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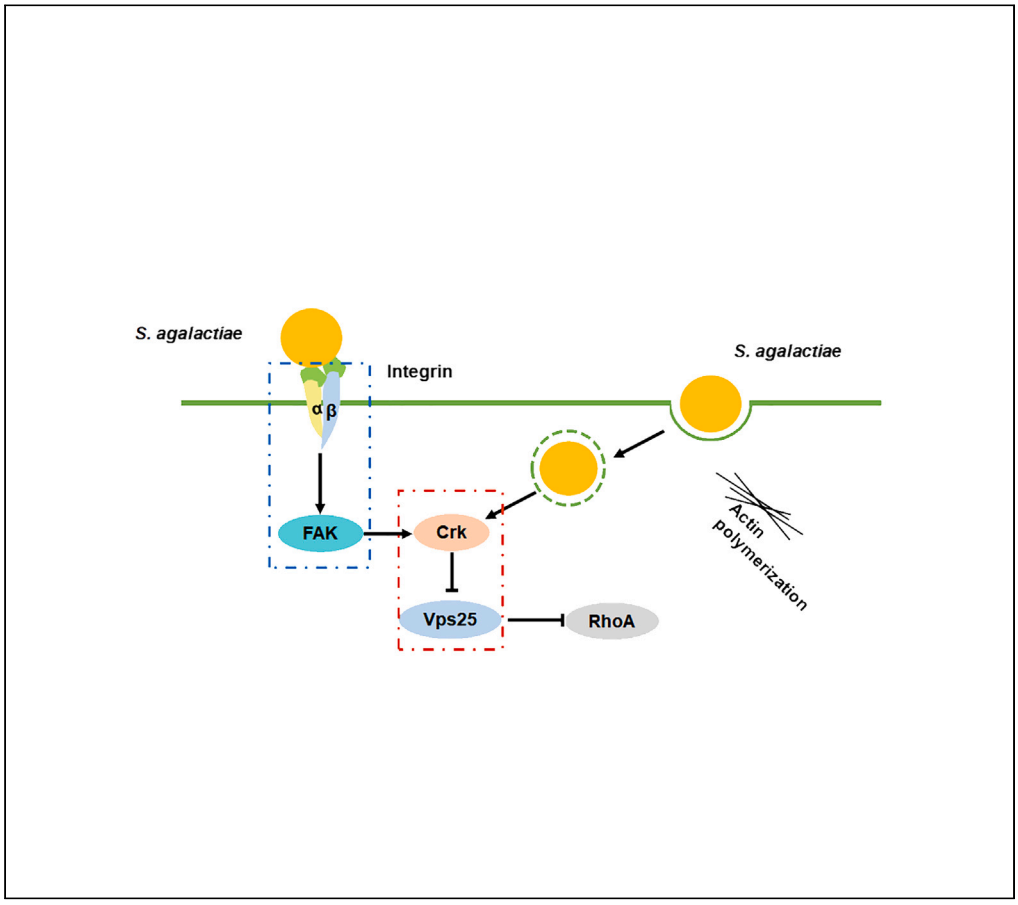

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Highlights

S. agalactiae infected BMECs through ECM laminin β2 and membrane integrin

Integrin/FAK signaling mediates intracellular infection of BMECs by *S. agalactiae*

Crk cooperates with Vps25 to defend against intracellular infection by *S. agalactiae*

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Crosstalk between integrin/FAK and Crk/Vps25 governs invasion of bovine mammary epithelial cells by *S. agalactiae*

Zhixin Guo,[1,2,4] Yuze Ma,[1,4] Zhibo Jia,[1] Liping Wang,[1] Xinyue Lu,[1] Yuhao Chen,[1,3] Yanfeng Wang,[1] Huifang Hao,[1] Shuixing Yu,[1,*] and Zhigang Wang[1,5,*]

SUMMARY

Streptococcus agalactiae (_S. agalactiae_) is a contagious obligate parasite of the udder in dairy cows. Here, we examined _S. agalactiae_-host interactions in bovine mammary epithelial cells (BMECs) _in vitro_. We found that _S. agalactiae_ infected BMECs through laminin β2 and integrin. Crk, Vps25, and RhoA were differentially expressed in _S. agalactiae_-infected cells. _S. agalactiae_ infection activated FAK and Crk. FAK deficiency decreased the number of intracellular _S. agalactiae_ and Crk activation. Knockdown of _Crk_ or _Vps25_ increased the level of intracellular _S. agalactiae_, whereas its overexpression had the opposite effect. RhoA expression and actin cytoskeleton were altered in _S. agalactiae_-infected BMECs. Crk and Vps25 interact in cells, and invaded _S. agalactiae_ also activates Crk, allowing it to cooperate with Vps25 to defend against intracellular infection by _S. agalactiae_. This study provides insights into the mechanism by which intracellular infection by _S. agalactiae_ is regulated in BMECs.

INTRODUCTION

As one of the most common diseases in dairy cows, bovine mastitis affects milk production and composition, lowering milk yield and the nutritional quality of milk.[1] *Streptococcus agalactiae*, also known as group B *Streptococcus* (GBS), is a contagious pathogen that causes intramammary infections in dairy cows;[2,3] this bacterium can internalize into bovine mammary epithelial cells (BMECs).[4] *S. agalactiae* expresses surface proteins (adhesins), such as fibrinogen (FBG)-binding protein A/B (FbsA/B), streptococcal fibronectin-binding protein A (SfbA), and laminin-binding protein (Lmb), that bind to host extracellular matrix (ECM) proteins, including fibrinogen (FBG), fibronectin (Fn), and laminin (Lm), which initiate the interaction of the bacteria with integrins on the surface of the host cell, allowing the bacteria to internalize into the host cell.[5–7] Integrins are the dominant receptors for ECM proteins and comprise α and β subunits.[8] Certain bacteria, including *S. agalactiae*, bind integrins via ECM proteins and use the former as receptors for internalization into host cells.[8–11] The classical integrin pathway is critical for this process.

Integrin-mediated uptake of microbial pathogens induces the formation of integrin-associated focal adhesions and their recruitment to the sites of bacterial attachment, and focal adhesion kinase (FAK) becomes activated during the internalization of pathogen into host cells, with subsequent reorganization of the actin cytoskeleton.[11–13] Integrin/FAK signaling mediates the internalization of microbial pathogens into host cells.[9] FAK is a nonreceptor tyrosine kinase that serves as a regulatory center for many signaling pathways,[14] and it integrates extracellular signals, such as integrins and mechanical pulling.[15,16] The C-terminal focal adhesion targeting (FAT) domain of FAK binds to naked protein (talin) and paxillin to localize FAK to integrin-enriched regions, altering the activity of Rho-GTPases[17] and rearranging the cytoskeleton.

