ISSN: 2157-2518
Journal of Carcinogenesis & Mutagenesis

OPEN ACCESS Freely available online

Research Article

L-plastin Expression in HCT 116 Colorectal Cells Increases Migration and ROS in an NADPH Oxidase-Dependent Manner

Eilis Foran[1], Michael Freeley[2], Aideen Long[1*]

[1]Department of Clinical Medicine, Trinity College Dublin and Trinity Translational Medicine Institute, Dublin, Republic of Ireland; [2]School of Biotechnology, Dublin City University, Glasnevin, Dublin, Republic of Ireland.

ABSTRACT

Objective: L-plastin is an action-bundling protein normally expressed in cells of haemopoietic origin but is also associated with malignant transformation. Aberrant expression of L-plastin expression correlates with tumour stage in colorectal cancers, and ectopic expression of L-plastin in colon cancer cell lines increases proliferation and migration. L-plastin is inducibly phosphorylated on Ser^5 in response to a range of stimuli but the role of phosphorylated L-plastin in colon cancer cells has not been characterised. The aim of this study was to analyse the effect of L-plastin Ser^5 phosphorylation on HCT 116 colon cancer cell proliferation and migration.

Methods: Wild-type L-plastin or a Ser^5 L-plastin mutant where serine was exchanged to a non-phosphorylatable alanine was expressed in HCT 116 cells. Cell proliferation was assessed through cell counting and cyclin D activation. Cell migration were assessed using transwell migration assays.

Results: We found that wild-type L-plastin was constitutively phosphorylated on Ser^5 when expressed in HCT 116 cells and promoted an increased proliferation rate of these cells. HCT 116 cells stably expressing wild-type L-plastin also increased the migratory capacity of these cells together with increased levels of reactive oxygen species. NADPH oxidases are the main intracellular sources of reactive oxygen species and inhibition of NADPH oxidase activity with the pharmacological inhibitor DPI decreased the migration of these cells. Interestingly, expression of an L-plastin mutant with a non-phosphorylatable alanine in place of Ser^5 did not promote increased cellular proliferation, indicating that phosphorylation is required for this process. In contrast, cellular migration and ROS production were independent of the phosphorylation status of Ser^5.

Conclusion: These data demonstrate that expression of L-plastin in HCT 116 colon cancer cells increases migration in a manner that is dependent on NADPH oxidase but independent of the Ser^5 phosphorylation status.

Keywords: Colon cancer; L-plastin; Phosphorylation; Reactive oxygen species; Migration

INTRODUCTION

L-plastin is an actin-bundling protein normally expressed in cells of haematopoietic origin where its function includes regulation of innate and adaptive immunity [1]. In particular, studies using L-plastin knockout mice, siRNA-mediated gene silencing and expression of nanobodies in target cells have demonstrated roles for this protein in the adhesion-dependent respiratory burst of neutrophils and killing of the bacterial pathogen *Staphylococcus aureus*, macrophage development and function, T-cell activation and motility and B-cell development, motility and antibody responses [2-12]. Interestingly, aberrant L-plastin expression was also reported in cultured carcinoma cell lines and was expressed in a range of human solid primary tumours compared to normal tissues including gastric cancer, osteosarcoma, oral squamous cell carcinoma, bladder cancer, breast cancer, prostate cancer, renal cancer, nasopharyngeal carcinoma stroma, laryngeal cancer and melanoma [13-28]. There is compelling evidence that aberrant expression of L-plastin in solid tumours plays a key role in the metastatic cascade. Firstly, L-plastin expression levels in patient tumour samples correlate with disease stage and progression in some (albeit not all) cancers [16-18,22,29,30]. Secondly, over-expression studies and gene silencing approaches in relevant cancer cell lines have shown that L-plastin regulates proliferation, migration and invasion *in-vitro* and metastatic colony formation *in-vivo* using animal models [15-17,22-34]. In colon cancer, for example, two studies have reported

Correspondence to: Aideen Long, Department of Clinical Medicine, Trinity College Dublin and Trinity Translational Medicine Institute, Dublin, Republic of Ireland, E-mail: longai@tcd.ie

Received: 1-Jun-2022, Manuscript No. JCM-22-16866; **Editor assigned:** 3-Jun-2022, Pre QC No. JCM-22-16866 (PQ); **Reviewed:** 17-Jun-2022, QC No. JCM-22-16866; **Revised:** 24-Jun-2022, Manuscript No. JCM-22-16866 (R); **Published:** 04-Jul-2022, DOI: 10.35248/2157-2518.22.13.390

Citation: Foran E, Freeley M, Long A (2022) L-plastin Expression in HCT 116 Colorectal Cells Increases Migration and ROS in an NADPH Oxidase-Dependent Manner. J Carcinog Mutagen. 13:390.

that L-plastin expression correlates with tumour grade/stage, size and lymph node metastasis [29,30]. In addition, the study of Otsuka et al. showed that L-plastin expression was absent in the SW480 cell line established from a patient with colorectal adenocarcinoma, whereas L-plastin expression was upregulated in the SW620 cell line established from a lymph node metastasis from the same patient, suggesting a role for this protein in the metastatic cascade. Our group subsequently demonstrated that expression of L-plastin in SW480 cells increased proliferation, invasion and loss of E-cadherin expression (a marker for epithelial-mesenchymal transition), providing novel evidence that this actin-bundling protein plays a key role in colorectal cancer metastasis [35].

