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Data Article

Data from ameloblast cell lines cultured in 3D using various gel substrates in the presence of ameloblastin

Gayathri Visakan, Jingtan Su, Janet Moradian-Oldak*

Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, United States

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ABSTRACT

This article contains data related to the research article in this issue titled ameloblastin promotes polarization of ameloblast cell lines in a 3D cell culture system (Visakan et al., 2022). In the process of amelogenesis, the organic matrix components are pivotal to the establishment of ameloblast-matrix adhesion. Here we employ immortalized ameloblast cell lines and analyse their morphological changes in 3D cell culture when cultured in the presence of recombinant enamel matrix proteins- ameloblastin and amelogenin compared with controls. The recombinant proteins that were purified using high-performance liquid chromatography (HPLC) were characterized using SDS-gel electrophoresis. A 3D-on-top culture technique was employed, and the cells were analysed 24 and 72 h post inoculation using fluorescent and confocal microscopy for qualitative and quantitative changes. Aspect ratio of cells was measured and used as the parameter to compare between test proteins and controls. Repeated measurements of aspect ratio were recorded to analyse for statistical significance. Additionally, three distinct gel substrates were studied to examine the effect of composition and stiffness of the substrate on cell behaviour. The cells in the 3D culture were fixed and labelled using an-

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* Corresponding author.
E-mail address: joldak@usc.edu (J. Moradian-Oldak).

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tibodies to junctional complex, polarity and tight junctional proteins following protocols for whole culture fixation. Co-localization between membrane and specific antibody labels were examined under confocal microscopy.

Specifications Table

Subject	Biological Sciences
Specific subject area	3D cell culture, cell engineering, extracellular matrix, cell adhesion, immunofluorescence labelling
Type of data	Image Graph Figure
How the data were acquired	Fluorescence microscopy using Keyence BZX810, cell aspect ratio measurements using Image Analyzer, (software version 1.1.1.8), Keyence, USA. Confocal laser scanning microscopy using Leica SP8 microscope and 3D image analysis using LAS-X version 1.8.1.13759. SDS-Gel elecrophoresis, statistical analysis using Origin 8.0.
Data format	Analysed
Description of data collection	3D cell culture of ameloblast cell lines- ALC and LS-8 were carried out in growth factor reduced Geltrex (Thermo), type I collagen (Gibco) and transglutaminase linked gelatin (ColTgel) (101Bio) based on published protocols, or manufacturer's recommendations [2]. Control and test proteins were introduced to the gels to examine their effects on the cells which were imaged using fluorescent microscopy and confocal laser scanning microscopy. 3D images were reconstructed from sequential Z stacks and cell aspect ratio measured and tabulated. Antibody labelling was performed with whole culture immunostaining against E-cadherin, Par-3 and claudin-1. Secondary antibodies conjugated with Alexa Fluor 488 and 647 were used for immunodetection.
Data source location	Institution: University of Southern California. City/Town/Region: Los Angeles, California. Country: United States of America.
Data accessibility	With the article
Related research article	*G.Visakan, J.Su, J.Moradian-Oldak, Ameloblastin promotes polarization of ameloblast cell lines in a 3-D cell culture system, Matrix Biol.* https://doi.org/10.1016/j.matbio.2021.11.002

Value of the Data

- Provides a framework for systematic assessment of morphological changes in cells in 3D by means of the aspect ratio measurements.
- Aids in providing the basis for the development of similar protocols for 3D culture of other cell types and using other gel substrates.
- Provides potential cell polarization markers that can be followed to examine cells' polarization status in 3D culture.
- Development of an animal-free robust in vitro system to observe changes in cell behaviour in response to matrix composition.

1. Data Description

Legend:

- AMBN- ameloblastin.
- AMEL- amelogenin.
- BSA- bovine serum albumin.
- ALC- ameloblast lineage cell.
- HPLC- high performance liquid chromatography.
- DiD- 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine perchlorate.

