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SHORT COMMUNICATION

Melanoma Spheroid Formation Involves Laminin-Associated Vasculogenic Mimicry

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Melanoma is a tumor where virulence is conferred on transition from flat (radial) to three-dimensional (tumorigenic) growth. Virulence of tumorigenic growth is governed by numerous attributes, including presence of self-renewing stem-like cells and related formation of patterned networks associated with the melanoma mitogen, laminin, a phenomenon known as vasculogenic mimicry. Vasculogenic mimicry is posited to contribute to melanoma perfusion and nutrition *in vivo*; we hypothesized that it may also play a role in stem cell—driven spheroid formation *in vitro*. Using a model of melanoma *in vitro* tumorigenesis, laminin-associated networks developed in association with three-dimensional melanoma spheroids. Real-time PCR analysis of laminin subunits showed that spheroids formed from anchorage-independent melanoma cells expressed increased α4 and β1 laminin chains and α4 laminin expression was confirmed by *in situ* hybridization. Association of laminin networks with melanoma stem cell—associated nestin and vascular endothelial growth factor receptor-1 also was documented. Moreover, knockdown of nestin gene expression impaired laminin expression and network formation within spheroids. Laminin networks were remarkably similar to those observed in melanoma xenografts in mice and to those seen in patient melanomas. These data indicate that vasculogenic mimicry—like laminin networks, in addition to their genesis *in vivo*, are integral to the extracellular architecture of melanoma spheroids *in vitro*, where they may serve as stimulatory scaffolds to support three-dimensional growth. *(Am J Pathol 2014, 184: 71—78; http://dx.doi.org/10.1016/j.ajpath.2013.09.020)*

Vasculogenic mimicry (VM) is a phenomenon that may be observed in aggressive melanoma cell lines that contain subpopulations of cells with an embryonic-like phenotype.[1] First described by Maniotis, Hendrix, and colleagues in 1999,[2] VM is defined by expression of endothelial genes by primitive tumor cells in association with net-like or tubular patterns of extracellular matrix deposition.[3,4] VM-like networks within melanoma are PAS-positive and, in patients, are associated with poor prognosis independent of Breslow depth.[5,6] In addition to PAS positivity, laminin expression is also an important biomarker associated with VM.[1,7] Inhibition of the laminin γ2 chain reduced the expression of vasculogenic mimicry—associated genes in melanoma cells.[8] Furthermore, when nonaggressive melanoma cells were plated onto collagen matrices preconditioned with laminin networks made by aggressive melanoma cells, they formed tubules and networks along the existing laminin scaffolds.[8] Genes involved in angiogenesis and vasculogenesis were up-regulated in aggressive melanoma cells.[9] These included vascular endothelial growth factor-C (*VEGF-C*), vascular endothelial (VE)-cadherin (*CD144*), tyrosine kinase with Ig and epidermal growth factor homology domains-1 (*TIE-1*), the laminin-5 γ2 chain, and ephrin-A2. The amount of VE-cadherin and TIE-1 seen on Western blots was greater in highly aggressive, as opposed to less aggressive, melanoma cell lines.[10] CD31 was not present. Melanoma cells with

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VE-cadherin knockdown lost the ability to form VM-like networks,[10] implicating their crucial role in VM.

We have previously confirmed laminin chain β2 networks in melanoma xenografts to be dependent on expression of vascular endothelial growth factor receptor-1 (VEGFR-1) by stem-like melanoma cells that coexpress endothelial markers VE-cadherin and TIE-1, but not CD31.[11] VM, as described by Hendrix and colleagues,[1,3] may be promoted by a hypoxia-driven *in vivo* pathway whereby aggressive cancer cells contribute to perfusion during periods of rapid growth via true anastomoses with authentic tumor vessels or by transporting fluid from sites of intralesional vascular leakage.[12,13]

