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Differential Mechanisms of Tumor Progression in Clones From a Single Heterogeneous Human Melanoma

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Abstract

We used vertical growth phase (VGP) human VMM5 melanoma cells to ask whether the tumor microenvironment could induce matrix metalloproteinase-1 (MMP-1) in vivo, and whether this induction correlated with metastasis. We isolated two clones from parental VMM5 cells: a low MMP-1 producing clone (C4) and high producing clone (C9). When these clones were injected orthotopically (intradermally) into nude mice, both were equally tumorigenic and produced equivalent and abundant amounts of MMP-1. However, the tumors from the C4 clones displayed different growth kinetics and distinct profiles of gene expression from the C9 population. The C4 tumors, which had low MMP-1 levels in vitro, appeared to rely on growth factors and cytokines in the microenvironment to increase MMP-1 expression in vivo, while MMP-1 levels remained constant in the C9 tumors. C9 cells, but not C4 cells, grew as spheres in culture and expressed higher levels of JARID 1B, a marker associated with melanoma initiating cells. We conclude that VMM5 melanoma cells exhibit striking intra-tumor heterogeneity, and that the tumorigenicity of these clones is driven by different molecular pathways. Our data suggest that there are multiple mechanisms for melanoma progression within a tumor, which may require different therapeutic strategies.

Malignant melanoma is one of the fastest growing cancers, and even a small superficial skin lesion can be deadly if it acquires the ability to invade into the dermis (www.cancer.org). Melanoma is thought to progress in a step-wise fashion, from pigmented nevus to dysplastic nevus to non-invasive but overtly malignant radial growth phase (RGP) and finally, to invasive and metastatic vertical growth phase (VGP) (Clark et al., 1975; Balch et al., 2004; Smalley et al., 2005). The mechanisms that convert non-metastatic RGP to VGP are not totally understood, but may involve enhanced signal transduction mediated by a mutation in BRAF (Huntington et al., 2004; Ryu et al., 2011), along with expression of the G protein coupled receptor protease activator receptor-1 (PAR-1) and the interstitial collagenase, matrix metalloproteinase-1 (MMP-1) (Brauer et al., 2011; Blackburn et al., 2007, 2009).

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MMP-1 is one of only a few enzymes active at neutral pH that can degrade the interstitial collagens, types I, II, and III, the body's most abundant proteins, and the destruction of these collagens is essential to the metastatic process (Brinckerhoff et al., 2000; Brinckerhoff and Matrisian, 2002). Indeed, high levels of MMP-1 expression correlate with increased metastasis and decreased patient survival (Airola et al., 1999; Noll et al., 2001; Nikkola et al., 2002; Ryu et al., 2011).

Recently, we confirmed and extended the role of MMP-1 in melanoma progression in two studies. First, using VGP VMM12 melanoma cells, a highly aggressive line that constitutively produces abundant levels of MMP-1, we silenced MMP-1 expression by stably transfecting cells with shRNAs. We found that primary tumor growth at the orthotopic (intradermal) site was not affected, but that silencing MMP-1 significantly reduced angiogenesis at the primary site and metastasis to the lung. Although degradation of the extracellular matrix is one mechanism for enhancing metastasis, we also found that MMP-1 cleaved PAR-1, initiating signal transduction pathways that activated a profile of genes involved in angiogenesis and metastasis (Boire et al., 2005; Blackburn et al., 2007, 2009). Conversely, we ectopically over-expressed MMP-1 in the RGP Bowes cell line and found increased tumorigenesis at the primary orthotopic site and increased metastasis to the lung (Blackburn et al., 2009).

In the present study, we expanded our investigations to another VGP melanoma cell line, VMM5 (Huntington et al., 2004). These tumor cells have been shown to display antigenic variability (Yamshchikov et al., 2005), and we hypothesized that they might also display intra-tumor heterogeneity, specifically with respect to MMP-1 expression. Thus, we began by asking whether MMP-1 expression could be enhanced in vivo in melanoma cells with lower levels of MMP-1 expression, and whether this induction correlated with an increase in metastasis. The VMM5 melanoma cells carry the BRAF^{V600E} mutation and produce MMP-1 constitutively, but at somewhat lower levels than VMM12 cells (Huntington et al., 2004). We cloned several lines from the parental VMM5 cells, selecting a high MMP-1 producing line and a low MMP-1 producing line. Interestingly, we found that when these clones were injected orthotopically into mice, both cell lines produced abundant amounts of MMP-1 and were equally tumorigenic. However, they displayed different growth kinetics and distinct profiles of gene expression. We conclude that VMM5 melanoma cells exhibit striking intra-tumor heterogeneity in their biologic characteristics, suggesting the existence of multiple mechanisms for melanoma progression.

