



Loss of the laminin subunit alpha-3 induces cell invasion and macrophage infiltration in cutaneous squamous cell carcinoma

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Summary

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Conflicts of interest

The authors declare they have no conflicts of interest.

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Background Cutaneous squamous cell carcinoma (cSCC) is a common cancer that invades the dermis through the basement membrane. The role of the basement membrane in poorly differentiated cSCC is not well understood.

Objectives To study the effect that loss of the laminin subunit alpha-3 (α 3) chain from the tumour microenvironment has on tumour invasion and inflammatory cell recruitment.

Methods We examined the role of the basement membrane proteins laminin subunits α 3, β 3 and γ 2 in SCC invasion and inflammatory cell recruitment using immunohistochemistry, short hairpin RNA knockdown, RNA-Seq, mouse xenograft models and patient tumour samples.

Results Analysis of SCC tumours and cell lines using antibodies specific to laminin chains α 3, β 3 and γ 2 identified a link between poorly differentiated SCC and reduced expression of laminin α 3 but not the other laminin subunits investigated. Knockdown of laminin α 3 increased tumour invasion both *in vitro* and *in vivo*. Western blot and immunohistochemical staining identified increased phosphorylated myosin light chain with loss of laminin α 3. Inhibition of ROCK (rho-associated protein kinase) but not Rac1 significantly reduced the invasive potential of laminin α 3 knockdown cells. Knockdown of laminin subunits α 3 and γ 2 increased monocyte recruitment to the tumour microenvironment. However, only the loss of laminin α 3 correlated with increased tumour-associated macrophages both in xenografted tumours and in patient tumour samples.

Conclusions These data provide evidence that loss of the laminin α 3 chain in cSCC has an effect on both the epithelial and immune components of cSCC, resulting in an aggressive tumour microenvironment.

What is already known about this topic?

- The tumour microenvironment, including the extracellular matrix, plays an important role in modulating tumour behaviour and inflammatory infiltration.
- Data on laminin expression in poorly differentiated squamous cell carcinomas (cSCCs) are limited.

What does this study add?

- Poorly differentiated cSCCs have significantly reduced laminin α 3 expression.
- Loss of laminin α 3 increases tumour invasion, amoeboid migration and ROCK (rho-associated protein kinase) signalling.
- Expression of laminin α 3 in patient tumours is inversely correlated with macrophage recruitment.
- Our results provide novel evidence for the role of laminin α 3 in the recruitment of tumour-associated macrophages in SCC.

What is the translational message?

- There is a potential prognostic value of laminin α 3 expression in predicting cSCC metastasis.
- There is potential for targeting ROCK activity in cSCCs lacking laminin α 3 to block tumour invasion.
- The role played by the laminin α 3 chain in macrophage polarization into tumour-associated macrophages presents additional targets for immunotherapy or immunoregulation in cSCC.

Cutaneous squamous cell carcinoma (cSCC) is the second most common skin cancer, with almost 1 million new cases of cSCC recorded in the USA each year (www.cancer.org). cSCC has a number of associated risk factors, including ultraviolet exposure, human papillomavirus infection and the immunocompromised state.^{1,2} There is a 4% risk of metastasis, which increases to 16% if the initial tumour is thicker than 6 mm.³

The tumour microenvironment is a complex assembly of different cell types, extracellular matrix (ECM) components and growth factors. The interplay between these components influences tumour growth, aggression and resistance to treatment.⁴ The ECM, and in particular the basement membrane, acts as a barrier to tumour invasion as well as a source of growth factors and signals when broken down by invading tumour cells. In the tumour, ECM and basement membrane components are secreted by nonmalignant and cancer cells, creating a unique tumour ECM.⁵ Both healthy and tumour ECM are made up of a mixture of secreted molecules including collagens, elastin, fibronectin and laminins.⁶

Laminins are secreted glycoproteins made up of three polypeptide chains, laminin subunits alpha (α), beta (β) and gamma (γ). Within the basement membrane they interact with collagen type IV, VII and XVII, agrin, nidogens and perlecan. The skin basement membrane contains a number of different laminin isoforms, with the most abundant being laminin 332, laminin 511 and laminin 311.

Mutations in the genes encoding laminin 332 subunits α , β and γ , LAMA3, LAMB3 and LAMG2, cause a congenital blistering disease, junctional epidermolysis bullosa (JEB).⁷ Chronic blistering and inflammation in JEB result in an increased incidence of cSCC.⁸ Laminin 332 is specifically degraded during normal tissue remodelling or tumour invasion by membrane type 1-matrix metalloproteinase (MT1-MMP), MMP2, and the proteinase BMP-1.^{9,10} Intact and cleaved laminin 332 are recognized by

cell-surface receptors, integrins α 3 β 1 and α 6 β 4, which are expressed on a range of cells including macrophages.¹¹

Changes in laminin 332 protein expression have been observed in a wide range of tumours, with both increases and decreases associated with poor prognosis.^{12–16} Numerous studies have focused on laminin 332 in SCC associating increased expression with poor prognosis; however, these studies have either focused on head and neck SCC or on a single laminin 332 chain.^{17–22}

By generating specific knockdowns of laminin 332 subunits in cSCC cell lines, we demonstrate that loss of α 3 but not β 3 or γ 2 chains increases cell invasiveness through activation of the ROCK (rho-associated protein kinase) signalling pathway, and that this is associated with increased monocyte recruitment into the tumour microenvironment and differentiation into M2 tumour-associated macrophages (TAMs).

Materials and methods

Detailed information regarding protocols can be found in Supplementary Methods (File S1; see Supporting Information). Antibodies are listed in Table S3 and quantitative polymerase chain reaction (qPCR) primers in Table S4 (see Supporting information).

Study approval

All animal experiments were performed in accordance with UK Home Office regulations and the European Legal Framework for the protection of animals used for scientific purposes (European Directive 86/609/EEC). The use of anonymized, archival human tumour specimens was conducted according to Declaration of Helsinki principles and approved by the City and East NRES Committee approval number 05/Q0603/9.

Immunostaining

Archival skin sections were obtained of formalin-fixed and paraffin-embedded human SCCs (Table S1; see Supporting Information). Tissue microarray blocks were generated previously from SCC.²³

Cell culture

Human cSCC keratinocyte cell lines SCC-IC1,²⁴ SCC-IC8, SCC-IC12, SCC-T1, SCC-T2 and SCC-T8 were cultured in Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 (3 : 1) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (200 mmol L⁻¹) and RM+ (0.4 μ g mL⁻¹ hydrocortisone, 5 μ g mL⁻¹ insulin, 10 ng mL⁻¹ epidermal growth factor, 5 μ g mL⁻¹ transferrin, 8.4 ng mL⁻¹ cholera toxin, and 13 ng mL⁻¹ liothyronine). Human foreskin fibroblasts were cultured in DMEM supplemented with 10% FCS and 1% L-glutamine (200 mmol L⁻¹). All cells were cultured at 37 °C and 5% CO₂. THP-1 cells were cultured in suspension in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine (200 mmol L⁻¹). SCC-IC1 cells were selected for laminin 332 knockdown as they expressed all three laminin subunits.

