

The official journal of

INTERNATIONAL FEDERATION OF PIGMENT CELL SOCIETIES · SOCIETY FOR MELANOMA RESEARCH

PIGMENT CELL & MELANOMA Research

Epac1 promotes melanoma metastasis via modification of heparan sulfate

Erdene Baljinnyam, Masanari Umemura,
Mariana S. De Lorenzo, Mizuka Iwatsubo,
Suzie Chen, James S. Goydos and Kousaku Iwatsubo

DOI: 10.1111/j.1755-148X.2011.00863.x

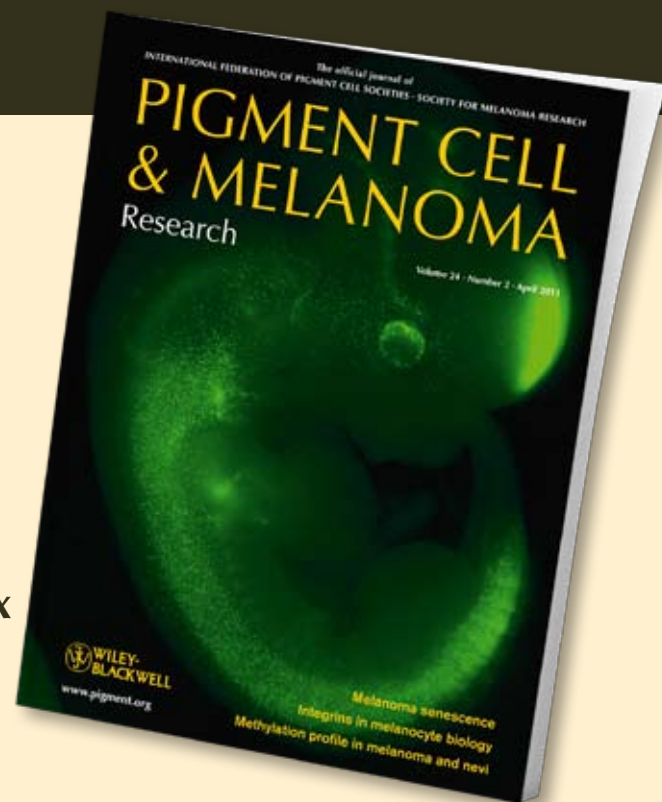
Volume 24, Issue 4, Pages 680–687

If you wish to order reprints of this article,
please see the guidelines [here](#)

Supporting Information for this article is freely available [here](#)

EMAIL ALERTS

Receive free email alerts and stay up-to-date on what is published
in Pigment Cell & Melanoma Research – [click here](#)



Submit your next paper to PCMR online at <http://mc.manuscriptcentral.com/pcmr>

Subscribe to PCMR and stay up-to-date with the only journal committed to publishing
basic research in melanoma and pigment cell biology

As a member of the IFPCS or the SMR you automatically get online access to PCMR. Sign up as
a member today at www.ifpcs.org or at www.societymelanomaresarch.org

To take out a personal subscription, please [click here](#)

More information about Pigment Cell & Melanoma Research at www.pigment.org

Epac1 promotes melanoma metastasis via modification of heparan sulfate

Erdene Baljinnyam¹, Masanari Umemura¹, Mariana S. De Lorenzo¹, Mizuka Iwatsubo¹, Suzie Chen², James S. Goydos³ and Kousaku Iwatsubo^{1,4}

1 Department of Cell Biology and Molecular Medicine, New Jersey Medical School-University of Medicine and Dentistry of New Jersey, Newark, NJ, USA **2** Department of Chemical Biology, Susan Lehman Cullen Laboratory of Cancer Research in the Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA **3** Division of Surgical Oncology, Department of Surgery, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ, USA **4** Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama, Japan

KEYWORDS melanoma/Epac/heparan sulfate/migration/NDST-1/tissue microarray/N-sulfation

PUBLICATION DATA Received 18 September 2010, revised and accepted for publication 14 April 2011, published online 19 April 2011

CORRESPONDENCE K. Iwatsubo, e-mail: iwatsuko@umdnj.edu

doi: 10.1111/j.1755-148X.2011.00863.x

Summary

Our previous report suggested the potential role of the exchange protein directly activated by cyclic AMP (Epac) in melanoma metastasis via heparan sulfate (HS)-mediated cell migration. In order to obtain conclusive evidence that Epac1 plays a critical role in modification of HS and melanoma metastasis, we extensively investigated expression and function of Epac1 in human melanoma samples and cell lines. We have found that, in human melanoma tissue microarray, protein expression of Epac1 was higher in metastatic melanoma than in primary melanoma. In addition, expression of Epac1 positively correlated with that of N-sulfated HS, and N-deacetylase/N-sulfotransferase-1 (NDST-1), an enzyme that increases N-sulfation of HS. Further, an Epac agonist increased, but ablation of Epac1 decreased, expressions of NDST-1, N-sulfated HS, and cell migration in various melanoma cell lines. Finally, C8161 cells with stable knockdown of Epac1 showed a decrease in cell migration, and metastasis in mice. These data suggest that Epac1 plays a critical role in melanoma metastasis presumably because of modification of HS.

Introduction

Melanoma causes the majority of skin cancer-related death, and is prevalent worldwide. The median life span of patients with advanced stage melanoma is less than a year because no therapies are effective once the tumor has spread to vital organs (Berwick and Wiggins, 2006). The tumor metastasis process is conventionally understood as the migration of individual cells that detach from the primary tumor, enter in the lymphatic vessels

or the bloodstream, attach to endothelial cells and undergo transendothelial extravasation, and proliferate in organs (Gaggioli and Sahai, 2007). Although numerous efforts have been focused on understanding the melanoma progression, the efforts to control melanoma cell migration/metastasis have been unsuccessful.

