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A short guide on the selection of melanocytes and melanoma cells' isolation procedures for cancer research

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ABSTRACT

The skin contains about 5% of melanin-producing cells called melanocytes. The analysis of melanocytes is of greatest interest for multiple fields of dermatology, especially those concerning the detailed molecular mechanism of melanogenesis and melanin transfer to keratinocytes. An effective and simple method aimed at the culture of melanocytes is also essential for the investigation regarding melanoma development, the deadliest skin cancer. The main purpose in the current melanoma research is focused on the better understanding of the metastasis process, which can lead to the development of new techniques concerning early diagnosis as well as an effective treatment. Because differences and similarities in cell properties can be observed simultaneously between the clinical samples, gaining general knowledge about the melanoma phenomenon, particularly in case of the unusual tumors, is a priority.

Unfortunately, due to the low percentage of melanocytes in the skin, the establishment of a pure melanocyte culture (without fibroblast and keratinocyte contamination) is difficult. For this reason, access to larger amounts of patient-derived cell models and established cell lines should be assessed by laboratories which are focused on the isolation of the cells of interest themselves. Here, we present an overview of the traditional as well as innovative procedures of isolation and culture of melanocytes and melanoma cells, which will be useful for the improvement of the existing laboratory protocols, as well as it may inspire novel laboratories to enlarge the spectrum of their scientific interest in melanoma research.

Keywords: melanocytes, melanoma, cell isolation, procedures

INTRODUCTION

Cancer remains as one of the biggest threats to human wellbeing, therefore novel diagnostic tools and procedures for its detection should be developed. The establishment of a quick but trustworthy test for the identification of cancer biomarkers in the human organism is intensively conducted by various scientists (Sobiepanek and Kobiela 2018). The availability of many different cell lines (from normal to the cancerous types being at different stages of progression) is of great importance for the researches performed *in vitro*. One of the main scientific objectives is focused on the better understanding of the metastasis process, what is indispensable for further development of novel techniques aimed at early diagnosis and effective treatment *in vivo*. To help with this issue and at the same time to better comprehend the metastasis process as well as to develop an effective treatment, access to larger numbers of melanoma models like primary cells, cell lines, mouse models and computer simulations (theoretical models) is essential. Since each model has its limitation (e.g. the *in vitro* models lack responsiveness from the immune system), application of several models at the same time of the study may help in a detailed understanding of cancer or its response to the proposed treatment (Madhunapantula et al. 2019). On the other hand, some cases of cancer may be unique (e.g. unusual or rare mutation, very aggressive tumor cells), thus cell isolation would be beneficial for further research and highly recommended. Furthermore, the isolation of the normal and cancerous cells from the same patient would be of tremendous value to widen the knowledge about tumor development and changes occurring between both types of cells. In this article, our interest will be focused on melanocytes and melanoma cells.

Melanocytes are the cells of neuroectodermal origin, which can be found in the skin, brain, eyes, ears, lungs, heart, hair and in some mucous membranes. Their broad spectrum of functions depends on their location in the human body, e.g. in the brain melanocytes may be involved in the process of detoxification; in the heart they play a role in the reduction of free radicals and anti- inflammatory processes; while in the skin,

hair and eyes melanocytes provide protection against the ultraviolet radiation due to melanin synthesis during melanogenesis. In the epidermis melanocytes constitute only 5% of all types of the skin cells, which contributes to about 1,200 melanocytes per mm^2 of the skin (independently of the human race), thus isolation and culture of the pure melanocytes may be of great challenge for many scientists (Marczyńska and Przybyło 2013, Wikramanayake et al. 2014, Rzepka et al. 2016, Johansen 2017).

Melanoma arises from melanocytes as a result of mutation/s and subsequent uncontrolled cell proliferation. Among skin cancers, melanoma represents the most malignant type, with the highest rate of mortality. According to the World Health Organization, nearly 288,000 of melanoma cases were diagnosed in 2018 worldwide and the occurrence of this disease in 2025 may be even greater and reach approximately 340,000 cases per year (nearly 20% more cases). The majority of the melanoma cells sustain their ability to melanin synthesis; thus, they form pigmented melanoma – typically brown or black. In rare cases with disrupted melanin synthesis, amelanotic melanoma may also occur. Many different clinical subtypes of melanoma have been described, but the most frequent are: acral lentiginous melanoma (ALM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and superficial spreading melanoma (SSM). On the other hand, the development of melanoma consists of six steps: (i) the common acquired melanocytic nevus, (ii) a melanocytic nevus with lentiginous melanocytic hyperplasia, (iii) a melanocytic nevus with aberrant diffe-rentiation and melanocytic nuclear atypia, (iv) the radial growth phase (RGP) of the primary melanoma, (v) the vertical growth phase (VGP) of the primary melanoma and (vi) metastatic melanoma (MM) (Forman et al. 2008, Dummer et al. 2016, Elder 2016, Sobiepanek et al. 2017).

Although, there are many commercially available cell lines of melanocytes and melanoma cells, the possibility of cell isolation directly from individuals allow for a comprehensive analysis of cell function and differentiation, as well as cance-rogenesis. The establishment of pure cultures of melanocytes and/or melanoma cells is indispensable for the researchers focused on the analysis of the *in vitro* relationship between melanocytes and keratinocytes (De Luca et al. 1988), mechanism of melanin production and its biosynthesis (Bao et al. 2015, Knapp and Iden 2020), melanocytes implementation in the 3D skin equivalents (Gledhill et al. 2015), as well as for the diagnostic procedure aimed at the differentiation melanomas from melanocytes *in vitro* (Fujiwara et al. 2019).