In vitro epidermal models

Type I collagen: Corning[®] Matrigel[®] organotypic gels were prepared as previously described²⁵ with modifications to generate large and small organotypic tumour models.

Xenografting of collagen: Matrigel[®] gels onto hairless SCID mice

In vitro organotypic cultures on type I collagen: Matrigel gels were performed as described above. After a 7-day incubation period in vitro, gels were xenografted subcutaneously onto Crl:SHO-PrkdcscidHrhrl mice. Mice were killed after 6 weeks, and gels were excised, halved, and snap frozen or fixed in formal saline for histological analysis.

Statistical methods

Data are expressed as mean \pm SEM of independent experiments. Means of experimental groups were compared using one-way ANOVA, and simultaneous multiple comparisons were then made using Tukey's multiple comparisons test. Differences were considered statistically significant for adjusted P-values less than 0.05.

Results

Reduced laminin $\alpha 3$ chain expression correlates with poorly differentiated tumours *in vivo*

Laminin 332 expression was quantified in cSCC tumours from 38 patients (Table S1). Serial sections were stained for

each subunit of laminin 332 and for involucrin as a marker of tumour differentiation.²⁶ Tumours were divided into two groups, low involucrin (poorly and moderately differentiated tumours) and high involucrin (moderately/well-differentiated tumours); tumour grading and involucrin staining level are shown in Table S1. A significantly lower level of $\alpha 3$ chain expression was observed in poorly and moderately differentiated SCCs ($P = 0.02$) (Figure 1a). No significant difference in $\beta 3$ (Figure 1b) or $\gamma 2$ (Figure 1c) chain expression was detected. Analysis of an independent gene expression dataset also demonstrated a decrease in expression of LAMA3 between well/moderately differentiated cSCC and poorly differentiated cSCC, but no decrease in expression of other laminin chains (Figure S1; see Supporting Information).

To further test the expression of laminin 332 in SCC, western blot analysis was carried out on six cutaneous SCC cell lines²⁷ isolated from immunocompetent (SCCIC1, SCCIC8 and SCCIC12) and immunocompromised patients (SCCT1, SCCT2 and SCCT8) (Figure 1d). Organotypic invasion assays were generated (Figure 1e) and levels of invasion and laminin $\alpha 3$ expression assessed (Figure 1f). The most invasive cell line, SCCIC8, showed the lowest expression of $\alpha 3$ chain both by western blot and staining, whereas the three least invasive cell lines (SCCT1, SCCT2 and SCCT8) had the highest expression (Figure 1g). SCCIC12 had low levels of $\alpha 3$ chain by western blot but in the organotypic invasion assay was not significantly invasive and demonstrated strong $\alpha 3$ chain staining (Figure 1f).

These data suggest that reduced expression of the laminin $\alpha 3$ chain is associated with increased invasive potential and decreased differentiation, indicating poor prognosis *in vivo*.

Cells lacking the laminin $\alpha 3$ chain are invasive in behaviour

To examine the effects of loss of each subunit of laminin 332 on SCC cell behaviour, we knocked down each subunit separately using short hairpin (sh)RNAs (Table S2; see Supporting Information) in the cell line SCCIC1. For each laminin subunit 3, shRNAs were tested and screened using qPCR (Figure S2; see Supporting Information). Only one shRNA was identified as giving significant knockdown; the knockdown was verified by western blot (Figure 2a) and no significant compensatory upregulation of other laminin subunits was observed (Figure S2). Phalloidin staining demonstrated that cells lacking the $\alpha 3$ chain of laminin 332 have increased cortical actin and a rounded morphology when compared with those lacking $\beta 3$ and $\gamma 2$ (Figure 2b). Cell proliferation was reduced in all of the subunit knockdowns when compared with control, although this difference was not significant (Figure 2c). A significant reduction in cell attachment ($P < 0.001$) (Figure 2d) and increased cell motility (Figure 2e) was observed with loss of laminin 332 $\alpha 3$ and $\gamma 2$ but not the $\beta 3$ chain. Cells lacking the $\alpha 3$ chain were more invasive when compared with control (Figure 2f).

