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Review paper

GENERAL CHARACTERISTICS OF THE MELANOGENESIS PROCESS WITH PARTICULAR EMPHASIS ON THE ROLE OF THE *PAX3* GENE

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Melanogenesis is a dynamic process of biological transformations leading to the formation of copolymeric dyes - melanins, which act as a protector against the ultraviolet radiation (UV) and reactive oxygen species (ROS). The process of these compounds formation is carried out by an enzymatic apparatus in specialized melanocyte organelles – melanosomes. Particularly from the point of view of biochemistry, melanins synthesis occurring depend to a large extent not only on genetic but also on environmental conditions. *Pax3* is an important candidate in research on genetic conditioning of animal colors pattern due to the fact that his gene expression product is a highly conserved transcription factor that during embryonic development is one of the elements responsible for regulating stem cell differentiation of the neural crest into melanocytes. Moreover during individual life *Pax3* is involved in the response of melanocytic units to UV radiation. Mutations/polymorphisms of the *Pax3* gene are the cause of the occurrence of some color varieties, as well as developmental disorders.

Keywords: *Pax3*, melanogenesis, melanocytes, melanoblast, coat color.

INTRODUCTION

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General characteristics of melanocytes

Melanocytes are neuroectoderm derived cells mainly specialized in the synthesis of melanin. During the embryonic period these cells migrate to their final destination as a precursor cells known as melanoblasts. Melanosomes, a membranous organelles LRO`s (lysosome-related organelles) are specialized in melanin synthesis. Melanosome precursors are endosomal organelles that collect the proteins enabling melanosomal matrix formation. Subsequently these organelles are transformed into premelanosomes into which the enzymes forming the melanin-producing machinery are transported from the trans-Golgi side (PILLAIYAR *et al*., 2017; ROK *et al*., 2012; SULAIMON and KITCHEL, 2003; RAPOSO and MARX, 2002).

Mature melanosomes are transported from the cell body of melanocytes to the tips of their dendrites. Movement of melanosomes is driven along microtubules and actin filaments. *B*idirectional transport along the microtubules is provided by a complexes of motor and linker proteins that allow for the formation of the melanosome-kinesin/dynein complex. KAP protein (kinesin accessory protein) connects with the light kinesin chains, mediating transport towards the positive microtubule, while dynein facilitates minus-end-directed transport (ROK *et al*., 2012; PARICHY *et al*., 2006). In the terminal part of the melanocyte`s dendrites, the transport is driven by F-actin and its specific protein complexes (WASMEIER *et al*., 2008).

Keratinocytes with melanocytes form a melanocytic unit (each melanocyte may contact with approximately 30-40 associated keratinocytes). Melanosomes located in the branched projections of melanocytes are transported to the keratinocytes. There are two reasons why melanosomes are localized in the cytoplasmic projections of the melanocytes (BRENNER and HEARING, 2008). First, to protect the cellular apparatus against highly reactive indole derivatives that are indirect metabolites of melanogenesis (RAPOSO and MARX, 2002). In addition, it allows the transport of melanosomes to keratinocytes (BOSSCHE *et al*. 2006). The process of transferring active melanosome to the keratinocyte is not fully explained, however, it is known that some proteins may be involved in that process including, lectin, E-cadherin, Snare and Rab proteins, as well as Rho protein with GTPase-activity. Four possible models of melanosome transfer have been described:

A process involving a cytophagocytosis mediated via PAR-2 (metabotropic receptor), which activation leads to local reorganization of the cytoskeleton and subsequent phagocytosis of the melanocytic branch projections (containing melanosomes) by keratinocyte.

By exocytosis, which involves melanosomal membrane fusion with the melanocytic membrane, resulting in the escape of melanin into the intercellular space, whereby phagocytosis is carried out by keratinocytes.

By membrane fusion that involves formation of filopodia that plays a role of transfer channel between melanocytes and keratinocytes.

By exocytosis of membrane follicles containing melanosomes, which by phagocytosis or membrane fusion enter the keratinocyte (MARCZYŃSKA and PRZYBYŁO, 2013; ROK *et al*., 2012; BOSSCHE *et al*., 2006;).