The exchange protein directly activated by cAMP (Epac), a guanine nucleotide exchange factor (De Rooij et al., 1998), has two isoforms, Epac1 and Epac2, which mediate cAMP signaling through activation of a

Significance

Biological function of Epac1 in melanoma has been largely unknown. We found, for the first time, that the protein expression of Epac1 is higher in metastatic melanoma than in primary melanoma in human melanoma samples. In addition, our data suggested that Epac1 regulates modification of HS as demonstrated by a positive correlation between Epac1 and N-sulfated HS. Further, ablation of Epac1 reduces melanoma metastasis in vivo in mice. As melanoma therapy has been unsuccessful because of its high metastatic potential, our findings would lead to a new therapy for this life-threatening disease.

small-molecular-weight G protein, Rap1 (Bos, 2006). In the cancer research field, reports demonstrated that Epac mediates cell adhesion of human ovarian carcinoma cells (Ovcar3) (Quilliam et al., 2002), apoptosis (Tiwari et al., 2004) and growth arrest (Grandoch et al., 2009) in B lymphoma cells, formation of embryonic vasculogenic networks in melanoma cells (Lissitzky et al., 2009), and proliferation of prostate carcinoma cells (Grandoch et al., 2009). We previously demonstrated that mRNA expression of Epac1 was higher in metastatic melanoma (MM) than in primary melanoma; however, protein expression of Epac1 has not been determined yet (Baljinnyam et al., 2010). We also demonstrated that Epac1 increases melanoma cell migration, and metastasis in mice. The potential mechanism by which Epac1 induces cell migration was associated with an increase in the expression of the N-deacetylase/N-sulfotransferase (NDST-1), a key enzyme for N-sulfation of glucosamine of heparan sulfate (HS) chain (Aikawa and Esko, 1999). N-sulfation of glucosamine is known to play a role in cell migration (O'sullivan et al., 2000) via syndecan-2 (Galante and Schwarzbauer, 2007), platelet derived growth factor (PDGF) receptors (Abramsson et al., 2007; Stenzel et al., 2009) or fibroblast growth factor receptors (Faham et al., 1996; Kreuger et al., 1999, 2002; Maccarana et al., 1993; Schlessinger et al., 2000). Accordingly, it is tempting to speculate that Epac1-rich melanoma cells produce HS with abundant N-sulfation via expression of NDST-1, which leads to increased cell migration and thus metastasis of melanoma.

In this study, we examined expressions of Epac1, NDST-1, and N-sulfated HS in human melanoma tissue microarray. In addition, the correlation among expressions of these proteins was analyzed. Also, in various melanoma cell lines, we tested the hypothesis whether Epac1 regulates cell migration, expressions of NDST-1 and N-sulfation of HS in multiple melanoma cell lines. Finally, we evaluated whether ablation of Epac1 reduces melanoma metastasis to distant organs in mice.

Results

Expression of Epac1 is higher in metastatic melanoma than in primary melanoma

We previously demonstrated that expression of Epac1 mRNA was increased in MM compared with primary melanoma (Baljinnyam et al., 2010); however, it has been unclear whether Epac1 is indeed increased in MM at the protein level. We thus performed quantification of protein expression of Epac1 as well as Epac2 with immunohistochemical staining in human melanoma tissue microarray (Figure 1 and Table 1). Protein expression of Epac1, but not Epac2, was significantly higher in MM than in primary melanoma, suggesting a role of Epac1 in melanoma metastasis, and further confirming our previous finding at the mRNA level.

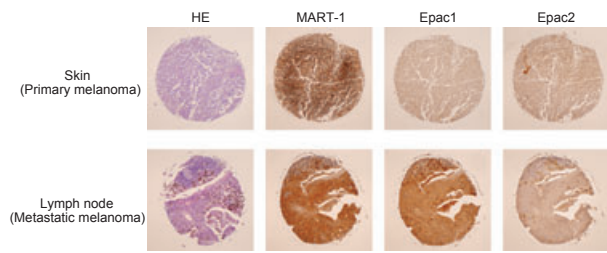


Figure 1. Expression of Epac in human melanoma tissues. Human melanoma tissue microarray was subjected to immunohistochemical analyses. Brown indicates positive staining. Representative images of immunohistochemical analysis for Epac1, Epac2 and MART-1, and of HE stainings are shown. There was a tendency toward higher expression of Epac1 in metastatic melanoma than in primary melanoma.

Expression of Epac1 positively correlates with that of NDST-1 and of N-sulfated HS

We next examined whether Epac1 increases expression of NDST-1 and thus N-sulfated HS in the same human melanoma tissue microarray. Both the expression of NDST-1 and that of N-sulfated HS were higher in MM than in primary melanoma (Figure 2A, Tables 2 and 3), suggesting involvement of N-sulfation of HS in melanoma progression. As our previous report demonstrated that Epac1 increases expression of NDST-1 (Baljinnyam et al., 2009), we examined whether there is a correlation between Epac1 and NDST-1. We then found a strong positive correlation between Epac1 and NDST-1 (Figure 2B), and in addition, between Epac1 and N-sulfated HS (Figure 2C). There was also a positive correlation between NDST-1 and N-sulfated HS as expected (Figure 2D). These data suggest that Epac1-mediated HS modification is involved, at least in part, in melanoma metastasis.

