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Differences in cell cycle progression between human melanoma cell lines.

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1. INTRODUCTION

1.1 HUMAN MELANOMA

Cancer is a diverse group of diseases characterized by the uncontrolled growth and spread of malignant cell [1]. Self sufficiency of growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and finally tissue invasion and metastasis are considered as the six characteristic changes that occur in carcinogenesis [2]. In addition to these characteristics, avoidance of immunosurveillance is considered to be the seventh hallmark of cancer [3]. Cancer is primarily the result of genetic or epigenetic changes in normal cells that give them a growth advantage. It is also generally recognized that a series of genetic changes are required in order for the development of cancer ; a single mutation is not sufficient for a normal cell to escape all of the regulatory processes that restrict it to a normal growth pattern [4]. Melanoma probably is the most aggressive cancer in humans, and remains one of the leading causes of cancer death in developed countries [5]. The main etiological risk factor for the development of melanoma is UV radiation, although hereditary reasons play a notable role in the progression of melanoma. Pigmentary traits, such as red hair, fair complexion, and a tendency to freckle have been show as the main risk factor

for the development of melanoma [6]. Clinically, melanoma is classified according to the thickness in millimeters, mitotic rate, presence of ulceration, penetration depth, location of existing metastases. Also, increasing age, male sex, and tumor location on the trunk, head, or neck also worsen prognosis [7]. The resistance of melanoma to therapy and its recurrence are related to the genetic heterogeneity and genomic instability of the tumor. For many years these genetic alterations were thought to be linked to the accumulation of random mutations in functionally differentiated cells which transform them into malignant cells that have lost their ability to differentiate and have develop drug resistance. Therefore, available treatments can induce objective tumor regression in a small percent of patient, but these responses are not always associated with improved long-term survival [8].

1.1.1 Development of melanoma from melanocyte to metastatic melanoma

Melanoma is neoplasm of melanocytic origin having the most rapid increase in incidence in many countries comparable to other tumors [9]. The development of melanoma and its progression is described in six steps: benign or common nevi without dysplastic changes, melanocytic nevus with lentiginous melanocytic hyperplasia, dysplastic nevi, radial-growth phase (RGP), of primary melanoma, vertical –growth phase (VGP), of

melanoma and metastatic melanoma (fig. 1), [9-13]. Of not, RGP melanoma cells extent upward into the epidermis, but remain in situ and lack the capacity to invade the dermis and metastasize. VGP melanoma invades the dermis and deeper structures and is metastatically competent [14-16]. Once metastasis to lymph node occur, the 5-year survival ranges from 13% to 69%, depending on the number of lymph nodes affected tumor burden [17]. With visceral metastasis, the 5-year survival drops to approximately 6%, and the median survival from time of diagnosis is 7.5 months [18]. Infact, metastatic cells have a highly unstable phenotype and can rapidly adapt to selective pressure, allowing the cell to survive even under the most unfavorable circumstances.

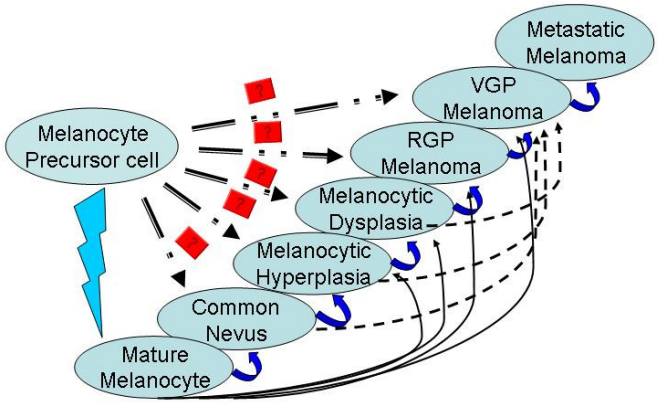

FIG. 1. The six steps of the development of melanoma and its progression. (1) Common acquired melanocytic nevus; (2) melanocytic nevus with lentiginous melanocytic hyperplasia; (3) melanocytic nevus with melanocytic dysplasia; (4) the radial growth phase of primary melanoma; (5) the vertical growth phase of primary melanoma; and (6) metastatic melanoma. The fact, that in 50% of all sporadic melanoma cases tumors arise without clinical precursor lesions, leads to the hypothesis that those lesions might derive directly from mature melanocytes (solid arrows) or melanocyte precursor cells(dashed arrows). Most melanomas arise within the epidermis (melanoma in situ) and then invade across the basement membrane region. There may in addition be rare melanomas that arise de novo—presumably from melanocyte precursor cells—in the dermis in a subset of nodular melanomas and other at least equally rare melanomas that arise in the dermis in association with a pre-existing congenital nevus(shown as dotted arrows from ''Common Nevus/Melanocytic Hyperplasia/ Melanocytic Dysplasia'' directly to ''VGP Melanoma'').[68,156]

1.1.2 Genomic instability in melanoma

Genomic instability and substantially altered cancer genomes are hallmark features of malignant neoplasms including melanoma [19,20]. Specifically, melanomas manifest extensive chromosomal rearramgements, such as translocation, chromosomal amplification, or deletion [21,22]. While such cumulative genomic alterations may lead to diverse melanoma

populations with differential cytogenetic abnormalities, uniform genomic characteristics between primary tumors and metastases form the same patient would suggest a clone progression of melanoma [23].The strongest genetic risk for the development of melanoma results from heritable alterations in cyclin-dependent kinase inhibitor 2A(CDKN2A) gene, which encodes two separate but related proteins, p16/INK4a and p14/ARF. These proteins help regulate cell division and apoptosis, both of which are necessary to maintain cellular homeostasis [24].Germline CDKN2A mutations were identified in 25-50% of familial melanoma kindreds. In sporadic primary melanoma, only a few mutations (0-25%) and homozygous deletions(10%) are found in this gene [25].However, this locus was found to carry UVB signature transversion in the sporadic primary melanomas , suggesting that UVB radiation may play a role in the etiology of melanoma development [26,27]. The importance of this locus in melanoma susceptibility was confirmed by studies showing that the penetrance of CDKN2A mutations significantly correlated with residence in a geographical location with a high population incidence rate of melanoma [28-31],and that CDKN2A mutation carriers have increased total nevus number and total nevus density- known risk factors for melanoma [32]. Other important genes include CDK4/6 and retinoblastoma (RB1), which encode downstream proteins in the same pathway as p16/INK4a and p14/ARF [23]. Oncogenic

mutations in either NRAS or BRAF are commonly associated with melanoma [33-35], and these mutations are also reported in 20-80% of melanocytic nevi [36-40]. Identification of individuals who may have a hereditary susceptibility for the development of melanoma is essential to provide on opportunity for primary prevention, and to target high risk groups for early diagnosis and treatment [41]. As the increase incidence of malignant melanoma with the completion of sequencing of the human genome, there have been increasing efforts to identify the "melanoma gene(s)".

1.1.3 Epidemiology of melanoma

Malignant melanoma continues to pose a substantial clinical challenge, and its risk may correlate with distinct skin pigmentation phenotypes. The protective role of melanin pigment indicated by incidence decrease of melanoma in black populations being 10 times less that white populations [40-42].In addition, epidemiological factors, such as intermittent ultraviolet radiation exposure concomitant with sunburns, particularly during childhood, significantly promote the susceptibility to melanoma [43,44]. The direct role of UVR in induction of squamous cells carcinomas (SCCs) and basal cell carcinomas (BCCs), clearly demonstrate by epidemiological

studies [41,45]. However, the relationship between sun exposure and melanoma is less evident. For example, melanomas can develop on sun protected areas of skin and in internal organs (for example in esophagus , colon, cervix),and they can be correlated with some genetic factors indicating that their induction and progression sometimes is not related with UV [46].Responses against environmental stresses including ultraviolet (UV) radiation are diverse in human skin phenotypes among racial/ethnic groups. It is well documented that black skin (alternatively called "African-American" or "dark" skin) is dramatically more resistant to the damaging effects of UV, including photocarcinogenesis and photoageing, that is white skin (alternatively called "Caucasian" or "light/fair"skin) [47-50]. Furthermore, the incidence of both melanoma and non melanoma skin cancers increases exponentially with age [51,52], the rate of increase with age is independent of the magnitude of risk due to the environmental carcinogen [53], this finding implies that age itself plays a major part in vulnerability to photo carcinogenesis. Ageing also influences that response to injury. In particular, there is an age associated decrease in the capacity to repair DNA [54,55], and a consequent increase in the rate of DNA mutations [56].

1.2 ULTRAVIOLET RADIATION

1.2.1 Definition and Characterization of UV radiations

UVR is located in the electromagnetic spectrum between the ionizing x-rays and the non-ionizing visible light (fig. 2). It spans a wavelength of 100-400 nanometers (nm) being non-ionizing and non-visible. Since biological effects of UVR vary greatly with wavelength, it has further been divided into the three subclasses: short wave ultraviolet (UVC; 200-280nm), mid-wave ultraviolet B (280-320nm) and long-wave ultraviolet A (UVA; 320-400 nm) [57,58]. Ultraviolet (UV) radiation has a few beneficial health effects like vitamin D3 formation or application in combination with drugs in the therapy of skin diseases including psoriasis and vitiligo, but it also causes many acute and chronic detrimental cutaneous effects, which may result in development of skin malignancies [59]. Although, UVR represents only a fraction of the solar radiation, it is responsible for the majority of its carcinogenic activity, UV photons can affects the DNA integrity, cell and tissue homeostasis, and induce mutation or affect expression of aplethora of genes including oncogenes and tumor suppressor gene [57,60-63].The ozone layer efficiently absorbs UV radiation up to about 310 nm thus it consumes all UVC radiation and most of UVB (95%). However, UVA is not absorbed at all [64]. Due to substantial damage to the protective ozone layer an increased amount of

UVB radiation is reaching the ground [65]. Moreover, UVB is about 20- fold less abundant than UVA, its energy is more efficiently absorbed by cellular molecules and is able to induced damages within cells and tissues at significantly lower doses than UVA [57,66]. Specifically, ultraviolet radiation causes genetic change in the skin, impairs cutaneous immune function, and induces the formation of DNA-damaging reactive oxygen species affects keratinocytes and melanocytes [67,68].

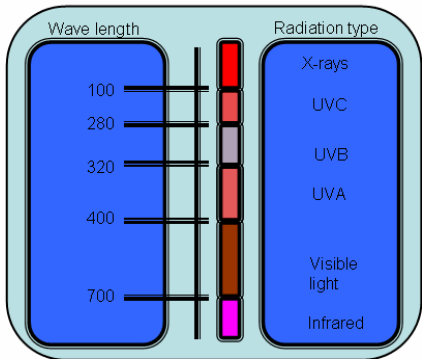

FIG. 2. Ultraviolet radiation is located in the electromagnetic spectrum between the ionizing x-ray and the non-ionizing visible light, spanning in the wavelength region of 100-400 nanometers. Ultraviolet radiation has been divided into the three subclasses: shortwave ultraviolet-C (UVC), mid-wave ultraviolet-B (UVB) and long-wave ultraviolet A (UVA) radiation [66].

