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

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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.

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 threefold 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 [15–17], 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 6×10^5 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 13,000 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 Biotechnology) 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). Labeled 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]. Gene ontology (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 6×10^4 cells per well and grown at 37 °C, 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, 6×10^4 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

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

	Biological process or molecular function					
	Proliferation	Death/apoptosis	ECM[a]	Adhesion	Migration	Endocytosis
Upregulated genes						
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						
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.

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 Fig. 1A and B, 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-labeled 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
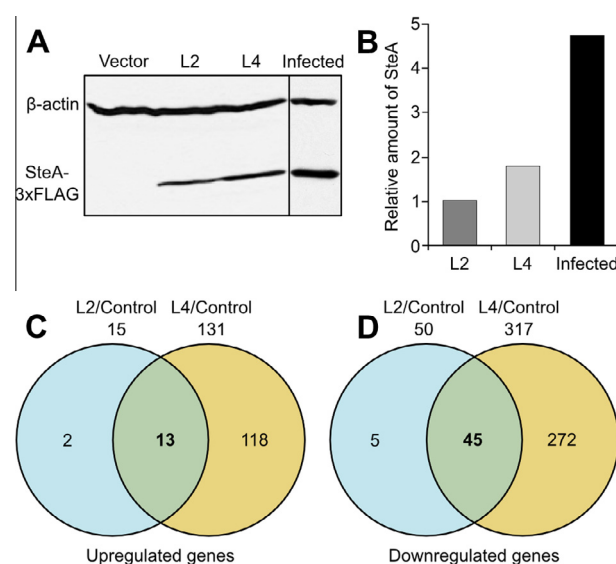

Fig. 1. Expression of SteA-3xFLAG in stably transfected HeLa cells (A) HeLa cells were stably transfected with plasmids pBABEpuro (Vector) and pBABEpuro-SteA-3xFLAG (SteA L2 and SteA L4) or were infected with *S. enterica* serovar Typhimurium strain SV5846 (Infected) and lysates were immunoblotted with 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.

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.

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).

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 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 anti-apoptotic 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.

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
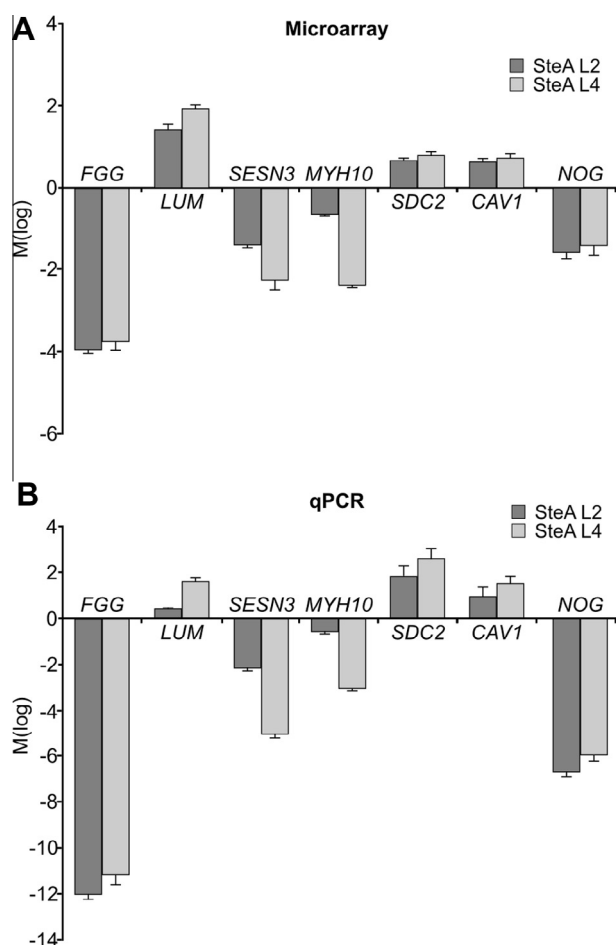

