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Differential Gene Expression and Tumorigenicity Analysis of Cultured Melanocyte Comparing Melanoma

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Original Article

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This study aimed to identify the optimal growth media for culturing human skin melanocytes for clinical applications and to assess their tumorigenic potential both *in vitro* and *in vivo*. Various growth media were tested to determine the most effective and safest for melanocyte culture, avoiding harmful growth factors such as TPA and colorant toxins. The study evaluated changes in RAF and *NRAS* gene expression through real-time PCR and gene sequencing of BRAF V600E and *NRAS* in exons 1 and 2, comparing these with melanoma. Melanocytes were subcutaneously injected into BALB/c nude mice to assess tumorigenic risk. Results indicated that a mixture of MGM-M2 supplemented with melanocyte growth factors provided the best outcomes in terms of cell proliferation and melanocyte count. Gene expression analysis revealed that HRAS and BRAF expressions in melanocytes at passage 6 showed less than 2-fold increases, whereas these genes were up-regulated by more than 3 and 8 folds, respectively, in melanoma cell lines. *NRAS* expression in melanocytes at passage 6 increased by 5-fold but remained lower than in melanoma cell lines. Gene sequencing of BRAF V600E and *NRAS* in exons 1 and 2 showed no mutations, and melanocytes injected into BALB/c nude mice exhibited no tumor formation risk. Furthermore, gene sequencing of *BRAF* and *NRAS* in the injected melanocytes 16 weeks' post-transplantation revealed no mutations. These findings suggest that while standard growth media protocols may elevate specific proto-oncogene expressions, they do not induce tumorigenic mutations in melanocytes, both *in vitro* and *in vivo*.

Keywords: Cultured melanocyte, Vitiligo, cell transplantation, tumorigenicity

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Introduction

Over the past two decades, melanocyte transplantation has emerged as a promising approach for treating vitiligo patches that are resistant to conventional therapies (1, 2). Both cultured and non-cultured melanocytes have been used for this purpose, but the transplantation of cultured melanocytes has become the preferred method, especially for extensively depigmented patches (3, 4, 5, 6). In a pioneering study, Lerner et al. first introduced the transplantation of autologous cultured melanocytes for vitiligo in 1987 (6, 7, 8). Since then, numerous clinical trials have demonstrated the safety and efficacy of autologous cultured melanocyte transplantation, despite ongoing safety concerns (8). It is generally believed that melanomas arise from the excessive proliferation of melanocyte precursors (9, 10). During in vitro melanocyte proliferation, growth factors and mitogens can accelerate cell cycle progression, potentially leading to DNA damage. This damage may trigger melanoma development either under cell culture conditions or after transplantation (9, 11). Melanoma, a malignant tumor originating from melanocytes, the pigment-producing cells in the skin is closely associated with genetic mutations, particularly in the *BRAF* and *NRAS* genes. These genes are crucial for cellular growth and differentiation. *BRAF*, a serine/threonine kinase, is frequently mutated in melanoma, with the *BRAF* V600E mutation being especially common. This mutation results in the constitutive activation of the MAPK/ERK signaling pathway, leading to uncontrolled cell proliferation and survival. *BRAF* mutations are present in approximately 40-60% of melanoma cases, making them a critical target for therapeutic intervention. *NRAS* encodes a GTPase that is involved in similar signaling pathways as *BRAF*. Mutations in *NRAS* activate the MAPK pathway, contributing to tumorigenesis. *NRAS* mutations are found in about 15-20% of melanoma cases and are associated with more aggressive forms of melanoma and poorer clinical outcomes. Understanding the roles of these genes is essential for developing targeted therapies and improving patient outcomes. This study investigates the effects of different growth media on the expression of *BRAF* and *NRAS* in cultured human melanocytes and assesses their potential tumorigenic risks. Lerner incubated the cells in a medium containing 12-O-tetradecanoyl-phorbol-13-acetate (TPA). While Czajkowski reported the safety of TPA in growth media for culturing purposes, other studies have indicated that TPA is a known tumor promoter. Its presence could potentially lead to malignant transformations of melanocytes under cell culture conditions (1, 7, 8, 9, 12). Melanocytes rely on the RAS/RAF/MEK/ERK signaling pathways to regulate critical processes such as proliferation, migration, differentiation, and apoptosis (6, 7, 13).

Mutations in the serine/threonine kinase *BRAF*, particularly at codon 600, have been reported in 25-80% of malignant melanomas (2, 3) and are considered early events in the development of melanocytic lesions (14). Additionally, mutations in *NRAS* at codons 12, 13, and 61 are observed in 15-30% of malignant melanomas, especially in tumor sites exposed to ultraviolet radiation. These mutations likely lead to the activation of MEK and subsequently ERK, contributing to oncogenesis (1, 9, 15). While several studies have assessed the in vitro tumorigenicity of various growth media, with or without the presence of TPA (6, 7), no research has yet evaluated the behavior of transplanted cells following transplantation. This study aimed to identify the optimal growth medium based on proliferation tests and functional activities for clinical use, and to assess the tumorigenic potential of cultured melanocytes under both in vitro and in vivo conditions. To achieve this, human skin melanocytes were isolated and cultured with various growth media. The study

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analyzed the cellular, molecular, and genetic changes occurring during the in vitro culture process. Subsequently, the cells were subcutaneously injected into BALB/c nude mice to evaluate the risk of tumorigenesis following transplantation.

Materials and methods

Isolation and Culture of Human Melanocytes in Various Growth Media

For this study, eight patients out of fourteen participants, all under 35 years of age with stable, generalized vitiligo resistant to conventional treatments, were selected for non-cultured melanocyte transplantation. Informed consent was obtained from all participants at the Royan Stem Cell Institute, Tehran, Iran, and the study was approved by the Institutional Review Board and Ethics Committee of the Institute.

A 2 × 2 cm partial thickness skin specimen was harvested from the buttock area of each patient. A 0.5 × 0.5 cm section of this specimen was cut and transferred to the laboratory for melanocyte isolation and culturing. The remaining skin sample was transferred to a clean room for further processing and transplantation. The skin specimens were washed, cut, and incubated for 15-18 hours at 4°C in a 1.2 U/ml Dispase II solution (Invitrogen, Karlsruhe, Germany). After this incubation, the epidermis was separated from the dermis. The epidermal sheets were then placed in 0.25% trypsin/EDTA (Invitrogen) at 37°C for 30 minutes. Following enzyme inhibition and cell washing (Promo Cell, Heidelberg, Germany), the cell suspension was transferred to MGM-M2 medium (Promo Cell). Four protocols were used for melanocyte culture:

Protocol A: Cell suspension cultured in MGM-M2 medium.

Protocol B: Cells cultured in MGM-M2 supplemented with mixed growth factors (Promo Cell, C-39420).

Protocol C: MGM-M2 medium supplemented with mixed growth factors and Melanocyte Growth Medium supplement, which included $CaCl_2$ (1.0 ml), BPE (2.0 ml), rhFGF-B (1.0 ml), rh-Insulin (1.0 ml), Hydrocortisone (0.5 ml), PMA (0.5 ml), GA-1000 (0.5 ml), and 2.5 ml of 4% FBS (Life Technologies, Grand Island, NY, USA).

Protocol D: MGM-M2 supplemented with mixed growth factors (Promo Cell) and 4% FBS (Life Technologies).

