2004

Characterization of novel melanoma-associated antigens.

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CHARACTERIZATION OF NOVEL MELANOMA-ASSOCIATED ANTIGENS

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2004

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Biochemistry and Molecular Genetics

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Title Characterization of Novel Melanoma-Associated Antigens

In this study, a serological identification of antigens by recombinant cDNA expression libraries (SEREX) was employed to identify a set of novel tumor antigens by screening serum from a late-stage melanoma patient against a cDNA library derived from human melanoma cell lines. The patient had undergone a nonspecific interleukin-12 (IL-12) immunotherapy (UAB clinical trial 9701) and had experienced a complete clinical response to this therapy. Using post-therapy serum from the patient was an attempt to increase opportunities to identify novel targets that may mediate tumor destruction based on this patient's clinical response. Seven novel candidate antigens specifically recognized by the humoral immune response of this patient were isolated. Of these seven candidates, the human homolog of *Drosophila* disc large protein (hDlg) was further characterized at the molecular level in melanomas. The immunoreactivity of hDlg was examined in sera derived from 21 additional melanoma patients undergoing immunotherapy clinical trials, a panel of 17 other melanoma patients treated with conventional methods, 128 ovarian patients, and 117 normal individuals. Three of 39 melanoma patients (8%) expressed serum IgG to the hDlg antigen. Consistent with real-time RT-PCR, Western blot analysis demonstrated that hDlg expression was low in melanoma compared to normal cells. Moreover, aberrantly spliced variants of hDlg were also detected in melanoma tumors and cell lines tested, but not in normal cells. Immunofluorescence analysis showed that

hDlg was primarily localized to intercellular junctions of epithelial cells and melanocytes, suggesting its physiologic role in cell-cell interaction. In contrast, subcellular localization of hDlg was aberrant in some melanoma cell lines and all melanoma tumor samples evaluated, with more hDlg protein exhibiting cytoplasmic localization. Frequent loss and/or aberrant localization of hDlg in melanomas suggests its tumor suppressor role in this cancer type. This possibility was supported by a colony-forming assay in which ectopic expression of hDlg in melanoma cell lines suppressed the ability of these cells to form colonies. The molecular characterizations performed in this study suggest that altered expression and cellular localization of hDlg contributes to the phenotype of malignant melanoma. These studies demonstrate the ability of SEREX to identify immunogenic proteins that have a potential role in tumorigenesis.

DEDICATION

I dedicate this dissertation to my late grandfather, Nit Nirachol, who inspired me to persevere, and to my mother, Pranom Hansakul, who has supported and encouraged me throughout my life to reach my potential and fulfill my goals.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AIDS	Acquired Immune Deficiency Syndrome
AJs	Adherens junctions
ALVAC hIL-12	Recombinant canarypox virus encoding human IL-12
APCs	Antigen presenting cells
APC	Adenomatous polyposis coli
APN	Aminopeptidase N
bFGF	Basic fibroblast growth factor
bp	Base pairs
CAM	Cell adhesion molecules
CDKs	Cyclin-dependent kinases
CMM	Cutaneous malignant melanoma
CREB	cAMP response element binding protein
CT	Cnacer/testis
CTLs	Cytotoxic T lymphocyte
Dlg	*Drosophila* disc-large protein
DPPIV	Dipeptidyl peptidase IV
Dsh	Cytoplasmic protein Dishevelled
DT	Delayed tanning
EMSA	Electromobility Shift Assay

ERK	Extracellular signal-regulated kinase or MAP kinase
ETBr	Endothelin-B receptor
ET-1	Endothelin-1 LIST OF ABBREVIATIONS (Continued)
FAMMM	Familial melanoma
FGFR	Fibroblast growth factor receptor
fsh	female sterile homeotic
GUK	Guanylate kinase homology domain
hDlg	The Human Homolog of *Drosophila* Disc Large Protein
HEM	Human epidermal melanocytes
HGF/SF	Hepatocyte growth factor/scatter factor
HPV	Human papillomavirus
IGF	Insulin-like growth factor
IPD	Immediate pigment darkening
KH	K-homologous
LOH	Loss of hyterozygosity
MAGUK	Membrane associated guanylate kinase family
MC1R	Melanocortin-1 receptor
MDCK	Madin-Darby canine kidney cells
MEFs	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase kinase or MAP kinase
MGMT	O-methylguanine DNA methylatransferase
MHC	Major histocompatibility complex

MITF	Microphthalamia-associated transcription factor
MMPs	Matrix metalloproteinases
M/SCF	Mast/stem cell factor
α-MSH	α-Melanocortin
NMD	Nonsense-mediated mRNA decay pathway
NSCLC	Human non-small-cell lung cancer
PBMC	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PHK	Primary human keratinocytes
PI3K	Phosphoinositide 3'-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PNPT1	Polyribonucleotide nucleo-tidyltransferase 1
PPT	polypyrimidine tract
PS2	Presenilin-2
PTPase	Tyrosine phosphatase
QRT-PCR	Quantitative real-time reverse-transcriptase polymerase chain reaction
RAF	MAP kinase kinase kinase or MEKK
RAR-β2	Retinoic acid receptors-β2
RAS/RAF/MAPK pathway	Ras/ Raf/ mitogen-activated protein kinase pathway
RASSF1A	RAS association domain family protein 1A

LIST OF ABBREVIATIONS (Continued)

Rb	Retinoblastoma gene product
RGP	Radial growth phase
Rn	Relative number
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
SAGE	Serial Analysis of Gene Expression
SEREX	Serological identification of antigens by recombinant
cDNA	Complementary DNA
siRNA/RNA_i	RNA interference
SR	Serine/arginine-rich
TAA	Tumor-associated antigens
TCF/LEF	T cell factor/lymphoid enhancer factor
$TCID_{50}$	Tissue Culture Infection Dose 50%
TIL	Tumor-infiltrating lymphocytes
TSLC1	Tumor suppressor in lung cancer-1
UVR	Ultraviolet radiation
VGP	Vertical growth phase
XP	Xeroderma pigmentosum

INTRODUCTION

Epidemiology of Melanoma

Cutaneous melanoma is the most fatal form of skin cancer and is a particular public health concern, because its incidence rates are rising throughout the world. In addition to the skin, melanoma may occur in the eye, as ocular or intraocular melanoma, and in mucous membranes; e.g., the vulva, lip, throat, and esophagus. In the United States, the incidence and mortality rates of melanoma have risen for many decades. The lifetime risk of an American developing invasive melanoma has increased from 1 in 1,500 in the 1930s to 1 in 74 in 2000 (1). The mortality rate from malignant melanoma has also risen about 2% annually since 1960 (1). In the year 2003, 54,200 new cases of melanoma were expected to be diagnosed, with estimated 7,600 deaths (2).

For primary melanoma, tumor thickness (depth into the skin) is the best prognostic indicator, and the survival rate drops quickly with increased tumor thickness. Deep melanomas tend to spread to local lymph nodes or distant sites through the lymphatic system and/or blood vessels. Most patients with disease localized to the skin can be cured with surgery. The majority of patients who experience the invasion and spread of melanoma to local lymph nodes cannot be cured with current therapies. Patients with lymph-node metastases have a 5-year survival rate of approximately 73.3%, compared to 93.3% in patients with no evidence of nodal metastases (3). Early diagnosis appears to be the most effective way of improving the cure rate for patients with melanoma.

Melanoma Environmental Risk Factors

Sunlight exposure

Epidemiologic studies of melanoma have shown that excessive sun exposure is the major environmental risk factor. The pattern of sunlight exposure associated with development of melanoma is intense, intermittent sun exposure rather than total lifetime exposure. People who have had at least one severe, blistering sunburn, especially in childhood, have an increased risk for melanoma (4, 5). Recreational activity leading to sunburn in adulthood is also associated with increased risk (6). Melanoma often occurs in the regions of the body subjected to intermittent exposure, such as the trunk, the lower legs, and the head and neck regions. In this connection, risk of melanoma is associated with geographical areas where the climate is sunny and hot; for example, melanoma is more common in people of Northern European ancestry in Australia, a tropical and subtropical country, than those in the British Isles (7, 8). In addition, light-skinned people who freckle or sunburn easily without tanning and have light eyes and hair are at a higher risk of developing melanoma than people with darker skin (9, 10). These people have less melanin to protect their skin from the cumulative damage produced by ultraviolet radiation (UVR). The incidence of melanoma has been most marked in older white populations. For the period 1992 to 1998, the reported incidence for white males and females rose 2.7% and 2.9% per year, respectively (11). Although melanoma is rare in dark-skinned people, when it does develop, it usually occurs in a body part that has less melanin, such as under the fingernails or toenails, or on the palms or soles.

Chemical and viral exposure

Other proposed environmental factors contributing to an increased risk of melanoma include exposure to vinyl chloride (12), petroleum industry products (13), and human papillomaviruses, such as HPV-16 and HPV-18 (14). Moreover, people with a weakened immune system, such as Acquired Immune Deficiency Syndrome (AIDS) patients and organ transplant patients on immunosuppressive drugs, are at a greater risk for developing melanoma.

Melanoma Genetic Risk Factors

A number of hereditary factors associated with increased risk of melanoma have been identified based on clinical, epidemiologic, and genetic studies.

Familial melanoma

Genetic predisposition of familial melanoma accounts for about 10% of all cases (15). The coexistence of melanoma and atypical nevi within families has been named familial atypical multiple mole melanoma (FAMMM) (16). Unlike ordinary moles (also called common acquired nevi), which are clusters of melanocytes, atypical moles are 5 mm or larger in size, have a flat appearance, and irregular and indistinct borders (Fig. 1). Germline mutations in the *CDKN2a* (INK4a/ARF) locus located in chromosome 9p21 were found in 40% of the FAMMM cases (16). Patients with familial melanoma are genetically at an increased risk of developing melanoma and other malignant neoplasms, such as pancreatic cancer, suggesting that melanoma may share predisposing genetic

defects with other relatively common cancers (16, 17). To date, the underlying gene defects for the majority of familial melanomas are still not known (18).

MC1R variants

The melanocortin-1 receptor (*MC1R*) gene codes for the α-melanocyte-stimulating hormone receptor, a receptor that responds to α-melanocortin (α-MSH) with increased proliferation and melanogenesis. The *MC1R* gene is one of more than 70 genes that are involved in melanocyte regulation and is of particular significance for human pigmentation because of its critical role in determining constitutive pigmentation and the ability to tan upon sun exposure (19, 20). The MC1R gene is highly polymorphic, suggesting its significance in a wide variation in human pigmentation among various ethnic populations (21, 22). Melanocytes with a nonfunctional MC1R have a reduced response to α-MSH and showed increased sensitivity to the apoptotic effect of UVR (23). α-MSH was shown to counteract the apoptotic effect of UVR and promote melanocyte survival (24, 25). Melanocytes with loss of MC1R function cannot benefit from the protection against apoptosis induced by α-MSH through the activation of functional MC1R.

Epidemiological studies found that melanocytes with genetic defects in the *MC1R* locus that encodes specific variants with a single amino acid substitution in the MC1R, particularly Arg142His, Arg160Trp, Asp294His, and Arg151Cys variants, are highly associated with red hair phenotype, poor tanning ability, and susceptibility to melanoma (20, 26). Other studies have shown that the MC1R genotype increases the risk for melanoma in families with inactivation of the *CDKN2a* locus, which engages the Rb and

p53 tumor suppressor pathways through its capacity to encode two distinct gene products, p16 ^{INK4a} and p14^{ARF} (27, 28).

Xeroderma pigmentosum (XP)

XP is a rare autosomal recessive syndrome associated with a defect in several genes that encode enzymes in DNA repair pathways (29). Patients with XP have a 1000-fold greater risk than normal individuals of developing melanoma and nonmelanoma skin cancer, because their skin is less able to repair the damage to their DNA that has been caused by the sun (29, 30).

Melanocyte Biology

Melanocytes are derived from neural cresT-cells and migrate to the basal layer of the epidermis

Melanomas are derived from the pigment-producing melanocytes following malignant transformation. Melanocyte progenitors (melanoblasts) originate from the neural crest and migrate during embryonic development to reach the basal layer of the epidermis of the skin, where they differentiate to mature melanocytes possessing the complete machinery to ensure melanin synthesis and distribution within the skin. As the largest organ of the human body, protecting the internal organs from various chemical and physical environmental insults, the skin is composed of two distinct layers, the epidermis and the dermis. The thin outer layer, the epidermis, is a stratified squamous keratinized epithelium, which is itself usually divided into several strata. These are, from deep to superficial, the stratum germinativum (or basal keratinocytes), the stratum spinosum, the stratum granulosum, the stratum lucidum, and the stratum corneum. These

layers are not entirely distinct but reflect a continuum of changes in the epithelial cells as they progress from the proliferative basal layer and differentiate to form keratins located at the surface of the epithelium. Melanocytes are found in the stratum germinativum of the epithemis and are evenly distributed in a ratio of 1 to 10 with keratinocytes (Fig. 2). The thicker underlying layer, the dermis, consists of a matrix of collagen and numerous cell types, including fibroblasts, dendritic and other immune cells, endothelial cells (vessels), sensory neurons, hair follicles, and sweat glands. Melanocytes are also found in the basal layer of epithelial cells in the hair bulb, where the melanin produced is passed to the cells of the hair, giving it its color.

Melanocytes produce melanin that provides photoprotection for themselves and neighboring keratinocytes

Melanocytes have a rounded cell body with long cytoplasmic arms (dendrites) extending from the cell body. These cytoplasmic arms pass between cells in the stratum germinativum and through the epithelial cells in the stratum spinosum. Melanocytes produce melanin, which is a dark brown pigment that makes a major contribution to the color of the skin and provides protection from ultraviolet rays of the sun. This brown pigment is synthesized within specialized intracellular granules called melanosomes. The melanin-containing granules at early stages of development are found in the cytoplasm of the cell body, with mature melanin granules being found predominantly in the cytoplasmic arms. These granules contain the specific enzymes required for converting tyrosine into melanin after a series of transformations. Two main types of melanin are produced, the eumelanins, which are black/brown, and the pheomelanins, which are yellow/red. In humans, total melanin, as well as the ratio of eumelanins to pheomelanins

in the skin, differ among individuals with different skin phenotypes (31). Eumelanin is thought to be superior to pheomelanin in its ability to quench reactive oxygen radicals and in its resistance to degradation by exposure to UVR, whereas pheomelanin acts as a photosensitizer that contributes to the generation of reactive oxygen radicals when exposed to UVR (32).

Although the melanin is produced in melanocytes, it is transferred to epidermal cells in the stratum germinativum and stratum spinosum of the epidermis in the form of melanin granules or melanosomes. The mature melanin granules migrate along the cytoplasmic arms whose tips abut onto the epidermal cells. At these sites the granules are transferred into the epidermal cells from the melanocytes by a little understood injection process known as cytocrine secretion. Once inside the keratinocytes in the stratum germinativum and stratum spinosum, the melanin granules become located in the supranuclear region of the cells where they are able to provide protection from penetrating ultraviolet rays of the sun to the nuclear material, in particular to the stem cells and proliferating cells located in the basal layers of the epidermis. For the cells located in the midstratum spinosum, the melanin granules fuse with lysosomes and the melanin is broken down. Thus, melanin is not usually observed in the more superficial cells of the stratum or in the more superficial strata. Surprisingly, melanocytes themselves generally contain less melanin than the other epidermal cells in the basal part of the epidermis in which the melanin granules are deposited.

The numbers of melanocytes within each area of the body is fairly constant, although variations are found among different regions of the body. The number of melanocytes is not influenced by sex or race. Differences in skin color are related to the

number and size of the melanosomes produced by the melanocytes rather than the number of melanocytes found in an individual. Darker-skinned individuals produce larger and more numerous melanosomes. The skin pigmentation response following UV irradiation comprises immediate pigment darkening (IPD) and delayed tanning (DT). IPD results from the alteration and redistribution of melanin already present in the skin by forming nuclear caps to protect epidermal basal cell nuclei from UV-induced damage. DT is associated with an increase in the activity and number of melanocytes. Single UV exposure increases only the activity of the melanocytes, while repeated UV exposure increases the number of melanocytes, melanocyte tyrosinase activity, and the number and size of melanosomes (33). Moreover, melanocyte dendrites elongate and branch, which accelerate the synthesis and transfer of melanosomes to the epidermis (33). These events correspond to melanocyte differentiation and play a central role in the tanning response.

Paracrine factors regulate melanocyte growth, differentiation, and melanogenesis

In normal skin, human keratinocytes synthesize multiple growth factors, such as α-MSH (34), endothelin-1(ET-1) (35), basic fibroblast growth factor (bFGF) (36, 37), and mast/stem cell factor (M/SCF) (38). In addition, cells of mesenchymal origin, such as fibroblasts, secrete hepatocyte growth factor/scatter factor (HGF/SF) (Fig. 3) (39). These growth factors function as paracrine factors that regulate normal functions of melanocytes in the epidermis, including proliferation and pigment production.

In response to these paracrine factors, human melanocytes express their respective receptors, such as MC1R (40), bFGF receptor, endothelin-B receptor (ETBr) (41), c-Met (the HGF/SF receptor), and c-kit (the M/SCF receptor) (42). Among these receptors,

MCR1, a Gαs-protein-coupled receptor with seven transmembrane domains, stimulates the cAMP/PKA pathway with α-MSH binding (40). In this pathway, cAMP activates PKA, and PKA phosphorylates and activates the transcription factor Ca^{2+}/ cAMP response element binding protein (CREB) which, when activated, binds to the CRE domain present in the microphthalamia-associated transcription factor (Mitf) promoter, thereby up-regulating its transcription. Mitf is an integral transcriptional regulator in melanocytes, as its expression controls expression of the three major pigment enzymes: tyrosinase, Tyrp1, and Dct, as well as other pigmentation factors.

Other activated receptor tyrosine kinases (RTKs), such as c-kit, c-Met, and bFGF receptor, mediate their growth stimulating effects through activation of the Ras/Raf/MAPK pathway (43), whereas activated endothelin-B receptor, a G-protein coupled receptor, stimulates protein kinase C (PKC) and increases intracellular calcium mobilization (44, 45). Through different sets of downstream effectors, activated RTKs and ETBr converge on the activation of MAP kinases ERK1/2, $p90^{rsk}$, and CREB, indicating a central role of these kinases in the mitogenic responses of normal human melanocytes (46, 47). Normal human melanocytes require multiple growth factors to proliferate *in vitro*, suggesting the importance of the crosstalk of different signaling pathways, particularly the tyrosine kinase, PKC, and cAMP pathways (48). Recently, α-MSH has been shown to interact synergistically with other growth factors, bFGF and ET-1, to potentiate CREB phosphorylation important in the pigment biosynthesis pathway (46).

Melanocytes interact with keratinocytes through E-cadherin-mediated adherens junctions

Keratinocytes are the dominant cellular partners of melanocytes in the epidermis and they control the growth, morphology, and antigenic phenotype of melanocytes (49, 50). In the absence of keratinocytes, melanocytes rapidly proliferate, fail to branch, and fail to express melanoma-associated cell adhesion molecules (CAM), such as MCAM/MUC18 (49, 51). Reintroduction of keratinocytes into culture can reverse these processes. The major adhesion mediator between keratinocytes and normal melanocytes is E-cadherin, and its loss appears during melanoma progression (52). Cadherins are a family of Ca^{2+}-dependent cell-cell adhesion molecules that play key roles in developmental processes and in the maintenance of tissue architecture (53-55). They are integral membrane glycoproteins with an extracellular domain, a single transmembrane domain, and a highly conserved cytoplasmic domain. Members of the classic cadherin subfamily include E-(epithelial), P-(placenta), and N-(neural)-cadherins, which are expressed in a cell-, tissue-, and development-specific manner (56, 57).

At the epidermal-dermal border where melanocytes are found as single cells scattered among keratinocytes, E-cadherins, the major cadherin in epithelial cells, localize in specialized regions of the plasma membrane termed adherens junctions (AJs). Direct contact between keratinocytes and melanocytes is mediated by homophilic interactions between neighboring extracellular domains of E-cadherins. The cytoplasmic domains of E-cadherin interact with β-catenin and γ-catenin (also called plakoglobin), which mediate a link between the cadherin complex and the actin filaments through their direct interaction with α-catenin (Fig. 4). Such a link through α-catenin confers stability to the cell-cell adherens junction. In addition to providing a physical link between cells,

E-cadherins have also been implicated as regulators of intracellular signaling through β-catenin and subsequent activation of target genes (58-60).

The human homolog of *Drosophila* disc large protein (hDlg) is a component of the adherens junctions important in maintaining cellular polarity and regulating proliferation

E-cadherins mediate adherens junctional complexes, which play a critical role in maintaining normal tissue architecture and regulating a number of biological processes, including cell polarity, differentiation, proliferation, and migration. Disruption of the E-cadherin-mediated complexes occurs in a variety of human tumors and appears to be an important step in the progression from tumor formation to invasion and metastasis. In *Drosophila* and *Caenorhabditis elegans*, genetic studies have revealed that the *Drosophila* disc-large (Dlg) protein and its *Caenorhabditis elegans* homolog are also required for proper assembly and function of adherens junctions, as well as for cell polarity and growth control (61, 62). These proteins belong to the membrane associated guanylate kinase (MAGUK) family commonly found at the plasma membrane of polarized epithelial cells. Their loss-of-function mutations result in abnormal adherens junction formation and invasive growth of epithelial cells (61, 62).

The human homolog of Dlg (hDlg) is also highly concentrated in the epithelial cell-cell junction sites and contains many protein-protein interaction domains, including three PDZ domains, an SH_3 domain, a GUK domain, and alternatively spliced insertions (Fig. 5). Previously, hDlg was suggested to regulate cadherin-mediated adhesion because of the observation that hDlg is closely associated with E-cadherin and adherens junctions in the intestinal epithelial cell line (63). More recently, RNA_i inhibition of hDlg

synthesis has been shown to decrease membrane-associated E-cadherin, further supporting its important role in the structural integrity of adherens junctions (64). Similar to its *Drosophila* homolog, hDlg appears to function as a negative regulator of epithelial cell growth, and it is reported to interact with a number of proteins implicated in the regulation of cell proliferation and differentiation, including PTEN (65), the TOPK/PBK mitotic kinase (66), and APC tumor suppressor (67). Overexpression of hDlg blocks cell cycle progression in NIH3T3 cells, possibly through direct interaction with APC (68).

UV-induced Molecular and Carcinogenic Responses of Melanocytes Involve Multiple Signaling Pathways

UV light has been implicated in the genesis of several forms of cutaneous malignancies, including malignant melanoma. Although UV-A light is more abundant in sunlight than UV-B light, the latter is responsible for several types of DNA lesions; it induces cyclobutane-pyrimidine dimers and pyrimidine-pyrimidine photoproducts by direct DNA absorption (69). DNA mutations result from incorrect repair of these lesions. Unlike UV-B, UV-A is poorly absorbed by the DNA molecule but it can damage DNA indirectly by the generation of reactive oxygen species (ROS) via these photo-synthesized reactions (70). UV-A has a longer wavelength and penetrates deeper than UV-B through the epidermal layers and the underlying dermis of human skin, so it can readily reach the melanocytes that reside on the epidermal-dermal junction.

UV irradiation has been shown to affect multiple signaling pathways, including the p53 pathway, pRb and p16 pathways, and RTKs-activated signal transduction pathways (Fig. 6). Deregulation of these pathways has been implicated in the molecular pathogenesis of melanoma. Although there is no direct association with UV irradiation,

aberrations of cadherin and Wnt/β-catenin pathways are associated with melanoma progression (Fig. 4).

p53 pathway

p53 and genomic stability. The role of p53 as a guardian of genomic stability involves: 1) cell cycle arrest at the G1/S transition, which inhibits the replication of damaged genomes and allows for repair of damaged DNA; 2) induction of apoptosis that destroys cells with irretrievably defective DNA; and 3) interaction with other molecules that participate in these mechanisms. Mutated p53 can no longer preserve genomic integrity and induce apoptosis in response to genotoxic stimuli, including UV radiation (71). p53 is the most frequent target of genetic alteration identified, and the mutant form is found in approximately 50% of all human cancers (72). However, alterations in p53 are detected in only ~1-5% and 11-25% of primary and metastatic melanomas, respectively (73-77). This suggests that wild-type p53 is probably kept in an inactive state, either by other factors or through post-translational mechanism(s), and that other genes/proteins associated with p53 may also play central roles, thereby functionally inactivating the p53 pathways.

p53 and induction of cell cycle arrest and DNA repair. The transcriptional activity of p53 is tightly regulated by interactions with its negative regulator, Hdm2 (Mdm2 in mouse) (78, 79), which induces ubiquitination of p53 and forces its export from the nucleus to the cytoplasm, where it is degraded (80, 81). p53 is also a direct transcriptional activator of Hdm2, activating the expression of the *HDM2* gene in an

autoregulatory feedback loop (79). After DNA damage induced by UVR, Hdm2 is negatively mediated by $p14^{ARF}$, one of the two distinct proteins encoded by the *CDKN2A* (*INK4a/ARF*) locus. $p14^{ARF}$ associates directly with Hdm2 to block its ability to interact productively with p53, both by localizing Hdm2 within the nucleolus and by inhibiting the E3 ubiquitin protein ligase activity of Hdm2. Consequently, the p53 is dissociated from Hdm2, resulting in the stabilization of p53 in the cells. The resulting accumulation of wild-type p53 protein in response to UVR drives expression of downstream proteins such as p21 protein (82, 83), the proliferating cell nuclear antigen (PCNA) (84), and GADD45 (82, 85), resulting in G1 phase arrest and attempts at DNA repair.

Hdm2 is commonly amplified in invasive and metastatic melanoma (76, 86), indicating that inhibition of p53 function by overexpression of Hdm2 probably plays a crucial role in melanoma development. Additionally, the amount of $p14^{ARF}$ protein staining is progressively decreased from benign melanocytic nevi to metastatic melanoma *in situ*, suggesting that its inactivation is important in the development of these tumors (87).

p53 and induction of apoptosis. In response to UV damage, p53 is increased and regulates downstream effectors, permitting cells to pause in the cell cycle to repair. In most cases, DNA damage is successfully repaired, restoring melanocytes to normalcy. However, irreparable DNA damage triggers p53-dependent apoptosis through the pro-apoptotic Bax protein (88). Bax is up-regulated and subsequently facilitates the release of apoptosis-inducing factor and cytochrome c from the mitochondria, thus activating the caspase cascade (89). The apoptotic action of Bax is inhibited by its heterodimerization

with the anti-apoptotic Bcl-2 protein; thus the ratio of Bcl2 to Bax determines if a cell will be targeted for apoptosis (90).

In this connection, normal melanocytes produce abundant levels of Bcl2 through the activation of the PI3K/Akt pathway so they (91) may resist apoptosis and survive with excessive DNA damage that might lead to malignant transformation (91-93). The majority of primary and metastatic melanomas exhibit reduced Bcl2 levels but are apparently resistant to apoptosis. This suggests that other anti-apoptotic regulators, such as Mcl-1 or Bcl-XL, may circumvent the normal cell death pathway, contributing to the pathogenesis and chemotherapeutic resistance in metastatic malignant melanoma (94).

p53 and UV-induced melanogenesis. Pigment enzymes tyrosinase and TRP1 have been implicated as downstream effectors of p53, both of which can be induced after UV radiation in a p53-dependent manner (95). These findings suggest that UV-induced melanin synthesis through the p53 pathway may provide a further level of protection against UV-induced cell cycle arrest by the same pathway (96, 97).

pRb and p16 pathways

In addition to activation of the p53 pathway, UV-induced G1 arrest also results from increased expression of the cyclin-cdk inhibitor $p16^{INK4a}$, one of the two distinct proteins encoded by the *CDKN2A* (*INK4a/ARF*) locus on chromosomal region 9p21-22. In response to UV irradiation, the increased p16 protein induces a G1 cell cycle arrest by competing with cyclin D for binding to cdk4 and inhibiting the formation of cyclinD-cdk4 complexes (98). This competition inhibits the phosphorylation of retinoblastoma

protein (Rb) by cyclin D-cdk4/cdk6 complexes, and sustains its activity in a hypophosphorylated state, acting as the gatekeeper of the G1 transition (99) and resulting in cell cycle arrest.

Inactivation of p16 may cause constitutive cdk4 activity and/or overexpression of cyclin D that result(s) in phosphorylation of Rb and the release from E2F transcription factor. This event facilitates unregulated cell cycle progression, leading to escape of the cells from G1 arrest and incomplete DNA repair. p16 is targeted by deletions or mutations in dysplastic naevi and metastatic melanoma, suggesting its association with the early transformation of benign lesions and with the later stages of malignant progression (100). Overall, mutations or deletions in $p16^{ink4a}$ are found in up to 40% of the melanoma-prone families and, to a lesser extent, in sporadic melanoma (101). Furthermore, other studies have also shown that loss of $p16^{ink4a}$ and activation of Ras are capable of inducing murine melanoma (102). Rb appears to be expressed at higher levels in melanomas than in benign naevi; therefore, it is unlikely that loss of Rb expression is an important factor in the pathogenesis of melanoma (103).

It is worth noting that missense mutations or deletions in the *CDKN2A* (*INK4a/ARF*) locus are found in the germline of 40% of familial melanoma kindreds (104, 105). This gene deletion is also common in ~50% of primary tumors and nearly all melanoma cell lines (106, 107). As stated earlier, this locus encodes two distinct proteins, $p16^{ink4a}$ and $p14^{ARF}$, both of which demonstrate tumor suppressor activity in genetically distinct anti-cancer pathways; i.e., the Rb pathway for $p16^{ink4a}$ and the p53 pathway for $p14^{ARF}$ (108, 109).

Tyrosine kinase receptor-activated signal transduction pathways

UV acts as an independent melanocyte mitogen (110) and, in fact, can directly activate cell surface growth factor receptors, including RTKs, in a ligand-independent fashion, leading to clustering and internalization (111, 112). As a consequence, the activated RTKs trigger the Ras/Raf/MAPK pathway (mitogen-activated protein kinase, also termed extracellular-signal-regulated kinases or ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. These two pathways contribute to survival and growth-stimulating signals in normal and, perhaps, in damaged melanocytes. UV activation and alterations of the components involved in these pathways are associated with unregulated cell proliferation and survival, leading to malignant transformation of melanocytes.