L-plastin is the only member of the plastin family of actin-bundling proteins known to undergo phosphorylation at Ser^5 in the N-terminal headpiece region in response to extracellular stimuli [1]. A variety of upstream kinases have been reported to directly or indirectly influence Ser^5 phosphorylation, including Protein Kinase A (PKA), Protein Kinase C (PKC) isoforms, Serum and Glucocorticoid Kinases (SGKs) and Ribosomal S6-kinases (RSKs) [1]. Phosphorylation of Ser^5 was reported to influence actin binding and actin bundling, based on the finding that mutation of Ser^5 to a non-phosphorylatable alanine (S5A) was more easily extracted following detergent-treatment of cells (i.e. decreased avidity for actin) [36]. Expression of the S5A mutant also perturbed its localisation to actin-rich structures when expressed in cells. Furthermore, the S5A mutant was defective in bundling actin filaments *in-vitro* [36]. Fluorescence Recovery After Photo Bleaching (FRAP) imaging studies subsequently demonstrated that Ser^5 phosphorylation increased the amount of F-actin in focal adhesions, thereby providing a link between post-translational regulation of L-plastin and adhesion/migration [37]. Whereas expression of wild-type L-plastin in transformed cell lines promoted migration and invasion, the same effects were not observed when the S5A mutant was expressed in parallel, demonstrating a mechanistic role for Ser5 phosphorylation in L-plastin-mediated tumour cell migration [28,36]. However, more recent studies have reported that an S5A L-plastin mutant was able to bind and bundle actin filaments as efficiently as the wild-type protein *in-vitro*, and that T-cells, B-cells and macrophages from a CRISPR-generated mutant mouse strain expressing endogenous L-plastin with an S5A mutation were largely similar to their wild-type (Ser^5) counterparts in terms of development, migration and activation [38,39]. These latter studies therefore suggest that L-plastin function may not be strictly dependent on Ser^5 phosphorylation.

In this study we have investigated the function of wild-type L-plastin and its S5A mutant in the HCT 116 colon cancer cell line, as it is unknown whether phosphorylation at this site specifically influences colon cancer cell proliferation and migration. Unlike most other transformed cell lines [14], HCT 116 cells do not aberrantly express L-plastin. We found that expression of L-plastin in HCT 116 cells increased cell proliferation and cyclin D1 levels and promoted cell migration *in-vitro*. Since L-plastin expression has been associated with NADPH oxidase-mediated Reactive Oxygen Species (ROS) in neutrophils we investigated whether such a role exists for L-plastin in colon cancer cells as NADPH oxidase/ROS play important roles in tumourigenesis [2,40,41]. We found that expression of L-plastin expression resulted in Rac1-GTP activation (an upstream activator of NADPH oxidase) and increased ROS production. Furthermore, Diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity, inhibited the L-plastin-mediated increase in cell migration. Interestingly, all effects bar cell proliferation and cyclin D1 activation were independent of Ser^5 phosphorylation. These results highlight a novel role for L-plastin in colon cancer progression *via* a mechanism involving NADPH oxidase and ROS production and demonstrate that such effects are largely independent of Ser^5 phosphorylation.

MATERIALS AND METHODS

Reagents and antibodies

Fugene 6™ transfection reagent was from Roche Diagnostics Ltd, UK. Zeocin was from Cayla, France. Monoclonal antibodies to L-plastin (LPL4A.1) were from Neomarkers, USA. Goat anti-mouse FITC and TRITC-labelled secondary antibodies were from Molecular Probes Inc, Eugene, OR.

Generation of L-plastin phospho-mutant constructs

PcDNA3.1/LPL containing the complete cDNA sequence for L-plastin expressed as a fusion protein encoding a C-terminal V5 epitope was purchased from Invitrogen BV, The Netherlands. The serine 5 residue on L-plastin was mutated to glutamic acid (S5E) or alanine (S5A) using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla). Primers containing the intended mutation were run in a QuikChange™ Site-Directed Mutagenesis (Stratagene) reaction with the L-plastin template. The product was then treated with DpnI, transformed into supercompetent bacterial cells and plated on low salt LB-Zeocin plates. A number of colonies were sequenced by the Agowa genomics company, Germany, and clones containing the intended mutations were selected and prepared using a Qiagen midi prep protocol and kit (Qiagen, UK).

Cell Lines, culture conditions and transfection procedure

The human colon carcinoma cell line HCT 116 was cultured in McCoy's 5A medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (Gibco-Brl, UK). PcDNA3.1/LPL containing the complete L-plastin cDNA insert, expressed as a fusion protein encoding a C-terminal V5 epitope, was obtained from Invitrogen BV, The Netherlands. HCT 116 cells were grown in 6-well plates and transfected with 1μg of pcDNA3.1/LPL (HCT 116-LPL), pcDNA3.1/LPL-S5E (HCT 116-S5E), PcDNA3.1/LPL-S5A (HCT 116-S5A) or with pcDNA3.1/GS (HCT 116-pcDNA3.1). For stable transfections, cells were seeded into 6-well plates 24 hours post-transfection. Selection of resistant clones was performed in medium containing 600μg/mL zeocin. Surviving colonies were picked, expanded and screened for the selection of stably transfected clones.

Immunoblot analysis

Expression of L-plastin protein was confirmed by Western blot using anti-LPL-4A.1 antibody (Neomarkers, USA). Cells were trypsinised, washed in warm PBS and resuspended in 1% NP40 lysis buffer. Lysates were acetone precipitated, resuspended in Laemmli buffer (0.0625 M Tris (pH 6.75), 2% SDS, 5% β-mercaptoethanol, 10% Glycerol, 0.001% bromophenol blue), boiled for 5 min, resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (Gelman Sciences, UK). Membranes were blocked with 5% milk powder in 1 × PBS-0.1% Tween-20, and were incubated overnight with anti-LPL-4A.1 mAb. Actin mAb (Sigma, UK) was used as a loading control in all experiments.

Immunoprecipitation

HCT 116 cells were stably transfected with pcDNA3.1/LPL-WT as previously described. Cells were lysed in 0.5 mL ice-cold RIPA lysis buffer for 30 minutes on ice. Unlysed cells and nuclei were removed by centrifugation at 1000 rpm for 3 minutes. Detergent-soluble lysates (5 × 106 cells in 0.5 ml lysis buffer) were pre-cleared with washed protein G–Sepharose beads for 2 h at 4°C with end-over-end mixing.

