signals are then interpreted by a software (39). This flow chart was illustrated by the author.*

The expression target genes can be studied using FISH on skin sections. There are different FISH protocols, varying with type of target retrieval, choice of probe, and detection method. There are several steps when performing FISH on skin sections, starting with 1) preparation of probes complementary to mRNAs of interest, 2) deparaffinize skin section in xylene, 3) rehydrate in graded alcohols, 4) target retrieval, 5) denaturation of probes and skin section, 6) incubation, 7) washes, and 8) detection (43). This method has sensitive detection and can be performed with oligonucleotide-, DNA-, or RNA probes. Several probes for each gene of interest (GOI) are

designed and tested to screen for the most suited ones. FISH can be performed on frozen skin sections or FFPE skin sections, although FFPE tissue sections are easier to store. FFPE skin sections are deparaffinized in xylene and rehydrated in graded alcohols. The target retrieval involves making mRNA more available for the probes. Pre-treatment of the skin section can be done with enzymatic digestion or heating (13, 43). mRNA may have secondary structures making them less available to with probes. Both the skin section and the probes are denatured to allow for hybridization of probes to target DNA or RNA (43). When analyzing gene expression, the skin sections are incubated with the probe mixture, allowing the probes to hybridize with complementary mRNA within the cells (13). Non-specific bound probes are washed away from the slides using a saline-sodium citrate buffer. Direct detection of gene expression implies measurements of fluorescence. Indirect detection is when skin sections are incubated with digoxigenin-labeled probes that are detected using an anti-digoxigenin antibodies labeled with a fluorophore. Indirect detection can also be done by incubating skin sections with biotinylated probes followed by avidin detection. Imaging of the slides is done with fluorescence microscopy (43).

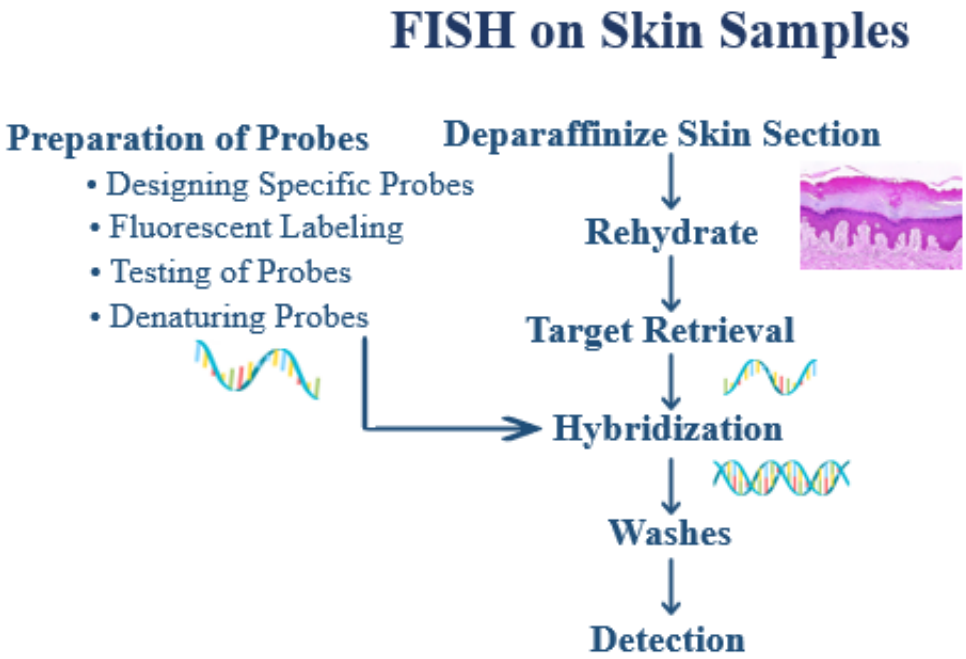


Figure 7. *Flow chart illustrating the sequential steps of FISH on skin sections. FFPE skin sections are deparaffinized and rehydrated before they are pre-treated to retrieve target structures, such as mRNA. Denaturation of probes and the skin section is important to allow for specific binding of probes to complementary mRNA during the hybridization step. Unbound probes are washed away, followed by detection of fluorescent signal (13). This flow chart is illustrated by the author.*

RT-qPCR is a technique that can be used to analyze target gene expression in skin samples. When developing a method for analyzing the expression of a given gene, it is important to find primers that meet the needs of the experimental design. If there are no available primers, it can be necessary to design them (39, 44). The Primer-BLAST page was developed by the National Center for Biotechnology Information (NCBI), and it is a tool for finding primers for the target PCR template using the software Primer3 and BLAST. The target gene's FASTA sequence is entered and parameters like primer length, primer melting temperatures (T_m), and PCR product length can be adjusted (44). Reverse transcriptase makes a complementary DNA strand from the mRNA present in the sample. The target cDNA is amplified using a set of specifically designed primers flanking the sequences of interest (39). There are two approaches for qPCR that are common and involves the use of SYBR Green or TaqMan Probes. SYBR Green is a dye that binds dsDNA. TaqMan probes are short sequences that are complementary to the target DNA in between where the primers bind. TaqMan probes contain a reporter dye that can emit light when excited by a laser (39). RT-qPCR involves the use of a computerized thermal cycler. Each PCR tube contains either SYBR green or TaqMan probes, and when illuminated by the laser they fluoresce, which is detected and interpreted by a computer. The signal intensity is equivalent to the amount of PCR product. The information is automatically plotted as the number of amplicons after each PCR cycle (39, 44). The amplicons are doubled with each cycle giving an exponential curve, but how steep the curve becomes depends on the amount of cDNA in the sample to start with (44).