MAPK pathway: mutations in RAS and BRAF. RTKs, such as ETBr and bFGFr, which are crucial modulators of melanocyte development and function, utilize the MAPK pathway to mediate their growth stimulating effects (113). In this cascade, activated RTKs mediate the activation of RAS, a small molecular weight GTP-binding protein that functions upstream of the MAPK pathway. Once activated, RAS phosphorylates RAF which, in turn, phosphorylates MEK (MAPKK or ERK kinase). Activated MEK sequentially phosphorylates ERK (MAPK) which, when activated, translocates to the nucleus and regulates expression of a number of genes associated with proliferation and differentiation, such as cyclin D (114). In addition to the MAPK pathway, BRAF was also shown to be an activation target of cAMP, a major component of the cAMP/PKA pathway involved in the melanogenesis and differentiation of melanocytes (115), and MAPK was reported to phosphorylate and activate Mitf (43).

These data indicate a potential link between BRAF activity and melanocyte differentiation.

Alterations in the components of this signaling pathway have been implicated in melanoma (116). Studies have shown somatic N-RAS mutations in 10%-37% of sporadic melanomas and in up to 95% of hereditary melanomas from patients carrying germline *CDKN2A* mutations (117, 118). In addition, somatic mutations in BRAF have been detected in nearly 70% of malignant melanomas, all of which are within the kinase domain, with a V599E substitution accounting for 80% of these (119, 120). Mutated BRAF proteins have elevated kinase activity and transforming activity (119). Moreover, the BRAF V559E mutation was expressed at a high frequency in about 89% of all forms of nevi (121). These findings implicate mutations in BRAF as early and critical events in the initiation of melanocyte transformation.

Another hallmark of human melanoma linked to the promotion and progression of melanoma is that autocrine/paracrine signaling loops of RTKs become autonomous through aberrantly expressed growth factors as well as their respective receptors of melanoma cells, which enable them to grow uncontrollably and acquire transforming activities (122). UV irradiation mediates such potent signaling loops by up-regulating growth factors such as endothelin-1 (41) and bFGF (123).

PI3K/Akt pathway. In addition to UV, the PI3K/Akt pathway is typically engaged in response to multiple mitogens that bind to receptor kinases at the plasma membrane and lead to the activation of the phosphoinositide 3-kinase (PI3K) (124). Once activated, PI3K converts the lipid PIP_2 into PIP_3. PIP_3 activates the protein kinase B/AKT which, in

turn, targets multiple factors involved in cell proliferation, migration, and survival. Regarding survival functions, AKT promotes the inactivation of the pro-apoptotic protein BAD (125) and the cell death pathway enzyme, caspase 9 (126). Additionally, PKB/Akt promotes cell survival by stimulating the expression of cellular genes via the CREB/CBP nuclear transduction pathway (127). A transcriptional factor Mitf was suggested to be activated by this pathway. In addition to controlling melanogenic gene expression and melanin synthesis, Mitf regulates the expression of the anti-apoptotic Bcl-2 protein, which is critical for melanocyte survival (24). Regarding cell proliferation, cyclin D1 transcription and E2F activity are mediated, at least in part, by the serine-threonine kinase Akt/PKB, resulting in cell cycle progression (128).

Collectively, the PI3K/Akt pathway likely plays a critical role in balancing UV-induced apoptotic signals, thereby preventing widespread skin cell death. Accordingly, the UV-induced PI3K/Akt pathway promotes the survival and proliferation of epidermal melanocytes, which is significant for photoprotection of the skin. Conversely, such activation may enhance survival of mutated cells, thereby promoting melanoma. Although no activating mutations have been identified in the Akt gene in melanoma (129, 130), blocking its function by targeting the PI3K (with the drugs wortmannin or LY294002, for example) inhibits cell proliferation and reduces the sensitivity of melanoma cells to UV radiation, pointing to the PI3K/Akt pathway as a putative candidate for therapeutic intervention in melanoma (131, 132).

Mammalian cells have also developed protective mechanisms to regulate Akt activity. The center of these mechanisms is PTEN, a phosphatase that targets PIP_3 and prevents the activation of Akt (133). Therefore, PTEN counteracts survival signals and

promotes apoptosis. One-third of primary melanomas and about 50% of metastatic melanoma cell lines showed reduced expression of PTEN as a result of allelic deletion, mutation (134), or transcriptional silencing (135), suggesting that inactivation of PTEN is a late, but frequent, event in melanomagenesis (136, 137).

Cadherin and Wnt/β-catenin pathways

β-catenin and cadherin-mediated adhesion. β-catenin forms complexes with the transmembrane adhesion protein E-cadherin, and helps to mediate intercellular adhesion. While this cadherin-bound pool of β-catenin has a crucial role in cell-cell adhesion, a cytoplasmic pool of β-catenin participates in the Wnt signal transduction pathway by interacting with the T-Cell factor/lymphoid enhancer factor Tcf/Lef as a cofactor, leading to transcriptional activation of its target genes, such as c-myc and cyclin D1. Loss of cadherin-catenin complexes through various mechanisms increases the free cytoplasmic pool of β-catenin, suggesting that the cadherin-bound pool of β-catenin can be released into the cytoplasm and made available for signaling (138, 139). Conversely, over-expression of E-cadherin reduces free cytoplasmic β-catenin by recruiting it to cell-cell junctions and thereby makes it unavailable for signaling in the nucleus (140, 141). Recently, β-catenin has been shown to be exchanged between its free and cadherin-bound state, supporting the possible crosstalk between the two pools (142). These data suggest that cadherin may act as a negative regulator of signaling β-catenin, as it binds β-catenin at the cell surface, thereby sequestering it from the nucleus.

Among several control mechanisms, the integrity of the cadherin-catenin complex is partly regulated by the tyrosine phosphorylation of β-catenin. For example, Src kinase

(139) phosphorylates tyrosine residues of β-catenin and causes dissociation of the cadherin-β-catenin complex and accumulation of free cytoplasmic β-catenin. Moreover, several studies show that activation of tyrosine kinases can increase β-catenin signaling, such as activation of the HGF/cMET receptor (143) and insulin-like growth factor (IGF) type II receptor (144). These phosphorylations appear to oppose the role of E-cadherin as a negative regulator of signaling β-catenin. However, such a role can be facilitated through a function of tyrosine phosphatase (PTPase) that antagonizes the effect of Src kinase and stabilizes the cadherin-catenin complex (145). Furthermore, serine/threonine phosphorylation of β-catenin (146) or E-cadherin (147) by casein kinase II also increases E-cadherin-β-catenin interactions and strengthens cell-cell adhesion.

Wnt pathway and possible interrelations with cadherin-mediated adhesion. In addition to modulation by cadherin, the amount of β-catenin is tightly regulated by Wnt signals, which are powerful regulators of differentiation and developmental processes. In the absence of Wnt signals, a multicomponent destruction complex containing GSK3β, axin, and APC promotes the phosphorylation of β-catenin at its N terminus and thereby targets it for degradation by the ubiquitin-proteasome pathway. Consequently, steady-state levels of free β-catenin outside the cadherin-catenin complex in the cell remain low, and formation of nuclear Tcf/β-catenin complexes is inhibited. Wnt signaling proceeds through Wnt ligands binding to their transmembrane receptor Frizzled, which activates the cytoplasmic protein Dishevelled. This activation leads to inhibition of the catenin destruction complex and accumulation of free cytoplasmic β-catenin, which translocates to the nucleus, where it interacts with and converts Tcf/Lef from a repressor to a

transcriptional activator. This leads to activation of Tcf/Lcf target genes influencing cell differentiation and development. With respect to cadherin-mediated adhesion, several lines of evidence suggest possible cross-talk between Wnt and the adhesion complex. Early studies demonstrated that an increased level of β-catenin induced by the Wnt signaling pathway led to the stabilization of β-catenin binding to cadherin at the plasma membrane and an increase in cell-cell adhesion (148). Loss and deregulation of E-cadherin expression in melanoma cells has been shown to involve up-regulation of its repressor Slug/Snail (149), which is reviewed to be another target gene of the Tcf/β-catenin complex (150). In addition, the Tcf/β-catenin complex itself binds and represses the E-cadherin promoter (151).

Alterations of the Wnt pathway in melanoma. Aberrant activation of the Wnt signaling pathway, which involves the formation of a persistent, transcriptionally active complex of Tcf/Lef-β-catenin, appears to be an important event in the genesis of a number of malignancies, including melanoma. Unusual messenger RNA splicing and missense mutations in the β-catenin gene (*CTNNB1*), which render the protein resistant to degradation by the APC-GSK 3β-axin complex and result in constitutive stabilization of β-catenin regardless of Wnt signaling, have been reported in a proportion of melanoma cell lines (152) and in a small fraction of primary melanomas (153, 154). Although these mutations appear rare in melanoma lesions, activation of β-catenin, as indicated by accumulation and nuclear localization, is frequent in melanoma (153-155). Using a combination of immunohistochemistry and RT-PCR, Demunter and colleagues (156) reported that down-regulation of membranous β-catenin was associated with an increased

amount of β-catenin RNA in primary or metastatic melanoma, suggesting that post-translational events, rather than *CTNNB1* mutations, are responsible for the altered distribution of β-catenin in melanoma. In addition, mutations in other components of the Wnt pathway, such as *APC* or *ICAT,* are rare in sporadic malignant melanomas. Rather, the expression of *ICAT* transcripts is commonly reduced or absent in this cancer. ICAT is a novel β-catenin-interacting protein that blocks the interaction between β-catenin and Tcf-4 and thereby antagonizes Wnt signaling. The findings with down-regulation of ICAT suggest that altered β-catenin/Tcf-4 regulation in the cell nucleus may be another mechanism important for melanoma progression (157).

Progression and Metastasis of Melanoma: Adhesion Factors, Membrane Peptidases, and Endoproteases

Based on clinical and histopathological features, melanoma commonly develops and progresses in a sequence of five stages (158, 159). The first step consists of structurally normal melanocytes forming common acquired and congenital nevi. The second step is dysplastic nevi (also called atypical moles and melanocytic dysplasia), with structural and architectural atypia thought to have a greater propensity for progression. These nevus cells cannot spontaneously transform in culture or develop tumors in immunodeficient mice (160). The third step is called the radial growth phase (RGP). Cells from RGP lesions can individually invade the dermis but have no capacity to metastasize. The vertical growth phase (VGP), the fourth step in progression, involves primary melanoma cells that acquire the ability to invade deeply into dermis and then into lymphatics and blood vessels, leading to the final step, metastatic melanoma with systemic dissemination into distant organ sites.

Melanoma is considered a disease of homeostatic imbalance in the skin. Normal skin homeostasis is maintained by dynamic interactions between melanocytes and epidermal keratinocytes as well as melanocytes and their microenvironment, such as fibroblasts, endothelial and immune cells, and extracellular matrix. Melanocytes adhere directly to keratinocytes, whereas communication between melanocytes and fibroblasts or endothelial cells occurs through soluble factors. Disruption of these homeostatic controls by alteration of some of the critical cellular components can lead to progression of melanoma. In the benign nevi stage, increased expression of cell surface receptors N-cadherin (161) and Mel-CAM/MUC18 (51) and reduced expression of E-cadherin (162) can be observed. Mel-CAM may play a major role in metastasis by mediating melanoma cell-cell interactions and melanoma-endothelial cell adhesion. Loss of dipeptidyl peptidase IV (DPPIV), which is expressed in normal melanocytes, occurs in the dysplastic nevi step (163, 164). In contrast to DPPIV, aminopeptidase N (APN) is not expressed by normal melanocytes but becomes increasingly prevalent as melanocytes transform to dysplastic nevi (165, 166). These peptidases are integral membrane proteins, with the catalytic site exposed at the external surface of the cell critical in the control of growth and differentiation of many cellular systems. As the cells progress from RGP to VGP, expression of invasive-related adhesion receptors, such as $\alpha_v\beta_3$ integrin, ICAM-1, and GD_2 ganglioside, is increased. In melanoma, $\alpha_v\beta_3$ integrin is currently the best molecular marker correlating with the change from RGP to VGP melanomas (167). In addition, activation of matrix metalloproteinases (MMPs), Ca^{2+}-dependent endoproteases, is also involved in promoting invasion and metastasis of tumor cells

through their ability to degrade the surrounding ECM proteins (168). Dynamic changes in expression of these components in melanoma progression are summarized in Fig. 7.

The E-cadherin to N-cadherin switch in melanoma progression

Melanomas develop in a sequence of steps. At present the genetic and biochemical alterations responsible for melanoma progression are not completely defined. Nevertheless, the very first step from a normal melanocyte to a benign lesion appears to be initiated by the escape of melanocytes from the keratinocyte-mediated regulation. The loss of such a control in melanocytes occurs in concert with down-regulation of E-cadherin expression (169). During melanoma development, in addition to reduced expression of E-cadherin, its (169) expression also becomes heterogeneous, gets diffusely distributed in the cytoplasm of the nevus cells and is predominantly absent in melanoma cells with acquired invasive characteristics (169, 170). Furthermore, in a skin reconstruction model, ectopic E-cadherin expression in melanoma cells renders them susceptible to keratinocyte-mediated control and inhibits their invasion into dermis by down-regulation of invasion-related adhesion receptors, such as MCAM/MUC18 and $\alpha_v\beta_3$ integrin, and by induction of apoptotic death (171). In melanoma cells, loss and deregulation of E-cadherin expression involve up-regulation of two transcriptional factors, snail (149) and SIP1 (172), and down-regulation of a third transcriptional factor AP2 (173), rather than deletions, mutations, and methylation of the E-cadherin gene (149). In addition, disruption of E-cadherin-containing AJs is suggested to contribute to melanoma progression through β-catenin released from the AJ pool, which then

translocates into the nucleus and induces a variety of genes that promote cell proliferation and invasion (60).

Melanocytic cells gain expression of N-cadherin during melanoma development, which mediates the adhesion of melanoma cells to one another and to N-cadherin-expressing cells in the stroma, including dermal fibroblasts and vascular endothelial cells (161, 174, 175). In normal skin, molecular cross-talk mediated by E-cadherin and gap junctional communication between keratinocytes and melanocytes maintains tissue homeostasis. During melanoma progression, down-regulation of E-cadherin with up-regulation of N-cadherin leads to a switch of communication partners from keratinocytes to dermal fibroblasts and endothelial cells. Through gap junctions, N-cadherin facilitates the transfer of stimulatory and inhibitory signals between melanoma cells and their microenvironment, supporting its role in promoting the migration and metastasis of melanoma cells to dermis. Although the functional implication of a cadherin switch remains to be elucidated, the observation that N-cadherin interacts with fibroblast growth factor receptor (FGFR), a member of RTKs, and enhances its tyrosine kinase signaling suggests that the cadherin switch may not only modulate tumor cell adhesion, but also stimulate classical signal transduction pathways, resulting in a shift from epithelial to fibroblastic phenotype with invasive characteristics (176, 177).

Immunotherapy for Melanoma and Melanoma Tumor Antigen Identification

The early stages of melanoma are curable with surgical treatment. However, the treatment options for metastatic melanoma are ineffective, as the tumors are radioresistant, chemoresistant and, hence, resistant to apoptosis (178, 179). The

development of effective prevention and intervention strategies for this aggressive disease remains a major challenge. There is growing evidence that the immune system interacts with tumor cells in such a way that may lead to eradication or control of tumor progression during the course of the disease. Some studies have shown that the proliferative activity of $CD8^+$ and $CD4^+$ T lymphocytes that infiltrate tumor cells reflects anti-tumor immunity and is associated with better prognosis and survival of patients (180-182). To mediate such immunity, $CD8^+$ T lymphocytes (CTLs) recognize tumor antigen epitopes presented by HLA class I [human leukocyte antigens, analogues to the surface major histocompatibility complex (MHC) class I molecules] in combination with certain costimulatory signals, whereas $CD4^+$ T lymphocytes recognize surface HLA class II-presented antigenic peptides on antigen-presenting cells (APCs) (183, 184). This discovery has led to the use of immunotherapy in an attempt to manipulate patient immune systems to eradicate cancer. For many years, various forms of immunotherapy, including nonspecific immunostimulation with cytokines or active specific immunotherapy ("cancer vaccines" or "tumor-antigen-based vaccines"), have been tested in clinical trials in attempts to enhance specific anticancer immunity. Based on the knowledge of the anti-tumor activity of $CD8^+$ and $CD4^+$ T lymphocytes through their specific recognition to tumor-associated antigens (TAA), identification of these antigens has become an active pursuit as a means to improve the effectiveness of cancer vaccines. Additional studies, including optimization of the delivery of these antigens by the use of viral vector systems or dendritic cells, and the increase of the immunogenicity of these antigens by the use of adjuvants and costimulatory molecules, have also been evaluated.

T-cell cloning approach

Melanoma is one of the most immunogenic solid tumors in which immuno-therapeutic strategies for treating patients in advanced stage have been investigated. In the early 1990s, the T-cell epitope cloning technique developed by Boon's research group was employed to identify melanoma-associated antigens (MAA) (185). In this approach, T-Cells derived from cancer patients were used to analyze tumor cDNA libraries for the expression of the target antigens. Therefore, the cDNA was transfected into a targeT-Cell presenting the appropriate HLA molecule, and then T-Cells were added to define the transfectant expressing the target antigen. This approach led to the identification of several antigens recognized by $CD8^+$ T-Cells. For example, melanoma antigen-1 or MAGE-1 (subsequently renamed MAGE-A1) was the first antigen clonally isolated by this technique (185). An intriguing feature of this antigen is its restricted expression in normal testis among normal tissues and its activation in a variety of cancer types in addition to melanoma. It is referred to as a cancer/testis (CT) antigen. Additional new CT antigens, such as the BAGE (186) and GAGE gene families (187), were subsequently identified following this T-cell epitope cloning strategy. Besides CT antigens, this technique also led to the identification of several melanocyte differentiation antigens, i.e., tyrosinase (188), Melan-A/MART-1 (189), gp 100 (190), and gp 75 (191). These differentiation antigens were expressed in most melanoma tumor samples and, among normal cells, only in melanocytes. In principle, this cloning approach, after some modification, can also be applied to the identification of target antigens recognized by $CD4^+$ T cells. Indeed, tumor reactive $CD4^+$ T-cell clones isolated from tumor-infiltrating lymphocytes (TIL) have been employed for epitope identification in the context of HLA

class II (192-194). Although the T-cell cloning approach leads to the identification of tumor antigens in melanomas as well as in other types of cancer, this strategy has the disadvantage that T-cell culturing is difficult and time-consuming. Moreover, the fact that the purification, subsequent stimulation, and outgrowth of T-cell clones are required might selectively amplify T-cell populations that are not relevant to *in vivo* tumor rejection. The conditions to obtain established tumor cell lines are also frequently difficult to meet in the case of certain tumor types. Overall, this strategy is difficult, laborious, and expensive.

SEREX approach and SEREX-defined antigens

In addition to the specific T-cell-based approach, serological identification of antigens by recombinant cDNA expression libraries, SEREX, developed by Pfreundschuh and his colleagues Sahin and Tureci (195), provides another route for defining immunogenic human tumor antigens. The SEREX approach is based on the rationale that immune recognition of tumor antigens is a concerted action between the cellular and humoral immune systems. As the development of high-titer IgG requires cognate $CD4^+$ T-helper cells, circulating tumor-associated antibodies may identify gene products recognized by at least the cognate T-cell helper. This strategy allows a systemic and unbiased search for antibody responses in autologous and allogeneic sera of cancer patients against antigenic proteins expressed by tumor cells. In this approach, a cDNA expression library is prepared from either fresh tumor specimens or tumor cell lines and cloned into λ phage expression vectors. The resulting recombinant phages are then used to transfect *E. coli*. Recombinant proteins expressed during the lytic infection of the

bacteria are transferred onto nitrocellulose membranes, which are immunoscreened with diluted and extensively pre-absorbed serum from the autologous patient. Clones reactive with a high-titer IgG humoral response are subcloned to monoclonality, thus allowing direct molecular characterization by DNA sequencing. Compared with the extensive manipulations required to identify tumor antigens based on T-cell reactivity, the SEREX method represents a comparatively rapid means of identifying potential TAAs in that it does not rely on T-cell lines recognizing autologous tumor and established tumor cell lines that could produce *in vitro* artifacts that might be associated with short- and long-term culture. The use of fresh tumor specimens in SEREX circumvents such artifacts and restricts the analysis to genes that are expressed by the tumor cell *in vivo*. Moreover, the use of the polyclonal patient's serum allows the identification of multiple antigens with one screening course. However, a limitation of this approach is the comparatively large number of antigenic proteins identified, some of which are not relevant to cancer.

Using SEREX, Chen et al. discovered a new CT antigen, NY-ESO-1 (196). Jager and colleagues also discovered NY-ESO-1 by using tumor-specific CTL or TIL derived from melanoma patients as probes and demonstrated that NY-ESO-1 is a target for both antibody and specific $CD8^+$ T lymphocyte responses (197, 198). The titer of anti-NY-ESO-1 antibodies appears to increase with progressive disease and decrease upon tumor regression (199). Antibodies specific for other CT antigens initially recognized by CTLs; e.g., MAGE-A1, MAGE-A3, and SSX-2, have also been detected in melanoma patient sera (200). Thus, it appears that SEREX can detect tumor antigens that elicit a CTL-mediated immune response and may be useful for analysis of the $CD4^+$ and $CD8^+$ T-cell repertoire against tumor antigens. In this regard, SEREX-defined antigens are used to

identify and determine peptide epitopes in the context of HLA class I or II molecules that are recognized by T lymphocytes in a strategy known as "reverse T-cell immunology." Several $CD4^+$-binding epitopes of NY-ESO-1 antigen have been identified by such a strategy (201). SEREX has led to the identification of a multitude of new tumor antigens in many different tumor entities, with more than 2,000 antigens deposited in the SEREX database of the Ludwig Institute for Cancer Research (http://www.licr.org/SEREX.html). According to their specificities, SEREX-defined antigens can be grouped into different classes: 1) CT antigens expressed in normal testis and various cancers (202); 2) melanocyte differentiation antigens, which are expressed in melanoma and also in normal melanocytes, but are antigenic in melanoma (203); 3) tumor-associated over-expressed gene products (204); 4) mutated gene products (205); 5) tumor-specific splice variants (206); and 6) viral antigens (207).

The pool of available tumor antigens has been greatly enlarged in recent years through the use of SEREX, which leads to new perspectives for the development of molecular vaccine strategies. The SEREX-defined tumor antigens facilitate the identification of epitopes recognized by tumor-specific T lymphocytes and therefore provide a molecular basis for polyvalent peptide-based and gene-therapeutic vaccine strategies in a wide variety of human neoplasms.

Rationale and Summary for This Dissertation Research

Although the systematic application of CTL- and antibody-based methods has led to an increasing number of antigens, only a few CT antigens known to date, e.g., NY-ESO-1, MAGE-A1, MAGE-A3, and SSX-2, are expressed at high frequencies in

melanomas, with the range of 15-35% (208-210), thus showing the clinical feature potentially useful for a widely applicable vaccine. Moreover, NY-ESO-1 appears to be the most immunogenic CT antigen, with serum antibody to NY-ESO-1 observed in 9.5% of melanoma patients. Humoral immune responses to other antigens are detected only in a small number of melanoma cases, with a range between 1-2% (MAGE-A1, -A3, SSX-2) (200). Clinical studies evaluating the immunological effects of these antigens are ongoing, most of them in metastatic melanoma patients. In addition to CT antigens, the differentiated antigens TRP-2 and tyrosinase are also induce spontaneous T-cell responses in melanoma patients, leading to the proposal that they can serve as targets of immune-mediated tumor rejection (211, 212). Therefore, the identification and molecular characterization of more appropriate tumor antigens that can elicit specific immune response in the tumor-bearing host may provide a basis for successful development of antigen-specific cancer vaccines.

With the main goal of achieving the identification of more relevant tumor antigens, we have applied SEREX to evaluate the humoral immune response elicited by tumor antigens in response to immunotherapy strategies. In this study, we performed SEREX using serum from patient 1, an advanced stage melanoma patient undergoing UAB clinical trial 9701, an active nonspecific melanoma immunotherapy involving intratumoral administration of recombinant canarypox viruses (called ALVAC) encoding the human interleukin 12 (ALVAC-hIL-12). This patient 1 experienced a complete clinical response and subsequent tumor regression to this therapy, which was likely attributable to induced immune responses to antigens within the patient's tumor nodules in response to ALVAC-hIL-12 treatment. This rationale was based on preclinical and

early clinical studies, which showed that hIL-12 has its potential roles in enhancing antitumor cell-mediated immunity (208, 213) and antibody responses (214, 215). Our focus on using serum from the patient with a complete clinical response in SEREX was to analyze the humoral immune response to tumor antigens present in the tumor patient, which might play a role in tumor rejection. This strategy applied to such a patient may increase opportunities to identify novel targets that are more specific for immunotherapy than using serum from a nonselected patient.

Using the serum of patient 1 from UAB clinical trial 9701 to screen a cDNA library derived from cultured human melanoma cell lines Mel 624 and Mel 888, SEREX analysis led to the identification of seven novel candidate antigens that were targets of the immune response induced by nonspecific immunotherapy using intratumoral injection of ALVAC-hIL-12. Humoral immune responses directed against the identified seven antigens were further evaluated in allogeneic sera from the 8 additional patients with metastatic melanoma who enrolled in UAB clinical trial 9701 and from the patients in UAB clinical trial 9705 (12 total). The serological analysis showed that most patients did not have circulating antibodies directed towards the isolated proteins. A notable exception was patient 9 from UAB trial 9705, whose serum was reactive with five of the isolated antigens, suggesting that these may represent shared tumor antigens. Importantly, the patient developed the antibodies against these five antigens during the treatment. Patient 2 from UAB trial 9701 exhibited antibody response to one of the seven antigens prior to the treatment.

Of the seven tumor antigens isolated, the human homolog of *Drosophila* disc large protein (hDlg) was further characterized at the molecular level in melanomas.

Serological reactivity of hDlg was analyzed using sera derived from allogeneic melanoma patients treated with conventional methods and from a large number of ovarian patients and normal individuals as well. The results showed that about 8% of melanoma patients had antibodies to the hDlg protein. Molecular characterization regarding expression analysis of hDlg transcript and protein in a panel of melanoma tumors and cell lines compared to normal human melanocytes revealed that hDlg mRNA and protein levels are frequently down-regulated in both melanoma tumors and cell lines. Aberrantly spliced variants of hDlg were also detected in melanoma tumors and cell lines tested, but not in normal cells. Furthermore, we demonstrated that subcellular localization of hDlg was aberrant in some melanoma cell lines and in all melanoma tumor samples evaluated. Based on the literature regarding on a potential role of hDlg as a tumor suppressor, together with our finding of frequent loss and/or aberrant localization in melanomas, we examined the role of hDlg in melanoma and found that hDlg acts as a tumor suppressor in this cancer type. Although the association between melanoma pathogenesis and immunogenicity of hDlg remains to be investigated, the characterization performed in this study suggests that altered expression and cellular localization of hDlg contributes to the phenotype of malignant melanoma.

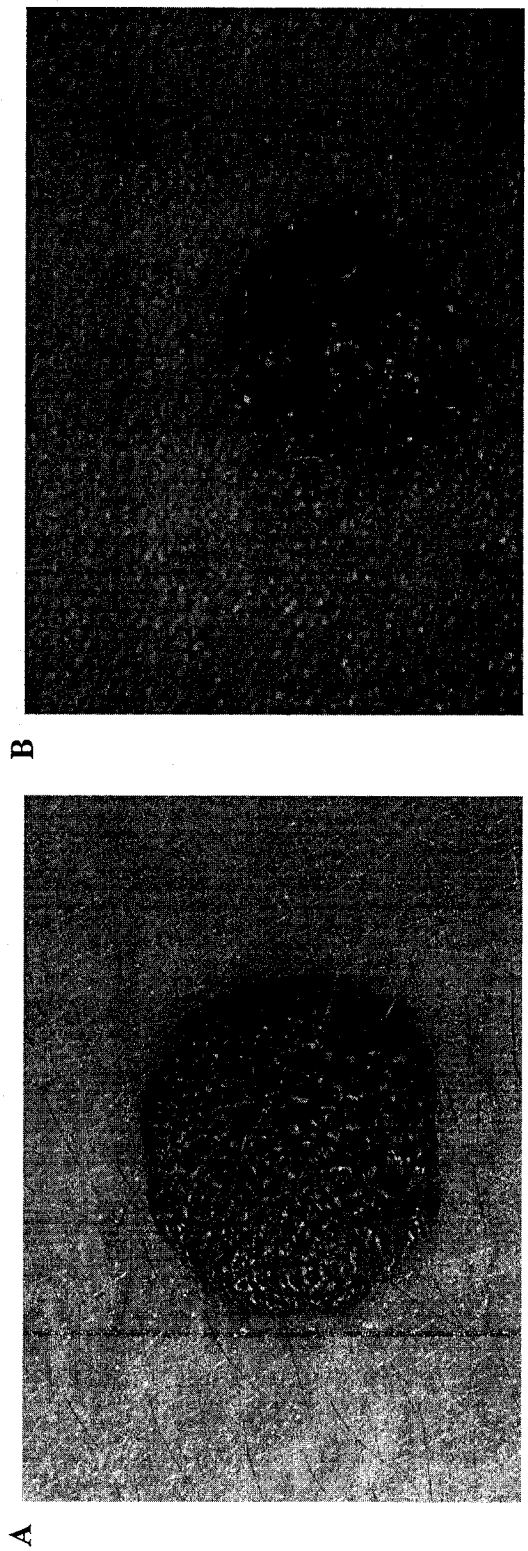

Fig. 1. *A*, common acquired nevi. *B*. atypical nevi.

