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ELABORATO DI LAUREA

ROLE OF THE CYTOSKELETON IN MELANOGENESIS

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Sommario

ABSTRACT

This thesis presents a critical analysis of the scientific article entitled *"PRP4 Promotes Skin Cancer by Inhibiting Production of Melanin, Blocking Influx of Extracellular Calcium, and Remodeling Cell Actin Cytoskeleton".*

The paper's objective was to investigate the mechanism by which the pre-mRNA processing factor 4B (PRP4) regulates melanin synthesis and its role in the progression of skin cancer in a murine melanoma-derived cell line (B16F10). They hypothesized that overexpression of PRP4 impinges on the signaling pathway involved in melanogenesis and underlies skin cancer development.

Upregulation of β-arrestin 1 and desensitization of the extracellular calcium-sensing receptor (CaSR), resulting in a reduced influx of extracellular Ca^{2+} ions, were also affected by PRP4 overexpression. It has also been proposed that PRP4 suppresses the expression of adenylyl cyclase (AC), thereby decreasing the production of cyclic adenosine monophosphate (cAMP) and modifying the actin cytoskeleton by regulating the expression of Ras homolog family member A (RhoA).

Based on their experimental findings, the researchers reported that the co-action of PRP4 on Ca^{2+} influx, melanin suppression and actin cytoskeleton modulation promotes the initiation of skin cancer.

ABBREVIATIONS

UVR: Ultraviolet radiation
ROS: Reactive oxygen species
MC1-R : Melanocortin 1 receptor
cAMP: Cyclic adenosine monophosphate
PKA: Protein kinase A
CREB: cAMP response element-binding protein
MITF: Melanocyte inducing transcription factor
MAPK: Mitogen-activated protein kinase
PLC: Phospholipase C
DAG: Diacylglycerol
PKCβ: Protein Kinase Cβ
NO: Nitric oxide
cGMP: Guanosine 3',5'-cyclic monophosphate
PKG: Protein kinase-G
a-MSH: Alpha-melanocyte stimulating hormone
TRP-1: Tyrosinase related protein 1
WNT : Wingless-related integration site
RhoA : Ras homolog family member 1
GDP: Guanosine diphosphate
GTP: Guanosine triphosphate
Rac1: Ras-related C3 botulinum toxin substrate 1
Cdc42: Cell division control protein 42 homolog
ROCK : Rho kinase
Rb: Retinoblastoma
PI-3K: Phosphoinositide 3-kinase
AKT: Protein kinase B
PRP4 : pre-mRNA processing factor 4B
CaSR: Calcium sensing receptor
EMT: Epithelial-mesenchymal transition
ARRB1: beta arrestin 1
TRPC1: Transient receptor potential canonical 1
TYR: Tyrosinase
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GPCR: G-protein-coupled receptor

INTRODUCTION: WHAT IS MELANOGENESIS, WHAT IS THE CYTOSKELETON AND WHAT IS THE RELATIONSHIP BETWEEN THE TWO?

Melanogenesis is defined as the production of the melanin pigments; these pigments are produced within melanocytes, dendritic cells derived from melanoblasts, their precursors, which are mainly found in the basal layer of the epidermis and hair follicles.

The molecular structure of melanin guarantees absorption of ultraviolet (UV) and visible light, providing defense against UVR from the sun and reducing their penetration through the epidermis.

Moreover, melanin offers protection from reactive oxygen species (ROS), formed through oxidative stress in cells, and is responsible for hair and skin pigmentation. In the skin, melanocytes are surrounded nearly by 36 keratinocytes, to which melanin pigments are transferred.

However, melanin is not only found in the skin; in fact scientists have determined the existence of three types of pigment in human, which are distributed in various bodily tissues. These three forms are eumelanin, pheomelanin and neuromelanin. Eumelanin, which comes in black and brown forms, is found predominantly in hair, skin, and eyes, contributing to the darker tones of these organs, while pheomelanin is a pigment that has a high Sulphur content and gives reddish-yellow hues to hair, eyes, and skin. It is found predominantly in the pinkish skin that constitutes nipples and genital organs.

Finally, neuromelanin is the polymeric pigment produced by catecholaminergic neurons in the brain and may have a protective function against harmful stimuli. Constitutive pigmentation, which can be affected by various regulatory factors, reflects the genetically determined level of melanin. This level depends on the number and size of melanosomes as well as the type of melanin and its distribution in keratinocytes.

In fact, the genetic polymorphisms responsible for phenotypic diversity of pigmentation, regard in particular melanocortin 1 receptor (MC1-R), a key protein involved in eumelanin synthesis: a mutation of MC1-R may lead to a decrease of eumelanogenesis and could alter pigment formation in response to UV radiation.

Since melanocytes are found in many different human tissues, both intrinsic and extrinsic stimuli can influence the beginning and development of pigmentation and melanogenesis.

Exposure to UVR and certain chemicals or drugs are examples of extrinsic factors, whereas intrinsic stimuli consist of the production of cytokines, prostaglandins and leukotrienes by melanocytes and keratinocytes, which possess an autocrine or paracrine impact on each other.

Within melanocytes, there are enzymatic chemical processes that spontaneously lead to melanin production; however, the influence of intrinsic or extrinsic factors can modify the activity of these processes by affecting the amount, intensity of production, and also the transfer of melanin from melanocytes to keratinocytes.
The main signaling pathways that control melanogenesis are shown in (Figure 1), and they are:

- cAMP/PKA/CREB/MITF cascade
- MAP kinases cascade
- PLC/DAG/PKCβ cascade
- NO/cGMP/PKG cascade