STATE OF ART

The skin is the largest organ of the human body, composed of the epidermis and the dermis built by progenitors and variously differentiated cells (De Falco et al. 2014). The main component of the outermost epidermal layer are keratinocytes. Keratinocytes undergo the differentiation process during which they maturate into corneocytes. These non-nucleated flattened and cornified cells, together with ceramides, cholesterol and free fatty acids form water impermeable layer located at the surface of the skin called *stratum corneum* (Sobiepanek et al. 2019). Other components of the epidermis are: the Langerhans cells (dendritic cells of the immune system of the integument), the Merkel cells (cells of the neural origin) and melanocytes. Melanocytes are located in the stratum basalis, the deepest layer of the epidermis located directly above the basement membrane, which separates the epidermis from the dermis (Sobiepanek et al. 2020). They are interspersed among the keratinocytes at a ratio of 1:30 depending on the body site (De Falco et al. 2014, Dehdashtian et al. 2018, Graham et al. 2019). The concept of a melanocyte interacting with a specified group of keratinocytes, creating the Epi-dermal Melanin Unit (EMU), was first proposed by Fitzpatrick and Breathnach in 1963. However, there are votes in favor to include the Langerhans cells in the physiological functional unit, which would be known as the keratinocyte-Langerhans-melanocyte (KLM) unit (Nordlund 2007).

In order to maintain the homeostasis *in vivo*, melanocytes require the presence of keratinocytes to which they bind through adhesion molecules called E-cadherins. The downregulation of their expression breaks the control of keratinocytes over melanocytes. In general, melanocytes have a small proliferative potential and rarely divide, therefore melanocyte overactivity leads to pathological conditions (Santiago-Walker et al. 2009, Marczyńska and Przybyło 2013). Moreover, changes in the cadherins expression pattern, from the E-cadherin into the

N-cadherin is observed during the epithelial-mesenchymal transition (EMT) that leads to the malignant transformation. The cadherin-switch allows for the mobility of melanocytes and for their interaction with fibroblasts, endothelial cells or themselves. This also leads to the penetration of the deeper layer of the dermis and to the further cell expansion through the blood and lymphatic vessels (Santiago-Walker et al. 2009, Marczyńska and Przybyło 2013).

Although, the molecular bases of melanoma progression are still the subject of an intensive research, it is associated with mutations in genes responsible for the proli-feration and apoptosis, as well as with epigenetic changes, loss of adhesion ability or production of the autocrine growth factors, which disturbs the signal transduction pathway in melanocytes. (Pokrywka and Lityńska 2012). The National Cancer Institute distinguishes four types of the patient-derived models (PDMs) for cancer research: the *in vitro* patient-derived tumor cell cultures (PDCs) and cancer-associated fibroblasts (CAFs), the *ex vivo* patient-derived organoids (PDOrg), as well as the *in vivo* patient-derived xenografts (PDXs). Several PDMs of melanoma and skin cancers are commercially available (6 cases of PDCs) or undergoing quality control like the Next-Generation Sequencing (NGS) and tumorigenicity verification (10 developed PDCs) (Evrard et al. 2019). The PDCs may reflect the characteristic features of the patient's tumor cells and can be used to deepen the molecular validation process e.g. gene expression profiling or immunohistochemical staining with the selected biomarkers. On the other hand, with the use of the optimal technique for melanocytes and melanoma cell isolation and culture, the variety of the available cell lines can be significantly increased (Bleijs et al. 2019). Therefore, here we summarize the spectrum of techniques used for melanocytes and melanoma cell isolation and culture described in literature and commonly used in laboratories. We believe this will be helpful for the improvement of the existing protocols or an inspiration for novel scientists who intend to perform their first experiments on melanocytes and melanoma cells.

SEARCH STRATEGY AND SELECTION CRITERIA

Literature analysis has been performed with the use of the PubMed database and the search term combinations of "melanocytes – melanoma – cell isolation – techniques – cell culture *in vitro*". For further analysis we have chosen articles in which melanocytes and/or melanoma cell isolation methods from human or animal tissues samples were well described. Moreover, during hand search in Google Scholar, some additional articles were also investigated for a better comprehension of the subject.

REVIEW AND DISCUSSION

For the *in vitro* research purpose, commercially available cell lines are frequently selected. Since 1978 over 350 human melanoma cell lines have been established and characterized in the Melanoma Research Center as well as in the Wistar Institute, which initiated major advances in melanoma research. These different cell lines, from each tumor progression stage (tab. 1), may be purchased from companies like ATCC, Biocompare, European Collection of Cell Cultures (ECACC), JCRB Cell Bank, Rockland Immunochemicals Inc. and Merck (formerly Sigma Aldrich). The establish-ment of a melanoma cell line is difficult and the success rate often depends on the stage of cancer progression. For example, the RGP melanoma cell lines are rarely developed, mainly due to the small specimen size available for isolation. On the other hand, the success rate for VGP melanomas culture ranges from 30-70% and also depends on the size of the lesion obtained during surgery. The highest success rate (75-80%) for the cell line establishment is observed for metastatic melanomas, in which the most common sources of samples are the lymph node metastases and the least common cutaneous nodules (Hsu et al. 1999). Despite the great diversity in melanoma cell lines which originnated from the different stages of melanoma progression, the establishment of the cell line models containing specific mutations, for example, in *BRAF* or *NRAS* genes, is also of great importance for scientific purposes (Schadendorf et al. 2018). An extremely valuable study can also be performed on the multiple types of cell lines isolated directly from the tumor and healthy samples of one patient. This model allows for the comparison between closely related cells that differ in morphology or in their invasive potential. Some of the related melanoma cell lines are presented in table 2.

Table 1. Examples of commercially available cell lines from different progression stages of melanoma

RGP site	VGP site	Lymph node MM	Solid tumor MM
SBC12 WM1552C WM1862 WM35	WM75 WM115 WM278 WM278 WM793 WM902B WM983A	A2058 GAK Hs 294T Ma-Mel-15 MeWo SK-MEL-1 WM266-4	451Lu A375 C32 CHL-1 Colo 794 G-361 WM1026

Table 2. Examples of the multiple cell lines developed from the same patient (Hsu et al. 1999)

Vertical Growth Phase	Metastatic Melanoma
WM75	WM373
WM115	WM165-1, WM165-2, WM239A, WM239B, WM266-1, WM266-2, WM266-3, WM266-4
WM278	WM1617
WM740V	WM858, WM873-1, WM873-2, WM873-3

In case of melanocytes, the commercially available cell lines are limited to two types of cells offered from each company: neonatal or adult skin. However, there is also a choice between cells obtained from lightly, moderately or darkly pigmented donors. Some of the established and commercially available melanocytes are listed in table 3. These cell lines are frequently used in cancer research studies. Unfortunately, the comparison of cells obtained from different donors may lead to a misinterpretation of the obtained results and mask the true mechanisms which melanoma pathogenesis undergoes. Due to this reason, the isolation of the cancerous and normal cells from the same patient becomes increasingly important.

