Evaluation of Cultured Melanocytes in Terms of Pigmentation, Genetic Stability and Tumorigenicity in order to Vitiligo patients Transplantation

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Abstract

Aims: Recently cultured melanocyte transplantation has been evolved as a promising modality against extensive depigmentation in vitiligo which are resistant to conventional treatments. Here, we aimed to find the best growth media for clinical purposes and to determine the tumorigenic activity of cultured melanocytes both *in vitro* and *in vivo*.

Main methods: Human skin melanocytes were isolated from 8 patients, cultured under various growth media till passages 6. The best growth media based on doubling time (DT) and cell counts at each passages, was determined. Also, changes in *BRAF*, *NRAS*, and *HRAS* genes expressions in cultured cells were assessed by real-time PCR and gene sequencing of BRAF V600E and NRAS in exons 1 and 2 as compared to melanoma cell lines. Karyotyping of the passage 6 melanocytes was done to assess the genetic stability. Cells of passages 5, 6 were subcutaneously injected into BALB/c nude mice to evaluate the risk of tumorigenesis after transplantation.

Key findings: A mixture of MGM-M2 supplemented with a combination of melanocyte growth factors two times plus 4% fetal bovine serum (FBS), had the best results in terms of PDT and melanocyte count during passages 0 to 6. Karyotypes of melanocytes at passages 5 and 6 in all patients displayed no structural alterations in the 15 metaphase plate spreads. *HRAS* and *BRAF* gene expressions of melanocytes at passage 6 showed <2-fold increases, while *HRAS* and *BRAF* gene expressions in melanoma cell lines were up-regulated to more than 3 and 8 folds, respectively. Furthermore, *NRAS* gene expression in melanocytes at passage 6, showed 5-fold change which was lower than *NRAS* gene expression in melanoma cell line. Gene sequencing of *BRAF* V600E and *NRAS* in exons 1 and 2 displayed no mutation. Melanocytes injected into BALB/c nude mice showed no risk of tumor formation. Also, gene sequencing of *BRAF* and *NRAS* in injected melanocytes displayed no mutation 16 weeks after transplantation.

Significance: Our results revealed that culturing human melanocytes using standard growth media protocols, may increase the expression of specific proto-oncogenes, however will not lead to tumorigenic mutations, in vitro and in vivo.

Introduction

Over the last two decades, melanocyte transplantation has been evolved as a promising modality against vitiligo patches which are resistant to conventional treatments. Both cultured and non-cultured melanocytes have been applied in vitiligo, however, in case of extensively depigmented patches, transplantation of cultured melanocytes is inevitable. Firstly, Lerner et al. introduced the transplantation of autologous cultured melanocytes for vitiligo in 1987. Since then, several clinical trials have shown the clinical safety and efficacy of transplantation of cultured autologous melanocyte in spite of marked safety concerns. It is thought that melanomas arise from excessive proliferation of melanocyte precursors and during this process, growth factors and mitogens used for *in vitro* proliferation of

melanocytes accelerate cell cycle progression that may lead to DNA damage and trigger the melanoma genesis under cell culture conditions or after transplantation. Lerner incubated the cells in a medium containing 12-O- tetradecanoyl phorbol- 13-acetate (TPA). Although Czajkowski reported the safety of using TPA in growth media for culturing purposes, other studies reported that TPA is a tumor promoter and its presence could lead to malignant transformations of melanocytes under cell culture conditions.

Melanocytes require the RAS/RAF/MEK/ERK signaling pathways to regulate proliferation, migration, differentiation, and apoptosis processes. Mutations in the serine/threonine kinase BRAF codon 600 have been reported in 25–80% of malignant melanomas and are considered early events in the development of melanocytic lesions. Also, mutations in the NRAS codons 12, 13, and 61 have been observed in 15–30% of malignant melanomas, particularly in tumor sites which are exposed to ultraviolet radiation. Activation of MEK and then ERK, is likely to occur in response to oncogenic *NRAS* and *BRAF* mutations.

So far, some studies assessed the *in vitro* tumorigenicity of various growth media in the presence or absence of TPA. However, there is no study evaluating the behavior of transplanted cells after transplantation.

In this study, we aimed to find the best growth media based on the results of proliferation tests and in terms of functional activities for clinical purposes and to determine the tumorigenic potential of cultured melanocytes under *in vitro* and *in vivo* conditions. For this purpose, we isolated human skin melanocytes, cultured them with various growth media and then determined the cellular, molecular, and genetic changes that might occur during an *in vitro* culture process. Afterwards, we subcutaneously injected the cells into BALB/c nude mice to evaluate the risk of tumorigenesis after transplantation.

Materials And Methods

Isolation and Culture of Human Melanocytes in Various Growth Media

We selected 8 patients of 14 subjects aging < 35 years old who had generalized, stable Vitiligo. These individuals showed resistance towards conventional treatments, and were candidate for non-cultured melanocyte transplantation. All patients provided written informed consent at Royan Institute, Tehran, Iran. The Institutional Review Board and Ethics Committee of Royan Institute approved this study. We harvested a 2×2 cm partial thickness skin specimen from the buttock area of each patient. We cut a 0.5×0.5 cm of the specimen and transferred it to the laboratory for melanocyte isolation and culturing process. The remained skin sample was transferred to the clean room for cell isolation and transplantation. The skin specimens were washed, cut, and incubated for 15–18 h at 4°C in a 1.2 U/ml Dispase II (Invitrogen, Karlsruhe, Germany) solution. Next, we separated the epidermis from the dermis and placed the epidermis sheets into 0.25% trypsin/EDTA (Invitrogen, Karlsruhe, Germany) at 37°C for 30 min. After enzyme inhibition and cell washing,

