bio-protocol

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Measurement of Cell Intrinsic TGF-β Activation Mediated by the Integrin αvβ8

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Abstract

Transforming growth factor beta (TGF-β) is a multi-functional cytokine that plays a significant role in multiple diseases, including fibrosis and tumor progression. Whilst the biologic effects of TGF-β are well characterized, it is unclear how TGF-β signaling is regulated to impart specific responses within certain cell types. One mechanism of regulation may be through TGF-β activation, since TGF-β is always expressed in a latent form (L-TGF-β). Campbell *et al.* (2020) recently presented a new structural model to demonstrate how the integrin $\alpha_v\beta_8$ might specifically control TGF-β activation and signaling. In this model, $\alpha_v\beta_8$ binds to cell surface L-TGF-β1 to induce a conformational change, which exposes mature TGF-β peptide to TGF-β receptors (TGF-βRs), allowing initiation of TGF-β signaling from within the latent complex. This model also predicts that TGF-β signaling would be directed specifically towards the TGF-β expressing cell surface. We sought to test the validity of the new structural model by creating a cell-based assay which utilizes luciferase TGF-β reporter cells (TMLC). TMLC cells express high levels of TGF-βRs, but do not express cell surface L-TGF-β. We modified TMLC reporter cells to express cell surface L-TGF-β1 in a mutant form, which prevents the release of mature TGF-β from the latent complex. The newly generated cell lines were then used in a novel functional assay to investigate whether integrin $\alpha_v\beta_8$ could potentiate cell intrinsic TGF-β signaling from within the latent complex *in vitro*.

Keywords: TGF-β, Integrin, Reporter assay

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Background

TGF-β is a potent cytokine with a plethora of biologic functions (Kubiczkova *et al.*, 2012; Chen and ten Dijke, 2016; Fenton *et al.*, 2017; French *et al.*, 2020). For instance, TGF-β suppresses immune responses by facilitating the differentiation and immunosuppressive function of regulatory T-cells (Treg) (Stockis *et al.*, 2017; Seed *et al.*, 2021). TGF-β also plays a pivotal role in vascular physiology, amongst many other reported roles (Parichatikanond *et al.*, 2020). Most cell types are known to express TGF-β and its receptors, so the mechanisms whereby TGF-β selectively and specifically functions within distinct cell types is not well understood. However, TGF-β is always expressed in a latent form (L-TGF-β), demonstrating that activation of TGF-β from L-TGF-β is an important event for the regulation of TGF-β function (Khalil, 1999). Therefore, understanding the mechanisms whereby TGF-β is activated may provide insights into how TGF-β signaling is controlled, even though it appears ubiquitous.

Many molecules have been associated with TGF-β activation, the most specific of which are reported to be the integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ (Munger *et al.*, 1998; Mu *et al.*, 2002). A new structural model of $\alpha_v\beta_8$ mediated TGF-β activation proposes that $\alpha_v\beta_8$ expressed on the surface of one cell activates TGF-β presented on the surface of an opposing cell, via the adaptor molecule glycoprotein A repetitions dominant (GARP) (Campbell *et al.*, 2020). In this case, TGF-β signaling is directed towards the TGF-β presenting cell. This model further predicts that conformational changes imparted on L-TGF-β1 by $\alpha_v\beta_8$ may expose mature TGF-β, so that TGF-βRs expressed on the same cell surface can bind to mature TGF-β when it is still present within the latent complex, thus allowing TGF-β signaling. Testing the validity of this model using cell-based assays is of biologic significance as TGF-β activation is commonly thought to require physical release of mature TGF-β from the latent complex, where it would be free to diffuse to other cell surfaces. Therefore, the new structural model could in part explain how TGF-β signaling is tightly controlled to function intrinsically within specific cell types (Campbell *et al.*, 2020).