After 3 days of culture, Geneticin (G-418 Solution, Sigma-Aldrich, St. Louis, MO) was added for an additional 3 days to remove fibroblasts and keratinocytes from all four groups. The media were changed every three days, and melanocytes were passaged when they reached 80-90% confluency. The culture medium was removed, and 1 ml of 0.125% trypsin/EDTA (Invitrogen) was added to each plate. The plates were incubated at 37°C for 2 minutes, then trypsin was neutralized, and the solution was centrifuged. The separated cells were distributed among the four media and cultured for at least six passages. Previous studies have indicated that passages 3 to 5 are adequate for clinical applications. Therefore, cells were selected from early passages (p1 – p6) to assess genetic stability and proto-oncogene expression in vitro and in vivo. Additionally, A375, NA8, and D10 cell lines were used as positive controls, while fibroblast cells served as negative controls. The cell lines were thawed from cryopreservation and propagated in DMEM-F12 medium (Life Technologies), supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, and 1% Penicillin-Streptomycin, as recommended by the ATCC.

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A-375 (A375) is a melanoma cell line, whose data is completely similar with that of ATCC:

(ATCC ® CRL-1619™) D10.G4.1 (ATCC® TIB-224™) and NA8-MEL (CVCL_S599) are also melanoma cell lines. All of the mentioned cell lines were received from a colleague, courtesy of Basel University. The fibroblasts were propagated in culture with DMEM-F12 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. Inclusion criteria included adults aged 20 years and older with vitiligo for at least one year and stable disease conditions without recent exacerbation or treatment with phototherapy or corticosteroids. Exclusion criteria comprised the presence of specific diseases, other skin conditions, and pregnancy. All procedures adhered to the guidelines set forth by the Institutional Review Board and Ethics Committee of Royan Stem Cell Institute, which also approved the study. This research was supported by Grant Number 8700099 from Royan Stem Cell Institute, Tehran, Iran.

Ethical approval for this study was obtained from the Institutional Review Board and Ethics Committee of Royan Stem Cell Institute under the ethical code IR.TUMS.VCR.REC.1400.2628. All patients provided written informed consent at Royan Stem Cell Institute, Tehran, Iran.

Immunofluorescence Staining of Melanocyte Markers

Immunofluorescence staining was performed to confirm the passage-6 melanocytes identity. Cultured melanocytes were fixed by 4% freshly buffered paraformaldehyde, washed with PBS and incubated with 10% goat serum, followed by incubation with primary antibody mouse anti-Melan-A (Sigma-Aldrich, M6570, St. Louis, MO, USA), S100 (Sigma-Aldrich, S2644 St. Louis, MO, USA), HMB-45 (ab878, Abcam, Cambridge, UK), and tyrosinase II (ab74073, Abcam, Cambridge, UK) at 4°C, overnight. The cells were washed with PBS and incubated with fluorescein is thiocyanate (FITC)-conjugated anti-mouse (F9006) for Melan-A, and HMB-45 and goat anti-rabbit for S100 and tyrosinase II for 60 minutes at room temperature. A375 and fibroblast cells were also stained for Melan-A and S100. Nuclei were counter-stained with five µg/ml of 4', 6-diamidino-2-phenylindole (DAPI) and Propiodin Iodine (PI), and analyzed by a fluorescent microscopy (Nikon, Tokyo, Japan).

Determination of Population Doubling Time (PDT) of Human Melanocytes in Culture

The population doubling time (PDT) in each protocol was assessed by seeding melanocytes from the primary culture and passages 1-6 of 10^4 cells per well in 12-well culture plates. PDT was calculated according to the following formula:

PDT = culture time (CT)/PDN Where PDN = log N/N0 × 3.31, N = Cell count at the end of the calculation period, and N_0 = Cell count at the culture initiation.

Karyotyping

Melanocytes of passages 4, 5, and 6 were examined for genetic stability. When the cells became 60% confluent, they were treated with Colcemid® (10 µg/ml) and incubated at 37°C for 45 minutes. For the hypotonic treatment, 13 ml of 0.056% KCl in distilled water was added and the cells were incubated at 37°C for 13 minutes. Next, the cells were fixed using a methanol: acetic acid (3:1) solution. To obtain G-bands, the slides were incubated at 60°C, overnight. The staining procedure was carried out using Giemsa/PBS$^+$ (1:10). Imaging and karyotyping were performed using CytoVision® software. Thus, 15 metaphase plates were counted, analyzed, and the representative metaphase cells were karyotyped. Karyotype analysis was also performed for A375, NA8, and D10 melanoma cells.

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RNA Extraction and Real-Time PCR

Total RNA was extracted from cultured melanocytes of passages one, three, and six, as well as melanoma cell lines like: A375 and D10 (passage 10), using a column-based RNeasy Mini Kit (Qiagen, Valencia, CA). Random hexamer primers were used for cDNA synthesis, performed with a Prime Script RT Reagent Kit (Perfect Real Time, Takara Biotechnology, Japan). The PCR reaction was performed using a Power SYBR® Green PCR Master Mix (Applied Bio systems, Foster City, CA) at the default setting on an ABI Biosystems StepOnePlus Real-Time PCR system with the following temperature profiles: denaturing at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Relative expression levels were determined based on collected data, such as cycle threshold (Ct) numbers. Real-time PCR assay was replicated 3 times for each sample, and the relative gene expression was calculated as 2-ΔCt, where ΔCt = Ct Target -Ct Reference. The GAPDH gene served as an internal control. Table 1 shows sequences of the primers used.

Table 1. The sequences of the primers used.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
NRAS	AGGGAGCAGATTAAGCGA	ACACCCTGTCTGGTCTTGG
HRAS	GAAGCAGGTGGTCATTGAT	GGCAAACACACACAGGAA
BRAF	CAAATTCTCACCAGTCCGT	ACCACGAAATCCTTGGTCT
GAPDH	CTCATTTCCTGGTATGACAACGA	CTTCCTCTTGTGCTCTTGCT

Outlined below are the primer sequences that we have used through the years in our STANDARD reactions. You may want or need to have these custom primers synthesized. They may differ a little from proprietary primers. That does not mean that primers purchased from conventional vendors will not work - we have simply found that this set of primers works well when sequencing with a wide array of vectors. If you wish to explore the custom primers option, we have some primer design tools listed on our Primer Guidelines page. Oligonucleotides are usually shipped in dry form. The dried DNA pellet becomes dislodged from the bottom of the tube during shipping and it can easily fly out of the tube when first opened, particularly as electrostatic attraction is present. For this reason:

DNA Extraction and Sequencing

To check the hotspot mutation of the *BRAF* gene at exon 15 and the *NRAS* gene at exons 1 and 2, DNA was extracted from passage-6 melanocytes, with passage-10 melanoma cell lines A375, and D10 as positive controls, using a QIAamp DNA Blood Mini Kit (Qiagen® 51306, Hilden, Germany), in accordance with the manufacturer's instructions. Primer pairs that targeted the human *BRAF* and *NRAS* genes were designed, and PCR was used to amplify the DNA region. The PCR products were submitted to conventional Sanger sequencing, to check for mutations in the cultured melanocytes and melanoma cell lines. Finally, samples were submitted to GenBank (BankIt) with accession numbers KY769663 and KY769668. Table 2 shows the samples and GenBank accession number list, and Table 3 portrays the sequences of the primers. Analysis and alignment of the data was performed by ChromasPro 2, CLC Sequence Viewer 6, and Gene Runner 5 software packages.