The beads were removed by centrifugation and pre-cleared lysates were subjected to immunoprecipitation with anti-LPL4A.1 monoclonal antibody, or an irrelevant IgG isotype control monoclonal (IE) antibody for 2 h on ice. The antibody–antigen complexes were then collected by incubating the lysates with washed protein G–Sepharose beads overnight at 4°C with end-over-end mixing. The immunoprecipitates were recovered by centrifugation and washed three times with ice-cold cell lysis buffer. The pellets were resuspended in SDS–PAGE sample buffer, boiled for 4 min, the beads were removed by centrifugation, and the immunoprecipitate supernatants were resolved on 10% SDS–PAGE gels. The phosphorylation status of wild-type L-plastin was determined using an antibody to phospho-L-plastin (a kind gift from Professor Eric Brown, UCSF). For determination of Rac1 activity, active Rac1 was immunoprecipitated using PAK1-GST agarose conjugate (Upstate) and detected by Western blot analysis as above using anti-Rac-1 (Transduction Labs).

Cell Proliferation Assays-Proliferation assays were performed on stably transfected cells (HCT 116-LPL, HCT 116-S5A or HCT 116-pcDNA3.1) by cell count. Briefly, for each cell line 1×10^5 cells were seeded into 6-well plates. Cells were trypsinized and counted with a haemocytometer at 24-hour intervals. Assays were carried out for 96 hours. All experiments were repeated at least 3 times.

Proliferation was also measured in cells transiently transfected with L-Plastin constructs using a chemiluminescent reporter gene assay system for detection of cyclin D1 activation. HCT 116 cells were plated at 1×10^5 cells/ml onto 6-well dishes. The following day, cells were transiently transfected with 0.4 µg of a Cyclin D1 Prpmoter-Luciferase-luciferase fusion construct (1745 CD1-Luc) a generous gift of Dr. Richard Pestell (Georgetown University)) and either 0.4 µg of pcDNA3.1-GS (control), pcDNA3.1-LPL/WT or pcDNA3.1-LPL/S5A using Fugene 6 transfection reagent (Roche Applied Science). 0.1 µg of B-gal vector (Promega, Madison WI)) was co-transfected with each of the above vectors. Luciferase and B-gal activities were measured by the Dual-Light reporter assay system (Promega, Madison, WI) on a Victor luminometer (Perkin Elmer, Wellesley MA).

Cell migration assay

Cell migration was measured using an 8µM BDBiocoat transwell filter (Becton Dickinson, Rutherford, NJ). Briefly, 5×10^5 cells (HCT 116-LPL, HCT 116-S5A or HCT 116-pcDNA3.1) were washed and suspended in serum free medium before seeding onto each filter in duplicate. Medium containing 20% FCS was added to the bottom of the plate. Cells were allowed to migrate through the filter for 24 hours, and filters were fixed and processed exactly according to manufacturer's instructions. All experiments were carried out three times.

Detection of Reactive Oxygen species (ROS)-ROS were detected in cells stably transfected with pcDNA3.1-GS (control), pcDNA3.1-LPL/WT or pcDNA3.1-LPL/S5A using the cell permeant reagent 2',7'-dichlorofluoroescin diacetate (H2DCFDA; Molecular Probes). Briefly, cells were seeded on black luminometry plates (10,000/well) to adhere overnight. Cells were washed twice in warm HBSS, serum-starved for 3 hours and then incubated in 100 ul of H2DCFDA (4 ug/ml) for 60 min. Cells were then washed and fluorescence detected using a Victor luminometer (Perkin Elmer, Wellesley MA), excitation 495 nm/emission 520 nm.

Immunofluorescence microscopy – HCT 116 cells stably transfected with L-plastin constructs were seeded into 8-well chamber slides (Nunc) and after 24 hours the cells were fixed with 4% paraformaldehyde for 15 min at 37°C, followed by permeabilisation with 0.3% v/v Triton X100 in PBS for 5 min at room temp. The slides were blocked with 3% BSA/PBS for 30 min before anti-L-plastin antibody (clone 4A.1) was added for 1 hour (diluted 1/100 in 3% BSA/PBS), followed by AlexaFluor goat anti-mouse 488 (1:500 dilution; Invitrogen) and phalloidin-TRITC (1:200 dilution; Sigma). The slides were incubated for 30 min at room temp before being washed and stored in PBS. The slides were analysed with a Zeiss LSM 510 confocal microscope.

RESULTS

Wild-type L-plastin is phosphorylated on Ser^5 when expressed in HCT 116 cells

In this study we used the HCT 116 colon cancer cell line to investigate the role of L-plastin Ser^5 phosphorylation on cell proliferation and migration. We used this cell line because they do not aberrantly express L-plastin as confirmed by Western blotting (data not shown). We first established the phosphorylation status of L-plastin in HCT 116 cells by stably transfecting the cells with a plasmid encoding the wild-type (WT) version of L-plastin. After overnight incubation to allow for L-plastin expression, the cells were serum starved and either left untreated or treated with the phorbol ester PMA which promotes L-plastin phosphorylation at Ser^5 [9,34,37]. The cells were lysed, L-plastin was immunoprecipitated and probed by Western blotting with an antibody specific for L-plastin when phosphorylated at Ser^5 [42]. We found that WT L-plastin was constitutively phosphorylated upon expression in HCT 116 cells, because L-plastin was phosphorylated at Ser^5 following expression and was only very modestly increased with PMA treatment (Figure 1A). We next wanted to determine if Ser^5 phosphorylation plays a role in these colon cancer cells and hence we generated L-plastin mutants where Ser^5 was exchanged to an Alanine (S5A mutant) or to a negatively-charged Glutamic acid (S5E mutant), both of which have been widely used as mimics of non-phosphorylated and phosphorylated L-plastin, respectively [28,36] (Figure 1B). We created stable cell lines of HCT 116 cells expressing either WT L-plastin, S5A L-plastin or S5E L-plastin and selected stable clones for further analysis that expressed the highest levels of L-plastin (Figure 1C). It should be noted however that the levels of L-plastin in these clones were much lower compared to the SW620 metastatic colorectal cancer cell line which was used as a positive control for Western blotting in these experiments (Figure 1C). Expression of these L-plastin constructs appeared to be functional, since expression of the WT and S5E L-plastin constructs were diffusely localized throughout the cell and F-actin staining was normal, whereas F-actin was mis-localised to distinct puncta within the cytoplasm of the cell in cells transfected with the S5A mutant (Supplementary Figure 1A and B).

