

Host H67D Genotype Affects Tumor Growth in Mouse Melanoma

Cody Weston^{1*}, William Hund¹, Anne Nixon¹, Elizabeth Neely¹, Becky Webb¹, Ahmed Alkhateeb² and James Connor¹

¹Department of Neurosurgery, Pennsylvania State University College of Medicine, USA

²School of Public Health, Harvard University, USA

Abstract

Background: We investigated the effects of the frequently polymorphic high iron gene (HFE), on tumor growth in a novel mouse model. We hypothesized that host mutations in the HFE protein modify tumor progression.

Methods: C57BL/6 mice possessing either H67D/H67D or WT/WT HFE genotype, aged 18 months, were injected subcutaneously with 4×10^6 cells of the B16F10 mouse melanoma cell line. After 2 weeks, mice were sacrificed and the tumors and plasma were collected. Animal methods were approved by our IACUC, (04-166). Tumors were analyzed by RT-PCR. In parallel, bone marrow derived macrophages were cultured to determine how B16F10 cell cultures react to conditioned media from macrophages of each genotype. ELISAs were performed for ferroportin, ferritin, secreted cytokine and chemokine content of macrophage media.

Results: H67D mice had significantly smaller tumors (t-test, $P = 0.02$) after two weeks. Exploratory qRT-PCR analysis of tumors revealed that the H67D host may suppress angiogenesis and growth. In culture, macrophages derived from H67D mice secrete higher levels of monocyte chemoattractant protein 1 (MCP1), suggesting a change in chemotactic signaling ($p = 0.02$). When conditioned media from the H67D macrophages were placed onto B16F10 cells in culture there was significantly less growth compared to conditioned media from WT macrophages ($p < 0.01$ MTT, $p < 0.01$ BrdU). In macrophage cell lysates, H67D is associated with lower levels of Ferroportin ($p = 0.03$).

Conclusions: Macrophages from H67D mice do not support tumor cell proliferation as well as WT controls. Our data reveal the importance of HFE genotype on tumor growth that may be related to macrophage function.

Keywords: Iron; HFE; Hemochromatosis; Melanoma; Tumor progression

Background

Although much clinical evidence exists to support a relationship between cancer and HFE genotype, the nature of this relationship and the mechanisms by which HFE function affect tumor progression are still unclear. HFE is an atypical MHC class I molecule that affects immune function [1,2] and HFE polymorphisms disrupt iron metabolism. It is already known that iron metabolism affects tumor progression [3]. Tumors require iron, and have increased ferritin and transferrin receptor 1 (TfR1) expression compared to normal tissue and stroma [4]. Clinically, we have previously reported that elevated ferritin, iron storage and transport protein, in serum are associated with poor prognosis in breast cancer [5]. Decreased levels of ferroportin in tumors, a protein responsible for iron export from cells, are linked to a more aggressive tumor phenotype in breast cancer [6]. Patients with hereditary hemochromatosis, who experience severe iron overload, showed a risk of liver cancer over two hundred times greater than matched controls. Hereditary hemochromatosis most often arises from mutations in the HFE gene and it has been commonly assumed that the cancers associated with the HFE mutation are related to excess iron accumulation [7].

Two HFE variants are extremely common: H63D and C282Y. Of these, H63D is the more common form, seen in approximately 13.5% of people in the United States [8]. C282Y is the rarer form, seen in approximately 5.4% of the United States population [8]. Functionally, both of these result in a loss of normal HFE activity, although their mechanisms differ. H63D leads to a failure of the HFE protein to inhibit the binding of transferrin to the transferrin receptor, whereas C282Y leads to a failure of the HFE protein to reach the cell membrane at all [9]. In both cases, there is evidence that the presence of mutant protein affects cell biology in ways that are not modeled by HFE knockout mouse models. For example, H63D has been shown to

increase ER stress, and C282Y has been shown to affect oxidative stress, mitochondrial membrane potential, and iron chelator response [10,11]. Therefore, the present study has chosen a mouse model exhibiting the H67D mutation, which is analogous to human H63D, to capture any effects of mutant protein expression. An investigation of C282Y would be equally valid, but H63D was chosen as a first step because of its comparatively high prevalence in human populations.

Because HFE polymorphisms affect so many cell types, it is necessary to perform nuanced studies to understand the mechanisms by which HFE modulates tumor progression. To date, most work has focused on the manipulation of HFE in tumor cells [12] and in population based studies [13-16]. In the present study, we sought to test the central hypothesis that host HFE genotype modifies tumor progression. This approach contrasts with the majority of existing work because most available models vary the genotype of the cancer cell while using a normal or immunocompromised mouse host. Here, we used the syngeneic B16F10 cell model of mouse melanoma, chosen because it is a well-studied syngeneic model of cancer used on mice with a C57BL/6 background. This strain was used to develop our novel H67D mouse line. Because of the influence of HFE on the immune system, the ability to use immunocompetent mice was crucial to modeling the host response to tumor accurately.