To construct a cell-based model to test the structural hypothesis, we employed the widely used TGF-β reporter cell line (TMLC) (Figure 1A). TMLC cells are a subclone of mink lung epithelial cells transfected to induce stable expression of a luciferase reporter gene fused to a truncated plasminogen activator inhibitor-1 (PAI-1) promoter sequence (Abe *et al.*, 1994; Campbell *et al.*, 2020). In addition to the reported abundance of TGF-βRs and high sensitivity and specificity for assessing TGF-β signaling, these cells do not present L-TGF-β at the cell surface (Abe *et al.*, 1994; Campbell *et al.*, 2020). This allows for the generation of a cell intrinsic reporter system using ectopic expression GARP and L-TGF-β1. GARP allows for cell surface presentation of L-TGF-β1 in TMLC cells (Figure 1A). Furthermore, we mutated the furin cleavage motif in L-TGF-β1 [to L-TGF-β(R249A)], which ensures that mature TGF-β remains covalently linked to latent peptides therefore preventing its release. We then subsequently assessed the ability of $\alpha_v\beta_8$ to activate cell intrinsic TGF-β from both wild-type (WT) and non-releasable forms of L-TGF-β1 (Figure 1A–D). These new models, accompanied by assessment of expression patterns in human tumors, provide functional support for the model proposed by Campbell *et al.* (Campbell *et al.*, 2020; Seed *et al.*, 2021), and a suitable *in vitro* cell based assay to measure cell intrinsic TGF-β signaling. It may be possible to adapt this protocol to test the ability of other known activators of TGF-β to initiate cell intrinsic TGF-β signaling.

Materials and Reagents

1. 96 well culture plate flat, white (Costar, catalog number: 3917)
2. 96 well immulon 4 HBX plates, flat (Fisher Scientific, catalog number: 3855)

> *Note: We have obtained comparable datasets if immulon 4HBX plates are substituted for standard 96 well cell culture plates (Corning, catalog number: 3599).*

3. 8-well, straight form multi-channel aspiration manifold (Drummond, catalog number: 3-000-093)
4. Multi-channel Pipette 30–300 µL (Gilson)
5. 15 mL Falcon tube (Corning, catalog number: 352097)
6. 1.5 mL sterile microcentrifuge tube (Eppendorf, EP022363344)
7. Sterile filter pipette tips (Axygen, catalog numbers: 350-rs, 250-rs, 30-rs)
8. Serological pipettes (Fisherbrand, catalog numbers: 13-676-10J and 13-676-10k)

9. Reagent reservoirs (Corning, catalog number: 4872)
10. Sterile Aeraseal cell culture plate sealer (EXCEL Scientific, catalog number: B-100)
11. Easyseal transparent plate sealers (Greiner Bio-One, catalog number: 676001)
12. Firefly luciferase assay kit 2.0 (Biotium, catalog number: 30085-2)
13. G418 sulfate (Geneticin) (Thermo Scientific, catalog number: 11811031)
14. Blasticidin-HCL (Corning, catalog number: 30-100-RB)
15. Puromycin dihydrochloride (Sigma-Aldrich, catalog number: P8833)
16. 0.25% trypsin-EDTA solution (CCF Media Production, catalog number: CCFGP003)
17. Dulbecco's Modification of Eagles Medium (DMEM) with 4.5 g/L glucose and L-glutamine, without sodium pyruvate (Fisher Scientific, catalog number: 10-017-CV)
18. Fetal bovine serum (FBS) qualified (Gibco, catalog number: 26140-079)
19. Penicillin/streptomycin 100× (CCF Media Production, catalog number: CCFGK004)
20. Amphotericin B (Fungizone) 100× (Fisher Scientific, catalog number: 12002-032)
21. Recombinant human TGF-β1 (R&D Systems, catalog number: 240-B-002/CF)
22. Phosphate buffered saline W/O calcium and magnesium (CCF Media Production, catalog number: CCFAL003-216T02)
23. Bovine serum albumin (BSA) essentially globulin free, low endotoxin (Sigma-Aldrich, catalog number: A2934-100G)
24. TMLC wild-type (WT), TMLC L-TGF-β1, TMLC L-TGF-β1(R249A) TMLC GARP, TMLC L-TGF-β1/GARP and TMLC L-TGF-β1(R249A)/GARP cell lines. Development of TMLC lines is described in Campbell *et al.* (2020). Available with MTA approval from Stephen Nishimura, Dept. Of Pathology, University of California San Francisco, San Francisco, California.
25. Recombinant Human $\alpha_v\beta_8$, $\alpha_v\beta_6$, and $\alpha_v\beta_3$ ectodomain. Available with an MTA approval from Stephen Nishimura, Dept. Of Pathology, University of California San Francisco, San Francisco, California. Development and purification of these reagents is described in Campbell *et al.* (2020).
26. MACS separation buffer (Miltenyi, catalog number: 130-091-221)
27. EZ-Link™ Sulfo-NHS-LC-Biotin (ThermoFisher Scientific, catalog number: 21335)
28. Anti-HA antibody (Genscript, catalog number: A01244-100)
29. Anti-latency associated peptide (LAP) antibody, clone AF246 (R&D Systems, catalog number: AF-246-NA)
30. TMLC basal media (see Recipes)
31. 5 mg/mL Blasticidin-HCl stock solution (see Recipes)
32. 50 mg/mL G418 sulfate stock solution (see Recipes)
33. 10 mg/mL puromycin dihydrochloride stock solution (see Recipes)
34. 20 µg/mL recombinant human TGF-β1 stock solution (see Recipes)
35. 1 µg/mL $\alpha_v\beta_3$ or $\alpha_v\beta_8$ ectodomain coating solution (see Recipes)
36. 1 µg/mL anti-LAP coating solution (see Recipes)
37. 1% BSA blocking solution (see Recipes)
38. 1× luciferase cell lysis buffer (see Recipes)
39. 1× luciferase assay buffer (see Recipes)