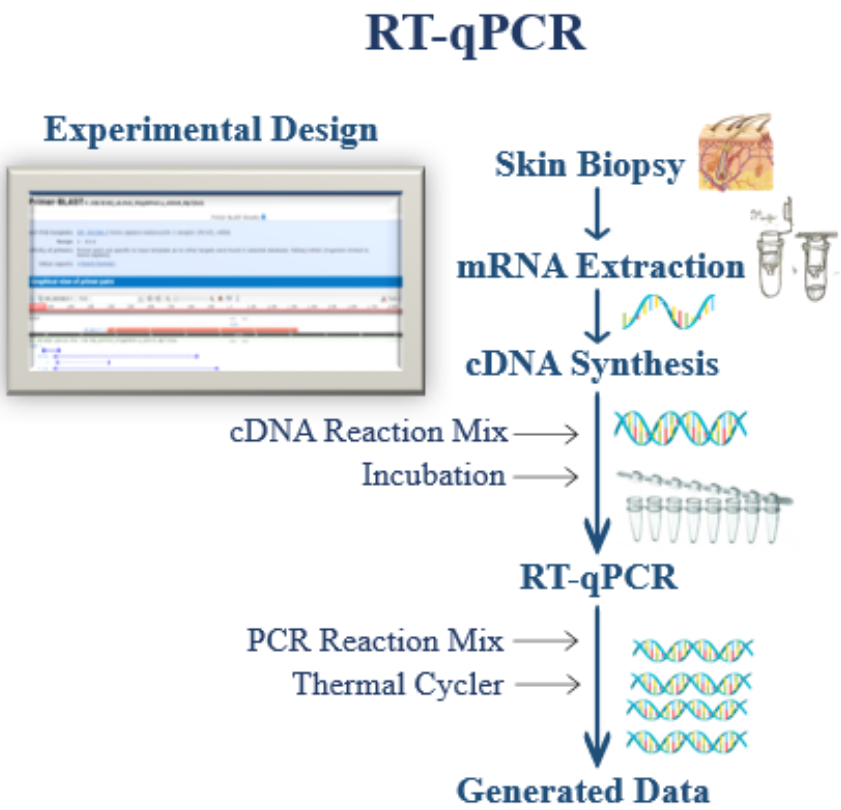


Figure 8. *Flow chart illustrating the sequential steps in performing RT-qPCR on skin samples. mRNA is extracted from the skin sample. The mRNA is then converted to cDNA by adding the isolated mRNA and the cDNA reaction mix to a PCR tube. The cDNA reaction mix contains reverse transcriptase, oligo(dT) primers, dNTPs, buffer, and RNase/DNase free water. The PCR tube is then incubated. When cDNA is synthesized, the cDNA can then be added to a new PCR tube together with the PCR master mix, as well as forward and reverse primers specific to the genes of interest and DNase free water. The PCR master mix contains thermostable DNA polymerase, dNTPs, and a buffer. It is common to use either SYBR Green or TaqMan probes as fluorescent labeling. The PCR strips are then placed in a thermal cycler, which detects the intensity of emitted fluorescens in real time (39, 44). This flow chart is illustrated by the author.*

5.3.3 Protein expression analysis

IHC can be used to study melanocytes. It is a frequently used method for investigating the progression of melanomas, but IHC is also a useful technique to study melanogenesis. There is a wide range of specific antibodies nowadays allowing researchers to study *in vivo* protein expression with IHC. Melanocytic antigens can be localized in frozen skin sections or FFPE skin sections utilizing conjugated secondary antibodies and immunofluorescence. IHC is commonly performed on FFPE tissue sections (65). Fluorescence emission and excitation wavelengths of conjugated fluorophores to secondary antibodies must be appropriate for the filters used in the fluorescens microscope. When multicolor staining, it is essential to exclude fluorophores with overlapping emission spectra (13). The sequential steps used in the indirect IHC method are 1) fixation of skin sections, 2) antigen retrieval, 3) blocking, 4) antibody labeling of primary- and secondary antibodies, and 5) visualization. There are also washing steps between each incubation. Antigen retrieval involves breaking cross-links between proteins and other structures caused by fixation of the tissue section. This makes the antigens more accessible to antibodies. There are several ways to retrieve antigens, e.g., physical treatment (ultrasound and heat) or chemical treatments (enzymatic digestion or denaturant treatment). In some cases, both physical and chemical treatments are used in combination (65). Blocking reduces the probability of false-positive staining. There is both a direct method and an indirect method for antibody labeling. The direct method is when the skin section is probed with primary antibodies only, and these are conjugated to either an enzyme or a fluorophore. The indirect method involves secondary conjugated antibodies (13, 41). IHC is a relatively easy technique to study protein expression, but antibodies are not always available for the target proteins (13). Antibodies used in IHC does not distinguish between variants of proteins *in situ*, as long as they are not designed to specifically do so (66). No immunoreactivity for a given protein could either mean that the translated protein is truncated, mutations rendering the protein structure altered, or a significant reduction in protein expression (13, 41).