***Corresponding author:** Cody Weston, Penn State Milton S. Hershey Medical Center, Mail Code H110, 500 University Drive, Hershey, PA 17033, USA, Tel: 717-531-3902; Fax: 717-531-0091; E-mail: codylweston@gmail.com

Received April 19, 2015; Accepted July 13, 2015; Published July 20, 2015

Citation: Weston C, Hund W, Nixon A, Neely E, Webb B, Alkhateeb A, et al. (2015) Host H67D Genotype Affects Tumor Growth in Mouse Melanoma. J Cancer Sci Ther 7: 216-223. doi:10.4172/1948-5956.1000353

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Materials and Methods

Cell culture

To model melanoma *in vitro* and *in vivo*, we used a B16F10 Luc G5 cell line (Xenogen, Cranbury, NJ). This was chosen because of its widespread use and its syngeneic relationship to C57BL/6, which allowed us to work with an immunocompetent model and utilize our novel mouse model carrying the most common HFE variant; H67D (homologous to human H63D). Cells were frozen in liquid nitrogen when not in active use and were grown in DMEM (Life Technologies, Grand Island, NY), with 10% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA) and 1% Penicillin-Streptomycin, (Life Technologies) in a humidity controlled incubator at 37 °C and 5% CO₂. When the cells reached confluence, they were subcultured by trypsin dissociation.

Mouse colony

Our laboratory maintains a colony of C57BL/6 mice with a combination of WT and H67D alleles for the HFE gene [17]. This colony has previously been shown to exhibit characteristics of hemochromatosis (increased hepatic iron loading, brain iron dyshomeostasis) and is superior to the commonly used HFE knockout models because it more closely approximates the human disease [18]. Specifically, our laboratory has shown some evidence of a gain of function resulting from HFE polymorphism that would not be reflected by the simple removal of functional HFE, including stress on the endoplasmic reticulum and alterations in cell proliferation [19,20]. The same study demonstrated that the H67D mutation leads to greater body weight, although the magnitude of this difference diminishes with age. All animal methods were approved by Penn State College of Medicine's IACUC, protocol 04-166.

Primary macrophage culture

To study the effects of HFE genotype on macrophage function, we isolated macrophages using standard bone marrow extraction and derivation techniques [21]. In brief, mice were sacrificed and their femurs and tibias removed. Bone marrow was extracted and a cell suspension was isolated using a 40 µm membrane. Whole bone marrow cell suspensions were plated on 100 mm x 20 mm round non-tissue-culture-treated dishes at a density of 8×10⁶ cells per well. To encourage the differentiation of precursors into macrophages, the culture medium was supplemented with M-CSF by adding 10% L929 cell supernatant. Because L929 cells secrete M-CSF, the use of supernatant from this cell line for primary macrophage culture is a well-established method for stimulating macrophage differentiation [21].

Macrophage cultures were titrated from their differentiation medium into a serum-free defined medium (Macrophage-SFM, Gibco) over a period of seven days. At this time, plates of macrophages were treated with 200 µM ferric ammonium citrate (FAC) for iron loading, with 75 µM deferoxamine (DFO) for iron chelation, or were left alone for 24 hours. After this 24-hour exposure, all media was aspirated, macrophages were washed, and 6 mL of fresh Macrophage-SFM was applied for 24 hours. This media was collected and used as macrophage conditioned media for further study. At the same time, the macrophages were lysed in RIPA buffer with phosphatase and protease inhibitors for further analysis.

Syngeneic tumor grafts and tumor collection

Subcutaneous tumors were formed in mice by injecting 4×10⁶ cells

at a concentration of 1×10⁶ cells per 50 µL of sterile PBS. These tumors were allowed to grow for two weeks, at which time the animals were sacrificed by lethal injection of ketamine/xylazine with secondary euthanasia by cardiac puncture and blood collection. The blood samples were collected via heparinized needle and spun down in heparinized tubes before storing at -80°C until analysis. The tumors were excised at this time, weighed, and subdivided for histology, protein-level, and RNA-level analysis. The histological samples were passively fixed in 4% Paraformaldehyde and embedded in paraffin. The protein samples were immediately frozen and stored at -80°C. The RNA samples were stored in RNALater (Qiagen) at room temperature as directed. All procedures involving animals were approved by Pennsylvania State University's Institutional Animal Care and Use Committee, protocol number 04-166.

Tumor analysis: Immunohistochemistry

To characterize the proliferation, iron handling, apoptosis, and immune infiltration of the tumors, immunohistochemical stains were performed. The staining was performed by the Penn State Hershey pathology core facility. Cell proliferation was assessed by staining for Ki67 (Cell signaling technologies, Danvers, MA, USA). An antibody to cleaved caspase 3 was used to assess apoptosis (Cell signaling technologies). These were evaluated qualitatively by 2 independent observers, who examined at least four high power fields (200x) from at least two slides from each tumor (n = 10 per genotype) while blinded to HFE genotype. To evaluate the density of microvessels, immunohistochemistry for CD31, a marker of endothelial cells, was performed. After this, two blinded observers counted microvessels manually. Briefly, low power magnification (40x) was used to identify areas of highest vascularization in each tissue sample, and four high power fields (200x) were counted and averaged to reach a quantitative estimate of vascular density. This technique was adapted from previous studies analyzing vascular density [22].

Tumor analysis: RT-PCR

In order to quantify mRNA expression in our tumor samples, we used the QIAGEN Cancer PathwayFinder Array in accordance with the manufacturer's recommendations. Tumors from both genotypes were incubated with/without 10 µg/mL S100B for 2 h and total RNA extracted using Qiazol (Qiagen). Gene expression of 84 genes representative of six biological pathways involved in tumor genesis (including 5 housekeeping genes for normalization; Rplp1, Hprt1, Rpl13a, Ldha and Actb) was assessed using the Mouse Cancer PathwayFinder™ RT2 Profiler™ PCR Array (Qiagen) according to the manufacturer's instructions and analyzed on an Applied Biosystems 7900 system. To validate the preliminary mRNA findings in a larger sample size, TaqMan assays (Applied Biosystems) were performed on four targets of interest, with Actb used as a loading control. In these cases, an N of 7 tumor samples from each group was used.

