

Plant Lectin Can Target Receptors Containing Sialic Acid, Exemplified by Podoplanin, to Inhibit Transformed Cell Growth and Migration

Jhon Alberto Ochoa-Alvarez^{1,2,9}, Harini Krishnan^{1,2,9}, Yongquan Shen², Nimish K. Acharya^{1,6}, Min Han^{1,6}, Dean E. McNulty³, Hitoki Hasegawa⁴, Toshinori Hyodo⁴, Takeshi Senga⁴, Jian-Guo Geng⁵, Mary Kosciuk⁶, Seung S. Shin⁷, James S. Goydos⁷, Dmitry Temiakov^{1,8}, Robert G. Nagele^{1,6}, Gary S. Goldberg^{1,2*}

1 Graduate School of Biomedical Sciences, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey, United States of America, **2** Department of Molecular Biology, School of Osteopathic Medicine, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey, United States of America, **3** Proteomics and Biological Mass Spectrometry, Molecular Discovery Research, GlaxoSmithKline, Collegeville, Pennsylvania, United States of America, **4** Division of Cancer Biology, School of Medicine, Nagoya University, Nagoya, Japan, **5** Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, Michigan, United States of America, **6** New Jersey Institute for Successful Aging, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey, United States of America, **7** Department of Surgery, Cancer Institute of New Jersey, New Brunswick, New Jersey, United States of America, **8** Department of Cell Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey, United States of America

Abstract

Cancer is a leading cause of death of men and women worldwide. Tumor cell motility contributes to metastatic invasion that causes the vast majority of cancer deaths. Extracellular receptors modified by α 2,3-sialic acids that promote this motility can serve as ideal chemotherapeutic targets. For example, the extracellular domain of the mucin receptor podoplanin (PDPN) is highly *O*-glycosylated with α 2,3-sialic acid linked to galactose. PDPN is activated by endogenous ligands to induce tumor cell motility and metastasis. Dietary lectins that target proteins containing α 2,3-sialic acid inhibit tumor cell growth. However, anti-cancer lectins that have been examined thus far target receptors that have not been identified. We report here that a lectin from the seeds of *Maackia amurensis* (MASL) with affinity for *O*-linked carbohydrate chains containing sialic acid targets PDPN to inhibit transformed cell growth and motility at nanomolar concentrations. Interestingly, the biological activity of this lectin survives gastrointestinal proteolysis and enters the cardiovascular system to inhibit melanoma cell growth, migration, and tumorigenesis. These studies demonstrate how lectins may be used to help develop dietary agents that target specific receptors to combat malignant cell growth.

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* E-mail: gary.goldberg@umdnj.edu

9 These authors contributed equally to this work.

Introduction

Over 1 in 4 people are diagnosed with cancer at some point in their life [1]. These cancers are the leading cause of death of men and women under 85 years old [2]. Tumor cell motility contributes to metastatic invasion that causes the vast majority of cancer deaths [3,4]. Extracellular receptors that promote this motility can serve as ideal chemotherapeutic targets [5–9]. Expression levels of receptors with α 2,3-sialic acid residues are closely associated with the invasive and metastatic potential of many cancers [10–12] including skin cancer [13,14]. Podoplanin (PDPN) represents one of these receptors.

PDPN is a unique transmembrane receptor that promotes tumor cell motility. PDPN expression can be induced by tumor promoters including TPA, oncogenic Ras, and Src [15–17]. For

example, we have reported that Src utilizes the focal adhesion adaptor protein Cas to induce PDPN expression in order to promote tumor cell migration [15]. PDPN regulates the activities of effectors including ezrin, Rho, and Cdc42 to mediate filopodia formation and promote tumor cell migration, invasion, and metastasis [5,18–22].

PDPN is found at the invasive front of many tumors, which is consistent with its role in promoting malignant invasion [5,20,23]. For example, PDPN expression is strongly induced in about 40% of breast cancers [20,24], 50% of oral cancers [23,25,26], and 80% of skin cancers [27,28]. The bulk of the PDPN protein, about 150 amino acids, lies outside of the cell and could serve as an ideal target to combat cancer invasion and metastasis [5,29].

The extracellular domain of PDPN is highly *O*-glycosylated with sialic acid, α 2,3 linked to galactose [5,30]. PDPN is activated by

endogenous ligands that bind to these extracellular carbohydrate moieties [31–33] to induce tumor cell motility and metastasis [20,33–35]. Thus, blocking this interaction should inhibit malignant progression. For instance, compounds blocking the action of galectins, which activate mucin receptors, can inhibit tumor cell metastasis [36,37].

Antibodies against PDPN can inhibit lung metastasis of transformed cells that express PDPN [38,39]. However, antibody therapy presents challenges in administration and toxicity [40,41]. Since PDPN is modified by α 2,3-sialic acid, it can be targeted by specific lectins [10,12–14,42].

Unlike antibodies, lectins can survive digestive processing and retain biological activity [43–46]. Indeed, dietary lectins can block the action of endogenous pro-metastatic lectins (such as galectins or selectins) to inhibit tumor cell growth [37,45–47]. For example, although its targets and mechanisms are not yet defined, Mistletoe lectin (viscumin) binds proteins containing α 2,3-sialic acid, has undergone successful clinical trials, and is widely used to treat melanoma in Europe [48–50]. However, anti-cancer lectins that have been examined thus far target receptors that have not been identified.

Here, we show that a lectin with affinity for *O*-linked carbohydrate chains containing sialic acid binds to PDPN to inhibit transformed cell growth and motility at submicromolar concentrations. This approach demonstrates how lectins may be used as dietary agents that target specific receptors to combat malignant cell growth.

Results

MASL targets PDPN on Src transformed cells

We have recently reported that the Src tyrosine kinase utilizes the adaptor protein Cas/BCAR1 to augment PDPN expression in order to promote tumor cell motility [15]. PDPN is also profoundly involved in the ability of transformed cells to escape contact normalization (growth control by surrounding nontransformed cells) and “break out” of their microenvironment to become malignant or metastatic [15]. Consistent with these previous reports, Src transformed cells expressed more PDPN (see **Figure 1a and 1b**) and migrated more than nontransformed controls (**Figure 1c**). As described above, the extracellular domain of PDPN is *O*-glycosylated with sialic acid, α 2,3 linked to galactose. Scholl et al have reported that PDPN associates with lectin from seeds of the legume tree *Maackia amurensis* (MASL) [30], which has an affinity for *O*-linked carbohydrate chains containing

sialic acid [51,52]. These observations led us to investigate the ability of MASL to target PDPN on transformed cells.

As shown in **Figure 2a**, MASL associated with PDPN on the membrane of Src transformed cells. Note that MASL did not target the membrane of cells that did not express PDPN, which, along with functional studies described below, attests to its preferential targeting. In addition, MASL associated with PDPN in lysates from transformed cells during affinity precipitation experiments as shown in **Figure 2b**.

