The Role of PMEPA1/TMEPAI in Lung Metastatic Colonization of MDA-MB-231 Cells

(MDA-MB-231 細胞の肺転移における PMEPA1/TMEPAI の役割)

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Abstract

I. Purpose

Metastasis is a process in which tumor initiating cells (TICs) disseminate from the primary tumor to settle in distant organs as dormant seeds that become reactivated after months or years to proliferate and form therein clinically overt cancer deposits, an upshot that results in clinical complications accounting for 90% of deaths in patients with solid tumors. The metastatic cascade is reflective of extensive epigenetic and genetic tweaks of heterogenous cell populations in the primary tumor that eventually favor the selection of rare subclones equipped with a competent set of molecular alterations capable of supporting their survival, adaptation then proliferation in the hostile and deadly microenvironment of distant organs. Therefore, identification of clinically relevant metastasis genes and the function of their protein product can provide insights to tackle key steps in the metastatic cascade at the molecular level.

PMEPA1/TMEPAI, a type Ib transmembrane protein whose transcription is upregulated by androgens, TGF-β signaling, Wnt signaling and EGF signaling, is implicated in regulation of important signaling pathways that are modified in oncogenic transformation including suppression of TGF-β/Smad and androgen signaling, as well as activation of PI3K/AKT signaling through negative regulation of PTEN. For its role in metastasis, reports are conflicting likely reflective of the molecular context in which PMEPA1 works. Hence, the purpose of this study was to experimentally investigate the role of PMEPA1 in metastasis of the human breast cancer cell line MDA-MB-231 to the lung with a focus on two important signaling pathways regulated by PMEPA1: TGF-β/Smad and PI3K/AKT signaling.

II. Materials and Methods

A CRISPR-Cas9 system was used to establish PMEPA1 knockout (KO) MDA-MB-231 cell clones. Two different single guide RNAs (sgRNA #1 and sgRNA #3) designed using the CRISPR Design Online Tool (https://crispr.dbcls.jp) were employed to induce a double-stranded DNA break (DSB) in Exon 2 of the human *PMEPA1* gene. Two cell clones (PMEPA1 KO 1-3 and PMEPA1 KO 3-5) were selected and expanded after screening for TGF-β-induced PMEPA1 protein expression levels that confirmed loss of PMEPA1 protein expression in PMEPA1 KO MDAMB231 cell lines. Luciferase (Luc)- or Green Fluorescence Protein (GFP)-expressing PMEPA1 KO or parental MDAMB231 cells were established using a lentivirus Luc- or GFP-expressing vector (CSII-CMV-MCS-IRES2-Bsd-Luc/GFP), respectively. PMEPA1 KO and parental MDA-MB-231 cell lines were used for investigation of PMEPA1 role in cell proliferation *in vivo* by subcutaneous injection of tumor cells into the left flank of female NOD/ShiJic-scid mice and *in vitro* both at high seeding density by cell proliferation assay and at low density by colony formation assay. Two experimental lung metastasis assays were performed either after subcutaneous or after tail vein injection of the tumor cells in age-matched female NOD/ShiJic-scid mice. When needed, the luciferase-labelled tumor cells were tracked *in vivo* by non-invasive bioluminescence imaging using In Vitro Imaging System (IVIS). Lung metastatic burden was assessed by microscopic examination of H&E-stained whole-lung sections at 3 different coronal levels for metastatic lung lesions. Total RNA, extracted from surgically resected tumor xenografts or from *in vitro* cell culture, was used to identify gene expression alterations after KO of PMEPA1

through quantitative real-time PCR (qRT-PCR) or RNA sequencing (RNA-seq). Gene set enrichment analysis (GSEA) was used to identify clinical association between altered expression of PMEPA1 and changes in PMEPA1-related signaling pathways in The Cancer Genome Atlas (TCGA) invasive breast cancer cases (BRCA). According to the experimental variable, statistical analysis was performed using Student T-test in Excel, One-way/ Welch's ANOVA and the appropriate Post Hoc test in SPSS (IBM, version 24) or Fisher's exact test in GraphPad Prism 7.

III. Results

The functional impact of PMEPA1 KO was validated by PMEPA1 KO-mediated promotion of TGF-β/Smad signaling as expected. The data initially showed suppression of *in vivo* tumor growth and lung metastasis after subcutaneous injection of the tumor cells in age-matched female NOD/ShiJic-scid mice but not of i*n vitro* monolayer cell proliferation at high seeding density in CRISPR-Cas9-mediated PMEPA1 KO MDA-MB-231 cell lines compared with parental MDA-MB-231 cells, a phenomenon that could not be attributed to molecular changes related to cell proliferation and/or survival such as *MYC* transcription that showed marginal *in vivo* downregulation and *in vitro* upregulation of *MYC* expression compared with those of the parental MDA-MB-231 cells. Rather, this difference reflects, at least partially, the PMEPA1 KO-mediated suppression of the proangiogenic factors VEGFA and IL8 which are salient for *in vivo* but not *in vitro* growing cells and are also substantial for initiation of lung metastasis through promotion of angiogenesis.

The data showed, however, suppression of i*n vitro* monolayer cell proliferation at low seeding density in CRISPR-Cas9-mediated PMEPA1 KO MDA-MB-231 cell

lines compared with parental MDA-MB-231 cells suggesting that PMEPA1 has a role in initiating and/or maintaining proliferation from single cells under low seeding density condition that is masked under high seeding density condition and that the discrepancy between *in vivo* tumor growth and *in vitro* cell proliferation in monolayer adherent cell culture condition of PMEPA1 KO cell lines at high seeding density probably reflects differences in other mediators in addition to those related to angiogenesis.