Host characterization: plasma analysis

To characterize the effects of HFE H67D genotype on serum proteins in a tumor-bearing animal, iTRAQ analyses were performed on the plasma samples obtained at the time of sacrifice. For analysis, the samples were paired from a common genotype to reduce the influence of individual biological variability. Subsequently, three WT and three H67D paired samples (representing a total of six animals from each genotype) were prepared and labeled according to the iTRAQ Multiplex (8-plex) Kit (Applied Biosystems). A control condition consisting of a pool from all of the samples was also prepared. The samples were then

sent to the Penn State College of Medicine Proteomics Core Facility, where MS and MS/MS spectra were taken of the SCX fractions separated on an Eksigent ChipLC and nanosprayed into a 5600 TripleTOF mass spectrometer. Using ProteinPilot software, the genotype-dependent differences in protein expression were flagged, and noteworthy results (greater than 1.5 fold differences after identification using Local False Discovery Rate estimation) are shown in Figure 3A. Tests of significant differences were not performed; these data are provided for the purposes of hypothesis generation.

Host characterization: macrophage characterization

ELISAs were performed for ferroportin and ferritin according to manufacturer's instructions (Cloud-Clone Corp, Houston, TX and Immunology Consultants Laboratory, Portland, OR). Briefly, macrophage cultures obtained as described above were plated at equal density in 1x macrophage serum-free medium (3000 cells per well, 9 wells per condition) for 24 hours, then their conditioned media were collected and the cells themselves were lysed using RIPA buffer. The lysates and media samples were used to determine cellular and secreted concentrations, respectively, of ferroportin and ferritin. Secreted cytokine and chemokine content of macrophage media was assessed with an ELISA array to detect IL-1B, IL-6, IL-10, IL-12, IL-17A, IFN-G, TNF-a, TGF-b1, MCP1, IL-13, GM-CSF, and IL-23. In this case, macrophages were plated at equal density in serum free media as described above, and their media extracted and tested after 24 hours, using 3 wells per genotype as well as a positive and negative control (Qiagen).

Host characterization: macrophage treatment of B16F10 cells

To determine if the macrophage secretome could recapitulate the observed genotype-mediated difference in tumor size *in vitro*, we treated B16F10 cells in culture with conditioned media from mouse bone-marrow-derived macrophages, collected as described above. First, B16F10 cells were plated in 96-well tissue culture plates at a density of 3000 cells per well, then allowed to settle for 24 hours before the media was aspirated, the cells were washed with PBS, and the media was replaced with 100 uL of conditioned media from the two macrophage types. After incubating for 48 hours in the presence of this media, MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and BrdU (5-Bromo-2-Deoxyuridine) assays (Roche, Basel, Switzerland and Calbiochem, Bilerica, Massachusetts, USA, respectively) were performed according to manufacturer's instructions. In this way, we were able to assess the effects of the macrophage-conditioned media on both cell proliferation and DNA turnover. In the case of the BrdU assay, an incorporation period of 24 hours was chosen based on past experience with this cell line.

Statistical analysis

All statistical analyses were performed using the Graphpad Prism (La Jolla, CA, USA) software package. Where comparing two groups, an independent two-tailed student's t-test was performed. Where comparing multiple groups in the same experiment, a one-way ANOVA was performed using Student-Newman-Keuls post-tests to identify significant contrasts within a statistically significant ANOVA.

