HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2014 February 01.

Published in final edited form as:

J Invest Dermatol. 2013 August ; 133(8): 2050–2058. doi:10.1038/jid.2013.114.

B7-H3 Associated with Tumor Progression and Epigenetic Regulatory Activity in Cutaneous Melanoma

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Abstract

B7-H3, a cell surface transmembrane glycoprotein, was assessed for its functional and prognostic role in cutaneous melanoma progression. B7-H3 expression in melanoma cells was shown to be related to specific downstream signal transduction events as well as associated with functional epigenetic activity. B7-H3 expression and prognostic utility was shown by RT-qPCR and IHC analysis on individual melanoma specimens and then verified in clinically annotated melanoma stage III and stage IV metastasis tissue microarrays in a double blind study. B7-H3 mRNA expression was shown to be significantly increased with stage of melanoma(P<0.0001) and significantly associated with melanoma-specific survival(MSS) in both stage III(P<0.0001) and stage IV(P<0.012) melanoma patients. B7-H3 expression was related to migration and invasion; overexpression B7-H3 increased migration and invasion while knockdown of B7-H3 reduced cell migration and invasion. MiR-29c expression was shown to inversely regulate B7-H3 expression. Furthermore, we demonstrated that melanoma B7-H3 expression was correlated to p-STAT3 activity level in melanoma tissues and cell lines. These studies demonstrate that B7-H3 is a significant factor in melanoma progression, and events of metastasis.

Keywords

B7-H3; melanoma; metastasis; miR-29c; prognosis; STAT3

Introduction

Melanoma is an aggressive form of skin cancer, having one of the highest increasing incidence rates of any cancer in Caucasians with a poor 5-year survival rate in patients with AJCC stage III and IV(Balch *et al*., 2009). It is difficult to predict melanoma recurrence

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Conflict of Interest: All authors disclose no conflicts of interest.

after surgical resection of early-stage metastatic melanomas. Studies have shown that immunoregulatory molecules can be expressed on human tumor cells and play important roles in both tumorigenesis and tumor progression(Balkwill, 2011; Flies and Chen, 2007; Goto *et al*., 2008; Hofmeyer *et al*., 2008; Sato *et al*., 2009; Shiao *et al*., 2011; Taube *et al*., 2012; Zang and Allison, 2007).

B7 family members are known to modulate host immunity. The prototype ligands of this family, B7-1 and B7-2, have well-defined roles as essential co-stimulatory molecules involved in T-cell regulation(Cao *et al*., 2011; Steinberger *et al*., 2004). In contrast, the roles of the other B7-H family molecules(i.e., B7-H3, and B7-H4) remain unclear(Crispen *et al*., 2008; Greenwald *et al*., 2005). One of the B7 family members, B7-H3(CD276), a type I transmembrane protein is expressed on dendritic monocytes, and macrophages and activates T-cells(Chapoval *et al*., 2001; Hofmeyer *et al*., 2008; Kobori *et al*., 2010). B7-H3 function is suggested to play a role in regulating immune responses of T-cells(Kobori *et al*., 2010; Leitner *et al*., 2009). B7-H3 expression on tumor cells, including melanoma, has been reported(Roth *et al*., 2007; Sun *et al*., 2006), and proposed to have involvement in tumor cell escaping immune surveillance and promoting immune tolerance(Hofmeyer *et al*., 2008; Petroff *et al*., 2005).

We investigated several significant melanoma-related regulatory factors associated with B7-H3; one of which, the downregulation of miR-29c has recently been implicated in melanoma progression by our group(Nguyen *et al*., 2010). In a recent report in neuroblastoma, miR-29 was shown to bind to the B7-H3 3′ UTR region(Xu *et al*., 2009). We *hypothesized* that B7-H3 expression on melanoma cells regulates mechanisms associated with tumor progression and its expression may be prognostic.

Results

B7-H3 mRNA expression in melanoma tissues

To examine B7-H3 expression relevance in melanomas, a RT-qPCR assay for B7-H3 mRNA analysis was developed on paraffin-embedded archival tissue(PEAT) melanomas. B7-H3 mRNA copy number distribution of nevi/benign lentigo melanocytes/normal skin(7 nevi/benign lentigo and 6 normal skin), primary, and metastatic melanomas are shown in Figure 1A. Mean(±SD) relative B7-H3 mRNA copies in tumors from AJCC stages I, II, III primary melanoma patients were $7.67 \times 10^{-4} \pm 1.29 \times 10^{-3}$ (n=22), $2.28 \times 10^{-3} \pm 3.12 \times 10^{-3}$(n=14), and $1.71 \times 10^{-3} \pm 2.86 \times 10^{-3}$(n=21), respectively. For AJCC stage III and IV metastatic tumors, B7-H3 mRNA copies were $4.76 \times 10^{-3} \pm 6.23 \times 10^{-3}$(N=23), and $5.10 \times 10^{-3} \pm 4.74 \times 10^{-3}$ (N=20), respectively.

B7-H3 mRNA copy number distribution of nevi/benign lentigo melanocytes/normal skin, primary, and metastatic melanomas are shown in Figure 1B. B7-H3 mRNA copy numbers were significantly higher ($p<0.05$) in melanomas compared to nevi/benign lentigo melanocytes/normal skin, and significantly increased with advancing stage. B7-H3 expression demonstrated to correlate to melanoma progression.

B7-H3 protein expression in melanomas

To examine B7-H3 protein expression, we performed immunohistochemistry(IHC) to evaluate primary melanomas(Stage II, n=18; Stage IIIp, n=12), stage III LN metastases(n=20), stage IV distant metastases(n=47), and nevi(n=17)(Figures 2A-D). There was trend of higher B7-H3 protein expression with disease stage(Stage II, p=0.0002; Stage III, p=0.0007; Stage IIIm, p<0.0001; Stage IV, p<0.0001; Figure 2E). B7-H3 was significantly enhanced in both primary and metastases compared to nevi(p<0.0001, Figure 2F). Patients were divided into two groups according to IHC intensity; tissues with a score of 2-3 were classified as the high B7-H3 expression group, while those scoring 0-1 were classified as the poor/no B7-H3 expression group. The level of B7-H3 expression in different stage IV organ site metastasis was not significantly different(Supplemental Figure 1). These results confirmed that B7-H3 expression levels were elevated in melanoma, as compared to nevi, and not related to the organ site of melanoma metastasis.