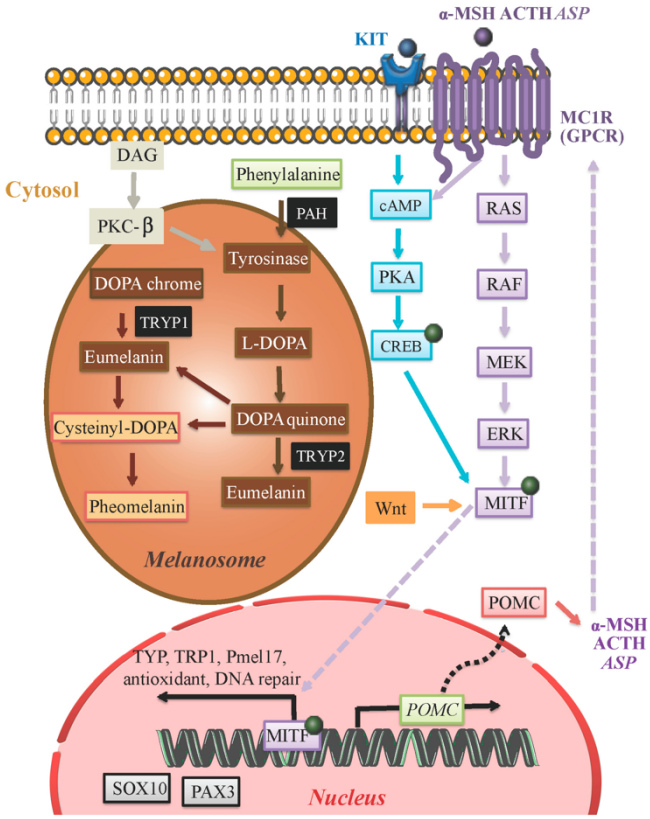

Figure 1. *Eumelanin and pheomelanin are synthesized within melanosomes of melanocytes by a series of reactions that are catalyzed by specific melanogenic enzymes (black). Production of these enzymes is driven by the MITF transcription factor whose activity is regulated by a number of signaling molecules including PKC (brown), cAMP (blue), MEK (purple), and WNT (orange). These signaling pathways are activated upstream by receptors such as KIT (ligand: SCF) and MC1R (ligands: α-MSH, ACTH and ASP). The MITF transcription factor drives the expression of a number of genes including SOX10 and PAX3. Protein kinase C (PKC); cyclic AMP (cAMP); MAPK/ERK Kinase (MEK); Wingless-related integration site (WNT); Stem Cell Factor (SCF); Melanocyte-specific melanocortin-1 receptor (MC1R); α-melanocyte-stimulating hormone (α-MSH); adrenocorticotropic hormone (ACTH); agonist stimulating protein (ASP). From: D'Mello S.A.N. et al., 2016*

The most important among these pathways is the first one, as it is affected by the pivotal hormone in stimulating melanogenesis: alpha-melanocyte stimulating hormone (α-MSH).
When α-MSH binds to MC1-R, it leads to the activation of adenylyl cyclase which stimulates the production of cAMP. This, in turn, induces the phosphorylation of CREB transcription factor by protein kinase A (PKA), and subsequently the activation of the melanocyte inducing transcription factor (MITF).
MITF positively controls the transcription of melanin-producing genes such as TRP-1 and TYR by binding to their promoter regions.

MITF expression can also be influenced by the Wingless-related integration site (WNT) pathway, that accomplishes a crucial role in various biological processes during development, tissue homeostasis and diseases.

However, there is another important element involved in regulating melanogenesis, and this is the cytoskeleton. The cytoskeleton is a dynamic system of different protein fibers that performs many important functions in the eukaryotic cell, notably the preservation of cell structure, internal organization, and mechanical support, in addition to its fundamental role in cell division and movement.

Three main types of filamentous proteins —actin filaments, intermediate filaments, and microtubules— make up its structure: these filaments are held together, connected to the plasma membrane and subcellular organelles by many accessory proteins.

Cytoskeleton dynamics are controlled by many molecules, including the essential Rho (RAS homolog) family proteins.

Rho family proteins range between 21 and 25 kDa in size; they typically contain a guanosine diphosphate/ guanosine triphosphate (GDP-/GTP-) binding domain, also known as G domain, which acts as a molecular switch, cycling between an active GTP-bound state and an inactive GDP-bound state, depending on the availability of guanosine triphosphate (GTP).

They have also a C-terminal hypervariable region that ends with a consensus sequence, and in some cases a N-terminal insertion with proline-rich motifs (Figure 2).

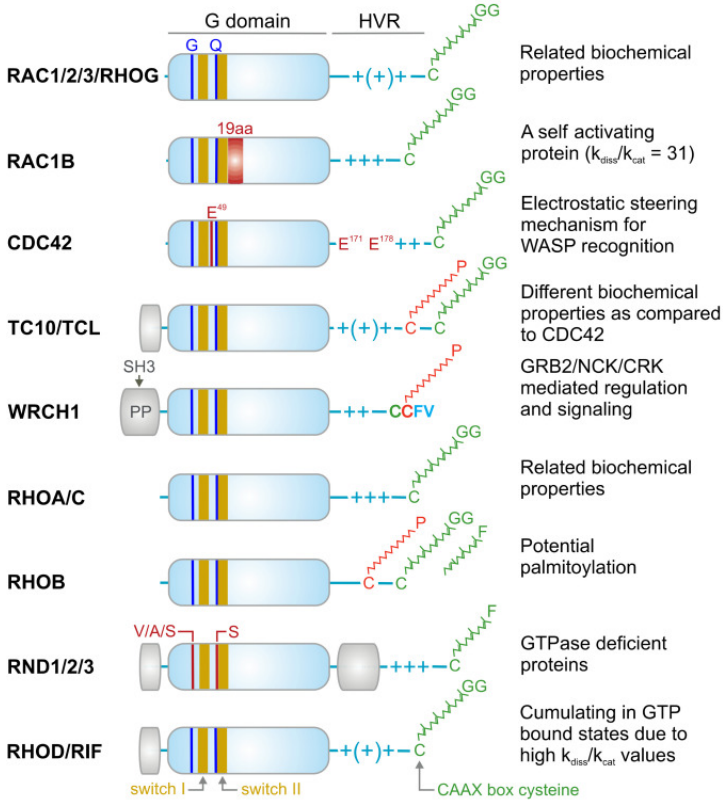

***Figure 2**. Domains, signature motifs, and post-translational modification of RHO GTPases. RHO GTPases contain a highly conserved G domain, which is responsible for GDP/GTP binding and GTP hydrolysis. […] The N-terminal insertion in WRCH1 contains proline-rich motifs responsible for interaction with SH3-contining adaptor proteins. […] The C-terminal hypervariable region (HVR) contains the terminal CAAX box, which undergoes posttranslational modification by geranylgeranylation (GG) or alternatively farnesylation (F) in the case of RHOB and the RND proteins at the conserved cysteine (green). From: Mosaddeghzadeh N. et al., 2021*

Rho GTPases control and regulate morphogenesis, movement, polarity, gene expression, cell division and cytoskeleton reorganization through interactions with downstream effector proteins. Among these proteins, RhoA, Rac1, and Cdc42 are the main ones involved in altering the cytoskeleton.

In particular, RhoA interacts with its downstream effector Rho kinase (ROCK) to promote the formation of stress fibers and focal adhesions, which are bundles of actin filaments and sites where cells adhere to the extracellular matrix. Then, Rac1 induces the formation of lamellipodia, which are thin and sheet-like protrusions at the leading edge of cell migration. They facilitate cell movement due to their abundant branched actin networks, which play a crucial role in cell motility.

Cdc42 is the last factor; it causes polymerization of actin filaments and the extension of finger-like filopodia at the cell surface, in addition to the development of cell polarity and asymmetric cell division.

In the specific context of melanogenesis, Rho GTPases play a significant influence in cell signaling and control of melanocyte differentiation.

It has been demonstrated that they are involved in the regulation of melanin synthesis by cAMP-PKA (cyclic AMP-protein kinase A) pathway, either acting on actin filaments and stress fibers, or on tyrosinase protein expression.

cAMP-PKA pathway can regulate the expression and activity of important transcription factors, including MITF.

Anyway, the impact of Rho GTPases on melanogenesis is not only mediated by their effects on the above-mentioned signaling pathways, but they show influences also in the shape and structure of melanocytes.

Particularly RhoA, if activated, can induce actomyosin contractility, leading to changes in melanocyte morphology and cell shape.

These alterations can affect the positioning and subcellular distribution of melanin-containing melanosomes within the cell, and they ensure that the appropriate structures are in place for the melanin transfer process to take place.

In fact, proper cytoskeletal organization must guarantee that melanocytes have the appropriate extensions from the perinuclear region to the dendrites, in order to transfer melanin to neighboring keratinocytes.

