Chapter 6

T-Cadherin Stimulates Melanoma Cell Proliferation and Mesenchymal Stromal Cell Recruitment, but Inhibits Angiogenesis in a Mouse Melanoma Model

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1. Introduction

Melanocytes are special pigment cells that reside predominantly in the skin and eyes. In the skin, melanocytes are located in the bottom layer (the stratum basale) of the skin's epidermis and in the hair follicles (Gray-Schopfer et al., 2001). Melanocytes produce melanins responsible for skin and hair color and perform protection function of the basal keratinocytes from ultraviolet light through synthesis and donation of melanin (Gray-Schopfer et al., 2001). Melanocytes maintain constant contact with the basal layer of the epidermis through direct interaction with basal keratinocytes and via secretion of soluble factors. Upon ultraviolet radiation, keratinocytes produce factors that control melanocyte proliferation, differentiation and motility (Gray-Schopfer et al., 2007). Melanocytes maintain during a lifetime a stable-ratio of 1:5 with basal keratinocytes (Fitzpatrick et al., 1979).

Initially, cutaneous melanocytes originate from neural crest cells and migrate into the skin during embryonic development. Neural crest cells start their migration from the neural tube shortly after the closure of the neural tube. These cells migrate along several well-defined pathways in a ventral direction from the neural tube through the somites. As the epithelial somites undergo a transition to form the dermatome (presumptive dermis), myotome (presumptive muscle), and sclerotome (presumptive vertebrae), most ventrally migrating neural crest cells invade the rostral half of each sclerotome and avoid the caudal (posterior) part of

each sclerotome (Rickmann et. al., 1985; Teillet et al., 1987; Serbedzua et al., 1989; Ranscht and Bronner, 1991).

Originated from a population of highly motile neural crest progenitors, melanocytes protect basal keratinocytes, in the skin. At the same time, they could become precursors of the most dangerous form of cancer - melanoma. Skin cancer including the most frequently occurring forms such as basal cell carcinoma, squamous cell carcinoma and melanoma, is one of the most common human malignancies. Today melanoma is one the fastest growing malignancies. The high propensity of melanoma to form metastasis is the most important feature that distinguishes melanoma from other types of skin cancers. According to the World Health Organization, melanoma accounts for only 25% of skin cancers. However, it is the most dangerous form of skin cancer leading to high mortality. If diagnosed early it can be successfully removed by surgical resection and about 80% of cases are cured this way (Gray-Schopfer et al., 2001). However, at progressed metastatic stages melanoma is highly resistant to currently existing therapies and has a very poor prognosis. This area requires future research to understand melanoma biology and develop new therapeutic solutions.

Melanoma begins as a benign naevus but can quickly progress to the malignant stage (Bar-Eli., 1997; Luca and Bar-Eli., 1998). Herewith melanocytes start to proliferate and spread, which can be limited to the epidermis (junctional naevus), or the dermis (dermal naevus) or both (compound naevus) (Gray-Schopfer et al., 2001). Naevi can progress to the radial-growth-phase (RGP) melanoma which is an intra-epidermal lesion with sporadic local microinvasion into the underlying derma. However, RGP melanoma can transform into the vertical-growth phase (VGP) melanoma with a higher invasive potential in which melanoma cells from tumor nodules or nests invade the underlying derma. Finally, melanoma can develop metastases after the vertical growth phase (Clark et al., 1984). Not all melanomas pass through each of these phases and can progress from isolated melanocytes or naevi, while both, RGP or VGP, can develop directly into metastatic malignant melanoma (Miller et al., 2006). Four main clinical subtypes of melanoma are described (Clark et al., 1984). Superficial spreading melanoma (SSM) is the most common form and it is associated with severe sunburns, especially at an early age (Ishihara et al., 2001; Gilchrest et al., 1999). In most cases, SSM is flat, with intra-epidermal microinvasion, particularly at the edges of the lesion. Nodular melanoma comprises raised nodules and has almost no flat parts. Acral lentiginous melanoma (ALM) is not linked to UV exposure and is usually found on the palms of the hands, soles of the feet and in the nail bed (Kuchelmeister et al., 2000). Lentigo maligna appears to be flat and is associated with chronic sun exposure in elderly people.

2. Cadherin-mediated adhesion in melanoma progression

Tumor progression is characterized by uncontrolled cell proliferation, high invasive potential into surrounding tissue and metastasis to distant organs. It is believed that this is largely due to disruption or dysfunction of intercellular contacts (Hanahan and Weinberg, 2000). Cadherins comprise a large family of Ca^{2+}-dependent adhesion molecules (Angst et al., 2001; Gumbin-

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er, 2005) and are involved in tissue and organ development durig embryogenesis and maintainance of the normal cell arrangement in the adult organism. The regulation of cadherin expression patterns and their activity at the neural crest-forming area plays a critical role in emigration of melanocyte precursors - neural crest cells from the neural tube (Nakagawa and Takeichi, 1998).

Cadherins play an important role in specific cell-cell adhesion, cell recognition and signaling (Angst et al., 2001; Gumbiner, 2005; Wheelock and Johnson, 2003). Cadherins are transmembrane glycoproteins with their extracellular part responsible for homotypic binding between the neighboring cells, while intracellular part is involved in anchoring cadherins to the cytoskeleton. Cadherins interact with the cytoskeleton via catenins (alfa and beta catenins and p120) and form a multicomponent complex which also comprises a number of regulatory molecules such as protein tyrosine kinase, protein tyrosine phosphatase and small GTPases (Perez-Moreno et al., 2003; Sallee et al., 2006; Gumbiner, 2005; Vincent et al., 2004; Rubina et al., 2007).

Disruption of cadherin adherent junctions and their dysfunction has been associated with tumor cell invasion and metastasis (Takeichi, 1993). In human carcinoma the loss of E-cadherin expression leads to dedifferentiation and increased invasiveness of carcinoma cells (Frixen et al., 1991). The change in the expression pattern of cadherins in melanocytes from E-cadherin, P-cadherin and desmoglein to N-cadherin is associated with melanocyte transformation and metastasis (Bonitsis et al., 2006). In normal skin, melanocytes project multiple extensions to keratinocytes and form cadherin adhesive contacts with the basal keratinocytes, which control maintain proliferation and correct positioning of melanocytes (Haas and Herlyng, 2005; Haass et al., 2005). E-cadherin is mainly responsible fot these cell-cell interaction. The loss of functional E-cadherin or its downregulation let melanocytes escape form keratinocyte control and correlates with high invasiveness and metastasis of the overlying melanoma cells (Hsu et al., 1996; Silye et al., 1998).

Downregulation of E-cadherin and/or its dysfunction is one of the earliest steps in the development of metastases in cutaneous melanoma (Johnson, 1999). E-cadherin expression is detected in cultured melanocytes and naevus cells, while its expression is often lost in cultured melanoma cells from the primary tumors or metastasis (Danen et al., 1996). In the radial growth phase melanoma cells could retain expression of membrane E-cadherin (Sanders et al., 1999). It was even noted that there is a correlation between E-cadherin expression and the depth of the primary tumor (Andersen et al., 2004). Experiments on re-expression of E-cadherin using adenoviral transfer of full length E-cadherin cDNA showed the reduction in tumorigenicity and decrease in proliferation rate of melanoma cells (Hsu et al., 2000). The mechanism of E-cadherin transcriptional downregulation in melanomas involves gene silencing by methylation or transrepression by the Snail protein from a superfamily of zinc-finger transcription factors (Tsutsumida et al., 2004). Thus it was suggested that E-cadherin could play an important role in the preventing the melanocytes transformation and limiting their proliferation (cited in Bonitsis et al., 2006).

Catenins are a group of cadherin-associated molecules and they were also suggested to be involved in malignant transformation of melanocytes. The change in catenin expression pattern was found in melanocytic naevus and melanomas, where the expression of alfa and

beta catenins was reduced or altered, while beta catenin was often overexpressed (Zhang and Hersey., 1999). The loss of E-cadherin in melanocytes may also indirectly influence the β-catenin cytoplasmic content and affects the β-catenin/wnt signaling pathways. Namely, the reduction in the membranous E-cadherin resulted in the accumulation of free cytoplasmic β-catenin which did not degrade in proteasomes and was translocated to the nucleus. Nuclear β-catenin could be involved in regulation of gene expression responsible for growth and metastasis control via β-catenin/wnt signaling pathways (McGary et al., 2002). Also the presence of the functional E-cadherin in melanocytes ensured their correct adhesion to keratinocytes and limited their motility and proliferation (Gruss et al., 2001; Tang et al., 1994).

Despite the majority of studies showed a correlation between the decreased E-cadherin expression and tumorigenicity of melanoma cells, there were data suggestintg that in some cases E-cadherin expression could be retained or even elevated in melanoma (Ruiter and van Muijen, 1998; Nishizawa et al., 2005). It was reported that membranous E-cadherin was present in the metastasizing melanomas and their corresponding lymph node metastasis (Silye et al., 1998).

The loss of E-cadherin expression in melanomas correlates with the increase in N-cadherin expression. This change contributed to the survival advantage of melanoma cells and their invasive and migratory properties (McGary et al., 2002). The shift in cadherin expression pattern was found both in vivo and in vitro (Hsu et al., 1996; Hsu et al., 2000). It was suggested that melanoma cells form N-cadherin adhesion contacts with fibroblasts, vascular endothelial cells, and adjacent melanoma cells (Li and Herlyn, 2000). The N-cadherin-mediated adhesion facilitated migration of melanoma cells over dermal fibroblasts and their transmigration into the vascular system (Haass and Herlyn, 2005; Li et al., 2002) and induced formation of communication gap junction between melanoma cells and the surrounding stroma (McGary et al., 2002). Anti-N-cadherin antibodies retarded the transendothelial migration of melanoma cells and induced their apoptosis, which linked the N-cadherin in the ability of melanoma cells for diapedesis (Li et al., 2002; Sandig et al., 1997; Voura et al., 1998). Surprisingly, adenoviral gene transfer of E-cadherin inhibited N-cadherin expression in melanoma cells and their survival and migration (Hsu et al., 2000). At the same time N-cadherin overexpression did not affect the endogenous E-cadherin expression (Li et al., 2001).

