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Identification of microRNAs involved in the spontaneous regression of cutaneous melanoma in pigs

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1300

Postinflammatory hyperpigmentation is not a post- but persistent inflammatory process that is influenced by ethnicity and causality

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Postinflammatory hyperpigmentation (PIH) is a common and difficult to treat condition resulting from numerous dermatologic conditions. Through surveys, photography, colorimeter ($L^* = 0$ [black] to 100 [white]) and spectrophotometer readings, as well as skin biopsies, this study aimed to better characterize PIH on a clinical and histopathological level. Twenty-three subjects were enrolled in the study (18 African-Americans [AA], 3 Asians (AN), 2 Caucasians(CN)). Acne was the most common cause of PIH in AA, followed by psoriasis/eczema in AN, and other causes in CN. AA were more likely to seek treatment for their PIH ($p=0.017$). ΔL^* between PIH and normal skin (NS) did not differ significantly by cause of PIH, but did differ by ethnicity (AA=4.27 + 3.79, AN=8.24 + 2.82, CN=9.71 +2.09; $p=0.0338$), with AA having the smallest ΔL^* followed by AN then CN. Spectrophotometer measurements of the absolute melanin in PIH skin was highest in AA (587.8 +52.2), followed by AN (494.0 + 43.7) then CN (470.7 + 1.5) while the relative melanin value did not differ significantly by cause of PIH or ethnicity. Skin biopsies taken from NS and PIH ($n=10$) were analyzed in a blinded manner by a dermatopathologist. Assessed semi-quantitatively, compared to NS, PIH showed significant increase (all $p<0.003$) in hyperkeratosis (44x), acanthosis (74x), elongation of rete ridges (44x), mid-dermal (16.5x) and interstitial inflammation (22x), plasma cells (19x), dermal fibrosis (17.5x), and dermal melanophages (1.5x, $p=0.0578$). In PIH compared with NS, a significantly greater number of cells stained positive for CD3 (1.7x; $p=0.0262$) and CD 20 (6.1x; $p=0.0068$) but not with CD68 or MITF. For acne PIH, persistent dermal inflammation indicates that the inflammatory process is ongoing even after the acne has resolved, suggesting continued anti-inflammatory treatment may be necessary. Further investigation into PIH will likely lead to better treatment approaches that will ultimately improve the clinical outcome of this condition.

1302

Identification of novel cd22- b cell subsets in melanoma with potential anti-tumour effects

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Melanoma is recognized as a highly immunogenic tumour characterized by the presence of large numbers of infiltrating immune cells. Recent publications have indicated a possible role for B lymphocytes in determining disease prognosis in patients with advanced stage melanoma. The aim of this study was therefore to investigate the phenotype of B lymphocyte subsets in melanoma and their possible roles in disease pathogenesis. Using immunohistochemistry we analysed skin biopsies containing primary or metastatic melanoma ($n=10$), as well as normal skin from healthy donors ($n=10$). Analysis of melanoma samples showed CD20+ B cell infiltrates predominantly within peri-tumoral and peri-vascular locations although small numbers were also seen within the tumour. B cells were rarely seen in normal skin. Using a combination of enzymatic tissue digestion and flow cytometric analysis, we identified novel CD20+CD22-CD27+ and CD20+CD22-CD27- B cell subsets which are enriched in biopsies of primary and metastatic melanoma ($n=6$) (mean % increase=11, $p=0.009$ for CD22-CD27+; mean % increase=20, $p=0.004$ for CD22-CD27-), and wide local excision/ peri-tumoral tissue ($n=6$) (mean % increase=22, $p=0.015$ for CD22-CD27+; mean % increase=22, $p=0.002$ for CD22-CD27-), compared with peripheral blood. Performing in-vitro stimulation assays on peripheral blood B cells using a variety of stimuli including CpG, CD40 ligand-expressing cells, PMA and Ionomycin, as well as a metastatic melanoma cell line (A375), we demonstrated production of the cytokines IL-4, interferon gamma (IFN- γ), and tumour necrosis factor alpha (TNF- α), but not IL-10, from these CD22-CD27+ and CD22-CD27- B cell subsets. Furthermore, co-culture experiments with the A375 melanoma cell line also showed an increase in the proportion of CD22- B cells relative to baseline. CD22 down-regulation has been described following B cell activation. We therefore postulate that these populations might represent an activated B cell subset with possible functional roles in the anti-melanoma anti-tumour response.

1304

Shedding of CD44 in human melanoma cells – the CD44 ectodomain is released by direct ADAM10-cleavage and binds to vimentin on neighboring cells

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The liberation of CD44 standard ectodomain (CD44-ED) plays a crucial role in the tumor biology of human malignant melanoma (MM). We had shown that the shed CD44-ED suppresses the proliferation of human MM induced by hyaluronan. This prompted us to identify the putative CD44 sheddase and to characterize the shedding mechanism in MM. Specifically; we aimed to identify a) the protease that cleaves the CD44-ED, b) the cleavage site within the CD44-ED and c) the putative interaction sites of CD44 and its protease. Finally, we investigated the fate of the released CD44-ED. We could identify ADAM10 as the critical protease in the shedding process of CD44. Inhibition and silencing of ADAM10 decreased CD44-ED shedding by MM significantly. Co-transfection of CHO-cells with CD44 and ADAM10 resulted in strong CD44-ED release of identical size as observed in MM. Moreover, we identified Ser 249 in the CD44 stem region of as the cleavage site for ADAM10 both in MM that shed CD44 naturally as well as in CD44 and ADAM10 co-transfected CHO. Bimolecular fluorescence complementation (BiFC) experiments verified a direct interaction of CD44 and ADAM10. Using a peptide library of ADAM10-derived peptides we could identify four main peptide motifs in the disintegrin-binding and cysteine-rich domains of ADAM10 to bind to the CD44-ED. Moreover, one of these peptides interfered with CD44-ED shedding from MM cells. Furthermore, we analyzed the biological impact of the shed CD44-ED on MM and stroma cells. After purifying and labelling of the shed CD44-ED, we could demonstrate its binding to MM and peritumoral fibroblasts. This binding did not depend on CD44 expression, on N-glycosylation or presence of hyaluronan. However, vimentin, recently shown to be of importance for MM progression, appears to interact with the shed CD44-ED as shown by co-localisation staining in MM. In conclusion, we could provide substantial new information on the structural basis of CD44 shedding, the putative fate and function of the shed CD44-ED in human melanoma.

1301

Regulation of melanin synthesis by S-palmitoylation of tyrosinase

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S-Palmitoylation is the attachment of a 16 carbon fully saturated lipid moiety to specific cysteine residue through a thioester bond and affects protein function, trafficking and localization via modulating membrane affinity and protein stability. In this study, we discovered that tyrosinase, a key enzyme of melanin synthesis pathway, is modified by S-palmitoylation in normal human melanocyte cells (NHEM) and Cys500 is a responsible S-palmitoylation site by resin-assisted capture to S-acylated proteins (acyl-RAC) and western blot analysis. In mammalian, palmitoylation reactions are mediated by 23 protein acyltransferases (PATs) family which commonly has Asp-His-His-Cys (DHHC) domain. Systematic screening for palmitoyl transferase activity of the DHHC proteins using DHHC and human tyrosinase transfected HEK293 cells and siRNA test for DHHCs using MNT-1 human melanoma cells showed DHHC-2, -3, -7, -15 are involved in tyrosinase palmitoylation. The palmitoylation inhibitor, 2-bromopalmitate (2-BP), treatment decreased ratio of palmitoylated tyrosinase and increased total tyrosinase level and melanin synthesis in a dose-dependent manner in NHEM. 2-BP did not increase tyrosinase mRNA expression but suppressed tyrosinase degradation. These results indicate that palmitoylation of tyrosinase affects its trafficking and/or stability. 2-BP also increased melanin contents in reconstructed human skin. In conclusion, S-palmitoylation of tyrosinase may have an important role in melanogenesis regulation via tyrosinase degradation.

1303

Apoptosis and caspase 3 stimulate melanoma tumor repopulation during vemurafenib and PD0325901 therapy

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Metastatic melanoma is aggressive and difficult to treat with our available drug regimens, despite promising advances such as vemurafenib, which targets the V600E BRAF mutation, and MEK inhibitors. We aim to define the role of apoptosis in melanoma tumor repopulation during these drug treatments. We propose a paradigm-changing hypothesis that dying cells undergoing apoptosis during the drug treatment of melanoma activate paracrine signaling events that promote the repopulation of surviving tumor cells. Caspase 3, which is a key executioner in apoptosis, may activate signals from dying cells to stimulate melanoma tumor repopulation. The concept of a role for apoptosis in tumor survival challenges the canon of apoptosis in cancer therapy. First, we created a caspase 3 reporter gene to study the kinetics of caspase 3 activation during melanoma drug treatment. In dying A375 cells, activated caspase 3 cleaves off a polyubiquitin domain so that the luciferase reporter is stabilized. A375 cells transfected with the reporter in vitro showed a significant increase in luciferase activity per cell after treatment with vemurafenib and PD0325901 (a MEK inhibitor). Second, we examined whether or not dying cells during the drug treatment of melanoma stimulate the growth of surviving tumor cells. In vitro, a small number of luciferase-labeled A375 cells seeded onto a layer of a larger number of feeder A375 cells lethally treated with vemurafenib or PD0325901 grew significantly faster than when seeded onto untreated feeder A375 cells. In order to determine if caspase 3 inhibition is a feasible way to inhibit melanoma regrowth after drug treatment, we developed caspase 3 dominant negative A375 cells. Experiments are underway to investigate the validity of our hypothesis in animal models of melanoma. We expect the characterization of a cell death-mediated mechanism of melanoma relapse after drug therapy to provide new targets to improve our current treatment regimens.

1305

Innate immune TLR2 and TLR3 participate in melanogenesis and melanosome transportation in human melanocytes

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In human skin, melanocytes localize in epidermis where innate immune stimuli such as microbe molecules affect the behavior of the cells. However, little knowledge is available in innate immune functions for melanogenesis. To investigate the influence of innate immunity on melanocytes, we stimulated normal human melanocytes with Toll-like receptor (TLR) ligands and measured extracellular and intracellular melanin and genes related to the melanogenesis. TLR2 ligand HKLM increased amount of extracellular melanin. Consistently with the increase in extracellular melanin, TLR2 ligands increased genes related to the melanogenesis such as *TYR*, *TYRP1*, and *DCT*. Interestingly, although TLR3 ligand Poly(I:C) increased amount of extracellular melanin, TLR3 ligands decrease the intracellular melanin, tyrosinase activity, and do not affected the genes related to the melanogenesis. Co-culture of melanocytes with keratinocytes showed the decrease in extracellular melanin and increase in intracellular melanin amount in keratinocytes under TLR2 and TLR3 stimulation. Immune fluorescence study with anti-Gp100 Ab confirmed the melanosome transportation to keratinocytes by TLR2 and TLR3 ligands. We further revealed that Rab27a, which mutations have been identified in partial albinism Griscelli syndrome type 2, was increased in mRNA and protein and accumulated to cell membrane by TLR3 ligands stimulation. Treatment of siRNA for Rab27a decreased Poly(I:C)-induced melanosome transfer to keratinocyte. Based on the recent observation that ultraviolet (UV) irradiation induces TLR3 activation (Nat Med 2012), we confirmed that UV also increased Rab27a mRNA and protein and induced Rab27a-localization to cell membrane in melanocyte. Along with these observations, Rab27a siRNA suppressed UV-induced Gp100 localization to cell membrane. These results suggested that TLR2 ligands increase release of melanin by inducing melanogenesis relating molecules, and TLR3 ligands as well as UV increase melanin release by activating melanosome transportation through Rab27a.

1306**Melanosome biogenesis protein complexes interact with the WASH complex**

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Biogenesis of melanosomes requires the targeting of specific membrane proteins and soluble cargoes to maturing organelles. One mechanism for this targeting is the recruitment and concentration of cargo into nascent vesicles by adaptor protein complexes, such as the adaptor protein complex-3 (AP-3). Mutations to AP-3 and related protein complexes including the BLOC-1 complex cause the Hermansky Pudlak Syndrome, which is characterized by hypopigmentation, platelet dysfunction, and pulmonary fibrosis. Adaptor proteins are recruited to membranes from the cytosol in part by specialized phospholipids, making lipid kinases key regulators of membrane traffic. One such regulator is the phosphatidylinositol-4-kinase type II α (PI4KII α), which binds to and regulates the function of the AP-3 and BLOC-1 complexes. In order to search for unrecognized regulators of this pathway we isolated PI4KII α protein complexes by immunoaffinity purification coupled with *in vivo* isotope tagging of the cell proteome and mass spectrometry (SILAC). We identified several regulators of the actin cytoskeleton, including the WASH complex, an Arp 2/3 activator, and the RhoA guanine exchange factor RhoGEF1. We confirmed these interactions by independent immunoaffinity chromatography experiments, sucrose velocity sedimentation, and deconvolution immunofluorescent microscopy. We found that PI4KII α is contained in endosomal tubules that elongate upon WASH complex depletion. In addition, BLOC-1 cargoes mislocalize in WASH complex deficiencies. We conclude that PI4KII α , a key enzyme regulating cytosolic coats required for melanosome biogenesis, interacts with RhoGEF1 and the WASH complex. Future work will continue to test the hypotheses that the WASH complex is required for AP-3- and/or BLOC-1-dependent cargo sorting and vesicle fission for melanosome biogenesis.

1308**Alcohol intake and risk of incident melanoma: A prospective study**

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Current evidence of an association between alcohol consumption and melanoma is equivocal. We investigated whether alcohol intake is associated with risk of cutaneous melanoma using data from 4,236,166 person-years of follow-up among US men and women in the Nurses' Health Study (NHS), NHSII, and Health Professionals' Follow-Up Study. Statistical analyses were performed using the Cox proportional hazards model and results are reported as multivariate-adjusted risk ratios per drink per day for all three cohorts combined. Covariates included age, body mass index (BMI), smoking status, sun sensitivity, average UVB flux at place of residence, number of severe sunburns, number of nevi, natural hair color, and family history. A total of 1,385 cases of invasive melanoma and 750 cases of melanoma *in situ* were documented during a mean follow-up time of 19.0 years. Total alcohol intake was associated with risk of invasive melanoma (RR 1.11, 95% CI: 1.00-1.24, $p=0.04$), but was not significantly associated with melanoma *in situ* (RR 1.16, 95% CI: 0.91-1.50, $p=0.23$). Beverage-specific analyses showed increased risk of invasive melanoma associated with white wine (RR 1.34, 95% CI: 1.11-1.62, $p=0.002$) and with non-light beer (RR 1.31, 95% CI: 1.05-1.64, $p=0.02$) after adjusting for total alcohol intake. Beverage-specific results for melanoma *in situ* also showed elevations in risk associated with white wine and with non-light beer, but did not reach statistical significance. Light beer, red wine, and liquor consumption were not associated with either invasive melanoma or melanoma *in situ* risk. There was no effect modification by age, smoking history, caffeine intake, physical activity, hair color, or BMI. Our findings support an association between alcohol intake and risk of melanoma, and suggest that the association between alcohol intake and melanoma may depend on beverage type.

1310**Melanoma cell regulation of fibroblast hyaluronan synthesis through the release of emmprin and stratifin: A role in modulation of tumour stroma, promoting tumour invasion**

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Background: A hyaluronan-rich stroma is associated with many growing and invading tumours and is predominantly synthesised by fibroblasts following stimulation by tumour cell-derived factors. We have examined the properties of fibroblast hyaluronan-stimulating factors released by the aggressive C8161 melanoma cell line. Methods: Serum-free conditioned medium taken from C8161 melanoma cell cultures was examined for its ability to stimulate fibroblast glycosaminoglycan synthesis by measuring the incorporation of ³H-glucosamine and by utilising a specific hyaluronan plate assay. The role of emmprin and stratifin were assessed by measuring their ability to stimulate fibroblast glycosaminoglycan and hyaluronan synthesis. C8161 melanoma cell-conditioned medium was then depleted of emmprin and stratifin and its effect on glycosaminoglycan and hyaluronan synthesis determined. Results: Preliminary characterisation of the active tumour cell-derived fibroblast hyaluronan-stimulating factors showed that they are retained by a 30kDa ultrafiltration membrane, and are protease-sensitive and heat-resistant. C8161 melanoma cell-conditioned medium concentrated 5-fold with a 30kDa cut-off membrane stimulated fibroblast hyaluronan synthesis by 45%. Emmprin and stratifin were shown to stimulate fibroblast hyaluronan synthesis 69% and 72% respectively at 0.75 μ g/ml. The hyaluronan-stimulating activity of C8161 melanoma cell-conditioned medium was decreased by 22% following emmprin depletion and 49% following stratifin depletion. Conclusion: C8161 melanoma cells release soluble factors including emmprin and stratifin that stimulate fibroblast hyaluronan synthesis which may in turn promote tumour cell growth and invasion.

1307**NVP-LDE225, a potent and selective smoothened antagonist possesses anti-melanoma activity both *in vitro* and *in vivo***

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Melanoma is one of the most aggressive cancers and its incidence is increasing worldwide. So far there are no curable therapies especially after metastasis. Due to frequent mutations in members of the mitogen-activated protein kinase (MAPK) signaling pathway, this pathway is constitutively active in melanoma. It has been shown that the SONIC HEDGEHOG (SHH)-GLI and MAPK signaling pathways regulate cell growth in many tumors including melanoma and interact with each other in the regulation of cell proliferation and survival. Here we show that the SHH-GLI pathway is active in human melanoma cell lines as they express downstream target of this pathway Gli1. Expression of Gli1 was significantly higher in human primary melanoma tissues harboring BRAFV600E mutation than those with wild type BRAF. Pharmacologic inhibition of BRAFV600E in human melanoma cell lines resulted in decreased expression of Gli1 thus demonstrating interaction of SHH-GLI and MAPK pathways. Inhibition of SHH-GLI pathway by the novel small molecule inhibitor of smoothened NVP-LDE225 was followed by inhibition of cell growth and induction of apoptosis in human melanoma cell lines, interestingly with both BRAFV600E and BRAFWild Type status. NVP-LDE225 was potent in reducing cell proliferation and inducing tumor growth arrest *in vitro* as well as *in vivo* and these effects were superior to the natural compound cyclopamine. Finally, we conclude that inhibition of SHH-GLI signaling pathway in human melanoma by the specific smoothened inhibitor NVP-LDE225 could have potential therapeutic application in human melanoma even in the absence of BRAFV600E mutation and warrants further investigations.

1309**Neural-associated melanoma progression genes are induced during melanocyte activation and proliferation**

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A number of neuronal genes have been associated with melanoma progression *in vivo*. These genes, however, were not identified in models comparing melanoma cell lines vs. cultured normal melanocytes. We have developed a novel approach to identify melanocyte specific transcripts associated with melanocyte activation and proliferation. Quiescent melanocytes were obtained by FACS sorting CD117+/Langerin- cells from human epidermis ($n=6$). Microarray analysis was performed on freshly isolated melanocytes, as well as cells derived from the same individual after 6 days in culture, when purified melanocytes have begun to proliferate. Transcriptional profiling of growing vs. quiescent melanocytes showed at least 4-fold induction of 80 genes related to cell cycle division, including RRM2, HMMR, cyclins and Aurora B (>50-fold, $p<0.05$). Interestingly, over 30 genes related to neuronal growth were upregulated as well (>2 fold), including SLC7A11, a cysteine and glutamate transporter (>400-fold); GMR1, glutamate receptor 1 (>11 fold). Notably, among the induced neuronal genes, several are known biomarkers for melanoma (e.g. S100B), while others are associated with melanoma progression (e.g. NF2, neuropilin-2, BDNF), or harbor high frequency mutations in melanoma (e.g. GRM1). In published transcriptional profiles of micro-dissected lesions of melanoma vs. nevi, a majority of the neuronal genes that we examined were increased in melanoma but not in dysplastic or common nevi. Melanocyte activation from a physiological quiescent state to a proliferating phase in melanomas is a defining step during malignant transformation. Unlike oncogenes that regulate cell divisions, neuronal genes expression is restricted to neural-crest derived cells. We thus believe that these highly expressed neuronal genes in proliferating melanocytes may represent excellent candidate molecules for further functional characterization during malignant transformation.

1311**Identification of microRNAs involved in the spontaneous regression of cutaneous melanoma in pigs**

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The Melanoma-bearing Libechov Minipig (MeLiM) has been characterized as a relevant model for cutaneous melanoma (CM) studies because of its similarities with human CM. However, Complete Spontaneous Regression (CSR) in human is extremely rare, whereas it is systematic in the MeLiM model, thus providing a great opportunity to study this phenomenon. MicroRNAs (miRNAs) are post-transcriptional silencers of gene expression involved in the development and progression of melanoma. In this study, we use small-RNA high-throughput sequencing to identify miRNAs differentially expressed in regressing CM compared to progressing ones. Four small-RNA libraries consisting of 2 pools of regressing CM ($n=3$ /pool) and 2 pools of progressing CM ($n=3$ /pool) were sequenced by Solexa technology and led to the identification of 549 miRNAs. Seventeen of these 549 miRNAs are differentially expressed (adjusted p -value < 0.01). The top five up-regulated and down-regulated miRNAs are respectively miR-195-5p, miR-126-3p, miR-27a-3p, miR-326-3p, miR-199a-5p and miR-18b-5p, miR-106a-5p, miR-451-5p, miR-363-3p, miR-144-3p. In a previous transcriptomic study, we demonstrated that CSR of melanoma in MeLiM is characterized by a down-regulation of cell-cycle related genes. Thus, we select several miRNAs to identify their impact on cell proliferation by MTT assay and cell cycle analysis by flow cytometry in human and mouse melanoma cell lines (WM-115 and B16F10). This work, which describes miRNAs involved in efficient regression of CM, should allow to identify potential prognosis biomarkers and also to highlight new pathways to target for future therapies.

1312

Modulation of MHC expression by BRAFV600E in melanoma

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To optimally integrate targeted kinase inhibitors and immunotherapies in the treatment of melanoma, it will be critical to understand how BRAFV600E mutation status and inhibition influence the expression of genes that govern cellular anti-tumor immune responses. Because major histocompatibility complex (MHC) molecules underpin interactions between tumor cells and lymphocytes, we investigated the impact of BRAFV600E-selective inhibitors on the induction of MHC molecules. We have found that treatment of A375 melanoma cells with vemurafenib enhances the induction of MHC class I and class II molecules by interferon (IFN)- γ and IFN- α 2b. Consistent with these findings, we have found that the forced over-expression of BRAFV600E has the opposite effect and can repress basal MHC class I levels in A375 cells. Additional studies revealed that vemurafenib-mediated enhancement of MHC induction by IFN- γ only occurred in cell lines homozygous for BRAFV600E and not in those with a heterozygous mutation. These findings suggest that BRAFV600E activity directly impacts the expression of MHC molecules and the response to type I and type II IFNs. Further, our data suggest that the effect of vemurafenib on immune-gene expression may depend on the zygosity of the BRAFV600E mutation, which is not routinely interrogated in patients with melanoma.

1314

Loss of ADAM-9 leads to modification of the extracellular matrix favoring tumor growth

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Remodeling of the extracellular matrix by proteases is a process that leads to modifications of the matrix, release of bound growth factors and cytokines. This process has been proven to be of particular importance during tumor development. Among metalloproteinase, ADAM-9 was shown to process matrices as laminin 1 and fibronectin thereby modulating tumor progression in liver. We recently found that expression of this enzyme in stromal fibroblasts during melanoma development regulates secretion of factors e.g. TIMP-1 which in turn modulate tumor cell proliferation and apoptosis. Using murine melanoma grafting in either WT or Adam-9-/- mice we have now analysed the matrix alterations produced by ADAM-9 in the peritumoral environment. Proteomic analysis was conducted on areas surrounding mouse tumors and revealed increased expression of matrix proteins such as decorin, collagen V and collagen type I in the peritumoral areas from Adam-9-/- as compared to WT mice. Using in vitro co-cultures of fibroblasts and murine melanoma cells, we could detect increased collagen type I and decorin secretion. Increased decorin but not collagen type I in Adam-9-/- fibroblasts was the result of altered de novo synthesis. Enhanced collagen type I protein was likely due to decreased degradation by collagenases in the peritumoral environment. Altered collagen density and organization around tumors may in turn modulate cellular proliferation. Indeed, in vivo B16F1 cell proliferation at the periphery of the tumors in Adam-9-/- animals compared to WT was increased. In addition, in vitro when increased collagen type I concentrations were supplied to B16F1 melanoma cells, their proliferation was enhanced reaching a plateau above which proliferation reduces steadily. Taken together, increased matrix expression at the tumor border in melanoma in the absence of ADAM-9 may result from both, altered synthesis and degradation thereby contributing to modulation of tumor cell proliferation.