(Promo Cell, c-24400 Heidelberg, Germany) we transferred the cell suspension into MGM-M2 (Promo Cell, c-24400 Heidelberg, Germany). We used four protocols for melanocytes culture. In protocol A, we cultured the cell suspension in MGM-M2 culture media. In protocol B, cells were cultured in MGM-M2 supplemented with mix growth factor (Promo Cell, C-39420 Heidelberg, Germany). In protocol C, we used MGM-M2 supplemented with mix growth factor: Melanocyte Growth Medium supplement included: CaCl2, 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; FBS, 2.5 ml. and 4% FBS (Life Technologies, Grand Island, NY, USA). Finally, in protocol D, we added MGM-M2 (Promo Cell), supplemented with mix growth factor (Promo Cell, C-39420). Two times and 4% FBS (Life Technologies). After 3 days of culture, cells were incubated with geneticin (G-418 Solution Sigma-Aldrich, St. Louis, MO) for 3 days for removal of fibroblasts and keratinocytes from all the four groups. Every three days, we changed the media. Next, we passaged the melanocytes when they became 80%-90% confluent. The culture medium was removed and 1 ml trypsin 0.125% EDTA (Invitrogen) was added to each plate. The plates were incubated at 37°C for 2 minutes. Afterwards, trypsin was neutralized and the solution was centrifuged. Separated cells were distributed among the four previously described media and cells were cultured for at least 6 passages. Previous studies showed that p3 to p5 of cultured melanocytes will be adequate for clinical applications. Consequently, we selected the cells from early passages (p1 – p6) to assess the genetic stability and proto-oncogenes, in vitro and in vivo. Furthermore, we cultured A375, NA8, and D10 cell lines as positive control cells and fibroblast cells as the negative control cells. These cells were thawed after cryopreservation and propagated in culture with DMEM-F12 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin as recommended by the ATCC. A-375 [A375] is a melanoma cell line all the data are similar with ATCC: (ATCC® CRL-1619™)

D10.G4.1 is a melanoma cell line: (ATCC® TIB-224™)

NA8-MEL is a melanoma cell line: (CVCL_S599) -

All of these cell lines were gifted from one of our colleagues in Basel University.

The fibroblasts were propagated in culture with DMEM-F12 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin.

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 isothiocyanate (FITC)-conjugated anti-mouse (F9006) for Melan-A, and HMB-45 and goat anti-rabbit for S100 and tyrosinase II for 60 min at room temperature. A375 and fibroblast cells were also stained for Melan-A and S100. Nuclei were counter-stained with 5

µg/ml of 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) and analyzed by a fluorescent microscopy (Nikon, Tokyo, Japan).

Determination of population doubling time (PDT) of Human Melanocytes in Culture

We assessed the population doubling time (PDT) in each protocol 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 N0 = 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 min. 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 min. Next, the cells were fixed using a methanol: acetic acid (3:1) solution. In order 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. We counted 15 metaphase plates, analyzed them, and karyotyped the representative metaphase cells. We also performed karyotype analyses for A375, NA8, and D10 melanoma cells.

RNA Extraction and Real-time PCR

Total RNA was extracted from cultured melanocytes of passages 1, 3, and 6 as well as melanoma cell lines A375 and D10) passage 10) using a column-based RNeasy Mini Kit (Qiagen, Valencia, CA). We used random hexamer primers for cDNA synthesis which was performed with a PrimeScript RT Reagent Kit (Perfect Real Time, Takara Biotechnology, Japan). The PCR reaction was performed using a Power SYBR® Green PCR Master Mix) Applied Biosystems, 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 min, 40 cycles at 95°C for 15 sec, and 60°C for 1 min. Relative expression levels were determined based on collected data such as cycle threshold (C_t) numbers. Real-time PCR assay was replicated three times for each sample and the relative gene expression was calculated as $2^{-\Delta\Delta Ct}$, where ΔCt = Ct Target -Ct Reference. The *GAPDH* gene served as an internal control. Table 1 shows the primer sequences.

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

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, and passage-10 melanoma cell lines A375 and D10 as positive controls, using a QIAamp DNA Blood Mini Kit (Qiagen® 51306, Hilden, Germany) according to 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. Table 3 shows the sequences of the primers. Analysis and alignment of the data was performed by ChromasPro 2, CLC Sequence Viewer 6, and Gene Runner 5 softwares.

Table 2
Samples and GenBank accession number list.

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

Table 3
The sequences of the primers used for sequencing.

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

Presence and Pigmentation Assessment of Cultured Melanocytes in Albino Mice

We assessed the presence and pigmentation of the cultured melanocytes by injecting the cultured cells in epidermal area of albino mice. Animals were obtained from Pasteur Laboratories, Tehran, Iran. All animal experiments were performed after obtaining the approval from the Institutional Animal Care and Use Committee at Royan Institute. We subcutaneously injected 2.0×106 cells/ml of passages 6 cultured melanocytes in epidermal site of 6 albino mice. We observed the pigmentation in mice weekly for up to 3 months. Furthermore, we took skin biopsies from pigmented sites. After tissue processing, H&E (Haematoxylin and Eosin) staining was carried out to evaluate morphological changes of the cells after transplantation. To confirm that cells were observed in the epidermis after transplantation, immunohistochemistry was performed on paraffin sections using antibodies against HMB45, Melan-A, and S100.