Little is known about the role of P-cadherin in the progression of malignant melanomas. P-cadherin is expressed in basal keratinocytes, melanocytes, in the cells of the basal and outer layers of skin appendages (Klymkowsky and Parr, 1995). As Similarly to E-cadherin, P-cadherin was thought to be involved in the regulation of melanocyte proliferation and migration (Klymkowsky and Parr, 1995). It was found that the soluble form of P-cadherin missing the transmembrane and the cytoplasmic part was expressed in melanoma cells (Bauer et al., 2005) and was associated with increasing tumor thickness and metastasis and reduced patient survival (Bachmann et al., 2005).

VE-cadherin is another member of the cadherin superfamily, which was shown to be involved in melanoma progression. VE-cadherin was found to be exclusively expressed on endothelial cells in normal vessels and mediated homophilic contacts between neighboring cells regulating endothelial barrier function (Dejana, 1996). VE-cadherin is essential for both the development and the maintenance of blood vessels in the adult organism. In the embryo, VE-cadherin

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appeared at a very early stage of vascular development in mesodermal cells of yolk sac mesenchyme; it was also expressed in progenitor cells during the early angioblast differentiation and endocardial development (Dejana et al., 2000; Cavallaro et al., 2006). At the later stages, VE-cadherin expression was restricted to the peripheral layer of blood islands that give rise to endothelial cells (Dejana et al., 2000).

VE-cadherin was also shown to be important for melanoma cell invasion and metastasis. At the beginning of diapedesis, endothelial cells located below the attached melanoma cells disassemble their VE-cadherin-mediated adhesion contacts. This allowed melanoma cell to penetrate through the VE-cadherin-negative regions in the endothelial cell monolayer and intercalate between endothelial cells. Subsequently, the endothelial cells surrounding the melanoma cells extended processes and spread over melanoma. The leading edges of the projections of endothelial cell expressed high levels of N-cadherin but not VE-cadherin. VE-cadherin expression was restored when the endothelial cells met and reformed cell-cell contacts above the melanoma cell (Voura et al., 1998). Highly aggressive human cutaneous and uveal melanoma cells were also found to express VE-cadherin in contrast to less aggressive cells (Hendrix et al., 2001). This expression contributed to the ability of melanoma cells to mimic endothelial cells and form patterned networks of vascular channels (Hendrix et al., 2001; Hendrix et al., 2003). These data indicated that melanoma cells cooperate with the endothelial cells in the process of invasion and that the regulated changes in the expression of cadherins played an essential role in melanoma growth and metastasis.

Thus, further studies are required to elucidate the biochemical and cellular mechanism of melanoma transformation and progression and the role of cadherins in this process. Lately, an atypical member of the cadherin superfamily – T-cadherin was was shown to be involved in melanoma progression. However, its role and mechanism of action were not completely understood.

3. T-cadherin in cancer

T-cadherin is a unique member of cadherin superfamily because it lacks the transmembrane and cytoplasmic domains and is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) moiety (Fredette and Ranscht, 1994; Fredette et al., 1996; Ranscht and Dours-Zimmermann 1991). Although T-cadherin contains five Ca^{2+}–binding domains on its N-terminal end it does not have the His-Ala-Val motif responsible for the recognition and binding of the classical cadherins. It was shown that T-cadherin can mediate week homophilic adhesion in aggregation assays *in vitro* (Vestal and Ranscht, 1992; Resink et al., 1999) but the lack of intracellular domain strongly suggested that T-cadherin is not involved in stable cell-cell adhesion. Moreover, T-cadherin was show to be absent from the adherent junctions and was located within lipid rafts in the plasma membrane (Philippova et al., 1998); was redistributed to the leading edge in migrating cells (Philippova et al., 2003). These imply T-cadherin as a navigation receptor involved in transduction of extracellular cues in migrating cells rather than an adhesion molecule.

Little is known about the biological role and underlying mechanisms of T-cadherin in malignant transformation and tumor progression. In some reports, T-cadherin was regarded as a tumor suppressor and its downregulation was associated with tumor progression. Downregulation of T-cadherin was shown to be associated also with tumorogenicity in breast (Riener et al., 2008), lung (Sato et al., 1998), and gallbladder cancers (Adachi et al., 2009). However, in other cancers such as ovarian, endometrial (Widschwendter et al., 2004; Suehiro et al., 2008) and osteosarcoma (Zucchini et al., 2004), decreased expression of T-cadherin positively correlated with patient survival. T-cadherin was upregulated in human invasive hepatocellular carcinomas (Riou et al., 2006) and astrocytomas (Gutmann et al., 2001).

T-cadherin is expressed in the basal layer of keratinocytes, in melanocytes and in vascular cells of the dermal blood vessels in the normal skin (Zhou et al., 2002; Kuphal et al., 2009; Rubina et al., 2012). However, its expression was consequently lost upon cell transformation and tumor progression in pre-malignant skin lesions and in non-melanoma skin cancer. In skin lesions, such as actinic keratosis T-cadherin was abundantly expressed in the atypical keratinocytes, while its expression varied in Bowen disease and was weaker than in the normal skin (Pfaff et al., 2010). Expression of T-cadherin was reduced in psoriatic samples (Zhou et al., 2003) and was down-regulated or completely absent in invasive cutaneous squamous cell carcinoma (Takeuchi et al., 2002a) and in basal cell carcinoma of the skin (Takeuchi et al., 2002b). Data obtained in our lab supported these findings and confirmed that T-cadherin expression was not changed if cells maintained the attachment to the basal membrane in the lesions characterized by slow or controlled keratinocytes growth (keratoacanthoma, psoriasis, actinic keratosis and superficial basalioma) (Rubina et al., 2012). However, T-cadherin expression in keratinocytes was downregulated upon tumor progression in basosquamous cell carcinoma, squamous cell carcinoma and in some cases of basal cell carcinoma, i.e. tumors with high proliferative, invasive, and metastatic potential (Rubina et al., 2012).

Apart from regulation of keratinocyte proliferation, T-cadherin may affect tumor progression through its direct involvement in neovascularization. While in the normal blood vessels T-cadherin was abundantly expressed in endothelial and mural cells (Ivanov et al., 2001), its expression was altered in tumor vessels in Lewis carcinoma lung metastasis and F9 endodermal teratocarcinoma and in human xenografts PC-3 prostate cancer or A673 rhabdomyosarcoma (Riou et al., 2006; Wyder et al., 2000). Inactivation of T-cadherin gene limited mammary tumor vascularization and reduced tumor growth in the mouse mammary tumor virus (MMTV)-polyoma virus middle T (PyV-mT) transgenic model (Hebbard et al., 2008). In human hepatocellular carcinoma (HCC) T-cadherin was also upregulated in intratumoral capillary endothelial cells and this increase correlated with tumor growth and metastasis (Adachi et al., 2006). Data obtained in our lab indicated that in pre-malignant skin lesions all blood vessels uniformly expressed T-cadherin (Rubina et al., 2012). The aberrant expression of T-cadherin and vascular markers was detected in aggressively developing skin tumors such as basosquamous cell carcinoma, squamous cell carcinoma and in some cases of basal cell carcinoma. We suggested that the high level of expression of T-cadherin in the normal keratinocytes and in benign tumors regulates the growth of blood vessels. Upon malignant transformation expression of T-cadherin was lost in tumor cells and altered on vascular cells. This caused the abnormality and excessive vascularization of the tumors (Rubina et al., 2012).

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The role of T-cadherin in melanoma progression and vascularization was addressed in a few studies and the results were contradictory. T-cadherin was expressed in normal human skin melanocytes (Kuphal et al., 2009; Bosserhoff et al., 2011). However, it was shown that the precursors of melanocytes did not express T-cadherin and invaded T-cadherin negative rostral parts of sclerotomes avoiding T-cadherin positive caudal parts of sclerotomes during neural crest cell migration (Ranscht and Bronner-Fraser, 1991). These results led to a hypothesis that T-cadherin is a navigating receptor that provides topographic guidance for migrating melanocyte precursors, and that de-differentiated or transformed melanocytes may loose T-cadherin expression. Indeed, T-cadherin expression was found to be diminished in melanocytes induced to de-differentiate to melanoblast-related cells and T-cadherin expression was undetectable in about 80% of human melanoma cell lines (Kuphal et al., 2009; Bosserhoff et al., 2011). While T-cadherin was expressed in benign naevus nests, its expression was lost in most tissue samples of human primary melanoma, lymph and visceral melanoma metastasis indicating the potential role of T-cadherin in melanoma progression (Kuphal et al., 2009). In addition, T-cadherin re-expression by stable transfection in human melanoma cells reduced the rate of tumor growth in the *nu/nu* mouse tumor model, decreased cell capacity for anchorage-independent growth, and for migration and invasion *in vitro* (Kuphal et al., 2009). However, it was shown in other studies that T-cadherin overexpression in endothelial cells stimulated intratumoral angiogenesis in tumor co-culture spheroid model with melanoma cells *in vitro* (Ghosh et al., 2007). Despite T-cadherin expression is lost in the majority of melanoma cell lines, 20% of melanomas still express T-cadherin (Kuphal et al., 2009; Bosserhoff et al., 2011) and possess invasive and metastatic potential.