Table 3. Examples of the commercially available melanocytes

Company	Melanocytes and their source
ATCC	Primary Epidermal Melanocytes; Normal, Human, Neonatal (HEMn) Primary Epidermal Melanocytes; Normal, Human, Adult (HEMa)
Cell Applications, INC. (distributor MERCK)	Human Epidermal Melanocytes: HEM, adult Human Epidermal Melanocytes: HEM, neonatal
Gibco™ Thermofisher Scientific	Human Epidermal Melanocytes, adult, lightly pigmented donor, (HEMa-LP) Human Epidermal Melanocytes, neonatal, lightly pigmented donor, (HEMn-LP) Human Epidermal Melanocytes, neonatal, moderately pigmented donor, (HEMn-MP) Human Epidermal Melanocytes, neonatal, darkly pigmented donor, (HEMn-DP)
PromoCell	Normal Human Epidermal Melanocytes (NHEM) Normal Human Epidermal Melanocytes 2 (NHEM 2)
Ximbio	Immortalised Human Melanocyte [PIG1] Cell Line

Ameri Research Inc. showed in 2016 that the total cost of the necessary instruments, consumables and reagents required for cell culture and isolation reached about 3.4 billion dollars and by the year of 2024 this cost may increase to even 11.5 billion dollars, what highlights the importance of the research using cell culture techniques (Ameri Research Inc.

webpage). In general, the isolation procedure consists of a few stages. First, (i) the tissue should be collected during surgery, then the sample should be stored in a dedicated transport media (ii), next the sample should be washed (iii), fragmented (iv) and digested (v) with the use of specific enzymes (e.g. collagenase, dispase, hyaluronidase, liberase or trypsin). After cell separation (vi), features of the isolated cells should be tested and confirmed by molecular methods. The most important and frequently used protocols implemented for melanocytes and melanoma cell isolation are summarized in table 4 and table 5.

Table 4. The described conditions for melanocyte isolation. The used abbreviations are: BBE – bovine brain extract, bFGF – basic fibroblast growth factor, BPE – bovine pituitary extract, CT – cholera toxin, EDTA – ethylenediaminetetraacetic acid, EGF – epidermal growth factor, FBS – fetal bovine serum, FCS – fetal calf serum, HBSS – Hanks Balanced Salt Solution, IBMX – isobutylmethyl xanthine, TPA – 12 tetradecanoylphorbol 13-acetate (otherwise known as PMA, phorbol 12-myristate 13-acetate)

The method of epidermis separation from the dermis (tissue origin)	The method of receiving a single cell suspension for seeding	The used culture medium	Reference
The tissue was incubated in 0.25% trypsin in PBS for 30 min at 37°C, next the epidermis was mechanically separated from the dermis (human)	A single-cell suspension was obtained by gentle agitation in the medium	McCoy's 5A medium with 10% CS, 1 nM CT and 0.33 µM IBMX	Tsuji and Karasek 1983
The tissue was incubated in Eagle's minimal essential medium without calcium (MEMS) with 0.25% trypsin, 200 U/ml penicillin, 100 µg/ml streptomycin and 100 ng/ml TPA at 4°C overnight (human)	The tissue was shaken vigorously in the melanocyte growth medium and the supernatant with cells was collected	Ham's F-10 medium with 10% Nu-serum, 2% CS, penicillin, streptomycin, 48 nM TPA, 2.5 nM CT and 0.1 mM IBMX	Halaban and Alfano 1984
The skin biopsy was minced and digested in 0.05% trypsin/0.01% EDTA at 37°C for 3 h (human)	The cells were collected from the received supernatant	Dulbecco-Vogt Eagle's and Ham's F12 medium (3:1) with 5% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 0.18 mM adenine, 0.4 mg/ml hydrocortisone, 1 nM CT, 10 ng/ml EGF, 20 pM triiodothyronine, 10 ng/ml PMA, 50 IU/ml BBE and 50 IU/ml penicillin/ streptomycin	De Luca et al. 1988
The tissue was cut into pieces and incubated in Eagle's minimum essential medium (EMEM) with 0.5% collagenase Type V 5% FBS for 1-2 h at 37°C (human)	The cell suspension was obtained by treating the tissue with 0.05% trypsin and 0.53 mM EDTA for 5-10 min at 37°C	EMEM with 10% FBS, 0.2 µg/m1 CT, 50 nM PMA, 0.05 mg/ml gentamicin, 2.5 µg/ml fungizone and keratinocyte serum-free medium	Tobin et al. 1995
The tissue was cut into pieces and digested in 2 mg/ml dispase at 4°C, mechanical separation of the epidermal tissue was performed with forceps (human)	The tissue was incubated in 0.25% trypsin at 37°C for 5 to 10 min	MCDB153 with 10 µg/ml bovine insulin, 10 µg/ml transferrin, 2.8 µg/ml hydrocortisone, 2 mM glutamine, 10 U/ml nystatin, 1 ng/ml vitamin E, 10 nM PMA, 100 ng/ml CT, 100 µg/ml bovine hypothalamic extract and 2% FCS chelex-treated	Goodall et al. 1994
The epidermis was separated from the dermis after an overnight incubation in 0.25% trypsin at 4°C (human)	undescribed	Medium 199 with 10 ng/ml EGF, 1 nM triiodothyronine, 10 µg/ml transferrin, 10 µg/ml insulin, 1.4 µM hydrocortisone, 1 nM CT, 10 ng/ml bFGF and 5-10% FBS	Hara et al. 1996
The tissue was cut into small pieces and incubated in 0.25% trypsin for 12 h at 4°C, and next the epidermis was mechanically separated from the dermis (sheep)	A single-cell suspension was derived by vigorous pipetting in 10% FCS from the epidermis	Melanocyte basal medium with 0.05 µg/ml amphotericin B, 1 ng/ml bFGF, 13 mg/ml BPE, 50 µg/ml gentamicin, 0.5 µg/ml hydrocortisone, 10 ng/ml PMA, 5 µg/ml bovine insulin, 1% FCS, CT	Sanchez Hanke et al. 2005
The tissue was digested with 0.2-0.4% dispase for 1-18 h depending on the thickness of the dermis (human)	The tissue was digested with 0.05% trypsin-EDTA	Melanocyte medium (254-CF)	Ghosh et al. 2008