Materials and Methods

Cell culture

VGP VMM5 melanoma cells were a gift from Dr. Craig L. Slingluff, Jr. (U. Virginia). These cells have been described previously (Huntington et al., 2004; Yamshchikov et al., 2005). Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin and streptomycin. VMM12 VGP melanoma cells (Huntington et al., 2004; Blackburn et al., 2007) were cultured similarly.

Sub-cloning of VMM5 cells

Parental VMM5 VGP melanoma cells were grown to confluence in a 6-well plate. Cells were trypsinized with 500 μ l of Trypsin (0.25%; 1 $\times$) to each well to obtain single cell suspensions. Fifteen centimeter plates, each with 30ml of DMEM/10% FBS and 1, 2, or 5 μ l of trypsinized cell suspension was added to each plate to ensure that each cell was separated from other cells, thereby allowing cells to form a single colony. Colonies were visible after 2–3 weeks. Individual clones were collected with cloning filter discs (PGC Scientific, Palm

Desert, CA) pre-soaked in 0.25% trypsin, and seeded into 6-well plates. Clones were maintained separately.

Measurement of mRNA

Total cellular RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) as described by the manufacturer. Reverse transcription was performed with 2 µg of total RNA using the iScript cDNA Synthesis kit (Bio Rad, Hercules, CA) according to the manufacturer's instructions. Cellular levels of mRNA were measured by qRT-PCR using the iQ SYBR Green Supermix (Bio Rad) according to the manufacturer's instructions. The primers for human MMP-1, TGFβ1, MCP-1, IL-6, and IL-8 are listed in Supplemental Table 1. All assays were carried out in triplicate, with machine duplicates. Gene expression was normalized to β2microglobulin (β2M), with the exception of MMP-1 expression. For MMP-1, a standard curve with a plasmid DNA was included in order to quantitate mRNA levels, which are reported as pg MMP-1/pg β2M. For other mRNAs, the data are reported as $2^{-\Delta \Delta (Ct)}$.

Western blot

Confluent cultures of C4, C9, and VMM12 cells in 6-well plates were placed in 1 ml serum-free medium containing 0.2% lactalbumin hydrolysate (LH) (Sigma, St. Louis, MO) (Huntington et al., 2004; Blackburn et al., 2007) for 24 or 48 h. Medium was collected and proteins from 1 ml of medium were precipitated with trichloroacetic acid at a final concentration of 3%. Pellets were washed in 95% ETOH/0.1 M KOAc and resuspended in 30 µl 2× Laemmli buffer. Twenty microliters were boiled for 5 min and proteins were resolved by SDS-PAGE. Proteins were transferred to a PVDF membrane and MMP-1 protein was visualized with anti-MMP-1 from Millipore AB6002 (Temecula, CA) at a dilution of 1:4,000.

In vitro proliferation of melanoma cells

C4 and C9 melanoma cells were seeded into 6-well plates in DMEM with 10% FBS at 5×10^4 cells/well. Cultures were trypsinized at 24, 46, 72, and 96 h and cell number was determined by counting each well in quadruplicates. Final cell count was the average of all counts for each time point. Trypan Blue staining was used to determine viability, which ranged from 90% to 98% at all time points for all cell lines.

Orthotopic injection of tumor cells

Cells were grown to confluence in DMEM 10% FBS. On the day of injection, C4 and C9 cells were trypsinized, washed 3× with sterile PBS, counted and resuspended in sterile PBS at 1×10^5 , 2×10^5 , or 5×10^5 cells/50 µl for intradermal injection into 6-week-old female nude mice (strain nu/nu, Charles River, Wilmington, MA). Eight mice were injected per group. Tumor diameter was determined weekly with a Vernier Caliper and tumor volume was calculated using the formula $(4/3)\pi r^3$. When the two measurements differed, the smaller radius measurement was squared and multiplied by the largest radius measurement. This measurement was then substituted for the r^3 portion of the formula (Wyatt et al., 2005). When tumors reached 10 mm diameter, mice were sacrificed.