From this evidence it is clear that Rho GTPases are the elements that closely link cytoskeleton with melanogenesis, as they influence melanocyte shape, intracellular transport of melanosomes, and the activity of critical transcription factors involved in melanin synthesis.

Under physiological conditions these pathways are perfectly controlled; nonetheless, when Rho GTPase is altered upon exposure to external stimuli (such as UV radiation, that is the greatest risk factor to which people are exposed), it can lead to pigmentary abnormalities or aberrant melanin distribution as observed in certain skin conditions and diseases.

Mutations on Rho GTPase have also been detected in melanoma, where melanocytes can acquire invasive growth since these specific enzymatic defects promote immune system evasion, amoeboid migration of cells and more aggressive phenotype.
In general, melanoma development begins with the proliferation of functionally normal melanocytes generating a dysplastic nevus with structural and cytological abnormalities at the basal stratum.
Proliferation is triggered by somatic mutations, mainly in the mitogen-activated protein kinase (MAPK) pathway.
At this point of the process, tumor cells enter the radial growth phase, the early stage of development where the cells present a horizontal spread in the epidermis leading to an inflammatory response also in the underlying dermis.
Subsequently the radial growth phase transitions into the vertical growth phase when the cells acquire the ability to breach the basement membrane and become invasive (Kumar V. et al., 2021).
Melanoma is usually more common on sun-exposed areas and notably among fair-skinned people, who are more likely to develop tumor than people with darker skin.
Sunlight is obviously not the exclusive risk factor, though, as melanomas can also develop in people with dark skin and in parts of the body that are not exposed to the sun.
According to estimates, 10-15% of melanomas occur in families, and many of these individuals also have dysplastic nevi.
Researchers identified mutations in numerous oncosuppressor-encoding genes in melanomas.
The first example relates to mutations in the retinoblastoma (Rb) oncosuppressor proteins, which have been discovered in both familial and sporadic melanomas.
Increases in the expression levels of RAS and PI-3K/AKT signal-transducing oncoproteins, are the second most frequent molecular changes in sporadic melanomas.
All these cellular alterations produce the same overall result: a rise in melanocyte proliferation that is unrestricted by oncogene-induced cellular senescence.
In the context of carcinogenesis, particularly, during the evolution of melanoma, cytoskeleton plays a significant role.
First and foremost, it is essential for the invasion of tumor cells and their transit through blood and lymphatic arteries, resulting in the invasion of neighbouring tissues and metastasis to other parts of the body. This process can be influenced by cytoskeletal proteins such as actin and microtubules, which form dynamic structures like lamellipodia and filopodia, allowing cells to move and become invasive.

The cytoskeleton has an effect on drug resistance as well: recent studies have suggested that the cytoskeleton may have a role in melanoma cells' resistance to pharmacological therapies (Muhammad Bilal Ahmed et al., 2021).
It has been demonstrated that antitumoral-resistant cells have aberrant actin cytoskeleton dynamics, influenced by the balanced expression of Rho GTPases which ultimately results in drug sensitivity in cancer cells.
Finally, the cytoskeleton can affect cell differentiation, the natural process by which a cell becomes specialized in form and function, and this can cause cells to lose their properties as healthy melanocytes and develop into an aggressive, less differentiated phenotype.
The cytoskeleton's role in melanoma progression is still unclear: however, the current evidence suggests that this protein network has a role in many aspects of tumor cell activity such as motility and invasion, changes in shape, cell division and angiogenesis.
Understanding how the cytoskeleton affects melanoma could lead to the discovery of new therapeutic targets and the development of more effective therapies for this type of skin cancer.

THE ARTICLE: HYPOTHESIS AND AIMS

The article analyzed in this thesis entitled *"PRP4 promotes skin cancer by inhibiting production of melanin, blocking influx of extracellular calcium and remodeling cell actin cytoskeleton",* is an experimental research paper published in the *International journal of Molecular Science* on 29 June 2021.
The study was performed and written by the authors Muhammad Bilal Ahmed, Salman UI Islam, while the experiments were designed by Young Sup Lee.
The authors focused on proving through various research that the pre-mRNA processing factor 4B (PRP4) plays a significant role in the regulation of melanogenesis in a murine melanoma cell line (B16F10).
Cells were transfected with a plasmid encoding for PRP4 and then incubated with alpha-melanocyte-stimulating hormone (α-MSH).
Researchers discovered that the (AC)-(cAMP)-tyrosinase signaling pathway was downregulated, leading to an inhibition of melanin production.
It has been demonstrated that PRP4 overexpression resulted in the downregulation of adenylyl cyclase (AC) expression, which could no longer convert ATP into cAMP, and modulated the actin cytoskeleton by suppressing the expression of Ras homolog family member A (RhoA).
These observations, in conjunction with the downregulation of extracellular calcium-sensing receptor (CaSR), which affects cellular proliferation,

differentiation, signaling pathways and homeostais during tumor growth, demonstrate the development of skin cancer in B16F10 cells by overexpression of PRP4.

PRP4 is the main topic of this article, as researchers aim to identify the pathways altered by its overexpression, particularly in the context of skin cancer.

PRP4 stands for pre-mRNA processing factor 4B, and it was initially discovered in *Schizosaccharomyces pombe* while seeking for crucial genes involved in pre-mRNA splicing.

Prp4 possesses a kinase domain that has homology with cyclin-dependent kinases and mitogen-activated protein kinases in addition to the RS domain that is located in the N-terminus and presents subdomains typically rich in Arg/Ser or Lys/His.

The following figure shows a representation of the Prp4 kinase's structure (Figure 3).

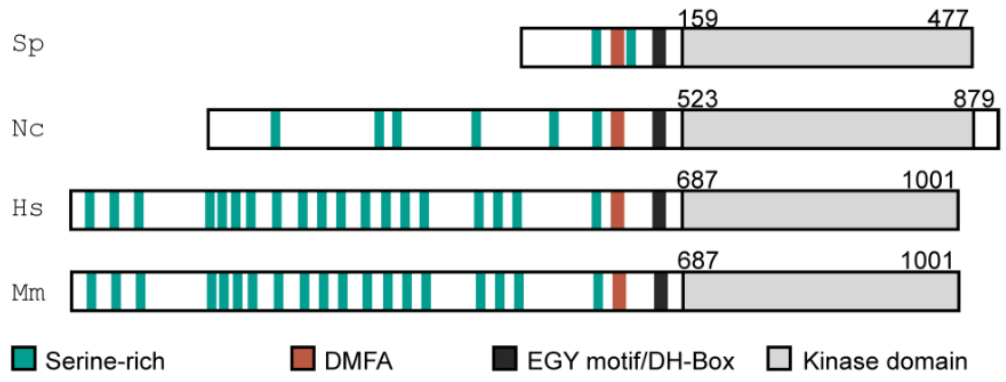

Figure 3. *Schematic representation of the Prp4 homologs from S. pombe, N. crassa, M. musculus and H. sapiens. Numbers on top indicate positions of the kinase domain and total length of the proteins, respectively. From: Lützelberger M. et al., 2012*

This protein plays a crucial role in pre-mRNA splicing, e.g., the critical stage in gene expression that involves the removal of non-coding introns from the precursor mRNA (pre-mRNA) and the joining of the remaining exons to create the mature mRNA.