MASL inhibits Src transformed cell growth and migration

We hypothesized that binding of MASL to PDPN would inhibit transformed cell migration. As shown in **Figure 2c**, MASL significantly inhibited transformed cell migration, with 385 nM and 770 nM inhibiting cell migration by over 25% and 50% respectively. In contrast, MASL did not inhibit the migration of nontransformed cells in a dose dependent fashion at these concentrations.

To verify the functional relevance of MASL targeting PDPN on cell migration, we investigated its effects on nontransformed cells transfected with PDPN or empty parental vector. Since PDPN expression is sufficient to promote cell migration [5,15,30], nontransformed cells transfected with PDPN migrated several fold more than control transfectants (**Figure 3a and 3b**). In addition, MASL reduced the migration of these nontransformed PDPN transfected cells in a dose dependent fashion (**Figure 3b**). For example, 385 nM MASL decreased the migration of PDPN transfectants by over 40%.

As shown in **Figure 3c**, in addition to inhibiting cell migration, MASL was also toxic to PDPN expressing cells in a dose dependent fashion. In contrast, MASL did not inhibit the viability of empty vector transfectants in an equally dose dependent fashion. For example, 1540 nM MASL decreased Trypan blue exclusion of PDPN transfectants by over 70%, but control transfectants by only about 30%.

MASL targets PDPN to inhibit melanoma cell growth and motility

Studies indicate that PDPN expression is strongly induced in about 80% of skin cancers [27,28]. Consistent with its role in tumor cell invasion and metastasis, malignant B16 melanoma cells expressed higher levels of PDPN (**Figure 4a**) and migrated significantly better (**Figure 4b**) than syngeneic nontransformed Melan-a melanocytes. As shown in **Figure 4c**, MASL effectively suppressed melanoma cell migration at concentrations of 308 nM

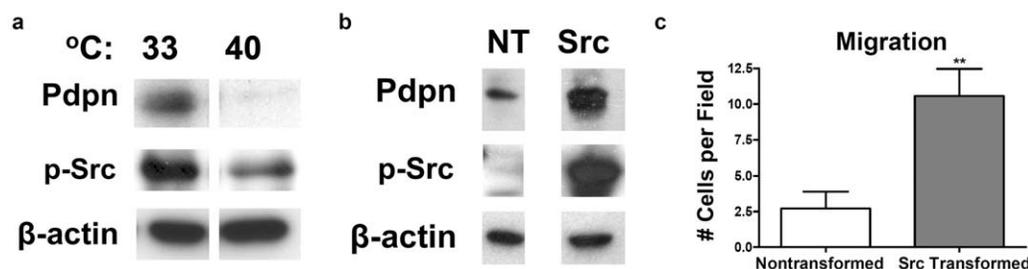


Figure 1. Src activation induces Pdpn expression and cell migration. (a) PDPN, active Src kinase (phosphorylated at Y-416), and β -actin were detected by Western blotting of protein (20 μ g per lane) from LA-25 cells (NRK cells containing temperature sensitive v-Src) grown overnight at 33°C (permissive temperature) or 40°C (non-permissive temperature). (b) PDPN, active Src (phosphorylated at Y416), and β -actin were detected by Western blotting of protein (15 μ g per lane) from nontransformed cells or Src transformed mouse embryonic cells (MEFs) as indicated. (c) Wound healing migration assays were performed on confluent monolayers of Src transformed or nontransformed cells. Data are shown as the number of cells that migrated into a 300 \times 300 micron area along the center of the wound in 24 hours (mean + SEM, n = 7). Double asterisks indicate $p < 0.01$ compared to untreated Src controls.

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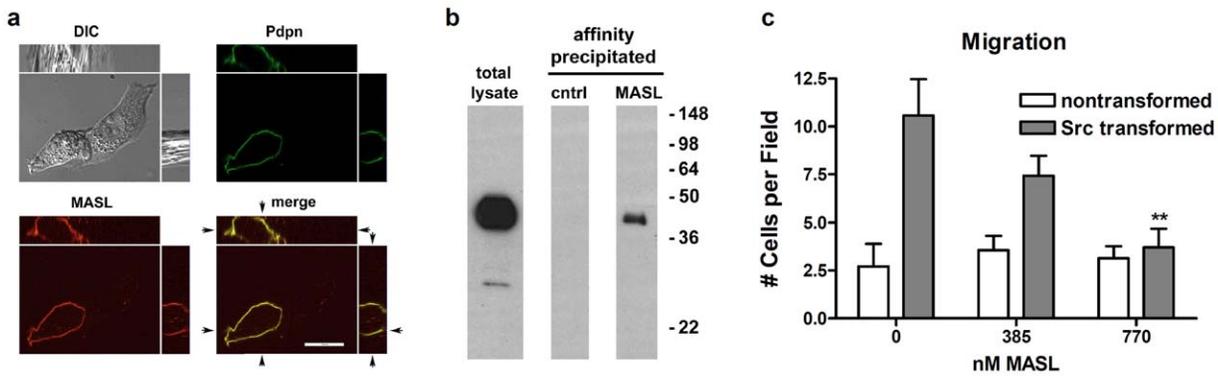


Figure 2. MASL associates with PDPN and inhibits migration of Src transformed cells. (a) Src transformed cells were exposed to MASL conjugated to HiLyte Fluor TR (red), and PDPN was detected by immunofluorescence microscopy (green). Colocalization of lectin and PDPN (yellow) is apparent in merged images, including orthogonal views of the z-axis which is 14 microns thick (bar = 20 microns). (b) Protein from Src transformed cells (750 μ g) was precipitated with agarose beads linked to MASL, or control beads, and examined for PDPN by Western blotting. Cell lysate (15 μ g/lane) were also examined as indicated. (c) Wound healing migration assays were performed on confluent monolayers of cells treated with concentrations of MASL as indicated. Data are shown as the number of cells that migrated into a 300x300 micron area along the center of the wound in 24 hours (mean + SEM, n = 7). Double asterisks indicate $p < 0.01$ compared to nontransformed cells. doi:10.1371/journal.pone.0041845.g002

or less. In addition to inhibiting melanoma cell migration, MASL also inhibited melanoma cell growth in a dose responsive manner (Figure 4d). Moreover, MASL was significantly more toxic to B16 melanoma cells than Melan-a cells ($p < 0.001$ by ANOVA).

Transwell chambers were used to further investigate the effects of MASL on melanoma cell growth and migration. As shown in Figure 4e, while 385 nM MASL was not significantly toxic to B16 melanoma cells ($p > 0.2$ compared to controls), migration through 8 micron pores was decreased by over 40 fold. These data indicate that MASL suppressed melanoma cell migration prior to inhibiting cell viability.