The data reported here are primarily from optimization experiments carried out in 3D culture in the presence of test recombinant proteins (AMBN, AMEL) and controls (BSA, heat denatured AMBN). Fig. 1 is a representative 3D reconstructed image of ALC cell clusters formed in the presence of AMBN viewed using cell membrane (DiD) and nuclear labelling (DAPI). Image reconstruction was performed from several individual 2D images taken at varying Z planes (0.4 µm pitch). In the cell clusters, DAPI labelling is restricted to one pole of the cells represented using white arrows in the merged image (Fig. 1c). Fig. 2 is the graphical representation of ALC aspect ratio data measured at 24 and 72 h timepoints from growth factor reduced Geltrex and type I collagen gels. At both timepoints, ALC form elongated cells characterized by increased aspect ratio only in the presence of AMBN or AMEL in both gel types. Fig. 2b represents a direct comparison of ALC aspect ratios in the two gel substrates showing no statistically significant difference in aspect ratios between the two gel types. Tables 1 and 2 contain raw data from cell width and height measurements and the aspect ratio calculations. Table 1 contains data obtained at the end of 72 h culture in GFR Geltrex and Table 2 from type I collagen, at the end of 72 h. Heat denaturation of AMBN by heating to 80 °C for 15 min was sufficient to reverse the effect of AMBN on cells, with aspect ratio values being comparable with those of control. Fig. 3 is a graphical representation of the effect of substrate stiffness on aspect ratio of cells cultured in soft (Fig. 3 a and b), stiff (Fig. 3 c and d) and medium (Fig. 3 e) ColTgel in the presence of test (AMBN) and control (heat denatured AMBN). In soft and stiff ColTgel, a response to AMBN in the form of cell elongation (increased aspect ratio) can be observed only at the end of 72 h (Fig. 3 b, d). At the end of 24 h, no cell elongation can be observed (Fig. 3 a, c). However, in medium ColTgel, ALC aspect ratio increases at the end of 24 h, comparable with what is observed with GFR Geltrex and collagen gels (Fig. 3 e). Fig. 4 comprises of fluorescent images and aspect ratio measurements of LS-8 cells cultured in the presence of AMBN and controls. LS-8 cells represent presecretory-stage ameloblasts [3]. LS-8 cells respond to AMBN in culture by cell clustering in comparison to the discrete cells obtained when cultured with Geltrex alone (Fig. 4 a, b). Measurements of LS-8 aspect ratio reveal a statistically significant increase in the presence of AMBN compared to the

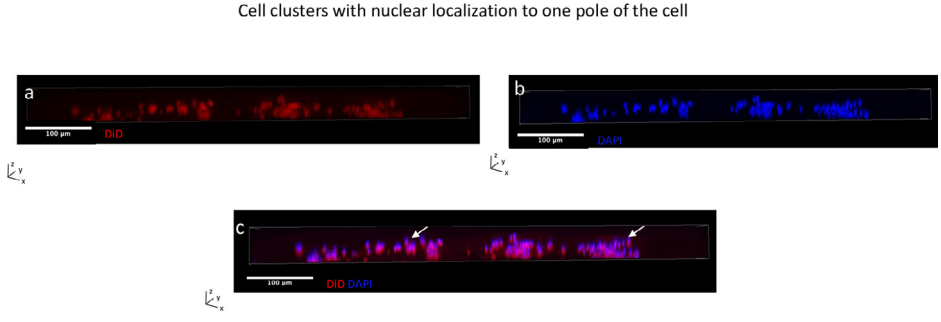

Fig. 1. 3D Z stack image of DiD (red) and DAPI (blue) labeling showing ALC cell polarization. Individual channels for membrane and nucleus shown in (a) and (b), respectively. Note that in the merged image, nucleus localization shown by DAPI is limited to one pole of the membrane shown in (white arrows in c).

