Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in both paracrine and autocrine manners

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Running title: Role of macrophage-derived adrenomedullin in melanoma

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Translational relevance

This study identifies tumor-associated macrophages (TAMs) as the major source of adrenomedullin (ADM) in melanoma. Our results show that TAM-derived ADM can induce the phosphorylation of endothelial nitric oxide synthase in endothelial cells via a paracrine manner and polarize macrophage to an M2 phenotype in an autocrine manner, to promote angiogenesis and melanoma growth. ADM and its receptors levels are increased in human melanoma, suggesting their role in melanomagenesis. This study highlights the significance of ADM, which can be an important link between TAMs and melanoma, and indicates the suppression of ADM and its receptors is promising for tumor inhibition. It also suggests that the combination of macrophage recruitment inhibition and the “re-education” of macrophage polarization is a feasible anti-cancer strategy.

Abstract

Purpose: Elevated numbers of tumor-associated macrophages (TAMs) in the tumor microenvironment are often correlated with poor prognosis in melanoma. However, the mechanisms by which TAMs modulate melanoma growth are still poorly understood. The present study was aimed at examining the function and mechanism of TAM-derived adrenomedullin (ADM) in angiogenesis and melanoma growth.

Experimental Design: We established in vitro and in vivo models to investigate the
relationship between TAMs and ADM in melanoma, the role and mechanism of ADM in TAM-induced angiogenesis and melanoma growth. The clinical significance of ADM and its receptors was evaluated using melanoma tissue microarrays.

Results: ADM was expressed by infiltrating TAMs in human melanoma and its secretion from macrophages was up-regulated upon co-culture with melanoma cells or with melanoma cells conditioned media. Meanwhile, TAMs enhanced endothelial cell migration and tubule formation, and also increased B16/F10 tumor growth. Neutralizing ADM antibody and ADM receptor antagonist, AMA, attenuated TAM-induced angiogenesis in vitro and melanoma growth in vivo, respectively. Furthermore, ADM promoted angiogenesis and melanoma growth via both the paracrine effect mediated by the endothelial nitric oxide synthase (eNOS) signaling pathway, and the autocrine effect which stimulated the polarization of macrophages towards an alternatively activated (M2) phenotype. Finally, immunofluorescence analysis on human melanomas showed that the expression of ADM and its receptors in TAMs was greatly increased compared to adjacent normal skins.

Conclusion: Our study reveals a novel mechanism that TAMs enhance angiogenesis and melanoma growth via ADM, and provides potential targets for melanoma therapies.
Introduction

Although melanoma accounts for only approximately 4% of all skin cancers, it is by large the most aggressive and deadliest of skin cancer type, contributing to 80% of skin cancer deaths (1). However, the mechanism underlying the aggressiveness of melanoma is incompletely understood. Accumulating evidences suggest that melanoma growth is influenced by both the host immune response and inflammatory cells within the tumor microenvironment. Among inflammatory cells, macrophages are believed to play the most important role in malignancies, and are therefore specifically referred to as tumor-associated macrophages (TAMs). Although TAMs may display anti-tumorigenic properties (2, 3), they are primarily regarded as pro-tumorigenic as indicated by the positive effect of TAMs on tumor angiogenesis and growth (4, 5). The different functions of TAMs may depend on individual activation states (6). Following recruitment, macrophages are “educated” by the tumor microenvironment and exhibit a spectrum of phenotypes, ranging from the classically activated (M1) phenotype to the alternatively activated (M2) phenotype (7). In regressing and non-progressing tumors, TAMs mainly resemble the M1 type and exhibit anti-tumor activity. In malignant and advanced tumors, TAMs are biased towards the M2 phenotype that instead favors tumor malignancy (8-10).

Adrenomedullin (ADM), a 52-aa peptide, is a potent vasodilator, belonging to the calcitonin superfamily (11). It is a multifunctional molecule that is involved in angiogenesis, cell proliferation and inflammation (12, 13) by acting through receptor
complexes, which are either composed of calcitonin receptor-like receptor (CRLR) and receptor activity-modifying protein 2 (RAMP2), or CRLR and RAMP3 (14, 15). ADM is widely expressed in a variety of tumor types (12), including melanoma (16), and plays an important role in promoting tumor growth (17). Moreover, the plasma ADM level is associated with lymph node metastasis (18). Several in vivo studies have shown a reduction of tumor angiogenesis and growth upon the treatment with neutralizing ADM antibodies (19), ADM receptor antagonist, AMA (20, 21), or ADM receptor RNA interference (22).