We employed siRNA to verify the effects of PDPN and MASL on melanoma cell growth and migration. PDPN siRNA effectively decreased B16 Pdpn expression levels (Figure 5a) and cell migration (Figure 5b). As shown in Figure 5c, this decreased PDPN expression resulted in a 25% decrease in MASL toxicity. These data indicate that while PDPN may not be the only receptor targeted by MASL on these melanoma cells, it is a functionally relevant receptor that can be targeted to prevent melanoma cell growth and migration.

MASL may induce caspase independent necrosis of melanoma cells

Some lectins used as potential anticancer agents, including Mistletoe lectins, contain a distinct N-glycosidase motif that acts as a ribosome inactivating peptide (RIP) [45]. Since MASL does not contain this peptide, inhibition of tumor cell viability by MASL did not result from RIP activity. In general, cell death can occur by apoptosis, necrosis, or autophagy [53,54]. MASL did not induce characteristics of autophagy such as vacuolarization or the formation of autophagosomes, or characteristics of apoptosis including nuclear chromatin condensation, membrane blebbing, or chromosomal DNA fragmentation. Instead, MASL induced morphological changes typical of necrosis including cell swelling, membrane rupture, and nuclei fragmentation.

Apoptosis usually results from the induction of caspase activity which cleaves poly(ADP-ribose) polymerase (PARP) into an 89 kD fragment [55,56]. As shown in Figure 6, while PARP cleavage was clearly detected in cells treated with puromycin which induces caspase mediated apoptosis [57], only slight PARP cleavage was seen in melanoma cells treated with MASL ($p > 0.2$ by ANOVA).

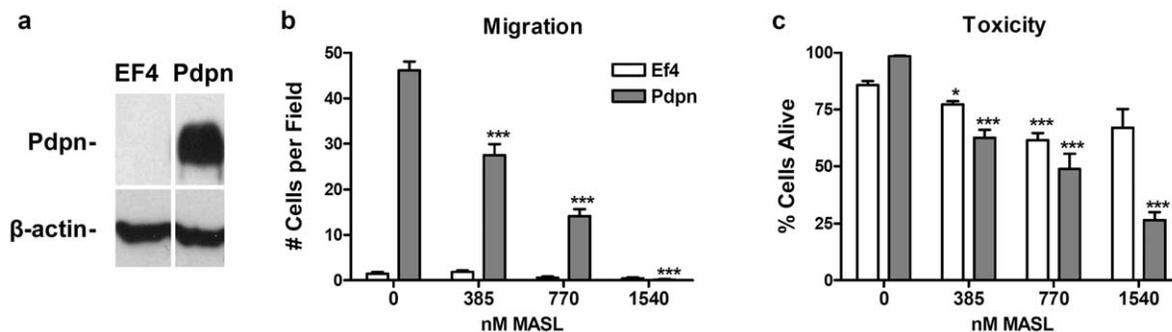


Figure 3. MASL targets PDPN to decrease cell migration and viability. (a) PDPN and β -actin were detected by Western blotting of protein (15 μ g per lane) from nontransformed mouse embryonic cells (MEFs) transfected with cDNA encoding PDPN or empty parental vector (EF4) as indicated. (b) Wound healing migration assays were performed on confluent monolayers treated with concentrations of MASL as indicated. Data are shown as the number of cells that migrated into a 300x300 micron area along the center of the wound in 24 hours (mean + SEM, n = 7). (c) MASL toxicity was evaluated by Trypan blue staining of cells, and shown as the percent of live cells from each well (mean + SEM, n = 4). Single and triple asterisks indicate $p < 0.05$ and $p < 0.001$, respectively, compared to untreated controls. doi:10.1371/journal.pone.0041845.g003

