Extracellular vesicle-mediated MFG-E8 localization in the extracellular matrix is required for its integrin-dependent function in mouse mammary epithelial cells

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Abstract

Milk fat globule-EGF factor 8 (MFG-E8) is a divalent-binding secretory protein possessing an Arg-Gly-Asp (RGD) motif and a phosphatidylserine (PS)-binding motif. This protein has been shown to be involved in mammary gland development and morphogenesis. Integrin binding activity is necessary for these MFG-E8-dependent cell processes. Although the target cells and molecules of MFG-E8 in the cellular microenvironment are important to understand its physiological function, its localization is largely unclear. Here, we found that mouse MFG-E8 localized to the basal lamina of the mammary gland during involution. In a model system of mammary COMMA-1D cells, exogenously and endogenously expressed MFG-E8 was deposited in the extracellular matrix (ECM) with membranous particles dependently on the PS-binding motifs in the discoidin domains that were essential for association ability to extracellular vesicles (EVs). These data revealed the basal MFG-E8 localization mechanism in which EVs served as a scaffold. Such an immobilized MFG-E8 associating with cell substrata but not soluble one in the culture media promoted integrin-dependent suppression of β-casein expression. These results suggest that MFG-E8 requires EVs to transduce cellular signals from the basolateral side of the adhesion cells by accumulating in ECM.

Introduction

The extracellular matrix (ECM) is a complex and dynamic meshwork consisting of various core macromolecules and numerous major and minor soluble molecules attaching to ECM, which not only mechanically supports the formation of multicellular structures and organs as the cellular scaffold but also determines cell behavior and properties through specific receptors on the cell surface (Bonnans *et al*. 2014; Gattazzo *et al*. 2014). Since cell-regulatory function of ECM is dependent on its molecular components, restricted distribution of the ECM components is important to maintain appropriate microenvironment for cells. Some of ECM proteins contain Arg-Gly-Asp (RGD) motifs such as a part of collagens, fibronectin, and vitronectin. The RGD motif is an integrin-binding sequence, which promotes cell attachment to ECM and cellular signal transduction through integrins and their intracellular binding proteins (Bonnans *et al*. 2014).

Recent proteomic analyses of the ECM proteins in various cells have identified a lot of minor components including milk fat globule-EGF factor 8 (MFG-E8) family proteins. MFG-E8 and EGF-like repeats and Discoidin I-Like domains 3 (EDIL3), as known as developmental endothelial locus-1, are homologous secretory proteins and they both have an evolutionally conserved RGD motif (Taylor *et al*. 1997; Hidai *et al*. 1998; Oshima *et al*. 2014). MFG-E8 was originally identified as a protein anchored onto the milk fat globule membrane (Stubbs *et al*. 1990). Its transcription level extremely increased during lactation and retained high during involution in which the alveolar mammary epithelial cells give rise to apoptosis followed by an active tissue remodeling including destruction of the basement membrane and alveolar structures (Nakatani *et al*. 2006; Macias & Hinck 2012). This protein was also expressed ubiquitously and isolated

as an ECM component in the aortic and pulmonary valves and in the aorta (Didangelos *et al*. 2010; Angel *et al*. 2011). MFG-E8 also localized to the junction between the inner and outer segments of the retinal tissue and to the basal domain of epididymal epithelial cells (Burgess *et al*. 2006; Raymond & Shur 2009), and bound to the elastic fiber of arteries via interaction with tropoelastin (Larsson *et al*. 2006). It was shown that the C-terminus tandem discoidin domains (C1, C2 domains) of MFG-E8 directly bound to type I collagen, resulting in promotion of collagen uptake by macrophages and decrease of severity of tissue fibrosis (Atabai *et al*. 2009; Wang *et al*. 2012). However the binding mechanism and target molecules of MFG-E8 to ECM are still largely unclear.

The C-terminus C2 domain of MFG-E8 has high binding activity toward anionic phospholipids, especially phosphatidylserine (PS) (Oshima *et al*. 2002; Shao *et al*. 2008; Ye *et al*. 2013). MFG-E8 thus specifically binds lipid bilayer exposing PS such as the cell membrane of apoptotic cells and extracellular vesicles (EVs) (Hanayama *et al*. 2002; Oshima *et al*. 2002; Théry *et al*. 2009; Raposo & Stoorvogel 2013). Cells employ the several types of EVs, such as exosomes, microvesicles, ectosomes, prostasomes, and matrix vesicles, for the intercellular communication, microenvironment formation, cell-cell contact, and extracellular sorting of the membrane and cytosolic components (Théry *et al*. 2009; Raposo & Stoorvogel 2013; Colombo *et al*. 2014; Shapiro *et al*. 2015). Previous studies have shown that some types of EVs interact with ECM. A well-known EV associating with ECM is matrix vesicle that initiates bone mineralization in vertebrates (Shapiro *et al*. 2015). Proteome analyses of matrix vesicles obtained from mouse osteoblasts and chicken embryonic bone revealed that MFG-E8 was a major component of matrix vesicles (Xiao *et al*. 2007; Balcerzak *et al*. 2008). Mouse and boar MFG-E8 were also present as the sperm membrane protein and bound to the ECM of the

unfertilized egg possibly through the C-domains (Ensslin *et al*. 1998; Ensslin & Shur 2003). Some of sperm surface proteins that are necessary for sperm mobility and fertilization ability are transferred from the epididymal fluid. MFG-E8 was identified as a component of EVs prepared from the epididymal fluid by the mass spectrometry analysis (Gatti *et al*. 2005).

