Global impact of *Salmonella* type III secretion effector SteA on host cells

Elena Cardenal-Muñoz[a], Gabriel Gutiérrez[b] and Francisco Ramos-Morales*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain

[a]e_cardenal@us.es

[b]ggpozo@us.es

*Corresponding author. Mailing address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Avda Reina Mercedes, 6, 41012 Sevilla, Spain. Phone: 34 95 455 7107. Fax: 34 95 455 7104. E-mail: framos@us.es

Abstract

Salmonella enterica is a Gram-negative bacterium that causes gastroenteritis, bacteremia and typhoid fever in several animal species including humans. Its virulence is greatly dependent on two type III secretion systems, encoded in pathogenicity islands 1 and 2. These systems translocate proteins called effectors into eukaryotic host cell. Effectors interfere with host signal transduction pathways to allow the internalization of pathogens and their survival and proliferation inside vacuoles. SteA is one of the few *Salmonella* effectors that are substrates of both type III secretion systems. Here, we used gene arrays and bioinformatics analysis to study the genetic response of human epithelial cells to SteA. We found that constitutive synthesis of SteA in HeLa cells leads to induction of genes related to extracellular matrix organization and regulation of cell proliferation and serine/threonine kinase signaling pathways. SteA also causes repression of genes related to immune processes and regulation of purine nucleotide synthesis and pathway-restricted SMAD protein phosphorylation. In addition, a cell biology approach revealed that epithelial cells expressing *steA* show altered cell morphology, and decreased cytotoxicity, cell-cell adhesion and migration.

Keywords

Salmonella enterica; type III secretion system; SteA; microarray; human epithelial cell; cell-cell adhesion.

Abbreviations

T3SS, type three secretion system; SPI, *Salmonella* pathogenicity island; NCBI, National Center for Biotechnology Information; LB, Luria-Bertani; FDR, false discovery rate; LDH, lactate dehydrogenase; qPCR, quantitative real-time PCR; HEMA, 2-hydroxyethyl methacrylate; GO, gene ontology.

1. Introduction

Many pathogenic Gram-negative bacteria possess type III secretion systems (T3SSs) for their interaction with the host. These systems allow delivery into eukaryotic host cells of effector proteins that direct the different stages of the infection at the cellular level [1]. *Salmonella enterica* possesses two distinct virulence-related T3SSs, T3SS1 and T3SS2, that are encoded by genes located in *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2), respectively [2]. T3SS1 is necessary for the invasion of non-phagocytic cells [3], whereas T3SS2 is induced after invasion and is essential for survival and replication within macrophages [4,5]. *S. enterica* injects more than thirty T3SS effectors to their host cells and some of them have been shown to manipulate cellular processes such as actin cytoskeleton organization, tight junction alterations, biogenesis of the *Salmonella*-containing vacuole and cell death [6]. However, the functions for many effectors are still unknown.

SteA was identified as a *S. enterica* serovar Typhimurium T3SS effector [7] that can be secreted to culture media and translocated into epithelial cells and macrophages through T3SS1 and T3SS2, depending on culture conditions, infected cell types and infection times. The first 10 amino acids of SteA act as a signal sequence for its translocation into the eukaryotic cell [8]. The gene *steA* is located outside SPI1 and SPI2, and its low GC content (43%) suggests horizontal acquisition, common in virulence-associated genes. We have previously shown that its expression is transcriptionally controlled by the bacterial redox status in a PhoQ/PhoP-dependent manner [9]. A *steA* null mutant is three-fold attenuated for BALB/c mice virulence after intraperitoneal infection [7] and SteA seems to be involved in the bacterial persistence during long time infection in 129X1/SvJ mice [10]. In the host cell, SteA localizes to the *trans*-Golgi network (TGN) and to *Salmonella*-induced membrane tubules containing the *trans*-Golgi marker GalT-mCherry [7,11]. A very recent report has shown that SteA participates in the control of *Salmonella*-containing vacuole membrane

dynamics [12]. This study also suggests that SteA should have additional roles, which remain to be elucidated, at earlier times of host cell infection.

Similarity to proteins with known activities has been useful in some cases to determine the function of a specific effector [13], but in the case of SteA no sequence similarities have been detected. Expression of individual effectors, like AvrA or SopB, in the budding yeast *Saccharomyces cerevisiae*, that serves as a simplified heterologous model [14], has been a productive approach to study the effects of these proteins on host cells [15,16,17], thereby helping in the discovery of their activities. Here, we use mammalian cells as a relevant model to specifically analyze the effect on the host transcriptome of the effector SteA. We show that in epithelial HeLa cells SteA leads to changes in the expression of genes related to extracellular matrix organization, cell proliferation, serine/threonine kinase signaling pathways, immune processes, regulation of purine nucleotide synthesis and pathway-restricted SMAD protein phosphorylation, and produces significant changes in cell death, adhesion, and migration.