During infection, dynamic host signaling pathways, gene expression, and signal molecules are stimulated to regulate pathogenic invasion and intracellular clearance, involving Crk (the CT10 regulator of kinase), ESCRT (the endosomal sorting complex that is required for transport) machinery, and RhoA-GTPase.[18–20] Crk proteins, including CrkI, CrkII, and CrkL, are v-Crk homologs and are all classified as chaperone proteins.[20] These proteins were originally demonstrated to be involved in apoptosis, adhesion, and migration.[21–23] Crk is a canonical adaptor, consisting of Src homologs 2 and 3 (SH2, SH3), and mediates several mechanisms of bacterial infection.[23,24] The phosphorylation of Crk at Tyr221 leads to reduced activity of Crk signaling.[22,23] The ESCRT machinery can be subdivided into three canonical subcomplexes and is associated with cell division, autophagy, viral replication, and host-pathogen interactions[25] and restricts bacterial growth.[26] Vps 25 (vacuolar protein sorting 25) is a subunit of ESCRT-II that is involved in activating this system and pathogenic infection.[25,27,28] Recent reports have shown that Vps25 participates in responses to bacterial proliferation[29] and viral replication.[30]

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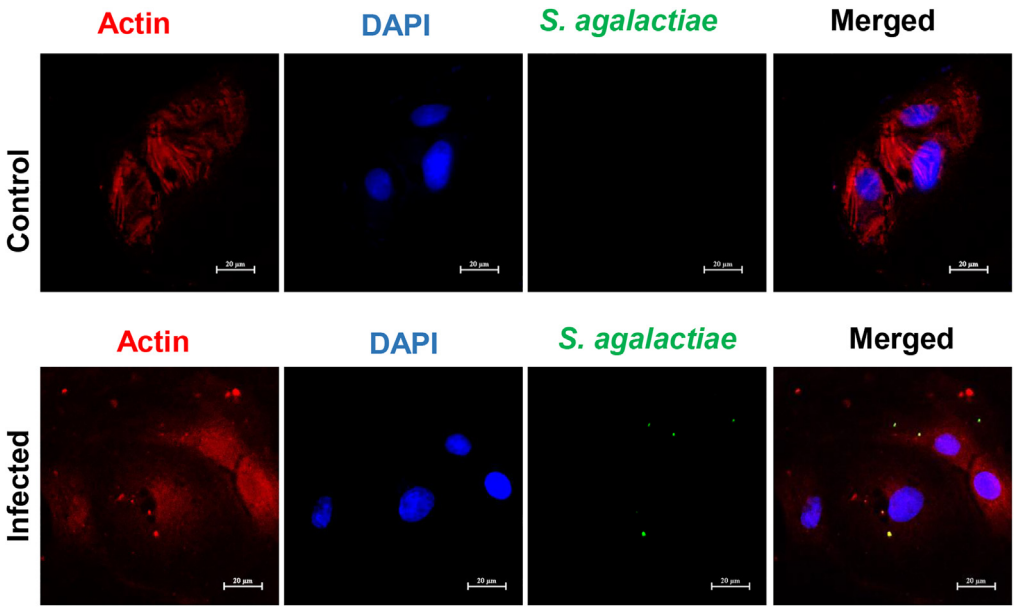

Figure 1. *S. agalactiae* invades BMECs

S. agalactiae was stained with CFSE (green) and used to infect BMECs at an MOI of 100 for 2 h, and the *S. agalactiae*-infected BMECs were maintained in culture medium with antibiotics and lysozyme to kill and lyse the extracellular bacteria. Nuclei were costained with DAPI (blue), and actin was stained with Alexa Fluor 594 Phalloidin (red), and cells were observed by laser scanning confocal microscopy. Scale bars represent 20 μm n = 3 independent experiments.

The actin cytoskeleton is a highly regulated complex in which there is a dynamic equilibrium between globular actin (G-actin) and filamentous actin (F-actin) that regulates such processes as phagocytosis, cell division, cell motility, and cell polarization.[31] The Rho family of small GTPases controls these actin cytoskeletal dynamics.[32] Actin cytoskeletal structures include cortical actin, stress fibers, lamellipodia, and microspikes, and RhoA induces actin stress fiber formation as a key regulator of actin cytoskeletal reorganization.[32,33] Changes in RhoA activity and synthesis are linked to reorganization of the actin cytoskeleton. RhoA proteins stimulate reorganization of the actin cytoskeleton through Rho kinases (ROCKs), which are major downstream effectors of RhoA.[34] Exploitation of a host cell's actin cytoskeleton is pivotal for many microbial pathogens to enter cells,[35] and the uptake of intracellular pathogens, including *Salmonella* and *Shigella* species, into nonphagocytic gut epithelial cells depends on the activation of Rho-mediated actin rearrangement.[36] Inhibition of integrin signaling effectors, such as protein tyrosine kinases—including focal adhesion kinase (FAK), Src, and RhoA—reduces intracellular invasion by *Orientia tsutsugamushi*.[37]

By adhering to the extracellular matrix, bacteria activate receptors on the cell membrane (e.g., integrin), stimulate downstream signaling pathways, and promote cytoskeletal rearrangement. The integrin/FAK pathway regulates this process, which mediates bacterial invasion of host cells. However, it is unknown whether the integrin/FAK pathway, Crk, and Vps 25 govern bacterial invasion of BMECs, and the relationship between these proteins is poorly understood. To determine the function and mechanism of the integrin/FAK pathway, Crk, and Vps 25 in invasion of BMECs by *S. agalactiae*, we examined the interplay between integrin/FAK signaling and the Crk/Vps25 axis in controlling infections. The results of this study provide insights into the mechanism by which *S. agalactiae* invades BMECs.

RESULTS

S. agalactiae infects bovine mammary epithelial cells through laminin β2

To determine whether *S. agalactiae* invades BMECs, we infected BMECs with this bacterium for 2 h at various MOIs: 10, 20, 40, 60, 80, and 100. The infected cells were maintained in medium with antibiotics and lysozyme to kill and lyse the bacteria, and the bacteria were evaluated intracellularly and extracellularly by bacterial colony count. Bacteria were present in the cell lysates but not culture medium, and the intracellular bacteria levels rose with increasing MOI (Table S1)—approximately 2.22×10^3 CFU/mL in the cell lysates at MOI 100. To further demonstrate the ability of *S. agalactiae* to invade BMECs, the bacteria, nucleus, and cytoskeleton were stained separately and observed under a laser scanning confocal microscope. As a results, labeled *S. agalactiae* was observed in BMECs (Figure 1) and MAC-T cells (Figure S1A). Thus, cells were infected with *S. agalactiae* for 2 h at MOI 100 in subsequent experiments. Further, to confirm the intracellular *S. agalactiae*, the MAC-T bovine mammary epithelial cells were infected with this bacterium, and *S. agalactiae* was also found in cytosolic vacuoles in MAC-T cells by TEM (Figure S1B). To complement the data from staining of bacteria, trypan blue was used to quench the extracellular CFSE-labeled *S. agalactiae*. By flow cytometry, compared with the control, trypan blue had no effect on the number of fluorescent cells in treatment group (Figure S1C). These results indicate that *S. agalactiae* was internalized by BMECs and MAC-T cells *in vitro*.

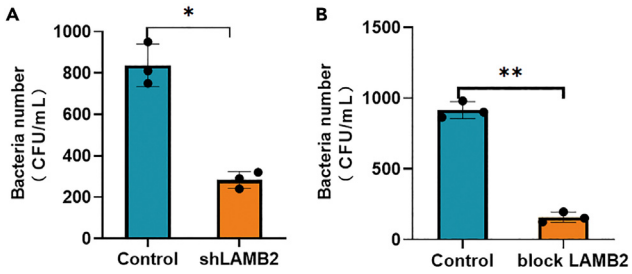

Figure 2. Laminin β2 mediates invasion of BMECs by *S. agalactiae*

(A) Intracellular bacterial count in *LAMBP2*-silenced BMECs infected with *S. agalactiae* for 2 h at MOI 100.

(B) Intracellular bacterial count in BMECs incubated with anti-laminin β2 and infected with *S. agalactiae*. *$p < 0.05$, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

To determine whether inhibition of phagocytosis was specifically restricted to the engulfment of *S. agalactiae*, BMECs were treated with Cytochalasin B, an inhibitor of actin, and intracellular bacteria were detected. The results showed that the number of intracellular bacteria were decreased in Cytochalasin B-treated cells (Figure S2A). To examine whether the bacterium proliferates in BMECs, we performed a gentamicin protection assay, and the results showed that *S. agalactiae* proliferated in cells after intracellular infection (Figure S2B). These results indicate that *S. agalactiae* invades BMECs and proliferates in cells.