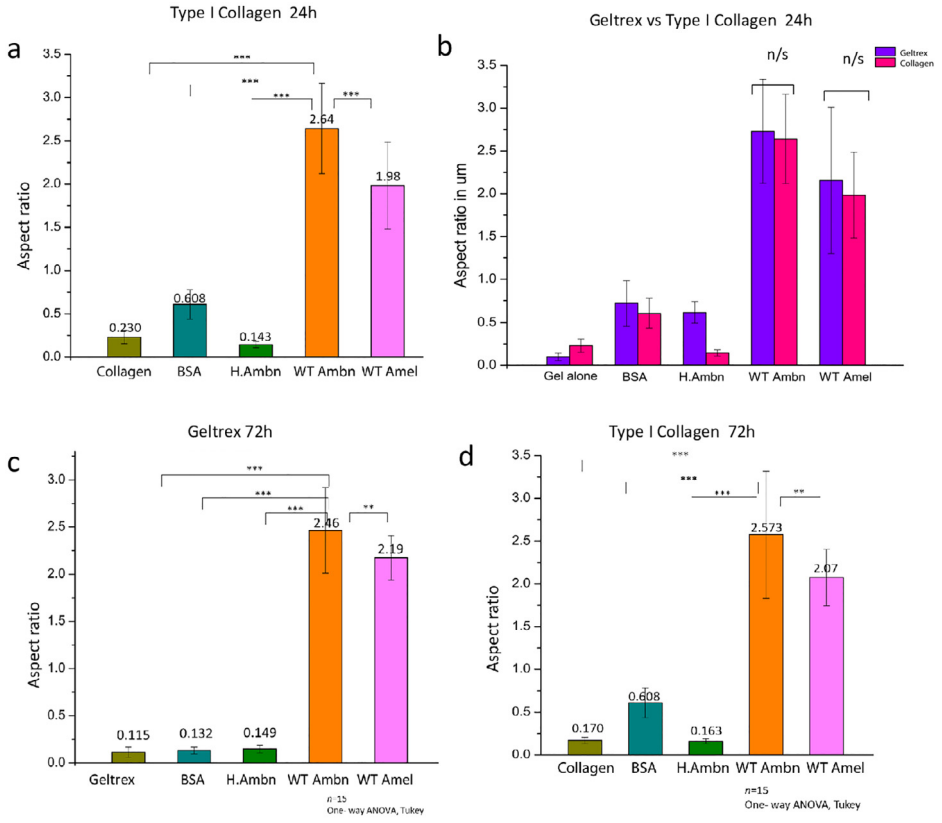

Fig. 2. ALC aspect ratio in type I collagen gel at the end of 24 h (a). Direct comparison between ALC aspect ratios obtained in type I collagen and GFR Geltrex, at the end of 24 h revealing no statistically significant difference (b). ALC aspect ratio at the end of 72 h in GFR Geltrex (c). ALC aspect ratio in type I collagen gel at the and 72 h (d). Note that the difference in ALC aspect ratio between the controls and EMPs remains consistent at the end of 72 h in collagen and Geltrex gels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

controls (Fig. 4 c). Fig. 5 is a confocal laser scanning 3D reconstructed image of elongated cells visualized using actin labelling with Alexa Fluor 488 conjugated phalloidin. Actin labelling using phalloidin confirms the cell elongation observed earlier using cell membrane dye, DiD. Surface rendering of the phalloidin labelled cells enables visualization of morphology (Fig. 5 b). Fig. 6 is a confocal image of a section of the ALC cell cluster viewed from two different points along Z axis showing differential labelling patterns for E-cadherin (Alexa Fluor 555) and DiD. Alexa Fluor 555 labelling indicating E-cadherin localization visible in Fig. 6 b (yellow arrow), and cell membrane labelling with DiD is also visible at this Z plane. However, in Fig. 6 f E-cadherin labelling is not discernible while DiD labelling is still visible at this Z depth (Fig. 6 h). Fig. 7 is a confocal image of secondary antibody controls for Alexa Fluor 488 and Alexa Fluor 647 fluorophores. No non-specific labelling observed with both Alexa Fluor 488 and 647. Fig. 8 is a confocal imaging displaying claudin-1 labelling pattern within the ALC clusters as visualized using Alexa Fluor 488 conjugated secondary to anti-claudin-1 antibody. Claudin-1 labelling restricted to one pole of the cell basal to nucleus position even when the cells are not highly elongated. Fig. 9 is a 12% SDS gel image stained with Coomassie Blue showing protein bands for recombinant AMBN and a standard ladder.