1316

A gamma secretase inhibitor synergistically kills melanoma cells in combination with ABT-737/ABT-263 through a Noxa-dependent mechanism

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Despite the recent, promising development of therapies targeting BRAF/MEK signaling, many melanoma patients still relapse. These therapies are also only meant for tumors with the specific mutations that are targeted by these treatments. Thus, developing other treatment options is still urgent for melanoma. Targeting Bcl-2 anti-apoptotic proteins, such as with the small molecule ABT-737, has been actively investigated for melanoma treatments. However, ABT-737 alone is not very effective in killing melanoma cells, since many tumors also rely on other Bcl-2 proteins, such as Mcl-1, whose over-expression can contribute to cell resistance to ABT-737 therapy. The pro-apoptotic protein Noxa, however, selectively inhibits Mcl-1 and is able to overcome this resistance. Gamma Secretase Inhibitors (GSIs) have been shown to induce Noxa in malignant melanoma cells. Thus in the present study, we tested the effects of the combination of ABT-737 with a GSI on malignant melanoma cells. We found that this combination strongly induced apoptosis, more than either drug alone, in multiple melanoma cell lines. Additionally, the combination dramatically increased the Noxa/Mcl-1 ratio. This is likely the main mechanism of killing since knocking-down Mcl-1 sensitized cells to ABT-737 while knocking-down Noxa protected cells from the combination treatment. Furthermore, the combination of GSI with the orally bioavailable equivalent of ABT-737, ABT-263, induced Noxa-dependent killing of melanoma cells very similarly to the combination of ABT-737 and GSI. Overall, our data suggests the combination of ABT-737/ABT263 with GSI is a promising treatment strategy for melanoma and is worthy of further investigation.

1313

Correlation of vitamin D receptor and E-cadherin expression in human melanomas

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E-cadherin plays an important physiological role in epithelial cell behavior and tissue formation. In cancer it regulates cell motility, invasive and metastatic potential. In many tumors, including melanomas, decreased expression of E-cadherin correlates with tumor progression. 1,25-dihydroxyvitamin D3 (calcitriol), acting through vitamin D receptor (VDR), regulates calcium-phosphate homeostasis and affects proliferation, differentiation, and apoptosis and protects DNA against oxidative damage, with net tumorostatic and anticarcinogenic effects. Our previous study demonstrated decreased VDR expression in more advanced stages of cutaneous melanoma, that correlated with decreased overall survival time. Since E-cadherin activity is regulated in calcium-dependent manner, we decided to analyze its expression in correlation to VDR. Using immunohistochemistry, we evaluated the expression of VDR and E-cadherin in 69 melanomas and 44 lymph node metastases obtained from 69 patients. In primary melanomas both cytoplasmic, and nuclear VDR expression positively correlated with E-cadherin immunostaining intensity, and high VDR expression was accompanied by significantly higher E-cadherin expression when compared to tumors with low or absent VDR levels. Furthermore, that tumors with high expression of E-cadherin showed brisk tumor-infiltrating lymphocytes response with highest E-cadherin levels in disease localized to the skin or having solely lymph nodes involvement (melanomas at pM0). In addition, patients with higher E-cadherin expression had longer overall survival. In metastases E-cadherin expression was significantly lower than in primary lesions. In conclusion, a positive correlation between E-cadherin and VDR expression in primary tumors may suggest that calcitriol can affect melanoma behavior through regulation of E-cadherin.

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Survivin promotion of melanoma metastasis requires upregulation of α 5 integrin

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Survivin is an apoptotic and mitotic regulator that is overexpressed in melanoma, and a poor prognostic marker in patients with metastatic disease. We recently showed that Survivin enhances melanoma cell motility through Akt-dependent upregulation of α 5 integrin. However, the functional role of Survivin in melanoma metastasis is not clearly understood. We found that overexpression of Survivin in LOX and YUSAC2 human melanoma cells increased colony formation in soft agar, and this effect was abrogated by knockdown of α 5 integrin by RNA interference. We employed melanoma cell xenografts to determine the in vivo effect of Survivin overexpression on melanoma metastasis. While Survivin overexpression did not affect primary tumor growth of YUSAC2 or LOX s.c. tumors, or indices of proliferation or apoptosis; it significantly increased expression of α 5 integrin in the primary tumors and formation of metastatic colonies in the lungs. Additionally, Survivin overexpression resulted in enhanced lung colony formation following i.v. injection of tumor cells. Importantly, in vivo inhibition of α 5 integrin via intraperitoneal injection of an α 5 β 1 integrin-blocking antibody significantly slowed tumor growth and reduced Survivin-enhanced pulmonary metastasis. Knockdown of α 5 integrin in cells prior to i.v. injection also blocked Survivin-enhanced lung colony formation. These findings support a direct role for Survivin in melanoma metastasis which requires α 5 integrin, and suggest that inhibitors of α 5 integrin may be useful in combating this process.

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Targeting hyperactivation of the AKT survival pathway to overcome therapy resistance of melanoma brain metastases

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Brain metastases are the most common cause of death in patients with metastatic melanoma, and the RAF-MEK-ERK and PI3K-AKT signaling pathways are key players in melanoma progression and drug resistance. The BRAF inhibitor vemurafenib significantly improved overall survival. However, brain metastases still limit the effectiveness of this therapy. In a series of patients, we observed that treatment with vemurafenib resulted in substantial regression of extracerebral metastases, but brain metastases developed. This study aimed to identify factors that contribute to treatment resistance in brain metastases. Matched brain and extracerebral metastases from melanoma patients had identical ERK, p-ERK, and AKT immunohistochemistry staining patterns, but there was hyperactivation of AKT (p-AKT) and loss of PTEN expression in the brain metastases. Mutation analysis revealed no differences in BRAF, NRAS, or KIT mutation status in matched brain and extracerebral metastases. In contrast, AKT, p-AKT, and PTEN expression was identical in monolayer cultures derived from melanoma brain and extracerebral metastases. Furthermore, melanoma cells stimulated by astrocyte-conditioned medium showed higher AKT activation and invasiveness than melanoma cells stimulated by fibroblast-conditioned medium. Inhibition of PI3K-AKT signaling resensitized melanoma cells isolated from a vemurafenib-resistant brain metastasis to vemurafenib. Brain-derived factors appear to induce hyperactivation of the AKT survival pathway and to promote the survival and drug resistance of melanoma cells in the brain. Thus, inhibition of PI3K-AKT signaling shows potential for enhancing and/or prolonging the antitumor effect of BRAF inhibitors or other anticancer agents in melanoma brain metastases.

1318**Vemurafenib potently induces endoplasmic reticulum stress-mediated apoptosis in BRAFV600E melanoma cells**

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The V600E mutation in the kinase BRAF is frequently detected in melanomas and results in constitutive activation of BRAF, which then promotes cell proliferation by the mitogen-activated protein kinase (MAPK) signaling pathway. Although the BRAFV600E kinase inhibitor vemurafenib has remarkable antitumor activity in patients with BRAFV600E-mutated melanoma, its effects are limited by the onset of drug resistance. We investigated the mechanisms of action and resistance to vemurafenib. In melanoma cell lines with the BRAFV600E mutation, vemurafenib decreased the abundance of anti-apoptotic proteins and induced intrinsic mitochondrial apoptosis. Vemurafenib-treated melanoma cells showed increased cytosolic concentration of calcium, a potential trigger for endoplasmic reticulum (ER) stress, which can lead to apoptosis. Consistent with an ER stress-induced response, vemurafenib decreased the abundance of the ER chaperone protein GRP78, increased the abundance of the spliced isoform of the transcription factor X-box protein 1 (XBP1), increased the phosphorylation of the translation initiation factor eIF2 α , and induced the expression of ER stress-related genes. Knockdown of the ER stress response protein ATF4 significantly reduced vemurafenib-induced apoptosis. Moreover, the ER stress inducer thapsigargin prevented invasive growth of tumors formed from vemurafenib-sensitive melanoma cells in vivo. In melanoma cells with low sensitivity or resistance to vemurafenib, combination treatment with thapsigargin augmented or induced apoptosis. Thus, thapsigargin or other inducers of ER stress may be useful in combination therapies to overcome vemurafenib resistance.

1320**Actinic lentigines from European and Japanese volunteers share similar alterations in gene expression profile and biological functions**

E Warrick,¹ C Duval,¹ S Nouveau,¹ C Benoit-Jay,¹ P Bastien,¹ S Deret,² A Morita,³ O de Lacharière¹ and F Bernerd¹ ¹ L'Oréal R&I, Clichy, France, ² Galderma R&D, Sophia-Antipolis, France and ³ Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan Actinic lentigines (AL) are benign skin hyperpigmented lesions associated with age and chronic sun exposure. It is now established that Asian and European skins differently age and that pigmented spots develop earlier in Asian skin. In order to better understand the aetiology of AL in both populations, the gene expression profile of AL from Japanese volunteers was analyzed and compared to previous results obtained on AL from European subjects. 12 Japanese women (aged 50-70, phototype III-IV) were included in this study. AL from the dorsal side of hands were selected on the same epiluminescence criteria (elongated honeycomb pattern) as in the European study. A whole genome transcriptomic study showed that 247 genes were differently expressed in AL versus adjacent non lesional skin (|Fold change (FC)|>1.5 and p-value <0.05). Interestingly, when comparing the Japanese and European AL profiles obtained with the same methodology, we observed that (1) 138 genes (56%) were significantly modulated in the same way in both populations (|FC|>1.5; p-value <0.05); (2) among remaining genes, more than 65% were also modulated in the same way, although with a lower FC (|FC|>1.25 in one population and |FC|>1.5 in the other one; p-value <0.05). These results show that AL lesions selected on the same clinical criteria from Japanese and European women share a common gene expression profile. In both populations, genes modulated in AL were involved in various biological functions, apart from the melanogenesis pathway which was not altered. Many genes involved in epidermal proliferation and differentiation, oxidative stress, inflammation and particularly in extracellular matrix organization were found modulated. In conclusion, European and Japanese AL share similar alterations of multiple actors involved in skin homeostasis, thus emphasizing the interest of a multi-targeted treatment for AL.

1322**Therapeutic potential of miR-26a in treating melanoma and identification of BAG4/SODD as a novel target of miR-26a**

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Dysregulation of miRNAs can be a major factor in the development of diseases, including cancer. This study aims to identify dysregulated miRNAs in melanoma cell lines compared to normal melanocytes and to use the results help developing novel treatment strategies for melanoma. Through analyses of miRNA microarrays and quantitative RT-PCR, we identified that miR-26a is strongly downregulated in melanoma compared to normal melanocytes. Upon reintroducing a miR-26a mimic, 5 out of 7 melanoma cell lines displayed an increased amount of apoptotic cell death, compared to the transfection controls. These results suggest that replacement of miR-26a could be used as a potential treatment strategy for melanoma. We also identified BAG4/SODD as a novel target of miR-26a and a possible mechanism for the killing observed here. Using immunoblot and 3' UTR dual luciferase reporter assays, we demonstrated that miR-26a can repress the expression of the BAG4 gene by binding to a site in the 3'UTR of the BAG4 transcript. Furthermore, knocking down BAG4 alone using siRNAs can induce apoptotic cell death in a subset of melanoma cell lines that are sensitive to miR-26a treatment. In summary, the replacement of miR-26a or blocking its novel target BAG4/SODD may provide new therapeutic strategies for treating melanomas.

1319**Blockade of phosphorylation of the transcription and translation factor YB-1 inhibits melanoma cell survival and invasion**

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Y-box binding protein 1 (YB-1) is an oncogenic transcription and translation factor and is overexpressed in several types of cancer. Our previous data indicated that YB-1 is upregulated and translocated to the nucleus during melanoma progression and that YB-1 is an important transcription factor regulating proliferation, survival, migration, invasion and chemosensitivity of melanoma cells. Furthermore, we showed that during melanoma progression in patient samples total YB-1 expression as well as phosphorylation at Ser102 in the YB-1 protein is increased. Nuclear translocation and transcriptional activation of YB-1 was reported to be mediated by Ser102 phosphorylation in the nucleic acid binding domain. We hypothesized that primarily the activation of YB-1 by phosphorylation and not total YB-1 abundance augments the functional effects of YB-1 as a transcription factor during melanoma progression towards metastasis. Indeed, the specific inhibition of Ser102 phosphorylation of YB-1 by a phospho-mimetic YB-1 decoy peptide blocks YB-1 transcriptional activity and significantly reduces melanoma cell proliferation as well as melanoma cell migration and invasion. Melanoma cells with overexpression of either a dominant-negative or a dominant-active mutant of YB-1 (Ser102Ala and Ser102Asp, respectively) were used in order to reassess the relevance of these findings and to evaluate their effects on the transcriptional and translational activity of YB-1.

1321**Identification of inhibitor of beta-catenin and Tcf-4 (ICAT) as a biomarker of malignant melanoma**

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We have previously shown that beta-catenin is an important signaling molecule in malignant melanoma mediating invasive and aggressive melanoma cell behavior during melanoma progression. Therefore, we evaluated the expression of Inhibitor of beta-catenin and Tcf-4 (ICAT) on transcript and protein level in melanoma biopsies on a melanoma tissue microarray, cryo-preserved melanoma tissue and melanoma cell lines. We found a significant reduction in the expression levels from nevi and primary melanoma to metastases. Moreover, the protein expression level in primary melanomas was inversely correlated to the survival of melanoma patients in an immunohistochemical analysis, being independent of tumor thickness. Additionally, we tested the potential of ICAT in inhibiting beta-catenin signaling activity in melanoma cells and evaluated its impact on melanoma cell proliferation, migration and invasion in different in vitro models. Our results propose that a low ICAT expression could be used as a predictive biomarker in malignant melanoma and represents an additional mechanism for the activation of beta-catenin in malignant melanoma. However, the identification of the exact biological function of ICAT in melanoma cells needs further investigation.

1323**Proanthocyanidins inhibit invasive potential of human melanoma cells by targeting β -catenin signaling**

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Melanoma is the leading cause of death from skin diseases due to its propensity to metastasize. Metastatic melanoma is often associated with activation of Wnt/ β -catenin signaling pathway. To develop newer and more effective strategies for the prevention of melanoma metastasis, we have examined the inhibitory effect of proanthocyanidins, bioactive components of grape seeds, on cell migration of metastasis-specific human melanoma cell lines (A375 and Hs294t) and assessed whether Wnt/ β -catenin signaling is the target of grape seed proanthocyanidins (GSPs). Using an in vitro cell migration as well as invasion assays, we found that treatment of human melanoma cell lines with GSPs resulted in inhibition of cell migration, which was associated with the degradation of cytosolic β -catenin, and subsequently reducing the nuclear accumulation of β -catenin and reducing the levels of matrix metalloproteinase (MMP)-2 and MMP-9 which are the down-stream targets of β -catenin. GSPs increased the: (i) levels of casein kinase 1 α , glycogen synthase kinase-3 β and phosphorylated- β -catenin on critical serine residues (Ser45, Ser33/37 and Thr41), and (ii) binding of β -transducin repeat-containing proteins (β -TrCP) with phospho forms of β -catenin in melanoma cells. To verify whether β -catenin is a potent molecular target of GSPs, the effect of GSPs was determined on β -catenin-activated (Mel1241) and β -catenin-inactivated (Mel1011) melanoma cells. Treatment of Mel1241 cells with GSPs or FH535, an inhibitor of Wnt/ β -catenin pathway, significantly inhibited cell migration of Mel1241 cells and simultaneously decreased accumulation of nuclear β -catenin and inhibition of MMP-2 and MMP-9 levels. However, this effect of GSPs and FH535 was not found in Mel1011 cells. These results indicate for the first time that proanthocyanidins inhibit the invasive potential of melanoma cells by targeting β -catenin signaling, and need to be further studied for chemoprevention or therapy of malignant melanoma in high risk human population.

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The circulating microRNA-221 as a new tumor marker of malignant melanoma

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Malignant melanoma (MM) is an aggressive neoplasm that can be fatal, especially at the metastatic stage, necessitating early diagnosis and better diagnostic tools. MicroRNA-221 (miR-221) is known to be abnormally expressed in MM cells, and it favors the induction of the malignant phenotype through down-modulation of p27Kip1/CDKN1B and the c-KIT receptor. This suggests that the serum level of miR-221 might increase in patients with MM and thus could be used as a new tumor marker. Serum samples were obtained from 94 MM patients and 20 healthy controls. MicroRNAs were purified from serum, and miR-221 levels were measured by quantitative real-time polymerase chain reaction. Circulating miR-221 was detectable and could be quantified in serum samples. MM patients had significantly higher miR-221 levels than healthy controls ($p < 0.0001$). Among the MM patients, the miR-221 levels were significantly increased in patients with stage I-IV MM compared to those with MM in situ ($p < 0.0001$), and the levels were correlated with tumor thickness ($p < 0.05$). Moreover, a longitudinal study revealed a tendency for the miR-221 levels to decrease after surgical removal of the primary tumor, and to increase again at recurrence. Serum levels of miR-221 may be useful not only for the diagnosis of MM, but also for the differentiating MM in situ from stage I-IV MM, and for evaluating tumor progression and monitoring patients during the follow-up period. In addition, considering that the serum levels of miR-221 were correlated with tumor thickness, miR-221 might also be useful as a prognostic marker for patients with MM.

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Pulsed ultrasound promotes melanoblast migration through upregulation of the M-CSF/FAK signaling pathway

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Repigmentation of vitiliginous lesions relies on the proliferation and migration of melanoblasts from hair follicles to the epidermis. Pulsed ultrasound has been demonstrated to have stimulatory effects on cell proliferation and migration and has been applied clinically to enhance tissue repair. To clarify the biological effects and signaling mechanisms of pulsed ultrasound on melanoblasts proliferation and migration, by using MTT, wound healing assay and cytokine profiling analysis respectively. Two melanoblast cell lines, the undifferentiated NCCmelb4 cells and the differentiated NCCmelan5 cells, were examined. We demonstrated that pulsed ultrasound increased cell migration in a dose-dependent manner ($p < 0.01$) without altering cell proliferation. Pulsed ultrasound enhanced autocrine secretion of macrophage colony stimulating factor (M-CSF) which subsequently activated the focal adhesion kinase (FAK) pathway to promote melanoblasts migration. FAK inhibitor 14 (1,2,4,5-benzenetetramine) or neutralizing M-CSF antibody blocked the stimulatory effect of pulsed ultrasound on melanoblast migration. Furthermore, conditioned medium from mouse embryonic fibroblasts NIH 3T3 treated with pulsed ultrasound could stimulate melanoblast migration through a paracrine effect. Our results provide a novel mechanism to promote migration of melanoblasts by pulsed ultrasound stimulation which may potentially served as a therapeutic adjunct for vitiligo.

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The analysis of the anti-tumor effect on melanoma by interferon beta

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The effect of local injection of interferon beta on melanoma has been reported. It is indicated that the mechanisms of anti-tumor effect of interferon beta on melanoma are both direct and indirect actions. To analysis the direct actions against melanoma cells, we assayed the susceptibility of nine melanoma cell lines to interferon beta, and the expressions of melanogenesis-associated genes and apoptosis-associated genes. The nine melanoma cell lines were modified by natural-type interferon beta, and harvested for real-time reverse transcription-PCR and cell count. In the modification of the concentration of 10^3 IU/ml, the ratio of modified cells number to control were 0.018 in RPMI-7951, 0.110 in A375, 0.131 in Hs294T, 0.149 in SK-MEL-28, 0.185 in SK-MEL-2, 0.206 in SH-4, 0.391 in C32, 0.462 in G361, 0.546 in SK-MEL-5 and 0.680 in SK-MEL-1. In the relatively susceptible melanoma cell lines, RPMI-7951, A375 and Hs294T, *PMEL* and *MLANA*, melanogenesis-associated genes, and *BIRC7*, apoptosis-associated gene, were markedly suppressed. We will examine the association between the susceptibility to interferon-beta and their suppression.

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Hidden players in vitiligo pathogenesis: Mitochondria?

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Vitiligo is still lacking exhaustive pathogenic description in the definition of the mechanism of the functional loss of epidermal melanocytes. We suggest that a metabolic alteration, possibly due to a mitochondrial involvement, takes place. An increased Cyclosporin A-dependent ROS production, associated with a loss of membrane potential ($\Delta\Psi_m$), and defective Complex I proteins activity and expression in vitro and ex vivo was observed in ontogenically different cell types, i.e. melanocytes, lymphocytes and fibroblasts. Accordingly to the defective ETC activity and pathological sensitivity to Complex I inhibitor, rotenone, we found a relevant decrease of ATP, a slight increase in lactate production, together with an increased mitochondrial mass as possible compensatory mechanism to ensure the necessary ATP amount. An over-expression of p53 with a subsequent activation of target genes including GAD45, p21 PML IGFBP3 at mRNA and protein level both in vitro and ex vivo was detected. An alteration of signal transduction pathways with hyperphosphorylated p38 and ERK was associated with a defective response to growth factors. GC-MS analyses of the membrane lipid constitution revealed an increased level of cholesterol and of lipid peroxidation products including oxysterol moieties. Finally the analysis of nuclear genes that encode mitochondrial proteins and the transcriptional profile of mtDNA provide support for the role of mitochondria in vitiligo pathogenesis. Overall our results suggest that in vitiligo cells exist a significant metabolic alteration and that mitochondria can be the source of a continuous intracellular oxidative stress, which makes the vitiligo melanocyte prone to senescence or susceptibility to external injuries.

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Forkhead Box M1 (FOXM1) transcription factor: A novel target in melanoma

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The elevated expressions of the transcription factor Forkhead box M1 (FOXM1) have been reported in various human cancers. PI3 kinase/Akt signaling pathway has been suggested to have a cross talk with the FOXM1, however the details are still unknown. Among normal adult human organs, FOXM1 is expressed only in the testis, thymus, small intestine and colon. Several studies reported FOXM1 to be overexpressed in human melanoma cell lines in mRNA and protein levels. However, there has been no previous study that examined the FOXM1 expression in melanoma lesions. We analyzed 20 benign nevi, 43 primary cutaneous melanomas and 13 metastatic melanomas by quantitative real-time reverse transcript PCR (RT-PCR) and immunohistochemistry. Metastatic melanoma samples showed significantly higher mRNA expression levels of FOXM1 than the nevi ($p = 0.004$). In the immunohistochemical analysis, FOXM1 was overexpressed in 53% of primary melanomas, 67% of metastatic melanoma and 10% of nevi. The patients with FOXM1 positive melanoma tended to develop recurrence earlier than those without. These results suggested the possibility that FOXM1 could be a diagnostic and prognostic marker of melanoma. Furthermore, we reported that down-regulation of FOXM1 by siRNA inhibited the proliferation of melanoma cells. Inactivation of Akt by PI3K inhibitor LY294002 and Akt inhibitor decreased the expression levels of FOXM1. And down-regulation of FOXM1 by siRNA did not affect the expression levels of Akt in melanoma cell line. These results suggested the possibility that FOXM1 is in the downstream of Akt pathway. In conclusion, FOXM1 could be a novel target of the treatment for melanoma.

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The effect of hypoxia on the melanogenesis in melanoma cells

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Melanin deposition in melanoma is differed in degree according to a case or a histological section. However, there is little understanding of the cause. To elucidate the cause of the variety of melanogenesis in melanoma, we analyzed the alteration of melanogenesis under the condition to mimic hypoxia. Three melanoma cell lines, SK-MEL-1, SH-4 and C32, were modified by an inducer of hypoxia-inducible factor- α . The modification induced melanogenesis in the three melanoma cell lines under the condition of the inhibition of cell growth, about 10% cell population compared to the control. Based on above data, it is suggested that hypoxia should induce melanogenesis in melanoma.

1330**Systems biological identification of effective and safe molecular targets for skin lightening**

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The process of skin color formation called melanogenesis is controlled by a complex molecular regulatory network in melanocytes and keratinocytes, which results in the variety of skin and hair pigmentation. When skin is exposed to ultraviolet radiation, keratinocytes excessively synthesize biochemical factors that affect melanocytes by paracrine regulatory mechanism, promoting melanin production. The melanogenesis pathway not only regulates melanin synthesis but also mediates cell fate decision. So, an attempt of skin color control by regulating the melanogenesis pathway may induce various skin diseases such as psoriasis, skin cancer, and sunburn. In this study, we employed a systems biological approach to investigate the complex regulatory mechanism underlying melanogenesis. We constructed the molecular regulatory network of melanogenesis with the focus on paracrine factor-mediated signaling pathway and Micropthalmia-associated transcription factor-related pathway as these two pathways are most central in skin pigmentation. By integrating all experimental evidence from literature, we established a Boolean network model that describes the dynamical characteristics of the network. From extensive mathematical simulations, we identified targets for skin lightening that are effective in reducing melanin production while having little effect in determining the cell fates of melanocytes or keratinocytes. We experimentally validated the skin lightening effect of these targets through biochemical experimentation with chemical inhibition such as beta-catenin inhibitors. Together, our study provides a novel insight into the complex regulatory mechanism of melanogenesis and the identification of both safe and effective target proteins for skin lightening.