To gain insights into the function of T-cadherin in melanoma progression and growth we first examined T-cadherin expression in the normal human skin melanocytes, melanoma cells and the blood vessels of the primary melanomas and melanoma metastasis in human samples and in the experimental models.

4. T-cadherin expression in human melanoma

We performed a comparative study of T-cadherin expression in normal skin and in melanoma samples. Tissue samples of primary human melanoma and metastasis obtained from patients undergoing surgical treatment were immediately frozen with liquid nitrogen and stored at -80°C. Human skin biopsies from healthy donors and melanoma samples of patients were immunostained with antibodies against T-cadherin and vascular cell markers and analyzed using fluorescent microscope.

Human skin biopsies from 6 healthy donors, 10 tissue samples of primary human melanoma and 12 samples of visceral melanoma metastasis were obtained from Blokhin Russian Cancer Research Center of Russian Academy of Medical Sciences. Consequent cryosections of the samples (7μm thick) were fixed in 4% paraformaldehyde (PRS Panreac, Spain), whashed and then incubated in a mixture of primary antibodies against T-cadherin (rabbit anti-human, ProSci, USA) or endothelial cells marker vWF (Von Willebrand factor, mouse anti-human, BD

Biosciences, USA), or melanoma (gp100) Ab-3 (Ab-3 is a mixture of Ab-1 (HMB45) and Ab-2 (HMB50) antibodies which are extremely sensitive and recognize differentiating melanocytes and melanomas, Lab Vision- Neomarkers, USA) – for 1 hour and subsequent extensive washing in PBS. Then sections were incubated in a mixture of secondary antibodies Alexa488-conjugated donkey anti-mouse and Alexa594-conjugated donkey anti-rabbit or Alexa488-conjugated donkey anti-rabbit and Alexa594-conjugated donkey anti-mouse (Molecular Probes, USA) (1μg/ml in PBS). Cell nuclei were counterstained with DAPI (Molecular Probes, USA). Images were obtained using Zeiss Axiovert 200M microscope equipped with CCD camera AxioCam HRc and Axiovision software (Zeiss, Germany) and further processed using Adobe PhotoShop software (Adobe Systems, USA).

We assessed the areas occupied by the T-cadherin-positive melanoma cells using MetaMorph 5.0 (Universal Imaging) and Adobe PhotoShop software (Adobe Systems). In a field of view, we determined the T-cadherin-positive areas and normalized to the DAPI-stained area unit of each section. For the quantification, T-cadherin-positive areas were counted in 4-5 fields of view (1.107 mm^2) on 10 random sections for each sample at 100x total magnification (10x objective).

In the normal skin T-cadherin was abundantly expressed in basal keratinocytes, in differentiating keratinocytes, in the stromal cells and in all blood vessels located in the underlying derma (Fig.1).

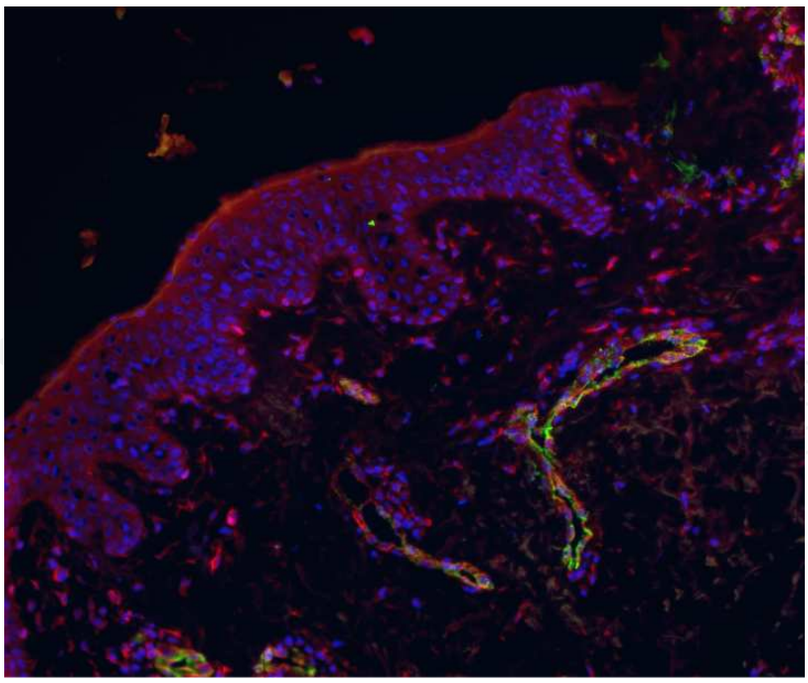

Figure 1. Double immunofluorescent staining of normal skin samples with antibodies against T-cadherin (red) and vWF (green). T-cadherin expression was detected in the basal keratinocytes, suprabasal layers, stromal cells and in the blood vessels (colocalization of T-cadherin and vWF is showed by the arrow). Nuclei were counterstained with DAPI (blue). Bars, 100 μm.

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The immunofluorescent staining with gp 100 antibodies demonstrated that differentiating melanocytes were located beneath the layer of epidermal basal keratinocytes and extended their processes over keratinocytes (Fig. 2A). We were able to show the expression of T-cadherin in differentiating melanocytes by the overlay of green and red fluorescence (Fig. 2A, B and C) and in mature melanocytes by mapping the red fluorescence that revealed T-cadherin expression (Fig. 2C) with a phase-contrast image where the dark cells corresponded to mature melanocytes (Fig. 2C and D).

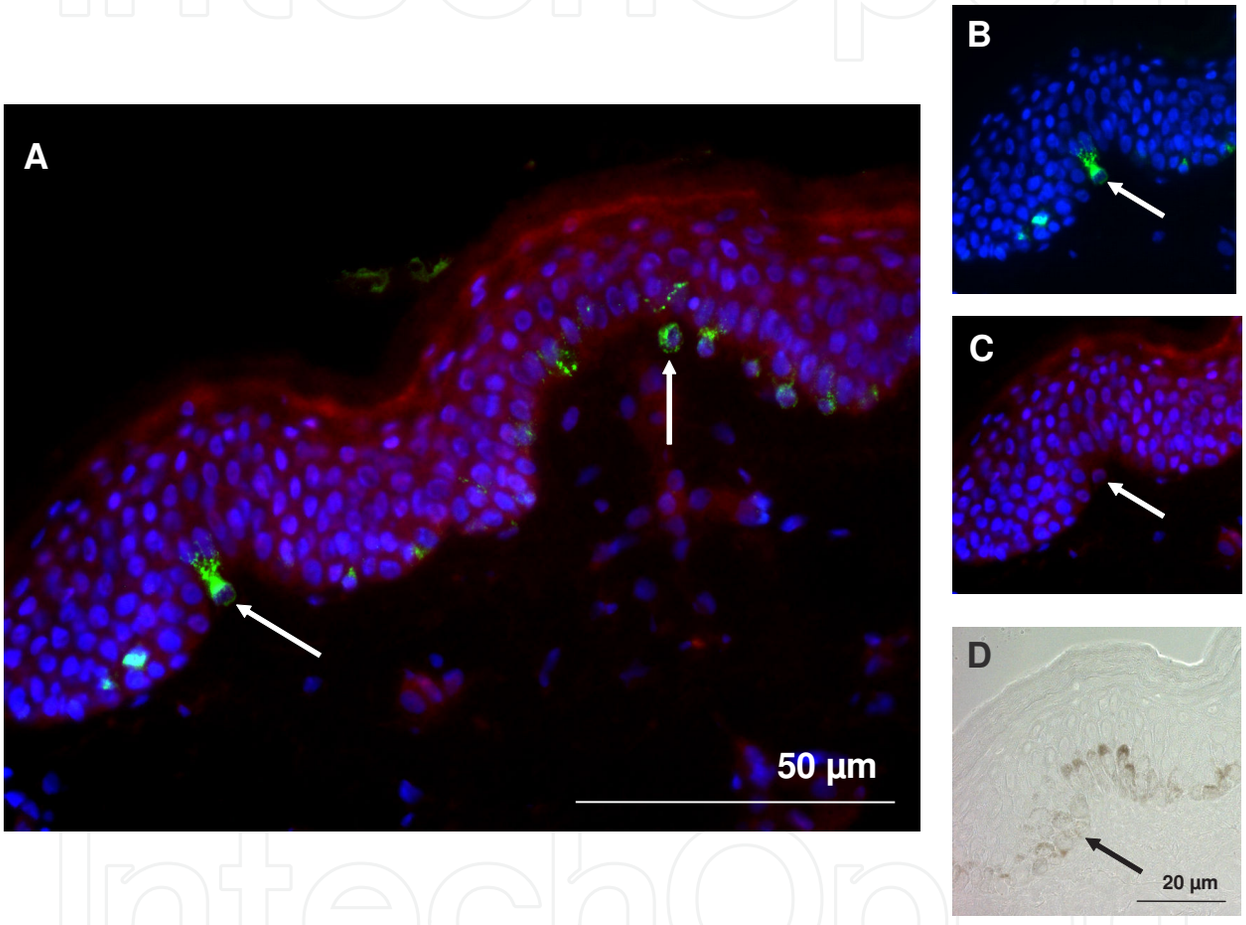

Figure 2. Double immunofluorescent staining of normal skin samples with antibodies against T-cadherin (red) (A, B, C) and gp 100 (green) (A, B). Figure D depicts phase contrast image. Figures B, C, D represent parallel frozen sections of the same sample. Differentiating melanocytes (green fluorescence in A, B) and mature melanocytes (phase contrast image with dark cells in D and corresponding immunofluorescent staining against T-cadherin – red fluorescence in C) all express T-cadherin. Nuclei were counterstained with DAPI (blue). Colocalization of T-cadherin and gp 100 is showed by the arrow. Bars, 100 µm.