There are two types of melanosomes such as: elliptical eumelanosomes, containing fibrillar matrix, synthesizing eumelanin of significant photoprotective function and protection against reactive oxygen species (ROS) and spherical feomelanosomes synthesizing pheomelanin. It should be noted that both eumelanosomes and feomelanosomes can be present in the melanocytes at the same time. Melanosomes transported to the keratinocytes form a protective structure over the cell nucleus. It protects the genetic material from damage resulting from UV radiation or oxygen radicals. Both UV and ROS can disrupt the RNA, DNA, protein and lipid structures, leading to disorders caused by dysfunction of these molecules (PILLAIYAR *et al*., 2017; ROK *et al*., 2012; SULAIMON and KITCHEL, 2003).

Outline of melanogenesis process

Melanogenesis is a series of a complex reactions, mostly enzymatic (Fig.1), leading to the formation of a pigment - a water-insoluble eumelanin and/or rich in sulfur amino acids pheomelanine. These two types of dyes are copolymers and each possess a different structure and function. The process of their formation is extremely dynamic and takes place in melanosomes. It should be pointed out that each intermediate compound can be used and transformed through different metabolic pathways. The direction of these changes strictly depends on chemical environmental parameters in which they occur and these include: activity of an enzymes catalyzing, various steps in enzymatic processes, substrate concentration (L-tyrosine), presence of sulfur compounds in melanosomes.

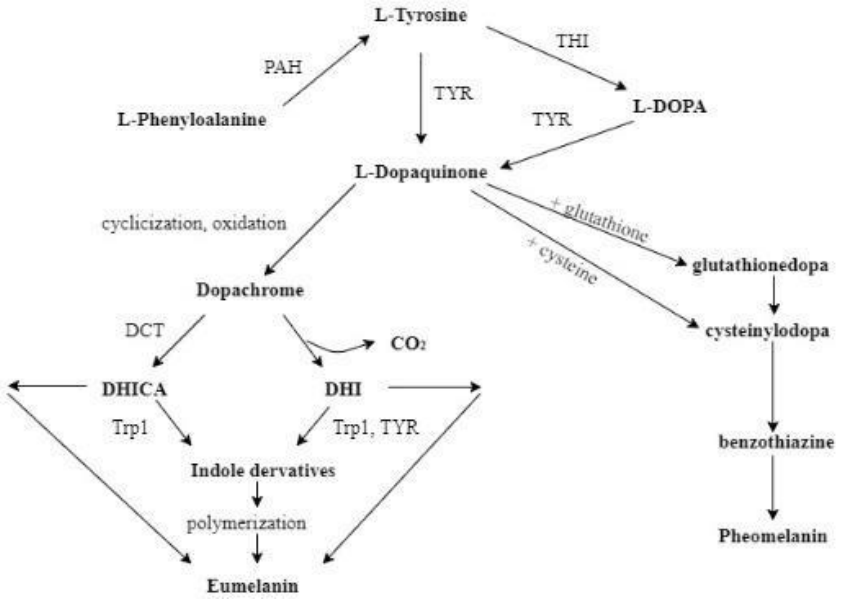

Figure 1. An outline of biochemical changes in melanogenesis; TYR - tyrosinase, TRP1 -Tyrosinase-related protein 1, DCT – dopachrome tautomerase, THI - tyrosine hydroxylase isoform 1, PAH - phenylalanine hydroxylase, DHICA - dihydroxyindol carbolic acid, DHI - 5,6-dihydroxyindolmelanin.

The substrate of the melanogenesis pathway, L-tyrosine, is formed through the transfer of hydroxyl group –OH on the aromatic ring of L-phenylalanine with the participation of cytoplasmic enzyme - phenylalanine hydroxylase (PAH). Further metabolism of L-tyrosine occurs in different metabolic pathways from DOPAquinone and leads to formation of two different classes of melanins.