Presence and Pigmentation Assessment of Cultured Melanocytes in Albino Mice

As the next step, the presence and pigmentation of the cultured melanocytes was assessed by injecting the cultured cells in epidermal area of albino mice. The animals were obtained from Pasteur Laboratory, Tehran, Iran, and all animal experiments were performed after obtaining the approval from the Institutional

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Animal Care and Use Committee at Royan Stem Cell Institute. 2.0 × 10^6 cells/mL of passage-6 cultured melanocytes were subcutaneously injected in the epidermal area of 6 albino mice, and the pigmentation in mice was observed weekly for up to 3 months. Furthermore, skin biopsies were taken from pigmented sites. After tissue processing, H&E (Hematoxylin and Eosin) staining was carried out to evaluate morphological changes of the cells after transplantation. To confirm that the cells were observed in the epidermis after transplantation, immunohistochemistry was performed on paraffin sections using antibodies against HMB45, Melan-A, and S100.

Table 2. Samples with Number List.

No.	Samples	Cell type	Aliases	GenBank accession #
1	MA	Melanocyte-A	Royan-MA	KY769663
2	MB	Melanocyte-B	Royan-MB	KY769664
3	MC	Melanocyte-C	Royan-MC	KY769665
4	MD	Melanocyte-D	Royan-MD	KY769666
5	A375	Melanoma	Royan-A375	KY769667
6	D10	Melanoma	Royan-D10	KY769668

A: These cultured melanocyte cells were validated in the Royan Laboratory Gene Bank. The Department of Molecular Biology at the Royan Institute checked all the samples, confirming their characteristics and real names.

Table 3.GenBank Accession Number List.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
BRAF-exon 15	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA
NRAS-exon 1	CAGGTTCTTGCTGGTGTGAAA	CTACCACTGGGCCTCACCTCTATGG
NRAS-exon 2	GTTATAGATGGTGAAACCTG	ATACACAGAGGAAGCCTTCG
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

DNA was extracted from passage-6 melanocytes and passage-10 melanoma cell lines A375 and D10 to check for BRAF exon 15 and NRAS exons 1 and 2 mutations. Using a QIAamp DNA Blood Mini Kit, PCR amplification and Sanger sequencing were performed. The samples were submitted to GenBank with accession numbers KY769663 and KY769668.

Safety Assessment of Cultured Melanocytes in BALB/c Nude Mice

The tumorigenicity of the cultured melanocytes was assessed by injecting the cultured cells obtained from eight patients into BALB/c nude mice, obtained from Pasteur Laboratory, Tehran, Iran, and all animal experiments were performed after obtaining the approval from the Institutional Animal Care and Use Committee at Royan Stem Cell Institute. 2.0 × 10^6 cells/ml of passages 5 and 6 cultured melanocytes were subcutaneously into 48 nude mice between their scapulae. Fibroblasts (2.0 ×10^6 cells/ml) and normal saline were separately injected S.C. (subcutaneously) in the back of four nude mice used as control negatives. A375, D10, and NA8 (n = 6) were also subcutaneously injected as positive controls, and tumor formation was monitored weekly for at least 16 weeks. After sixteen weeks, all the animals were put down, and the tissue from the injection sites of three mice in the melanocyte-injected group and one mouse form the positive control was harvested, extracting the DNA. PCR analysis was then performed to amplify the DNA region. PCR products were submitted to conventional Sanger sequencing to check for the *BRAF* gene mutation at codon 600 (V600E) in exon 15 in tissues that were obtained from the injected mice.

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Statistical Analysis

All data were presented as mean ± standard deviation (SD) and were analyzed by ANOVA and PostHoc Bonferroni, to assess differences among means. $P < 0.05$ was regarded as statistically significant. In addition, data were expressed as median and inter quartile range. Descriptive statistics was performed on all data, and the homogeneity of variance and normal distribution between all groups was calculated. Differences between groups were analyzed by unpaired t-test, Mann–Whitney U-test and Kruskal–Wallis test, based on descriptive statistics. All analyses were performed with SPSS V. 20.0 software (IBM, Armonk, NY*).* $P < 0.05$ (two-tailed) was considered significant in terms of descriptive statistics.

Results

Morphology and Immunofluorescence Staining of Confirmed Cultured Melanocytes

In all protocols, the cultured melanocyte cells from passages one to six had bi-polar or tripolar multidendritic morphologies, in contrast to melanoma cell lines, which had polygonal and epithelioid-like morphologies (Figure 1A). Immunofluorescence staining confirmed the expressions of Melan-A, S100 protein, HMB-45, and tyrosinase II in the cytoplasm of all passages one to six of cultured melanocytes, using each of the four protocols explained. The expressions of Melan-A, S100 protein, HMB-45, and tyrosinase II were confirmed by Image J software in over 90 percent of cultured melanocytes. Furthermore, A375, NA8, and D10 melanoma cell lines expressed Melan-A and S100 surface markers, yet the cells were negative for HMB-45 and Tyrosinase II markers. All the fibroblasts lacked melanocyte surface markers (Figure 1B). PDT Assessments Showed that Protocols C and D to Be the Most Appropriate.

The mean PDT of Passage-One of cultured melanocytes using Protocols A, B, C, and D were 34.8, 33.0, 24.7, and 22.9 hours, respectively as depicted in (Figure 1C). These values were persistent from passages 1 to 6, while at passage six, PDT for Protocols C and D were 23.6 and 22.0 hours, being significantly lower than those of Protocols A and B (39.1 and 36.2 hours, respectively) ($P < 0.001$). Proliferation of cultured melanocytes from primary culture to passage 3, following utilization of all protocols, showed non-significant consistent growth ranging from 10,000 to 20,000 cells/ml. However, melanocytes cultured cells using Protocols C and D exhibited a sudden increase in number, especially in passages 4 and 5 (i.e., 40,000 to 60,000 cells/ml, respectively), in contrast to that achieved through Protocols A and B, where the number of cells remained unchanged until passage six of cultured melanocytes (Figure 1D).

By adopting standardized procedures, maintaining rigorous quality control, and fostering collaborative validation, researchers can mitigate the impact of variations in protocol implementation and subjective interpretations, leading to more consistent and reliable scientific results.

Karyotype Analysis Displayed No Structural Alterations.

Karyotypes of passages-5 and -6 melanocytes obtained from 8 patients displayed no structural alterations in the 15 metaphase plate spreads. All chromosomes were normal and none showed instability. In contrast, the positive control melanoma cell lines NA8, A375, and D10 displayed numerous structural alterations, including deletions, duplications, and rearrangements, as well as aneuploidy and polyploidy The NA8 melanoma cell line depicted a modal distribution of chromosome numbers 59-61 (hypo triploid), A375 cells a modal distribution of chromosome number 62 (hypo triploid), and D10 cells a bimodal distribution of

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chromosomes 46–48 near triploid (Table 4). Here, we assessed tumorigenic activity by evaluating cell proliferation rates, survival rates, and metastatic potential across passages P0 to P6. Karyotype analysis of 50 metaphases per passage per patient revealed no structural alterations in melanocytes from P1 to P6, unlike cell lines that showed various chromosomal alterations (Figure 2A).