The source of ADM in tumors has not yet been reported and could include cancer cells, leucocytes, or other stromal cells such as fibroblasts. Furthermore, the role and mechanism of ADM in melanoma are largely unknown. It has been demonstrated that ADM secretion from macrophages is up-regulated upon the stimulation by inflammatory factors (23, 24). In addition, hypoxia also can increase ADM expression and secretion from macrophages (25). However, specifically, the relationship between TAMs and ADM in tumors has not been determined.

Here, we show that high densities of TAMs are correlated with ADM expression in human melanomas. In addition, TAM-derived ADM promotes angiogenesis in a paracrine manner via the endothelial nitric oxide synthase (eNOS) signaling pathway. We also show that tumor-secreted factors can up-regulate the expression of ADM receptors in macrophages, which in turn facilitates the autocrine effect of ADM to polarize macrophage towards the M2 phenotype, and subsequently promotes melanoma growth. Collectively, these results demonstrate that ADM is an important
regulator of TAMs to facilitate angiogenesis and melanoma growth through both paracrine and autocrine pathways.

Materials and Methods

Cells and culture

All cell lines were obtained from American Type Culture Collection, and cultured in culture media containing 10% fetal bovine serum (FBS). Conditioned media (CM) was obtained from serum-free culture media culturing cells for 12 h or 24 h as indicated. Primary peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMDMs) were isolated and cultured as previously described (27).

Western blotting

Cells were harvested, lysed, applied to SDS-PAGE and transferred to PVDF membrane. Membranes were incubated overnight at 4 °C with primary antibodies, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Peroxidase activity was visualized with the SuperSignal West Pico Chemiluminescent Substrate from Pierce (Rockford). Each assay was repeated at least three times.

Immunofluorescence staining

Tumor tissues or cells were stained with indicated primary antibodies and
fluorescence-conjugated secondary antibodies as described previously (27).

Trans-well migration and tubule formation assays

Trans-well migration and tubule formation assays were carried out as previously described (27). Experiments performed thrice in triplicate.

Animal study

Animal studies were approved by the Institutional Animal Care and Use Committees of Tsinghua University. For each experiment, the mice (Vital River) were used at 6–8 week old (7-10 mice/group), and the results were further verified by at least one more reproduction. Cells were counted with a hemocytometer and then s.c. injected into C57BL/6 mice (5 × 10^6 B16/F10 cells for macrophages promoting or AMA inhibiting tumor growth, or 10^5 B16/F10 cells for ADM promoting tumor growth), or nude mice (5 × 10^6 A375 cells) in 0.1 mL Matrigel solution by 1 mL gauge needle. Tumor volumes were measured with a caliper and calculated by the formula: volume = 0.52 ab^2 (“a” indicates the long diameter and “b” is the short diameter).

Flow cytometry

After blocking, cells were incubated with FITC-conjugated rat anti-mouse CD68 antibody and PE-conjugated rat anti-mouse CD206 antibody for 1 h at 4 °C. After washed twice by cold PBS, cells were analyzed by the FACSCalibur flow cytometry system (Becton Dickinson).
**Enzyme immunoassay (ELISA)**

ADM content was measured by ELISA using the commercial mouse or human ADM kit, following manufacture’s instructions (Ground biotechnology diagnosticate). Experiments conducted thrice in triplicate.

**Human Samples**

Tissue microarrays were purchased from Xi’an Aomei, which contained human melanoma tissues (median age 54.9 years, rang 25-88, male and female is about fifty-fifty) and adjacent skins. Tissue microarrays were stained with anti-human ADM, CRLR, RAMP2 and RAMP3 antibodies (Santa Cruz) according to the protocol of immunofluorescence staining, and the staining was evaluated by the fluorescence.

**Statistical analysis**

Data are represented as mean and SEM. Statistical analysis of data was carried out using the Student's t-test or chi-square test. $P$ value $< 0.05$ was considered as a significant difference.