Results

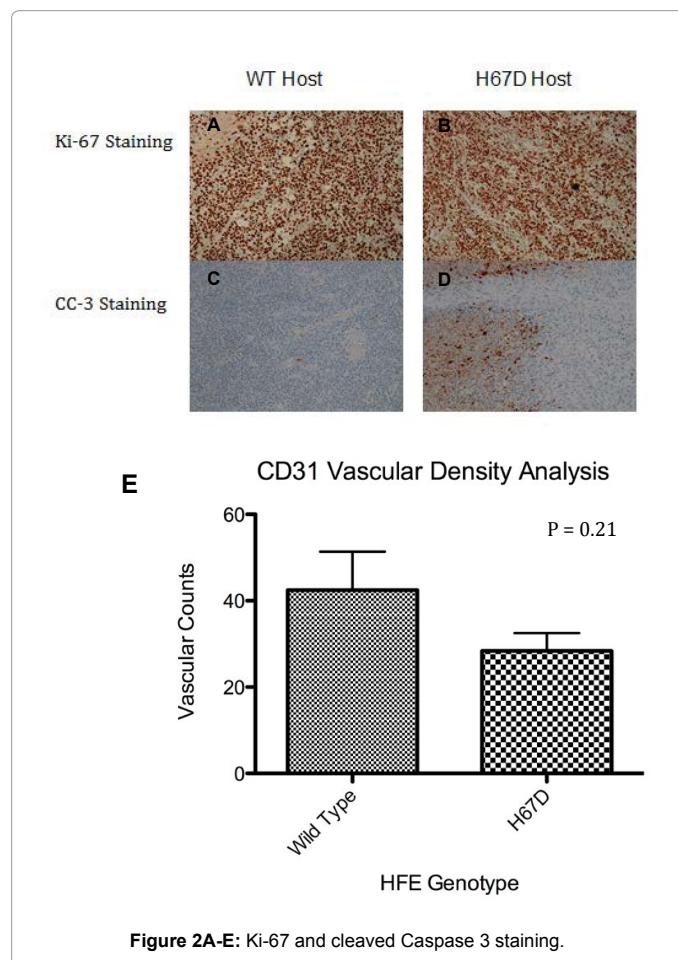
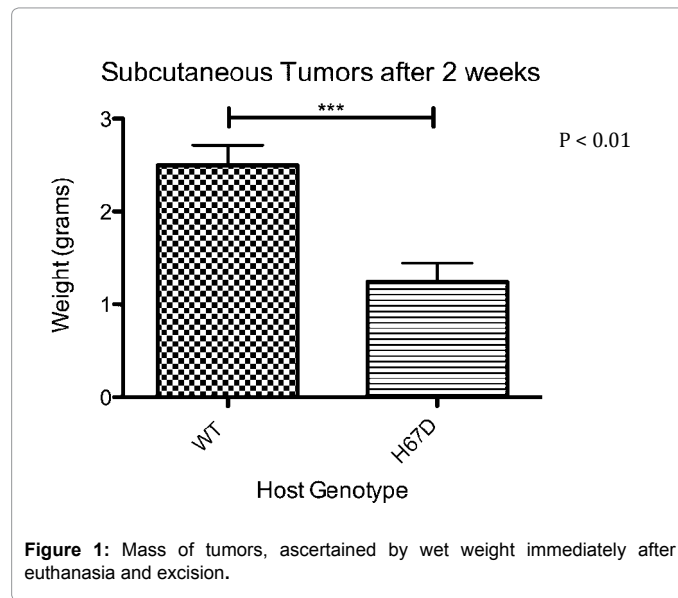
In vivo tumor analysis

Incubation of B16F10 cells in the H67D/H67D mouse yields a

smaller subcutaneous tumor compared to its wild type littermate ($P < 0.001$, Figure 1).

Immunohistochemistry results

Immunohistochemical staining of tumor sections revealed



extremely high proliferation rates (80-90% positive cells) as determined by ki67 staining, with no visibly detectable differences between H67D and WT hosts (Figure 2A, 2B). At the same time, cleaved caspase 3 staining showed an apparent genotype-mediated increase, indicating an increase in apoptosis in tumors borne by H67D hosts (Figure 2C, 2D).

Quantitation of vascular density using CD31 IHC showed a 33% decrease in vascular density in the H67D tumors relative to wild type but the difference did not reach statistical significance (Figure 2E).

Examination of F4/80 and CD3 staining for macrophage and T-cell infiltration, respectively, revealed that both cell types were present in the tumors, although there were no apparent differences in cell quantity between genotypes.

Tumor analysis – QPCR results

The RT²-PCR Cancer Pathfinder Array identified four transcripts that were differentially expressed in tumors using a cutoff of 2-fold (Figure 3A) although follow-up specific validation assays found no significant differences. Snai2 (Snail Family Zinc Finger 2) showed $\Delta\Delta C_T$ values of 0.6311 ± 0.8880 (Mean \pm SEM) for WT and -1.517 ± 0.5535 (Mean \pm SEM) for H67D relative to a calibrator sample, with an N of 7 tumor samples in each group (P = 0.06). Apaf1 (Apoptotic Peptidase Activating Factor 1) showed $\Delta\Delta C_T$ values of 0.9960 ± 0.5789 (Mean \pm SEM) for WT and -0.9018 ± 0.7537 (Mean \pm SEM) for H67D relative to a calibrator sample, with an N of 7 tumor samples in each group (P = 0.07). Fgf2 (Fibroblast growth factor 2) showed $\Delta\Delta C_T$ values

of -0.03800 ± 1.028 (Mean \pm SEM) for WT and -1.110 ± 0.8196 (Mean \pm SEM) for H67D relative to a calibrator sample, with an N of 7 tumor samples in each group (P = 0.44). Angpt1 (Angiopoietin 1) showed $\Delta\Delta C_T$ values of -0.7659 ± 0.7639 (Mean \pm SEM) for WT and $-1.835 \pm 0.1.115$ (Mean \pm SEM) for H67D relative to a calibrator sample, with an N of 7 tumor samples in each group (P = 0.46).

Plasma proteomic results

Comparison of plasma samples between the two mouse genotypes identified several plasma proteins differentially expressed between the two host genotypes (Table 1). Plasma from H67D mice showed genotype mediated changes in inflammatory proteins such as $\alpha 1$ antitrypsin (2.5 fold difference), inter--trypsin inhibitor heavy chain H1 (1.62 fold difference), plasminogen (1.61 fold difference), carboxypeptidase N catalytic chain precursor (1.53 fold difference), fibrinogen (0.7 fold difference). The adhesion protein vitronectin (1.66 fold difference) was found to be elevated in WT hosts. Several proteins associated with Immune activation differed including complement C5 (2.3 fold difference), CD5 (1.93 fold difference), H2 class 1 histocompatibility antigen (1.77 fold difference), CD44 (0.63 fold difference), and calreticulin (0.57 fold difference). Proteins implicated in cholesterol and lipid metabolism were affected by HFE genotype such as phosphatidylinositol-glycan-specific phospholipase D (1.51 fold difference), apolipoprotein C-1 (3.85 fold difference), and apolipoprotein E4 (0.41 fold difference). Sulfhydryl oxidase 1 isoform b (1.62 fold difference), a protein involved in redox homeostasis, was higher in H67D plasma compared to WT plasma. Two cell survival signals HSP-90 (Heat Shock Protein 90) (0.48 fold difference) and HSP-70 (Heat Shock Protein 70) (0.7 fold difference), were elevated in WT plasma compared to H67D. In addition, proteins involved in glycolysis were elevated in WT plasma including triosephosphate isomerase (0.46 fold difference) and G6P isomerase (0.74 fold difference).