MFG-E8 deposition in the tissue ECM has sometimes been related to the progression of some diseases (Häggqvist *et al*. 1999; Fu *et al*. 2009; Wang *et al*. 2012). Although MFG-E8 has an integrin-binding motif and its localization in ECM was associated with EVs in some cases, the accumulation mechanism and inherent function of MFG-E8 in the tissue ECM are largely unknown. Here, we discovered transient localization of mouse MFG-E8 to the basal lamina of mammary epithelial cells in the involution phase. Both long and short variants of mouse MFG-E8 (Oshima *et al*. 1999), MFG-E8-L and MFG-E8-S, were deposited with some membranous particles in the basal cell substrata when they were expressed in the cultured cells including a mouse normal mammary epithelial cell line, COMMA-1D (Danielson *et al*. 1984). This substratum localization required the PS-binding motifs in the discoidin domains but not the RGD motif. Beta-casein expression induced by lactogenic hormones was suppressed when COMMA-1D cell and HC11 cell, which is a clonal cell line isolated from polyclonal COMMA-1D cell line (Ball *et al*. 1988), were cultured on the cell substratum containing exogenously overexpressed-MFG-E8. This suppression was dependent on the RGD motif of MFG-E8. Importantly access of milk MFG-E8 from apical side was insufficient for depression of β-casein expression. From these results, we suggest that MFG-E8 is retained in the extracellular cell substratum coupling with some EVs in various types of adherent cells, which is essential to exert its integrin-dependent physiological function.

Results

MFG-E8 localizing in ECM of the involuting mammary gland and cultured cells

In mammary gland involution, MFG-E8 was expressed in a high level as lactation (Nakatani *et al*. 2006). MFG-E8 was secreted into the apical luminal space from the lactating mouse mammary epithelial cells as a soluble form and a sarface protein of milk fat globules (Fig. 1A). In this piriod MFG-E8 never colocalized with collagen IV, indicating no basal localization (Fig. 1E). Fourty eight hours after weaning is a period when the mouse mammary gland transits from reversible early involution phase to irreversible late phase (Watson 2006). In this period MFG-E8 signals coexisted with collagen IV (Fig. 1G). Some of these signals appeared in a punctate pattern (Fig. 1G"). These results indicate that MFG-E8 transiently localizes to the basal lamina of the mammary epithelial cells during involuion.

We next examined deposition of MFG-E8 proteins in the substratum of cultured cells where the ECM proteins accumulated. Western blot analysis revealed several bands corresponding to MFG-E8-L glycoforms (66, 56, and 51 kDa) and a single band of MFG-E8-S (51 kDa) in the ECM fraction deposited on the culture dishes when MFG-E8-L and -S were expressed in COS-7 cells (Fig. 2). To clearly define the domains necessary for the deposition of MFG-E8 in the cellular substratum, domain-deletion mutants were transfected. Lack of either the C1 or C2 domain largely reduced ECM deposition. To verify this deposition further, MFG-E8 in the cell culture substratum was analyzed by immunocytochemistry. Figure 3A shows typical fluorescence micrographs of MFG-E8 in the cell substrata of COS-7 cells expressing MFG-E8 and its mutants. Footprint-like patterns were observed when MFG-E8-L and -S were expressed. MFG-E8 expressed in HEK293T cells was equally localized in the cell substratum. Interestingly, MFG-E8

hardly colocalized with collagen IV although both was deposited in the cellular substratum originating from the same cells (Fig 3B). Consistent with the Western blot analysis, both the ΔC1 and ΔC2 mutants could not be tethered to the cell substrata (Fig. 3A). These results show that the C-domains are essential for MFG-E8 to be retained in ECM.

ECM deposition of MFG-E8 requires phospholipid-binding activity

The C-domains of MFG-E8 belong to the discoidin family and were shown to have binding ability to PS and type I collagen (Oshima *et al*. 2002; Atabai *et al*. 2009). We thus speculated two possibilities that MFG-E8 directly bound to some ECM components or indirectly through some secreted membranous structures containing phospholipids, such as EVs, attaching to ECM. To investigate the contribution of membranous structures, cell substrata were treated with 1% Triton X-100 and methanol to wash out the lipid-based molecules. After treatment, footprint-like pattern of MFG-E8-L in the ECM effectively disappeared though collagen IV was unaffected (Fig. 3C). Detergent-sensitive MFG-E8 localization in the cell substratum was also observed when cells were cultured in the EV-free medium (Fig. 3D). A former structural analysis of the MFG-E8 C2 domain revealed the clusters of hydrophobic residues and glycine next to the positively charged amino acid exposed to the aqueous outside, which were termed spikes (Shao *et al*. 2008). These spikes have been suggested as the essential motifs for direct binging with PS (Shao *et al*. 2008; Ye *et al*. 2013). To elucidate the contribution of PS for basal MFG-E8 deposition, the PS-binding motif mutants of MFG-E8 were constructed (Fig. 4A). Mutations in the spike 1 and the spike 3 motifs partially diminished the binding activity to the EVs and PS, and the spike 1,3 mutant completely lost this