The first stage of melanogenesis is common for both types of melanin. It is based on the enzymatic transformation of the amino acid in a highly reactive dopaquinone. There are two pathways of this transformation depending upon initial L-tyrosine amino acid concentration. At a lower concentrations of L-tyrosine (micromolar) the transformation takes place in two steps through an intermediate formed by the enzyme THI (tyrosine hydroxylase isoform 1). In contrast, in the presence of higher concentrations of amino acids (millimoles), this process is catalyzed by the main enzyme of melanogenesis – tyrosinase. The direction of further transformation depends on the presence of thiol compounds. With the appropriate concentration of cysteine and/or glutathione, these amino acids may be added to the DOPAquinone and may form cysteinyloDOPA and/or glutathioneDOPA. The oxidation of these compounds results in the formation of benzothiazine derivatives and eventually into a photodegradable pheomelanin, without a protective function and harmful to melanocytes due to the generation of radicals causing oxidative damage to cell elements.

The absence or low concentration of thiol compounds determines the spontaneous process of intramolecular cyclization and oxidation of DOPAquinone to DOPAchrome, which further transformations depend on the presence of dopachrome tautomerase enzyme (DCT). When DCT is present in the DOPAchrom cell, it is transformed into DHICA, which is one of the monomers of eumelanin. The absence or low activity of the DCT enzyme causes spontaneous decarboxylation of DOPAchrom to another eumelanin monomer - DHI. Both DHI and DHICA may undergo further enzymatic changes involving tyrosinase and/or protein related to tyrosinase (TRP1). These compounds are oxidized and cyclized to indole derivatives, which together with DHICA and DHI polymerize into the final product – eumelanin.

Eumelanin protects cells against UV degradation and free radicals (eumelanin has similar effects as peroxide dismutase). The qualitative composition of the eumelanin depends on the biological and chemical conditions of the melanocytes. These include presence and the rate of enzyme activity catalyzing the process of melanogenesis as well as the concentration of microelements (Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}) (RZEPKA *et al.*, 2016; MARCZYŃSKA and PRZYBYŁO, 2013; ITO, 2003).

The role of Pax3 in melanogenesis.

Generally Pax proteins are involved in many various cellular processes including, proliferation, migration, inhibition of apoptosis, as well as regulator of precursor cell differentiation, and organogenesis. The above-mentioned functions of Pax proteins are realized via molecular interactions with enhancers and/or promoters of the target genes or through interacting with other transcription factors or modulators of protein function (KUBIC *et al.*, 2008).

Pax3 is one of the key transcriptional factors regulating the process of melanocyte differentiation both during embryo development as well as in mature organism. This is due to the ability of Pax3 protein to stimulate neural crest stem cell differentiation by activating the *MITF* gene expression, the product of which is called the main transcriptional factor of melanogenesis. At the same time, Pax3 protein inhibits the final differentiation of melanoblasts by inhibiting the expression of *DCT* gene. Pax3 inhibits cell development until the melanoblasts will reach the

targeted region. Inhibition of the final differentiation during migration to the destination area is likely to protect a developing organism from potentially harmful intermediate metabolites of melanogenesis.

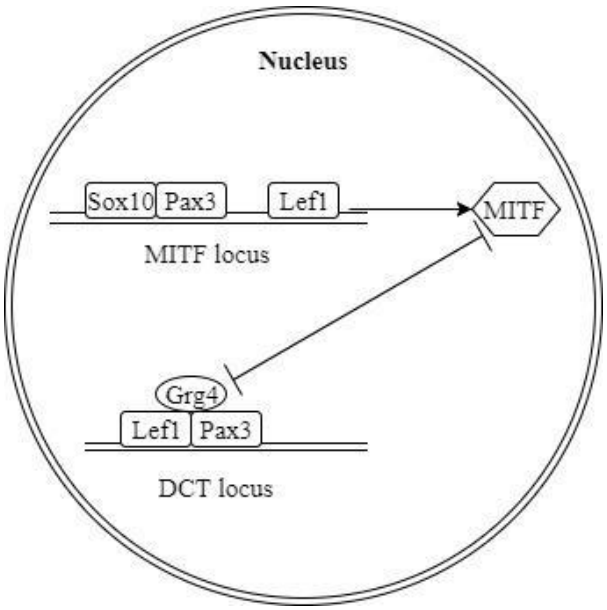

Figure 2. Scheme describing an activation of *MITF* gene expression and inhibition of *DCT* gene expression; Sox10/Pax3/Lef1 transcription activating complex, Lef1/Grg4/Pax3 transcriptional inhibitory complex; Sox10 - SRY-related HMG-box 10, Pax3 - Paired box protein 3, Lef1 - lymphoid enhancer binding factor 1, Grg4 - Groucho-related protein.