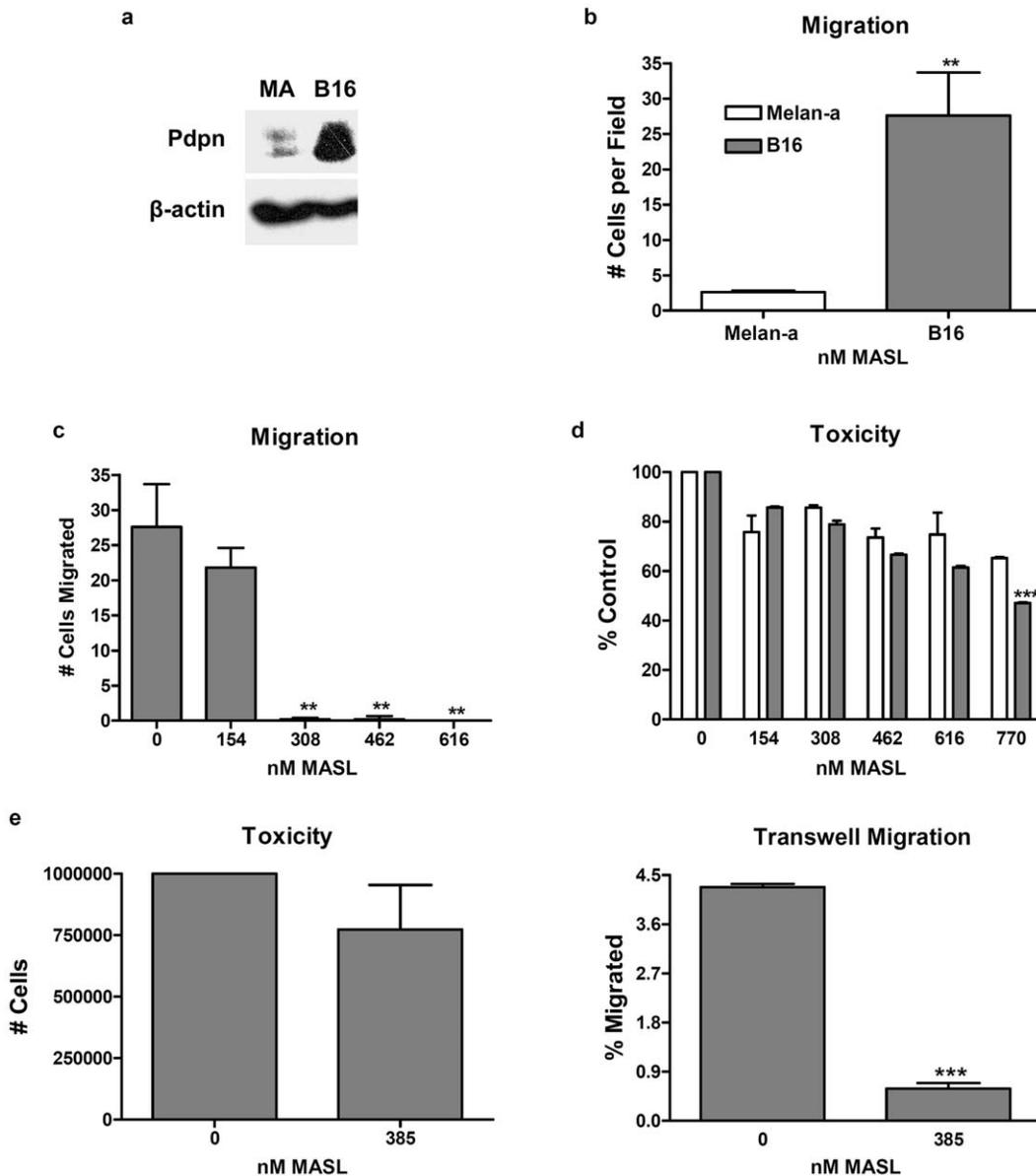


Figure 4. MASL inhibits melanoma cell motility and viability. (a) PDPN and β -actin were detected by Western blotting of protein (15 μ g per lane) from Melan-a melanocytes and B16 melanoma cells. (b) Wound healing migration assays were performed on confluent MelanA and B16 monolayers. Data are shown as the number of cells that migrated into a 400 \times 500 micron area along the center of the wound in 24 hours (mean + SEM, n = 5). (c) Wound healing migration assays were performed on confluent monolayers treated with MASL as indicated. Data are shown as the number of cells that migrated into a 400 \times 500 micron area along the center of the wound in 24 hours (mean + SEM, n = 5). (d) Melan-a and B16 cells were treated with MASL, and cell viability was evaluated by Alamar blue assay. Data are shown as percent of nontreated cells (mean + SEM, n = 2). (e) Melanoma cell viability and Transwell migration assays were performed on 600,000 cells plated on cell culture inserts containing membranes with an 8 micron pore size in 6-well plates. Cell viability was evaluated by Alamar blue assay and shown as percent of nontreated cells, while Transwell migration was measured after 24 hours as the percent of cells found on the underside of the membrane (mean + SEM, n = 2). Double and triple asterisks indicate $p < 0.01$ and $p < 0.001$ compared to corresponding treatments of untreated cells or Melan-a cells in panels d and e, respectively. doi:10.1371/journal.pone.0041845.g004

These observations suggest that MASL can reduce tumor cell growth by caspase independent necrotic mechanisms. This property may be particularly advantageous when applied to malignancies that resist apoptosis induced by classical cancer agents.

Dietary MASL inhibits melanoma cell migration and tumorigenesis

As described above, some dietary lectins can survive digestive processing to combat cancer *in vivo* [43–46]. Serum was taken from

mice fed MASL and then examined for its effects on melanoma cell migration *in vitro* to determine if this dietary lectin can survive gastrointestinal proteolysis to remain biologically active in the circulatory system. As shown in **Figure 7**, serum from mice fed 100 or 200 mg/kg MASL inhibited melanoma cell migration by about 30% or over 80% compared to serum from mice fed no MASL, respectively. These data indicate that dietary administration of MASL can result in biologically relevant levels of circulating product that are sufficient to inhibit melanoma cell migration.

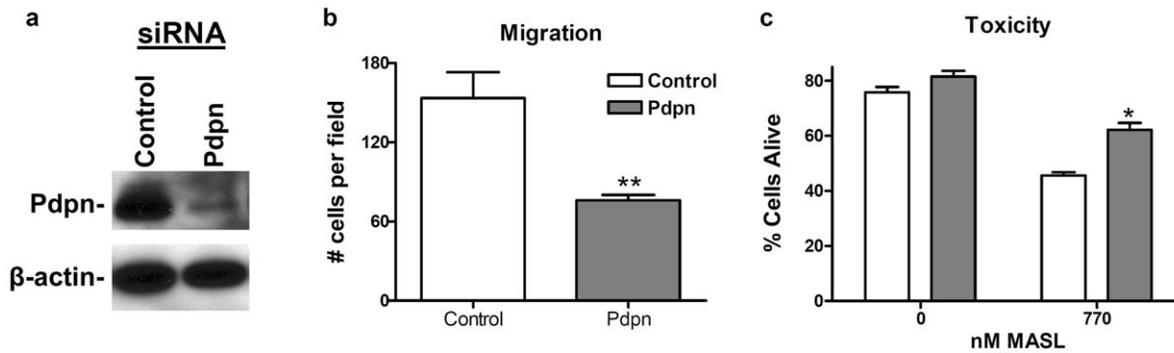


Figure 5. MASL targets PDPN to inhibit melanoma cell growth. (a) PDPN and β -actin were detected by Western blotting of protein (5 μ g per lane) from B16 melanoma cells transfected with control siRNA or siRNA targeted against PDPN, as indicated. (b) Wound healing migration assays were performed on confluent B16 monolayers transfected with control siRNA or siRNA targeted against PDPN, as indicated. Data are shown as the number of cells that migrated into a 500 \times 400 micron area along the center of the wound in 24 hours (mean + SEM, n=4). (c) MASL toxicity was evaluated by Trypan blue staining of cells, and shown as the percent of live cells from each well (mean + SEM, n=2). Single, double, and triple asterisks indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to nontransformed cells, untreated Src transformed cells, or control transfectants as indicated. doi:10.1371/journal.pone.0041845.g005

Since MASL, or at least its biological activity, was resistant to gastrointestinal proteolysis we examined the effects of dietary MASL on tumor cell growth *in vivo*. As shown in **Figure 8a**, oral administration of 25 mg/kg of MASL once a week inhibited the subcutaneous growth of melanoma cells in mice by approximately 50% ($p < 0.05$ by ANOVA). Moreover, we found no adverse effects on mouse health or physiology over the course of these experiments based on animal behavior, weight, and organ analysis after dissection.

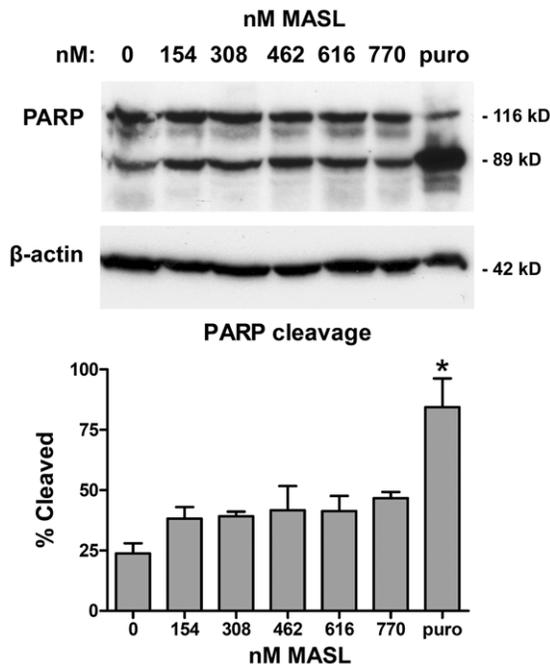


Figure 6. Effects of MASL on PARP cleavage. (top) PARP and β -actin were examined by Western blotting of protein from B16 melanoma cells treated for 24 hours with MASL or 37 μ M puromycin as indicated. (bottom) Signal was quantitated by image densitometry (NIH Image) and shown as the percent of cleaved PARP compared to total PARP (mean + SEM, n=2). doi:10.1371/journal.pone.0041845.g006

As expected, PDPN expression was evident in melanoma cells of tumors *in vivo*. As shown in **Figure 8b**, PDPN expression was more intense in tumor cells from control animals than animals treated with MASL. In addition, tumor cells from MASL treated animals appeared more epithelial in nature, with a relatively flattened morphology and more restricted PDPN staining than tumors from untreated animals.