Equipment

1. 37°C humidified incubator, 5% CO_2
2. Jouan C4i benchtop centrifuge or equivalent, for spinning 15–50 mL tubes (Thermo Scientific, model: Jouan C4i)
3. Flow cytometer (BD Biosciences, model: LSR II)
4. Cell sorter (BD Biosciences, model: FACS ARIA II)
5. Luciferase assay plate reader (Promega, model: Glomax Explorer)
6. Luna automated cell counter or equivalent (Luna, model: L10001-LG)
7. Hoefer RED ROCKER rocking platform or equivalent (Hoefer Scientific Instruments)

Software

1. GraphPad Prism 9 (https://www.graphpad.com/scientific-software/prism/)

Procedure

A. Maintenance of stable TMLC reporter cell lines

Note: For all cell culture steps, use sterile filter tips and sterile tubes (see Materials and Reagents).

1. Grow TMLC cells in T75 flasks (or equivalent) with DMEM (4.5 g/L glucose and L-glutamine, without sodium pyruvate) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% amphotericin B. To maintain expression of the luciferase TGF-β reporter cassette, also supplement the media with 1 mg/mL G418.
   TMLC cells expressing the various components of L-TGF-β1 or GARP are cultured using the following antibiotic selection markers (also see Recipes section):
   - TMLC L-TGF-β1 or TMLC L-TGF-β1(R249A): TMLC basal media with 1 mg/mL G418 and 10 µg/mL puromycin.
   - TMLC L-TGF-β1/GARP or TMLC L-TGF-β1(R249A)/GARP: TMLC basal media with 1mg/mL G418, 10 µg/mL puromycin, and 20 µg/mL blasticidin.
2. When 70–90% confluent, detach TMLC cells using 0.25% trypsin EDTA at 37°C for 2 min. Trypsin should be inactivated using at least an equal volume of basal media.
3. Sub-culture cells at a density of 1:20 twice weekly. Spinning and washing of cells is not necessary for routine cell culture. In preparation for functional assays, 70–90% confluent cells are sub-cultured at 1:5 density for 48 h, in the absence of antibiotic selection.

   Note: We have noticed a significant reduction in the performance of TMLC cells if allowed to reach confluency. For further information on cell line maintenance and quality control, please refer to the "notes on reproducibility" and "cell line QC sections" in the Notes section.