Next, to examine whether *S. agalactiae* invades BMECs through laminin β2, *LAMB2*, the laminin β2-encoding gene, was silenced by shRNA in BMECs (Figure S3), and the cells were infected with *S. agalactiae* for 2 h at MOI 100. By colony count, the number of intracellular *S. agalactiae* in infected cells declined significantly compared with control (Figure 2A) ($p < 0.05$). Further, to confirm that *S. agalactiae* invasion was mediated by laminin β2, BMECs were incubated with an antibody to laminin β2 and infected with *S. agalactiae*. The number of intracellular *S. agalactiae* in antibody-blocking cells decreased significantly versus the control (Figure 2B) ($p < 0.05$). These data indicate that laminin β2 mediates the invasion of BMECs by *S. agalactiae*.

S. agalactiae invasion and FAK activation are dependent on integrin in BMECs

To examine the function of integrin during internalization of *S. agalactiae* by BMECs, the integrin-specific inhibitor RGD was used to pretreat BMECs for 24 h, after which they were infected by *S. agalactiae*. To exclude the effects of RGD on BMEC proliferation, we first performed MTT assay to measure cell proliferation, showing that RGD treatment is harmless to BMECs at 2.5 μM–10 μM for 24 h (Figure S4) ($p > 0.05$). Thus, the BMECs were pretreated with 5 μM RGD for 24 h and then infected by *S. agalactiae* for 2 h at MOI 100. The results showed that RGD decreased the intracellular bacteria number compared with the control (Figure 3A) ($p < 0.01$). Further, to confirm the function of integrin, ITGB1, the gene that encodes integrin subunit β1, was silenced by shRNA in BMECs (Figure S5). As a result, infection by *S. agalactiae* was impaired versus control (Figure 3B) ($p < 0.01$). These data indicate that integrin is vital in the invasion of BMECs by *S. agalactiae*.

Integrins are large, membrane-spanning, heterodimeric proteins, the ectodomains of which can interact with certain matrix proteins (receptors for pathogenic adhesion), whereas their cytoplasmic domains initiate the assembly of large signaling complexes that lead to the recruitment and activation of FAK, constituting the integrin/FAK pathway.[9] To determine the function of integrin in FAK activation in *S. agalactiae*-infected BMECs, BMECs were pretreated with 5 μM RGD for 24 h and then infected with *S. agalactiae* for 2 h. By western blot, *S. agalactiae* infection enhanced FAK phosphorylation (Tyr397) (Figure 3C), suggesting that *S. agalactiae*-induced FAK activation depends on integrin in BMECs.

Differential expression of invasion-related proteins in *S. agalactiae*-infected BMECs

To identify important proteins that are involved in the invasion of BMECs by *S. agalactiae*, a comparative proteomic analysis was performed. BMECs were infected with *S. agalactiae* for 2 h at MOI 100. Then, protein extracts of the infected or uninfected BMECs were subjected to proteomic analysis. Proteins with a log2 ratio ≥ 1 and FDR ≤ 0.01 were considered to be significant differentially expressed proteins (DEPs). As a result, 170 DEPs were identified (uniprot database, https://www.uniprot.org/) (Table S2) and subjected to KEGG pathway annotation (https://www.kegg.jp/) (Table S3; Figure S6). By functional screening, the 170 DEPs were mainly distributed in MAPK signaling pathway, metabolic pathways, and PI3K-Akt signaling pathway, of which 37 DEPs are closely associated with invasion of *S. agalactiae* and could be analyzed by KEGG and GO, 16 DEPs were significantly upregulated, and 21 DEPs were significantly downregulated on *S. agalactiae* infection, including upregulated Crk and downregulated vps25 and RhoA. Further analysis showed that Crk, vps25, and RhoA were related to the MAPK pathway, endocytosis, and cytoskeletal rearrangement, respectively. Thus, Crk, Vps25 and RhoA were selected for further validation and subsequent experiments. By western blot, Crk was upregulated in *S. agalactiae*-infected BMECs, whereas Vps25 and RhoA were downregulated compared with control (Figure 4), and these patterns were also observed in *S. agalactiae*-infected MAC-T cells (Figure S7), consistent with the proteomic analysis.

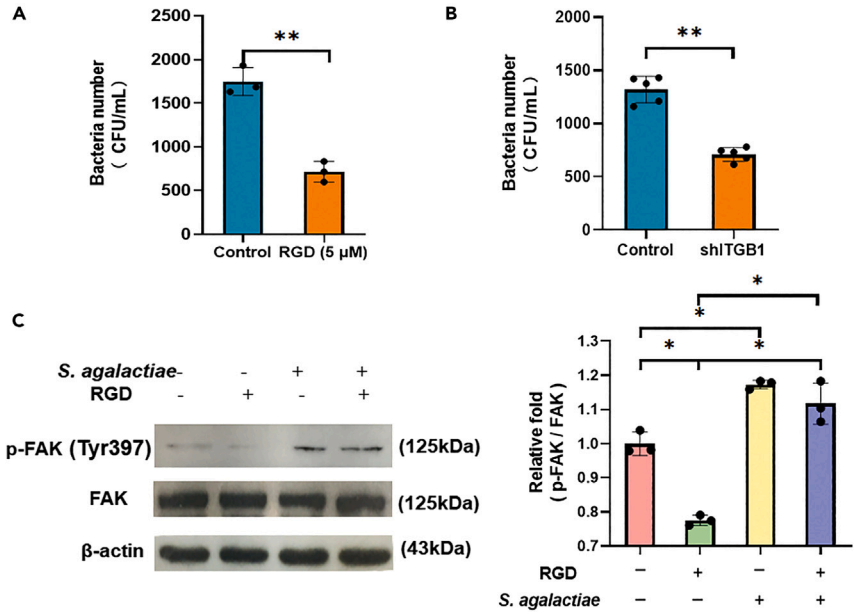

Figure 3. Integrin mediates invasion of BMECs by *S. agalactiae* and FAK activation

(A) BMECs were pretreated with 5 μM RGD for 24 h and infected with *S. agalactiae* for 2 h at MOI 100.

(B) Silencing of *ITGB1* impairs *S. agalactiae* infection.

(C) *S. agalactiae* infection enhances FAK phosphorylation (Tyr397), whereas RGD inhibits it. *$p < 0.05$, **$p < 0.01$, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

FAK mediates internalization of *S. agalactiae* and Crk phosphorylation in BMECs

Our data demonstrated that FAK is activated by *S. agalactiae* in BMECs (Figure 2C). Moreover, Crk was upregulated in the proteomic analysis. Previous reports have shown that Crk is essential for the interaction between host cells and pathogens[20–22] and that the phosphorylation of Crk at Tyr221 negatively regulates Crk signaling.[22,23] Thus, we hypothesized that FAK is important in the internalization of *S. agalactiae* and is associated with Crk in BMECs.

To examine the functions of FAK in the internalization of *S. agalactiae* and Crk activation, BMECs were pretreated with TAE226, a specific inhibitor of FAK, for 24 h and infected with *S. agalactiae* for 2 h at MOI 100. By colony count, the number of intracellular *S. agalactiae* declined significantly compared with the control (Figure 5A) ($p < 0.01$). Further, we examined the phosphorylation of FAK at Tyr397 and Crk and its phosphorylation at Tyr221 by western blot. The results showed that FAK was inhibited by TAE226 and stimulated by *S. agalactiae* (Figure 5B), whereas the phosphorylation of Crk at Tyr221 was stimulated by TAE226, whereas inhibited by the bacteria, and this phosphorylation at Tyr221 was alleviated by TAE226 (Figure 5B). These data indicate that FAK and Crk were activated by *S. agalactiae* and FAK negatively regulates Crk phosphorylation at Tyr221 or that FAK positively regulates Crk activity. Further, RhoA expression upregulated in TAE226-treated cells, while downregulated in *S. agalactiae*-invaded cells (Figure 5B), indicating the reorganization of actin cytoskeleton was arranged due to the bacteria invasion.