1.2.2 UV damage and DNA repairs

The UV component of sunlight causes skin damage and increases the risk for skin cancers such as melanoma. It appears

that melanoma risk is typically associated with intermittent, intense sun exposure rather than cumulative sun exposure. The exact mechanism and wavelengths of UV light that are most critical remain controversial, but both UVA and UVB have been implicated [69,70]. To exert its biological effects, UV light energetic photons must be first transmitted through skin layers and absorbed by a cellular molecule (chromophore, photosensitizer). UV radiation induced damage via two different mechanism :(1) direct absorption of the incident light by the cellular components, resulting in excited state formation and subsequent chemical reaction, this kind of injury is typical for DNA bases (2). In direct, absorption include photosensitization mechanisms, where the light is absorbed by endogenous (or exogenous) sensitizers. The excited photo sensitizers can induce cellular damage by electron transfer and hydrogen abstraction processes to yield free radicals or energy transfer with O_2 to yield the reactive excited state, single oxygen [71]. When UV-induced mutation affect critical genes encoding protein or enzymes contributing to DNA repair, cell cycle control or apoptosis, it is likely that cumulative or subsequent DNA alteration are not sufficiently eradicated. Disrupted function of such regulative proteins are strongly connected with early stages of skin carcinogenesis [72]. Thus, UV- fingerprint mutations can be abundantly detected in the well characterized and pathogenically important tumor suppressor gene p53 from

squamous and basal cell carcinoma of human skin [73,74]. More recent observations suggest that another DNA repair system the methyl-derived mammalian mismatch repair (MMR) may also be attributable to the multistep tumorigenesis of UV- associated skin cancer. Microsatellite instability (MSI), caused by replication errors of small repetitive DNA sequences can be detected in epithelial and melanotic skin tumors [75], and is characterized by length changes at those repetitive loci scattered throughout the genome [76,77]. Tumor cells that display MSI are typically defective in posttranscriptional MMR providing a direct link between insufficient mispairing DNA repair and genetic instability [78,79]. Concomitant replication errors in different tumor suppressor and growth regulatory genes are supposed to the genetic mechanism of tumorigenesis in those cells. Mutations in MMR genes are etiologically responsible for hereditary nonpolyposis colon cancer. Functional MMR alterations are furthermore associated with visceral malignancies and the occurrence of sebaceous skin tumor, keratoacanthomas and less frequently squamous cell carcinomas in the rare autosomal dominant. Muir- Torre syndrome . Underlying mutations were found in the hMSH2 and hMLH1 gene [80-82]. The most important protein involved in early UV- induced carcinogenesis of SCC appears to be the tumor suppressor p53. p53 is an essential and well defined transcription factor regulation cell cycle control and apoptosis

[83]. UV specific p53 mutations can be found in 75-80% of AK and in more than 90% of cutaneous squamous cell carcinoma [72,73,84]. These UV- fingerprint mutations of p53 mutations appear to correspond predominantly to UVB radiation, as UVA-induced carcinomas in hairless mice reveal p53 mutations only in 15% [85]. Recent data suggest that the MMR protein hMMR protein hMSH2 is a novel p53 regulated target gene indicating a direct involvement of p53 in DNA repair mechanisms [86]. Moreover, both p53 and MMR proteins reside in a large multisubunit complex of tumor suppressors, DNA damage sensors, and signal transducers named BASC for BRCA1-associated genomic surveillance complex.

1.3 MISMATCH REPAIR GENES

Mismatch repair genes present several functions relating to genetic stabilization, such as correcting errors in DNA synthesis, ensuring fidelity of genetic recombination or participating in the initial steps of apoptotic responses to different classes of DNA damage[87]. Since the discovery of the major human genes with DNA mismatch repair function, mutations in five of them have been correlated with susceptibility to Lynch syndrome: mutS homolog 2 (*MSH2*); mutL homolog 1 (*MLH1*); mutS homolog 6 (*MSH6*); postmeiotic

segregation increased 2 (*PMS2*); and postmeiotic segregation increased 1(*PMS1*) [88]. Germline abnormalities in *MLH1* and *MSH2* genes are found in more than 90% of HNPCC mutation carriers [90], 50% relating to hMLH1, 40% to hMSH2 and 10% distributed among the others [89]. The DNA mismatch repair (MMR) system play a critical role in maintaining genomic integrity in both prokaryotes and eukaryotes[92].Together with base-base mismatch, which are caused by errors of DNA polymerases that escape their proofreading function, IDLs (insertion/deletion loops) are addressed by the mismatch repair (MMR) system, which degrades the error- containing section of newly synthesized strand and therefore provides the DNA polymerase with another chance to generate an error- free copy of the template sequence. In the absence of MMR, IDLs , and base-base mismatches remain uncorrected, which results in a mutator phenotype that is accompanied by MSI and eventually, in cancer [93]. The MMR machinery has to satisfy two criteria: first, it must efficiently recognize bases- base mismatches and IDLs, second, it must direct the repair machinery to the newly synthesized DNA strand, which carries the erroneous genetic information. How these tasks are fulfilled was first elucidated in *E.coli*, where studies of the mutator strains mutS, mutL, mutH and uvrD culminated in the reconstitution of this prototypic MMR system from individual purified components [94]. The situation in eukaryotes is more complex than in *E.coli*. Of the

five MutS homologues (MSH) that have been identified in human cells, hMSH2, hMSH3 and hMSH6 participate in MMR in the form of heterodimers [95]. The most abundant mismatch binding factor is composed of hMSH2 and hMSH6. This factor, which is often to as MutSα , initiates the repair of base-base mismatches and IDLs of one or two extra helical nucleotides [96-98],whereas the repair of larger IDLs is initiated by MutSβ , which is a heterodimer of hMSH2 and hMSH3. However, hMSH4-hMSH5 operates only in meiosis1 and has no role in MMR [91]. The MutL proteins are ATPases of the GHKL (gyrase/Hsp90/histidine-kinase/MutL) family [99],with the ATPase situated in the N-terminal domain and the dimerization domain at the C-terminus. The complex that is composed of hMLH1 and hPMS2-Mutlα- has the most important role in the MMR, as cells that lack either protein exhipt mutator phenotypes and MSI that is comparable to cells that are mutated in hMSH2 [93,100]. MutLβ ,which is composed of hMLH1 and hPMS1, could conceivably fulfill this function. Although this heterodimer could not be shown to participate in MMR in vitro. Similarly, MutLγ, which is composed of hMLH1 and hMLH3, and which has hitherto been believed to predominantly involved in meiotic recombination, might also have a backup role in mammalian MMR [101,102,90].

1.4 TUMOR SUPPRESSOR GENES

Tumor suppressor genes are normal genes that slow down cell division, repair mistakes, and tell cells when to die. When tumor suppressor genes don't work property, cells can grow out of control, which can lead to cancer. Damage to tumor suppressor genes contributes to a large number of different types of tumors. Mutation in tumor suppressor genes can arise spontaneously by exposure to a mutagenic substance such as ultraviolet or certain chemicals. In such cases, only the mutated cell and its descendants will be affected, Mutations can also be inherited from a parent or arise early in development, In these case, almost all the cells of the body will inherit the same mutation. A mutation in a single tumor suppressor gene is usually not enough to cause cancer. This is because each cell contains two copies of each gene, one inherited from each parent. Most cancer causing mutations cause a loss of function in the mutated gene. Often, having even one functional copy is enough to prevent disease, and two mutations are needed for cancer to develop. This is known as the "two-hit" model of carcinogenesis. There are a growing number of genes that have been identified as having some function as function as tumor suppressor gene (es.P53,BRCA1,BRCA2,APC and RB1)[103,104]. In fact, tumor suppressor genes participate in a variety of critical and highly conserved cell function, including regulation of the

cell cycle and apoptosis, differentiation, surveillance of genomic integrity and repair of DNA errors, signal transduction, and cell adhesion. Moreover, Tumor suppressor genes can be separated into 2 major categories: gatekeepers and caretakers. Gatekeepers directly inhibit tumor growth or promote tumor death. Inactivation of these genes contributes directly to cancer formation and progression, while inactivation of caretakers does not directly promote the growth of tumor. Rather, inactivation of caretakers leads to genetic instability that indirectly promotes growth by causing an increased mutation rate [104]. The increase in genetic instability greatly accelerates the development of cancer. Thus, mutation of caretaker genes lead to accelerated conversion of a normal cell to a neoplastic cell [105].

1.4.1 p53 gene

The p53 tumor suppressor belongs to a small family of related proteins that includes two other members p63 and p73[106]. Although structurally and functionally related, p63 and 73 have clear role in normal development, whereas p53 seems to have evolved in higher organisms to prevent tumor development. p53 is activated in response to several malignancy-associated stress signals, resulting in the inhibition

of tumor-cell growth [107,108]. Several responses can be provoked by p53, including cell-cycle arrest, senescence, differentiation and apoptosis, with the option chosen being dependent on many factors that are both intrinsic and extrinsic to the cell. P53 also contributes to the repair of genotoxic damage, potentially allowing for the release of the rehabilitated cell back into the proliferating pool [109]. However, p53 function in cancers can be lost various mechanisms, including lesions that prevent activation of p53,mutations within the TP53 gene (which encodes p53) itself or mutations of downstream mediators of p53 function. Analysis of many tumors has shown that TP53 is mutated in about half of all cancer, resulting in loss of apoptotic function. From the data available, it would seem that only 5% of TP53 mutation are found in the regulatory domains (amino terminus, amino acid 1-99; carboxyl terminus, amino acid 301-393). Whereas 95% of the mutation occur in the central region of TP53, which is responsible for sequence-specific DNA binding (amino acid 100-300) (Fig. 3) [110,111].