Dietary MASL inhibits the formation of blood channels lined by melanoma cells

Although its molecular target has not been defined, Mistletoe lectin is used as an anticancer agent that can reduce tumor vascularization *in vivo* [46,58,59]. Aggressive melanoma cells are known for their ability to form blood vessels lined with tumor cells by a process referred to as “vasculogenic mimicry” [60]. Interestingly, as shown in **Figure 8b and 8c**, these blood filled vascular spaces lined by tumor cells were much smaller and less

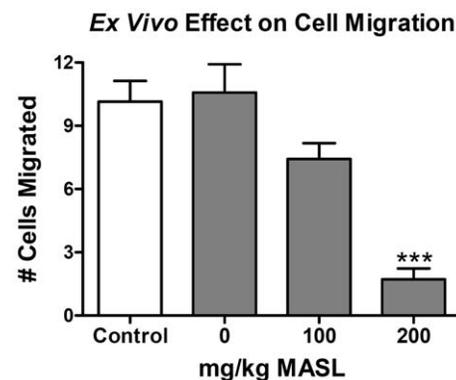


Figure 7. Dietary MASL bioactivity survives gastrointestinal proteolysis to enter the cardiovascular circulatory system and inhibit melanoma cell migration. Wound healing migration assays were performed on confluent monolayers of B16 melanoma cells treated with serum from mice fed MASL to achieve doses of 1, 100 mg/kg, or 200 mg/kg, or without mouse serum (controls) as indicated. Data are shown as the number of cells that migrated into a 200 \times 300 micron area along the center of the wound in 24 hours (mean + SEM, n=7). Triple asterisks indicate $p < 0.001$ compared to controls. doi:10.1371/journal.pone.0041845.g007

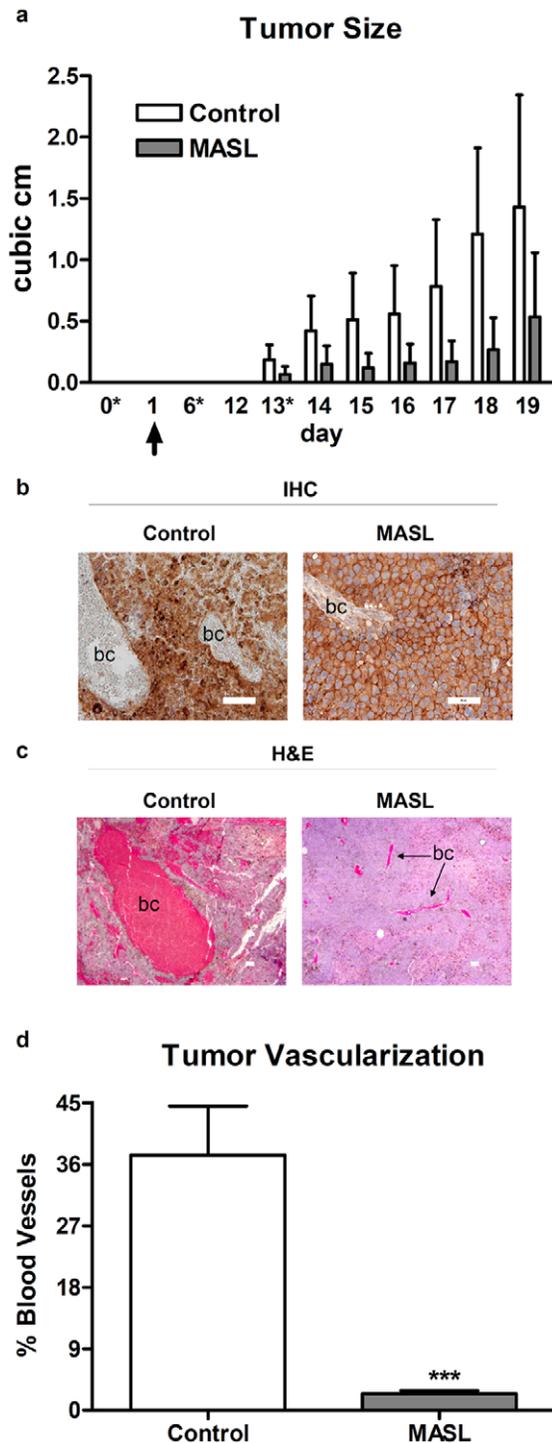


Figure 8. Dietary MASL inhibits melanoma growth *in vivo*. (a) Mice were fed MASL to achieve a dosage of 0 or 25 mg/kg once weekly (indicated by asterisks) and inoculated subcutaneously with B16 melanoma cells (100,000 cells per mouse on day indicated by arrow). Tumors were measured daily by caliper, and data is shown as cubic cm (mean + SEM, n = 4). (b) Tumors were evaluated for PDPN expression by IHC as indicated (bar = 50 microns). "bc" indicates blood filled vascular spaces lined by tumor cells. (c) Tumors were examined by hematoxylin and eosin staining (H&E) to visualize vascularization and morphology. (d) Tumor vascularization was quantified as the percent of each field (0.8 mm²) occupied by blood vessels and shown as mean + SEM (n = 7). doi:10.1371/journal.pone.0041845.g008

numerous in tumors from MASL treated mice than from control animals.

Human melanoma cells express PDPN and respond to MASL

We examined cell lines and clinical specimens to further investigate PDPN expression and MASL sensitivity in human melanoma cells. As shown in **Figure 9a**, PDPN expression was drastically increased in primary and metastatic melanoma specimens from every patient that we examined. As with mouse melanoma cells, PDPN expression was also much higher in human melanoma cell lines than normal skin. In addition, as with mouse cells, MASL significantly suppressed human melanoma cell growth and migration at concentrations of less than 300 nM (**Figure 9b and 9c**). Moreover, as with mouse cells, MASL inhibited human melanoma cell motility prior to effecting cell growth. For example, 308 nM MASL inhibited HT-144 cell migration by over 10 fold, but cell growth by less than 40%.