Harvesting and processing of tumor tissues

Mice were sacrificed by inhalation of isofluorane and cervical dislocation. Tumors were excised; a portion was placed in formalin for histology, another portion was snap frozen at -70°C for subsequent RNA isolation, another section was cut into 2 mm³ pieces for explant cultures in serum-free medium, and a final section was processed for isolation of tumor cells to be re-cultured in vitro. The lungs were removed and frozen at -70°C for evidence of

tumor metastasis by analysis of tissue for human *Alu I* DNA sequences by PCR (Tester et al., 2004; Blackburn et al., 2007, 2009).

Formalin-fixed sections were stained with Masson Trichrome for demonstrating deposition of interstitial collagens within the tumors. The snap-frozen tissue was processed for RNA using the Qiagen RNA Isolation Kit following the manufacturer's instructions. The explants were placed in 24-well culture dishes with 1 ml of serum-free LH medium/well. Approximately 4–6 explants were placed into each well. Each tumor was cultured separately. After 48 h, the medium was centrifuged to remove debris and stored at -70°C . Explants were blotted dry and weighed. To establish cultures from the excised tumors, fragments of tumor were digested with bacterial collagenase (4mg/ml) for 60 min at 37°C . The suspension was filtered through a $70\text{ }\mu\text{M}$ cell strainer (BD Bioscience, Durham, NC). The cells were pelleted by centrifugation and then cultured at 37°C in DMEM with 10% FBS.

MMP-I ELISA

The serum-free medium from the explant cultures was assayed for MMP-1 protein using the Fluorokine E Assay (R&D, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 150 μl of culture medium was assayed and compared to values from a standard curve. Nanograms of total and active MMP-1 protein from the ELISA were normalized to milligrams of explant weight; assays were carried out in triplicate. Data were pooled from individual tumors.

Immunoassay for cytokines and growth factors secreted by tumor explants: LUMINEX

Aliquots of serum-free medium from the explant cultures were tested by immunoassay by the Immune Monitoring Laboratory at Dartmouth-Hitchcock Medical Center, using Bio-Plex human cytokine multiplex kits (Bio-Rad, Inc., Hercules, CA); fluorescence intensity was measured using a Bio-Plex array reader. Bio-Plex Manager software with five-parametric-curve fitting was used for data analysis.

PCR analysis of lung metastases

Human DNA in mouse lung was measured by PCR. DNA was extracted using the Puregene Tissue Core Kit A (Qiagen). Realtime PCR to amplify human *Alu I* sequences was performed on 100ng of lung DNA as previously described (Tester et al., 2004; Blackburn et al., 2007, 2009). Primers are listed in Supplemental Table 1. The data are presented as pg human *Alu* DNA/100ng mouse lung DNA, with murine $\beta 2\text{M}$ as a marker. Each data point is the average of three separate PCR reactions on the lung of one mouse.

Sphere formation assay

The C4 and C9 cell lines were cultured as adherent cells in DMEM supplemented with 10% FBS. Once these adherent cells were confluent, they were washed three times with HBSS, harvested with Cell Stripper (Cellgro, Manassas, VA) and pelleted. Cell pellets were resuspended with DMEM/F-12 medium with N2-supplement (Invitrogen, Grand Island, NY) (Perego et al., 2010) and plated in 12-well plates at a density of 50 cells per ml and 10 cells per μl . After 5–7 days the wells were imaged. Cell Stripper was used to harvest the C4 adherent cells from the plate and to dissociate the C9 spheres.

Flow cytometry

The C4 and C9 cell lines were grown in DMEM/F-12 medium with N2-supplement, and harvested as described above. Harvested single cell suspensions were fixed in 80% ethanol (5 min) and then permeabilized in 0.1% PBS-TWEEN (20 min) before staining with rabbit

anti-human polyclonal JARID 1B(1 µg/ml; Novus Biologicals, Littleton, CO) and PE-conjugated goat anti-rabbit IgG secondary (1:500 dilution; Jackson Immunobiologicals, West Grove, PA) in 1% BSA in PBS. Cells were resuspended in 0.5% PFA and scanned on a FACSCalibur. Data were analyzed with FlowJo software.