4. Cryopreserve TMLC cells at a density of 2×10^6 cells/mL in a solution of 90% FBS and 10% DMSO, using standard protocols (At a rate of cooling: 1°C per minute, to -80°C. Transfer cells to liquid nitrogen storage within 1 week).

B. Preparation of Assay plates

Note: For transferring all reagents, we recommend using sterile filter pipette tips and sterile tubes (see Materials and Reagents).

1. Prepare stock solutions of recombinant Human $\alpha_v\beta_8$ ectodomain and negative controls, including BSA, $\alpha_v\beta_3$ (which does not activate TGF-β), and anti-LAP at a concentration of 1 µg/mL in phosphate buffered saline (PBS) supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} (see Recipes section).

   Note: Anti-LAP binds to L-TGF-β1 and was therefore used to control for both the binding and subsequent cell adhesion affects that $\alpha_v\beta_8$ may have, in addition to its ability to promote TGF-β activation.

2. Plate solutions onto immulon 4 HBX 96-well plates (50 µL/well is sufficient) as in Figure 1C, and cover the plate with a transparent plate sealer. Incubate on a rocking platform at 37°C for 1 h.
3. Wash cells twice with 300 µL of PBS, and block wells in PBS solution supplemented with 1% BSA, using a multichannel pipette and reagent reservoirs. Incubate the assay plate at room temperature for 1 h.

Note: Plates can be stored at 4°C up to 48 h before the assay start date. However, it is recommended to use the plates on the same day, for purposes of reproducibility.

C. Cell line preparation and cell seeding

1. Forty eight hours prior to TGF-β activation assays, split 70–90% confluent TMLC lines at 1:5 density. At this stage, culture cells in TMLC basal media only.

Note: For continued use of cell lines, also maintain some cells in relevant reselection media.

2. On the day of the assay, detach cells using 0.25% trypsin EDTA as described above.

Note: Cells should not be confluent.

3. Inactivate trypsin using an equal volume of TMLC basal media, and transfer cell suspensions to labelled 15 mL Falcon tubes.
4. Pellet cell suspensions by centrifugation at 400 × *g* for 5 min. After centrifugation, aspirate supernatant and resuspend cells in TMLC basal media by gentle vortexing for 5 s. Count viable cells using a LUNA cell counter or equivalent.
5. Prepare standard curves in a serial 3-fold dilution series, starting from 16 ng/mL* rTGF-β1 in basal media using a multi-channel pipette (This can be performed within the assay plate, see Figure 1C). The final volume of standards in the assay plate should be 100 µL.

*Note: *For clarity, when an equal volume of TMLC cell suspension is subsequently added to each well, this dilutes each standard by 50%, matching the dilution series depicted in Figure 1C. Standard curves are essential to control for differences in TGF-β sensitivity between TMLC cell lines.*

6. Dilute TMLC cell suspensions to a concentration of 1.5×10^5 cells/mL in TMLC basal media, and plate 100 µL of cell suspension/well onto immulon 4 HBX 96-well assay plates, using a multi-channel pipette and reagent reservoir (an example of an assay plate format is shown in Figure 1C–D).

Note: Whilst TMLC cells do not easily clump, it is recommended to pipette up and down three times before transferring cells from the reagent reservoir to the assay plate.

7. Cover plates with Aeraseal plate covers, and incubate under standard cell culture conditions (37°C, 5% CO_2, in a humidified cell culture incubator) for 16–18 h.

Note: For assay reproducibility, ensure that incubation times are consistent between experimental replicates.

D. Assay development

1. Ensure that luciferase assay lysis buffer, luciferase assay buffer and PBS are equilibrated to room temperature prior to assay development.
2. Prepare cells for lysis by aspiration of media using a multi-channel vacuum manifold. Immediately wash cells twice with 300 µL of PBS, using a multi-channel pipette and reagent reservoir. It is not necessary to maintain aseptic technique from this point.

   Note: TMLC cells adhere strongly to culture plastic, and all aspiration steps can be performed with a vacuum manifold for efficiency.

3. Lyse cells in 100 µL of luciferase assay lysis buffer, and incubate on a rocking platform at room temperature for 30 min.