The initiation of neural crest stem cell differentiation occurs when three proteins linked to the regulatory cis region of the *MITF* gene, namely: Pax3, Sox10 (SRY-related HMG-box) and Lef1 (lymphoid enhancer binding factor 1) - *β*-catenin dependent protein. The above-mentioned complex of proteins activates the expression of the *MITF* gene which results in the expression of a number of genes involved in melanogenesis, thus the differentiation of stem cells of the neural crest.

The Pax3 protein binding domain interacts with the HMG region of the Sox10 transcription factor, thereby enabling synergistic activation of the *MITF* gene expression. MITF transcription factor regulates expression of *Tyr*, *DCT* and *Trp1* genes. The products of above mentioned genes are called molecular markers of melanogenesis due to the fact that their presence in the cell indicates the course of the melanin synthesis process in the mature melanocytes. At the same time, a complex of Pax3/Lef1/Grg4 (groucho-related proteins, Grg proteins are transcriptional corepressors not directly associated with DNA) is formed, which has a greater affinity with the region of the *DCT* gene promoter than the MITF transcriptional factor. As a result, despite the expression of MITF protein, there is no expression of the *DCT* gene and no complete differentiation of melanoblasts (Fig.2) It is important to note that the melanocytes which express *Pax3* gene also express antiapoptotic *Bcl-Xl* gene (LIPKA and CHARON, 2015;

HATHAWAY and HAQUE, 2011; MEDIC and ZIMAN, 2010; KUBIC *et al.*, 2008; PLUMMER *et al.*, 2008).

Pax3 in melanoblast differentiation during embryonic period

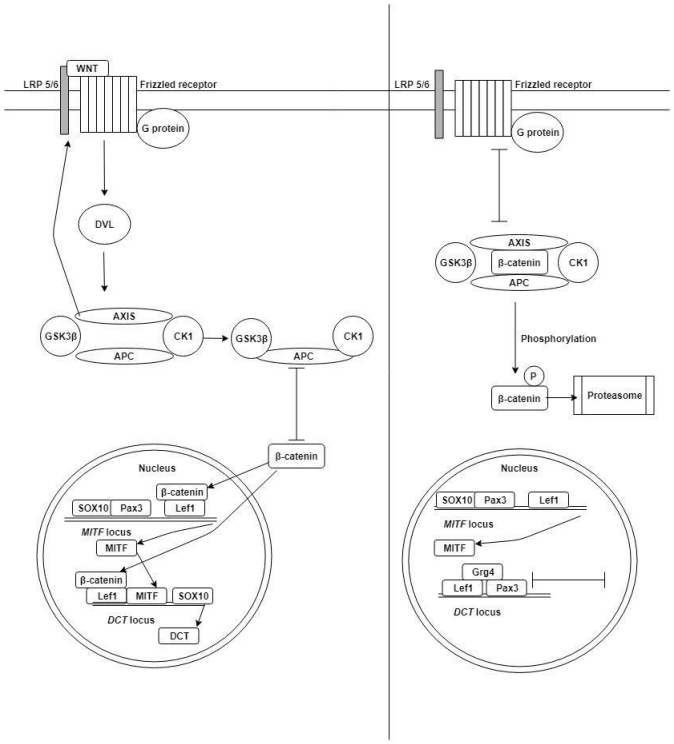

Figure 3. Frizzled receptor in the process of melanoblasts differentiation, GSK-3β /APC/Axin/CK1 - degradation complex, LRP - Low-density lipoprotein-related receptors, P - phosphate group, GSK3-β - Glycogen synthase kinase 3 beta, CK1 - Casein kinase 1, APC - Adenomatous polyposis coli.