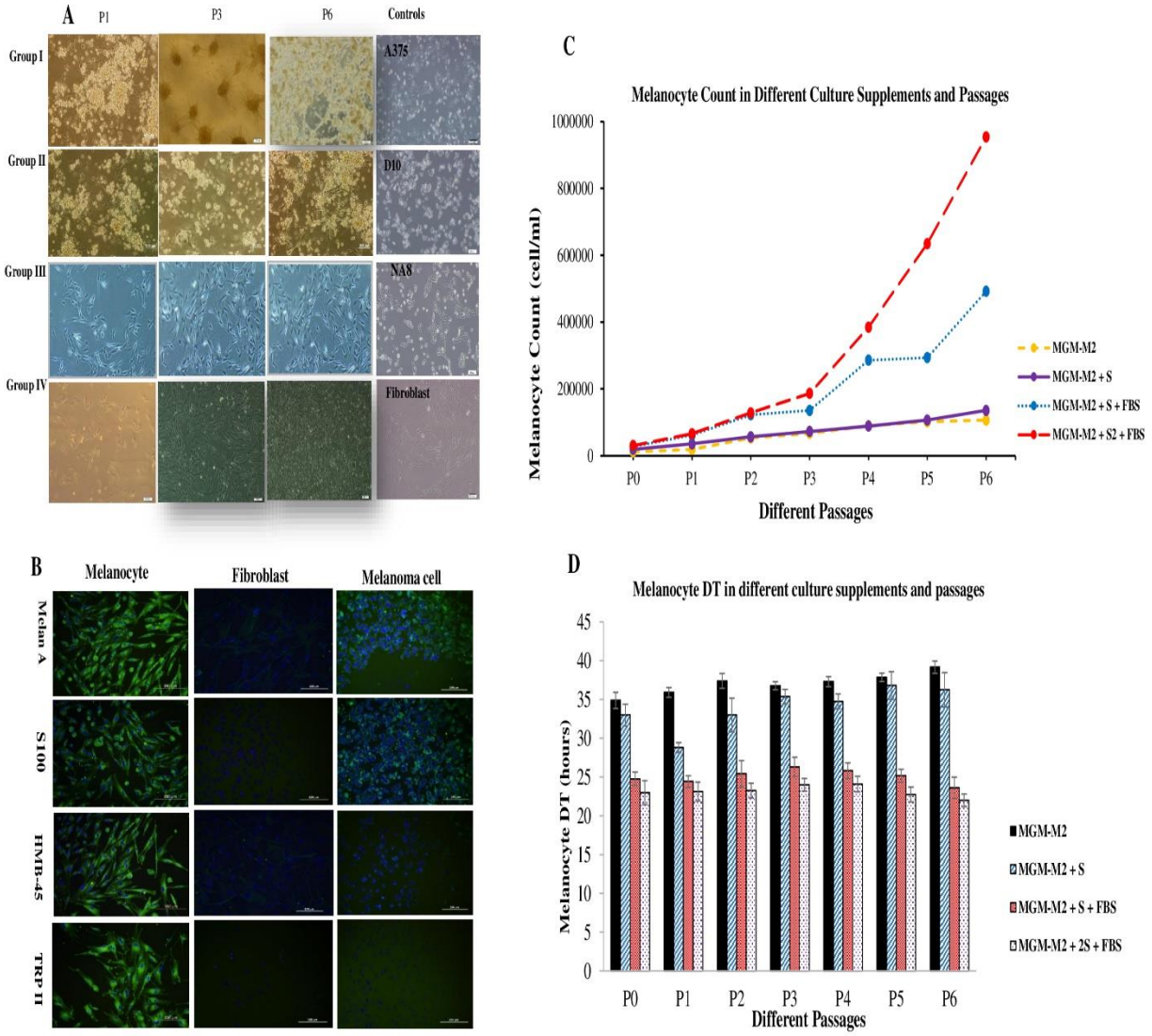

Fig. 1. Characteristics of Cultured Human Adult Melanocytes. (A) Phase contrast images of cells shows melanocyte cultured under four conditions (i.e., four groups): I) melanocyte growth medium (MGM-M2); II) MGM-M2 + supplementary mixed growth factors; III) MGM-M2 + supplementary mixed growth factors + 4% FBS; and IV) MGM-M2 + supplementary mixed growth factors were added twice growth factors + 4% FBS. The cells had bi- or tripolar multidendritic morphologies. The melanoma cell lines (A375, D10 and NA8) showed polygonal and epithelioid-like morphologies. Scale Bar: 100 µm. (B) Immunofluorescence staining showed that passage-6 melanocyte cultured in MGM-M2 + supplementary mixed growth factors + 4% FBS expressed Melan-A, S100, HMB-45, and TRPII markers. A375 cells expressed Melan-A and S100 in their cytoplasm. The cultured fibroblasts did not express any melanocytic markers. Nuclei were stained with PI (red) and DAPI (blue). Scale Bar: 100 µm. (C) Population doubling time (PDT) per hour for cultured melanocytes in four groups in primary culture and at passages 1-6. The data was expressed as mean ± SD of three replicates. *** $P < 0.001$ showed significant differences between groups 1 and 2 and group 3, *** $P < 0.001$ showed significant differences between groups 1 and 2 and group 4. (D) Cultured melanocyte counts from primary culture to passage 6 in all groups.

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Table 4. The Assessment of Melanocyte and Melanoma Chromosomal Structure.

Cell line Name	Karyotype	Chromosomal instability signs
NA8	Modal no: 59-61 (Hypotriploid)	59-61,XX,-2[4],t(2;13)(p11.2;q11)[4],-3[4],t(3;17)(p25;q25.2)[4],t(3;17)(p27;q25.2)[4], +4[4],del(4)(q33)[2],del(4)(q33)[1],dup(4)(q21q24)[4],+5[4],del(5)(p15)[4],rev(5)(p?)[4],6[4],del(7)(q31)[4],der(7)t(7;10)(q32;q23)[4],8[4],+10[4],del(10)(p13)[4],del(10)(p13)[4],add(11)(p11.2)[4],der(12)t(1;17)(q21;p11.1)[4],der(12)t(9;12)(q21;p13)[2],add(13)(p13)[3],der(13)t(5;13)(q11.2;p13)[2],-14[3], add(14)(q32)[2],t(14;14)(q10;q10)[1], -15[3],add(15)(q13)[14],der(15)t(15;21)(p13;q11.2)[4],-16[2],-18[2],add(18)(q23)[4],-19 [2],-21,-22[2],+mar, mar.
A375	Modal no: 62 (Hypotriploid)	62,XX,+1[3],+del(2)(q32)[4],+3[4],+5[4],der(6)t(1:6)(q12:q13)[4],+7[1],+8[4],+9[3], +10[2],inv(11)(q12q22)[2],+12[4],+13[3],+14[4],+15[4],+16[4], add(16)(q24) [1],+17[3],+18[3],add(19)(q13.4)[4],+20[4],-22[3],+mar[1],+X[1][cp4].
D10	Modal No: 46-48 (Near triploid)	46-48,XX,i(1)(q10)[1],+der(2) t(2:X)(q21;q3)[3],dup(5)(q32;q34)[4],der(7)(q32)[4],-9[4],del(10)(p12;3)[4],t(12;15)(q10;q10),add(13)(p13)[4],der(13)t(1;13)(p13;q12)[4],der(14)t(1;14)(p11.2;p13)[4],+5[4],del(16)(p12)[4],-17[1],+20[1],-X[4][CP4].