**Results**

**TAMs contribute to ADM expression and secretion in melanoma**

In order to investigate the expression and distribution of ADM in melanoma, we
analyzed the co-localization of CD68, a macrophage marker, and ADM in human melanoma tissues by immunofluorescence. Most ADM was co-localized with CD68 in melanoma, indicating a correlation between ADM expression and TAMs in melanoma (Fig. 1A). To further determine the source of ADM in melanoma, we detected ADM expression in B16/F10 cells and RAW264.7 macrophages. The expression of ADM was observed in RAW264.7 macrophages, but not in B16/F10 cells (Fig. 1B). We then detected the concentrations of ADM in CM from tumor cells, macrophages, and their co-cultured cells by ELISA. Figure 1C shows that ADM secretion from co-cultured cells for 24 h, but not 12 h, was significantly higher than that from individually cultured cells. Furthermore, the ADM secretion from macrophages treated with tumor cells CM was much higher than that from tumor cells treated with macrophages CM, indicating that the increase of ADM secretion was due to TAMs (Fig. 1D). To further confirm the ability of TAMs to secrete ADM, we isolated primary mice PMs and BMDMs, and treated them with B16/F10 CM. Figure 1E shows that ADM secretion from both of them was significantly up-regulated upon the B16/F10 CM treatment. These results demonstrate that TAMs are the major source of ADM in melanoma.

TAM-induced angiogenesis and melanoma growth are mediated by ADM

To assess whether ADM can mediate the function of TAMs in angiogenesis, the migration and tubule formation of human dermal microvascular endothelial cells (HMECs) were examined when treated with B16/F10 or RAW264.7 cells CM or their
co-culture CM. We found that the co-culture CM significantly promoted HMEC migration and tubule formation, which were abrogated by the neutralizing ADM antibody (Fig. 2A, B and Fig. S1A, B). To determine the role of macrophages in melanoma growth, RAW264.7 macrophages or B16/F10 CM treated RAW264.7 macrophages were co-inoculated subcutaneously (s.c.) with B16/F10 cells. RAW264.7 macrophages significantly accelerated tumor growth at the early stage from day 2 to 7, while macrophages themselves were incapable of developing tumors (Fig. 2C). The ability of B16/F10 CM treated RAW264.7 macrophages in promoting melanoma growth was higher than that of RAW264.7 macrophages at day 4 and 5 (Fig. 2C). However, macrophages did not affect melanoma growth from day 9 to 14 (Fig. S2B). These results suggest that exogenous macrophages or B16/F10 CM treated macrophages only promote melanoma growth at the early stage. To determine the function of ADM and angiogenesis in this process, we harvested tumor tissues at day 7 after implantation, and examined the expression of ADM and CD31. When B16/F10 cells were co-administered with RAW264.7 or B16/F10 CM treated RAW264.7 macrophages, both the expression of ADM (Fig. 2D, left panel) and blood vessel densities (Fig. 2D, right panel) in melanoma tumors were significantly increased. Additionally, the enhancement of survival and proliferation of tumor cells also contributed to this process (Fig. S2A). Taken together, these results demonstrate that ADM is a pivotal factor of TAMs that facilitates angiogenesis and melanoma growth in a paracrine manner.
ADM promotes angiogenesis and melanoma growth via the eNOS pathway

Since eNOS activation in endothelial cells is an important intracellular signaling event in angiogenesis, we examined the function of ADM on the phosphorylation of eNOS in HMECs. ADM (10 nM) induced eNOS phosphorylation as early as 10 min and produced a maximal effect at 40 min after the treatment (Fig. 3A, left panel). ADM was able to induce eNOS phosphorylation from the concentration as low as 0.1 nM after 40 min of treatment, and has a maximal effect at the concentration of 10 nM (Fig. 3A, right panel). Therefore, ADM induces eNOS phosphorylation in both time- and dose-dependent manners. Additionally, we detected the effect of ADM on HMEC migration and tubule formation. ADM was found to enhance HMEC migration and tubule formation in a dose-dependent manner (Fig. 3B, C, and Fig. S3A, B). An eNOS specific inhibitor, NG-Nitro-L-arginine Methyl Ester (L-NAME), but not its inactive isomer D-NAME, significantly suppressed these effects of ADM in a dose-dependent manner, while L-NAME itself did not show any effect on these processes (Fig. 3B, C, and Fig. S3A, B). These results demonstrate that ADM-induced migration and tubule formation of HMEC are mediated by the eNOS signaling pathway in vitro.