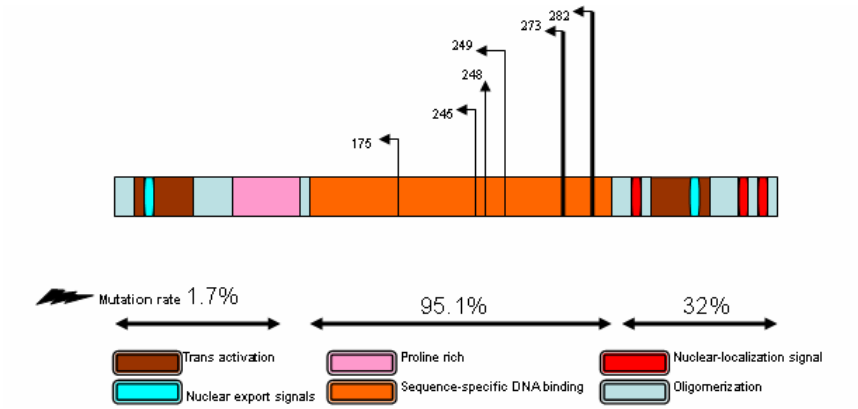

FIG.3. p53 structure and location of tumor-associated mutation. p53 is a transcription factor that contains several well-defined domains core and a carboxyl-terminal region that contains oligomerization sequences and nuclear-localization signal. Nuclear export of p53 regulated by signals in the amino and carboxyl termini. Interaction of proteins such as MDM2 or p300/CBP with the amino terminus of p53 can lead to modification such as acetylation or ubiquitylation in the carboxyl terminus.

Mutations of the p53 gene have been reported in more than 50% of all cancer types [112,113], and in approximately 20% of melanoma cell line [114,115], and 5% of metastatic melanoma [116]. Also less than 1% of primary melanoma [117]. Also there are multiple of evidence support that: (1) p53 is the most commonly mutated gene in human cancer, (2) individual who inherit a p53 mutation are highly tumor prone , usually developing malignancy during childhood or a young adult [118]. This suggests that p53 mutation may not be the major underlying cause in the development of melanoma, but instead have a role in the progression and invasiveness of this cancer

type [119]. p53 is a transcription factor that directly activates the expression of genes that contain p53-binding sites within their regulatory regions [120-123]. However, the principal role of p53 is in the induction of the apoptotic cascade. In addition to inducing genes that drive apoptosis,p53 can also activate the expression of genes that inhibit survival signaling,(Fig.4), and can repress gene expression and act independently of the regulation of transcription-functions that have also been implicated in the induction of the full apoptotic response.

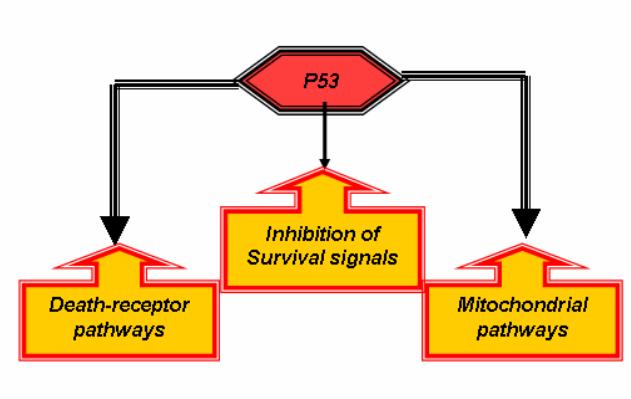

FIG. 4. Several apoptotic pathways are activated by p53. p53 can induced the expression of numerous apoptotic gene that contribute to the activation of both death-receptor and mitochondrial apoptotic pathways. P53 can also affect the efficiency of survival signaling

Detailed studies of tumor–derived p53 mutants showed that the tumor-suppression function of p53 is best correlated with its ability to induce apoptosis, and that the ability of p53 to

repress gene expression is tightly linked to this function [124-127]. Although p53 can be a potent activator of cell death, induction of p53 dose not necessarily initiate a full apoptotic a full apoptotic response [109,128].

1.4.2 BRCA1 gene

The BRCA1 tumor suppressor gene encodes a phosphoprotein involved in many cellular key function including DNA repair, transcription regulation, cell-cycle control and apoptosis. Germline mutations in BRCA1 are present in nearly 50% of inherited breast cancer cases, and the acquisition of a single defective allele leads to an elevated predisposition to both breast and ovarian cancer [129,155,156]. Evidence suggests that BRCA1 may also be mutated in some sporadic breast cancer tumors [130].A common feature of cancer cells is a profound increase in genome instability. Accordingly, cells defective for BRCA1 exhibit elevated levels of chromosome aberrations, such as DNA breaks and chromatid exchanges, enhanced sensitivity to agents that damage DNA and defects in cell-cycle checkpoint function. Such changes are often associated with defects or loss of proteins involved in the detection and repair of DNA damage. It has therefore been proposed that BRCA1 functions in the DNA-damage response as a caretaker of

the genome [131]. Since its discovery, many studies have addressed the function of BRCA1 with a view to understanding how it contributes to the maintenance of genome stability and how defects in this process result in cancer progression. These studies have led to a series of discoveries that implicate BRCA1 in a multitude of different cellular processes. For example, there is now a large body of evidence supporting a direct role for BRCA1 in the repair of DNA damage by HR (homologous recombination). Not only is BRCA1 recruited to sites of DNA damage where it co-localizes with other proteins involved in the repair of DNA DSBs (double strand breaks) by HR, such as BRCA2 and Rad51 [132], but cells lacking functional BRCA1 are highly impaired for the homology-directed repair of defined DSBs, introduced into the genome by a restriction endonuclease [133]. BRCA1 has also been shown to be required for the activation of both S- and G2/M-phase cell-cycle arrest after DNA damage, the latter being dependent on prior phosphorylation of BRCA1 by the master checkpoint kinase ATM (ataxia telangiectasia mutated) [134]. Furthermore, BRCA1 is thought to be involved in transcriptional regulation. BRCA1 not only associates with RNA polymerase II [133], but also has been shown to activate the transcription of several genes. Finally, BRCA1 has been implicated in two types of gene silencing: the inactivation of the X chromosome, and meiotic sex chromosome inactivation [135,137].

The BRCA1 gene is organized in 24 exons encoding a protein of 1863 amino acids. Whereas conservation of the BRCA1 amino acid sequence varies among species, the N- and C-termini of the protein are highly conserved from nematode worms to humans [136]. These regions comprise two recognizable domains in BRCA1: a RING (really interesting new gene) finger domain at the N-terminus, and two C-terminal BRCT (BRCA1 C-terminal) domains (first identified in BRCA1, but subsequently found in a variety of DNA-repair proteins). Both domains mediate interactions with other proteins that may be important for BRCA1 function. In fact, BRCA1 associates with a myriad of different proteins , whose interactions are not limited to the RING and BRCT domains. These proteins include, (1) component of the basal transcription machinery, such as, RNA helicase A and RNA pol II [138,139], (2) generalized transcriptional coactivators, such as, p300, CBP, Brg1 [140,141], and corepressor, such as, RbA p46,RbAp48,histone deacetylases-1,2, and CtIP [142,143], (3) tumor suppressors, such as, p53, RB1, BRCA2 [144,145], (4) steroid hormone receptors estrogen receptor-α , and androgen receptor [146], (5) DNA repair proteins, such as, Rad50, Rad51, MSH2 and MLH1 [147-149], (6) other sequence-specific transcription factors, such as, c-Myc, Oct-1, and NF-YA [150,151], and (7) cell cycle regulatory proteins, such as BARD1, E2F1, cyclins [152,153]. These interactions are summarized in (Fig. 5). [154].

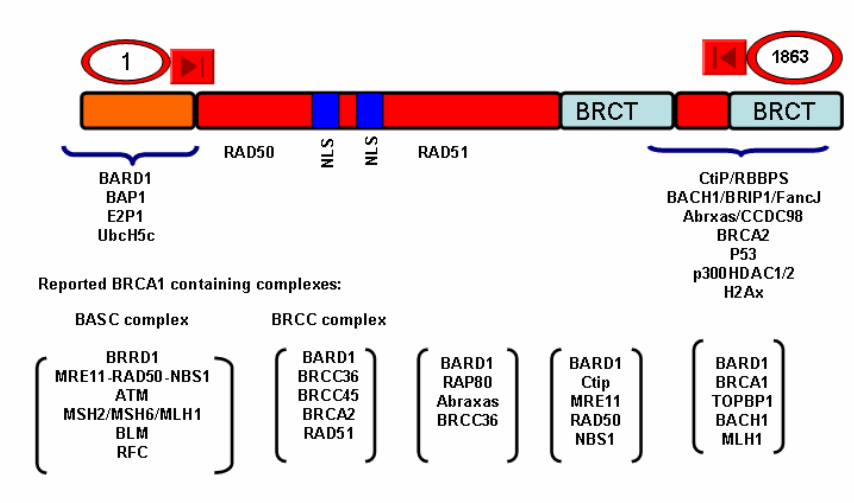

Fig. 5. BRCA1-interacting proteins. Structure of full-length human BRCA1 protein. Colored rectangles represent the previously characterized RING and BRCT domains, as well as two nuclear localization signals (NLS). BRCA1-binding partners are annotated below the domain in which the interaction is known to occur. Reported BRCA1-containing complexes are annotated at the bottom. BAP1, BRCA1-associated protein 1; BACH1/BRIP1, BRCA1-interacting protein-associated C-terminal helicase 1; BASC, BRCA1-associated genome surveillance complex; BLM, Bloom's syndrome protein; BRCC, BRCA1/BRCA2-containing complex; MLH, MutL homologue; MSH, MutS homologue; RBBP8, pRb-interacting protein 8; RFC, replication factor C; TOPBP1, topoisomerase II-binding Protein 1.

Although BRCA1 has been shown to be involved in a large variety of processes and make many different physical interactions, there is very little mechanistic detail addressing its molecular function. Since each of these domains may have distinct structural and biochemical characteristics, it conceivable that BRCA1 might perform different functions in each one of them[154]. However, the identification of several large complexes in which BRCA1 resides with many other DNA-

repair-associated proteins provides many avenues for future discovery.

2. Material and Methods

2.1 Cell Culture

Human melanoma cells, Colo38 and SK-MEL28, p53 mutant, and SK-MEL93 were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin (Sigma) at 37°C in a 5% CO_2/95% air atmosphere.

HEK293T (Human Embryonic Kidney cells) were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% FBS and 1% streptomycin/penicillin.

2.2 RNA Interference

The siRNA duplexes were synthesized by Sigma. The sequence targeting BRCA1 gene was :5'-ccggccctaagtttacttctctaaactcgagtttagagaagtaaacttagggttttt-3'. The non-silencing siRNA was purchased from Sigma and used as mock controls. The schematic representation of lentiviral vectors (pLK0.1/$^{empty\ vector}$, pLK0.1/$^{non\text{-}target}$shRNA, pLK0.1/BRCA1$^{3'UTR}$shRNA and pLK0.1/$^{Turbo\ GFP}$) utilized are represent in Fig. 6, Fig. 7, Fig. 8 and Fig. 9, and show the

locations of major identifiable landmarks on DNA like restriction enzyme sites, gene of interest, plasmid name and length, etc.