1332**What are the origins of dermal melanophages?**

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It is well known that melanophages, possibly derived from macrophages or histiocytes that ingest melanosomes, are involved in dermal melanin deposition. Here we propose that dermal fibroblasts are also involved in dermal pigmentation, in addition to melanophages originated from macrophages. We recently developed a co-culture system consisting of normal human melanocytes and fibroblasts separated by a microporous membrane filter that enabled melanocyte dendrites to be reached to the membrane filtered-another side where fibroblasts reside. Electron microscopic observation revealed that melanocyte dendrites penetrated through the membrane pores produced multiple melanosome-containing globules and released them into the extracellular space, followed by the incorporation of the globules in fibroblasts. The comparative study of degradation and distribution pattern of the multiple melanosome-containing globules incorporated into the cytoplasm of keratinocytes, fibroblasts and macrophages showed that the globules incorporated in fibroblasts were little altered, while the globules incorporated in keratinocytes and macrophages were severely degraded and the individual melanosomes were distributed in perinuclear area only in keratinocytes. These results suggest that dermal fibroblasts in vivo may ingest multiple melanosome-containing globules dropped down into the dermis and may keep them for prolonged times in their cytoplasm due to their inactive degradation capability, leading to the characteristic behavior of dermal melanophages which reside in the dermis for long years and cause clinical pigmentary disorders.

1334**Artemisinic acid inhibits melanogenesis through downregulation of C/EBP α -dependent expression of HMG-CoA reductase gene**

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Cholesterol is associated with the regulation of melanogenesis which is the major physiological defense against solar irradiation. The present study was designed to determine the effects of artemisinic acid on melanogenesis and its mechanisms of action in human epidermal melanocytes. In this study, we found that artemisinic acid inhibited melanin content. The mRNA levels of micropthalmia-associated transcription factor (MITF) and its downstream genes tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 were reduced by artemisinic acid treatment. Additionally, the mRNA levels of melanogenesis-related genes (c-KIT, stem cell factor (SCF), and macrophage migration inhibitory factor (MIF)) were down-regulated by artemisinic acid. Furthermore, cAMP production and protein kinase A (PKA) activity were suppressed by artemisinic acid. Moreover, attempts to elucidate a possible mechanism underlying the artemisinic acid-mediated effects revealed that artemisinic acid regulated melanogenesis by inhibiting cholesterol synthesis through downregulation of the hydroxymethylglutaryl CoA (HMG CoA) reductase gene, which was mediated through reduced expression of the CCAAT/enhancer-binding protein (C/EBP) α gene. Taken together, these findings indicate that the inhibition of melanogenesis by artemisinic acid occurs through reduced expression of the HMG CoA reductase gene, which is mediated by C/EBP α inhibition and suggest that artemisinic acid may be useful as a hyperpigmentation inhibitor.

1331**Introduction of the reprogramming factors into B16 murine melanoma cells attenuated in vivo metastasis through promoting mesenchymal-epithelial transition**

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It was reported that mesenchymal-epithelial transition (MET) was observed in induced pluripotent stem (iPS) cells following introduction of the Yamanaka's reprogramming factors (RFs) to dermal fibroblasts. This prompted us to study whether fibroblast-like cancer cells, which showed epithelial-mesenchymal transition (EMT), could regain the epithelial properties by introduction of RFs (Oct3/4, Sox2, Klf4, c-Myc and Lin28). Murine and human SCC cells, that had undergone EMT, showed a morphological change to 'epithelial-like' by expression of RF genes. Examination of adhesion molecules, such as cadherins, also supported the notion that they were under MET. Furthermore, the in vitro invasion activity and gene expressions of MMPs were diminished in the RF-transfected SCC cells. These results suggest that the EMT-associated malignant nature of SCC cells was reversed by introduction of RFs. To investigate whether RFs attenuate tumor malignancy in vivo, we used murine melanoma B16 cells. As seen in the SCC cell lines, introduction of B16 cells with RFs resulted in cell morphological change to polygonal shape with well-established intercellular adhesion. Further, melanogenesis of B16 cells expressing RF genes was inhibited as indicated by reduced gene expressions of MART-1, MC1R and tyrosinase. To study the metastatic potential in vivo, the RFs-introduced or the parental B16 cells (1×10^6) were inoculated into C57BL6 mice via tail vein. Fourteen days later, the parental B16 cells developed multiple foci of lung metastases. In contrast, the RFs-introduced B16 cells failed to generate obvious metastatic tumors in the lungs. Histological analysis revealed that lungs showed much fewer numbers and smaller size of metastatic foci in mice inoculated with the RFs-B16 cells than those with the parental B16 cells. Those results suggest that the reprogramming factors could reverse the malignant nature of melanoma through promoting MET and attenuate the metastatic potential in vivo.

1333**Patients with cutaneous malignant melanoma increase their sun exposure in the years following diagnosis**

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The purpose of the study was to assess sun behaviour in the years following diagnosis of cutaneous malignant melanoma (CMM). The study was a three-year follow-up, observational case-control study. Twenty-one patients with CMM, and 21 controls matched to patients by gender, age, occupation, and constitutive skin type participated. Ultraviolet radiation (UVR) exposure was assessed the first and the second summer (N=20) and the first and the third summer (N=22) after diagnosis. Data from 40 participants were analysed. UVR exposure was assessed by personal electronic UVR dosimeters measuring time-related UVR in standard erythema dose (SED). Corresponding sun diaries were filled in daily (mean: 74 days per participant each participation year). Patients' UVR dose increased during the 3-year follow-up; The increase was 1.2 SED per day on holidays each year (95% CI: 0.2 to 2.2, P=0.02), 2.3 SED per day on holidays with body exposure each year (95% CI: 0.8 to 3.7, P=0.006), and 1.9 SED per day on days abroad each year (95% CI: 0.3 to 3.5, P=0.02). This development was significantly different from that of controls, whose UVR doses were independent of year of follow-up. Days with sunscreen and body exposure did not change significantly during the years of follow-up in either patients or controls and there was no significant difference between groups in the second and third year of follow-up. Our findings suggest that patients with CMM do not maintain a cautious sun behaviour in connection with an increase in UVR exposure, especially abroad and on holidays.

1335**Elucidating the temporal dynamics of NF- κ B activation by TNF- α in melanoma**

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Melanoma is a heterogeneous disease resulting from dysregulation of molecular pathways, leading to poorly controlled tumor growth and metastasis if the cancer is not surgically excised in time. NF- κ B is a critical transcription factor which underlies several key pathways of cellular growth, though its contribution in melanoma is not well elucidated. TNF- α is a cytokine which activates NF- κ B by targeting the I κ B inhibitor to the proteasome for degradation. Recent work has suggested that the melanoma stem-like state is directly dependent on TNF- α activity, further stressing the importance of this pathway in melanoma. We have found that TNF- α activation of NF- κ B causes transcription of several genes, including A20, NFKBIA, and NFKBIE, which is regulated in a temporal fashion and is concentration dependent. Furthermore, this activation can be modulated through pharmacologic agents including bortezomib, which blocks proteasome degradation of I κ B, thereby delaying or preventing TNF- α activity. We have also generated a fluorescent lentiviral-based reporter system for directly observing NF- κ B protein expression and localization (through a p65-fluorescent protein fusion) in the cytoplasm and nucleus in order to better elucidate the dynamics of NF- κ B activity in melanoma and are concurrently working to model these dynamics. The NF- κ B pathway represents an important aspect in melanoma pathogenesis and our work serves to elucidate the mechanism of this activation which may be useful in developing novel strategies to block growth.

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ATG7-dependent autophagy protects melanocytes against premature senescence and contributes to the control of the Nrf2 stress response

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Autophagy is a catabolic process used by cells to degrade and recycle sub-cellular components including proteins and organelles. Here we study the contribution of autophagy to melanocyte biology. Based on our observation that both human and murine melanocytes (MCs) constitutively undergo autophagy in vitro we generated a mouse strain which is deficient in autophagy specifically in melanocytes by crossing mice carrying a floxed ATG7 gene (*ATG7^{fl/fl}*) with mice expressing the Cre recombinase under the control of the tyrosinase promoter. This led to an efficient suppression of autophagy-associated lipidation of LC3 in MCs of *ATG7^{fl/fl};Tyr::Cre* mice. Whereas mutant mice had no overt phenotype, autophagy deficiency consistently led to a reduction of melanin in hair by 10-15% as compared to control animals. Analysis of MCs derived from mutant mice revealed that in vitro they produced the same amounts of melanin as MCs from control mice but they showed a strongly decreased proliferation rate and premature growth arrest. Both in vivo and in vitro, autophagy-deficient MCs strongly accumulated p62, the carrier protein which delivers cargo to autophagosomes. This accumulation was accompanied by enhanced expression of the nuclear factor erythroid 2-related factor 2 (Nrf2) target genes including, NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase Mu 1 (GSTM1) both at the mRNA and protein levels. In summary, the results of our study suggest that whereas ATG7-dependent autophagy is not essential for melanogenesis, its inhibition strongly impacts the proliferative capacity of MC and dysregulates the catabolism of protein aggregates and Nrf2 signalling in these cells.

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Comprehensive promoter methylation analysis identifies epigenetic silencing of MAPK13 in primary cutaneous melanoma

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The development and progression of cutaneous melanoma is not only driven by genetic alterations, but also by epigenetic alterations such as aberrant DNA methylation. In this study we performed genome-wide DNA methylation analysis of primary cutaneous melanoma and benign melanocytic naevus interrogating 14,495 genes using beadchip technology. This first comprehensive view of promoter methylation in primary cutaneous melanoma revealed an array of recurrent DNA methylation alterations with potential diagnostic applications. Among 106 frequently hypermethylated genes there were many novel methylation targets and tumor suppressor genes. Highly recurrent methylation of several genes including HOXA9 and MAPK13 was established using bisulphite melting curve analysis. There were 45 significantly hypomethylated genes in melanoma with the HLA-DRA gene being the most frequently affected. The identified methylation targets have potential value as molecular diagnostic biomarkers to distinguish melanoma from benign melanocytic tumors. A causal role for aberrant DNA methylation in the malignant progression of melanoma is supported by the finding of promoter hypermethylation of several established tumor suppressor genes. Promoter hypermethylation of MAPK13, encoding the p38 delta isoform, was present in 67% of primary and 85% of metastatic melanomas. Restoration of MAPK13 expression in melanoma cells exhibiting epigenetic silencing of this gene reduced proliferation, indicative of tumor suppressive functions. This study demonstrates that DNA methylation alterations are widespread in melanoma and suggests that epigenetic silencing of MAPK13 contributes to melanoma progression.

1340

Decreased melanogenesis through p53 by heme oxygenase-1 inhibitor in normal human melanocytes

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Heme oxygenase (HO)-1 is one of phase 2 enzymes of antioxidant and plays roles in anti-apoptosis and in the growth of several solid tumors. p53 has a role in skin pigmentation and may impact on melanoma at all stages. In this study, we aimed to know the effect of HO-1 on melanogenesis through p53 in normal human melanocytes (hM). hM were cultured from foreskin. hM cultures at 80% confluency were incubated with Zinc Protoporphyrin IX (ZnPP IX), HO-1 inhibitor. Melanin content was detected with ELISA, and Western blot analysis and RT-PCR of tyrosinase, MITF were performed after ZnPP IX treatment. After ZnPP IX treatment, melanin content, tyrosinase and MITF protein, mRNA levels were decreased. After UVB exposure of hM, HO-1 and p53 expression were increased. However, p53 expression was markedly decreased after UVB exposure in ZnPP IX pre-treated hM. These results suggest that melanogenesis is inhibited by ZnPP IX through decreased melanin production, tyrosinase and MITF protein and mRNA levels. Furthermore, those inhibitory effects of ZnPP IX may be dependent on p53 in normal human melanocytes. Therefore, HO-1 may play an important role in melanogenesis.

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Glucosamine attenuates endothelin-1 and stem cell factor-stimulated melanogenesis by abrogating the increased levels of MITF protein due to O-N-acetylglucosaminylation and subsequent down-regulated activation of CREB but not of ERK in normal human melanocytes

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Glucosamine (GlcN) abrogates the stimulation of pigmentation induced by endothelin-1 (EDN1) and stem cell factor (SCF) in pigmented human epidermal equivalents. In this study, we characterized the molecular mechanisms involved using normal human melanocytes (NHMs). The EDN1+SCF stimulated expression of tyrosinase, tyrosinase-related protein-1, dopachrome tautomerase and PMEL17 at the transcriptional and translational levels was significantly abrogated by 24-72 hr pretreatment with GlcN, and this was accompanied by the reduced expression of MITF at the transcriptional and translational levels. In GlcN-treated NHMs, there was a marked deficiency in the EDN1+SCF-stimulated phosphorylation of CREB but not of ERK compared with untreated NHMs. The abrogated level of stimulated phosphorylation of CREB was associated with a preceding diminished level of CREB protein (but not its mRNA) after incubation with GlcN prior to the EDN1+SCF stimulation. Interestingly, GlcN treatment for 24 hr distinctly increased alloxan (an inhibitor of O-linked N-acetylglucosaminyltransferase) suppressible O-N-acetylglucosaminylation of many proteins within NHMs including CREB and MITF but not ERK. Since O-N-acetylglucosaminylation is associated with susceptibility to proteasome degradation via the modulation of protein ubiquitination, those findings suggest a possible involvement of O-N-acetylglucosaminylation in the CREB protein deficiency. The silencing of CREB mRNA markedly abrogated the EDN1+SCF stimulated expression of MITF and the melanocyte-specific proteins in concert with the attenuated stimulation of CREB phosphorylation. The sum of these findings indicates that GlcN attenuates EDN1 and SCF-stimulated melanogenesis by abrogating the increased levels of MITF protein due to O-N-acetylglucosaminylation and the subsequent down-regulated activation of CREB but not of ERK in NHMs.

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Tobacco smoke-induced skin pigmentation is mediated by the Wnt/ β -catenin pathway and the aryl hydrocarbon receptor signaling

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It is widely recognized that tobacco smoke causes skin pigmentation. Few studies, however, have directly evaluated the mechanisms of the changes in smoker's skin pigmentation. In the present study, we investigated whether tobacco smoke enhances skin pigmentation by culturing normal human epidermal melanocytes with water-soluble tobacco smoke extract. When cultured with tobacco smoke extract, melanocytes grew to a large size and produced more melanins significantly. Although UVB-irradiated melanocytes produced equivalent amount of melanins, no morphological changes were observed. We evaluated melanocyte activations by quantitating microphthalmia-associated transcription factor (MITF) expressions by real-time PCR. MITF expressions were significantly and dose-dependently increased by tobacco smoke extract, and under the conditions with equally UVB radiations, MITF activations were more significantly. The Wnt/ β -catenin signaling pathway seemed to mediate these melanocyte activations by tobacco smoke, because β -catenin expressions were increased only by tobacco smoke not by UVB irradiations. These results indicate that UVB-induced melanogenesis might be mediated by another pathway, for example the α -MSH or the SCF/c-kit pathway. Immunocytochemical studies revealed that these activated melanocytes actively-expressed the aryl hydrocarbon receptors around the nuclear membrane. This tobacco smoke-induced MITF activation was inhibited by RNA silencing of the aryl hydrocarbon receptors. These findings of the present study indicate that the activation of human melanocytes caused by tobacco smoking is mainly mediated by the Wnt/ β -Catenin pathway and the aryl hydrocarbon receptor signaling. This study is the first evidence that tobacco smoke enhances pigmentation in vitro, and of its β -catenin and aryl hydrocarbon receptor-mediated mechanisms inside human melanocytes.

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Immunohistochemistry for histone H3 lysine 9 methyltransferase and demethylase proteins in human malignant melanomas

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Methylation and demethylation of histone H3 lysine 9 (H3K9) play a role in the transcriptional regulation of several cancer-related genes, and are closely associated with malignant tumor behavior. A novel study has recently demonstrated that SETDB1, a member of the H3K9 methyltransferases, accelerates tumor formation significantly in a zebrafish-melanoma model. However, the expression of H3K9 methyltransferases including SETDB1 and demethylases has not been systematically examined in samples of human melanoma. Here we used immunohistochemistry to examine the expression of the H3K9 methyltransferases, EHMT2 and SETDB1, and a H3K9 demethylase, LSD1, in 67 patients with malignant melanoma. Overexpression of EHMT2, SETDB1 and LSD1 was observed in 14 (21%), 38 (57%) and 53 (79%) of the 67 patients, respectively. A significant relationship was observed between overexpression of EHMT2 or SETDB1 and aggressive tumor behavior such as lymph node metastasis and/or distant metastasis ($P < 0.05$), whereas no significant relationship was evident for LSD1 immunoreactivity. Univariate log-rank tests demonstrated that patients with malignant melanoma overexpressing EHMT2 had a poorer outcome ($P < 0.001$), whereas overexpression of SETDB1 or LSD1 had no prognostic impact. These results suggest that overexpression of EHMT2 might be a prognostic marker in patients with malignant melanoma.

1342**ADAM protease inhibitor regulates chemokine expression in human keratinocytes, and modulates melanogenesis in human melanocytes**

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The ADAMs (a disintegrin and metalloprotease) are the family of proteases involved in ectodomain shedding. It has become clear in recent years that ADAM proteases have important roles in skin homeostasis and in skin diseases. One of the most widely studied ADAM17, is also known as tumor necrosis factor- α (TNF- α) converting enzyme (TACE). ADAM17 plays a critical role in the ectodomain shedding of many soluble proteins, including TNF- α TNF receptor (TNFR), epidermal growth factor (EGF) receptor ligands, Kit ligand (KITL), and its receptor KIT. Members of EGF family are the most important factors in skin wound healing. In addition, EGF receptor (EGFR) and TNFR signaling is known to be involved in the regulation of chemokine expression in the skin wound. KITL and its receptor KIT play important roles in melanocyte development, survival, proliferation, and melanogenesis. Recently, the deletion mutation in ADAM17 has been reported in patients with autosomal recessive neonatal inflammatory skin and bowel lesions. Adam17 knockout mice revealed a disorganized distribution and structure of hair follicles, which contain hairs with irregular pigment deposition. In humans, ADAM17 is also known candidate gene that regulates pigmentation in East Asians. We therefore examined the effect of ADAM protease inhibitor on the expression of chemokines in human keratinocytes using real-time RT-PCR and ELISA, and on the modulation of melanogenesis in human melanocytes using western blotting, real-time RT-PCR, and electron microscopy. We show that ADAM protease inhibitor up-regulates cytokine-induced chemokine expression in keratinocytes, and modulates melanogenesis in melanocytes. Our observations suggest that inhibition of ADAM protease inhibitor induces more severe inflammation in skin disease by enhancing the chemokine expression.

1344**Genetic studies in Japanese patients with oculocutaneous albinism (OCA)**

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More than one hundred fifty melanogenesis-associated genes have now been identified that directly or indirectly play important roles in the melanin biosynthesis pathway. Mutations in some of these genes result in hereditary pigment disorders. Patients with OCA are characterized by reduced skin and hair pigmentation. OCA types can be classified into two groups: non-syndromic and syndromic. Non-syndromic OCA arises from a reduction in melanogenesis. Syndromic types include a number of conditions in addition to OCA, such as bleeding diathesis, e.g. Hermansky-Pudlak syndrome (HPS). OCA1 and OCA2 account for approximately 40% and 50%, respectively, of OCA cases worldwide. The overall frequency of OCA2 in the United States has been reported as approximately 1 per 36,000. However, among the Ibo people of Nigeria, the prevalence was 1/1,100. Whereas OCA2 is much less frequent among Caucasians, OCA1 is uncommon in African Americans and Africans. Thus, the predominant OCA subtype is dependent on the ethnic group in a region. Here, we report frequencies of OCA subtypes among Japanese. We performed molecular analysis of *TYR*, *P*, *TYRP1*, *SLC45A2*, *HPS1*, and *HPS4* in 101 patients with OCA, who visited us or were introduced to our hospital in these 6 years, and here, reviewed the clinical and genetic characteristics. After obtaining informed consent, genomic DNA was extracted from peripheral blood and used as a template for mutational screening using SSCP/HD analysis. The result revealed that OCA1 (16 families, 15.8%), OCA4 (17 families, 16.8%), and HPS1 (17 families, 16.8%) were frequent types, and OCA2 was present at less than 4% (4 families). Furthermore, two patients with OCA3 and two with HPS4, which both had not been reported in Japanese so far, were newly identified respectively. While, in 43 families (42.6%) any pathological mutations were not identified in these 6 genes, indicating new loci might be exist for the new OCA subtypes. Other genes must be investigated to identify new major loci for Japanese OCA.

1346**Possible dermal involvement in vitiligo pathogenesis**

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Vitiligo is a multifactorial disease where the functional loss of epidermal melanocytes gives rise to the appearance of cutaneous white lesions. Most of the metabolic defects featuring vitiligo have been detected in melanocytes even if several authors reported the overall cutaneous involvement. Keratinocytes are characterized by altered index of redox equilibrium mitochondrial function and vacuolization; in the dermal compartment, high level of tenascin expression related to the defective adhesion of melanocytes has been described. We have investigated the presence of similar metabolic defects on dermal fibroblasts to provide further support for a generalized cellular defect. We observed altered arrangement of membrane lipid, mainly cholesterol and cardiolipin, associated with index of oxidative stress, such as ROS hyperproduction and membrane lipoperoxidation. An associated deregulation of intracellular signalling, involving the degree and the duration of p38, ERK1/2, and JNK phosphorylation was also detected. An increased sensitivity to pro-oxidant evaluated as cell viability, ROS production and level of ERK phosphorylation was observed in vitiligo fibroblasts with respect to normal ones. In normal fibroblasts the enrichment in membrane cholesterol reproduces a vitiligo-like signalling profile suggesting that membrane lipid arrangement can be crucial to induce the functional impairment. In conclusion, our data indicate that in vitiligo a metabolic impairment can be detected in several cell types, probably related to an altered lipid-mediated signalling.

1343**Screening of natural medicine effects on melanogenesis in normal human melanocytes**

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Melanocytes play a major role in preventing cellular and DNA damage upon ultraviolet radiation by generating melanosins. Melanogenesis depends on three major enzymes, tyrosinase, tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase (DCT, TYRP2). TYRP1, a melanosomal enzyme involved in pigmentation, affects melanocyte proliferation. In the present study, we screened 109 natural medicines, most of which are prescribed in 294 formulas approved by the Ministry of Health, Labor, and Welfare of Japan as traditional Japanese medicines (Kampo), and identified natural medicines that suppressed or enhanced melanogenesis. Methanol extracts of the 109 natural medicines were prepared and added to normal human cultured melanocytes for 24-h culture. The effects of each extract on TYRP1 mRNA expression and cell morphology were then evaluated. The mRNA expression was assessed by real-time polymerase chain reaction. Some extracts exhibited no melanocyte cytotoxicity, whereas other natural medicine extracts were cytotoxic at 20 μ g/ml. Cytotoxicity was excluded by lower glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Among the 109 extracts tested, we selected 5 extracts that increased TYRP1, including Quercus Cortex, and 10 extracts that decreased TYRP1, including Trichosanthis Semen, for further detailed analysis. TYRP1 levels were increased between 2.0- and 3.1-fold by the 5 melanogenesis-enhancing extracts (ratio of TYRP1 to GAPDH mRNA expression of the corresponding control = 1) and decreased between 0.28- and 0.46-fold by the 10 melanogenesis-suppressing extracts. Some of the extracts with enhancing effects induced a large number of melanosins in vitro. Based on a relatively large-scale screening of natural medicines, we found some natural medicines with potent suppressing or activating effects on melanocytes that are potentially useful for controlling skin melanogenesis.

1345**Phototoxicity of melanin in skin cells upon visible light**

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Strategies to protect the skin against excess of exposition to solar light are constantly changing, but usually do not consider the effect of visible light. This is a problem that concerns a wide range of the human population, because sun exposition is responsible to accelerated skin aging and skin cancer. Previous report have shown that type IV (but not type II) skin become darker under exposition with visible light, suggesting the role of melanin in the phototoxicity of visible light. We decided to study the photosensitization of melanin under visible light excitation, by quantifying the yield and life time of singlet oxygen (1O_2). 1O_2 generation was quantified by its emission in the Near Infrared region (1270nm). We have shown that melanin produces 1O_2 under visible excitation, depending on how open the biopolymer structure is (urea, partial hydrolysis and oxidation, increases the level of 1O_2 production). Besides generating 1O_2 , melanin is also able to suppress this species by functioning as a sacrificial agent (formation of a hydroperoxide at the C3 position of the indol ring). These processes can explain the hair bleaching and the damage in the hair cuticle by visible light. The role of pigmentation and phototoxicity of visible light was also investigated in melanocompetent cells. Pigmentation was induced in B16 cells with 0.5 mM L-tyrosine and 10 mM NH₄Cl and quantified in μ g melanin/ μ g of total protein (Bradford). We were able to correlate the higher phototoxicity (cell viability decreased to 30% and 50% at 36 and 72 J.cm⁻² of visible light, respectively), with higher level of melanin (5x more melanin) and higher 1O_2 generation (50% increase). Cell death mechanism varied from autophagy to apoptosis and necrosis depending on the light dose. These changes are being correlated with different types of DNA damage induced by the phototoxicity of melanin under visible light excitation. In this report we unravel a mechanistic explanation for photoaging activated by visible light.