L-plastin expression increases proliferation and cyclin D1 activation in a manner dependent on Ser^5 phosphorylation

Proliferation rates for HCT 116 cells expressing WT, S5A or S5E L-plastin were determined by cell counting every 24 hours. No change in cell proliferation rates were observed until 72 hours post-seeding, when HCT 116 cells expressing WT L-plastin exhibited increased proliferation compared with control HCT 116 cells transfected with empty plasmid (Figure 2A). Whereas expression of the S5E L-plastin mutant resulted in similar levels of proliferation compared to cells transfected with WT L-plastin, proliferation was lower in cells expressing the S5A L-plastin mutant (Figure 2A). This result indicates that L-plastin expression promotes colon cancer cell proliferation, a finding that is consistent with our previous study in the SW480 colon cancer cell line [35]. Furthermore, the increase in proliferation mediated by L-plastin expression is dependent on phosphorylation

at Ser[5]. Because cell proliferation is regulated by cyclin D1-mediated transition from the G1 phase to the S phase of the cell cycle, we assessed the effect of these L-plastin constructs on cyclin D1 activation in HCT 116 cells using a reporter system. Similar to the proliferation assay, expression of WT and S5E L-plastin promoted cyclin D1 activation, whereas the levels of cyclin D1 were lower in cells transfected with the L-plastin S5A mutant (Figure 2B). Because L-plastin was constitutively phosphorylated at Ser[5] upon expression in HCT 116 cells, we did not assess the effect of the S5E L-plastin mutant in further experiments unless where indicated.

L-plastin expression increases migration and reactive oxygen species in an NADPH oxidase-dependent manner

HCT 116 cells stably transfected with L-plastin constructs were assessed for migration towards cell culture medium supplemented with FCS using transwell assays. Expression of WT L-plastin increased HCT 116 migration compared to control cells transfected with empty plasmid (Figure 3A). Curiously however, expression of the L-plastin S5A mutant resulted in levels of migration that were similar to WT L-plastin. This implies that while L-plastin-mediated cell proliferation and cyclin D1 activation are dependent on phosphorylation of Ser[5], L-plastin-mediated migration is independent of Ser[5] phosphorylation. To investigate the mechanism of this L-plastin-induced increase in HCT 116 cell migration in more detail, we investigated whether L-plastin expression resulted in the production of ROS. We pursued this for two reasons, namely because L-plastin expression has been shown to regulate the production of ROS in neutrophils *via* NADPH oxidases and such highly-reactive oxygen species have been implicated in cell proliferation, invasion and metastasis of cancer cells which suggests that L-plastin-mediated ROS production may be a novel mechanism of colon cancer pathogenesis [2,40,41]. Expression of either the WT L-plastin or the S5A mutant in HCT 116 cells resulted in significantly increased levels of ROS when compared to cells transfected with control (empty) plasmid (Figure 3B). Rac1 is a GTPase that promotes cellular migration *via* polymerization of the actin cytoskeleton 44 but also regulates activation of NADPH oxidases which are the main intracellular sources of ROS production [43]. Consistent with a role for L-plastin in ROS production, we found that L-plastin expression resulted in Rac1 activation (measured by GTP-loading) and that this was independent of the phosphorylation status of Ser[5] phosphorylation (Figure 3C). These results demonstrate a novel role for L-plastin expression in ROS production in colon cancer cells. Finally, we demonstrated that Diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity, significantly decreased the migration of HCT 116 cells transfected with WT L-plastin or S5A L-plastin (reversed to levels of mock-transfected cells) with no effect on mock-transfected cells (Figure 3D). These results indicate that expression of L-plastin in HCT 116 colon cancer cells increases their migratory capacity *via* Rac1/NADPH oxidase-mediated increase in ROS and that this is independent of L-plastin Ser[5] phosphorylation status.