   Note: To reduce intra-plate variability, it is not recommended to scrape the wells manually.

4. Transfer 80 µL of lysate from each well of the immulon 4 HBX plate into a 96-well cell culture white plate (Costar 3917), using a multi-channel pipette.
5. Record luciferase activity using a Glomax Explorer (Promega), with the following settings:
   Injection volume of luciferase assay buffer (100 µL), wait 2 s, integrations (10) interval (0.3 s).

E. Data analysis and expected results

1. Subtract the raw relative light unit (rLU) data recorded in the BSA control wells from all other rLU values. This allows for removal of the background signal for each cell line.

   Note: Subtracting the BSA control data for each cell line is important due to variance in background readings between cell lines.

2. Transform background subtracted rLU data to represent nanograms per mililiter of active TGF-β, by interpolating from the standard curves of known TGF-β treatments (Figure 2B).

   Note: This allows for more accurate comparison of the amount of TGF-β signaling between different TMLC lines, as it also controls for observed differences in TGF-β sensitivity.

3. Present the data using GraphPad prism 7 or above, as depicted in Figure 2D.

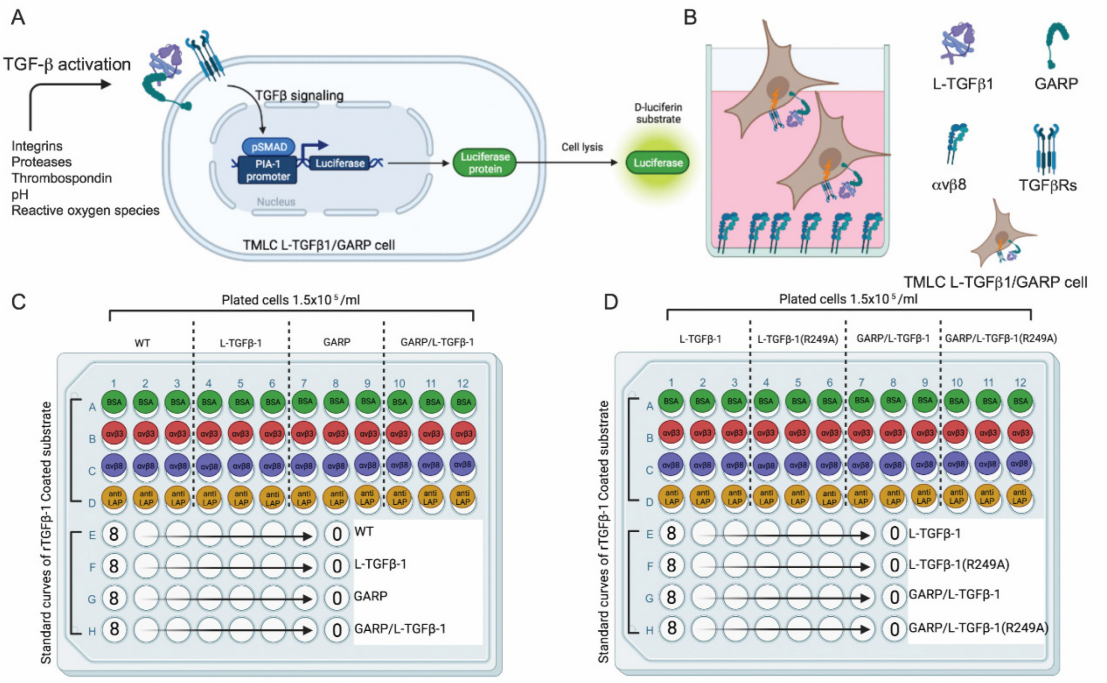

Figure 1. Assay design to assess the ability of recombinant $\alpha_v\beta_8$ ectodomain for promoting cell intrinsic TGF-β signaling, from both releasable and non-releasable forms of L-TGF-β1.