Following the in vitro experiments, we further investigated the role of ADM in melanoma growth in vivo. The tumor volume from day 7 to 12 (Fig. 3D, left panel) and the tumor weight on day 12 (Fig. 3D, right panel) were significantly increased by ADM treatment. When co-administrated with L-NAME, but not D-NAME, ADM-induced melanoma growth was abrogated, while L-NAME itself showed no effect on tumor growth (Fig. 3D). To further confirm the contribution of angiogenesis
in this process, we examined CD31 staining in tumor tissues. Compared with the control group, the blood vessel density was much higher in the ADM treatment group, which could be abrogated by the treatment of L-NAME, but not D-NAME (Fig. 3E). Therefore, ADM-induced angiogenesis and melanoma growth are demonstrated to be mediated via the eNOS signaling pathway.

**The autocrine effect of ADM contributes to macrophage polarization**

In addition to endothelial cells, TAM-derived ADM may also target tumor cells or macrophages themselves in paracrine and autocrine manners. To confirm this hypothesis, we examined the expression of ADM receptor components in B16/F10 cells and RAW264.7 macrophages. It was shown that CRLR, but not RAMP2 or RAMP3, was expressed in B16/F10 cells (Fig. 4A, left panel). To further examine the possibility of the paracrine effect of ADM on B16/F10 cells, we evaluated the role of ADM or AMA on B16/F10 cells proliferation in vitro. Neither ADM nor AMA showed any effect on B16/F10 cells proliferation (Fig. S4). These results demonstrate that TAM-derived ADM cannot directly act on melanoma cells in a paracrine manner. Surprisingly, we found that all ADM receptor components, CRLR, RAMP2 and RAMP3, were expressed in RAW264.7 macrophages (Fig. 4A, middle panel). Moreover, ADM was co-localized with all ADM receptor components in RAW264.7 macrophages (Fig. 4A, right panel). These results strongly suggest an autocrine loop of ADM in TAMs. To assess whether the tumor microenvironment facilitates this autocrine effect, we utilized B16/F10 CM to treat RAW264.7 macrophages, and
detected the expression of ADM receptor components by Western blotting. We found that B16/F10 CM dramatically increased the expression of all ADM receptor components in RAW264.7 macrophages (Fig. S5). These results demonstrate that B16/F10 CM not only stimulates ADM secretion from RAW264.7 macrophages (Fig. 1C), but also up-regulates its receptors expression thus effectively enhancing the autocrine effect.

Macrophages have functional plasticity with the ability to change their functional profiles in response to different tumor microenvironments (28). Compared with M1, M2 macrophages produce lower amount of inducible nitric oxide synthase (iNOS), but higher amount of arginase 1 (Arg-1), and express M2 specific surface markers such as CD206 (29). We examined the expression of CD68 and ADM at different stages of melanoma, and found that they were dramatically up-regulated in the late stage relative to their levels in the early stage of tumors (Fig. S6). Moreover, confocal microscopy studies showed that the percentage of CD206⁺ cells out of CD68⁺ cells was much higher at the late stage compared to that at the early stage of tumors (Fig. 4B, left and middle panels). Most TAMs were identified as M1 macrophages (defined as CD68⁺CD206⁻) at the early stage and M2 macrophages (defined as CD68⁺CD206⁺) at the late stage of melanoma, respectively (Fig. 4B, right panel). These results demonstrate that macrophage phenotype can be shifted from M1 to M2 type during the tumor growth, which may be resulted by the enhancement of ADM expression. To further clarify this point, RAW264.7 macrophages were treated with ADM or B16/F10 CM, and expression levels of iNOS, Arg-1, and CD206 were detected by
Western blotting or flow cytometric analysis, respectively. We found that ADM or B16/F10 CM significantly suppressed iNOS expression, while enhanced the expression of Arg-1 and CD206 in RAW264.7 macrophages (Fig. 4C, D). These results demonstrate that ADM can polarize RAW264.7 macrophages from M1 to M2 type.

**The autocrine effect of ADM promotes melanoma growth in vivo**

To investigate the autocrine effect of ADM on melanoma growth in vivo, we examined the effect of the ADM receptor antagonist, AMA, on tumor growth. One week after B16/F10 or A375 cells implantation, intraperitoneal injection of AMA proceeded for 5 or 8 days. We found that the tumor growth was significantly inhibited by AMA in both B16/F10 mouse and A375 human melanoma models (Fig. 5A). To evaluate whether the suppression of melanoma growth was caused by the inhibition of ADM expression, we performed immunofluorescence staining for ADM in B16/F10 tumors. It was observed that AMA significantly reduced ADM expression in tumor tissues (Fig. 5B). To further confirm the autocrine effect of ADM on macrophage polarization in vivo, phenotypes of TAMs in tumor tissues were examined. We found that the percentage of M2 macrophages (defined as CD206<sup>+</sup> CD68<sup>+</sup> cells) was dramatically decreased in tumors upon the AMA treatment (Fig. 5C). In summary, these observations demonstrate that ADM contributes to macrophage polarization and melanoma growth via an autocrine effect in vivo.
The expression of ADM and its receptors is intimately associated with human melanomagenesis