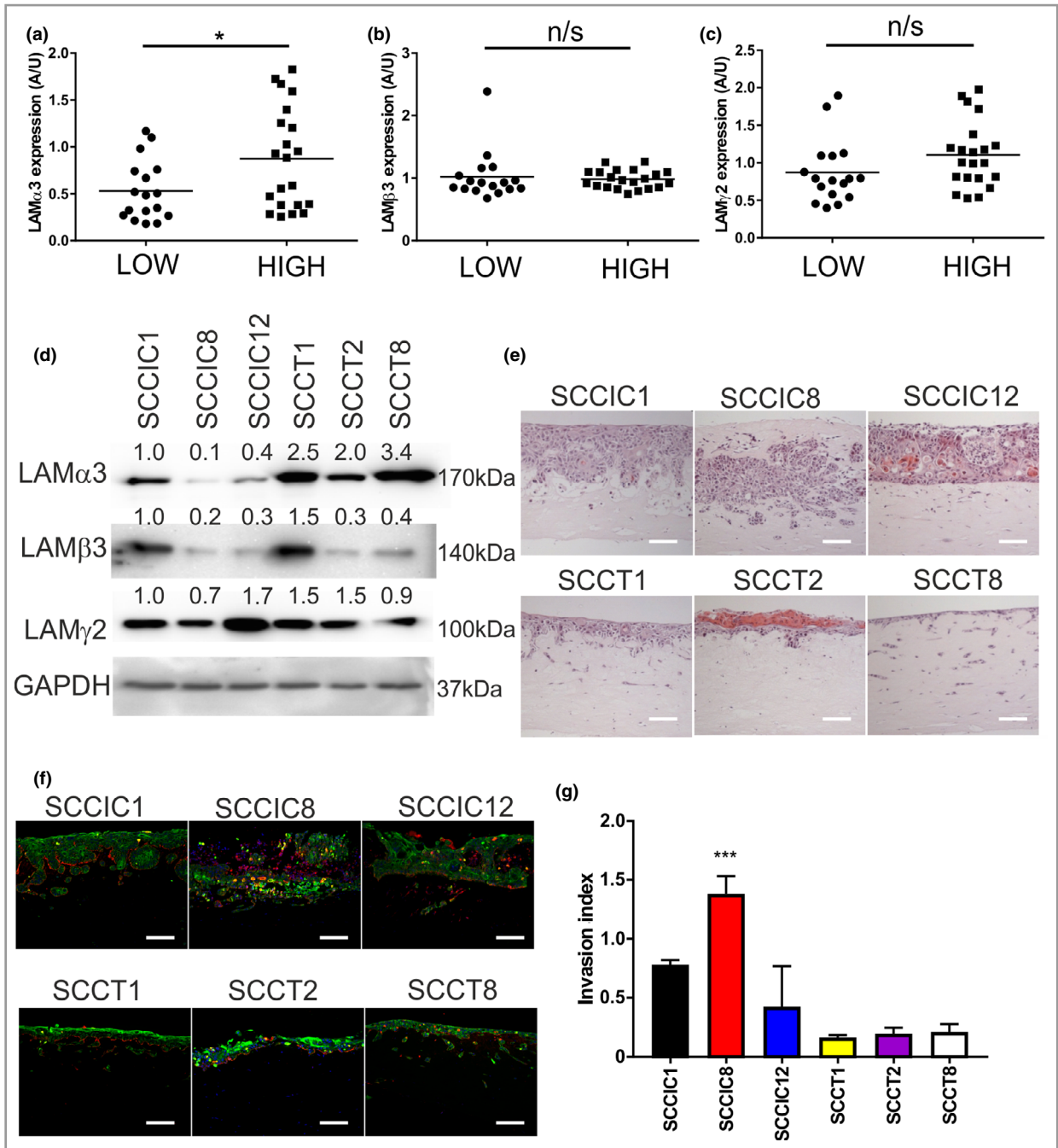


Figure 1 Poorly differentiated squamous cell carcinomas (SCCs) have low levels of laminin (LAM) α 3, which is associated with a more aggressive phenotype *in vitro*. CellProfiler²⁹ was used to quantify the expression level of proteins in SCC samples based on integrated fluorescence intensity; expression levels are shown as arbitrary units. (a–c) Quantification of the expression of LAM α 3 (a), LAM β 3 (b) and LAM γ 2 (c) in SCC, with tumours grouped by involucrin expression into low (poorly differentiated) and high (well differentiated) ($n = 38$). (d) Western blot analysis of each chain of laminin 332 in six SCC cell lines; blot representative of $n = 3$ repeats. (e) Haematoxylin and eosin staining of organotypic invasion assays showing invasion of six SCC cell lines. (f) Staining of sections of organotypic invasion assays with antibodies to pancytokeratin (green) and LAM α 3 (red) with 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (blue). (g) Quantification of invasion of six SCC cell lines in a type I collagen: Matrigel[®] organotypic invasion assay. Scale bars = 100 μ m. A/U, arbitrary units. * $P > 0.05$, *** $P \leq 0.01$; n/s, not significant.

Xenograft tumours were generated as described previously²⁸ and assessed for tumour invasion, tumour size, epithelial to mesenchymal transition (EMT) markers (Figure 3a) and MMP expression (Figure S3; see Supporting Information). Tumour

invasion was quantified by counting individual tumour islands adjacent to the main body of the tumour (Figure 3b). Tumours with loss of α 3 chain expression were significantly more invasive *in vivo*, with an increased number of small