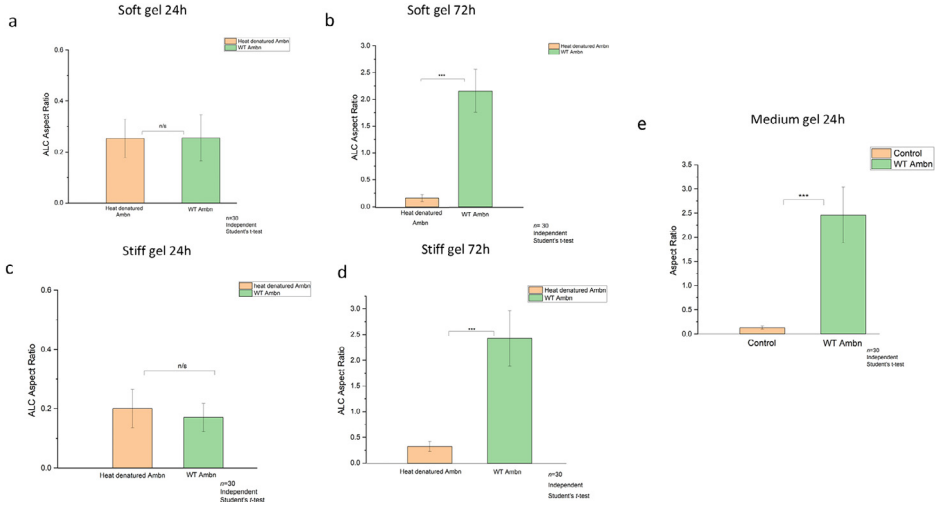

Fig. 3. Data from transglutaminase crosslinked gelatin gels (ColTGel); Soft gel (a, b) Stiff gel (c, d) and Medium gel (e). Note that with the soft and stiff gels, ALC aspect ratio does not increase at the end of 24 h (a, c). Whereas with the medium gel, aspect ratio is enhanced at the end of 24 h (e). Independent Student's *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

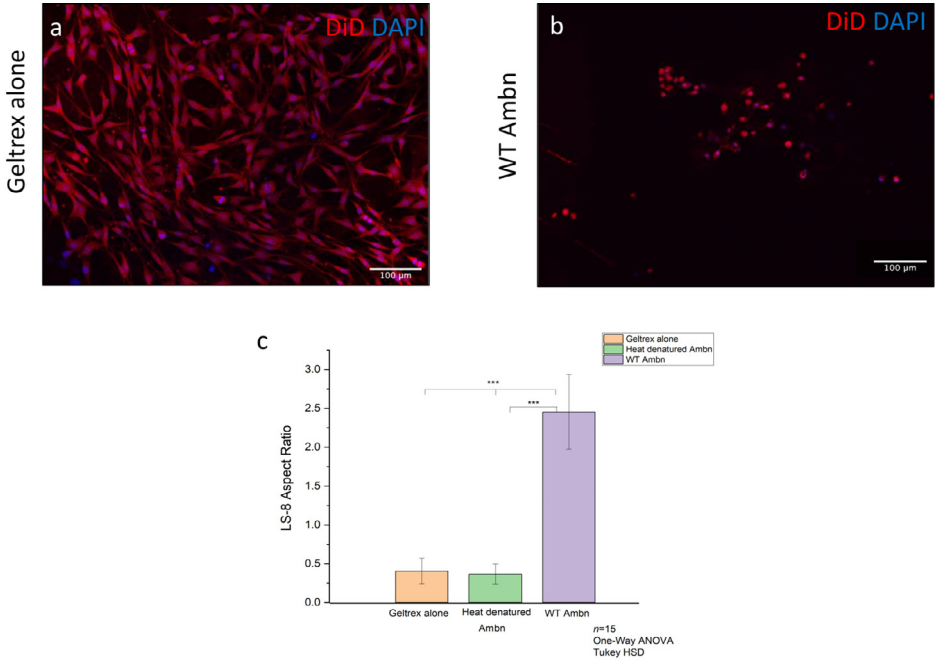

Fig. 4. Fluorescent images of LS-8 cells in Geltrex showing the presence of discrete cells in the control (a) and the formation of cell clusters with AMBN (b). Measurement of cell aspect ratio reveals a statistically significantly increased aspect ratio in the presence of AMBN (c). One way ANOVA, Tukey HSD * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

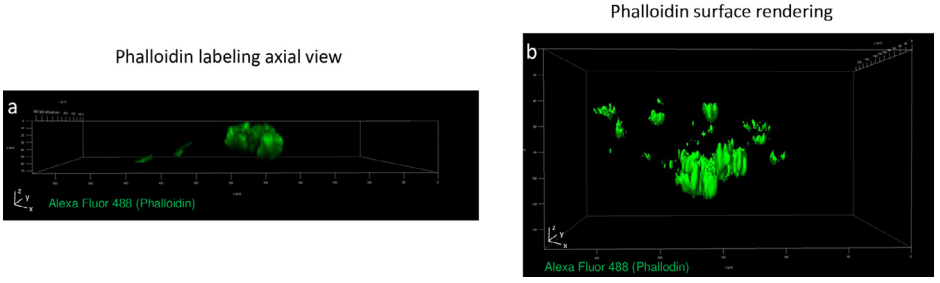

Fig. 5. Confocal laser scanning microscope axial images of ALC cell clusters labelled with Alexa Fluor 488 conjugated Phalloidin. 3D reconstruction and surface rendering reveal the presence of elongated cells within the clusters (b).