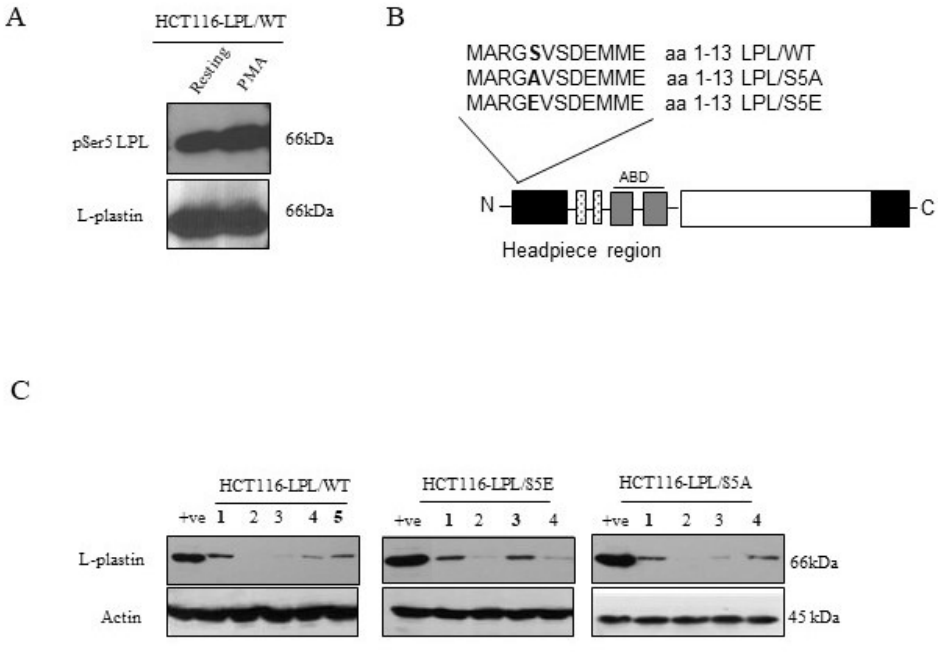

Figure 1: Expression and charaterisation of phospho-status of wild-type L-plastin and expression of phosphorylation mutants in HCT 116 cells. (A) HCT 116-LPL cells express constitutively phosphorylated L-plastin. HCT 116 cells were stably transfected with wild-type L-plastin, immunoprecipitated with anti-L-plastin antibody and immunoblotted with either anti-phospho-Ser[5]-L-plastin (top) or anti-L-plastin (bottom). (B) Domain structure of the fimbrin family and the N-terminal amino acid sequence of L-plastin. The fimbrin family is characterized by two EF hand calcium-binding domains in the N-terminal headpiece region and two -actinin-type actin-binding domains (ABD). The amino acid sequences of WT L-plastin, and the phospho mutants (S5E and S5A) are indicated. (C) Establishment of stable HCT 116-LPL/WT, HCT 116/S5E and HCT 116/S5A human colorectal-carcinoma cell lines. HCT 116 cells were transfected with either empty vector (pcDNA3.1) or vector containing the full cDNA coding sequence to L-plastin, L-plastin-S5E or L-plastin-S5A and stable clones were selected in the presence of 600 µg ml−1 zeocin for 15 days. Parental, control and HCT 116-LPL/WT, HCT 116-LPL/S5E and HCT 116-LPL/S5A cells were solubilized in lysis buffer containing 1% Nonidet P-40 and immunoblotted with antibodies to L-plastin. ß-actin was included in order to verify equal loading.

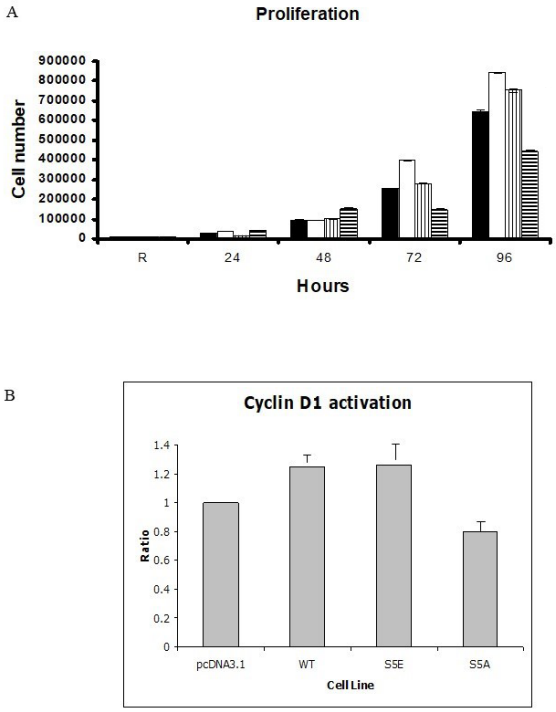

Figure 2: Expression of L-plastin/WT increased proliferation rate of HCT 116 cells. (A) For each cell line (HCT 116-LPL/WT, HCT 116-LPL/S5A or control HCT 116 cells transfected with empty vector pcDNA3.1), 1×10^5 cells were seeded into 6-well plates. Cells were trypsinized and counted with a haemocytometer at 24-hour intervals. Data are presented as a single experiment and are representative of 3 independent experiments. (B) Cyclin D1 activation was measured using a dual light reporter system as described in the Materials and Methods. Activation in HCT 116-LPL/WT, HCT 116-LPL/S5A and HCT 116-LPL/S5E cells was normalized to cells expressing the pcDNA3.1 vector. **Note:** (■) Ctrl; (□) WT; (▥) S5E; (▤) S5A