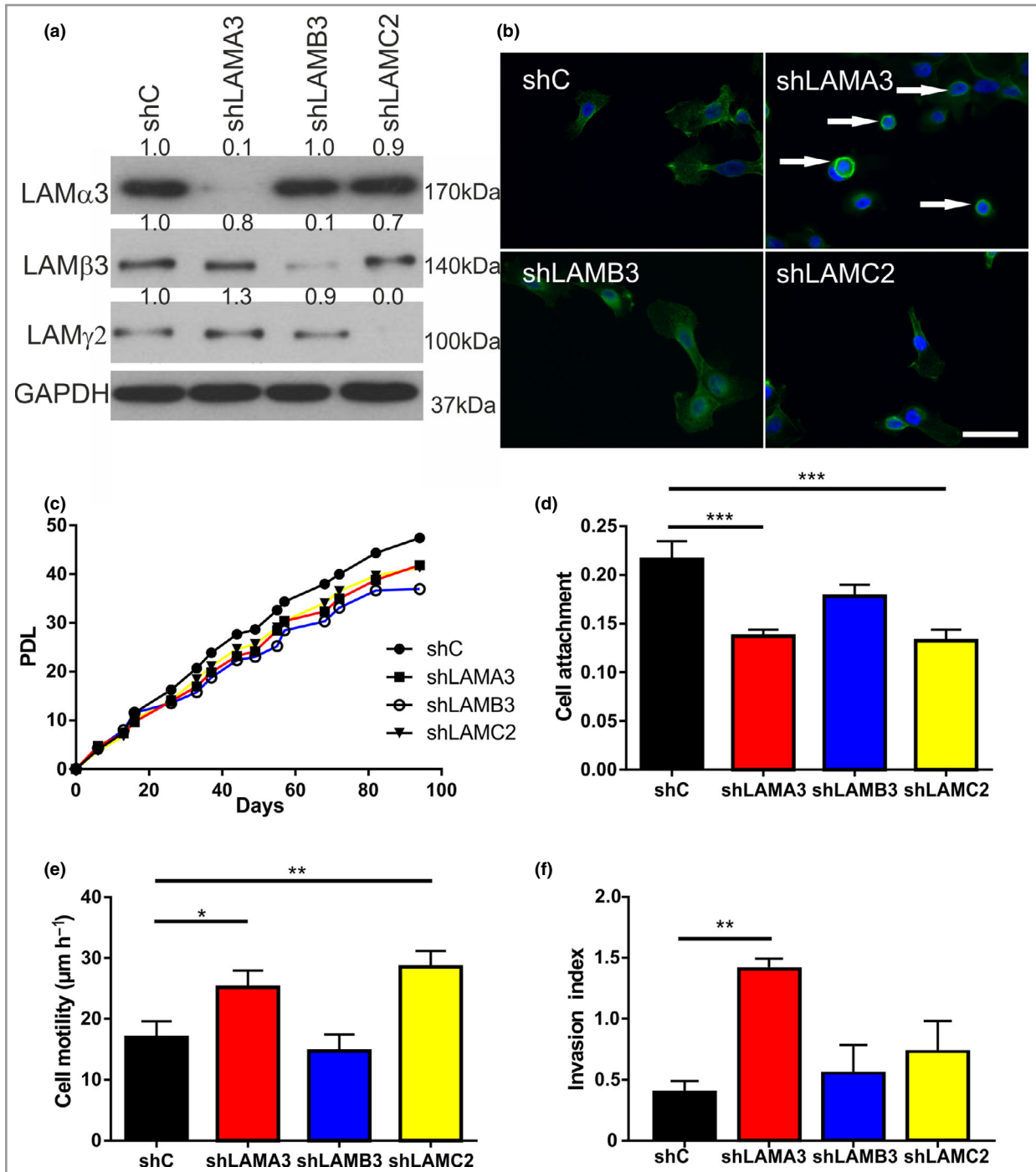


Figure 2 Short hairpin (sh)RNA-mediated knockdown of laminin (LAM)A3 increases cell motility and invasion. (a) Western blot analysis of cells stably transduced with SMARTvector™ lentiviral particles of three different shRNA clones (shLAMA3, shLAMB3 and shLAMC2) targeting each chain of laminin 332. Nontargeting shRNA particles, shC, were used as a negative control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control of protein loading. Blot representative of $n = 4$ repeats. (b) Actin staining of shLAMA3, shLAMB3 and shLAMC2 cells 24 h after trypsinization; arrows indicate cells with cortical actin. (c) Population-doubling levels (PDL) of shLAMA3, shLAMB3 and shLAMC2 cells. (d) Adhesion of shC, shLAMA3, shLAMB3 and shLAMC2 cells; $n = 3$ from three independent experiments. (e) Motility of shC, shLAMA3, shLAMB3 and shLAMC2 cells in $\mu\text{m h}^{-1}$. (f) Quantification of invasion of shC, shLAMA3, shLAMB3 and shLAMC2 cells in a type I collagen: Matrigel® organotypic invasion assay. Scale bars = 50 μm . * $p > 0.05$, ** $p \leq 0.05$, *** $p \leq 0.01$.

tumour islands, and were also significantly larger than control tumours (Figure 3c). In tumours with loss of the $\gamma 2$ chain, tongues of invading cells rather than invading islands were

observed, suggestive of collective migration. In tumours lacking the $\beta 3$ chain, a little invasion was observed *in vivo*, and these tumours appeared to be more differentiated and

significantly larger than control tumours (Figure 3c). Cells with loss of $\alpha 3$ and $\gamma 2$ chains demonstrated significantly increased vimentin (Figure 3d) and decreased E-cadherin expression (Figure 3e), suggesting either EMT or epithelial to amoeboid transition (EAT). Significantly reduced expression of MMP-1 and MMP-7, shown by reduced staining, was observed in the xenografted tumours in all three knockdown tumour cell lines compared with control (Figure S3). These data suggest that the invasive behaviour of cells lacking $\alpha 3$ chain expression in laminin 332 is independent of MMP regulation.

Loss of $\alpha 3$ chain expression activates rho-associated protein kinase/myosin light chain activation *in vitro* and *in vivo*

Tumour invasion and motility require alteration of the cellular cytoskeleton, allowing cells to interact with and move through the ECM. Two closely linked signalling pathways are known to be important in cancer cell motility, Rho-ROCK and Rac-PAK. Western blot analysis of control and knockdown cells identified an increase in phosphorylated (p)FAK Y397 (1.5 fold), pSrc (2.2 fold), myosin light chain (MLC) (1.5 fold)

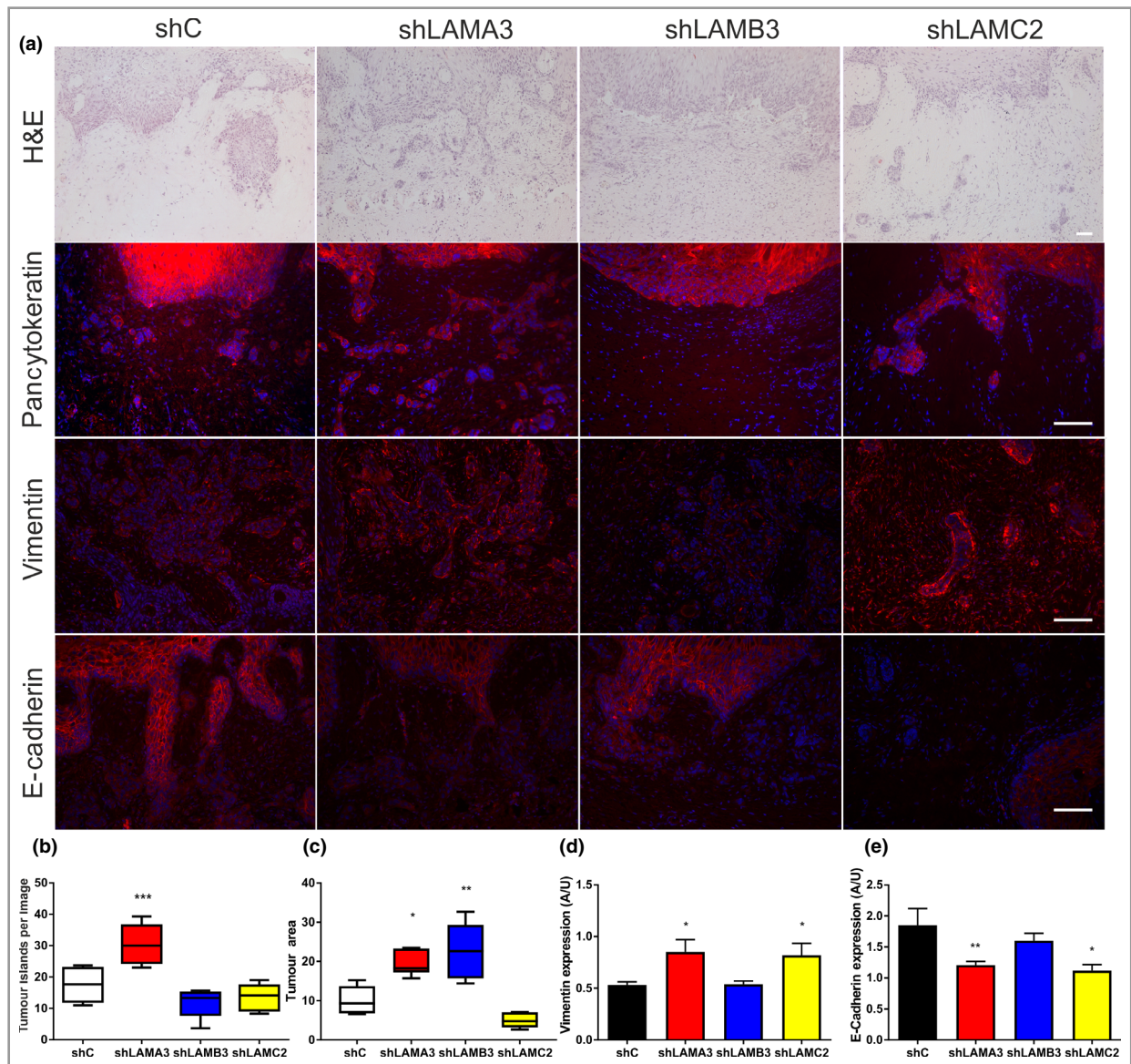


Figure 3 *In vivo* tumour invasion on loss of the laminin (LAM) $\alpha 3$ chain in laminin 332. (a) Staining of sections of short hairpin (sh)C, shLAMA3, shLAMB3 and shLAMC2 SCC-IC1 xenografts with haematoxylin and eosin (H&E) and antibodies to pancytokeratin, vimentin and E-cadherin. (b) Quantification of *in vivo* tumour invasion based on the number of individual invading tumour islands ($n = 7$). (c) Quantification of *in vivo* tumour area ($n = 7$). Quantification of staining for vimentin (d) and E-cadherin (e). Scale bars = 100 μm . A/U, arbitrary units. * $P > 0.05$, *** $P \leq 0.001$, ** $P \leq 0.01$.