1347**Enriched tumor vascularity and high density mast cells correlate with a poor prognosis in melanoma**

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Peritumoral Mast Cell Density (MCD) has been correlated with the extent of angiogenesis in several tumor types and may favor tumor progression. This study aims to evaluate the prognostic significance of Microvascular Density (MVD) and peritumoral (MCD) in melanoma. We investigated 55 primary melanomas, in which we evaluated several parameters, including number of mast cells and CD31-positive microvessels in consecutive tissue sections; peritumoral MCD was calculated by averaging the number of Giemsa positive cells in the entire area of the melanocytic lesions. MVD was obtained by averaging the number of CD31-stained vascular structures in ten non-overlapping hot-spot fields from tumor area. These measurements yielded a significant correlation between MVD and MCD. Also, we noted significant differences in MVD and MCD between relapsed and non-relapsed patients. Furthermore, high MVD and MCD correlated well with poor relapse-free periods and overall survival of melanoma patients. In conclusion, high MCD and MVD correlate with poor prognosis in melanoma patients, suggesting a relationship between presence of peritumoral mast cells and angiogenesis with an aggressive behavior in these tumors.

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The relative ratio of Nestin compared to HMB-45 can have prognostic significance in oral malignant melanoma

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The purpose of this study is to examine the prognostic significance of stem cell marker Nestin and melanoma-associated antibody HMB-45 for regional lymph node metastasis, distant metastasis and stage advance in oral malignant melanoma. We investigated a total of 39 cases of oral malignant melanoma between March 1999 and October 2012. The radial and vertical growth areas were stained immunohistochemically for Nestin and HMB-45. The following clinicopathological variables were evaluated: age, gender, tumor site, invasion depth, regional lymph node metastasis-free survival(RFS), distant metastasis-free survival(DFS), and stage advance-free survival(SFS). Several parameters were analyzed for RFS, DFS, and SFS using the Kaplan–Meier method and the multivariate Cox proportional hazards model. The level of Nestin expression in the radial growth area was associated with RFS(p=0.029). The absolute level of Nestin in the vertical growth was not associated with RFS or DFS, whereas the relative level of Nestin compared with HMB-45 in the vertical growth was associated with RFS(p=0.016) and DFS(p<0.001). Both the absolute and relative level of Nestin in the vertical growth were associated with SFS(p=0.018 and <0.001, respectively), according to anatomic stages of mucosal melanoma defined in 2010 AJCC. However, only the relative level of Nestin compared to HMB-45 was associated with SFS(p<0.001) on the classification of stages similar to cutaneous melanoma, which was confirmed by the multivariate Cox regression analysis. It is suggested that we should evaluate the relative Nestin-staining expression compared with HMB-45 in vertical growth area of oral malignant melanomas to predict the regional lymph node metastases, distant metastasis and stage advance.

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Intratumoral lymphatic and blood microvessel density can predict the occurrence of metastases in melanoma patients

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Angiogenesis is the key factor for growth and invasion of tumors, including melanoma. On the other hand, cutaneous melanoma is distinguished by its propensity for early metastatic spread via lymphatic vessels. Lymph node metastasis remains the major determinant for staging and management of melanoma patients. The aim of this study was to analyze whether intratumoral blood (MVD) and lymphatic (LMVD) microvessel density in primary cutaneous melanoma (PCM) can predict the occurrence of sentinel lymph node (SLN) and visceral metastases. To analyze tumour angiogenesis and lymphangiogenesis, we performed double immunohistochemical stains for the vascular marker CD31 and for the lymphatic-specific marker D2-40 in 104 PCM samples, including 71 (68.2 %) superficial spreading (SSM) and 33 (31.8 %) nodular melanomas (NM). The results were correlated with histological tumor parameters and clinical outcome. Significant difference of intratumoral MVD and LMVD between SSM and NM was found. Significantly higher intratumoral MVD and LMVD were found in NM than in SSM (p<0.001 and p<0.003, respectively). Higher intratumoral MVD was positively correlated with SLN metastasis (p=0.001), and higher MVD and LMVD were positively correlated with the occurrence of visceral metastases (p<0.001 and p=0.019, respectively). Higher MVD and LMVD were also positively correlated with Breslow stage (p<0.001), mitotic rate (p<0.001), presence of tumor ulceration (p<0.001). Our results show that the amount of tumor angiogenesis and lymphangiogenesis, assessed by intratumoral MVD and LMVD, are of prognostic significance in PCM. Assessment of intratumoral MVD and LMVD in primary melanoma may be an effective approach in selecting patients with the risk of disease progression and early metastatic disease.

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Melanomas resist T-cell therapy through inflammation-induced reversible dedifferentiation

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Adoptive cell therapies (ACT) with melanocyte-specific CTL can achieve remissions in patients with metastatic melanomas but tumors frequently relapse. Hypotheses explaining resistance to ACT include the selection of antigen-deficient tumor cell variants and the loss of CTL effector functions in the tumor microenvironment. We established an effective ACT protocol with gp100-specific pmel-1 TCRtg CTL in Hgf-Cdk4 mice to gain further insights into the mechanisms underlying tumor relapse. A booster vaccination with Ad-gp100 could expand persistent memory CTL and delay tumor relapse indicating that therapy resistance was partly due to loss of CTL effector functions. However, histopathology of relapsing melanomas also showed focal absence of gp100 expression associated with increased immune cell infiltration. Serial transplantations revealed that melanoma cells switched between differentiated and dedifferentiated phenotypes in response to CTL driven inflammation. Immunofluorescence for gp100 and the neural crest marker Ngr1 and FACS sorting showed that mouse and human melanoma cells exist in a dynamic equilibrium between differentiated and dedifferentiated subpopulations. We identified TNF as a crucial factor that reversibly shifts melanoma cells towards a dedifferentiated state. Thus, TNF-exposed melanoma cells were poorly recognized by melanocyte-specific CTL. In contrast, recognition by CTL specific for non-melanocytic antigens was unaffected. Our results demonstrate that phenotypic plasticity of melanoma cells in an inflammatory microenvironment contributes to tumor relapse after initially successful ACT. We propose that future ACT protocols should simultaneously target melanocytic and non-melanocytic antigens to ensure broad recognition of differentiated and dedifferentiated melanoma cells and include strategies to sustain CTL effector functions by blocking immune-inhibitory mechanisms in the tumor microenvironment.

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Melanoma incidence in children and adolescents: Decreasing trends from 2000-2009 in the U.S

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The purpose of this study examines melanoma incidence patterns in children and adolescents from 1973-2009 in the U.S. Surveillance, Epidemiology, and End Results (SEER-9) registries data were used to calculate annual melanoma incidence from 1973-2009 for ages 0-4, 5-9, 10-14, and 15-19 years. SEER-18 registries were also used to calculate melanoma incidence for these ages from 2000-2009, as SEER-18 provided the most cases with detailed, clinically relevant information. Incidence rates were stratified by gender, tumor site, Breslow depth, ulceration status, lymph node involvement, and distant metastases. Age-adjusted incidence trends were calculated, generating the annual percent change. From 1973-2002, melanoma incidence rates increased in adolescents (ages 15-19), peaked in 2002, then trended downward. In 10-14 year olds, rates increased from 1973-2004, peaked in 2004, then trended downward. In 5-9 year olds, rates seemed stable from 1973-2001, peaked in 2001, then decreased. In 0-4 year olds, rates peaked in 1986, but otherwise were stable from 1973-2009. SEER-18 data from 2000-2009 showed a statistically significant decreasing trend in melanoma incidence in 15-19 year olds (p=0.04). Significant decreasing melanoma incidence trends (p<0.05) were also found in: males age 15-19; nodular melanoma in 10-14 year olds; truncal melanoma in 15-19 year olds; melanoma with thin depth (0.01-1.00mm) in 15-19 year olds; melanoma without ulceration in 15-19 year olds; and melanoma without distant metastases in 15-19 year olds. In conclusion, melanoma incidence increased in children (age 10-14) and adolescents (age 15-19) from 1973-2004 and 2002, respectively, then decreased. From 2000-2009 overall, incidence significantly decreased in 15-19 year olds. This decreasing trend was significant in males and melanomas with better prognostic indicators. In contrast, past pediatric and adult studies reported increasing melanoma incidence over broader time periods. Decreasing trends, however, have been reported in other countries for children and adults.

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New candidate oncomiRs for malignant melanoma

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Malignant melanoma is a highly aggressive tumor, which is often treatment-resistant in the metastatic stage. MicroRNAs (miRNAs) are a large family of non-coding RNAs, which have been shown to both enhance and inhibit tumorigenesis. To get an overview of the role of miRNAs in melanoma biology, we screened the expression of 670 miRNAs in a set of primary and metastatic melanoma patient samples using miRNA Taqman® technology. We identified miR-638, miR-126*, miR-454 miR-30e* and miR-30e which had significantly higher expression in lymph node and distant cutaneous metastases as compared with primary melanomas. We further focused on top candidate miRNA miR-638. Interestingly, there was no expression of miR-638 in freshly isolated primary melanocytes, which suggested that miR-638 might play an important role both in initial cellular transformation and metastatic progression. Expression of miR-638 directly correlated with the increasing tumor thickness of primary tumors which further substantiated our findings. Overexpression of miR-638 significantly enhanced the tumorigenic properties of melanoma cells including proliferation, clonogenicity, migration and anchorage-independent growth. These results suggested that miR-638 is indeed an oncogenic miRNA (oncomiR) in malignant melanoma. In line with this, genome-wide cDNA microarray analysis using miR-638 overexpressing and knockdown melanoma cells revealed a number of tumor suppressors including TP53INP2, BTG2, PLEKHM1 and ZNF185 to be targeted by miR-638. Interestingly, pathway analysis for miR-638 regulated genes showed an enrichment of p53-dependent apoptosis and phagocytosis pathways. Indeed, knockdown of miR-638 in human melanoma cells induced a dramatic p53-Bax-Casp3 mediated apoptosis which might in future be exploited for melanoma therapy. Taken together, our findings demonstrate that miR-638 enhances the oncogenic and metastatic properties of melanoma cells and that it might be a target for melanoma treatment.

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Differential expression of SOX9 and SOX10 in melanoma

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Melanoma is the most aggressive skin cancer worldwide. Although melanoma is curable when detected in early stages of progression, patients diagnosed with late stage and metastatic melanomas have very poor survival. The difficulty in treating late stage and metastatic melanoma patients has been attributed to the heterogeneity of melanoma. However the molecular mechanisms of establishing heterogeneity are currently unknown. Our group recently described a model for melanoma heterogeneity. Briefly, this model hypothesizes that melanoma cells are capable of switching between two gene signatures that define the phenotype of the cell. We found that SOX10 and SOX9 are differentially expressed between the two phenotypes. SOX10 and SOX9 are normally coexpressed in normal melanocytes but in melanoma cell cultures, their expression seems to be mutually exclusive. The SOX10 positive melanoma cell cultures have a high proliferative capacity and low invasive capacity, in contrast to the SOX9 positive melanoma cell cultures which have a low proliferative capacity and high invasive capacity. The SOX10 population had many melanocytic genes upregulated like MITF, TYR, and DCT, whereas the SOX9 population had many mesenchymal genes upregulated like FGF2, TGFB1, and WNT5a. Knockdown of SOX10 leads to expression of SOX9 and decreased proliferation capacity. Knockdown of SOX9 leads to expression of SOX10 and decreased invasive capacity. Staining of SOX10 and SOX9 in human melanoma tumors revealed heterogeneous staining, suggesting both populations are needed for melanoma tumor progression. These results suggest that the SOX10 and SOX9 negatively regulate each other for differential functions of proliferation and invasion in melanoma progression.

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MicroRNA expression profiling identifies potential serum biomarkers for non-segmental vitiligo

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It is widely believed that non-segmental vitiligo (NSV) results from the autoimmune destruction of melanocytes. MicroRNAs (miRNAs), a class of small non-coding RNAs that negatively regulate gene expression, are detected in the serum and involved in autoimmune diseases. Given that miRNAs play a pivotal role in the regulation of immune response and regulate melanocyte apoptosis, we hypothesize that miRNAs derived from melanocytes and immune cells during NSV development could be detected in serum and may serve as novel non-invasive biomarkers for vitiligo. To test this hypothesis, we first examined serum miRNA gene expression profiles in vitiligo mice using TaqMan RT-PCR arrays, and we found that 7 serum miRNAs were changed in vitiligo mice compared to B6 control mice. Next, the serum miRNA expression profiles from 10 NSV patients and 20 healthy controls were investigated, and we have identified 31 miRNAs associated with NSV, of which miR-146a, miR-191, and miR-26a were changed in both vitiligo mice and NSV patients. The value of AUC for 12 the most up-regulated and down-regulated miRNAs were between 0.80 and 0.98. Furthermore, miR-16, miR-19b, and miR-720, appeared to be the best serum biomarkers distinguishing NSV from healthy controls, while miR-574-3p is associated with NSV progression. Our study suggests that miRNAs may be involved in NSV development and that serum miRNAs could serve as novel promising biomarkers for NSV diagnosis and progression.

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The effect of validamycin A on tyrosinase: Inhibition kinetics and computational simulation

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Based on the structural property of validamycin A, we investigated validamycin A as a tyrosinase inhibitor in this study. We found that the reversible inhibition of tyrosinase by validamycin A was occurred in a mixed-type manner with $K_i = 5.893 \pm 0.038$ mM, which was studied by integrating of kinetics and computational simulations. Time-interval tyrosinase studies showed that the inhibition followed first-order kinetics with 2 phases. ANS-binding fluorescence measurements showed that validamycin A induced changes in the tertiary protein structure of tyrosinase. To obtain further insights, the computational docking and molecular dynamics were applied, and the results indicated that HIS85, HIS244, GLU256, HIS259, and ASN260 of tyrosinase interacted with validamycin A. This strategy of predicting tyrosinase inhibition based on hydroxyl group number might be useful in the design and screening of potential tyrosinase inhibitors. Key Words: validamycin A; tyrosinase; inhibition kinetics; molecular dynamics; docking simulation.

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Hippo pathway oncogenic effectors YAP and TAZ in cutaneous melanoma

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YAP and its paralog protein TAZ are downstream effectors of the Hippo pathway in mammals. Both are amplified in many human cancers and promote cell proliferation and epithelial-mesenchymal transition. To date, little is known about the status of the Hippo pathway in cutaneous melanoma. We thus undertook a broad analysis of Hippo pathway components expression in human melanoma cell lines and tumors, and characterized the capacity of YAP and TAZ to control melanoma cell behavior. YAP and TAZ immunostaining revealed mixed cytoplasmic and nuclear staining for both proteins in benign nevi as well as in human melanoma samples (superficial spreading melanoma, nodular melanoma and malignant melanoma in situ). Quantitative PCR and Western analyses identified variable expression of Hippo kinases LATS1/2 and MST1/2 and their effectors YAP1/2 and TAZ across a panel of human melanoma cell lines, irrespective of their BRAF mutation status. Overall, TAZ mRNA levels were about 10-fold higher than those for YAP2, and a 100-fold higher than those for YAP1. Western analysis revealed constitutive phosphorylation of LATS, MST, YAP and TAZ in several cell lines, indicating functional Hippo signaling. Stable knockdown of TAZ by means of lentiviral expression of a specific shRNA in four distinct cell lines did not alter their morphological phenotype in culture, yet dramatically reduced the expression of CCN2/CTGF, a classical Hippo target, as well as anchorage-independent growth and capacity to invade Matrigel. TAZ knockdown in 1205Lu cells also reduced invasion in a model of skin reconstruct. YAP knockdown had similar effects, albeit to a lesser extent. Inversely, YAP (and TAZ) overexpression increased melanoma cell invasiveness, associated with increased TEAD-dependent transcription and CCN2/CTGF expression. Together, these results indicate that both YAP and TAZ contribute to the invasive capacity in melanoma cells.

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Intronic miR-330 targets tyrosinase and induces depigmentation

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MicroRNAs (miRNAs) are small non coding RNA molecules (22nt) that extensively regulate gene expression at the posttranscriptional level. It has been shown that miRNAs control many biological processes including pigmentation. Skin pigmentation is complex and includes many proteins orchestrating a multistep process that finally results in melanin production and distribution throughout the epidermis to generate skin and hair color. Tyrosinase (TYR) is the rate-limiting and key enzyme using tyrosine as substrate to produce dopaquinone. This quinone has the potentiality to generate melanin. Impaired tyrosinase production leads to type I oculocutaneous albinism (OCA1), the complete or partial absence of pigment in the skin, hair and eyes, underlining the importance of TYR in the pigmentation process. We identified miR-330-5p to target tyrosinase mRNA using in silico prediction. To test this potentiality, we treated pigmented human melanoma cells and normal human epidermal melanocytes (NHEMs) with miR-330-5p mimics for 12 days, we found TYR mRNA and protein levels subsequently repressed, and a reduced amount of melanin. We validated the TYR 3'UTR/miR-330-5p predicted binding site by cloning it into a reporter vector and performing luciferase-activity assays. The mature miRNA miR-330-5p is derived from the miR-330 gene located on Chr 19q13.32. In humans, out of the 2042 acknowledged mir, mir-330 is one of 686 intronic miRNAs known to date. It is situated in the first intron of the EML2 gene that codes for echinoderm microtubule associated protein like 2. In this respect, we investigated the coregulation of miR-330 and EML2. Little is known about the role of EML2, except it may modify the assembly dynamics of microtubules. Interestingly, EML2 was lately identified to be downregulated in a murine model for iris pigment dispersion syndrome using a transcriptomic screen.

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Computational prediction for the protein interaction of Tyrosinase: Protein experimental interactome MAP

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Tyrosinase (EC 1.14.18.1) is a diversely distributed enzyme in various organisms with physiological roles related to pigment production. Tyrosinase have gained the attention of researchers due to its biological functions and potential applications. In this regard, studies on the partner proteins of tyrosinase are important. In this study, we predicted the 3D structure of human tyrosinase and simulated the protein-protein interactions between tyrosinase and binding partners by using PEIMAP algorithm. As a result, we found that tyrosinase was predicted to bind with G protein related proteins, potassium voltage-gated channel related proteins, and vesicle/sorting related proteins. Especially, GIPC1, GIPC2, GIPC3, TYRP1, and DCT were predicted to bind with tyrosinase primarily. 103 interacting proteins were secondarily bound to these five interacting proteins in PEIMAP network of tyrosinase. The melanogenesis was also newly predicted by associating the predicted binding proteins. We simulated the protein-protein bindings for probing the residues of each complex facing toward the predicted site of interaction with tyrosinase. Among the interacting residues, some of residues were predicted to docking to the active site of tyrosinase which could affect the activity directly. Our computational predictions will be useful for elucidating the protein-protein interactions of tyrosinase and studying its binding mechanisms and melanin biosynthesis pathway. Key Words: tyrosinase; simulation; protein-protein interaction; PEIMAP; interactomics.

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Live imaging analysis of melanosome transfer using lipophilic tracer

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Melanosome transfer is an important biological activity for skin pigmentation. The mechanism remains controversial. In order to elucidate the mechanism of melanosome transfer, we visualize the delivery of melanosome in the skin-constitutive cells. For melanosome, pigment globules (PGs) were collected from culture medium of human primary melanocyte and were labeled with Vybrant™ Cell labeling solution (Dil). The labelled-melanosomes were co-cultured with primary keratinocyte, primary fibroblast and a macrophage cell line, and then the melanosome transfer was visualized. From the result of the imaging analysis, we hypothesize the process of melanosome transfer using the PGs as melanosome as described below. 1) The pseudopods of keratinocyte trap the PGs. 2) The keratinocyte engulf the PGs by the cell membrane to form phagosome. 3) The engulfed PGs are degraded, leading to the internalization of the vesicle, a size of which is similar to that of human melanosome. The macrophage cells take up the PGs in a similar manner to the keratinocyte. These results indicate that the PGs could be ingested by phagocytosis in the keratinocyte and the macrophage. On the other hand, primary fibroblasts take up the PGs in a different manner from that observed in the keratinocytes. The fibroblasts actively incorporate the PGs into the cytoplasm. However, the pseudopods were not formed for trapping the PGs. Furthermore, the degradation of the PGs and internalization of the vesicle were not seen in the fibroblasts. Thus, we found that the uptake of PGs could be observed in the three kinds of the cells, however, the process of the uptake is different between the keratinocyte and fibroblast.

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Integrated microarray analysis reveals COL11A1, CXCL13 and PTPRF as three new potential biomarkers of metastatic melanoma

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A review of the literature reveals poor congruence between gene signatures of different published microarray-based cancer studies. To address this issue we have developed a novel framework methodology, based on a genome-wide relative significance (GWRS) and genome-wide global significance (GWGS) model, for the systematic analysis of microarray data. Five melanoma microarray datasets, involving a total of 24,185 probe sets, comparing normal/benign nevi with metastatic melanoma and published between 2000 and 2011, were investigated using this methodology. After application of the 'wrapper approach' for gene selection, 200 genes were identified as an initial broad signature. Based on the close interactions of some of these selected genes with well-known melanoma driver genes (i.e. NRAS, BRAF, c-KIT, MITF), we propose a new signaling network significantly involved in the MAPK, Ca2+, and WNT signaling pathways. A new 12-gene signature (i.e., EGFR, FGFR2, FGFR3, IL8, PTPRF, TNC, CXCL13, COL11A1, CHP2, SHC4, PPP2R2C, and WNT4) was defined as a potential diagnostic biomarker for metastatic melanoma. Of them, CXCL13, COL11A, PTPRF and SHC4 in the MAPK signaling pathway were found to be overexpressed in metastatic melanoma cells in vitro and in situ. While SHC4 has been reported previously to be associated to melanoma, this is the first time an experimental validation of CXCL13, COL11A1, PTPRF as new biomarkers for metastatic melanoma is reported. Further investigation into the role of these 12 genes in a revised signaling network for metastatic melanoma should provide new insights into the underlying biological mechanisms driving the progression of melanoma.

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ATM is a marker of oxidative stress in melanocytes of the canities-affected human scalp hair bulb

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The ATM (ataxia telangiectasia mutated) gene encodes a protein kinase that acts as a cellular sensor and provides a checkpoint for DNA damage repair. Recent evidence suggests a role for ATM in protecting cells from damage caused by oxidative stress, and may also protect melanocyte stem cells from premature differentiation in mouse hair follicles. We have previously shown that ATM is expressed exclusively in melanocytes within the hair follicle, and that loss of ATM expression in these cells is associated with hair graying in human scalp tissue. In this study we examined the expression of ATM in hair follicle melanocytes isolated and cultivated from human anagen scalp follicles (20y male & 56y female). ATM was expressed in the nuclei of melanocytes grown under basal culture conditions. When cells were incubated with 40µM hydrogen peroxide (H₂O₂) to induce oxidative stress, a 2-fold increase in ATM expression (over basal levels) was detected by immunoblotting densitometry in hair follicle melanocytes isolated from the young donor compared with a 4-fold increase in cells from the older donor. This increased ATM expression was returned to basal levels by co-incubation with Vitamin E (10⁻³M)/Quercetin (10⁻⁶M) antioxidant mixture. Selective siRNA knockdown of ATM gene expression in hair follicle melanocytes prior to H₂O₂ incubation sensitized these cells to treatment and produced visible areas of cell detachment and death compared to controls. The latter was more prevalent in hair melanocytes established from the older versus the younger donor. In conclusion we show that ATM (a canities-associated hair bulb biomarker) is also a marker of oxidative stress in hair follicle melanocytes. ATM appears to play an important role in protecting human hair follicle melanocytes from oxidative stress/damage, and maintenance of its expression may be important for hair bulb melanocyte survival in canities-prone scalp hair follicles.

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EMMPRIN mediates VEGF activation of VEGFR-2 in melanoma cells

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EMMPRIN's expression in melanoma tissue was reported to be predictive of poor prognosis. Here we demonstrate that EMMPRIN up-regulated VEGF receptor-2 (VEGFR-2) in two different primary melanoma cell lines and consequently increased migration and proliferation of these cells while inhibiting their apoptosis. siRNA inhibition of VEGFR-2 expression abrogated these EMMPRIN effects. EMMPRIN regulation of VEGFR-2 was mediated through the over-expression of HIF-2α and its translocation to the nucleus where it forms heterodimers with HIF-1β. These results were supported by an in vivo correlation between the expression of EMMPRIN with that of VEGFR-2 in human melanoma tissues as well as with the extent of HIF-2α localization in the nucleus. They demonstrate a novel mechanism by which EMMPRIN promotes tumor progression through HIF-2α/VEGFR-2 mediated mechanism, with an autocrine role in melanoma cell malignancy. As VEGF/VEGFR2 system has been implicated in regulation of melanoma cells migration, proliferation and apoptosis, the role of EMMPRIN in the regulation of these cellular properties was investigated. Blocking EMMPRIN by siRNA have shown that VEGF enhancement of melanoma cell migration requires EMMPRIN. The inhibition of EMMPRIN in cancer may thus simultaneously target both the VEGFR-2/VEGF system and the matrix degrading proteases to block tumor cell growth and invasion.