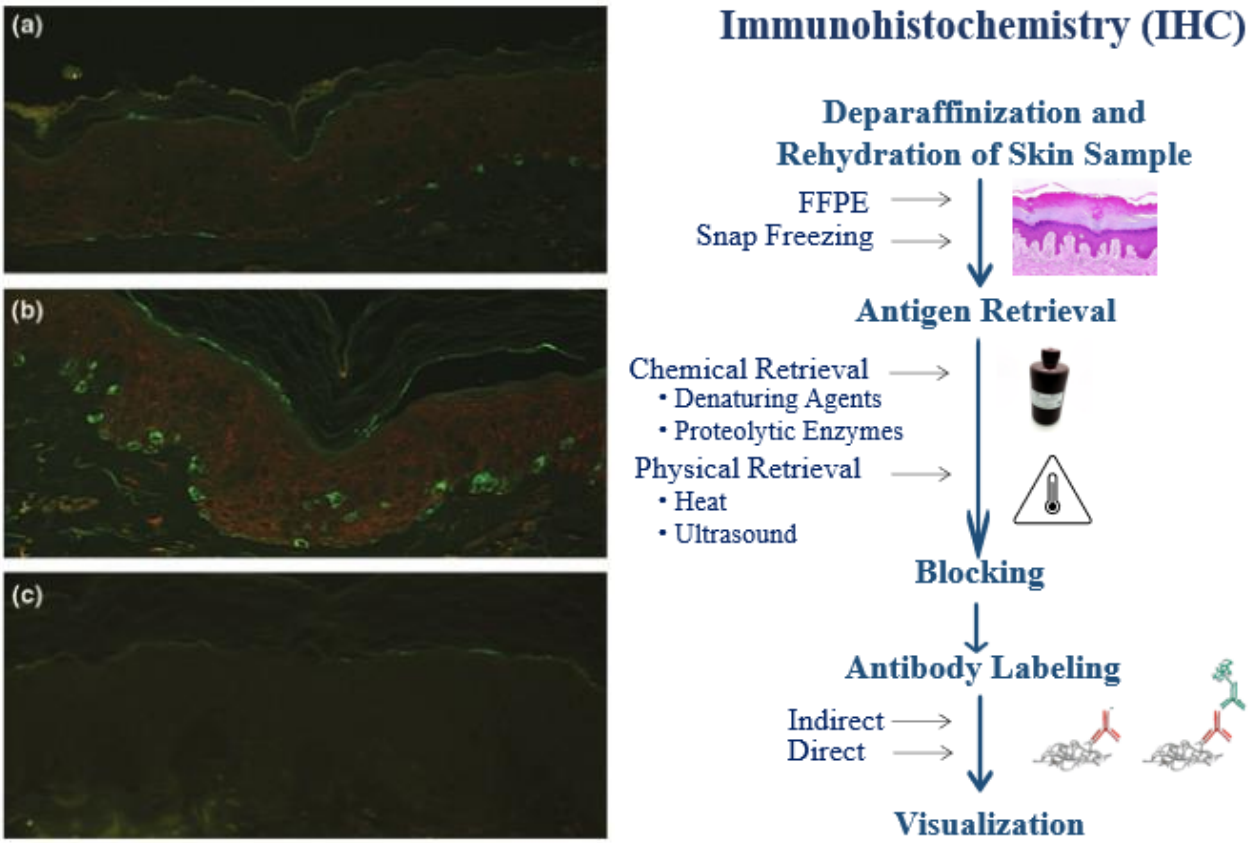


Figure 9. *T. Passeron et al. (13) used IHC to show increased protein expression of endothelin 1 (red) and MAR1 (green) after exposure to UVR. (a) skin unexposed to UVR, (b) skin exposed to UVR and (c) control (only stained with secondary antibodies). Copyright 2022 Wiley. Used with permission from T. Passeron et al., Immunohistochemistry and in situ hybridization in the study of human skin melanocytes, Experimental Dermatology, John Wiley and Sons. The flow chart on the right is illustrated by the author and shows the major sequential steps in IHC. The skin sections are fixed using FFPE or snap-freezing. Fixation retains antigenicity and structure. Antigen retrieval can be done with chemical treatment or physical treatment, or in combination. Blocking is important to prevent false-positive staining. There are two methods of antibody labeling, direct and indirect. There are several washing steps involved in IHC that are not illustrated on this flow chart (13, 65).*

Western blotting is a useful tool when the aim is to examine the relative protein expression of different versions of the same protein due to post-transcriptional modifications, e.g., alternative splicing, glycosylation and phosphorylation (46). Quantitative western blot experiments can be used to analyze protein expression in skin samples. For patients with depigmentation, it would be interesting to assess the protein expression of lesioned areas, and the non-lesioned areas could be

used as reference. Western blotting can be useful to screen for altered protein expression under specific conditions, such as assessing the response to different drugs (66). The sequential steps in performing western blot analysis on skin samples are 1) skin biopsy, 2) protein isolation and sample preparation, 3) separation of proteins on SDS-PAGE, 4) transfer of protein bands to a nitrocellulose membrane, 5) probing with primary antibodies against proteins of interest, 6) washing step, 7) incubation with secondary conjugated antibodies, 8) washing step, and 9) applying substrate buffer and detection (39, 46). Selecting a suitable normalization method is important so that the observed fold changes are not artifacts when compared to reference signals. Housekeeping proteins (HKPs) are used as loading controls, and it is important that the expression of HKPs is consistent with different experimental conditions (46).

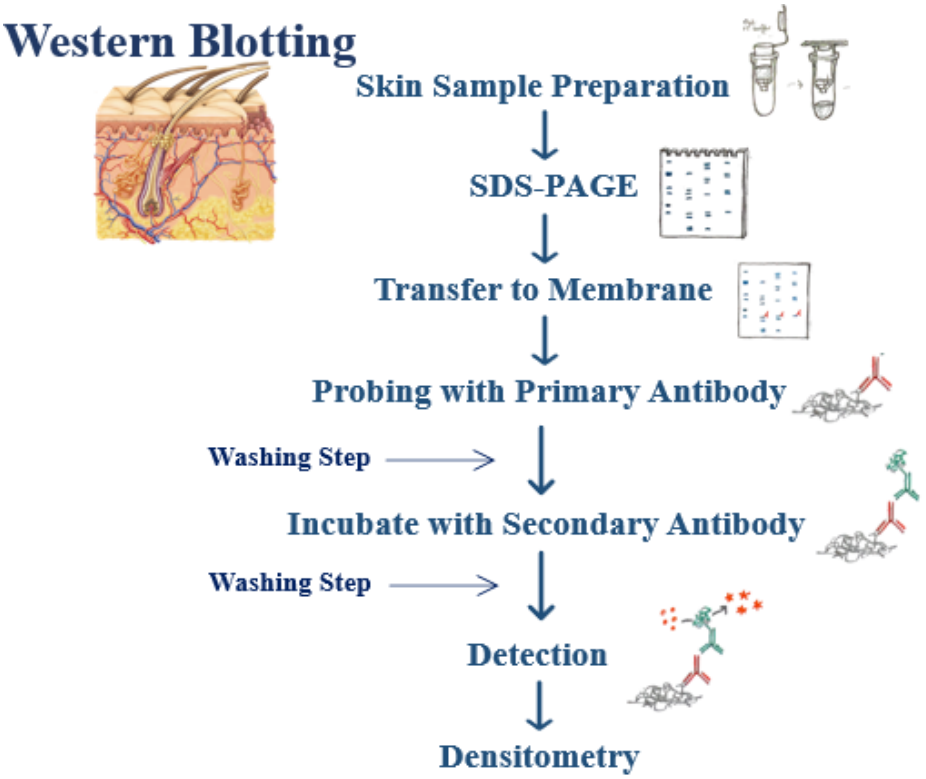


Figure 10. *Flow chart showing the sequential steps in western blot analysis of skin a sample. The process starts with collection and preparation of a skin sample. Proteins are separated on SDS-PAGE. The respective bands are transferred to a nitrocellulose membrane, which is then probed with primary antibodies. Antibodies that are not bound to any antigen will we washed away. The membrane is incubated with secondary conjugated antibodies with specificity towards the primary antibodies. Excess and unbound antibodies are washed away. A substrate buffer is added, and the signal is detected (46, 47). The figure is illustrated by the author.*