Prp4 is defined as "*the first kinase being involved in the regulation of pre-mRNA splicing in fungi and mammals"* (Lützelberger M. et al., 2012). It was demonstrated to regulate alternative splicing via phosphorylation and activate spliceosomes, as well as interact with various target structures, including transcription factors, chromatin remodeling factors, spindle checkpoint proteins, and cytoskeleton proteins, often altering their function. For example, PRP4 mutations or dysregulation can result in pre-mRNA buildup, abnormal splicing events or can impair the G1/S transition during the cell cycle due to the loss of catalytic activity and the accumulation of pre-mRNA.

PRP4 can also determine drug resistance in cancer cell lines as it affects the assembly of the actin cytoskeleton by regulating the activity of Rho family proteins and initiates the epithelial-mesenchymal transition (EMT, the essential process through which cells lose cell junctions and reorganize the expression of their genes, taking on the characteristics of metastatic cancer cells) via phosphorylation of p53 protein and loss of E-cadherin that mediates cell adhesion.
In this study scientists investigated the role of PRP4 in controlling cell morphology and migration as well as in promoting cutaneous melanoma through the suppression of melanin formation: they decided to focus on PRP4 since previous analyses had already highlighted its role in carcinogenesis.
Therefore, based on this research, it was investigated whether PRP4 affects the regulation of melanin production in order to understand the potential role of this factor in the development of skin cancer, and if so, by what mechanism.

MATERIALS AND METHODS

Materials and methods are treated directly from: Ahmed M.B. et al., 2021.

1. Chemicals and Reagents

Cell line applied was B16F10, a murine melanoma cell line from a C57BL/6J mouse. As a medium for cell culture, they used Dulbecco's modified Eagle's medium (DMEM) with an increased concentration of vitamins and amino acids compared to Basal Medium Eagle (BME); and fetal bovine serum (FBS) rich in growth-promoting factors.
Penicillin/streptomycin were added to the culture medium to prevent bacterial contamination.
PRP4 cDNA clone, ARRB1 cDNA clone and siRNA-TRPC1 were used for plasmid transfections together with Xfect – a transfection reagent useful for increasing transfection efficiency of plasmid DNA into mammalian cells – and Lipofectamine RNAiMAX Transfection Reagent – a proprietary RNAi-specific cationic lipid formulation designed specifically for the delivery of siRNA and miRNA into all cell type – .
Cal-520 AM assay kit, 1 mM Ca^{2+} supplemented buffer, cAMP assay kit, Bradford protein assay kit were used to carry out the analysis, along with electrophoresis reagents and antibodies for Western Blot [PRP4, β-actin, ARRB1, Tyrosinase, CaSR, adenylyl cyclase] and for cell signaling technology [calmodulin, RhoA, anti-mouse IgG-HRP-linked, and anti-rabbit IgG-HRP-linked].
ECL Prime detection reagent – characterized by stable signal emission – was employed for chemiluminescent Western blotting detection on nitrocellulose membrane.

Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole) and Actin-stain™ 488 Phalloidin were used for nuclei and cytoskeleton staining.
SuperScript III Reverse Transcriptase is a genetically engineered MMLV enzyme used for the generation of first strand cDNA in PCR procedure.
α-MSH was purchased from Sigma Aldrich.
Researchers used all chemicals and reagents following the manufacturer's instructions.

2. Cell Culture and Transfections
B16F10 cells were cultured in DMEM enriched with 10% FBS and 2% penicillin–streptomycin and were cultured at 37 °C in a humidified environment, 5% CO_2.

3. Plasmid Transfection and Gene Knock Down by siRNA
PRP4 overexpression was accomplished using the Xfect Transfection Reagent for transient transfection, whereas RNAiMAX was used in accordance with the manufacturer's instructions to transfect siRNA interference targeting PRP4, ARRB1, and TRPC1. Likewise, gene overexpression and downregulation were confirmed especially by Western blot using a specific antibody to quantify the proteins encoded by the gene in question, agarose gel electrophoresis and SDS-PAGE.

4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
For the RT-PCR procedure, scientists used the SuperScript III First-strand synthesis kit for cDNA synthesis from total RNA. Next, RNase H was used to eliminate any RNA residues from the synthesized cDNA.
PCR was performed using the following primers:

TARGET	PRIMER FORWARD	PRIMER REVERSE
PRP4	5' -AGGGATCGAAGCTGGAAATA-3'	5' -TGACCTCTGAGTCATCT-GTGG-3'
CASR	5'-CTGAAGAGAAGGCAACGCTATG-3'	5'- GGGCAACAAAACTCAAGGT -3'
ARRB1	5'-CGGATGCTTTCTCGTCTC-3'	5'-ACCCATCATCATTGTGCC-3'
CALMODULIN	5'-CCCTCTGTCCACACACAAAG-3'	5'-TTGATGGTGTGCTCAAGTCC-3'
TRPC1	5'-CCTCCTTGTTCTGTTTTCCTTC-3	5'-GTGTCATTGCTTTGCTGTTC-3'
TYR	5'- GCTGCAGGAGCCTTCTTTCTC-3'	5'-AAGACGCTGCACTGCTGGTCT-3'
GAPDH	5'-AGGGCTGCTTTTAACTCTGGT-3'	5'-CCCCACTTGATTTTGGAGGGA-3'

PCR cycles were run as follows: a first cycle was run at 98 °C for 3 min, then 30-35 cycles were run at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. The amplified PCR products were examined through 2% agarose gel electrophoresis and EcoDye Nucleic Acid Staining Solution.

They used Wise Capture I-1000 software in order to capture images.

5. Relative qRT-PCR Analysis

Relative quantification of RT-qPCR is a process used to find changes in expression of the target genes compared to a reference one.

Total RNA was extracted using the Trizol reagent, cDNA was produced using the SuperScript® VILO™ cDNA synthesis kit, and the analysis was carried out using a NanoQ spectrophotometer.

The final volume (20 µL) of the master mix contained SYBR Green I, template DNA and primers (the same used on the Eco TM Real-Time PCR system). The qPCR cycle consisted of denaturation at 95 °C for 15 min, annealing and extension at 60 °C, repeated for 40 cycles.

6. F-Actin Staining

Alexa Fluor 488 phalloidin was used to stain and visualize actin filaments. After the growing medium was removed, the cells were fixed with paraformaldehyde, permeabilized with 0.2% Triton X-100 and then the Alexa Fluor 488 phalloidin solution diluted with albumin was added to the cells. Finally, ZEISS LSM 800 confocal microscope was used to observe the cytoskeleton. Cells were always washed 2 to 6 times with PBS between each passage of the procedure.

7. Cal-520 AM Assay

Cal-520 AM Assay is a rugged assay tool based on fluorescence that allows the detection of intracellular calcium mobilization.

DMEM with Ca^{2+} and fetal bovine serum was used to cultivate B16F10 cells.

After being transfected with siRNAs, cells were incubated in full serum DMEM media. Following that, cells were loaded with Cal-520 AM dye inHHBS buffer with Pluronic F-127, an indicator that increases in fluorescence in response to Ca^{2+} binding. After washing the cells with HHBS Solution, they finally obtained images of calcium-dependent fluorescence by using a FITC- ZEISS LSM 800 confocal microscope.