2. Materials and methods

2.1. Bacterial strains and bacterial culture

Bacterial strains used were *Escherichia coli* DH5α [18] and *S. enterica* serovar Typhimurium SV5846 [8], a derivative of strain 14028 carrying a *steA*::3xFLAG chromosomal fusion. The standard bacterial culture medium was Luria-Bertani (LB) broth. Solid LB contained agar 1.5% final concentration. Antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml.

2.2 Plasmid construction, DNA amplification, and sequencing

Plasmid pIZ1963 is a derivative of pBABEpuro [19] coding for SteA with a C-terminal 3xFLAG tag. To construct this plasmid, *steA*::3xFLAG was amplified from strain SV5846 using primers steApcdnadir and steAflagxho3, digested with *Bam*HI and *Xho*I and ligated together with vector pBABEpuro previously digested with *Bam*HI and *Sal*I. Amplification reactions and confirmation by sequencing were carried out as previously described [8]. Primers are listed in Table S1.

2.3 Mammalian cell culture, transfection and lysis

HeLa cells (ECAC no. 93021013) were cultured, transfected, and lysed as previously described [20]. For stable transfection, HeLa cells were electroporated with pBABEpuro or its derivative and selection was started 24 h after electroporation in medium containing 1 μg/ml puromycin (InvivoGen).

2.4 Bacterial infection of cultured cells and analysis of SteA translocation

HeLa cells were plated in 6-well plates at $6x10^5$ cells per well and incubated for 24 h. *Salmonella* infections were carried out as previously described [8]. The cell culture was

washed twice with PBS 1 h post-infection and overlaid with DMEM containing 100 μg/ml gentamicin. One hour later the concentration of gentamicin was lowered to 16 μg/ml after an additional wash with PBS. Infected mammalian cells were lysed 6 h post-infection with Nonidet P-40 buffer as described [8]. The extract was centrifuged at 13000 rpm for 20 min and the supernatant was filtered and analyzed by immunoblot.

2.5 Electrophoresis, immunoblot, and antibodies

Proteins in cell extracts were resolved by SDS-PAGE. The gel was blotted onto a nitrocellulose membrane (Amersham) and probed with mouse monoclonal anti-FLAG M2 primary antibodies (1:5000; Sigma) and mouse monoclonal anti-β-actin C4 (1:5000; Santa Cruz Biotech) as loading control. Goat anti-mouse horseradish peroxidase-conjugated antibodies (1:5000; BioRad) were used as secondary antibodies. Detection was via chemiluminescence procedures (Pierce).

2.6 RNA preparation, gene array processing, and statistical analysis

Total RNA from HeLa cells stably transfected with pBABEpuro or its derivative encoding the SteA-3xFLAG fusion was isolated in triplicate using 1 ml of TRIzol reagent (Invitrogen) according to the protocol supplied by the manufacturer. An additional purification step was carried out by using the RNeasy Min Elute Cleanup Kit (Qiagen). Biotinylated single-stranded cDNA was prepared from 100 ng per sample of total intact RNA extracted from 9 independent samples (3 from HeLa pBABEpuro, 3 from HeLa pBABEpuro-SteA-3xFLAG L2, and 3 from HeLa pBABEpuro-SteA-3xFLAG L4). Labelled cDNA was hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix) following the manufacturer's instructions. The arrays were scanned in a 3000 7G Scanner from Affymetrix. Image analysis, fluorescent data quantification and quality control was carried out with Affymetrix software. All procedures and preliminary data analysis, including fluorescent data processing,

normalization using Robust Multi-array Average (RMA) algorithms, and annotations, were performed at the Genomics Unit of the Andalusian Center for Molecular Biology and Regenerative Medicine (CABIMER, Seville, Spain). Fold change was calculated for each cell line relative to the control line transfected with pBABEpuro. Statistical significance (*p* value) was calculated by empirical Bayes moderated t-test based on the results of three arrays per condition. The false discovery rate (FDR) for each *p* value was also calculated. Genes that changed with an FDR-adjusted *p* value higher than 0.05 were removed from subsequent analysis. The microarray data used in this analysis is available from NCBI's Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE51043.

2.7 Function prediction of gene sets

To predict the functional association networks of the activated and repressed genes from the microarray, we used the web-based software application tool GeneMANIA [21]. GO terms associated to genes in Table 1 were searched using AmiGO version 1.8 [22].

2.8 Quantitative real-time PCR (qPCR)

The protocol for qPCR was previously described [9]. DNA primers are indicated in Table S1. Gene expression levels were normalized to transcripts of *BCAT1*, gene that appeared not affected by the presence of SteA in the microarray.

2.9 Proliferation assays

HeLa cells were seeded in 6-well plates at a density of $6x10^4$ cells per well and grown at 37ºC, 5% CO_2 in DMEM. At 24 h intervals, the cells from a well per cell line were trypsinized and counted on a haemocytometer

2.10 Cell death, cell adhesion, and cell migration assays

Cell death was measured using a previously described protocol based on lactate dehydrogenase (LDH) release in the cultures [20]. Cell adhesion was determined as previously described [23]. For cell-cell adhesion assays, $6x10^4$ cells/ml were seeded onto 6-well plates coated with poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma) in order to prevent cell-matrix interactions. After 48 h of culture in suspension, cells were photographed on an inverted microscope. For the migration assay (wound closure assay), we followed the protocol previously described [24].