In vitro macrophage treatment of B16f10 cells

The unexpected diminished growth of tumors in H67D host

A: Genes differentially expressed in WT and H67D tumors.

Gene	Fold Change WT Vs. H67D
Apaf1	5.06
Fgf2	3.00
Snai2	2.16
Angpt1	2.07

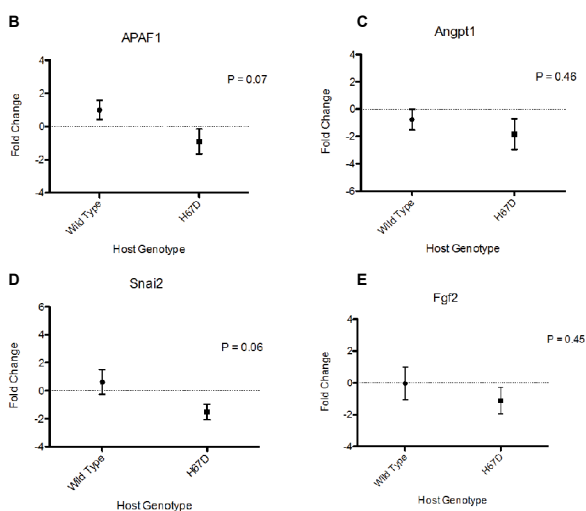


Figure 3: TaqMan qRT-PCR assays.

Higher in H67D	
Protein/Peptide	Fold difference
$\alpha 1$ antitrypsin	2.5
vitronectin	1.66
$\beta 2$ -microglobulin	1.76
ceruloplasmin isoform a precursor	1.75
plasminogen	1.61
inter-alpha-trypsin inhibitor heavy chain H1	1.62
complement C5	2.3
H-2 class 1 histocompatibility antigen	1.77
phosphatidylinositol-glycan-specific phospholipase D	1.51
carboxypeptidase N catalytic chain precursor	1.53
apolipoprotein C-1	3.85
CD5	1.93
sulfhydryl oxidase 1 isoform b	1.62
Higher in WT	
Protein/Peptide	Fold difference
fibrinogen	0.7
apolipoprotein E4	0.41
HSP-90 alpha	0.48
HSP70	0.7
triosephosphate isomerase	0.46
G6P isomerase	0.74
calreticulin	0.57
CD44	0.63

Table 1: Proteins differentially expressed in H67D and WT plasma.

animals suggested a mechanism more complex than organism-wide iron overload. Because macrophages are known to be iron-poor in clinical hemochromatosis populations [23] and because macrophages are thought to be the major suppliers of needed iron to tumors [24], we hypothesized that the macrophages of H67D homozygotes were less effective at supporting tumor growth compared to their WT counterparts. To test this hypothesis, we isolated bone-marrow-derived macrophages from both mouse types using the methods described above. *In vitro* exposure of B16F10 cells to the conditioned media from these macrophages showed an effect that resembled what was seen *in vivo*. Specifically, MTT assays showed that WT macrophage conditioned media led to greater proliferation than H67D macrophage conditioned media ($P < 0.05$, Figure 4A). Similarly, BrdU assays showed that WT macrophage conditioned media led to greater DNA turnover than H67D macrophage conditioned media ($P < 0.05$, Figure 4B).

Macrophage characterization

Because of the effects of macrophage conditioned media on B16F10 cell growth in culture, altered macrophage iron handling was thought to be contributing to the observed effect of HFE genotype on tumor growth. To better understand the differences in iron handling and inflammatory signaling between WT and H67D homozygous

macrophages, cell lysates and conditioned media were analyzed by ELISA. HFE genotype did not significantly affect baseline ferritin levels of secretion or expression. After pretreatment with 200 μM FAC, there was a statistically significant difference between H67D and WT intracellular ferritin levels ($P < 0.05$, SNK post-test). Although both H67D and WT macrophages responded to iron loading by increasing both intracellular and secreted ferritin, the H67D macrophages expressed more cellular ferritin in response to iron loading compared to the WT (ANOVA Main Effect $p < 0.01$ for conditioned media, $p < 0.01$ for lysates) (Figure 5A). At the same time, ferritin secretion was not significantly different between the two macrophage genotypes when loaded with iron ($p > 0.05$) (Figure 5B). In the cell lysates there was a significantly lower level of ferroportin in the H67D macrophages ($p = 0.03$) (Figure 5C). No significant differences were observed in macrophage viability between the two genotypes.