To confirm these data, FAK was knocked down by shRNA in MCF-10A nonneoplastic breast epithelial cells (Figure S8). The number of intracellular *S. agalactiae* in FAK-silenced cells decreased significantly versus control (Figure 6A) ($p < 0.05$), and the phosphorylation of Crk (Tyr221) was enhanced by shFAK, whereas inhibited by *S. agalactiae* (Figure 5B). Further, RhoA expression was upregulated by FAK silencing but downregulated by *S. agalactiae* (Figure 6B). These data demonstrate that FAK mediates the internalization of *S. agalactiae*, positively regulates Crk activation, and negatively regulates RhoA expression in mammary epithelial cells.

Crk cooperates with Vps25 to prevent *S. agalactiae* invasion and regulates the expression of RhoA

Based on the comparative proteomic analysis, Crk, Vps25, and RhoA are DEPs that are related to *S. agalactiae* infection in BMECs. The data above also showed that *S. agalactiae* infection suppresses Crk (Tyr221) phosphorylation and indicated that Crk is activated by this bacterium (Figures 4B and 5B). Thus, we hypothesized that Crk regulates the internalization of *S. agalactiae* by BMECs. We first knocked down Crk by shRNA in BMECs (Figure S9) and infected them with *S. agalactiae*. The number of *S. agalactiae* increased significantly in Crk-silenced cells compared with control (Figure 7A) ($p < 0.01$). Further, on overexpression of Crk in BMECs (Figure S10), the number of *S. agalactiae* fell significantly in the cells versus control (Figure 7B) ($p < 0.01$). These data indicate that Crk is a negative regulator during bacterial invasion.

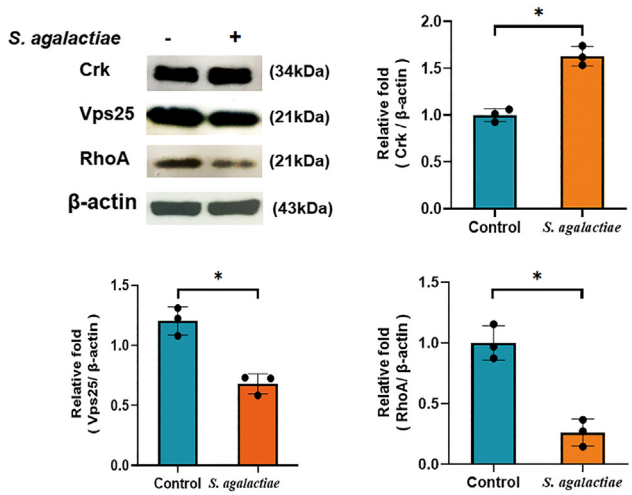

Figure 4. Verification of differential expressed proteins in *S. agalactiae*-infected BMECs identified by comparative proteomic analysis

Crk, Vps25, and RhoA were examined by western blot in *S. agalactiae*-infected BMECs. *p < 0.05, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

We then examined the function of Crk in the expression of Vps25 and RhoA in Crk-silenced and *S. agalactiae*-infected cells. The expression of Vps25 and RhoA decreased in both cell types compared with control (Figure 7C), suggesting that Crk prevents *S. agalactiae* invasion and regulates the expression of Vps25 and RhoA in BMECs.

Next, to determine the function of Vps25 in *S. agalactiae* infection, a targeting shRNA was designed to silence Vps25 in BMECs (Figure S11), after which the cells were infected with *S. agalactiae*. The number of *S. agalactiae* rose significantly in Vps25-silenced cells compared with control (Figure 8A) ($p < 0.01$). On overexpression of Vps25 in BMECs (Figure S12), the number of *S. agalactiae* decreased significantly versus control (Figure 8B) ($p < 0.05$). These data indicate that Vps25 prevents *S. agalactiae* invasion. Further, by western blot, Vps25 was significantly downregulated in Vps25-silenced and *S. agalactiae*-infected cells, and shVps25 and *S. agalactiae* infection altered the expression of RhoA (Figure 8C). These data suggest that Vps25 opposes intracellular *S. agalactiae*. Furthermore, to eliminate the possibility of *S. agalactiae* adhesion to affect the expression of FAK, *p*-Crk, and Vps25, we first examined adhesion of *S. agalactiae* to BMECs and found that the bacteria only adhered to BMECs but failed to internalize within 15 min (Table S4). Next, BMECs were infected with *S. agalactiae* for 15 min at MOI 100, and then the cells were cultured for 2 h in a medium with antibiotics and lysozyme. By Western blot, there was no significant difference between the bacterial adhesion group and the control (Figure S13), indicating that *S. agalactiae* adhesion has no effect on the expression of FAK, *p*-Crk, and Vps25.

Based on the data above, we reasoned that Crk and Vps25 cooperate to resist the internalization of *S. agalactiae*. By coimmunoprecipitation, Crk bound Vps25 (Figure 8D), suggesting that Crk interacts with Vps25 in BMECs. These data demonstrate that Crk cooperates with Vps25 to prevent *S. agalactiae* invasion and regulates the expression of RhoA in BMECs.

DISCUSSION

Bovine mastitis is the most frequent disease in dairy cows[38] and an economic burden for farmers and adversely affects the global dairy industry. Subclinical mastitis is characterized by increases in milk bacterial pathogens and somatic cell count (SCC) in response to bacterial infections, decreasing milk yield and altering milk composition.[39,40] However, the molecular mechanisms of subclinical mastitis remain unknown, because the cause of chronic inflammation of the udder in cows is complex, in which many pathogens are involved. *S. agalactiae* is a contagious obligate parasite of the bovine mammary gland,[41,42] and intramammary infections (IMIs) that are caused by *S. agalactiae* primarily induce subclinical mastitis and elevate the SCC in cows.[42] *S. agalactiae* can parasitize the mammary tissue or be internalized by mammary cells, such as mammary epithelial cells,[4,43] when an intramammary infection occurs. The bacteria that invade these cells are more difficult to be killed by drugs and for the host immune system to eliminate them, and they can colonize the cells, leading to chronic infection of the cow udder. Recent reports have demonstrated important proteins that are related to the inflammatory response and the harmful effects of subclinical mastitis due to intracellular *S. agalactiae* infection in dairy cows.[4,44] In this study, we confirmed that *S. agalactiae* can internalize into BMECs and identified significant DEPs that respond to bacterial infection, including Crk, vps25, and RhoA.

Pathogenic bacteria use surface-bound adhesins to enter into specific interactions with extracellular matrix proteins and membrane receptors of host cells to infect nonphagocytic cells, leading to cytoskeletal rearrangements that allow the bacteria to enter the cell.[45] Integrins are heterodimeric transmembrane proteins in mammals and are the dominant receptors for the ECM that are critical for pathogenic bacteria-cell adhesion, mediating cellular internalization of the bacteria.[9,11] For example, integrins mediate the invasion of BMECs by *Staphylococcus aureus*, another main pathogen of mastitis in dairy cows.[46,47] In fact, *S. agalactiae* also exploits integrins when invading human endothelial

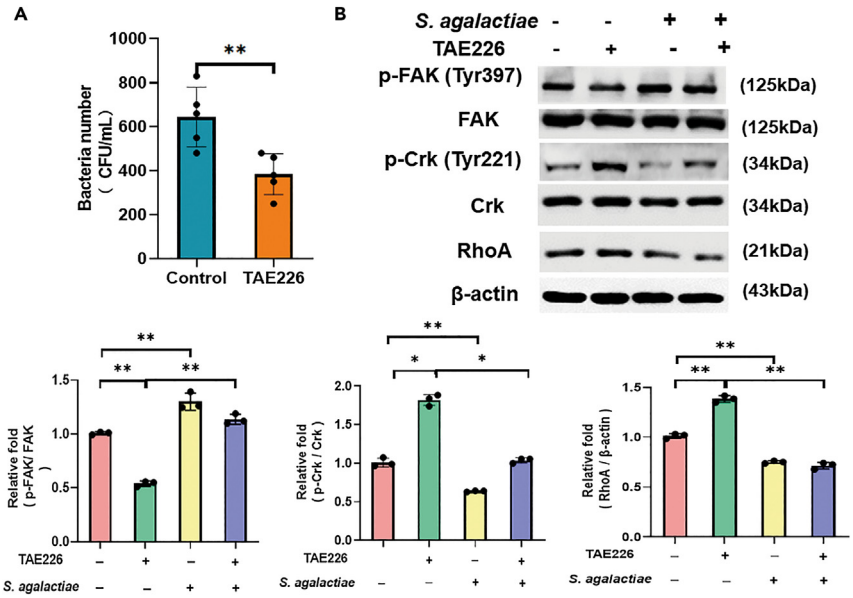

Figure 5. TAE226 inhibits *S. agalactiae* internalization and enhances Crk phosphorylation in BMECs

BMECs were pretreated with TAE226 for 24 h and infected with *S. agalactiae* for 2 h at MOI 100.