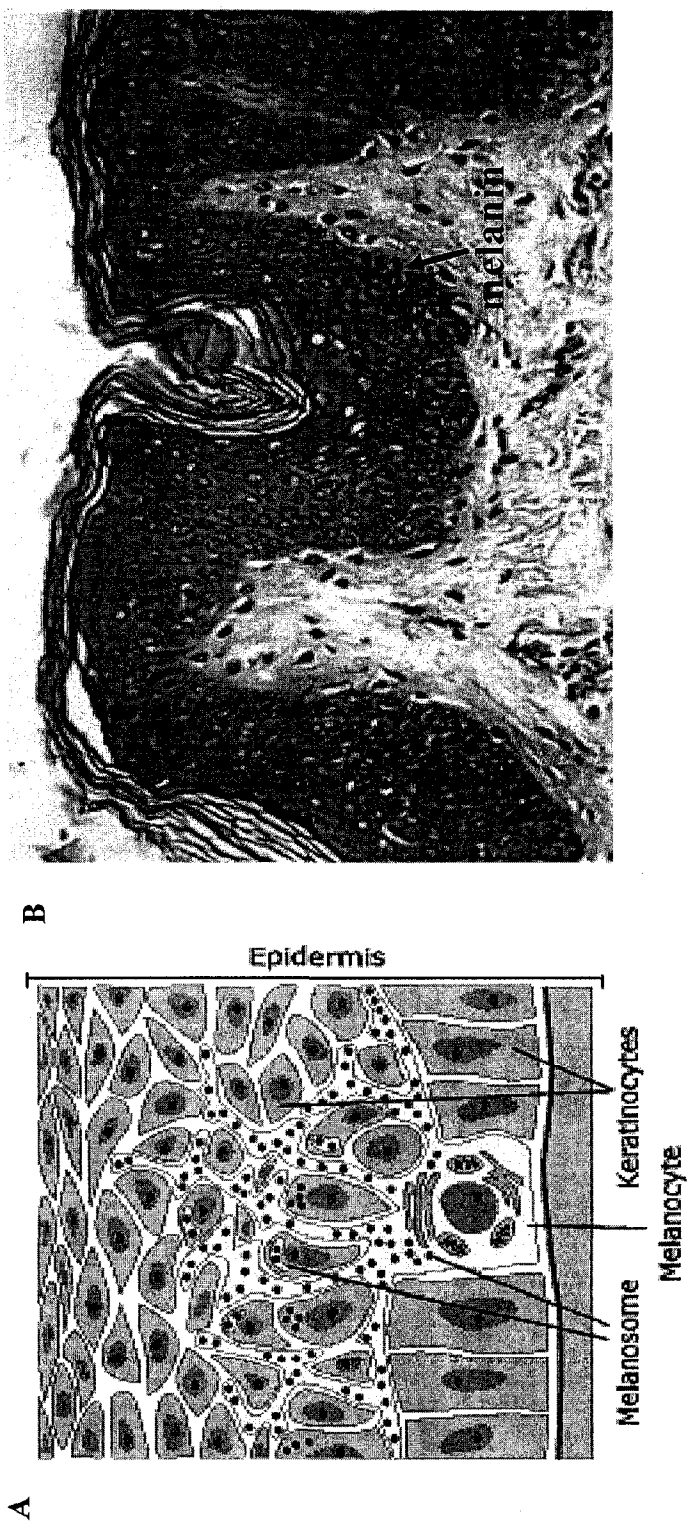

Fig. 2. *A.* melanocyte structure in the epidermis. *B.* melanin distribution in the skin.

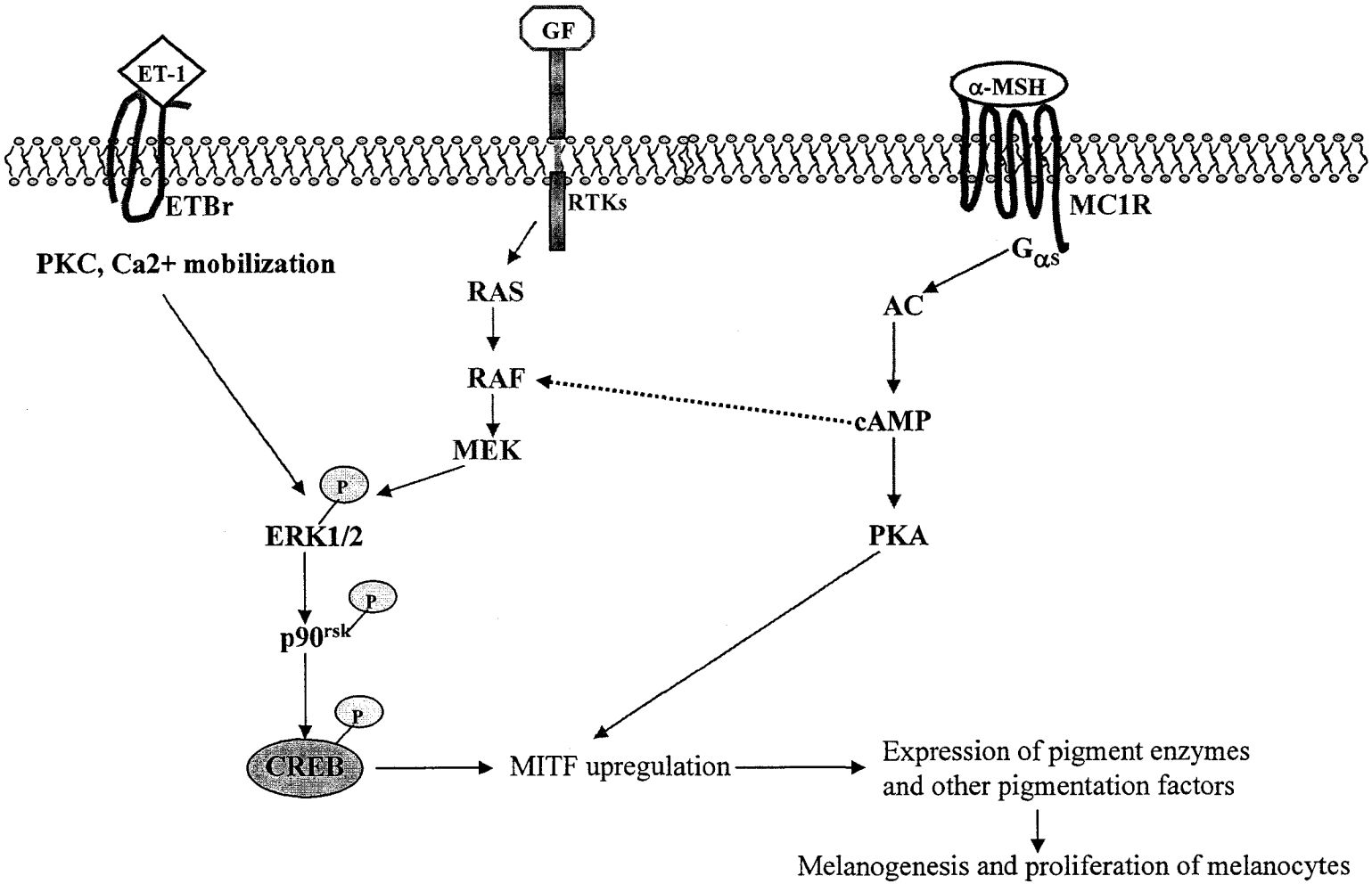

Fig. 3. Signaling cascade of paracrine factors in melanogenesis and proliferation of melanocytes.

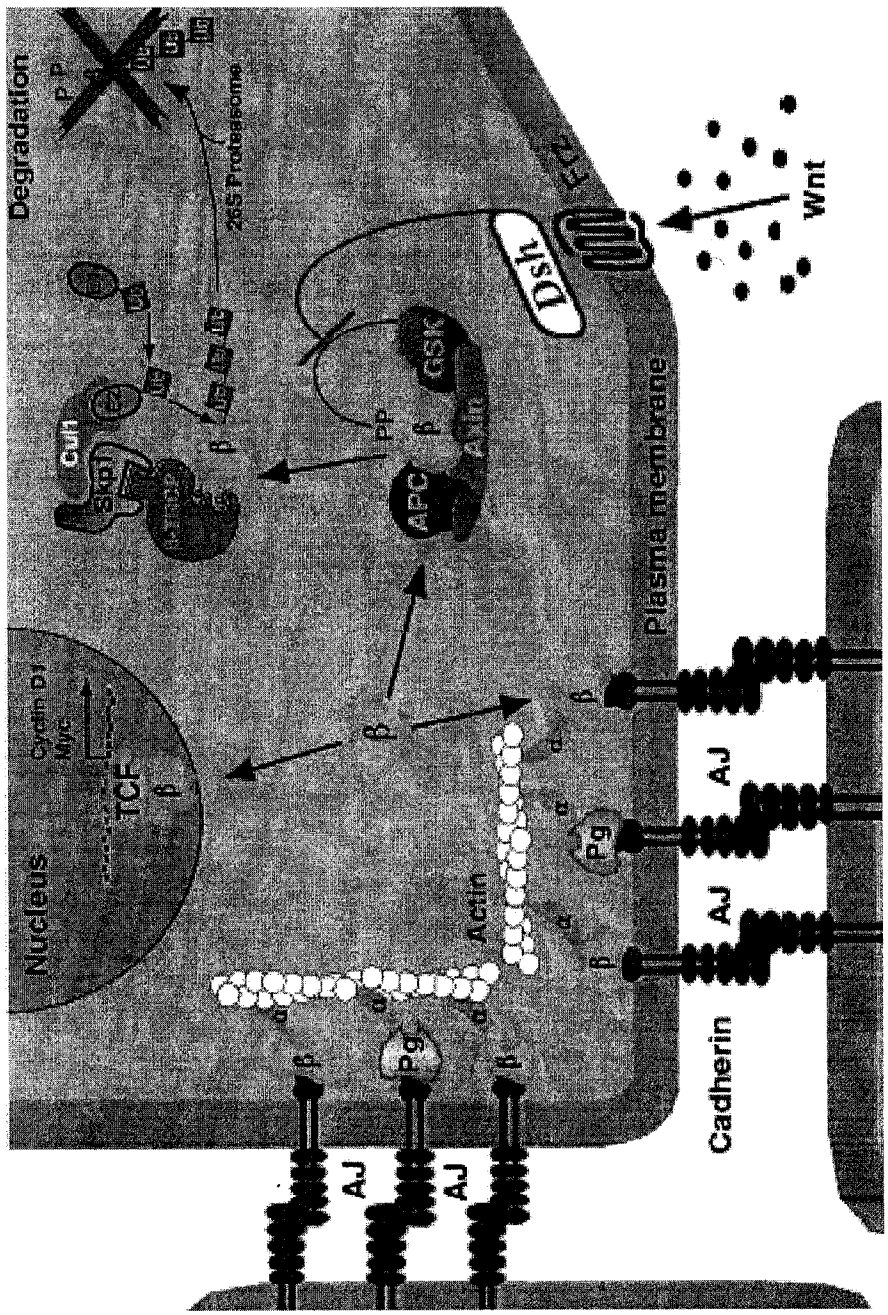

Fig. 4. Adherens junctions and the Wnt/β-catenin signaling pathway.

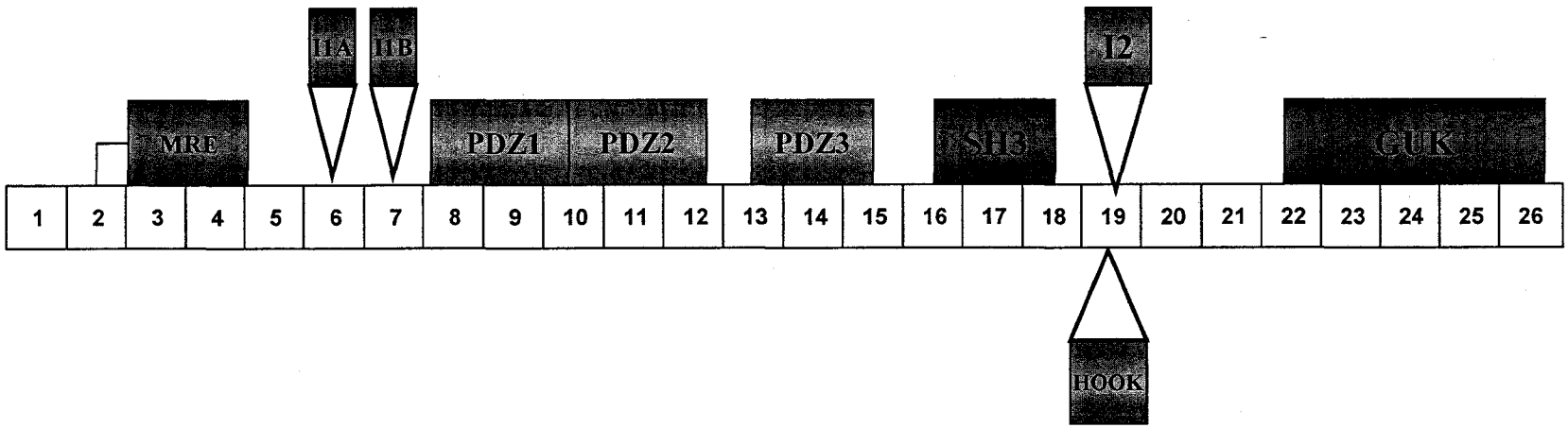

Fig. 5. Schematic diagram of hDlg protein represents MRE, PDZ, SH3, and GUK domains. Three alternative domains encoded by alternatively spliced exons are designated as I1, I2, and I3, respectively.

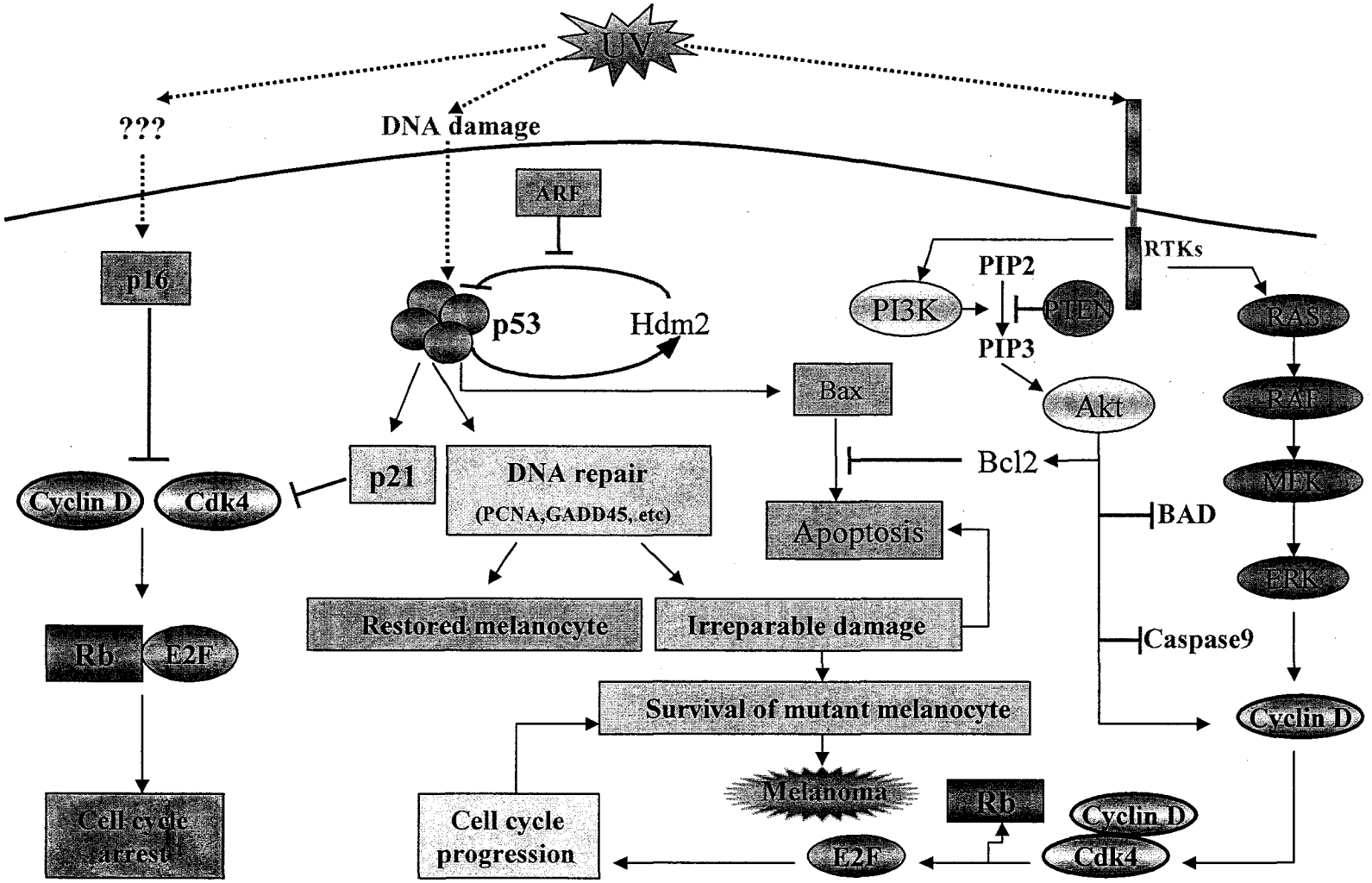

Fig. 6. UV-induced signaling pathways: pRb and p16, p53, PI3K/Akt, and MAPK pathways.

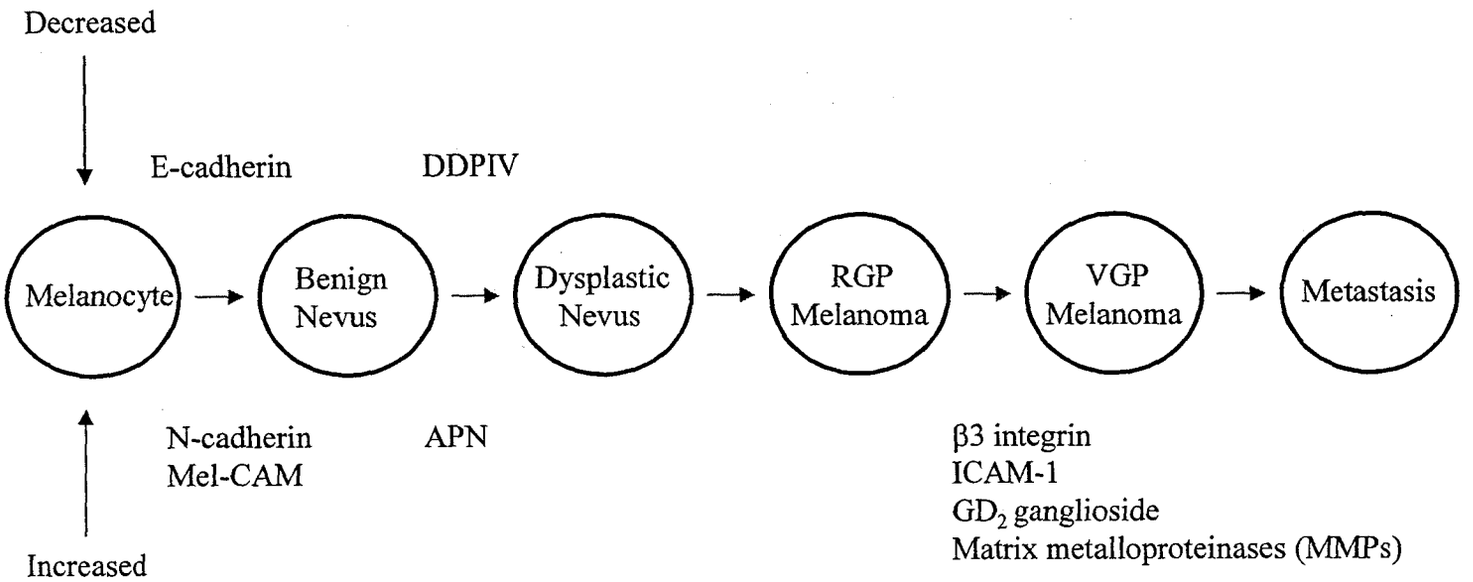

Fig. 7. Dynamic changes in melanoma progression.

SERIOLOGICAL IDENTIFICATION OF MELANOMA ANTIGENS IN A PATIENT RESPONDING TO ALVAC hIL-12

by

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In preparation for *International Journal of Cancer*

Format adapted for dissertation

ABSTRACT

An understanding of the proteins that are important in mediating clinical responses following nonspecific immunotherapy remains incomplete. To identify novel antigenic proteins in melanoma, the humoral immune response of a patient undergoing a phase Ib immunotherapy trial for advanced stage disease was studied. The patient experienced a complete clinical response following intratumoral injection with a Canarypox virus encoding human IL-12 (ALVAC hIL-12). Post-therapy serum from the patient was used to screen a cDNA library derived from two human melanoma cell lines. Seven immunologically reactive clones were isolated and sequenced. None of these represent previously reported melanoma antigens. The patient did not have detectable antibodies to these proteins prior to therapy, indicating that the humoral immune response was induced by the treatment. Isotype analysis of the antibodies elicited to these proteins revealed a mature, T-cell dependent antibody response. Of 21 additional patients receiving ALVAC hIL-12 or ALVAC-encoding human B7.1, one patient exhibited induction of humoral immunity to five of the seven identified proteins, and another patient exhibited humoral reactivity to one of the seven proteins. Serological identification of immunogenic proteins provides a means to identify, at the molecular level, the immune response induced by nonspecific immunotherapy.

INTRODUCTION

The incidence of melanoma in North America has increased dramatically over the past 50 years (1). While detection and treatment of early stage melanoma results in a high cure rate, advanced stage disease is often fatal. Surgical resection and chemo-

therapeutic regimes have been standard treatments for advanced metastatic disease, but with limited success (2). Laboratory and clinical studies have indicated that melanoma may be amenable to immunotherapy (3-7), offering new possibilities for the treatment of advanced stage disease.

A number of experimental immunotherapies, both specific and nonspecific, have been developed for treatment of advanced melanoma (8-13). While these studies strongly suggest that clinical responsiveness is mediated by the immune system, the specificity of the response at the molecular level in many cases remains to be defined. With the identification of melanoma-associated antigens, including MART-1/Melan-A (14, 15), gp100 (16), and tyrosinase (17), it is possible to evaluate patient immune responses to known antigenic proteins and peptides subsequent to immunotherapy, providing a small number of assessiable endpoints. However, approaches that monitor immune responses to known antigens ignore cryptic tumor antigens that may be important mediators in the anti-tumor immune response. A more complete characterization of the targets of the cellular and humoral immune response elicited by nonspecific immunotherapy may provide a basis for the development of more effective cancer vaccines.

Humoral immune responses to a number of known tumor-associated antigens have been described (18-24). Although the clinical implications of circulating antibodies to autologous tumor proteins is not clear (25-27), these antibodies represent a valuable reagent for the identification of novel candidate tumor antigens (28, 29). The catalogue of candidate tumor antigens has been greatly expanded in recent years through the use of serological identification of antigens by recombinant cDNA cloning expression (SEREX), an efficient and rapid technique for the identification of novel antigens (28, 30,

31). A potential drawback of this approach is the comparatively large number of antigenic proteins identified. As a means to identify immunogenic proteins that may be more relevant to the process of tumor rejection, we have applied SEREX to evaluate the immune response to a tumor immunotherapy strategy. In this study, we performed SEREX using serum from an advanced stage melanoma patient who experienced a complete clinical response to intratumoral injection of ALVAC-hIL-12. SEREX analysis led to the identification of seven novel candidate antigens that were targets of the induced immune response.

MATERIALS AND METHODS

Clinical Trial

The two Institutional Review Board-approved, Phase Ib clinical trials relevant to this study, NCI Protocol #T97-0005 (University of Alabama at Birmingham [UAB] 9701) and NCI Protocol #T97-0046 (UAB 9705), are described elsewhere (Triozzi *et al.*, manuscript in preparation). All patients provided informed consent. Briefly, patients with surgically incurable melanoma were treated with intratumoral injections of a canary poxvirus (ALVAC)-encoding human IL-12 (UAB 9701) or human B7.1 with or without IL-h12 (UAB 9705). In UAB clinical trial 9701, accessible tumor nodules were injected with ALVAC-hIL-12 at a dose of 1-2 x 10^6 Tissue Culture Infection Dose 50% ($TCID_{50}$) on days 1, 4, 8, and 11. Clinical trial 9705 involved the injection of ALVAC hB7.1 at a dose of 2.5 x 10^9 pfu on days 1, 4, 8, and 11 into accessible nodules in group 1 patients, and injection of both ALVAC hIL-12 and ALVAC–hB7.1 in the same tumor nodules for group 2 patients. Whole blood was collected from patients on day 0 (pre-treatment), day

18, and day 43 in Vaccutainer SST Gel and Clot Activator tubes (Becton Dickinson, Franklin Lakes, NJ). Tubes were spun at 4,000 x *g* for 10 min and sera were collected and stored at –70°C.

cDNA Library Construction and Immunoscreening

Total RNA from Mel 624 and Mel 888 human melanoma cell lines (kindly provided by Stephen Rosenberg, National Cancer Institute, and grown in RPMI 1640 with 10% FBS) was isolated using the Stat-60 reagent (TelTest B), and mRNA was isolated using an poly(A^+) mRNA isolation kit (5 Prime – 3 Prime, Boulder, CO). mRNA from the cell lines was pooled and a cDNA library was constructed in the Zap Express vector (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Briefly, mRNA was reverse transcribed using an oligo (dT) primer with an internal *XhoI* site. After second strand synthesis, cDNA fragments were cloned into the directional λZap Express vector, packaged, and used to infect *E. coli* cells. Before amplification, the library titer was 6.8 x 10^6 primary recombinants. As a preliminary characterization of the library, inserts from random plaques were amplified by polymerase chain reaction (PCR), and insert sizes ranged from 400 base pairs to 5 kb with > 90% recombinants (data not shown).

For immunoscreening, recombinant plaques were plated at a density of ~20,000 plaques per 150-mm plate, and protein expression was induced using nitrocellulose filters (Millipore) saturated with isopropyl β-D-thiogalactoside (5'Prime- 3' Prime, Inc Boulder, CO). Filters were lifted, washed with dH_2O, blocked with 2% bovine serum albumin in Tris Buffered Saline [20 mM tris (hydroxymethyl)aminomethane (Tris) (pH 7.5) and 150

mM NaCl] with 0.05% Tween (TBST). Human sera were pre-absorbed with *E. coli* phage lysate (Stratagene) and diluted 1:250 for screening. After incubating filters with primary sera for 1 hr at room temperature, the filters were washed with TBST and incubated with alkaline phosphate-conjugated goat anti-human immunoglobulin (H+L) Abs (Jackson Labs) at a dilution of 1:5000 for 1 hr at room temperature. After washing, a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phos-phate colorimetric substrate was used to identify positive clones. Positive plaques were purified to clonality for further study.

Filter Lift Assays

Sera from patients in UAB clinical trials 9701 and 9705 collected on day 43 were used at a dilution of 1:250 to screen positive clones previously recovered, as described. Isolated clones were mixed in a 50/50 ratio with a no insert control to determine reactivity.

Isotype Analysis

Analysis of the immunoglobulin isotypes were performed by using alkaline phosphatase-conjugated, isotype-specific antibodies (Clonotyping System, Southern Biotechnology Associates, Birmingham, AL). Positively identified clones from the library were plated in a 50/50 mixture with a negative insert. Filters were incubated with the primary patient sera, washed, and incubated with alkaline phosphatase-conjugated goat anti-human secondary antibody specific for IgG1, IgG2, IgG3, IgG4, IgA, or IgM,

respectively, as secondary antibodies at a dilution of 1:1000 in the previously mentioned screening protocol.

DNA Sequencing

Phagemid DNA from positive plaques was rescued by *in vivo* excision using a helper phage system (Stratagene). Purified DNA was sequenced at the UAB Sequencing Core Facility using internal T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3') primer sites. Some additional sequencing was performed by the Iowa State University Sequencing Facility. Sequences were searched against the National Center for Biotechnology Information databases using the BLASTN program. In some cases, partial sequencing revealed identity with previously reported sequences, and the identity of the unsequenced portion of the clone was confirmed through restriction analysis.

RESULTS

Patient 1 Experienced a Complete Clinical Response

Patient one was an 87-year-old female with stage III recurrent melanoma. The patient had multiple recurrent melanoma lesions on her right thigh and had undergone previous surgical resections in the six months prior to entry into the clinical study. This patient received four intratumoral injections of ALVAC hIL-12 at a dose of 10^6 $TCID_{50}$. Four days after the second injection (day 8), Grade 2 local toxicity was observed for warmth and tenderness surrounding the ALVAC hIL-12 injected nodules consisting of 10-cm diameter patches of erythema (Fig. 1). The patient experienced Grade 2 fever and

chills beginning approximately 3 hr after the third injection. On day 11, an asymptomatic rash over the entire back and buttocks was noted, which eventually subsided by day 18. The patient also developed several 1-cm diameter tender right inguinal nodes draining the sites of ALVAC hIL-12 injection on her right thigh that spontaneously resolved by day 18. Analysis of serum IFN-γ levels revealed an increase in serum levels at days 8, 9, and 12 of treatment. The patient experienced complete regression of all tumor nodules and has remained tumor-free for more than 2 years.