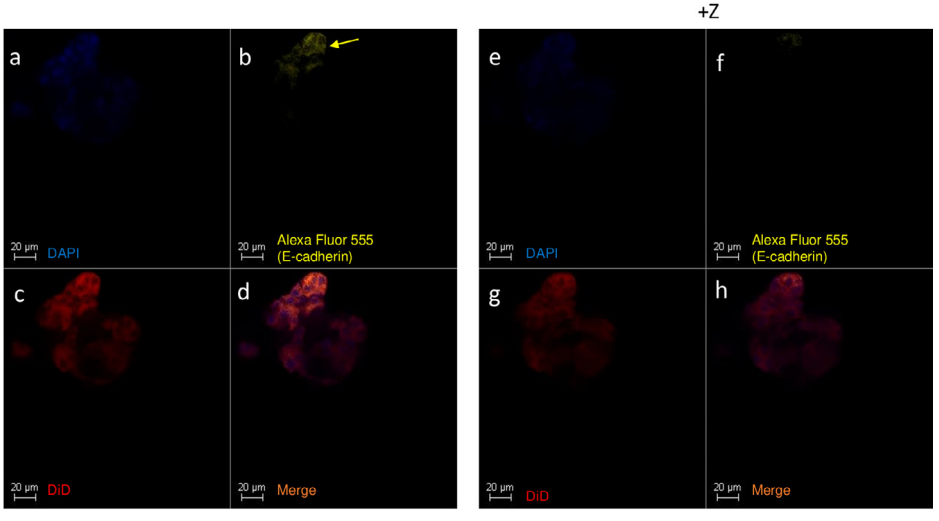

Fig. 6. 2D range imaging of E-cadherin membrane double labelled ALC cluster. E-cadherin labelled with Alexa Fluor 555 (yellow), cell membrane with DiD (red) and nucleus with DAPI (blue). 2D images of a representative cell cluster at a certain Z distance from the surface (a–d). The same cluster at a greater Z distance from the surface the of the gel (e–h). Note that the Alexa Fluor 555 signal (yellow arrow in b) representative of E-cadherin labeling disappears with increasing Z depth (f), whereas the DiD signal is still present at both Z depths (c, g).

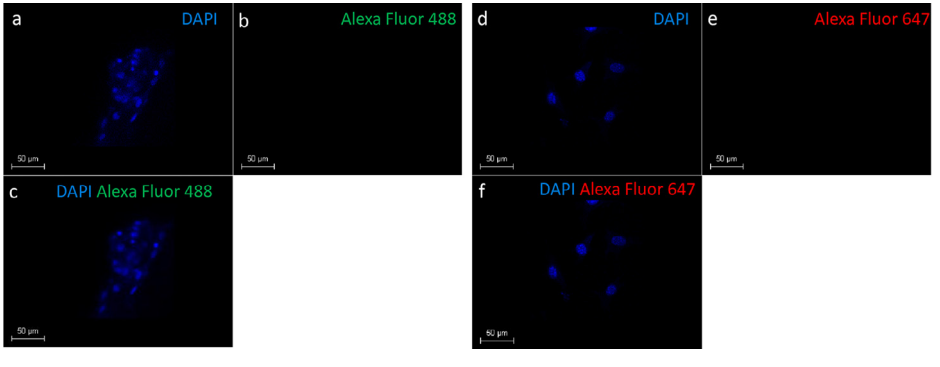

Fig. 7. Confocal secondary antibody controls for Alexa Fluor 488 (a–c) and Alexa Fluor 647 (d–f) secondary antibodies.

Table 1
ALC cell aspect ratios with and without added proteins after 72 h in GFR Geltrex (raw data). S. No.: Sample No.