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Biomarkers of canities-affected human scalp gray hair follicles

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Canities (graying) is an age-linked loss of melanin from hair. While the cause(s) of the associated deletion of melanogenically-active melanocytes from affected human scalp anagen hair bulbs is unknown, endo/exogenous oxidative stress appears to be involved. Here we examined, by immunohistochemistry, the degenerating follicular melanin unit in aging human scalp of 22 healthy individuals (22-70yr). Over 20 markers were examined within the following categories: melanocyte-specific, DNA repair/damage, senescence, cell cycle, apoptosis, and oxidative stress. Results of this study revealed a reduction in melanocyte-specific marker expression proportionate with the extent of canities. A major finding of this study was the strong and exclusive expression of ATM within surviving melanocytes in canities-affected hair bulbs, the frequency of which correlated with pigmentation status. ATM phosphorylates several key proteins to activate the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. Moreover, increased levels of GADD45 and 8-OHdG were also detected within some bulbar melanocytes, although lacked a clear association with donor age or canities extent. The former multi-protein interactive stress sensor modulates the cellular response to genotoxic/physiological stress, while levels of 8-Oxo-2'-deoxyguanosine expression (major product of DNA oxidation) are a good marker for tissue oxidative stress. Unexpectedly, we found no specific evidence of increased expression of other well-known markers of oxidative stress, senescence or DNA damage/repair in the canities-affected melanocytes compared to surrounding bulbar keratinocytes, although some markers did show basal expression in the hair follicle. In conclusion, we identify here ATM, GADD45 and glutathione peroxidase 1 immunoreactivity as important biomarkers of melanocyte status in canities-affected human scalp hair follicles, which may provide protection against canities via the DNA/Damage repair pathway.

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Periorbital hyperchromia in a caucasian and chinese study population

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A large proportion of the population shows signs of periorbital hyperchromia or dark under eye circles. Despite the prevalence of DUEC its etiology is poorly defined. We non-invasively characterised the under-eye area of respectively 140 and 320 Caucasian and Chinese volunteers, with or without periorbital hyperchromia. People were classified in three groups according to the clinical manifestation of periorbital hyperchromia. As expected colour contrast (Visia-CR), defined as the grey value of the cheek bone minus the grey value at the under-eye area, gradually increased at increasing clinical severity for both ethnicities. Further the colour contrast was inversely correlated with the chronological age in the Chinese (r=-0.23; p=0.00002) and Caucasian population (R=-0.19, p=0.02). Additional evaluation of the data indicated a negative correlation between the grey values of both the cheek and the eye and the chronological age for the Chinese study population (cheek: p<10⁻⁴, r=-0.52; eye: p<10⁻⁴, r=-0.26). The slope of the trend line was steeper on the cheek than on the under-eye area (-0.314 and -0.196 grey value units/year respectively) indicating that the difference in grey value, and thus overall skin tone, between both sites decreased with age. This agrees with the observation that in the Chinese study population, there was a statistical significant difference in chronological age between the groups with different degrees of periorbital hyperchromia, with the lowest age in the group of the highest severity. It can be concluded that age dependent changes in skin colour vary depending on the site of the face. This contributes to age dependent changes in visibility of periorbital hyperchromia in the Chinese study population.

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Peri-tumoural expression of pro-autophagic Ambra-1 as a prognostic biomarker for melanoma

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Currently the overriding prognostic indicator for melanoma is disease stage at diagnosis; however, current staging criteria are unable to predict the subset of patients with high risk lesions that eventually progress. Loss of autophagy, the principle lysosomal mediated process for the degradation/recycling of damaged organelles and excess proteins is associated with tumourigenesis. Protein regulators of autophagy including pro-autophagic Ambra-1 thus present ideal targets for biomarker development. The aim of the current study was to correlate peri-tumoural expression of Ambra-1 with melanoma development and progression. Immunohistochemical analysis of peri-tumoural epidermal Ambra-1 expression was determined in a retrospective cohort of 149 melanocytic naevi or primary melanomas derived from differing disease stage and correlated with patient outcome over 7 years. Results demonstrated consistent expression of Ambra-1 in normal epidermis, and the epidermis overlying benign naevi, while expression varied in the epidermis overlying primary melanomas. A decrease or complete loss of Ambra-1 overlying all stage tumours was associated with a significant risk of disease progression (Hazard Ratio 3.95, 95% Confidence Interval (CI) 1.45 - 10.75, P = 0.007) and the development of tumour ulceration. Multivariate analysis highlighted the loss of Ambra-1 epidermal expression with increased risk of metastases in AJCC stage I melanomas (Hazard Ratio 4.4 (95% CI 1.07- 18.03, P = 0.03), collectively suggesting a decrease in epidermal Ambra-1 expression is associated with the loss of autophagy and epidermal barrier dysfunction and breakdown. Loss of epidermal Ambra-1 expression thus provides independent prognostic value over-and-above the current American Joint Committee on Cancer (AJCC) staging criteria, allowing early identification and adjuvant therapeutic intervention of patients with melanoma at greatest risk of disease progression.

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MC1R variants increase melanoma risk independent from previous ultraviolet radiation

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Only recently, an ultraviolet-radiation independent mechanism of melanoma development was identified in red-coated mice with decreased MC1R signaling ability, indicating an ultraviolet-radiation independent mechanism in melanoma development. To test this hypothesis in a clinical setting, we evaluated the role of MC1R and melanoma risk independently from actinic skin damage and sunburns. 1791 participants from our M3-Study were included in the analyses. MC1R variant carriers had a 1.76 fold increased risk for melanoma compared to wildtype, and risk was increased with the number of MC1R variants (OR 2.15 for ≥ 2 variants and OR 1.29 for carriers of one variant compared to wildtype). When we adjusted these results for sex, age, sunburns in the past and signs of actinic skin damage, the associations remained significant (OR for R/R 1.64, OR for R/r 2.63, OR for R/0 1.84 and OR for r/r 1.55 when adjusted for facial actinic skin damage, OR for R/r 2.35 and OR for R/0 1.46 when adjusted for dorsal actinic skin damage and OR for R/r 2.54, OR for R/0 1.75 and OR for r/r 1.54 when adjusted for actinic skin damage on the hands). We conclude that risk of melanoma in MC1R variant carriers is determined by other factors than ultraviolet radiation and that further studies are required to elucidate the cause of melanoma development in these high risk individuals.

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GD1b, GT1b, GQ1b gangliosides mediate the progression of melanoma

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Gangliosides influence tumoral development through various mechanisms. The authors aimed to evaluate the involvement of GD1b, GT1b, GQ1b gangliosides in modulating the proliferation of malignant melanocytes, the capacity of these glycosphingolipids antigens to elaborate an antitumoral immune response and their possible influence on angiogenesis. The study included 128 adult patients with primary melanoma evaluated before the surgical removal of the tumor and 48 healthy volunteers. We determined the content of GD1b, GT1b, GQ1b gangliosides in tumoral tissue and IL8 serum concentration (proangiogenic factor); evaluated the status of antiGD1b, antiGT1b, antiGQ1b antibodies in melanoma patients. Tumoral gangliosides were quantified by thin layer chromatography. Serum levels of IL8 were determined by ELISA method, and anti-gangliosides antibodies were analyzed by IMMUNODOT method. GD1b, GT1b, GQ1b gangliosides are minor components expressed in melanoma cells. These biomolecules represented less than 5% from total gangliosidic sialic acid determined in tumor. Detectable values of GD1b, GT1b, GQ1b gangliosides were observed in less of 30% of specimens. Autoantibodies against these 3 gangliosides, most of IgM class, were found in 19.53 % from melanoma cases. Serum concentration of IL8 was significantly increased in patients with melanoma compared to control (103.25 \pm 55.93pg/ml versus 12.03 \pm 10.35pg/ml). The statistical analysis showed a significant negative correlation between the content of GD1b, GT1b, GQ1b gangliosides in tumoral tissue and serum level of IL8 ($r=-0.58$, $p<0.05$, $CI=95\%$). A negative correlation, but without statistical significance, was observed when compared anti-gangliosides antibodies status with IL8 levels ($r=-0.20$, $p>0.05$, $CI=95\%$). In conclusion, GD1b, GT1b, GQ1b gangliosides could influence the evolution of melanoma patients by stimulating an efficient antitumor immune response or by inhibiting IL8 synthesis and secretion.

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PD-1 expression by melanoma cells modulates antitumor immunity and promotes cancer progression

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The immunologic checkpoint receptor, programmed death-1 (PD-1), plays a key role in thwarting antitumor immunity and PD-1 pathway blockade has shown promising clinical results in patients with advanced melanoma. Current understanding holds that PD-1 is predominantly expressed by immune cells and that PD-1 inhibition can overcome tumor immune evasion by reactivating exhausted antitumor immune responses. Here, we demonstrate constitutive PD-1 expression by a wide array of human and murine melanoma cell lines and identify critical roles of melanoma-expressed PD-1 in promoting regulatory T-cell (Treg) induction and tumor growth. Melanoma-specific shRNA-mediated knockdown (KD) of PD-1 inhibited CD3-dependent proliferation of peripheral T-cells isolated from B16 melanoma-bearing mice. Furthermore, compared to control-B16 tumors, PD-1 KD melanomas revealed significantly decreased Treg frequencies among tumor-infiltrating lymphocytes (TILs). Moreover, melanoma-specific PD-1 KD markedly inhibited tumor growth in syngeneic C57BL/6 recipient mice (by > 80%). Systemic administration of a monoclonal antibody directed at PD-1 exerted similar tumor-inhibitory effects. Consistent with these findings, B16 melanoma variants engineered to overexpress PD-1 demonstrated > 5-fold increased tumorigenicity compared to control B16 melanomas. Additionally, Treg numbers were significantly increased among TILs and in draining lymph nodes of syngeneic PD-1-overexpressing versus control B16 melanoma-bearing mice. Our findings establish melanoma-expressed PD-1 as a novel tumor growth-promoting immunoregulatory mechanism and identify melanoma-specific PD-1 signaling as a previously unrecognized target for tumor therapy.

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Insulin-like growth factor II mRNA-binding protein 3 (IMP-3) expression promotes tumor migration, invasion and predicts poor prognosis in melanoma

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Insulin-like growth factor-II messenger RNA (mRNA)-binding protein-3 (IMP-3) is a member of insulin-like growth factor-II mRNA-binding protein family. Recently, IMP-3 has been found to be a novel progression marker in malignant melanoma. A total of 82 patients with melanoma were analyzed in this study. IMP-3 positivity was associated with older age at diagnosis (≥ 65 years old) ($P=0.022$) but did not correlate with sex, ulceration, lymph node metastasis, tumor thickness, stage or subtypes of melanoma. Stratified by positive ($n=59$) and negative IMP-3 expression ($n=23$), the 5-year overall survival rate of melanoma patient was 33.9% and 60.9%, respectively. Patients with positive IMP-3 expression had a significantly worse overall survival by the Kaplan-Meier analysis ($P=0.0124$). Univariate analysis by the Cox regression model showed that lymph node metastasis, advanced stage and IMP-3 expression predicted a poor outcome. The *in vitro* study confirmed that melanoma cells with higher migratory/invasive abilities have higher expression of IMP-3. Overexpression of IMP-3 in MeWo cells enhanced their migratory ability, while depletion of IMP-3 expression by RNA interference in A2058 melanoma cell line reduced the migration/invasion abilities. Larger xenograft tumors developed from implantation of MeWo-IMP-3 cells than MeWo-vec cells in tumorigenicity assay. IMP-3 overexpression also increased the number of lymph node metastasis. Microarray and RT-qPCR studies found that IMP-3 over-expressing MeWo cells had increased *HMG2c*, *c-myc*, *ETS-1* and *CCL20* transcript levels compared with the vector controls. These results provide evidence of direct involvement of IMP-3 in regulating melanoma biological behavior.

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DC-HIL expressed by osteoblasts mediates these cells' ability to inhibit hematopoietic stem cell proliferation in an integrin-dependent manner

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Having shown the cell surface receptor DC-HIL to be expressed constitutively by skin-resident cells (Langerhans cells, melanocytes, melanoma cells) and inducibly by bone marrow (BM)-derived cells (macrophages and myeloid suppressor cells), we discovered its 2 functions: (1) mediator of adhesion to keratinocytes and endothelial cells; and (2) suppressor of T cell activation by binding syndecan-4 on T cells. In surveying DC-HIL expression in BM cells via RT-PCR, FACS and immunofluorescence staining, we found osteoblasts (Ob) to be the sole constitutively expressing cells. Given that Ob critically inhibit hematopoietic stem/progenitor cells (HSPC) proliferation via cell adhesion, we posited a role for DC-HIL in this process and asked whether our blocking Ab to DC-HIL (UTX) (vs. control IgG) can interfere with Ob/HSPC interactions. Addition of UTX to primary cocultures of Ob and GFP⁺Lineage^{neg} HSPC increased the number of adherent HSPC transiently (by 36% within 3h, but not by 6h), as measured by CFU-GM. Longer treatment with UTX (1wk) led to more HSPC (234 \pm 44%, $p=0.02$) and to shriveling and tight aggregation of Ob. Substitution of recombinant DC-HIL protein (for UTX Ab) produced similar results. All outcomes were abrogated by addition of the integrin inhibitor RGD peptide. We then asked whether UTX treatment of mice causes HSPC proliferation. Naive mice ($n=5$) were injected *i.p.* with UTX (200 μ g/mouse) on alternate days a total of 5 times, and examined for CFU-GM. UTX (vs. control IgG) significantly increased HSPC in BM by 150 \pm 25% and in peripheral blood by 633 \pm 267% ($p<0.05$). Thus, Ob express DC-HIL, which regulates Ob/HSPC adhesion in an integrin-dependent manner, to inhibit HSPC proliferation. This novel function of DC-HIL coupled with a previous finding showing soluble DC-HIL receptor to be shed by melanoma cells into circulation, fortifies the concept of interconnectedness between skin and BM, with pathogenic and therapeutic implications for neoplastic and inflammatory skin diseases.

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MITF regulates the proliferative architecture of the melanoma microenvironment

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Dysregulated proliferation is a hallmark of tumour cells. However, solid cancers are typically composed of irregular zones containing mixtures of actively cycling and quiescent cells, and other areas where cells invade into the surrounding tissues. Although considerable insight has been attained into the molecular mechanisms underlying aberrant cell cycle progression of cancer cells, we have limited understanding as to what regulates the positioning of proliferating cells within the complex microenvironment of a tumour. In addition, the relationship between proliferative and invasive potential of cancer cells is unclear. In this study, we show that the microphthalmia-associated transcription factor (MITF) regulates the compartment-specific distribution of proliferating melanoma cells *in situ*. We made use of the fluorescent ubiquitination-based cell cycle indicator (FUCCI), which facilitates the identification of individual proliferating cells in G1 versus S/G2/M cell cycle phases, in combination with confocal and multiphoton microscopy to track melanoma cell proliferation in real-time. While MITFhi melanomas revealed a phenotype characterized by random distribution of proliferating cells, MITFlow melanomas were composed of proliferation hot-spots and large areas of G1-arrested cells. MITF did not, however, impact on the duration of cell cycle phases of individual melanoma cells. Finally, cell-tracking analyses revealed that migratory speed and directionality as well as invasive behaviour were independent of whether melanoma cells resided in G1 or S/G2/M cell cycle phases. Our data uncover the reliance of the melanoma proliferation architecture on MITF, and further reveal a surprising lack of correlation between cell cycle progression and migration of melanoma cells.

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Peri-orbital dark circles in Brazilian women: Risk factors and hyperspectral image analysis
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Peri-orbital dark circles (DUEC) are a cosmetic concern world-wide, and have been attributed to hyperpigmentation from allergy or atopic dermatitis, blood stasis, structural shadowing effects, and a thinner epidermis/dermis under the eye. It is of interest to better understand risk factors and the relative contribution of melanin, blood and epidermal/dermal factors. We surveyed lifestyle and health factors of 495 Brazilian women and evaluated subjects' DUEC severity by a dermatologist expert assessment. Hyperspectral image analysis was used to determine the relative contribution of melanin, blood volume, degree of tissue oxygen saturation, and dermal scattering. Spectral Imaging indicated that melanin was the dominant correlate with DUEC severity, and oxygen saturation was secondary. Spectrophotometric color evaluation terms ΔL^* and ΔE^* were the most significant instrumental parameters correlated with visual assessment of DUEC severity. We found that family history was the most significant risk factor for DUEC. The average age of onset was 24 years, and a younger onset correlated with higher severity scores. Asthma was highly significantly associated with DUEC score, but self-reported allergy was not. Fitzpatrick skin type was not correlated with the risk or severity of DUEC. Although consumers world-wide believe that lack of sleep makes their DUEC worse, sleep quantity and quality were not correlated to DUEC scores in this study. No measure of sleep quantity or sleep quality correlated with physician assessed DUEC severity. Objective assessment modalities for physiological factors responsible for DUEC are critical to effective therapy for this condition, and can help predict treatment success.

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Involvement of Src kinase in fisetin mediated melanoma growth inhibition: Studies in 3-D melanoma model

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Melanoma, a leading cause of skin cancer, typically progresses through an initial, minimally invasive radial growth phase followed by the invasive vertical growth phase, and finally, metastasis. Activation of Src kinase with subsequent promotion of epithelial-mesenchymal transition (EMT) has been implicated in tumor growth and invasion. We previously showed that the dietary flavonoid fisetin inhibited melanoma growth in vitro and in vivo. Here, we studied the molecular mechanisms involved in fisetin-mediated decrease in melanoma cell motility and growth inhibition. Monolayer cell culture studies showed that fisetin (10-40 μ M) inhibited the growth of A375 melanoma cells and suppressed invasion across the matrigel. This was associated with decreased phosphorylation of FAK and Src kinases and disruption of the functionally interdependent FAK/Src interaction. In addition, fisetin reduced the expression of matrix-metalloproteinases -2 and -9, positive regulators of tumor invasion and metastasis. Fisetin modulated the expression of EMT markers with a decrease in N-cadherin and Vimentin, and a concomitant increase in E-cadherin protein expression. To establish the human relevance of these findings, we determined the effect of fisetin in 3-D multilayered melanoma constructs that closely simulate human skin. The constructs treated with fisetin showed significantly less melanocytic lesions in the vertical growth phase as opposed to untreated controls that showed large tumor nests and many invading disseminated cells, with progression of melanoma from radial to vertical growth phase. Fisetin-treated constructs showed diminished staining of phosphorylated Src and FAK, with moderate to strong E-cadherin expression, in radially growing melanoma cells. Our findings indicate an important role of FAK/Src signaling in melanoma progression and identify it as a novel target of fisetin. We propose that fisetin has the potential to be developed as an anti-melanoma agent that can be used alone or in combination with other known agents.

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7,3',4'-Trihydroxyisoflavone, a metabolite of the soy isoflavone daidzein, suppresses α -MSH-induced Melanogenesis by targeting Melanocortin 1 receptor

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7,3',4'-Trihydroxyisoflavone (7,3',4'-THIF) is a metabolite of daidzein which is a representative isoflavone found in soybean. Recent studies suggested that 7,3',4'-THIF exerts a hypopigmentary effect in B16F10 cells, however, its underlying molecular mechanisms and specific target remain unknown. Here, we found that 7,3',4'-THIF, but not daidzein, inhibited α -melanocyte-stimulating hormone (MSH)-induced intracellular and extracellular melanin production in B16F10 cells. The expression of tyrosinase and tyrosinase-related protein-1 (TRP-1) was also suppressed by 7,3',4'-THIF treatment at non-cytotoxic concentrations. Microphthalmia-associated transcription factor (MITF) and cAMP response element-binding (CREB), crucial transcription factors involved in tyrosinase expression, were activated by α -MSH and 7,3',4'-THIF abolished this activation. 7,3',4'-THIF inhibited α -MSH-induced dephosphorylation of AKT and phosphorylation of p38 kinase and cAMP-dependent protein kinase (PKA). cAMP and pull-down assay revealed that 7,3',4'-THIF strongly inhibited forskolin-induced intracellular cAMP production and bound directly with melanocortin 1 receptor (MC1R). Moreover, 7,3',4'-THIF inhibited α -MSH-induced intracellular melanin production in human epidermal melanocytes (HEMs). Collectively, these results demonstrate that 7,3',4'-THIF targets MC1R directly, resulting in the suppression of melanin production, suggesting a protective role for 7,3',4'-THIF against melanogenesis.

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Specific KIT mutation is present in melanomas with dark homogeneous streaks pattern
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Primary cutaneous melanomas are classified as superficial spreading (SSM), lentigo maligna (LMM), acral lentiginous, and nodular melanoma. Also subcategorized as chronic and non-chronic/intermittent sun damaged. BRAF, NRAS, and KIT are the major growth promoting mutated genes. However, for a significant fraction of melanomas the growth promoting mutation is unknown. BRAF is prevalent in SSM/intermittent-sun damaged whereas KIT is in LMM/chronic-sun damaged; but there is not a precise correlation with tumor subtype. We hypothesize that the underlying growth promoting mutations drive unique surface growth patterns. Dermoscopy allows for improved visualization of melanoma growth patterns and may better segregate molecular subtypes. We reviewed dermoscopic images of cutaneous melanomas removed at University of Miami and Skin and Cancer Associates. Four consistent dermoscopic patterns were identified and chosen for genetic analysis: negative network, rhomboidal, polygonal, and dark homogenous streaks pattern. Three melanomas per pattern were analyzed for BRAF, NRAS, HRAS, KIT and GNAQ mutations. Dark homogenous streaks pattern melanomas revealed KIT exon 17 mutations, located within or adjacent to the catalytic region of the second kinase domain. The expected frequency of KIT mutations in melanoma is 4.3%, of all KIT mutations the frequency of mutations in exon 17 is 4.4%. The chance of this occurring in 3 tumors prospectively segregated is 1 in 6.8 billion; highly suggestive that the mutation is correlated with the dermoscopic pattern. Despite the high prevalence of BRAF and NRAS mutations in melanomas as a whole, the other tested patterns were wild type suggesting that BRAF and NRAS may be enriched in more advanced tumors. In summary, our results show that specific dermoscopic patterns can be identified that correlate with specific gene mutations. Also dermoscopic patterns reveal melanomas that lack common mutations and therefore may allow for the detection of new driving mutations.

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Lack of clinical response of recalcitrant melasma to triple combination cream (Tretinoin, Fluocinolona and Hidroquinone) due to abnormal concentration of melanin

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Nearly thirty percent of the patients with Melasma do not show any response to conventional treatment with Hydroquinone. This is hypothesized due to atypical melanin arrangement and concentration. The purpose of this study is to use Raman spectroscopy and Diffuse Reflectance Spectroscopy to investigate abnormal molecular structure and concentration of melanin in patients with recalcitrant melasma lesions pre-treatment and post-treatment with tretinoin, fluocinolona and hidroquinone (Tri-Luma). The study enrolled 31 subjects (27 females, 4 males) ages 23 to 52 years (Median 38.7), who met the inclusion criteria specifying patients with melasma. A detailed questionnaire was prepared to record the subjects' histories, sunlight exposures, related medicine administrations, former treatments, and cosmetics usage. Raman and diffuse reflectance spectra measurements were taken from melasma lesions, non lesional facial photoexposed skin area, and non photoexposed skin area. Raman spectroscopy results show that patients non responsive to treatment demonstrate differences in the molecular structure of their skin compared to people that showed improvement with treatment, the difference in the former are consistent with increased melanin concentration and are evident through a Principal Component Analysis of the Raman spectra.

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A progression pattern of subungual melanoma

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 Subungual melanoma is a rare subtype of malignant melanoma. It usually occurs in the nail matrix of nail unit. However, a progression pattern of subungual melanoma from nail matrix is unknown. We reviewed 63 cases of subungual melanoma in our hospital from 1997 to 2012. We analyzed 21 cases of subungual melanoma that include each part of the nail unit: proximal nail fold, nail matrix, nail bed, and hyponychium. We analyzed the histological features in each anatomical region of the nail unit and the prognosis of patients. We classified 21 cases of subungual melanoma according to the presence of dermal invasion in each part of the nail unit. Subungual melanoma in situ in all part of nail unit were 4 cases and subungual melanoma with dermal invasion more than one part of nail unit were 17 cases. We observed 4 cases with dermal invasion in one more parts of nail unit except nail matrix. In 13 cases with dermal invasion involved the nail matrix, the dermal invasion affected more than two parts of nail unit. There was no case of dermal invasion in nail matrix only. The depth of dermal invasion of nail matrix involved cases was deeper than that of the nail matrix sparing cases and the 31% of cases with dermal invasion in the nail matrix had distant metastasis. In this study, we found that dermal invasion of subungual melanoma in nail matrix occur less than in other parts of nail unit. Our result suggested that the nail matrix may have an ability to resist to invasion.