Results

Isolation of clones expressing different levels of MMP-1 mRNA and protein

To study potential heterogeneity in VMM5 melanoma cells, we cloned the parental cells by limiting dilution. We isolated six clones, characterized them by Western blot and qRT-PCR for MMP-1 expression, and compared these levels to those seen with the MMP-1-producing melanoma cell line, VMM12 (Blackburn et al., 2007) (Fig. 1A,B). The figures show varying levels of MMP-1 expression in all clones tested, with Clone 9 (C9) expressing the highest (slightly higher than VMM12) and Clone 4 (C4) expressing the lowest. The levels of mRNA and protein correlate with each other (Brinckerhoff and Matrisian, 2002), and MMP-1 protein is in latent form, indicating that these cells do not express a proteinase that can activate proMMP-1. We chose the C4 and C9 cells for comparative studies on tumorigenesis and induction of MMP-1 in vivo.

Growth of C4 and C9 melanoma clones in vitro and in vivo

In vitro, the C4 and C9 cells displayed identical growth rates (Supplemental Fig. 1A). When the clones were injected orthotopically (intradermally) into nude mice, the tumor incidence was similar in both the C4 and C9 tumors, with an incidence of 86% for 1×10^5 and 2×10^5 (Fig. 2A,B) and 100% for 5×10^5 cells (data not shown). However, they grew at different rates, whether 1×10^5 , 2×10^5 , or 5×10^5 cells were injected (Fig. 2A,B; Supplemental Fig. 1B). In all instances, the C9 tumors grew more rapidly than the C4 tumors, necessitating sacrifice at earlier times due to the large size of the primary tumor. We also noted greater variability in tumor volume among the C9 tumors, suggesting that there might be a sub-population of cells in the C9 tumors that controlled cell proliferation (see below). In contrast, the C4 tumors were more uniform in their growth rate, which was substantially slower than the C9 tumors. Taken together, these data support the concept that both the C4 and C9 cells are equally tumorigenic but that there are differences in their growth kinetics.

Characterization of C4 and C9 tumors: MMP-1 expression and metastatic ability

The tumor tissue and explants were analyzed for expression of MMP-1 mRNA and protein, respectively (Fig. 3A,B). We found that in contrast to the in vitro studies (Fig. 1), both the C4 and C9 tumors produced abundant levels of MMP-1 mRNA (Fig. 3A). This was confirmed at the level of protein, by assaying explant medium for MMP-1 by ELISA (Fig. 3B). Two points are noteworthy. First, both C4 and C9 tumors produced substantial amounts of MMP-1 protein, ranging from 14 to 18 ng MMP-1/mg tumor explant, and, importantly, some MMP-1 (2–4 ng/mg explant) was in its enzymatically/biologically active form (Fig. 3B). Second, even though the cultured C4 cells produced less MMP-1 than the C9 cells, before injection into mice, the C4 tumors produced more MMP-1 than the C9 tumors. These findings suggest that within the tumor microenvironment, there are mechanisms that induce MMP-1 expression in the C4 melanoma tumors and that activate latent MMP-1 in both the C4 and C9 tumors (Benbow et al., 1999; Blackburn et al., 2007, 2009; Melnikova and Bar-Eli, 2009; Braeuer et al., 2011).

At time of sacrifice, the lungs were removed to determine if primary tumors had metastasized. A lobe was processed for detection of human *Alu* sequences (repetitive DNA sequences) as a marker for metastasis (Blackburn et al., 2007, 2009). We found that approximately 50% of primary tumors showed some evidence of lung metastasis (Fig. 4 and

Supplemental Fig. 1C). Surprisingly, even with comparatively large inoculum of 5×10^5 cells, there was no increased incidence of metastasis in the C4 or C9 tumors, perhaps because these tumors were fast growing and mice were sacrificed before metastasis could occur. Nonetheless, neither the C4 nor C9 tumors were highly metastatic, which may reflect an inherent property of the tumor originally derived from the patient (Yamshchikov et al., 2005) (see the Discussion Section).