(A) Colony count of *S. agalactiae*.

(B) Western blot of phosphorylation of FAK at Tyr397 and Crk at Tyr221. *p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

cells,[48,49] but it is unknown whether it uses them to internalize into BMECs. In this study, we confirmed that laminin β2 and integrin are vital in the invasion of BMECs by *S. agalactiae* and mediate *S. agalactiae*-induced FAK activation in BMECs, as in *S. agalactiae*-infected human endothelial cells[50] and *Staphylococcus aureus*-infected murine osteoblasts.[51] Integrin/FAK signaling has significant function during the infection of BMECs by *S. agalactiae*.

FAK is an important modulator of integrin-dependent signaling and is stimulated by pathogenic infection, thus participating in remodeling the actin cytoskeleton during the entry of invasive pathogens into nonphagocytic cells.[52] FAK deficiency affects resistance to invasion by *S. agalactiae* in human endothelial cells[50] and by *S. aureus* in murine osteoblasts.[51] In the current study, we confirmed that FAK mediates the internalization of *S. agalactiae* in BMECs, and the number of intracellular *S. agalactiae* in FAK-deficient cells is significantly decreased. In addition, the phosphorylation of Crk at Tyr221 was inhibited by *S. agalactiae*, which was alleviated by an FAK inhibitor. Notably, Crk Tyr221 phosphorylation induces an inhibitory state in Crk;[22] thus, suppression of Crk phosphorylation (Tyr221) means promoting its active state. FAK positively regulates Crk activity by impairing Crk phosphorylation (Tyr221). FAK is an activator of Crk during *S. agalactiae* infection in of BMECs.

Crk is closely related to bacterial invasion as an articulation protein, contributing to host cell and bacterial interactions,[24] and Vps25 is an important component of the ESCRTs, which are associated with pathogenic infection.[25] The interaction between Crk and Vps25 has not been examined—only that Crk and an ESCRT II component (EAP45) have increased levels in virus-infected cells[18] and that Vps25 participates in classical swine fever virus (CSFV) endocytosis.[53] In our study, proteomic analysis and western blot have shown that the activity of Vps25 was inhibited during *S. agalactiae* infection. The intracellular number of *S. agalactiae* increased significantly in *Vps25*-silenced cells, while the opposite in Vps25-overexpressed cells. *S. agalactiae* infection decreased Crk (Tyr221) phosphorylation (enhancing its activity), and Crk positively regulates the expression of Vps25 in BMECs, indicating that Crk cooperates with Vps25 to defend against *S. agalactiae* infection in BMECs. Earlier reports have implicated Crk in invasion by *Salmonella*, *Shigella*, and *Yersinia*.[24,54,55] The reasons for this difference between species and hosts remain to be determined.

RhoA is a key regulator of reorganization of the actin cytoskeleton.[32,33] Changes in RhoA activity and synthesis lead to cytoskeletal reorganization,[56] and alterations in RhoA and the cytoskeleton are exploited by pathogens to enter cells.[36,37,57] Integrin-binding ligand enhances α5β1 integrin activation and initiates RhoA,[58] and loss of laminin β1 (LAMB1) affects the dysregulation of actin.[59] Integrins and their dominant ECM ligands are associated with RhoA and cytoskeletal reorganization. FAK regulates actin polymerization during sperm capacitation via ERK2/RhoA signaling.[60] Crk associates with ERM (ezrin-radixin-moesin) proteins, activates RhoA, and promotes cell motility.[61] In our study, we found that the invasion of BMECs by *S. agalactiae* decreased the expression of RhoA, led to reorganization of the actin cytoskeleton, and that deficiency of integrin, FAK, Crk, and Vps25 alters RhoA expression in BMECs. These data indicate that RhoA mediates the cytoskeletal rearrangement during infection of BMECs by *S. agalactiae*.

In summary, *S. agalactiae* causes intracellular infections in BMECs *in vitro*, mediated by the ECM laminin β2 and membrane integrin, which are the dominant receptors for the ECM proteins. The signaling adaptor Crk; an important component of ESCRT, Vps25; and RhoA, a key

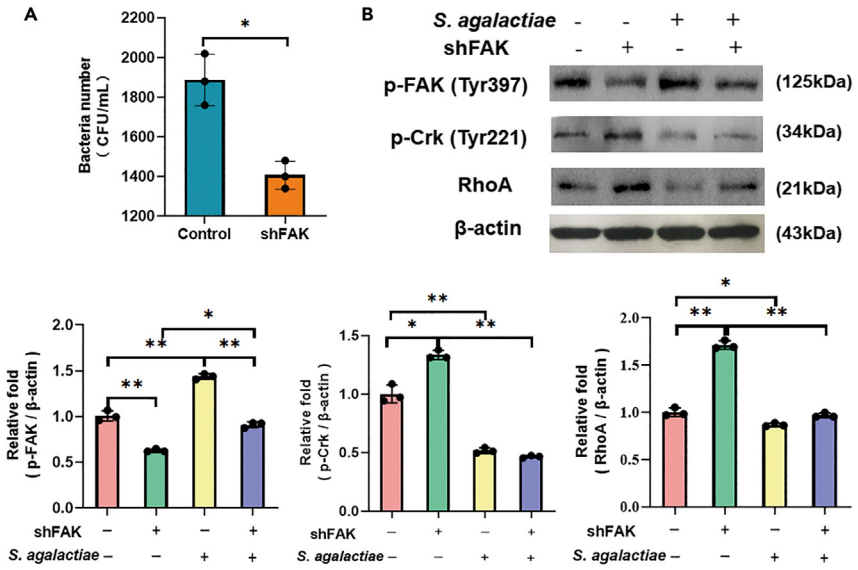

Figure 6. Knockdown of *FAK* decreases the number of intracellular *S. agalactiae* and enhances the phosphorylation of Crk in MCF-10-A cells

FAK was silenced by a targeting shRNA in MCF-10A nonneoplastic breast epithelial cells, and the cells were infected with *S. agalactiae* for 2 h at MOI 100.

(A) Knockdown of *FAK* decreases the number of *S. agalactiae* in MCF-10A cells compared with control.

(B) Knockdown of *FAK* enhances the phosphorylation of Crk at Tyr221 and alters the expression of RhoA in MCF-10A cells. *p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

regulator of actin cytoskeleton reorganization, are differentially expressed in *S. agalactiae*-infected cells. *S. agalactiae* infection induces integrin/FAK signaling, and FAK mediates the internalization of this pathogen into BMECs. FAK deficiency enhances Crk phosphorylation, leading to its inactive state. Crk acts as a negative regulator during bacterial internalization and positively regulates the expression of Vps25. Vps25 cooperates with Crk to defend against intracellular infection of BMECs by *S. agalactiae*. Integrin/FAK signaling is beneficial to *S. agalactiae* infection, whereas Crk/Vps25 axis is an unfavorable effector for this bacterium infection in BMECs. Crosstalk between integrin/FAK signaling and the Crk/Vps25 axis controls the internalization of *S. agalactiae* into BMECs *in vitro*. This study demonstrates synergy between Crk and Vps25 to prevent bacterial infection, providing insights into the mechanism by which intracellular *S. agalactiae* infections are regulated in BMECs.