2.11 Statistical analysis

Student's *t* test was used to analyze differences in growth, cytotoxicity percentage, and relative migration. *p* values of 0.05 or less were considered significant.

3. Results and Discussion

3.1 cDNA microarray analysis of gene expression in HeLa cells in response to SteA

To investigate the consequences of the presence of the *Salmonella* T3SS effector SteA in human epithelial cells, we generated three stably transfected HeLa cell lines, one with the empty vector pBABEpuro and two with a derivative of the same vector encoding a SteA-3xFLAG fusion (lines SteA L2 and and SteA L4). The expression level of tagged SteA in these cell clones was determined by Western blot analysis and compared to the level of SteA translocated into HeLa cells during in vitro infections with *S. enterica* serovar Typhimurium strain SV5846. As seen in Figs. 1A and 1B, the level of SteA was higher in L4 than in L2. Importantly, in both cell lines the levels were lower than after translocation from *Salmonella*. This result suggests that the effects of SteA studied in this work are not due to a non-physiological level of expression of the effector. Next, we performed microarray hybridization with RNA from stably transfected cells. Biotin-labelled cDNAs prepared from total RNA were hybridized to microarray chips containing 28,869 sequenced human genes. The number of genes that changed in response to SteA expression in a statistically significant manner (FDR-adjusted p value < 0.05) is represented in Fig. 1C (upregulated genes) and Fig. 1D (downregulated genes) using Venn diagrams. As a result of this analysis we decided to focus on 58 genes (0.2 % of the whole human gene number analyzed) that showed differential expression in both of the cell lines expressing SteA (L2 and L4) when compared to the control cell line. Of these, 13 genes showed higher expression while 45 genes showed lower expression. These genes are listed in Table S2. A general conclusion that can be drawn from the microarray analysis is that the presence of SteA within HeLa cells causes more gene repression than activation. This is true for both stably transfected cell lines, L2 and L4. The

fact that differential expression is more pronounced in L4, correlates with a higher level of SteA in this cell line.

Figure 1

3.2 Validation of microarray data

To validate microarray results we selected 7 differentially expressed genes and performed qPCR assays. These experiments were carried out using new samples, independent from the ones used for the microarray. The gene expression changes measured by qPCR were in agreement with microarray data (Fig. 2).

Figure 2

3.3 Functions of the SteA-regulated genes

To get insight into the cell functions affected by SteA, differentially expressed genes were used to search the GeneMANIA prediction server [21]. GeneMANIA uses a very large set of functional interaction data to find genes related to the list of query genes. Interaction data include co-expression, physical interactions, co-localization, shared protein domains, genetic interactions, and pathways. The resultant networks for the up- and downregulated genes are shown in Fig. S1. GeneMANIA also computes gene ontology (GO) enrichment on the resulting set of genes (query list plus related genes). The results of these analyses are shown in Table S3, for upregulated genes, and Table S4, for downregulated genes. From this study, we conclude that genes activated by SteA expression in HeLa epithelial cells are mainly related to extracellular matrix organization, cell proliferation and serine/threonine kinase signaling, whereas genes repressed by SteA are mainly involved in immune processes, regulation of purine nucleotide biosynthetic process and regulation of the pathway-restricted

SMAD protein phosphorylation. Additional functions affected by these genes that may be relevant in the context of *Salmonella* infections are shown in Table 1.

3.4 SteA decreases epithelial cell death

Since SteA has not been shown to have a nuclear localization, a direct function as a transcriptional regulator is unlikely. However, the observed alterations suggest that SteA significantly interferes with several host transduction pathways. Therefore, we decided to use data obtained in the transcriptomic analysis as a guide to study phenotypic alterations induced by SteA in HeLa cells. The functional analysis shown in the previous section revealed several genes whose expression could affect cell proliferation (Table 1). However, we were unable to detect differences in proliferation rates between HeLa pBABEpuro cells and HeLa pBABEpuro-SteA-3xFLAG cells under the experimental conditions used (Fig. 3A).

Some of the genes upregulated (*CTGF, C5AR1, MAP2K6*) or downregulated (*COL15A1, CYFIP2, IER3, NFE2L2, NOG, TGFB1, UNC13B*) in the presence of SteA are involved in cell death. Thus, we decided to compare cell death in cultures of *steA* and vector-transfected cells. Fig. 3B shows a significant decrease in cytotoxicity in SteA expressing cells as determined by LDH release. This result suggests that SteA, together with other effectors like AvrA and SopB, whose antiapoptotic activities are already known [25,26], could contribute to the delay in cell death that is observed during infections of epithelial cells. This is probably beneficial for the pathogen because it allows the establishment of a stable intracellular niche and contributes to avoid adaptive immunity.