Final melanoblast differentiation in target area occurs as a result of stimulation of the metabotropic (G-protein coupled receptor) receptor Frizzled by a WNT molecule. WNT signaling molecules are a family of 19 conservative glycoproteins that are agonists of the Frizzled receptors family. The Frizzled receptor interacts with the transmembrane LRP5/6 lipoprotein molecule involved in the stabilization of the bound WNT signaling molecule and signal transduction. When the receptor is not stimulated, the *β*-catenin which is a key molecule for the expression of the *DCT* gene, is degraded in the proteasome. The *β*-catenin degradation process involves AXIN/APC proteins forming the degradation scaffold and GSK3/CK1, which are responsible for the phosphorylation of the *β*-catenin molecules. Phosphorylated β-catenin is ubiquitinated by the dedicated ubiquitin ligase complex E3 that results in degradation of *β*-catenin in the 26S proteasome. The stimulation of Frizzled receptor by his agonist causes activation of the Dishevelled protein (DVL), protein G and LRP5/6 coreceptor. The DVL

protein recruits the AXIN/GSK3-*β* complex, which, together with CK1 phosphorylates characteristic amino acid motif in the cytoplasmic part of the LRP5/6 coreceptor, which allows the AXIN protein to be bound by the C-end of the LPR5/6 coreceptor and thus break down the AXIN/APC/GSK3/CK1 degradation complex (Fig.3) (DOLATSHAD *et al*., 2015; STAMOS and WEIS, 2013; HATHAWAY and HAQUE, 2011; ANGERS and MOON, 2009).

Pax3 also interacts with the region of the *Trp1* gene promoter, causing its expression and thus directs the precursor cell towards functional melanocyte (GALIBERT *et al*., 1999).

Pax3 in final melanoblast differentiation in mature organism

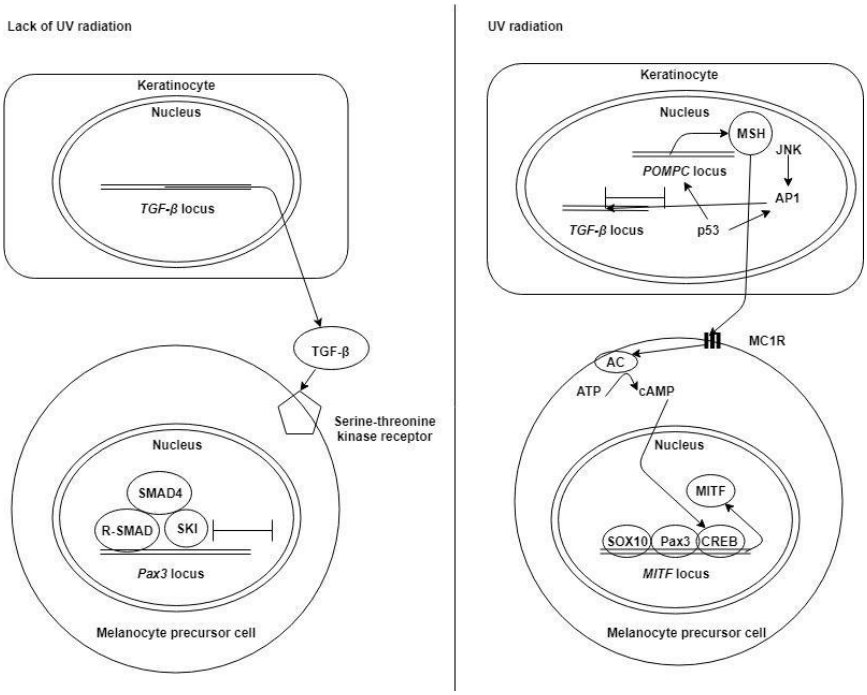

Figure 4. The scheme of the influence of UV radiation on melanocytic units; TGF-β-Transforming growth factor beta, Smad4/R-Smad/SKI - proteins complex inhibiting gene expression of Pax3, MSH-melanotropin, MC1R- melanotropin receptor, JNK - c-Jun N-terminal kinases, AC - adenylyl cyclase.