Characterization of C4 and C9 tumors: matrix deposition and profiles of gene expression

The differences in the kinetics of tumor growth between C4 and C9 tumors (Fig. 2) suggest that different mechanisms are controlling progression of the C9 versus the C4 tumors. Therefore, we considered three potential reasons for the differences in growth kinetics. First, C9 tumors may constitutively express MMP-1, due to autocrine signaling, whereas C4 tumors do not, and therefore, may require cytokines and growth factors within the tumor microenvironment to up regulate MMP-1 in situ. Second, there might be inherent differences in the patterns of gene expression between the two tumor types, which may regulate MMP-1 expression as well as other genes (Nesbit et al., 2001; Melnikova and Bar-Eli, 2009; Ara and DeClerck, 2010; Braeuer et al., 2011; Diaz-Valdes et al., 2011; Lederle et al., 2011). Thirdly, perhaps the C4 and C9 tumors have different proportions of cells that display characteristics associated with melanoma initiating cells (MICs; cancer stem cells) (Fang et al., 2005; Cheli et al., 2011; Held and Bosenberg, 2010; Hoek and Goding, 2010; Perego et al., 2010; Roesch et al., 2010; Frank et al., 2011).

To begin to investigate differences in gene expression, when the mice were sacrificed, a portion of the primary tumor was processed for histologic analysis. Interestingly, staining of tissue sections with Masson Trichrome revealed deposits of collagen within the C4 tumors (Fig. 5A, arrows), which were absent in the C9 tumors (Fig. 5B). Further, the C4 tumors also expressed higher levels of TGF β mRNA (Fig. 5C), an inducer of matrix synthesis in melanomas (Berking et al., 2001; Diaz-Valdes et al., 2011). The fact that we noted collagen deposition in the C4 tumors in the face of active MMP-1, which degrades interstitial collagens, suggests that the rate of matrix deposition in these tumors is greater than the rate of degradation, thereby resulting in a net increase in extracellular matrix.

To examine other differences in the pattern of genes expressed in the C4 and C9 tumors, we profiled the serum-free medium from the tumor explants for secreted cytokines and growth factors (Table 1). Both the C4 and C9 explants expressed similar levels of proteins, such as FGF2, Exotaxin, PDGF, and VEGF. In contrast, the C4 explants had higher levels of G-CSF and GM-CSF, as well as elevated expression of MCP-1, IL-8, and IL-6, three proteins that constitute a cohort of cytokines associated with inflammation and an innate immune response to tumors (Yamamoto et al., 2000; Nesbit et al., 2001; Ara and DeClerck, 2010; Braeuer et al., 2011; Diaz-Valdes et al., 2011; Lederle et al., 2011) (see the Discussion Section). Specifically, the C4 tumors expressed about 10-fold more IL-6 and MCP-1, and 2-fold more IL-8, at the level of protein, and these proteins are highlighted (bold) in the table. Importantly, these differences are mirrored at the level of the level of mRNA (Fig. 6A–C).

Also noteworthy, we saw different levels of gene expression in the cultured cells before they were injected into mice compared to levels in cultures that were re-established from the excised tumors. Before injection, MCP-1 expression was considerably higher in the C4 and C9 cells compared to cells that were re-established in culture from the corresponding excised tumors (Supplemental Fig. 2A). In addition, before injection, the C4 and C9 cells expressed similar levels of IL-8 and TGF β mRNA, but the cultures of C9 cells derived from the tumors expressed significantly lower levels of IL-8 and TGF β (Supplemental Fig. 2 B–C). These findings highlight discrepancies between our in vitro and in vivo data, and illustrate the potential limitations of relying only on in vitro studies.

Melanoma initiating cells (MICs), or “cancer stem cells”

It is possible that the different growth kinetics between the C4 and C9 tumors could be due to the expression of gene(s) associated with the presence of a subpopulation of MICs, or “cancer stem cells” (Fang et al., 2005; Quintana et al., 2008; Cheli et al., 2011; Held and Bosenberg, 2010; Hoek and Goding, 2010; Perego et al., 2010; Roesch et al., 2010; Frank et al., 2011). Indeed, a variety of markers have been associated with the ability of only a few tumor cells to form a tumor when injected into immune-compromised mice, and one characteristic is the ability of the tumor cells to grow as spheres in vitro, under appropriate growth conditions. MICs have also been shown to express specific markers, such as the H3K4 demethylase, JARID 1B, which appears to increase under hypoxic conditions, such as within a sphere (Roesch et al., 2010).