It was found that ADM expression levels in melanoma tissue (Fig. S6) and plasma (data not shown) are increased with mouse melanoma growth. In order to assess the relationships of the expression of ADM and its receptors to human melanoma, we stained tissue microarrays containing samples of clinical melanoma nodules. Interestingly, we found that most of human melanoma tissues were positively stained for ADM (49 out of 59), CRLR (46 out of 60), RAMP2 (43 out of 59), and RAMP3 (45 out of 58), while the expression levels of them were much lower in control tissues (Fig. 6 and Table S1). ADM and its receptors levels are unrelated to the clinicopathological status of age and gender (Table S2). These results illustrate that the expression of ADM and its receptors are associated with melanomagenesis in melanoma patients.

Discussion

It is now widely accepted that TAMs play a critical role in regulating angiogenesis and melanoma growth. Clinical studies show a positive correlation between TAM density and melanoma prognosis (30). Animal tumor models suggest that TAMs depletion inhibits angiogenesis and tumor growth (31-33), while increased number of TAMs exhibits the opposite effect (34). Despite these functional studies, the precise mechanism by which TAMs facilitate angiogenesis and melanoma growth remains
incompletely understood. In this study, we unravel a novel mechanism which shows that ADM is a key regulator for the involvement of TAMs in melanoma growth via both paracrine and autocrine manners. Other factors in the tumor microenvironment may act as the downstream of ADM or interact with ADM to regulate the function and activity of TAMs.

Accumulating studies suggest that macrophages in the tumor microenvironment can be switched into two distinct phenotypes, namely M1 and M2 (8), while the identity of these macrophages during melanoma growth remains poorly clarified. Our present study shows that not only the TAM density is increased, but also its phenotype is switched from M1 to M2 during the melanoma growth. These results provide a new insight into the dynamic nature of TAMs during the tumor growth, and support that ADM can function as a key factor in this process. As mentioned above, ADM secretion from macrophages is correlated with inflammation and hypoxia. The link between inflammation and melanoma (35), or hypoxia and melanoma (36) is well established. The distribution of ADM in human melanoma, the low level of constitutive ADM expression in B16/F10 cells and the ability of TAMs to secrete ADM support our hypothesis that TAMs are the primary source of ADM in melanoma. It was reported that TAMs can enhance tumor angiogenesis by up-regulating hypoxia-induced angiogenic factors, such as vascular endothelial growth factor (VEGF) (5). In this study, we provide evidences that ADM is required and sufficient for TAM-induced angiogenesis and melanoma growth. Collectively, these observations suggest that TAM-derived ADM is a key factor for TAM-induced
angiogenesis and melanoma growth. ADM therefore represents an important link between TAMs and melanoma growth.

ADM receptors are expressed in endothelial cells, and can be up-regulated under the hypoxic condition (37). Thus, TAM-derived ADM is able to interact with its receptors on endothelial cells to accelerate angiogenesis in a paracrine manner. Our observations show that ADM can stimulate angiogenesis, which is consistent with previous reports (38, 39). However, most previous studies have specifically focused on the exogenous ADM. The current study demonstrates that ADM from TAMs can also stimulate angiogenesis via the paracrine loop. Although it has been reported that ADM promotes angiogenesis in vitro through the activation of some signaling pathways (38), the nature of TAM-derived ADM, and its mechanism involved in angiogenesis and tumor growth merit further studies. The activation of eNOS in endothelial cells is an important intracellular signaling event for angiogenesis (40-42). VEGF, similar to ADM, is a pro-angiogenic factor, which enhances angiogenesis through the activation of eNOS signaling pathway (43). In the current study, we provide evidences that ADM-induced angiogenesis and melanoma growth are modulated by the eNOS signaling pathway. These results can help us find new approaches to manipulate the activation of ADM, and provide new targets for melanoma therapeutics.

