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REVIEW

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Recent Advances in Studies of Skin Color and Skin Cancer

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The relationship between skin color and skin cancer is well established: the less melanin in one's skin the greater the risk for developing skin cancer. This review is in two parts. First, we summarize the current understanding of the cutaneous pigmentary system and trace melanin from its synthesis in the pigment cell melanosomes through its transfer to keratinocytes. We also present new methods for reducing melanin content in hyper-pigmented areas of skin such as solar lentigenes, melasma, and post-inflammatory hyperpigmentation. Second, we present evidence that at least one mechanism for the development of metastatic melanoma and other solid tumors is fusion and hybridization of leucocytes such as macrophages with primary tumor cells. In this scenario, hybrid cells express both the chemotactic motility of the leucocyte and the de-regulated cell division of the tumor cell, causing the cells to migrate a deadly journey to lymph nodes, distant organs, and tissues.

INTRODUCTION: SKIN COLOR

Melanin Biosynthesis

Melanin is synthesized in melanocytes (pigment cells) in sub-cellular organelles known as melanosomes. In the process of skin pigmentation melanosomes are transferred from melanocytes in the basal layer of the epidermis to overlying keratinocytes (Figure 1). The transfer involves a unique biological process involving organelle donation from one cell to another and is a crucial step in skin pigmentation. Individuals with defects in transfer can have markedly reduced skin melanin content. Melanosome transfer begins with attachment of melanocyte dendrites to keratinocytes followed by transfer of melanosomes through the melanocyte dendrites into the keratinocytes and finally trafficking of the melanin within keratinocytes to the supra-nuclear area of the cell. There is growing information on melanocyte to keratinocyte transfer regarding cell biology, cytokine, and hormonal signaling pathways, and the role of various peptides and proteins such as proopiomelanocortin (POMC, melanocyte stimulating hormone, MSH) [1-11].

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Abbreviations: PMOC, proopiomelanocortin; MSH, melanocyte stimulating hormone; CRH, corticotropin releasing hormone; UVR, ultraviolet radiation.

Keywords: skin color, skin cancer, melanin transfer, cytidine as melanin inhibitor, leucocyte-tumor cell hybrids

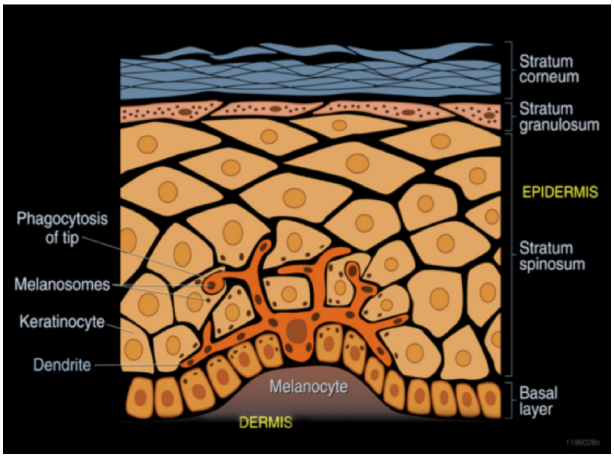

Figure 1. Shown is a diagram of a melanocyte in the basal layer of the epidermis transferring melanosomes to overlying keratinocytes.

MELANOGENESIS AFFECTS BEHAVIOR OF NORMAL MELANOCYTES, MELANOMA CELLS, AND SURROUNDING CELLS

Slominski and co-workers did a number of studies on the effects of melanogenesis and melanogenis regulatory systems on the behavior of both normal melanocytes and melanoma cells. They proposed that normal epidermal MC also are "sensory" and regulatory cells operating in the context of a regulatory network for the maintenance of human epidermal homeostasis [12].

They showed that skin expression levels of the POMC gene and POMC/corticotropin releasing hormone (CRH) peptides are not static but are determined by such factors as the physiological changes associated with hair cycle, ultraviolet radiation, immune cytokine release, or the presence of various cutaneous pathological states [13-15]. In melanoma cells melanogenesis elicits up-regulation of HIF-1-alpha [16].

In agreement with Sarna *et al*. [17] they showed that the physical/mechanical affects of loading melanoma cells with melanosomes attenuates metastasis [18]. Slominski *et al*. further showed that increased elasticity is strongly with the metastatic phenotype of melanoma cells [19].

Skin Color and UVR Damage

Ultraviolet radiation (UVR) is the chief risk factor in the induction of skin cancer. Cutaneous melanin is the most important protective factor since melanin is not only a UV absorbent but also has antioxidant and radical scavenging activities. It is well established that there is a lower incidence for skin cancer in individuals with dark skin compared to those with fair skin. Below (Figure 2a) is a photograph of an African child with albinism. If the skin is not protected from UVR, such individuals are highly susceptible to basal cell and squamous cell carcinomas, although curiously the increase in melanoma does not change substantially. Human skin is repeatedly exposed to various DNA-damaging environmental influences and therefore requires numerous endogenous mechanisms to protect against, reduce and/or repair such damage. These mechanisms include increasing epidermal thickness, DNA repair mechanisms and apoptosis, antioxidant enzymes and, last but not least, skin pigmentation. UVR is the major environmental factor that influences the function and survival of many cell types and is regarded as the main causative factor in the induction of the skin tumors above [20].