Ablation of Epac1 reduces N-sulfation of HS

In order to confirm the role of Epac1 in modification of HS, we examined whether inhibition of Epac1 reduces the expression of NDST-1, and thus N-sulfation of HS. C8161 cells with stable knockdown of Epac1 [C8161/Epac1(-)] showed lower expression of NDST-1 (Figure 3A) than control cells (C8161/control). In addition, HS ELISA showed decreased N-sulfated HS amount in C8161/Epac1(-) cells (Figure 3B). Further, we performed immunocytochemistry for N-sulfated HS in co-culture of C8161/control and C8161/Epac1(-) cells (Figure 3C), and compared the degree of N-sulfation of HS. Expression of N-sulfated HS was lower in C8161/Epac1(-) cells which have relatively low level of Epac1 (Figure 3D, white arrow), than in C8161/control cells which have relatively high level of Epac1 (Figure 3D, red arrow). By contrast, total HS was not different between these cell lines (Figure 3D). Further, in human primary melanoma tissue, N-sulfated HS was obvious in Epac1-rich melanoma area compared with

Table 1. Staining intensity of Epac1 and Epac2 in primary and metastatic melanoma tissues

		Weak (ISS: <1.5)	Moderate (ISS: 1.5–2.5)	Strong (ISS: 2.5<)	P versus primary melanoma
Epac1	Primary	37/127 (29.2%)	68/127 (53.5%)	22/127 (17.3%)	0.01
	Metastasis	4/62 (6.4%)	13/62 (21.0%)	45/62 (72.6%)	
Epac2	Primary	28/127 (22.1%)	68/127 (53.5%)	31/127 (24.4%)	0.45
	Metastasis	19/62 (30.6%)	28/62 (45.2%)	15/62 (24.2%)	

The immunostaining scores (ISS), ranging from 1.1 to 11 (Epac1), and 0.8 to 10.2 (Epac2), were categorized as weak (<1.5), moderate (1.5–2.5), or strong (2.5<).

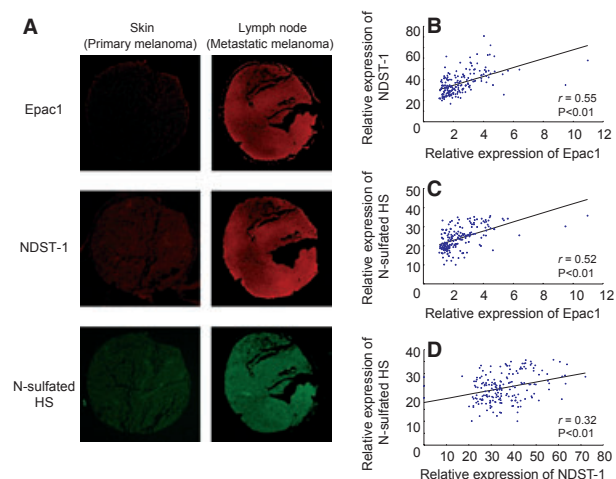


Figure 2. Correlation of Epac1, NDST-1, and N-sulfated heparan sulfate (HS) in melanoma. (A) Expression of Epac1, NDST-1, and N-sulfated HS in primary and metastatic melanoma tissues. Representative images of immunohistochemical analysis for Epac1 (red), NDST-1 (red), and N-sulfated HS (green) are shown. Expressions of Epac1, NDST-1, and N-sulfated HS were higher in metastatic melanoma than in primary melanoma. (B–D) Analyses of a correlation between Epac1 and NDST-1 (B), Epac1 and N-sulfated HS (C), and NDST-1 and N-sulfated HS (D) are shown. Expression levels are shown as blue dots. Pearson correlation coefficients (r) and P-values (P) are indicated.

Epac1-poor area (Figure 3E). These data strongly suggest that Epac1 increases expression of NDST-1, and thus the N-sulfation of HS in melanoma.

Epac1 increases cell migration, expression of NDST-1 and N-sulfated HS

We next tested the hypothesis whether the effects of Epac1 are universal among various melanoma cell lines. As shown in Figure 4A, 8-(4-Methoxyphenylthio)-

2'-*O*-methyladenosine-3',5'-cyclic monophosphate (8-pMeOPT), an Epac-specific agonist, significantly increased cell migration in all melanoma cell lines (Figure 4A) with the exception of the C8161 cell line. In C8161 cells, it is plausible that the Epac-signaling is already saturated under basal conditions by the abundant expression of Epac1 (Figure 4B). We next examined whether stimulation of endogenous Epac increases expression of NDST-1, and thus N-sulfation of HS in cell lines from different progression stages, i.e., WM1552C cells [radial growth phase (RGP)], WM1361A [vertical growth phase (VGP)], SK-Mel-2 and C8161 (MM) cells. Expression of NDST-1 was enhanced by 8-pMeOPT (Figure 4C) in WM1552C, WM1361A and SK-Mel-2 cells, paralleled with increased N-sulfated HS (Figure 4D) but without changes of Epac1 itself (Figure S2). In C8161 cells, again, 8-pMeOPT did not change NDST-1 expression and N-sulfated HS. Considering the 15- to 20-fold higher amount of basal N-sulfated HS in C8161 cells, it could be explained by the saturated Epac1's effect in this cell line. Further, when Epac1 was overexpressed in Epac1-poor melanoma cell lines (Figure 4B), NDST-1 expression was clearly increased (Figure S1), confirming the major role of Epac in NDST-1 expression. All these evidences confirm the idea that the effects of Epac1 on cell migration, NDST-1 expression and the resultant HS modification are universal among various melanoma cell lines.

Ablation of Epac1 reduces cell migration and metastasis

We next examined whether ablation of Epac1 reduces cell migration and thus metastasis to distant organs. C8161/Epac1(-) showed reduced cell migration (Figure 5A), and metastasis to the lungs in mice (Figure 5B,C) compared with C8161/control cells, suggesting the involvement of endogenous Epac1 in melanoma metastasis.

Table 2. Staining intensity of NDST-1 in primary and metastatic melanoma tissues

	Weak (ISS: <30)	Moderate (ISS: 30–50)	Strong (ISS: 50<)	P versus primary melanoma
Primary	41/127 (32.3%)	74/127 (58.2%)	12/127 (9.5%)	<0.01
Metastasis	3/62 (4.8%)	42/62 (67.7%)	17/62 (27.5%)	

The immunostaining scores, ranging from 20.1 to 80.8, were categorized as weak (<30), moderate (30–50), or strong (50<).