2.3 Preparation of lentiviral supernatants and transduction of SK-MEL93 cells

5 x 10^6 293T cells were grown on 10-cm plates to 70-80% confluence and co-transfected with 10 µg siRNA lentiviral DNA (pLK0.1/$^{empty\ vector}$, pLK0.1/BRCA1$^{3'UTR}$shRNA) (Sigma-Aldrich), 2 µg VSV-G plasmid DNA and 18 µg packaging viral CMV delta 8.9 plasmid, using the calcium phosphate precipitation method. After the addition of fresh culture medium 8 hr later, the cells were cultured for an additional 2 days. The medium was harvested 48 hr post-transfection, and filtered through a 0.45 µm filter. The supernatants from 293T cultures were used to cross-transduce SK-MEL93 cells in the presence of 8 µg/ml polybrene (Sigma-Aldrich) and subsequently clones were selected by puromycin (1µg/ml) (Sigma-Aldrich) for an additional 2 days and treated with UV-B. The transduction efficiency was calculated by the green fluorescent protein (GFP) expression and was observed under a fluorescent microscope. The efficiency of RNA interference was monitored by Western blotting analysis. Fig. 10 summarizes

schematic representation of the preparation of lentiviral supernatants and transduction of SK-MEL93 cells.

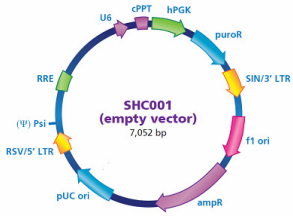

Fig. 6

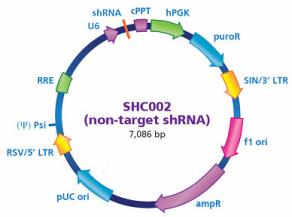

Fig. 7

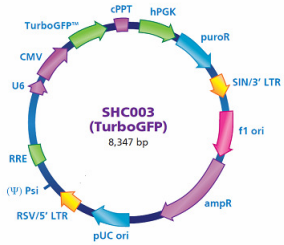

Fig. 8

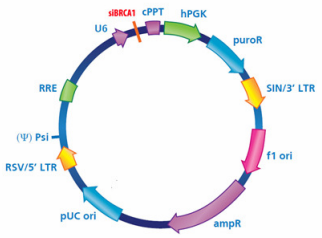

Fig. 9

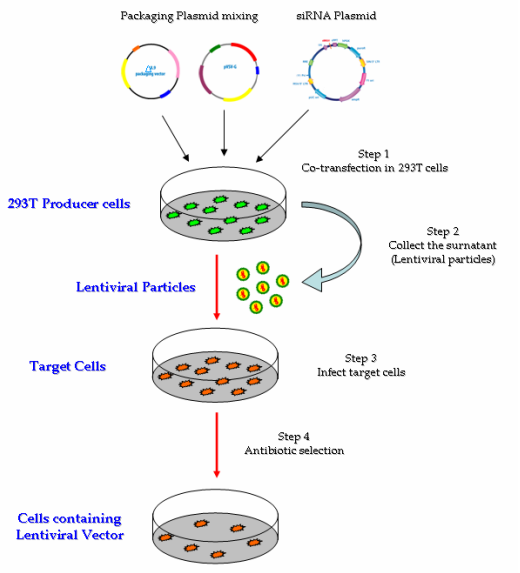

Fig. 10

2.4 UV-B irradiation

Different human melanoma cell lines (Colo38, SK-MEL93, SK-MEL28, SK-Mel93/^{sh}BRCA1 and SK-Mel93/^{sh}plKO.1) were cultured for triplicate experiments. Media was removed from 70 to 80% confluent cell cultures, cells were rinsed with phosphate-buffered saline and exposed to UV-B (230V, 50Hz) using a Vilber Lourmat, FLX-35M at indicated doses. Medium was added immediately to continue culture until designated time points. Cells at 0hr were a non-irradiation control.

2.5 Preparation of protein extracts

For preparing whole-cell extracts, cells were washed in ice-cold PBS, harvested, and re-suspended in whole-cell extract buffer (50mM Tris-HCl; pH 8, 150mM NaCl, 1mM EDTA, 1mM DDT, 1mM PMSF, proteinase inhibitor Complete; Roche, Mannheim, Germany). After sonication on ice (two times for 10 seconds) the homogenates were centrifuged (10.000 g, 10 min at 4°C), and the clear supernatants were stored at -80°C. Protein concentrations were determined using the Bradford method (Bradford, 1976). Bradford reagent (200 µl; 0.01% G240 brilliant blue (Saba), 5% ethanol, 10% H_3PO_4, 85% dH_2O) was added to 10 µl of a 1: 10 dilution of the protein extracts. Following 15 min incubation in the dark, the absorption was measured by photometry at 595 nm. The protein concentration was

determined using a calibration curve with BSA protein, taken in parallel.

2.6 Western blot analysis

Samples of 40-80 µg of protein total extracts were separated on a 6 or 8% SDS-polyacrylamide gel. Separated protein were blotted onto a nitrocellulose transfer membrane (Sigma) in a Bio-Rad blot cell for 2 h at 50Volt using buffer consisting of 25mM Tris-HCl, 192mM glycine. The membranes were blocked for 1 h at room temperature in 5% (wt/vol) milk powder in TBS (150mM HCl, 20mM Tris pH 7.6) containing 0.1% Tween 20 (TBS–Tween) and incubated overnight at 4°C with the primary antibody (1 : 200) in 5% (wt/vol) milk powder or BSA in TBS–Tween. The membranes were washed three times for 10 min in TBS–Tween each, incubated for 1 h with a horseradish-peroxidase coupled secondary antibody (dilution 1:5000-1:10000) (Santa Cruz Biotechnology) in TBS–Tween and washed again three times for 10 min in TBS–Tween. For developing the membranes, a chemiluminescence detection system (Santa Cruz Biotechnology, Heidelberg, Germany) was used. The antibodies used were anti- BRCA1 (C-20), anti-p53 (Bp-53-12), anti-MLH1 (H-300), anti-MSH2 (N-19) and γ-tubulin (Santa Cruz Biotechnology, Heidelberg, Germany).

2.7 RNA extraction and semiquantitative reverse transcription-PCR.

Total RNA extraction for semiquantitative reverse transcription-PCR (RT-PCR) was done from three different human melanoma cell lines (Colo38, SK-MEL93 and SK-MEL28) at 80% to 90% confluence with TRIzol reagent (Life Technologies) according to the manufacturer's protocol. A total of 5 µg DNase-treated RNA was reverse transcribed into first-strand cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexanucleotide primers. cDNA (2 µL) was amplified for BRCA1, hMSH2, hMLH1 and p53 genes with the following primers:

BRCA1: Forward 5′-ggcaacttattgcagtgtg-3′
Reverse 5′-tccccatcatgtgagtcatc-3′.
hMSH2: Forward 5′-gccattttggagaaaggaca-3′
Reverse 5′-ctcacatggcacaaaacacc-3′.
hMLH1: Forward 5′-gctgatgttaggacactacc -3′
Reverse 5′-aggaattggagcccaggagc -3′.
p53: Forward 5′-cggacgatattgaacaatg -3′
Reverse 5′- ggaactgttacacatgtag -3′.

A human glyceraldehyde 3-phosphate dehydrogenase cDNA fragment was amplified as the internal control for the amount of cDNA in the PCR with the following primers:

GAPDH: Forward 5′-tgatgacatcaagaaggtggtgaag-3′
Reverse 5′-tccttggaggccatgtgggccat-3′

Following nested PCR amplification, the products were separated by agarose gel and stained by ethidium bromide.

2.8 Flow cytometry

Cells, were collected by centrifugation at 200g for 10 min and fixed with 70% ethanol at + 4°C for 24h. The cell cycle was evaluated by flow cytometry using propidium iodide (50 □g/ml) staining (Sigma), after prior incubation with 13 k-units/ml RNase, (Sigma) (20 min incubation at 37°C) on a FACS-Calibur flow cytometer (Becton-Dickinson). A total of 30000 events were evaluated using the ModFit LT 3.0 Programme.

3. Results

3.1 Effect of UVB-irradiation in three human melanoma cell lines: Colo 38, SK-MEL93 and SK-MEL28.

Recently, in vitro and in vivo laboratory investigations have shown that the DNA repair system modulates in melanoma cells UVB-induced DNA repair, cell cycle progression and apoptosis (159). To investigate the susceptibility of melanoma to UVB-irradiation, we used three human melanoma cell lines (Colo38, SK-MEL93 and SK-MEL28).

We first, examined the expression of BRCA1, hMSH2, hMLH1 and p53 proteins by Western Blot analysis in Colo38, SK-MEL93 and SK-MEL28 cell lines. As shown in Fig. 11, we found equal amounts of BRCA1 and hMLH1 proteins in the three cell lines. Conversely, hMSH2 protein was over-expressed in Colo38 cell line, while p53 protein was over-expressed in Colo38 and SK-MEL28 cell lines. Moreover, BRCA1, hMSH2, hMLH1 and p53 over-expression was assessed by RT-PCR carried out on an aliquot of RNA done from Colo38, SK-MEL93 and SK-MEL28 cell lines. As shown in Fig. 12, two independent assays confirmed the results obtained by Western Blot analysis. Glyceraldehyde 3-phosphate dehydrogenase cDNA was used as control of quantify and quality of RNA preparation.

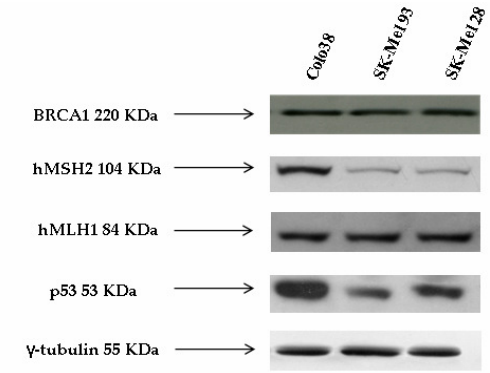

Fig.11. Western blot analysis of BRCA1, hMSH2, hMLH1 and p53 in different melanoma cell lines. γ-tubulin immunoblot analysis was performed to ensure equal levels of protein loading.

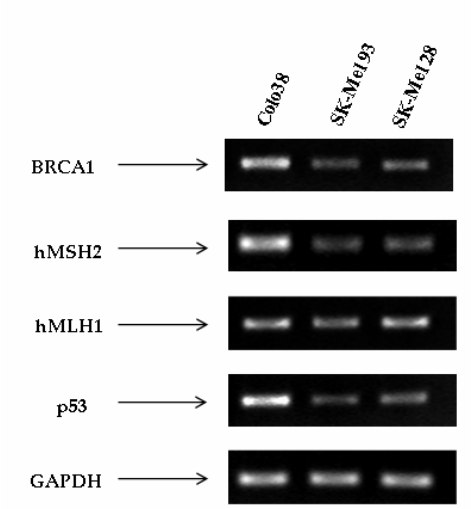

Fig. 12. Reverse transcription-PCR was performed on total RNA from different melanoma cell lines. Following nested PCR amplification, the products were separated by agarose gel and stained by ethidium bromide. Amplification of GAPDH cDNA served as a control for quantity and quality of RNA preparations.