We found that in N-2 supplemented media (Perego et al., 2010) the C9 cells, but not the C4 cells, readily grew as spheres (Fig. 7A,B), and that the two cell lines retained this differential morphology over five passages (data not shown). Further, flow cytometry revealed a higher proportion of JARID 1B-expressing cells, with nearly 36% of the C9 cells expressing JARID 1B, compared to 13% of the C4 cells (Fig. 7C). Thus, our data are consistent with the hypothesis that the C9 cells harbor a more substantial sub-population of cells that are able to express characteristics that have been associated with MICs.

Expression of the transcription factor MITF (Cheli et al., 2011) ATP-binding cassette ABCB5 (Frank et al., 2011) and of CD133 and CD34 (Fang et al., 2005; Monzani et al., 2007; Held et al., 2010) have also been linked to MICs. Therefore, we tested melanoma cells derived from C4 and C9 tumors for increased expression of these proteins. Both the C4 and C9 cells showed only minimal expression of these markers and expression was not increased under sphere-forming conditions (data not shown). Thus, our data are in keeping with the literature where melanomas can express a few selected markers that have been associated with MICs.

Discussion

In this article, we describe the tumorigenic and metastatic potential of two heterogeneous clones of malignant melanoma cells, derived from a single parental VMM5 tumor. These clones were selected in vitro for their differential expression of MMP-1 mRNA and protein, with the C4 clone producing low levels and the C9 cells producing high levels. The rationale for selecting these clones is based on our previous work (Benbow et al., 1999; Noll et al., 2001; Huntington et al., 2004; Blackburn et al., 2007, 2009) and that of others (Airola et al., 1999; Curran and Murray, 2000; Nikkola et al., 2002; Ryu et al., 2011), which has shown important functions for MMP-1 as a mediator of melanoma invasion and metastasis (Blackburn et al., 2007; Blackburn et al., 2009; Airola et al., 1999; Curran and Murray, 2000; Nikkola et al., 2002; Ryu et al., 2011).

Our goals for the present studies were to extend our work to an additional VGP melanoma cell line, one with documented antigenic variability (Yamshchikov et al., 2005), and to test whether the parental tumor also displayed clonal heterogeneity. Initially we asked whether MMP-1 expression could be induced in vivo, with a subsequent increase in tumorigenic/metastatic potential. However, despite substantial differences in MMP-1 expression in vitro, both clones displayed similar levels of MMP-1 in vivo, and were equally tumorigenic and metastatic. Nonetheless, they exhibited differences in their kinetics of tumor growth and in their profiles of gene expression, indicating heterogeneity within the parental tumor and suggesting distinct mechanisms for tumor progression.

The *in vivo* difference in growth kinetics of the two clones is quite striking, with the C4 tumors displaying a lag in growth, but eventually developing into sizable tumors. Most likely, these differences in growth kinetics result from multiple mechanisms. For example, C4 and C9 excised tumors had similar levels of MMP-1 mRNA and similar amounts of enzymatically active MMP-1 enzyme, thus suggesting that the tumor microenvironment contained growth factors and cytokines that both induced MMP-1 expression in the C4 tumors and activated proMMP-1 in both tumors. Possibly, these proteinases include thrombin and plasmin, which are commonly found in the tumor microenvironment (Brinckerhoff et al., 2000; Iida and McCarthy, 2007; Blackburn and Brinckerhoff, 2008; Braeuer et al., 2011; Lederle et al., 2011), and MMP-3 (stromelysin), which is also known to activate latent MMP-1 (Benbow et al., 1999; Brinckerhoff and Matrisian, 2002). We speculate that the growth curve of the C4 tumors is co-incident with the induction of MMP-1, and that the process of inducing and activating MMP-1 in the C4 cells may have contributed to tumorigenesis and progression (Blackburn et al., 2009; Ryu et al., 2011). Further, since the parental VMM5 (Huntington et al., 2004) and both the C4 and C9 clones (unpublished observations) harbor mutant BRAF^{V600E} this signal transduction pathway may be stimulated *in vivo* and target the MMP-1 promoter, thereby contributing to elevated levels of MMP-1 (Ryu et al., 2011).

However, despite the presence of activated MMP-1, neither the C4 nor the C9 tumors were highly metastatic. While somewhat curious, this behavior is consistent with a previous report, in which the parental tumor line was only locally metastatic in the human host (Yamshchikov et al., 2005). This previous report also notes heterogeneity within the tumor over a 16-year span in the patient, with continuing changes in tumor antigenicity. The authors concluded that immune editing and immunological adaptation in this patient represents a host response to counteract the tumor variants developed by the heterogeneous tumor as it attempted to escape from the host immune system.