The tissue was minced into 0.2×0.5 cm pieces, digested in 0.25% trypsin solution or in 0.05% trypsin in 0.02% EDTA at 4°C for 20 h, next the tissue was incubated at 37°C for 2 h, the mechanical separation of the epidermal tissue was performed with forceps (alpaca)	The epidermal tissue was digested in 0.25% trypsin in 0.02% EDTA for 8 min at 37°C and vigorous pipetting in Melanocyte Basal Medium + 10% CS and filtration through a 200-pore steel sifter with a pore diameter of 76 μm	Melanocyte basal medium supplemented with 0.2 μg/ml CT, 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone, 50 μg/ml BPE, 0.5 μg/ml hydrocortisone, 1 ng/ml bFGF, 5 μg/ml insulin and 10 ng/ml TPA	Bai et al. 2010
The tissue was cut into small stripes and digested in 1.07 U/ml Dispase II in HBSS at 4°C overnight, next the mechanical separation of the epidermal tissue was performed with forceps (fox)	Mechanical blowing was performed and cell suspension was obtained with a cell sieve (200 meshes)	Keratinocyte serum free medium (K-SFM) with 10% FBS and 1% penicillin- streptomycin, EGF and BPE (for 3 days); next the full medium was supplemented with PMA (for 7-10 days)	Bao et al. 2015
The subcutaneous adipose tissue was manually removed from the dermis with a scalpel, the remaining tissue was cut into small pieces and incubated in 2.4 U/ml dispase in PBS for 1.5 h, next the epidermis was mechanically separated from the dermis (human)	The tissue was treated with 0.25% trypsin and 0.53 mM EDTA for 10 min at 37°C and next the suspension was filtered through a 200 m filter	Medium 254 supplemented with a human melanocyte growth supplement (HMGS)	Zhang et al. 2017
The mechanical removal of the subcutaneous tissue, mincing and digestion with trypsin (human)	The cells were collected from the received supernatant	DermaLife medium	Fujiwara et al. 2019
The tissue was incubated in RPMI with 10%FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 nM sodium pyruvate, 10 nM non-essential amino acids and 5 mg/ml Dispase II at 4°C overnight (mouse)	The tissue incubated in TrypLE for 20 min at room temperature	RPMI-1640 with 200 nM TPA and 200 pM CT	Knapp and Iden 2020

Table 5. The described conditions for melanoma isolation. The used abbreviations are: CT – cholera toxin, FBS – fetal bovine serum, FCS – fetal calf serum, G418 – geneticin, PMA – phorbol 12- myristate 13-acetate

Applied method of melanoma cell isolation (tissue origin)	The used culture medium	Reference
The tissue was digested with dispase (1:2) in RMPI for 3 h at 37°C, and next digested with collagenase (1:100) in Tris-buffered saline containing $CaCl_2$ for 2 h at 37°C, the cell suspension was filtrated through 70- μm nylon strainers (horse)	RPMI-1640 with 10% FCS, 5 mM glutamine, 1 mM sodium pyruvate, 10,000 U/ml penicillin and 10 mg/ml streptomycin	Chapman et al. 2009
The tissue was cut into small pieces and digested with 235 U/ml collagenase and 850 U/ml hyaluronidase in RPMI+1%FBS for 2 h at 37°C, and next the suspension was filtered through 70- and 40-μm strainers (human)	RPMI-1640 with 10% FBS	Luo et al. 2013
The tissue was digested with 60 μg/ml liberase Blendzyme TM mix in Medium 199 for 1 h at 37°C and filtered through 70 μm nylon mesh (human)	Medium 199	Boiko 2013
The tissue was digested with 200 U/ml collagenase type IV for 20 min at 37°C, and next incubated with 0.05% trypsin-0.5 mM EDTA as well as 100 U/ml DNase solution for 2 h at 37°C. The suspension was filtered through a 40 μm strainer (human)	80% MCDB153 and 20% L15 supplemented with 2% FBS, 5 μg/ml insulin and 1.68 mM $CaCl_2$	Slipicevic et al. 2014
The tissue was digested with trypsin and collagenase (human)	Ham F12 medium with 10 ng/ml CT, 100 nM PMA, 100 μg/ml G418 and 50 μg/ml gentamicin	Weidmann et al. 2017
The tissue was mechanically disaggregated and next digested with 2 mg/ml collagenase type II for 2 h at 37°C, the suspension was filtered with a 70 μm nylon strainer (dog)	RPMI-1640 Glutamax growth medium with 10% FCS and 100 U/ml penicillin and 0.1 mg/ml streptomycin	Segaoula et al. 2018
The tissue was mechanically disaggregated (human)	DMEM with 10% FBS, 2 mM glutamine and a 1% penicillin/ streptomycin mixture	Heitzer et al. 2019
The mechanical removal of the subcutaneous tissue, mincing and digestion with trypsin (human)	DermaLife medium	Fujiwara et al. 2019