Table 3. Staining intensity of N-sulfated heparan sulfate in primary and metastatic melanoma tissues

	Weak (ISS: <15)	Moderate (ISS: 15–25)	Strong (ISS: 25<)	P versus primary melanoma
Primary	7/127 (5.5%)	75/127 (59.1%)	45/127 (35.4%)	<0.05
Metastasis	2/62 (3.2%)	22/62 (35.5%)	38/62 (61.3%)	

The immunostaining scores, ranging from 10 to 57.6, were categorized as weak (<15), moderate (15–25), or strong (25<).

Discussion

Our conclusion in the present study is that Epac1 plays a major role in melanoma metastasis by modification of HS. Human melanoma tissue microarray showed increased expression of Epac1, NDST-1 and N-sulfated HS, and positive correlation between these molecules. Stimulation of Epac increased, and ablation of Epac1 decreased, cell migration and N-sulfation of HS in various melanoma cell lines. Further, ablation of Epac1 markedly reduced melanoma metastasis in mice. These data suggest the major role of Epac1 in melanoma progression through modification of HS.

Despite extensive research on the role of Epac over a decade, little attention has been paid to changes in its expression in malignant cells. It was reported that, in B-cell chronic lymphocytic leukemia cells, mRNA expression of Epac1 was increased, leading to a slight reduction of apoptosis (Tiwari et al., 2004). We previously demonstrated that Epac1 expression was

increased in regional dermal and distant MM, but not in lymph node metastasis. Non-significant change in lymph node metastasis was probably due to a small number of samples (Baljinnyam et al., 2010). Also, the limitation of our previous study was the demonstration of Epac1 expression at the mRNA level, but not at the protein level. In this study, we demonstrated that Epac1 protein is higher in MM than in primary melanoma. In addition, C8161 cells from highly MM (Welch et al., 1991; Yohem et al., 1991) showed abundant expression of Epac1 accompanied by elevation of N-sulfation of HS and basal cell migration. Further, data from the current (Figure 4B) and the previous (Baljinnyam et al., 2010) studies showed a tendency toward higher Epac1 expression in MM cell lines, i.e., cells from VGP or MM cells, than in non-MM, i.e., cells from RGP cells, and melanocytes. These findings suggest that expression of Epac1 plays a key role in obtaining metastatic potential in melanoma.

Human tissue microarray showed that the expression of Epac1 positively correlated with that of N-sulfated

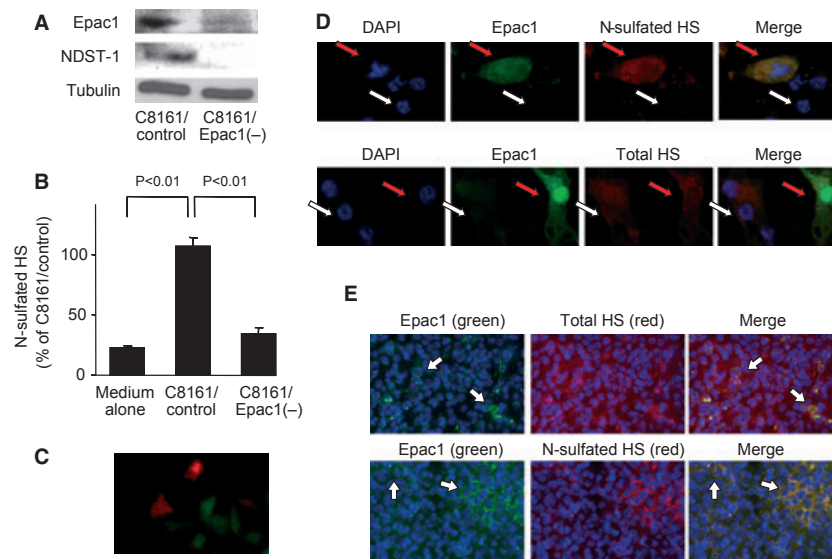


Figure 3. Ablation of Epac1 reduces expression of NDST-1 and N-sulfated HS in melanoma. (A) Western blots for Epac1 and NDST-1 from C8161/control or C8161/Epac1(–) cells are shown. Ablation of Epac1 reduced protein expression of Epac1 as well as NDST-1. (B) HS ELISA shows that ablation of Epac1 reduced the amount of N-sulfated HS. $n = 4$. (C) Co-culture of C8161/control expressing RFP (red) and C8161/Epac1(–) expressing GFP (green), which were subjected to immunocytochemistry shown in D. (D) Immunocytochemistry for Epac1 (green) and N-sulfated or total N-sulfated HS (red) in co-culture of C8161/control and C8161/Epac1(–) cells. C8161/Epac1(–) cells which are considered as Epac1-poor cells (white arrows), showed lower signal intensity of N-sulfated HS than C8161/control cells which are considered as Epac1-rich cells (red arrows). Total HS was not different between C8161/control and C8161/Epac1(–) cells. (E) Primary skin melanoma tissue was subjected to immunohistochemistry with antibodies against Epac1 (green) and total or N-sulfated HS (red). Epac1-rich melanoma cells (white arrows) showed more abundant N-sulfated HS than Epac1-poor melanoma cells, whereas there was no difference in total HS.