activity (Fig. 4B-D). Mutant analyses of the C1 domains of the coagulation factor V and factor VIII have revealed their contribution to membrane binding (Saleh *et al*. 2004; Meems *et al*. 2009). The C1 domain of MFG-E8 contains the putative conserved PS binding motifs (Oshima *et al*. 2014). The ΔC1 mutant also failed to accumulate in the EV fraction analogously to the other spike mutants (Fig 4E). When these mutants were expressed in HEK293T cells, the spike 1 mutant and the spike 3 mutant were weakly retained in the cell substrata and the spike 1,3 mutant in the ECM was almost undetectable as same as the ΔC2 mutant was (Fig. 5). The binding profile of the each mutant to EVs was significantly similar to that to the cell substrata (Figs 4C and 5C). These results strongly imply that deposition of MFG-E8 in ECM is mediated by EVs. Integrins loaded on exosomes were shown to determine the attachment affinity of the vesicles to the specific ECM during cancer metastasis (Hoshino *et al*. 2015). To address the requirement of integrins for interaction between MFG-E8 and EVs, two RGD motif mutants were analyzed. Both the KGD and RGE mutants were detected in the ECM fractions as well as the wild type MFG-E8 isoforms (Figs 2, 3A, and 3E). These results show that integrin binding ability is dispensable for MFG-E8 localization in ECM.

Integrin-dependent suppression of β-casein gene expression in mammary epithelial cells cultured on the MFG-E8-deposited substrata

To understand the function of MFG-E8 accumulated in the mammary basal lamina, we used cultured mammary epithelial cell lines. At first, localization of MFG-E8 in the cell substratum was investigated in the mouse mammary epithelial cell lines established from a primary culture of normal mammary tissue. MFG-E8 protein secreted from COMMA-1D cells was bound to the EVs but not to the cell membrane (Fig. 6A).

MFG-E8 in the EV fraction was degraded by proteinase K without permeabilization but cytosolic Caveolin-1 was not, indicating that MFG-E8 secreted from the mammary epithelial cells bound to the outer surface of EVs (Fig. 6B). Endogenous MFG-E8-L and -S proteins were clearly detected in the cell substrata prepared from wild type COMMA-1D and HC11 cells. The MFG-E8 proteins in the ECM of stable transfectant clones increased compared to those of wild type COMMA-1D cells and the mock transformant (Fig. 6C). More expression of the KGD mutant than MFG-E8-L must be ascribed to its clonal cell property rather than the mutant protein property. To clarify the associated localization between MFG-E8 and EVs in ECM, membranous structures in the ECM of COMMA-1D cells stably expressing MFG-E8 were stained with a cell-membrane-labeling fluorescent dye, PKH67. Most of the punctate MFG-E8 signals in ECM were colocalized with membranous particles (Fig. 6D middle panels). These MFG-E8 and PKH67 signals were quite similar to those of EVs secreted into the culture medium (Fig. 6D upper panels). Cell substrata of COMMA-1D contained detergent-resistant membrane structures with which all of MFG-E8 signals were associated after Triton X-100 treatment while most of the membranous particles was washed out with MFG-E8 as shown in Figure 3C (Fig. 6D lower panels).

The components of ECM and integrins coordinately govern differentiation of mammary epithelial cells and the tissue-specific gene expression with lactogenic hormones (Akhtar & Streuli 2006). Thus, we examined the contribution of MFG-E8 to maintenance of functional differentiation of mammary epithelial cell by monitoring β-casein expression. When β-casein was induced by lactogenic hormones in wild type COMMA-1D cells and a stable MFG-E8-L transformant clone, this transformant expressed less β-casein mRNA than the wild type (Fig. S1A). To evaluate whether the

ECM containing MFG-E8 protein has an ability to suppress β-casein expression, wild-type COMMA-1D cells were treated with the lactogenic hormones on the conditioned dishes where the wild type or the transformant COMMA-1D cells had formerly been cultured and then removed (Fig. S1B). When COMMA-1D cells were induced on the conditioned dishes prepared from the transformants overexpressing MFG-E8-L and -S, their β-casein expression levels were reduced by approximately 40% as compared to the control. There was no difference in β-casein reduction levels between MFG-E8-L and -S. To avoid the possibility that this reduction of β-casein was due to the ECM compositions specifically expressed in each cloned cell instead of MFG-E8 itself, HC11 cells were also cultured on the conditioned dish from HEK293T cells transiently expressing MFG-E8-L. Equivalently β-casein mRNA expression was diminished by 40% in this situation (Fig. 7A). There was no apparent change in the quantity and quality of the ECM proteins in HEK293T cells transiently transformed with MFG-E8-L in comparison with the mock transfectant (Fig. S2). MFG-E8 can transduce cell signaling through αvβ3 and αvβ5 integrins and regulate various cellular events including suppression of gene expression (Taylor *et al*. 1997; Aziz *et al*. 2009). To investigate the contribution of integrins to the transcriptional suppression of β-casein by MFG-E8, HC11 cells were cultured on the conditioned dish including the RGD motif mutant MFG-E8. Mutation in the RGD motif led to a loss of the suppressive activity for β-casein expression (Fig. 7B). These results indicate that MFG-E8 in ECM plays a role in suppression of gene expression in an integrin-dependent manner. MFG-E8 is primarily secreted as one of soluble proteins in milk serum (whey) and then gradually binds to the surfaces of the milk fat globule membranes retained in the luminal space of lactating mammary gland (Nakatani *et al*. 2013; Yasueda *et al*. 2015). In order to evaluate the suppressive ability of MFG-E8