Isolation and Sequence Analysis of Reactive Clones

A cDNA library from the melanoma cell lines Mel 624 and Mel 888 was synthesized containing 6.8 x 10^6 primary recombinants. The amplified library was screened with a 1:250 dilution of the patient's serum from day 43 post-initiation of ALVAC hIL-12 treatment. Sixty-eight immunoreactive clones were isolated initially from a screen of approximately 500,000 clones. Clones were further processed if there was no detectable reactivity with a minimum of 10 normal (non-diseased) sera. Inserts encoding for the same protein are reported as a single positive clone (longest cDNA reported). Seven independent immunologically reactive proteins were subjected to DNA sequence analysis, and the identities of the clones are shown in Table 1. Clone 3.1 corresponds to the full-length coding sequence of KIAA0663, a previously identified, uncharacterized coding sequence isolated from a brain cDNA library (32). KIAA0663 was reported to have a wide range of expression in tissues, including brain, heart, lung, testis, skeletal muscle, kidney, pancreas, spleen, and ovary (32). Clone 3.14 contains a sequence identical to a region of the human homologue of the Drosophila discs large

Table 1 *Identity of clones isolated by SEREX screening of a melanoma cDNA library*

Clone No.	Identity	Size, bp	Accession No.	Function
3.1	KIAA0663	619	NM_014827	unknown
3.14	Human homolog of Drosophila discs large protein (DLG1)	2980	U13897	tumor suppressor, adherens junction
5.16	hnRNP-E1	598	X78137	posttranslational regulation
5.17	KIAA0555	861	NM_014790	unknown
5.23	Homo sapiens chromosome 5 clone CTD-2320O4	3512	AC008942	unknown
5.28	Polyribonucleotide nucleotidyltransferase 1 (PNPT1)	2635	NM_033109	cellular growth regulation
5.31	RING3/Bromodomain-containing protein 2 (BRD2)	1495	X96670	developmental and growth control

tumor suppressor (hDlg) (33). Clone 5.16 corresponds to bp 51-1310 of the human hnRNP-E1 gene encoding a poly (rC)-binding protein (34), and it encompasses the majority of the coding sequence. Clone 5.17 represents the majority of the predicted coding region of KIAA0555 protein (35), containing bp 364-3193 of the published sequence. Clone 5.28 encodes polyribonucleotide nucleotidyltransferase 1 (PNPT1) gene. Clone 5.31 is the *Homo sapiens* RING3 gene (36). The insert corresponds to base pairs 4595-6533 of the published sequence, containing a large portion of exon 4, and the complete sequences of exons 5, 6, and 7, as well as the corresponding intronic sequences. This protein was previously reported to be antigenic in human lung cancer (37).

Antibody Responses Were Induced by the ALVAC hIL-12 Treatment

To determine whether the ALVAC-IL-12 immunotherapy resulted in induction of a humoral immune response to the isolated proteins, the patient's pretreatment sera (day

0), and sera from day 18 and day 43 post-treatment, were used in a filter-lift assay of isolated clones (Fig. 2). Each protein was reactive with sera from day 18 in addition to day 43, while no reactivity was detected with serum from day 0. This suggests that the ALVAC-IL-12 injection elicited an immune response to these proteins. Serum from day 640 was available and found to be reactive with Clones 5.17 and 5.23 only, while the remaining five clones were not reactive (Table 2).

Isotype Analysis

To further characterize the nature of the humoral immune response induced by ALVAC-IL-12, isotype analysis of the reactive antibodies was performed using day 43 serum (Table 3). All isolated clones were isotype IgG1, indicating a CD4+ T-cell-dependent humoral response. Antibodies to several clones also included the IgG3 and IgA isotypes. Class switching was thus apparent for all reactive antibodies.

Several of the Isolated Clones Were Immunologically Reactive With Additional Patients

The seven immunologically reactive clones were then screened against sera from the other patients enrolled in UAB clinical trial 9701 (8 additional patients) and patients in UAB clinical trial 9705 (12 total patients) (Table 4). Most patients did not have circulating antibodies directed towards the isolated proteins. A notable exception was patient 9 from UAB trial 9705, who was reactive with five of the isolated clones, suggesting that these may represent shared tumor antigens. Patient 9 (9705) had no detectable antibody response at day 0 (data not shown) to the five clones he was reactive with at days 18 and 43. Patient 2 (9701) was reactive with clone 5.17 at all three time

Table 2 *Timeline of antibody response induced by ALVAC hIL-12*

Clone	Day 0	Day 18	Day 43	Day 640
3.1	-	+	+	-
3.14	-	+	+	-
5.16	-	+	+	-
5.17	-	+	+	+
5.23	-	+	+	+
5.28	-	+	+	-
5.31	-	+	+	-

Clones isolated with patient 1 day 43 serum were screened with patient 1 serum from days 0, 18, and 640 to assess onset of humoral response.

Table 3 *Isotype analysis of humoral immune response*

Clone	IgG1	IgG2	IgG3	IgG4	IgM	IgA
3.1	+	-	-	-	-	-
3.14	+	-	+	-	-	+
5.16	+	-	+	-	+	-
5.17	+	-	+	-	+	+
5.23	+	-	+	-	-	-
5.28	+	-	-	-	-	-
5.31	+	-	+	-	-	-

Antibody isotype was determined using patient 1 serum from day 43.
+ indicates a positive immunological reaction in a 50/50 mixture with a negative insert clone, - indicates no reactive difference using the specific isotype in the 50/50 mixture assay.

Table 4 *Clone reactivity amongst patients*

Clone	Patient number	
	9701	9705[a]
3.1	1	
3.14	1	9
5.16	1	9
5.17	1, 2	9
5.23	1	9
5.28	1	
5.31	1	9

Clones isolated with patient 1 serum were screened with day 43 sera from all patients undergoing two clinical trials at UAB (UAB 9701 and UAB 9705).
a- refers to the UAB clinical trial number.

time points assayed (days 0, 18, and 43) (data not shown). The HLA type of patient 9 is A10, while that of patient 1 is A2 and patient 2 is A2.

DISCUSSION

In a clinical trial of nonspecific immunotherapy for advanced stage melanoma, patient 1 experienced a prolonged remission following a Phase Ib trial examining the safety and toxicity of ALVAC hIL-12 administration. To better understand which proteins may be important in mediating this clinical response, we used a SEREX approach to identify immunogenic proteins recognized by the patient. Seven novel candidate melanoma-associated antigens were isolated in an allogeneic screen of a cDNA library derived from the melanoma cell lines. Our results demonstrate the induction of a high titer, mature antibody response to these proteins subsequent to intratumoral injection of the ALVAC hIL-12. SEREX provides a rapid means of identifying the targets of an immune response induced by this therapy.

None of the seven isolated clones has previously been reported as a melanoma-associated antigen. Interestingly, three of the seven isolated clones (3.1, 5.17, and 5.23) encode proteins of unknown function. Two of these proteins (3.1 and 5.17) were previously identified as transcripts from human brain with large open reading frames (32, 35). Both are widely expressed, as demonstrated by RT-PCR analysis of normal human tissues (32, 35), and the basis of antigencity for these proteins remains to be determined.

Of the remaining isolated clones, clone 5.16 codes for a nuclear protein (hnRNP-E1) thought to be involved in RNA regulation and transport (38). The presence of antibodies against this widely expressed nuclear protein might suggest a general

autoimmune response. However, day 43 serum from patient 1 was not positive in clinical assays for anti-nuclear antibodies or rheumatoid factor (data not shown). This, along with the high titer, IgG isotype of the response, indicates that the antibody response was specific for this antigen. Although some antigens identified by SEREX are known autoantigens, these do not constitute the majority of identified proteins. Further, it has been suggested that the presence of antibodies to specific nuclear proteins may have prognostic implications in some tumor types (39). Thus this antigen may be relevant in melanoma.

Clone 5.31 encodes a portion of the RING3 protein, the human homologue of the female sterile homeotic (fsh) gene in *Drosophila*, a developmental regulator shown to be associated with the establishment of segments in the early embryo (40). In humans, RING3 is a mitogen-activated nuclear kinase, a member of a newly described family of bromodomain-containing proteins that transactivate the promoter of a number of the E2F family of transcription factors (41). This nuclear kinase activity is increased upon cellular proliferation and is implicated in human development and growth control. Furthermore, RING3 was previously identified as immunogenic in lung cancer (37), and it is highly expressed in melanoma cell lines (data not shown), suggesting that it may represent a shared tumor antigen.

Clone 3.14 encodes a portion of the human homolog of *Drosophila* disc large protein (hDlg). This protein is a member of the membrane-associated guanylate kinase homologues and is normally localized to adherens junction in epithelial cells. These proteins are localized to the membrane-cytoskeleton interface, where they have both structural and signaling roles. Hdlg interacts with the tumor suppressor gene,

adenomatous polyposis coli (APC) (42-44), and overexpression of the hDlg in eukaryotic cell lines suppresses cell proliferation by blocking cell cycle progression from G0/G1 to S phase (44). Somatic mutations in this protein have been identified in breast tumors (45), and we have shown that the protein is frequently down-regulated and mislocalized in malignant melanomas (PH, manuscript submitted).

It is interesting to note that one additional patient (patient 9, UAB 9705) had an induced immune response to several of the clones isolated using patient 1 serum (Table 4). In contrast to patient 1, this individual did not experience an objective clinical response and, in fact, had progressive disease. This observation is consistent with reports of variable clinical outcome for patients with antibodies to various TAAs (25, 26, 46). Several differences between patient 1 (9701) and patient 9 (9705) may also have contributed to the disparity in clinical outcomes. Patient 9 had a larger tumor burden and more progressive disease at the start of treatment (stage IV). While both patients had a humoral response to the isolated antigens after the therapy, the extent of the cell-mediated immune response has not yet been determined. Patients 1 and 9 also had differing HLA types, which may have influenced CTL reactivity and anti-tumor response. Furthermore, the status of the antigen presentation capabilities of the corresponding patient's tumors is not known, a critical component for generating an anti-tumor CTL response (47-55). Finally, the two patients received different recombinant ALVAC viruses: patient 1 received ALVAC-encoding IL-12, and patient 9 received ALVAC-encoding human B7.1. Further analysis of cellular-mediated immune responses to these antigens may help to clarify differing clinical outcomes. Interestingly, patient 2 serum was also reactive to clone 5.17. However, the antibody response was present prior

to the start of immunotherapy, indicating a previously established humoral immune response to this particular clone.

The ease and rapidity of this serological screening method demonstrates its potential value in defining the targets of nonspecific immunotherapies. SEREX is a relatively rapid means to characterize the humoral immune response subsequent to immunotherapy, providing a better understanding of the immune response at the molecular level. Characterization of potentially immunogenic proteins expressed by tumors will likely facilitate the development of new immunotherapy strategies, focused on eliciting effective humoral and cellular immune responses to well-defined targets (2).

ACKNOWLEDGMENTS

This work was supported by NIH R21 CA78498 (TVS). P.H. was supported by a scholarship from the Royal Thai Government.

Fig. 1. Patient melanoma nodule after ALVAC hIL-12 injection. A melanoma nodule injected with 1 x 10^6 $TCID_{50}$ is shown. A large area of inflammation, erythema, and tenderness spontaneous resolved and was accompanied by regression of the tumor nodule and a complete clinical response.

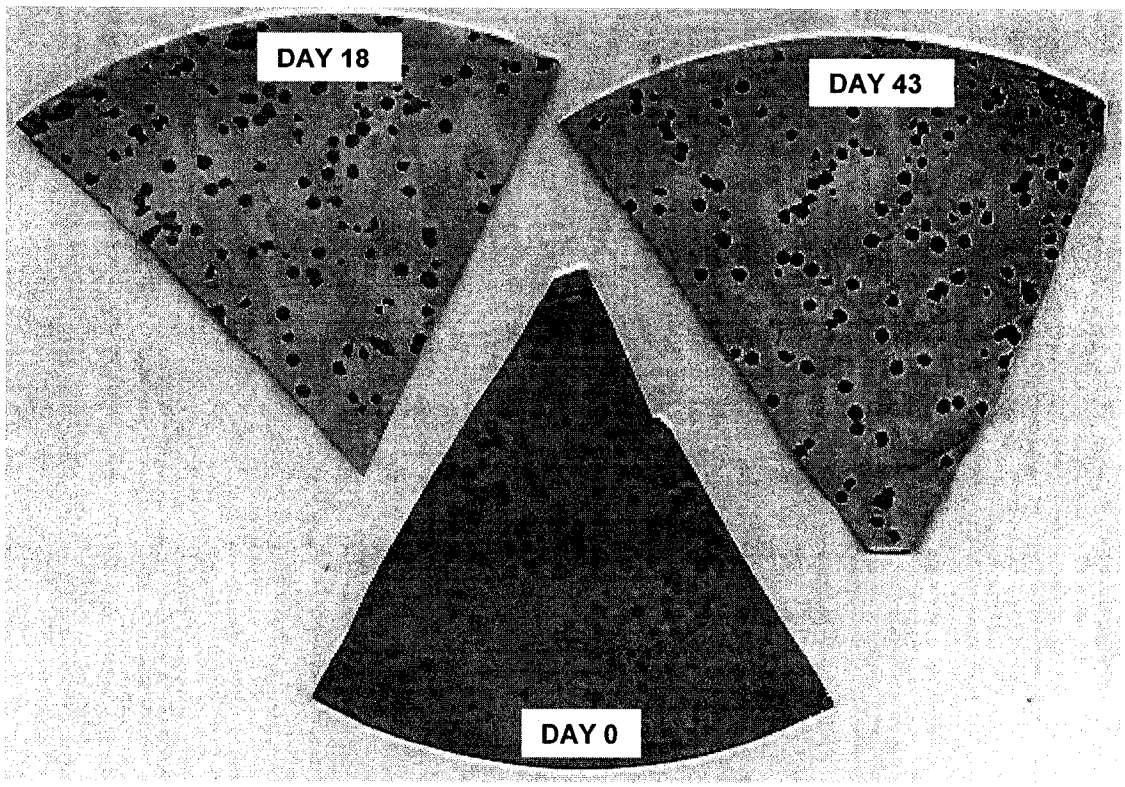

Fig. 2. Antibody responses to isolated clones were induced by ALVAC hIL-12 therapy. Filter lift assays were performed using patient 1 sera collected on days 0 (pretreatment), 18, and 43. Preabsorbed and diluted sera were allowed to react with filters lifted from plates containing a 50/50 mixture of the positive clone and a no-insert control. In all cases, pretreatment sera showed no specific reactivity with the clones, despite extended color development. In contrast, sera collected on days 18 and 43 were strongly reactive with the clones.

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THE HUMAN HOMOLOG OF DROSOPHILA DISC LARGE PROTEIN (HDLG) IS FREQUENTLY DOWN-REGULATED AND MISLOCALIZED IN MALIGNANT MELANOMA

by

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In preparation for *Cancer Research*

Format adapted for dissertation

ABSTRACT

The *Drosophila* Disc large protein (hdlg) participates in maintaining epithelial structure and acts as a negative regulator of cell growth. The human homolog of this protein, hDlg, localizes to sites of intercellular contact and interacts with several structural and growth regulatory proteins. We previously identified hDlg as a melanoma-associated antigen. In this study, hDlg expression was studied in malignant melanoma. Quantitation of hDlg mRNA expression in normal cells, melanoma cell lines, and melanoma tumor samples demonstrated reduced or absent expression in 7 of 9 melanoma cell lines and in 12 of 12 melanomas compared to normal human melanocytes. Moreover, several aberrantly spliced variants of the hDlg gene were detected in melanoma cell lines and tumor samples by RT-PCR and sequencing. Western blot analysis demonstrated that hDlg protein expression was low in melanoma compared to normal cells. Subcellular localization of hDlg was examined in melanocytes, raft cultures, cell lines, and tumors. In normal cells, hDlg was primarily localized to the membrane, particularly at points of intercellular contact. In contrast, little or no membrane-localized hDlg was observed in all melanoma tumor samples evaluated, with the protein exhibiting cytoplasmic localization. Loss of total cellular hDlg and/or aberrant localization in melanoma suggests a potential role for this protein as a tumor suppressor. Consistent with this, ectopic expression of hDlg in melanoma cell lines suppressed colony formation. These results indicate that altered expression and cellular localization of hDlg contributes to the phenotype of malignant melanoma.

INTRODUCTION

In 2003, an estimated 50,000 new cases of malignant melanoma were diagnosed in the United States, resulting in more than 7,000 deaths (1), and both the incidence and mortality of melanoma have markedly increased over the past decades (2). The genetic and biological changes that lead to the development and progression of malignant melanoma are not yet fully characterized (3), but include frequent alterations of the *RAS* and *WNT* signaling pathways (4-8). We have identified the human homolog of the *Drosophila* discs-large (*dlg*) tumor suppressor protein (hDlg) as an antigenic protein in human melanoma by screening of a melanoma cDNA expression library with sera from melanoma patients[1]. In *Drosophila*, the *dlg* tumor suppressor protein is localized at the septate junctions and is required for proper epithelial structure, apicobasal polarity, and growth regulation (9, 10). Loss-of-function *dlg* mutations disrupT-cell polarity, cell adhesion, and cellular growth control, resulting in neoplastic overgrowth of imaginal disc epithelial cells and embryonic lethality (9, 10). Moreover, in combination with an activated *Ras* gene, loss of *dlg* or its associated proteins is sufficient to promote metastatic behavior, including down-regulation of E-cadherin expression, basement membrane degradation, invasion, and secondary tumor formation in *Drosophila* (11).

Regulation of epithelial polarity and growth appears to be a conserved function in the mammalian orthologs of *dlg,* including the human ortholog, hDlg, encoded by *DLG1* (NM_004087). hDlg is a member of membrane-associated guanylate kinase (MAGUK) scaffolding proteins containing three PDZ domains, an SH3 domain, and a C-terminal guanylate kinase homology domain (GUK). PDZ domains can participate in homotypic associations with other PDZ-containing proteins or recognize the carboxy-terminal

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T/SXV consensus sequence of target proteins, recruiting proteins, and organizing supramolecular complexes (12). These complexes typically localize to the membrane-cytoskeleton interface in regions of cell-cell contact, where they serve a role in cell signaling. In human epithelial cells, hDlg localizes to the adherens junctions, associating with the 4.1/ezrin, radixin, moesin (ERM) family of cytoskeletal proteins (13-15). E-cadherin induced cell-cell adhesion mediates the recruitment of the rat ortholog of hDlg, SAP97, to the lateral plasma membrane and promotes its association with the cortical actin cytoskeleton in the formation of adherens complexes (16). In addition to actin and protein 4.1 ERM, hDlg interacts with other MAGUK family members, including hCASK (17, 18), DLG2, and DLG3 (19), and this association is also important in mediating its membrane localization in epithelial cells (18). In intestinal epithelial cells, hDlg links E-cadherin with phosphatidyliositol 3-kinase (PI3K), promoting adherens junctions stability and a differentiated phenotype (20).

Similar to its *Drosophila* homolog, hDlg functions as a negative regulator of epithelial cell growth, and it is reported to interact with a number of proteins that control cell proliferation, including PTEN (21) and the TOPK/PBK mitotic kinase (22). Over-expression of hDlg blocks cell cycle progression in NIH3T3 cells; the PDZ, SH3, and GUK domains are required for this growth inhibitory activity (23). hDlg may exert its cell cycle suppressing effect through direct interaction with the adenomatous polyposis coli (APC) tumor suppressor (24), an important component of the Wnt signaling pathway. It may also interact elsewhere in the Wnt pathway through direct binding to Frizzled receptors (25). A role for hDlg in controlling proliferation is further supported by evidence that it is a common target for viral transforming proteins. Normally,

intracellular levels of hDlg are regulated via the ubiquitin-proteasome pathway, with cell contact-dependent stabilization of hDlg occurring when cultured cells reach high density (26). In cells infected with high-risk human papillomavirus (HPV), the E6 oncoprotein targets hDlg for proteasome-mediated degradation (27). The resultant down-regulation of hDlg levels and loss of cell contact-dependent stabilization are strongly associated with highly malignant phenotypes in HPV-associated cervical neoplasia (28). Other viral transforming proteins that directly interact with and disrupt the function of hDlg include adenovirus E4 ORF1 oncoprotein (29) and human T-cell leukemia virus type 1 Tax oncoprotein (30). Recent studies suggest that loss of hDlg expression may represent an important step in the development of non-viral-induced epithelial cancers as well, including gastric (31) and breast cancer (32). In the present study, we analyzed hDlg expression in human melanoma and determined that it is frequently down-regulated and mislocalized in this tumor type. Loss of full-length hDlg mRNA transcripts was found in conjunction with the appearance of aberrant splice variants. Forced expression of full-length hDlg in melanoma cell lines suppressed cell growth, supporting a potential role for this protein as a tumor suppressor in melanoma.

MATERIALS AND METHODS

Cells and Culture Conditions

Human melanoma cell lines SK-Mel-37, SK-Mel-170, SK-Mel-241, SK-Mel-249, SK-Mel-257, and SK-Mel-258, previously described (33) and kindly provided by Dr. Jane Fountain, National Cancer Institute, Bethesda, MD; Mel624 and Mel888, kindly provided by Dr. Steven Rosenberg, National Cancer Institute; and SK-Mel-28 and A-375,

American Type Culture Collection, Manassas, VA were cultured in Dulbecco's Modified Eagle's Medium with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. Human epidermal melanocytes (HEM), primary cells isolated from normal human neonatal foreskin (Cell Applications, Inc., San Diego, CA) were cultured in melanocyte growth medium (Cell Applications, Inc.). Primary human keratinocytes (PHK) were grown in serum-free keratinocyte medium (Invitrogen-Life Technologies, Inc., Carlsbad, CA). Cultured human skin fibroblasts were maintained in Eagle's Minimum Essential Medium with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum.

Raft Cultures

Organotypic (raft) cultures of PHK were prepared as previously described (34, 35). Briefly, 2 x 10^5 PHKs were seeded onto a dermal equivalent collagen bed containing rat tail type 1 collagen (BD Biosciences, San Jose, CA) and mouse fibroblasts. After overnight incubation as a submerged culture in a 1:1 mixture of raft culture media and serum-free keratinocyte medium (Invitrogen-Life Technologies, Inc.), the rafts were raised to the air-medium interface. For melanoma cell line rafts, cells were cultured as described previously and seeded on the collagen beds at the same density as PHK. After culturing overnight in a 1:1 mixture of raft culture media and RPMI 1640 with 10% FBS, they were raised to air-media interface. All raft cultures were grown at the air-medium interface for 10 days, then fixed in 10% buffered formalin for 1 hr and embedded in paraffin. Paraffin blocks of raft cultures were cut into four micron sections for analysis.

Tumor Samples and Tissue RNA Samples

Tumor specimens were obtained from patients with Stage III or IV melanoma who were enrolled in clinical trials at the University of Alabama at Birmingham. The tumor specimens were obtained after informed consent with the permission and approval of the Institutional Review Board for Human Experimentation of the University of Alabama at Birmingham. Tumors were excised, immediately frozen, and stored at -70°C. Total RNA from normal human testis, skin, liver, and prostate were purchased from Clontech (BD Biosciences, Palo Alto, CA).

Quantitative Real-Time PCR (QRT-PCR)

Quantitative real-time reverse-transcriptase polymerase chain reaction (QRT-PCR) was performed using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Total RNA was isolated from frozen tissues and cell lines using RNA STAT60 (Tel-Test, Friendswood, TX) and purified from residual protein using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration was calculated spectrophotometrically and the quality of the RNA assessed by denaturing agarose gel electrophoresis. For QRT-PCR analysis, 25 ng of total RNA was used in a 50-μl reaction mixture. The primers and probes for 18S ribosomal RNA and *DLG1* were purchased from Applied Biosystems and used as directed by the manufacturer (Applied Biosciences Assay ID Hs00177739_m1, probe corresponding to exon 19 of NM_004087). One-step RT-PCR was performed (Applied Biosystems) (48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min). Amplification of 18S ribosomal RNA served as the endogenous control for

normalization of samples. Expression of the hDlg mRNA normalized with 18S RNA expression within the corresponding sample was expressed as ΔCt. The difference between ΔCt of each sample and that of primary melanocytes as the calibrator was calculated (ΔΔCt = ΔCt of sample - ΔCt of melanocytes). The relative number (Rn) of hDlg mRNA copies compared to that of melanocytes was calculated by $2^{-\Delta\Delta Ct}$ and shown as relative units. The samples were run in duplicate, and each sample was run at least three times.

Reverse-Transcriptase PCR and Sequence Analysis

Total cellular RNA was purified from cell lines and melanoma tumor samples as described above. One μg of total RNA was reverse transcribed for 45 min at 45°C, followed by 95°C for 15 min, and the resulting cDNA in the reaction was sequentially amplified using one-step RT-PCR kit (Qiagen, Inc.) with the *DLG1*-specific primers (5'-TTGGAAACGGCACTGCTG-3' and 5'-CCAGAGGAAAGGGCAAAG-3'). Amplification conditions were as follows: 35 cycles of 94°C for 1 min, 60°C for 1 min, 68°C for 3 min, and the final extension reaction at 68°C for 10 min. 2.5-5.0 μl of the first round amplification products were used as template for the second round using nested primers specific for *DLG1* (5'-CACCATGCCGGTCCGGAAGCAA-3' and 5'-TAGCT TTTCTTTTGC CGGAACCCA-3') and Platinum *Taq* DNA Polymerase (Invitrogen-Life Technologies, Inc.). Amplification with primers specific for β-actin was used to confirm the integrity of the RNA (not shown). Amplified PCR products were visualized by agarose gel electrophoresis and isolated by gel extraction for DNA sequencing analysis.

To examine previously described alternatively spliced isoforms, I1 variants (36) were amplified using the primers 5'-ATCGTTCAAAGCCGTCTG-3' and 5'-GACCC-GCAATCTTCCATC-3', and I2 and I3 variants (36) were amplified with 5'-TCCCTCTATGTCAGAGCC-3' and 5'-TTTGCCCTTTCCTGCTAC-3'. Using a one-step RT-PCR kit (Qiagen, Inc.), 1 μg of total RNA was reverse transcribed for 30 min at 50°C, followed by 95°C for 15 min, and cDNA was sequentially subjected to PCR for 30 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 1 min, and the final extension reaction at 70°C for 10 min.

Western Blot Analysis

Normal primary cells and melanoma cells were washed in PBS and treated with a lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 8.0), including protease cocktail (Roche Diagnostics Corp., Indianapolis, IN). Surgical specimens frozen in liquid nitrogen were pulverized by mortar and pestle and added to the lysis buffer described above. The lysates were incubated on ice for 20 min and then centrifuged. The protein content of the supernatant was measured using a bicinchoninic acid protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of lysates (20-50 μg) were electrophoresed on 7.5% polyacrylamide gel (Biorad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience, Inc., Keene, NH). After blocking with TBS containing 0.05% Tween 20 (TBS-T) and 5% milk for 1 hr at room temperature, the filters were incubated with anti-hDlg antibody [mAb clone 2D11 (anti-SAP97), 2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA] and anti-β actin (mAb clone Ali12-28, 50 ng/ml, Abcam, Inc, Cambridge, MA) at 4°C

overnight, washed in TBS-T, and then incubated with horseradish peroxidase-labeled antimouse IgG (1:5000 dilution, Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were detected using the Amersham ECL System according to the manufacturer's instructions.

Immunofluorescence Analysis

To study hDlg localization in cultured primary cells and melanoma cell lines, cells were seeded onto collagen I-coated one-well culture slides (BD Biosciences Discovery Labware, Bedford, MA) for 5-10 days before fixation. To examine hDlg localization in mixed cultures of melanocytes and keratinocytes, melanocytes were seeded on subconfluent cultures of differentiated keratinocytes grown on collagen I-coated culture slides and cultured for 5-7 days before fixation. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT, rinsed three times with PBS, permeabilized for 10 min with cold (-20°C) acetone, and rinsed three times with PBS. Samples were blocked for 1 hr with 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS and then incubated with anti-hDlg antibody (mAb clone 2D11, 10-16 μg/ml, Santa Cruz Biotechnology) overnight at 4°C. Slides were washed three times with PBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc., Eugene, OR, 1:1000 dilution) for 1 hr at RT. Slides were subsequently rinsed with PBS, and mounted with permanent aqueous mounting medium (Biomeda, Foster City, CA). Control sections were incubated in the absence of anti-hDlg antibody and did not show fluorescent staining (data not shown). Images were captured on a Zeiss fluorescent microscope (Axioplan 2 Imaging System).

For paraffin-embedded raft cultures, sections were deparaffinized and rehydrated. Epitope retrieval was achieved by heating the sample at 95°C in preheated citrate buffer (10 mM citric acid, pH 6.0) for 10-20 min, followed by slow cooling to RT for 20 min. Immunofluorescence staining was as described previously. Paraffin-embedded human foreskin samples were sectioned and deparaffinized, rehydrated, and boiled in 1 mM EDTA, pH 8.0, for 15 min, followed by cooling at RT for 20 min. Immunofluorescence double staining was performed using the anti-tyrosinase mAb (IgG_{2b}) at 2 μg/ml (NeoMarkers, Inc., Fremont, CA) and the anti-hDlg mAb (IgG_1) at 10 μg/ml (2D11, Santa Cruz Biotechnology, Inc.) for primary antibodies. The double staining was detected using Alexa Fluor 488-conjugated goat anti-mouse IgG_1 (to detect anti-hDlg mAb) and Alexa Fluor 594-conjugated goat anti-mouse IgG_{2b} (to detect anti-tyrosinase mAb) secondary antibodies at a 1:500 dilution (Molecular Probes, Inc.) for 1 hr at RT. For frozen surgical specimens that had been embedded with OCT compound (Fisher Scientific, Pittsburgh, PA), sections were fixed 4% paraformaldehyde and stained as above, but without antigen retrieval.

Transfection and Colony Formation Assay

The hDlg coding sequence was amplified by reverse transcription-PCR using total RNA of the adult human skin (Stratagene, La Jolla, CA) with hDlg-specific primers (5'-CACCATGCCGGTCCGGAAGCAA-3", 5'-TAGCTTTTCTTTTGCCGGAACCCA-3'), subcloned into pcDNA3.1/V5-His-TOPO TA vector (Invitrogen Life-Technologies, Inc.), and verified by sequence analysis and by transient transfection and detection with the anti-V5 antibody (Invitrogen Life-Technologies, Inc.). For a colony-formation assay,

SK-Mel-258, Mel624, and A375 cells (1 x 10^6 cells / 6-well plate) were transfected with 25 μg of either empty plasmid control or hDlg expression plasmid using the Lipofectamine reagent (Invitrogen Life-Technologies, Inc.) following the manufacturer's instructions. Twenty-four hours after transfection, cells were plated at varying densities (20,000-40,000 cells) into 6-well plates and grown for 14 days in media supplemented with 1.2 mg/ml of G418 (Geneticin, Cellgro by Mediatech, Herndon, VA). To count G418 resistant colonies, the cells were washed with PBS, and fixed and stained with a crystal violet solution (10% acetic acid, 50% methanol, and 0.15% crystal violet). The number of colonies that survived G418 selection on the vector control dishes was defined as 100% in this assay. The assay was performed two times in triplicate.