Limitations of the study

Our work on the *S. agalactiae*-host interactions in bovine mammary epithelial cells (BMECs) illustrated the crosstalk between integrin/FAK signaling, and Crk/Vps25 axis governs invasion of bovine mammary epithelial cells by *S. agalactiae*. *S. agalactiae* infected BMECs by ECM laminin β2 and membrane integrin, and integrin/FAK signaling is beneficial to *S. agalactiae* infection, whereas Crk/Vps25 axis is an unfavorable effector for this bacterium infection in BMECs. However, this work has some limitations, including the use of other cell models, the design of mouse model, and the limitation of experimental validation. Firstly, the role of Crk/Vps25 axis in invasion of nonlactating cells by *S. agalactiae* is unknown. Secondly, data obtained from *S. agalactiae*-BMECs model need to be verified by animal model. Finally, further studies are essential to testify the role of integrin/FAK signaling and Crk/Vps25 axis in internalization of BMECs by pathogens.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
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  - Cell culture
  - *S. agalactiae* infection of BMECs

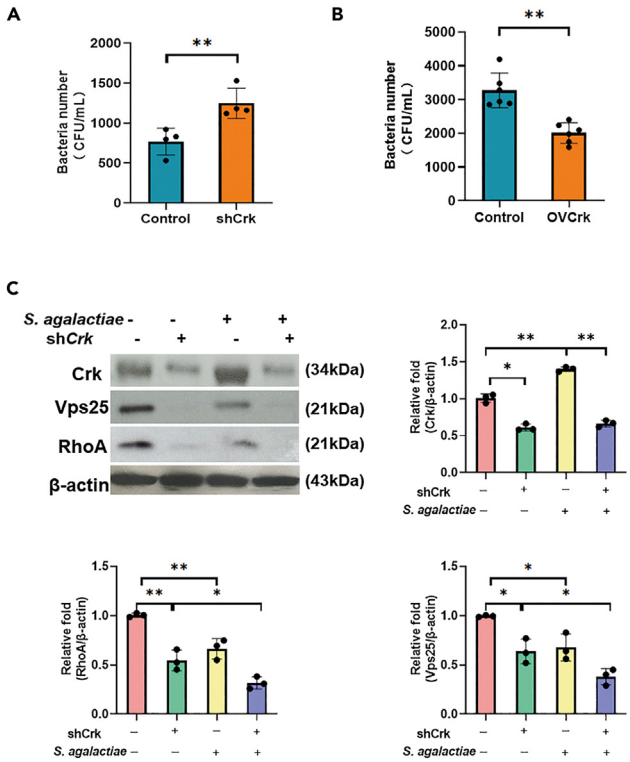

Figure 7. Crk prevents *S. agalactiae* invasion and regulates the expression of Vps25 and RhoA in BMECs

Crk was silenced or overexpressed in BMECs, and the cells were infected with *S. agalactiae* for 2 h at MOI 100.

(A and B) (A) Knockdown of *Crk* increases the number of intracellular *S. agalactiae* in BMECs, whereas its overexpression decreases it compared with control (B).

(C) Knockdown of *Crk* downregulates Vps25 and RhoA. *p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

- Bacterial colony count
- Staining of bacteria and BMECs
- Gentamicin protection assay
- Transmission electron microscopy (TEM)
- Trypan blue staining assay
- Cytochalasin B treatment
- Anti- laminin β2 blocking assay
- Adhesion assays
- Comparative proteomic analysis
- Western blot analysis
- MTT
- Transfection *in vitro*
- RT-qPCR
- Coimmunoprecipitation

● QUANTIFICATION AND STATISTICAL ANALYSIS

- Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107884.

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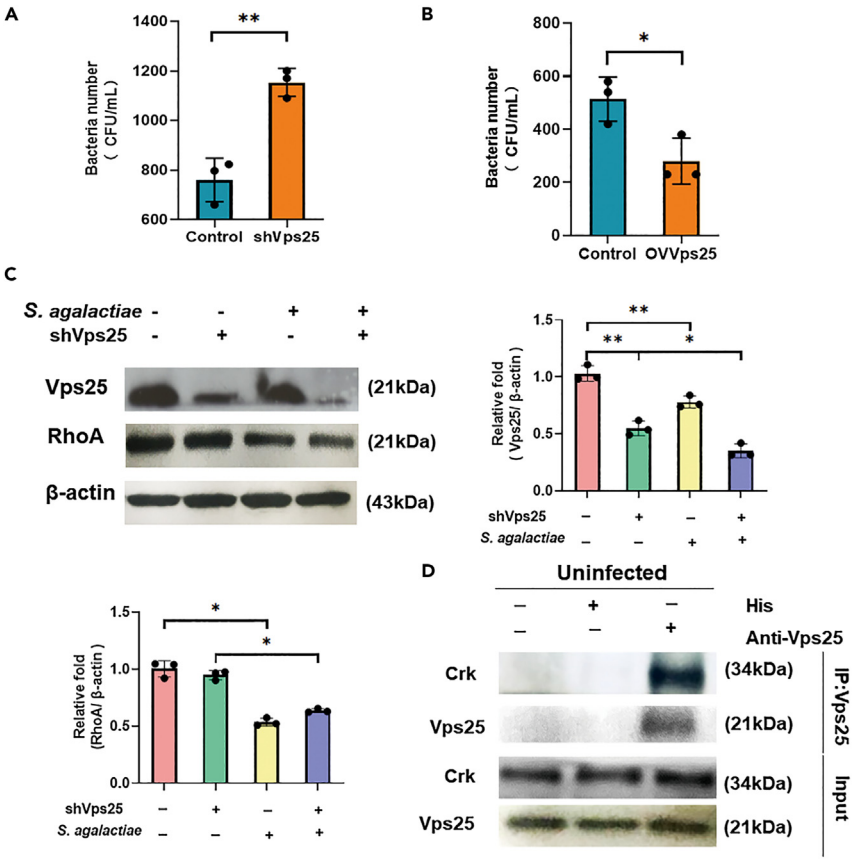

Figure 8. Vps25 protects against *S. agalactiae* invasion and cooperates with Crk in BMECs

Vps25 was silenced or overexpressed in BMECs, and the cells were infected with *S. agalactiae* for 2 h at MOI 100.

(A and B) (A) Knockdown of *Vps25* increases the number of intracellular *S. agalactiae* in BMECs, whereas its overexpression decreases it, compared with control (B).

(C) Knockdown of *Vps25* decreases the expression of RhoA.