Safety Assessment of Cultured Melanocytes in BALB/c Nude Mice

We assessed the tumorigenicity of the cultured melanocytes by injecting the cultured cells obtained from eight patients into BALB/c nude mice. Animals were obtained from Pasteur Laboratories, Tehran, Iran. All animal experiments were performed after obtaining the approval from the Institutional Animal Care and Use Committee at Royan Institute. We subcutaneously injected 2.0×10^6 cells/ml of passages 5 and 6 cultured melanocytes into 48 nude mice between their scapulae. Fibroblasts (2.0×10^6 cells/ml) and normal saline were separately injected S.C. (subcutaneous) in the back of four nude mice used as control negatives. A375, D10, and NA8 as positive controls (n = 6) were subcutaneously injected. Tumor formation was monitored weekly for at least 16 weeks.

After 16 weeks, all the animals were sacrificed and we harvested tissue from the injection sites of three mice in the melanocyte-injected group and one mouse form the positive control. DNA was extracted from the tissues. We performed PCR analysis 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.

Statistical Analysis

All data are presented as mean ± standard deviation (SD) and were analyzed by ANOVA and PostHoc Bonferroni to assess differences among means. A $P < 0.05$ was regarded as statistically significant.

Results

Morphology and Immunofluorescence Staining of Human Cultured Melanocytes

In all protocols, the cultured cells of passages 1-6 had bi- or tri-polar 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 passages1, 3 and 6 melanocytes cultured using each of the four protocols. The expressions of Melan-A, S100 protein, HMB-45, and tyrosinase II were confirmed by Image J software in > 90% of cultured melanocytes. Furthermore, A375, NA8, and D10 melanoma cell lines expressed Melan-A and S100 surface markers, however, 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 A and B are the most appropriate

The mean PDT of passage-1melanocytes cultured using protocols A, B, C, and D were 34.8, 33.0, 24.7, and 22.9 hours, respectively. As pointed out in Figure 2, these values were persistent from passage 1 to passage 6, while at passage 6, PDT for protocols C and D were 23.6 and 22.0 hours which were significantly lower than those of protocols A and B (39.1 and 36.2 hours, respectively) ($P<0.001$). As shown in (Figure 2 A) 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, cells cultured using protocols C and D exhibited a sudden increase in number especially in passages-4 5 (i.e. 40,000 to 60,000 cells/ml, respectively) in contrast to that achieved following utilization of protocols A and B, in which the number of cells remained unchanged till passage 6. (Figure 2 B)

Karyotype Analysis

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 showed numerous structural alterations that included deletions, duplications, and rearrangements, as well as aneuploidy and polyploidy. The NA8 melanoma cell line displayed a modal distribution of chromosome numbers 59-61 (hypotriploid), A375 cells displayed a modal distribution of chromosome number 62 (hypotriploid),

and D10 cells displayed a bimodal distribution of chromosomes 46–48 (near triploid, Figure 3A and Table 4).

Table 4

Karyotype results detected in the melanoma cell lines.

Cell line	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].

Gene Expression Analysis

BRAF, *NRAS*, and *HRAS* gene expressions in A375 and D10 melanoma cell lines and cultured melanocytes were assessed by RT-PCR. We observed <1-fold increases in *HRAS* gene in passage 3 of cultured melanocytes. Also, in passages 1 and 6, expression levels of *HRAS* gene showed less than 2-fold increases. Moreover, BRAF gene in cultured melanocyte of passages 1, 3, and 6 showed 2-fold increases. Interestingly, passage 3 showed the lowest level of BRAF gene expression. In comparison, BRAF gene expression of melanoma cell lines A375 and D10 showed 8 fold increase which is of crucial importance in melanomas ($p<0.05$). Also, the data showed 1-fold increase in the expression of *NRAS* gene of passages 1 and 3 of cultured melanocytes Overall, the *NRAS* gene expression was significantly increased (up to 5 fold) in passage 6, in melanoma cell lines A375 and D10, *NRAS* gene expression showed a 6-fold increase. (Figure 3B)

Gene Sequencing

We investigated probable mutations in *BRAF* exon 15 and *NRAS* exons 1 and 2 in cultured melanocytes (passages 5 and 6) Sequencing analysis showed that in melanoma cell lines A375 and D10, *BRAF* gene

had a T to A transversion at nucleotide position (*BRAF* c.1799T>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 (Figures 4 A-C). DNA sequence of the *NRAS* gene in exons 1 and 2 did not show any mutations neither in the cultured melanocytes nor the A375 and D10 melanoma cell lines (Figures 5 A-C). Genome sequencing of cultured melanocyte showed that all nucleotides were in correct positions and no mutations were seen. All passage-6 melanocytes obtained from eight patients were analyzed and no wrong nucleotide was seen.

Presence and Pigmentation of Cultured Melanocytes in Albino Mice

(Figure 6A) shows the punctuated pigmentation on injected site of the skin, which indicates the functionality of cultured cells after transplantation. The histological assessment showed intra-epidermal existence of the transplanted cells. The immunohistochemistry results showed bioactive cells after transplantation. The results showed that 96% (19 of 20) of cells were positive for Melan-A, 92% (18 of 20) of cells were positive for S-100, and 81% (15 of 20) of cells were positive for HMB45 in 20 melanocytes counted. The Immunostaining results confirmed the presence of cells in epidermis. (Figure 6B)

Tumorigenicity Assessment of Cultured Melanocytes in BALB/c Nude Mice

Cultured melanocytes of passages 5 and 6 were injected into BALB/c nude mice. Also, melanoma cell lines namely, A375, D10 and NA8 and fibroblasts were injected as positive and negative controls, respectively. We monitored the mice weekly over a period of 16 weeks, it was observed that 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 mice that received A375, D10 and NA8 cells (Figure 7A).