In another example, shown below (Figure 2b) are two individuals of about the same age. The woman on the left has fair skin and red hair with little melanin and her skin shows widespread sun damage on her face. The skin of the man on the right contains considerable melanin and there is no noticeable sun damage. The difference between red/blond hair is due to the pheomelanin to eumelanin ratios [21].

Cytidine Inhibits of Melanin Biosynthesis and Transfer to Keratinocytes

While the major protein regulators are glycoproteins, little is known about the roles of glycosylation in the process. Accordingly, we assembled a panel of biotinylated lectins as markers for specific glycosylation structures and used lectin histochemistry to analyze staining patterns in biopsies of human skin and co-cultures of human melanocytes and keratinocytes. One of the lectins, Elderberry Bark Lectin (EBL) showed specific staining of melanocytes and highlighted melanocyte dendrites. Melanocytes were identified in culture by their dark pigmentation and elongated dendrites. Keratinocytes were non-pigmented with punctate dendrites. Figure 3A shows a melanocyte in contact with a keratinocyte. The melanocyte plasma membrane, including dendrites, stains strongly with EBL. At points of contact with the keratinocyte, the melanocyte dendrite extends numerous filopodia that also stain with EBL (blue asterisk). A higher power view is shown in Figure 3B [21]. EBL is known to bind to the structure Neu5Ac(alpha(2,6)Gal/GalNAc)—which is the terminal sequence for some membrane-associated glycoconjugates in various biological systems [21]. Our results demonstrated that the Neu5Ac(alpha(2,6)Gal/GalNAc) sequence is at the terminus of glycans on melanocyte dendrites where it is likely to be involved with melanosome transfer to keratinocytes.

Cytidine Inhibits of EBL Binding Melanocyte-Keratinocyte Co-Cultures

Figure 2a. A child with albinism in central Africa. Due to of the lack of melanin, the boy in the foreground is far more likely to develop skin cancers than the women with dark skin as seen in the background. The photo is courtesy of Dr. Sidney Klaus.

Figure 2b. Effects of solar radiation on a fair-skinned, red headed female with very little melanin and considerable sun damage to her skin versus a male with heavily melanized skin and no apparent sun damage.

We tested for inhibitors that might affect melanocytes and keratinocytes in co-culture. Of a number of molecules showing inhibition in cell culture cytidine, a sialyltransferase inhibitor was quite effective (Figure 4). Untreated co-cultures had highly melanized melanocytes surrounded by keratinocytes. The melanocytes had close contacts with neighboring keratinocytes over large portions of the plasma membranes. The keratinocytes in direct contact with the melanocyte (nuclei with yellow asterisks) contained numerous cytoplasmic melanin granules transferred from the melanocyte. Keratinocytes not in contact with the melanocyte (nuclei with red asterisks) contained notably fewer melanosomes (Figure 4A). In contrast a representative field from a co-culture treated with cytidine showed a marked reduction in melanocyte-keratinocyte contacts and a reduction of melanosomes in both cell types (Figure 4B) [21]. These effects of EBL were seen in multiple fields, including the changes in cellular morphology. We did not test whether EBL caused an increase of melanosomes into the culture medium.

When cells were removed from the culture plates and melanin was solubilized, cytidine showed a strong inhibition of melanin content (Figure 5). Together, these results indicated that cytidine inhibited EBL binding, melanin transfer, unexpectedly, melanin synthesis [22].

Excess or uneven skin pigmentation such as seen in melasma, post-inflammatory hyperpigmentation, and solar lentigenes can cause severe anxiety and depression in affected individuals. As seen below, these findings point to new possibilities for reducing hyperpigmentation through cytidine based inhibition of the synthesis and function of oligosaccharides regulating the pigmentary system.

Cytidine Decreases Melanin Content in a Reconstituted Three-Dimensional Human Epidermal Model

The efficacy of cytidine was studied in MelanoDerm™ skin equivalents, in comparison with the positive control kojic acid and the vehicle control. Both kojic acid and cytidine demonstrated a significant reduction in melanin content relative to the vehicle control. These experiments conclude that cytidine can effectively reduce melanin content in a skin equivalence assay and suggests that cytidine may be a good candidate as a lightening agent for human skin [22] (Figure 6).