Previous studies demonstrated that T-cadherin expression is reduced during cancer progression. Thus, we examined T-cadherin expression in melanoma samples of patients undergoing surgical treatment. Consequent cryosections of 10 tissue samples of the primary human melanoma and 12 samples of visceral metastasis, including two cases of primary melanoma that developed metastasis within a year were double immunostained with antibodies against

melanoma cell marker gp 100, T-cadherin, endothelial cell marker vWF and analyzed using fluorescent microscope.

The tissue staining with subsequent image analysis showed that in the primary melanoma 60% of the sections was occupied by melanoma cells uniformly expressing T-cadherin (Fig.3A), while 30% of the sections contained areas with heterogeneous, mosaic pattern of T-cadherin expression where some melanoma cells were T-cadherin positive and some cells - T-cadherin negative (Fig.3B and 3C), and the rest 10% exhibited no T-cadherin expression.

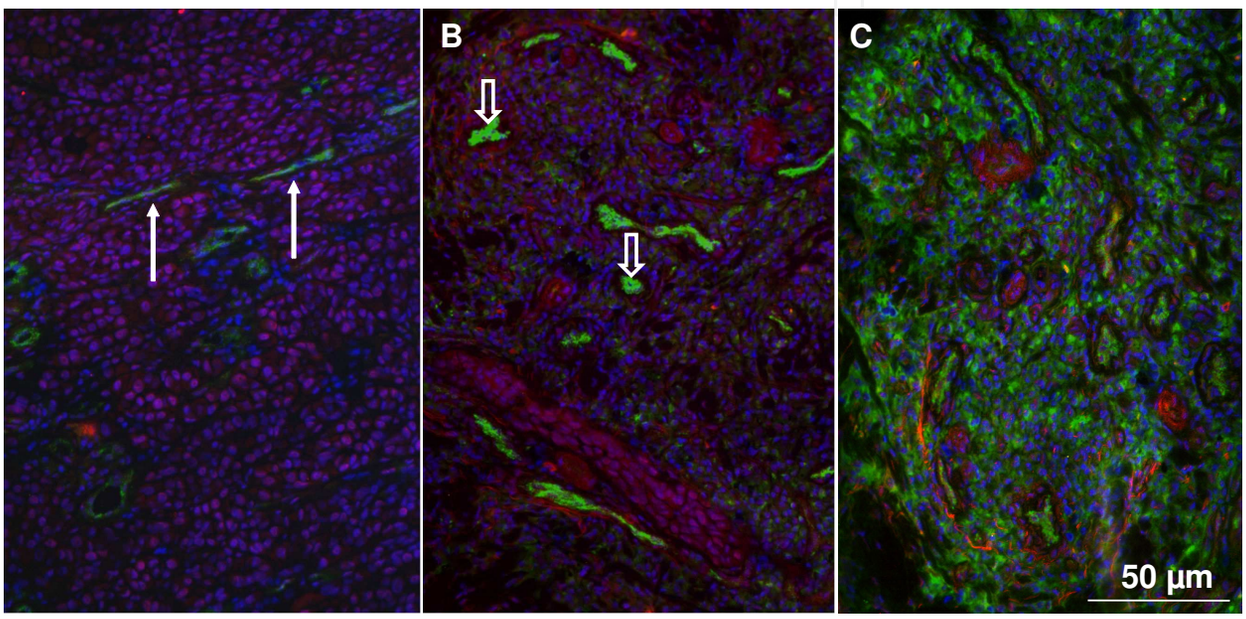

Figure 3. Double immunofluorescent staining of the primary melanoma sample with antibodies against T-cadherin (red) (A, B, C) and vWF (green) (A, B) or gp 100 (green) (C). Nuclei were counterstained with DAPI (blue). Blood vessels that expressed T-cadherin are showed by the arrow in A, blood vessels with no T-cdherin are marked by an empty arrow in B. Bars, 50 µm.

Tumor growth and progression require blood supply. There are three mechanisms by which solid tumors acquire their blood supply. First, tumor and the surrounding stromal cells secrete angiogenic factors that stimulate vessel growth and recruitment into the tumor from the preexisting vessels in a well described process of angiogenesis (Folkman et al., 1971; Folkman et al., 1992, Folkman 1995). Asahara and colleagues demonstrated the incorporation of circulating endothelial progenitors form the peripheral blood into sites of ischemia-induced angiogenesis (Asahara et al., 1997). Beyond these two mechanisms, aggressive primary and metastatic melanomas are capable of generating microcirculatory channels composed of extracellular matrix and lined by tumor cells that express VE-cadherin (Hendrix et al., 2001; Maniotis et al., 1999). These vascular channels (lacunas) allow blood flow. However, lacunas are not strictly vasculogenic or angiogenic because they are formed by melanoma cells (Folberg et al., 2002; Maniotis et al., 1999). Therefore the name "vasculogenic mimicry" was assigned to the process by which melanoma cells generate non-endothelial cell-lined channels (Maniotis

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et al., 1999). This vasculogenic mimicry adds challenges to the practical surgery and requires new cancer diagnostic and treatment strategies.

Our data indicate that the areas devoid of T-cadherin in the primary melanomas were more intensively vascularized than the areas with high level of T-cadherin expression. 80% of vWF-positive blood vessels also expressed T-cadherin (arrows in Fig.3A), while no T-cadherin could be detected in the rest of the blood vessels (arrows in Fig.3A, 4A). Besides vWF-positive blood vessels in the primary melanomas (Fig.4A) and in the visceral metastasis, vascular channels devoid of endothelial cells were found. Interestingly, vascular channels were lined up by the cells expressing T-cadherin (Fig.4A, C).

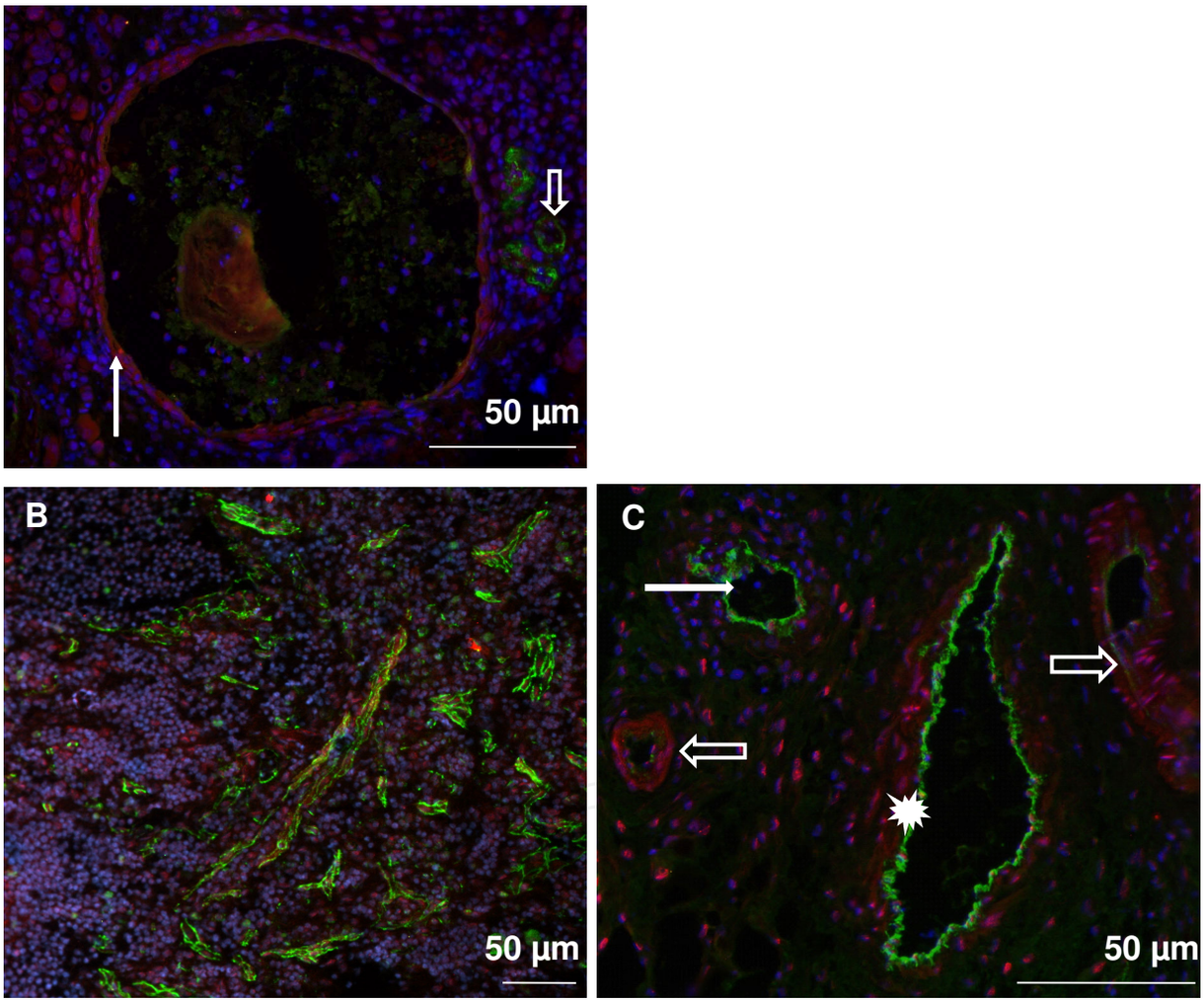

Figure 4. Double immunofluorescent staining of the primary melanoma (A) and visceral metastasis samples (B, C) with antibodies against T-cadherin (red) and vWF (green). (A) Large lacuna can be seen in the center of the image of the primary melanoma (showed by an arrow). The walls of the lacunas in the primary melanomas (A) and metastasis (B, C) were lined up by T-cadherin positive melanoma cells (red). Blood vessels of the primary melanomas and metastasis were heterogeneous. In (A) vWF-positive blood vessels with no T-cadherin expression could be seen located in the immediate vicinity of the lacuna (green) fluorescence, marked by an empty arrow in A): in (B and asterisk in C) both variants of vWF-positive blood vessels are presented: T-cadherin positive and T-cadherin negative (arrows in A and C, respectively). Nuclei were counterstained with DAPI (blue). Bars, 50 µm.