and pMLC (1.9 fold) expression in cells with loss of the $\alpha 3$ chain compared with control (Figure 4a). The ratio of pMLC to MLC was 1.3 in cells with loss of the $\alpha 3$ chain, compared with 1 in control or other laminin chain knockdowns. Increased MLC expression was also observed in cells with transient short interfering RNA-mediated loss of the $\alpha 3$ chain and was normalized when cells were grown on recombinant laminin 332 (Figure S4; see Supporting Information). Previously, studies have shown that laminin 332 binding to $\beta 1$ integrin promotes RAC1 GTPase-driven cell migration in SCC.¹⁷ In contrast, we observed downregulation of RAC, PAK and pPAK in cells lacking the $\alpha 3$ chain of laminin 332 (Figure 4a). Immunofluorescence staining of tumour xenografts revealed a

significant increase in expression of both MLC and pMLC *in vivo* on loss of the $\alpha 3$ chain of laminin 332 (Figure 4b, c).

To confirm the role of ROCK kinase signalling in the invasive behaviour of cells with loss of the $\alpha 3$ chain, transwell invasion assays were performed (Figure 4d). This demonstrated invasive behaviour for cells lacking $\alpha 3$ rather than $\beta 3$ and $\gamma 2$ chains of laminin 332 and the shC controls ($P < 0.05$). Addition of the ROCK kinase inhibitor H1152 (Figure 4d) significantly abrogated the increased invasive behaviour of cells lacking the $\alpha 3$ chain of laminin 332. Further confirmation of the role of ROCK but not RAC in the invasive potential of cells lacking the $\alpha 3$ chain of laminin was generated using organotypic invasion assays in the presence of

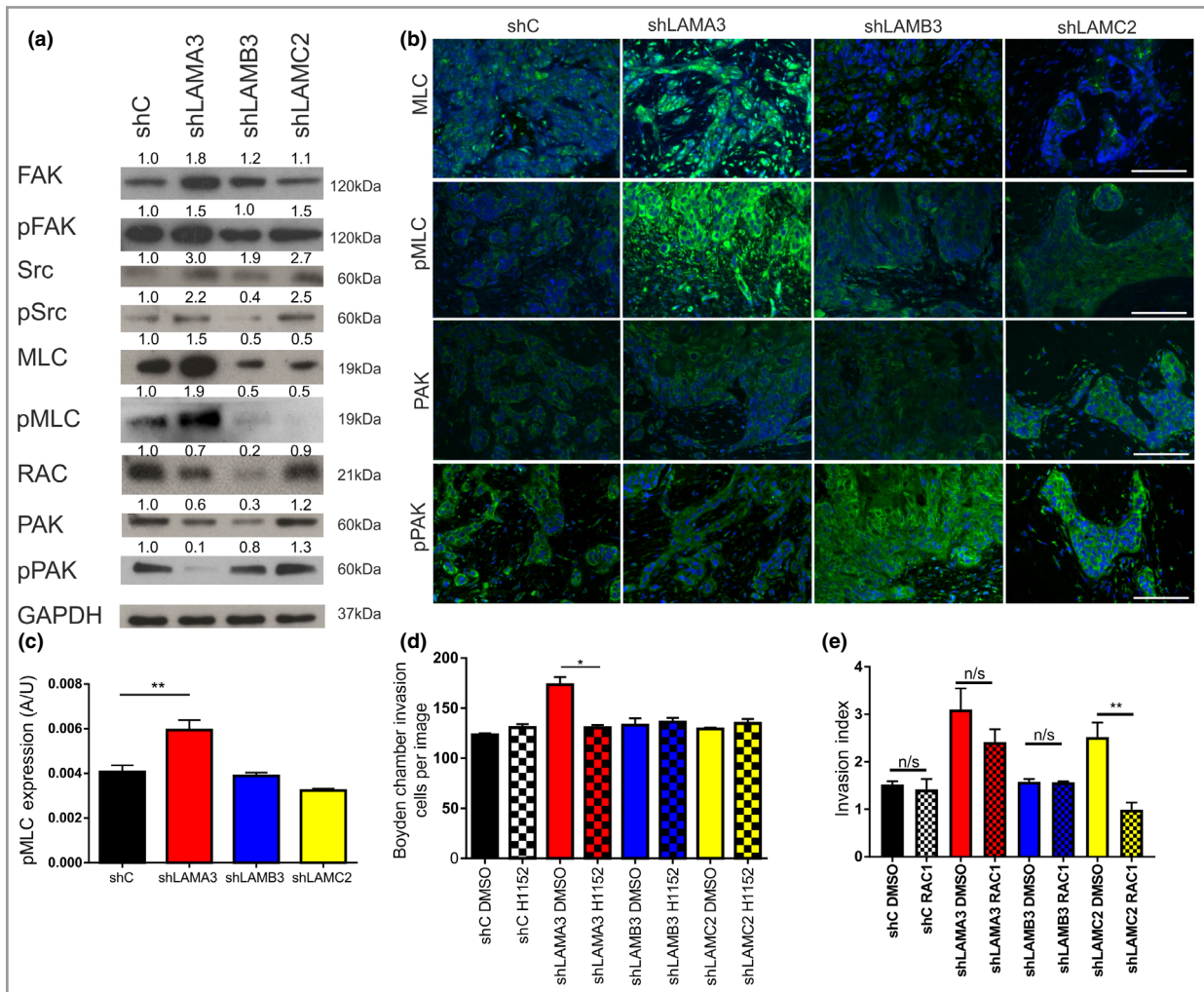


Figure 4 Loss of the laminin (LAM) $\alpha 3$ chain in laminin 332 activates the ROCK (rho-associated protein kinase) signalling pathway in cutaneous squamous cell carcinoma (cSCC). (a) Western blot analysis of FAK, phosphorylated (p)FAK Y397, Src, pSRC Y416, myosin light chain (MLC), pMLC, RAC, PAK and pPAK in short hairpin (sh)C, shLAMA3, shLAMB3 and shLAMC2 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control of protein loading; blot representative of $n = 3$ repeats. (b) Immunofluorescence staining of sections of shC, shLAMA3, shLAMB3 and shLAMC2 SCC-IC1 organotypic invasion assays for MLC, pMLC, PAK and pPAK. (c) Quantification of pMLC staining ($n = 7$). (d) Transwell invasion of treated shC, shLAMA3 and shLAMC2 cells through a Matrigel[®]-coated tissue culture insert with the ROCK inhibitor H1152 or dimethyl sulfoxide (DMSO) control. (e) Organotypic invasion of shC, shLAMA3 and shLAMC2 cells in a type I collagen: Matrigel[®] organotypic invasion assay, treated with the RAC inhibitor RAC1 or DMSO control ($n = 3$). Scale bars = 100 μm . * $P > 0.05$, ** $P \leq 0.05$; n/s, not significant.