Size and appearance of tumors were similar to those of melanoma tumors, however, following transplantation of cultured melanocyte into nude mice, no tumors, nodules or any palpable tissue were seen. This monitoring was continued for four months. To make sure, we investigated possible *BRAF* mutation at codon 600 (V600E) after 16 weeks of transplantation in biopsy samples obtained from mice with melanoma and cultured melanocytes groups. The *BRAF* V600E mutation that resulted in an amino acid substitution from valine to glutamic acid, was found in all of the samples from the melanoma groups. However, gene sequencing of the *BRAF* gene in nude mice that received the melanocytes showed normal results. And 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 expressed antibodies. (Figures 7 B-D).

Discussion

To produce adequate numbers of the cells for clinical purposes, cells should be expanded while maintaining their genomic stability and functionality. Given the increasing use of cultured melanocyte transplantation for generalized Vitiligo, finding the ideal growth media which requires less culturing time but results in higher melanocytes yield in culture with no tumorigenicity risk, is critical. Safety concerns have been raised as studies proposed that some components present in culturing media may induce gene mutation and tumor formation. So far, different techniques have been used to find a safe media for culturing melanocytes by elimination of tumor promoters.

In this study, we attempted to culture human adult melanocytes in different growth media using safe supplements to find the best growth media and check the safety of the cells, *in vitro* and *in vivo*.

First time, Funan Hu et al in 1956 performed a study to find the normal morphology of human melanocytes of benign pigmented nevi and foreskin. Concerning melanocytes morphology, they reported dark and spindle-shaped dendritic cells containing pigmented granules. In this study, cultured melanocytes showed typical morphology from passage 0 to 6 and after transplantation. Immunofluorescence staining of Melan-A, S100, HMB-45, and tyrosinase II markers confirmed the melanocytic identity of the cultured cells.

In the next step, molecular and conventional karyotyping were done to assess the genetic stability of the melanocytes after *in vitro* expansion. As serial passages of melanocytes can potentially lead to changes in chromosomal arrangements and a transformed karyotype, karyological analysis of the cells at different passages was done but showed no clinically significant numerical or structural chromosomal abnormalities in the cultured melanocytes compared to the melanoma cell lines. These results were consistent with those reported by Herlyn et al.

We found that the *HRAS*, *NRAS*, and *BRAF* genes which are the most common causes of early stage melanoma transformation, were not mutated in cultured melanocytes following a series of passages.

In the cancer transformation of melanoma, *RAS* family genes play a remarkable role, especially when mutations occur at codons 11, 12, 13, and 18 in exon 1, and codons 59 and 61 in exon 2 of the *NRAS* gene. Importantly, *BRAF* functions as an immediate downstream effector of *RAS*, a mutation at codon 599 or 600 (V600E) in exon 15, lead to a glutamic acid to valine substitution, activating *BRAF* and stimulating constitutive MEK-ERK signaling in cells which is frequently observed in melanoma.

In this study, for the first time, we measured the changes in *BRAF*, *HRAS* and *NRAS* expression levels in cultured melanocytes of different passages and compared the results with those of melanoma cell lines. We found that *HRAS* and *BRAF* gene expression levels of melanocytes at passage 6 showed < 2 fold increase, while melanoma cell lines gene expression levels should 3-8-fold increases in as shown by real-time PCR assay. Also, *NRAS* gene expression of melanocytes at passage 6 showed a 5-fold increase which was lower than that of melanoma cell line's *NRAS* gene expression.

Although we observed some alterations in *BRAF*, *NRAS* and *HRAS* gene expression levels during culturing process, gene sequencing analysis of cultured melanocytes at passage 6 revealed no mutation in the *BRAF* gene at codon 600 (V600E) in exon 15 in contrast to melanoma cells. This was in line with the findings reported by Czajkowski. Moreover, the DNA sequence of the *NRAS* gene in exons 1 and 2 did not show any mutations in cultured melanocytes and in the A375 and D10 melanoma cell lines.

In order to address the tumorigenicity potential of cultured cells, we transplanted the passages 5 and 6 cultured melanocytes and malignant melanoma cells into 48 athymic mice. The results showed 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 indicate that culture of human melanocytes for a limited number of population doublings, in a growth media which contains physiologic compounds in the absence of TPA or other tumor promoters, will not induce tumorigenicity under *in vitro* and *in vivo* conditions. This study provides strong evidence supporting the safety of cultured melanocytes transplantation for generalized vitiligo and other hypo- pigmented disorders, when conventional treatments are not effective. Our results revealed that culturing human melanocytes using standard growth media protocols, may increase the expression of specific proto-oncogenes, however will not lead to tumorigenic mutations, in vitro and in vivo.

Declarations

Ethical Approval

Ethical approval to report this case was obtained from The Institutional Review Board and Ethics Committee of Royan Institute, Tehran, Iran.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the The Institutional Review Board and Ethics Committee of Royan Institute, Tehran, Iran (NO: 8700099) All patients provided written informed consent at Royan Institute, Tehran, Iran. The Institutional Review Board and Ethics Committee of Royan Institute approved this study.

Statement of Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article. All patients provided written informed consent at Royan Institute, Tehran, Iran.