8. cAMP Assay

PRP4 was transfected into B16F10 cells which were subsequentially collected and lysed. After that, a cAMP assay kit based on sensitive colorimetric competitive ELISA technique, was used to measure the intracellular levels of cAMP. The assay was repeated three times.

9. Animal Study Protocol

The experimental protocols of Kyungpook National University were applied to 6 male BALB/c-n mice. Two mice were housed in each cage, with a stable temperature and under 12-hour light/dark cycles. Researchers employed an insulin syringe to implant parental/PRP4-transfected B16F106 cells into 6-week-old mice. They monitored tumor volumes every week through a Vernier caliper and using the formula: V = 4/3πW2L (short diameter2 × long diameter [mm3]). 30–45 days after cells' post implantation they excised tumor with scissors.

10. Measurement of Melanin Content

After cell seeding and incubation, serum-free optimum media was used to replace the original one.

Following PRP4 transfection and α-MSH incubation, cells were washed in PBS and then dissolved in 10% DMSO and NaOH. Melanin absorbance at 405 nm was then quantified using a spectrophotometric microplate reader.

11. Western Blot

Western blot is the assay used by the experimenters to confirm the results obtained at the end of each experiment, to quantify the proteins examined each time.

In particular, the evidence from Western blot analysis of adenylate cyclase, RhoA, and TYR in cells transfected with PRP4 and siRNA-PRP4 was the most relevant, showing that overexpression of PRP4 respectively modified cell morphology and inhibited melanin production.

In each sample, the solubilized proteins subjected to Western blot, came from both PRP4 and siRNA-PRP4-transfected cultured cells.

The analyses were conducted according to the following protocol:

First, the sample was prepared by extraction, centrifugation, resuspension in cell lysis buffer and quantification with Bio-Rad Protein Assay. Protein denaturation took place in SDS sample buffer containing Tris-HCl SDS, glycerol, and β-mercaptoethanol. Samples were separated on a 10-12% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane blocked with albumin.

Researchers used Clarity ECL Western Blotting Substrate to reveal chemiluminescent signals resulting from antibody-antigen binding.

12. Statistical Analysis

Every experiment and sample were set up and run-in triplicate. Data are reported as mean ± standard deviation (SD). Differences between groups were evaluated using a Student t-test, and p-values < 0.05 were considered statistically significant.

RESULTS

First, researchers looked at PRP4's function *in vivo* by injecting six BALB/c-n mice with B16F10 cells. B16F10 is a murine cell line isolated from skin tissue of a mouse with melanoma, and it is usually employed in oncology research, particularly in the study of melanoma biology, metastasis, and response to drug treatment.

The mice in question belong to strain C57BL/6J, the most common inbred strain (obtained from cross-breeding of consanguineous individuals) used in laboratory.

The PRP4-transiently transfected B16F10 cells and the parental B16F10 cells were injected into every animal either on the left or right side.

Up to 50 days after implantation, tumor diameters were routinely assessed (using the formula described in Materials and Methods) at 3- or 4-day intervals. PRP4-transfected B16F10 cells exhibited accelerated tumor growth in comparison to parental ones (Figure 4A). This finding supported the theory that PRP4 encourages the growth of skin cancer (Figure 4B).

The effect of PRP4 on the melanin content of B16F10 cells was also investigated. To this aim, the researchers took in consideration the AC-cAMP-MITF-tyrosinase signaling pathway: they treated B16F10 cells with α-MSH to induce MITF activation by increased cAMP levels, and then they transfected them with PRP4.

They noticed that PRP4 overexpression blocked the α-MSH-induced melanin production in B16F10 cells, along with the downregulation of mRNA and protein levels of tyrosinase (Figures 4C, 4E).

To confirm to this evidence, there is the result that siRNA-PRP4 transfected cells did not influence the production of melanin and TYR levels (Figures 4B, 4D, 4F), because of the function of short interfering RNA that silenced PRP4 genes.