the RAC inhibitor RAC-1 (Figure 4e). The RAC inhibitor did not significantly decrease invasion of cells lacking the $\alpha 3$ chain of laminin; however, it did decrease that of cells lacking the $\gamma 2$ chain (Figure 4e). These findings indicate a role for ROCK kinase but not Rac-PAK signalling in laminin $\alpha 3$ chain-regulated tumour invasion both *in vitro* and *in vivo*.

Knockdown of $\alpha 3$ and $\gamma 2$ chains of laminin 332 increases monocyte recruitment but only loss of laminin $\alpha 3$ increases macrophage differentiation

Transcriptomic analysis of knockdown cells that each lacked one of the laminin 332 subunits ($\alpha 3$ or $\beta 3$ or $\gamma 2$) was carried out. Enriched gene subsets with unique gene expression patterns that matched phenotypic changes were identified (Figure S5; see Supporting Information). Over-representation analysis of RNA-Seq gene expression data in cells lacking the $\alpha 3$ chain of laminin 332 identified upregulation of an inflammatory cell recruitment cluster (Figure 5a).

This cluster included genes known to be important in mononuclear cell activation and proliferation. This would include B and T cells as well as monocytes. The xenograft model we used was developed in SCID Crl:SHO-Prkdc^{scid}Hr^{hr} mice, which have no adaptive immune system but do have normal myeloid cells. Monocyte infiltration into the xenograft tumour microenvironment was assessed by immunofluorescence staining for the mouse monocyte marker LyC

(Figure 5b) and quantified using CellProfiler²⁹ (Figure 5c). This revealed an increase in the number of monocytes on loss of the $\alpha 3$ and $\gamma 2$ chains of laminin 332. To identify the proinflammatory factor that increased monocyte recruitment, conditioned media were analysed by enzyme-linked immunosorbent assay (ELISA) for CCL2 (Figure 5d). A significant increase in levels of CCL2 was observed only in cells lacking the $\alpha 3$ and $\gamma 2$ chains of laminin 332. Analysis of stained tumour xenografts for expression of TWIST, a known regulator of CCL2 expression (Figure S6; see Supporting Information), demonstrated a significant increase in TWIST expression in tumours with loss of either the $\alpha 3$ or $\gamma 2$ chains of laminin 332 (Figure 5e). Within the tumour microenvironment, monocytes differentiate into macrophages. Staining of tumour xenografts for the mouse macrophage marker F4/80 revealed a significant increase in the number of F4/80⁺ cells in tumours lacking the $\alpha 3$ chain but not in tumours lacking the $\gamma 2$ chain, suggesting a role for the $\alpha 3$ chain in regulation of macrophage differentiation (Figure 5f).

We had previously identified patient SCC tumours with high and low expression levels of the $\alpha 3$ chain of laminin 332 (Figure 1a). Tumours were stained for laminin $\alpha 3$ and CD68 (Figure 6a). Macrophage numbers per tumour were counted and compared with $\alpha 3$ chain expression levels (Figure 6b). A significant inverse correlation between macrophage numbers and $\alpha 3$ chain expression levels was identified. Macrophage differentiation was investigated in mouse xenograft

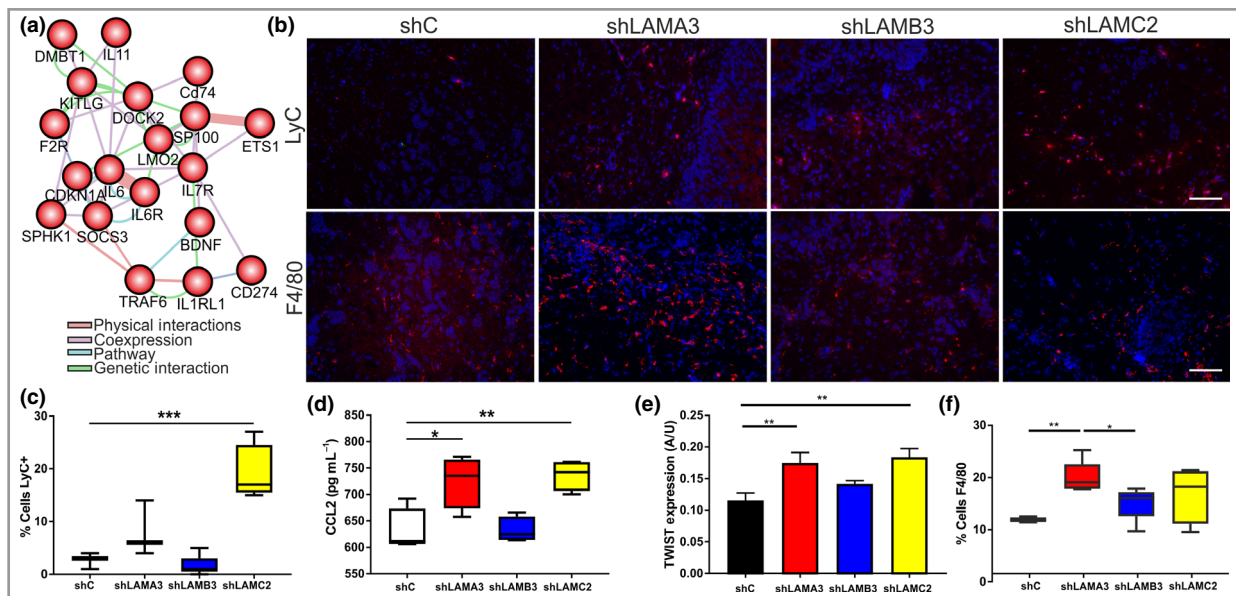


Figure 5 Loss of the laminin (LAM) $\alpha 3$ and $\gamma 2$ chains in laminin 332 drives monocyte recruitment via CCL2 but increased macrophage differentiation is only seen with loss of the $\alpha 3$ chain. (a) Inflammatory genes identified by RNA-Seq analysis upregulated in short hairpin (sh) LAMA3 by 1.5 fold or higher. (b) Immunofluorescence staining of sections of shC, shLAMA3, shLAMB3 and shLAMC2 xenografts for the mouse monocyte marker LyC and the mouse macrophage marker F4/80. (c) Quantification of monocyte recruitment in xenograft tumours ($n = 7$). (d) Enzyme-linked immunosorbent assay analysis of conditioned media from shC, shLAMA3, shLAMB3 and shLAMC2 SCC cells for CCL2, the major monocyte recruitment factor ($n = 3$). (e) Analysis of immunofluorescence staining of sections of shC, shLAMA3, shLAMB3 and shLAMC2 xenografts for TWIST ($n = 7$). (f) Quantification of tumour-associated macrophages in each xenograft ($n = 7$). Scale bars = 100 μm . A/U, arbitrary units. * $p > 0.05$, ** $p \leq 0.05$, *** $p \leq 0.01$.