UV radiation plays an important role in the regulation of melanocytic units. It activates paracrine signal pathways between keratinocytes and melanocytes that are responsible for the processes of melanocytes precursor cells differentiation, melanin synthesis and transport of melanosomes to keratinocytes. If there is no stimulation of keratinocytes by ultraviolet light, on the basis of an unknown mechanism, beta transformation factor gene (*TGF-β*) is expressed, which binds to the serine-treoninine kinases receptors (presents in the melanocyte`s cell

membrane) that results in transduction of the signal via phosphorylation of R-SMAD proteins (receptor-activated-SMAD). This allows to oligomerize SMAD molecules and thus formed repressor complex consists of Smad proteins: R-Smad/Smad4 and SKI factor (MACIAS *et al*., 2015; HATHAWAY and HAQUE, 2011, MOUSTAKAS, 2008). The resulting complex has an inhibitory effect on the expression of the *Pax3* gene and as a result the precursor cells does not differentiate. Keratinocyte stimulation via UV light leads to JNK (c-Jun N-terminal kinases) kinase and p53 protein activation. JNK synergistically with p53 activates the AP1 protein complex that inhibits the expression of the *TGF-β* gene, simultaneously the activation of p53 leads to activation of a signaling cascade POMC/MSH (Proopiomelanocortin)/(melanotropin) which in turn result in the secretion of MSH into the intercellular space, where it interacts with the receptor, metabotropic MC1R. Activation of the MC1R receptor leads to dissociation of the trimeric G protein, where alpha part activates the membrane enzyme - adenyl cyclase, which converts ATP to cAMP. cAMP is a key intermediate element of the intracellular cascade that activates the *MITF* gene expression by binding to a CREB protein (cAMP response element) included in the complex of CRE*B*/Pax3/SOX10 located in the regulatory region of the *MITF* gene (Fig.4). The presence of the transcription factor-MITF activates the transcription of a number of genes (*Tyr*, *DCT*, and *Trp1*) whose products are essential components of the melanogenesis process (Fig.1) (HATHAWAY and HAQUE, 2011; MOUSTAKAS, 2008; YANG *et al*., 2008; MASSAGUÉ *et al*., 2005).

The effects of Pax3 gene mutations on melanogenesis.

Neural crest is a unique structure of embryonic, multipotent stem cells. During development these cell undergo differentiation, proliferation, and finally migrate to target sites. Neural crest stem cells are precursors of many types of specialized cells such as: melanocytes, osteocytes, chondrocytes, neurons, smooth myocytes, odontoblasts, endocrine cells, and glial cells. The fate of the stem cell largely depends on the activity of specific transcription factors, including Pax3 proteins (Hou and Pavan 2008). *Pax3* gene mutations may cause a developmental impairments associated with abnormal Pax3 protein structure. The overall range of mutation effects of this gene includes disorders related to the differentiation/survival and migration of the neural crest stem cells.

In humans, the *Pax3* gene is located on the long arm of the chromosome 2 in the 35 region (2q35). Up to now, about 70 point mutations have been identified, including nonsense, missense mutations, deletions, mutations in the RNA assembly sites and chromosomal aberrations in the form of *Pax3* and *FKHR* fusion (HU *et al*., 2013; OTRĘBA *et al*., 2013). Changes in the gene sequence leads to disorders in the structure of each protein that in turn result in a loss or limitation of his functions. One of the molecular effects of the mutation is the dysfunction of the Pax3 protein caused by the loss of homeodomain (HD) where the NLS (nuclear location signal) signaling region is located. Otherwise, this alteration does not specifically affect the transport of the protein to the nucleus. This is due to the relatively small molecular weight (about 21 kDa) of the protein allowing less efficient but still sufficient transport of the transcription factor Pax3 to the cell nucleus by a passive diffusion. However, the loss of HD hinders for the interaction of the *Pax3* protein with the regulatory sequence of the *MITF* gene (OTRĘBA *et al*., 2013).

Mutations in the *Pax3* gene are considered to be the main cause of a Waardenburg syndrome type I and III (WS I and WS III). WS is caused by disorders elicited by abnormal development of melanocytes. WS is characterized by a hearing loss and a pigmented hair, skin

and eye disorders (OTRĘBA *et al*., 2013; READ and NEWTON, 1997). WS I is inherited in autosomal dominant pattern and is characterized by pigmented disorders of the scalp, chest and head, iris heterochromatic, hearing impairment. WS III, depends on the type of mutation in the *Pax3* gene, and may be autosomal, dominant or recessive.WS III may be caused by a SNP or a deletion of the *Pax3* gene region along with the associated genes. Similarly to WS I, WS III characterizes a hypopigmentation and deafness. In addition, people with genetic defects have muscular and skeletal disorders and heart defects. Probably homozygous mutations or complex heterozygous mutations may be lethal in the embryonic stage or during early childhood (JALILIAN *et al*., 2015; OTRĘBA *et al*., 2013).