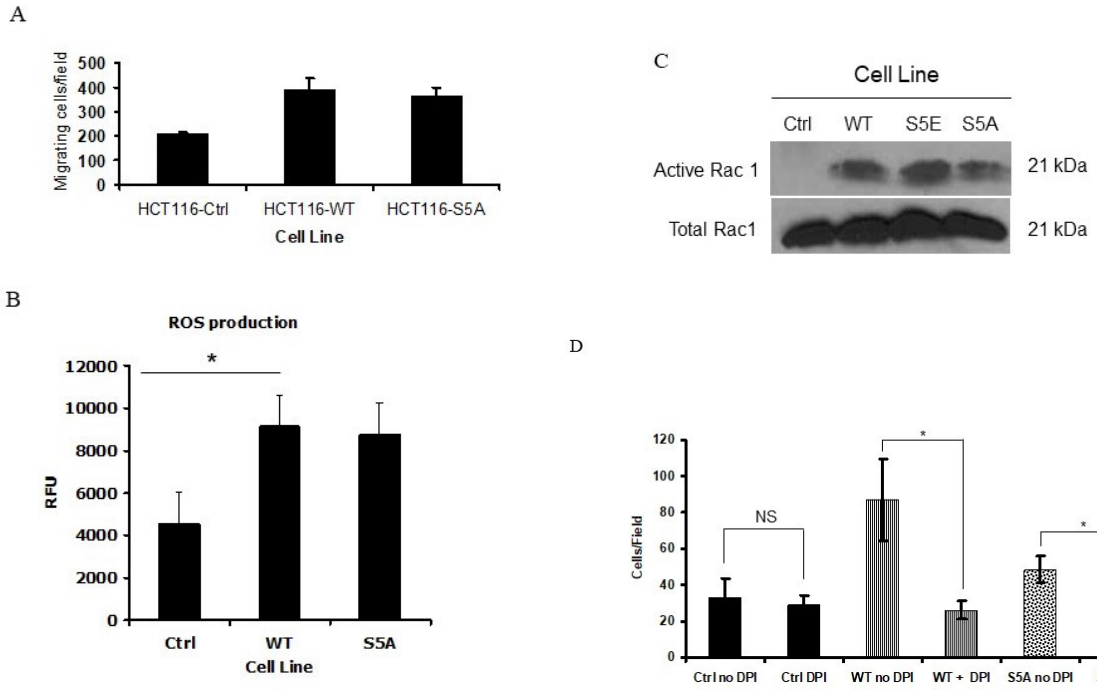

Figure 3: Expression of L-plastin stimulates migration of HCT 116 cells through a Trans well filter in a NADPH oxidase dependent manner. (A) 5×10^5 cells were seeded onto each filter (8 μM) and allowed to migrate through the filter for 24 hours when they were stained and counted. Graph illustrates results from three migration assays ±S.D. (B) ROS levels were measured in control HCT 116 cells (expressing pcDNA3.1) and cells stably expressing pcDNA3.1-LPL/WT and HCT 116-LPL/S5A cells. A one-way ANOVA with Bonferroni posttest, showed the means are significantly different ($p=0.0453$). (C) HCT 116 cells were transiently transfected with pcDNA3.1 (ctrl), WT-, S5E- or S5A-L-plastin. 24 hrs post-transfection, total lysates were harvested, subjected to immune precipitation with PAK1-GST to pull-down active/GTP-loaded Rac1, and Western blotted for Rac1. Total lysates were blotted for Rac1 as a loading control. (D) Migration of control (HCT 116-pcDNA3.1), HCT 116-LPL/WT and HCT 116-LPL/S5A cells in the presence and absence of the NADPH oxidase inhibitor, Diphenyleneiodonium (DPI). (*denotes $p<0.001$).

DISCUSSION

In this study we have demonstrated that expression of L-plastin increases cell proliferation and cyclin D1 activation in HCT 116 colon cancer cells. We also showed that expression of this actin-bundling protein promoted migration and ROS production, and that the L-plastin-mediated increase in migration was inhibited by a pharmacological inhibitor of NADPH oxidases which are considered the main intracellular sources of ROS. Therefore, this study corroborates a role for L-plastin in colon cancer cell proliferation and provides a novel mechanistic link for L-plastin-mediated migration through ROS production *via* Rac1-GTP and NADPH oxidase.

Aberrant L-plastin expression in solid tumours has long been known to influence several of the hallmarks of cancer particularly limitless replicative potential and tissue invasion/metastasis [44]. For example, Zhang et al. 1999 first demonstrated that antisense-mediated silencing of L-plastin in prostate cancer cell lines reduced their growth rate, whereas we demonstrated that expression of L-plastin in the SW480 colon cancer cell line increased cell proliferation [35]. These studies were the first to document that that L-plastin positively influences replicative potential [35]. Other reports have since demonstrated that expression of L-plastin is associated with increased proliferation of osteosarcoma cells and gastric cancer cells whereas knockdown of L-plastin expression decreases cell proliferation in osteosarcoma cells oral squamous cell carcinoma cells and a breast cancer cell line [22]. Our results presented in this study corroborate these findings by demonstrating that expression of L-plastin in HCT 116 colon cancer cells increases cell proliferation. It should be noted however that the molecular mechanisms driving L-plastin-mediated proliferation are not well understood. Ge et al., only recently highlighted that L-plastin-mediated cell proliferation in osteosarcoma cells correlated with cyclin D1 up-regulation, a finding that we have demonstrated in this study here. These studies point to a role for L-plastin in cell proliferation *via* progression from the G1 phase of the cell cycle to the S phase (DNA replication). Nonetheless, further studies will be required to elucidate in more detail the mechanistic role of L-plastin in cyclin D1 expression and cell cycle progression.

It is also well established using *in-vitro* cell line models and animal studies that L-plastin promotes migration/invasion and metastasis, which is another defining hallmark of cancer cells [44]. Manipulation of L-plastin expression in diverse cancer cell lines has clearly shown a key role for this actin-bundling protein in migration and invasion (see Introduction). This migratory/invasive phenotype is thought to occur primarily *via* bundling and stabilization of actin filaments (which localize to cellular structures that promote migration/invasion into tissues including focal adhesions, lamellipodia, filopodia, dorsal ruffles, microspikes, podosomes and invadopodia) epithelial-mesenchymal transition and production of matrix metalloproteases that degrade the extracellular matrix [16,28,34,36,37,45]. Interestingly, Ge et al. additionally demonstrated that L-plastin promotes epithelial-mesenchymal transition (e.g. loss of E-cadherin) in osteosarcoma cell lines *via* degradation of neuregulin receptor degradating protein 1 (NDRP1; a RING-Type E3 Ubiquitin Transferase) and activation of the JAK2/STAT3 pathway. This study highlights the complex pathways influencing migration and invasion that are activated in response to aberrant expression of L-plastin.

Our results presented in this study here further underscore this complexity as we demonstrate for the first time in a cancer cell line that L-plastin expression promotes ROS production which is known to play a key role in tumour migration and invasion. We investigated this novel mechanism of L-plastin-mediated migration following reports in neutrophils that L-plastin is required for NAPDH oxidase activation and ROS-mediated pathogen killing and that optimally phosphorylated L-plastin (i.e. highly phosphorylated L-plastin induced by treatment of cells with the phosphatase inhibitor calyculin A) may be involved in activation of NAPDH oxidase in these cells [40]. Indeed, we found that ectopic expression of L-plastin in HCT 116 colon cancer cells stimulated ROS production and that a pharmacological inhibitor of NADPH oxidase blocked the L-plastin-mediated increase in migration. Interestingly, it has been recently reported that NADPH oxidase-induced ROS production in cancer cells diminishes the actin-bundling activity of L-plastin *via* oxidation of Cys101, which forms a disulphide bridge with Cys42 and is primarily restricted to the periphery of the cell [45]. The finding that ROS modulates L-plastin and that L-plastin stimulates ROS production (our results) strongly suggests that cancer cells operate a feed-forward ROS loop for initiation and modulation of actin-based migration.

Phosphorylation of L-plastin on Ser^5 is considered as an 'activating' switch that facilitates actin-bundling and actin localization in cells, thus actively contributing to cellular migration, invasion and metastasis [28,32,36,37]. We therefore investigated a role for Ser^5 L-plastin phosphorylation for the first time in colon cancer proliferation and migration. Our results clearly demonstrated that phosphorylation was required for proliferation and cyclin D1 expression, whereas migration, Rac1 activation and ROS production were increased by L-plastin independently of Ser^5 phosphorylation. However, it should be noted that roles for Ser^5 phosphorylation in cancer cell migration appear to be context dependent, as phosphorylation of Ser^5 was required for integrin-mediated migration and cellular invasion *in-vitro* but was not required for chemotaxis in response to chemokines or cell culture medium supplemented with foetal calf serum [28,36]. Our finding that expression of the S5A L-plastin mutant does not affect HCT 116 migration towards foetal calf serum-supplemented cell culture medium is consistent with these previous results. Nonetheless, we cannot rule out that phosphorylation of other sites on L-plastin, including Thr89 which was shown to regulate T-cell motility influences L-plastin-mediated tumour migration and actin dynamics [46]. In summary, our study uncovers a novel role for L-plastin in colon cancer migration *via* a mechanism involving NADPH oxidase and ROS production.