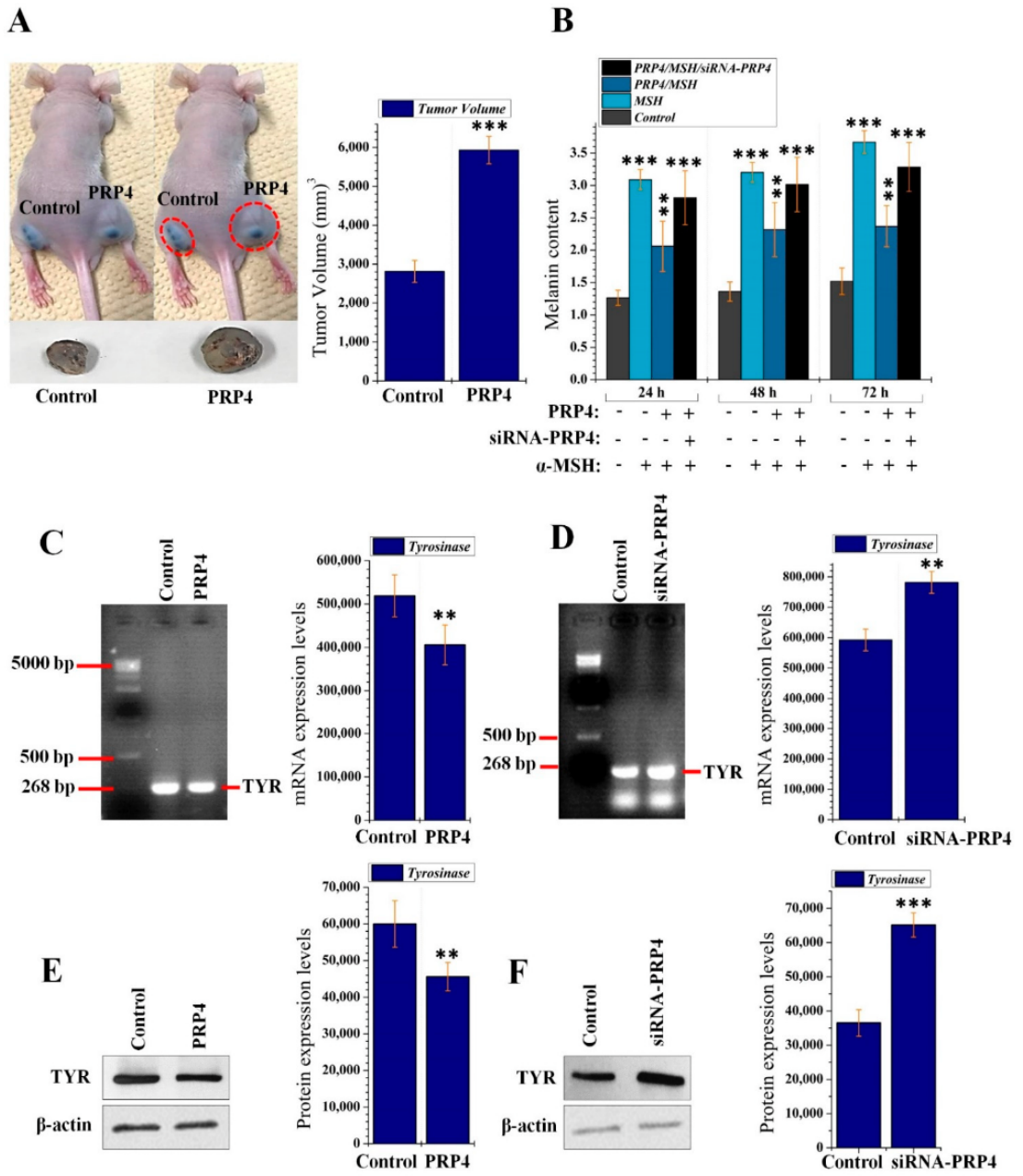

Figure 4: *PRP4 inhibits the production of melanin in B16F10 cells, which subsequently leads to the promotion of skin cancer. (**A**) Nude mice (n = 6) received parental and PRP4-transfected B16F10 cells into the left and right flank, respectively, by subcutaneous injection. Data were collected from 3 independent experiments. *** p < 0.001. For clear visibility of the tumors in vivo, the same mouse was photographed twice; the tumors are encircled in the latter set of photographs. (**B**) Melanin production in PRP4 transfected B16F10 melanoma cells. The cells were transfected with 5 µg PRP4 for 24, 48, and 72 h, and α-MSH was used as a positive control. Data were collected from 3 independent experiments. ** p < 0.01, *** p < 0.001. (**C,D**) Triplicate total RNA samples from control and the PRP4 and siRNA-PRP4 transfected cells were analyzed by RT-PCR. ** p < 0.01. (**E,F**) Solubilized proteins (20 µg) from PRP4 and siRNA-PRP4 transfected cultured cells were subjected to Western blot analysis using the corresponding antibodies for detection of respective TYR protein. The blot was simultaneously incubated with an antiproteins antibody to show that each electrophoretic lane was loaded with the same amount of protein. ß-actin was used as a standard. Data are presented as mean ± standard deviation (SD) of at least three independent experiments (n = 3). ** p < 0.01, *** p < 0.001. Treatment or transfection related abbreviations: PRP4 = PRP4 cDNA plasmid transfection; siRNA-PRP4 = transfection of siRNA-PRP4; α-MSH = 100 nm treatment of α-MSH; TYR = tyrosine. From: Ahmed, M.B. et al., 2021*

The authors also studied how PRP4 changes the morphology of B16F10 cells.
First of all, they found that PRP4 overexpression or silencing through siRNA, caused alterations in AC and consequently in cAMP synthesis.
Overexpression of PRP4 resulted in lowered expression of AC, thereby blocking cAMP production; PRP4 knockdown, reestablished the expression of AC together with cAMP (Figures 5A,5B).
Since previous studies have demonstrated that cAMP regulates RhoA and causes morphological changes in the cells, the authors verified whether PRP4 could influence also RhoA activity and signaling. RhoA activity appeared downregulated by PRP4 expression, while RhoA expression levels seemed to be almost unaffected with the use of siRNA-PRP4 (Figure 5C).
Given this evidence, scientists finally analyzed the actin cytoskeleton of the cells via Alexa Fluor™ 488 Phalloidin and observed clear changes in the formation of actin stress fibers under the expression of PRP4 rather than siRNA-PRP4.
With this evidence, the authors stated that PRP4 controlled the actin cytoskeleton and morphology of B16F10 cells, causing redistribution of actin filaments and changing cell morphology from an agglomerated, flattened shape to a round shape without harming the cells' nucleus (Figure 5D). These results imply that PRP4 modifies the AC-cAMP-RhoA pathway to change the morphology of B16F10.

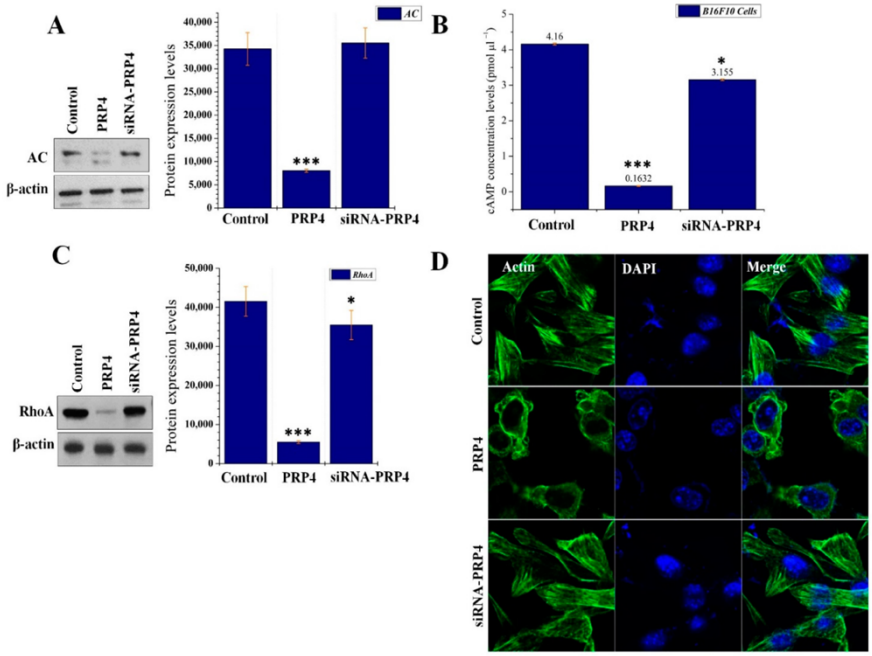

***Figure 5**. PRP4 alters the morphology of B16F10 cells. (**A,C**) Solubilized proteins (20 µg) from PRP4 and siRNA-PRP4-transfected cultured cells were subjected to Western blot analysis using the corresponding antibodies for detection of respective adenylyl cyclase and RhoA proteins. The blot was simultaneously incubated with an anti-proteins antibody to show that each electrophoretic lane was loaded with the same amount of protein. β-actin was used as a standard. * $p < 0.05$, *** $p < 0.001$. (**B**) Corresponding cells were observed for cAMP levels using a cAMP assay kit, and plated cells were measured in a microplate reader (OD 450 nm). * $p < 0.05$, *** $p < 0.001$. (**D**) PRP4 and siRNA-PRP4 transfected B16F10 cells were stained with Alexa Fluor™ 488 Phalloidin to observe the structure of actin stress fiber formation. Nuclei were counterstained with DAPI, and cells were imaged using a confocal microscope (Carl Zeiss). From: Ahmed, M.B. et al., 2021*

In addition, researchers conducted investigations on Ca^{2+} which controls cAMP synthesis both directly and indirectly (via calmodulin), leading to melanin production through the AC-cAMP-MITF-tyrosine signaling pathway. First, an experiment was performed to show that PRP4 appropriately promotes CaSR desensitization – the condition whereby a receptor no longer responds to its ligand – by upregulating ARRB1: scientists focused on CaSR because it is the crucial protein for the regulation of calcium levels in the body, whose concentration influences cell signaling, motility and cell division. They examined the protein level of ARRB1 by means of Western blot analysis upon PRP4 overexpression on B16F10 cells. In fact, β-arrestin plays a key role in receptor desensitization by binding to the phosphorylated receptor and inducing its degradation or clathrin-mediated internalization. In the presence of PRP4 overexpression, ARRB1 appeared increased both at the mRNA and protein level (Figures 6A,6B), and it led to a downregulation of CaSR in response to desensitization, as shown in (Figures 6C,6D).