secreted into the luminal space, milk serum samples prepared from the wild type and *MFG-E8*-deficient mice were added to the culture media during hormone induction. In this experiment, the final concentration of diluted MFG-E8 was approximately 1.2 nM in the culture media, and this was quite abundant compared to the amounts of MFG-E8 expressed in the ECM and culture supernatant of HEK293T cells (Fig. S3). No significant difference in β-casein expression was observed between the wild type and the MFG-E8-deficient milk serum (Fig. 7C). This indicated that addition of MFG-E8 to the culture medium was not sufficient for abrogation of hormone dependent β-casein production. Integrin β5 localized in the basal side of HC11 cells and was costained with MFG-E8 there, but not in the apical side (Fig. 7D).

Discussion

Preceding studies have suggested that the integrin-binding activity was prerequisite for MFG-E8 activity in various physiological processes of the adhesive cells, such as mammary gland morphogenesis (Ensslin & Shur 2007), neovascularization (Silvestre *et al*. 2005), induction of proliferation and invasion of vascular smooth muscle cells within the adult arterial wall (Fu *et al*. 2009; Wang *et al*. 2012), and maintenance of epididymal epithelium integrity (Raymond & Shur 2009). Although integrins generally localize in the basolateral region of the adhesive cells to interact with the ECM components, detailed localization of MFG-E8 in the epithelial and endothelial tissues was not understood clearly. On the other hand, MFG-E8 has been well known as one of major components of various EVs (Oshima *et al*. 2002; Xiao *et al*. 2007; Balcerzak *et al*. 2008; Théry *et al*. 2009). Previous studies have reported that some EVs associate with the ECM (Clayton *et al*. 2004; Shapiro *et al*. 2015). In this study, we found that accumulation of MFG-E8 in the cell substratum was dependent on the PS-binding motifs in the C-domains that were essential for its EV association. The C1 domain deletion mutant lost the EV-binding affinity and was absent from the cell substratum, indicating that the C1 domain in addition to the C2 domain was necessary for MFG-E8 to bind to the EVs and to be retained in the ECM. We also showed that MFG-E8 and some membranous particles coexisted in the cell substratum (Fig. 6D). Based on these results, we suggest that MFG-E8 can be accumulated in ECM by associating with some EVs. Although the property of EVs to which MFG-E8 preferably binds is yet unclear, the amount of PS on the vesicle surface must be critical (Théry *et al*. 2009; Colombo *et al*. 2014). Because proteinase K treatment without detergent completely digested MFG-E8 in the EV fraction (Fig. 6B), MFG-E8 protein was expected on the outside of EVs. Thus, the C-domains of MFG-E8

associate with PS existing in the outer leaflet of EVs, and its N-terminal EGF domains containing the integrin-binding motif are exposed toward the outer side from EVs, which can transduce cell signals by binding to αvβ3 and αvβ5 integrins localized in the basolateral region of the target cells (Andersen *et al*. 1997; Taylor *et al*. 1997). Multivalent accumulation of MFG-E8 proteins on EVs is possibly beneficial to elicit efficient signal transduction by inducing clustering of integrins (Iwamoto & Calderwood 2015). We here identified accumulation of MFG-E8 in the basal lamina of mammary epithelial cells in the involution period (Fig 1). This result is consistent with a preceding proteomic analysis that identified MFG-E8 as an ECM component of the involuting rat mammary gland (O'Brien *et al*. 2012). Although how MFG-E8 translocates to the basal lamina in the involution period is unclear, we speculate that MFG-E8 gains access to mammary ECM by diffusion through the loosened epithelial sheet or by the altered vectorial intracellular transport to the basolateral side. Extracellular vesicles are abundant in the milk serum. However, whether EVs are requisite for MFG-E8 localization in the mammary basal lamina is still not convince.

There are a lot of types of the EVs released from various tissues, referred to as exosomes, microvesicles, shedding vesicles, and so on (Théry *et al*. 2009; Raposo & Stoorvogel 2013; Shapiro *et al*. 2015). MFG-E8 was deposited in HEK293T cell substratum even when the cells were cultured in the EV-free medium. Thus, contribution of membranous particles originated from the cells is sufficient for localization of MFG-E8 in the cell substratum. Targeting of the EVs to ECM must be largely dependent on the vesicle surface components. Matrix vesicles contain several proteins that tightly bind to the type II and X collagens (Wu *et al*. 1991). Invasive tumor cells released exosomes prior to metastasis and the destination of these exosomes was determined by the sets of

integrins loaded on the exosomes, which bound to the ECM proteins specifically expressed in the specific tissues (Hoshino *et al*. 2015).