5.4 Analytical bias

For gene expression studies, it is crucial to extract mRNA rapidly after sample collection to prevent that the gene expression is altered significantly or that RNA is degraded. If the isolation of RNA and/or synthesis of cDNA is halted, it is important to preserve all the mRNA by snap-freezing the sample at -80 ℃ or by utilizing RNAlater reagents (42). RNAlater keeps RNA stable for minimum a week at room temperature, a month at 4 ℃, and even longer at -20 ℃ (41). With different methods for fixating skin sections there are some essential variables to be aware of, e.g., conservation of cell shape, altered antigens and membrane permeabilization (13).

When performing FISH on skin sections, it is important to avoid making bubbles when applying the probe mixture, which could result in heterogenous staining or false negatives. Samples should be stored in a humidifying chamber during incubation to prevent the tissue sections from drying (13). For RT-qPCR it is important to choose adequate reference genes for standardization. mRNA profiles can be perturbed by the collection and processing conditions of the sample. Quantification of RNA in the sample makes it possible to assure that each sample has approximately the same RNA-concentration before proceeding with PCR. The MIQE guidelines give a solid introduction to how to both perform and interpret qPCR data (45). There is a similar guidance for conducting experiments with DNA microarray, known as the MIAME-guidelines These guidelines allow researchers easy interpretation of obtained data and to produce results that can be verified independently (67).

Blocking is important in IHC to reduce the probability of false positives. The presence of specific endogenous enzymes may lead to false positives. It is therefore essential to block endogenous enzymes to assure that they do not react with the substrate later in the procedure. Endogenous antibodies from B-lymphocytes, for example, may cross-react with the commercial antibodies used in the procedure, which can lead to high background staining (13, 65). For successful IHC analysis it is important with skin sections of good quality, optimized procedures, and specific secondary antibodies (13). When analyzing protein expression on skin slides with IHC, negative and positive controls should be performed with each run. Positive controls are specimens that are known to contain the proteins that are being analyzed, while negative controls are skin sections undergoing the same procedure as the skin section being analyzed without the addition of primary antibodies (65). When conducting western blot analysis on skin samples, it is important

to validate that the interaction between antibodies and antigens are specific. Determining the sample's dilution factor is essential for loading in the linear range for each antibody. It is also important to select an adequate normalization method to assure that the observed fold changes of target proteins are not artifacts compared to reference signals (46). HKPs can be used as loading controls, and it is critical that the expression of HKPs is consistent despite the given experimental conditions. Data normalization is essential to identify experimental errors and to evaluate which corrective measures should take place. The density of bands can be affected by 1) improper treatment of specimens (varying expression and degradation of proteins), 2) inadequate composition of protease inhibitors, detergents and salts in the lysis buffer, and 3) insufficient homogenization (46, 47).

6 Conclusion

There are many regulatory mechanisms that not only affect the differentiation and development of melanocytes, but also the synthesis of melanin (4). Melanocyte related pathologies often have a dysregulated melanogenesis and development of melanocytes. Producing melanin requires a set of enzymes, but also a wide range of factors regulating their function (1, 2, 18). It is important to study mutations in melanogenic genes and their respective phenotypes to further understand the implications of pathogenic variants. Genome sequencing, transcriptome analysis and gene expression analysis are important fields of study to promote a better understanding of the intricate communication and molecular cues affecting melanogenesis (41, 42, 64). Further study of transcriptional and posttranscriptional mechanisms, as well as miRNAs is crucial to understand changes in phenotype (38).

Analysis of candidate genes has a priori bias when it comes to selecting which genes to study. It is not necessarily the candidate genes that have been chosen for a study that is causal for a given condition. In many situations the cause is multifactorial or polygenic. Retrieving information about genetic variants through WGS, WES or RNA-seq is considered unbiased (41, 64). Genetic variants and varying gene expression cause a wide spectrum of phenotypic diversity. It is essential to generate a comprehensive profile of both the genotype and the transcriptome to understand phenotypic abnormalities (40-42). Downregulated expression of hormones,

transcription factors, enzymes, phosphatases, kinases, or transport proteins could have a negative effect on the pigmentation process leading to the progression of depigmented macules (1, 4). The pigmentation process relies on myriad molecular processes and the fine balance between them (1). When analyzing gene- and protein expression, it will be recommended that biopsies are obtained from both lesioned and non-lesioned areas. This makes it possible to compare the transcriptome, gene- and protein expression between affected areas and areas that are not seemingly affected.

