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Use of multicellular tumor spheroids to dissect endothelial cell–tumor cell interactions: A role for T-cadherin in tumor angiogenesis

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Abstract **This study addresses establishment of an "in vitro" melanoma angiogenesis model using multicellular tumor spheroids (MCTS) of differentiated (HBL) or undifferentiated (NA8) melanoma cell lines. DNA microarray assay and qRT-PCR indicated upregulation of pro-angiogenic factors IL-8, VEGF, Ephrin A1 and ANGPTL4 in NA8-MCTSs (vs. monolayers) whereas these were absent in MCTS and monolayer cultures of HBL. Upon co-culture with endothelial cell line HMEC-1 NA8-MCTS attract, whereas HBL-MCTS repulse, HMEC-1. Overexpression of T-cadherin in HMEC-1 leads to their increased invasion and network formation within NA8-MCTS. Given an appropriate angiogenic tumor microenvironment, T-cadherin upregulation on endothelial cells may potentiate intratumoral angiogenesis.**
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Keywords: Melanoma cell; Tumor spheroid; Angiogenesis; T-cadherin

1. Introduction

Tumor microenvironment is a dynamic composition where cell–cell contacts and cell-extracellular matrix adhesion take place and soluble factor gradients are operational. These events play critical roles in tumor initiation, progression and metastasis. Proliferation in structured three-dimensional (3D) architectures appears to represent a pre-requisite for cancer development. Single cell suspensions of tumor cells are frequently unable to produce life threatening cancer outgrowth, while being able to induce specific immune responses, as opposed to solid tumor fragments [1]. Our previous analyses of gene expression profiles of melanoma cell lines growing in physically different microenvironments, namely in standard 2D monolayer conditions or as 3D multicellular tumor spheroids (MCTSs), showed that structural modifications of the architecture of tumor cell cultures alter expression of a number of genes potentially involved in melanoma progression and dissemination [2–4]. Additionally, MCTSs are poorly immunorecognized by cytotoxic T lymphocytes (CTL) specific for tumor-associated antigens [2–4] suggesting that 3D tumor architecture also affects antigen-specific functions of CTL.

Angiogenesis represents a prerequisite for tumor growth, invasion, progression and metastasis [5]. Cancer cells respond to conditions of hypoxia within the tumor by producing pro-angiogenic factors causing endothelial cell (EC) recruitment and proliferation (reviewed in [6]). Since MCTSs resemble in vivo cancers in their capacity to develop necrotic areas far from nutrient and oxygen supplies, they are considered a useful model for the study of solid tumor angiogenesis. Several studies attempted to investigate tumor angiogenesis by either co-culture of MCTSs with EC monolayers or EC spheroids in vitro or implantation of MCTSs in vivo in animals (reviewed in [7,8]); the majority of these studies have investigated the influence of MCTSs on EC phenotypes and demonstrated that EC angiogenic behaviour and characteristics of the neovessels can be dictated by the nature of the tumor cell. Tumor-penetrating blood vessels differ morphologically and biochemically from vessels in normal organs [9]. However, surface molecules unequivocally specific for ECs lining tumor-invading blood vessels have not yet been identified. Nevertheless, also surface proteins that are substantially overexpressed by tumor-infiltrating blood vessels have enormous clinical importance. For example, the cell surface adhesion molecules integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are overexpressed in the tumor vasculature, and a number of inhibitors are currently being evaluated in clinical trials (reviewed in [10,11]). Another cell surface adhesion molecule, T-cadherin (T-cad), is upregulated in tumor vasculature from murine lung metastases [12] and human metastatic hepatocarcinomas [13,14]. Whereas the role of integrins in tumor angiogenesis is well established (reviewed in [15]), angiogenic properties of T-cad, an atypical glycosylphosphatidylinositol-anchored member of the cadherin superfamily, have only recently been appreciated. In vitro T-cad induces angiogenic phenotypes, facilitates EC migration [16], and stimulates in-gel outgrowth of endothelial sprouts in EC-spheroid and heart tissue models of angiogenesis [17]. In vivo, myoblast-mediated delivery of recombinant soluble