Geltrex only (control)				with BSA (negative control)			
S.No.	Z	XY	Aspect Ratio	S.No.	Z	XY	Aspect Ratio
1	2	17	0.117	1	3	21	0.142
2	1	20	0.050	2	5	25	0.200
3	3	20	0.150	3	3	24	0.125
4	2	28	0.071	4	3	21	0.142
5	3	21	0.142	5	3	23	0.130
6	2	24	0.083	6	4	22	0.181
7	2	15	0.133	7	3	21	0.142
8	2	17	0.117	8	3	22	0.136
9	2	27	0.074	9	2	30	0.066
10	2	17	0.117	10	3	20	0.150
11	2	26	0.076	11	2	25	0.080
12	3	11	0.272	12	3	19	0.157
13	3	27	0.111	13	3	29	0.103
14	2	15	0.133	14	2	21	0.095
15	2	27	0.074	15	3	24	0.125
with heat denatured AMBN (negative control)				with AMBN			
1	3	25	0.120	1	9	4	2.25
2	3	20	0.150	2	12	6	2.00
3	3	19	0.157	3	16	6	2.66
4	3	20	0.150	4	14	6	2.33
5	4	29	0.137	5	10	5	2.00
6	3	20	0.150	6	8	4	2.00
7	3	22	0.136	7	17	7	2.42
8	3	24	0.125	8	12	6	2.00
9	3	24	0.125	9	14	5	2.80
10	3	18	0.166	10	12	6	2.00
11	4	16	0.250	11	14	5	2.80
12	3	24	0.125	12	12	5	2.40
13	4	19	0.210	13	14	5	2.80
14	4	25	0.160	14	12	4	3.00
15	2	24	0.083	15	14	4	3.50
with AMEL							
1	12	5	2.40				
2	15	7	2.14				
3	14	6	2.33				
4	12	6	2.00				
5	13	5	2.60				
6	11	5	2.20				
7	13	7	1.85				
8	9	4	2.25				
9	13	6	2.16				
10	10	5	2.00				
11	12	6	2.00				
12	11	5	2.20				
13	13	7	1.857				
14	13	5	2.60				
15	10	5	2.00				

2. Experimental Design, Materials and Methods

2.1. Enamel Matrix Proteins Expression and Purification

Recombinant amelogenin and ameloblastin were expressed and purified as per previously described in established protocols [4,5]. Briefly, recombinant mouse amelogenin was expressed in BL21 *Escherichia coli*. Cells were lysed, protein concentrated using ammonium sulphate precipi-

Table 2
ALC cell aspect ratios with and without added proteins after 72 h in type I collagen (raw data). S. No.: Sample No.

Type I Collagen (Control)				with BSA (negative control)			
S. No.	Z	XY	Aspect Ratio	S. No.	Z	XY	Aspect Ratio
1	4	31	0.129	1	7	11	0.636
2	4	21	0.190	2	8	10	0.800
3	4	18	0.222	3	8	11	0.727
4	4	22	0.181	4	5	19	0.263
5	4	20	0.200	5	5	7	0.714
6	5	23	0.217	6	9	13	0.692
7	3	20	0.150	7	7	16	0.437
8	4	23	0.173	8	6	14	0.428
9	3	21	0.142	9	8	16	0.500
10	4	23	0.173	10	7	9	0.777
11	4	22	0.181	11	7	12	0.583
12	4	18	0.222	12	8	9	0.888
13	3	22	0.136	13	5	12	0.416
14	2	23	0.086	14	4	6	0.666
15	3	20	0.150	15	6	10	0.600
With heat denatured AMBN (negative control)				with AMBN			
1	3	17	0.176	1	10	5	2.00
2	3	23	0.130	2	12	6	2.00
3	3	17	0.176	3	20	10	2.00
4	4	20	0.200	4	15	8	1.87
5	3	18	0.166	5	12	6	2.00
6	3	22	0.136	6	15	4	3.75
7	3	20	0.150	7	10	5	2.20
8	4	22	0.181	8	13	5	2.60
9	4	22	0.181	9	15	7	2.14
10	3	23	0.130	10	12	5	2.40
11	3	20	0.150	11	12	3	4.00
12	3	14	0.214	12	14	4	3.50
13	4	24	0.166	13	10	3	3.33
14	3	24	0.125	14	10	5	2.00
15	3	25	0.120	15	12	4	3.00
with AMEL							
1	2	17	0.117				
2	1	20	0.050				
3	3	20	0.150				
4	2	28	0.071				
5	3	21	0.142				
6	2	24	0.083				
7	2	15	0.133				
8	2	17	0.117				
9	2	27	0.074				
10	2	17	0.117				
11	2	26	0.076				
12	3	11	0.272				
13	3	27	0.111				
14	2	15	0.133				
15	2	27	0.074				

tation and purified using reverse phase HPLC. Recombinant full-length mouse ameloblastin was expressed in BL21 *Escherichia coli*. Cells were lysed, and the protein was initially affinity purified using Ni-NTA agarose columns (Qiagen). It was then dialyzed against 10K MWCO snakeskin dialysis membrane (ThermoFisher) and purified using reverse phase HPLC after removal of histidine, thioredoxin and S-tags using enterokinase (New England Biolabs) at 37 °C. Characterization of recombinant proteins was performed using 12% SDS gel electrophoresis. 10 µl of protein was mixed with 5x SDS-loading buffer and heated at 80 °C for 15 min. Protein sample was then cen-