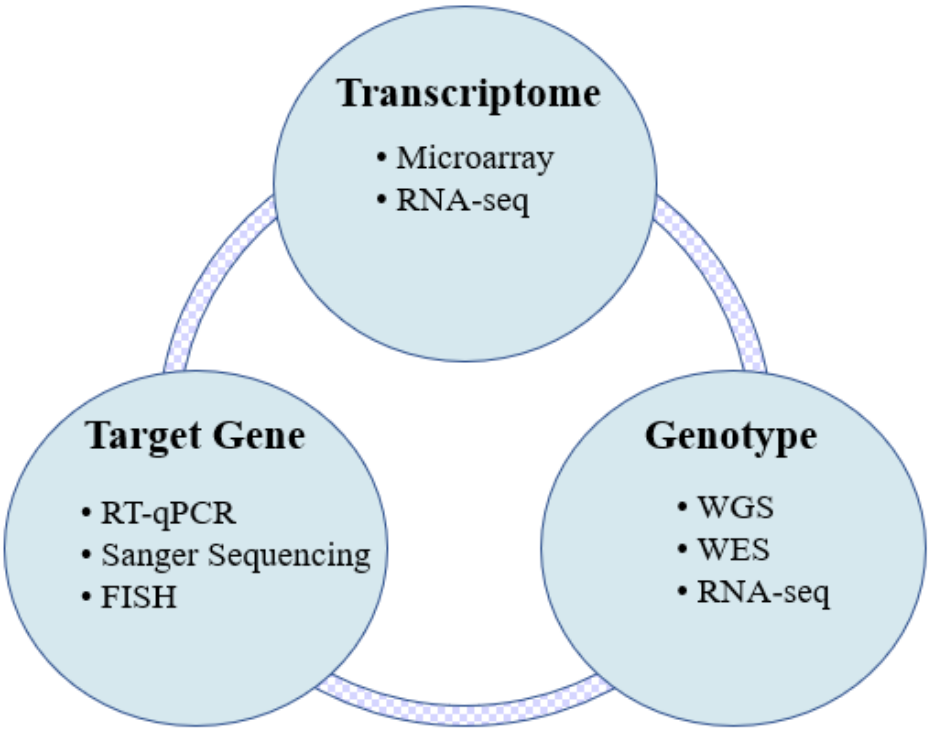


Figure 11. *Illustrates the different methods for investigating the cause of unknown depigmentation disorders. The transcriptome can be analyzed using microarray analysis or RNA-seq. Genotyping can be conducted by sequencing the whole genome (WGS) or selectively the protein-coding regions (WES and RNA-seq), and may give information about different variants of pigmentation related genes. Genes considered likely causal can be studied with RT-qPCR, Sanger sequencing or FISH (40-42, 44). This figure is illustrated by the author.*

There are different analyzing techniques that are applicable when investigating the cause of depigmentation. Genotyping through sequencing the whole genome or only the protein-coding regions may give information on different variants. These variants can be assessed using functional proteomics software programs that determine the probability and extent of affected

molecular functions (64). Analyzing the transcriptome through RNA-seq or microarray give valuable information about both different variants and gene expression. When analyzing unknown causes of depigmentation disorders, it will be recommended to start with analyzing the genotype or the transcriptome to search for variants or altered expression of pigmentation related genes. If microarray or RNA-seq shows altered expression, it would be of interest to confirm these results through an independent analysis of target gene expression (41, 42). Gene variants found through genotyping or altered expression of genes found with microarray or RNA-seq can then be analyzed using target gene expression analysis, such as RT-qPCR or FISH (13, 44). When analyzing target gene expression, it is important to analyze the protein counterpart using protein expression techniques, e.g., IHC and Western blot. Gene expression does not necessary correlate with protein expression. Detection of mRNA provides no information on whether or not it will be translated into a protein. Protein quantification methods like Western blot and IHC do not corroborate mRNA data obtained from RT-qPCR, microarray or FISH (13, 45). It is important to perform quantitative detection of both proteins and mRNAs of interest. IHC and FISH combined allows studying mRNA and protein levels of the target gene simultaneously (13). This will highlight whether there are factors halting the translational process leading to a reduced protein expression. Designing a method for analyzing gene expression of target genes could be of diagnostic value in possible future cases of depigmentation. This could be the design of RT-qPCR to assess expression of culprit genes.

When the cause of depigmentation disorder is detected, it is possible to develop innovative techniques for diagnosing future cases of depigmentation disorders. Analyzing the cause of depigmentation disorders makes it possible to understand the molecular foundations of the condition, and to develop novel therapeutics than can improve a patient’s quality of life.

7 References

1. D'Mello S, Finlay G, Baguley B, Askarian-Amiri M. Signaling Pathways in Melanogenesis. International Journal of Molecular Sciences. 2016;17(7):1144.
2. Cichorek M, Wachulska M, Stasiewicz A, Tymińska A. Skin melanocytes: biology and development. Advances in Dermatology and Allergology. 2013;1:30-41.
3. Saleem MD, Oussedik E, Schoch JJ, Berger AC, Picardo M. Acquired disorders with depigmentation: A systematic approach to vitiliginoid conditions. Journal of the American Academy of Dermatology. 2019;80(5):1215-31.e6.
4. Videira IFDS, Moura DFL, Magina S. Mechanisms regulating melanogenesis*. Anais Brasileiros de Dermatologia. 2013;88(1):76-83.
5. Bennett DC, Lamoreux ML. The Color Loci of Mice - A Genetic Century. Pigment Cell Research. 2003;16(4):333-44.
6. Fuller BB, Spaulding DT, Smith DR. Regulation of the Catalytic Activity of Preexisting Tyrosinase in Black and Caucasian Human Melanocyte Cell Cultures. Experimental Cell Research. 2001;262(2):197-208.
7. Plonka PM, Passeron T, Brenner M, Tobin DJ, Shibahara S, Thomas A, et al. What are melanocytesreallydoing all day long…? Experimental Dermatology. 2009;18(9):799-819.
8. Haass NK, Herlyn M. Normal Human Melanocyte Homeostasis as a Paradigm for Understanding Melanoma. Journal of Investigative Dermatology Symposium Proceedings. 2005;10(2):153-63.
9. O?Rahilly R, Müller F. The development of the neural crest in the human. Journal of Anatomy. 2007;211(3):335-51.
10. Ernfors P. Cellular origin and developmental mechanisms during the formation of skin melanocytes. Experimental Cell Research. 2010;316(8):1397-407.
11. Whiteman DC, Parsons PG, Green AC. Determinants of melanocyte density in adult human skin. Archives of Dermatological Research. 1999;291(9):511-6.
12. Rizos H, Becker TM, Holland EA, editors. Cell cycle regulation in the melanocyte2003.
13. Passeron T, Coelho SG, Miyamura Y, Takahashi K, Hearing VJ. Immunohistochemistry and in situ hybridization in the study of human skin melanocytes. Experimental Dermatology. 2007;16(3):162-70.
14. Borovanský J, Riley P. Melanins and Melanosomes: Biosynthesis, Biogenesis, Physiological, and Pathological Functions. 2011. p. 343-81.
15. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin Pigmentation in Mammalian Skin and Its Hormonal Regulation. Physiological Reviews. 2004;84(4):1155-228.