B7-H3 expression in melanoma Tissue Microarrays(TMAs) and prognostic utility

We next assessed the prognostic value of B7-H3 in metastasis using stage III & IV tumor tissue TMAs. B7-H3 expression level of regional LN metastases and distant organ metastases was assessed by IHC(Figure 3A) and correlated with melanoma-specific survival(MSS). Patients were divided into two groups based on their B7-H3 expression levels as described above. B7-H3 expression was a significant predictor of MSS in the stage III TMA(p<0.0001)(Figure 3B) and in the stage IV TMA(p=0.012)(Figure 3C). The verification of IHC analyses on the melanoma TMAs confirmed the findings of the individual melanoma PEAT as well as demonstrated prognostic value of B7-H3 in disease outcome for both stage III and IV patients.

To further confirm the role of B7-H3 in prognosis of different stage of metastatic melanoma, we analyzed B7-H3 protein expression by IHC in autologous pairs of stage III melanoma LN metastases and metachronous stage IV distant metastases from 32 patients within the stage IV TMA. Stage IV metastases showed significantly higher expression of B7-H3(p=0.042) when compared to their autologous paired stage III metastasis, further verifying elevation of B7-H3 expression in melanoma progression(Figure 3D).

B7-H3 expression on melanoma cells

B7-H3 protein expression in melanoma was verified by various approaches. Immunofluorescent staining of melanoma lines showed strong B7-H3 protein expression on the cell surface(Figure 4A). B7-H3 immunofluorescent analysis of melanocytes showed no detection. We then assessed melanoma cell lines M-1, M-101, M-111, M-12, M-14, JK-0346 and Mel-B, along with two donor PBLs as negative controls by flow cytometry. Flow cytometric analysis showed that B7-H3 protein was highly expressed on the cell surface of all melanoma lines(n=7)(Figure 4B, Supplemental Figure 2).

Western blot analysis confirmed the expression of B7-H3 protein in melanoma. Melanoma cell lines M-101, M14, M24, Wm266-4, JH-1173, along with three frozen metastases from stage III and IV patients were assessed. B7-H3 protein expression was confirmed in both

melanoma lines and tissues(Figure 4C) but not in melanocytes supporting PEAT IHC and mRNA results.

MiR-29c regulates B7-H3

MiR-29c can have tumor suppressor function in that it is significantly downregulated during melanoma progression(Nguyen *et al*., 2010). MiR-29c was shown to directly target B7-H3 3′ untranslated region (3′UTR)(Figure 5A). We focused on miR-29c levels in melanomas relative to B7-H3. B7-H3 IHC was performed on the same LN specimens in the TMAs as those previously assessed by reverse transcription and real-time PCR(RT-qPCR) for miR-29c expression. MiR-29c expression level was found inversely correlated to B7-H3 protein level (Spearman's correlation ρ=-0.403, $p<0.0001$; n=90)(Figure 5B).

To investigate the regulatory role of miR-29c in melanoma B7-H3 expression, transfection studies were carried out on M14, M101 and Wm266-4, which have high B7-H3 expression. Transfection of miR-29c mimic significantly reduced B7-H3 expression (Figure 5C, Supplemental Figures 3A and 3B). B7-H3 mRNA and protein expression in M14 and M101 transfected with miR-29c mimic were reduced >3 fold compared to that in M14 and M101 transfected with miR-29c negative control(Figure 5D and 5F, Supplemental Figures 3C and 3D).

STAT3, p-STAT expression were related to B7-H3

Recent studies demonstrate that poor melanoma prognosis is associated with elevated phosphorylated STAT3(p-STAT3) expression(Jensen *et al*., 2011). We therefore explored the association between p-STAT3 and B7-H3 expression. IHC staining of p-STAT3 in B7-H3 high(score 3) LN metastases(n=10) was compared to those in B7-H3 low(score 0-1) LN metastases(n=10). Expression of p-STAT3 was significantly ($p=0.029$) higher in B7-H3 high expression compared to B7-H3 low expression melanomas. Our results demonstrated an association of p-STAT3 and B7-H3 expression(Figure 5E). Results from the miR-29c transfection studies demonstrated miR-29c significantly reduced STAT3 and p-STAT3 expression in the M14, M101 and Wm266-4(Figure 5F, Supplemental Figure 3D).

To further confirm whether p-STAT3 was related to B7-H3 expression, we assessed level of stat3 and p-STAT3, and also the level of their downstream target cyclin D1 in M14 B7-H3 shRNA stable cell clones. It was shown in Figure 5G that level of STAT3, p-STAT3 and cyclin D1 was reduced on knockdown of B7-H3 in melanoma cells. These results suggested B7-H3 influences p-STAT3 activity supporting our other findings above.

B7-H3 function in melanoma cells

To explore the functions of B7-H3 in melanoma cell lines, B7-H3 expression was assessed by Western blotting. We found all melanoma lines except M219 expressed high levels of B7-H3 (Figure 6A left). To further examine B7-H3 relevance, we knocked-downed B7-H3 by B7-H3 shRNA in M14, and overexpressed B7-H3 by transfecting B7-H3 expression vector in M219. B7-H3 knock-down M14 stable clones (M14 B7-H3 shRNA) and B7-H3 overexpression M219 stable clones(M219 B7-H3) were established (Figure 6A middle and right). Cell functional studies were carried out using these specific modified cell lines.

Initially, we explored B7-H3 role in cell growth, and found that B7-H3 had no effect on cell growth (data not shown). Secondly, we examined the effect of B7-H3 on cell migration and invasion. B7-H3 expression enhanced cell migration and invasion (Figure 6B and 6C). Finally, we performed soft agar and Matrigel 3D cell culture. Cells which were seeded on day one were showed in Supplemental Figure 4. As shown in Figures 6D and 6E, B7-H3 did not have any effect on colony formation in soft agar, which indicated that anchorage-independent growth was not affected by B7-H3, whereas B7-H3 enhanced cell growth in 3D culture, which further confirmed that B7-H3 was related to cell invasion. These results indicated that B7-H3 enhanced cell migration and invasion and had no significant effect on cell growth.

Discussion

We have demonstrated B7-H3 cell surface expression by melanoma cells using multiple approaches to support our *hypothesis* that its differential expression in advancing stages of melanoma is prognostic. To our knowledge, this is a previously unreported study to demonstrate that B7-H3 expression is related to cutaneous melanoma progression and prognostic significance, as well as integrated with tumor cell metastasis related regulatory factors. Our study demonstrates that B7-H3 expression is significantly related to melanoma progression and prognosis using large cohorts of well clinically annotated specimens with long-term follow-up.

Studies carried out to assess tumor-infiltrating lymphocytes CD3+/CD8+/CD4+ by IHC in different stages of melanomas show there is no significant correlation to B7-H3 expression(data not shown). This suggests that B7-H3 may not have a significant immunoregulatory role on melanoma cells. The analysis of tumor-infiltrating lymphocytes or ratio to tumor cells in relation to cancer cell B7-H3 expression has also been inconclusive (Kobori *et al*., 2010).