Consent for publication

Not applicable

Availability of data and material

Some data analyzed during this study are included in this published article, and the others are not publicly available due limited amount of figures and tables but are available from the corresponding author.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions:

Seyed Jalal Zargar and Nasser Aghdami conceived and designed the original protocol. All authors were involved in amending the protocol. Atefeh Shahbazi coordinated the study. Atefeh Shahbazi performed the cell isolation, culturing process and in vitro study Seyed Jalal Zargar and Amir Bajouri were involved in in vivo experiment. Data entry and analysis were performed by Atefeh Shahbazi. Atefeh Shahbazi wrote the first draft of the manuscript. Seyed Jalal Zargar and Nasser Aghdami supervised the study. All authors contributed to subsequent and final drafts. All authors read and approved the final manuscript.

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Figures

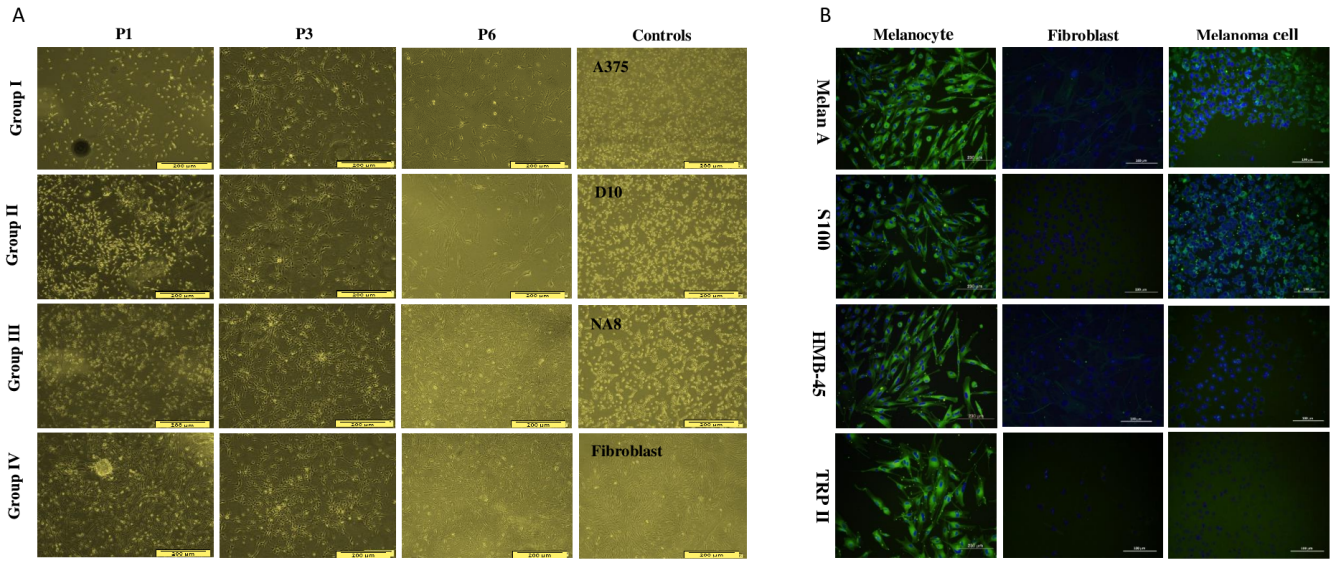

Figure 1

Characteristics of cultured human adult melanocytes. (A) Phase contrast images of cells shows melanocytes cultured under four conditions (i.e. four groups): i) melanocyte growth medium (MGM-M2), ii) MGM-M2 + supplementary mix growth factors, iii) MGM-M2 + supplementary mix growth factors + 4% FBS, and iv) MGM-M2 + Supplementary mix growth factors were added two times growth factors + 4% FBS. The cells have 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 melanocytes cultured in MGM-M2 + supplementary mix growth factors + 4% FBS expressed Melan-A, S100, HMB-45, and tyrosinase II 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.