MASL consists of 2 subunits of identical primary amino acid sequence

Lectins are a complex family of proteins. MASL is a tetramer formed by 32 kD and 37 kD subunits. The 32 kD subunit is called *Maackia amurensis* hemagglutinin (MAH) and the 37 kD subunit is called *Maackia amurensis* leucoagglutinin (MAL) or *Maackia amurensis* mitogen (MAM) [52,61,62]. LC-MS-MS analysis shown in **Figure 10** indicates that both of these subunits consist of the same primary amino acid sequence that contains a single cysteine toward the carboxyl terminal at position 243. Unique molecular weights and other properties of these subunits are likely due to specific modifications including asparagine glycosylation and deamidation events. The presence of a cysteine residue in MASL has been reported for the 37 kD subunit (MAL) [62]. In contrast, sequences reported for the 32 kD subunit (MAH) do not contain a cysteine, but contain a serine in its place [52,61,62]. Our finding that both 32 kD and 37 kD subunits contain this cysteine explains their dimerization into respective 64 kD and 74 kD subunits.

Discussion

Lectins are promising anticancer agents that can be administered orally [47,50]. For example, dietary Mistletoe lectin has been shown to inhibit lymphoma growth in mice and is used as an adjuvant therapy to treat various forms of cancer in people [43,59]. Plant lectins are resistant to gastrointestinal proteolysis and breakdown by gut bacteria. Wang et al have shown that intact and bioactive peanut agglutinin (PNA) reaches concentrations of approximately 50nM in the serum of people within hours after ingesting 200g of peanuts [63]. Our data indicate that similar levels of lectin, or its bioactive digestion products, may be achieved from ingestion of MASL. These results also indicate that properties of MASL may be used to help develop reagents to prevent or combat melanoma.

Melanoma cell motility contributes to metastatic invasion that causes the vast majority of skin cancer deaths [4]. Robust PDPN expression was found 100% of the 8 melanoma samples examined from cancer patients in this study (see **Figure 9a**). These data are in contrast to previous studies reporting PDPN expression in less than 20% of spindle cell and other types of melanomas [64,65]. This may be the result of different experimental methods. For example, the NZ1 antibody used here targets an approximately 12 amino acid epitope in the PDPN PLAG domain with a dissociation constant (K_D) below 1 nM, while antibody used for these previous studies targets a larger epitope with lower affinity [66,67]. However, PDPN staining seen in our patient samples may

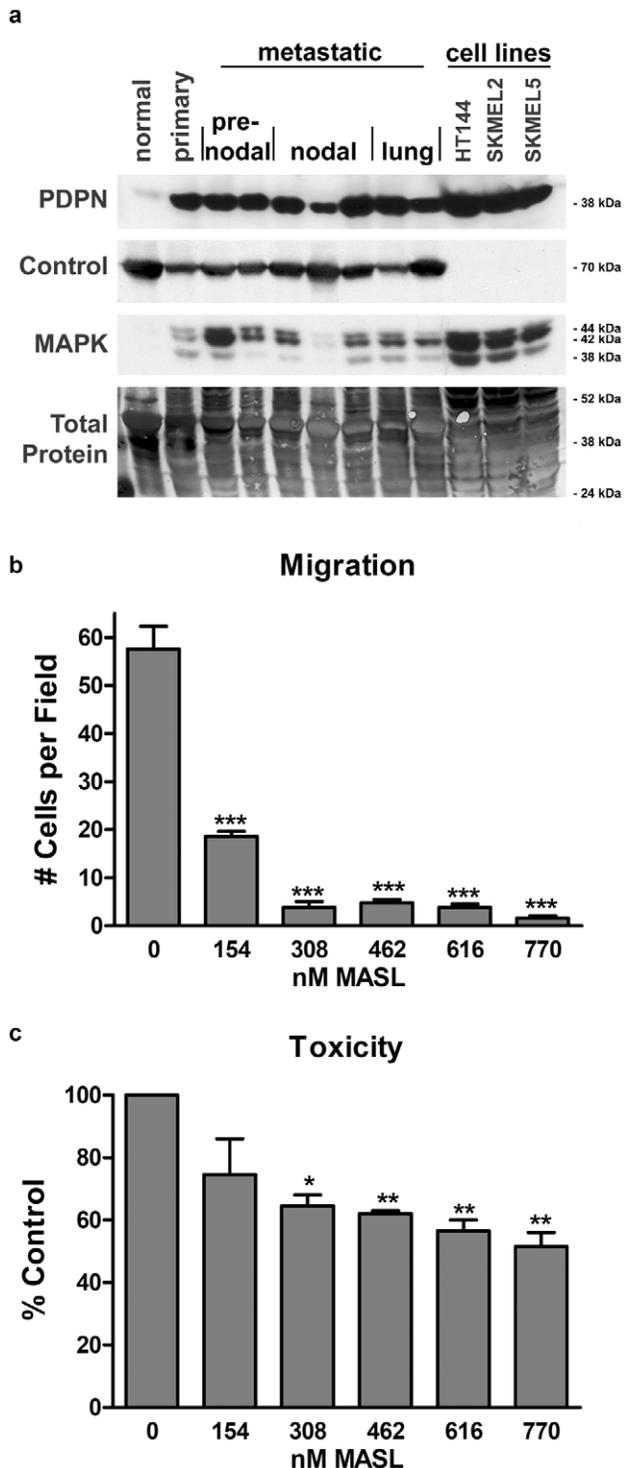


Figure 9. Human melanoma cells express PDPN and respond to MASL. (a) PDPN was detected by Western blotting of protein (15 μ g per lane) from a variety of human specimens including normal skin, primary melanoma, melanoma in transit prior to lymph node metastasis, nodal metastasis, lung metastasis, and three melanoma cell lines (HT-144, SK-MEL-2, SK-MEL-5). Detection of MAPK and nonspecific bands by Western blotting and India ink staining of membranes is also shown to verify equal loading of these samples from different patients and cell lines. (b) Wound healing migration assays were performed on confluent HT-144 monolayers treated with MASL as indicated. Data are shown as the number of cells that migrated into a 400 \times 500 micron area along the center of the wound in 24 hours (mean

+ SEM, n=5). (d) HT-144 cells were treated with MASL, and cell viability was evaluated by Alamar blue assay. Data are shown as percent of nontreated cells (mean + SEM, n=2). Single, double, and triple asterisks indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to untreated cells, respectively.

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have been due to cancer associated fibroblasts (CAFs), which would not be distinguished from actual melanoma cells by Western blotting. Indeed, PDPN expression in CAFs has been correlated with tumor aggression in cancers including mammary carcinoma [68] and lung adenocarcinoma [69–71]. In any case, robust PDPN expression was seen in all of the melanoma cell lines that we examined, indicating that melanoma cells themselves express PDPN (see **Figures 4 and 9**).