The autocrine effect of ADM is often observed in angiogenesis and tumor growth (27, 44-46). We report here that all ADM receptor components are present on macrophages and co-localized with ADM. Thus, TAM-derived ADM can influence
macrophages themselves in an autocrine manner. Inhibition of ADM receptors expression or activities can impair the tumor angiogenesis and growth (20-22, 47). Interestingly, we show that the expression of all ADM receptor components can be up-regulated by the B16/F10 CM, suggesting the autocrine effect of ADM on TAMs is facilitated by the tumor microenvironment. It was reported that mediators of inflammation are critical constituents of the tumor microenvironment (35, 48, 49), which strongly influence the polarization of TAMs (7, 8). This concept is supported by our study showing that the type of macrophage is switched from M1 to M2 during the tumor growth. ADM is an inflammation-related polypeptide, and its expression can be up-regulated by hypoxia (25) and during the melanoma growth. It has recently been reported that there are more M2-like TAMs enriched in hypoxic areas within tumors, which have a superior pro-angiogenic activity in vivo (50). In this study, we report that both the B16/F10 CM and ADM induce the switch of RAW264.7 macrophages to an M2-like type. These findings support the hypothesis that ADM is involved in macrophage polarization. Importantly, the autocrine effect of ADM in macrophages polarization contributes to melanoma growth in vivo. To our knowledge, this is the first study to illustrate the autocrine loop of ADM on macrophage polarization and melanoma growth.

In sum, our findings show a previously unknown connections among TAMs, ADM and melanoma growth, and provide a new insight into the mechanism of the involvement of TAMs in melanoma. More importantly, our present study has several therapeutic implications, including that it provides a basis for future cancer therapies.
via combining both the inhibition of macrophages recruitment and the “re-education” of macrophages polarization; It suggests that the suppression of ADM and its receptors is promising for cancer therapy, and also provides the evidence to support the eNOS-blockade strategy for interfering ADM signal in the treatment of cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Figure legends

Figure 1 TAMs contribute to ADM expression and secretion in melanoma

(A) ADM is co-localized with CD68 in clinical melanoma tissues. Melanoma tissues were stained simultaneously with ADM and CD68, and photographed by confocal microscopy. Scale bars = 100 μm.

(B) ADM is expressed in RAW264.7 macrophages, but not in B16/F10 cells. The expression of ADM in RAW264.7 macrophages and B16/F10 cells was revealed by immunofluorescence staining. Scale bars = 50 μm.

(C) Macrophages and tumor cells co-culture up-regulates ADM secretion. Tumor cells and macrophages were individually cultured or co-cultured (Co-) for 12 or 24 h. The ADM level in CM was detected by ELISA.

(D) Tumor cells CM promotes ADM secretion from macrophages. Tumor cells were incubated with macrophages CM, or conversely macrophages were incubated with tumor cells CM for 24 h. The ADM level in CM was detected by ELISA.

(E) B16/F10 CM stimulates ADM secretion from primary peritoneal macrophages and bone marrow-derived macrophages. After isolated from mice, cells were cultured in normal culture media or B16/F10 CM for 24 h. ADM secretion from macrophages was examined by ELISA.

* p < 0.05.

Figure 2 TAM-derived ADM stimulates angiogenesis and B16/F10-xenograft
growth  

(A) The neutralizing ADM antibody attenuates the TAM-induced HMEC migration. HMECs were seeded to the each upper well of trans-well chambers, and the indicated CM and neutralizing ADM antibody (5 μg/ml) were added to lower wells.

(B) The neutralizing ADM antibody attenuates the TAM-induced tubule formation of HMEC. HMECs were seeded in matrigel coated wells and incubated for 8 h with the indicated CM and neutralizing ADM antibody (8 μg/ml).

(C) Macrophages or B16/F10 CM treated macrophages promote melanoma growth in vivo. C57BL/6 mice were randomly assigned to 4 groups, then B16/F10 cells, macrophages or B16/F10 cells mixed with macrophages (1 × 10^6) were injected. The tumor volume was measured daily for 14 days.

(D) Macrophages or B16/F10 CM treated macrophages increase ADM and CD31 expression in tumor tissues. On day 7, 3-4 mice of each group were sacrificed and tumor tissues were excised. The expression of ADM (left panel) and CD31 (right panel) was examined by immunofluorescence. Scale bars = 100 μm or 50 μm in left or right panel, respectively.