My experiments revealed that both parental and PMEPA1 KO MDA-MB-231 cells could comparably provide growth signals enough to further drive cell proliferation. At high seeding density, GFP-expressing PMEPA1 KO MDA-MB-231 cells had a stronger response than this of GFP-expressing parental cells to the growth signals provided at high seeding density by the surrounding Non-GFP-expressing population of either PMEPA1 KO or parental cell, respectively. Yet, GFP-expressing PMEPA1 KO MDA-MB-231 cells kept showing less capacity than parental cells to proliferate from single cells even on switching the surrounding Non-GFP-expressing population either to parental or to PMEPA1 KO MDA-MB-231 cells, respectively, supporting the observation that KO of PMEPA1 does not affect the growth signals provided by non-GFP-expressing cells at high seeding density. Collectively, the data suggest that the slow cell proliferation associated with KO of PMEPA1 in MDA-MB-231 cells at low seeding density is related to changes in intrinsic molecular properties. Further analysis revealed a salient role of enhancing PI3K/AKT signaling, in contrast to the marginal role of suppressing TGF-β/Smad signaling, in driving single cell proliferation either of parental or of PMEPA1 KO MDA-MB-231 cells at low seeding density. In TCGA invasive breast cancer cases (BRCA), upregulation of PMEPA1 was clinically associated with suppression of PTEN activity. My data showed also

that PMEPA1 KO MDA-MB-231 cells have tendency to form smaller metastatic lung lesions than parental MDA-MB-231 cells after tail vein injection of tumor cells.

IV. Discussion

Impaired growth of PMEPA1 KO cell line-derived tumor xenografts is consistent with several reports that dominantly indicate a tumorigenic function of PMEPA1. PMEPA1 KO-mediated induction of TGF-β signaling alone could not sufficiently explain this *in vivo* phenotype since MDA-MB-231 cell growth is poorly inhibited by TGF-β. Rather, the discrepancy between *in vivo* tumor growth and metastasis on the one hand and *in vitro* cell proliferation in monolayer adherent cell culture condition of PMEPA1 KO cell lines at high seeding density on the other hand partially reflects the marked impact of PMEPA1 KO-mediated suppression of critical proangiogenic mediators, including *IL8* and *VEGFA*, and therefore impaired angiogenesis on *in vivo* tumor growth and on metastasis which is negligible on *in vitro* proliferating cells that evenly receive nutrients from enriched medium.

The *in vitro* proliferation from single cells mimics a critical step in the early metastatic colonization of distant organs: Naturally, aggressive tumors shed thousands of cancer cells in the circulation every day. However, most of them perish and only few are competent enough to survive then to proliferate again after a latency period. Hence, the role of PMEPA1 in initiating and/or maintaining proliferation from single cells at low density suggests that PMEPA1 supports metastasis not only through maintaining transcription of important proangiogenic mediators and promotion of angiogenesis, but also probably through initiation of proliferation from single cells early during distant organ colonization. Indeed, PMEPA1 KO MDA-MB-231 cells

tended to form smaller metastatic lung lesions than those of parental MDA-MB-231 cells after tail vein injection of tumor cells. Yet, the mechanism is still elusive.

The data showed that both parental and PMEPA1 KO MDA-MB-231 cells could comparably provide growth signals enough to further drive cell proliferation at high seeding density suggesting insignificant impact of PMEPA1 knockout on supportive growth signals provided by MDA-MB-231 cells at high seeding sensity, a conclusion that was further supported on tracking proliferation of parental and of PMEPA1 KO cell colonies after switching the surrounding cells to PMEPA1 KO or to parental cells, respectively, that resulted in insignificant difference. As a result, my focus was directed to the role of PMEPA1 in modulating intrinsic molecular properties.

The data attest a critical role of PI3K/AKT signaling in the proliferation of both parental and PMEPA1 KO MDA-MB-231 cells at low seeding density. Therefore, PMEPA1 KO-mediated attenuation of PI3K/AKT signaling probably contributes to the reduction of proliferation from single cells at low seeding density in PMEPA1 KO MDA-MB-231 cells. At the clinical level, upregulation of PMEPA1 was associated with suppression of PTEN activity. Nevertheless, these data are still short on a direct evidence to support this putative mechanism. For early metastatic colonization, amplification of AKT signaling is a protective response that safeguards metastatic cancer cell survival in distant organs.

Although the experiments, so far, did not figure out how PMEPA1 KO MDA-MB-231 compensate at high seeding density for their impaired proliferation at low seeding density, the current findings showed that PMEPA1 KO MDA-MB-231 cells have a stronger response than parental cells to the provided growth signals at high seeding density, a trait that enables them to greatly neutralize molecular differences

that suppress their proliferation at low seeding density and to almost catch up with the proliferation rate of parental cells.

V. Conclusion

To summarize, the data show suppression of *in vivo* tumor growth and lung metastasis and of i*n vitro* monolayer cell proliferation at low, but not at high, seeding density in CRISPR-Cas9-mediated PMEPA1 KO MDA-MB-231 cell lines compared with parental MDA-MB-231 cells. This discrepancy reflects, at least partially, the PMEPA1 KO-mediated suppression of the proangiogenic factors VEGFA and IL8 which are salient for *in vivo* but not *in vitro* growing cells and are also substantial for initiation of lung metastasis. In addition, PMEPA1 KO MDA-MB-231 cells tended to have smaller metastatic lung lesions than those of parental MDA-MB-231 cells in lung metastatic colonization assay after tail vein injection of tumor cells. My data also put a high gloss on the role of PI3K/AKT signaling in proliferation of both parental and PMEPA1 KO cells at low seeding density. The data also reveal that PMEPA1 KO MDA-MB-231 cells have a stronger response than parental cell to the provided growth signals at high seeding density that enable them to catch up with the proliferation rate of parental cells although the molecular mechanism is yet to be identified.