Our data support the concept of intra-tumor heterogeneity, as we show diversity in gene expression between the C4 and C9 clones. The C4 clone consistently expressed a cohort of genes (MCP-1, IL-6, IL-8) that have been linked to an autocrine signaling loop in squamous cell carcinoma (Lederle et al., 2011) and that are associated with an inflammatory response and innate immunity (Yamamoto et al., 2000; Nesbit et al., 2001; Ara and DeClerck, 2010; Braeuer et al., 2011; Diaz-Valdes et al., 2011; Lederle et al., 2011). These cytokines are thought to act on the tumor cells to induce MMP-1, enhance angiogenesis and influence matrix composition through a reciprocal signal transduction loop that links TGF β , MCP-1, and MMP-1 (Yamamoto et al., 2000; Berking et al., 2001; Nesbit et al., 2001; Iida and McCarthy, 2007).

In addition, it is likely that this network of secreted cytokines modulates host responses in the tumor microenvironment (Li et al., 2009). Indeed, the deposition of collagen matrix seen in the C4 tumors, together with increased expression of TGF β , exemplifies the ability of an altered microenvironment to enhance tumor growth (Iida and McCarthy, 2007; Li et al., 2009; Diaz-Valdes et al., 2011). Further, the secretion of IL-6, IL-8 and MCP-1 is known to stimulate host stromal fibroblasts to secrete their own profile of chemokines, cytokines, and MMPs, and to modify the microenvironment so that it is permissive for melanoma invasion (Li et al., 2009). Thus, there are multiple mechanisms by which the C4 tumors could enhance their tumorigenicity *in vivo*.

In contrast, the C9 tumors consistently expressed cytokines at significantly lower levels, underscoring the genotypic diversity between the C4 and C9 tumors and suggesting that other mechanisms are driving tumorigenesis. One possibility relates to the expression of genes that have been linked to MICs. The topic of MICs is controversial since numerous

studies have generated different results, depending on the melanoma cell line(s), the assays used and the markers being examined (Fang et al., 2005; Monzani et al., 2007; Quintana et al., 2008; Cheli et al., 2011; Held and Bosenberg, 2010; Held et al., 2010; Hoek and Goding, 2010; Perego et al., 2010; Roesch et al., 2010; Frank et al., 2011). The controversy centers over whether a fraction of melanoma cells embodies true stem cell characteristics or whether the melanoma cells are simply displaying “phenotypic plasticity,” that is, the ability to (temporarily) adopt an MIC identity.

Growth as non-adherent spherical cells is a phenotype that has often been associated with MICs. Similarly, a host of stem-related markers have been observed in a variety of melanomas: including CD20, CD24, CD44, CD133, ABCB5, MITF, and JARID1B. In our studies, we have demonstrated expression of two of these characteristics in the C9 tumors, compared to C4: sphere-forming ability (Fang et al., 2005) and increased JARID1B (Roesch et al., 2010). Whether or not there is a sub-population of cells within the C9 tumors that are true cancer stem cells seems less relevant than the fact that their C4 counterparts do not share these characteristics. Indeed, Roesch et al. (2010) have suggested that JARID1B expression by a sub-population of melanoma cells may be essential for continuous tumor growth, but that expression of JARID1B is dynamically regulated and does not follow the classical hierarchical cancer stem cell model.

Regardless of the expression of MIC-like markers in the C9 tumors and the expression of a distinct profile of cytokines and growth factors by the C4 tumors, in vivo both clones express similar levels of (active) MMP-1, and both clones are equally tumorigenic and metastatic in nude mice, although the kinetics of tumor growth differ considerably. This suggests that the two clones use different mechanisms of tumor progression. A corollary to this point is that therapeutic treatments that target one pathway may allow the emergence of another clone(s), which harbors an alternative route(s) for tumor growth and progression. While this is not necessarily a new concept, our data on the heterogeneity of two clones derived from a parental tumor underscore this point. Understanding the mechanisms controlling intra-tumor diversity and heterogeneity and developing multi-targeted therapies may be essential to preventing the progression of VGP melanomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

MMP-1 expression by VMM5 clones. VMM12 cells and clones isolated from parental VMM5 cells were grown to confluence in DMEM/10% FCS. Cells were washed three times in HBSS and placed in 2 ml serum-free DMEM/LH for 24 h. MMP-1 in serum-free medium was detected by Western blot and MMP-1 mRNA was measured by qRT-PCR. A: MMP-1 protein. B: MMP-1 mRNA. Quantitation of MMP-1 proteins levels relative to VMM12 MMP-1 protein levels.