Our data indicated that T-cadherin expression in human melanoma was gradually reduced upon melanoma progression. While nearly 60% of melanoma cells in the primary melanomas expressed T-cadherin, 30% of the sections exhibited a mosaic pattern of T-cadherin expression in melanoma cells and 10% of the sections were T-cadherin negative. In metastasis, 60% of the sections were occupied by melanoma cells with heterogeneous T-cadherin expression (mosaic pattern) and the number of T-cadherin-positive cells was reduced to 30% (Fig. 5). These results were in agreement with the data obtained by Kuphal (Kuphal et al., 2009) who detected T-cadherin expression in melanocytes in the healthy skin and intratumoral capillaries of the primary melanoma samples while T-cadherin expression was lost in melanoma cells.

The same pattern was previously described for pre-cancer skin lesions and non-melanoma skin cancer: T-cadherin expression was gradually lost upon malignization. The aberrant expression of T-cadherin was also detected in the blood vessels and correlated with the histological features and invasive behavior of more aggressive tumors (Rubina et al., 2012).

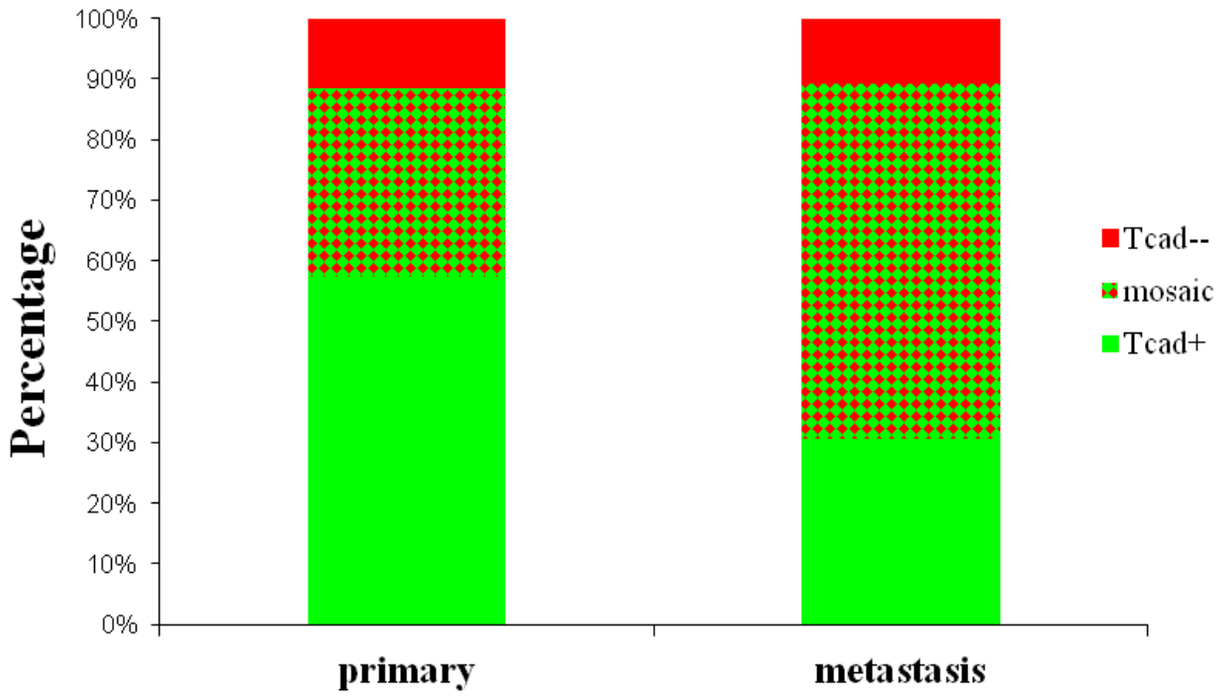

Figure 5. Loss of the expression of T-cadherin in melanocytes during metastasis.

To elucidate the potential role of T-cadherin in melanoma progression and growth, we used an established model of highly aggressive murine melanoma B16F10 in BDF1 mice. The murine melanoma B16F10 with lung metastases is considered as an adequate model for testing the efficacy of new anticancer therapies in preclinical evaluations and the forecasting model of human disseminated melanoma in anticancer screening (Teicher and Andrews, 2004).

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5. The effect of T-cadherin expression on melanoma cell proliferation and apoptosis

To assess the effect of T-cadherin expression on melanoma cell proliferation we generated stable cell lines of murine malignant melanoma B16F10 cells (ATCC® №CRL-6475™) by transfecting the cells with pcDNA-Tcad using Lipofectamine™ 2000 reagent (Invitrogen, USA. As a control, luciferase cDNA fragment in the antisense orientation was cloned into the pcDNA 3.1 vector. The cells were cultured and transfected as described before (Yurlova et al., 2010). For stable cell line generation transfected cells were cloned and selected by incubation with 2 mg/ml G418 (Invitrogen, USA). For some experiments we also used the polyclonal mouse melanoma B16F10 cell cultures obtained after transfection and subsequent selection with G418 without cloning. Expression of T-cadherin in polyclonal mouse melanoma cell cultures and melanoma cell clones was examined by Western blotting. Three clones of B16F10 melanoma cells with different level of T-cadherin expression was chosen: a control clone with no T-cadherin (T-), clone with low T-cadherin expression (T+) and clone with high T-cadherin expression (T++) (Yurlova et al., in press).

To investigate the effect of T-cadherin expression on melanoma cell proliferation, cells were seeded on 6-well plates and harvested by trypsinization after 24, 48 and 72 h in culture and counted with the Countess® (Invitrogen, USA). After 3 days, the number of T-cadherin-expressing cells (T+ and T++) was 44-48% higher compared to the control (Fig. 6A). We obtained similar results using the impedance measurement with xCELLigence system: the cell index was continuously monitored with 1 min interval for 5 hrs and with 10 min interval for 96 hrs. (Fig. 6B). The xCELLigence system (Roche, USA) monitors cellular events in real time and measures electrical impedance across microelectrodes integrated on the bottom of E-Plates. Thus, our data indicated that T-cadherin expression in mouse B16F10 melanoma cells increased their proliferation *in vitro*.

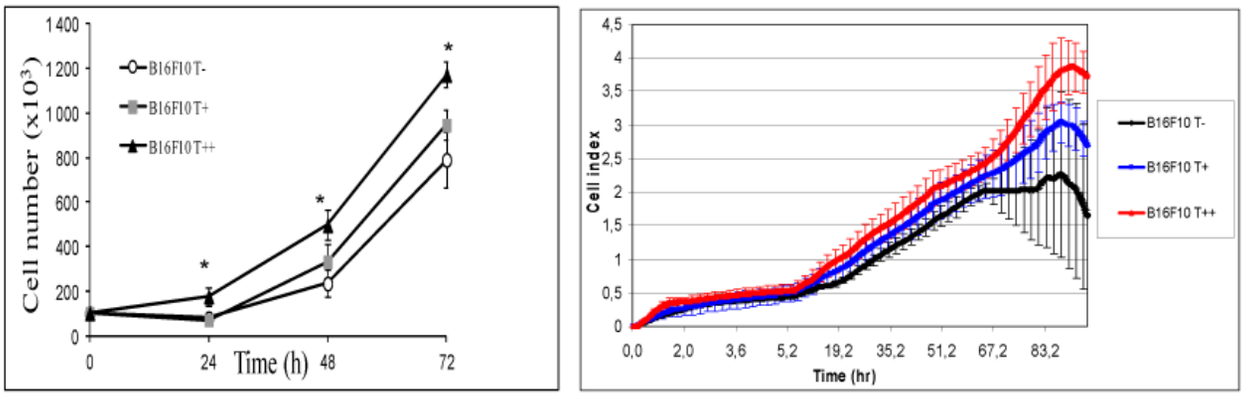

Figure 6. T-cadherin expression stimulated proliferation of B16F10 cells *in vitro*. (A) Cells were incubated in RPMI 1640 medium containing 10% FBS for indicated periods of time, harvested and counted using CellCounter. (B) The cell proliferation assay was performed with xCELLigence system. Attachment of 5000 cells per chamber was monitored with the impedance measurement of the xCELLigence System for 96 hrs. The cell indexes of B16F10 T- (control cells, black), T+ (cells with low level of T-cadherin, blue) and T++ (cells with high level of T-cadherin, red) are shown. Results are presented as the means ± SEM of three independent experiments.

To evaluate the effect of T-cadherin expression on apoptosis of B16F10 cells we performed staining with Annexin V. The B16F10 cell clones were resuspended in 200 μl of Annexin V binding buffer (BD Biosciences, USA), incubated with 5 μl of Annexin V-Phycoerythrin (BD Biosciences, USA) and 10 μl of 7-AAD (BD Biosciences, USA) for 15 min in the dark. The percentage of apoptotic cells was evaluated by flow cytometry (FACSCanto II™, BD Biosciences, USA). The fraction and absolute number (data not shown) of apoptotic and dead cells in T-cadherin-expressing clones were not significantly different from the control.