Dietary lectins can block the action of endogenous pro-metastatic lectins (such as galectins or selectins) to inhibit tumor cell growth, and can be used as medicinal cancer treatments [37,45–47]. While MASL has been shown to decrease viability of different types of transformed cells including melanoma [72,73], its targets and effects on cell migration have not been reported. Our data indicate that MASL be used to target PDPN in order to combat tumor cell growth and migration. Consistent with PDPN acting as a functionally relevant target, these results indicate that MASL inhibits cell motility prior to inhibiting cell viability. For example, 308 nM MASL suppressed melanoma cell migration by over 99%, while inhibiting cell viability by about 20% within the same time period (see **Figure 4**).

Although some lectins may nonspecifically bind to many glycoproteins, experiments with cloned *Maackia amurensis* lectin indicate that it can accurately target specific glycoproteins expressed by human cells [74,75]. Our data indicate that MASL, which has a high affinity for O-linked carbohydrate chains containing sialic acid [51,52], targets PDPN to inhibit transformed cell growth and motility at nanomolar concentrations. Interestingly, *Maackia amurensis* has been used as a medicinal plant for several centuries to treat ailments including cancer in parts of Asia [76,77]. However, as with many traditional medicines, clinical efficacy has been hindered by a lack of mechanistic understanding. Here, we describe how MASL can serve as a potent bioactive plant medicine that targets PDPN to combat cancer.

PDPN expression is relatively low in most normal cells, and found predominantly in tissues including retina, kidney podocytes, lymphatic endothelium, and lung alveolar epithelium [5,78]. Indeed, as shown in **Figure 11**, PDPN expression in normal tissues is somewhat similar to that of VEGFR2 which serves as a useful chemotherapeutic target [79,80]. Interestingly, PDPN and VEGFR2 are both receptors that promote cell migration and are suppressed during contact normalization by surrounding non-transformed cells in the microenvironment [15].

Our data demonstrate that MASL targets PDPN to inhibit tumor cell growth and motility. However, MASL is bound to target other receptors containing similar sialic acid motifs. In addition, like other reagents, MASL may produce “off target” effects in a clinical setting. However, lectin pharmacology is complex and MASL may undergo enzymatic modification to form bioactive compounds *in vivo*. For example, although legumes including lentils and peanuts contain significant amounts of agglutinating lectins, their ingestion does not result in toxic platelet aggregation [50]. Moreover, MASL caused no notable toxic effects on organ morphology or animal behavior in our studies which followed mice treated for about 3 weeks. As stated above, *Maackia amurensis* has been used as a medicinal plant for several centuries to treat ailments including cancer in parts of Asia [76,77]. These treatments would result in oral administration of hundreds of mg

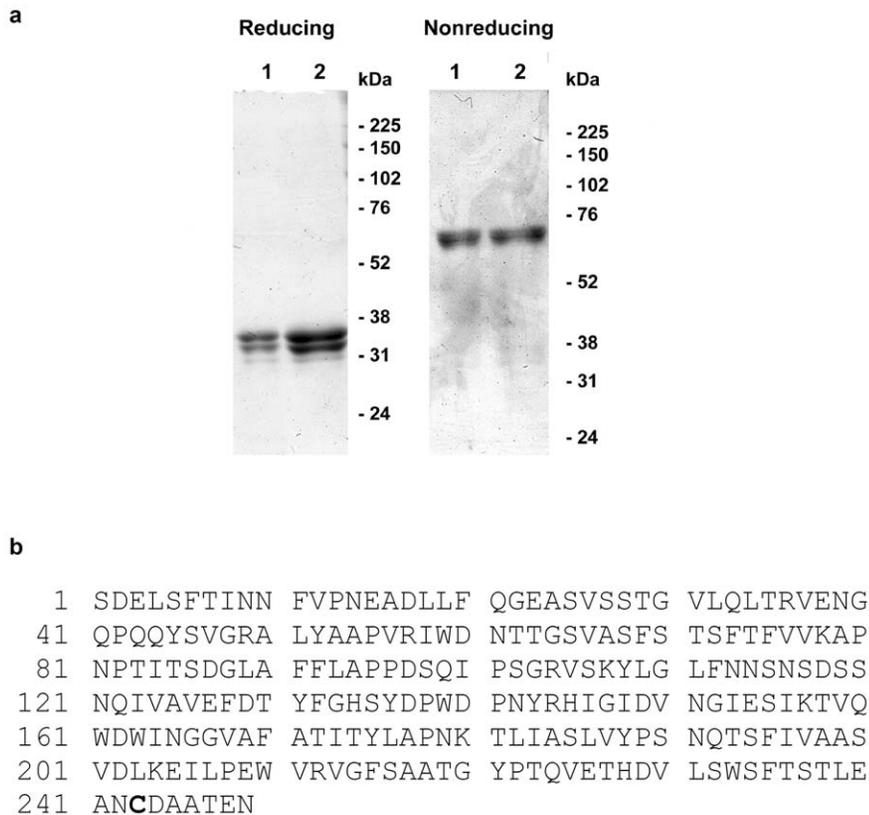


Figure 10. Analysis of MASL protein subunits. (a) MASL from Sigma (lane 1) or Sentrimed (lane 2) was analyzed by reducing and nonreducing SDS-PAGE. (b) Coomassie stained bands were excised, extracted and digested with trypsin, reduced and alkylated with iodoacetamide, and sequenced using data dependent acquisition by LC-MS/MS on a Thermo Scientific LTQ Orbitrap XL. All peptides were identical in primary sequence and contained a single cysteine at residue 243 (bold in figure). doi:10.1371/journal.pone.0041845.g010

of MASL per dosage, and have not resulted in reports of toxicity [81]. However, future studies will clearly be needed to further investigate the potential toxic effects of MASL or other lectins that may be considered for clinical use.

Taken together, results from this study suggest that lectins exemplified by MASL could significantly expand our limited arsenal of targeted cancer treatments, particularly anticancer agents that can be administered orally. This paradigm may lead to novel skin cancer treatments, and pave the way to treat other cancers with elevated PDPN expression levels including breast [20,24], glioma [21], and oral cancer [25,26]. However, as stated above, while PDPN served as the model target of this investigation, other receptors with α 2,3-sialic acid residues are increased in melanoma and a variety of other cancers, and may also be targeted to some extent by MASL [10,12–14,42].

Materials and Methods

Ethics Statement

Use of mice to obtain serum was approved by Committee of Animal Experiment in Nagoya University Graduate School of Medicine (Approved ID: 23132). Mouse experimental protocols were approved by the University of Medicine and Dentistry School of Osteopathic Medicine Institutional Animal Care and Use Committee (Approved ID: 10350).