Fig. 2.

Growth curves of C4 and C9 melanoma cells injected into nude mice. C4 and C9 cells were grown to confluence in DMEM/10% FBS. Cells were washed three times in HBSS and removed from the culture dish with trypsin. Cells were counted, resuspended in sterile PBS and then injected intradermally into nude mice. A: 100,000 cells injected. B: 200,000 cells injected.

Fig. 3.

MMP-1 expression in C4 and C9 tumors. Mice were sacrificed when tumors reached 10 mm diameter. Tumors were excised and a portion was frozen for extraction of RNA and measurement of MMP-1 mRNA by qRT-PCR. Another portion was cut into 2mm fragments for culture as explants in serum-free DMEM/LH. After 28 h medium was removed and analyzed for latent and active MMP-1 by ELISA. A: MMP-1 RNA levels in tumor tissue; $P < 0.0001$. B: Latent and active MMP-1 levels released by tumor explants into serum free media measured by ELISA.

Fig. 4.

Lung metastases of C4 and C9 tumors. At time of sacrifice, the left lobe of lung was removed and frozen. DNA was extracted and human *Alu* I sequences were quantified by PCR. Values are normalized to 100ng mouse lung DNA.

Fig. 5.

Matrix deposition in C4 and C9 tumors. Mice were sacrificed when tumors reached 10 mm diameter. Tumors were excised and a portion was fixed in formalin for histology, and another portion was frozen at -70°C for extraction of RNA. Tumor sections were stained with Masson Trichrome, which stains the interstitial collagens, and RNA was analyzed for the presence of human TGFβI mRNA. A,B: Masson Trichrome stain for collagen in C4 and C9 tumors, respectively. Magnification is 20×. C: TGFβ mRNA in C4 and C9 tumor tissue; $P < 0.001$.

Fig. 6. Gene expression profile of MCP-1, IL-6, and IL-8 in C4 and C9 tumors. Mice were sacrificed when tumors reached 10 mm diameter. A portion of the tumors was frozen at -70°C for extraction of RNA. Tumor tissue was analyzed for mRNA by q-RT-PCR. A: MCP-1 mRNA; $P = 0.0038$. B: IL-6 mRNA; $P = 0.0124$. C: IL-8 mRNA; $P = 0.0005$.

Fig. 7.

Expression of cancer stem cell characteristics in C4 and C9 cells. A,B: Ability to grow under sphere-forming condition. C4 and C9 cells were cultures for 7 days in medium supplemented with N2. Images were taken and show that C4 cells are mainly adherent and C9 cells are mainly spheres. Magnification is 5 $\times$. C,D: Flow cytometry for JARID 1B protein in C4 cells and C9 spheres, respectively. —=unstained; _____=stained.

[Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcp>]

Table 1
Cytokine and growth factor production by explants of tumor tissue

Analyte	C4 tumor (pg/mg explant)	C9 tumor (pg/mg explant)
Eotaxin	20.99 ± 2.13	19.21 ± 6.46
FGF2	5.81 ± 2.04	5.29 ± 2.02
Fractalkine	67.45 ± 10.32	75.02 ± 21.13
G-CSF	83.71 ± 46.14	11.41 ± 5.87
GM-CSF	16.9 ± 2.88	8.26 ± 2.94
GRO	986.1 ± 255.4	543.89 ± 179.38
IL-6	31.5 ± 8.0	2.0 ± 1.45
IL-8	1760.6 ± 389.29	1182.62 ± 334
IL-10	ND	ND
MCP-1	2002.65 ± 465.95	173.8 ± 55.8
PDGF AA	36.08 ± 3.72	34.91 ± 8.57
PDGF AB	5.25 ± 0.94	8.86 ± 2.08
TNF-α	2.63 ± 0.66	5.47 ± 1.27
VEGF	186.33 ± 39.61	289.6 ± 5

ND, not detected.