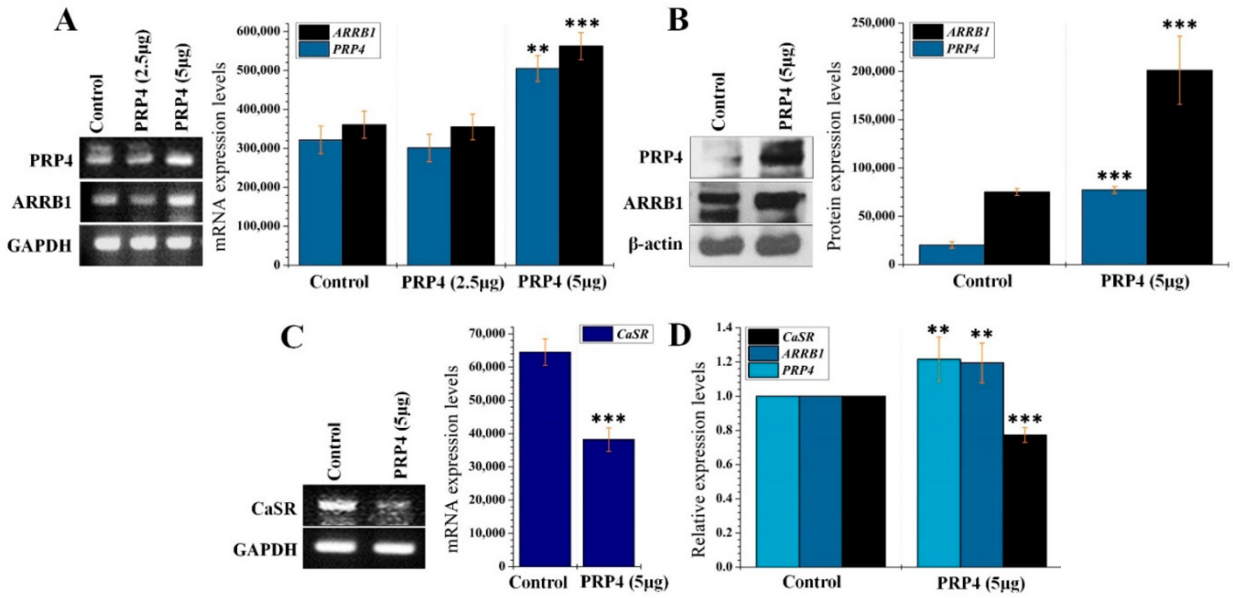

Figure 6. *PRP4 regulates ARRB1–CaSR pathway. (**A**) Triplicate total RNA samples from control and the PRP4-transfected were analyzed by RT-PCR. *** p < 0.001. (**B**) Solubilized proteins (20 μg) from PRP4 transfected growing cells were subjected to Western blot analysis using the corresponding antibodies for detection of respective proteins. The blot was simultaneously incubated with an antiproteins antibody to show that each electrophoretic lane was loaded with the same amount of protein. β-actin was used as a standard. *** p < 0.001. (**C**) Triplicate total RNA samples from control and the PRP4-transfected were analyzed for CaSR by RT-PCR. *** p < 0.001. (**D**) Gene expressions were quantified by using quantitative Real-Time PCR (qPCR). ** p < 0.01, *** p < 0.001.From: Ahmed, M.B. et al., 2021*

Scientists used PRP4- siRNA-mediated knockdown to prove that ARRB1 expression is decreased in the absence of PRP4. To further confirm these findings, they used qPCR and Western blot analysis through antibodies targeting proteins of interest (Arrb1, Prp4, CaSR, Calmodulin).

Researchers found that PRP4 and ARRB1 block the influx of Ca^{2+} through desensitization of the Calcium Sensing Receptor (CaSR). Overexpressing PRP4 and ARRB1 reduced intracellular Ca^{2+} levels, while silencing them with siRNAs did not affect Ca^{2+} concentration (Figure 7A). Analyzing mRNA level of calmodulin – a calcium binding protein that mediates the Ca^{2+} regulation of several physiological processes –, it seemed to be downregulated by PRP4, confirming the link between PRP4, Ca^{2+} concentration, and calmodulin levels (Figures 7B,7C).

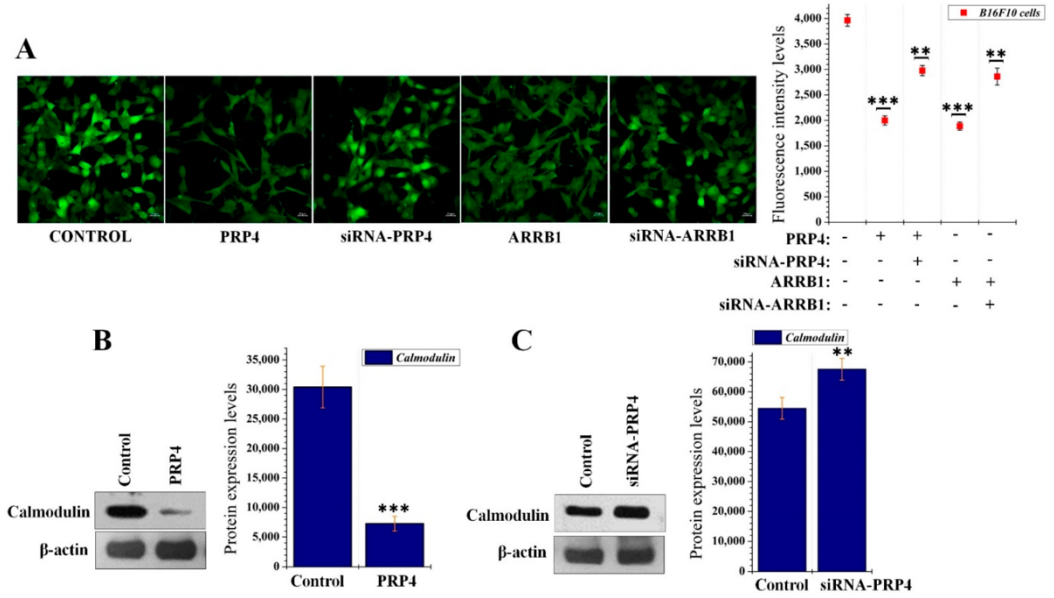

***Figure 7**. PRP4 reduces the influx of Ca2+ and decreases the expression of calmodulin. (**A**) PRP4, quadrant 4. ARRB1, or siRNA-ARRB1-transfected B16F10 cells were stained with Cal-520 AM to observe the intracellular calcium ions levels through fluorescence intensity levels. **p < 0.01, *** p < 0.001. (**B**,**C**) PRP4 and siRNA-PRP4-transfected B16F10 cells were analyzed with Western blotting to observe calmodulin (CALM1) expression. Data are presented as mean ± standard deviation (SD) of at least three independent experiments. ** p < 0.01, *** p < 0.001. Treatment or transfection related abbreviations: ARRB1= transfection of ARRB1 plasmid. From: Ahmed, M.B. et al., 2021*

Additionally, they tried to identify which ion channels contribute to CaSR-induced Ca^{2+} influx in B16F10 cells. By examining the gene expression of different ion channels and conducting two separate experiments with si-RNA-PRP4 and siRNA-TRPC1 transfected cells, they found that PRP4-regulated CaSR-induced Ca^{2+} influx occurs via the TRPC1 channel.