A Clinical Evaluation of the Lightening Effect of Cytidine on Hyperpigmented Skin

A randomized, vehicle-controlled study was conducted for 12 weeks on healthy Korean female subjects (Figure 7, Appendix A). Cytidine was formulated into the lotion at concentrations of 2%, 3%, and 4% (w/w) and compared to the vehicle control formulation. The clinical outcomes were evaluated by performing visual assessment grading, measuring melanin index, skin brightness, and skin color parameters. *In vitro* skin penetration studies were conducted using Franz cell chambers for the 2% cytidine test formulation [23]. The test group showed significant improvements in the visual assessment scores, melanin index, skin brightness, and skin color compared to the control group. Thus, this randomized, double-blind, 12-week clinical study successfully demonstrated the efficacy of cytidine on skin depigmentation in a dose-dependent manner, restoring the color in the application area to its original hue.

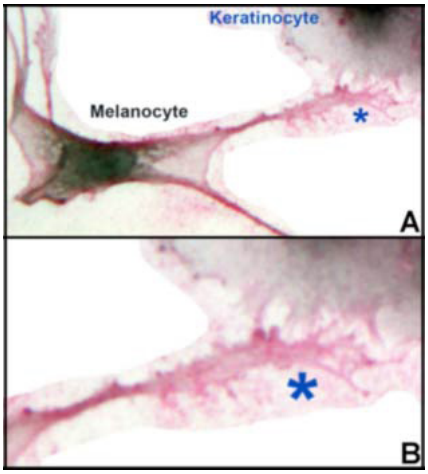

Figure 3. A photograph of a melanocyte in contact with a keratinocyte in co-culture. Cultures were stained with elderberry bark lectin by standard histochemical techniques using a red chromagen and photographed through a Zeiss light microscope. **A**. Low power photo showing EBL staining of melanocyte plasma membrane. **B**. High power of the filopodial contact points (blue asterisk) [21].

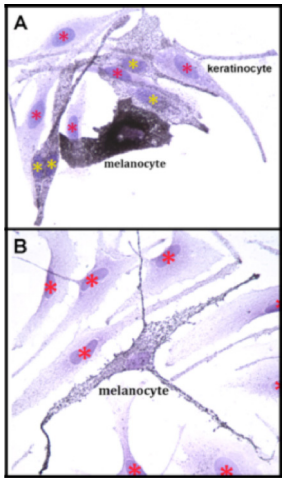

Figure 4. Effects of cytidine on melanocyte/keratinocyte interactions and melanosome transfer. Cells were incubated 72 h with cytidine, fixed in paraformaldehyde, and stained for melanin with the Fontana-Masson silver stain. **A**. Untreated control. **B**. Cytidine 50 μM [21].

CONCLUSIONS AND OUTLOOK: SKIN PIGMENTATION

There has been much progress in understanding the pathways for melanin biosynthesis and transfer to keratinocytes, however much remains, for example, the precise events following UVR exposure and generation of mutations, as well as understanding the complex signaling networks and how they interact. The discovery that cytidine can reduce melanin content in hyper-pigmented regions of skin such in those in solar lentigenes, melasma, and post-inflammatory hyperpigmentation is important because it could help in the emotional well-being of some individuals.

SKIN CANCER

Background

The predominant cause of cancer death is not the original tumor but metastases to distant organs and tissues. Leukocyte–cancer cell fusion and hybrid formation as an initiator of metastasis was proposed more than a century ago by the German anthropologist and pathologist Prof. Otto Aichel [24]. Aichel's prescient concept has since been confirmed in more than 50 animal models and more recently by our group in two patients with renal cell carcinoma and three patients with malignant melanoma. Leukocyte–tumor cell fusion is a unifying explanation for metastasis. While primary tumors arise in a wide variety of tissues representing not a single disease but hundreds of different diseases, metastatic cancer may be only one disease arising from a common, non-mutational event: fusion of primary tumor cells with leukocytes. From our studies it seems hybrid formation is a major pathway for metastasis. Aichel not only provided an explanation for metastasis but he also foresaw cancer epigenetics. His idea that a new hybrid cell would form with characteristics of both "mother cells" in today's terminology would refer to gene expression patterns from both fusion partners in the same cell. The hybrids would express the leukocyte traits of motility, chemotaxis, and homing and the de-regulated cell division of the cancer cell. Accordingly, we have been studying cancer patients who had previously received an allogeneic bone marrow transplant (BMT), usually for leukemia or lymphoma, and then later developed a solid tumor such as melanoma. By analyzing tumor cells for both donor and patient DNA, we reasoned that these cells were likely to be leukocyte–tumor cell hybrids [25].