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Abbreviations: MCTS, multicellular tumor spheroids; EC, endothelial cell; HMEC-1, human microvascular EC line; T-cad, T-cadherin; CTL, cytotoxic T lymphocytes

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T-cad to mouse skeletal muscle facilitates VEGF-induced angiogenesis [17]. The role for T-cad in tumor angiogenesis has not been directly investigated.

By using co-cultures of melanoma MCTSs and microvascular ECs, we (1) examined the angiogenic potential of melanoma cells and (2) investigated the effects of T-cad expression on tumor angiogenesis. We found that melanoma cell lines at different stages of differentiation are endowed with distinct angiogenic properties. Furthermore, we show that upregulation of T-cad in ECs promotes their penetration and formation of network structures within MCTSs from poorly differentiated melanoma cells.

2. Materials and methods

2.1. Cell culture

Melanoma cell lines were cultured in RPMI 1640 containing non-essential aminoacids, sodium pyruvate, glutamine, HEPES buffer and antibiotics and supplemented with 10% fetal calf serum [3,4]. Multicellular tumor spheroids (MCTSs) of melanoma cells were prepared in plastic culture plates precoated with 50 mg/ml poly-2-hydroxyethyl methacrylate (polyHEMA, Sigma–Aldrich. St. Louis, MO, USA) as described before [3,4]. Human microvascular EC line, HMEC-1 (PromoCell GmbH, Heidelberg, Germany), was cultured in EC growth medium containing growth supplement (PromoCell) and 10% fetal calf serum. Co-cultures of HMEC-1 and melanoma MTCSs used media specific for either cell type at a 1:1 ratio.

2.2. Real-time RT-PCR

Total cellular RNA was extracted from melanoma cells cultured in 2D or as MCTSs and reverse transcribed. cDNA was amplified in qRT-PCR assays as detailed before [4] in the presence of primers and probes specific for Melan-A/MART-1, gp100 and tyrosinase, IL-8, VEGF1 and VEGF2. Expression of house keeping gene GAPDH was used as reference.

2.3. Co-culture and microscopy imaging

In initial experiments 3-day cultured MCTSs were placed above HMEC-1 monolayer cultures and the behaviour of HMEC-1 cells monitored under a phase contrast Olympus IX-50 inverted microscope equipped with a digital camera. Subsequent experiments focused on invasion of HMECs into MCTSs. Tumor cells prior to preparation of MCTSs and HMEC-1 were differentially stained either with PKH26 red fluorescent cell linker (Sigma–Aldrich) or CFSE (Molecular Probes, OR, USA). HMEC-1 were seeded over labelled 3-day cultured MCTSs, and images captured using laser scanning confocal microscope Zeiss LSM 510 at different time points.

2.4. Overexpression of T-cadherin and analysis of intra-MCTS angiogenesis

T-cadherin was overexpressed in HMEC-1 cells by adenoviral mediated gene transfer as detailed before [18]. After overnight infection with empty- or T-cad-containing adenovectors, HMEC-1 were seeded above MCTSs. After 6 days MCTSs were collected, fixed in 4% formaldehyde, washed in PBS, and embedded in paraffin [4]. Microtome sections were examined using a Zeiss Axiophot fluorescent microscope (Zeiss, Feldbach, Switzerland) and images recorded using a digital camera and AnalySIS software (Soft Imaging System GmbH, Münster, Germany).