RESULTS

Expression of hDlg Transcripts is Reduced in Most Human Melanoma Cell Lines and Tumors Compared to Normal Melanocytes

The expression of hDlg mRNA transcripts in primary cultured cells and tissues, melanoma cell lines, and primary tumors was evaluated by QRT-PCR. The 18S ribosomal RNA was used as an internal control because of its consistent expression between normal cells and tumors (37). Primary cultured melanocytes were used as a reference. QRT-PCR revealed that melanocytes expressed the highest levels hDlg mRNA compared to other normal tissues, with intermediate levels of hDlg transcripts in PHK, fibroblasts, and skin, and comparatively low levels in testis, liver, peripheral blood mononuclear cells (PBMC), and prostate (Fig. 1). A majority of melanoma cell lines (7 of 9) showed hDlg reduced transcript levels (25-60% of the transcript level of primary melanocytes, Fig. 1). Moreover, tumors from 12 patients with malignant melanoma

expressed hDlg transcripts levels that were ≤ 50% of levels in normal melanocytes (Fig. 1). Thus, low expression of hDlg transcripts is frequent in both melanoma cell lines and tumors.

Identification of Aberrant Splice Variants in Melanoma Cell Lines and Tumors

Expression of hDlg transcripts was next investigated by conventional reverse-transcriptase (RT) PCR. To amplify the entire coding region, primers were synthesized corresponding to exon 2, 5' to the translational start site, and exon 26, following the stop codon. A second round of PCR with nested primers was necessary to obtain a visible amplification product from melanoma cell lines and tumors. PCR amplification products of the expected size (~2.8 kb) were detected in all samples, although at low levels in some melanoma cell lines and tumor samples (Fig. 2*A*). In addition, several smaller than expected amplification products were detected in melanoma cell lines and tumors, but not in normal melanocytes, skin (Fig. 2*A*) or PHK (not shown). The smaller amplification products were isolated by gel extraction and sequenced directly. Sequence analysis showed that these bands represent alternatively spliced hDlg mRNA transcripts. A summary of the spliced variants observed in melanoma cell lines and tumors tested is presented diagrammatically in Fig. 2*B*. Most of these spliced transcripts diverged from the full-length sequence at exon 5, using either the expected splice donor site or an internal splice donor site, and joined aberrantly to various downstream exons. All of these alternatively spliced transcripts result in the skipping of multiple downstream exons, a shift in reading frame, and a premature stop codon. Translation of the spliced

transcripts is predicted to produce truncated proteins with most of the PDZ domains, SH3 and GUK domains deleted, and with an altered carboxy-terminal sequence.

Expression of hDlg Protein is Low in Melanoma Cell Lines and Tumors

To determine if the reduction in mRNA levels correlated with a reduction in hDlg protein levels in melanoma cell lines and tumors, protein expression was analyzed by Western blot using the commercially available hDlg monoclonal antibody 2D11, which reacts with the N-terminal 229 amino acid peptide (exons 2-8). Immunoblots of melanocytes probed with 2D11 showed a doublet at a molecular mass of approximately 120 kDa (Fig. 3*A*), as previously described by others (26, 28, 38). Consistent with the QRT-PCR data, the majority of melanoma cell lines expressed low levels of hDlg compared to normal cells, including melanocytes, except for two cell lines, SKM28 and SKM241, which expressed hDlg at levels comparable to those in melanocytes (Fig. 3*A*). Western blot analyses of melanoma tumor samples were also consistent with QRT-PCR studies, with reduced expression of hDlg in all primary tumors compared to normal melanocytes, PHK, and fibroblasts (Fig. 3*B*). A band smaller than the reported size of 120 kDa for hDlg was detected only in the cell line SKM249, with no smaller than expected products detected in the tumor samples. This suggests that most protein products of alternatively spliced transcripts are either unstable or produced at levels below those detectable by Western blot analysis; alternatively, the monoclonal antibody may not be able to recognize some truncated hDlg products. The results demonstrate that expression of full-length hDlg protein is down-regulated in melanoma.

hDlg Localization in Normal Keratinocytes, Melanocytes, and Foreskin

Localization of hDlg appears to be critical to its function, with the loss of hDlg at sites of intercellular contact associated with highly malignant phenotypes in cervical neoplasia (28). Therefore, hDlg localization in normal cells and melanomas was investigated by immunofluorescence staining. In PHK, hDlg was detected at sites of cell-cell contact (Fig. 4*A*), similar to what has been previously reported in epithelial cells (13, 16, 28). In primary cultured human melanocytes, prominent staining of hDlg was restricted to the sites of intercellular contacts but not at the edges of cells lacking cell-cell contact (Fig. 4*B*). Moreover, in cultures of normal melanocytes mixed with keratinocytes, hDlg was concentrated at the sites of contact between these two cell types (data not shown), consistent with localization of hDlg to adherens junctions formed between melanocytes and keratinocytes. Localization of hDlg was also examined in organotypic raft cultures of normal keratinocytes, which mimic *in vivo*-like skin structure and maintain physiological cell-cell interaction (39). Again, hDlg was detected uniformly around the cell periphery at sites of cell-cell contact in the rafts (Fig. 4*C*). This junctional localization of hDlg was also found in raft cultures of normal melanocytes seeded with keratinocytes (data not shown) and in human foreskin tissue sections, where melanocytes were distributed among keratinocytes in the basal layer of the epidermis (Fig. 4*D*).

Localization of hDlg in Melanoma Cell Lines, Raft Cultures, and Melanoma Tumor Samples

In most human melanoma cell lines, hDlg immunofluorescence signal was low, as expected based on Western blot analysis. In the cell lines A375 and SKM28, some localization of hDlg at points of cell-cell contact was detected (not shown); however,

these cell lines also showed perinuclear staining of hDlg, which was not detected in normal cells. In other cell lines, including SKM170 with low levels of hDlg transcript and protein expression (not shown) and SKM241 with comparatively high levels of expression (Fig. 5*A*), hDlg was detectable, but showed an altered subcellular localization. The protein was present in the cytoplasm but was not observed at the membrane. Melanoma cell lines were grown in raft cultures to study hDlg localization in a 3-dimensional structure. SKM28 raft cultures showed some membrane localization but predominantly cytoplasmic hDlg staining. In SKM624 raft cultures, the membrane staining of hDlg was completely absent, with the protein again exhibiting cytoplasmic localization (Fig. 5*B*). The studies were extended to melanoma tumor samples. Localization of hDlg at sites intercellular contact was greatly diminished in tumor cells, whereas hDlg was strongly localized at the cell-cell junctions in adjacent normal epidermis (Fig. 5*C*). Six tumor samples were examined by immunofluorescence, and all showed predominantly cytoplasmic hDlg staining (Fig. 5*D* and data not shown).

Expression Analysis of Previously Reported Isoforms of hDlg (I1, I2, I3)

Alternatively spliced exons of hDlg, different from those shown in Fig. 2, have been reported in normal tissues (13, 36), and the isoforms encoded by the spliced variants were proposed to be important in determining the localization of the protein (36, 40). The previously reported insertions include I1A, I1B, I2, and I3. Located in the N-terminal region, I1A and I1B are 99- and 54-bp insertions, respectively, which correspond to exons 6 and 7 of NM_004087. I2 (exon 19 of NM_004087) and I3, alternatively referred to as isoform 1 (accession # U13897) and isoform 2 (accession # U13896) consist of 36-

bp and 102-bp insertions (36), respectively, located in the region between the SH3 and GUK domains. The presence of I2 versus I3 appears to be mutually exclusive.

To determine whether differences in expression of these previously described hDlg isoforms might explain differential localization of the protein in normal compared to tumor cells, we investigated expression of I1-, I2- and I3-containing transcripts in melanomas compared to normal melanocytes and skin. The I1 sequence was amplified from total RNA extracts of melanoma cell lines and melanoma tumor samples by RT-PCR, using I1-specific primers as shown in Fig. 6*A*. Three amplification products were detected in all normal and tumors samples tested (Fig. 6*B*, upper panel). Sequence analysis of these amplification products demonstrated the three different isoforms of hDlg: isoforms containing I1A and I1B together, I1B without I1A (I1B only), and neither I1A nor I1B. Approximately equal amounts of these three isoforms were found in normal skin, cultured melanocytes, 9 of 9 melanoma cell lines, and 11 of 11 primary melanomas (not all shown).

The proportion of I2 versus I3 sequences in melanomas was similarly compared to normal melanocytes and skin, using primers specific for the region indicated in Fig. 6A. Two amplification products were detected, and sequence analysis showed that the upper and lower bands represent insertions of I3 and I2, respectively (Fig. 6*B*, lower panel). Both transcripts were present in normal skin, cultured melanocytes, and all melanoma cell lines (n = 9) and tumor samples (n = 11) evaluated (not all shown). I3 variants were present at slightly higher levels than I2 variants in both normal tissues and melanoma. We thus found no significant differences in the expression of transcripts containing I1, I2 or I3 between normal cells and melanoma, suggesting that the altered

cellular localization of hDlg in melanoma compared to normal cells is not likely to due to differences in expression of these isoforms.

Growth Inhibitory Effects of hDlg on Melanoma Cells

Because of the evidence supporting a role for hDlg as a tumor suppressor, together with frequent loss of total cellular hDlg at both the mRNA and protein levels in melanomas, we next evaluated the consequences of forced expression of hDlg in melanoma cell lines by colony-forming assay. The hDlg full-length cDNA (containing I1B and I3) in the mammalian expression vector pcDNA3.1/V5 was transfected into SKM 258, SKM624, and A375, and hDlg expression was confirmed by Western blot analysis using the epitope tag (not shown). Cells transfected with either the hDlg cDNA or a control vector were selected by culturing cells in the presence of G418. After 2 weeks, the G418-resistant colonies were stained and counted (Fig. 7*A*). Cells transfected with the hDlg expression plasmid formed fewer than half the G418-resistant colonies compared to those transfected with the vector alone (Fig 7*B*), suggesting that ectopic expression of hDlg can suppress the growth of melanoma cells.

DISCUSSION

During tumor progression, crucial steps in the acquisition of the malignant and invasive phenotype include deregulation of cell proliferation and loss of polarity and cell-cell adhesion. Adherens junctions are cadherin-based sites of intercellular interaction that serve as organizational regions and are important in regulating the available levels of key signal transducing factors, including β-catenin (41, 42). E-cadherin is the major adhesion

molecule mediating the interaction between keratinocytes and epidermal melanocytes, and loss or mislocalization of E-cadherin and its associated catenins is associated with a malignant phenotype in melanoma (8, 43). Disruption of the cadherin-catenin adhesion system may contribute to the ability of tumor cells to proliferate and migrate, promoting metastases (44, 45). A role for hDlg in this process is suggested by its localization to adherens junctions in normal cells and its known interaction with key signaling proteins, including the APC-β-catenin complex and PI3K (20, 24). The current study demonstrates frequent changes in the expression and cellular localization of hDlg in malignant melanoma. Overall, full-length mRNA and protein levels are reduced, and the remaining hDlg is localized primarily to the cytoplasm. Whether changes in hDlg expression precede or promote metastatic progression remains to be determined, but loss of cell polarity markers is a hallmark in the transition to the mesenchymal phenotype that characterizes malignan T-cells (46).

hDlg protein levels are dynamically regulated, with the protein stabilization and localization to the membrane junction occurring upon cell-cell interaction. In normal cells, hDlg protein concentrations are regulated through controlled degradation by the ubiquitin-proteasome pathway. The protein is marked for degradation by hyperphosphorylation, which promotes its association with the β-TrCP ubiquitin ligase receptor of the $SCF^{\beta\text{-}TrCp}$ ubiquitin ligase complex (38). Thus, similar to β-catenin, hDlg ubiquitination provides a phosphorylation-dependent mechanism to modulate protein levels rapidly. In cells infected with high-risk HPV, the E6 oncogenic protein induces the loss of hDlg by targeting it for degradation by the same proteasomal pathway (27, 47), usurping an existing regulatory pathway. An alternative mechanism of hDlg loss has

been suggested in human breast cancer, where somatic mutation of hDlg was documented in some tumor samples (32). As is the case for other tumor suppressor proteins, a number of mechanisms may operate to down-regulate protein expression.

In the melanoma samples studied here, low hDlg levels were associated with decreased levels of full-length hDlg transcripts. The overall levels of hDlg mRNA were low, and aberrantly spliced messages were detected specifically in melanoma cell lines and tumor samples. These aberrant transcripts included deletions of the regions encoding the major protein-interacting domains of hDlg and would not be expected to produce functional proteins. Aberrant splicing and exon skipping have been proposed as means of inactivating other genes implicated in tumorigenesis, including p53 (48, 49) and interferon regulatory factor-1 in chronic myeloid leukemia (50). As is the case with hDlg, high levels of aberrant splice products were detected, with a parallel reduction in the normal full-length message. A broader, genome-wide analysis of mRNA transcript splicing in normal versus tumor cells indicates that cancer-specific splice variants are common, and likely represent important causes of disrupted tumor suppressor function (51). The mechanism(s) responsible for transcriptional down-regulation and aberrant splicing of hDlg transcripts have yet to be determined, but a number of plausible scenarios exist. The gene encoding hDlg, *DLG1*, is located on chromosome 3q29. Chromosome 3 loss occurs in approximately 25% of malignant melanomas of the skin (52), and this may contribute to the low level of hDlg transcripts in some cell lines and melanoma samples. Both *cis* and *trans*-acting mechanisms can induce exon skipping, and a variety of mechanisms have been implicated in human disease. Gross rearrangements of DNA, including intragenic deletion or genomic rearrangement (53), might result in exon

skipping. Point mutations in exonic sequences or intronic sequences, including changes in intronic polypyrimidine tracts, splice donor, and splice acceptor sites may promote exon skipping (54). Up-regulation of transacting factors that bind to specific exons is another means by which exon skipping can be induced (55). It is intriguing that the majority of aberrant splices sequenced in the current study diverged from the full-length sequence at exon 5 (Fig. 2*C*), suggesting the importance of sequences in this region in determining splice patterns. This exon is also the site of alternative exon usage in normal cells (13) and Fig. 6, suggesting that the genomic sequences in this region may be prone to exon skipping (56). Additional studies will be needed to delineate the means by which exon skipping of the hDlg transcript is induced in malignant melanoma.

In summary, although the precise role of hDlg in normal melanocytes and malignant melanoma is not yet fully understood, loss of expression and inappropriate localization of the protein is a frequent occurrence in malignant melanomas. As an integral part of the adherens complexes with a key role in maintaining cellular polarity and regulating proliferation, loss of hDlg is likely an important step in development of the malignant phenotype. A more complete understanding of the causes and consequences of hDlg loss will be critical to fully elucidating the cellular processes involved in the transition from normal melanocyte to malignant melanoma.

ACKNOWLEDGEMENTS

We thank Hsu-Kun Wang and Zdenek Novak for their assistance in culturing primary cells. We are grateful to Dr. Denise Shaw for her helpful discussions and comments on the manuscript.

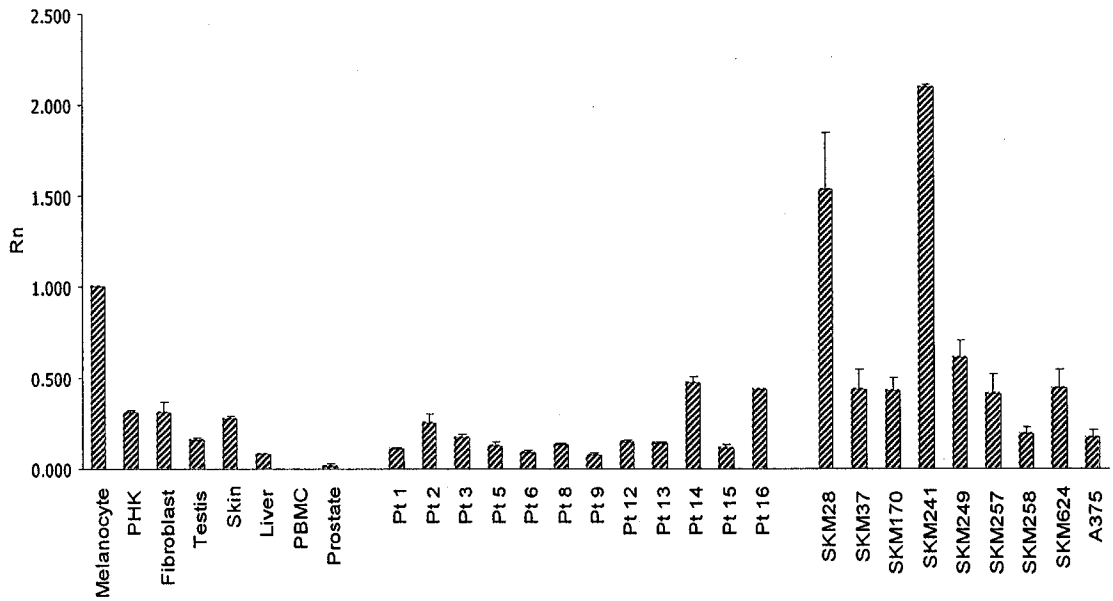
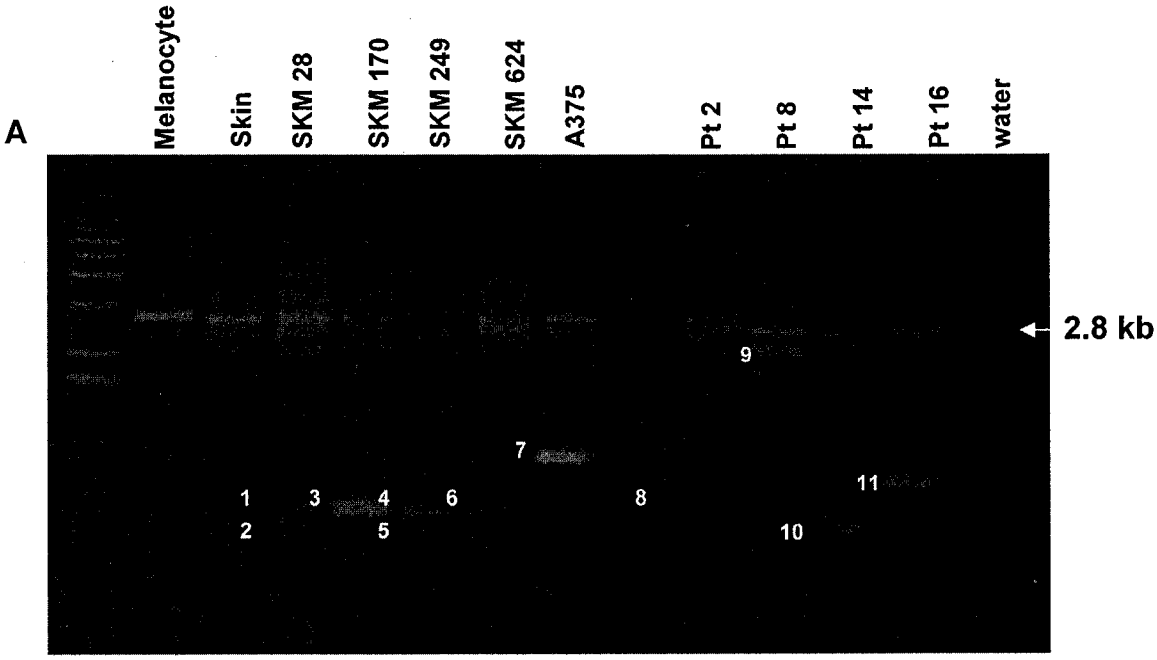


Fig. 1. Real-time quantitative PCR analysis of hDlg transcripts in primary normal cells and tissues, melanoma cell lines, and melanoma tumor samples. Expression relative to normal human melanocytes is shown and was determined as described in "Materials and Methods." hDlg transcripts in RNA from normal cells (melanocytes, PHK, fibroblasts, and PBMC), and tissues (testis, skin, liver, and prostate) is compared to transcript levels in tumors from 12 patients with metastatic melanoma and melanoma cell lines. Each sample was run in duplicate, at least three times, and standard deviation bars are shown.

Fig. 2. hDlg transcripts are aberrantly spliced in melanoma cell lines and tumor samples. *A*, Total RNA from cultured normal melanocytes, skin, melanoma cell lines, and melanoma tumor samples was reverse transcribed, amplified with hDlg-specific primers, and resolved on a 1% agarose gel. The expected size of the amplification product is 2.8 kb, indicated by the arrow. Additional, smaller than expected amplification products were detected in melanoma cell lines and tumors, and are labeled 1-11. M = DNA size standard (1-kb ladder, Invitrogen Life-Technologies, Inc.) *B*, Characterization of exon skipping in hDlg transcripts. Diagrammatic representation of full-length hDlg transcript (FL) and encoded protein domains, with the arrow indicating the translational start site. The spliced transcripts identified in melanoma cell lines and patient tumor samples are indicated in the diagrams below, with the band number corresponding to the bands indicated in 2*A*. The white boxes indicate the presence of the expected open reading frame, whereas shaded boxes indicate a shift in reading frame in downstream exons. Bold-edged exons indicate the use of an internal splice donor site or acceptor site. Exon sizes are not drawn to scale.



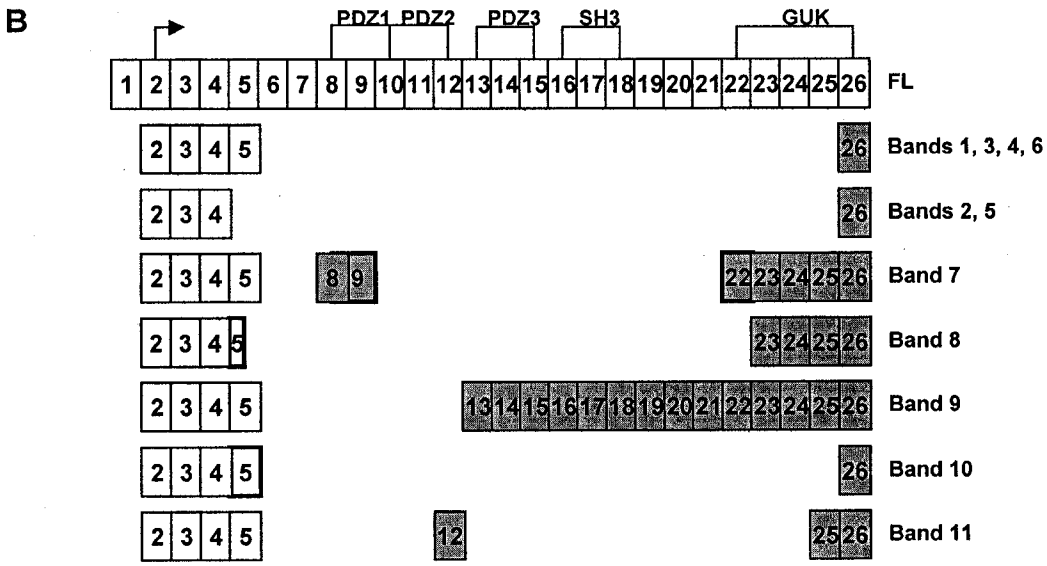
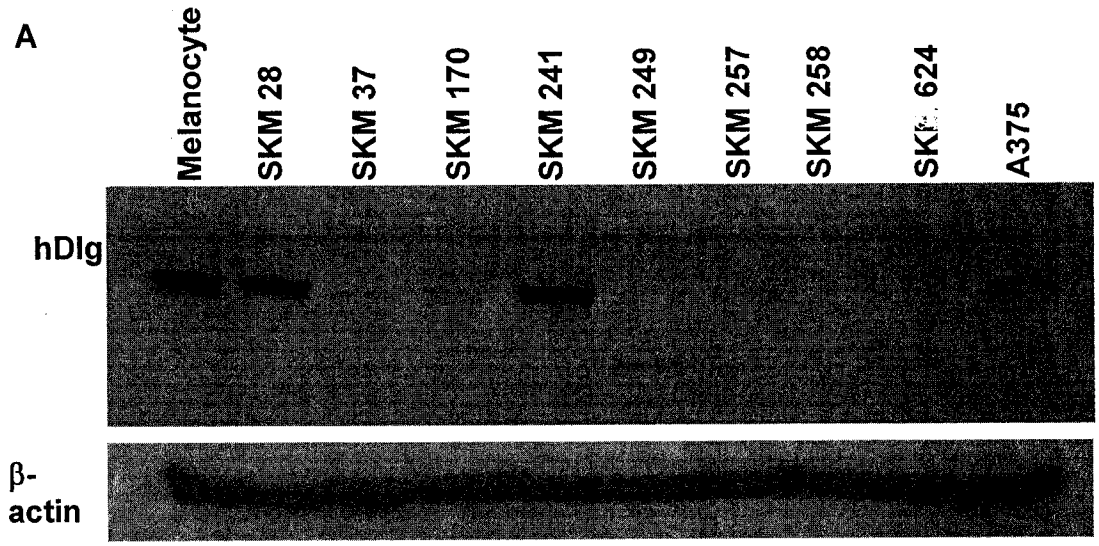
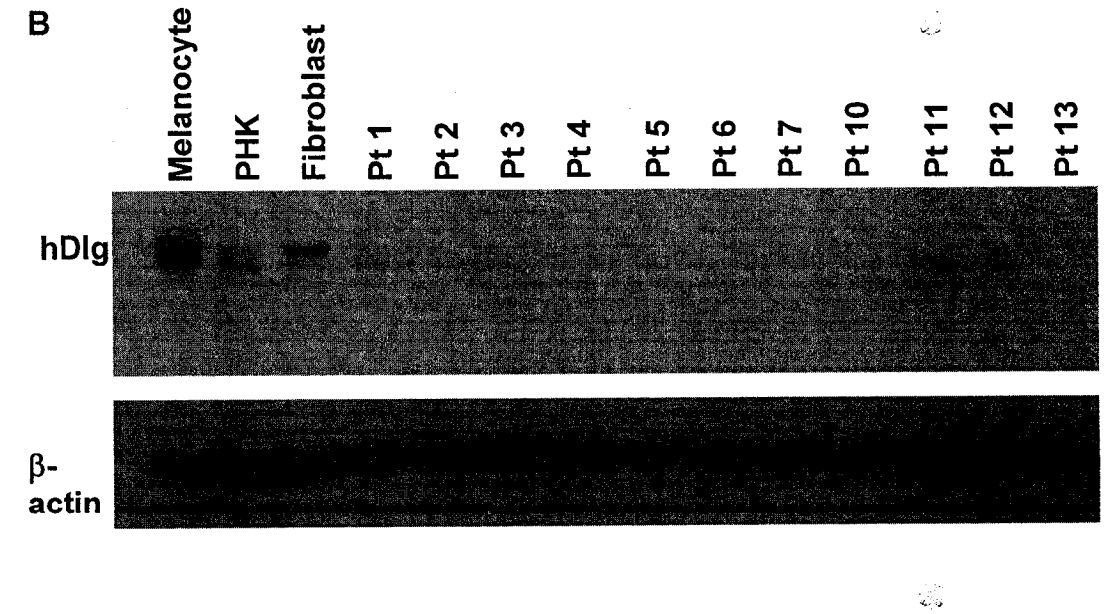


Fig. 3. hDlg protein expression is decreased in melanoma compared to normal melanocytes, primary human keratinocytes, and fibroblasts. Cell lysates were separated on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody specific for hDlg. Melanoma cell lines (*A*), and melanoma tumor samples (*B*), were compared to normal cells. A monoclonal antibody to b-actin was simultaneously applied to demonstrate approximately equal loading of the cell lysates.





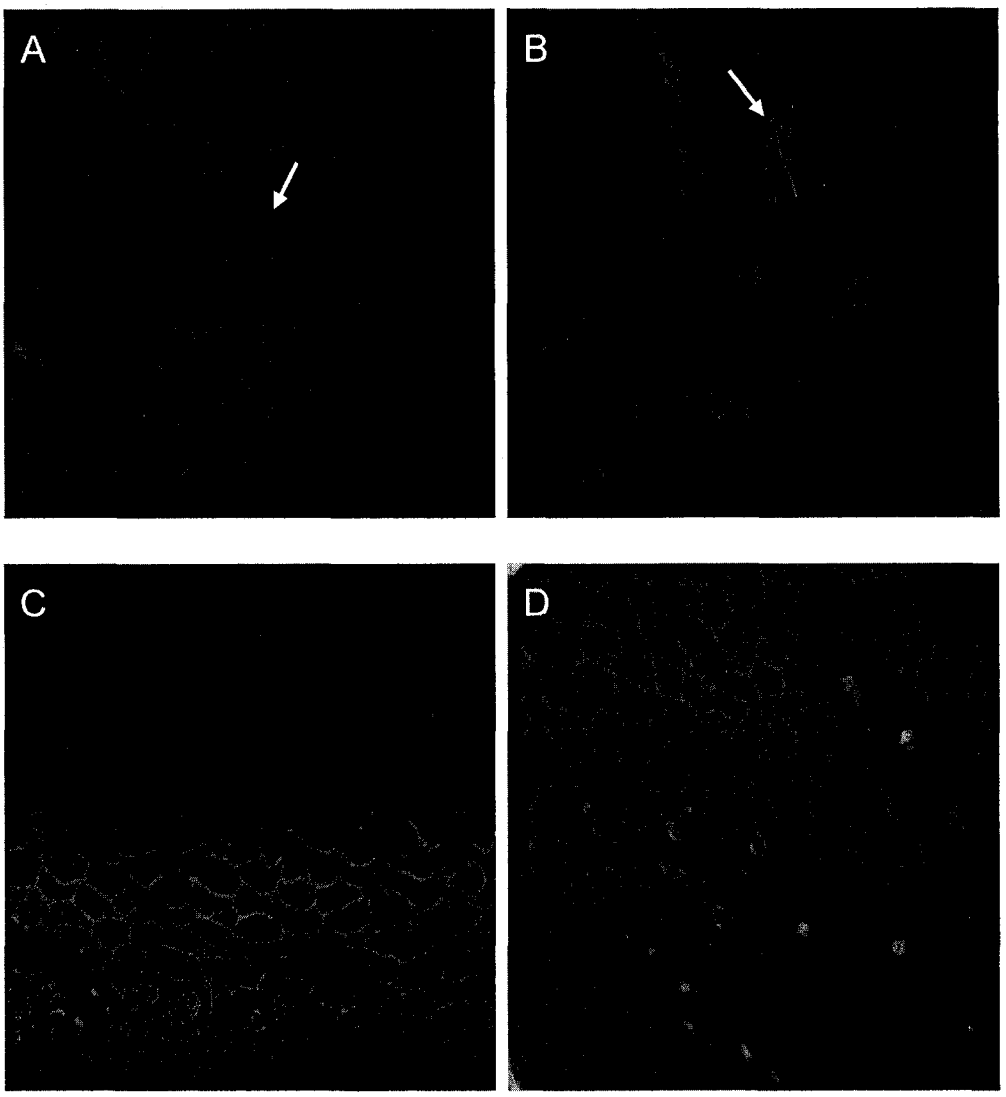


Fig. 4. hDlg is localized to the cell membrane in normal cultured cells, raft cultures, and human foreskin. Immunofluorescent detection of hDlg in cultured cells (*A*), PHK and (*B*), melanocytes, with areas of intercellular contact and high hDlg expression indicated by the arrows. (*C*) Localization of hDlg in a raft culture of PHK and (*D*), in human foreskin. In all samples, hDlg is *green*. In the foreskin sample (*D*), melanocytes were detected by staining for tyrosinase and detected by Alexa Fluor 594 (*red*).