Experimental Evidence

The first detection of leukocyte–cancer cell fusion and hybrid formation in a patient with melanoma using forensic short tandem repeat (STR) length polymorphisms to distinguish donor and patient genomes. The first evidence for leukocyte–cancer cell hybrids in a human using DNA genotyping methods came from our study of a patient who had received an allogeneic BMT for lymphoma and later developed a melanoma brain metastasis with a donor–patient hybrid genome [26]. Tumor cells were isolated by laser microdissection and sections were analyzed throughout the tumor, using forensic short tandem repeat (STR) length polymorphisms to distin-

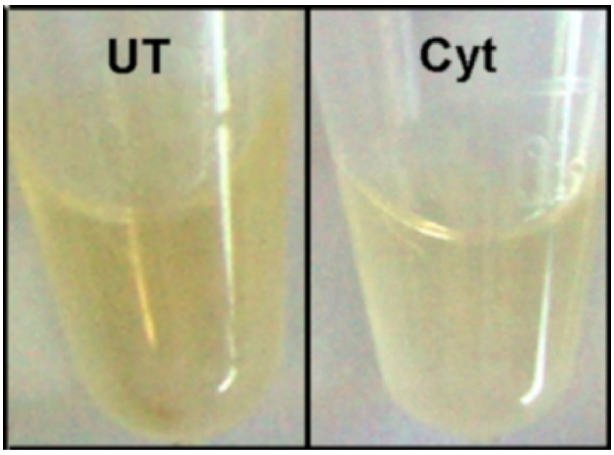

Figure 5. Effects of L-cytidine (Cyt, 50 μM) on melanin content of human melanocyte-keratinocyte co-cultures compared to an untreated control (UT). Cultures were incubated 72 h with each agent, pelleted by centrifugation, and melanin was solubilized for quantitation via spectrophotometry [21].

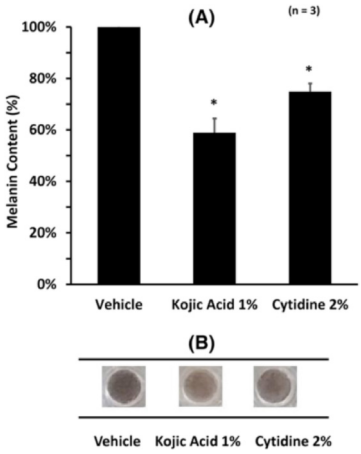

Figure 6. MelanoDerm™ tissues were treated with vehicle control, 1% kojic acid, and 2% cytidine for 14 days in triplicates. A. A graph demonstrating changes in melanin content. Data are presented as the percentage control (*p < 0.05), B Macroscopic images of the Melano Derm™ on day 14 [22].

guish donor and patient genomes. Tumor and pretransplant blood lymphocyte DNAs were analyzed for donor and patient alleles at 14 autosomal STR loci and the sex chromosomes. Eight of these loci were informative and indicated the presence of donor–patient hybrids. Figure 8 (Appendix A) shows these loci with peaks from the electropherograms designated by asterisks with the following colors: black (donor and patient), red (donor only), and blue (patient only). Both donor and patient alleles were present in tumor cells throughout the tumor (sample numbers) and the tumor appeared to consist largely if not solely of bone-marrow-derived cell (BMDC)–tumor cell hybrids. Moreover, similar allelic ratios for each locus in sections throughout the tumor indicated a clonal origin of the metastasis and suggested that the tumor was generated from a prior fusion event between a single donor BMDC and patient tumor cell. We therefore conclude that the tumor-initiating cell was a BMDC–tumor cell hybrid [27].

The second evidence for leukocyte–cancer cell hybrids came from a man who, eight years following an allogeneic BMT from his brother for treatment of chronic myelogenous leukemia, developed a nodular malignant melanoma on the upper back with spread to an axillary sentinel lymph node [26]. Combining laser microdissection with detection of STR length polymorphisms, we were able to distinguish donor and patient genomes. Tumor and pretransplant blood lymphocyte DNAs were analyzed for donor and patient alleles at 15 autosomal STR loci and the sex chromosomes. DNA analyses of the primary melanoma and the nodal metastasis revealed that they exhibited alleles at each STR locus that were consistent with both the patient and donor. The doses varied between these samples, indicative of the relative amounts of genomic DNA derived from the patient and donor. Figure 9 (Appendix A) shows genotyping results using short tandem repeats (STRs) at each of the loci of DNA from donor (D), patient (P), primary tumor, and lymph node metastasis. As with the prior cases, the evidence supports fusion and hybridization between donor and patient cells as the initiator of metastasis in this patient [26].

This information opens many potential targets for the development of new therapies, for example: (1) inhibition of the fusion process itself regarding events such as membrane attachment and heterokaryon formation; (2) inhibition of the hybridization processes involving integration of parental fusion partner genes into hybrid genomes; and (3) prevention of post-hybridization events involving activation of genes that control cell migration, chemotaxis, intravasation, extravasation, and migration to lymph nodes and distant metastases.