Figure 3

3.5 SteA induces changes in morphology, adhesion and migration of epithelial cells

Strikingly, HeLa cells expressing SteA were more rounded and formed smaller colonies than control cells (Fig. 4A). We reasoned that these morphological alterations could be explained by the observed changes in the level of expression of genes related to cell adhesion (*CNTNAP3, COL15A1, CTGF, CYFIP2, MPDZ, TGFB1*), production or organization of the extracellular matrix (*COL15A1, CTGF, EFEMP1, FURIN, LUM, SDC2, TGFB1*), and cell migration (*CAV1,CTGF, MYH10, SLC7A11, TGFB1, TNS1*) (Table 1). To test these ideas we investigated adhesion and migration of *steA*-transfected cells. Interestingly, although adhesion to plastic substrate or extracellular matrix proteins (collagen I or vitronectin) was not altered in these cells (data not shown), cell-cell adhesion was clearly disrupted. To carry out the later experiments, cells were cultured in poly-HEMA treated plates. In this way, cells are not able to adhere to the plate and grow as a suspension but they can bind to other cells. Under these conditions, control cells formed dense aggregates whereas *steA* transfected cells were more loosely dispersed in the media, indicating a defect in intercellular binding (Fig. 4B). In addition, cells constitutively producing the *Salmonella* effector SteA showed reduced migration ability in a wound closure assay (Figs. 4C and 4D). Taken together, these results suggest that the presence of SteA in epithelial host cells alters their morphology through a modification of their adhesion and migration properties.

Figure 4

The direct approach used here to investigate the impact of the *Salmonella* effector SteA on host cells has significant advantages. Since SteA is the only effector that is present in stably transfected HeLa cells all the effects observed should be ascribed to this effector. There are no interferences from other effectors. Actually, one of the hurdles in the study of the functions of T3SS effectors is the fact that sometimes they act redundantly or in opposite ways [27]. Functional redundancy or overlap can explain why mutants lacking only one effector are often as virulent as the wild type. Also, conditions for expression and

translocation of a particular effector are not always completely defined since they can depend on diverse factors including: *Salmonella* strain or serovar, culture conditions previous to infection, host cell type, and time post-infection. Our approach ensures constitutive expression throughout the experiments. Using this approach we have been successful in identifying changes in host cell gene expression and phenotypes triggered by SteA. Overall, the results presented here suggest that the T3SS effector SteA can have a significant impact in the biology of *Salmonella*-infected host cells. Our results also open the possibility of studying in detail the role during infection of individual host genes whose expression is affected by SteA.

Acknowledgements

We are grateful to Josep Casadesús (University of Seville) for discussions and suggestions and to Eloísa Andújar and Mónica Pérez-Alegre of the Genomics Unit (CABIMER) for assistance in experiments performed at this facility. This work was supported by the Spanish Ministry of Science and Innovation and the European Regional Development Fund (grant number SAF2010-15015), and Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía, Spain (grant number P08-CVI-03487). E.C.M. was the recipient of a predoctoral fellowship from the Junta de Andalucía.

References

[1] G.R. Cornelis, The type III secretion injectisome, Nat Rev Microbiol 4 (2006) 811-825.

[2] J.A. Ibarra, O. Steele-Mortimer, *Salmonella*--the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival, Cell Microbiol 11 (2009) 1579-1586.

[3] J.E. Galán, R. Curtiss, 3rd, Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells, Proc Natl Acad Sci U S A 86 (1989) 6383-6387.

[4] H. Ochman, F.C. Soncini, F. Solomon, E.A. Groisman, Identification of a pathogenicity island required for *Salmonella* survival in host cells, Proc Natl Acad Sci U S A 93 (1996) 7800-7804.

[5] J.E. Shea, M. Hensel, C. Gleeson, D.W. Holden, Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*, Proc Natl Acad Sci U S A 93 (1996) 2593-2597.

[6] F. Ramos-Morales, Impact of *Salmonella enterica* type III secretion system effectors on the eukaryotic host cell, ISRN Cell Biology 2012 (2012) Article ID 787934.

[7] K. Geddes, M. Worley, G. Niemann, F. Heffron, Identification of new secreted effectors in *Salmonella enterica* serovar Typhimurium, Infect Immun 73 (2005) 6260-6271.

[8] E. Cardenal-Muñoz, F. Ramos-Morales, Analysis of the expression, secretion and translocation of the *Salmonella* enterica type III secretion system effector SteA, PLoS One 6 (2011) e26930.

[9] E. Cardenal-Muñoz, F. Ramos-Morales, DsbA and MgrB regulate *steA* expression through the two-component system PhoQ/PhoP in *Salmonella enterica*, J Bacteriol 195 (2013) 2368-2378.

[10] T.D. Lawley, K. Chan, L.J. Thompson, C.C. Kim, G.R. Govoni, D.M. Monack, Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse, PLoS Pathog 2 (2006) e11.