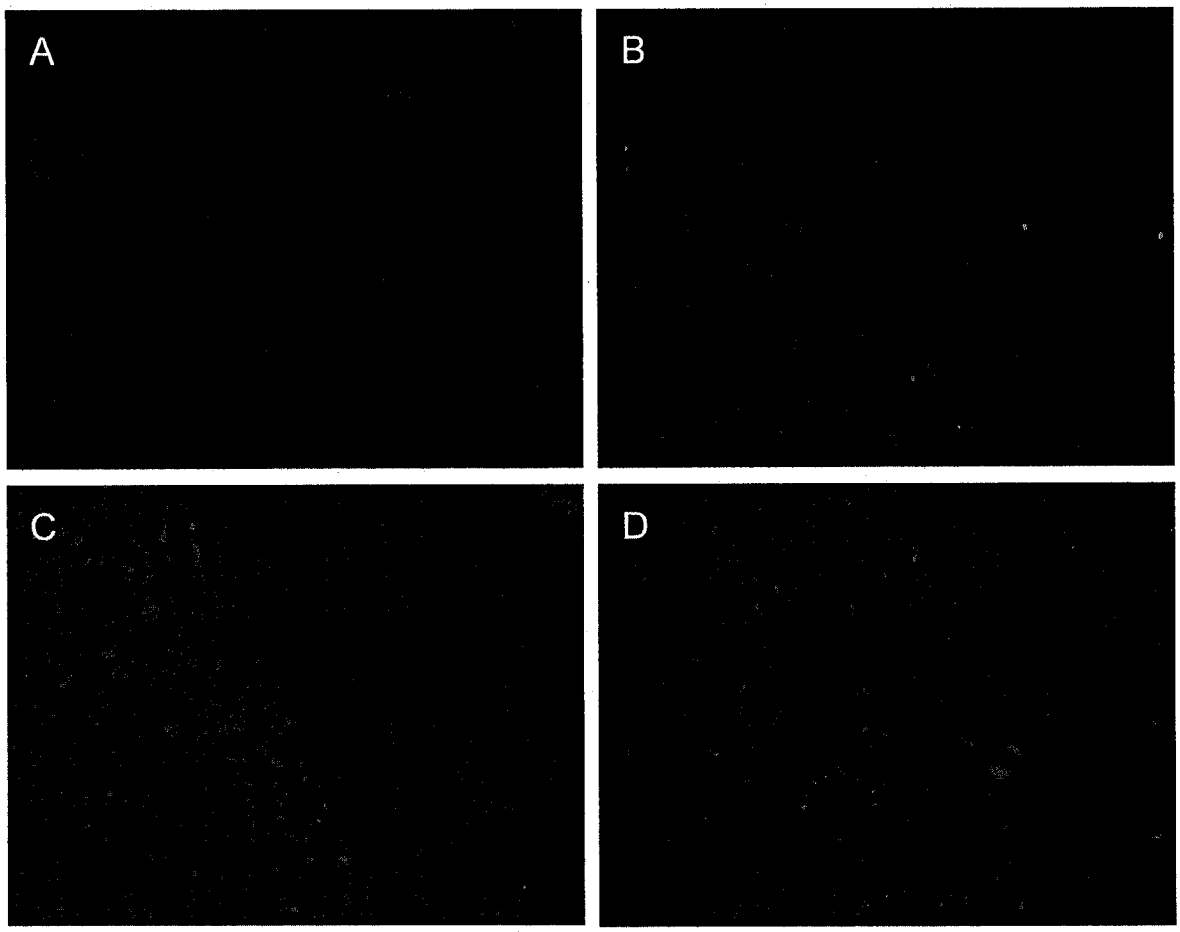


Fig. 5. hDlg protein is decreased and mislocalized in melanoma cell lines grown in culture and as rafts, and in melanoma tumor samples. Immunoflurorescent detection of hDlg in the melanoma cell line SKM 241 (*A*), and in SKM 624 grown on a raft culture (*B*). In patient 5 tumor (*C*), an area of normal epidermis (right) showing membrane localization of hDlg is present overlying the tumor. (*D*), Patient 12 tumor.

Fig. 6. Expression of alternative splice variants (I1A, I1B, I2, and I3) is not altered in melanoma compared to normal melanocytes and skin. *A*, Diagram of hDlg showing location of alternatively spliced insertions (I1A, I1B, I2, and I3) as well as primers (black arrows) used for PCR amplification. *B*, Amplification products from normal cells, melanoma cell lines (SKM28, SKM170, SKM241, SKM258, SKM624, A375) and melanoma tumor samples (Pt 1, 2, 5, 12, 16). The upper panel shows products generated following amplification of the region including I1A and I1B, while the lower panel shows the amplification products following amplification of the region including I2 and I3. All products were confirmed by DNA sequence analysis.

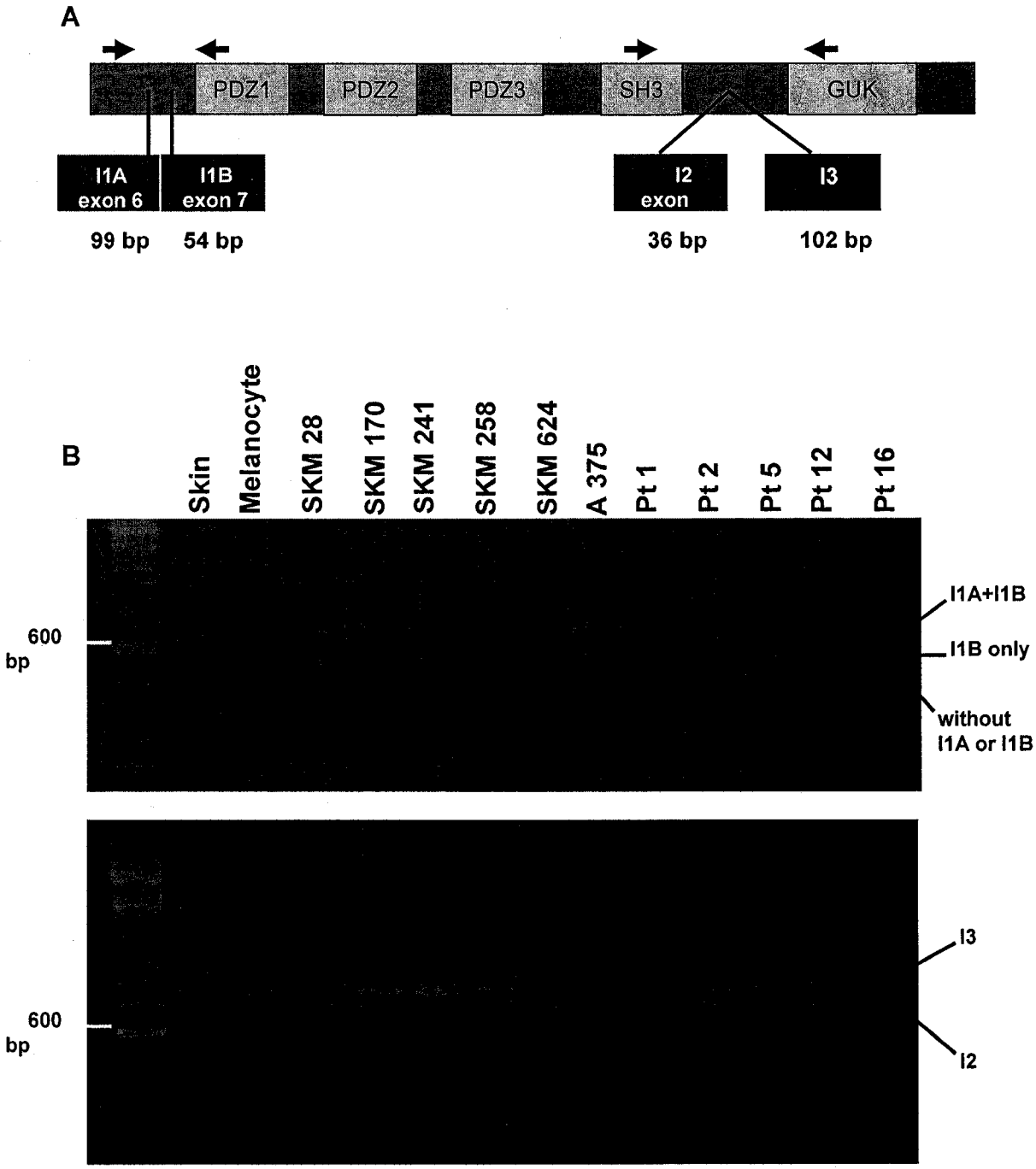
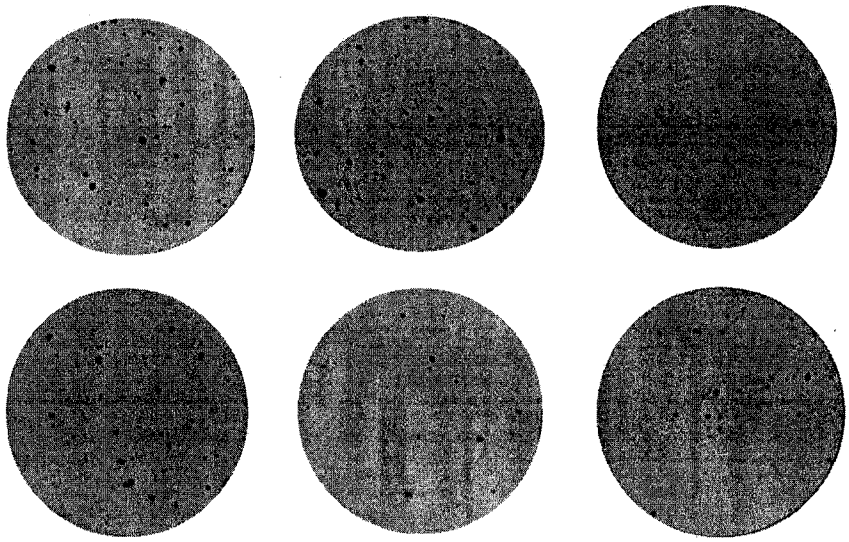


Fig. 7. Suppression of colony formation in melanoma cell lines by forced expression of hDlg. *A*, Melanoma cell lines SKM258, SKM624, and A375 were transfected with a plasmid DNA encoding hDlg or an empty vector and selected for the presence of the plasmid by culturing in G418. Fourteen days later, cell colonies were stained with crystal violet and counted. *B*, the number of hDlg transfectant colonies is shown relative to the number of vector control transfectants. The experiment was repeated twice, with each sample in duplicate, and standard deviation bars are shown.

A



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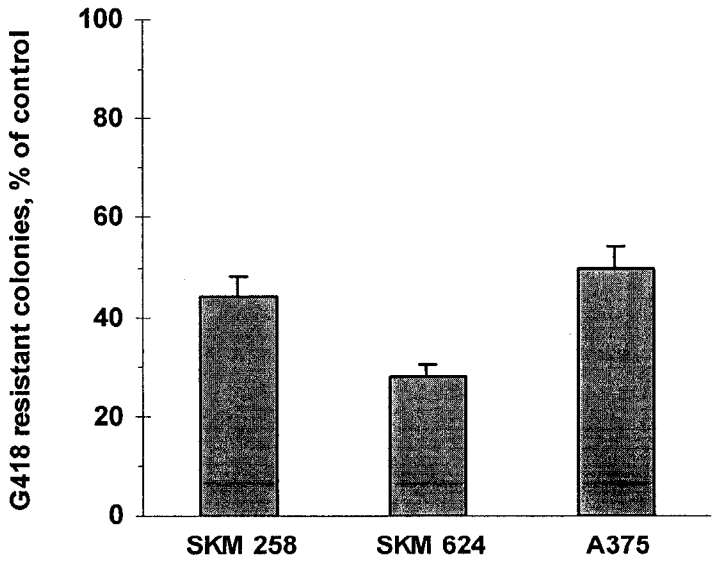

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TSLC1 EXPRESSION IS FREQUENTLY LOST IN MALIGNANT MELANOMA

by

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In preparation for *The Journal of Investigative Dermatology*

Format adapted for dissertation

ABSTRACT

Allelic loss of 11q23 has been associated with melanoma progression, but no tumor suppressor genes in this region have been identified in melanoma. *TSLC1* (tumor suppressor in lung cancer-1) was identified on chromosome 11q23.2 as a novel tumor suppressor in human non-small-cell lung cancer (NSCLC) by functional complementation. *TSLC1* encodes an adhesion molecule of the Ig superfamily involved in intercellular adhesion. Expression of *TSCL1* is reduced or lost in several tumor types, including lung cancer, squamous cell carcinoma of the head and neck, and cervical cancer, but it has not been studied in melanoma, so we evaluated the expression of *TSLC1* in melanoma cell lines and tumor samples. Reduced expression of *TSLC1* transcripts was observed by reverse transcription-PCR in three of four melanoma tumor samples compared to normal melanocytes. Western blot analysis demonstrated that TSLC1 expression was reduced or absent in 7 of the 11 melanoma cell lines investigated and in 6 of 10 metastatic melanoma tumors. Immunofluorescence detection of TSLC1 in melanoma tumor sections yielded findings consistent with those of Western blot analysis. In addition, ectopic expression of TSLC1 in the melanoma cell line A375 suppressed growth *in vitro*, supporting a tumor suppressor role for TSLC1 in melanoma.

INTRODUCTION

In 2003, cutaneous malignant melanoma (CMM) was estimated to be the fifth most common cancer among males and the seventh most common cancer among females in the United States (1). Both the incidence and mortality of melanoma have markedly increased over the past two decades. Early stage melanoma is highly curable by surgical

intervention, but metastatic melanoma is one of the most aggressive and chemoresistant tumors. The underlying genetic alterations contributing to this malignancy are not yet completely defined. To date, mutations in only a few genes, such as *BRAF*, *RAS*, and *CDK2A*, occur in a significant portion of melanomas (2-5). The identification of other genes important in controlling growth in melanoma remains an important objective.

Allelic loss in chromosomal band 11q23 was previously reported to be associated with multiple progression steps of melanoma (6) and with a less favorable clinical prognosis (7). *TSLC1* [the tumor suppressor in lung cancer-1, also known as nectin-like molecule 2, IGSF4, RA175/SgIGSF(8), Accession #NM_014333] was identified on chromosome 11q23.2 as a tumor suppressor gene by functional complementation analysis. Transfer of the 11q23.2 chromosomal region into a human non-small-cell lung cancer (NSCLC) cell line, A549 (which normally does not express TSLC1), completely suppresses their tumorigenicity in nude mice (9). *TSLC1* encodes a member of the immunoglobulin superfamily proteins comprising three immunoglobulin-like extracellular domains, a single hydrophobic membrane-spanning α-helix, and a short cytoplasmic domain (10). TSLC1 is an *N*-linked glycosylated protein which is expressed at the cell-attachment site and localizes along the entire cell membrane in polarized epithelial cells (11). TSLC1 forms homodimers through *cis*-interaction at the cell membranes, and it mediates intracellular adhesion through Ca^{2+}- and Mg^{2+}-independent homophilic *trans*-interaction (11). The cytoplasmic portion of TSLC1 contains two important motifs, a protein 4.1 (FERM)-binding motif adjacent to the transmembrane domain and a PDZ-interacting motif at the carboxyterminus (12, 13). TSLC1 interacts directly with DAL-1, another lung cancer tumor suppressor, through its 4.1-binding motif

(14). Recently, TSLC1 was shown to associate directly with MPP3, one of the human homologs of the *Drosophila Discs large* tumor suppressor, through its PDZ-binding motif (15). The cytoplasmic domains of TSLC1 are critical for tumor suppressor activity *in vivo*, cell-cell adhesion, and proliferation in an anchorage-dependent manner (16). Truncation of the cytoplasmic domains of TSLC1 abrogates its tumor suppressor function in A549 cells, which results in a considerably increased growth rate of such tumor cells in nude mice (16).

Loss of TSLC1 expression occurs frequently in lung cancer cell lines, is associated with tumorigenicity and metastasis (17), and is predictive of poor outcome in human adenocarcinoma of the lung (18). In addition to NSCLC, TSLC1 is reduced or absent in several cancer types, including hepatocellular carcinoma (17), pancreatic cancer (19), cervical neoplasia (20), esophageal squamous cell carcinoma (21), and gastric cancers (22). Allelic loss at 11q23 has been implicated in metastatic melanoma, but the role of TSLC1, a candidate tumor suppressor in this locus, has not yet been studied in melanoma. We report that TSLC1 expression is frequently lost in human melanoma cell lines and tumors, and that restoration of TSLC1 expression suppresses the colony-forming ability of A375 melanoma cells.

MATERIALS AND METHODS

Cells and Culture Conditions

Human melanoma cell lines SK-Mel-37, SK-Mel-170, SK-Mel-241, SK-Mel-249, SK- Mel-257, SK-Mel-258, and MelJuso, previously described (23) and kindly provided by Dr. Jane Fountain, National Cancer Institute, Bethesda, MD; Mel624 and

Mel888, kindly provided by Dr. Steven Rosenberg, National Cancer Institute; and SK-Mel-28 and A-375, American Type Culture Collection, Manassas, VA, were cultured in Dulbecco's Modified Eagle's Medium with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. Human epidermal melanocytes (HEM), primary cells isolated from normal human neonatal foreskin (Cell Applications, San Diego, CA), were cultured in melanocyte growth medium (Cell Applications). Cultured human skin fibroblasts were maintained in Eagle's Minimum Essential Medium with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum.

Tumor Samples and Tissue RNA Samples

Tumor specimens were obtained from patients with Stage III or IV melanoma who were enrolled in clinical trials at the University of Alabama at Birmingham (UAB). The tumor specimens were obtained after informed consent with the permission and approval of the Institutional Review Board for Human Experimentation of UAB. Tumors were excised, immediately frozen, and stored at -70^{o}C.

Reverse-transcription PCR

Total RNA was isolated from frozen tissues and cell lines using RNA STAT60 (Tel-Test, Friendswood, TX) and purified from residual protein using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration was calculated spectrophotometrically, and the quality of the RNA assessed by denaturing agarose gel electrophoresis. RT-PCR was performed

using the Gene Amp PCR kit (Roche Molecular Systems, Branchburg, NJ) as described in the manufacturer's protocol, using 1 μg of total RNA as template with random hexamers for RT priming. Negative controls without the addition of RT enzyme were performed for each RNA sample. For PCR, *TSLC1*-specific primers (the forward primer 5'-CATCACAGTCCTGGTCCCACCACGTAATCT-3' and the reverse primer 5'-AATAG-GGCCAGTTGGACACCTCATTGAAAC-3') were added at a concentration of 0.3 μM each and amplification carried out for 35 cycles with an annealing temperature of 65°C. RNA from normal cultured melanocytes was used as the positive control. Amplification with primers specific for GAPDH (the forward primer 5'-TGGTATCGTGGAAG-GACTCATGAC-3', the reverse primer 5'ATGCCAGTGAGC TTCCCGTTCAGC-3') was used as an internal control.

Western Blot Analysis

Normal primary cells and melanoma cells were extracted with lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 8.0) including protease cocktail (Roche Diagnostics, Indianapolis, IN). Surgical specimens frozen in liquid nitrogen were pulverized by mortar and pestle and added to the lysis buffer described above. The lysates were incubated on ice for 20 min and then centrifuged. The protein content of the supernatant was measured using a bicinchoninic acid protein assay reagent (Pierce Biotechnology, Rockford, IL). Equal amounts of lysates (20-50 μg) were electrophoresed on 7.5% polyacrylamide gel (Biorad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH). After blocking with TBS containing 0.05% Tween 20 (TBS-T) and 5% milk for 1 hr at

room temperature, the filters were incubated with anti-TSLC1 [a rabbit polyclonal Ab against 18 synthetic polypeptides of the C-terminus of TSLC1, (18), final concentration 1 μg/ml] and anti-β actin [mAb clone Ali12-28, 50 ng/ml, Abcam, Cambridge, MA] at 4°C overnight, washed in TBS-T, and then incubated with horseradish peroxidase-labeled anti-mouse and anti-rabbit IgG (1:5000 dilution, Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were detected using the Amersham ECL System according to the manufacturer's instructions.

Immunofluorescence analysis

Frozen surgical specimens that had been embedded with OCT compound (Fisher Scientific, Pittsburgh, PA) were sectioned. The sections were fixed with 4% paraformaldehyde, blocked for 1 hr with 10% normal goat serum (Jackson Immuno-Research Laboratories, West Grove, PA) in PBS, and then incubated with anti-TSLC1 antisera (18) (2 μg/ml) overnight at 4°C. Slides were washed three times with PBS and incubated with Texas Red-X-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, 1 μg/ml) for 1 hr at RT. Slides were subsequently rinsed with PBS and mounted with permanent aqueous mounting medium (Biomedia, Foster City, CA).

For localization studies of ectopic TSLC1 expression, A375 cells that had been seeded on collagen I-coated culture slides and cultured for 2-3 days were transfected with a pcDNA3.1 plasmid. After being washed, cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT, rinsed three times with PBS, permeabilized for 10 min with cold (-20°C) acetone, and rinsed three times with PBS. Cells were blocked for 1 hr with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in

PBS and then incubated with anti-TSLC1 antisera (2 μg/ml) overnight at 4°C. Immunofluorescence staining was performed as described above.

Transfection and Colony Formation Assay

The TSLC1 coding sequence was subcloned into pcDNA3.1 (Invitrogen Life-Technologies) and protein expression verified by transient transfection and detection with the anti-TSLC1 antibody. For colony-formation assays, A375 cells (1 x 10^6 cells / 6-well plate) were transfected with 10 μg of either empty plasmid control or TSLC1 expression plasmid using the Lipofectamine reagent (Invitrogen Life-Technologies) following the manufacturer's instructions. Twenty-four hours after transfection, cells were plated at varying densities (20,000-40,000 cells) into 6-well plates and grown for 14 days in media supplemented with 1.0 mg/ml of G418 (Geneticin, Cellgro by Mediatech, Herndon, VA). To count G418-resistant colonies, the cells were washed with PBS, fixed and stained with a crystal violet solution (10% acetic acid, 50% methanol, and 0.15% crystal violet). The number of colonies that survived G418 selection on the vector control dishes was defined as 100% in this assay. The assay was performed two times in duplicate.

RESULTS

TSLC1 mRNA Expression is Reduced in Melanoma Tumor Samples

We first examined the expression of TSLC1 mRNA in total RNA isolated from melanoma cell lines and melanoma tumor tissues by RT-PCR and compared it to that of cultured normal human melanocytes and fibroblasts (Fig. 1). In this qualitative assay, loss of TSLC1 mRNA was not observed in eight melanoma cell lines evaluated (not all

data shown). In contrast, the decrease of TSLC1 transcripts was readily detected in three of the four melanoma tumor RNA samples evaluated.

TSLC1 Protein is Reduced or Absent in Most Melanoma Cell Lines and Tumors

We next analyzed TSLC1 protein expression by Western blot analysis using polyclonal antisera specific for TSCL1. Consistent with previous reports, the mobility size of the TSLC1 proteins ranged from 80 to 110 kd due to the extent of glycosylation. Reduced or undetectable levels of TSLC1 protein were detected in 7 of the 11 melanoma cell lines (64%) compared to normal melanocytes (Fig. 2*A*). Further, TSLC1 was absent in six of seven melanoma tumor samples (Fig. 2*B*). Only one of the tumor samples, from patient 5, showed TSLC1 protein levels comparable to that in normal melanocytes. This level of protein was consistent with mRNA levels by RT-PCR (Fig. 1). These results demonstrated frequent loss of TSLC1 expression in both melanoma cell lines and tumors.

Immunofluorescence detection of TSLC1 in melanoma tumor sections yielded similar findings (Fig. 3). Tumor sections from patient 5 (9705) were strongly positive for TSLC1 protein, consistent with Western blot analysis. Evaluation of three different tumor samples that had shown little or no TSLC1 expression by Western blot analysis also showed little or no expression by immunofluorescence analysis.

Growth Suppression of A375 by Ectopic TSLC1 *in vitro*

The finding that TSLC1 expression is frequently lost in melanoma suggests that down-regulation of TSLC1 may contribute to the malignancy of melanoma. To test this possibility, we evaluated whether restoration of TSLC1 expression in melanoma cell lines

can suppress their growth. A375, a melanoma cell line expressing very low levels of TSLC1 as shown by Western blot analysis, was transfected with either the TSLC1 full-length cDNA in the mammalian expression vector pcDNA3.1 or a control pcDNA3.1 empty vector. Western blot analyses showed that A375 cells transfected with TSLC1 expression plasmid had readily detected TSLC1 protein levels compared to these A375 cells transfected with the empty vector control (Fig. 4*A*). To determine the subcellular localization of the ectopic TSLC protein, we performed immunofluorescence staining. TSLC1 protein was apparent in the transfected cells and was primarily localized to the cell membrane at the sites of cell-cell contact, consistent with the proposed role of TSCL1 in adhesion (Fig. 4*B*). Some TSLC1 protein was also detected in the cytoplasm.

The consequence of ectopic TSLC1 expression on the growth of A375 cells was examined by an assay of colony-forming ability. The two groups of the transfected A375 cells were selected by culturing cells in the presence of G418. After 2 weeks, the G418-resistant colonies were stained and counted (Fig. 4*C*). The results showed that the ectopic TSLC1-transfected A375 cells formed two-fold fewer G418-resistant colonies than did the mock-transfected cells, indicating that restoration of TSLC1 expression by transfection of the TSLC1 full-length cDNA construct suppresses colony formation of A375 cells (Fig. 4*D*). This growth inhibitory effect of ectopic TSLC1 appeared to be specific to the cells expressing reduced or absent TSLC1, as ectopic TSLC1 expressed in SKM 624 cells did not show any inhibitory effect on colony formation of these cells (Fig. 4*D*).

DISCUSSION

TSLC1 has recently been recognized to be an important tumor suppressor gene in human cancers. Although initially identified in non-small-cell lung cancer, it is now clear that loss of TSLC1 expression may be a frequent event in several types of cancer. Loss of TSLC1 mRNA expression was reported in advanced NSCLC (24), pancreatic cancer (19), cervical neoplasia (20), esophageal squamous cell carcinoma (21), gastric cancers (22), and prostate cancers (25). In this study, we studied *TSLC1* expression in melanoma and found that TSLC mRNA levels decreased in most malignant melanoma samples evaluated. A decrease in mRNA level was detected in three of four tested patient tumor samples, suggesting that the transcriptional control is likely to be involved in the reduced TSLC1 transcripts in melanoma. In other tumor types, TSLC1 mRNA loss was shown to be mediated by hypermethylation of the TSLC1 promoter region (19-22, 24-26). Interestingly, decreased TSLC1 mRNA was not detected in most melanoma cell lines tested, despite the fact that several of these cell lines had reduced or absent TSLC1 protein, as revealed by Western blot analyses. These data suggest that the post-transcriptional control might also participate in the expression of TSLC1 protein in melanoma, in addition to the transcriptional mechanism. Analysis of TSCL1 cDNA in these samples for the presence of mutations may be informative in this regard. Alternatively, the nonquantitative nature of RT-PCR may not clearly demonstrate more subtle decreases in TSLC1 mRNA expression. Quantitative real-time PCR may allow a more accurate determination of mRNA levels.

Nevertheless, Western blot analysis of both melanoma cell lines and tumors showed that loss of TSLC1 protein expression is a frequent event in melanoma. The

tumor samples evaluated were all from patients with advanced stage disease, and it will be of interest to determine if loss of TSLC1 is an early or late event. Allelic loss at 11q23, which encompasses the *TSLC1* locus, is frequently found in melanoma at the metastatic stage, supporting this possibility (27-29). The loss or reduction of TSLC1 expression was reported to be associated with the advanced stages of lung adenocarcinoma (18). Further studies would be required to elucidate potential mechanisms that may cause TSLC1 expression to be shut off in this cancer type, such as a loss of hyterozygosity (LOH) of TSCL1 or the hypermethylation of TSLC1 promoter regions. Hypermethylation of promoter regions of other known and candidate tumor suppressor genes; e.g., retinoic acid receptors-β2 (RAR-β2) (70%), RAS association domain family protein 1A (RASSF1A) (57%), and O-methylguanine DNA methylatransferase MGMT (34%) were previously reported in melanoma cell lines and metastatic melanomas (30).

Masuda and colleagues reported that TSLC1 protein was involved in cell-cell adhesion using Madin-Darby canine kidney (MDCK) cells (11). In the present study, TSLC1-transfected A375 melanoma cells showed TSLC1 protein distributed along the cell membranes when the cells reached confluence, suggesting that the TSLC1 protein is also associated with cell-cell adhesion in melanoma. Enforced expression of TSLC1 was associated with growth suppression. The molecular function of TSLC1 remains to be determined, but its implication in cell-cell adhesion and association with other known tumor suppressor proteins, including DAL-1 (14) and MPP3 (15), suggests that loss of TSLC1 may disrupt normal cell-cell interactions. As alterations of E-cadherin are also known to be involved in progression of human cancers, including melanoma (31), loss of

TSLC1 expression may lead melanoma cells to invade or metastasize through disruption of cell-cell interactions.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Truman Grayson and Connie Jenkins. This work was supported by National Cancer Institute R21CA78498 (TVS). P.H. was supported by a scholarship from the Royal Thai Government.