The table shows the positive control melanoma cell lines NA8, A375, and D10 displayed numerous structural alterations, including deletions, duplications, and rearrangements, as well as aneuploidy and polyploidy. The NA8 melanoma cell line exhibited a modal distribution of chromosome numbers ranging from 59 to 61 (hypo triploid), while A375 cells displayed a modal distribution of chromosome number 62 (hypo triploid). The D10 cells showed a bimodal distribution of chromosomes, with numbers ranging from 46 to 48, indicating near triploid. This chromosomal analysis highlights the significant genetic instability within these melanoma cell lines, contrasting sharply with the stable chromosomal profiles observed in cultured melanocytes

The limitations of the karyotyping results include potential issues with sample size, passage number, detection techniques, specificity of positive controls, and lack of functional analysis. Addressing these limitations through larger sample sizes, extended culture periods, complementary genetic analyses, and functional studies can provide a more comprehensive understanding of chromosomal stability and instability in melanocytes and melanoma cell lines.

Gene Expression and Gene Sequencing Analysis

The *BRAF*, *NRAS*, and *HRAS* gene expressions in A375 and D10 melanoma cell lines and cultured melanocytes were assessed through RT-PCR, and less than 1-time increases in *HRAS* gene in passage 3 of cultured melanocytes was observed. In passages 1 and 6, moreover, expression levels of *HRAS* gene showed

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less than double increases. Furthermore, the *BRAF* gene in cultured melanocyte of passages 1, 3, and 6 displayed double increases. Interestingly, passage 3 depicted the lowest level of the *BRAF* gene expression. In comparison, the *BRAF* gene expression of melanoma cell lines A375 and D10 showed an 8-time increase, which was of crucial importance in melanomas ($P < 0.05$). Also, the data showed a 1-time increase in the expression of the *NRAS* gene of passages 1 and 3 of cultured melanocytes. Overall, the *NRAS* gene expression was significantly increased (up to 5 times) in passage 6 in melanoma cell lines A375 and D10, while the expression of the same gene showed a 6-time increase (Figure 2B).

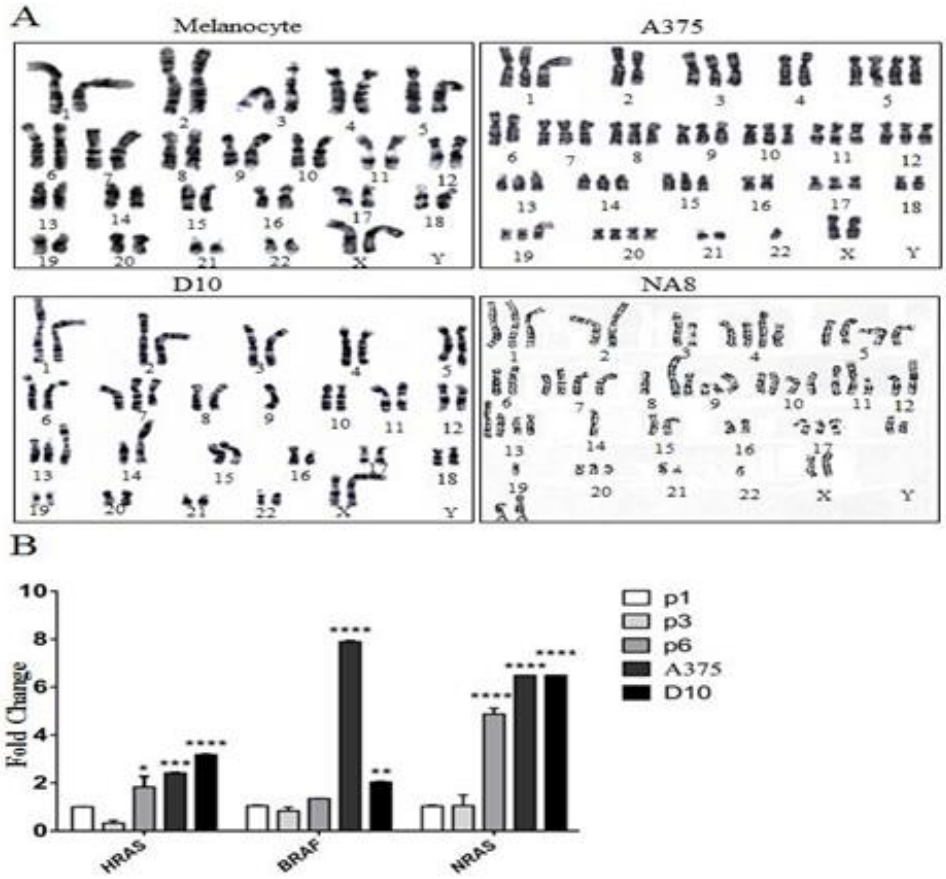

Fig. 2. Molecular and conventional karyotype evaluations of genetic stability of the expanded melanocytes and melanoma cell lines. (A) Cultured melanocytes presented a normal karyotype. Numerous structural alterations including deletions, duplications, rearrangements, aneuploidy, and polyploidy were detected in A375, D10 and NA8. (B) Quantitative real-time PCR (qRT-PCR) of cultured melanocytes at passages 1, 3, and 6 for cell lines A375 and D10. The data were expressed as mean ± SD of 3 replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ showed significant differences as compared to melanoma cells.

The probable mutations in the *BRAF* exon 15 and *NRAS* exons 1 and 2 were also investigated in cultured melanocytes (passages 5 and 6). Sequencing analysis showed that in melanoma cell lines A375 and D10, the *BRAF* gene had A to E trans version at nucleotide position (*BRAF* c. 1799 T > A), or amino acid position 600, which resulted in an amino acid substitution from Valine to Glutamic Acid at codon 600 (V600E). None of the cultured melanocyte chromatograms had mutations at V600E (Figure 3 A-B-C). DNA sequence of the *NRAS* gene in exons 1 and 2 did not depict any mutations, either in the cultured melanocytes, or in the A375

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and D10 melanoma cell lines (Figure 4 A-B-C). Genome sequencing of cultured melanocyte showed that in all six passages of melanocytes obtained from eight patients analyzed, all nucleotides were in correct positions and no mutations were observed (no wrong nucleotide).