Thus, in contrast to the earlier study by Kuphal (Kuphal et al., 2009) who found no effect of T-cadherin expression on human melanoma cell proliferation, the current study showed that the expression of T-cadherin in mouse melanoma cells stimulated their proliferation *in vitro* and had no effect on the apoptosis.

6. Effect of T-cadherin expression on tumor growth and metastatic potentials of B16F10 clones in vivo

Animal studies were conducted according to the guidelines of the Institutional Animal Care and Use committee of Cardiology Research Center (permit number 385.06.2009). In order to establish a relationship between T-cadherin expression level and increased proliferation of melanoma cells and melanoma growth in vivo, we injected the clones of B16F10 cells with different level of T-cadherin expression into BDF1 mice. $1x10^6$ B16F10 cells (T-, T+, or T++ clones) in 0.3 ml of serum-free media were injected subcutaneously in BDF1 mice (n=11 in each group). 28 days after the injection, animals were sacrificed and primary tumors and lungs were collected. The measurement of the tumors was performed 4 times a week over 28 days. The tissue samples were embedded in Tissue-Tek (Sakura, USA), frozen in liquid nitrogen and stored at -80°C. The cryosections (6 μm) were fixed in 4% formaldehyde. To analyze the necrosis areas, cryosections were stained with Mayer's hematoxylin. Vascularization and stroma content was analyzed using the following primary antibodies: anti-T-cadherin, anti-CD31 to visualize endothelial cells (1:100, BD Biosciences, USA), anti-NG2 to visualize pericytes (1:100, Abcam, USA), anti-CD90 to visualize MSCs (1:100, BD Biosciences, USA). For negative controls, non-specific IgGs were used in similar concentration. Cell nuclei were counterstained with DAPI (Sigma-Aldrich, USA). The sections were analyzed using Leica AF6000 microscope and MetaMorph 5.0 (Universal Imaging, USA). For statistics, five view fields on five random sections for each tumor sample were used. The blood vessels were separated into three groups: capillaries (CD31-positive vessels without lumen and with length <20 μm); medium vessels (CD31-positive vessels with length 20-40 μm), and large vessels (with diameter >40 μm). The contribution of the stroma into the growth of primary tumor was determined as the area of CD90-positive cells in a field normalized to the DAPI-stained area of each cryosection. To evaluate the necrosis area we calculated the number of cryosections containing necrosis foci and normalized it to the total number of assessed sections.

The growth kinetic of tumors was compared within the next 4 weeks and then the histopathology was studied. The T+ and T++ B16F10 cells generated tumors 2.5 and 3 times larger than

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control B16F10 cells, correspondingly (Fig. 7A). Difference between the control and T-cadherin-expressing tumor volumes was statistically significant. The histological analysis showed that tumors formed by the control and T-cadherin-expressing B16F10 clones had different morphology. The tumors generated by the T-cadherin-expressing melanoma clones contained wider areas of necrosis (Fig. 7B).

We also compared the spontaneous metastatic potential of the B16F10 clones. 28 days after injection, tumor-bearing mice were sacrificed and their lungs were analyzed. We found that T-cadherin overexpression enhanced spontaneous metastatic activity of B16F10 cells. The B16F10 clones with high level of T-cadherin (T++) formed metastasis in 54.5% cases in comparison to 18.2% and 9.1% formed by T+ and T- cells, respectively. Taken together, these results suggested that the expression of T-cadherin stimulated growth of the primary tumors *in vivo* and enhanced their invasion and metastatic potential.

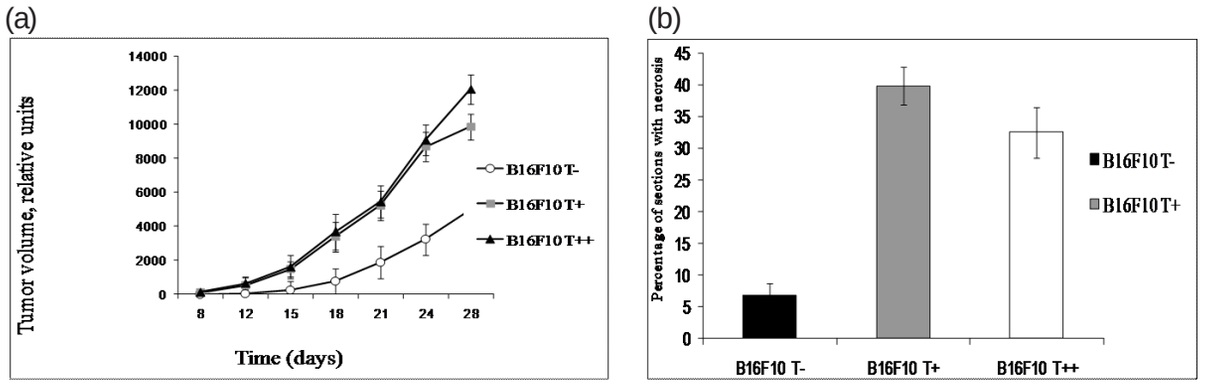

Figure 7. T-cadherin expression in B16F10 cells stimulates tumor growth *in vivo*. (A) 1x10^6 of B16F10 cells (T-, T+, or T++) were injected subcutaneously in BDF1 mice (n=11). The tumor volume was measured every 3 day after the tumor cell inoculation. Data represent the mean ± SEM. (B) The necrosis area was calculated as the number of cryosections containing necrosis foci normalized to the total number of sections. Results are the means ± SEM, $p<0.05$.

7. Effect of T-cadherin expression on tumor vascularization

Because T-cadherin was shown to inhibit angiogenesis in some models (Rubina et al., 2007) we examined the effect of T-cadherin on the neovascularization of B16F10 primary melanoma sites. For that, cryosections of primary melanomas were stained with anti-CD31 antibody to visualize endothelial cells in the blood vessels penetrating the tumor. The quantitative evaluation revealed a 1.3-1.5-fold decrease in the number of medium size vessels and 1.5-2-fold reduction in capillaries in T-cadherin-expressing primary melanomas compared to the control (Fig. 8). There was no detected difference in the amount of large or stable vessels using double immunofluorescent staining with anti-CD31 and anti-NG2 (marker of pericytes and smooth muscle cells) antibodies (data not shown).

Similar results were obtained using melanoma primary tumors formed by polyclonal mouse melanoma cell culture (data not shown). We concluded that the effects of T-cadherin were not related to the individual features of the selected clones.

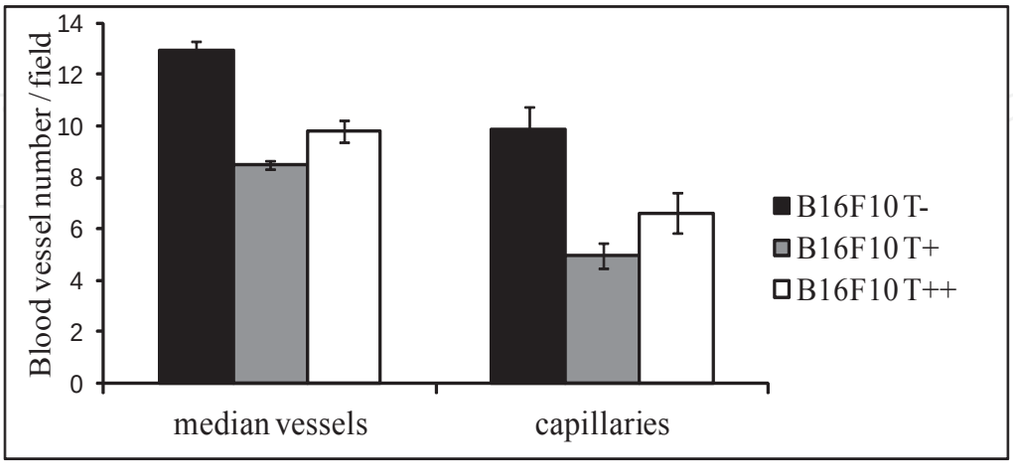

Figure 8. T-cadherin expression in B16F10 cells caused the reduction in tumor vascularization. 28 days after the melanoma cell inoculation, sections of primary tumors formed by the B16F10 T-, T+ or T++ cells were stained with anti-CD31 and anti-T-cadherin antibodies. The quantitative assessment of the blood vessels from 30 random fields of five independent tumors is presented. The results are the means ± SEM of three independent experiments, $p<0.05$.

These results suggested that T-cadherin overexpression in B16F10 melanoma cells suppresses tumor neovascularization by limiting tumor neoangiogenesis.

8. Effect of T-cadherin expression on host stroma

Over the past decade it was discovered that heterogeneous population of progenitor cells known as multipotent stromal cells or mesenchymal stem cells (MSCs) derived from the bone marrow or adipose tissue exhibited a marked tropism for tumors (Klopp et al., 2011). Circulating in the blood stream, MSC from the bone marrow or resident mesenchymal stromal cells could engraft within the tumor microenvironment and incorporate into the stroma of solid tumors as tumor-associated fibroblasts and contribute to the growth of the primary tumor sites (Mishra et al., 2008; Spaeth et al., 2008). MSCs can also act as pericytes-like cells and potentiate tumor growth, vascularization and metastasis. The mechanism by which MSCs support the tumor growth and progression is in the intercellular interactions with tumor cells and the release of the paracrine signals (Spaeth et al., 2009). MSCs themselves are likely to respond to chemoattractants similar to many immune cells that migrate to injury or inflammation site (Spaeth et al., 2008).