We demonstrated an inverse correlation between miR-29c and B7-H3 expression, and a role of miR-29c in B7-H3 regulation during melanoma progression. These findings are novel demonstrating the functional regulatory effect by miR29c of B7-H3 expression in melanoma cells. The functional studies strongly indicate that miR-29c has a regulating role in B7-H3 expression in relation to melanoma progression. Based on our results, we demonstrate that miR-29c plays an important role in mediating progression of cutaneous melanoma through regulating B7-H3. Our studies demonstrated that B7-H3 expression is related to cell migration and invasion of melanoma cells using knockdown and upregulated B7-H3 melanoma cells. These findings support the functional relation of B7-H3 expression relation to cutaneous melanoma progression.

p-STAT3 has various important roles in cancer including melanoma progression(Jensen *et al*., 2011; Lee *et al*., 2010). Previous studies have shown that downregulation of B7-H3 reduced phosphorylation of both STAT3 and its upstream activator JAK2, whereas overexpression of B7-H3 activated JAK2/STAT signaling. Activation of STAT3 regulates key cell cycle molecules downstream such as cyclin D1(Agarwal *et al*., 2007). We demonstrated downregulation of p-STAT3 and cyclin D1 through B7-H3 knockdown

experiments. This confirmed studies in breast cancer whereby B7-H3 knockdwon reduces p-STAT3 activity(Liu *et al*., 2011).

Ipilimumab, an anti-CTLA4 monoclonal Ab, can control melanoma progression(Amaria *et al*., 2011; Hodi *et al*., 2010; O'Day *et al*., 2007). Recently, studies on anti-PD1(receptor of B7-H1, another B7-H family member) have been successful in early clinical studies(Ascierto *et al*., 2010). These clinical studies indicate the importance of targeting the B7-H family. Development of therapeutics targeting B7-H3 in melanoma may be useful for intervention. Regulation of B7-H3 ligand binding a receptor is still not understood. Identification of the B7-H3 receptor in melanomas may allow the development of targeted therapies blocking the B7-H3 signaling pathway. Recently, the TREM(triggering receptor expressed in murine myeloid cells) like transcript 2 was found to bind B7-H3(Hashiguchi *et al*., 2008). However, these findings have not been verified in humans and are controversial(Leitner *et al*., 2009). In summary, we demonstrated that B7-H3 expression on metastatic melanoma cells significantly correlated with tumor progression and poor MSS in patients, and associated with several melanoma regulating pathways. The studies also demonstrated B7-H3 affected functional activity of B7-H3 positive cells independent of immunoregulatory events.

Materials and Methods

Cells lines and tissues

Melanoma cell lines(M-1, M-101, M-111, M-12, M-14, JK-0346 Mel-B, JH-1173, Wm266-4) were cultured in RPMI 1640(Life Technologies, Grand Island, NY) as previously described(Goto *et al*., 2008). Melanocyte primary cultures were grown as suggested by the manufacturer(Lonza Inc, Newington, NH).

For protein expression by IHC analysis, PEAT specimens of primary melanomas(n=30) and metastatic melanomas(n=67) were assessed. PEAT specimens of nevi were used as controls. For mRNA analysis, PEAT specimens of a separate cohort consisting of primary and metastatic melanomas were assessed. All studies were conducted blinded to the pathology and clinical status of patients. PEAT specimens were obtained under a research protocol approved by the Western IRB.

Melanoma TMAs

As previously described(Camp *et al*., 2008), two melanoma TMAs were constructed by the department of pathology (Dr. D. Rimm, Yale University) using AJCC stage III and IV melanomas from JWCI with annotated clinical outcome. Melanomas from each PEAT block was identified; cores measuring 0.6mm in diameter were made and the TMA was constructed as previously described(Nguyen *et al*., 2010). The AJCC Stage III TMA included 139 metastatic melanoma containing LN, 10 melanoma lines, 12 histopathology negative LN(cancer-free), and 2 liver tissues to be used as controls. The AJCC Stage IV melanoma TMA included 268 distant organ metastases and 39 paired stage III/LN metastases from 169 melanoma patients, as well as 29 normal tissues from each respective

organ(cancer-free). Duplicated cores of all specimens and controls were included in all TMAs. TMA results were analyzed by two independent investigators.

IHC analysis of B7-H3 expression

PEAT sections were deparaffinized with xylene and rehydrated with ethanol, prior to antigen retrieval(Diagnostic BioSystems, Pleasanton, CA). Endogenous peroxidase activity was inactivated by incubation in 3.0% H_2O_2(20min). Non-specific binding sites were blocked for 30min with Protein Block serum-free(Dako, Carpinteria, CA). The tissue sections were incubated at room temperature with a goat anti-human B7-H3 polyclonal Ab(100μg/ml; R&D Systems, Minneapolis, MN) diluted(1:50). After washing with phosphate-buffered saline(PBS) containing 0.1%Tween 20(PBST), the reaction for B7-H3 was developed using a labeled streptavidin biotin(LSAB) method(LSAB+ Kit; Dako) and visualized using VIP Substrate Kit(Vector Laboratories, Burlingame, CA)(Yoshimura *et al*., 2011). The negative controls consisted of sections treated with goat serum alone(Santa Cruz Biotechnology, Santa Cruz, CA).

The melanoma AJCC stage II/III/IV and normal tissue IHC analysis for B7-H3 was scored(0, 1, 2, 3) by two independent investigators. The IHC for B7-H3 protein expression was classified as score 0(absent), score 1(weak), score 2(moderate) and score 3(strong) by light microscopy.

IHC for p-STAT3

p-STAT3 IHC was conducted on 5μm stage III melanoma metastasis sections(n=20). IHC was conducted following melanin bleaching after modification(Momose *et al*., 2011). In brief, following deparaffinization and rehydration, tissue was bleached with 3%H_2O_2, then subjected to antigen retrieval(Vector Labs). Tissue sections were then blocked with goat serum(10%) for 1hr followed by incubation with rabbit anti-pY705-STAT3 monoclonal Ab(Cell Signaling, Danvers, MA) overnight at 4°C. Ab staining was amplified with VECTASTAIN Elite ABC Kit(Vector Labs). After development with Dako Diaminobenzidine(DAB) substrate, sections were counterstained with hematoxylin and mounted. P-STAT3 stained tissues were assessed and scored using the same method as used for B7-H3, on a scoring scale of 0(none), 1, 2, and 3(strongest).