An animal model that mimics the course of WS in humans is Splotch (Sp) mice having a mutated *Pax3* gene located in the first chromosome. Changes observed in murine models with minor deviations correspond to those observed in humans with mutations in the *Pax3* gene. It has been shown, that the differences observed among mouse and humans are the result of generalized genetic differences and not the direct effect of the mutations of the *Pax3* gene (TASSABEHJI *et al*., 1994). Mice with a mutation in the third intron, probably due to aberration of the alternative splicing process, produce four alternative mRNA molecules, three of which are incomplete, and the final transcriptional product is devoid of the paired domain (EPSTEIN *et al*., 1993). Heterozygous Sp mutants are characterized by a lower Pax3 protein activity resulting in phenotypic coat white spotting (CHALEPAKIS *et al*., 1994). Mouse embryos possessing two mutant alleles of the Pax3 gene (homozygotes) usually die an about 13 day of embryonic development, probably due to severe defects in the neural crest.

In horses coat splashed white or white spotting (characterized by the appearance of white spots of different sizes on the base coat) is an autosomal dominant trait. Results of studies performed on horses (Quarter Horse) clearly suggest that mutation in the *Pax3* and / or *MITF* genes causes splashed white coat. In horses, the presence of a missense mutation in the *Pax3* gene, involving the substitution of 209 nucleotide G>A, results in a change in the amino acid cysteine to tyrosine in the polypeptide chain of the Pax3 protein. It should be noted that the splashed white coat is only present in the heterozygous individuals and no homozygous individuals are observed. Probably a homozygous in terms of mutation may result in fatal changes during embryonic or fetal development (like mouse Sp). Crossbreeding of white spotting horses is not recommended as they may be carriers of a defective allele of the *Pax3* gene, resulting in up to 25% chance of a lethal homozygous pattern in the offspring. In addition, this mutation may result in iris heterochromia and less frequent deafness.

A similar phenotypic effect can be observed the *MITF* gene mutation in gene`s promotor region, what can result in two different types of coat colors: mottled and macchiato. Individuals homozygous in terms of mutation characterized by an extraordinary phenotypic effect of complete absence of pigment, while individuals heterozygous phenotypic effects are very similar to the effects seen with the presence of the mutant *Pax3* allele. There are also cases of animals carrying the faulty variants of both the *Pax3* and *MITF* genes in that case the phenotype is almost the same as in the presence of only one defective allele (LIPKA and CHARON, 2015; HAUSWIRTH *et al*., 2012).

The known function and significance of the *Pax3* gene in the process of melanogenesis urged to determine his structure in the various animals species. A number of *Pax3* variations were observed in American mink (Neovison vison) and some of them are associated with the coat color, what once again proved the role of the *Pax3* gene in the described process (KMIEĆ *et al*., 2019, KMIEĆ *et al*., 2018).

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OPŠTE KARAKTERISTIKE PROCESA MELANOGENEZE SA POSEBNIM OSVRTOM NA ULOGU *PAX3* GENA

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Izvod

Melanogeneza je dinamički proces bioloških transformacija koje dovode do formiranja kopolimernih boja - melanina, koji deluju kao zaštitnik od ultravioletnog zračenja (UV) i reaktivnih vrsta kiseonika (ROS). Proces formiranja ovih jedinjenja vrši enzimski aparat u specijalnim melanocitnim organelima – melanozomima. Posebno sa stanovišta biohemije, sinteza melanina zavisi u velikoj meri ne samo od genetičkih već i od spoljašnjih uslova. *Pax3* je važan kandidat u istraživanju genetskog kondicioniranja uzorka životinjskih boja zbog činjenice da je njegov proizvod ekspresije gena visoko konzervisan transkripcioni faktor koji je tokom embrionalnog razvoja jedan od elemenata odgovornih za regulisanje diferencijacije neuronskih grebena u melanocite. Štaviše, tokom individualnog života *Pak3* je uključen u odgovor melanocitnih jedinica na UV zračenje. Mutacije / polimorfizmi *Pak3* gena su uzrok pojave nekih boja, kao i razvojnih poremećaja.

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