A. Cartoon depiction of TMLC TGF-β reporting cells. TMLC are a stable clone of mink epithelial cells overexpressing the PAI-1 promoter sequence fused to a luciferase reporter cassette (Abe *et al.*, 1994). TMLC cells express high levels of TGF-βRs which are able to initiate TGF-β signaling on binding to mature TGF-β. Downstream of TGF-β signaling, pSMAD drives the expression of luciferase, and TGF-β signaling can be reported following cell lysis and assessment of luciferase activity using luciferase assay buffer (containing D-luciferin substrate). To build a cell intrinsic TGF-β signaling model, we overexpressed the adaptor molecule GARP and/or L-TGF-β1, or the non-releasable form (R249A). **B.** Cartoon depiction of TMLC L-TGF-β1/GARP cells in culture with immobilized $\alpha_v\beta_8$ ectodomain. In this case, the TMLC L-TGF-β1/GARP cells (or controls) were plated onto immobilized integrin ectodomain (or control substrates), and the subsequent luciferase activity of the cells measured. **C.** Assay format to assess whether $\alpha_v\beta_8$ can support cell intrinsic TGF-β activation. Negative controls, including BSA protein (green), $\alpha_v\beta_3$ ectodomain (red), and anti-LAP (yellow), were immobilized alongside $\alpha_v\beta_8$ ectodomain (purple), as described in the assay procedure section. TMLC WT, TMLC GARP alone, and TMLC L-TGF-β1 alone control cells were then plated onto the various substrates in triplicate, so that they could be compared to the signal generated in the TMLC L-TGF-β1/GARP test cells. **D.** Assay format to assess the ability of $\alpha_v\beta_8$ to promote cell intrinsic TGF-β signaling of non-releasable TGF-β. Here, the assay was modified to include controls of TMLC L-TGF-β1 alone, TMLC L-TGF-β1(R249A) alone, and TMLC L-TGF-β1/GARP, so that they could be compared to the signal generated in TMLC L-TGF-β1(R249A)/GARP cells. In all cases, a unique standard curve of each cell type treated with known concentrations of recombinant human TGF-β1 (in 3-fold serial dilution, from 8 ng/mL) was used, so that TGF-β responsiveness could be normalized between cell lines, and data could be presented as concentration (ng/mL) of TGF-β1 signaling (Abe *et al.*, 1994; Campbell *et al.*, 2020).

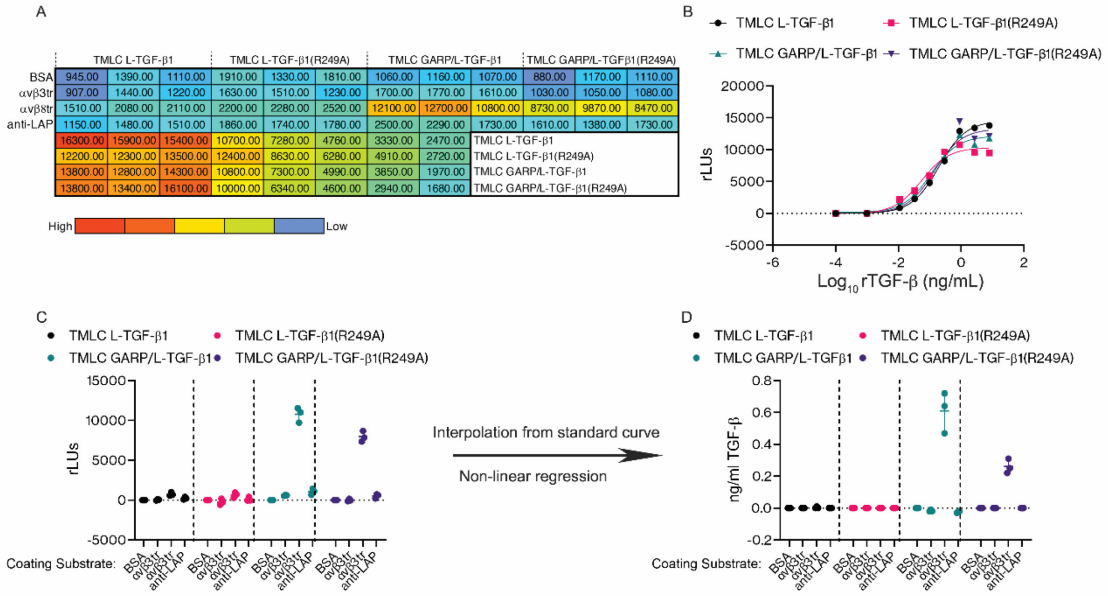

Figure 2. An exemple of raw data and data analysis workflow from an assay plate prepared identically to Figure 1D.