Flow cytometry

Flow cytometric analysis was performed using the BD FACS Calibur System(BD Biosciences, San Jose, CA). After washing in buffer containing 2%FBS, melanoma cells(10^5) were incubated at 4°C(1hr) with phycoerythrin(PE)-conjugated mouse monoclonal anti-human B7-H3 Ab(R& D Systems), followed by washing three times, then resuspended in PBS for flow cytometry, and analyzed using Quest software(BD Biosciences). Respective isotype-matched Ab was used as a negative control.

Immunofluorescence

Melanoma cells were cultured on Lab-Tek II chamber slides(Nalge Nunc, Thermo Fisher Scientific) and fixed with paraformaldehyde(4%) in PBS(10min) after washing in PBS. Melanoma cells were blocked by bovine serum albumin(5%) for 30min and stained using

mouse monoclonal anti-human B7-H3 Ab(R&D Systems) and PE-conjugated goat anti-mouse secondary Ab(Santa Cruz) at room temperature for 1hr. Slides were mounted with Vectashield mounting medium containing 4′, 6-diamidino-2-phenylindole(DAPI) for nuclear staining(Vector Labs). Cells were analyzed using a Nikon Eclipse Ti fluorescence microscope.

RNA extraction

Tri-Reagent(Molecular Research Center Inc., Cincinnati, OH) was used to extract total RNA from cultured cells(Koyanagi *et al*., 2006). For RNA extraction of individual PEAT specimens, 10 sections of 10μm-thick tissues were deparaffinized with xylene and washed with ethanol. Total RNA from PEAT specimens was extracted, isolated, purified, quantified as previously described(Koyanagi *et al*., 2006).

Primers and probes

Primer and probe sequences of B7-H3 were designed to assess B7-H3 mRNA expression in PEAT specimens. The forward primer, fluorescence resonance energy transfer probe sequence, and reverse primer were as follows: B7-H3, 5′-GACAGCAAAGAAGATGATGGA-3′(forward), 5′-FAM-CCTCCCTACAGCTCCTACCCTCTGG-BHQ-1-3′(probe), 5′-ACCTGTCAGAGC AGGATGC-3′(reverse). The glyceraldehyde-3-phosphate dehydrogenase(GAPDH) forward primer, fluorescence resonance energy transfer probe sequence, and reverse primer were as follows: 5′-GGGTGTGAACCATGAGAAGT-3′(forward), 5′-FAM-CAGCAATGCCTCCTGCACCACCAA-BHQ-1-3′(probe), and 5′-GACTGTGGTCATGAGTCCT-3′(reverse).

RT-qPCR assay

All reverse transcription reactions of RNA were performed as previously described (Koyanagi *et al*., 2006). The RT-qPCR assay was performed with the iCycler iQ Real-Time Thermocycler Detection System(Bio-Rad Laboratories, Hercules, CA). For each reaction, complementary DNA from 250ng of RNA was used with the PCR reaction mixture. Samples were amplified with a precycling hold at 95°C(10min), followed by 40 cycles of denaturation for each marker at 95°C(60sec), annealing at 63°C(60sec, B7-H3; 55°C, GAPDH), and extension at 72°C(60sec). Specific gene plasmids for external controls of each gene were synthesized. Standard curves for each assay were generated using a threshold cycle of dilutions of plasmid containing the target gene template(10^6-10^1 copies). Copy number analysis was performed using the iCycler iQ Real-Time Software(Bio-Rad). Each sample was assessed in duplicates with positive(M14 cells) and reagent controls for RT-qPCR assays.

To demonstrate correlation between miR-29c and B7-H3, RT-qPCR miR-29c expression data from a previous study(Nguyen *et al*., 2010) were compared to B7-H3 expression of the same tissue specimens. B7-H3 IHC score was assigned using the following scoring system: 0-none, 1-weak, 2-moderate, 3-strong. Correlation was demonstrated using Spearman's rank correlation test over the Pearson's correlation test as data demonstrated skewness. The

analysis was carried out in concordant with the minimum information for publication of quantitative realtime PCR experiments(MIQE) guidelines(Bustin *et al*., 2009).

Western blotting

Protein concentrations were determined using the Pierce BCA assay(Thermo Scientific). Western blot was performed as previously described(Narita *et al*., 2009). Membranes were immunoblotted overnight with primary rabbit polyclonal anti-B7-H3 Ab and cyclin D1 Ab(1:1000, Santa Cruz), rabbit polyclonal anti-STAT3 and anti-p-STAT3 Ab(1:1000, Cell Signaling) respectively.

After immunoblotting, the membranes were washed 3× with PBST and followed by 1hr incubation with horseradish peroxidase-conjugated goat anti-rabbit Ab(1:5000, Santa Cruz) or horseradish peroxidase-conjugated rabbit anti-mouse Ab(1:5000, Santa Cruz), respectively. Immunoreactive bands were visualized with the SuperSignal West Dura Extended Substrate kit(Thermo Scientific) and the densities of protein bands were quantified by Alpha Ease FCTM software(Version 3.1.2, Alpha Innotech Corp)

Transfection of miR-29c

M14 cells, M101 and Wm266-4 were transfected in 60mm dishes using the Jetprime™ Transfection Reagent(VWR International, Radnor, PA). Cells were seeded at 2.5–3.0 × 10^5 cells/60mm dishes and transfected with 100nmol/L(final) of miR-29c mimic and negative control(Thermo Fisher Dharmacon). Dharmacon miRIDIAN microRNA mimic is a double stranded RNA oligonucleotide, chemically modified with the Dharmacon ON-TARGET modifications. Dharmacon miRIDIAN negative control is a non-human miRNA with minimal sequence identity with human miRNAs. After transfection(24 hrs), cells were treated with 1 mg/mL Pronase E(E. Merck)(30min; 37°C) to strip off B7-H3 protein on the cell surface. Newly expressed B7-H3 protein levels 48hrs after Pronase treatment as measured by phycoerythrin(PE)-conjugated monoclonal mouse anti-human B7-H3 Ab immunofluorescence staining followed by flow cytometry analyses. Cells transfected(72hrs) with 100nmol/L(final concentration) of miR-29c mimic or negative control were lysed in cold lysis buffer, followed by western blotting.

Development of B7-H3-knockdown cells

The B7-H3 shRNA 1 sequence is CCGGCTCTGAAACACTCTGACAGCACTCG AGTGCTGTCAGAGTGTTTCAGAGTTTTTTG. The B7-H3 shRNA 2 sequence is CCGGCTAGCCTTAATACTGGCCTTTCTCGAGAAAGGCCAGTATTAAGGCTAGTT TTTTG. The vector is a lentiviral construct pLKO.1-Puro (Sigma). A shRNA which does not match any known human cDNA was used as a control. Cells were stably transduced with B7-H3 shRNAs or the control shRNA construct and selected in 5 μg/ml puromycin as suggested by manufacturer's instructions. Pooled populations of knockdown cells were used for the experiments.