Next, we exposed Colo38, SK-MEL93 and SK-MEL28 cells to 10mJ/cm^2 UV-B-irradiation and examined the expression of BRCA1, hMSH2, hMLH1 and p53 proteins by Western Blot analysis at different time (0,30-24 h). As shown in Fig 13, 14 and

15 we found equal amounts of hMLH1 protein in the three cell lines. Similar results, with equal amounts of BRCA1, hMSH2 and p53 proteins, were observed with the cell lines Colo38 and SK-MEL28 (Fig. 13 and 14). Conversely, the expression of BRCA1, hMSH2 and p53 proteins, in the SK-MEL93 was found increased at 3h after UV-B-irradiation (Fig. 15).

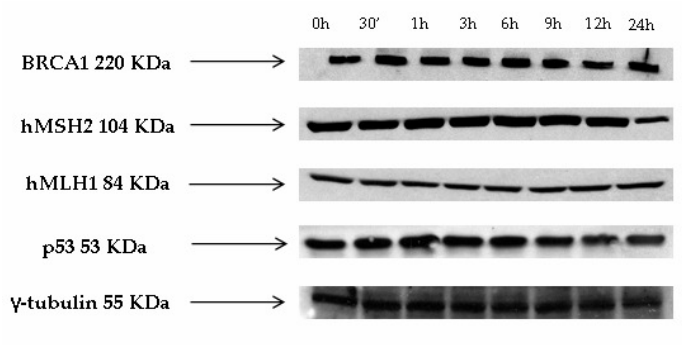

Fig 13. Western Blot analysis of BRCA1, hMSH2, hMLH1 and p53 protein levels in Colo38 cells after treatment with UVB 10mJ/cm^2, for the indicated time periods. γ-tubulin was used as control for loading.

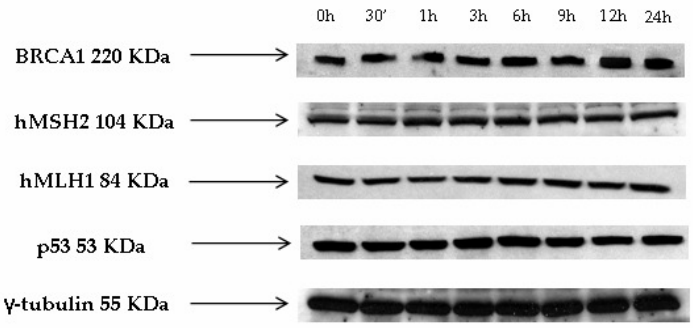

Fig 14. Western Blot analysis of BRCA1, hMSH2, hMLH1 and p53 protein levels in SK-Mel28 cells after treatment with UVB 10mJ/cm^2, for the indicated time periods. γ-tubulin was used as control for loading.

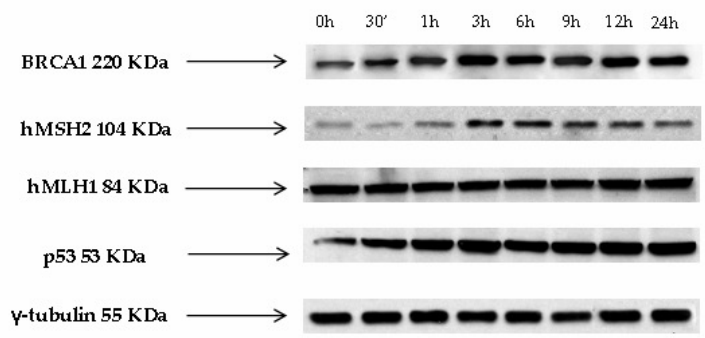

Fig 15. Western Blot analysis of BRCA1, hMSH2, hMLH1 and p53 protein levels in SK-Mel93 cells after treatment with UVB 10mJ/cm^2, for the indicated time periods. γ-tubulin was used as control for loading.

3.2 Effect of UV-b-irradiation on cell cycle progression in Colo38, SK-MEL93 and SK-MEL28 cell lines.

To evaluate the effect of UV-B on the cell cycle of each cell lines (Colo38, SK-MEL93 and SK-MEL28), DNA content was serially observed after UV-B-irradiation (10mJ/cm^2) by flow cytometry. As show in Fig. 16 Panel A, Colo38 cells seemed to be synchronized at the G1/S boundary phase until 12h after 10mJ/cm^2 UV-B-irradiation. From 12 to 24h a decrease in the fraction of S cells and a reciprocal increase of cells in Sub-G1 phase was observed. Similar comportment was observed in the SK-MEL28 cell line (Fig. 16 Panel C) although did not seemed to be synchronized and the increase of cells in Sub-G1 phase was observed already to 9h after UVB irradiation. While, the SK-MEL93 cell line did not seemed to be synchronized but a rapid

increase (about 35%) of cells in Sub-G1 phase was observed already 6h after UVB irradiation (Fig.16 Panel B).

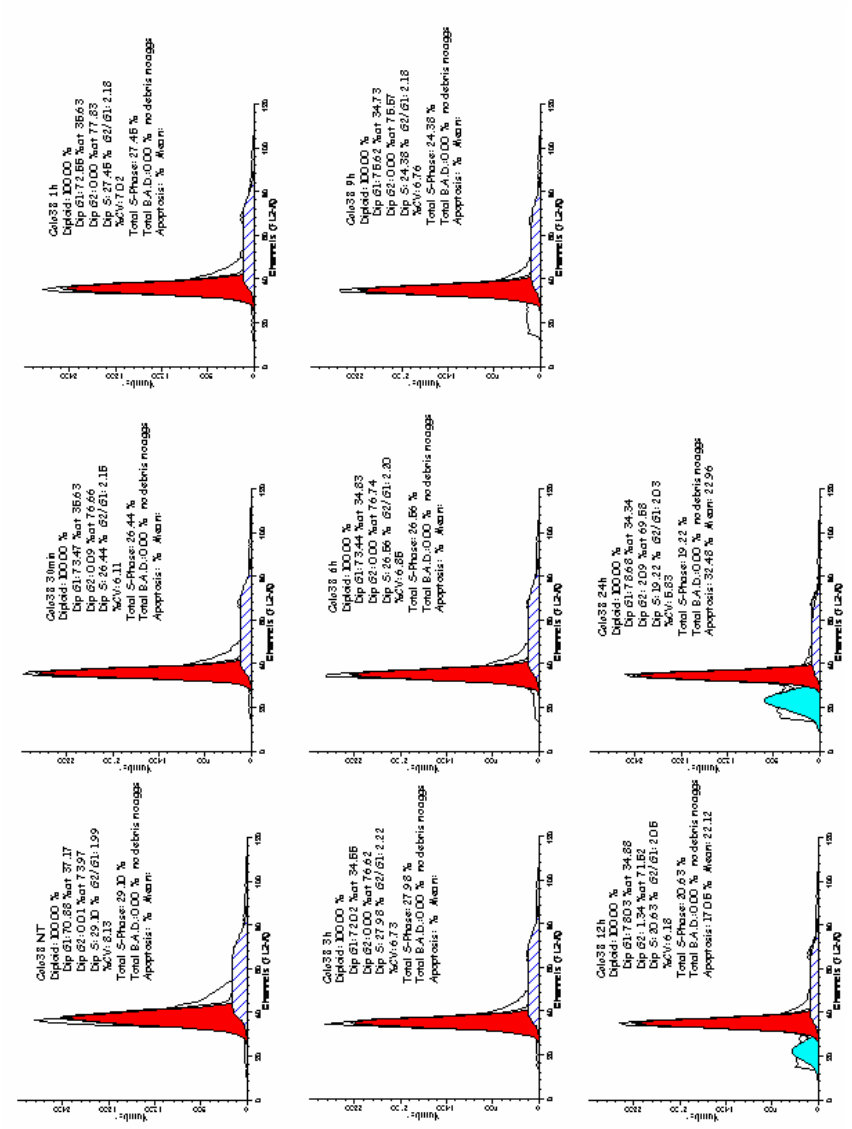

Fig. 16 ***Panel A.*** Cell cycle analysis of Colo38 cell line after exposure to UVB damage (10mJ/cm^2). Data were analyzed with ModFit LT 3.0 software.

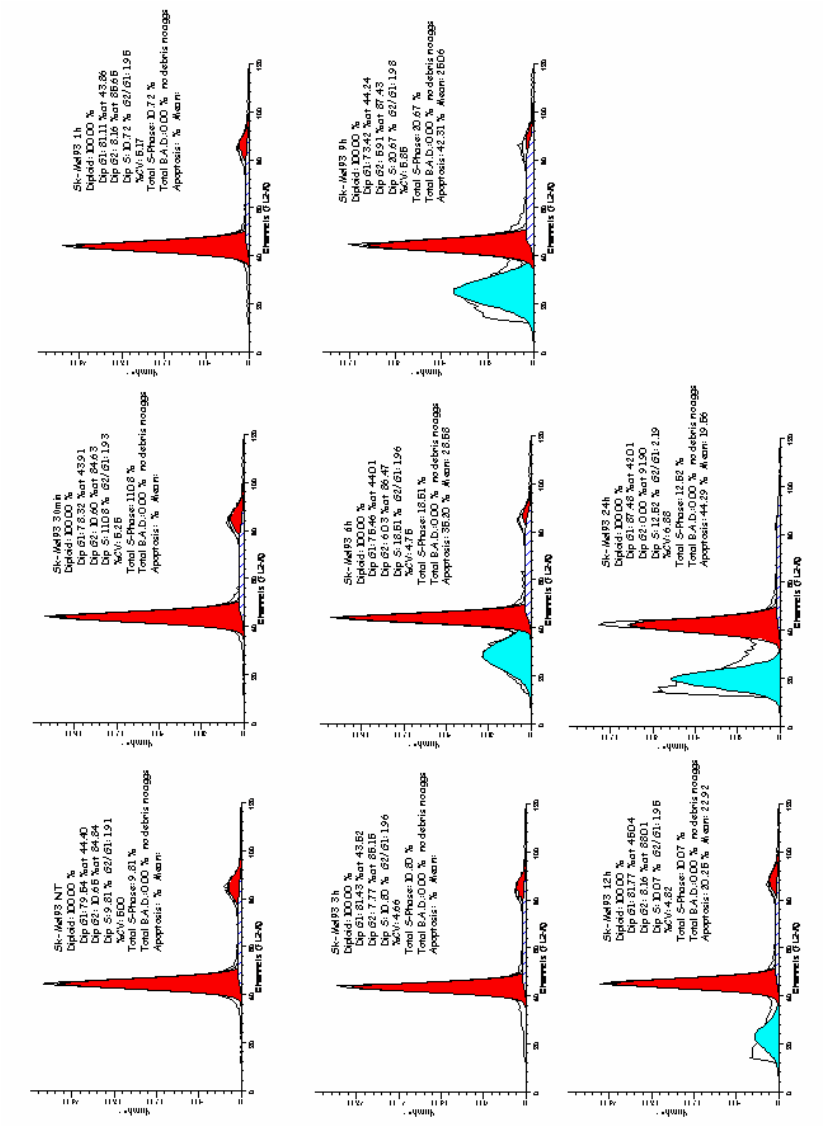

Fig. 16 ***Panel B*** Cell cycle analysis of SK-Mel93 cell line after exposure to UVB damage (10mJ/cm^2). Data were analyzed with ModFit LT 3.0 software.