The study in which the cells are isolated from the tissue can begin as soon as the consent of the local Bioethics Committee for these experiments is acquired. The researcher may proceed with the following steps of the isolation procedure and adapt it to his/her needs or capabilities:

a) tissue collection during the surgery and its transport

The first two steps of the process require tissue collection after surgery (after a written consent is signed by the patient) and its transport to the destinated laboratory. The most common sources of normal melanocytes are newborn foreskin or adult skin removed for cosmetic as well as health reasons (Halaban 2005). On the other hand, the lymph nodes are the most frequent source of melanoma cells in comparison with cutaneous tissue. Typically the tissue is stored in a medium containing a mixture of an antibiotic-antimycotic solution (e.g. 400 U/ml penicillin, 400 µg/ml streptomycin or 10 µg/ml Amphotericin B) and subsequently transported to the laboratory within 24-48 hours while stored at 4°C. Prolongation of the transport time may affect the quality of the tissue sample, and in consequence, the success rate of the subsequent cell isolation and culture (McLeod and Mason 1995, Ghosh et al. 2008, Bai et al. 2010).

b) tissue preparation for cell isolation and the disaggregation step

Large tissue fragments should be rinsed with an antiseptic solution (e.g. 2.5 mg/ml iodine solution, 70% ethanol) for 5-10 minutes. If the tissue fragments are very small, medium with 10-times greater concentration of an antibiotic solution should be used for quick rinsing only (rinsing with the antiseptic solution, like in case of the large tissue fragments, will fix the cells). Subsequently, the tissue should be washed with a Phosphate-Buffered Saline (PBS) or Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} or Mg^{2+} ions for 5-10 minutes. The subcutaneous fat should be removed from the tissue with the use of a scalpel and the tissue should be cut into smaller pieces. The size of the tissue fragment recommended for the enzymatic digestion is 1 cm x 1 cm, while for the mechanical disaggregation 1 mm x 1 mm. Furthermore, the incubation of the tissue fragments with a buffer containing ethylenediaminetetraacetic acid (EDTA; 1-50 mM depending on the cell type) may decrease the adhesion of naturally adherent cells and allow to obtain a single cell suspension (McLeod and Mason 1995, Ghosh et al. 2008, Godwin et al. 2014, Heitzer et al. 2019, Reichard and Asosingh 2019).

In order to separate the epidermis from the dermis, the enzymatic digestion (mainly with dispase and/or collagenase; for up to 2 hours at 37°C or 24 hours at 4°C) is frequently implemented. After separation of the layers, melanocytes can be found within the basal layer of the epidermis. To obtain cell suspension, the epidermis should be shortly washed with PBS or DPBS and incubated with a typsin/EDTA solution or a TrypLE reagent (up to 30 minutes at 37°C). Next, the enzyme should be inactivated and cell suspension should be filtered through a cell strainer, typically nylon or polyethylenterephthalat (PET) meshes (McLeod and Mason 1995, Halaban 2005, Godwin et al. 2014). The mesh size should be adjusted to the cell type; in case of melanocytes and melanoma cells the mesh size should be approximately 200 µm and 70 µm, respectively (Bai et al. 2010, Boiko 2013). Before cell seeding or specific cell separation, suspension after filtration should be centrifuged at 1000 g for 5-10 minutes. Next, the pellet with cells should be resuspended in the appropriate medium and cells may be counted before approaching the next step (Bai et al. 2010, Zhang et al. 2017).

Another approach in order to obtain cell culture is to attach the tissue fragment to the culture dish, the so-called skin explants method, however it is rarely used (Tobin et al. 1995).

c) the separation of different cell types and cell culture

Once cell suspension is assessed, the desired method of cell separation may be implemented without the cell culture. Cells coupled with fluorochromes or magnetic particles, may be separated by the use of the flow cytometry analysis: the fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), respectively (Hu et al. 2016). For this purpose, the presence of particular biomarkers expressed on the cell surface should be known to the investigators. For example, in order to sort cells of melanotic origin, a CD117 marker (the transmembrane tyrosine kinase growth factor receptor) is frequently used (Fujiwara et al. 2019). Other biomarkers specific for the detection of melanoma cells are CD45, CD31,

CD2, CD3, Glycophorin A, EpCAM (Epithelial Cell Adhesion Molecule) (Boiko 2013). After sorting, the obtained melanocytes or melanoma cells may be cultured or used directly for the planned experiments.

Cells may be seeded into 6-well plates (usually at a density of 1×10^5 cells per well) in a complete growth medium. For melanocytes and melanoma cell cultures, many different commercially available media may be used like DMEM (Dulbecco's Modified Eagle Medium), RPMI-1640 (Roswell Park Memorial Institute Medium), Ham's F10, Ham's F12, Medium 199 or MCDB153, almost all supplemented with fetal bovine serum (FBS, 1-10%) or fetal calf serum (FCS, 1-10%) and an antibiotic mixture (see Tables 4 and 5). In some cases, the ready-to-use media were applied like DermaLife (for both cell types) or Medium 254 supplemented with HMGS (in case of melanocytes only). For the stimulation of melanocyte growth *in vitro* four mitogens may be added to the growth medium: TPA (12-O-tetradecanoylphorbol 13-acetate; otherwise known as phorbol 12-myristate 13-acetate, PMA), cholera toxin (CT), endothelin 1 (ET1) and stem cell factor (SCF) (Bai et al. 2010, Godwin et al. 2014). Cholera toxin decreases the growth of cells typically stimulated by serum added to the medium. Therefore, in the presence of CT, the growth of fibroblasts, the most frequent contaminant of the melanocytes culture, is signify-cantly reduced. However, in the case of metastatic melanoma cultures, the addition of the CT will influence their morphology. On the other hand, fibroblasts can proliferate in the TPA-supplemented medium even in the presence of CT, that is why a selective combination of the growth factors is required to minimalize the proliferation of the contaminant cells and stimulate the desired cells (Halaban 2005).