Development of B7-H3-overexpressing cells

The human B7-H3 cDNA vector was purchased from Origene Company. Stable cell lines, after transfection, were selected with 800μg/ml G418. Pooled populations were used in all experiments.

Cell growth and soft agar colony formation

Cell proliferation and viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. 3D cell culture was performed using Matrigel matrix (BD Biosciences) in 96-well plate according to manufacturer's instruction. A soft agar colony formation assay was performed using six-well culture plates.

Cell migration and invasion assay

Briefly, 10^4 cells were plated on the top of the Boyden chamber inserts(BD Biosciences). Serum (5%) was used as the chemoattractant. To rule out the effect of cell proliferation, 2 μg/ml mitomycin C was added to the cells. Cells on the lower surface of the inserts were stained and counted using a light microscope. For invasion assays, inserts were coated with a thin layer of Matrigel basement membrane matrix.

Biostatistical analysis

For PEAT specimens, the Wilcoxon rank sum test was used to assess the difference in B7-H3 mRNA copy numbers between AJCC stage-linked melanoma and nevi/benign lentigo melanocytes/normal skin tissues. The Kruskal-Wallis test was used to identify AJCC stage-related differences in B7-H3 mRNA copy numbers. Increased expression of B7-H3 in distant organ metastasis relative to their matched lymph node metastasis was assessed by the paired t-Test. MSS analyses were conducted using the Kaplan-Meier method and comparisons among groups were performed using log-rank tests. Correlation analysis between miR-29c mRNA expression and B7-H3 IHC was performed using Spearman's correlation coefficient. Statistical calculations were performed using SAS statistical software(SAS); $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, Ruth and Martin H. Weil Fund, and the Award Number P0 CA029605 Project II and Core C from the NIH, NCI.

References

Agarwal C, Tyagi A, Kaur M, Agarwal R. Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells. Carcinogenesis. 2007; 28:1463–1470. [PubMed: 17341659]

Amaria RN, Lewis KD, Gonzalez R. Therapeutic options in cutaneous melanoma: latest developments. Ther Adv Med Oncol. 2011; 3:245–251. [PubMed: 21957431]

Ascierto PA, Simeone E, Sznol M, Fu YX, Melero I. Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. Semin Oncol. 2010; 37:508–516. [PubMed: 21074066]

Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009; 27:6199–6206. [PubMed: 19917835]

Balkwill FR. The chemokine system and cancer. J Pathol. 2011; 226:148–157. [PubMed: 21989643]

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55:611–622. [PubMed: 19246619]

Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. J Clin Oncol. 2008; 26:5630–5637. [PubMed: 18936473]

Cao Y, Zhang L, Ritprajak P, Tsushima F, Youngnak-Piboonratanakit P, Kamimura Y, et al. Immunoregulatory molecule B7-H1 (CD274) contributes to skin carcinogenesis. Cancer Res. 2011; 71:4737–4741. [PubMed: 21730022]

Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. Nat Immunol. 2001; 2:269–274. [PubMed: 11224528]

Crispen PL, Sheinin Y, Roth TJ, Lohse CM, Kuntz SM, Frigola X, et al. Tumor cell and tumor vasculature expression of B7-H3 predict survival in clear cell renal cell carcinoma. Clin Cancer Res. 2008; 14:5150–5157. [PubMed: 18694993]

Flies DB, Chen L. The new B7s: playing a pivotal role in tumor immunity. J Immunother. 2007; 30:251–260. [PubMed: 17414316]

Goto Y, Arigami T, Kitago M, Nguyen SL, Narita N, Ferrone S, et al. Activation of Tolllike receptors 2, 3, and 4 on human melanoma cells induces inflammatory factors. Mol Cancer Ther. 2008; 7:3642–3653. [PubMed: 19001446]

Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005; 23:515–548. [PubMed: 15771580]

Hashiguchi M, Kobori H, Ritprajak P, Kamimura Y, Kozono H, Azuma M. Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. Proc Natl Acad Sci U S A. 2008; 105:10495–10500. [PubMed: 18650384]

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010; 363:711–723. [PubMed: 20525992]

Hofmeyer KA, Ray A, Zang X. The contrasting role of B7-H3. Proc Natl Acad Sci U S A. 2008; 105:10277–10278. [PubMed: 18650376]

Jensen TO, Schmidt H, Moller HJ, Donskov F, Hoyer M, Sjoegren P, et al. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. Cancer. 2011

Kobori H, Hashiguchi M, Piao J, Kato M, Ritprajak P, Azuma M. Enhancement of effector CD8+ T-cell function by tumour-associated B7-H3 and modulation of its counter-receptor triggering receptor expressed on myeloid cell-like transcript 2 at tumour sites. Immunology. 2010; 130:363–373. [PubMed: 20141543]

Koyanagi K, O'Day SJ, Gonzalez R, Lewis K, Robinson WA, Amatruda TT, et al. Microphthalmia transcription factor as a molecular marker for circulating tumor cell detection in blood of melanoma patients. Clin Cancer Res. 2006; 12:1137–1143. [PubMed: 16489066]

Lee H, Deng J, Kujawski M, Yang C, Liu Y, Herrmann A, et al. STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. Nat Med. 2010; 16:1421–1428. [PubMed: 21102457]

Leitner J, Klauser C, Pickl WF, Stockl J, Majdic O, Bardet AF, et al. B7-H3 is a potent inhibitor of human T-cell activation: No evidence for B7-H3 and TREML2 interaction. Eur J Immunol. 2009; 39:1754–1764. [PubMed: 19544488]

Liu H, Tekle C, Chen YW, Kristian A, Zhao Y, Zhou M, et al. B7-H3 silencing increases paclitaxel sensitivity by abrogating Jak2/Stat3 phosphorylation. Mol Cancer Ther. 2011; 10:960–971. [PubMed: 21518725]

Momose M, Ota H, Hayama M. Re-evaluation of melanin bleaching using warm diluted hydrogen peroxide for histopathological analysis. Pathol Int. 2011; 61:345–350. [PubMed: 21615609]

Narita N, Tanemura A, Murali R, Scolyer RA, Huang S, Arigami T, et al. Functional RET G691S polymorphism in cutaneous malignant melanoma. Oncogene. 2009; 28:3058–3068. [PubMed: 19561646]