Fig. 2. Microarray and qPCR results for selected differentially expressed genes. (A) Microarray results of 7 differentially expressed genes in HeLa pBABEpuro-SteA-3xFLAG (SteA L2 and SteA L4) compared to HeLa pBABEpuro. (B) qPCR results showing mRNA relative amount of the same genes in the same strains. 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.
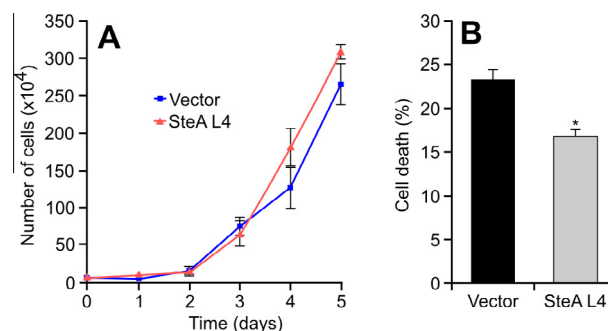

Fig. 3. Effect of SteA on cell death. (A) Proliferation of HeLa cells expressing SteA-3xFLAG (SteA L4) compared to control cells (Vector) during a period of 5 days. (B) HeLa cells stably transfected with pBABEpuro (Vector) and pBABEpuro-SteA-3xFLAG (SteA L4) were cultured in serum-free medium for 24 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.
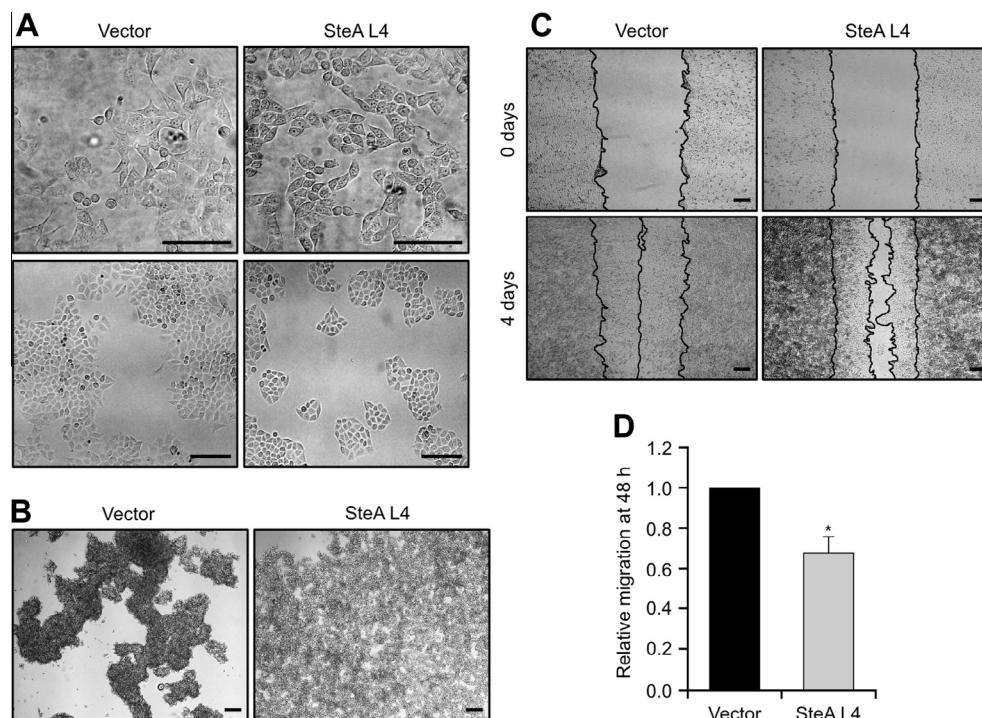
Fig. 4. HeLa cells expressing SteA have a defect in cell–cell adhesion and cell migration. (A) HeLa cells stably transfected with pBABEpuro (Vector) and pBABEpuro-SteA-3xFLAG (SteA L4) were observed under an inverted microscope to detect differences in cellular shape (upper panels) and in cell colony 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.

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 (Fig. 4C and D). 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.

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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.056.

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