[11] S.B. Van Engelenburg, A.E. Palmer, Imaging type-III secretion reveals dynamics and spatial segregation of *Salmonella* effectors, Nat Methods 7 (2010) 325-330.

[12] L. Domingues, D.W. Holden, L.J. Mota, The *Salmonella* effector SteA contributes to the control of membrane dynamics of *Salmonella*-containing vacuoles, Infect Immun (2014) in press. http://dx.doi.org/10.1128/IAI.01385-13

[13] P. Dean, Functional domains and motifs of bacterial type III effector proteins and their roles in infection, FEMS Microbiol Rev 35 (2011) 1100-1125.

[14] R.H. Valdivia, Modeling the function of bacterial virulence factors in *Saccharomyces cerevisiae*, Eukaryot Cell 3 (2004) 827-834.

[15] E. Bosis, D. Salomon, G. Sessa, A simple yeast-based strategy to identify host cellular processes targeted by bacterial effector proteins, PLoS One 6 (2011) e27698.

[16] F. Du, J.E. Galán, Selective inhibition of type III secretion activated signaling by the *Salmonella* effector AvrA, PLoS Pathog 5 (2009) e1000595.

[17] I.R. Rodríguez-Escudero, R., Cid, V. J.; Molina, M., Inhibition of Cdc42-dependent signalling in *Saccharomyces cerevisiae* by phosphatase-dead SigD/SopB from *Salmonella typhimurium*., Microbiology 152 (2006) 3437-3452.

[18] D. Hanahan, Studies on transformation of *Escherichia coli* with plasmids, J Mol Biol 166 (1983) 557-580.

[19] J.P. Morgenstern, H. Land, Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line, Nucleic Acids Res 18 (1990) 3587-3596.

[20] J. Bernal-Bayard, F. Ramos-Morales, *Salmonella* type III secretion effector SlrP is an E3 ubiquitin ligase for mammalian thioredoxin, J Biol Chem 284 (2009) 27587-27595.

[21] K. Zuberi, M. Franz, H. Rodriguez, J. Montojo, C.T. Lopes, G.D. Bader, Q. Morris, GeneMANIA prediction server 2013 update, Nucleic Acids Res 41 (2013) W115-122.

[22] S. Carbon, A. Ireland, C.J. Mungall, S. Shu, B. Marshall, S. Lewis, AmiGO: online access to ontology and annotation data, Bioinformatics 25 (2009) 288-289.

[23] S. Mueller, Cadenas, E., Schönthal, A. H., p21WAF1 regulates anchorage-independent growth of HCT116 colon carcinoma cells via E-cadherin expression., Cancer Res 60 (2000) 156-163.

[24] W. Kleeberger, Bova, G. S., Nielsen, M. E., Herawi, M., Chuang, A. Y., Epstein, J. I., Berman, D. M., Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis., Cancer Res 67 (2007) 9199-9206.

[25] O. Steele-Mortimer, L.A. Knodler, S.L. Marcus, M.P. Scheid, B. Goh, C.G. Pfeifer, V. Duronio, B.B. Finlay, Activation of Akt/protein kinase B in epithelial cells by the *Salmonella typhimurium* effector SigD, J Biol Chem 275 (2000) 37718-37724.

[26] H. Wu, R.M. Jones, A.S. Neish, The *Salmonella* effector AvrA mediates bacterial intracellular survival during infection in vivo, Cell Microbiol 14 (2012) 28-39.

[27] J.E. Galán, Common themes in the design and function of bacterial effectors, Cell Host Microbe 5 (2009) 571-579.

Figure legends

Fig. 1. Expression of SteA-3xFLAG in stably transfected HeLa cells (A) HeLa cells were stably transfected with plasmids pBABEpuro (Vector) and pBABEpuro-SteA-3xFLAG (SteA L2 and SteA L4) or were infected with *S. enterica* serovar Typhimurium strain SV5846 (Infected) and lysates were immunoblotted with monoclonal anti-FLAG antibodies and with antibodies against β-actin as a loading control. (B) Quantification of SteA-3xFLAG with respect to β-actin and normalization regarding the SteA L2 quantification. Means and standard deviations from two independent experiments are represented. (C and D) Venn diagrams of genes with differential expression between HeLa cells expressing SteA-3xFLAG and control HeLa cells. Number of significantly (FDR-adjusted p value < 0.05) upregulated (C) or downregulated (D) genes in clones expressing SteA-3xFLAG compared to the control (L2/Control and L4/Control) is shown.

Fig. 2 Microarray and qPCR results for selected differentially expressed genes. (A) Microarray results of 7 differentially expressed genes in HeLa pBABEpuro-SteA-3xFLAG (SteA L2 and SteA L4) compared to HeLa pBABEpuro. (B) qPCR results showing mRNA relative amount of the same genes in the same strains. Means and standard deviations from three experiments performed in triplicate are represented. Gene names are indicated. M (log) is the $\log_2$ of the ratio L2/control or L4/control for each gene

Fig. 3. Effect of SteA on cell death. (A) Proliferation of HeLa cells expressing SteA-3xFLAG (SteA L4) compared to control cells (Vector) during a period of 5 days. (B) HeLa cells stably transfected with pBABEpuro (Vector) and pBABEpuro-SteA-3xFLAG (SteA L4) were cultured in serum-free medium for 24 h and cell death was determined by LDH release. Means from representative experiments carried out in triplicate are represented. Error bars

represent the standard deviations. The asterisk indicates a significant difference ($p < 0.01$) by the Student's t test.