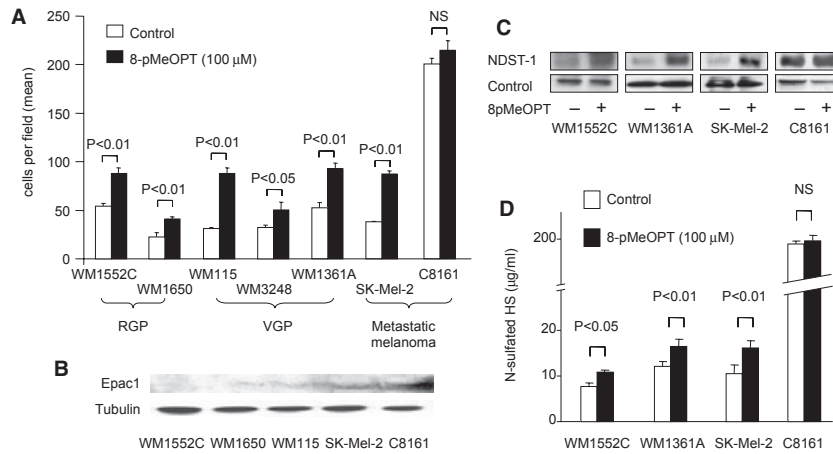


Figure 4. Endogenous Epac increases cell migration, expression of NDST-1 and N-sulfated HS in various melanoma cell lines. (A) Migration assay was performed in the presence or absence of 8-pMeOPT. 8-pMeOPT increased cell migration in all melanoma cell lines with the exception of C8161 cells. $n = 4$. (B) Western blot analysis showed abundant expression of Epac1 in C8161 cells. (C) Western blot analysis showed increased NDST-1 expression by incubation with 8-pMeOPT (100 μ M) for 48–72 h. Loading controls (Control) are actin for WM1552C cells and tubulin for the other cell lines. (D) HS ELISA showed that 8-pMeOPT increased N-sulfated HS in WM1552C, WM1361A and SK-Mel-2 cells but not in C8161 cells. C8161 cells showed abundant basal N-sulfated HS (\sim 20-fold) compared with other cell lines. $n = 4$.

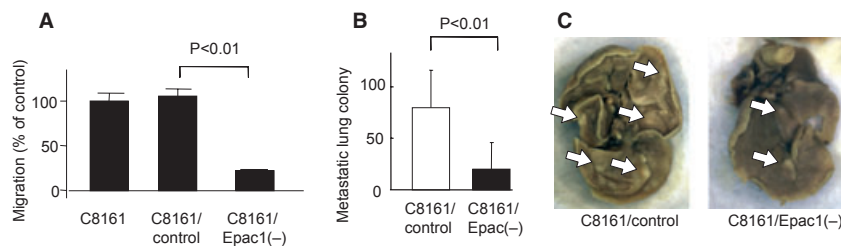


Figure 5. Ablation of Epac1 reduces metastasis in vivo in mice. (A) Cell migration assay demonstrated that C8161/Epac1(–) showed lower basal cell migration than C8161/control. $n = 4$. (B) Ablation of Epac1 reduced melanoma metastasis in mice. Cells were intravenously injected into the tail vein. Three weeks after the injection, the lungs were fixed and then subjected to counting of the number of metastatic colonies. $n = 4$. (C) Representative lung images after Bouin-picric acid fixation are shown. Arrows indicate white metastatic colonies.

HS. In addition, the Epac agonist increased, but ablation of Epac1 decreased, the expression of NDST-1, and N-sulfated HS (Figures 3B and 4C,D) (Baljinnyam et al., 2009). Further, ablation of NDST-1 and degradation of HS abolished Epac-induced melanoma cell migration (Baljinnyam et al., 2009). These data strongly indicate the involvement of HS modification in Epac1-induced cell migration and thus metastasis in melanoma; however, the detailed mechanism by which N-sulfation of HS regulates these processes also remains unclear. We previously showed that syndecan-2, a membrane HS proteoglycan, is involved in Epac-induced cell migration (Baljinnyam et al., 2009). In addition, another report demonstrated that N-sulfation of syndecan-2 is critical for fibronectin assembly (Galante and Schwarzbauer, 2007), suggesting that N-sulfation of HS in syndecan-2 may play a role in Epac1-induced cell migration. Furthermore, previous studies demonstrated that N-sulfation of HS is critical for agonists' binding to PDGF receptors (Abramsson et al., 2007; Stenzel et al., 2009) or FGF

receptors (Faham et al., 1996; Kreuger et al., 1999, 2002; Maccarana et al., 1993; Schlessinger et al., 2000), indicating that future studies should focus on these signaling pathways in Epac1-induced cell migration. PDGF and FGF receptors are also known to be accelerators for melanoma proliferation (Furuhashi et al., 2004; Nesbit et al., 1999; Ugurel et al., 2005), angiogenesis (Sharma et al., 1998; Westphal et al., 2000), and cell migration/metastasis (Chalkiadaki et al., 2009; Westphal et al., 2000). Because Epac1 expression varies even within MM cell lines (Figure 4B) (Baljinnyam et al., 2010), it is tempting to speculate that Epac1-rich melanoma cells can produce N-sulfation-rich HS, increases cell proliferation, angiogenesis, and cell migration of adjacent, Epac1-poor melanoma cells via PDGF or FGF signaling. Meanwhile, our previous report showed that Epac1 regulates cell migration also via inositol triphosphate (IP3)/Ca²⁺ pathway in SK-Mel-2 cells (Baljinnyam et al., 2010). In C8161 cells, Epac agonist-induced calcium elevation was also observed, and it was negated

by ablation of Epac1, an IP3 antagonist, and ablation of IP3 receptor 1 (unpublished data), suggesting that Epac1-IP3/Ca²⁺ receptor pathway may also be involved, at least in part, in cell migration of C8161 cells.

Our study provided important insights regarding the expression and physiological roles of Epac1, NDST-1 and N-sulfation of HS in melanoma progression. In order to bring our findings to bedside, it is necessary to develop a small molecule that can selectively inhibit Epac1. Also, extensive studies should be performed to investigate whether the role of Epac1 is universal in other types of cancer, and whether inhibition of Epac1 is effective to attenuate progression of those cancers.