3. Results

3.1. Selection of melanoma cell lines

Melanoma cell frequently express genes encoding enzymes shared by untransformed melanocytes, mostly involved in the melanogenesis process. A number of these gene products encompass antigenic peptides recognized by CD4+ or CD8+ T cells in a HLA restricted manner [19]. Notably, concordant loss of expression of these so-called differentiation antigens is frequently observed in metastatic lesions [20]. To identify cellular reagents "bona fide" representative of different tumor stages, we screened a panel ($n = 15$) of melanoma cell lines for expression of gp100, Melan-A/MART-1 and tyrosinase differentiation antigens. We identified one cell line, HBL, characterized by concordant expression of these genes and one cell line, NA8, where no specific transcripts could be amplified (Fig. 1A). Both NA8 and HBL cell lines derive from metastatic melanoma and have widely been used in tumor immunology studies [2,21–23].

3.2. Tumor spheroid model system

Proliferation of both NA8 and HBL cells in 2D cultures reaches a plateau within 7 days, whereas MCTS culture follows a slow sigmoid growth curve [3,4]. After 10–12 days of culture necrotic cores appear within the MCTSs, resulting in hollow centers with large, compact cells typically detectable in the periphery (Fig. 1B). Appearance of necrotic cores might be due to severe hypoxia (oxygen deprivation), low pH, and nutrient starvation [6].

3.3. NA8-tumor spheroids express proangiogenic molecules

We previously reported on high throughput gene expression profiles in monolayer cultures and MCTSs from NA8 [4] and HBL [3] melanoma cells. Here, to specifically evaluate the pro-angiogenic properties of NA8 and HBL cells we comparatively analysed in greater detail the expression of a number of genes whose products are known to promote angiogenesis in cells cultured in standard monolayers and in MCTS. We found that genes encoding proangiogenic factors IL-8, VEGF, Ephrin A1 and Angiopoietin-like 4 protein (ANGPTL4) were upregulated in NA8-MCTSs vs. monolayer cultures, but absent, with the exception of ANGPTL4, in HBL cultures (Fig. 2A). HBL-MCTS exhibited upregulation of Semaphorin 6D mRNA whereas this gene was not expressed in NA8-MCTS.

To validate the expression profile of genes encoding pro-angiogenic factors we performed real-time quantitative RT-PCR experiments. Data shown in Fig. 2B confirm the upregulation of IL-8, VEGF1 and VEGF2 gene expression in NA8 cells sampled after 3-day culture in MCTS vs. monolayer cultures.

3.4. Endothelial cells are attracted by NA8-tumor spheroids but repulsed by HBL-tumor spheroids

Prompted by gene expression data we performed co-culture experiments in which MCTSs from NA8 and HBL cell lines, representative of poorly or highly differentiated melanoma cells, respectively, were placed onto confluent HMEC-1 monolayers. Periodic observation by phase contrast microscopy revealed distinct effects of the two types of melanoma spheroids on HMEC-1. HMEC-1 were repulsed by HBL-MCTS within 6 h (Fig. 3A) but remained as an intact monolayer upon co-culture with NA8-MCTS HMEC-1 (Fig. 3B).

To further investigate the differential response of HMEC-1 to NA8-MCTS and HBL-MCTS we performed co-culture experiments following differential staining of melanoma cells and HMEC-1 with either red or green fluorescent dyes, respectively. In these experiments HMEC-1 (green) were seeded