In some cases, the co-culture may be separated due to the differential trypsin digestion as it is noted in case of keratinocytes-melanocytes cell culture (Ścieżyńska et al. 2019). The application of low concentrated trypsin (e.g. 0.05%) for a short time (up to 5 minutes) allows to collect only melanocytes or melanoma cells present in the culture. In order to detach keratinocytes, a longer time of incubation (10-15 minutes) with trypsin is needed (Fujiwara et al. 2019). Unfortunately, the separation of melanocytes from fibroblasts causes more difficulties (a similar trypsinization time) and requires cell incubation with a selective agent like geneticin (G418). It is an aminoglycoside antibiotic, which eliminates the quickly dividing cells (in this case fibroblasts) from the mixed cell culture by blocking the polypeptide synthesis. The effective concentration of geneticin is described usually as 100 μg/ml which should be added to the cell culture in the full growth medium for 2-3 days (Halaban and Alfano 1984, Zhang et al. 2017). Next, pure cell cultures may undergo experiments which confirm their melanotic origin.

d) confirmation of the cells' origin

To confirm the origin of the cell, specific biomarkers characteristic for each cell type should be selected. In this step the biomarkers must be expressed by the cells, in contrast to the cell sorting methods where the biomarkers had to be present on the cell surface. For this reason, the real-time polymerase chain reaction (qPCR) as well as immunohistochemical (IHC) or immunofluorescent (IF) cell staining methods are typically used. The selection of genes and proteins is strictly determined due to the possible false positive results. If cells express melanocyte markers (genes *MLANA, MITF, DCT, TYR;* positive staining of the S100 protein family or of the HMB45-antigen), they are qualified as cells of melanocytic origin (Li et al. 2012, Weinstein et al. 2014). In case of the accompanying cells, their presence in the culture can be confirmed by biomarkers like the cytokeratin family for keratinocytes (Guo and Jahoda 2009) and the collagen family for fibroblasts (Nissen 2019).

Another frequently used approach is based on the detection of tyrosinase activity in the cultured cells of melanocytic origin, a specific biochemical marker of these cells. The DOPA assay allows not only to confirm the purity of the melanocyte/melanoma culture but also the phenotypic characteristics of the cells. To determine the ratio between melanocytes and keratinocytes in co-culture experiments, cells must be grown on coverslips, then fixed, permeabilized, subjected to the reaction with L-DOPA and scored for positivity (brown stained cells) under the optical microscope (De Luca 1988, Ghosh et al. 2008, Bai et al. 2010).

The presence of mycoplasma contamination in the established cell lines should be checked before cell banking or further cell culturing. A frequently applied method to detect most

species of mycoplasma (*M. fermentans, M. hyorhinis, M. arginini, M. orale, M. salivarium, M. hominis, M. pulmonis, M. arthridtidis, M. bovis, M. pneumoniae, M. pirum, M. capricolum*) is PCR due to its sensitivity and specificity. The second approved method is DNA staining with DAPI (4,6-diamidino-2-phenylindole-dihydrochloride), Hoechst 33258 or Hoechst 33342. Although it is an easy and rapid test, the interpretation of results in some cases can be difficult and require experience (Nikfarjam and Farzaneh 2012).

e) cell banking

As soon as the origin and purity of the isolated cells is confirmed, they should be frozen before direct experiments are performed. This step is important due to the fact that cells have a limited lifespan in culture (Hayflick 1965). Melanocytes derived from newborn foreskin can proliferate up to 18 passages in culture, whereas cells derived from adult skin only up to 5 passages. Some components of the growth medium may also prolong the lifespan of these cells before the signs of senescence appear (e.g. bFGF, HGF/SF and ETl) (Halaban 2005). As a consequence, only cells at a low number of passages are suitable for functional studies and the sooner the cells will be frozen, the more experiments can be performed with their use (Dell'Anna and Cario-André 2019).

Cells may be stored in liquid nitrogen for a long period of time and may be sub-sequently, easily transported in dry ice. The recommended concentration of dimethyl sulfoxide (DMSO) in the freezing medium (full growth medium) is 7.5% (v/v), while cell density should be in the range of $0.5\text{-}1\times10^6$ cells/ml (Godwin et al. 2014).

SHORT CONCLUSION

Although the isolation of cells is time-consuming, it is highly recommended in case of unique cell types or cells with low commercial access (e.g. melanocytes). Primary cells allow for a better understanding of cell interactions, their function and changes occurring during cell differentiation or tumor development. The isolation of melano-cytes and melanoma cells may be performed according to several protocols. The basic steps include: tissue collection during surgery, securing the sample in proper conditions for their transport, preparation of the tissue for subsequent fragmentation, digestion, cell separation, cell culture and confirmation of the cell origin. Each step may be adjusted to the researchers needs based on the described conditions.

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REFERENCES

Bai R., Sen A., Yu Z., Yang G., Wang H., Fan R., Lv L., Lee K.-B., Smith G.W., Dong C**., Validation of Methods for Isolation and Culture of Alpaca Melanocytes: A Novel Tool for *In vitro* Studies of Mechanisms Controlling Coat Color**, Asian Australas. J. Anim. Sci 23, 2010, 430-436.

Bao J., Wang L., Wang G., Liu X., Yang F., **Isolation and Culture of Melanocytes from the Arctic Fox (Alopex Lagopus)**, Italian Journal of Animal Science 14, 2015, 4005.

Bleijs M., Wetering M., Clevers H., Drost J., **Xenograft and organoid model systems in cancer research**, EMBO J 38, 2019.

Boiko A.D., **Isolation of Melanoma Tumor-Initiating Cells from Surgical Tissues**, in: Has, C., Sitaru, C. (Eds.), Molecular Dermatology, Methods in Molecular Biology. Humana Press, Totowa, NJ, 2013, 253-259.

Chapman S.W.K., Metzger N., Grest P., Feige K., von Rechenberg B., Auer J.A., Hottiger M.O., **Isolation, establishment, and characterization of ex vivo equine melanoma cell cultures**, In Vitro Cell.Dev.Biol.-Animal 45, 2009, 152-162.