Fig. 4. HeLa cells expressing SteA have a defect in cell-cell adhesion and cell migration. (A) HeLa cells stably transfected with pBABEpuro (Vector) and pBABEpuro-SteA-3xFLAG (SteA L4) were observed under an inverted microscope to detect differences in cellular shape (upper panels) and in cell colony shape (lower panels). (B) HeLa cells were transferred to poly-HEMA-coated plates. After 48 h of culture in suspension, cells were photographed under an inverted microscope. Depicted are control cells HeLa pBABEpuro (Vector), which form dense aggregates, and HeLa pBABEpuro-SteA-3xFLAG (SteA L4), which adhere to each other only very weakly. (C) Representative images of wound-healing assays showing that HeLa pBABEpuro (Vector) closed the wound 4 days after wounding, whereas HeLa pBABEpuro-SteA-3xFLAG (SteA L4) did not. Wounds are outlined. (D) Wound widths measured 48 h after wounding and normalized to the value obtained for HeLa pBABEpuro are graphed, showing means and standard deviations from six experiments. The asterisk indicates a significant difference ($p < 0.01$). Scale bars, 200 μm.

Table 1
Selected biological functions associated to a subset of differentially expressed genes in *steA*-transfected HeLa cells

Upregulated genes	Biological process or molecular function					
	Proliferation	Death/apoptosis	ECM[a]	Adhesion	Migration	Endocytosis
CAV1	X				X	X
CCND1	X					
CTGF	X	X	X	X	X	
C5AR1	X	X				
C5orf23 (NPR3)	X					
EFEMP1			X			
LUM			X			
MAP2K6		X				
SDC2			X			
Downregulated genes	Biological process or molecular function					
	Proliferation	Death/apoptosis	ECM[a]	Adhesion	Migration	Endocytosis
AKR1C2	X					
ALDH3A1	X					
CNTNAP3				X		
COL15A1		X	X	X		
CYFIP2		X		X		
FURIN	X		X			
IER3		X				
MPDZ				X		
MYH10	X				X	
NFE2L2		X				
NOG	X	X				
RARRES1	X					
SLC7A11					X	
TGFB1	X	X	X	X	X	
TNS1					X	
UNC13B		X				

[a]ECM: extracellular matrix regulation. X indicates that this GO term appears associated to this gene.

Figure 1

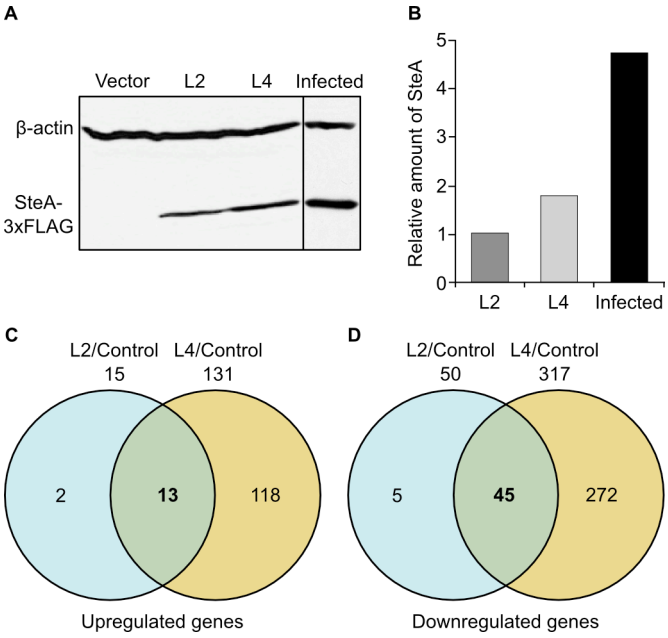

A
Vector
L2
L4
Infected
β-actin
SteA-
3xFLAG
B
Relative amount of SteA
0
1
2
3
4
5
L2
L4
Infected
C
L2/Control
15
L4/Control
131
2
13
118
Upregulated genes
D
L2/Control
50
L4/Control
317
5
45
272
Downregulated genes