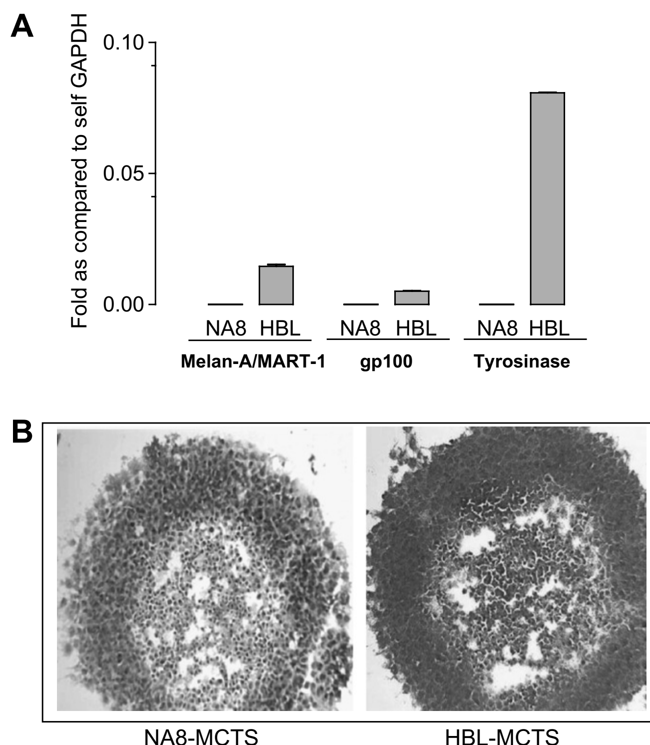
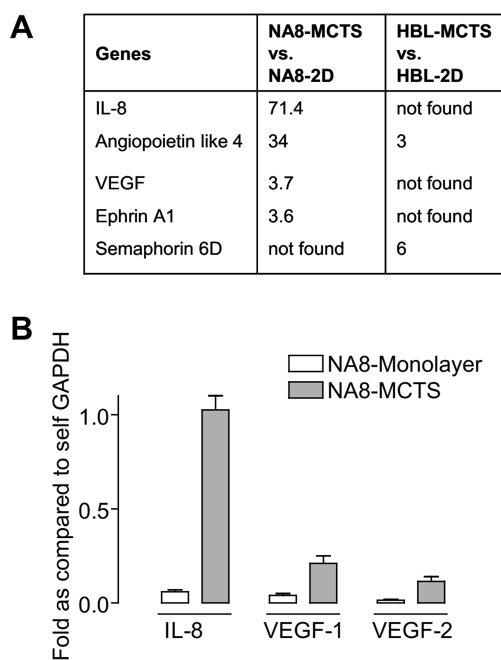


Fig. 1. Expression of differentiation markers in HBL and NA8 melanoma cell lines and morphological features of MCTSs from HBL and NA8 melanoma cells. Expression of Melan-A/MART-1, gp100 and tyrosinase genes in 2D cultures of HBL and NA8 cells was evaluated by qRT-PCR (A). Hematoxylin/eosin-stained sections of 10-day cultures of NA8-MCTS and HBL-MCTS show the appearance of necrotic cores (B).


A

Genes	NA8-MCTS vs. NA8-2D	HBL-MCTS vs. HBL-2D
IL-8	71.4	not found
Angiopoietin like 4	34	3
VEGF	3.7	not found
Ephrin A1	3.6	not found
Semaphorin 6D	not found	6

Fig. 2. Upregulation of genes encoding pro-angiogenic factors in NA8-MCTS. Architecture-dependent modulation of genes encoding factors regulating angiogenesis as obtained from DNA microarray analysis in 3-day MCTS and 2D cultures of NA8 and HBL cells (A). Values refer to increases in expression in MCTSs relative to expression in 2D. qRT-PCR evaluation of gene expression in NA8 cells cultured as monolayers (open bars) or MCTSs (closed bars) for 3 days (B).

above tumor spheroids (red), and images were captured under confocal microscopy after 3 days of co-culture. The images clearly revealed deflection from HBL-MCTS (Fig. 3C) but penetration of HMEC-1 into NA8-MCTS (Fig. 3D). Thus spheroids composed of NA8 melanoma cells attract endothelial cells whereas spheroids composed of HBL melanoma cells repulse them.