Several other groups have now entered this field and much progress is being reported [28]. For example, Berndt *et al*. showed that fusion between a leukocyte or fibroblast and a cancer cell fusion can cause aneuploidy and drug resistance. With loss of different chromosomes hybrid cells can have different genotypes [29]. Through the Cre-loxP-system, Searles *et al*. found that cells can rapidly deliver DNA to macrophages and fibroblasts producing hybrids. Such cells showed aneuploidy and increased clonal diversity, as well as developing chemo-resistance [30]. Mohr *et al*. investigated the factors and conditions through which plasma membranes between two different cells fuse, creating a single cell. They found that the cytokine TNF-α under hypoxia is a potent inducer of cell fusion in human MDA-MB-435 and MDA-MB-231 breast

cancer cells [31]. Gast *et al*. recently developed methods for detecting hybrids in peripheral blood of human cancer patients that correlate with disease stage and predict overall survival. They pointed out that such hybrids might be used as biomarkers to assess disease progression [32].

Macrophage Traits in Metastatic Cancer Cells

Many macrophage-like traits are expressed by metastatic cancer cells. For example, Kemény *et al*. showed that melanoma cells spontaneously fusing with macrophages and fibroblasts *in vitro* can express the phenotypes of both cell types [33]. Broncy and Paterlini-Bréchot reviewed evidence that circulating cancer cells expressed both epithelial and macrophage-specific markers [34]. These included CD14+/CD11c+ cells of myeloid lineage. B7-H4 is a cell surface antigen (encoded by the VTCN1 gene, meaning V-set domain containing T cell activation inhibitor 1 which interacts with ligands bound to receptors on the surface of T cells and has been correlated with tumor progression). CD163 protein is a member of the scavenger receptor cysteine-rich superfamily and is exclusively expressed at the cell surface by monocytes and macrophages. CD146 is the Melanoma Cell Adhesion Molecule (MCAM) that is expressed in the cytoplasm of adipose and stromal progenitor cells. The CD68 protein is a trans-membrane glycoprotein expressed by human monocytes and tissue macrophages. CD45 is the protein tyrosine phosphatase receptor type C (PTPRC) that is a trans-membrane receptor expressed by mature leukocytes. The CD14 protein is a cell surface antigen expressed on monocytes and macrophages, but also present on other subtypes of myeloid cells such as dendritic cells. CD11b is the integrin subunit alpha M (ITGAM) and CD11c to the integrin subunit alpha X (ITGAX) that are both parts of leukocyte-specific integrins. CD133 is prominin 1, a transmembrane glycoprotein which localizes to membrane protrusions and is often expressed on adult stem cells, where it is thought to function in maintaining stem cell properties by suppressing differentiation. CD204 refers to the macrophage scavenger receptor 1 (MSR1) which is a macrophage-specific trimeric integral membrane glycoprotein. CD206 is the mannose receptor C-type 1 (MRC1) a type I membrane receptor mediating endocytosis of glycoproteins by macrophages. Cytokeratins are intermediate filaments in epithelial tissues and used as a marker for epithelial cells. The epithelial cell adhesion molecule (EpCAM) is a membrane protein on most normal epithelial cells. It is a homotypic calcium-independent cell adhesion molecule. Vimentin is a type III intermediate filament protein which is responsible for maintaining cell shape and integrity of the cytoplasm in mesenchymal cells but has also recently been associated with tumor cells when expressed at the cell surface (*i.e.* cell surface vimentin, (CSV)) presenting with enlarged nuclei, CD45+ and exhibiting cytoplasmic staining by cytokeratins 8, 18, and 19 and epithelial cell adhesion molecule (EpCAM) [34].

Shabo, Svanvik *et al*. showed that macrophage traits in cancer cells are induced by macrophage-cancer cell fusion and cannot be explained simply by cellular interactions. They showed that tumor cell expression of the macrophage marker CD163 is related to poor prognosis in patients with breast cancer, colorectal cancer, and urinary bladder cancer [35-40]. Leukocyte–cancer cell fusion as a source of myeloid traits in cancer has also been discussed by Pawelek *et al*. as well as many other laboratories [25,40-48]. Following macrophage–cancer cell fusion, the resultant hybrid cells acquired new abilities to promote angiogenesis, matrix alterations, motility, chemotaxis, and immune signaling pathways. Macrophage–tumor cell fusion could explain the aneuploidy, plasticity, and heterogeneity of malignant melanoma and it could also account for epidermal–mesenchymal transition in tumor progression since macrophages are of mesodermal origin. There is considerable evidence that fusion between macrophages or other phagocytes and cancer cells causes epigenetic reprograming. Following fusion *in vitro* between weakly metastatic Cloudman S91 mouse melanoma cells and mouse or human macrophages, more than half of the resulting hybrids were more metastatic that the parental cell line. The metastatic hybrids showed increased expression of a number of macrophage-like molecules including SPARC, SNAIL, MET, MITF; integrin subunits α3, α5, α6, αv, β1, β3 [28], GnT-V (β1,6-acetylglucosaminyltransferase-V) and its enzymatic products β1,6-branched oligosaccharides conjugated to N-glycoproteins, cell-surface LAMP1, high levels of autophagy, acquired hormone inducible chemotaxis, and expression of c-Met pro-oncogene. These traits are all associated with tumor progression and poor outcome in a number of cancers [25,40-48].