tumours using double staining for markers for M1 (nitric oxide synthase) (Figure 6c) and M2 (arginase) (Figure 6d) macrophages coupled with F4/80. A significantly reduced number of M1 macrophages (Figure 6c) and significantly increased number of M2 macrophages (Figure 6d) were observed in tumours with knockdown of the $\alpha 3$ chain when compared with other tumours.

To identify the factor increasing macrophage M2 differentiation, analysis by ELISA of conditioned media for interleukin (IL)-13 revealed significantly increased IL-13 in cells lacking the $\alpha 3$ chain of laminin 332 (Figure 6e) when compared with control and other knockdown cells. To confirm that the levels of IL-13 in the media were sufficient to induce macrophage differentiation, an *in vitro* macrophage differentiation assay was performed. Significantly more CD68-positive cells were seen in wells seeded with cells lacking the $\alpha 3$ chain of laminin 332.

Discussion

In this study we show that in cSCC, poor tumour differentiation is linked with loss of expression of the $\alpha 3$ chain but not $\beta 3$ or $\gamma 2$ chains of laminin 332. Poor tumour differentiation is a reported risk factor for tumour recurrence, metastasis and disease-related death.² Cell lines with reduced laminin $\alpha 3$ chain expression showed increased invasiveness both *in vitro* and *in vivo*, with recruitment of TAMs *in vivo*.

Laminins are believed to be secreted only as heterotrimers after intracellular assembly,³⁰ thus loss of any subunit of laminin 332 should prevent secretion of the others, giving the same phenotype irrespective of which chain was knocked down. However, there is evidence for the secretion of both $\alpha 3$ and $\gamma 2$ ^{31,32} chains as monomers, suggesting a mechanism for the subunit-specific differences we observed in this study.