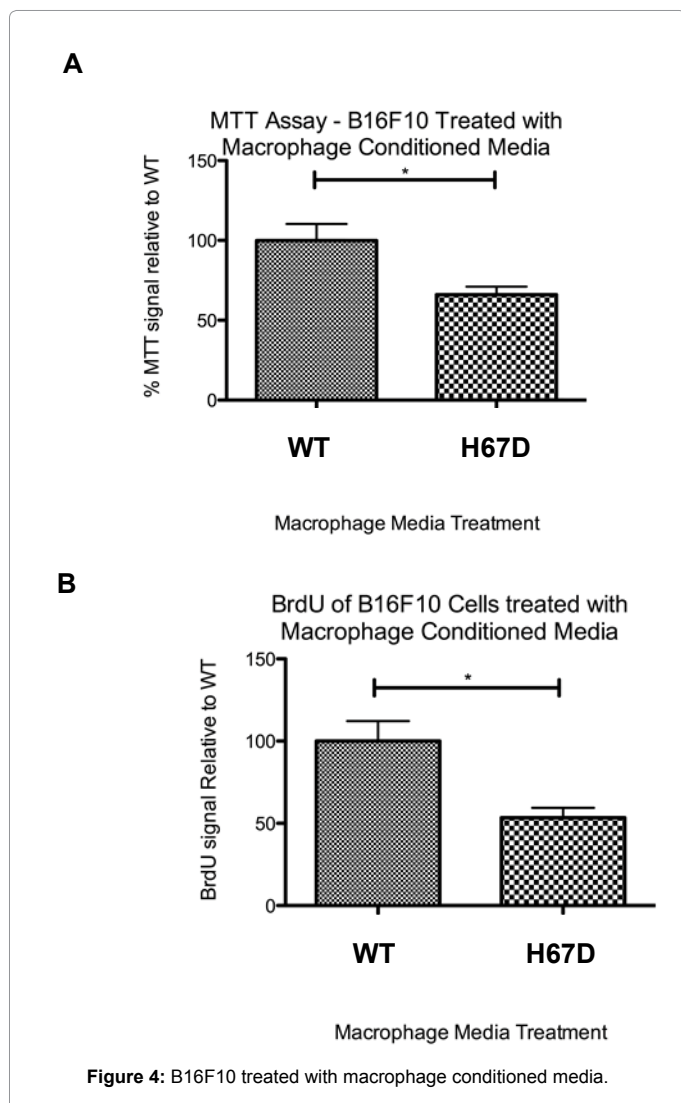
MCP1 is elevated in H67D macrophage secretions

MCP1 levels were significantly higher in secretions from H67D macrophages compared to WT macrophages ($P = 0.02$ Figure 6). Levels of other cytokines and chemokines tested (IL1B, IL6, IL10, IL12, IL17A, IFN- γ , TNF-, TGF- β 1, IL13, GM-CSF, and IL-23) were not significantly different.

Discussion

The results of the present study demonstrate that tumors formed from B16F10 Luc G5 melanoma cells are smaller in mice carrying the H67D HFE gene variant compared to wild type. The analysis of tumors indicated no difference in cell proliferation and only trend-level suggestions of decreased angiogenesis and increased apoptosis that did not reach statistical significance; perhaps because the study was terminated at two weeks. Longer incubation times were not practical due to the tumor burden. Tumor associated macrophages appeared to be present at similar density in tumors associated with both host genotypes. The salient question associated with these findings is whether difference in tumor growth is driven by the tumor microenvironment, extratumoral factors or interplay between both. To interrogate extratumoral factors, we performed a proteomic analysis of plasma proteins and found differences in proteomic profiles that could be categorized according to functional groups. Analysis of these suggested changes in immune activation and cell survival signals that could account for some of the differences in tumor growth between genotypes. In an attempt to model the tumor microenvironment *in vitro*, we cultured bone marrow derived macrophages and found that conditioned media from the macrophages of the H67D mice were less capable of supporting B16F10 Luc G5 cells than wild type, similar to what was observed *in vivo*. The macrophage culture studies suggest that altered iron handling by macrophages as a result of expressing the HFE mutant protein may be responsible for the differences in tumor growth.

The trend toward a decrease in angiogenesis in the tumors from the H67D HFE mice was demonstrated both by immunohistochemistry for CD31, a protein marker of endothelial cells and by gene expression analysis that showed decreased expression of AngPT1. Angpt1 is a growth factor that supports angiogenesis and is already being investigated as a therapeutic target in cancer [25]. This indicates a host-tumor interaction is taking place in which WT host conditions are supporting angiogenesis to a greater extent than H67D host conditions. The expression of Ki67 was similar in the tumors from both genotypes indicating that cell proliferation was not affected by genotype but rather, based on the increase in cleaved caspase 3 staining seen in the H67D mouse tumors, it appears that there is an increase in cell death