Figure 2

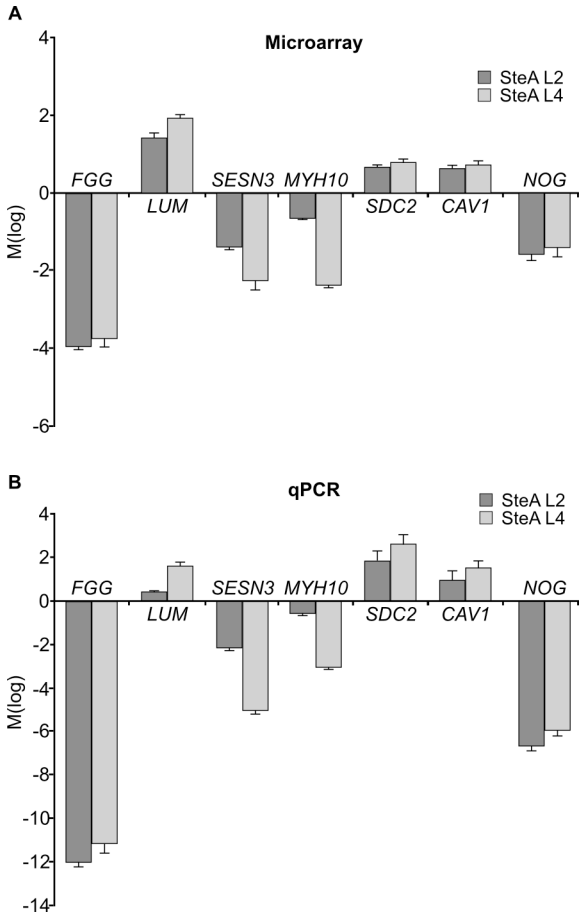

A
Microarray
SteA L2
SteA L4
M(log)
4
2
0
-2
-4
-6
FGG
LUM
SESN3
MYH10
SDC2
CAV1
NOG
B
qPCR
SteA L2
SteA L4
M(log)
4
2
0
-2
-4
-6
-8
-10
-12
-14
FGG
LUM
SESN3
MYH10
SDC2
CAV1
NOG

Figure 3

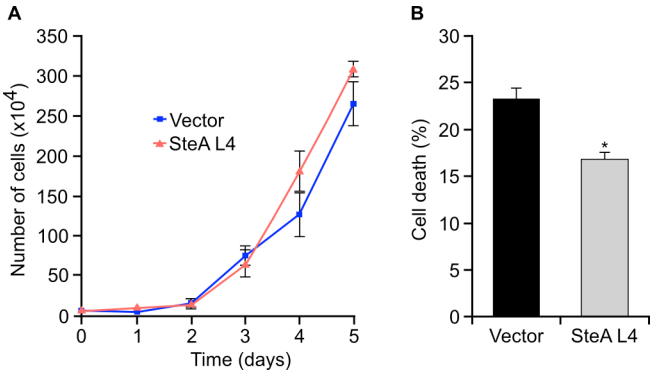

A
Number of cells (x10^4)
350
300
250
200
150
100
50
0
Vector
SteA L4
0
1
2
3
4
5
Time (days)
B
Cell death (%)
30
25
20
15
10
5
0
*
Vector
SteA L4