c

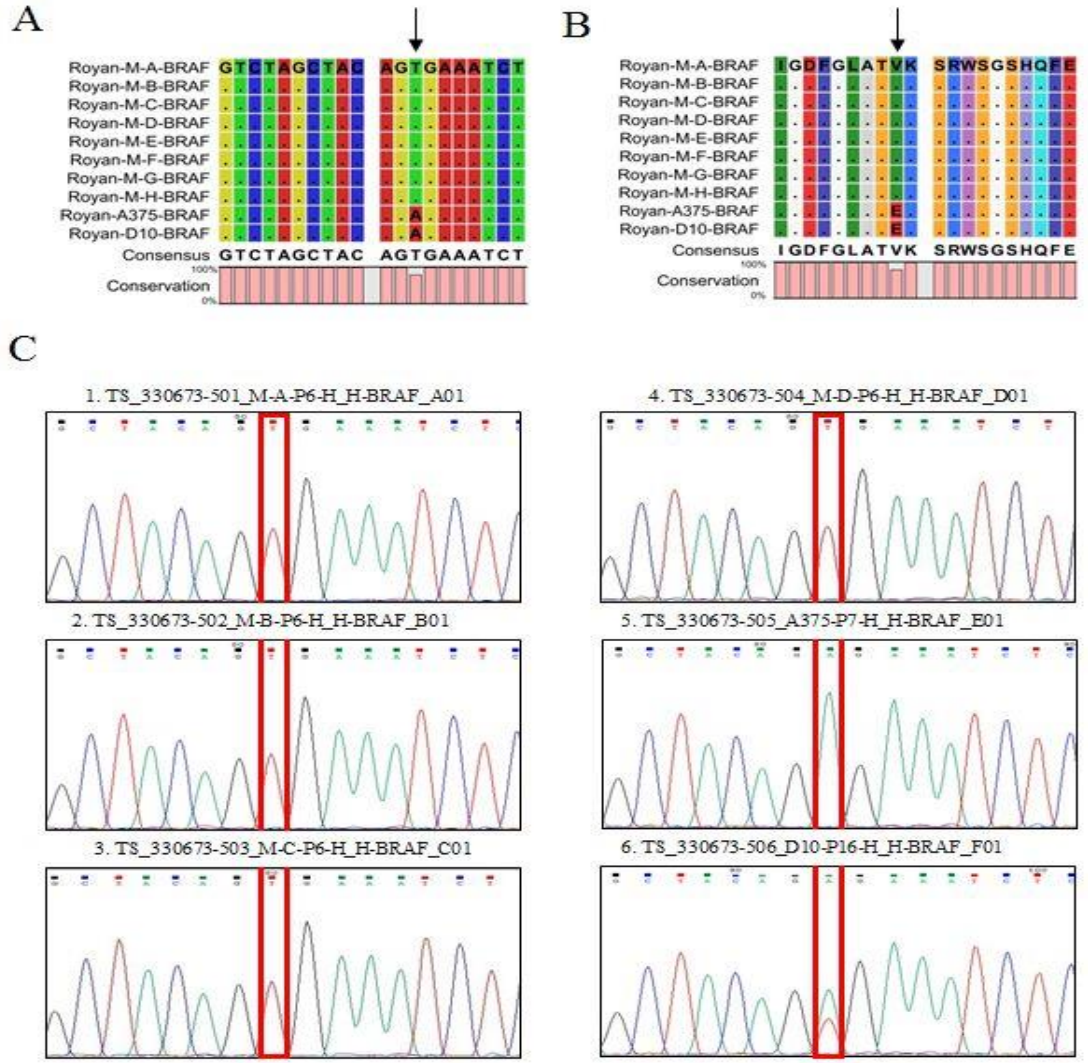

Fig. 3. Multiple-sequence alignments and DNA sequence chromatograms of the *BRAF* gene from melanoma and melanocytes samples. (A) Nucleic acid multiple-sequence alignment in the *BRAF* gene. (B) Amino acid multiple-sequence alignment in the *BRAF* gene from melanoma and melanocyte samples revealed codon 600 to be the location of the c.1799T>A mutation. (C) DNA sequence chromatograms of the *BRAF* gene from melanoma and melanocyte samples. There was a single base T > A transition in exon 15 of the *BRAF* that caused a conservative valine to glutamic acid substitution at codon 600 (V600E).

The gene expression and sequencing analysis provided insights into the differences between cultured melanocytes and melanoma cell lines regarding *BRAF* and *NRAS* gene expression and mutation status. However, limitations such as passage-specific variations, sample size constraints, and the absence of functional validation must be addressed to obtain a more comprehensive understanding of the genetic and functional implications of these findings. Expanding the analysis to include additional passages, larger sample sizes, and functional assays could enhance the interpretation and relevance of these results.

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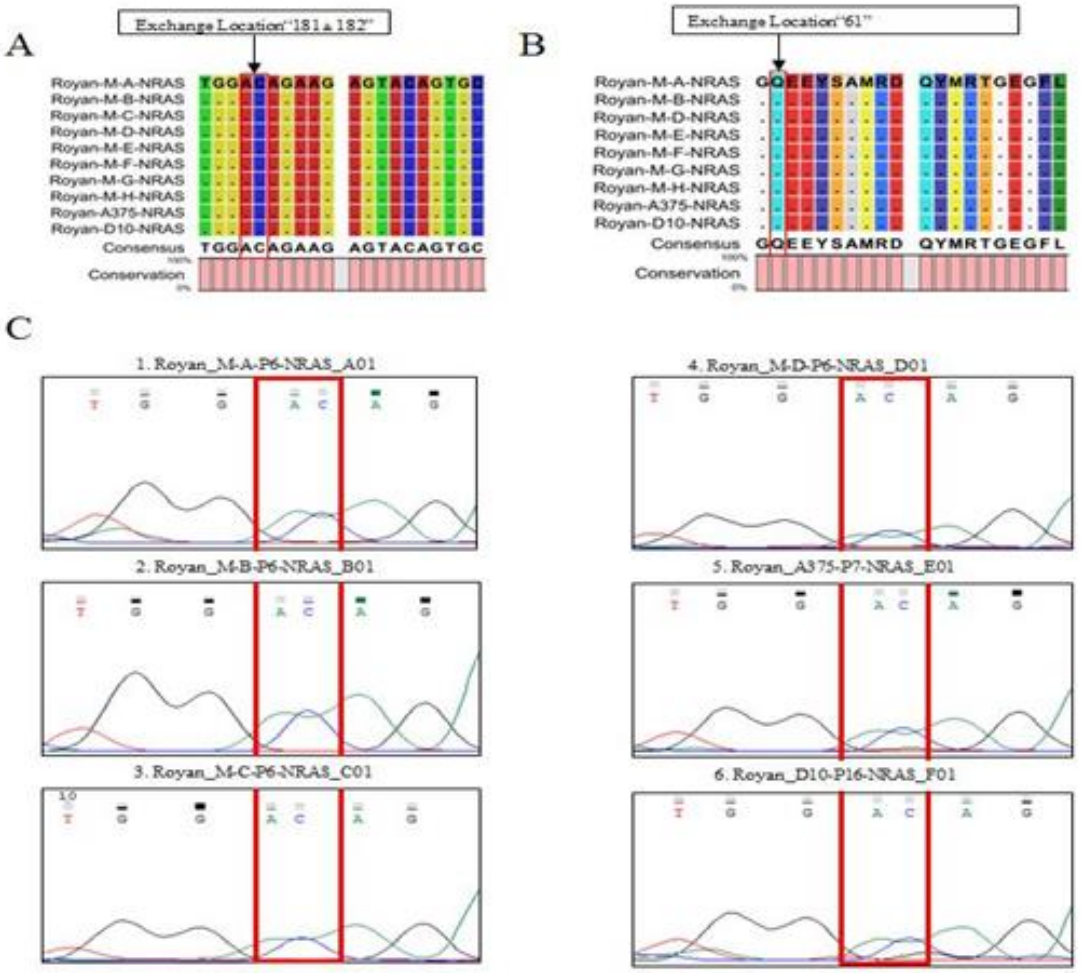

Fig. 4. Multiple-sequence alignments and DNA sequence chromatograms of the *NRAS* gene from the melanoma and melanocyte samples. (A) Nucleic acid multiple-sequence alignment of the *NRAS* gene. (B) Amino acid multiple-sequence alignment of the *NRAS* gene from melanoma and melanocyte samples, without any exchanges in locations Q61K or C181A and Q61R or A182G. (C) DNA sequence chromatograms of the *NRAS* gene from melanoma and melanocyte samples.