De Falco M., Pisano M.M., De Luca A., **Embryology and Anatomy of the Skin**, in: Baldi, A., Pasquali, P., Spugnini, E.P. (Eds.), Skin Cancer: A Practical Approach, Current Clinical Pathology, Springer, New York, NY, 2014, 1-15.

De Luca M., D'Anna F., Bondanza S., Franzi A.T., Cancedda R., **Human epithelial cells induce human melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis**, The Journal of Cell Biology 107, 1988, 1919-1926.

Dehdashtian A., Stringer T.P., Warren A.J., Mu E.W., Amirlak B., Shahabi L., **Anatomy and Physiology of the Skin**, in: Riker, A.I. (Ed.), Melanoma: A Modern Multidisciplinary Approach, Springer International Publishing, Cham, 2018, 15-26.

Dell'Anna M.L., Cario-André M., ***In Vitro* Study of Vitiligo**, in: Picardo, M., Taïeb, A. (Eds.), Vitiligo, Springer International Publishing, Cham, 2019, 225-236.

Dummer R., Siano M., Hunger R.E., Lindenblatt N., Braun R., Michielin O., Mihic-Probst D., von Moos R., Najafi Y., Guckenberger M., Arnold A., **The updated Swiss guidelines 2016 for the treatment and follow-up of cutaneous melanoma,** Swiss Med Wkly 146, 2016, w14279.

Elder D.E., **Melanoma progression**, Pathology 48, 2016, 147-154.

Evrard Y.A., Alcoser S.Y., et al., **Comparison of PDX, PDC, and PDOrg models from the National Cancer Institute's Patient-Derived Models Repository (PDMR)**, 2019.

Forman S.B., Ferringer T.C., Peckham S.J., Dalton S.R., Sasaki G.T., Libow L.F., Elston D.M., **Is superficial spreading melanoma still the most common form of malignant melanoma?** Journal of the American Academy of Dermatology 58, 2008, 1013-1020.

Fujiwara S., Nagai H., Jimbo H., Jimbo N., Tanaka T., Inoie M., Nishigori C., **Gene Expression and Methylation Analysis in Melanomas and Melanocytes From the Same Patient: Loss of NPM2 Expression Is a Potential Immunohistochemical Marker for Melanoma**, Front. Oncol. 8, 2019, 675.

Ghosh D., Shenoy S., Kuchroo P., **Cultured Melanocytes: From Skin Biopsy to Transplantation**, Cell Transplant 17, 2008, 351-360.

Gledhill K., Guo Z., Umegaki-Arao N., Higgins C.A., Itoh M., Christiano A.M., **Melanin Transfer in Human 3D Skin Equivalents Generated Exclusively from Induced Pluripotent Stem Cells**, PLoS ONE 10, 2015, e0136713.

Godwin L.S., Castle J.T., Kohli J.S., Goff P.S., Cairney C.J., Keith W.N., Sviderskaya E.V., Bennett D.C., **Isolation, Culture, and Transfection of Melanocytes**, Current Protocols in Cell Biology 63, 2014.

Goodall T., Buffey J.A., Rennie I.G., Benson M., Parsons M.A., Faulkner M.K., MacNeil S., **Effect of melanocyte stimulating hormone on human cultured choroidal melanocytes, uveal melanoma cells, and retinal epithelial cells**, Invest. Ophthalmol. Vis. Sci. 35, 1994, 826-837.

Graham H.K., Eckersley A., Ozols M., Mellody K.T., Sherratt M.J., **Human Skin: Composition, Structure and Visualisation Methods**, in: Limbert, G. (Ed.), Skin Biophysics: From Experimental Characterisation to Advanced Modelling, Studies in Mechanobiology, Tissue Engineering and Biomaterials, Springer International Publishing, Cham, 2019, 1-18.

Guo A., Jahoda C.A.B., **An improved method of human keratinocyte culture from skin explants: cell expansion is linked to markers of activated progenitor cells**, Experimental Dermatology 18, 2009, 720-726.

Halaban R., **Culture of Melanocytes from Normal, Benign and Malignant Lesions**, in: Pfragner, R., Freshney, R.I. (Eds.), Culture of Human Tumor Cells, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2005, 289-318.

Halaban R., Alfano F.D., **Selective elimination of fibroblasts from cultures of normal human melanocytes**, In Vitro 20, 1984, 447-450.

Hara M., Toyoda M., Yaar M., Bhawan J., Avila E.M., Penner I.R., Gilchrest B.A**., Innervation of melanocytes in human skin**, The Journal of Experimental Medicine 184, 1996, 1385-1395.

Hayflick L., **The limited in vitro lifetime of human diploid cell strains**, Experimental Cell Research 37, 1965, 614-636.

Heitzer E., Groenewoud A., Meditz K., Lohberger B., Liegl-Atzwanger B., Prokesch A., Kashofer K., Behrens D., Haybaeck J., Kolb-Lenz D., Koefeler H., Riedl S., Schaider H., Fischer C., Snaar-Jagalska B.E., de'Jong D., Szuhai K., Zweytick D., Rinner B., **Human melanoma brain metastases cell line MUG-Mel1, isolated clones and their detailed characterization**, Sci Rep 9, 2019, 4096.

Hsu M.-Y., Elder D.E., Herlyn M., **Melanoma: The Wistar Melanoma (WM) Cell Lines**, in: Masters, J.R.W., Palsson, B. (Eds.), Human Cell Culture: Cancer Cell Lines Part 1, Human Cell Culture, Springer Netherlands, Dordrecht, 1999, 259-274.

Hu P., Zhang W., Xin H., Deng G., **Single Cell Isolation and Analysis**, Front. Cell Dev. Biol. 4, 2016.

Johansen C., **Generation and Culturing of Primary Human Keratinocytes from Adult Skin**, Journal of Visualized Experiments 130: 56863, 2017.

Knapp S.K., Iden S., **Melanocyte differentiation and epidermal pigmentation is regulated by polarity proteins (preprint)**, Cell Biology, 2020.