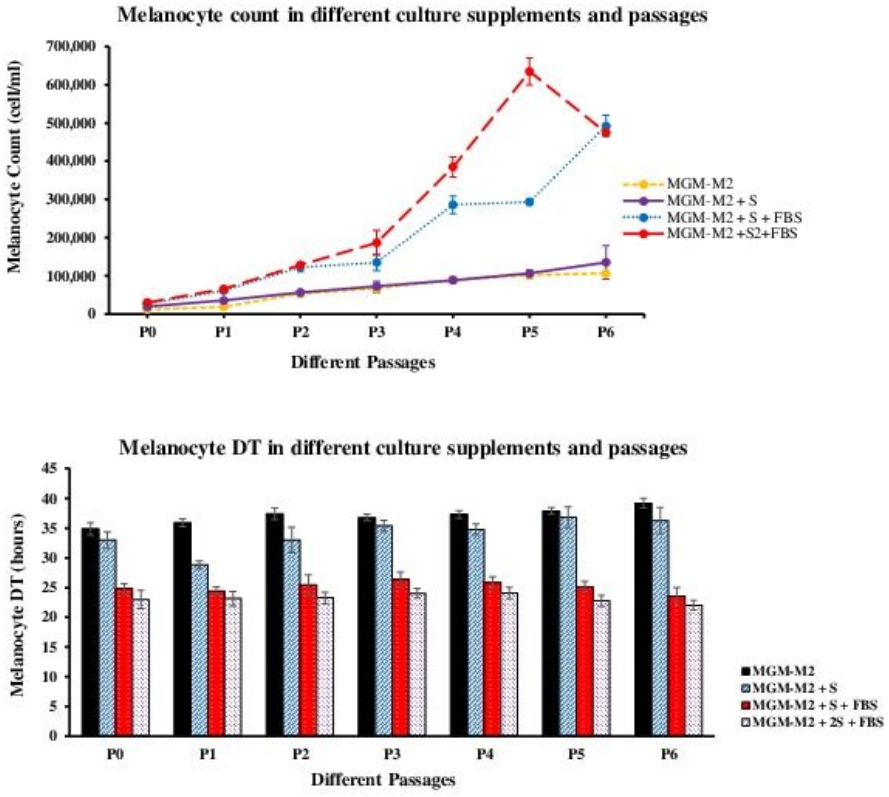

Figure 2

(A) Population doubling time (PDT) per hour for cultured melanocytes in four groups in primary culture and at passages 1-6. The data are expressed as mean ± SD of three replicates. ### P<0.001 shows significant differences between groups 1 and 2 and group 3, *** P<0.001 shows significant differences between groups 1 and 2 and group 4. (B) Cultured melanocytes counts from primary culture to passage 6 in all four groups.

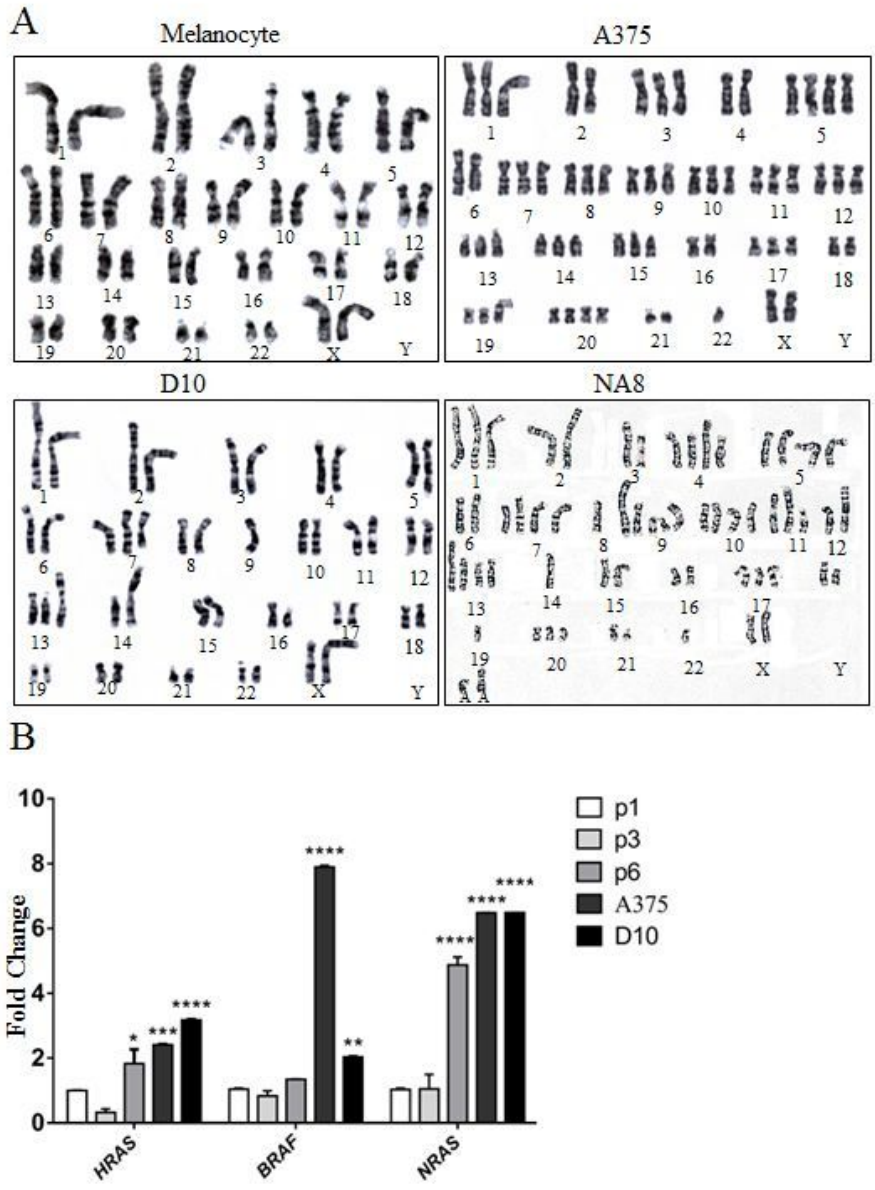

Figure 3

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 are expressed as mean ± SD of 3 replicates. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 show significant differences as compared to.

Figure 4

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 that codon 600 is 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).