Presence and Pigmentation of Cultured Melanocytes in Albino Mice

Figure 5A portrays the punctuated pigmentation on injected site of the skin, indicating the functionality of cultured cells after transplantation. The histological assessment showed intra-epidermal existence of the transplanted cells. The immunohistochemistry results depicted bioactive cells after transplantation. The results showed that 96% (19 out of 20) of cells were positive for Melan-A, 92% (18 out of 20) of cells were positive for S-100, and 81% (15 out of 20) of cells were positive for HMB45 in the total 20 melanocytes counted. The immunostaining results further confirmed the presence of cells in epidermis (Figure 5B).

The results demonstrate that the cultured melanocytes transplanted into albino mice were successfully integrated into the skin and functional, as evidenced by the punctuated pigmentation, intra-epidermal localization, and high levels of specific melanocyte marker expression. These findings support the effectiveness of the transplantation procedure and the functional capability of the cultured melanocytes in contributing to skin pigmentation.

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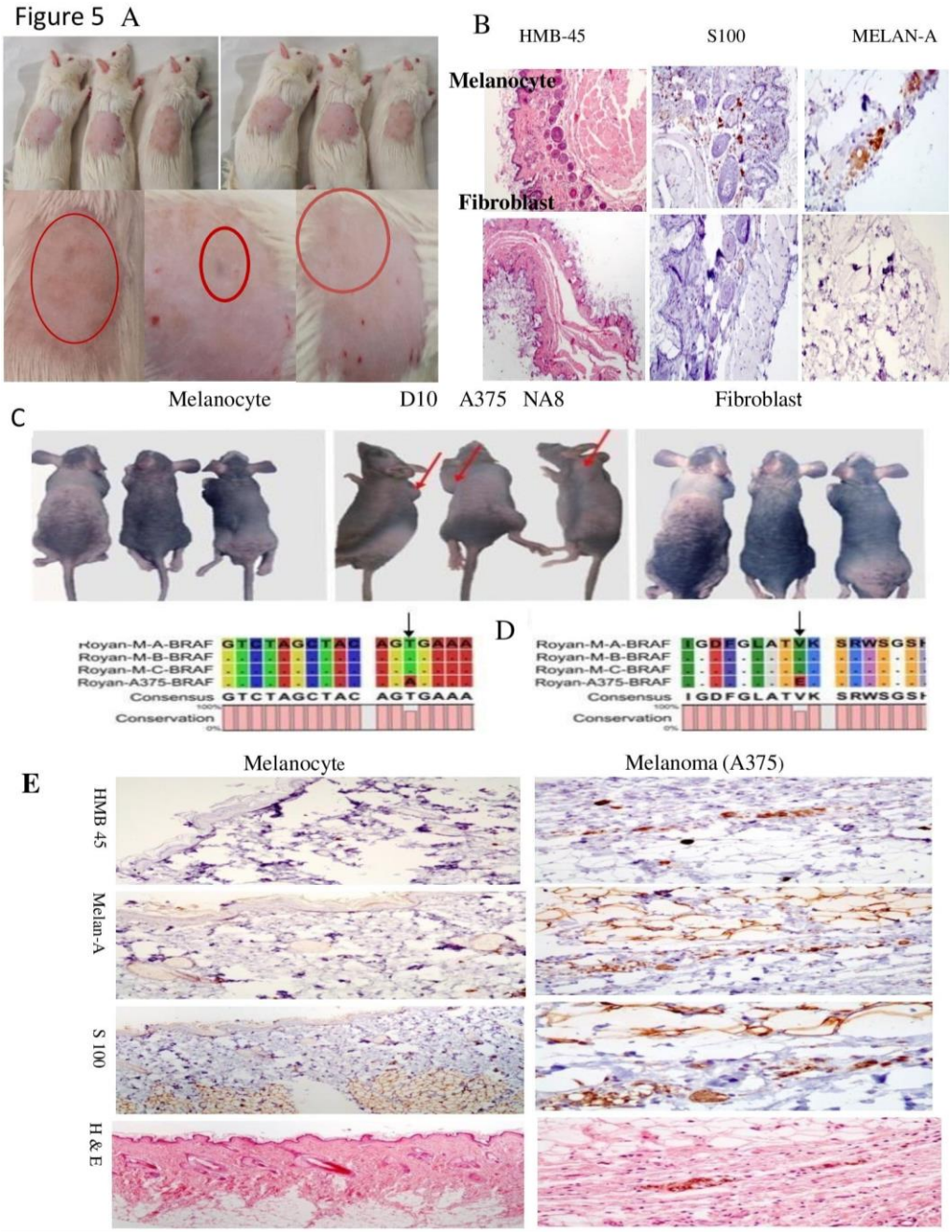

Fig. 5. (A) shows the punctuated pigmentation on injected site of the skin of BALB/c nude mice, C57/B6, which indicates the functionality of cultured cells after transplantation. The histological assessment showed intra-epidermal existence of the transplanted cells. (B) The immunohistochemistry results depicted bioactive cells after transplantation. The results showed that 96% (19 out of 20) of cells were positive for Melan-A, 92% (18 out of 20) of cells were positive for S-100, and 81% (15 out of 20) of cells were positive for HMB45 in the total 20 melanocytes counted. The immunostaining results confirmed the presence of cells in epidermis (C). Photographs of BALB/c nude mice injected with cultured melanocytes, melanoma cell lines, and fibroblast. Sequence alignments and DNA sequence chromatograms of the *BRAF* gene after transplantation. Cultured melanocytes and fibroblasts showed no signs of tumor formation after 16 weeks, whereas the areas injected with A375, D10, and NA8 generated palpable tumors. Red arrows depicted the palpable tumors. (D) Nucleic acid multiple-sequence alignment of the *BRAF* gene. Amino acid multiple-sequence alignment of the *BRAF* gene from melanoma and melanocyte samples showing the presence of a mutation (c. 1799 T > A) at codon 600. (D) DNA sequence chromatograms of the *BRAF* gene from A375 and melanocyte samples. (E) Immunofluorescence staining on the skin of nude mice after cultured melanocyte transplantation and melanoma cell lines showed that melanoma expressed Melan-A, S100, HMB-45 markers.

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No visible Evidence of Tumor Formation in BALB/c Nude Mice

Cultured melanocytes of passages 5 and 6 were injected into BALB/c nude mice. Furthermore, melanoma cell lines, namely A375, D10 and NA8, and fibroblasts were injected as positive and negative controls, respectively. The mice were monitored weekly over a period of 16 weeks, and it was observed that the mice injected either with cultured melanocytes (cultured melanocytes/animal, n = 48) or fibroblasts had no visible evidence of tumor formation, whereas palpable tumors were readily detected at the injection site on the scapula in the mice that received A375, D10 and NA8 cells (Figure 5C). Size and appearance of tumors were similar to those of melanoma tumors, yet no tumors, nodules or any palpable tissue were observed following transplantation of cultured melanocyte into nude mice. The monitoring was continued for four months. To make sure, the possible *BRAF* mutation at codon 600 (V600E) was investigated after 16 weeks of transplantation in biopsy samples obtained from mice with melanoma and cultured melanocytes groups. The *BRAF* V600E mutation, which resulted in an amino acid substitution from valine to glutamic acid, was found in all samples from the melanoma groups. However, gene sequencing of the *BRAF* gene in nude mice that received the melanocytes showed normal results. Moreover, the result of histopathological tissues analyses suggested that following subcutaneous injection, the integration of the cultured melanocytes of passages 5 and 6 into the subcutaneous tissue occurred without non-transformed cells and no pigmentation. In contrast, transplantation of human melanoma cell lines showed cells expressing antibodies (Figure 5D-5E).