Li L., Fukunaga-Kalabis M., Herlyn M., **Isolation and Cultivation of Dermal Stem Cells that Differentiate into Functional Epidermal Melanocytes**, in: Mitry, R.R., Hughes, R.D. (Eds.), Human Cell Culture Protocols, Methods in Molecular Biology, Humana Press, Totowa, NJ, 2012, 15-29.

Luo Y., Nguyen N., Fujita M., **Isolation of Human Melanoma Stem Cells Using ALDH as a Marker**, Current Protocols in Stem Cell Biology 26, 2013.

Madhunapantula S.R.V., Patel T.N., Annageldiyev C., Sharma A., **Animal (mouse) models of melanoma**, in: Animal Models in Cancer Drug Discovery, Academic Press, 2019, 401-417.

Marczyńska D., Przybyło M., **Melanocyty – komórki barwnikowe o wielu obliczach**, Kosmos 62, 2013, 491-499.

McLeod S.D., Mason R.S., **Isolation of enriched human melanocyte cultures from fetal, newborn and adult skin**, Methods Cell Sci 17, 1995, 187-193.

Nikfarjam L., Farzaneh P., **Prevention and Detection of Mycoplasma Contamination in Cell Culture**, Cell Journal 13, 4, 2012, 203-12.

Nissen N.I., Karsdal M., Willumsen N., **Collagens and Cancer associated fibroblasts in the reactive stroma and its relation to Cancer biology**, J Exp Clin Cancer Res 38, 2019, 115.

Nordlund J.J., **The Melanocyte and the Epidermal Melanin Unit: An Expanded Concept**, Dermatologic Clinics 25, 3, 2007, 271-81.

Pokrywka M., Lityńska A., **Celując w czerniaka**, Targeting the melanoma 39, 2012.

Reichard A., Asosingh K., **Best Practices for Preparing a Single Cell Suspension from Solid Tissues for Flow Cytometry**, Cytometry 95, 2019, 219-226.

Rzepka Z., Buszman E., Beberok A., Wrześniok D., **Od tyrozyny do melaniny: ścieżki sygnalizacyjne i czynniki regulujące melanogenezę**, Postepy Hig Med Dosw 70, 2016, 695-708.

Sanchez Hanke M., Kief S., Leuwer R., Koch U., Moll I., Brandner J.M., **In vitro Isolation and Cell Culture of Vestibular Inner Ear Melanocytes**, Audiol Neurotol 10, 2005, 191-200.

Santiago-Walker A., Li L., Haass N.K., Herlyn M., **Melanocytes: From Morphology to Application**, Skin Pharmacol Physiol 22, 2009, 114-121.

Schadendorf D., van Akkooi A.C.J., Berking C., Griewank K.G., Gutzmer R., Hauschild A., Stang A., Roesch A., Ugurel S., **Melanoma**, Lancet 392, 2018, 971–984.

Scolyer R.A., Long G.V., Thompson J.F., **Evolving concepts in melanoma classification and their relevance to multidisciplinary melanoma patient care**, Molecular Oncology 5, 2011, 124-136.

Segaoula Z., Primot A., Lepretre F., Hedan B., Bouchaert E., Minier K., Marescaux L., Serres F., Galiègue-Zouitina S., André C., Quesnel B., Thuru X., Tierny D., **Isolation and characterization of two canine melanoma cell lines: new models for comparative oncology**, BMC Cancer 18, 2018, 1219.

Slipicevic A., Somasundaram R., Sproesser K., Herlyn M., **Isolation of Melanoma Cell Subpopulations Using Negative Selection**, in: Thurin, M., Marincola, F.M. (Eds.), Molecular Diagnostics for Melanoma, Methods in Molecular Biology, Humana Press, Totowa, NJ, 2014, 501-512.

Sobiepanek A., Baran J., Milner-Krawczyk M., Kobiela T., **Different Types of Surface modification used for Improving the Adhesion and Interactions of Skin Cells**, OAJBS 2, 2020, 275-278.

Sobiepanek A., Galus R., Kobiela T., **Application of the tape stripping method in the research on the skin condition and its diseases**, Review and Research on Cancer Treatment 5, 2019, 4-14.

Sobiepanek A., Kobiela T., **Application of biosensors in cancer research**, Review and Research on Cancer Treatment 4, 2018, 4-12.

Sobiepanek A., Milner-Krawczyk M., Lekka M., Kobiela T., **AFM and QCM-D as tools for the distinction of melanoma cells with a different metastatic potential**, Biosensors and Bioelectronics, 93, 2017, 274-281.

Ścieżyńska A., Nogowska A., Sikorska M., Konys J., Karpińska A., Komorowski M., Ołdak M., Malejczyk J., **Isolation and culture of human primary keratinocytes – a methods review**, Exp Dermatol, 2019, 1-6.

Tobin D.J., Colen S.R., Bystryn J.-C., **Isolation and Long-Term Culture of Human Hair-Follicle Melanocytes**, Journal of Investigative Dermatology 104, 1995, 86-89.

Tsuji T., Karasek M., **A Procedure for the Isolation of Primary Cultures of Melanocytes from Newborn and Adult Human Skin**, Journal of Investigative Dermatology 81, 1983, 179-180.

Weidmann C., Pomerleau J., Trudel-Vandal L., Landreville S., **Differential responses of choroidal melanocytes and uveal melanoma cells to low oxygen conditions,** Mol. Vis. 23, 2017, 103-115.

Weinstein D., Leininger J., Hamby C., Safai B., **Diagnostic and prognostic biomarkers in melanoma,** J Clin Aesthet Dermatol 7, 2014, 13-24.

Wikramanayake T.C., Stojadinovic O., Tomic-Canic M., **Epidermal Differentiation in Barrier Maintenance and Wound Healing**, Advances in Wound Care, 2014, 3: 272-80.

Zhang R., Liu Q., Cheng S., Zhu J., **The Clonal Growth Behavior of Melanocytes Derived From Melanocyte Stem Cells in Glabrous Skin**, JDC 1, 2017.