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The p53 tumor suppressor acts as tumor promoter in vemurafenib resistant melanoma cells
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 Melanoma is an aggressive malignancy whose incidence is increasing very rapidly worldwide. In melanoma cells, the MAP Kinase signaling pathway is often activated by mutations in BRAF or NRAS. In particular, BRAF is mutated in about 60% of cutaneous MM. The BRAF inhibitor Vemurafenib is now the first line treatment for patients with metastatic melanoma with a V600E BRAF mutation. However the occurrence of secondary resistance hampers the efficacy of this drug after 6 to 8 months of treatment. Mechanisms of resistance are the object of an intense research activity. Resistance to BRAFi can involve re-activation of the MAP kinase pathway by post-translational modifications of BRAF, by activation of NRAS upstream of BRAF or MEK, downstream of BRAF. Additional pathways including those activated by IGF1R, PDGFR, cKIT or the PI3K pathway can also account for resistance. However, little is known about the role of p53 tumor suppressor gene family in melanoma resistance to BRAFi. p53 is rarely mutated in melanoma, but is inactivated by conformational change and is unable to activate cell cycle arrest and apoptosis. We have explored the role of p53 in this context using cell lines from naïve patients and from patients who developed resistance to BRAFi and found that p53 displays a counter-intuitive role as a tumor promoter in BRAFi resistant cells. Melanoma cells with acquired resistance to BRAFi inhibitors re-activate p53 transcriptional activity to promote synthesis of Kit ligand (KITLG), MSH1, actinA and other mediators of invasion and resistance to BRAFi. These soluble factors act in an autocrine and paracrine way to promote resistance to BRAFi and proliferation. Interestingly, the mdm2 inhibitor Nutlin-3 can efficiently kill BRAFi resistant cells reacquiring p53 transcriptional activity.

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Investigation of the role of amyloid precursor protein in ocular melanoma using goldfish (*Carassius Auratus*) model
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 Amyloid precursor protein, or APP, is recognized as a critical factor in the pathogenesis of Alzheimer's disease, while its physiological roles and other pathological functions remain poorly understood. Recent studies on the physiological functions of APP have focused on its soluble N-terminal ectodomain (or sAPP), which displays structure resemblance and functional characteristics of a growth factor. In skin, sAPP from melanocytes and/or keratinocytes stimulate melanocytes with an increased lamellipodia dynamics and melanin release. Full-length APP, located in the membrane of endoplasmic reticulum and melanosomes in melanocytes, mediates melanosome transport along microtubules interacting with motor protein kinesin. However, the role of APP in transformation from melanocytes to melanoma has not been known until recently. Studies suggest that APP, together with EGFR, is involved in the metastatic potential of melanoma. Using confocal microscopy and Western blot analyses, we found that human skin melanocytes express abundant APP but almost undetectable EGFR, however, human skin melanoma cells (A375, WM-266-4, WM115) express both APP and EGFR. Western blot analysis also showed that mTOR pathway is constitutively activated in melanoma cells. Similar to human skin melanoma, the mechanism of ocular melanoma remains unclear, while the incidence of ocular melanoma is consistently on the rise. We hypothesize that both EGFR and APP are associated with ocular melanoma. Using confocal microscopy, we investigated the expression of APP in sections of golden fish eyes. Preliminary data showed that APP is expressed in the retina area of the golden fish eyes. Western blot data confirm this observation. On-going studies using transmission electron microscope will clarify the relationship between EGFR and APP in golden fish eyes. Our studies will provide insights into understanding of the molecular role of APP in ocular melanoma.

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Soluble adenylyl cyclase regulates melanosome production and decay in human melanocytes
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 Cyclic AMP (cAMP) is a key signaling molecule for the transcription of melanogenesis enzymes. We study soluble adenylyl cyclase (sAC), a novel enzymatic source of melanocytic cAMP. sAC, unlike transmembrane adenylyl cyclases, is localized to the nucleus, cytoplasm and mitochondria rather than the plasma membrane. sAC is regulated by changes in cellular pH and is known to regulate vacuolar-type H⁺-ATPase (V-ATPase), a protein essential for the acidification and maturation of organelles such as the melanosome. We previously showed that pharmacological or genetic inhibition of sAC increased cellular melanin content, an intriguing result because cAMP has been well characterized as an inducer of melanogenesis. Our new findings suggest that sAC inhibition raises melanin levels by impairing melanosome degradation. sAC inhibition induced an altered phenotype relative to untreated cells, producing membranous and vacuolar structures within their cell bodies. Electron microscope studies demonstrated an elevation in total melanosome number and the accumulation of large atypical double membrane enclosed structures containing melanin. These large structures are consistent with autophagolysosomes, and our laboratory has recently shown that sAC is essential for normal autophagy in neurons, hepatocytes and fibroblasts. Pharmacological and genetic inhibition of sAC led to increased numbers of late stage melanosomes, including Stage III and IV melanosomes, while untreated cells had less total melanosomes and a greater proportion of Stage I and II melanosomes. More autophagolysosomes were present in sAC inhibited cells. Our results suggest that sAC inhibition in melanocytes impairs melanosome degradation and subsequent production. This melanosome production cycle is essential to human skin pigmentation, and the sequestration of chemotherapy in melanosomes is thought to be an important mechanism of melanoma drug resistance. sAC's emerging role in autophagolysosome degradation pathways may offer new mechanisms to regulate melanosome turnover.

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A two gene serum epigenetic signature detects metastatic melanoma with high sensitivity and specificity
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 Cutaneous melanoma is an aggressive malignancy with a very poor prognosis once metastatic. This study aims to develop a portfolio of biomarkers of diagnostic and prognostic usefulness that will facilitate early treatment of metastatic disease. Cancer-associated gene promoter methylation is recognized as an important mechanism in tumour progression. Identification of cancer-specific methylation markers in melanoma patients' tissues and blood may help us to better understand the molecular mechanisms of melanoma, to develop prognostic biomarkers and to develop new targeted therapies. We have identified TFPI2 and Dab2 methylated in both primary and metastatic melanomas, and increased methylation levels of TFPI2 and Dab2 significantly correlate with melanoma progression. TFPI2 and Dab2 are also promising candidates for serum epigenetics with apparent correlation between detectable methylated DNA in patient's blood and poor outcome in terms of metastatic melanoma. The two gene serum epigenetic signature detects metastatic melanoma with high sensitivity and specificity. These data support a role for epigenetic silencing of TFPI2 and Dab2 in melanoma. Detection of promoter hypermethylated TFPI2 and Dab2 in patient serum samples suggests 'serum epigenetics' could provide novel biomarkers with clinical utility for early detection of metastasis.

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Vimentin protects against endoplasmic reticulum stress induced by arsenite in human skin melanoma cells
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 Arsenite has been shown to induce apoptosis of cancer cells and has been used in the treatment of leukemia. While the studies of the molecular mechanism of arsenite action have been intensive, less has been known about the ER stress induced by arsenite. We studied the effect of arsenite on the growth, survival and cell migration in human melanoma cells (WM-266-4 and A375). We found that arsenite inhibits cell migration and induces cell death. Confocal microscopic data demonstrated that arsenite does not inhibit the over-activation of EGFR/mTOR pathway in human melanoma cells. Melanoma cells (WM266-4), resistant to H2O2 treatment, do not produce ROS in response to arsenite treatment. Arsenite does not inhibit constitutive activation of 56 ribosomal protein. We also observed that arsenite induces calreticulin or CRT translocation in human melanoma cells but not in human melanocytes. Previous studies have shown that vimentin, a major intermediate filament protein, is over-expressed in malignant melanoma, and over-expression of vimentin in cancer cells correlates well with tumor growth, invasion, and poor prognosis. However, the role of vimentin in cancer metastasis remains to be elucidated. Recent studies have shown that vimentin interacts with ERK and p38 leading to downstream component activation. In this study, interestingly, we found that arsenite induces vimentin polarization in ER. Our results suggest that this vimentin encampment of ER induced by arsenite may be a protective measure under arsenite treatment. Our results further support the notion that vimentin can be considered as potential biomarker for predicting melanoma metastasis and thus as a potential molecular target for melanoma.

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RASSF1A protein expression despite DNA methylation in melanocytic nevi
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 DNA methylation of the gene promoter region is closely correlated with loss of downstream gene transcription. Hypermethylation of Ras association domain family member 1 (RASSF1A) gene has been detected in 36%–41% melanomas, and immunohistochemical analyses have revealed that RASSF1A protein is downregulated in melanoma tissues. Therefore, RASSF1A gene is considered as a melanoma suppressor gene. In ESDR 2011, we reported that RASSF1A gene hypermethylation was detected in 5 (12%) of 43 melanocytic nevi using methylation-specific PCR. This study aimed to evaluate the correlation between RASSF1A gene hypermethylation and loss of RASSF1A protein expression using a sample set previously reported by us. The pathological tissues were stained with the anti-RASSF1A antibody using the immunostaining technique. The stained nevi were classified into four groups on the basis of staining intensity: negative, mild, moderate and severe. Consequently, the number of nevi in each of the four groups was 0, 7, 31 and 5, respectively. Five nevi were hypermethylated; none exhibited negative staining, 1 exhibited mild staining, 3 exhibited moderate staining and 1 exhibited strong staining. There was no correlation between methylation status and RASSF1A protein expression in this study. This discrepancy can be attributed to the hypersensitivity of methylation-specific PCR, which can detect even partial methylation. In conclusion, loss of RASSF1A protein expression, particularly that resulting in DNA methylation, is rarely associated with melanocytic nevi.

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Potential approach for treatment of melasma by regulation of epidermal stem cells

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Recently, it is reported that pendulous melanocytes can be a characteristic feature of melasma which is related with reduced expression of type IV collagen. Histologically, melasma is characterized by increased melanin in epidermal layer but melasma skin often has more melanins and melanophages in the dermis. These findings suggest that melasma is not only a disease of melanocytes but also a disease of surrounding cells and environment such as basement membrane. Stem cells are under the control of surrounding environment. Already mentioned, disruption of basement membrane is frequently observed in melasma. In addition, microenvironment changes will affect epidermal stem cells because basement membrane will provide niches for the epidermal stem cells. Thus, strategies for manipulating "cell to extracellular matrix interactions" hold promise in preventing aging effects or controlling skin disease such as melasma. In this presentation, the effects of "various external factors" and "epigenetic factor" on epidermal stem cells will be presented. In three dimensional skin models, these factors such as GHK, hyaluronic acid, and some flavonoids, increased the expression of p63 and integrin expression along the basement membrane. Our results clearly suggested that stem cell activator can be useful in controlling skin aging and skin diseases including melasma.

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Epigenetic control of tumor suppressors and oncogenes by melanoma antigens (MAGE) and KRAB-zinc finger transcription factors (KZFs)

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MAGE proteins are not expressed in somatic tissues but are often expressed in malignancies, making them ideal targets. MAGE expression in melanoma predicts poor prognosis and positive sentinel lymph nodes independent of Breslow thickness. MAGE proteins suppress p53 and promote DNA repair, but the mechanisms of MAGE effects have not been completely characterized. KZFs repress genes by guiding KAP1, a ubiquitin ligase and scaffolding protein, to specific chromatin sites where KAP1 recruits HP1, NuRD and SETDB1, inducing H3K9me3 and chromatin condensation. We have shown that MAGE-A3 and C2 bind to KAP1 and decrease KAP1 binding and specific gene repression at some sites by increasing KAP1 mediated ubiquitination and degradation of KZFs. At other sites, MAGE increase KAP1 binding and gene repression. Using KRAB-Gal4-DBD-fusion proteins we found gene activation or repression is dependent on the structure of the KRAB domain of individual KZFs. To determine global effects of MAGE expression we performed ChIP and ChIP-seq with anti-KAP1 antibodies and HEK293T cells expressing ectopic MAGE-C2, and found MAGE-C2 alters KAP1 target gene profiles. We validated MAGE effects for selected affected genes including BRAF and ID1 using immunoblotting, loss of function mutants, knockdown of endogenous MAGE in melanoma lines, ChIP for H3K9me3, reverse transcription qRT-PCR, and functional assays. Finally, we determined MAGE effects on BRAF associated proliferation. Melanocyte proliferation induced by oncogenic BRAF is limited by p16-mediated senescence that can be overcome by ID1, an oncogene regulated by MAGE via K51, a KZF tumor suppressor. We found MAGE-A3 and C2 knockdown decreases BRAF and ID1 mRNA, and increases senescence in melanoma cells. We conclude that MAGE-A3 and C2 are master regulators of KAP1-KZNF gene repression and contribute to melanoma progression by increasing oncogene expression and decreasing tumor suppression, creating an internal milieu favorable for melanoma growth.

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Molecular profiling of dysplastic nevi identifies genes with progression towards primary cutaneous melanomas

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Dysplastic nevi (DN) have altered clinical and histologic features from common melanocytic nevi (CMN). It is also widely viewed that DN have clinical/cellular features progressing towards malignant melanoma (MM). However, molecular distinctions between CMN and DN as well as molecular markers of cancer progression are not established. In this study, we obtained fresh frozen biopsies of CMN, DN, and primary cutaneous MM. Different sections were prepared in order to characterize histologic features of each lesion by immunohistochemistry (IHC) as well as diagnostic pathology. mRNA was also extracted from frozen sections with high quality, therefore being suitable for Affymetrix gene arrays and/or RT-PCR experiments. A series of different statistical approaches was used to identify expressed genes with altered expression in CMN vs. DN, CMN vs. MM, and DN vs. MM, as well as gene sets that could be used for molecular classification of each condition. Using principal component analysis and other techniques, there was clear separation of CMN and MM mRNA profiles, but as a category, DN were intermediate lesions, with a few individual lesions having profiles that overlapped the melanoma region. At the level of individual genes, a large series of DN genes had intermediate levels of expression between CMN and MM ("progression genes"), while other genes, e.g., MAGE-A3, -A6, -A12, PRAME, or ABCB5, clearly separated DN from MM ("melanoma-specific" genes). Importantly, some "melanoma-specific" gene products were detected focally in atypical cellular regions of DN by IHC. Our combined results suggest 1) that DN are progressive neoplasms towards melanomas and 2) that variability in gene expression within individual DN lesions might be used to classify a level of molecular dysplasia that could be used eventually for clinical management of DN patients.

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Loss of Noxa and Puma have inverse effects on melanomagenesis in a predisposed, genetically-defined mouse melanoma model

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Studies to evaluate the contribution of the intrinsic apoptosis pathway to melanoma biology and drug resistance are often compromised by the in vitro culture systems and xenograft models utilised. While such models have their advantages, adaptation of cells to culture in vitro and subsequent engraftment risks further selection for defective apoptosis. Furthermore, some important aspects are missing in vitro, as cells are not grown in their natural environment or with an intact immune system. Genetically defined, predisposed mouse models obviate each of these problems. In this project, an established mouse model of melanoma (Cdkn2a^{-/-}, Tyr-HRAS) was utilised, which mimics lesions common in human melanoma. The BH3-only proteins Noxa and Puma were chosen as appropriate exemplars for intervention in the apoptosis pathway as there is evidence supporting their involvement in melanomagenesis and progression. The melanoma model mice were thus crossed with either Puma^{-/-} or Noxa^{-/-} lines. These models were used to investigate the effect of loss of Puma or Noxa on melanoma biology, and its resistance to treatment. Mice of all three cohorts developed melanomas and other tumours. Loss of Noxa delayed melanomagenesis, decreased tumour penetration and number of melanomas but – once tumours were established – accelerated melanoma growth compared to the control and Puma^{-/-} cohorts. We speculate that loss of Noxa may be rescued by induction of other BH3-only pro-apoptotic proteins e.g. Puma, which could be responsible for the delayed melanomagenesis.

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The melanoma surface glycome

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Most mammalian proteins are glycosylated. N-linked glycans are attached to the amino acid asparagine and O-linked glycans are attached to either serine or threonine. Not all asparagines, serines and threonines will have a glycan attached to them and there are thousands of possibly glycan modifications. A particular protein can be glycosylated differently when expressed in different cell types or in the same cell over time. Since protein glycosylation plays an important role in cellular functions such as migration and proliferation, we hypothesized that the cell surface glycome of a malignant cell line will evolve as selective pressure is applied to increase its tumorigenicity. As an initial screen for alterations in cell surface glycans we characterized the lectin-binding profile of different malignant melanoma cell lines. This was accomplished by using a large panel of fluorescently-labeled lectins to stain the melanoma cell lines, which were then analyzed by flow cytometry. Using the B16 murine model of melanoma, we successfully demonstrate profound alterations of the cell surface glycome between B16 melanoma cell lines that differ in their tumorigenicity. Specifically, there is an increase in high mannose and a corresponding decrease in Galβ1-3GalNAcα1-Ser/Thr-containing glycans on the surface of the more tumorigenic B16 cell line. These results demonstrate that clonal populations with similar cell surface proteomes can have profoundly different glycosylation profiles. We speculate how these glycan modifications can increase the in vivo tumorigenicity of the melanoma cell lines.

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Identification of a drug resistance signature by proteome analysis

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Drug resistance is a major obstacle in melanoma treatment. Recognition of specific resistance patterns, the understanding of the patho-physiology of drug resistance and identification of remaining options for individual melanoma treatment would greatly improve therapeutic success. We performed mass spectrometry-based proteome profiling of A375 melanoma cells and HeLa cells characterized as sensitive to cisplatin in comparison to cisplatin resistant M24met and TMF1 melanoma cells. Cells were fractionated into cytoplasm, nuclei and secretome and the proteome profiles classified according to Gene Ontology. The cisplatin resistant cells displayed increased expression of lysosomal as well as Ca²⁺ ion binding and cell adherence proteins. These findings were confirmed using Lysotracker Red staining and cell adhesion assays with a panel of extracellular matrix proteins. To discriminate specific survival proteins, we selected constitutively expressed proteins of resistant M24met cells which were found expressed upon challenging the sensitive A375 cells. Using the CPL/MUW proteome database, the selected lysosomal, cell adherence and survival proteins apparently specifying resistant cells were narrowed down to 47 proteins representing a potential resistance signature. These were tested against our proteomics database comprising more than 200 different cell types/cell states for its predictive power. We provide evidence that this signature enables the automated assignment of resistance features as readout from proteome profiles of any human cell type. Proteome profiling and bioinformatic processing may thus support the understanding of drug resistance mechanism, eventually guiding patient tailored therapy.

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To explore the relationship of tyrp1 gene and melanoma tumor growth by piggyBac transposon mutagenesis

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To date, the malignant melanoma remains one of the most treatment-refractory malignancies. Among the several subtypes of melanoma, the amelanotic melanoma is particularly difficult to recognize due to the decrease or absence of pigmentation and its diagnosis may be delayed. Although the prognosis of amelanotic melanoma is similar to that of melanotic lesions, the recent research has identified an aggressive subgroup of melanoma constitutes of an amelanotic appearance without an associated nevus. Besides, it has been documented in a microarray study that TYRP1 (tyrosinase-related protein 1) mRNA expression in melanoma metastases correlates with clinical outcome. TYRP1 is a melanocyte-specific enzyme involved in melanogenesis, which is encoded by the TYRP1 gene. TYRP1 also influences melanocyte proliferation and melanocyte cell death. Interestingly, we have established a PiggyBac transposon mutagenesis library of murine B16 melanoma for phenotype screening. In this library, a tyrp1 loss-of-function depigmented clone mimicking amelanotic melanoma was selected. The loss-of-function transposon was inserted between exon 2 and exon 3 of tyrp1 gene. In comparison with the parental B16 melanoma cell line, the trapped clone had lower tyrp1 mRNA expression and displayed apparent depigmentation that could be distinguished grossly. The further examination showed increased proliferation capability of the tyrp1 loss-of-function clone. Meanwhile, there was no significant change of the migration ability. For the drug screening, there was no altered response to the chemotherapeutic agent (dacarbazine and cisplatin) and the target therapy (BRAF inhibitor, LGX818, and MEK inhibitor, MEK162). These results suggest that the characterization of the tyrp1 loss-of-function melanoma has implications for investigating amelanotic melanoma.

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Differential cellular localization of activating transcription factor 3 (ATF3) in melanoma progression

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Melanoma is an aggressive malignancy with a relatively high metastatic rate, responsible for almost 60% of lethal skin tumors. In spite recent progress in cancer therapy, melanoma still lacks effective treatment and tumor recurrence is frequent after chemotherapy. ATF3 is a member of the ATF/cyclic AMP response element-binding family of transcription factors and functions as a modulator of proliferation. It has been found to be over-expressed in various carcinomas viz. breast, prostate, Hodgkin lymphoma and invasive SCC. However, it is unknown whether differential cellular expression of ATF3 contributes to the pathogenesis of melanoma. Recently, ATF3 was shown to be upregulated after high-dose chemotherapy and treatment caused increased nuclear expression in melanoma cells (Pigment Cell Melanoma Res. 2012; 25(4):454-65). To address the role of ATF3 in different stages of malignant melanoma, we have performed semiquantitative immunohistochemical analysis of ATF-3 in a tissue microarray containing benign nevi (n=59), primary melanomas without metastasis (n=22) or with metastasis (n=17), and melanoma metastases (n=32 cases). Melanoma and metastatic malignant melanoma had larger percentage of cells expressing ATF3 (p=0.04) and significantly more intense cytoplasmic immunopositivity (p=0.05) compared to nevi. Also, primary melanoma had higher cytoplasmic (p=0.02) and nuclear [both for percentage (p=0.03) and intensity (p=0.03)] expression of ATF3, in comparison to nevi. Although there were no significant differences between primaries with or without metastasis, primary melanomas with subsequent metastasis had a larger number of tumor cells with cytoplasmic expression of ATF3 than did primary lesions without metastasis. In conclusion, differential cellular expression and localization of ATF3 protein appears to correlate with disease progression and might partially explain the chemoresistance seen in many patients with melanoma.

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Dermatologists and dermatopathologists have different levels of concern that partially sampled melanocytic lesions carrying a microscopic diagnosis of dysplastic nevus may actually be melanoma

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Dysplastic nevi represent a major diagnostic and therapeutic conundrum for dermatologists and dermatopathologists, and a firm consensus on both definitions and management remains elusive. We sought to understand whether some of the discrepancies in proposed dysplastic nevus management stem from differing beliefs regarding the histopathological meaning of a dysplastic nevus and its potential to represent putative melanoma. Surveys were sent to 1,100 members of the American Academy of Dermatology (AAD), representing practicing clinical dermatologists in the United States (483 returned, participation rate 45.9%), and all 856 members of the American Society of Dermatopathology (ASDP), representing dermatopathologists (533 returned, participation rate 63.0%). While 61.8% of ASDP members cited concern that a residual dysplastic nevus could actually represent a melanoma, only 31.9% of AAD members felt this was a concern (p<0.01), with a similar discrepancy in the proportion of respondents citing that reason as the single most important reason for excision (cited by 37.4% of ASDP members vs 19.6% of AAD members (p<0.01). These findings suggest that there is a large discrepancy between dermatologists and dermatopathologists regarding whether a histopathological diagnosis of a dysplastic nevus excludes the possibility that the lesion represents melanoma. These findings have important implications regarding public policy decisions in dysplastic nevus management and patient education, and highlight the need for close communication between clinical dermatologists and dermatopathologists.

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High-definition optical coherence tomography imaging of melanocytic lesions. Correlation with dermoscopy, reflectance confocal microscopy and histopathology

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High-definition OCT (HD-OCT) is a new non-invasive in vivo imaging technique with cellular resolution based on the principle of conventional OCT. The aim of this study was to evaluate HD-OCT for its feasibility to identify micro-architectural patterns and cytologic feature analyses of melanocytic lesions in comparison with histopathology, dermoscopy and Reflectance Confocal Microscopy (RCM). 26 pigmented skin lesions of 26 volunteers were imaged with HD-OCT. These images were correlated to histopathology, dermoscopy and RCM. Identification of micro-architectural patterns and cytologic features of pigmented cells in the epidermis, papillary dermis and superficial reticular dermis was possible by HD-OCT. An good correlation of HD-OCT aspects with the corresponding histopathologic features, RCM features and dermoscopic patterns was observed. In conclusion, HD-OCT provides morphological imaging with sufficient resolution and penetration depth to discriminate micro-architectural patterns and cytologic features of pigmented cells in epidermis and dermis. It offers the possibility of additional structural information complementary to that of RCM and dermoscopy.

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Macrophage-derived interferon-gamma exerts a downstream response signal in human malignant melanoma cells

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A case-series study of primary malignant melanoma patients was initiated to investigate whether pro-tumorigenic macrophage-derived IFN- γ signaling occurs in humans as has been proposed in a mouse melanomagenesis model. Biopsy specimens of fresh-frozen primary melanoma tissue were analyzed for macrophages, T-cells, and melanocytes using immunofluorescent labeling and confocal microscopy with specific cell and cytokine markers. Preliminary data identified 33% of tumor infiltrating CD68+ macrophages co-localized with IFN- γ (Pearson's Correlation = 0.35 \pm 0.13; n=10) in patients with primary melanoma (Stage 0-III). Additionally, 51% of infiltrating CD3+ T-cells also co-localized with IFN- γ (Pearson's Correlation = 0.58 \pm 0.12; n=10). This pattern was consistently observed in both superficial spreading and lentigo maligna melanoma subtypes. In the majority of melanoma samples evaluated, the predominant IFN- γ signal in closest proximity to melanoma cells was associated with CD68+ macrophages, not CD3+ T-cells. Furthermore, malignant melanoma cells were double positive for downstream IFN- γ response elements, including monokine induced by gamma interferon (MIG/CXCL9) and phosphorylated signal transducer and activator of transcription (P-STAT1). The CXCR3 receptor to MIG/CXCL9 was also highly expressed in melanoma cells. Melanocytes in psoriasis tissue did not co-localize with downstream IFN- γ response elements. Together, these findings reveal that IFN- γ -secreting macrophages and lymphocytes infiltrate the melanoma tumor microenvironment and localize within the tissue. High levels of IFN- γ exert downstream signaling from melanoma cells, as evidenced by MIG/CXCL9 and P-STAT1 co-localization. Melanoma cells themselves concomitantly display the CXCR3 receptor and are able to respond to MIG/CXCL9 signaling in autocrine and paracrine fashions. These results confirm active IFN- γ signaling in primary human melanoma, prompting further investigation of a pro-tumorigenic role of IFN- γ in malignant melanoma.