When mammary epithelial cell lines were plated on the conditioned dishes where MFG-E8 was abundant, induction of β-casein expression by lactogenic hormones declined dependently on the integrin-binding activity of MFG-E8. Importantly, both conditioned dishes prepared from COMMA-1D cells and HEK293T cells weakened β-casein expression identically. This suggested that the decline of β-casein transcription was just owing to MFG-E8 but not to the other EV and ECM components endogenously expressed in the donor cells. In the cultured cells MFG-E8 possibly evokes integrin-mediated STAT3 activation that abrogate milk gene expression (Watson 2006; Jinushi *et al*. 2008), or competes some other RGD proteins supporting β-casein expression (Jones *et al*. 1995; Aziz *et al*. 2009). Addition of milk serum containing MFG-E8 to the culture media of HC11 cells did not depress β-casein expression. This result suggests that the basolateral approach of MFG-E8 to the target polarized epithelial cells is critically important to exert its integrin-mediated function. The amount of MFG-E8 protein deposited in HEK293T cell substrate was sufficient to suppress β-casein expression in HC11 cells although this was several thousand times less than that in milk serum. From these results, we deduce that MFG-E8 secreted into the apical luminal space of mammary duct in a lactation period is not able to modulate milk gene expression in vivo. This was consistent with the fact that casein protein level in the lactating *MFG-E8*-deficient mouse was comparable to that in the wild type mouse (Hanayama & Nagata 2005). We examined milk protein concentrations in the involuting mammary gland of the *MFG-E8*-deficient mouse. Two days after pup's withdrawal, milk protein levels of the *MFG-E8*-deficient mouse were comparable with those of the wild type (Fig. S4). Although MFG-E8 in cell

substratum decreased β-casein transcription in vitro, MFG-E8 seems not to be pivotal in the direct control of milk protein expression in the involuting mammary gland. Further study is necessary to elucidate MFG-E8 function with EVs in the basal space in vivo. Recently EDIL3 was identified as an EV component discharged from cancer cells, which was related to tumor invasion (Lee *et al*. 2016). Interestingly, MFG-E8 associating with EVs was also elevated in the sera of patients suffering from breast tumor metastasis and prostate cancer (Larocca *et al*. 1991; Soki *et al*. 2014). Age-associated accumulation of MFG-E8 in the arterial wall was reported to promote invasive ability and proliferation of vascular smooth muscle cells and induce arterial wall remodeling, as well (Fu *et al*. 2009; Wang *et al*. 2012). Thus, understanding of the mechanism for MFG-E8 accumulation in tissue ECM that is important for cell migration and development must be subservient for exploitation of novel therapy treating tumor metastasis and age-dependent vascular diseases.

Experimental procedures

Expression plasmids

Expression plasmids containing MFG-E8-L, -S, and C-domain-deletion mutants (ΔC1, ΔC2) were described previously (Oshima *et al*. 2002). Amino acid of Arg^{87} of MFG-E8-L was substituted to Lys for the KGD mutant, and Asp^{89} to Glu for the RGE mutant. The spike1 and the spike3 mutants were designed as shown in figure 4A (Shao *et al*. 2008; Ye *et al*. 2013). The spike 1,3 mutant contains both spike1 and 3 mutations. All mutant forms of MFG-E8 were subcloned into the mammalian expression vector pEF1/myc-His (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Cell culture

Mouse normal mammary epithelial cell lines, COMMA-1D and HC11, were kindly provided from Dr Medina and Dr Groner, respectively (Danielson *et al*. 1984; Ball *et al*. 1988). Mammary epithelial cell lines, monkey kidney fibroblast-like cell line COS-7, and human embryonic kidney derived cell line HEK293T were cultured in DMEM (Sigma-Aldrich, St. Louis, Missouri, USA) containing 10% heat-inactivated fetal calf serum (FCS), penicillin at 100 units/ml, streptomycin at 100 μg/ml at 37˚C under humidified 5% CO_2 and 95% air.

Transfection and clonal selection

Expression plasmids pEF1/myc-His containing MFG-E8 cDNAs were transfected into cells by using Polyethylenimine Max (Polysciences, Warrington, Pennsylvania, USA). Empty expression vector was used for mock transfection as a negative control. To obtain the stable expression clones, COMMA-1D cells were transfected with the expression

plasmids and then selected in the culture medium containing 500 μg/ml G418 (Thermo Fisher Scientific). MFG-E8 expression in colonizing clones was evaluated by Western blotting and RT-PCR analysis.

Phospholipid-binding assay

The ELISA-based phospholipid-binding assay was performed as described previously (Oshima *et al*. 2002). Briefly, 50 μl of cell culture supernatants collected from MFG-E8 expressing cells was mixed with Tween 20 at a final concentration of 0.5% and applied to the 96-well plate coated beforehand with PS (Sigma-Aldrich), phosphatidylcholine (PC) (Sigma-Aldrich), and methanol. After overnight incubation at 4˚C, bound MFG-E8 proteins were detected with anti-mouse MFG-E8 antiserum (Aoki *et al*. 1997).

Purification of EV and plasma membrane fractions

For the EV preparation, cells were cultured for 2~4 days in serum-free DMEM and then the culture supernatants were clarified by sequential centrifugation at 1,200 x *g* for 10 min and 15,000 x *g* for 10 min to eliminate apoptotic cells and debris. Samples were then ultracentrifuged at 100,000 x *g* for 1 h at 4˚C. The resulting supernatants were recovered, while the pelleted EVs were washed twice with Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) and resuspended in 100 μl of buffers containing 10 μg/ml leupeptin. Total proteins in the cell culture supernatants were precipitated with trichloroacetate for Western blot analysis.