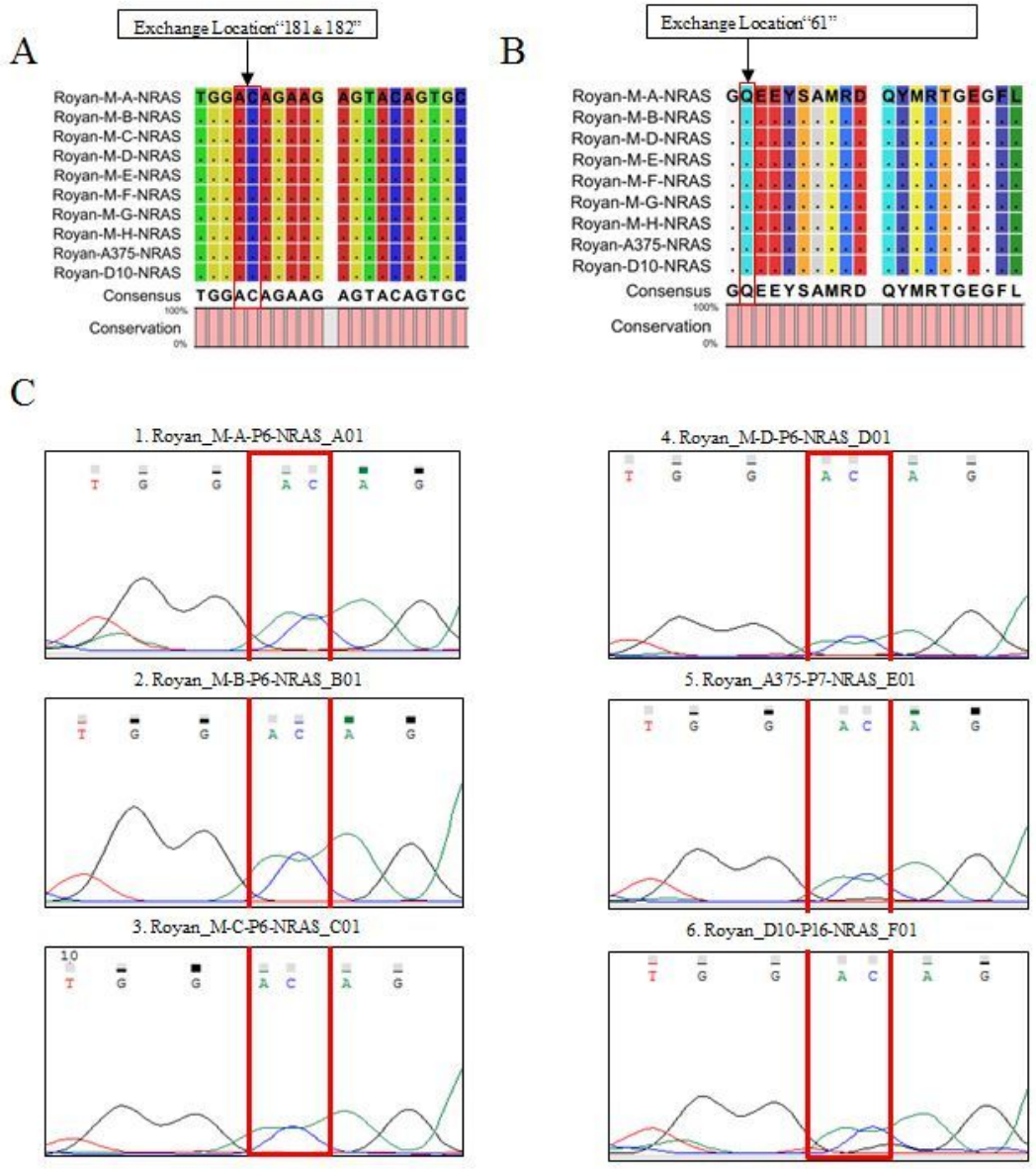

Figure 5

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.

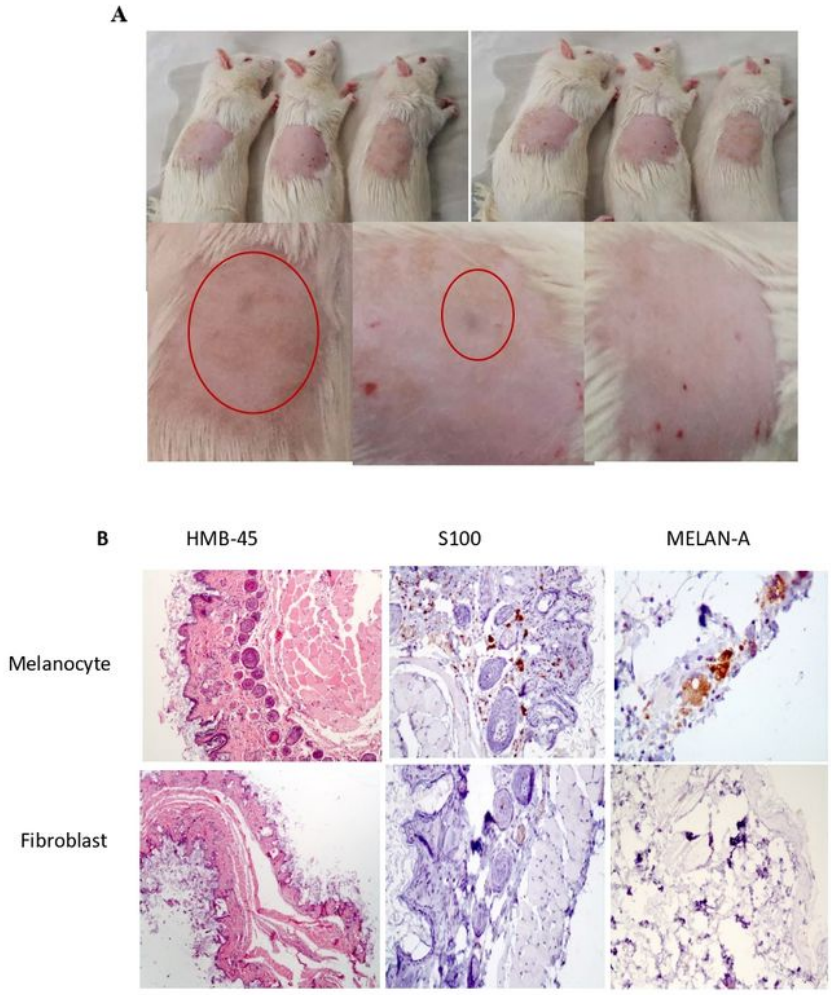

Figure 6

(Figure 6A) shows the punctuated pigmentation on injected site of the skin of Balb/c, C57/B6