Methods

Reagents and cell lines

8-pMeOPT was purchased from Biolog (Bremen, Germany). Other reagents were purchased from Sigma (St. Louis, MO, USA) unless specified. Antibodies against Epac1, Epac2, and α -tubulin, were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Antibody against N-sulfated Heparan sulfate (HepSS) was obtained from Seikagaku BioBusiness Corporation (Tokyo, Japan). Antibody against NDST-1 was purchased from Abnova (Jhongli City, Taoyuan, Taiwan). Antibody against melanoma antigen recognized by T cells-1 (MART-1) was purchased from Invitrogen (Carlsbad, CA, USA). SK-Mel-2 cell line was obtained from American Type Culture Collection. C8161 melanoma cell line was kindly provided by Dr Mary JC Hendrix. WM1552C, WM1650, WM115, WM3248, and WM1361A cell lines were kindly provided by Dr Meenhard Herlyn (Wistar institute). Cells were maintained in MEM containing 10% FBS and 1% penicillin-streptomycin.

Melanoma tissue arrays

Human melanoma tissue microarray (ME2082) was purchased from US Biomax Inc (Rockville, MD, USA). The microarray contained 128 cases of primary malignant melanoma, 64 metastatic malignant melanoma, and single core per case. Primary melanoma included 62 skin melanomas, 21 melanomas of gastric tract tissues, 12 eye melanomas, seven melanomas from the nose, and eight melanomas from various tissues such as fibrous or soft or striated muscle. Metastatic melanomas included 56 lymph node metastases, one brain metastasis, two spleens, one bone, and one parotid gland metastasis. One of the primary melanomas (eye) and two of metastatic melanomas (lymph node) were eliminated because of abundant melanin production or tissue core damage. Each array spot was 1.5 mm in diameter and 5 μ m in thickness. Detailed information for these arrays can be viewed at <http://www.biomax.us/tissue-arrays/Melanoma/ME2082>.

Immunohistochemical staining

Enzymatic immunohistochemical staining was performed as we described previously (Baljinnyam et al., 2010; Iwatsubo et al., 2007). Briefly, paraffin-embedded tissue microarray sections were submitted to deparaffinization in xylene, followed by treatment with a graded series of alcohols (100, 95, and 80% ethanol [v/v] in double-distilled H₂O) and rehydration in PBS (pH 7.5). For antigen retrieval, the sections were submerged in a boiling temperature citrate buffer (pH 6.0) for 15 min. After washing in PBS, endogenous peroxidases were blocked with 3% hydroxyl peroxide (H₂O₂) in PBS for 10 min, followed by 3 washes in PBS. The sections were blocked with Histostain Plus 3rd gen IHC detection kit (Invitrogen)

in a humidified chamber for 20 min followed by incubation with the primary antibodies against Epac1, Epac2, MART-1 at 37°C for 1 h. After washing with PBS, the slides were incubated with biotinylated goat anti-mouse IgG for 30 min at room temperature, followed by streptavidin-HRP incubation for 30 min at room temperature. The chromogenic reaction was visualized by incubation with stable 3,3'-diaminobenzidine (DAB) solution (Invitrogen) for 10 min or until color change was observed under a microscope. The sections were rinsed with distilled water, counterstained with hematoxylin solution for 1 min, and mounted with Vectashield mounting media (Vector Labs, Burlingame, CA, USA). Negative control samples were exposed to secondary antibody alone.

Fluorescent immunohistochemistry in tissue microarray, human melanoma tissue, and C8161 cell lines for Epac1, NDST-1 and N-sulfated HS was performed as we described previously (Baljinnyam et al., 2010; Iwatsubo et al., 2007) with some modifications for C8161 cell lines (Thompson et al., 2009). The paraffin-embedded tissue microarray slides of human melanoma tissues, or co-culture of C8161/Epac1(-) and C8161/control were fixed, permeabilized as described earlier. The samples were blocked with the Image-iT FX signal enhancer (Invitrogen) to prevent non-specific staining and incubated sequentially with primary antibodies for Epac1, NDST-1, total HS (3G10; Seikagaku) or N-sulfated HS (HepSS, Seikagaku for tissue microarray and human melanoma tissue, and HS4C3V kindly provided by Dr von Kuppevelt for C8161 cell lines) followed by exposure to respective secondary antibodies. For total HS, slides were exposed to heparitinase I digestion before incubation with the 1st antibody (Pan et al., 2008). Alexa Fluor 488- and 594-conjugated goat anti-rabbit and anti-mouse antibodies (Molecular Probes, Invitrogen) were used. The slides were mounted using Prolong Gold mounting media with DAPI.

Evaluation of immunostaining

Immunostaining was evaluated as we previously described (Baljinnyam et al., 2010) with some modifications (Zhang et al., 2007). The images of each tissue core on the microarrays, with exclusion of samples with diffuse distribution of melanin (final number of samples: primary melanoma, 127; MM, 62), were captured by a video camera mounted on a microscope (Nikon Eclipse 80i, Nikon Instruments Inc., Melville, NY, USA). All pictures were taken at the same settings. The optical density of each image was obtained using the IMAGE J (NIH). The immunostaining intensity of each sample was quantified as the average optical density readings of 50 randomly selected malignant tumor areas. The immunostaining score for each specimen was calculated as the average immunostaining intensity of tumor area minus the average immunostaining intensity readings of stromal connective tissue area on the same specimen, which served as a blank control. Thus, the relative staining intensities of all tissue cores were normalized to account for background staining for further comparison. The immunostaining scores were categorized as weak, moderate, or strong (Tables 1–3).

HS ELISA

HS content was determined with HS ELISA kit (Seikagaku) as we previously described (Baljinnyam et al., 2009). Cells were collected and disrupted by sonication followed by centrifugation at 13 000 *g* for 10 min. The supernatants were collected and diluted six times and incubated for 18 h at 4°C in the plates coated with HS antibody (10E4) which recognizes N-sulfated glucosamine. The secondary reaction with horseradish peroxidase-conjugated streptavidin-biotinylated antibody was carried out for 1 h at room temperature. After color development and stop reaction, OD was measured at 350/630 nm.