16. Costin G-E, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. The FASEB Journal. 2007;21(4):976-94.
17. Tsatmali M, Ancans J, Thody AJ. Melanocyte Function and Its Control by Melanocortin Peptides. Journal of Histochemistry & Cytochemistry. 2002;50(2):125-33.
18. Pavan WJ, Sturm RA. The Genetics of Human Skin and Hair Pigmentation. Annual Review of Genomics and Human Genetics. 2019;20(1):41-72.
19. Wiriyasermkul P, Moriyama S, Nagamori S. Membrane transport proteins in melanosomes: Regulation of ions for pigmentation. Biochim Biophys Acta Biomembr. 2020;1862(12):183318-.
20. Park HY, Kosmadaki M, Yaar M, Gilchrest BA. Cellular mechanisms regulating human melanogenesis. Cellular and Molecular Life Sciences. 2009;66(9):1493-506.
21. Schallreuter KU, Kothari S, Chavan B, Spencer JD. Regulation of melanogenesis - controversies and new concepts. Experimental Dermatology. 2008;17(5):395-404.
22. Hearing VJ. Determination of Melanin Synthetic Pathways. Journal of Investigative Dermatology. 2011;131:E8-E11.
23. Simon JD, Peles D, Wakamatsu K, Ito S. Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell & Melanoma Research. 2009;22(5):563-79.
24. Del Marmol V, Beermann F. Tyrosinase and related proteins in mammalian pigmentation. FEBS Letters. 1996;381(3):165-8.
25. Seiberg M. Keratinocyte-Melanocyte Interactions During Melanosome Transfer. Pigment Cell Research. 2001;14(4):236-42.
26. Choi W, Kolbe L, Hearing VJ. Characterization of the bioactive motif of neuregulin-1, a fibroblast-derived paracrine factor that regulates the constitutive color and the function of melanocytes in human skin. Pigment Cell & Melanoma Research. 2012;25(4):477-81.
27. Wang Z, Coleman DJ, Bajaj G, Liang X, Ganguli-Indra G, Indra AK. RXRα Ablation in Epidermal Keratinocytes Enhances UVR-Induced DNA Damage, Apoptosis, and Proliferation of Keratinocytes and Melanocytes. Journal of Investigative Dermatology. 2011;131(1):177-87.
28. Slominski A, Zmijewski MA, Pawelek J. L-tyrosine and L-dihydroxyphenylalanine as hormone-like regulators of melanocyte functions. Pigment Cell & Melanoma Research. 2012;25(1):14-27.
29. Rouzaud F, Kadekaro AL, Abdel-Malek ZA, Hearing VJ. MC1R and the response of melanocytes to ultraviolet radiation. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2005;571(1):133-52.
30. Eller MS, Ostrom K, Gilchrest BA. DNA damage enhances melanogenesis. Proceedings of the National Academy of Sciences. 1996;93(3):1087-92.

31. Seneschal J, Boniface K, D'Arino A, Picardo M. An update on Vitiligo pathogenesis. Pigment Cell & Melanoma Research. 2021;34(2):236-43.
32. Wang Y, Li S, Li C. Perspectives of New Advances in the Pathogenesis of Vitiligo: From Oxidative Stress to Autoimmunity. Medical Science Monitor. 2019;25:1017-23.
33. Richmond JM, Bangari DS, Essien KI, Currimbhoy SD, Groom JR, Pandya AG, et al. Keratinocyte-Derived Chemokines Orchestrate T-Cell Positioning in the Epidermis during Vitiligo and May Serve as Biomarkers of Disease. Journal of Investigative Dermatology. 2017;137(2):350-8.
34. Bhawan J, Bhutani LK. Keratinocyte damage in vitiligo. Journal of Cutaneous Pathology. 1983;10(3):207-12.
35. Kovacs D, Bastonini E, Ottaviani M, Cota C, Migliano E, Dell'Anna ML, et al. Vitiligo Skin: Exploring the Dermal Compartment. Journal of Investigative Dermatology. 2018;138(2):394-404.
36. Abdallah M, El-Mofty M, Anbar T, Rasheed H, Esmat S, Al-Tawdy A, et al. CXCL-10 and Interleukin-6 are reliable serum markers for vitiligo activity: A multicenter cross-sectional study. Pigment Cell & Melanoma Research. 2018;31(2):330-6.
37. Tulic MK, Cavazza E, Cheli Y, Jacquel A, Luci C, Cardot-Leccia N, et al. Innate lymphocyte-induced CXCR3B-mediated melanocyte apoptosis is a potential initiator of T-cell autoreactivity in vitiligo. Nature Communications. 2019;10(1).
38. Mansuri MS, Singh M, Dwivedi M, Laddha NC, Marfatia YS, Begum R. MicroRNA profiling reveals differentially expressed microRNA signatures from the skin of patients with nonsegmental vitiligo. British Journal of Dermatology. 2014;171(5):1263-7.
39. Thieman WJ, Palladino mA. Chapter 3 Recombinant DNA Technology and Genomics. Introduction to Biotechnology. 4 ed: Pearson Education; 2019. p. 80-124.
40. Burian AN, Zhao W, Lo TW, Thurtle-Schmidt DM. Genome sequencing guide: An introductory toolbox to whole-genome analysis methods. Biochemistry and Molecular Biology Education. 2021;49(5):815-25.
41. Saeidian AH, Youssefian L, Vahidnezhad H, Uitto J. Research Techniques Made Simple: Whole-Transcriptome Sequencing by RNA-Seq for Diagnosis of Monogenic Disorders. Journal of Investigative Dermatology. 2020;140(6):1117-26.e1.
42. Cordero F, Botta M, Calogero RA. Microarray data analysis and mining approaches. Briefings in Functional Genomics and Proteomics. 2008;6(4):265-81.
43. Ramos TDP, Amorim LMF. Molecular biology techniques for loss of heterozygosity detection: the glioma example. J Bras Patol Med Lab. 2015;51(3):189-96.