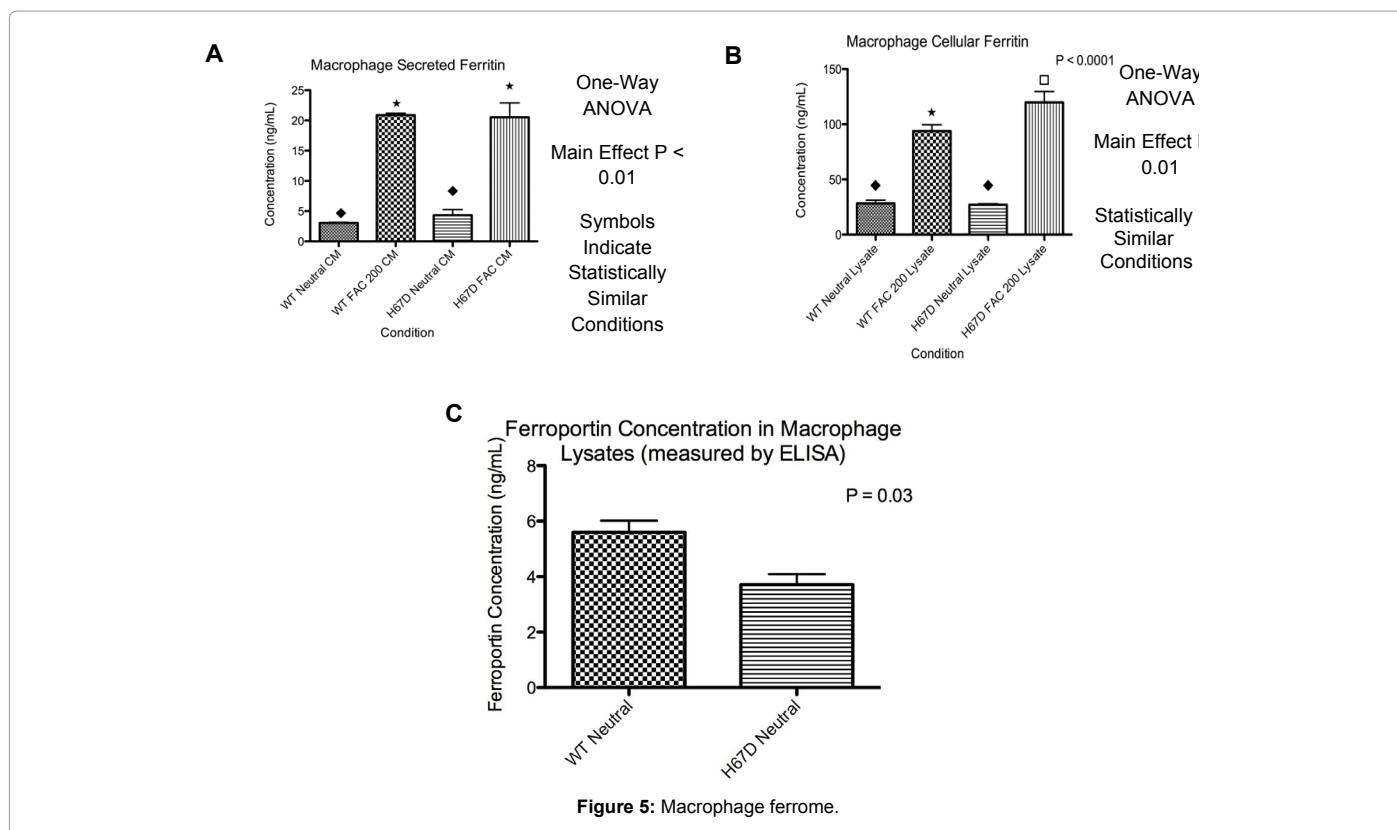


Figure 5: Macrophage ferronome.

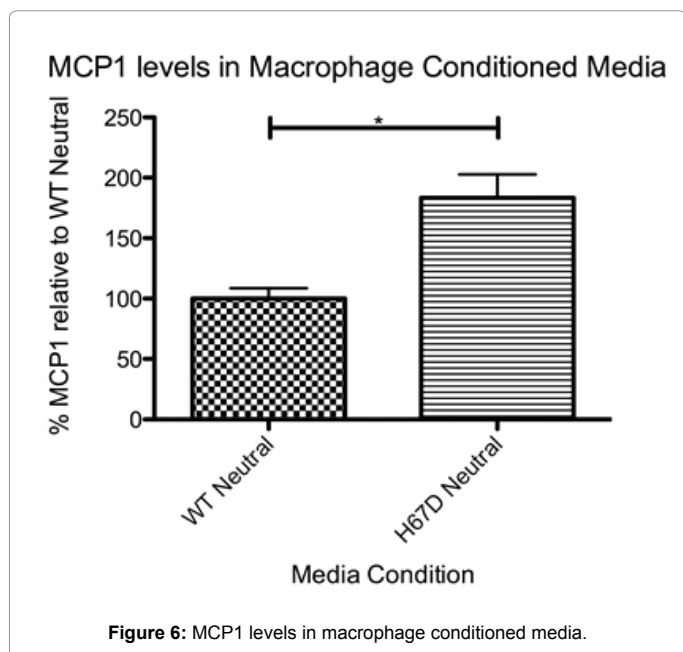


Figure 6: MCP1 levels in macrophage conditioned media.

underlying the difference in size. Increased cell death would be consistent with a poor nutrient supply because of the decreased angiogenesis in the tumors in the H67D mice. In the gene array study, we found APAF1 was elevated in the tumors in the WT mice compared to HFE mutant mice. APAF1 is a known regulator of apoptosis in cancers, including melanoma [26]. However, melanomas typically show downregulation of APAF1, so the lower APAF1 levels seen in H67D organisms, which

possessed smaller tumors, was counterintuitive [27,28]. Furthermore, the H67D hosted tumors appear to undergo more apoptosis while WT hosted tumors express more APAF1. The literature does, however, indicate that APAF RNA levels are not necessarily correlated to activity [29]. The SNAI2 transcript, associated with the slug protein, is associated with increased invasiveness and metastatic propensity in melanomas and other cancers, including gliomas and breast cancers [30,31]. The subcutaneous xenograft model is not designed to assess features related to metastasis, though the direction of expression suggests that the H67D host genotype may foster less invasive tumors in addition to less overall growth.