CONCLUSION

We have demonstrated for the first time that L-plastin regulates ROS production and cellular migration in a colorectal cancer cell line in a manner that is dependent on NADPH activity. This process was independent of phosphorylation of Ser5 in the headpiece region of L-plastin. This study therefore provides new mechanistic insights into the cellular drivers of ROS and colon cancer tumourigenesis.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from The Health Research Board in Ireland and Cancer Research Ireland.

DISCLOSURE STATEMENT

The authors have no conflict of interest.

REFERENCES

1. Schaffner-Reckinger E, Machado RAC. The actin-bundling protein l-plastin-a double-edged sword: Beneficial for the immune response, maleficent in cancer. Int Rev Cell Mol Biol. 2020;355:109–154.
2. Chen H, Mocsai A, Zhang H, Ding R, Hiroshi Morisaki J, White M,

et al. Role for plastin in host defense distinguishes integrin signaling from cell adhesion and spreading. Immunity. 2003; 19(1): 95-104.

3. De Clercq S, Boucherie C, Vandekerckhove J, Gettemans J, Guillabert A. L-plastin nanobodies perturb matrix degradation, podosome formation, stability and lifetime in thp-1 macrophages. 2013;8(11):e78108.

4. Deady LE, Todd EM, Davis CG, Zhou JY, Topcagic N, Edelson BT et al. L-plastin is essential for alveolar macrophage production and control of pulmonary pneumococcal infection. Infect Immun. 2014;82(5):1982-1993

5. Todd EM, Zhou JY, Szasz TP, Deady LE, D'Angelo JA, Cheung MD et al. Alveolar macrophage development in mice requires L-plastin for cellular localization in alveoli. Blood. 2016; 128(24):2785-2796.

6. Zhou JY, Szasz TP, Stewart-Hutchinson PJ, Sivapalan J, Todd EM, Deady LE, et al. L-Plastin promotes podosome longevity and supports macrophage motility. Mol Immunol. 2016;78:79-88.

7. Morley SC, Wang C, Lo W, Lio CJ, Zinselmeyer BH, Miller MJ, et al. The actin-bundling protein L-plastin dissociates CCR7 proximal signaling from CCR7-induced motility. J Immunol. 2010; 184(7):3628-3638.

8. Wang C, Morley SC, Donermeyer D, Peng I, Lee WP, Devoss J, et al. Actin-bundling protein l-plastin regulates t cell activation. J Immunol. 2010; 185(12):7487-7497.

9. Freeley M, O'Dowd F, Paul T, Kashanin D, Davies A, Kelleher D, et al. L-plastin regulates polarization and migration in chemokine-stimulated human T lymphocytes. J Immunol. 2012; 188(12):6357-6370.

10. Clercq SD, Zwaenepoel O, Martens E, Vandekerckhove J, Guillabert A, Gettemans J, et al. Nanobody-induced perturbation of lfa-1/l-plastin phosphorylation impairs mtoc docking, immune synapse formation and t cell activation. Cell Mol Life Sci. 2013;70(5):909-922

11. Todd EM, Deady LE, Morley SC. The actin-bundling protein l-plastin is essential for marginal zone B cell development. J Immunol. 2011;187(6):3015-3025.

12. Todd EM, Deady LE, Morley SC. Intrinsic T- and B-cell defects impair T-cell-dependent antibody responses in mice lacking the actin-bundling protein l-plastin. Eur J Immunol. 2013;43(7):1735-1744.

13. Lin CS, Park T, Chen ZP, Leavitt J. Human plastin genes.Comparative gene structure, chromosome location, and differential expression in normal and neoplastic cells. J Biol Chem.1993; 268(4):2781-2792,

14. Park T, Chen ZP, Leavitt J. Activation of the leukocyte plastin gene occurs in most human cancer cells. Cancer Res. 1994;54(7):1775-1781.

15. Teng YS, Chen WY, Yan ZB, Lv YP, Liu YG, Mao FY,et al. L-plastin promotes gastric cancer growth and metastasis in a helicobacter pylori caga-erk-sp1-dependent manner. Mol Cancer Res. 2021;19(6):968-978.

16. Ge X, Liu W, Zhao W, Feng S, Duan A, Ji C, et al. Exosomal transfer of lcp1 promotes osteosarcoma cell tumorigenesis and metastasis by activating the jak2/stat3 signaling pathway. Mol Ther Nucleic Acids. 2020;21:900-915.

17. Koide N, Kasamatsu A, Sakamoto YE, Ishida S, Shimizu T, Kimura Y, et al. Evidence for critical role of lymphocyte cytosolic protein 1 in oral cancer. Sci Rep. 2017;7:43379.

18. Harris LD, Cerda JDL, Tuziak T,Rosen D, Xiao L, Shen Y, et al. Analysis of the expression of biomarkers in urinary bladder cancer using a tissue microarray. Mol Carcinog. 2008;47(9):678-685.

19. Fang ZQ, Zang WD, Chen R, Ye BW, Wang XW, Yi SH, et al. Gene expression profile and enrichment pathways in different stages of bladder cancer. Genet Mol Res. 2013;12(2):1479-1489.

20. Lapillonne A, Coué O, Friederich E, Nicolas A, Maestro LD, Louvard D, et al. Expression patterns of l-plastin isoform in normal and carcinomatous breast tissues. Anticancer Res. 2000;2(5A):3177-3182.

21. Alldridge L, Metodieva G, Greenwood C, Janabi KA, Thwaites L, Sauven P, et al. Proteome profiling of breast tumors by gel electrophoresis and nanoscale electrospray ionization mass spectrometry. J Proteome Res. 2008; 7(4):1458-1469.