A. Relative light units (rLU) readings following assay development. Data is presented in heat map format (high rLU values in red low values in blue). **B.** A standard curve of known rTGF-β1 concentrations were prepared for all four TMLC lines used in this assay, allowing for quantitative analysis of TGF-β signaling and for normalization of TGF-β sensitivity between cell lines. **C.** Data from A is presented in graphical format, after subtracting the BSA control wells from each sample. **D.** BSA background subtracted rLU data was then converted to ng/mL of TGF-β signaling for each cell line, by interpolating from individual standard curves as shown in (B). Data sets with more than one technical replicate are presented as mean ± standard deviation (SD).

Notes

Supporting observations

During the development of these assays, we sought to assess whether mature TGF-β was released from the surface of TMLC L-TGF-β1/GARP cells after activation by integrins. We took two approaches to address this. Firstly, we retained the conditioned media from the assays performed in this protocol and re-plated them onto new TMLC WT cells. This allowed us to assess the concentration of liberated mature TGF-β under each assay condition. Secondly, we performed the assays described above using permeable transwell cell culture inserts. This enabled us to immobilize two populations of TMLC cells: one population that can attach directly to the integrin, and one population of cells separated by the permeable insert, allowing measurement of TGF-β liberation throughout the assay. When TMLC TGF-β signaling is assessed in these supporting assays, it provides insights into the amount of mature TGF-β that is liberated after $\alpha_v\beta_8$ mediated TGF-β activation (see Campbell *et al.*, 2020; Seed *et al.*, 2021).

Other assay formats

We have successfully substituted immobilized integrin ectodomain for $\alpha_v\beta_8$ expressing cells. If $\alpha_v\beta_8$ was expressed ectopically in an $\alpha_v\beta_8$ null cell-line, then background was subtracted by incubation of TMLC cells with mock transfected cells. Cell lines expressing endogenous $\alpha_v\beta_8$ were tested by comparing scrambled short homologous ribonucleic acid (shRNA) transduced cells to ITGB8 shRNA transduced cells, or using an $\alpha_v\beta_8$ specific inhibitor. Assays using $\alpha_v\beta_8$ expressing cells in co-culture with TMLC cells were plated onto standard 96-well culture plates (Corning, catalog number: 3599), where 3×10^5 integrin expressing cells were plated with 1.5×10^5 TMLC reporter cells (Seed *et al.*, 2021).

The integrin $\alpha_v\beta_6$ also functions to activate TGF-β, and we have found that $\alpha_v\beta_6$ supports cell intrinsic TGF-β activation in a comparable manner to $\alpha_v\beta_8$ in these assays.

Notes on reproducibility

In addition to ensuring that all lines express similar levels of L-TGF-β and/or GARP, we recommend limiting the subculture of TMLC lines to five successive 1:20 passages, as this decreases the chance of developing natural variation in TGF-β reporter sensitivity between cell lines. To maintain cell stocks, it is useful to freeze multiple vials at each passage. If any TMLC lines reach confluency, it is recommended to resuscitate new vials of all cell lines used. To ensure accuracy of results, wherever possible, perform assays with three technical replicates as inter-well variability is common in these assays. It is also useful to record cell morphology using a microscope immediately prior to assay development, as changes in morphology between assays have been found to correlate with unexpected results. Therefore, we do not recommend performing all steps in the 96 well cell culture white plates.