The results indicate that cultured melanocytes, when injected into BALB/c nude mice, do not form tumors, and there is no evidence of malignancy or abnormal pigmentation. This suggests that the cultured melanocytes from passages 5 and 6 are non-transformed and lack tumorigenic potential. On the other hand, the melanoma cell lines exhibited typical melanoma characteristics, including tumor formation and the presence of the BRAF V600E mutation, validating their use as positive controls. The findings provide strong evidence supporting the non-tumorigenic nature of the cultured melanocytes and highlight the contrast between benign melanocytes and malignant melanoma cells (17).

Discussion

To produce sufficient numbers of cells for clinical applications, it is essential to expand the cells while preserving their genomic stability and functionality (15, 16). With the increasing use of cultured melanocyte transplantation for generalized vitiligo, identifying the optimal growth medium is crucial. This medium should minimize culturing time while maximizing melanocyte yield and eliminating any risk of tumorigenicity (7, 8, 16, 17, 18). Safety concerns have emerged, as some studies suggest that certain components in culturing media may induce gene mutations and tumor formation. Various techniques have been employed to identify a safe medium for culturing melanocytes by eliminating tumor promoters (7, 17, 18). The present study aimed to culture human adult melanocytes in different growth media, utilizing safe supplements to determine the optimal growth medium and assess cell safety both in vitro and in vivo. In 1956, Funan Hu et al. conducted the first study to describe the normal morphology of human melanocytes from benign pigmented nevi and foreskin. They observed that melanocytes appeared as dark, spindle-shaped dendritic cells containing pigmented granules (18, 19). In this study, cultured melanocytes maintained typical

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morphology from passage 0 to passage 6 and after transplantation. Immunofluorescence staining with Melan-A, S100, HMB-45, and Tyrosinase II markers confirmed the melanocytic identity of the cultured cells. Molecular and conventional karyotyping was then performed to evaluate the genetic stability of the melanocytes following in vitro expansion. Although serial passages can potentially lead to chromosomal rearrangements and a transformed karyotype, karyological analysis at different passages revealed no clinically significant numerical or structural chromosomal abnormalities in the cultured melanocytes compared to melanoma cell lines. These findings are consistent with those reported by Herlyn *et al*. (18, 19). The observed increase in *NRAS* gene expression in melanocytes at passage 6 may result from various factors. Prolonged culture conditions can induce genetic and epigenetic changes, including the up regulation of oncogenes like NRAS, due to cellular aging, selection pressure, and adaptive responses to the in vitro environment. Additionally, the accumulation of mutations and the need to support increased cellular proliferation may contribute to higher *NRAS* expression. These observations underscore the importance of monitoring gene expression across multiple passages to ensure the reliability of in vitro studies. Our study also found that the *HRAS*, *NRAS*, and *BRAF* genes—common drivers of early-stage melanoma transformation (20) were not mutated in melanocytes after several passages. The *RAS* family genes are known to significantly contribute to melanoma transformation, particularly with mutations at codons 11, 12, 13, and 18 in exon 1, and codons 59 and 61 in exon 2 of the NRAS gene (13). The BRAF gene, functioning as an immediate downstream effector of RAS, is often mutated at codons 599 or 600 (V600E) in exon 15. This mutation results in a substitution of glutamic acid with valine, activating BRAF and stimulating constitutive MEK-ERK signaling, which is frequently observed in melanoma (13, 21, 22). Melanoma displays a range of histologic features that can mimic various other tumor types, complicating diagnosis. Real-time PCR and gene expression analysis are crucial for distinguishing gene expressions. Our data highlight the context-dependent activity of RAS and RAF isoforms, which explains the absence of NRAS mutations in melanocytes. Understanding these nuances is essential for the successful transplantation of cultured melanocytes into vitiligo patients. The RAS signaling pathway is critical for the intracellular transduction of mitogenic signals from activated growth factor receptors. Our study found no abnormalities in cultured melanocytes and developed methods to assess tumorigenicity, focusing on cells from primary culture through to the sixth passage. These melanocytes continued to grow and age without exposure to mutagenic growth factors, differing from findings in other studies. Despite elevated RAF or RAS signaling after six passages, injections, cell sequencing, and karyotype analyses indicated no tumorigenic potential, highlighting the safety and therapeutic promise of cultured melanocytes for treating vitiligo. This study represents the first measurement of changes in BRAF, HRAS, and NRAS expression levels in cultured melanocytes across different passages, with results compared to melanoma cell lines. The study found that HRAS and BRAF gene expression levels in melanocytes at passage 6 increased by less than double, whereas melanoma cell lines exhibited 3- to 8-fold increases, as shown by real-time PCR assays. Additionally, *NRAS* gene expression in melanocytes at passage 6 increased fivefold, which was lower compared to the *NRAS* expression in melanoma cell lines. Although some changes in *BRAF*, *NRAS*, and *HRAS* gene expression levels were observed during culturing, gene sequencing analysis of melanocytes at passage 6 revealed no mutations in the *BRAF* gene at codon 600 (V600E) in exon 15, unlike in melanoma cells. These results are consistent with

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those reported by Czajkowski (23). Furthermore; the DNA sequence of the *NRAS* gene in exons 1 and 2 did not depict any mutations in cultured melanocytes and in the A375 and D10 melanoma cell lines. To address the tumorigenicity potential of cultured cells, the passage-5 and -6 cultured melanocytes and malignant melanoma cells were transplanted into 48 athymic mice, which resulted in no tumor formation, no alterations in karyotyping nor gene mutations in cultured melanocytes after transplantation, in contrast to the mice injected with melanoma cells. The results of this study also indicated that culture of human melanocytes for a limited number of population doublings, in a growth medium, which contained physiologic compounds in the absence of TPA or other tumor promoters, would not induce tumorigenicity under *in vitro* and *in vivo* conditions. This study provided strong evidence supporting the safety of cultured melanocytes transplantation for generalized vitiligo and other hypo-pigmented disorders, when conventional treatments were not sufficiently effective. The results of the present study revealed that culturing human melanocytes using standard growth media protocols might increase the expression of specific proto-oncogenes, but would not lead to tumorigenic mutations, *in vitro* and *in vivo*.

Ethical Approval and Consent to Participate: Human and Animal Rights

The ethical approval to report this case was obtained from Institutional Review Board and Ethics Committee of Royan Stem Cell Institute. Moreover, all patients were provided with written informed consent at Royan Stem Cell Institute, Tehran, Iran. Moreover, the ethical code followed for the purpose of the present study was IR.TUMS.VCR.REC. 1400.2628. The inclusion criteria for participation in this research were adults at a minimum age of 20; patients suffering from vitiligo for a period of at least one year; and stable disease conditions (no recent exacerbation) without using any other treatment, such as phototherapy or corticosteroid drugs. The exclusion criteria involved any special diseases (any acute or chronic conditions that would limit the ability of the patient to participate in the study), other skin problems and pregnancy. All procedures in this study were conducted in accordance with the approval and guidelines of the Institutional Review Board and Ethics Committee of Royan Stem Cell Institute, following the ethical code IR.TUMS.VCR.REC. 1400.2628. In addition, all patients were provided with written informed consent. The Institutional Review Board and Ethics Committee of Royan Stem Cell Institute approved this study. The Grant Number regarding the present study was 8700099 from Royan Stem Cell Institute, Tehran, Iran.

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