22. Pillar N, Polsky LA, Shomron N. Dual inhibition of abce1 and lcp1 by microrna-96 results in an additive effect in breast cancer mouse model. Oncotarget. 2019;10(21):2086-2094.

23. Zheng J, Ganguly NR, Powell WC, Burman PR. Suppression of prostate carcinoma cell invasion by expression of antisense l-plastin gene. Am J Pathol. 1999;155(1):115-122.

24. Chen C, Cai Q, He W, Lam TB, Lin J, Zhao Y, et al. Ap4 modulated by the pi3k/akt pathway promotes prostate cancer proliferation and metastasis of prostate cancer *via* upregulating l-plastin. Cell Death Dis. 2017; 8(10):3060.

25. Kim DS, Choi YP, Kang S, Gao MQ, Kim B, Park HR, et al. Panel of candidate biomarkers for renal cell carcinoma. J Proteome Res. 2010;9(7):3710-3719.

26. Li LMX, Xiao ZQ, Liu YF, Chen YH, Li C, Zhang PF, et al. Quantitative proteomic analysis of differential proteins in the stroma of nasopharyngeal carcinoma and normal nasopharyngeal epithelial tissue. J Cell Biochem. 2009;106(4):570-579.

27. Klobučar M, Sedić M, Gehrig P, Grossmann J, Bilić M, Bilić LK, et al. Basement membrane protein ladinin-1 and the mif-cd44-β1 integrin signaling axis are implicated in laryngeal cancer metastasis. Biochim Biophys Acta. 2016;1862(10):1938-1954.

28. Klemke M, Rafael MT, Wabnitz GH, Weschenfelder T, Konstandin MH, Garbi N, et al. Phosphorylation of ectopically expressed L-plastin enhances invasiveness of human melanoma cells. Int J Cancer. 2007;120(12):2590-2599.

29. Otsuka M, Kato M, Yoshikawa T, Chen H, Brown EJ, Masuho Y, et al. Differential expression of the L-plastin gene in human colorectal cancer progression and metastasis. Biochem Biophys Res Commun. 2001;289(4):876-881.

30. Li J, Zhao R. Expression and clinical significance of l-plastin in colorectal carcinoma. J Gastrointest Surg. 2011;15(11):1982-1988.

31. Chaijan S, Roytrakul S, Mutirangura A, Leelawat K. Matrigel induces l-plastin expression and promotes l-plastin-dependent invasion in human cholangiocarcinoma cells. Oncol Lett. 2014;8(3):993-1000.

32. Riplinger SM, Wabnitz GH, Kirchgessner H, Jahraus B, Lasitschka F, Schulte B, et al. Metastasis of prostate cancer and melanoma cells in a preclinical *in vivo* mouse model is enhanced by l-plastin expression and phosphorylation. Mol Cancer. 2014;13(10):10.

33. Lommel MJ, Trairatphisan P, Gäbler K, Laurini C, Muller A, Kaoma T, et al. L-plastin ser^{5} phosphorylation in breast cancer cells and *in vitro* is mediated by rsk downstream of the erk/mapk pathway. FASEB J. 2016;30(3):1218-1233.

34. Machado RAC, Stojevski D, Landtsheer SD, Lucarelli P, Baron A, Sauter T, et al. L-plastin ser^{5} phosphorylation is modulated by the pi3k/sgk pathway and promotes breast cancer cell invasiveness. Cell Commun Signal. 2021;19(1):22.

35. Foran E, McWilliam P, Kelleher D, Croke DT, Long A. The leukocyte protein L-plastin induces proliferation, invasion and loss of E-cadherin expression in colon cancer cells. Int J Cancer. 2014; 118(8):2098-2104.

36. Janji B, Giganti A, Corte VD, Catillon M, Bruyneel E, Lentz D, et al. Phosphorylation on ser^{5} increases the f-actin-binding activity of l-plastin and promotes its targeting to sites of actin assembly in cells. J Cell Sci. 2006; 119(9):1947-1960.

37. Tanoury ZA, Reckinger ES, Halavatyi A, Hoffmann C, Moes M, Hadzic E, et al. Quantitative kinetic study of the actin-bundling protein l-plastin and of its impact on actin turn-over. PloS One. 2010; 5(2):9210.

38. Schwebach CL, Agrawal R, Lindert S, Kudryashova E, Kudryashov DS. The roles of actin-binding domains 1 and 2 in the calcium-dependent regulation of actin filament bundling by human plastins. J Mol Biol. 2017; 429(16):2490-2508.

39. Anaya EP, Lin X, Todd EM, Szasz TP, Morley SC. Novel mouse model reveals that serine phosphorylation of l-plastin is essential for effective splenic clearance of pneumococcus. J Immunol. 202; 206(9):2135-2145

40. Oshizawa T, Yamaguchi T, Suzuki K, Yamamoto Y, Hayakawa T. Possible involvement of optimally phosphorylated l-plastin in activation of superoxide-generating nadph oxidase. J Biochem. 2003; 134(6):827-834.

41. Skonieczna M, Hejmo T, Poterala-Hejmo A, Cieslar-Pobuda A, Buldak RJ. NADPH oxidases: insights into selected functions and mechanisms of action in cancer and stem cells. Oxid Med Cell Longev. 2017; 9420539.

42. Wang J, Brown EJ. Immune complex-induced integrin activation and l-plastin phosphorylation require protein kinase a. J Biol Chem. 1999; 274(34):24349-24356.

43. Hordijk PL. Regulation of NADPH oxidases: the role of rac proteins. Circ Res. 2006; 98(4): 453-462.

44. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100(1):57-70.

45. Balta E, Hardt R, Liang J, Kirchgessner H, Orlik C, Jahraus B et al. Spatial oxidation of l-plastin downmodulates actin-based functions of tumor cells. Nat Commun. 2019; 10(1):4073.

46. Xu X, Wang X, Todd EM, Jaeger ER, Vella JL, Mooren OL et al. Mst1 kinase regulates the actin-bundling protein l-plastin to promote T cell migration. J Immunol. 2016; 197(5):1683-1691.