3.5. Overexpression of T-cadherin in endothelial cells increases invasion/migration into NA8 tumor spheroids

Taking advantage of the model thus established, we used NA8 and HBL melanoma cells to investigate whether upregulation of T-cadherin expression on HMEC-1 might directly affect their interaction with MCTS. HMEC-1 infected with either empty-adenovirus (HMEC-empty-Adv) or T-cad-adenovirus (HMEC-T-cad-Adv) and labelled with a red fluorochrome were seeded above green fluorochrome-labelled tumor spheroids, and co-cultures monitored under confocal microscopy. Invasion/migration of HMEC-1 into NA8-MCTS was evident after 6 days of co-culture (Fig. 4). Most importantly, invasion/migration of HMEC-T-cad-Adv into NA8-MCTS (Fig. 4C) was much more pronounced as compared to control HMEC-empty-Adv (Fig. 4A), yielding an image of almost uniform distribution within the spheroid. To further evaluate the organization of HMEC-1 after invasion, microtome sections of NA8-MCTS following 10 days co-culture with fluorochrome-labelled HMEC-empty-Adv or HMEC-T-cad-Adv were prepared and examined by fluorescence microscopy. Micrographs revealed the prominent formation of a

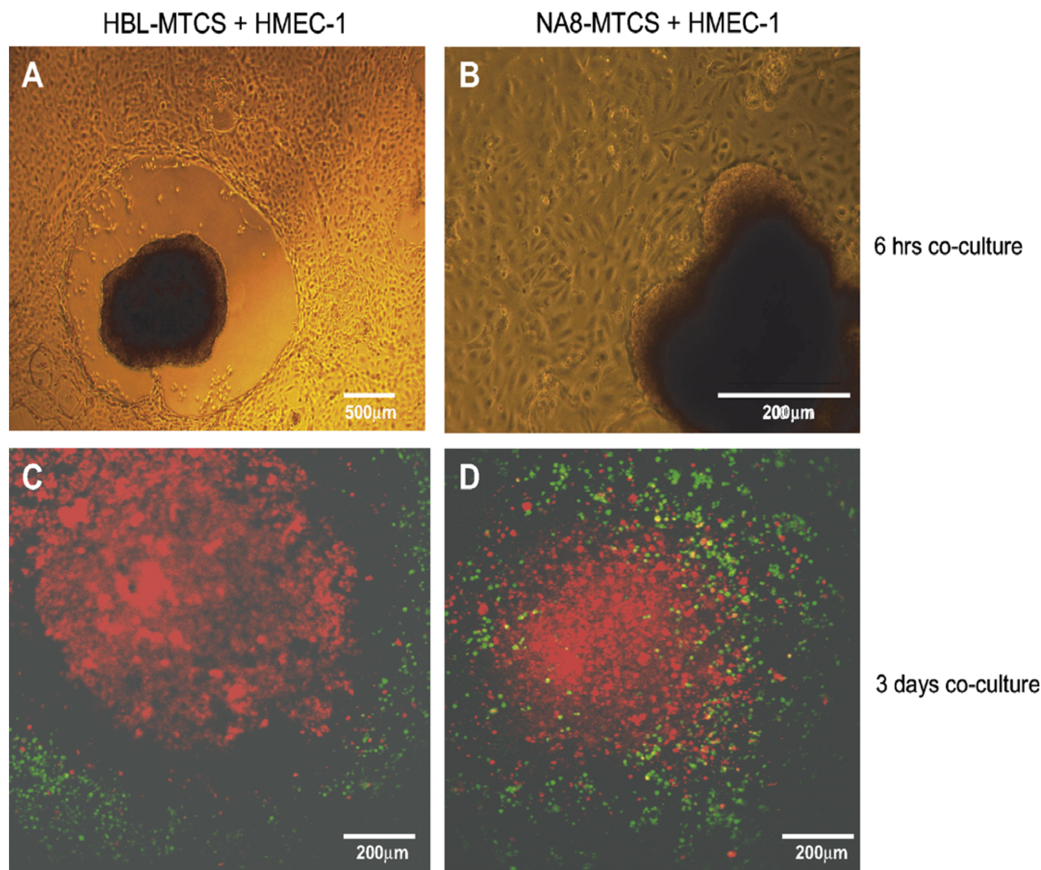


Fig. 3. Endothelial cells invade NA8-MCTS but not HBL-MCTS. HBL-MCTSs (A) or NA8-MCTSs (B) were placed upon HMEC-1 monolayers and phase-contrast microscopy images taken after 6 h of co-culture. HMEC-1 pre-stained with CFSE (green fluorescence) were seeded (at 10000 cells) on top of individual PKH26 (red fluorescence) pre-stained HBL-MCTS (C) or NA8-MCTSs (D) confocal microscopy images were captured after 3-days.

network-like pattern of interconnecting tubular structures by T-cad overexpressing HMEC-1 within NA8-MCTS (Fig. 4D).

The inability of HMEC-1 to invade HBL tumors was not overcome by overexpression of T-cad (data not shown). Since neither NA8-MCTS nor HBL-MCTS expressed T-cad (data not shown), the repulsive effects of HBL-MCTS are unlikely to be due to homophilic T-cadherin interactions and contact inhibition [16,24–26].

4. Discussion