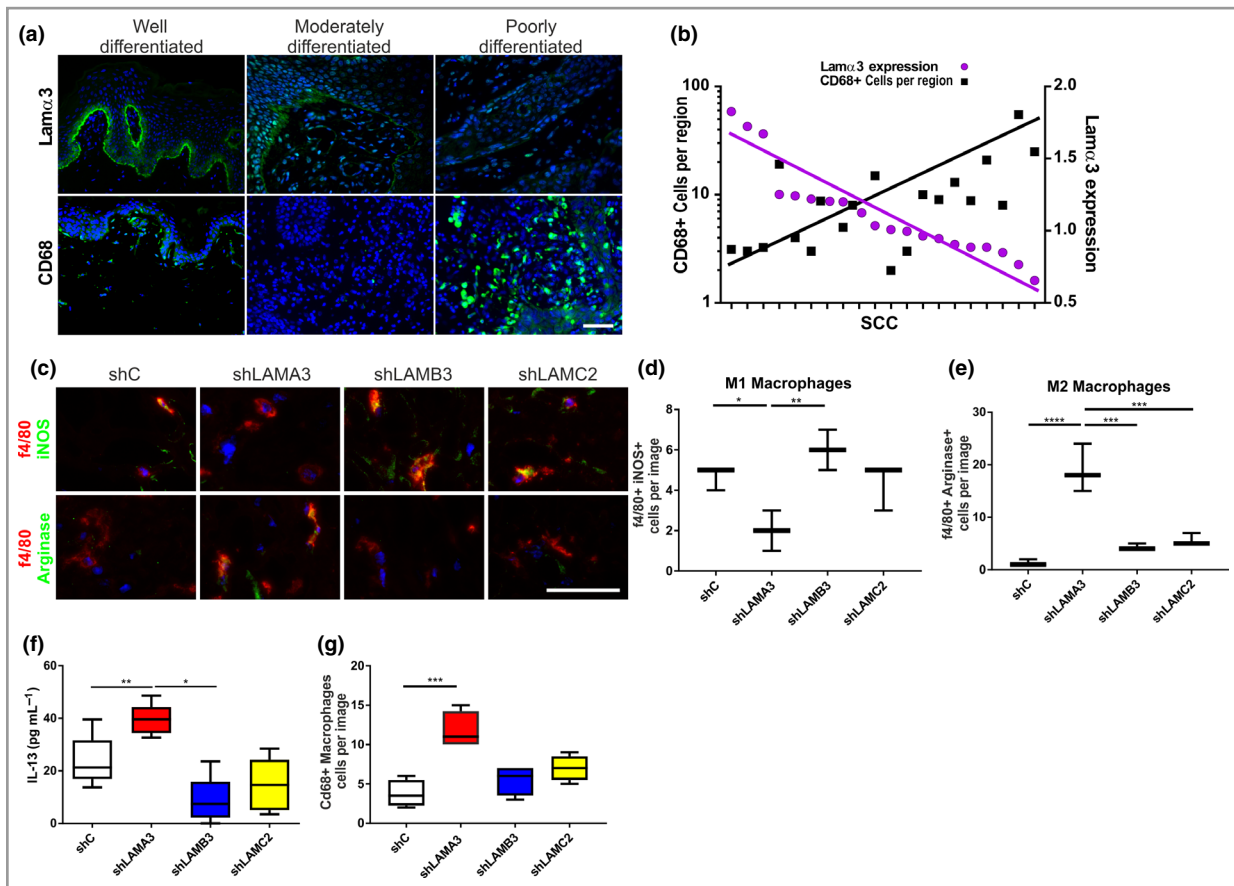


Figure 6 Loss of the laminin (LAM) $\alpha 3$ chain in laminin 332 drives macrophage recruitment in cutaneous squamous cell carcinoma (cSCC) and interleukin (IL)-13-mediated M2 macrophage differentiation. (a) Representative immunofluorescence staining of SCC tumour sections with antibodies against Lam $\alpha 3$ and CD68 ($n = 20$). (b) Correlation between Lam $\alpha 3$ expression and recruitment of CD68-positive cells (Spearman's correlation coefficient = -0.588 , $P = 0.006$; $n = 20$). (c) Representative immunofluorescence staining of short hairpin (sh)C, shLAMA3, shLAMB3 and shLAMC2 xenograft sections with antibodies against F4/80 and either nitric oxide synthase (iNOS) or arginase ($n = 3$). (d) Quantification of M1 macrophage differentiation in sections of shC, shLAMA3, shLAMB3 and shLAMC2 xenografts; M1 macrophages are F4/80⁺ and iNOS⁺. (e) Quantification of M2 macrophage differentiation in sections of shC, shLAMA3, shLAMB3 and shLAMC2 xenografts; M2 macrophages are F4/80⁺ and Arginase⁺. (f) Enzyme-linked immunosorbent assay analysis of conditioned media from shC, shLAMA3, shLAMB3 and shLAMC2 SCC cells for IL-13 ($n = 3$). (g) *In vitro* macrophage differentiation by conditioned media from shC, shLAMA3, shLAMB3 and shLAMC2 SCC cells ($n = 3$). Scale bars = 100 μm . * $P > 0.05$, ** $P \leq 0.05$, *** $P \leq 0.01$, **** $P \leq 0.001$.

Laminin 332 expression has been shown to be increased in colorectal carcinoma, breast cancer, prostate carcinoma, and oral SCC.^{12–16,33} These studies did not investigate the expression pattern of each subunit of laminin 332 separately, and therefore may have missed the chain-specific effects we have identified in this study. Within SCC, a number of studies have investigated laminin 332 function and have correlated that increased expression is observed in well-differentiated tumours when compared with premalignant tissue.^{18–21} These studies lack a significant number of poorly differentiated SCC samples and therefore were unable to investigate changes in laminin 332 expression in more aggressive SCC tumours. Our data confirm that laminin 332 expression is not decreased in well-differentiated SCC; however, we demonstrate for the first time that reduced expression of the $\alpha 3$ chain of laminin 332 correlates with poor differentiation status of tumours.

Xenografts generated from cells lacking the $\alpha 3$ chain of laminin 332 were significantly more invasive *in vitro* and had reduced expression of integrin $\alpha 3$. Loss of integrin $\alpha 3$ has previously been linked with increased keratinocyte activation, motility and FAK signalling.³⁴ We established that tumour invasion in the absence of the $\alpha 3$ chain of laminin 332 did not elevate the expression of MMPs, which are normally required for tumour invasion via EMT.³⁵ An alternative method of cancer invasion is suggested through amoeboid migration and EAT. Amoeboid migration does not require expression of MMPs and is characterized by activation of ROCK signalling within the cells.³⁶ An increase in pMLC³⁷ and inhibition of cell invasion by ROCK inhibition confirms the role of ROCK signalling in those cells lacking the $\alpha 3$ chain of laminin 332.

Previously it has been shown that LAMA3 expression can be controlled by epigenetic modification in a number of tumour types.³⁸ Hypermethylation of the LAMA3 promoter is associated with more aggressive lung and breast cancers. Within SCC, mutations in LAMA3 were identified in 20% of poorly differentiated tumours but they were not considered as driver mutations in SCC development.³⁹ A recent LAMA3 knockout mouse model exhibited a JEB-like phenotype but it does not spontaneously generate tumours.⁴⁰ Therefore, LAMA3 itself does not appear to be an oncogene; however, loss of $\alpha 3$ chain expression in existing tumour cells does allow them to become more invasive through activation of the ROCK signalling pathway.

TAMs have been shown to have tumour-promoting functions^{41–44} and in SCC they promote tumour progression and aggression,⁴⁵ which may be through the secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta.⁴⁶ We observed recruitment of monocytes to the xenografted tumour microenvironment through TWIST-regulated increased secretion of CCL2⁴⁷ with loss of both the $\alpha 3$ and $\gamma 2$ chains of laminin. However, only in tumours with loss of the $\alpha 3$ chain did we see a significant increase in macrophage number, linking loss of $\alpha 3$ with macrophage differentiation. Importantly, loss of the $\alpha 3$ chain in laminin 332 increased IL-13 secretion and promoted an increase in M2

macrophages in the tumour microenvironment.⁴⁸ Reduction of $\alpha 3$ chain expression in poorly differentiated cSCC increased recruitment of macrophages to the tumour environment, suggesting tumour aggression (Figure 6b).

Basement membrane proteins have previously been investigated for their immunoregulatory properties.⁴⁹ This study is the first to demonstrate that loss of basement membrane proteins in the tumour microenvironment induces an inflammatory infiltrate and regulates differentiation. In poorly differentiated cSCC with a higher risk of metastasis, loss of the $\alpha 3$ chain of laminin 332 appears to be an important feature of the cellular milieu. The role played by the $\alpha 3$ chain in macrophage polarization into M2 macrophages presents additional targets for immunotherapy or immunoregulation in cSCC.⁵⁰ M2 macrophages have been shown to recruit regulatory T cells, T-helper lymphocytes, eosinophils and basophils to the tumour microenvironment.⁵¹ Altering the recruitment or the polarity of TAMs has been suggested as a novel tumour therapy.⁵² Our study suggests that increasing expression of the $\alpha 3$ chain would reduce the number of TAMs and the polarity of those still recruited. Importantly, we demonstrate specific functions for laminin 332 subunits, suggesting a more complex role for this molecule in defining the extracellular niche.

A limitation of this study was the observational nature of the correlation between laminin $\alpha 3$ chain expression and macrophage infiltration in human cSCC. Future work should focus on the mechanism for loss of laminin in aggressive cSCC, restoration of normal expression levels and the prognostic value of laminin $\alpha 3$ chain expression in predicting SCC metastasis.

In summary, this study provides a mechanistic insight into invasive cSCC, identifying a new role for laminin 332 in cSCC aggression via activation of ROCK signalling and macrophage recruitment to the tumour microenvironment.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

File S1 Supplementary methods including manufacturer details.

Figure S1 Laminin chain expression in cutaneous squamous cell carcinoma (cSCC).

Figure S2 Confirmation of lentiviral knockdown and laminin chain compensation.

Figure S3 Matrix metalloproteinase (MMP) expression in tumour xenografts.

Figure S4 Myosin light chain (MLC) expression in short interfering (si)RNA-treated cells and on recombinant laminin 332.

Figure S5 RNA-Seq analysis.

Figure S6 TWIST expression in tumour xenografts.

Table S1 Squamous cell carcinoma (SCC) tumour samples.

Table S2 Short hairpin (sh)RNA clones and their target sequences and short interfering (si)RNA sequences.

Table S3 Antibody table.

Table S4 Quantitative polymerase chain reaction (qPCR) primers.