The plasma membrane fraction was prepared from a stable MFG-E8-L expression clone and parental COMMA-1D cells, which were cultured for 2 days in the culture medium containing FCS and then for 4 days in serum-free DMEM containing 5

μg/ml insulin (Sigma-Aldrich) in three 100 mm tissue culture dishes (4 x 10^6 cells/dish). The cells were washed with TBS, scraped from the culture dish, and homogenized with Dounce homogenizer on ice in 1.5 ml of the isolation buffer containing 5 mM Tris-HCl (pH7.4), 250 mM sucrose and 1 mM $MgCl_2$. The homogenates were centrifuged for 30 min at 4˚C and 10,000 x *g* to remove nuclei and mitochondria. The microsome fractions containing plasma membrane were then precipitated by centrifugation at 100,000 x *g* for 1 h and washed twice with TBS. Finally, the pellets were suspended in 50 μl of TBS containing 10 μg/ml leupeptin.

Treatment of EVs with proteinase K

Two hundreds and sixty nanogram of proteins in the EV fraction derived from the COMMA-1D clone stably expressing MFG-E8-L were treated with proteinase K at a concentration of 0.5 mg/ml for 1 h on ice in the presence or the absence of 1% Triton X-100. Proteolysis was stopped by adding phenylmethylsulfonyl fluoride at a final concentration of 12.5 mM and further incubation for 5 min on ice.

Preparation and analyses of cell substratum proteins remaining on culture dishes

COS7 and HEK293T cells transiently transfected with the MFG-E8 expression plasmids were cultured for 2 days in the serum free media. Alternatively, HEK293T cells were cultured during transfection and cell substratum preparation in the EV-free media containing exosome-depleted FCS, Exo-FBS™ (System Biosciences, Palo Alto, California, USA). Wild-type COMMA-1D cells and the stable MFG-E8 transformants (2.5 x 10^6 cells/100 mm dish) were cultured for 3 days in DMEM containing 10% FCS and then for 2 days in serum free DMEM containing 5 μg/ml insulin. The cells were then

completely removed from the dishes by incubating and gently flushing with PBS containing 1 mM EDTA, collected by centrifugation at 1,000 x g for 10 min, and lysed on ice for 10 min with the lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Substratum proteins remaining on the culture dishes were provided to Western blotting and immunohistochemistry.

SDS-PAGE and Western blot analysis

Substratum proteins on the culture dishes were solubilized with Laemmli sample buffer. Proteins in ECM, EVs, plasma membrane fractions, and cell lysates were separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membrane was blocked and then sequentially incubated with primary antibodies and peroxidase-conjugated secondary antibody. Proteins probed with antibodies were visualized with an enhanced chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) and Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, Massachusetts, USA). For silver stain, polyacrylamide gel was stained with Silver stain II kit (Wako, Osaka, Japan). Human HSC70 was detected with rat monoclonal antibody 1B5 (Enzo Life Sciences, Farmingdale, New York, USA). Mouse HSC70 was detected with rabbit polyclonal antibody ADA-SPA-757 (Enzo Life Sciences).

Immunohistochemistry

C57BL/6 mice were purchased from Japan SLC. *Mfge8* null C57BL/6 (*Mfge8*$^{-/-}$) mice were kindly given by Dr Shigekazu Nagata (Hanayama *et al*. 2004). The mice were fed

laboratory chow (Japan SLC, Hamamatsu, Japan) and cared for according to Nagoya University guidelines for animal study. For forced induction of mammary involution, the pups were removed from the mother at day 10 of lactation and then mammary glands were dissected after 2 days. Mammary glands were fixed in 4% paraformaldehyde/PBS overnight, soaked in 20% sucrose/PBS, embedded in Tissue-Tek OCT compound (Sakura finetek, Tokyo, Japan), and sectioned at 10 μm. After blocking with TBS containing 0.25% gelatin for 1 h, the sections were incubated overnight at 4˚C with hamster anti-mouse MFG-E8 monoclonal antibody 18A2-G10 (MBL, Nagoya, Japan) and rabbit anti-human collagen IV polyclonal antibody ab6586 (Abcam, Cambridge, UK), followed by incubation for 50 min with the fluorescent dye-conjugated secondary antibodies and Alexa Fluor 568 Phalloidin (Thermo Fisher Scientific).

COS7 cells and HEK293T cells were transfected with the plasmids containing MFG-E8 and its mutant forms or an empty plasmid, and cultured for 24 to 48 hours on coverslips coated with poly-L-Lys. Cell substrata were prepared as described above. Cells and cell substrata on the cover glasses were incubated in TBS with or without 1% Triton X-100 on ice for 15 min and fixed with 4% paraformaldehyde/PBS for 10 min. Alternatively, cells and cell substrata were incubated with methanol at -20˚C for 10 min. For EV staining, cell substrata were incubated with PKH67 Green Fluorescent Cell Linker (Sigma-Aldrich) after detergent treatment according to manufacturer's instruction. After blocking with TBS containing 0.25% gelatin for 1 h or PBS containing 1% bovine serum albumin, the specimens were incubated overnight at 4˚C with the rabbit antiserum specific for MFG-E8, hamster anti-mouse MFG-E8 monoclonal antibody 18A2-G10, rabbit anti-human collagen IV polyclonal antibody, and rabbit anti-human integrin β5 polyclonal antibody SAB4501589 (Sigma-Aldrich) followed by incubation for 3 hours

with the fluorescent dye-conjugated secondary antibodies and 500 nM TOTO-3 (Thermo Fisher Scientific). Images were acquired by using laser-scanning confocal microscopes, MRC-1024 (Bio-Rad, Hercules, California, USA), LSM5 PASCAL (Carl Zeiss, Oberkochen, Germany), and FV1000 (Olympus, Tokyo, Japan). Colors and sizes of the acquired images were modified using Adobe Photoshop CS5 (Adobe, San Jose, California, USA) and ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