SKIN CANCER CONCLUSIONS AND OUTLOOK

Thus, there is now considerable evidence from several sources that fusion and hybridization of phagocytes such as macrophages with cancer cells creates metastatic cells. Our group has demonstrated this in three patients with melanoma and two with renal cell carcinoma. In addition, several labs have made immunological observations that metastatic cancer cells exhibit many macrophage traits. Thus, it seems safe to say that this is at least one mechanism for metastasis. This confirms the century-old proposal of Prof. Otto Aichel that in retrospect was prescient indeed, especially considering that he had only a microscope with which to work. For the first

time, we can glimpse the engine that drives metastasis. A scheme for this is shown in Figure 10 (Appendix A). This information opens many potential targets for the development of new therapies, for example: (1) inhibition of the fusion process itself regarding events such as membrane attachment and heterokaryon formation; (2) inhibition of the hybridization processes involving integration of parental fusion partner genes into hybrid genomes; and (3) prevention of post-hybridization events involving activation of genes that control cell migration, chemotaxis, intravasation, extravasation, and migration to lymph nodes and distant metastases.

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APPENDIX A

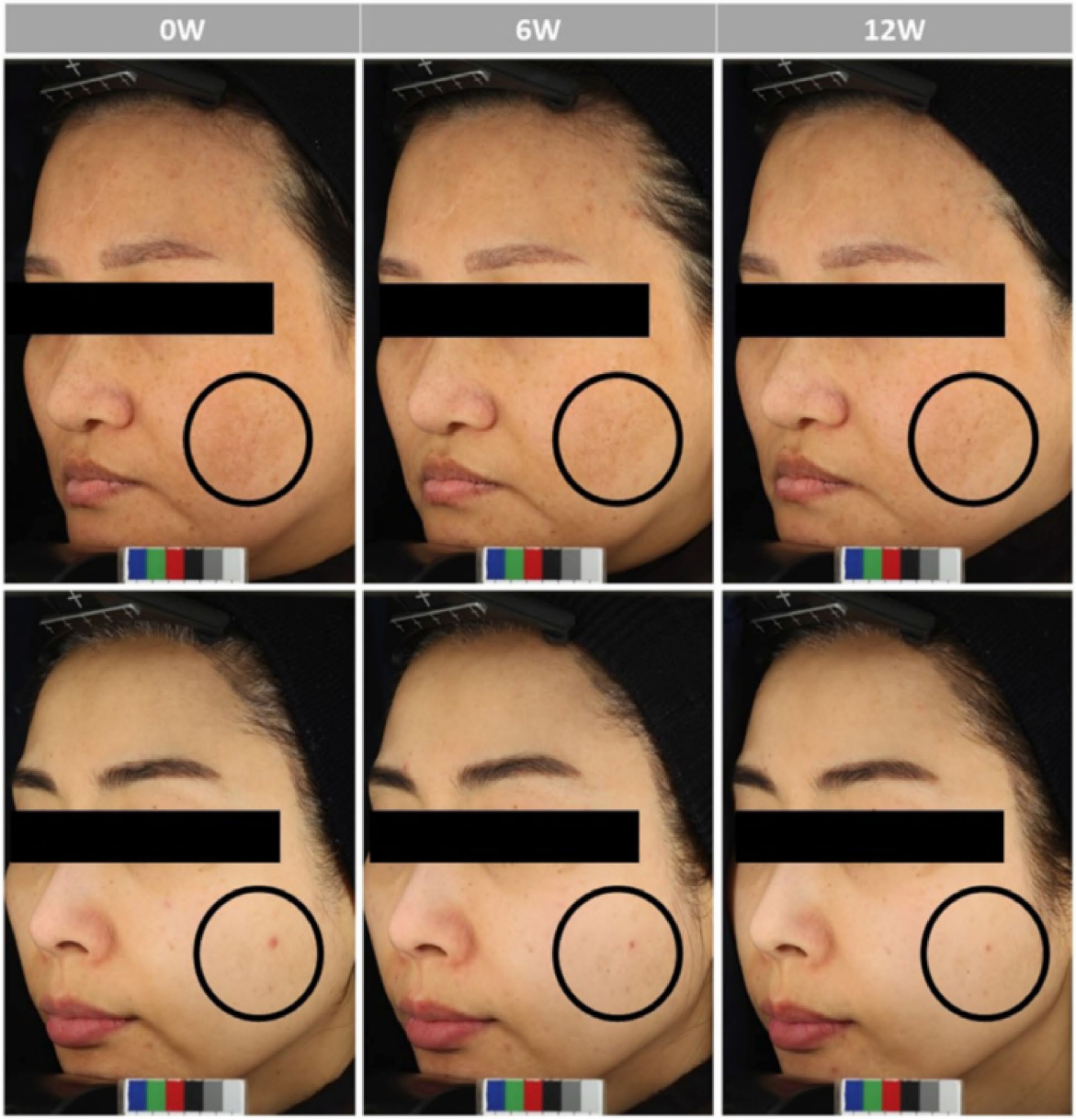

Figure 7. Photographic analyses showing skin lightening by cytidine (2% w/w) formulation in a time-dependent fashion over the 12-wk test period. The circles demonstrate the areas in question [23].