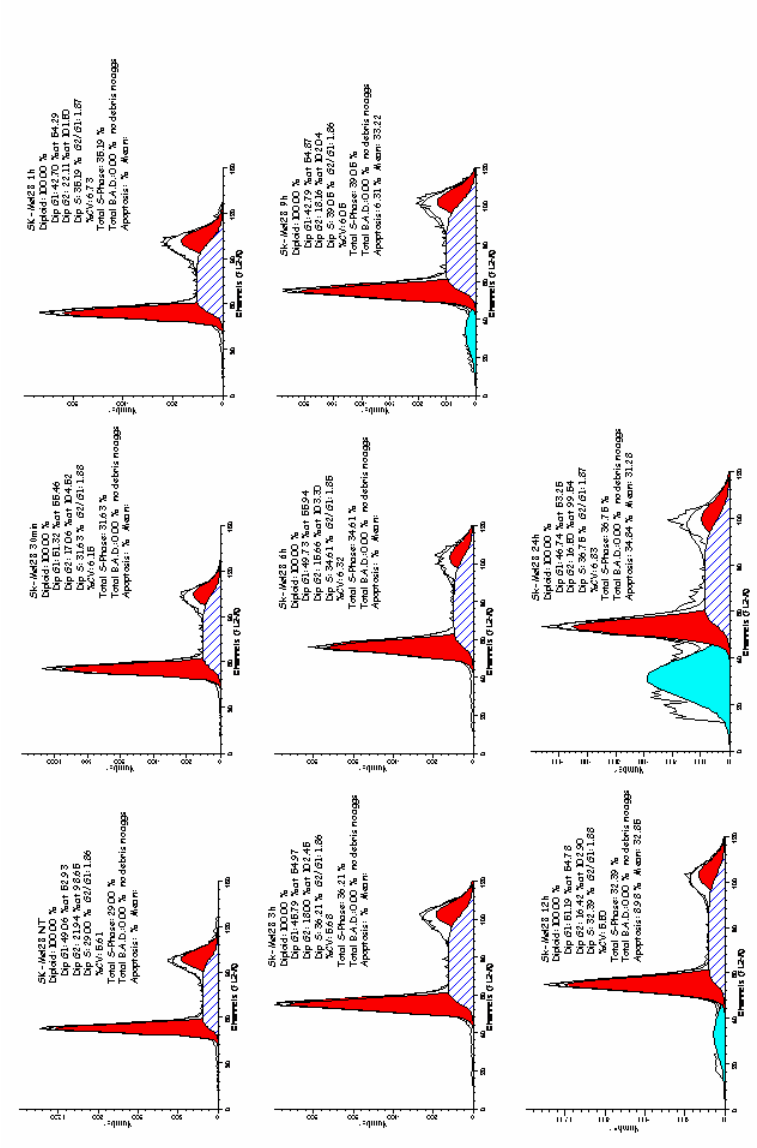

Fig. 16. ***Panel C.*** Cell cycle analysis of SK-Mel28 cell line after exposure to UVB damage (10mJ/cm²). Data were analyzed with ModFit LT 3.0 software.

3.3 Transduction of BRCA1 shRNA (BRCA1$^{3'UTR}$ siRNA) into SK-MEL93 cells results in modulation of UV-B-induced cell cycle arrest

To determine whether BRCA1 is involved in signalling UV-B-mediated effects, we used siRNA technology to inhibit its expression. We generated stable knocked down BRCA1 into SK-MEL93 cells, using shRNA lentiviral specific for BRCA1 (BRCA1$^{3'UTR}$ shRNA) (see Material and Methods). As shown in Fig. 17, BRCA1 protein expression was not detectable in SK-Mel93/^{sh}BRCA1 cells when compared with cells infected with the empty lentiviral vector (SK-Mel93/^{sh}plKO.1).

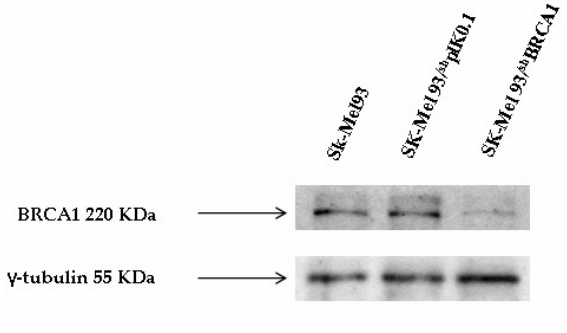

Fig. 17. Western blotting analysis of BRCA1 protein level after infection of SK-Mel93 cells with pLK0.1/^{sh}RNA (SK-Mel93/^{sh}RNA), or pLK0.1/^{sh}BRCA1 (SK-Mel93/^{sh}BRCA1). γ-tubulin was used as control for loading.

Then, SK-Mel93/^{sh}BRCA1 cells were treated with UV-B-irradiation at different time (0,30-24h), and performed Western

Blotting analysis using antibodies against MSH2 and p53 proteins. We found no significantly changes in the expression levels of MSH2 and p53 proteins (Fig.18).

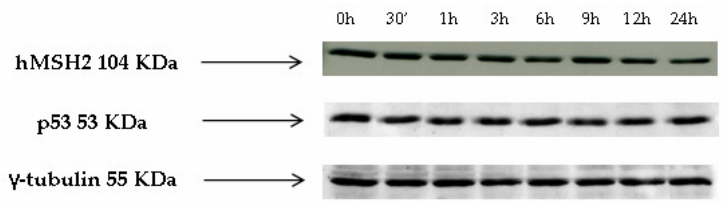

Fig 18. Western Blot analysis of hMSH2 and p53 protein levels in SK-Mel93/^{sh}BRCA1 cells after treatment with UVB 10mJ/cm^2, for the indicated time periods. γ-tubulin was used as control for loading.

On the contrary, the levels of MSH2 and p53 proteins in SK-Mel93/shplKO.1 cells were to an extent comparable to that of parental cells (SK-Mel93) (Fig.19).

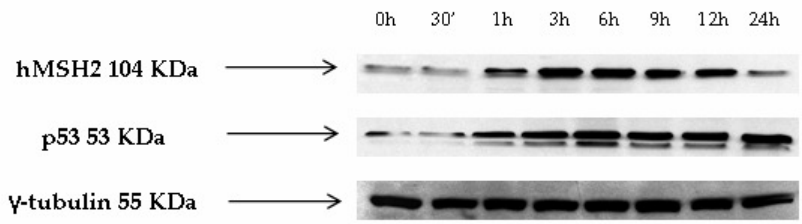

Fig 19. Western Blot analysis of hMSH2 and p53 protein levels in SK-Mel93/^{sh}plKO.1 cells after treatment with UVB 10mJ/cm^2, for the indicated time periods. γ-tubulin was used as control for loading.

To enforce the idea that BRCA1 protein may be intimately linked to pathway caused by UV-B and evaluate the effect of UV-B on the cell cycle of SK-Mel93/^{sh}BRCA1 cell line,

DNA content was serially observed after UV-B-irradiation (10mJ/cm^2) by flow cytometry. As show in Fig. 20 Panel A, SK-Mel93/^{sh}BRCA1 cells seemed an increase (about 15%) of cells in Sub-G1 phase already 12h after UVB irradiation compared with cells infected with the empty lentiviral vector (SK-Mel93/^{sh}plKO.1) (Fig. 20 Panel B).

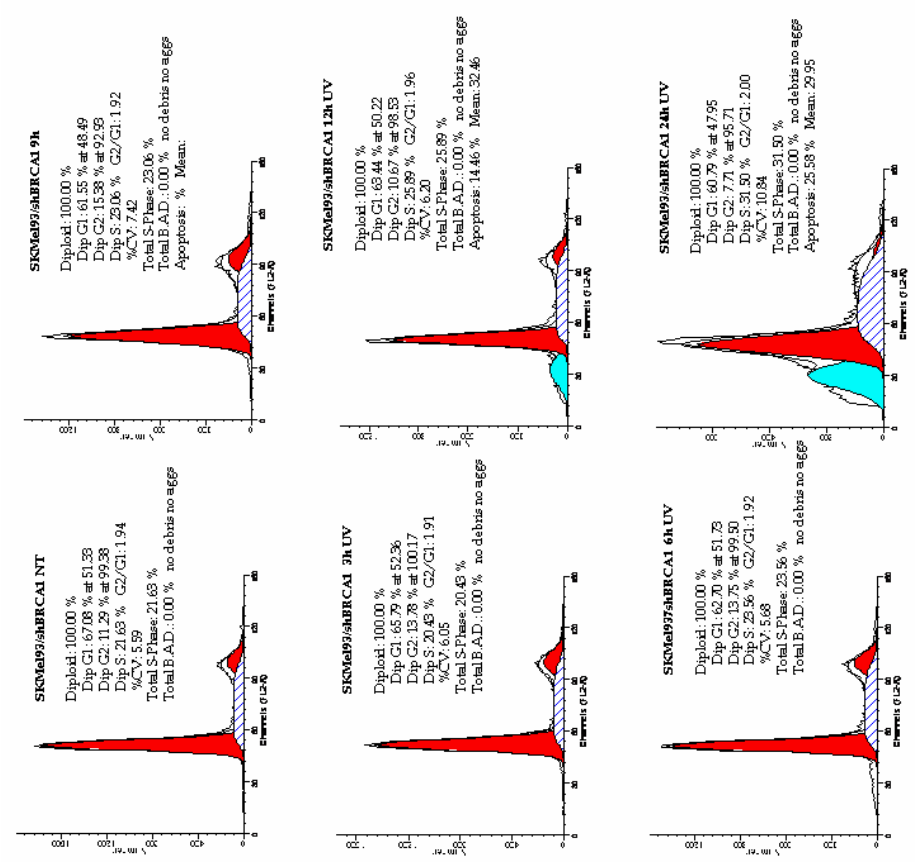

Fig. 20. ***Panel A.*** Cell cycle analysis of SK-Mel93/^{sh}BRCA1 cell line after exposure to UVB damage (10mJ/cm^2). Data were analyzed with ModFit LT 3.0 software.