Nguyen T, Kuo C, Nicholl MB, Sim MS, Turner RR, Morton DL, et al. Downregulation of microRNA-29c is associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma. Epigenetics. 2010; 6:388–394. [PubMed: 21081840]

O'Day SJ, Hamid O, Urba WJ. Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): a novel strategy for the treatment of melanoma and other malignancies. Cancer. 2007; 110:2614–2627. [PubMed: 18000991]

Petroff MG, Kharatyan E, Torry DS, Holets L. The immunomodulatory proteins B7-DC, B7-H2, and B7-H3 are differentially expressed across gestation in the human placenta. Am J Pathol. 2005; 167:465–473. [PubMed: 16049332]

Roth TJ, Sheinin Y, Lohse CM, Kuntz SM, Frigola X, Inman BA, et al. B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. Cancer Res. 2007; 67:7893–7900. [PubMed: 17686830]

Sato Y, Goto Y, Narita N, Hoon DS. Cancer Cells Expressing Toll-like Receptors and the Tumor Microenvironment. Cancer Microenviron. 2009; 2(1):205–214. [PubMed: 19685283]

Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumors: new targets for therapy. Genes Dev. 2011; 25:2559–2572. [PubMed: 22190457]

Steinberger P, Majdic O, Derdak SV, Pfistershammer K, Kirchberger S, Klauser C, et al. Molecular characterization of human 4Ig-B7-H3, a member of the B7 family with four Ig-like domains. J Immunol. 2004; 172:2352–2359. [PubMed: 14764704]

Sun Y, Wang Y, Zhao J, Gu M, Giscombe R, Lefvert AK, et al. B7-H3 and B7-H4 expression in non-small-cell lung cancer. Lung Cancer. 2006; 53:143–151. [PubMed: 16782226]

Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with b7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med. 2012; 4:127ra137.

Xu H, Cheung IY, Guo HF, Cheung NK. MicroRNA miR-29 modulates expression of immunoinhibitory molecule B7-H3: potential implications for immune based therapy of human solid tumors. Cancer Res. 2009; 69:6275–6281. [PubMed: 19584290]

Yoshimura T, Nagahara M, Kuo C, Turner RR, Soon-Shiong P, Hoon DS. Lymphovascular invasion of colorectal cancer is correlated to SPARC expression in the tumor stromal microenvironment. Epigenetics. 2011; 6:1001–1011. [PubMed: 21725199]

Zang X, Allison JP. The B7 family and cancer therapy: costimulation and coinhibition. Clin Cancer Res. 2007; 13:5271–5279. [PubMed: 17875755]

Abbreviations

DAB	Diaminobenzidine
DAPI	4′, 6-diamidino-2-phenylindole
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LN	lymph node
LSAB	labeled streptavidin biotin
MSS	melanoma-specific survival
PBS	phosphate-buffered saline

PE	phycoerythrin
PEAT	paraffin-embedded archival tissue
p-STAT3	phosphorylated STAT3
TMA	tissue microarrays

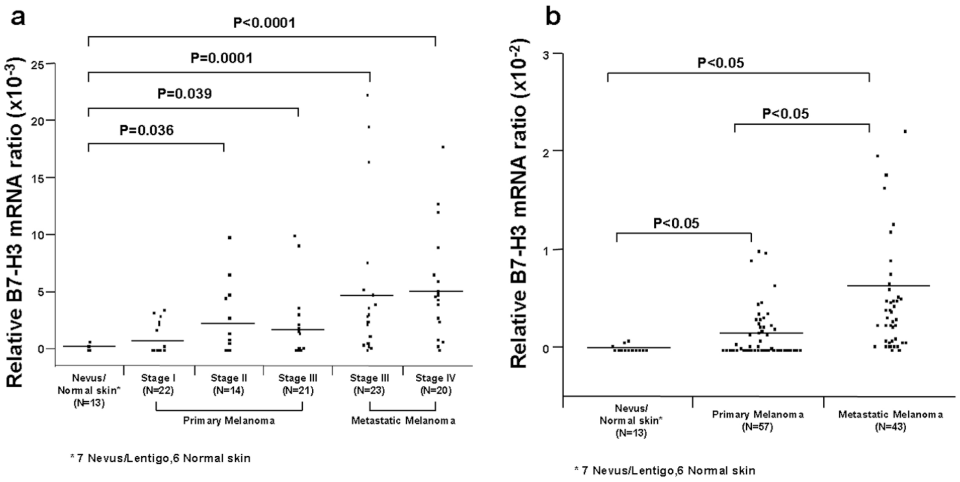

Figure 1. B7-H3 mRNA expression in melanomas

A: B7-H3 mRNA expression in AJCC Stage I, II, III primary melanomas, Stage III. Stage IV metastatic melanomas, and nevi/benign lentigo melanocytes/normal skin tissues. **B**: B7-H3 mRNA expression in primary melanomas, metastatic melanomas, and nevi/benign lentigo melanocyte/normal skin. Horizontal bars indicate mean relative B7-H3 copy numbers.

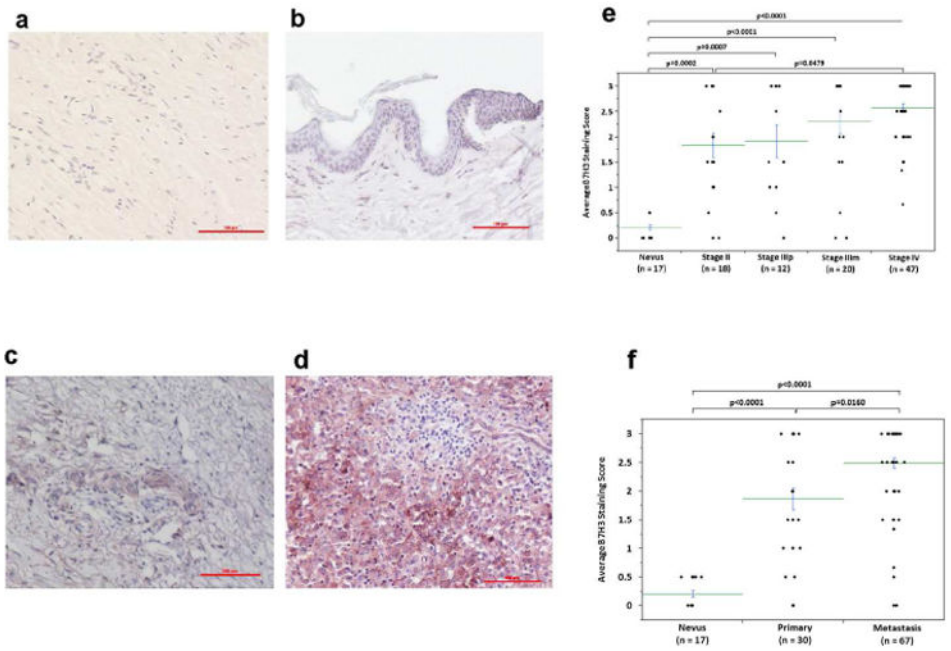

Figure 2. IHC staining of B7-H3 of melanomas

A-D: Representative IHC staining of B7-H3. **A**: Negative control (primary antibody isotype control). **B**: Nevus. **C**: Primary melanomas. **D**: Metastatic melanomas. **E**: B7-H3 expression(IHC score) in primary melanomas AJCC Stage II, III, metastatic melanoma AJCC Stage III, IV, and nevus. **F**: B7-H3 IHC score in primary melanomas, metastatic melanomas, and nevus. Scale bar = 100μm.