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Comparative proteomic analysis reveals unique tumor protein composition among the melanoma subtypes pure desmoplastic and superficial spreading melanoma

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Pure desmoplastic melanoma (pDM) is a rare subtype of melanoma characterized by malignant spindle melanocytes surrounded by a fibro-collagenous stroma. pDM accounts for less than 1% of all melanoma diagnoses. While locally aggressive, pDM has significantly lower incidence of lymph nodal metastasis compared with case-matched controls of superficial spreading melanoma (SSM), and portends a more favorable survival prognosis independent of all other tumor characteristics. Comparative proteome characterization of these two divergent patterns of melanocytic malignancies thus has the potential to enhance current pathological prognostication and may add new targets to melanoma intervention. We sought to determine a protein signature associated with potential toward metastasis. Formaldehyde-fixed, paraffin-embedded case-matched pDM and SSM, with paired benign intradermal melanocytic nevi (IMN) as control were purified via laser-capture microdissection and subsequently analyzed by two-dimensional electrophoresis coupled with nano-LC-MS/MS on an LTQ Orbitrap Velos. Data were searched using MASCOT protein discovery software and ArrayTrack for pathway and ontology. We chose the 120 proteins found to be statistically significantly differentially expressed between pDM and SSM for further analysis. Supporting a paradigm of reduced metastatic potential in pDM, we found increased expression of proteins involved with wound-healing, cell-cell adhesion, and lesion stability, including desmoglein 1, plakophilin 1, and junctional plakoglobin (p<0.05). Protein signatures of SSM were indicative of increased tumor mass expansion and DNA-replication, including at least 10 proteins of the heat-shock family (p<0.05). We have identified differential protein signatures for melanoma subtypes. Further prospective validation studies are warranted to evaluate their utility for prognostic and potentially therapeutic purposes.

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Dietary fat intakes are associated with the risk for severe solar lentigines in middle-aged caucasian women

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Solar lentigines are a hallmark of photodamage. Numerous studies have focused on the possible role of diet to counter UV induced skin damage, however the possible involvement of lipid intakes has been poorly investigated. The aim of this study was to investigate the links between daily fatty acid intakes and the severity of solar lentigines. A cross-sectional study was conducted on 279 middle aged Caucasian women from the SU.VI.MAX cohort. Solar lentigines were assessed on forehead and cheeks by a dermatologist using a validated 6-grade scale. Besides, the fat diet was determined using ten 24-h dietary record questionnaires. Intakes of total lipids, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (linoleic, α -linolenic, arachidonic, eicosapentaenoic, n-3 docosapentaenoic, and docosahexaenoic acids) have been estimated by sources. After adjustment on possible confounders, a significant association was found between the severity of solar lentigines and the dietary intakes of monounsaturated fatty acids and arachidonic acids. Women with high intakes of monounsaturated fatty acids and arachidonic acids presented a lower severity of solar lentigines. A similar tendency was found with linoleic acids intakes. These findings suggest a possible role of dietary fatty acids on hyperpigmentation disorders related to sun exposure. Further epidemiological studies are still necessary to confirm our findings and to gain further insight into underlying mechanisms.

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Effective modulation of human skin pigmentation by inhibitors of human tyrosinase

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Hyperpigmentary disorders like melasma, actinic lentigines and post-inflammatory hyperpigmentation are a major cosmetic concern. The most prominent target for inhibitors of hyperpigmentation is tyrosinase, however, most inhibitors lack clinical efficacy. We evaluated the inhibition of skin pigmentation by well known skin whitening actives like hydroquinone, arbutin, kojic acid, and resorcinol derivatives. We compared the inhibition of human tyrosinase activity in a biochemical assay as well as inhibition of melanin production in MelanoDermTM skin model culture. In vivo efficacy was tested in clinical studies. Arbutin and hydroquinone only weakly inhibit human tyrosinase with a half maximal inhibitory concentration (IC50) in the millimolar range, while kojic acid is ten-times more potent. By far the most potent inhibitor of human tyrosinase is 4-n-butylresorcinol with an IC50 of 21 μ M. The IC50 values of 4-n-hexylresorcinol and 4-phenyl-ethylresorcinol were both markedly higher than those for the n-butyl derivative. Molecular modeling experiments revealed that 4-n-butylresorcinol blocks the active site of human tyrosinase most effectively. In artificial skin models, arbutin was least active with an IC50 for inhibition of melanin production >5000 μ M, kojic acid inhibited with an IC50 >400 μ M. Hydroquinone inhibited melanin production with an IC50 below 40 μ M, probably due to a mechanism different from tyrosinase inhibition. Again, 4-n-butylresorcinol was the most potent inhibitor with an IC50 of 13.5 μ M. In vivo efficacy of 4-n-butyl-resorcinol was confirmed in clinical studies. Subjects with age spots on the forearm treated age spots with a formula containing 4-n-butylresorcinol, vehicle-treated spots served as controls. Within eight weeks, 4-n-butylresorcinol reduced visibly the appearance of age spots. A second study showed that 4-n-butylresorcinol was more effective than 4-n-hexylresorcinol and 4-phenylethylresorcinol.

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A new mouse model for studying the development of melanomas

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Genetic and environmental factors act in concert to deregulate melanocytic stem cells, and can ultimately lead to their transformation and progression into metastatic melanoma. Elucidating the mechanisms which govern spontaneous transformation of pigmented cells has been challenging, due in part to the paucity of mouse models that faithfully recapitulate this process. We have developed a mouse model of melanoma with progression to lymph node involvement. Treatment of C3H/HeN mice with dimethylbenz(a)anthracene (DMBA) followed by TPA induces the formation of dysplastic pigmented skin lesions that appear at 9 weeks. Histologic examination of tissues taken at 25 weeks showed that DMBA/TPA treated mice developed nests of melanocytic cells in their draining lymph nodes, while age matched untreated mice did not. Histology and immunofluorescent staining confirmed that pigmented cells in skin and lymph nodes were of melanocytic origin rather than pigment laden macrophages, since cells in melanobleached serial sections stained positive for S100 and Melan-A (Mart-1) but not CD11b or CD116. Moreover, tyrosinase, gp100 and Melan-A expression in lymph node-derived cDNA from treated mice was observed by real-time PCR analysis. Furthermore, proliferative cells could be routinely cultured from skin pigmented lesions and draining lymph nodes. The cell lines expressed melanoma markers HMB45 and S100, but not keratinocyte proteins keratin 1 or 14. Functional analyses indicated that cell lines demonstrated anchorage-independent growth in soft agar colony formation assays, and were able to grow progressively as transplantable tumors in nude mice. This model may prove to be useful in identifying genes that regulate susceptibility to carcinogen-induced pigmented lesions in skin and lymph nodes, and may identify novel targets for developing metastatic melanoma therapeutics.

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Macromolecular changes in melanoma cells after treatment with vemurafenib observed by a mid-infrared sensor system

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Vemurafenib is a B-Raf-kinase inhibitor which is used in the first line treatment of metastatic melanoma with a V600E BRAF mutation. In the present work, the effects of vemurafenib on M14 melanoma cells harboring the BRAFV600E mutation were investigated at the biomolecular level using a mid-infrared sensor system. The measurement method is based on the CH₂-symmetric and CH₂-antisymmetric stretches of tumour cells at wavelengths 3.51 μ m and 3.42 μ m, respectively. The CH₂-stretch ratio is calculated by dividing the functional absorbance values from the CH₂-symmetric stretch with the CH₂-antisymmetric stretch. 2x10⁵ M14 melanoma cells were prepared on 6x6 mm² CaF₂ sample slides and incubated for 8, 18, and 24 hours with 500 nM vemurafenib. Four different 1.5mm diameter spots per CaF₂ slide were measured. Antiproliferative effects of vemurafenib were determined by a [³H] cell proliferation assay. M14 melanoma cells were seeded in triplicates and treated with increasing concentrations of vemurafenib (from 0 nM to 10.000 nM). The IC50 value of vemurafenib was calculated to be 237 nM for M14 melanoma cells. A significant increase in the CH₂ stretch ratio was seen in M14 melanoma cells incubated with 500 nM vemurafenib after 18 and 24 hours (0.82 \pm 0.09 and 1.2 \pm 0.03, respectively) compared to untreated melanoma cells (0.63 \pm 0.09). These results show that vemurafenib leads to a biochemical modification in the inner-membrane lipid region and to internal movements of its molecules containing CH₂ that can be measured with the mid-infrared sensor system. The mid-infrared sensor system provides a promising method for analyzing the influence of vemurafenib related structural changes in melanoma cells.

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SOX2 maintains growth and specifies the high risk phenotype of melanoma cells

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Malignant melanoma is associated with poor prognosis, however with a heterogeneous display of local invasion and distant metastatic spread. The heterogeneity is partly attributable to variant oncogenic cascade of driver mutations, and also likely epigenetic and involve, for example, involving differential tumor marker expression patterns within a localized cancer clone. Using step-wise oncogenic transformation of immortal melanocytes and in keeping with the notion that stem-like abilities may regulate tumor-initiation and metastasis, we show the increased expression of the stem cell transcription factor SOX2 co-existent with acquired tumor-associated end-points. Importantly, SOX2 levels suppress expression of the melanocyte master regulator MITF and, conversely, knockdown of SOX2 leads to increased MITF expression. Moreover, SOX2 over-expression defines a gene expression signature that enriches for tumor initiating cell features and suppressed differentiation. This SOX2 signature can stratify clinical primary melanoma specimens with high histopathological risk. Additionally, melanoma cells depend on SOX2 function for growth and survival, while melanocytes do not, suggesting that its expression confers an Achilles' heel to melanomas, and constitute a potential therapeutic target.

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Effects of BRAF inhibitors on glucose utilization in melanoma *in vitro*

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Our current research is aimed at investigating changes in metabolism induced by pharmacotherapy in malignant melanoma. Roughly 50% of melanomas harbor a BRAF activating mutation. In 2011 the FDA approved vemurafenib, the first mutant BRAF-targeting inhibitor for metastatic melanoma, based on significant increases in progression free and overall survival. Unfortunately, resistance emerges after several months in the vast majority of patients. Markedly decreased FDG-PET signal is observed in nearly all BRAF-mutated melanomas after 14 days of therapy. Due to the dependence of FDG-PET imaging on tumor glucose uptake and utilization, the direct effects of vemurafenib on tumor metabolism represents a possible mechanism of action and an important field for further research. Using flow cytometry and the fluorescent glucose analog 2-NBDG, we have demonstrated that there is a rapid, apoptosis-independent decrease in glucose uptake in vemurafenib-treated human melanoma lines that are sensitive to the drug, which is reversible upon drug removal. In contrast, there is no change in glucose uptake in vemurafenib-resistant lines. This association between mutant BRAF inhibition and metabolic phenotype suggests that alterations in metabolism may constitute a primary mechanism of action of vemurafenib. Furthermore, preliminary data shows that several vemurafenib-resistant lines display a significant increase in oxygen consumption with vemurafenib treatment despite minimal loss of proliferative capacity, suggesting that metabolic adaptation may represent a potential mechanism of resistance. If a strong mechanistic link between BRAF inhibition and decreased glucose utilization can be fully characterized, it will represent an important advancement of our understanding of the mechanism of action of BRAF inhibitors, as well as open up potential new, metabolically-targeted methods for overcoming resistance based on multi-drug treatment regimens.

1402**Differential expression of melanocyte markers in the human epidermis and hair follicle**

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The goal of the current study was to determine the location of melanocyte (MC) precursor and of differentiated MC populations in human interfollicular epidermis (IE) and hair follicles (HF). We used transverse paraffin skin sections from five Caucasian adult females undergoing elective breast reduction surgery. We tested four regions, the IE and three defined portions of the HF, infundibulum, bulge and bulb. Triple immunofluorescence was used, with two primary MC antibodies [either anti-c-Kit or anti-dopachrome tautomerase (DCT) combined with anti-PAX3 or anti-tyrosinase (TYR)], and one primary epithelial cell antibody (anti-K14). The co-expression of c-Kit or DCT with PAX3 (marker of immature MC) was considered a less differentiated phenotype, compared to those MCs that showed co-localization of c-Kit or DCT with TYR (marker expressed in mature MC). The MC counts were compared to the total number of cells in the basal epidermis or external sheath of the HF. The differences in percentages for each site/marker were analyzed with two-way ANOVA. c-Kit was significantly more abundant in all regions ($p < 0.05$) compared to the other markers. Its expression was maximal at the bulb. We found the expression of all other markers to be higher in the IE. The more differentiated MCs co-expressing c-Kit or DCT with TYR were significantly higher represented in the IE than the other regions tested ($p < 0.05$); they decreased in a gradual manner with progressive depth along the follicular infundibulum, being absent at the bulge. In all three regions of the HF, the c-Kit+ or DCT+ MCs that co-localized with PAX3 were more numerous than those co-expressing c-Kit or DCT with TYR. The expression pattern of tested markers indicated a gradual differentiation of MCs as they progress from the bulge to the IE. The identification of the precursor and differentiated MC populations can be used for further characterization of MC transformation in pathologic states.

1404**Genome-wide miRNA expression profiles identify serum miRNAs as potential noninvasive biomarkers for malignant melanoma**

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OBJECTIVE Circulating blood has been found to contain ample amounts of microRNA (miRNAs), which can be detected and may serve as biomarkers for a variety of diseases. We hypothesize that some of these circulating miRNAs could be a novel class of blood-based biomarkers for malignant melanoma (MM). **RESEARCH DESIGN AND METHODS** Serum RNA samples were isolated from 34 melanoma patients and 34 age- and gender-matched healthy control subjects. Serum miRNA expression profiles were performed by TaqMan® real-time RT-PCR MicroRNA Arrays and analyzed with Statminer®. **RESULTS** 435 miRNAs were detected in the overall cohort. The expressions of 10 miRNAs were significantly changed in MM patients compared to healthy controls with AUC greater than 0.75, of which miR-151-3p, miR-17-5p, miR-590-5p, miR-191, and miR-146a showed significantly increased expression levels, while miR-30b, miR-30c, miR-139-5p, miR-99b, and miR-150 showed significantly decreased expression levels. A combination of 6 miRNAs, miR-99b, miR-139-5p, miR-30c, miR-590-5p, miR-191, miR-151-3p yielded an AUC of 0.85. **CONCLUSIONS** The results from our study provide an insight into the pattern of serum miRNA expression in MM, which could serve as potential MM-specific biomarkers for the diagnosis, prognosis and therapy response.

1406**Imaging mass spectrometry - a novel ancillary method for the diagnosis of malignant melanoma**

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Previously, using imaging mass spectrometry (IMS) we found proteomic differences between Spitz nevus (SN), a type of benign melanocytic nevus, and Spitzoid malignant melanoma (SMM), melanoma that resembles histologically SN. The objective was to investigate whether IMS can help diagnose difficult Atypical Spitzoid Neoplasms (ASN), cases in which a diagnosis of benign SN or malignant SMM cannot be made with certainty. 26 cases of ASN from the Yale Spitzoid Neoplasm Repository and 7 other institutions were analyzed by IMS. A diagnosis of either SN or SMM was rendered by IMS, which was then correlated with the histopathologic diagnosis and clinical outcome. In 20/26 (77%) cases of ASN the diagnosis provided by IMS was SN. In all 20 cases the clinical outcome demonstrated no recurrence, metastases, or death (mean follow-up: 6.5 years). The remaining 6/26 (23%) cases were diagnosed by IMS as SMM. In 5 cases there was either a sentinel lymph node (SLN) or other lymph nodes metastases. In one ASN case, in which SMM was favored by both histopathology and IMS, one SLN was negative and patient remained ANED at 3.5-years. All 6 patients diagnosed as SMM by IMS were ANED (mean follow-up: 4.3 years). In conclusion, based on clinical outcome and lymph node metastasis data, the sensitivity of IMS for the diagnosis of SMM is 100% and the specificity is 95%. Therefore, IMS analysis can provide a reliable advancement in our ability to differentiate benign and malignant melanocytic lesions.

1403**Melanoma-associated macrophages express molecules inhibiting innate immune responses**

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Melanomas, as well as other solid tumors, are infiltrated by tumor-associated macrophages (TAMs). These cells are believed to play a role in tumor progression and metastasis as their amount within the cancer heralds a poor prognosis. We isolated TAMs from human melanoma metastases (n=3) and compared them to other human myeloid/antigen-presenting cells (dermal CD14+ macrophages, dermal CD1a+ dendritic cells, Langerhans cells, peripheral blood monocytes, myeloid and plasmacytoid dendritic cells) by gene expression analysis. In unsupervised clustering analyses these cells displayed a unique gene signature with certain similarities to tissue-resident dermal macrophages. Among the differentially expressed genes we observed a highly upregulated expression of Mer, which was validated at the protein level. Mer belongs to the family of Tyro3, Axl and Mer (TAM) - receptor tyrosine kinases, which play an important role in the phagocytosis of apoptotic cells and function as inhibitors of innate immune responses. Additionally, we observed an upregulation of liver x receptor α and β , which were described to play a role in the induction of Mer expression. To determine the role of Mer in TAMs and their possible relationship to the suppression of an effective anti-tumor immune response, we established an in vitro model, trying to mimic the in vivo situation as closely as possible. Therefore we generated spheroids from melanoma cell lines in collagen type I gel and seeded sorted CD14+ (CD16-163-206-Mer-) peripheral blood monocytes on top. These cells were then allowed to migrate into the spheroids. After seven days monocytes had infiltrated the spheroids and differentiated into cells resembling ex-vivo isolated TAMs (CD14+CD163+CD206+), including the upregulated expression of Mer. This model should allow us to better understand the role of TAMs on anti-tumor immune responses and, in a more general fashion, to study the cellular and molecular events occurring in cancer-host cell interactions.

1405**Role of neuropeptides in melanoma**

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Melanoma is a malignant growth of melanocytes which originates from multipotent neural crest cells. In addition to the known role of α -melanocyte-stimulating hormone (α -MSH) in its pathophysiology, our group has postulated the involvement of neuropeptide Y (NPY) and galanin. In this study, we investigate the association of NPY, galanin and α -MSH with markers of apoptosis, invasion, inflammation and prognosis in cutaneous melanoma. An observational study using 68 paraffin-embedded tissue samples of melanomas confirmed histologically was performed. Using immunohistochemistry, we quantified the percentage of positive cells expressing NPY, α -MSH, galanin, caspase 3, cadherin-N, CD31, VEGFR-3 as well as CD4+ and CD8+ T lymphocytes. Histological variables with proven prognostic value were assessed. SPSS (non-parametric tests) was used for statistical analysis. 29 nodular melanomas (NM), 23 superficial spreading melanomas (SSM), 13 lentigo maligna (LM) and 3 LM melanoma were included. Whereas galanin and α -MSH were significantly more expressed by SSM, NPY expression was modestly increased in NM. Immunostaining revealed a moderate positive correlation between NPY and galanin with that of α -MSH (0.5). Galanin had a statistically significant positive correlation with caspase-3 (0.6), N-cadherin (0.5), VEGFR-3 (0.6) and negative with Breslow index (0.5), number of mitosis (0.5) and mm of ulceration (0.3). α -MSH correlates positively with caspase-3 (0.5) and VEGFR-3 (0.4). NPY showed a low correlation index with caspase-3 (0.4). Importantly, NPY expression was the only neuropeptide significantly associated with metastasis and more expressed in those melanomas which resulted in death. Our study demonstrates that expression of NPY in melanoma is associated with bad prognosis, whereas galanin is inversely correlated.

1407**Identification of a chemokine profile associated to melanoma progression**

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Metastasis in melanoma requires a complex sequence of interconnected processes such as angiogenesis, lymphangiogenesis, tumor and host immunology and tumor cell migration. In this regard, chemokines are important mediators in the above mentioned steps. In a spontaneously metastasizing xenotransplantation melanoma mouse model, the chemokine profiles of normal skin, primary tumors and metastatic and tumor-free lymph nodes were compared. Twelve chemokines were found to have differently regulated mRNA expression levels. Expression of these chemokines was subsequently analyzed in FFPE samples of human stage T1 to T4 melanomas using RT-PCR. Interestingly, a chemokine pattern primarily reflecting differences in biological behavior and not only the different tumor stages could be identified. From the upregulated chemokines, we focused in those involved in the metastatic process through cell migration and angiogenesis/lymphangiogenesis. Chemokines were overexpressed in cell lines with diverse metastatic behavior including two different cell lines isolated from the same patient. These cell lines were injected into the skin of CB17-SCID mice. Characteristic macroscopic tumor morphology along with microscopic findings revealed a more invasive phenotype in the overexpressing cell lines. A marked increase in neutrophil infiltration and tumor lymphangiogenesis in the overexpressing tumors compared to controls could potentially explain the increment in lymph node and lung metastasis.

1408

Antibodies of the IgG4 subclass impaired immune responses against melanoma

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The presence and roles of host-induced antibodies in cancer are insufficiently understood. Since the presence of the immunomodulatory cytokine IL-10 can drive Th2-biased inflammation to induce production of IgG4, we investigated the presence and functional significance of IgG4 auto-antibodies in melanoma. We analyzed the distribution of B cells, cytokines and IgG subclasses in melanoma patients skin, lymph and blood specimens and in healthy individuals skin and blood and examined the propensity of cancer cells to induce Th2 cytokines and trigger IgG4 ex vivo. Consistent with Th2 inflammation, we detected CD22+ and IgG4+ infiltrating cells accumulating in melanoma lesions, IL-10, IL-4 and tumour-reactive IgG4. B cells secreted increased VEGF and IgG4, while tumor cells enhanced IL-10 secretion leading to an enhanced production of IgG4 in co-culture. To gain insights into the functional significance of IgG4 subclass antibodies, we engineered tumor-specific IgG1 and IgG4 antibodies and evaluated their functions in vitro and in a human melanoma xenograft mouse model. Despite accumulation in tumors, and in contrast to IgG1, tumor antigen-specific IgG4 was ineffective in triggering effector cell-mediated tumor killing in vitro and in vivo. IgG4 blockade was mediated through reduction of FcγRI signalling. Introduction of IgG4 significantly impaired the potency of tumoricidal IgG1 in a human melanoma xenograft model. Finally, we found an inverse correlation between IgG4 serum levels and survival in a 33 patient cohort, suggesting the merits of further evaluations for this antibody subclass as a potential biomarker in melanoma. Our findings demonstrate that IgG4 promoted by tumor-induced Th2-biased inflammation may impair anti-tumor immunity, and these insights may provide the basis for the design of novel therapeutic approaches to counteract IgG4 immunoregulatory mechanisms.

1410

The receptor tyrosine kinase, Axl, as a therapeutic target in malignant melanoma

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Malignant melanoma is the most aggressive form of skin cancer. Early stage melanoma is cured by excision, however despite promising results from BRAF inhibitors and immunotherapy, metastatic melanoma remains an incurable, fatal disease. Multiple mechanisms contribute to the development of drug resistance in melanoma, including altered expression of oncogenes or tumor suppressors, and enhanced extracellular efflux of a broad spectrum of hydrophobic, cytotoxic drugs by ABC transporters. Axl is a receptor tyrosine kinase upregulated in a variety of tumours including melanoma. We have demonstrated previously that Axl depletion in cutaneous SCC inhibits EMT and stem cell properties and inhibits tumour initiation in vivo suggesting that Axl contributes to chemotherapy resistance. The purpose of this study is to explore the role of Axl as a therapeutic target in malignant melanoma. To investigate this hypothesis, we induced transient Axl knockdown in primary (SbCl2, Mel224) and metastatic (SkMel2, WM1361) melanoma cell lines. We show variable expression of Axl in melanoma cell lines by Western blotting. Quantitative-PCR and FACS analysis demonstrate expression of putative stem cell markers in Axl-overexpressing melanoma cell lines, such as CD44, CD271, CD133 and some ABC transporters (ABCB1, ABCB5, ABCG2). We are currently investigating the correlation of Axl and putative stem cell marker expression as well as the relationship between Axl and ABC transporter expression using lentiviral stable knockdown of Axl and functional assays. In conclusion, our preliminary data suggest that Axl may play a role in stemness and therapeutic resistance in malignant melanoma.