We addressed the establishment of an "in vitro" melanoma angiogenesis model. To at least in part take into account the high heterogeneity of cancer cells, we selected a cell line, HBL, characterized by expression of differentiation markers, and a cell line, NA8, where this was undetectable. Hypoxia and acidosis are microenvironmental hallmarks of solid tumors, and our 3D MCTS model system closely simulates such a microenvironment, as manifested by the eventual development of an inner necrotic core upon prolonged (>10 days) culture. Expression of pro and anti-angiogenic genes can be triggered by metabolic stress. Our DNA microarray assay indicated upregulation of several angiogenic factors, such as IL-8, VEGF, Ephrin A1 and ANGPTL4 in NA8-derived MCTSs (vs. 2D cultures) after only 3 days culture, which is long in advance of formation of a necrotic core. None of these angiogenic factors, with the exception of ANGPTL4 were detectable in either 2D or 3D cultures of the relatively differentiated HBL-MCTS. Interestingly, HBL-MCTSs exhibited upregulation of Semaphorin 6D whereas expression of this gene was absent in the relatively undifferentiated NA8-MCTSs.

Consistent with the expression of angiogenic regulators in the two melanoma-cell derived MCTSs, our investigation of the angiogenic potential of MCTSs by co-culture with HMEC-1 revealed pro-angiogenic properties for undifferentiated NA8-derived MCTSs but repulsive properties for differentiated HBL-derived MCTSs. Proangiogenic activity of IL8 was first demonstrated by Koch et al. [27] and the role of IL8 in tumor angiogenesis has since been well studied (reviewed in [28]). Neutralizing antibody to IL8 inhibited tumor angiogenesis and concomitant metastasis in melanoma [29]. Li et al. reported IL8 induced endothelial cell survival via anti-apoptotic Bcl family members resulting in increased angiogenesis [30]. IL8 was reported to induce angiogenesis in HIF-1/VEGF dependent fashion [31]. VEGF is one of the key regulators of angiogenesis. Low extracellular pH can cause stress-induced alteration in gene expression, such as upregulation of VEGF and IL-8 in tumor cells in vitro [32]. ANGPTL4 is one of the pro-angiogenic factors induced by hypoxia regulating angiogenesis and metastasis [33].