44. Thornton B, Basu C. Real-time PCR (qPCR) primer design using free online software. Biochemistry and Molecular Biology Education. 2011;39(2):145-54.
45. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry. 2009;55(4):611-22.
46. Taylor SC, Posch A. The Design of a Quantitative Western Blot Experiment. BioMed Research International. 2014;2014:1-8.
47. Mishra M, Tiwari S, Gomes AV. Protein purification and analysis: next generation Western blotting techniques. Expert Review of Proteomics. 2017;14(11):1037-53.
48. Bennett DC, Medrano EE. Molecular Regulation of Melanocyte Senescence. Pigment Cell Research. 2002;15(4):242-50.
49. Parra EJ. Human pigmentation variation: Evolution, genetic basis, and implications for public health. American Journal of Physical Anthropology. 2007;134(S45):85-105.
50. Kauser S, Westgate GE, Green MR, Tobin DJ. Human Hair Follicle and Epidermal Melanocytes Exhibit Striking Differences in Their Aging Profile which Involves Catalase. Journal of Investigative Dermatology. 2011;131(4):979-82.
51. Le Pape E, Passeron T, Giubellino A, Valencia JC, Wolber R, Hearing VJ. Microarray analysis sheds light on the dedifferentiating role of agouti signal protein in murine melanocytes via the Mc1r. Proceedings of the National Academy of Sciences. 2009;106(6):1802-7.
52. ClinVar [Internet]. 2022. Available from: https://www.ncbi.nlm.nih.gov/clinvar/.
53. Yamaguchi Y, Hearing VJ. Physiological factors that regulate skin pigmentation. BioFactors. 2009;35(2):193-9.
54. Lasseaux E, Plaisant C, Michaud V, Pennamen P, Trimouille A, Gaston L, et al. Molecular characterization of a series of 990 index patients with albinism. Pigment Cell & Melanoma Research. 2018;31(4):466-74.
55. Ambrosio AL, Boyle JA, Aradi AE, Christian KA, Di Pietro SM. TPC2 controls pigmentation by regulating melanosome pH and size. Proceedings of the National Academy of Sciences. 2016;113(20):5622-7.
56. Potterf SB, Mollaaghababa R, Hou L, Southard-Smith EM, Hornyak TJ, Arnheiter H, et al. Analysis of SOX10 Function in Neural Crest-Derived Melanocyte Development: SOX10-Dependent Transcriptional Control of Dopachrome Tautomerase. Developmental Biology. 2001;237(2):245-57.
57. Cheung M, Briscoe J. Neural crest development is regulated by the transcription factor Sox9. Development. 2003;130(23):5681-93.

58. Levy C, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol Med. 2006;12(9):406-14.
59. Levy C, Nechushtan H, Razin E. A New Role for the STAT3 Inhibitor, PIAS3. Journal of Biological Chemistry. 2002;277(3):1962-6.
60. Takeda K, Yasumoto K-I, Takada R, Takada S, Watanabe K-I, Udono T, et al. Induction of Melanocyte-specific Microphthalmia-associated Transcription Factor by Wnt-3a. Journal of Biological Chemistry. 2000;275(19):14013-6.
61. Park H-Y, Wu C, Yonemoto L, Murphy-Smith M, Wu H, Christina, et al. MITF mediates cAMP-induced protein kinase C-β expression in human melanocytes. Biochemical Journal. 2006;395(3):571-8.
62. Bae-Harboe Y-SC, Park H-Y. Tyrosinase: A Central Regulatory Protein for Cutaneous Pigmentation. Journal of Investigative Dermatology. 2012;132(12):2678-80.
63. Bondurand N. Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. Human Molecular Genetics. 2000;9(13):1907-17.
64. Lappalainen T, Scott AJ, Brandt M, Hall IM. Genomic Analysis in the Age of Human Genome Sequencing. Cell. 2019;177(1):70-84.
65. Magaki S, Hojat SA, Wei B, So A, Yong WH. An Introduction to the Performance of Immunohistochemistry. Springer New York; 2019. p. 289-98.
66. Duerr J. Immunohistochemistry. WormBook. 2006.
67. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nature Genetics. 2001;29(4):365-71.