Short hairpin RNA transduction

Short hairpin RNA (shRNA) transductions with lentivirus (Santa Cruz Biotechnology) were performed as we previously described (Baljinnyam et al., 2010). C8161 cells were incubated with 8 $\mu\text{g}/\text{ml}$ of Polybrene (Santa Cruz Biotechnology) and lentiviral particles harboring shRNA for Epac1 followed by selection with puromycin dihydrochloride (Santa Cruz Biotechnology) for 1 week. Fresh puromycin-containing medium was replaced every 3–4 days. Established cell lines are as follows: C8161 cells with control shRNA (C8161/control), with Epac1 shRNA [C8161/Epac1(-)].

Western blot analysis

Western blot analysis was performed as we previously described (Baljinnyam et al., 2010; Iwatsubo et al., 2004). Cells were lysed and sonicated in the lysis buffer. Equal amounts of protein were subjected to SDS-PAGE. After protein separation by electrophoresis, samples were transferred to Immobilon-P membrane (Millipore, Danvers, MA, USA) followed by immunoblotting with the antibodies.

Migration assay

Migration assay was performed using the 24-well Boyden chambers (8 μm pores, BD Biosciences, San Jose, CA, USA) as we previously described (Baljinnyam et al., 2009, 2010). The cells were plated at a density of 1×10^6 cells/ $100 \mu\text{l}$ in the inserts, and incubated for 3 h at 37°C followed by staining using the Diff-Quick kit (Dade Behring). Pictures were taken with a microscope, and migrated cells were counted with IMAGE J software using 10 randomly chosen fields.

Experimental metastasis assay

Experimental metastasis assay was performed as we previously described (Baljinnyam et al., 2009). Cells (1×10^6 cells/ 0.1 ml medium) were injected into the tail vein of female 4–6 weeks BALB/c athymic nude mice (Charles River, Wilmington, MA, USA). Three weeks after the injection, mice were sacrificed, and the lungs were subjected immersion with Bouin solution (75% picric acid, 20% formaldehyde, and 5% glacial acetic acid). The number of metastatic colonies on the lung surface was counted under a stereomicroscope.

Statistics

Statistical comparisons among groups were performed using Student's *t* test one-factor ANOVA with Bonferroni post hoc test. For quantification of immunohistochemical staining in the melanoma tissue arrays, Mann–Whitney's *U* test was used to compare the immunostaining scores. For the analysis of the association between Epac1 and NDST-1, Epac1 and N-sulfated HS, and NDST-1 and N-sulfated HS, was performed with Pearson's correlation coefficient test. A *P*-value of <0.05 was considered significant.

Acknowledgement

This study was supported by the American Heart Association (SDG 0835596D), the Foundation of UMDNJ and Melanoma Research Foundation (K. Iwatsubo).

References

Abramsson, A., Kurup, S., Busse, M. et al. (2007). Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. *Genes Dev.* **21**, 316–331.

Aikawa, J.-I., and Esko, J.D. (1999). Molecular cloning and expression of a third member of the heparan sulfate/heparin GlcNAc N-deacetylase/N-sulfotransferase family. *J. Biol. Chem.* **274**, 2690–2695.

Baljinnyam, E., Iwatsubo, K., Kurotani, R., Wang, X., Ulucan, C., Iwatsubo, M., Lagunoff, D., and Ishikawa, Y. (2009). Epac increases melanoma cell migration by a heparan sulfate-related mechanism. *Am. J. Physiol. Cell Physiol.* **297**, C802–C813.

Baljinnyam, E., De Lorenzo, M.S., Xie, L.H., Iwatsubo, M., Chen, S., Goydos, J.S., Nowycky, M.C., and Iwatsubo, K. (2010). Exchange protein directly activated by cyclic AMP increases melanoma cell migration by a Ca^{2+} -dependent mechanism. *Cancer Res.* **70**, 5607–5617.

Berwick, M., and Wiggins, C. (2006). The current epidemiology of cutaneous malignant melanoma. *Front. Biosci.* **11**, 1244–1254.

Bos, J.L. (2006). Epac proteins: multi-purpose cAMP targets. *Trends Biochem. Sci.* **31**, 680–686.

Chalkiadaki, G., Nikitovic, D., Berdiaki, A., Sifaki, M., Krasagakis, K., Katonis, P., Karamanos, N.K., and Tzanakakis, G.N. (2009). Fibroblast growth factor-2 modulates melanoma adhesion and migration through a syndecan-4-dependent mechanism. *Int. J. Biochem. Cell Biol.* **41**, 1323–1331.

De Rooij, J., Zwartkruis, F.J.T., Verheijen, M.H.G., Cool, R.H., Nijman, S.M.B., Wittinghofer, A., and Bos, J.L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**, 474–477.

Faham, S., Hileman, R.E., Fromm, J.R., Linhardt, R.J., and Rees, D.C. (1996). Heparin structure and interactions with basic fibroblast growth factor. *Science* **271**, 1116–1120.

Furuhashi, M., Sjoblom, T., Abramsson, A. et al. (2004). Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. *Cancer Res.* **64**, 2725–2733.

Gaggioli, C., and Sahai, E. (2007). Melanoma invasion – current knowledge and future directions. *Pigment Cell Res.* **20**, 161–172.

Galante, L.L., and Schwarzbauer, J.E. (2007). Requirements for sulfate transport and the diastrophic dysplasia sulfate transporter in fibronectin matrix assembly. *J. Cell Biol.* **179**, 999–1009.

Grandoch, M., Rose, A., Ter Braak, M., Jendrossek, V., Rubben, H., Fischer, J.W., Schmidt, M., and Weber, A.A. (2009). Epac inhibits migration and proliferation of human prostate carcinoma cells. *Br. J. Cancer* **101**, 2038–2042.