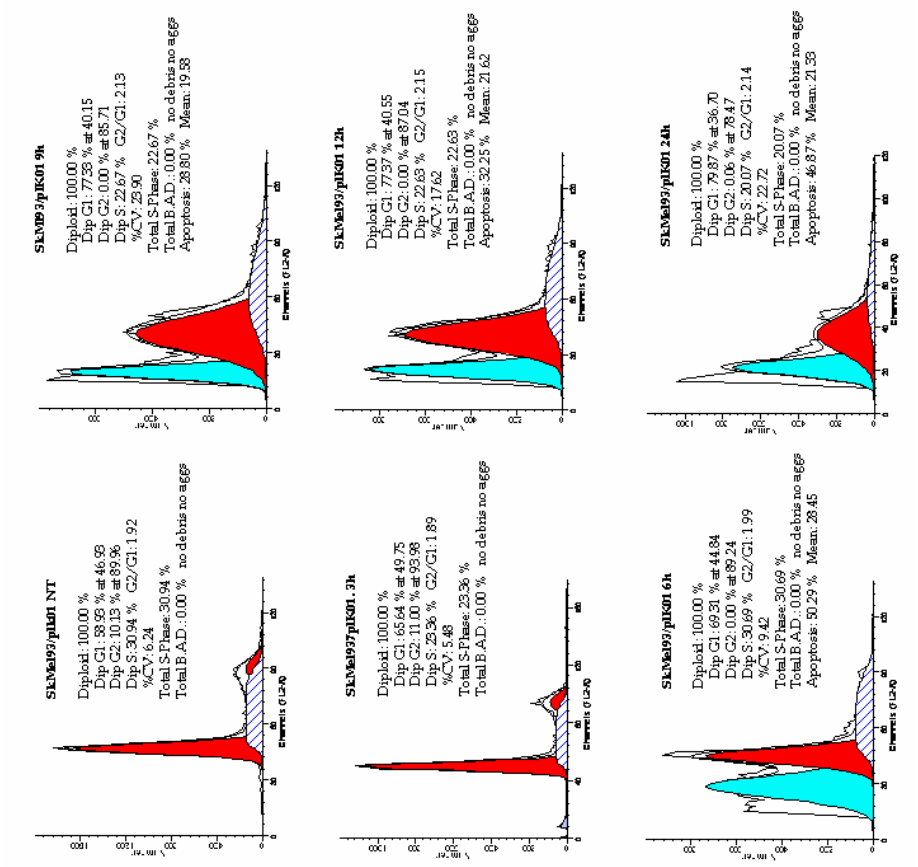

Fig. 20. ***Panel B.*** Cell cycle analysis of SK-Mel93/^{sh}plK0.1 cell line after exposure to UVB damage (10mJ/cm^2). Data were analyzed with ModFit LT 3.0 software.

4. Discussion

Melanoma is a malignant tumor type characterized by a poor prognosis partly due to ineffective radiotherapy and chemotherapy (160,161, CCR2005 myc), although radiotherapy is widely applied for treatment of melanoma patients. Recently, it has been reported that several molecular factors, such as those involved in DNA repair or in the cell cycle, modulate in melanoma cells UV-B induced DNA repair, cell progression and apoptosis. In particular, it was suggested that the mismatch repair system is an initial step of the damage signalling and repair cascade. Additional, increasing evidence indicate an important function of hMSH2 for other pathways that are of importance for UV-induced melanomagenesis, including cell cycle regulation and modulating the apoptotic response of cells following UV-exposure (162). Moreover, the hMSH2 gene has been identified as a possible novel p53 regulated target gene, indicating a direct involvement of p53 in repair mechanisms via DNA binding of a mismatch repair gene. In this context, BRCA1 tumor suppressor gene, known to play a central role in controlling cell progression and apoptosis, seems to be one possible candidate (163).

In our work, we have evaluated the effect of BRCA1 and of other its molecular partners (MSH2, MLH1and p53) after UV-B-irradiation in a panel of human malignant melanoma cells.

We exposed Colo38, SK-MEL93 and SK-MEL28 cells to 10mJ/cm^2 UV-B-irradiation and examined the expression of BRCA1, hMSH2, hMLH1 and p53 proteins by Western Blot analysis at different time (0,30-24h). The expression of hMLH1 protein in the three cell lines shows no alteration. Similar results, with equal amounts of BRCA1, hMSH2 and p53 proteins, were observed with the cell lines Colo 38 and SK-MEL28. Conversely, the expression of BRCA1, hMSH2 and p53 proteins, in the SK-MEL93 was found increased at 3h after UV-B-irradiation.

To evaluate the effect of UV-B on the cell cycle of each cell lines (Colo38, SK-MEL93 and SK-MEL28), DNA content was serially observed after UV-B-irradiation (10mJ/cm^2) by flow cytometry. Colo38 cells seemed to be synchronized at the G1/S boundary phase until 12h after 10mJ/cm^2 UV-B-irradiation. From 12 to 24h a decrease in the fraction of S cells and a reciprocal increase of cells in Sub-G1 phase was observed. Similar comportment was observed in the SK-MEL28 cell line although did not seemed to be synchronized and the increase of cells in Sub-G1 phase was observed already to 9h after UVB irradiation. While, the SK-MEL93 cell line did not seemed to be synchronized but a rapid increase (about 35%) of cells in Sub-G1 phase was observed already 6h after UVB irradiation. On the other hand, the three human melanoma cell lines shown a

different p53 status: Colo38 and SK-MEL28 are mutant p53 cell lines, while SK-MEL93 is wild type p53 cell line (162).

Based on our results, we speculate that BRCA1 over-expression and the increase of cells in Sub-G1 phase, after UVB irradiation may be intimately linked to specific human melanoma cell line (SK-MEL93). In concordance with this hypothesis, we generated stable knocked down BRCA1 into SK-MEL93 cells, using shRNA lentiviral specific for BRCA1 ($BRCA1^{3'UTR}$ shRNA) (see Material and Methods). BRCA1 protein expression was not detectable in SK-Mel93/^{sh}BRCA1 cells when compared with cells infected with the empty lentiviral vector (SK-Mel93/^{sh}plKO.1). Then, SK-Mel93/^{sh}BRCA1 cells were treated with UV-B-irradiation at different time (0,30-24h), and performed Western Blotting analysis using antibodies against hMSH2 and p53 proteins. We found no significantly changes in the expression levels of hMSH2 and p53 proteins. Moreover, we evaluated the effect of UV-B on the cell cycle of SK-Mel93/^{sh}BRCA1 cell line and found that SK-Mel93/^{sh}BRCA1 cells seemed an increase (about 15%) of cells in Sub-G1 phase already 12h after UVB irradiation compared with cells infected with the empty lentiviral vector (SK-Mel93/^{sh}plKO.1). In conclusion, our findings support the hypothesis that BRCA1 expression modulates UV-B-induced effects on cell cycle progression.

Taken together, our data lend support to the general hypothesis of an important role played by BRCA1 providing

new insights into the molecular mechanisms underlying UV-induced melanomagenesis, with a special focus on the cascade of events triggered in a specific human melanoma cell line (SK-MEL93).

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Communication and Participation in International Conferences and Courses

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DNA damage induced a post-translational modification of mismatch protein hMLH1

F.Romeo, **N.Ahmad,** *M.DI Sanzo, D. Scumaci, M. Saccomanno,G.Cuda, M.C. Faniiello,B. Quaresima and F.S. Costanzo.*

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PP1-13
BRCA1-mediated stabilization of MLH1 DNA mismatch repair protein in response to adryamicin-induced DNA damage

F. Romeo, M. D. Sanzo, A. Nasar, G. Cuda, M. C. Faniello, B. Quaresima and F. S. Costanzo

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Introduction: The tumor suppressor BRCA1 is involved in many cellular processes (DNA repair, transcription regulation, cell-cycle control and apoptosis). Most of these functions are strictly related to the ability of BRCA1 to interact with the other partners of a multimeric complex called BASC. Among these components, an important role is played by MLH1. The induction of MLH1 protein was observed in multiple cell lines in response to several DNA-damaging agents indicating that stabilization of MLH1 protein is a general response to DNA damage. The aim of our study was to demonstrate that BRCA1 mediates the stabilization of MLH1 in response to adryamicin-induced DNA damage.

Methods: In this study were used MCF7, HCT116 and HCT116 $p53^{-/-}$, HCC1937$^{-/-}$, HCC1937/^{wt}BRCA1 cells treated and untreated with adryamicin (600 ng/ml). Western Blot and RT-PCR analysis were performed. Immunoprecipitation (IP) was performed on total extract from MCF7. shRNA. Specific shRNA for BRCA1 was used for silencing endogenous BRCA1 in MCF7 cells.

Results: We found that hMLH1 and hBRCA1 activated proteins interact by adryamicin treatment in time-dependent manner. Moreover, after 6-hour exposure to adryamicin an increase of p27, transcriptional target of BRCA1, was observed.

Conclusions: Our results support the hypothesis that BRCA1-mediated stabilization of MLH1 in response to adryamicin-induced DNA damage.

roteins contained one or several phosphorylation-depen-
-3-3-binding sites indicating a potential direct interaction
-3-3ε. Accordingly of survival function of 14-3-3ε isoform,
ectopic 14-3-3ε expression delay cell death and knock-
f this isoform sensitized cells to C2-ceramide induced
is. Finally, these biochemical and functional analysis
4-3-3ε isoform as a survival factor during C2-ceramide-
apoptosis and characterized novel C2-ceramide regulated
14-3-3ε interacting proteins related with processes that control life or death in Hela cells.

P1–82
Computational analysis of miRNA targets and CpG islands in human genes

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MicroRNAs (miRNAs) are small noncoding RNAs (single-stranded RNA molecules of about 21–23 nucleotides in length) that regulate gene expression by targeting messenger RNA (mRNA) transcripts. CpG islands are genomic regions that contain a high frequency of CG dinucleotides. Both, CpG islands and miRNA are involved in the epigenetic landscape, i.e., heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. CpG islands are DNA sequence features which typically occur at or near the transcription start site of genes, particularly housekeeping genes; and cytosines in such an arrangement tend to be methylated. However, miRNAs, are epigenetic factors acting at post-transcriptional level, through their targets in mRNAs. The aim of this work is to explore the possible relationship between miRNA targets and CpG islands in human genes. Genomic data have been retrieved from NCBI the current *Homo sapiens* Build 36.3. We have used the MySQL relational database management system, MySQL and Perl scripts. First, taking into account all human genes, the overlapping percentage of gene sequence (including the flanking regions) by CpG islands were evaluate. The plot CpG-gene interactions vs number of miRNA targets, clearly shows an inverse correlation. In order to identify whether this behaviour plays a significant role in genes which are under an epigenetic regulation, we have analysed the incidence of CpG islands and miRNA targets in hypomethylated and hypermethylated in specific genes of colon cancer; and in a set of human experimentally identified imprinted genes. Project AGL2007-65678/ALI.