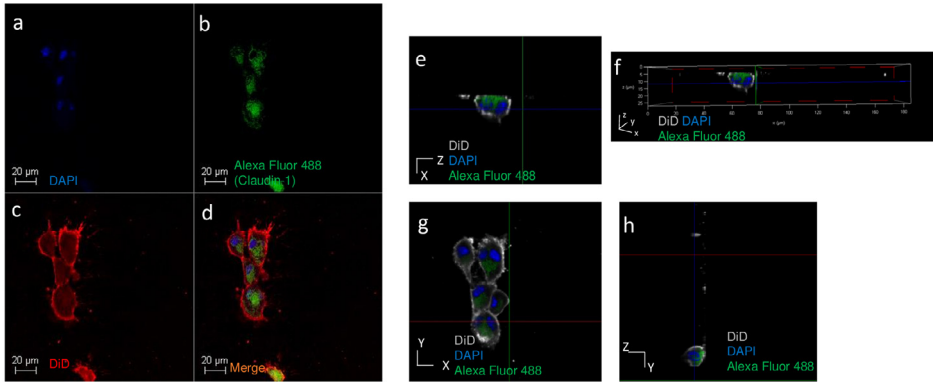

Fig. 8. Confocal images of ALC clusters labelled by DAPI (a), Alexa Fluor 488 labeled claudin-1 (cldn-1) distribution is cytoplasmic in reference to the membrane labeled with DiD as seen in (b, d). XZ (e), XY (g) and YZ (h) plane sections of the same cluster showing the cytoplasmic positioning of cldn-1 label within the cells. Blue (DAPI; nucleus), Green (Alexa Fluor 488; cldn-1); Red (DiD; membrane) Grey pseudo color (DiD; membrane).

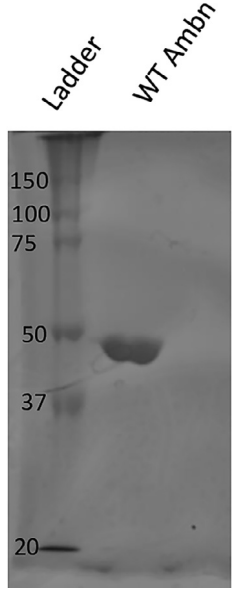

Fig. 9. SDS-PAGE of purified recombinant full-length AMBN expressed in *E.coli,* stained with Coomassie blue.

trifuged and loaded onto denaturing SDS-PAGE gels. Upon completion, gels were stained with Coomassie blue.

2.2. Heat Denaturation

Recombinant mouse ameloblastin was heat denatured by heating in cell culture grade PBS to 80 °C for 15 min in a heating block.

2.3. 2D Culture of Ameloblast Cell Lines

ALC were obtained from Prof. Toshiro Sugiyama (Japan) [6] and LS-8 cells were from Prof. Malcolm Snead (University of Southern California, USA) [7]. Cells from passages 4 and above were cultured in 13 mm culture dishes with modified Dulbecco's Modified Medium (DMEM, Gibco, Thermo) supplemented with 10% fetal bovine serum (FBS, Thermo) and 1% penicillin/streptomycin. This supplemented DMEM is referred to as cell culture media. Cultures were maintained under 5.0% CO_2 at 37 °C until they achieved 80% or greater confluence.

2.4. 3D Cell Culture with Geltrex, Collagen and Gelatin Substrates

Three different gel substrates were employed in these experiments- growth factor reduced (GFR) Geltrex (ThermoFisher), type I collagen (rat tail, Gibco) and transglutaminase crosslinked gelatin (ColTgel, 101Bio). A 3D-on-top type culture technique was employed where differential cell contact surfaces could be created with the test and control proteins immobilized on one end of the gel. The 3D culture procedure was carried out based on published protocols for GFR Geltrex [2] and were adapted for collagen and gelatin substrates. Glass- bottomed 96-well plates (Mattek) that were prechilled in the refrigerator were used for the 3D culture experiments. 20 µg/ml protein was prepared by diluting appropriate volumes of test (AMBN, AMEL) and control proteins (BSA, heat denatured AMBN) in cell culture grade PBS (ThermoFisher). 6 µl of protein solution was then pipetted onto the surface of the glass bottomed plates and incubated for 15 min at room temperature. Ice-cold GFR Geltrex was then pipetted onto the protein coated wells at a volume of 18 µl/well Plates were then incubated at 37 °C in the cell culture incubator for 30 min to allow Geltrex to polymerize. Simultaneously, a 10% Geltrex top-coat was prepared by dissolving ice-cold Geltrex in prechilled cell culture media. This 10% coat was maintained on ice until ready for use.