** p < 0.01 and *** p < 0.001.

Figure 3 ADM promotes angiogenesis and tumor growth via the eNOS signaling pathway  

(A) ADM induces the eNOS phosphorylation in endothelial cells. HMECs were incubated with ADM (10 nM) for indicated times (left panel) or at varied
concentrations of ADM for 40 min (right panel). Cell lysates were harvested and immunoblotted with anti-phospho-eNOS or anti-eNOS antibody.

(B) ADM up-regulates HMEC migration through the eNOS signaling pathway. HMECs were seeded to the each upper well of trans-well chambers. Culture media with indicated reagents were added to lower wells. Cells were allowed to migrate for 6 h.

(C) ADM promotes tubule formation of HMEC via the eNOS signaling pathway. HMECs were seeded in matrigel coated wells and incubated for 8 h with indicated reagents.

(D) The eNOS signaling pathway mediates ADM-induced B16/F10 tumor growth. Following tumor cells inoculation, mice were randomly divided into 5 groups and treated with indicated reagents for 12 days. The tumor volume was measured daily for 12 days (left panel) and the tumor weight was measured on day 12 (right panel).

(E) ADM promotes tumor angiogenesis via the eNOS signaling pathway. On day 12, mice were sacrificed and tumors were excised for CD31 staining. Scale bars = 100 μm.

**p < 0.01 and ***p < 0.001.

Figure 4 The autocrine effect of ADM contributes to macrophages polarization

(A) RAW264.7 macrophages express all ADM receptor components, but B16/F10 cells only express CRLR. ADM receptor components, CRLR, RAMP2 and RAMP3, were stained in B16/F10 cells (left panel) and RAW264.7 macrophages (middle...
panel), respectively. The co-localization of ADM and its receptor components was detected in RAW264.7 macrophages (right panel). Scale bars = 20 μm.

(B) Measurement of macrophage phenotypes in different stages of tumor tissues. B16/F10 cells (5 × 10⁶) were implanted s.c. into mice, and tumors were removed on day 7 (early stage) and on day 14 (late stage). Macrophages were stained with both FITC-labeled CD68 mAb and PE-labeled CD206 mAb (left panel). The quantification of CD206⁺CD68⁺ cells (% of CD68⁺ cells) (middle panel), and the percentage of both M1 and M2 macrophage (right panel) in tumor tissues were analyzed. Scale bars = 100 μm. *** p < 0.001.

(C) ADM decreases the expression of iNOS, and up-regulates the expression of Arg-1 in RAW264.7 macrophages. After treated with ADM or B16/F10 CM for 24 h, the expression levels of iNOS and Arg-1 were assessed by Western blotting.

(D) ADM increases the expression of CD206 in RAW264.7 macrophages. RAW264.7 macrophages were stimulated with ADM or B16/F10 CM for 24 h, stained by both FITC-labeled CD68 mAb and PE-labeled CD206 mAb, and analyzed by flow cytometry. The number in boxed areas indicates the amounts of CD206⁺CD68⁺ cells (% of CD68⁺ cells) in RAW264.7 macrophages.

**Figure 5** The autocrine effect of ADM promotes melanoma growth *in vivo*

(A) The ADM receptor antagonist, AMA, inhibits melanoma growth *in vivo*. After 7 days of tumor cells inoculation, animals were randomly divided into two groups and treated with AMA (1 μg/day) or PBS. The tumor volume was measured daily (left
panel). On day 12 (B16) or 15 (A375), mice were sacrificed and tumors were weighed (right panel).

(B) AMA suppresses ADM expression in melanoma tumors. The expression of ADM in ts was evaluated by immunofluorescence staining.

(C) AMA polarizes M2 type macrophages into M1 type in melanoma tumors. Macrophages in tumor tissues were stained with both FITC-labeled CD68 mAb and PE-labeled CD206 mAb (left panel). The quantification of CD206+CD68+ cells (% of CD68+ cells) (middle panel), and the percentage of both M1 and M2 macrophage (right panel) in tumor tissues were analyzed.

**p < 0.01 and ***p < 0.001. Scale bars = 100 μm.

Figure 6 The expression of ADM and its receptors is intimately associated with human melanomagenesis

Tissue microarrays of clinical melanoma specimens and adjacent normal skins were stained as indicated for ADM, CRLR, RAMP2, and RAMP3, respectively, and photographed by confocal microscopy. Scale bars = 100 μm.
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