Cell line QC analysis

To ensure cell lines are expressing consistent levels of L-TGF-β1 and the cell surface adaptor protein GARP, cell lines should be routinely screened and sorted using a flow cytometer and cell sorter. Green fluorescent protein (GFP) is used a surrogate marker for L-TGF-β1 expression, Anti-LAP (clone AF246) is used to characterize TGF-β1 cell surface expression, and anti-HA (clone 5E11D8) to detect GARP expression. For normalization of cell surface expression, cells can be sorted based on GFP positivity and cell surface latency associated peptide (LAP) positivity. In all cases, cells are stained in MACS buffer, using appropriate antibodies for 15 minutes on ice, before washing and staining with appropriate fluorophore-conjugated antibodies.

To prove that TMLC GARP/L-TGF-β1(R249A) cells express a non-cleavable form of L-TGF-β1, L-TGF-β1(R249A)/GARP expressing, or WT L-TGF-β1/GARP TMLC cells were surface biotinylated using EZ-link sulfo-NHS biotin, and immunoprecipitated using anti-HA, resolved by a 4–12% gradient sodium dodecylsufate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, immunoblotted, probed with streptavidin-HRP, and detected by chemiluminescence, essentially as described (Mu *et al.*, 2002). This assay confirms the association of GARP with WT L-TGF-β1, L-TGF-β1(R249A), and absence of cleavage of LAP from mature TGFβ1 in the L-TGF-β1(R249A)/GARP TMLC cells (this paragraph was adapted from Campbell *et al.*, 2020).

Recipes

Note: We recommend filter sterilization of all reagents used in cell culture using a 0.22-μm filter.

1. **TMLC basal media**
   440 mL of Dulbecco's Modification of Eagles Medium (DMEM) with 4.5 g/L glucose and L-glutamine, without sodium pyruvate
   50 mL of FBS Qualified
   5 mL of 100× Penicillin/Streptomycin
   5 mL of 100× Amphotericin B

2. **5 mg/mL Blasticidin-HCl stock solution**
   Dissolve contents of 50 mg Blasticidin-HCl bottle in 10 mL of cell culture grade H_2O. Store for a maximum of 2 weeks at 4°C (dilute 1:500 in basal media).

3. **50 mg/mL G418 sulfate stock solution**
   Dissolve contents of 1 g G418 sulfate bottle in 20 mL of cell culture grade H_2O, and store protected from light at 4°C for up to 1 month (dilute 1:400 in basal media).

4. **10 mg/mL puromycin dihydrochloride stock solution**
   Dissolve contents of 10 mg Puromycin dihydrochloride bottle in 1 mL of cell culture grade H_2O. Store protected from light at 4°C for up to 1 month (dilute 1:1,000 in basal media).

5. **20 µg/mL recombinant human TGF-β1 stock solution**
   Dissolve 2 µg vial in 100 µL of sterile 4 mM HCl containing 1 mg/mL bovine serum albumin. Dilute 1:1,250 into TMLC basal media to prepare the 16 ng/mL TGF-β standard curve top dose. Use of TGF-β reconstitution media is critical to achieve accurate concentrations.

6. **1 µg/mL $\alpha_v\beta_3$ or $\alpha_v\beta_8$ ectodomain coating solution**
   Dilute recombinant integrins in PBS supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+}, to a working concentration of 1 µg/mL (see Campbell *et al.*, 2020 and Takasaka *et al.*, 2018, for more information on recombinant integrins).

7. **1 µg/mL anti-LAP coating solution**
   Prepare a stock solution of 200 µg/mL anti-LAP antibody, clone AF246 by diluting it in sterile PBS. Prepare 1 µg/mL working stock (or of a desired stoichiometric equivalent concentration as $\alpha_v\beta_8$) by diluting in PBS supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+}.

8. **1% BSA blocking solution**
   Dissolve 1 g of BSA powder in 100 mL of cell culture grade PBS without calcium and magnesium.

9. **1× luciferase cell lysis buffer**
   Dilute one part lysis buffer in four parts deionized H_2O, and use immediately for cell lysis.

10. **1× luciferase assay buffer**
    Prepare D-luciferin by adding 1 mL of H_2O to 10 mg D-luciferin (this can be stored at –20°C and thawed up to five times).
    Add 20 µL of D-luciferin to each mililiter of luciferase assay buffer, to make a 1× working solution – always protect the solution from light.

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Competing interests

The authors declare no competing interests.

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