1412

An epidermal model containing functional melanocytes for skin lightening and pigmentation disorder studies

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Considerable interest exists in evaluating cosmetic and/or skin care pharmaceutical formulations which cause lightening of the skin. These products are utilized to cosmetically alter one's natural skin color or to combat skin pigmentation disorders such as melasma, post-inflammatory hyperpigmentation, and other hyperpigmentation lesions. In order to aid in the development and testing of such products, we have developed a melanogenesis/whitening protocol using MelanoDermTM, a highly differentiated, three-dimensional tissue culture model of human epidermis that contains normal human melanocytes (NHM) and keratinocytes (NHK). The use of this 3D model along with the whitening protocol can provide invaluable in vitro data as an early screening tool for raw materials prior to the commencement of costly clinical trials. Skin lightening protocols have been produced containing NHM of varying skin phenotypes which follow the expected pigmentation level of the donor tissue, i.e. black>Asian>Caucasian. Tissues are treated topically three times a week over a two to three week treatment period to mimic consumer application. Several over-the-counter skin lightening products were evaluated in cultures containing NHM from black and Asian donors. Over the two to three-week period, control cultures became increasingly pigmented with retention of normal epithelial morphology. In contrast, tissues treated topically with cosmetic skin lightening agents containing Kojic acid, magnesium ascorbyl phosphate or ascorbyl glucoside remained distinctly lighter than control cultures. The skin lightening effect on treated tissues was quantitatively evaluated for melanin content using both a soluble melanin assay and spectrophotometric measurement. Treated tissues showed varied although significant changes in overall melanin content (reductions of 12-21%) compared to water-treated tissues. The results described herein suggest that this model will be useful to study melanogenesis, skin lightening, and other pigmentation phenomena of skin in vitro.

1409

An in vivo model of postinflammatory hyperpigmentation

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Postinflammatory hyperpigmentation (PIH) is an acquired hypermelanosis that occurs after cutaneous inflammation or injury. The study objective was to validate an in vivo model for PIH by comparing clinical and histological characteristics of trichloroacetic acid (TCA, 35%) induced PIH with PIH resulting from truncal acne. Thirty subjects completed the study. Subjects served as their own control. Four TCA treated sites and four acne pustules were followed for eight weeks. Subjects were evaluated with photography, diffuse reflectance spectroscopy (DRS), colorimetry, and a 6-point Investigator's Global Assessment (IGA) scale (0: absence of PIH; 5: the most severe PIH). Four mm punch biopsies were obtained: one from representative acne pustule and one from TCA treated site at the first visit (D1) and a second set 4 weeks later (D28). IGA scores for both acne-PIH and TCA-induced PIH were similar at 2 weeks and reached a plateau at day 28 with average scores of 2.9 and 3.0, respectively. On D1 acne pustules were characterized by perifollicular neutrophilic inflammation, whereas TCA lesions had full thickness epidermal necrosis with perivascular and/or perifollicular lymphocytic inflammation. By D28, both acne and TCA-induced PIH showed perifollicular/perivascular lymphocytic inflammation and dermal fibrosis. IGA erythema scores were virtually identical for both types of PIH at D7, 14, 42 and 56. Dermal melanophages were present in both TCA and acne-induced PIH lesions. The similarity of IGA scores, and the observation that chronic inflammation along with fibrosis was found in both acne and TCA-induced PIH suggest that TCA-induced PIH is reproducible model with which to study risk factors and treatment modalities for PIH. Differences in cellular infiltrate did not seem to distinguish traumatic from inflammatory-induced PIH outcome in predisposed individuals. Dermal melanophages may be an important component of PIH under both experimental conditions.

1411

Mechanism of sirtuin inhibition mediated anti-proliferative response in human melanoma cells: A proteomics approach

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Melanoma is a lethal skin cancer and its incidence continues to rise in the developed world. Therefore, novel mechanism-based strategies are needed for the management of this disease. Sirtuins, the NAD(+)-dependent class III histone deacetylase (HDAC), have been implicated in a variety of physiological processes and pathological conditions. The role and functional significance of SIRT1 in cancer development and progression is a subject of intense research. We have shown that 1) SIRT1 is overexpressed in human melanoma cells and tissues; 2) Tenovin-1, a small molecule inhibitor of SIRT1 (as well as SIRT2) resulted in decreased growth, viability, and clonogenic survival of melanoma cells, possibly via an activation of p53 (J Invest Dermatol; 132 Suppl-1: S130, 2012). Here, employing a proteomics approach using gel free Liquid Chromatography-Mass Spectrometry (LC-MS) method, we attempted to identify the downstream molecular targets of sirtuin inhibition in melanoma cells. The human melanoma G361 cells were treated with Tenovin-1 (25 μM; 48 h) followed by protein extraction, "in liquid" trypsin digestion, and peptide analyses by LC-MS/MS using a Agilent 1100 nanoflow system. Raw MS/MS data was searched against uniprot_human_FR_CP_090710 amino acid sequence database using Mascot search engine 2.2.07 (Matrix Science, London). As shown by the differentially expressed proteins data, we found 14 proteins (from a total of 1092) with >2 fold differential expressions with statistical significance (p < 0.05). These proteins were globally related to RNA processing-, mitochondrial checkpoint-, cell cycle- and apoptosis-related pathways. The data were validated using real time QPCR- and immunoblot- analyses. Our data identified the mitotic checkpoint protein BUB3, as a novel downstream target of SIRT1 and/or SIRT2 in melanoma cells. Further studies are in-progress to confirm our data and to ascertain the functional relevance of BUB3 as a downstream sirtuin-target.

1413

The crucial role of Sox10-Sox9 axis in melanoma development

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Patients with giant congenital nevi are at high risk for developing melanoma, the most aggressive skin cancer. The precise molecular and cellular mechanisms of this malignancy remain to be further characterized. We have recently identified the crucial role of the neural crest transcriptional factor Sox10 in melanoma formation and demonstrated that interfering with Sox10 levels counteracts NrasQ61K-driven melanoma in vitro and in vivo. Microarray analysis of the putative Sox10 target genes revealed that another member of the Sox gene family, Sox9, was upregulated upon Sox10 loss, suggesting a potential role of Sox9 in preventing melanoma development. To address this, we have conditionally inactivated both Sox10 and Sox9 genes in the melanocytic lineage of Tyr::NrasQ61K mice. The lack of Sox9 restored the characteristic hyperpigmentation phenotype in the Tyr::NrasQ61K mice. Furthermore, in the vast majority of human melanoma samples analyzed expression of Sox10 and Sox9 was mutually exclusive. Taken together these findings establish the importance of the Sox10-Sox9 axis as functional regulator of melanoma development.

1414**PDE4 interacts with FAK to control melanoma invasion**

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The cAMP pathway is both important for normal melanocyte biology and implicated in the risk of melanoma development. Evidence suggest that the cAMP pathway is inhibited in melanoma, favouring tumor development. We have shown that transformation of melanocytes by oncogenic BRAF inhibits cAMP signalling. This inhibition implicates members of the phosphodiesterase (PDE) superfamily, enzymes that physiologically degrade cAMP. More specifically, we have identified the PDE4 subgroup as a major suppressor of cAMP signalling in melanoma using a range of PDE inhibitors. The combination of PDE4 inhibitors and α -MSH stimulated CREB protein phosphorylation, indicating that the cAMP/PKA-signalling pathway in response to α -MSH was restored in these cells. Interestingly, inhibition of PDE4 activity in BRAF mutated melanoma inhibits invasion but does not inhibit proliferation. This is different from what we have previously shown in RAS mutated melanoma, where reactivating the cAMP pathway induced apoptosis. Moreover, PDE inhibitors induced only transient CREB phosphorylation in BRAF mutated melanoma and did not induce MITF or tyrosinase. However, we show that PDE4 interacts with FAK through the scaffold protein RACK1. We have developed cell-permeable peptides to inhibit PDE4D interaction with RACK1 and are testing their effects on melanoma invasion. As FAK has been shown to promote cancer cell invasion, our results have implications for the understanding of the aggressive melanoma phenotype. PDE4 isoforms may therefore serve as a new therapeutic target for the treatment of aggressive melanoma and may prevent the emergence of melanoma clones with enhanced metastatic capabilities.

1416**Expression of heat shock proteins 60, 70 and 90 in melanoma and benign melanocytic nevi**

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Cutaneous melanoma is the most important type of skin cancer due to its implications on the morbidity and mortality of patients. Heat shock proteins (HSP) are involved in the folding of several functional cellular proteins, including malignant cells. We studied the relation between the expression of HSP 60, 70, 90 in melanoma and benign melanocytic nevi. Eight specimens of malignant melanoma and five of benign melanocytic nevi were stained immunohistochemically for HSP 60, 70, 90. The evaluation was performed using a histochemical score, which included the intensity and percentage of staining. For HSP 60 and 70, the median values were higher in the melanoma group compared to the benign melanocytic nevi group, however, no statistically significant relationship was found ($p=0.118$, $p=0.149$). For HSP 90, the median value was also higher in the melanoma group, the results being statistically significant ($p=0.012$). No statistically significant relationship was found between HSP 60, 70, 90 and melanoma ulceration, Clark level and Breslow index. Our pilot study demonstrates that expression of HSP 60, 70 and 90 is to a small extent higher in melanoma compared to benign melanocytic nevi, with HSP 90 being the most reliable tumor-associated marker.

1418**Role of hypoxia in melanoma stem cells and progression**

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Hypoxia is playing an increasing role in cancer stemness and progression. Melanoma is still incompletely understood and for a high number of patients this disease is still life threatening. In our work we focused on hypoxia in melanoma stem cells and progression addressing isolated stem cells from cultured melanoma cells as well as growth under low oxygen condition. Furthermore we were looking at tissue from patients that have primary tumours and metastasis compared to nevi and correlated suggested melanoma stem cell markers (ALDH1, CD133, p75 NGF, ABCB5) with hypoxia or antioxidant markers (HIF-1 α , HIF-2 α , NRF2, SOD1, VEGF).

1415**Eradication of melanoma by intratumoral injection of attenuated vaccinia virus requires CD8+ T cells and combination of anti-CTLA-4 blockade and virotherapy enhances therapeutic efficacy in advanced melanoma**

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Advanced melanoma is largely refractory to conventional therapies, including chemotherapy and radiotherapy. Immunological approaches to melanoma treatment have been proven to be beneficial in animal models and human clinical trials and deserve to be actively explored. Poxviruses are large cytoplasmic DNA viruses that hold promises as vectors for immunotherapy and as oncolytic agents. Vaccinia virulence factor E3 consists of an N-terminal Z-DNA binding domain and a C-terminal dsRNA binding domain. Mutant vaccinia with E3 deletion is non-pathogenic in mice. Δ E3L infection of melanoma cells induces apoptosis partly through degradation of anti-apoptotic protein MCL-1, and intratumoral injection of Δ E3L into intradermally implanted poorly immunogenic murine B16 melanomas results in the regression of the tumors at the injection sites as well as rejection of tumor challenge at the contralateral side. Immunological studies revealed that intratumoral injection of Δ E3L virus results in the generation of tumor-specific CD8+ T cells and anti-melanoma antibodies. Cell depletion studies indicate that CD8+ T cells are critical for tumor eradication after intratumoral injection of Δ E3L virus. Combination of intratumoral injection of Δ E3L virus and anti-CTLA-4 antibody results in more efficient eradication of advanced melanoma in mice. Our results indicate that attenuated vaccinia viruses have the potential to be developed as novel therapeutics for melanoma.

1417**Metabotropic glutamate receptor 6 signaling enhances TRPM1 calcium channel function and increases melanin content in human melanocytes**

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Transient receptor potential, melastatin 1 (TRPM1), a member of the TRPM subfamily of TRP proteins, is a calcium channel expressed in melanocytes and the retinal ON bipolar cells. In epidermal melanocytes, TRPM1 expression is associated with proliferation and melanin pigmentation. TRPM1 plays a critical role in the synaptic transmission from photoreceptors to ON bipolar cells. Mutations in TRPM1 cause complete congenital stationary night blindness with no discernible skin phenotype. In the retina, TRPM1 activity is negatively coupled to metabotropic glutamate receptor 6 (mGluR6) signaling through G α and TRPM1 mutations result in the loss of responsiveness of TRPM1 to mGluR6 signaling. Here, we show that human melanocytes express mGluR6 and treatment of melanocytes with L-AP4, an mGluR6-selective agonist, enhances Ca²⁺ uptake. Knockdown of TRPM1 or mGluR6 by shRNA abolished L-AP4-induced Ca²⁺ influx and TRPM1 currents showing that TRPM1 activity in melanocytes is positively coupled to mGluR6 signaling. G α o protein is absent in melanocytes. However, forced expression of G α restored negative coupling of TRPM1 to mGluR6 signaling, but treatment with and pertussis toxin, an inhibitor of Gi/Go proteins, did not affect basal or mGluR6-induced Ca²⁺ uptake. Additionally, chronic stimulation of mGluR6 altered melanocyte morphology and increased melanin content. Our data provide a possible explanation for lack of pigmentary defects in TRPM1 mutants and suggest that selective expression of G α subunit proteins can produce cell type-specific TRPM mutant phenotypes. These data also suggest differences in coupling of TRPM1 function to mGluR6 signaling explain different cellular responses to glutamate in the retina and the skin.

1419**Hypomelanotic macules in tuberous sclerosis complex is linked to Th17 cell infiltration with possible impairment of cytoplasmic autophagy via mTOR pathway**

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Background Hypomelanotic macular lesions are occasionally observed in tuberous sclerosis complex (TSC) with continuous up-regulation of mammalian target of rapamycin (mTOR) signaling by mutations in tuberlin and/or hamartin. However, pathomechanisms of hypomelanotic lesion formation in TSC has been poorly recognized. Objectives and Methods We conducted immunohistochemical and electron microscopic analysis on the hypomelanotic lesion in TSC samples obtained from 9 cases to clarify the underlying pathomechanism. In vitro, normal human melanocytes (NHM) treated with several cytokines were observed ultrastructurally and investigated autophagically. Results Immunohistochemically, the number of Melan A positive cells did not change in contrast to increased and activated CD1a+ Langerhans cells in TSC hypomelanotic lesions compared with normal lesions. Unexpected infiltration of Th17 cells was observed in 7 out of 9 cases with less significant infiltration of CD8+ T cells and activated STAT3 in the lesional keratinocytes. In the electron microscopic analysis, numbers of stage III / IV small melanosomes were different among each melanocyte and keratinocyte. Distribution of melanin pigment was uneven in contrast to complete loss of melanin in vitiligo vulgaris or senile leukoderma. Intriguingly, NHMs treated with TNF- α were ultrastructurally similar to the lesional melanocytes of hypomelanotic macule in TSC. Moreover, autophagic related-p62 proteins were significantly accumulated in NHMs by TNF- α . Conclusions Present observations suggest that Th17 cell-related cytokines with constitutive activation of mTOR play robust role in induction of hypomelanotic macules in TSC through impaired function of autophagy resulting in abnormal melanosome maturations.

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Inhibition of polo-like kinase 1 (Plk1) induces a senescence-like phenotype in human melanoma cells

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Polo-like kinase 1 (Plk1), a member of a highly conserved family of serine/threonine kinases, is well-known for its regulatory role during mitosis. Plk1 is a critical regulator of centrosome maturation, kinetochore recruitment, bipolar spindle formation and cytokinesis. The overexpression of Plk1 has been correlated with poor disease prognosis and aggressiveness in a variety of cancers, which has made Plk1 an emerging druggable target for cancer management. We earlier demonstrated that Plk1 is overexpressed in human melanoma and a targeted depletion of Plk1 through lentiviral shRNA or a small-molecule inhibitor resulted in a mitotic catastrophe and apoptosis in human melanoma cells (J Invest Dermatol 129: 2843-53, 2009). In order to further define the mechanisms of Plk1 inhibition mediated responses in melanoma, in this study, we assessed the effects of BI6727, a second generation Plk1 small molecule inhibitor, in human melanoma A375 cells. In accordance with our previous studies, employing western blot analysis and caspase activity assays, we found that BI6727-mediated inhibition of Plk1 resulted in apoptosis of melanoma cells. Interestingly, employing immunofluorescence analysis, we also found that Plk1 inhibition resulted in a subpopulation of A375 cells with a distinct senescence-like phenotype, as characterized by an enlarged morphology and increased micronuclei formation. In addition, cell cycle analysis revealed that Plk1 inhibition caused an increased accumulation of cells in S phase, in addition to typically seen G2/M phase arrest. Further, BI6727 (100 nM; 48 hours) treatment resulted in a remarkable increase in senescence-like growth arrest characterized by induction of senescence-associated beta-galactosidase staining. Furthermore, the cells removed from above treatment protocol after 48 hours and seeded in growth medium were unable to form colonies for 14 days. To our knowledge, this is the first study providing an evidence of induction of senescence-like growth arrest by Plk1 inhibition in melanoma cells.

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Cytotoxic effects of hyper-inflammatory macrophages on melanoma survival in vitro

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Despite advances in scientific knowledge, metastatic melanoma remains extremely difficult to treat resulting in a five-year survival rate of less than 20%. Recent studies have shown that HMG-CoA reductase inhibitors (statins) and PPAR gamma agonists (glitazones) independently inhibit the growth of melanoma cells via apoptosis and cell cycle arrest in vitro. Work in our laboratory has focused on a novel pharmacologic method using statins and glitazones to generate polarized hyper-inflammatory macrophages characterized by hyper-induced levels of tumor necrosis factor alpha and inducible nitric oxide synthase. In our current study, murine B16 melanoma cells were cultured with and without bone marrow derived macrophages (BMDMs) in vitro for 48 hours. The cultures were treated with mevastatin, rosiglitazone, or both. Qualitative examination of the cultures with light microscopy revealed large reductions in the B16 monolayer when treated with mevastatin and mevastatin with rosiglitazone. In addition, B16 morphology was altered and displayed fewer cell divisions and dendritic processes. B16 viability was quantitatively demonstrated with double-negative annexin and propidium iodide staining with flow cytometry. Mevastatin, and mevastatin plus rosiglitazone significantly reduced the viability of B16 alone or co-cultured with BMDMs. Compared to B16 control, mevastatin and rosiglitazone reduced the number of viable B16 by 26.7% (n=7; p=5.57x10⁻⁵). A greater reduction was seen in B16 co-cultured with BMDMs. Compared to the B16 plus BMDMs co-culture control, mevastatin and rosiglitazone reduced B16 viability by 58.4% (n=11; p=2.71x10⁻⁷). Isolated BMDMs primed with mevastatin and rosiglitazone prior to co-culturing with B16 and without the addition of mevastatin and rosiglitazone to the co-culture did not significantly reduce B16 viability. Thus, robust B16 cytotoxicity requires exposure of both BMDMs and B16 to mevastatin and rosiglitazone. In summary, mevastatin and rosiglitazone inhibit B16 proliferation while increasing the vulnerability of B16 to hyper-inflammatory macrophages.

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Higher-order chromatin organization of melanocyte-specific gene loci in human melanocytes

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Higher-order chromatin structure and nuclear compartmentalization of transcriptionally active and inactive genomic domains and transcription machinery play an important role in regulation of gene expression. Melanocytes and keratinocytes are highly specialized cells in the skin, whose functions are controlled by distinct lineage-specific gene expression programs. By three-dimensional fluorescence in situ hybridization (3D-FISH) and confocal microscopy, we show that higher-order chromatin structure and intra-nuclear positioning of the genomic loci containing melanocyte-specific genes tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and MLANA in the primary human melanocytes differ very substantially, compared to the primary keratinocytes. In primary human keratinocytes, all three melanocyte-specific loci were located at the nuclear periphery closely to the nuclear membrane. However, in melanocytes TYR, TYRP1 and MLANA gene loci were located significantly more internally, compared to keratinocytes. Furthermore, 3D-FISH and RNA-FISH data revealed that in human hair follicle, active melanin synthesis in the epidermal/hair bulb melanocytes is associated with relocation of TYR and MLANA genes toward the nuclear interior, compared to amelanotic melanocytes. Finally, by using an inducible lac operator/lac repressor system that allowed experimentally re-localize MLANA gene loci from the nuclear interior to the inner nuclear membrane, we showed that MLANA tethering to the nuclear periphery resulted in significant decrease of MLANA gene expression in melanocytes. Thus, these data demonstrate that spatial arrangement of the melanocyte-specific genes in the nucleus is functionally important for pigment production and suggest a role for higher-order chromatin remodelling factors in the control of execution of lineage-specific program in melanocytes.

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Combined targeting of MEK and PI3K/mTOR effector pathways effectively inhibits NRAS mutant melanoma in vitro and in vivo

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Activating mutations in the NRAS gene are common genetic events in malignant melanoma being found in 15-25% of cases. NRAS is thought to activate both RAF and PI3K signaling in melanoma cells. We studied the influence of different components on the MEK/ERK and PI3K/mTOR signaling cascades in NRAS mutant melanoma cells. In general, these cells were more sensitive to MEK inhibition compared to inhibition in the PI3K/mTOR cascade. Combined targeting of MEK and PI3K was superior to MEK and mTOR1,2 inhibition in all NRAS mutant melanoma cell lines tested, suggesting that PI3K signaling is more important for cell survival in NRAS mutant melanoma when MEK is inhibited. However, targeting of PI3K/mTOR1,2 in combination with MEK inhibitors is necessary to effectively abolish growth of NRAS melanoma cells in vitro and to regress xenografted NRAS mutant melanoma. We showed that MEK and PI3K/mTOR1,2 inhibition is synergistic in 10 human NRAS mutant melanoma cell lines. Expression analysis confirms that combined MEK and PI3K/mTOR1,2 inhibition predominantly influences genes in the RAS pathway and growth factor receptor (GFR) pathways, which signal through MEK/ERK and PI3K/mTOR. The combination also affected genes involved in cell division such as DBF4, CDC7, MAP2K6, FLT1 and NME1, supporting the substantial decrease in cyclin D1 seen by MEK1+PI3K/mTOR1,2. Furthermore, the combination caused the up-regulation of tumor suppressor genes such as RB1CC1, STK11 and pro-apoptosis genes such as CAB31, MAP3K10, DAPK3 and MAP3K9. Our results suggest that combined targeting of the MEK/ERK and PI3K/mTOR pathways has anti-tumor activity and might serve as a therapeutic option in the treatment of NRAS mutant melanoma, for which there are currently no effective therapies.

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Distinct properties of melanocyte progenitors from the hair follicle bulge and secondary hair germ

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Mammalian coat pigmentation is maintained by melanocyte stem cells (MSCs), which generate a large number of differentiated melanocytes during each hair follicle (HF) cycle. Previous studies have defined a quiescent population of MSCs in the lower region of the HF where quiescent follicular epithelial stem cells reside. Two distinct sets of melanocyte progenitor cells, one located in the bulge/lower permanent portion (LPP) of the HF as described previously and another restricted to the secondary hair germ (SHG) region, differ in their ability to reconstitute the pigmentary unit during HF anagen. Cells isolated from a strain with melanocyte progenitors enriched in SHG cells generate stably pigmented HFs more efficiently than cells from a strain with melanocyte progenitors enriched in the bulge/LPP cells. To understand more clearly molecular differences between bulge/LPP and SHG melanocyte progenitors, we used a more recently developed strain of transgenic mice which express GFP constitutively in melanoblasts, melanocytes, and melanocyte progenitors. We separate the bulge/LPP and the SHG populations using fluorescence-activated cell sorting (FACS) with GFP and anti-CD34. Analysis of FACS-separated cells using quantitative RT-PCR confirms high levels of Dct and low levels of Krt14 expression in both CD34+GFP+ bulge/LPP and CD34-GFP+ SHG cells. CD34+GFP+ bulge/LPP cells have a high Cd34/Cdh3 (encoding P-cadherin) ratio which is reversed in CD34-GFP+ SHG cells, demonstrating that the expression of these markers is transcriptionally regulated. We also find that the melanocyte differentiation genes Tyr, Tyrp1, and Pmel17 are expressed more highly in CD34-GFP+ SHG cells, confirming the notion that they occupy a more advanced state of melanocyte differentiation. The use of CD34 to separate bulge/LPP and SHG HF melanocyte progenitors should facilitate understanding the relationship between these two subsets and assist in dissecting their functional properties.

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The TRPA1 agonist allyl isothiocyanate, an inducer of neurogenic inflammation, acts as a model substance for the investigation of postinflammatory hyperpigmentation, leading to increased melanin levels in vivo and in vitro

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Postinflammatory Hyperpigmentation (PIH) is an acquired dyspigmentation, often being a stress factor for those concerned when found in exposed areas. Only few models have been shown to induce PIH *in vivo*, but none is available that can induce PIH *in vivo* and *in vitro*. The chemical substance allyl isothiocyanate (AITC) binds to the TRPA1 receptor on sensory nerve fibers and causes a neurogenic inflammation of the skin, resulting in a not yet described tanning reaction. We sought to characterize this AITC-induced response *in vivo* and confirm it as a melanin based pigmentation reaction, conducting a kinetic study with 12 subjects and comparing results with responses in primary melanocytes *in vitro*. The inflammatory reaction was confirmed by increased levels of proinflammatory mediators such as IL-1α, IL-6 and IL-8. Five and 16 days after AITC application, epidermal samples showed increased amounts of eu- and pheo-melanin in AITC treated areas, supported by increased transcript levels for several melanosomal proteins at 5 days after AITC application. Amongst several regulated soluble factors in suction blister fluid was prostaglandin E2 for which an influence in PIH has been discussed as it leads to the activation of tyrosinase *in vitro*. These findings further indicate a central role for PGE2 in PIH formation. This effect is likely to be amplified by the proinflammatory factor TNFα, which was strongly down-regulated during the first 24 hours. As TNFα inhibited the synthesis of melanin in further *in vitro* experiments, and is described to counteract αMSH, these results suggest yet another mechanism that may favor melanin formation *in vivo* through the down-regulation of TNFα.