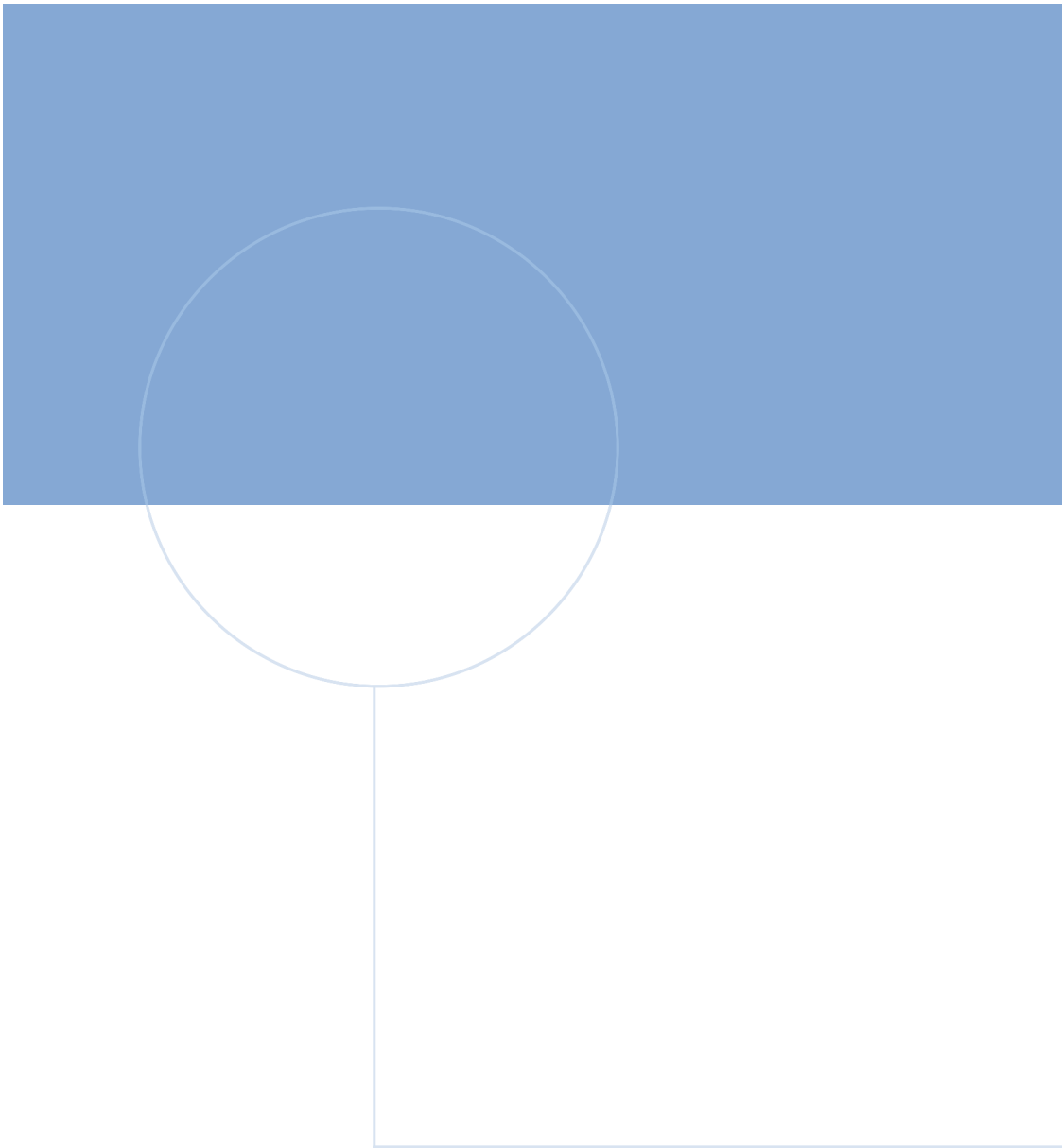
8 Appendix

The gene and protein names in table 1 have been mentioned in the literary study. Gene names and approved abbreviations of gene names are retrieved from HGNC. Approved names of proteins are retrieved from *InterPro*. Which type of CREB involved in the transcription of melanogenic enzymes was not specified in any of the articles mentioning its role. The subtype of protein kinase A (PKA) involved in the signaling pathway or V-ATP in the melanosomal membrane was also not mentioned in any of the articles used in this literary study, and is therefore referred to as just CREB, PKA, V-ATP. ACTH and α-MSH are cleavage products of the proopiomelanocortin (COLI) protein and are therefore not considered separate genes.

Table 1: Approved names of genes and proteins

Gene name from HGNC	**Approved abbreviation**	**Protein name from *InterPro***
Tyrosinase	*TYR*	TYRO
Tyrosinase related protein 1	*TYRP1*	TYRP1
Dopachrome tautomerase	*DCT*	TYRP2
Leucine rich melanocyte differentiation associated	*LRMDA*	LRMDA
OCA2 melanosomal transmembrane protein	*OCA2*	P-protein
HECT and RLD domain containing E3 ubiquitine protein ligase 2	*HERC2*	HERC2
Helicase like transcription factor	*HLTF*	HLTF
Solute carrier family 45 member 2	*SLC45A2*	S45A2
Solute carrier family 24 member 5	*SLC24A5*	NCKX5
Two pore segment channel 2	*TPCN2*	TPC2
ATPase copper transporting alpha	*ATP7A*	ATP7A
Cystinosin, lysosomal cysteine transporter	*CTNS*	CTNS
KIT proto-oncogene, receptor tyrosine kinase	*KIT*	KIT
KIT ligand	*KITLG*	SCF
Basonuclin 2	*BNC2*	BNC2
Proopiomelanocortin	*POMC*	COLI
Agouti signaling protein	*ASIP*	ASIP
Melanocortin 1 receptor	*MC1R*	MCHR
Melanocyte inducing transcription factor	*MITF*	MITF
Wnt family member 1	*WNT1*	WNT1
Wnt family member 3A	*WNT3A*	WNT3A
Protein kinase C beta	*PRKCB*	KPCB
SRY-box transcription factor 9	*SOX9*	SOX9
SRY-box transcription factor 10	*SOX10*	SOX10
Paired box 3	*PAX3*	PAX3
Premelanosome protein	*PMEL*	PMEL
Attractin	*ATRN*	ATRN

RAB27A, member RAS oncogene family	*RAB27A*	RB27A
Myosin VA	*MYO5A*	MYO5A
Myosin VIIA	*MYO7A*	MYO7A
Endothelin 3	*EDN3*	EDN3
Endothelin receptor type B	*EDNRB*	EDNRB
Receptor for activated C kinase 1	*RACK1*	RACK1
One cut homeobox 2	*ONECUT2*	ONEC2
Lymphoid enhancer binding factor 1	*LEF1*	LEF1
Protein inhibitor of activated STAT 3	*PIAS3*	PIAS3
Melan-A	*MLANA*	MAR1
Melanophilin	*MLPH*	MELPH
HNF1 homeobox A	*HNF1A*	HNF1A
BCL2 apoptosis regulator	*BCL2*	BCL2
F2R like trypsin receptor 1	*F2RL1*	PAR2
Interferon gamma	*IFNG*	IFNG
Tumor necrosis factor	*TNF*	TNFA
Interleukin 1 alpha	*IL1A*	IL1A
Interleukin 6	*IL6*	IL6
Nuclear factor, erythroid 2	*NFE2*	NEF2
Glutamate-cysteine ligase catalytic subunit	*GCLC*	GSH1
NAD(P)H quinone dehydrogenase 1	*NQO1*	NQO1
Glutamate-cysteine ligase modifier subunit	*GCLM*	GSH0
Diablo IAP-binding mitochondrial protein	*DIABLO*	DBLOH
C-X-C motif chemokine ligand 10	*CXCL10*	CXCL9
High motility group box 1	*HMGB1*	HMGB1
CD80 molecule	*CD80*	CD80
CD40 molecule	*CD40*	TNR5
Intracellular adhesion molecule 1	*ICAM1*	ICAM1
Class II major histocompatibility complex transactivator	*CIITA*	C2TA
RELA proto-oncogene, NF-kB subunit	*RELA*	TF65
Mitogen-activated protein kinase 1	*MAPK1*	MK01
Mitogen-activated protein kinase 14	*MAPK14*	MK14
Tumor protein p53	*TP53*	P53



NTNU

Kunnskap for en bedre verden