APPENDIX A

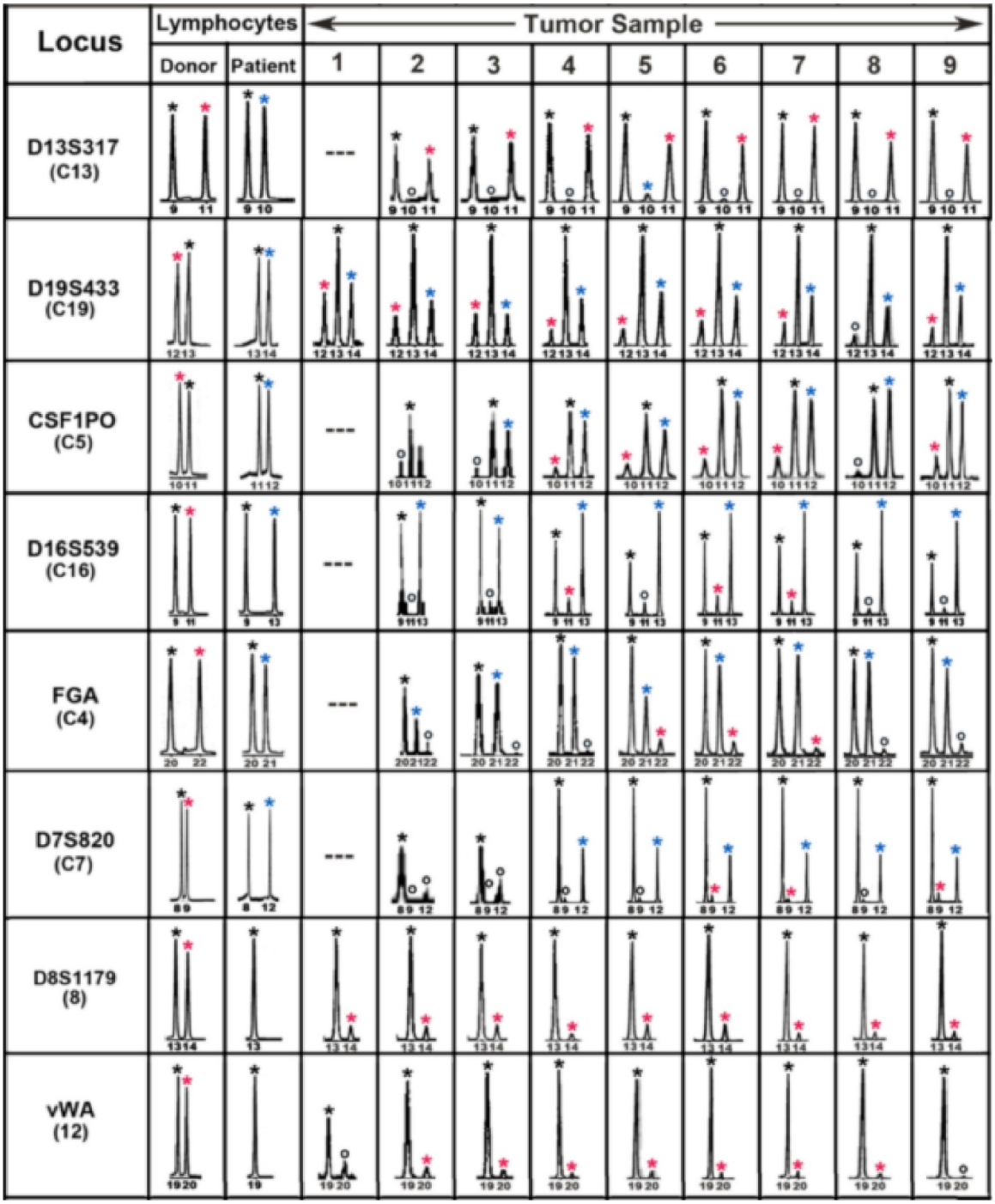

Figure 8. Forensic STR analyses of the MH3 melanoma along with donor and patient pre-BMT lymphocytes. Shown are "informative" loci exhibiting donor- and patient-specific alleles in pre-BMT lymphocytes. Tumor loci are listed in order of relative abundance of the donor-specific alleles (red asterisk) compared to patient-specific (blue asterisk) and shared alleles (black asterisk). Allele peaks, 50 relative fluorescence units were censored as "no call" (open circles). Loci with no detectable alleles after PCR amplification (—). (Used with the permission of the journal PLOS One [26].)