Hormonal induction on the conditioned dishes

COMMA-1D cells, and the MFG-E8-L and -S stable transformants were seeded in 60 mm dishes (3 x 10^5 cells/dish), cultured for 3 days, and then completely removed by incubating and gently flushing with PBS containing 1 mM EDTA. Wild type COMMA-1D cells (1 x 10^6 cells/dish) were seeded onto the remaining culture dishes with cell substrata (conditioned dishes), and induced with three lactogenic hormones as described previously (Oshima *et al*. 1999). Otherwise, HEK293T cells were transiently transfected with the MFG-E8 and its mutant expression vectors in the 6 well microplates (2 x 10^5 cells/well). After 3days culture, conditioned culture plates were prepared as described above. HC11 cells (5 x 10^5 cells/well) were precultured on these plates with EGF (10 ng/ml)(Sigma-Aldrich) and insulin (5 μg/ml) (Sigma-Aldrich) for 24 hours and then induced with the lactogenic hormones (Taverna *et al*. 1991). Milk samples were collected from the three wild type and the three *MFG-E8*-deficient mice on days from 10 to 14 of lactation and then centrifuged tree times at 15,000 g for 15 min at 4 ˚C to separate fat globule fraction, milk serum and casein fractions. Milk serum fractions were diluted up to 1000-fold in the 1.5 ml of culture media during hormone induction. The original concentration of MFG-E8 in the wild milk serum was estimated at approximately 1.2 μM

using immunoblotting.

Quantitative and semi-quantitative PCR analysis

Total RNA was prepared using TRIzol® (Thermo Fisher Scientific) from the cultured COMMA-1D and HC11 cells, and cDNA was then synthesized using Superscript II reverse transcriptase (Thermo Fisher Scientific) and random hexamers according to the manufacturer's instructions. Amplification of MFG-E8, β-casein, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by PCR was performed as described previously (Oshima *et al*. 1999). Semi-quantitative expression levels of β-casein and GAPDH were analyzed using ImageJ. SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD) and StepOne real-time PCR system (Thermo Fisher Scientific) were used for the quantitative expression analysis. Sense and antisense primers for mouse β-casein (M26940) used in semi-quantitative PCR were 5'-ATG-AAG-GTC-TTC-ATC-CT-3' and 5'-AGA-AGT-TCT-AGG-TAC-TGC-3', and in real time PCR 5'-CCA-TGA-AGG-TCT-TCA-TCC-TC-3' and 5'-CAT-GAG-ATT-CAC-CTT-CTG-AAG-3'. Primers for mouse GAPDH (M3259) used in semi-quantitative PCR were 5'- ACA-AAA-TGG-TGA-AGG-TCG-GT-3' and 5'-TCC-AGG-GTT-TCT-TAC-TCC-TT-3', and in real time PCR 5'-GAC-TTC-AAC-AGC-AAC-TCC-CAC-3' and 5'- TCC-ACC-ACC-CTG-TTG-CTG-TA-3'.

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Figure Legends

Figure 1. Accumulation of MFG-E8 in the basal lamina of mouse mammary epithelial cells in the early phase of involution. (A-D) Sections of mammary glands from the wild type (WT) and *Mfge8*$^{-/-}$ mice on the day 10 of lactation (L10) and the day 2 of involution (I2) were stained with anti-MFG-E8 (green), anti-collagen IV (magenta), and phalloidin (cyan). Scale bar indicates 50 μm. (E-H) Areas surrounded by white-dotted lines in A-D are enlarged. The images of immunofluorescence and differential interference contrast are shown. White arrowheads indicate luminal MFG-E8 signals on the milk fat globules. White arrows indicate MFG-E8 localizing in the basal lamina. Scale bar indicates 10 μm.

Figure 2. Identification of MFG-E8 proteins in the extracellular matrix fraction. COS7 cells were transfected with plasmids containing cDNA of MFG-E8-S, MFG-E8-L, KGD mutant, ΔC1 mutant, and ΔC2 mutant. Empty vector was used for mock transfection. ECM proteins that remained on the cell culture dishes and cell lysates were analyzed by Western blotting with the antiserum specific for MFG-E8. Positions of molecular-mass standards are indicated on the left.

Figure 3. Footprint-like deposition of MFG-E8 in the cell scaffold. MFG-E8 in the cell substratum (ECM) was immunocytochemically stained. (A) COS-7 cells were transfected with the wild type forms (MFG-E8-L and -S) and the mutant forms of MFG-E8 (KGD, ΔC1, and ΔC2). After removal of cells, remaining cell substrata were fixed, and stained with anti-MFG-E8 antiserum followed by fluorochrome-labeled secondary antibodies. (B) Cell substratum of HEK293T cells transfected with MFG-E8-L was

stained with anti-MFG-E8 and anti-collagen IV antibodies. (C) Before fixation, the ECM fractions of HEK293T cells transfected with MFG-E8-L were treated with PBS, 1% Triton X-100, and methanol. Cells were treated with methanol. The ECM fractions and cells were stained with anti-MFG-E8 and anti-collagen IV (Col IV) antibodies. The images of immunofluorescence and differential interference contrast (DIC) are shown. (D) ECM fractions were prepared from HEK293T expressing MFG-E8-L cultured in the EV-free media and then treated with 1% Triton X-100 or PBS. MFG-E8 and collagen IV in the ECM were stained. (E) ECM fractions of HEK293T cells transfected with the wild type and RGE mutant of MFG-E8 were stained with anti-MFG-E8 antiserum. Scale bars indicate 20 μm in A and E and 10 μm in B, C and D.