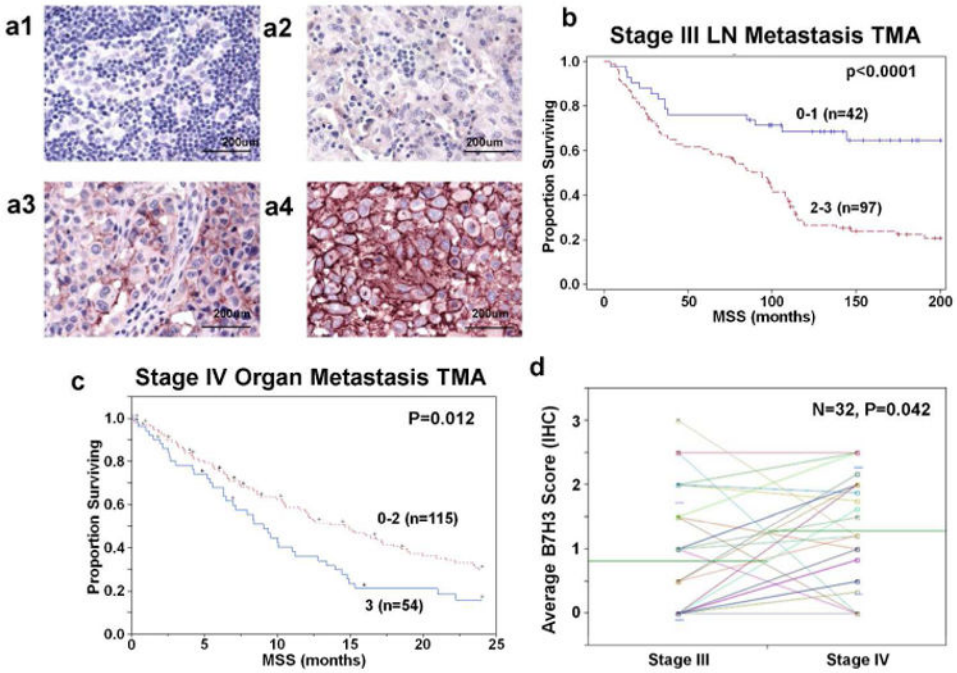

Figure 3. Melanoma-specific survival in stage III and stage IV melanoma(TMA) Patients

A: Representative TMA IHC staining of B7-H3. (**A1**) LN metastasis with absent expression of B7-H3. (**A2**) LN metastasis with weak expression of B7-H3. (**A3**) LN metastasis with moderate expression of B7-H3. (**A4**) LN metastasis with strong expression of B7-H3. In Figure **A1, A2, A3** and **A4**, scale bars indicate 200μm in length. Magnification: 100X. **B**: Stage III TMA(n=139) MSS comparing B7-H3 IHC score of 0-1(no-weak) versus 2-3(moderate-strong). **C**: Stage IV TMA(n=169) MSS comparing B7-H3 IHC score of 0-2(no-moderate) versus 3(strong). **D**: Analysis of B7-H3 IHC score between paired matched stage III LN metastases and stage IV distant metastases from the same patient in the stage IV TMA. Horizontal lines represent average B7-H3 score among each stage.

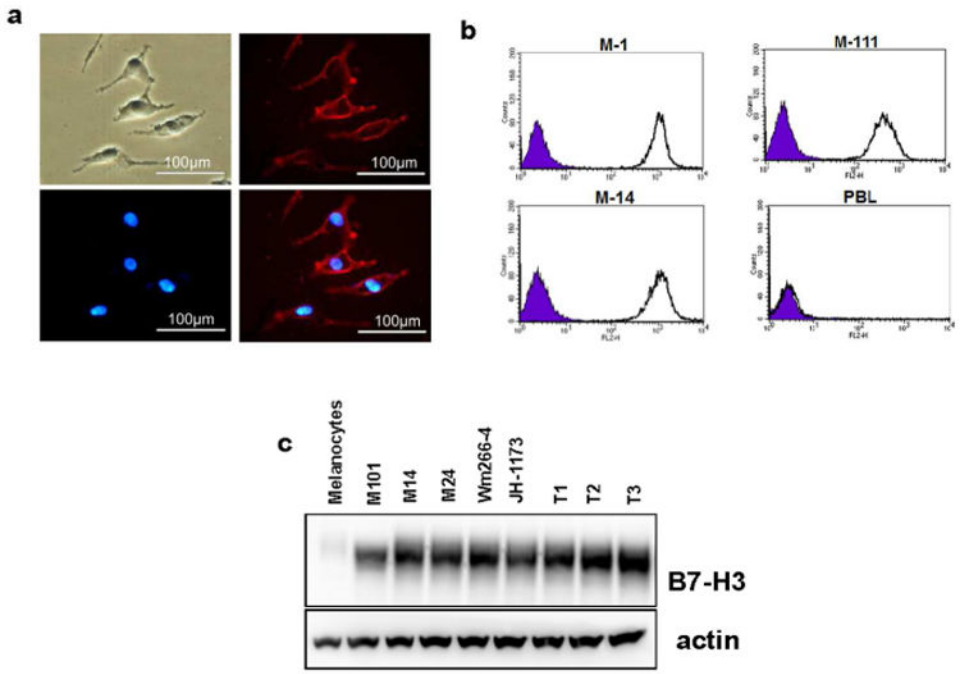

Figure 4. B7-H3 expression in melanoma cells

A: Immunofluorescent IHC staining of a melanoma cell line(M14). Top left: Bright field; Top right: B7-H3; Bottom left: DAPI; Bottom right; Merged DAPI + B7-H3. Magnification: 400X. **B**: Flow cytometry analysis of B7-H3 expression(open histogram) versus Ab isotype controls(shaded histogram) in melanoma cell lines and normal PBL. **C**: Western blotting analysis of melanoma cell lines and tissues (T1∼T3). Scale bar = 100μm.