(D) Coimmunoprecipitation of Vps25 and Crk with anti-Vps25. N1 denotes no antibody, and N2 is anti-his. Total protein lysates of BMECs were used as a positive control. *p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey's method. n = 3 independent experiments. Error bar indicates SD.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Crk	abcam	Cat#ab45136; RRID: AB_881843
Rabbit anti-p-Crk (Tyr221)	abcam	Cat#ab76227; RRID: AB_2245325
Goat anti-Vps25	abcam	Cat#ab42500; RRID: AB_778893
Rabbit anti-RhoA	cst	Cat#67B9
Rabbit anti-FAK	Santa	Cat#sc-557; RRID: AB_2253252
Rabbit anti-p-FAK (Tyr397)	Becton Dickinson	Cat#611806; RRID: AB_399286
Mouse anti-β-actin	Sigma-Aldrich	Cat#A5441; RRID: AB_476744
Rabbit anti-Goat IgG-HRP	absin	Cat#Abs20005ss
Rabbit anti-Integrin beta 1	abcam	Cat#ab179471; RRID: AB_2773020
Rabbit anti-Laminin beta 2	abcam	Cat#ab91481; RRID: AB_10563652
FITC-conjugated anti-rabbit IgG	Jackson Immuno Research Laboratories	Cat#115-095-003; RRID: AB_2338589
FITC-conjugated anti-mouse IgG	Jackson Immuno Research Laboratories	Cat#115-095-146; RRID: AB_2338599
goat anti-rabbit secondary antibodidy	Abcam	Cat# ab136817
goat anti-mouse secondary antibodidy	Abcam	Cat# ab205719; RRID: AB_2755049
Bacterial and virus strains		
Streptococcus agalactiae	ATCC	ATCC27956
Chemicals, peptides, and recombinant proteins		
RGD	Selleck	Cat#99896-85-2
TAE226	MCE	Cat#HY-13203
CFSE [5 (6)-carboxyfluorescein diacetate N-succinimidyl ester]	MCE	Cat#HY-D0938
progesterone	Sigma-Aldrich	Cat#V900699
L-Glutamine	Sigma-Aldrich	Cat#G8540
bovine insulin	Sigma-Aldrich	Cat#I6634
epidermal growth factor	Sigma-Aldrich	Cat#E4127
transferrin	Sigma-Aldrich	Cat#T8158
Triton X-100	Triton X-100	Cat#T8787
Hydrocortisone	Solarbio	Cat#507A051
penicillin	Solarbio	Cat#A8180
Alexa Fluor™ 594 Phalloidin	Invitrogen	Cat#A12381
Lipofectamine™2000	Invitrogen	Cat#11668500
DAPI	Beyotime	Cat#C1005
Trypsin	TransGen	Cat#FG301-01
4% paraformaldehyde	Biosharp	Cat#143174
Gentamicin	N/A	N/A
Lysozyme	TIANGEN	N/A
Experimental models: Cell lines		
BMECs	mammary tissue of Chinese Holstein cows	N/A
MCF-10A	ATCC	ATCC number : CLR-10317™
MAC-T	Shunran (Shanghai) Biotechnology Co., Ltd.	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
shFAK：5′-aaGCCCTCAACCAGGGATTATGATTCAAGAGA TCATAATCCCTGGTTGAGGGCtt-3′	This paper	pGPU6/GFP/Neo
shCrk：5′-aaGCAGGGTAGTGGAGTGATTCTTTCAAGAGAA GAATCACTCCACTACCCTGCtt-3′	This paper	pGPU6/GFP/Neo
shVps25 ：5′-aa GCCAGAACAACTCCGTGTTCATTCAAGAG ATGAACACGGAGTTGTTCTGGCtt-3′	This paper	pGPU6/GFP/Neo
shITGB1：5′-CACCGCTTAATATGTGGAGGAAATGTTCAAG AGACATTTCCTCCACATATTAAGCTTTTTTG-3′	This paper	pGPH1/GFP/Neo
sh LAMB2:5′- aaCGCTACATCTCAAGCTGGTGTTCAAGAGA CACCAGCTTGAGATGTAGCtt-3′	This paper	pRNAT-U6.1/Neo
Software and algorithms		
GraphPad Prism 8.0.2	Graphpad Software	https://www.graphpad.com/
ImageJ	NIH	RRID:SCR_003070
FlowJo	BD Biosciences	https://www.flowjo.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhigang Wang (lswzg@imu.edu.cn).

Materials availability

Plasmids and cell lines generated in this study are available from the lead contact upon request.

Data and code availability

- This study did not generate new unique reagents, cell lines, or mouse lines.
- Data reported in this paper and any additional information required to reanalyze the data reported in this paper are available from the lead contact upon request.
- Data from genomic and proteomic analyses mentioned in this paper are presented in the Supplementary file. Additional data reported in this article will be shared.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics statement

All experimental procedures with animals were conducted according to the guidelines for the care and use of experimental animals per the Inner Mongolia University Animal Care and Use Committee.

METHOD DETAILS

Cell culture

Primary BMECs were isolated from adherent cultures of mammary tissue of Chinese Holstein cows, after their slaughter on a commercial cattle slaughter farm.[62,63] Briefly, mammary tissue from slaughtered cows was surgically excised and placed in sterile, ice-cold phosphate-buffered saline (PBS) that was spiked with 300 U/mL penicillin G and 100 mg/mL streptomycin (Sigma-Aldrich, Inc., USA) for immediate transport to the laboratory. The mammary tissue was trimmed of visible fat and connective tissue and rinsed several times with PBS until the solution was clear and milk-free. The mammary tissue was cut into small pieces (1×1×1 mm^3), and primary cell cultures were established, from which BMECs were isolated. Isolated and purified BMECs were maintained in DMEM/F12 (Hyclone Laboratories, Inc., Logan, UT, USA) with 10% fetal bovine serum. Primary BMECs of second-generation (P2) and fourth-generation (P4) that were in the logarithmic growth phase were used for all assays. MCF-10A non-neoplastic breast epithelial cells were grown in DMEM/F12 (Hyclone Laboratories, Inc. Logan, UT, USA), supplemented with 10% heat-inactivated fetal bovine serum. MAC-T bovine mammary epithelial cells were cultured in growth medium consisting of DMEM (Hyclone Laboratories, Inc., Logan, UT, USA), which was supplemented with 10% fetal bovine serum. All cells were cultured in humidified air with 5% CO_2 at 37°C.

S. agalactiae infection of BMECs

BMECs were seeded in 6-well plates, incubated until 80% confluence, and infected with *S. agalactiae* for 2 h at MOI 100 in serum free medium; the cells were cultured sequentially in medium that contained gentamicin (100 μg/mL) and lysozyme (1 mg/mL) for 2 h, or penicillin (5 mg/mL) and lysozyme (1 mg/mL) for 2 h, to kill and lyse extracellular bacteria. Intracellularly infected cells were harvested with trypsin and centrifuged to remove the supernatant.

Bacterial colony count

S. agalactiae (ATCC27956) was cultured to the logarithmic growth phase. The multiplicity of infection (MOI) was set to 10, 20, 40, 60, 80, and 100 for infecting cells. BMECs were infected with S. agalactiae for 2 h at the indicated MOI, and extracellular bacteria were killed and lysed with antibiotics and lysozyme. BMECs were collected and lysed, and the number of bacteria in the cells and culture medium was determined by spread plate counting in Table S1.

Staining of bacteria and BMECs

Bacteria and BMECs were stained with Alexa Fluor® 594 Phalloidin.[64] Briefly, BMECs were placed on slides and incubated overnight. *S. agalactiae* were washed with PBS and stained with CFSE at room temperature for 20 min. These stained bacteria were then used to infect BMECs at an MOI of 100 for 2 h, and the BMECs were maintained in culture medium with antibiotics and lysozyme to kill and lyse extracellular bacteria. Next, the BMECs were washed 3 times with PBS and fixed with 4% paraformaldehyde for 20 min. The BMECs were then permeabilized with 1% Triton X-100, stained with Alexa Fluor 594 Phalloidin for 1 h in the dark, washed 3 times with PBS, and stained with DAPI for 3 min to assess nuclear morphology. The stained cells were analyzed and imaged by laser scanning confocal microscopy (LSCM) (NIKON AIR, Nikon Corp., Tokyo, Japan).

Gentamicin protection assay

BMECs were seeded in 12-well plates, incubated until 80% confluence. Cells were infected with *S. agalactiae* at MOI of 100 for 2 h, and the cells were washed 3 times with PBS and cultured in media containing 200 μg/mL gentamicin for 3 h, 4 h and 5 h after infection, respectively. The cells were harvested with trypsin and washed 3 times with PBS, and lysed. The number of bacteria in the cells was determined by spread plate counting.