Laminin is a heterotrimeric protein composed of various combinations of one of five α, three β, and three γ subunits. Laminin serves as an important basement membrane protein, with laminin 411 (α4, β1, γ1) involved in formation of vascular basement membranes. Various laminin isoforms are expressed by melanoma cell lines and patient melanomas.[8,14,15] Throughout this paper, unless a specific laminin chain or heterotrimer is designated, we use laminin as a generic term for the protein family or when the specific laminin isoforms are unknown. Moreover, studies have implicated a correlation between laminin expression and melanoma virulence, although the biological basis for this association remains conjectural. It is known, however, that extracellular laminin (either secreted by keratinocytes, endothelial cells, or added exogenously) promotes melanoma mitogenesis, growth, and migration.[16–20] Indeed, particular regions within laminin chains have been found to enhance melanoma metastasis to organs such as lung and liver.[21,22]

In this study, we used melanoma spheriods as a model for three-dimensional tumorigenesis in an effort to study laminin-associated VM.[23–26] Although previous pioneering work has established the ability of several melanoma cell lines to produce VM on radial proliferation on laminin gels,[2] examination of three-dimensional models that recapitulate the expansile tumorigenic growth phase of melanoma is lacking. Melanoma spheroids have been used as assays for stem cell activity and have been implicated in melanoma self-renewal and tumorigenesis.[27,28] We hypothesized that not only is VM integral for melanoma nutrition and perfusion but also plays an important role in stem cell–driven spheroid formation, a potential three-dimensional *in vitro* surrogate for early tumorigenic growth. The data presented support this hypothesis and establish melanoma spheroids as a novel model for exploring the pathobiology of VM.

Materials and Methods

Cell Lines and Cell Culture

Human melanoma cell A2058 and A375 were originally obtained from American Type Culture Collection (Manassas, VA). Melanoma cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) and 200 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37°C, 5% CO_2. Viable cells were counted by Trypan blue exclusion assay under a hemocytometer.

Melanoma Spheroid Cultures

Liquefied Matrigel (50 μL; Millipore, Billerica, MA) was spotted onto a Petri dish and allowed to set at 37°C for 2 hours; 5×10^3 A2058 viable cells per 10 μL complete medium were loaded over Matrigel, incubated at 37°C, 5% CO_2, for 4 hours for attachment, and then overlaid with complete medium and cultured for 7 to 10 days. Melanoma spheroids over Matrigel were fixed with formalin and embedded. Perpendicular sections were used for histological analysis.

Melanoma spheroid culture in suspension was performed at low cell-plating density (2000 viable cells per 6-well or 1000 viable cells per 24-well) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine in the ultra-low attachment plate (Corning, Acton, MA) at 37°C containing 5% CO_2 for 14 to 21 days. Spheroids were fed with 0.5 mL of fresh medium twice weekly. They were collected, fixed, and processed for histological analysis using a Cellient Automated Cell Block System (Hologic, Marlborough, MA). Epithelial growth factor and fibroblast growth factor-2 were not used for Matrigel or suspension cultures because they may alter nestin expression.[29,30] Methylcellulose was used in the media for all spheroid cultures to prevent aggregation.[31]

shRNA Gene Knockdown

A lentivirus-based shRNA method was used to knock down the gene expression of nestin. shRNA clones (TRCN000014728, TRCN000014729, TRCN000014730, TRCN000014731, and TRCN000014732) specifically targeting nestin were purchased (Sigma-Aldrich). shRNA lentivirus was produced in HEK293T cells by cotransfecting shRNA lentiviral backbone and packaging vectors psPAX2, pMD2.VSV-G as described before.[32] Supernatant was collected and filtered through 0.45-μm filters and used to transfect target cells. A nontargeting, scramble vector (SHC002; Sigma-Aldrich) was used as vector control. Efficacy of nestin gene knockdown (nestin KD) was confirmed by quantitative real-time PCR and Western blot. Among the five shRNA clones examined, TRCN000014728, which targeted the 3′-untranslated region of nestin, had the highest KD efficiency and was routinely used in this study.

Quantitative Real-Time RT-PCR

Total RNA from A2058 adherent cells and spheroid-forming cells, including nestin KD and vector controls,