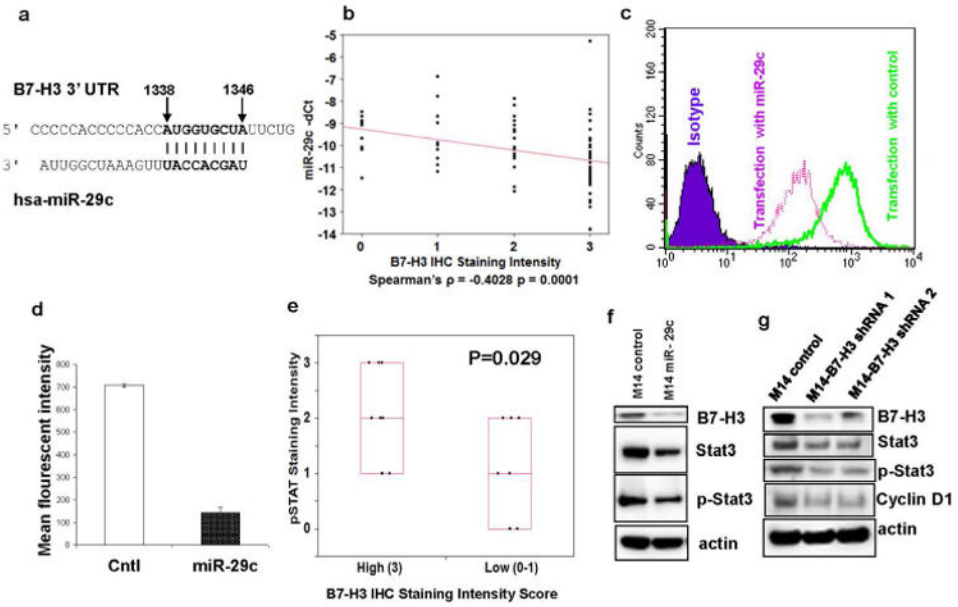

Figure 5. MiR-29c regulated B7-H3 expression

A: Binding site of miR-29c(bottom strand) to B7-H3 mRNA 3′UTR position from 1338 to 1346(top strand) (9 bp in length). **B**: B7-H3 IHC staining score vs. miR-29c mRNA expression in melanoma TMA samples (paired T-test analysis). **C**: Flow cytometry analysis of B7-H3 expression in a melanoma cell line with transfection of miR-29c mimic and with a negative miRNA control. **D**: Quantitation of B7-H3 expression change after transfection with miR-29c. **E**: Relationship of IHC staining score of p-STAT3 versus B7-H3 in metastatic melanoma PEAT. **F**: Western blot of STAT3 and p-STAT3 in a melanoma cell line transfected with miR-29c mimic and with negative miRNA control. **G**. Western blot of STAT3, p-STAT3 and cyclin D1 in a melanoma cell lines transfected with B7-H3 shRNA or with shRNA control.

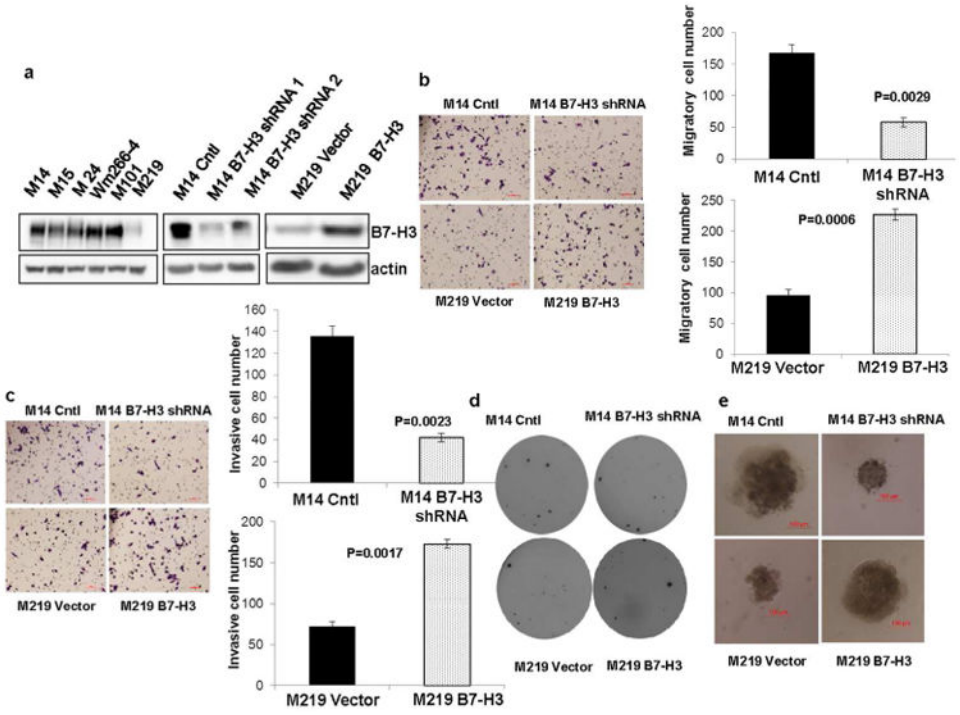

Figure 6. B7-H3 functional studies

A: Immunoblots of B7-H3 expression in melanoma lines untreated(left), M14 Cntl(Control) vs M14 B7-H3 shRNA cells (middle) and M219 Vector vs M219 B7-H3 cells (right). **B**: Migration assay of M14 Cntl vs M14 B7-H3shRNA cells (top), and M219 Vector vs M219 B7-H3 cells (bottom). **C**: Invasion assay of M14 Cntl vs M14 B7-H3shRNA cells (top) and M219 Vector vs M219 B7-H3 cells (bottom). The data was presented as mean ± SD (Right). **D**: Colony formation of M14 Cntl vs M14 B7-H3 shRNA cells (top) and M219 Vector vs M219 B7-H3 cells (bottom) in soft agar after 18 days culture. **E**: The growth of M14 Cntl vs M14 B7-H3 shRNA cells (top) and M219 Vector vs M219 B7-H3 cells (bottom) in 3D Matrigel. (M14 Cntl, M14 B7-H3 shRNA refer to cells transfected with shRNA control and B7-H3 shRNA respectively; M219 Vector, M219 B7-H3 refer to cells transfected with empty vector and B7-H3 expression plasmid respectively). Student's t-test was used to assess differences between the groups. The data was presented as mean ± SD (Right). Scale bar = 100μm.