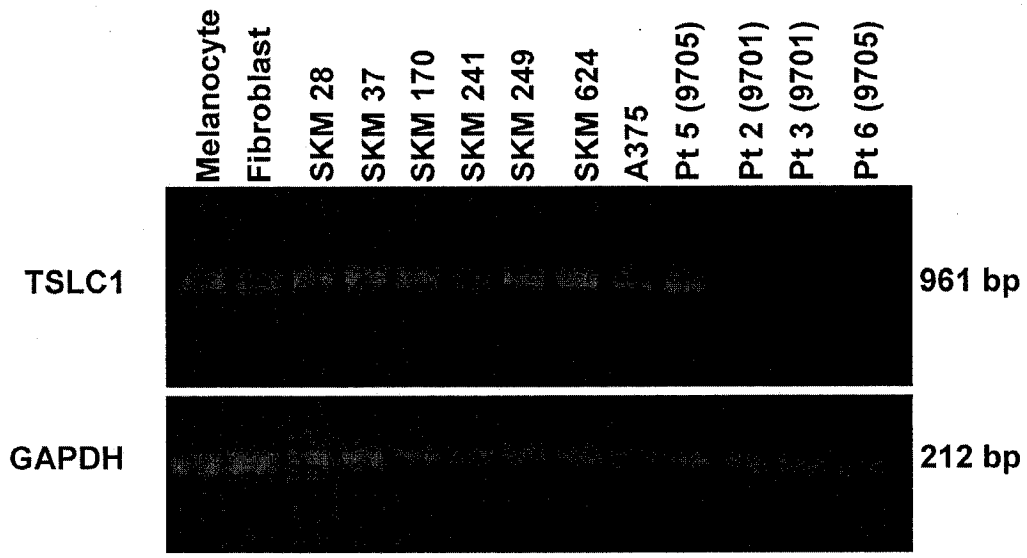
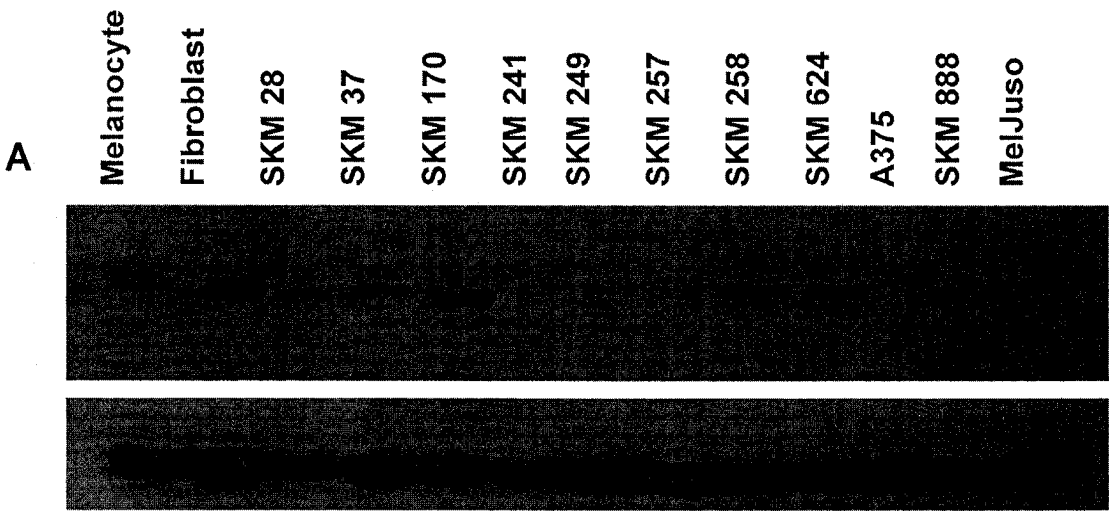
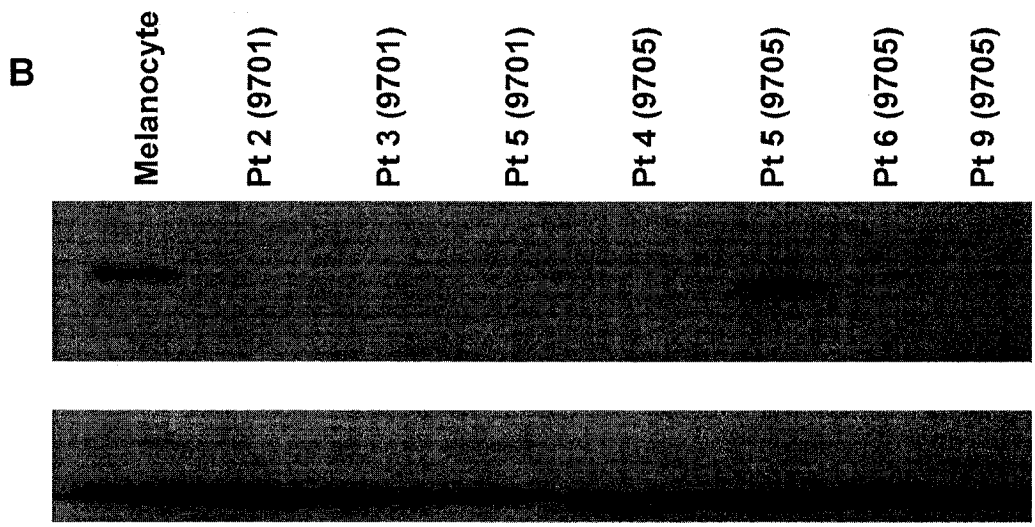


Fig. 1. RT-PCR analysis of TSCL1 mRNA expression in normal melanocytes and fibroblasts, and in melanoma cell lines and tumor samples. Total RNA from these samples was reverse-transcribed, amplified with TSLC1-specific primers, and resolved on a 1% agarose gel. The expected size of the amplification product is 961 b bp. Amplification of GAPDH mRNA was used as a control to assess amounts of cDNA used in each reaction with the expected size of 221 bp.

Fig. 2. Western blot analysis of TSLC1 protein expression in normal melanocytes and fibroblasts, and in melanoma cell lines and tumor samples. Cell lysates were separated on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a polyclonal antibody specific for TSLC1. Melanoma cell lines (*A*) and melanoma tumor samples (*B*) were compared to normal cells. A monoclonal antibody to β-actin was simultaneously applied to demonstrate approximately equal loading of the cell lysates.





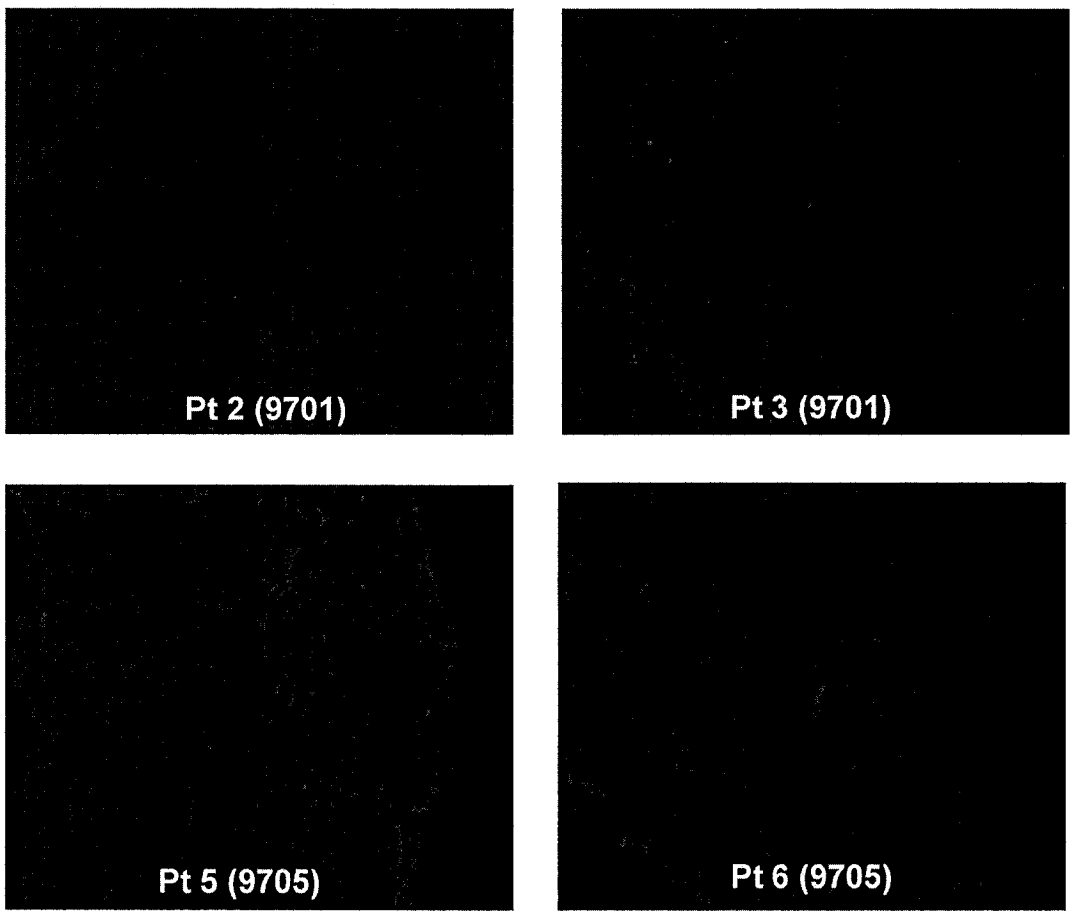


Fig. 3. Immunofluorescence analysis of TSLC1 expression in melanoma tumor samples. Patient tumor samples of metastatic melanomas were sectioned and incubated with polyclonal antisera specific for TSLC1. Binding of the antibody was detected with a Texas Red-X-conjugated goat anti-rabbit secondary antibody.

Fig. 4. Colony formation assay for growth inhibitory effect of ectopic TSLC1 protein in melanoma cell lines. (*A*) TSLC1 expression in pcDNA3.1- and pcDNA 3.1/TSLC1 transiently transfected A375 cells. (*B*) Subcellular localization of ectopic TSLC1 in A375 cells. (*C*) Effect of ectopic TSLC1 on colony formation of A375 and SKM 624 cells. (*D*) Relative number of G418 resistant cells in A375 and SKM 624 cells.

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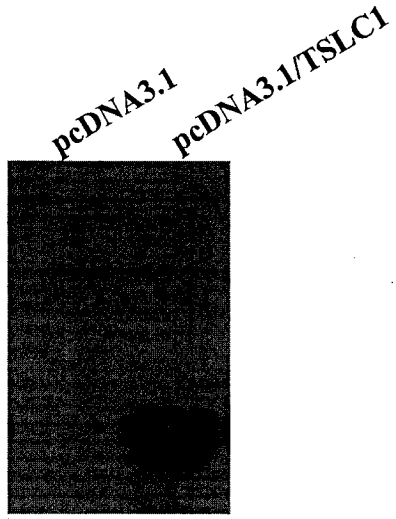

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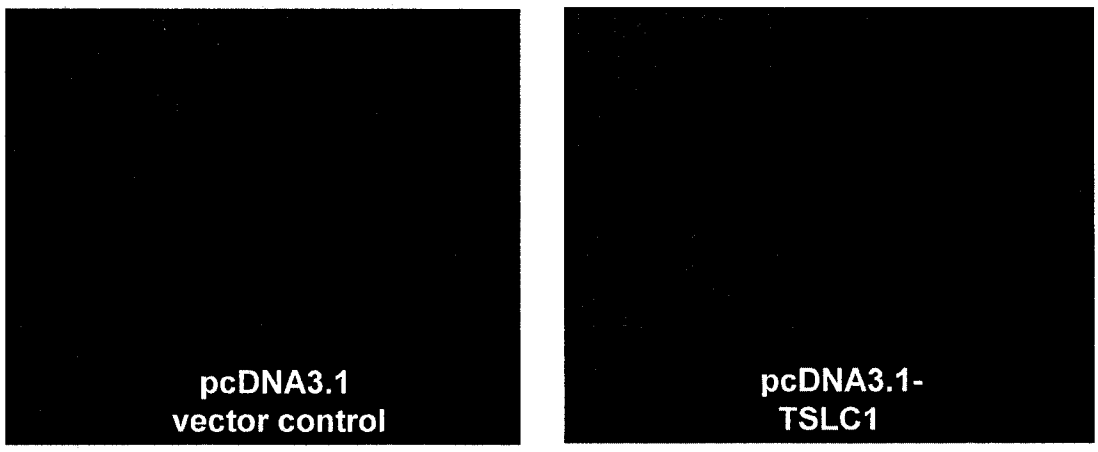

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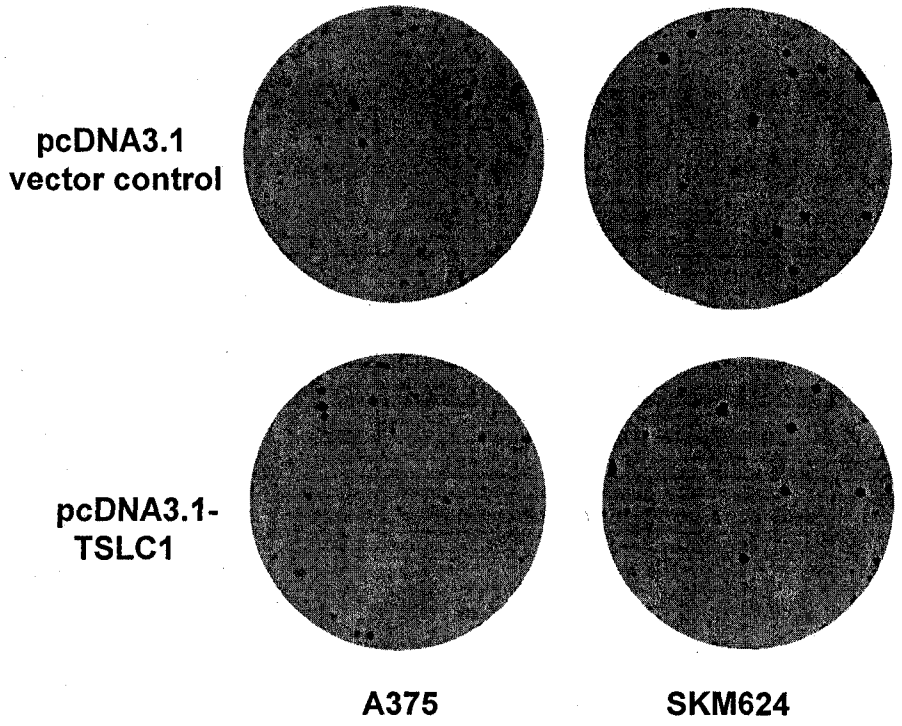

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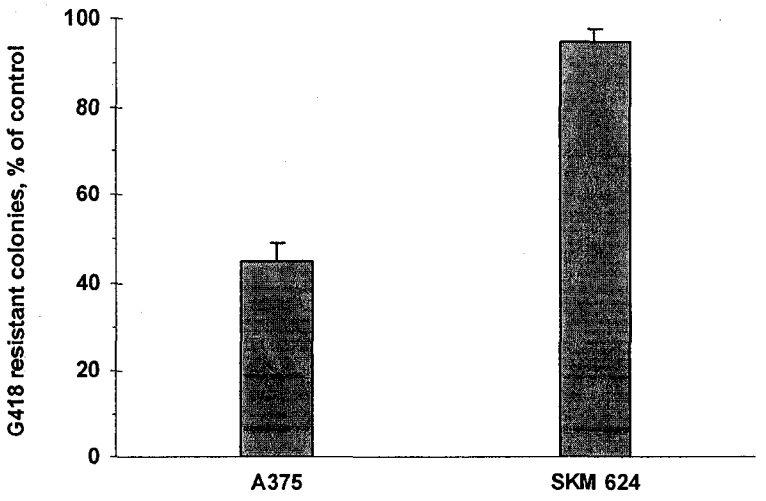

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DISCUSSION

By screening the SKM 624 and SKM 888 melanoma cell line cDNA expression library with serum of a melanoma patient experiencing a complete clinical response to active nonspecific immunotherapy, we have identified a total of 7 immunogenic proteins. Of these, six represented known genes in the GenBank database and one was a previously uncharacterized gene. All of the genes isolated in this study are present in many tissues. At least two genes appear to have a potential relevance to cancer based on previous studies and our present studies. Other research groups have shown that RING3, one of the two genes, encodes a human mitogen-activated nuclear kinase, a member of a newly described family of bromodomain-containing proteins that transactivate the promoter of a number of the E2F family members of transcription factors (216). This nuclear kinase activity is increased upon cellular proliferation and is implicated in human development and growth control. Its *Drosophila* homolog, female sterile homeotic (fsh), is a developmental regulator associated with the establishment of segments in the early embryo (217). With regard to the pathogenetic relevance to cancer, RING3 may be important in acute and chronic lymphocytic human leukemias since its kinase activity is markedly elevated in such patients (217). The RING3 transcript was found also to be highly expressed in melanoma cell lines (218). Further studies involving 1) quantitative evaluation of RING3 expression in normal tissues and in either primary or metastatic melanomas compared with normal human melanocytes, and 2) a large-scale survey for anti-RING3 antibodies from healthy controls and melanoma patients would provide

significant information on the potential of RING3 as a marker and a therapeutic target for metastatic melanoma.

The other gene product with previously reported biological relevance is hDlg. In *Drosophila*, the *dlg* tumor suppressor protein was discovered as a critical regulator of cell polarity, adhesion, proliferation, and migration processes (219, 220). As a consequence, it is anticipated that its human homolog might also play a similar role in vertebrate pathologies. Growing evidence now provides significant indications of a role for hDlg in the control of cell proliferation (68) and in the pathogenesis of some human malignancies, including cervical neoplasias (221, 222), mammary ductal carcinoma (223), and gastric carcinoma (224). Our present study reveals the biologic relevance of hDlg in melanoma.

With respect to the other four genes, hnRNP-E1 (clone 5.16), polyribonucleotide nucleo-tidyltransferase 1 (PNPT1) (clone 5.18), and KIAA0663 (clone 3.1), and KIAA0555 (clone 5.17), the biological role of two of them, the hnRNP-E1 and PNPT1 gene products, have been reported (225, 226), whereas the function of KIAA0663 and KIAA0555 gene products remains undefined (227, 228). hnRNP-E1, also known as PCBP-1 (poly [rC]-binding protein 1) encodes an RNA-binding protein containing three K-homologous (KH) domains believed to play a key role in the post-translational regulation of gene expression (226). Moreover, hnRNP-E1 mRNA is detected in many human tissues (229). *PNPT1* is a type I interferon-inducible gene encoding an RNA-degradation enzyme, and its ectopic expression in melanoma cells results in growth suppression in human melanoma cells through the molecular mechanisms of G1 phase arrest and apoptosis, together with a marked reduction in the S phase (225). Moreover,

inhibition of *PNPT1* by antisense strategy significantly rescues these melanoma cells from growth cessation, further supporting its potential role in regulating cellular growth (225). Whether these genes have a physiological relevance to tumorigenesis remains a question. Analyzing the expression profiles of these genes electronically may provide answers to some extent quickly and efficiently. This electronic search could be carried out through NCBI databases and online analysis tools, which are available publicly. Among these, Serial Analysis of Gene Expression (SAGE) is a technique designed to take advantage of high-throughput sequencing technology to obtain a quantitative profile of cellular gene expression. SAGE quantifies a "tag," usually 10 bp in length, which represents a gene transcript, and its analytical aspect involves mapping the tags to known genes and deciphering the degree of expression. In theory, the higher the tag count number, the more prevalent the transcript would be in the cell analyzed. Importantly, a comparison of the expression of any given SAGE tag from each of the four transcripts between two libraries, i.e., tumor libraries versus their corresponding normal tissue libraries, could provide some insight into the differing expression between the two cellular states. The genes with such a difference might represent cancer-related genes. The SAGE map on the Human Map Viewer and other useful products, such as xProfiler (which compares expression from two libraries), are located on the SAGE map website at: http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd=expsetup. However, SAGE is limited to the transcript level and not applicable to analysis for other information regarding mutations, splice variants, or protein quantification.

The novel melanoma antigen found to be of greatest interest was hDlg. Our finding of its immunogenicity in metastatic melanoma prompted us to evaluate the role of

hDlg in this cancer by examining the expression of hDlg at both mRNA and protein levels in melanoma cell lines and tumors, as well as in normal human cells and tissues. Our data show that hDlg expression at both levels is greatly reduced in the majority of tested melanoma cell lines and all tested tumors with metastatic disease. The loss of total cellular hDlg protein has previously been reported in invasive cervical neoplasia (221). Reduced or absent expression of hDlg in melanomas, particularly in patient 9 of UAB trial 9705, one of the two patients who had induced anti-hDlg antibodies after the immunotherapy [manuscript 2, Fig. 1, patient 13], suggests that some other mechanisms may cause the immunogenicity of hDlg rather than the over-expression of hDlg antigen, resulting in anti-hDlg antibodies being induced in this patient. The other patient who also developed anti-hDlg antibody response is patient 1 of UAB trial 9701. The information on the expression level of hDlg in this patient could not be provided because the patient's specimens were not available. As a result, the over-expression of hDlg antigen could not be ruled out as the cause of hDlg immunogenicity in this patient. However, it is worth noting that all 12 tested melanoma tissues from the patients in the two clinical trials showed low levels of hDlg transcript and/or protein. Moreover, 6 of the 12 patients who were evaluated for both mRNA and protein levels showed the reduced hDlg expression at both mRNA and protein levels. Therefore, it is more likely that low mRNA and protein levels of hDlg would be detected in patient 1 of UAB trial 9701 as well.

The findings that aberrantly spliced transcripts of the hDlg gene are expressed in some melanoma cell lines and tumors, but not in normal cells, led us to speculate that the hDlg products encoded by these aberrantly spliced transcripts may be immunogenic. This speculation is based on the two following reasons: 1) abnormal transcripts skipped

several downstream exons and, therefore, would be predicted to produce truncated proteins with frameshifts, mutated peptide sequences at their C-terminus, and a premature termination codon; and 2) these aberrant products could be rapidly degraded, allowing the antigenic peptides derived from these degraded proteins to be presented by HLA class I or class II for priming and activation of $CD8^+$ T and $CD4^+$ T cells, respectively. Antigen presentation to $CD4^+$ T cells by both APCs and B cells is critical for the production of an antibody response. Previous reports have shown that normal hDlg in cells that reach confluence is stabilized at sites of cell-cell contact, but it is degraded via the proteasome-mediated degradation process in isolated cells and undifferentiated cancer cells (230). It can be speculated that truncated hDlg proteins with several important domains deleted would not be localized to adherens junctions, but would likely be degraded instead, facilitating induction of humoral and cellular immune responses. An indication supporting the possibility of degradation is that most products of aberrantly spliced transcripts were not detected by Western blot analysis, suggesting that these proteins may be unstable. However, it is also possible that the aberrant products may be produced at levels below those detectable by Western blot analysis. Corresponding to this possibility, low levels of the aberrant transcripts were detected in our tested samples, because a nested-RT-PCR was necessary to obtain visible amplification of these products. A mechanism by which cells have evolved to rid themselves of aberrant transcripts by the nonsense-mediated mRNA decay (NMD) pathway that rapidly degrades mRNA with premature translation termination codons (231) might be an explanation for our results. This mechanism prevents synthesis of incomplete and

potentially deleterious proteins. The possibility that the monoclonal hDlg antibody may not be able to recognize some truncated products could not be excluded.

Evaluating CD8+ and antibody responses specific for aberrantly spliced transcripts will be important in that it would not only provide insight into the molecular basis of hDlg immunogenicity, but also evaluate whether these aberrant hDlg antigens could be attractive targets for cancer vaccines. These aberrant hDlg products have potential in this regard because they are tumor specific, likely to exist in a broad population of patients based on our data, and less likely to develop antigen-loss variants if mutations are involved in the proliferation and survival of tumor cells.

To evaluate CD8+ responses specific to the aberrant transcripts, dendritic cells could be pulsed with peptides derived from aberrant transcripts or transfected with the gene encoding the aberrant proteins in order to generate endogenously processed epitopes. These dendritic cells could next be cultured with T cells derived from peripheral blood mononuclear cells (PBMCs) or TILs of the patients expressing anti-hDlg antibodies to test for the generation of CD8+ CTLs reactive to these aberrant proteins. However, this method is limited in that dendritic cells should be obtained from the blood of patients who express the appropriate HLA restricting allele (232). Moreover, dendritic cells are generally short-lived and not easily transfected.

To evaluate anti-hDlg antibodies specific for the aberrant transcripts, various bacterial recombinant proteins of such transcripts would be probed with the patients' sera using dot blot analysis. Wild-type hDlg protein could also be used as a control in this analysis as a comparison. It is possible that an antibody response directed against the aberrantly spliced hDlg may also crossreact with the wild-type hDlg. If it does so, this

could explain why the isolated clone 3.14 (a partial hDlg) did not contain an altered sequence in the excised cDNA expression vector.

Our data show that expression of hDlg protein is greatly reduced in most of the tested melanoma cell lines and in all of the melanoma tumors compared to expression of hDlg protein in normal human melanocytes. Moreover, the down-regulation of hDlg expression occurs at the RNA level, as indicated by the low levels of hDlg mRNA in these samples. Low expression levels of hDlg protein were previously reported in other cancer types, and the mechanisms responsible for these findings were also addressed. For example, in invasive phenotypes of cervical neoplasia, the oncogenic high-risk human papillomavirus (HPV) E6 protein decreases hDlg protein levels by targeting hDlg for degradation via the ubiquitin-proteasome pathway, which appears to also function in regulating hDlg levels in normal cells (221, 233). Another mechanism is the loss of heterozygosity and somatic mutations of the *hDlg* gene reported in non-viral-induced mammary ductal carcinoma, affecting the amount of the hDlg protein (223). In some melanoma samples, low expression levels of full-length hDlg transcript and protein might be explained by chromosome 3 loss, which occurs in approximately 25% of malignant melanomas of the skin (234). However, in parallel with a low level of full-length hDlg transcript, we also detected novel, aberrantly spliced messages specifically in melanoma cell lines and tumors. These results suggest that aberrant splicing may constitute another mechanism of hDlg down-regulation in melanoma.

Most of the transcript variants diverged from the full-length sequence at exon 5, which was aberrantly spliced to various downstream exons, leading to skipping of multiple downstream exons. These splices resulted in a shift in reading frame, leading to

altered C-terminal sequence and premature stop. In addition to these aberrant variants of hDlg found in our study, alternatively spliced isoforms of hDlg in which alternative exons 6 and 7 are included have been previously reported in normal human tissues. This suggests that the flanking introns of the exons 6 and 7 may be prone to undergo exon skipping and determine splicing patterns. Alternative splicing is a versatile form of genetic control in which a common pre-mRNA is processed into multiple mRNA isoforms differing in their precise combination of exon sequences, and exon skipping is the most common form of this process. Alternative splicing occurs in a large proportion of human genes, about 42% or more, generating proteomic diversity from a limited number of human genes (235). Alternative splicing appears to be associated with *cis*-regulatory elements within the exons (236) or introns (237), and trans-acting factors that interact with them, such as the serine/arginine-rich (SR) protein family and SR-related proteins. Differences in the activities or amount of general splicing factors and/or gene-specific splicing regulators, and differences in sets of *cis*-acting elements, play a vital role in the selection of splice sites and the generation of differential patterns of splicing (238).

The frequent expression of aberrantly spliced transcripts in a set of melanoma samples suggests that the fidelity of alternative splicing is altered in this cancer and that the aberrant splicing and exon skipping may account for the loss of hDlg expression. In fact, aberrant splicing has been shown to inactivate gene expression of other tumor suppressor genes, such as p53 (239), APC (240), and WT-1 (241). It would be important to elucidate the mechanism(s) responsible for aberrant splicing of hDlg transcripts in melanoma. In the majority of inherited diseases, altered pre-mRNA splicing results from mutations; e.g., missense, nonsense, and translationally silent mutations located in

noncoding regions, such as consensus 5' and 3' splice sites, branch sites, and polypyrimidine tract (PPT), or in *cis*-acting elements that can be either exonic or intronic (240, 241). Large genomic deletions or rearrangements could also cause the production of the aberrant mRNA splicing (242). In the case of hDlg, sequence analysis of genomic DNA in exons 5 and other skipped exons, as well as their flanking introns, could be performed to identify splicing mutations that may cause the aberrant mRNA splicing of this gene. In addition, as multiple exons are skipped in the hDlg transcript variants, large genomic deletions could also be investigated by Southern blot analysis.

If no splicing mutations were revealed at the genomic DNA level, it would be reasonable to next look at alterations in the *trans*-acting factors. A few diseases caused by alterations in the *trans*-acting factors required for RNA processing were previously reported. For example, increased levels of splicing factor HMGA1 induces aberrant exon 5 splicing of Presenilin-2 (PS2) pre-mRNA in sporadic Alzheimer's disease (AD) (243). To identify the *trans*-acting factors for DLG1 pre-mRNA splicing, genomic regions of exons and introns 5-6 of the DLG1 could be analyzed for sequences known to bind specific SR proteins as well as for other possible RNA *cis*-elements. Once the potential *cis*-elements were identified, the *trans*-activating factors that bind to such sequences could be further examined by binding assays. In this assay, a tagged RNA oligonucleotide sequence for *cis*-elements would be incubated with nuclear extracts and UV cross-linked, producing several protein-RNA complexes that could be sequentially analyzed by electromobility shift assay (EMSA). These *trans*-acting proteins could be identified and tested for their expression profiles in normal cells and tissues as well as

melanoma cell lines and tumors. Mini gene constructs could be used to look at splicing in the presence of high or low concentrations of those factors.