The HFE genotype impacts the basic physiology of the whole organism and thus our model provided the opportunity to explore extratumoral contributions to the tumor growth. The plasma protein profile revealed differences between the H67D and WT mice. In general, the proteins that were identified as different included those involved in inflammation and immune signaling which is consistent with the role of HFE as a protein involved in both iron regulation and the immune system [32]. Consistent with the genotype-specific difference in apoptosis, HSP-90 α and HSP-70, both associated with cell survival, the stabilization of mutant proteins, and the inhibition of apoptosis, were found to be higher in WT compared to H67D plasma. It is likely that these proteins are related to the relative lack of apoptosis in the tumors of WT mice [33].

Several of the protein changes in plasma suggest altered immune activation. CD5, H-2 class1 histocompatibility antigen, complement C5, and β 2 microglobulin are markers that indicate different aspects of immune system function that were elevated in H67D. CD5 is a marker for T-cells, and it is already known that HFE genotype can affect T-cell populations [34]. The similar infiltration of T-cells in the tumors

regardless of genotype suggests that T-cells are not responsible for the differences in tumor size although we cannot rule out that the T-cells are differentially activated. Arguing for this differential activation, H-2 class 1 histocompatibility antigen and β 2 microglobulin are involved in antigen presentation, which indirectly affects T-cell function. Complement C5 plays a role in chemotaxis and the complement membrane attack complex. These results are consistent with the smaller tumor size in the H67D mice because they suggest a more highly activated immune system.

CD44 and calreticulin were reduced in H67D compared to WT plasma. CD44 is a marker of lymphocyte activation and homing, but is also a marker for certain cancer stem cells and tumor metastasis, and so may be elevated in WT plasma as a result of the increased tumor growth in WT animals [35]. Calreticulin is expressed on many cancer cells and promotes macrophage phagocytosis. The increased presence of cleaved caspase 3 staining in the H67D mice may be related to the decreased calreticulin as macrophages may not be effectively clearing dead and dying cells in the tumors in the H67D mice [36].

In addition to the immune-related proteins mentioned above, several inflammatory markers were elevated in H67D including α 1 antitrypsin, plasminogen, inter--trypsin inhibitor heavy chain H1, and carboxypeptidase N catalytic chain precursor. The increase in these proteins is modest but *in toto* is consistent with an inflammatory milieu that may be hostile to tumor growth [37].

The changes in plasma protein profile suggest the possibility that tumor associated macrophages and other infiltrating cells of the immune system into the tumor microenvironment may be differentially activated depending on host HFE genotype. To investigate possibility that the cells, specifically macrophages, are inherently different, we cultured bone derived macrophages from animals of each genotype. The results revealed that macrophages carrying the HFE mutant protein were less capable of supporting B16F10 Luc G5 cells in culture indicating that there are inherent differences in macrophages based on HFE genotype. We demonstrated that there are differences in iron handling in the macrophages based on HFE genotype which is consistent with reports in the literature [38]. The differences in iron handling could directly impact tumor growth in the tumor microenvironment [39]. An intriguing observation was the increased MCP-1 secretion by the H67D macrophages. MCP-1 is a chemokine associated with inflammation and the infiltration of T-cells and macrophages so this differential secretion is consistent with the alterations in immune signaling and inflammatory molecules seen between the two genotypes.

Conclusions

We have demonstrated that host genotype impacts growth of tumors. As for mechanism, the tumors in the H67D mice trend toward exhibiting fewer angiogenic factors (Angpt1 and Fgf2), which in turn leads to decreased angiogenesis which leads to a lack of nutrient provision and waste removal, leading to an apparent, although not statistically significant, increase in apoptosis, as evidenced by the cleaved caspase 3. Moreover, HSP proteins known to inhibit apoptosis and promote cell survival are increased in the plasma of WT mice. There is a plasma profile of increased inflammation in the H67D mice when coupled with potentially increased immune system activation would be consistent with smaller tumor growth. The plasma protein analysis revealed genotype specific changes that support the differences in tumor growth. At the same time, the cell culture data reveal that there are inherent differences in macrophages that are consistent with the differences in tumor growth. The tumor growth period in this

study was short but we chose to interrogate the system after tumors were clearly established. Longer term studies are warranted but may mask some of the early events in establishing differences in tumor growth. Thus, our data suggest that the host genotype creates a milieu of humoral factors and microenvironmental conditions that result in the significant alterations to early tumor growth rates.

Acknowledgements

The authors wish to thank Becky Webb and Beth Neely for their assistance in animal handling and mouse colony management, and the entirety of the George M. Leader Laboratory for their feedback in the development and presentation of this work.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

Cody Weston: Drafted the manuscript, designed and performed experiments, conducted statistical analysis.

William Hund: Designed and performed experiments, edited manuscript.

Ahmed Alkhateeb: Designed and performed experiments, edited manuscript.

Anne Nixon: Designed and performed experiments, conducted statistical analysis, edited manuscript.

James Connor, Ph.D.: Edited manuscript, assisted with experimental design.

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