DISCUSSION

The overall objective of the research is to demonstrate the initial hypothesis of scientists who claim that PRP4 promotes skin cancer in B16F10 cells and are interested in investigating the mechanisms by which this occurs.

The current study builds upon earlier investigation conducted by the authors, as documented in the 2017 publication *"PRPF overexpression induces drug resistance through actin cytoskeleton rearrangement and epithelial-mesenchymal*

transition". The previous work demonstrated that PRP4 contributes to drug resistance in tumor cells, leading to alterations in the cytoskeleton and the promotion of EMT (a key step in tumor growth) in cancer cell lines. With this in mind, Muhammad Bilal Ahmed and his colleagues decided to investigate the impact of PRP4 on melanoma development in particular, focusing on the production of melanin in response to PRP4 as a key element in skin cancer protection.

Another important aspect of oncological research is the influence of calcium, which is closely linked to the morphological and migratory properties of cells.

Studies in recent years have shown that Ca^{2+} is deregulated in cancer, and Min Pi et al. demonstrated that arrestin, which binds the phosphorylated receptor and blocks its signaling, intervenes in the desensitization of the calcium-sensing receptor.

On the basis of this evidence, the authors also decided to focus on the link between the presence of arrestin and CaSR desensitization, which leads to the downregulation of AC, cAMP and, consequently, the regulation of RhoA, a protein that causes morphological changes in cells (Oishi A., et al., 2012).

To carry out the experiments, they used B16F10 cells, a cell line with a spindle-shaped and epithelial-like cell morphology that has been used extensively in previous studies of melanogenesis, depigmentation, tumor metastasis and various measurements of cytotoxicity in the skin model.

This was an appropriate choice as the B16F10 cell line consists of cells derived from the skin tissue of a mouse with melanoma, making it an ideal study model for the skin cancer research that the authors wanted to conduct.

Furthermore, this cell line is a suitable transfection host, which renders it perfect for use in skin cancer research and specifically for the studies described above, where transfection is widely applied.

The authors then used a wide range of biological experiments.

Firstly, they utilized a subcutaneous tumor xenograft model by injecting B16F10 cells into BALB/c-n mice for the *in vivo* studies. This model is applicable for cancer research as the immunodeficient mice allow for the tumors to take root subcutaneously.

In each experiment, the expression of PRP4 was increased by transient transfection of overexpression plasmid into B16F10 cells. Subsequently, the protein levels, (and often mRNA levels as well), were measured by Western blot and compared with the control to determine whether there was a true under expression of the protein in question.

After applying this process to each sample, the expression level of PRP4 was inhibited by transfecting cells with PRP4-siRNA, using the gene silencing method,

and confirming PRP4-mediated downregulation of the protein under investigation by Western blot.
In fact, siRNAs are able to silence genes by binding to a single target mRNA sequence.
Each transfection with PRP4 was performed at different concentrations, to determine the concentration at which the procedure is most effective.
Regarding PRP4-influenced calcium channel gene expression, RT-PCR was employed to assess the mRNA levels of TRPC1, demonstrating that calcium influx into the cell is facilitated by the opening of TRPC1 channel.
The hypothesis derived from the initial results was subsequently supported via assessment of the fluorescence intensity with Cal 520 AM (a fluorogenic calcium-sensitive dye) of intracellular calcium ion levels in cells transfected with siRNA-TRPC1. Another method of fundamental importance in observing changes in the cytoskeleton was Actin-stain™ 488 Phalloidin, a high-affinity filamentous actin probe conjugated with the green fluorescent dye Alexa Fluor 488.

After conducting the experiments and analyzing the data, the authors reached the conclusion that the tumor in mice injected with cells transfected with PRP4, exhibited a greater increase in size. This was confirmed by measuring the volume of the tumor mass. However, they did not examine whether the neoplasia had spread to the lymph nodes or whether it had metastasized, although this comparison of parameters would have been essential to corroborate the finding that tumor growth is more prominent in the presence of PRP4.
They also found that PRP4 inhibits melanin production by downregulating the AC-cAMP-MITF-tyrosinase pathway, a result supported by the evidence presented.
From the experimental results, it was concluded that PRP4 regulates the ARRB1-CaSR pathway, resulting in ARRB1 overexpression. This overexpression, in turn, may lead to CaSR desensitization. It is important to note, however, that the authors based the conclusion of desensitization on previous studies and did not conduct experiments to confirm whether the receptor was actually desensitized. For instance, they did not analyze calcium activity, receptor signaling or receptor internalization in their experiments. They could have used β-arrestin recruitment-based assays, which are often used as reporters of GPCR activation, or G protein-coupled receptor interaction assays, which look at the interaction between the membrane receptor and its ligand.
Levels of calmodulin – an intermediary protein that senses calcium levels and transmits signals to calcium-sensitive enzymes, ion channels and other proteins – seemed to be downregulated by PRP4 expression, which enhanced ARRB1-mediated CaSR desensitization, but the authors only analyzed mRNA levels by Western blot, not protein levels.

The last considerations they drew on Ca^{2+} concern the channel through which ion influx occurs, which was demonstrated to be TRPC1.
Finally, there is a lack of control even in the experiments where the authors claimed that PRP4 modifies cell morphology by regulating the AC-cAMP-RhoA pathway: in fact, they only observed changes in the actin cytoskeleton by means of Phalloidin, as well as alterations in the expression levels of RhoA and AC, and cAMP concentration. However, no quantitative results regarding these changes were provided. To arrive at this conclusion, an analysis of cell morphology would have been necessary, measuring volume, area and length, and observing changes in shape between the parental cells and cells that were transfected with PRP4.
Authors also conducted Western blot analyses of the protein expression levels of AC and RhoA (after PRP4 overexpression) since they have been shown to correlate with the remodeling of the cell structure. Nevertheless, an error was made in selecting the control: β-actin was chosen as the standard because it is usually suitable for this purpose, being a housekeeping protein that is unaffected by most cellular treatments.
However, in this case β-actin has been identified as one of the primary proteins responsible for altering the cytoskeleton, and the experimental procedures specifically involved manipulating it. Therefore, using it as a control could result in fallacious outcomes.
For this purpose, it would have been more appropriate to use a protein not involved in the pathway under examination, but still highly conserved such as GADPH.

The authors have displayed great diligence in investigating the role of PRP4 and the various mechanisms involved in its overexpression in cancer development, but the methodological limitations mentioned above weaken the evidence and make it only partially convincing. Further investigation and improved controls would be necessary to confirm the results.
The other limitation of the study is the fact that researchers did not directly propose the practical implications of the findings.
To maximize the potential benefits for future research, it would be advantageous to explore the research's applicative possibilities extensively. This would help to ensure that the obtained results have a concrete impact on the field of science and medicine, instead of just being a theoretical finding.
Actually, presented discoveries could make a significant contribution to both the research field and clinical/therapeutic practice: in fact, there is an urgent need to find new therapeutic approaches for drug-resistant metastatic melanomas, which currently exhibit resistance to conventional chemotherapy and radiotherapy.

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