Contact between tumor and ECs may promote toxic effects. In a study on co-culture of MCTS of malignant melanoma cells (ST-ML-12) and ECs Offner et al. observed EC death;

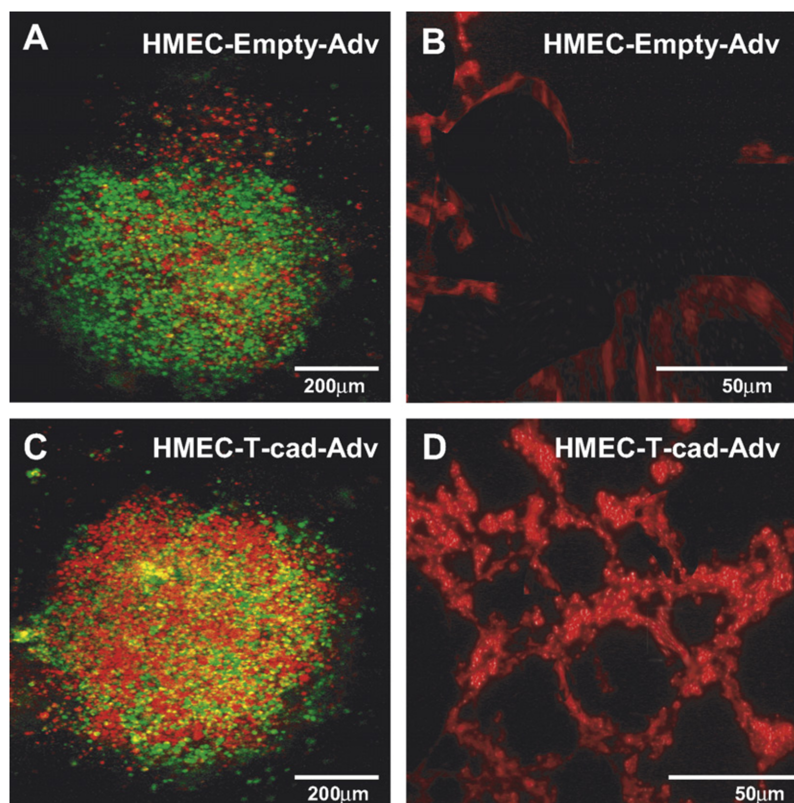


Fig. 4. T-cadherin facilitates endothelial cell invasiveness and network formation within NA8-MCTS. HMEC-empty-Adv or HMEC-T-cad-Adv were stained with PKH 26 (red fluorescence) and seeded on top of NA8-MCTS either prestained (A, C) or not (B, D) with CFSE (green fluorescence). Confocal microscopy images (A, C) were captured after 6 days co-culture. Fluorescence microscopy images of microtome sections of MCTS (B, D) were taken after 10 days co-culture.

an involvement of reactive oxygen species was proven by inclusion of antioxidants, which rescued endothelial apoptosis [34]. The repulsive effect of HBL-MCTSs in our experiments does not reflect EC death. We might speculate that the repulsive effect of HBL-MCTSs could be due to their expression of Semaphorin 6D. A role for Semaphorin 6D in the regulation of angiogenesis has not yet been reported, but Semaphorin 3F has been reported as a potent for chemorepulsant ECs. Implantation of Semaphorin 3F-transfected cells into mice produced a chemorepulsive effect on ECs and eventually decreased tumor metastasis [35].

The MCTS model proposed here can be exploited to address interactions between EC and tumor cells in the presence of altered expression of EC molecules. Evidence is mounting to support a pro-angiogenic role for EC-expressed T-cad. Studies in human have demonstrated upregulation of T-cad in tumor vasculature in vivo [12–14]. Further upregulation of T-cad has been shown to induce angiogenesis in both in vitro and in vivo models [17,18]. However, a recent study recently using the Matrigel-plug model of angiogenesis reported that implantation of L929 cells overexpressing T-cadherin inhibited angiogenesis [26]. Here, we compared the 3D-angiogenic outcome following co-culture of MCTSs with control and T-cad overexpressing HMEC-1. Overexpression of T-cad in HMEC-1 led to increased invasion/network formation within undifferentiated NA8-MCTSs but did not overcome the repulsive effects of more differentiated HBL-MCTSs, thereby suggesting that, given an appropriate angiogenic tumor microenvironment, T-cad upregulation on EC may potentiate intratumoral angiogenesis.

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