APPENDIX A

STR Locus	Primary Tumor	Lymph Node	Patient sample	Donor Sample
D8S1179	13,15	13,15	13,15	13
D21S11	28,29,30,30.2	28,29,30,30.2	28,29	30,30.2
D7S820	11,12	11,12,14	12,14	11
CSF1PO	9,11	10,11,12	11,12	9,10
D3S1358	15,16,18	16,18	16,18	15,16
TH01	6,7,9	6,7,9	6	7,9
D13S317	8,9,12	8,12	12	8,9
D16S539	13	11,13	11,13	13
D2S1338	16,17,18	17,19	17,19	16,18
D19S433	13,15,16	13,15,16	15,16	13,16
vWA	17,18,19	17,18	17,18	18,19
TPOX	8,9,11	8,9	8,9	8,11
D18S51	12	12,20	12,20	15,18
Amelogenin	X,Y	X,Y	X,Y	X,Y
D5S818	9,11,12	9,11,12	11	9,12
FGA	21,22,24	21,24	21,24	22,25

Figure 9. STR genotyping of DNA from donor (D), patient (P), primary tumor, and lymph node metastasis. STR units: number of tandem repeats of the locus-specific tetranucleotide sequence [18]. (Used with the permission of the journal PLOS One [26].)

APPENDIX A

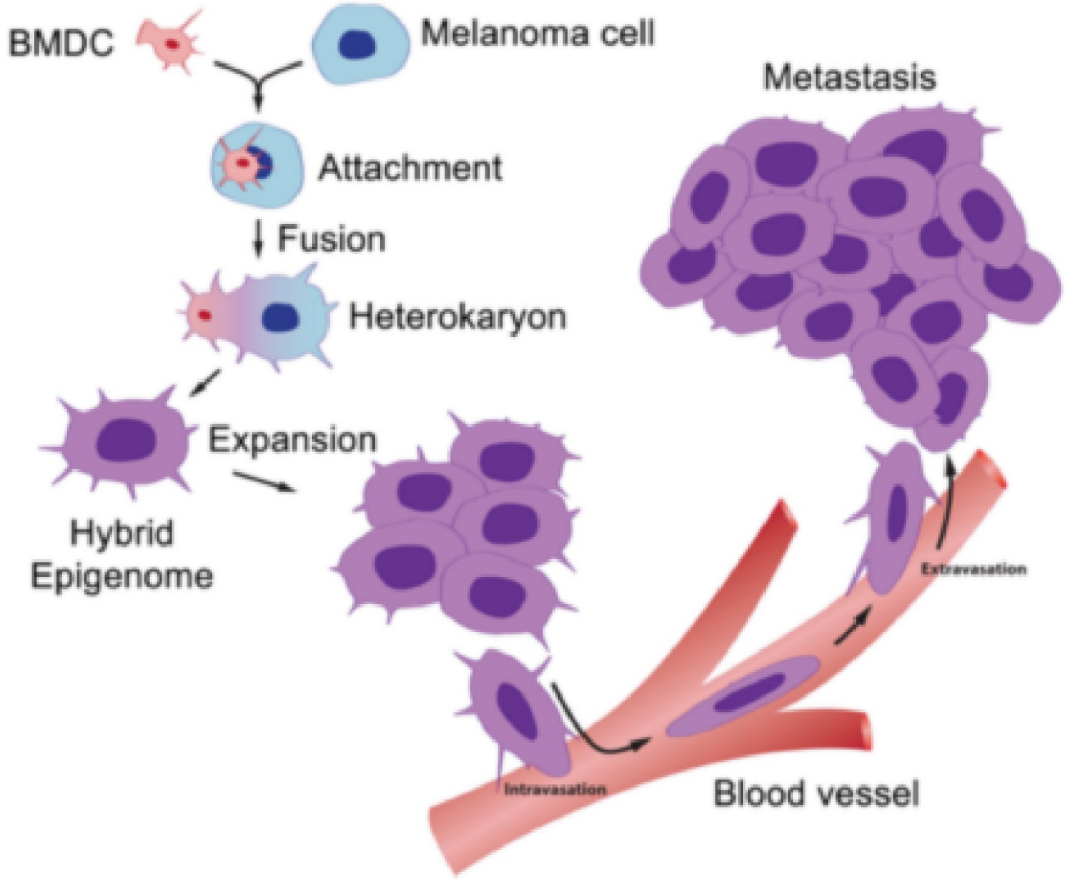

Figure 10. The leukocyte-cancer cell fusion hypothesis. A leucocyte (red) such as a macrophage approaches a cancer cell (blue). The outer cell membranes of the two cells become attached. Fusion occurs with the formation of a bi-nucleated heterokaryon having a nucleus from each of the fusion partners. The heterokaryon goes through genomic hybridization creating a melanoma-BMDC hybrid with co-expressed epigenomes, conferring deregulated cell division and metastatic competence to the hybrid. (Used with the permission of the journal PLOS One [26]).