P1–83
DNA damage induces a post-translational modification of the mismatch protein hMLH1

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DNA mismatch repair (MMR) system contributes to the maintenance of the genomic stability in both prokaryotes and eukaryotes, through the correction of replication errors, the suppression of recombination between non identical, but homologous sequences, and the activation of cell cycle arrest and apoptosis in response to DNA damage. Moreover, the inactivation of the MMR pathway is linked to cancer predisposition. Recent evidence supports a potential role of MMR system in signalling the DNA damage response, in particular, has been observed the induction of hMLH1 in response to several DNA-damaging agents, including cisplatin, adriamycin and MNNG. Furthermore, the stabilization of the remaining hMutL (hPMS1 and hPMS2) proteins and their nuclear compartmentalization in response to DNA damage requires hMLH1. Here, we examined the induction of hMLH1 protein in response to adryamicin-induced DNA damage. We have used the MCF7 cells, treated and untreated with adriamycin, and performed Western blot and RT-PCR assays. We found that hMLH1 and BRCA1 (molecular partner of hMLH1) were activated by adryamicin treatment in time-dependent manner. On the basis of these results specific shRNA for BRCA1 was used for silencing endogenous BRCA1 in MCF7 cells. We found that hMLH1 stabilization is mediated by BRCA1 in response to DNA damage. To further uncover a potential link between hMLH1/BRCA1, we looked for post-translational modifications of hMLH1, like Ser/Thr phosphorylation. Using immunoprecipitation and 2D gel approaches we found a post-translational modification of hMLH1. Moreover, the post-translational modifications of hMLH1 protein is currently under investigation by LC/MS-MS approaches.

P1–84
Functional genomic resolution of pharmacogenetic nexus between metabolic syndrome and morphogenesis

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We tested the hypothesis of common genetic factors forming a *nexus between morphogenesis and metabolic syndrome*. We utilized metabolic and transcriptomic profiling of a unique set of rat models with previously ascertained disparity in both limb development and metabolic syndrome features. Adult male rats (n = 12/strain) of spontaneously hypertensive rat (SHR) and congenic SHR.PD-(D8Rat42-D8Arb23)/Cub (SHR-*Lx*) strains, differing in 1.4 Mb region of chromosome 8, were fed a high sucrose diet (HSD) for 2 weeks and subsequently treated with RA (15 mg/kg) for 16 days, while still on HSD. *We contrasted metabolic* (insulin sensitivity, adipokines, free fatty acids, triacylglycerol and cholesterol in 20 lipoprotein fractions) *and transcriptomic* (Affymetrix Rat Exon 1.0 ST, liver) *profiles* between SHR and SHR-*Lx* under conditions of standard, HSD and HSD + RA administration. We observed noticeable *distinction in effect of RA* between SHR and SHR-*Lx* strains. SHR-*Lx* reacted with significant impairment of glucose tolerance and less favorable distribution of cholesterol and triaylglycrols into the lipoprotein fractions compared to SHR. Significant interactions between strain and diet/RA factors were found for free fatty acid and insulin levels. Transcriptomic data corroborated the metabolic profile as they revealed a concerted shift in distinct pathways between the strains in response to RA. We demonstrated interaction of retinoic acid with a 14- gene region of rat chromosome 8, *affecting concurrently the features of metabolic syndrome and, as previously shown, the limb morphogenesis.*Our results support the notion of *interconnection of morphogenetic and metabolic*

A proteomics approach to identify changes in protein profiles in serum of Familial Adenomatous Polyposis patients

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Background: Familial Adenomatous Polyposis (FAP) is one of the most important clinical hereditary forms of inherited susceptibility to colorectal cancer and is characterized by a high degree of phenotypic heterogeneity. We have used a mass spectrometry driven-proteomic strategy to identify serum molecules that are capable to distinguish distinct forms of FAP (carpeting and diffuse polyps) and with respect to healthy individuals.

Methods: -*Subjects and serum sample preparation*: Serum samples from FAP patients and from unaffected individuals were collected after informed consent. Blood was drawn by venipuncture and placed on ice. The samples were centrifuged within 2 hours of collection at 1.400 x g for 10 min. - *Depletion of HSA and IgG from Serum Samples*: The depletion procedure was performed using ProteoExtractTM HSA/IgG Removal Kit, according to the manifacturer's instruction. SDS-PAGE analysis was performed to evaluate albumin and IgG removal.The gel was stained with colloidal blue stain - *Labelling samples with cleavable ICAT reagents*: The proteins were precipitated at –20°C over night and then centrifuged at 13.000 x g for 15 min at 4 °C and dissolved in 50mM Tris and 0,1% SDS buffer pH 8.5. The complex protein samples were reduced, alkylated, digested, cleaned-up and purified according to the protocols supplied in the Cleavable ICAT Reagent Kit for protein Labelling. -*Proteomic Analysis*: Chromatography was performed on an Ultimate nano LC system from LC Packings. MS detection was performed on a QSTAR XL hybrid LC-MS/MS from Applied Biosystems. Qualitative and quantitative LC-MS/MS information was processed by the ProICAT® software. The identification of protein obtained from a single peptide hit was verified manually. -*Bioinformatic Analysis*: The ProICAT® protein identification process has been boosted through a bioinformatics module, named EiPeptiDi. Moreover, the Clementine data mining platform is used to perform classification experiments. -*Western Blotting analysis*: 50 µg of serum biomarker candidate proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated with the specific antibodies.

Results: Significant differences were highlighted in the expression of serum proteins of FAP patients versus healthy donors. In particular, two proteins (alpha-2-HS-glycoprotein and apoliprotein D) appear to be down-regulated (about 0.5 and 0.7-fold respectively) in carpeting versus diffuse FAP patients, and versus healthy donors, while alpha-2-antiplasmin is up-regulated (about 1.4-fold). Moreover, one protein (vitronectin) was found up-regulated (more than 1.4-fold) in diffuse FAP patients versus carpeting FAP and versus healthy donors. Finally, two candidates (Haptoglobin and alpha-1-acid glycoprotein 1) were found up-regulated in 2 out of 3 carpeting FAP patients. To strengthen the findings of mass spectrometry, western blotting analysis was performed on sera from patients using antibodies against the six biomarker candidate proteins.

Conclusions: These data identify a specific proteomic signature in serum of FAP patients with distinct morphological classes and may significantly contribute to clarify the mechanisms underlying the FAP pathophysiology, providing, at the same time, the opportunity to design alternative screening strategies in at risk subjects.

P64) SPECIFIC CHANGES IN THE GENE EXPRESSION PROFILING PRODUCED BY THE BRCA1 5083DEL19 FOUNDER MUTATION

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We have recently identified a founder mutation in the BRCA1 gene (5083del19) in Calabrian families with hereditary breast/ovary tumors. This mutation is highly prevalent in patients undergoing genetic counseling (72% of the overall BRCA1 gene mutations identified by our group) and it is harbored by at least 10% of the overall breast tumors occurring in Calabria.

The aim of our study was to compare by microarray analysis the transcripts of stably transfected HeLa cells with either exogenous wild-type BRCA1 (HeBRCA1) or mutated 5083del19 allele (HeBRCA1/5083del19).

We found an array of genes with increased and decreased expression in HeBRCA1/5083del19 versus HepcDNA3.1/empty, in HeBRCA1/5083del19 versus HeBRCA1 and in HeBRCA1 versus HepcDNA3.1/empty; among these transcripts are genes coding for proteins or enzymes related to cell growth, cell cycle, apoptosis, signal transduction, transcription, immune system, and stress.

In particular, we focused our attention to periostin gene as it shows a 76-fold increase in HeBRCA1/5083del19 versus HeBRCA1 and a 72-fold increase in HeBRCA1/5083del19 versus HepcDNA3.1/empty. Other candidates are currently under investigation. Periostin has been described as a mesenchyme-specific gene whose over expression by human breast cancers leads to significant enhancement of tumor progression and angiogenesis.

Supporting evidence for microarray results was collected by RT-PCR and Western blot analysis performed on parental and transfected cells. These independent assays confirmed that periostin expression is increased, both at mRNA and protein level, in HeLa cells carrying the mutated 5083del19 allele (HeBRCA1/5083del19). Conversely, down-regulation was observed for both genes in HeLa cells over-expressing wild-type BRCA1.

Our findings support a molecular basis for the pathogenic role of BRCA1 mutations in breast carcinogenesis, highlighting the function of this protein in the modulation of the expression of genes involved in various cellular processes.

MADDALENA DI SANZO POSTER SESSION 1

p53 regulates negatively the transcription of the H ferritin gene

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Ferritin, the major iron storage protein, is composed of 24 subunit of two types, named H (heavy) and L (light). The promoter of the gene coding for the H subunit consists of approximately 150 bp, from the transcription start, in which are located two cis-elements. The distal element, at -132 bp from the transcription start, is recognized by Sp1 and the proximal element, at -62 bp from the transcription start, is recognized by transcription factor NF-Y. It has been demonstrated that NF-Y binds the co-activator p300 which acts as from molecular anchor recruiting on the promoter activators (c-Jun) or inhibitors (E1A) of the transcription. p53 regulates negatively the transcription of the genes interacting with the transcriptional factor NF-Y. In this study we have investigated the potential involvement of p53 in the control of the H ferritin gene transcription. We have demonstrated that p53 overexpression represses the H ferritin promoter through inhibition of the transcriptional effects of NF-Y. Transient transfection analysis showed that p53 down-regulates the H ferritin promoter, while in presence of the small interfering RNA p53, the H ferritin promoter was not down-regulated by p53. Chromatin immunoprecipitation (ChIP) analysis found that p53 is bound to the NF-Y on the H ferritin promoter. Moreover, the co-transfection with a construct containing the region Carboxyl-terminal region of p53 reduced the ferritin promoter basal activity, while the co-transfection with a p53 mutant where all lysines of the C-terminal are replaced by Arginines, abolishes the inhibition effect. These data suggest that the ferritin gene is a target of p53 and that it will be important to clarify the physiological significance of H ferritin suppression by p53 to fully appreciate the role of this protein involved in a number of biological functions including cell, growth control, in the regulation of iron homeostasis and metabolism.

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