Transmission electron microscopy (TEM)

MAC-T bovine mammary epithelial cells were infected by bacteria (*S. agalactiae*) for 2 h at an MOI of 100, and the extracellular bacteria were lysed by gentamicin and lysozyme. Briefly, the infected MAC-T cells were washed with PBS 3 times centrifuged for 10 min at 3000×*g* at 4°C, and fixed with 2.5% glutaraldehyde overnight. Finally, the cells were embedded in 4% AGAR and fixed with 2.5% glutaraldehyde overnight. The samples were examined by TEM (Hitachi HT7700, Hitachi, Ltd., Tokyo, Japan) to detect intracellular bacteria.[64]

Trypan blue staining assay

MAC-T cells were infected with *S. agalactiae* for 2 h at an MOI of 100, and the cells were maintained in culture medium with gentamicin and lysozyme for 2 h to kill and lyse extracellular bacteria. Next, the cells were washed 3 times with PBS and stained with trypan blue for 3 min. Next, the trypan blue-stained cells and control were analyzed by flow cytometry (BD LSRFortessa, Biosciences, Becton, Dickinson and Company, New Jersey, USA).

Cytochalasin B treatment

BMECs were treated with 10 μg/mL Cytochalasin B for 30 min, and then cells were infected with *S. agalactiae* for 2 h at an MOI of 100. The cells were placed on ice and washing the cultures three times to remove non-internalized bacteria. The extracellular bacteria were killed and lysed by gentamicin and lysozyme. BMECs were lysed, and the number of intracellular bacteria was determined by spread plate method.

Anti- laminin β2 blocking assay

BMECs were seeded in 6-well plates and incubated until 80% confluence. The cells were then pretreated with anti-laminin β2 for 1 h, infected with *S. agalactiae* for 2 h at MOI 100, and cultured sequentially in medium that contained gentamicin and lysozyme for 2 h to kill and lyse extracellular bacteria. The number of intracellular bacteria was determined by spread plate counting.

Adhesion assays

Adhesion assay of *S. agalactiae* was achieved in two phases. First, BMECs were infected with *S. agalactiae* at MOI 100 for 15, 30 and 60 min, respectively. End of infection, BMECs were continued and maintained for 2 h in medium with gentamicin and lysozyme. After incubation, cells were harvested with trypsin and washed three times with PBS to remove extracellular dead bacteria, and then lysed. The number of intracellular bacteria was determined by spread plate counting in Table S4. Second, BMECs were infected with *S. agalactiae* for 15 min at MOI 100, and then the cells were cultured for 2 h in a medium with gentamicin and lysozyme. After incubation, BMECs were harvested with trypsin and

washed three times with PBS to remove non-adherent bacteria, and then lysed using lysis buffer. The expression of p-Crk, Vps25 and FAK was detected by western blot.

Comparative proteomic analysis

Three groups of BMECs were independently infected by bacteria (S. agalactiae) for 2 h at an MOI of 100, and the extracellular bacteria were killed and lysed with antibiotics and lysozyme. The control cells and infected BMECs were washed with PBS 3 times and centrifuged for 10 min at 3000×g and 4°C. Total proteins were extracted from control and *S. agalactiae*-infected BMECs separately. Then, proteins from infected and uninfected BMECs were extracted and mixed respectively and were subjected to proteomic analysis. Protein concentration was measured and SDS-PAGE was performed to ensure that the quality of the samples conformed to iTRAQ assay standards. The abundance of proteins in the control group was compared with that in the bacteria-infected group to generate a control: infection ratio. Proteins were considered to be differentially expressed if they met the following criteria: log2 ratio ≥ 1 and FDR ≤ 0.01. Differentially expressed proteins and KEGG pathway annotation were analyzed in Tables S2 and S3.

Western blot analysis

Proteins and phosphorylated proteins were examined by western blot.[62,63] Briefly, control and bacteria-infected BMECs were collected with trypsin and lysed in cell lysis buffer. Equal quantities (40 μg) of proteins were subjected to electrophoresis and transferred to polyvinylidene fluoride membrane, which was then incubated with primary antibodies. Signals were detected with peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) reagent using the Western Blotting System (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The bands were quantified on a Gel-Pro Analyzer 4.0 (Media Cybernetics, USA).

MTT

BMECs were seeded into 96-well plates at 6×10^3 cells per well 24 h before drug treatment. Then cells were incubated with and treated with various concentrations of RGD (Arg-Gly-Asp peptides) for 24 h to evaluate the inhibitory efficiency of RGD on cell metabolic activity and formazan accumulation in cells. The different volumes of the DMSO were added to the corresponding control group media, and the concentration of DMSO in the final solution did not exceed 0.5% (v/v). The medium with RGD was absorbed, and fresh medium was added. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma-Aldrich, Inc. St. Louis, MO, USA) was added to each well and incubated for 4 h at 37°C. The solution was absorbed, and formazan product was dissolved by adding 100 μL DMSO to each well and incubating it for 10 min at 37°C. MTT absorbance was measured at 490/630 nm with a spectrophotometer set (Thermo, Multiskan SX 353, USA). This assay was used to evaluate the efficacy of RGD against cell proliferation.

Transfection *in vitro*

The pRNAT-U6.1/Neo-shLAMB2, pGPH1/GFP/Neo-shITGB1, pGPU6/GFP/Neo-shCrk, pGPU6/GFP/Neo-shVps25, pIRES2-EGFP-Crk, and pIRES2-EGFP-Vps25 plasmids were transfected into BMECs using Lipofectamine TM2000 (Invitrogen, Carlsbad, New Mexico, USA) per the manufacturer's instructions, whereas pGPU6/GFP/Neo-shFAK was transfected into MCF-10A cells. Transfectants were selected with G418 (Hyclone Laboratories, Inc. Logan, UT, USA) for 48 h and imaged under a ZEISS AX10 fluorescence microscope (Carl Zeiss Microscopy, LLC One Zeiss Drive, Thornwood, NY 10594 USA).

RT-qPCR

RT-qPCR was performed to measure *LAMB2*, *ITGB1*, *Crk*, and *Vps25* in BMECs and *FAK* in MCF-10A cells. Cells (6×10^5 cells/well in a 6-well plate) were transfected with vectors for RNA interference or overexpression. Total RNA was extracted using RNAiso Plus per the manufacturer's instructions (9109, TaKaRa Co. Ltd., Dalian, China). Briefly, the cells were washed with PBS and lysed in RNAiso Plus, and chloroform was added to the cell lysates for homogenization; the top aqueous layer was transferred to a new tube after centrifugation, and isopropanol was added to the supernatant and mixed well. Total RNA was precipitated by centrifugation, and the pellet was dissolved in RNase-free water.

Total RNA was reversed-transcribed using the EasyScript one-step gDNA Removal and cDNA Synthesis SuperMix kit according the manufacturer's instructions (Code#AE311-03, Transgen Co. Ltd., China). qPCR was performed with the KAPA SYBR FAST qPCR Kit Optimized for LightCycler 480 (KAPA BIOSYSTEMS, Inc., Boston, MA, USA). qPCR primers for *LAMB2*, *ITGB1*, *FAK*, *Crk*, and *Vps25* were designed according to the sequences in GenBank (The primer sequences in Table S5). *ACTB* was selected as the internal control gene. $2^{-\Delta\Delta CT}$ values were calculated to determine expression levels. Three independent experiments were performed, and the results were analyzed by student's t-test.

Coimmunoprecipitation

BMECs were collected, washed 3 times with cold PBS, and dissolved in cell lysis buffer. Then, equivalent amounts of protein lysate were incubated with anti-Vps25 (1:50) or anti-His (1:50) (negative control) and inactive resin (negative control) using the Co-Immunoprecipitation Kit per the manufacturer's protocols (Cat# 17-500, Millipore, Billerica, MD, USA). Equivalent amounts of protein lysate were also evaluated by western blot as a positive control for Vps25 and Crk, as reported.[63]

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical analyses were conducted using SPSS PASW Statistics for Windows, v18.0 (SPSS Inc.: Chicago, IL, USA). Data were analyzed using standard parametric statistics and one-way ANOVA, followed by Tukey's method. Data are expressed as mean $\pm$ SD. The results are presented as the average of at least 3 independent experiments. Western blot results were quantified on a Gel-Pro Analyzer 4.0 (Media Cybernetics, USA). Statistical significance was accepted when $p \leq 0.05$.