Figure 4

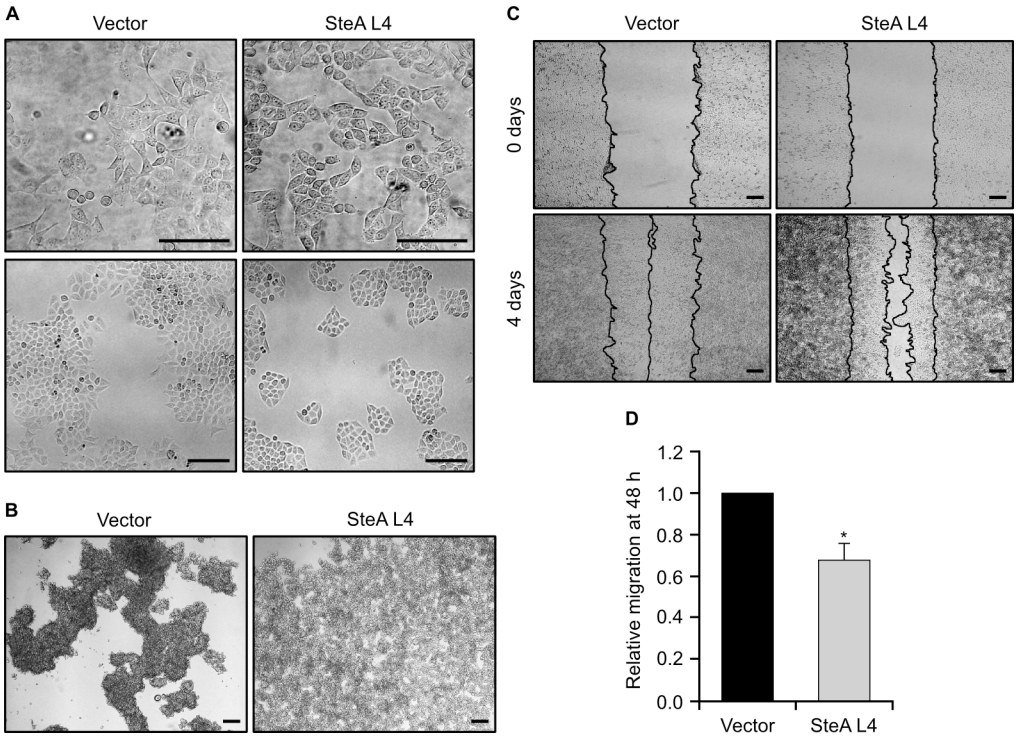
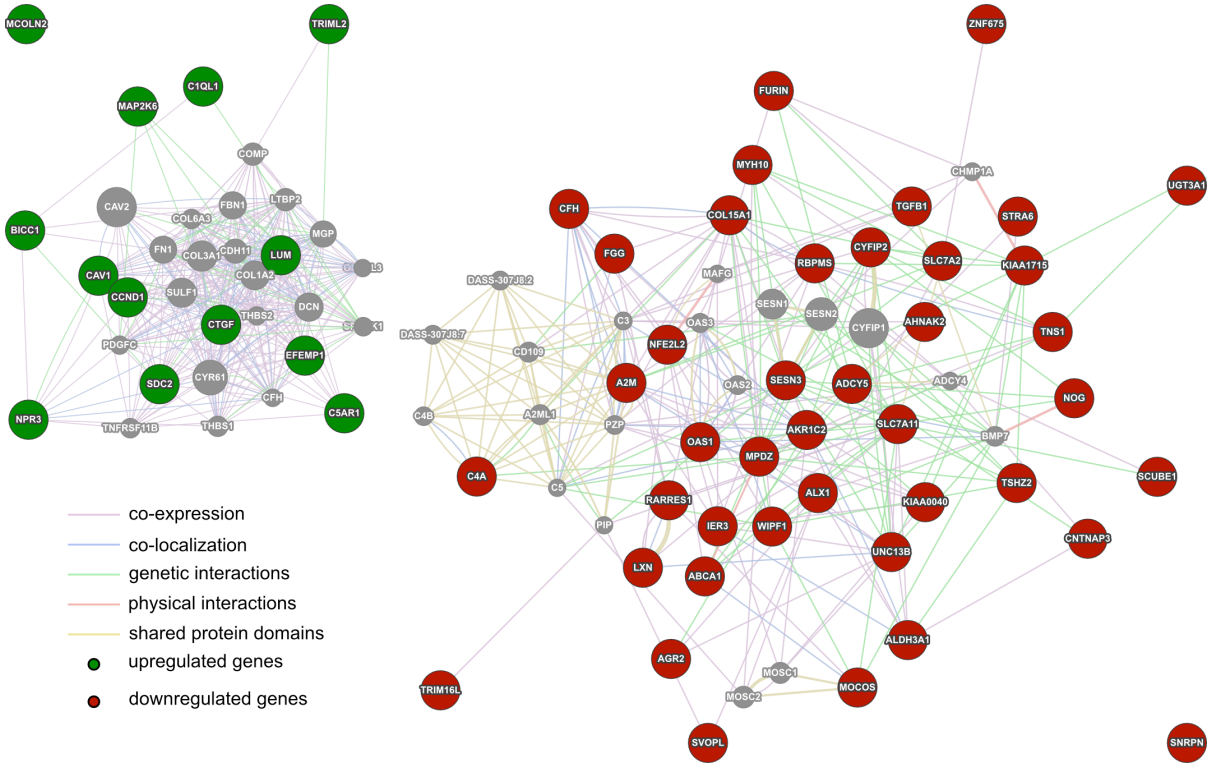

A
Vector
SteA L4
B
Vector
SteA L4
C
Vector
SteA L4
0 days
4 days
D
Relative migration at 48 h
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Vector
SteA L4

Figure S1


MCOLN2
TRIML2
C1QL1
MAP2K6
COMP
CAV2
FBN1
LTBP2
BICC1
COL6A3
MGP
FN1
COL3A1
CDH11
LUM
CAV1
COL1A2
CCND1
SULF1
DCN
CTGF
THBS2
PDGFC
EFEMP1
SDC2
CYR61
CFH
NPR3
TNFRSF11B
THBS1
C5AR1
co-expression
co-localization
genetic interactions
physical interactions
shared protein domains
upregulated genes
downregulated genes
ZNF675
FURIN
MYH10
CHMP1A
UGT3A1
CFH
COL15A1
TGFB1
STRA6
FGG
RBPMS
CYFIP2
SLC7A2
KIAA1715
DASS-307J8.2
MAFG
SESN1
SESN2
CYFIP1
AHNAK2
TNS1
C3
OAS3
DASS-307J8.7
NFE2L2
CD109
A2M
OAS2
SESN3
ADCY5
ADCY4
NOG
C4B
A2ML1
PZP
AKR1C2
SLC7A11
BMP7
OAS1
MPDZ
C4A
C5
ALX1
TSHZ2
SCUBE1
RARRES1
KIAA0040
PIP
IER3
WIPF1
CNTNAP3
UNC13B
LXN
ABCA1
ALDH3A1
AGR2
MOSC1
TRIM16L
MOSC2
MOCOS
SVOPL
SNRPN