Another mechanism for aberrant splicing might be the loss of the accuracy and efficiency of the general splicing machinery and/or gene-specific splicing regulators associated with metastatic melanomas. This possibility is supported by the recent report indicating that abnormal splice forms appear to be a frequent event and cancer specific. This mechanism is likely to represent an important cause of disrupted tumor suppressor function (244). It is also possible that more than one mechanism may be involved in the aberrant splicing of DLG1 pre-mRNA.

With respect to the biological role of hDlg, growing evidence of several structural and growth regulatory proteins interacting with hDlg suggests that hDlg performs both structural and signaling functions at cell-cell contact sites of epithelial cells, controlling both cell polarity and proliferation. In normal intestinal epithelial cells, hDlg expression is required for the recruitment of PI3K to the E-cadherin-adhesion complex and for the organization of the actin cytoskeleton at the adherens junctions. hDlg and PI3K may be implicated in a number of biological processes of E-cadherin, including cell growth and differentiation (64). hDlg, also assists the growth inhibitory activity of APC, probably through the APC-β-catenin complex in the Wnt pathway (68). Loss of hDlg expression in normal intestinal epithelial cells severely alters E-cadherin junction stability, decreases cortical actin cytoskeleton localized to adherens junctions, prevents the recruitment of PI3K to such junctions, and inhibits cell differentiation (64). Moreover, disruption of E-cadherin-mediated AJs seems to play a critical role during tumor progression, in that it promotes the ability of tumor cells to proliferate and migrate, leading to an invasive/

metastastic state (245). These findings suggest that the loss of hDlg may represent a critical event in tumor progression. Consistent with this idea, our studies show frequent loss of hDlg at sites of intercellular contact in melanoma cell lines, raft cultures, and melanoma tumor samples, with concomitant increases in the hDlg signal in the cytoplasm. Loss of hDlg at the AJ sites is reported to be also associated with high-grade neoplasias and invasive carcinomas, representing a crucial step in the progression of cervical cancer (221, 222). However, direct evidence is lacking that the loss of hDlg disrupts AJs and contributes to invasion and metastases in malignant melanoma. An interesting next step would be to determine the molecular consequences of hDlg down-regulation in the progression of melanoma.

To investigate the relationship between hDlg alterations and melanoma progression, the altered expression level and cellular localization of hDlg could be assessed in melanoma patients with different clinical stages of disease (the American Joint Committee on Cancer, AJCC), including dysplastic nevi, primary melanomas with radial growth phase, and vertical growth phase, as well as melanoma metastases. If the hypothesis that hDlg loss is important in the invasive/metastatic state is correct, than the decreased expression of total cellular hDlg and its loss at the sites of cell-cell contact would be more likely to correlate with advanced stages of disease.

Next, the molecular consequences of hDlg loss could be determined by using the organotypic raft culture system of melan-a cells together with keratinocytes, which could allow for the analysis of the histopathologic characteristics of tumor progression. Melan-a is an immortal line of non-tumorigenic mouse melanocytes and possesses all of the characteristics of normal melanocytes. Melan-a cells have recently been produced to

stably express an activated ^{V559E}BRAF, presumably representing an early transformation event (246). The ^{V559E}BRAF, an activated version of BRAF, could be used for an oncogenic background that is likely to be required for the initiation of melanocytic neoplasia in melan-a cells. The potential of ^{V559E}BRAF in this aspect is based on previous reports that ^{V559E}BRAF is the most common BRAF mutation, expressed in 80% of nevi, which suggests that the V559E mutation is a critical step in the initiation of melanocyte transformation (121). Using the organotypic raft culture model, a comparison of melan-a cells with Dlg loss and melan-a cells with Dlg loss plus activated BRAF might reveal a correlation between Dlg loss and tumor progression. If the hDlg loss is a late event, it would be expected that only melan-a BRAF cells would show a phenotype with loss of Dlg. Expected consequences would include the loss of E-cadherin, and the disruption of AJs, mimicking an invasive phenotype. In *Drosophila* studies, inactivation of *dlg* gene in combination with another oncogenic ^{G12V}RAS expression is also sufficient to promote metastatic behavior (247).

Four sets of melan-a cell lines could be established and grown on raft culture with keratinocytes: 1) melan-a cells; 2) melan-a cells stably expressing low level of Dlg; 3) melan-a cells stably expressing ^{V559E}BRAF (246); and 4) melan-a cells stably expressing ^{V559E}BRAF and low levels of Dlg. To decrease the Dlg level, melan-a cells or ^{V559E}BRAF stably transfected melan-a cells would be transfected with siRNAs directed toward the Dlg gene sequence to inhibit Dlg protein synthesis, generating melan-a cells with Dlg loss (set 2) or melan-a cells stably expressing ^{V559E}BRAF with Dlg loss (set 4). siRNAs capable of down-regulating human Dlg have been described (64). The two sets of melan-a cells would be examined for the specific decrease of Dlg in comparison with the

untreated melan-a cells (set 1) using Western blot analysis. Again, if our hypothesis is correct, it would be anticipated that invasive/ metastatic characteristics would be detected in melan-a raft with an activating ^{V559E}BRAF background and Dlg loss (set 4), compared to the non-transformed (set 1) or BRAF-transformed melan-a rafts (set 3). Disruption of AJs could be assessed by immunofluorescent localization of several AJ components, including E-cadherin, N-cadherin, β-catenin, and α-catenin, along with that of hDlg. These components would not be expected to localize precisely along cell-cell boundaries where Dlg is absent, but they would be expected to localize diffusely throughout the cells instead, leading to the absence of AJ formations observed in melan-a raft with ^{V559E}BRAF background and Dlg loss (set 4).

Furthermore, E-cadherin loss, together with melanocytes separating from keratinocytes and interacting with each other, may be illustrated by staining for E-cadherin together with keratinocyte and melanocyte markers. More aggressive morphology, in which melanocytic lesions appear to increase N-cadherin expression and deeply invade collagen-fibroblast matrices where they interact with other cells that express N-cadherin, such as fibroblasts, may also be detected by staining for N-cadherin and a fibroblast marker. Additionally, the intensity and localization of other signaling proteins known to be associated with Dlg, such as F-actin cytoskeleton, APC, p85 subunit of PI3K, PTEN, and TOPK/PBK, could be analyzed by immunofluorescence for any alterations compared with their normal localization in raft culture controls. This approach might be able to uncover genes or signaling pathways affected by the loss of Dlg, which contributes to metastatic melanoma.

For melan-a cells with hDlg loss in the absence of a ^{V559E}BRAF background (set 2), hDlg loss only may be enough to alter E-cadherin, PI3K, and F-actin localization to cell-cell contact sites, leading to unstable and disrupted AJ assembly in the raft set 2. Alternatively, Dlg deficiency only may not be able to cause these alterations, because there may be other tumor suppressors that could compensate for the lack of Dlg function in regulating the localization and assembly of AJ components at sites of cell-cell contact. Nevertheless, hDlg loss alone would not be likely to promote the invasive/metastatic phenotypes in the melan-a raft set 2, but it would be likely to if it is accompanied with the synergistic action of oncogenic activation that could drive to melanocyte immortalization and transformation. It is also possible that melan-a raft with Dlg loss (set 2) and melan-a raft with activated BRAF and Dlg loss (set 4) might show the same histopathologic results, and this would suggest that additional genetic events beyond BRAF activation and Dlg deficiency are required for progression to metastatic disease. Moreover, the tumor suppressor function of Dlg may be compensated for by other members of the MAGUK family, inhibiting progression to invasive/metastatic phenotypes of melanocytes.

An additional technique to examine the molecular consequences of Dlg in raft cultures is to assess gene expression changes induced by Dlg loss. These gene expression profiles that may be transcriptionally affected following Dlg loss may be identified through gene-array expression analysis, in which two sets of array membranes spotted with the cDNA of well-characterized mouse genes related to melanoma could be prepared, one of which could be probed with cDNA probes synthesized from RNA isolated from melan-a cells with ^{V559E}BRAF activation and Dlg loss (set 4) and the other

of which could be probed with cDNA probes synthesized from RNA isolated from melan-a cells with only activated ^{V559E}BRAF (set 3). The comparison between these two sets of the gene-array expression may provide an overview of the key factors that may be involved in Dlg loss, and investigation of these altered genes may disclose the precise molecular consequences following Dlg loss that could lead to the progression of melanoma.

Another method to examine the molecular consequences of Dlg loss would entail the use of Dlg-deficient primary mouse embryonic fibroblasts (MEFs) from the Dlg insertion mutant mice (248). Based on previous literature, the loss of cell growth control in *Drosophila dlg* mutants clearly shows that this Dlg protein possesses tumor suppressor activity, and growing evidence suggests that this role is likely to be conserved in mammalian homologs of *dlg*. In this regard, our *in vitro* studies reveal that ectopic expression of hDlg in human melanoma cell lines expressing low levels of hDlg suppresses the colony-forming ability of these tumor cells, supporting the hDlg role as a tumor suppressor in melanoma for the first time. To date, no definite proof exists for the tumor suppressor function of the mammalian homologs *in vivo*. Mice homozygous for a partially deleted *dlg* knockout have cleft palates and developmental abnormalities and die perinatally, thus precluding further assessment of their function in tumorigenesis (248). Nevertheless, the use of MEFs from these Dlg insertion mutant mice would be an alternative way to determine the molecular effects of Dlg loss on AJs and on tumorigenicity in comparison with primary control cells containing a normal level of Dlg.

As Dlg contributes to the proper formation of AJs that have a direct role in contact-dependent inhibition of epithelial cell proliferation (249, 250), it would be

important to first determine whether Dlg deficiency in Dlg-deficient MEFs could prevent the cells from undergoing contact-dependent growth arrest when they reach confluence compared with wild-type MEFs. In this analysis, two sets of wild-type and Dlg-deficient MEFs would be cultured subconfluently and confluently, respectively. The two sets of these cells would be analyzed for the proliferation rate by measuring growth rate and S-phase entry. *In vitro* growth rate would be determined using cell number versus time during the logarithmic growth phase, and the percentage of cells present in S phase would also be determined by cell cycle analysis using flow cytometry. Dlg-deficient MEFs may be likely to exhibit a loss of contact-dependent growth arrest, such as a persistent proliferation and a significant percentage of the cells in S phase after reaching confluence. If this phenotype was found in the Dlg-deficient MEFs, fully transformed characteristics of these cells could next be tested by an anchorage-independent growth assay for the ability of the cells to form colonies in soft agar and by *in vivo* growth assay for their ability to form tumors in nude mice.

To determine whether Dlg is involved in the stability of AJ structures, the expression of AJ components E-cadherin, N-cadherin, β-catenin, and α-catenin could be examined in confluent wild-type and Dlg-deficient MEFs. Additionally, AJ structures and the localization of these components in Dlg-deficient MEFs could be revealed by immunofluorescence, compared with the staining results of these AJ components in confluent wild-type MEFs. If the loss of AJ structures at sites of cell-cell contact and the mislocalization of the AJ components were found in Dlg-deficient MEFs, retroviral constructs encoding wild-type full-length Dlg could be used to transduce Dlg-deficient MEFs to increase Dlg to normal level and the biologic consequences of its ectopic

expression in these cells; e.g., restoration of contact-dependent inhibition of proliferation and AJ formation could be analyzed. Moreover, the effect of hDlg loss on tumor metastasis could be investigated in athymic nude mice. In this analysis, nonmetastatic murine melanoma cells, in which the hDlg expression level is reduced by RNA_i, would be subcutaneously injected into nude mice. Mice injected with control nonmetastatic cells would serve as controls. The ability of the RNA_i-treated nonmetastatic melanoma cells to enhance tumor growth and develop metastatic colonies distantly in nude mice would be evaluated and compared with that of the untreated control cells. Nonmetastatic mouse melanoma clones, e.g. B16 clone C1-2 (251) and K1735 clones Cl3 and Cl10, (252) have been previously established. Collectively, these studies would further our understanding of the consequence of Dlg loss in melanoma and its role in promoting cancer progression.

The present study demonstrates that SEREX is a powerful method in identifying tumor-associated antigens, and the use of antibodies from the metastatic melanoma patient responsive to the nonspecific immunotherapy IL-12 as a valuable reagent is a key to obtain more potentially immunogenic antigens with their potential in tumorigenesis. hDlg is an example of SEREX-defined targets in response to non-specific melanoma immunotherapy, and it appears to play a functional role in the pathogenesis of melanoma, based on its molecular characterizations gained in this study. Further characterization of both humoral and CTL responses for the aberrantly spliced hDlg proteins may show these to be novel potential targets for active immunotherapies.

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APPENDIX A

UBIQUILIN 1, AN ADDITIONAL PUTATIVE MELANOMA-ASSOCIATED ANTIGEN

Ubiquilin 1, an Additional Putative Melanoma-Associated Antigen

In addition to patient 1's (9701) serum, patient 5's (9701) serum was also initially used to search for putative tumor antigens. Patient 5 (9701) was a female with advanced stage IV disease in her mid 20s who had experienced a partial clinical response to ALVAC-hIL-12 treatment. Approximately 5 x 10^5 recombinant clones of a cDNA library derived from cultured human melanoma cell lines Mel 624 and Mel 888 were screened with serum collected at day 43 of treatment from patient 5. Clone 3.3T, which represented a portion of Homo sapiens ubiquilin1 (UBQLN1 isoform 1, Genbank accession no. NM_013438) was isolated as a novel melanoma-associated antigen.

The full-length ubiquilin 1 gene encodes a protein of 595 residues (66 kD) and belongs to a family of proteins that contain an N-terminal ubiquitin-like (UB, or UBQ, or UBL) domain and a C-terminal ubiquitin-associated (UBA) domain. Ubiquilin 1 physically associates with both proteasomes and ubiquitin ligases, and thus is thought to functionally link the ubiquitination machinery to the proteasome to affect *in vivo* protein degradation (1). Ubiquilin 1 modulates accumulation of presenilin proteins and is found in lesions associated with Alzheimer's and Parkinson's diseases (2). Its rat homologue, DA41, is associated with a candidate tumor-suppressor DAN protein, and a complex of these two proteins might play a crucial role in the regulation of the cell cycle progression (3).

As anti-ubiquilin 1 antibodies were detected in multiple patients with melanoma and ovarian cancer in our preliminary data, ubiquilin 1 was of further interest for use as a tumor marker for diagnosis and disease monitoring. To achieve this aim, enzyme-linked immunoassay (ELISA) assay was established for large-scale serological analysis by using

purified recombinant partial ubiquilin 1 (clone 3.3T) protein as the antigenic target. To generate recombinant 3.3T protein for this analysis, the entire coding sequence of 3.3T was cloned into pTrcHis TOPO TA vector (Invitrogen Life-Technologies, Inc.) in-frame for expression as HisG fusion proteins in *Escherichia* coli, and the protein was purified on a large scale by Ni^{2+}-NTA column. The assay was applied to test 167 serum samples from cancer patients with melanoma or ovarian cancer and also 117 normal serum samples. The result of this serological analysis is summarized in Table A1.

Table A1 *ELISA reactivity of sera from healthy donors and cancer patients: Test for Ubiquilin 1 antibody*

Sera	ELISA, positive/ total
Healthy individuals	0/117 (0%)
Melanoma	8/39 (20.5%)
- Clinical ALVAC therapy (patients with advanced stages)	3/22
- Conventional therapy (patients with advanced stages)	5/17
Ovarian cancer	7/128 (5.5%)
- Clinical trial for recurrent cancer (patients with advanced stages)	6/49
- Pacific Ovarian Cancer Research Consortium, POCRC (patients with early stages or benign ovarian individuals)	1/79

Based on this large-scale survey, an immune response to ubiquilin 1 was observed in only 1 of 79 patients from POCRC with early stages or benign ovarian cancer, indicating that the antibody response to ubiquilin 1 is infrequent in patients with early stages. Moreover, in our survey of 167 cancer patients, with the exception of the patient from POCRC, 15 patients developed ubiquilin 1 antibodies were on experimental trials, meaning that they had failed chemotherapy and were in very late stages of diseases. These data support that humoral immunity to ubiquilin 1 may occur late in disease; thus it would not be a good tumor marker for early detection. However, ubiquilin 1 may be

useful to be included in a format of microarray screening because ubiquilin-specific antibodies are shown to be highly specific to disease. None of the 117 healthy individuals had the ubiquilin 1 antibody.

Ubiquilin 3 was not the target of the anti-ubiquilin antibodies

Although clone 3.3T cDNA is homologous to ubiquilin 1, isoform 1, the amino acid sequence of this clone showed about 90% identity to ubiquilin 3 protein (Fig. A1). Because of these highly homologous conserved regions and the fact that ubiquilin 3 is reported to have testis-restricted expression (4), which is a characteristic of the cancer-testis antigen class of tumor antigens, ubiquilin 3, instead of ubiquilin 1, might be the target of anti-ubiquilin antibodies developed in our tested patients with melanoma and ovarian cancer, both of which may cross react to clone 3.3T.

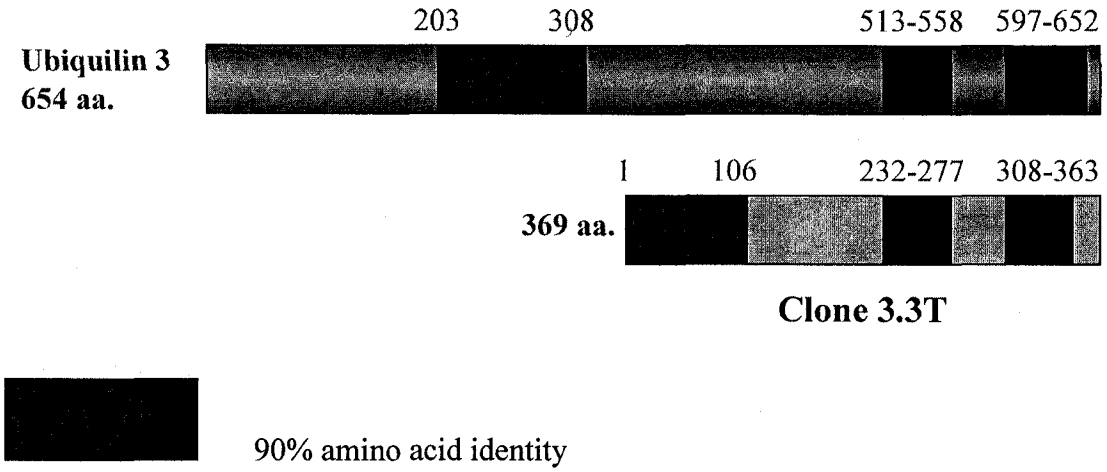

Fig. A1. Alignment of amino acid sequence of clone 3.3T and ubiquilin 3.

To test this possibility, ubiquilin 3 mRNA expression was tested in some melanoma tumors and cell lines, as well as in various types of cancer cell lines and

tissues, by regular RT-PCR or QRT-PCR techniques using testis mRNA expression as reference. The tumor cell lines and tissues, that were tested for ubiquilin 3 mRNA expression, are listed in Table A2.

Table A2 *A summary of tumor cell lines and tissues tested for ubiquilin 3 mRNA expression*

Method	Samples	Results
RT-PCR	5 melanoma tumors 10 melanoma cell lines 10 ovarian tumors 4 ovarian cancer cell lines 4 breast tumors 4 breast cancer cell lines 2 head and neck tumors 3 cervical cancer cell lines 1 lung cancer cell line 1 colon cancer cell line	no ubiquilin 3 mRNA detected
QRT-PCR	8 melanoma tumors 5 melanoma cell lines	no ubiquilin 3 mRNA detected

The results showed that no ubiquilin 3 transcripts were detected in our tested tumor cell lines and tissues, indicating that ubiquilin 3 mRNA were not aberrantly expressed in these tumor samples. Thus, it is not likely that anti-ubiquilin antibodies in our tested patients with melanoma and ovarian cancer could be developed against ubiquilin 3.

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APPENDIX B

SEROLOGICAL STUDIES OF HDLG

Serological studies of hDlg

The immunogenicity of clone 3.14 or a partial hDlg was further characterized on a large scale using serum samples from cancer patients and normal individuals by ELISA assay. In this assay, recombinant partial hDlg (clone 3.14) protein was made into pTrcHis TOPO TA expression vector (Invitrogen Life-Technologies, Inc.) and used as the antigenic target. An unrelated recombinant protein made in the same expression system was used as a control antigen. The frequency analysis of hDlg antibody would indicate the immunogenic potential of hDlg antigen and evaluate whether hDlg could be an attractive target for cancer vaccines (Table B1).

Table B1 *ELISA reactivity of sera from healthy donors and cancer patients: Test for hDlg antibody*

Sera	ELISA, positive/ total
Healthy individuals	0/117 (0%)
Melanoma	3/39 (8%)
- Clinical ALVAC therapy (patients with advanced stages)	2/22
- Conventional therapy (patients with advanced stages)	1/17
Ovarian cancer	0/128 (0%)
- Clinical trial for recurrent cancer (advanced stages)	0/49
- Pacific Ovarian Cancer Research Consortium, POCRC (patients with early stages or benign ovarian individuals)	0/79

In addition to sera from the two immunotherapeutic trials, spontaneous antibodies to hDlg were found at low percent, with 1 out of 17 positive in patients treated with conven-tional therapy. hDlg antibody appears to be specific to melanoma, based on our finding that about 8% of melanoma patients in our small number of melanoma samples had antibodies to hDlg, whereas the patients with ovarian cancer did not. Furthermore, antibodies to hDlg are very specific to cancer, because none of the 117 healthy persons

had antibodies against this protein. Importantly, this is the first time that hDlg was identified as a melanoma-associated antigen.

APPENDIX C

TSLC1 DOES NOT INTERACT WITH HDLG

TSLC1 does not interact with hDlg

As hDlg consists of multiple domains which interact with several cellular proteins, it is able to play roles through its binding partners in maintaining proper epithelial structure and in transducing signals that regulate cell growth and differentiation (1-6). The number of reports describing binding partners of hDlg has been rapidly growing (1-6).

TSLC1, a tumor suppressor gene in human cancers, became our interest in this aspect because its product was reported to directly associate with DAL-1 (7), another tumor suppressor protein containing a FERM domain that belongs to the protein 4.1 superfamily, (8). The protein 4.1 superfamily is known to anchor for several transmembrane proteins to the actin cytoskeleton through their FERM domains and their spectrin-actin binding domains (8, 9). Interestingly, 4.1R protein, one of the four distinct members of the protein 4.1 superfamily, was reported to play a critical role in recruiting hDlg to the lateral membrane in epithelial cells (1, 10). Moreover, TSLC1 was shown recently to interact directly with DLG3, one of the members of the MAGUK through its PDZ-interacting motif (11), and the DLG3 was previously reported to interact with the MRE domain of hDlg (2). From these findings, it was interesting to see whether the whole complex of hDlg and TSLC1 proteins would colocalize in normal cells but be disrupted in melanoma.

Immunofluorescence analysis of melanoma patient tumor sections to study colocalization of hDlg and TSLC1 showed that both proteins are cytoplasmic in these tumor samples, but there did not seem to be strong overlap in their localization (Fig. C1). Moreover, immunoprecipitation of normal cell extracts with anti-hDlg antibody could not

detect coprecipitated TSLC1, indicating that TSLC1 does not directly associate with hDlg (Fig. C2).

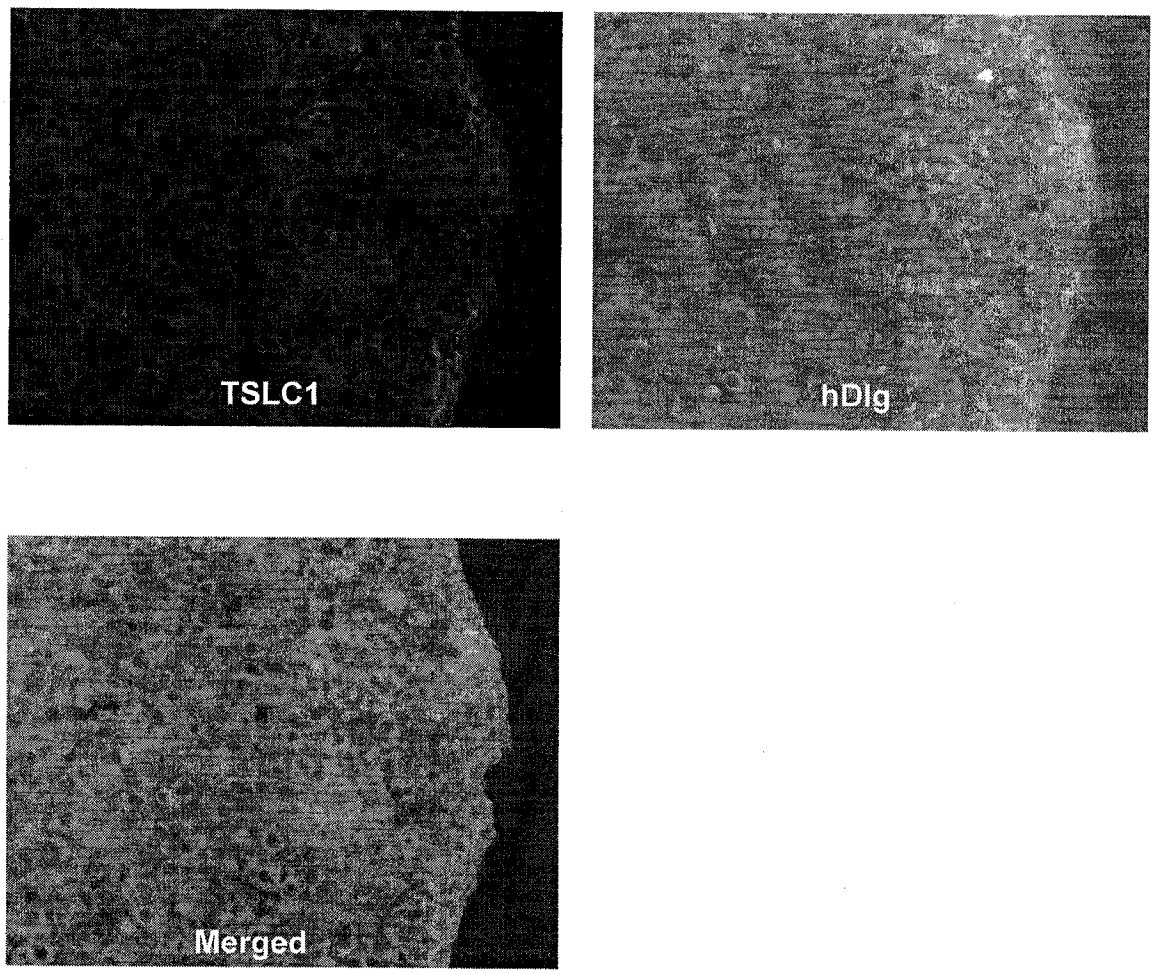

Fig. C1. Immunofluorescence analysis of TSLC1 and hDlg localization in melanoma tumor samples.

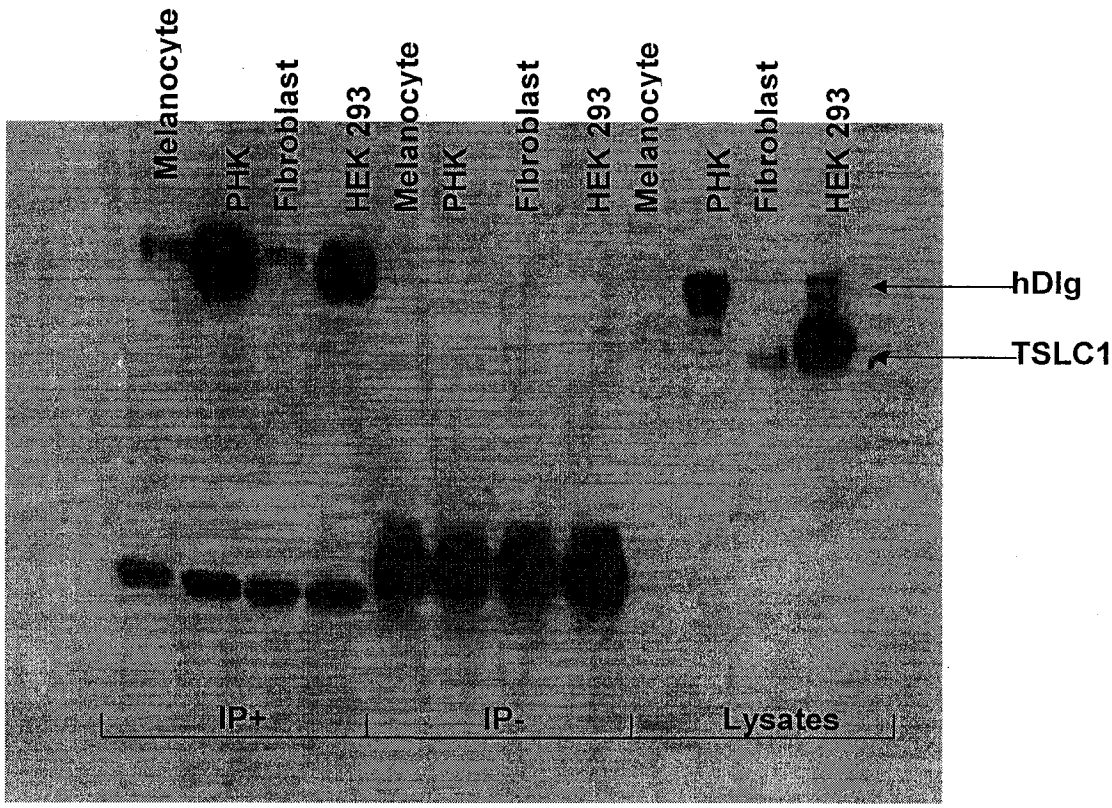

Fig. C2. Immunoprecipitation analysis of TSLC1 in human normal cells using anti-hDlg mAb (2D11). The whole cell extracts (Lysates), or the extracts that were immunoprecipitated with anti-hDlg mAb (IP+) or with normal IgG (IP-), were detected by Western blotting with anti-TSLC pAb (CC2) and also with 2D11 to show that hDlg was present in all tested normal lysates and immunoprecipitated by 2D11.

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UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

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Title of Dissertation Characterization of Novel Melanoma Associated Antigens

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