To examine whether T-cadherin expression influences the recruitment/proliferation of stromal cells in the model of mouse melanoma growth and progression, cryosections of primary tumor sites were stained with anti-CD90 antibody to visualize the activated stroma (Campioni et al., 2008) (Fig. 9A). Immunofluorescent analysis revealed a 2.4–2.9-fold increase in the CD90

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positive areas in the T-cadherin-expressing tumor samples compared to the controls (Fig. 9B). CD90-positive cells were arranged in a form of cell aggregates among the tumor cells or located perivascular around CD31-positive vessels structures. Thus, for the first time we revealed that the expression of T-cadherin stimulated the recruitment of CD90-positive cells to the primary tumor site. The CD90-positive cells were represented mainly by MSCs. However, some populations of neutrophils, T cells and monocytes could also express CD90 and be recruited to the tumors (Rege and Hagood, 2006).

To support the *in vivo* data on the T-cadherin-mediated recruitment of the stromal cells to the growing tumor, we established a transwell assay in which MSCs were co-cultured with the melanoma clones with different T-cadherin expression. The transwell system allowed exchange of the medium in the absence of direct interaction between cells in the lower and upper chambers. The MSCs were isolated from subcutaneous adipose tissue of the inguinal region of CBA/C57BL male mice and cultured until the 2nd passage as described before (Rubina et al., 2009). To assess the effect of T-cadherin expression in melanoma cells on their ability to induce MSC migration, MSCs were seeded in the upper chamber and melanoma cell clones were seeded in the lower chamber. The MSCs were allowed to migrate across the collagene-covered membrane and the conditioned medium from the B16F10 clones served as a chemoattractant. We found that migration of MSCs towards the conditioned medium from the T++ B16F10 cells was at least 1.5-fold higher than towards the T+ or T- B16F10 cells (Fig. 10).

We did not detect the shedding of T-cadherin into the conditioned medium from the T-cadherin expressing B16F10 clones using Western blotting (data not shown). Thus, we suggested that the observed effects on MSCs migration were mediated by secretion of chemoattractants and growth factors into the conditioned medium by the T-cadherin expressing melanoma cells. To prove that we studied the expression of angiogenec factors, extracellular matrix proteins, adhesion molecules and chemokines using the PCR Array assay (SABiosciences, USA) and quantitative PCR.

9. T-cadherin expression changes the gene expression pattern in B16F10 melanoma cells

For PCR Array assay, total RNA was isolated from B16F10 clones using RNeasy Mini kit. 1 μg of total RNA was treated with DNase, cDNA was prepared using RT2 First Strand kit (SABiosciences, USA). For each experiment, cDNA sample was mixed with RT2 qPCR Master mix and distributed across the PCR array 96-well plates. After cycling with real-time PCR (IQ5 PCR platform, Bio Rad, USA), obtained amplification data (fold-changes in Ct values) was analyzed with SABiosciences software. RNA expression of each gene was normalized using 5 housekeeping genes. The relative expression of each gene, compared to expression in the control B16F10 clone was calculated on the website using ΔΔCt method. A gene was considered as differentially regulated if the difference was >2-fold compared with the control clone.

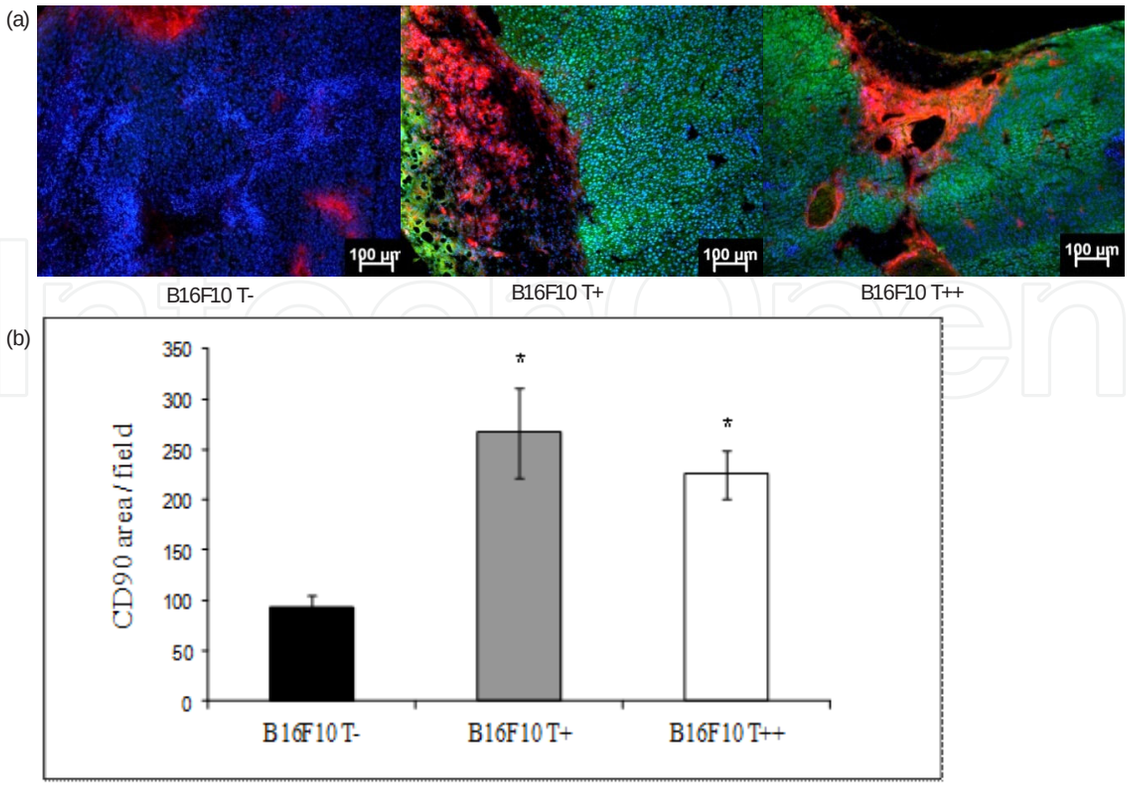

Figure 9. The effect of T-cadherin expression in B16F10 cells on MSC recruitment. (A) 28 days after melanoma cell inoculation, sections of the primary tumor formed by the B16F10 T- (left), T+ (middle), or T++ (right) cells were stained with anti-CD90 (red) and with anti-T-cadherin (green) antibody. Bars - 100 μm. (B) The contribution of the stroma into the growth of primary tumor was determined as an area of CD90-positive cells in a field normalized to the DAPI-stained area unit of each cryosection using program MetaMorph 5.0. Results are the means ± SEM of three independent experiments, $p<0.05$.

For quantitative PCR the RNeasy Mini Kit (Qiagen, Germany) was used to extract the total RNA. cDNA were prepared using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, USA). The primers were obtained from Sintol (Russia). Real-time qPCR analysis was performed with SYBR Green I on Rotor – Gene™ 3000 (Corbett Research, UK). The gene expression was normalized to the expression of β-actin and GAPDH. The primer specificity was confirmed by melting curve analysis. The qRT-PCR was repeated five times.

We performed quantitative PCR and PCR Array Assay of melanoma cells and revealed that T-cadherin expression in B16F10 melanoma cells resulted the increase in expression of chemokines CXCL10, CCL5, CXCL11 and CCL7, which were earlier implicated in the growth and metastasis of different neuroectodermal tumors (Somasundaram and Herlyn, 2009). In a screen of several human and mouse melanoma cell lines, it was detected that the expression of chemokine receptors CCR7, CCR10, CXCR1, CXCR2 and CXCR4 could dramatically increase the rate of metastases (Longo-Imedio et al., 2005; Simonetti et al., 2006; Singh et al., 2009; Wiley et al., 2001). In the present study, the up-regulation of CXCL10, CCL5, CXCL11 and CCL7 genes was found to be correlated with the increase in spontaneous metastatic activity in the B16F10 mouse melanoma model.

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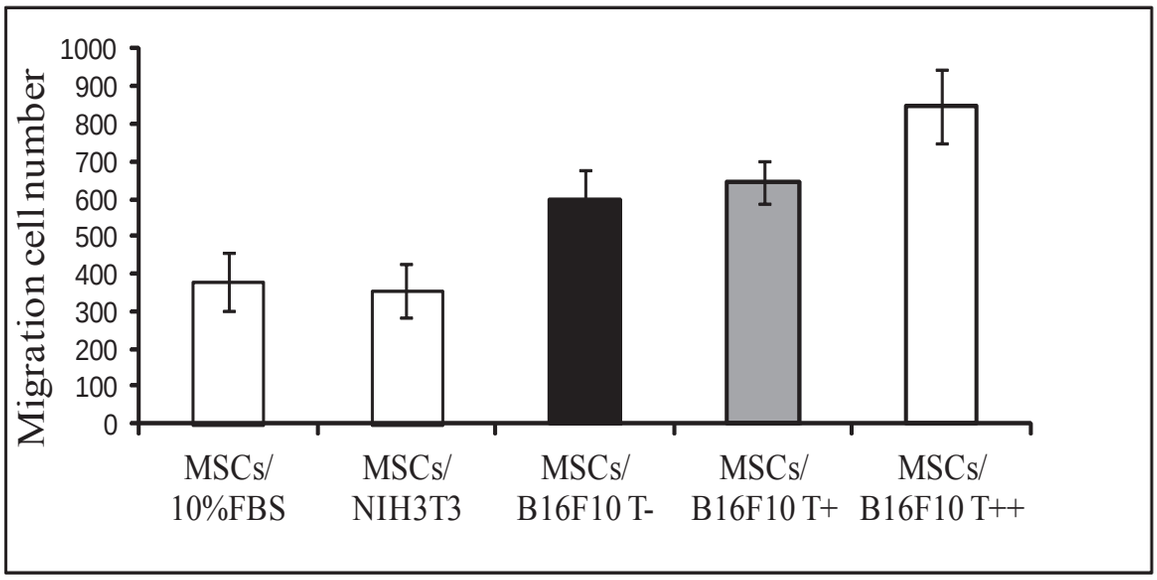

Figure 10. The effect of conditioned medium from the B16F10 cell clones with different T-cadherin expression on migration of MSCs. Migrated MSCs were quantified after fixation and hematoxylin staining of the membrane. The results are the means ± SEM of three independent experiments performed in duplicates, $p<0.05$.