Figure 4. Loss of EV-binding activity of the PS-binding motif mutants. (A) Schematic structure of MFG-E8 PS-binding motif mutants. Some essential residues of the PS-binding motifs, spike 1 and spike 3, in the C2 domain were replaced to alanine. SS; signal sequence, P/T; proline/threonine-rich domain. (B) Vesicle-binding activities of the PS-binding motif mutants. Extracellular vesicles were collected from the culture supernatants of HEK293T cells transiently expressing MFG-E8 and PS-binding motif mutants. MFG-E8 and its mutant proteins in each fraction were analyzed. Sup; supernatant fraction of 100,000 x *g* ultracentrifugation, EV; precipitate fraction of 100,000 x *g* ultracentrifugation. (C) Index values of EV binding were calculated by dividing the band intensity of MFG-E8 in the EV fraction shown in B by that in the supernatant fraction. Loaded proteins were normalized by HSC70. (D) Phospholipid-binding activity. The culture supernatants of MFG-E8 expressing cells were subjected to the ELISA-based phospholipid-binding assay. Note that the spike 1,3 mutant completely

lacked the PS-binding ability. Error bars indicate s.e.m (n=5). (E) Extracellular vesicle-binding assay of the ΔC1 mutant expressed in COS7 cells and COMMA-1D cells. Extracellular vesicles in the culture supernatants were obtained by ultracentrifugation.

Figure 5. Requirement of PS-binding activity for MFG-E8 deposition in the ECM. Immunocytochemical staining of MFG-E8 and its mutants in HEK293T cells (A) and in ECM (B). (A) The cells transiently expressing MFG-E8 were fixed, and permeabilized. MFG-E8 and nuclei were stained. (B) Cell substrata were fixed and stained without permeabilization. The spike 1 mutant and the spike 3 mutant showed subtle residual signals. Scale bars indicate 50 μm. (C) Relative intensities of MFG-E8 signals in the ECM. Total fluorescent intensities in three independent images were measured. Error bars indicate s.e.m.

Figure 6. Endogenous and exogenous MFG-E8 deposition in the ECM of mammary epithelial cell lines. (A) MFG-E8 and EV marker HSC70 in 10 μg of membrane-associated proteins prepared from COMMA-1D cells stably expressing MFG-E8-L were analyzed. CM; cell membrane, EV; extracellular vesicle. Single and double asterisks (* and **) respectively indicate the positions of exogenous MFG-E8-L and endogenous MFG-E8-S. (B) Topological analysis of MFG-E8 on the EV fraction. The proteins in the EV fraction from the MFG-E8-L transformant were incubated with or without proteinase K, in the presence or the absence of 1% Triton X-100. Degradation of MFG-E8 and caveolin-1 was analyzed by Western blotting. (C) MFG-E8 deposition in the ECM of HC11 cell, COMMA-1D cell, and MFG-E8 transformants was analyzed. (D) Immunocytochemical analysis of EVs and MFG-E8 in ECM. Cell substrata were

prepared from COMMA-1D clone highly expressing MFG-E8-L KGD mutant. Extracellular vesicles were purified by ultracentrifugation and then placed on the poly-L-lysine coated coverslips. After treatment with Triton X-100 or PBS and subsequent fixation, cell substrata and EVs were stained with PKH67 (green) and anti-MFG-E8 (magenta). Areas enclosed by the dotted white open squares in the left panels are enlarged in the right panels. Scale bars indicate 20 μm in the left panels and 4 μm in the right panels.

Figure 7. Integrin-dependent repression of β-casein gene expression by MFG-E8. (A) HC11 cells were induced with the lactogenic hormones (LH) on the conditioned ECM produced by HEK293T cells ectopically expressing MFG-E8-L. Expression levels of β-casein were compared using real-time PCR from tree independent experiments. (B) Quantitative comparison of β-casein expression in HC11 cells, which was induced with lactogenic hormones on the conditioned ECM prepared from HEK293T cells expressing the integrin-binding motif mutant (RGE), was obtained from four independent experiments. (C) HC11 cells were induced with the lactogenic hormones in the culture medium containing the milk serum (whey) obtained from the wild type (WT) or *Mfge8*$^{-/-}$ (KO) mice. Quantitative comparison of β-casein expression was acquired from three independent experiments. Statistical significance was evaluated by Student's test (two-tailed). Error bars indicate s.e.m. (D) Integrin β5 in HC11 cells was stained with a membrane marker PKH67 (left panels) and MFG-E8 (right panels). Integrin β5 was absent from the apical membrane (arrowheads in the left panels). Lower row of the right panels shows a single focal plane of the basal side indicated by a dotted white line in the top panel. Scale bars indicate 10 μm in the left panels and 20 μm in the right panels.