Cell culture

Mouse embryonic cells transfected with v-Src, PDPN, or empty parental vectors have been previously described [15]. LA25 cells

harboring a temperature sensitive v-Src construct were grown as described [82]. B16F10, SK-MEL-2, SK-MEL-5, and HT-144 melanoma cells were obtained from ATCC. Melan-a melanocytes were provided by Richard Niles [83]. Clones were not taken from any cell lines, thus minimizing potential effects of clonal variation. To knockdown Pdpn expression in B16 cells, cells were transfected with 200nM of nontargeting control siRNA (Dharmacon D-001810-10-05) or 200nM podoplanin siRNA (Dharmacon L-048117-01-0005) with Lipofectamine 2000 (Invitrogen 11668-027) as described [15]. For some experiments, cells were treated with MASL (Sigma L8025, Sentrimed MASL) or puromycin (Sigma P8833).

Western blotting

Human tissue collected by the Honest Broker Program was excised and flash frozen in liquid nitrogen at the time of excision during surgery. Western blotting was performed as previously described [15,84–86]. Protein was resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore IH1079562), and incubated with antisera specific for PDPN (Sigma NZ1 P0085, Santa Cruz Biotechnology SC23564, or University of Iowa Developmental Studies Hybridoma Bank 8.1.1), active Src kinase (Cell Signaling Technology 2101), PARP (Cell Signaling Technology 9542), MAPK (Cell Signaling Technology 9102), or β -actin (Sigma A1978). Primary antiserum was recognized by appropriate secondary antiserum conjugated to horseradish peroxidase and detected using Enhanced Chemiluminescence (Millipore WBKLS0500 or ThermoScientific 32106). After blotting, mem-



Figure 11. Comparison of Pdpn and Vegfr2 (Kdr) mRNA expression in human tissues. Values from Affymetrix probe sets representing Pdpn and Vegfr2 are shown as presented on BioGPS (<http://biogps.gnf.org>). doi:10.1371/journal.pone.0041845.g011

branes were stained with India ink to verify equal loading and transfer.

Immunofluorescence microscopy

Cells (300,000 per dish) were cultured on 35-mm poly-D-lysine-coated glass-bottomed culture dishes (MatTek P35GC-1.5-14-C) for 24 hours. MASL was conjugated to Hilyte Fluor TR (Anaspec 72052) and incubated with cells for 15 min at 37°C. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 minutes, washed thrice with 0.1% Tween 20 in PBS

followed by 1% bovine serum albumin (BSA) in PBS for 30 minutes, incubated with PDPN antiserum (1:100) overnight at 4°C, washed, and then labeled with goat anti-syrian hamster IgG conjugated to Alexa Fluor 488 (Molecular Probes A21110). Cells were visualized on a Zeiss Axiovert microscope as described [15,85].

Affinity precipitation

Cells were lysed in lysis buffer (20 mM Tris.Cl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1mM PMSF) on ice for 30 min, clarified by centrifugation, diluted to 1 mg/ml in PBS supplemented with

1mM PMSF and 10 mM MgCl₂, and incubated with MASL conjugated to agarose beads, or empty beads as controls (Thermo Scientific 20501), on ice for 3 hours. Beads were then washed 4 times with PBS, and eluted in SDS-PAGE sample buffer at 95°C for 5 minutes. Eluted protein was examined along with total cell lysates by Western blotting.

Cell migration and toxicity assays

Cell monolayers were scratched and incubated with MASL for 24 hours to assess cell migration by wound healing assays, which were quantitated as the number of cells that entered an area of the wound indicated in Figure Legends as described [15,84,85]. Alamar blue (Invitrogen DAL1025) was added to cells 24 hours after MASL treatment, and incubated for an additional 8 hours to assay viability, which was detected by fluorescence measurements (ex/em 570/600 nm) as directed by the manufacturer (Invitrogen) in a Tecan GENios microplate fluorescence spectrophotometer. Cells were also stained with 0.2% trypan blue and counted with a hemocytometer to evaluate cytotoxicity. For Transwell migration assays, 600,000 cells were plated in 6-well cluster plates on cell culture inserts with an 8 micron pore size (Transwell-Clear, Costar) and grown for 24 hours. Cells were then released separately from the top of the membrane and the bottom of the membrane. Transwell migration was then quantitated as the percent of cells found on the underside of the membrane as described [15]. All experiments were performed on parallel cultures to control for variations in cell culture conditions.

Ex vivo effects of dietary MASL on cell migration

Mice (C57BL/6) were fed with a 200 mg food pellet containing MASL to achieve doses of 0, 100, or 200 mg/kg 1 hour before blood was taken from subclavian vein. Blood was allowed to coagulate 1 hour at room temperature and clarified by centrifugation for 10 minutes at 20,000 g to obtain serum. This serum was added to cell culture medium (DMEM+10%FBS) to a final concentration of 20%. B16 cell monolayers were scratched, washed with DMEM, and then incubated with these media, or

control medium not fortified with mouse serum, for 24 hours to assess their effects on cell migration by wound healing assays, which were quantitated as the number of cells that entered an area of the wound indicated in Figure Legends as described [15,84,85]. Use of mice to obtain serum was approved by Committee of Animal Experiment in Nagoya University Graduate School of Medicine (Approved ID: 23132).

In vivo tumorigenesis

Mice (C57BL/6) were fed with a 200 mg food pellet containing MASL to achieve doses of 0 or 25 mg/kg once weekly, starting the day before subcutaneous inoculation with 100,000 B16 cells in 0.1 ml PBS. Tumor volumes were measured blindly by a trained physician with a single caliper daily. Mice were sacrificed 18 days after inoculation and dissected. Tumors were fixed in formalin, paraffin embedded, sectioned (5 microns), and processed for hematoxylin/eosin staining or immunohistochemistry to detect PDPN expression with 8.1.1 monoclonal antibody as described [15,85,87]. Samples were analyzed on a Zeiss Axiovert microscope with Axiovision software as described [15,85]. Mouse experimental protocols were approved by the UMDNJ-SOM Institutional Animal Care and Use Committee (Approved ID: 10350).

MASL mass spectroscopy

MASL subunits were resolved by reducing and nonreducing SDS-PAGE and stained by Coomassie dye. Bands were then extracted and digested with trypsin. Extracted protein was reduced and alkylated with iodoacetamide, and sequenced using data dependent acquisition by LC-MS/MS on a Thermo Scientific LTQ Orbitrap XL.

Author Contributions

Conceived and designed the experiments: GSG YS DT RGN SSS JSG. Performed the experiments: JAO-A HK YS NKA MH DEM HH TH TS J-GG MK DT RGN GSG SSS. Analyzed the data: JAO-A HK YS MH DEM TS J-GG DT RGN GSG. Contributed reagents/materials/analysis tools: J-GG JSG. Wrote the paper: GSG HK JAO-A.

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