Iwatsubo, K., Minamisawa, S., Tsunematsu, T., Nakagome, M., Toya, Y., Tomlinson, J.E., Umemura, S., Scarborough, R.M., Levy, D.E., and Ishikawa, Y. (2004). Direct inhibition of type 5 adenylyl cyclase prevents myocardial apoptosis without functional deterioration. *J. Biol. Chem.* **279**, 40938–40945.

Iwatsubo, K., Suzuki, S., Li, C. et al. (2007). Dopamine induces apoptosis in young, but not in neonatal, neurons via Ca^{2+} -dependent signal. *Am. J. Physiol. Cell Physiol.* **293**, C1498–C1508.

Kreuger, J., Prydz, K., Pettersson, R.F., Lindahl, U., and Salmivirta, M. (1999). Characterization of fibroblast growth factor 1 binding heparan sulfate domain. *Glycobiology* **9**, 723–729.

Kreuger, J., Matsumoto, T., Vanwildemeersch, M., Sasaki, T., Timpl, R., Claesson-Welsh, L., Spillmann, D., and Lindahl, U. (2002). Role of heparan sulfate domain organization in endostatin inhibition of endothelial cell function. *EMBO J.* **21**, 6303–6311.

Lissitzky, J.C., Parriaux, D., Ristorcelli, E., Verine, A., Lombardo, D., and Verrando, P. (2009). Cyclic AMP signaling as a mediator of vasculogenic mimicry in aggressive human melanoma cells in vitro. *Cancer Res.* **69**, 802–809.

Maccarana, M., Casu, B., and Lindahl, U. (1993). Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* **268**, 23898–23905.

- Nesbit, M., Nesbit, H.K., Bennett, J., Andl, T., Hsu, M.Y., DeJesus, E., Mcbrian, M., Gupta, A.R., Eck, S.L., and Herlyn, M. (1999). Basic fibroblast growth factor induces a transformed phenotype in normal human melanocytes. *Oncogene* *18*, 6469–6476.
- O'sullivan, G.M., Boswell, C.M., and Halliday, G.M. (2000). Langerhans cell migration is modulated by N-sulfated glucosamine moieties in heparin. *Exp. Dermatol.* *9*, 25–33.
- Pan, Y., Carbe, C., Powers, A., Zhang, E.E., Esko, J.D., Grobe, K., Feng, G.S., and Zhang, X. (2008). Bud specific N-sulfation of heparan sulfate regulates Shp2-dependent FGF signaling during lacrimal gland induction. *Development* *135*, 301–310.
- Quilliam, L.A., Rebhun, J.F., and Castro, A.F. (2002). A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog. Nucleic Acid Res. Mol. Biol.* *71*, 391–444.
- Schlessinger, J., Plotnikov, A.N., Ibrahim, O.A., Eliseenkova, A.V., Yeh, B.K., Yayon, A., Linhardt, R.J., and Mohammadi, M. (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* *6*, 743–750.
- Sharma, B., Handler, M., Eichstetter, I., Whitelock, J.M., Nugent, M.A., and Iozzo, R.V. (1998). Antisense targeting of perlecan blocks tumor growth and angiogenesis in vivo. *J. Clin. Invest.* *102*, 1599–1608.
- Stenzel, D., Nye, E., Nisancioglu, M., Adams, R.H., Yamaguchi, Y., and Gerhardt, H. (2009). Peripheral mural cell recruitment requires cell-autonomous heparan sulfate. *Blood* *114*, 915–924.
- Thompson, S.M., Fernig, D.G., Jesudason, E.C., Losty, P.D., Van De Westerlo, E.M., Van Kuppevelt, T.H., and Turnbull, J.E. (2009). Heparan sulfate phage display antibodies identify distinct epitopes with complex binding characteristics: insights into protein binding specificities. *J. Biol. Chem.* *284*, 35621–35631.
- Tiwari, S., Felekkis, K., Moon, E.Y., Flies, A., Sherr, D.H., and Lerner, A. (2004). Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. *Blood* *103*, 2661–2667.
- Ugurel, S., Hildenbrand, R., Zimpfer, A. et al. (2005). Lack of clinical efficacy of imatinib in metastatic melanoma. *Br. J. Cancer* *92*, 1398–1405.
- Welch, D.R., Bisi, J.E., Miller, B.E., Conaway, D., Sefror, E.A., Yohem, K.H., Gilmore, L.B., Sefror, R.E., Nakajima, M., and Hendrix, M.J. (1991). Characterization of a highly invasive and spontaneously metastatic human malignant melanoma cell line. *Int. J. Cancer* *47*, 227–237.
- Westphal, J.R., Van't Hulenaar, R., Peek, R., Willems, R.W., Crickard, K., Crickard, U., Askaa, J., Clemmensen, I., Ruiter, D.J., and De Waal, R.M. (2000). Angiogenic balance in human melanoma: expression of VEGF, bFGF, IL-8, PDGF and angiostatin in relation to vascular density of xenografts in vivo. *Int. J. Cancer* *86*, 768–776.
- Yohem, K.H., Clothier, J.L., Montague, S.L., Geary, R.J., Winters, A.L. 3rd, Hendrix, M.J., and Welch, D.R. (1991). Inhibition of tumor cell invasion by verapamil. *Pigment Cell Res.* *4*, 225–233.
- Zhang, J., Park, S.I., Artime, M.C., Summy, J.M., Shah, A.N., Bomser, J.A., Dorfleitner, A., Flynn, D.C., and Gallick, G.E. (2007). AFAP-110 is overexpressed in prostate cancer and contributes to tumorigenic growth by regulating focal contacts. *J. Clin. Invest.* *117*, 2962–2973.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Epac1 overexpression increases NDST-1 expression in melanoma.

Figure S2. Epac agonist does not change Epac1 expression.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.