Gene	Annealing temperature	Forward 5'-3'	Reverse 5'-3'
GAPDH	60	GACCCCTTCATTGACCTCAACTAC	TGGTGGTGCAGGATGCATTGCTGA
β-actin	61	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
VEGF A	60	AGAGCAGAAGTCCCATGAAGTGA	TCAATCGGACGGCAGTAGCT
PDGF B	58,5	TCTCTGCTGCTACCTGCGTCTGG	GTGTGCTCGGGTCATGTTCAAGTC
HGF	60	TCATTGGTAAAGGAGGCAGCTATA	CTGGCATTTGATGCCACTCTTA
MMP2	53	AGTTCCCGTTCCGCTTCC	GACACATGGGGCACCTTCTG
EGF	60	CCTGCCCCCTTCCTAGTTTTC	CTCCGTTCTGTTGGTCTACCC
uPAR	60	CGTTACCTCGAGTGTGCGTCCTG	AGCCTCGGGTGTAGTCCTCATCCT
c-Met	60	CAACGAGAGCTGTACCTTGACCTTA	GCGGGACCAACTGTGCAT
uPA	53	GAATGCGCCTGCTGTC	AGGGTCGCTTCTGGTTGTC
MMP9	61	GCGGTGTGGGGCGAGGTG	CCAGGGGGAAAGGCGTGTG

Table 1. Sequences of primers used in qRT-PCR.

In normal conditions, the blood vessel growth is strictly controlled by the balance between pro-angiogenic and and anti-angiogenic factors. At the same time, tumor progression is accompanied by neoangiogenesis due to enhanced production of pro-angiogenic molecules by tumor and stromal cells (Hanahan and Folkman, 1996). To elucidate the mechanisms

responsible for T-cadherin-mediated suppression of angiogenesis in primary melanoma we performed qRT PCR and PCR Arrays. No difference in the mRNA expression level of the main angiogenic growth factors such as VEGF A, HGF, bFGF, EGF, PDGF B, TGFβ between control and T-cadherin-expressing clones was revealed. However, PCR Array analysis revealed that T-cadherin expression in B16F10 cells resulted in upregulation of mRNA of such antiangiogenic molecules as CXCL 10 (Strieter et al., 1995); angiopoietin 2 (Cao et al., 2007); procollagen type XVIIIα1 - a precursor of the angiogenesis inhibitor endostatin (O'Reilly et al., 1997) and chromogranin A - a precursor of angiogenesis inhibitor vasostatin-1 (Belloni et al., 2007). Angiopoietin 2 acts together with VEGF A in initiating blood vessel growth through inhibition of the interactions between endothelial and perivascular cells and destabiliziation of blood vessels. However, in the absence of VEGF A, angiopoietin 2 suppresses angiogenesis and promotes vessel regression (Holash et al., 1999). Since in the present study VEGF A level was not changed after the expression of T-cadherin, the elevated angiopoietin 2 expression could act in reducing the number of newly formed vessels. We also found that the T-cadherin-expressing B16F10 cells demonstrated decreased expression of angiogenic molecules TGFα (Leker et al., 2009) and Tie 1 (Sato et al., 1995). Thus, the PCR Array analysis indicated that the balance between the pro-angiogenic and anti-angiogenic factors was shifted towards the latter, which could reduce the number of medium size vessels and capillaries in vivo.

It is well known that MSCs secrete many growth factors and cytokines and their production increases in hypoxic conditions (Rubina et al., 2009; Martin-Rendon 2007). Among them is HGF/SF, which is a potent stimulator of DNA synthesis and growth in normal human melanocytes and melanoma cells (Halaban et al., 1993). Thus, it was shown that overexpression of the proto-oncogene c-Met (HGFR - HGF receptor) is tightly correlated with human melanoma progression from the radial to the vertical stage (Natali et al., 1993). In the present study we found that control B16F10 melanoma cells expressed low levels of c-Met and no HGF. Our quantitative PCR analysis demonstrated that T-cadherin overexpression resulted in 6-fold increase in the content of c-Met mRNA in melanoma cells. This data suggested that one of the mechanisms by which T-cadherin could be able to affect the growth of B16F10 melanoma cells is the regulation of c-Met/HGF signaling pathway. We speculated that the melanoma cells expressing T-cadherin could secrete the high levels of chemokines resulting in MSCs recruitment to the primary tumor site. MSCs in hypoxic conditions are known to increase the production of HGF (Rubina et al., 2009). Thus, in hypoxic conditions of the primary tumor the recruited MSCs could produce the high levels of HGF, which upon binding to c-Met on melanoma cells could cause their increased proliferation and invasion

Apparently, invasive and metastasizing cancers are characterized by the change in integrin expression pattern (Makrilia et al., 2009). Thus, the overexpressions of integrins such as α3, α5 and α1 or their single subunits were shown to be involved in melanoma growth and progression (Kuphal et al., 2005). So we compared the expression level of certain integrins in the control and T-cadherin expressing melanoma cells using PCR Array. We revealed the upregulation of mRNA expression of α5, αV, αE, and β3 integrins upon T-cadherinsion. This correlated with the increase in the metastatic activity of those cells and possibly contributed to melanoma progression.

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It was shown that the overexpression of some integrins can induce matrix metalloproteinases (MMPs) expression in melanoma cells or their activation (Khatib et al., 2001; Sil et al., 2011). Several MMPs including MMP-1, -2, -3, -7, -9, -13, -14, -15, -16 as well as uPA were implicated in human melanoma progression, invasion and metastasis (Bianchini et al., 2006; Ria et al., 2010). Using qRT-PCR we examined the expression of mRNA of MMPs with gelatinase activity and uPA. No differences in MMP2, MMP9 and uPA expression in the control and T-cadhering positive B16F10 cell clones were revealed. Further PCR The array analysis established that MMP14 was the only protease with enhanced expression detected in the T-cadherin expressing clone T++.

Melanoma cells express multiple isoforms of laminin that were shown to mediate cell attachment and invasion via integrin receptors using laminin as a substrate (Oikawa et al., 2011). In addition, expression of fibronectin was correlated with the acquisition of invasive and metastatic behavior of human melanoma (Gaggioli et al., 2007). Using PCR Array analysis we found that T-cadherin overexpressing melanoma cells exhibited the elevated level of fibronectin 1 and laminin $\alpha 3$ expression suggesting their role in the increased metastatic potential of these cells. The obtained data indicated that T-cadherin expression affects the expression of certain genes involved in regulation of melanoma growth and progression.

In contrast to the results obtained in the present study, the re-expression of T-cadherin by stable transfection in human melanoma cells reduced the rate of tumor growth in the nu/nu mouse tumor model, decreased cell capacity for anchorage-independent growth, migration and invasion in vitro, while cell proliferation was not affected (Kuphal et al., 2009).This discrepancy could be due to the differences in the experimental conditions of the models (highly aggressive murine melanoma B16F10 in the BDF1 mice versus human melanoma cells injected into immunodeficient nu/nu mice). The difference could also be explained by the distinct signaling pathways and spectrum growth factors and receptors expressed by mouse and human melanoma.

10. Conclusions

In the present study, we found that T-cadherin is expressed in normal epidermal keratinocytes, vascular cells of the dermal blood vessels and melanocytes in the human skin. However, upon malignant transformation we observed mosaic pattern of T-cadherin expression in primary melanomas and partial or complete loss of T-cadherin in melanoma metastasis. These data are in accordance with the earlier published results and confirmed the correlation between tumor progression and the loss of T-cadherin expression. It was previously reported that 80% of the human melanoma cell lines did not express T-cadherin and re-expression of T-cadherin reduced the tumorigenicity of these cell lines in nu/nu mouse model. However, 20% of human melanoma cell lines abundantly expressed T-cadherin and possessed invasive and metastatic potential. This prompted us to use a well-described model of highly aggressive murine melanoma B16F10 in BDF1 mice and examine the effect of T-cadherin expression in melanoma cells on their proliferation, tumor growth, invasive and metastatic potential and neovascula-

rization. We showed that overexpression of T-cadherin in melanoma B16F10 cells resulted in the increased tumor growth and metastasi as well as the recruitment of MSC into the primary site. We suggested that in response to the chemoattractants (chemokines) produced by the T-cadherin-expressing tumors, the stromal cells migrated into the primary site and produced HGF. In return, HGF triggered the HGF/c-Met signaling cascade in T-cadherin-expressing melanoma cells that could lead to their increased proliferation and metastasis. The elevated expression level of prooncogenic integrins, the extracellular matrix components and MMP14 in these cells could be contributing factors to enhanced metastatic and invasive potential. However, T-cadherin expression in melanoma cells exerted inhibitory effect on vascularization of the primary tumors, which is likely to be due to the switch in the balance of pro- and antiangiogenic molecules. The established link between the expression of T-cadherin and pathological processes that trigger neovascularization and tumor progression are particularly important in search for new approaches for inhibiting metastasis of the less curable tumors such as disseminated melanoma of the skin. Further investigations studies are needed to identify the role of T-cadherin in the initiation of tumor progression associated with the regulation of neoangiogenesis. Our studies provided new evidence on the role of tumor microenvironment and will help to identify the critical points for suppressing the blood supply at the early stages of tumor progression. We concluded that the expression of T-cadherin in melanoma cells underlies a novel mechanism of stem cell tropism to malignant solid tumors, which may be important for the development of the optimal stem cell-based therapy. Investigation of such mechanisms is an important task in finding new targets for cancer treatment.

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