, which indicates the functionality of cultured cells after transplantation. The histological assessment showed intra-epidermal existence of the transplanted cells. The immunohistochemistry results showed bioactive cells after transplantation. The results showed that 96% (19 of 20) of cells were positive for Melan-A, 92% (18 of 20) of cells were positive for S-100, and 81% (15 of 20) of cells were positive for HMB45 in 20 melanocytes counted. The Immunostaining results confirmed the presence of cells in epidermis. (Figure 6B)

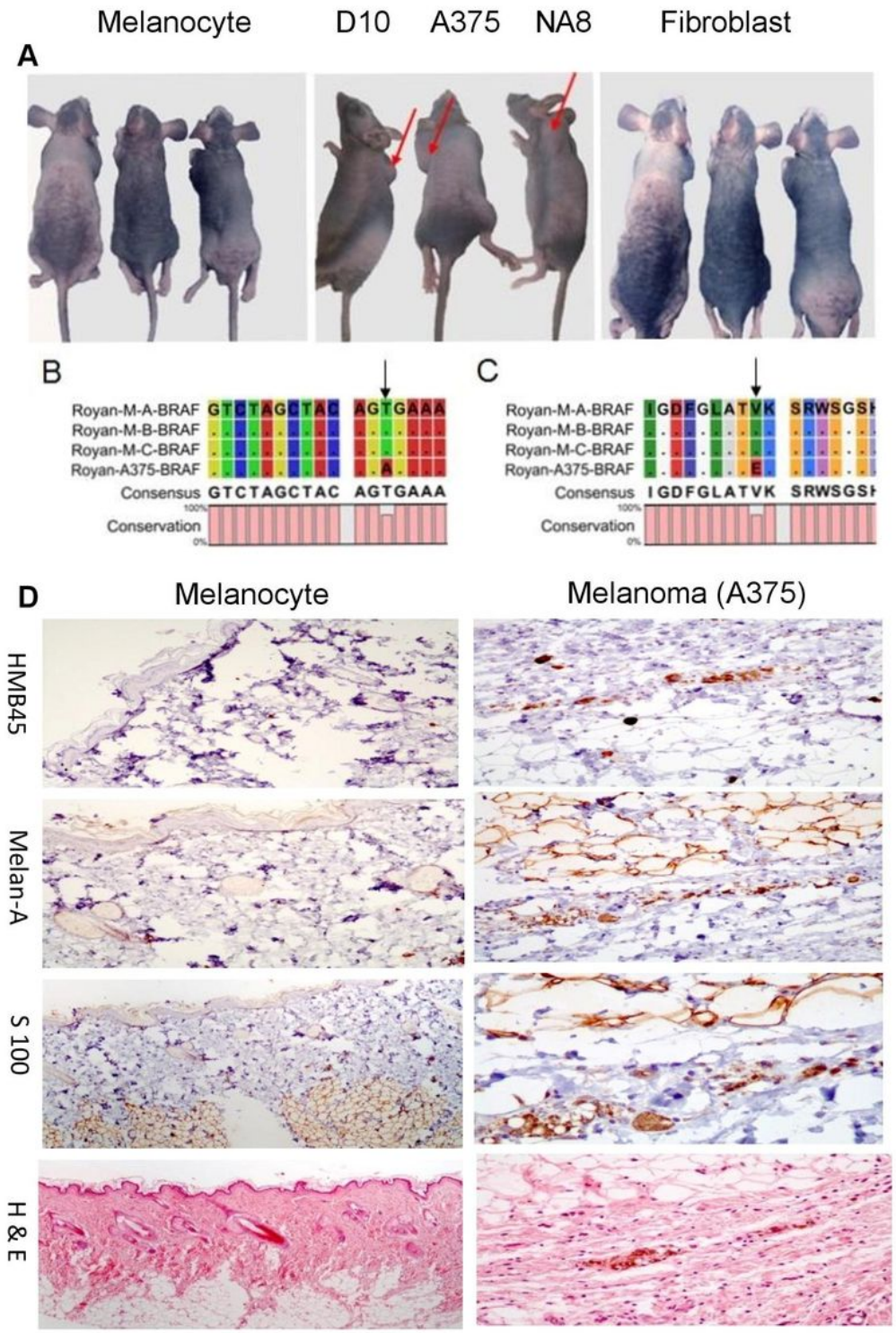

Figure 7

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. (A) Cultured melanocytes and fibroblasts showed no signs of tumor formation after 16 weeks whereas sites injected with A375, D10, and NA8 generated palpable tumors. Red arrows show the palpable tumors. (B) Nucleic acid multiple-sequence alignment of the *BRAF* gene. (C) Amino acid

multiple-sequence alignment of the *BRAF* gene from melanoma and melanocyte samples showed the presence of a mutation (c.1799T>A) at codon 600. (D) DNA sequence chromatograms of the *BRAF* gene from A375 and melanocyte samples.

(D) Immunofluorescence staining on skin nude mice after cultured melanocyte transplantation and melanoma cell lines showed that melanoma expressed Melan-A, S100, HMB-45 markers.