2.5. Cell Seeding

Upon initiation of gel polymerization, cells were detached from the plates using 0.25% Trypsin (ThermoFisher) for 30 s–2 min (depending on cell type). Trypsin was then inactivated using cell culture media and the detached cells were centrifuged at a low speed of 200 g to obtain a soft pellet. The cell pellet was then resuspended in cell culture media from which working concentrations of 3.5×10^4 cells/well were prepared by resuspending cells in half total volume of media. The working concentration of cells was achieved based on previously published literature [2,8,9]. Cells were pipetted into each well at 30 µl/ well. The plate was then agitated gently along the X and Y planes and incubated at 37 °C for 60 min to allow attachment of cells to the gel. At the end of 60 min, 30 µl of 10% Geltrex in media was added to each well thereby completing the 3D-on-top type culture. Plates were incubated for 24–72 h under 5.0% CO_2 at 37 °C.

2.6. Fixation

The method used for immunostaining involved whole culture fixation and staining without removal of the cells from the gel. At the end of 24 and 72 h, the cells were fixed using 25% glutaraldehyde for 15 min at room temperature. The wells were then washed gently with cell culture grade phosphate buffer saline (PBS) thrice to remove excess glutaraldehyde. PBS was added to the wells and the plates were stored in 4 °C until ready for use.

2.7. Immunofluorescence

All steps were carried out in dark. Cell membrane labeling was carried out using DiD, diluted 1:1000. The plates were incubated at 37 °C overnight. DiD was rinsed thrice with cell culture grade PBS in the dark and incubated with 1:1000 DAPI for 5 min and washed thrice. Only DiD and DAPI labelling was carried out for fluorescent microscopy. Cell permeabilization prior to antibody labeling was carried out using 0.1% TritonX-100 for 5 min at room temperature. The following primary antibodies were used: Alexa Fluor 555 conjugated mouse monoclonal anti-E-cadherin (610181 BD Biosciences; 1:400), Alexa Fluor 488 conjugated phalloidin (sc-363791 Santa Cruz Biotechnology; 1:200), rabbit polyclonal anti-claudin-1 (28674-1-AP Abcam; 1:400). The secondary antibodies used for detection are Alexa Fluor 488 (Jackson Immuno; 1:400) and Alexa Fluor 647 (Jackson Immuno; 1:400). The permeabilized cells were blocked using 3% fetal bovine serum (FBS) (for E-cadherin), 5% rabbit serum (for claudin-1) as per manufacturer's recommendations for 30 min. With E-cadherin, Alexa Fluor 555 conjugated mouse monoclonal antibody was used. To reduce the non-specific binding of mouse-on-mouse antibody, an additional blocking step involving monovalent unconjugated anti-mouse Fab fragments (715-007-003 Jackson Immuno) were used. Fab dilutions were freshly prepared for each experiment and added to each well at a working concentration of 30 ug/ml for 60 min at room temperature. The cells were then incubated with E-cadherin Ab (1:400) at room temperature for 60 min. No additional secondary antibodies were used for E-cadherin labeling. For claudin-1 labelling, cells were blocked using 5% rabbit serum for 30 min. The cells were washed and incubated with 1:400 claudin-1 primary antibody overnight at 4 °C and subsequently with 1:400 Alexa Fluor 488 conjugated secondary for 60 min at room temperature.

2.8. Fluorescent Microscopy and Cell Aspect Ratio Measurement

Fluorescent microscopy was carried out using Keyence BZX810 inverted fluorescent light microscope with an objective PlanApo λ NA 0.75. Sequential Z stacks were recorded with an optical sectioning pitch of 0.4 µm. Z stacks were 3-dimesionally reconstructed using Keyence ImageViewer (software version 1.1.1.8). The 3D measure tool was used to measure cell width (XY) and height (Z). These measurements were used to calculate the cell aspect ratio which was defined as: Cell Aspect Ratio = Cell height (Z)/Cell width (XY). Using this formula, cells that had elongated recorded aspect ratio > 1 and planar cells recorded aspect ratio values < 1 with $n = 30$ for each group. Aspect ratio measurements were carried out for cells cultured in the presence of test and control proteins.

2.9. Confocal Imaging

Confocal imaging was performed using Lecia SP8 confocal microscope with an oil immersion objective HCX PL APO CS $\times$ 60 (NA 1.4). Sequential Z stacks were recorded with an optical pitch of 0.5 µm. Detection of Alexa Fluor 555 was performed at 556 to 595 nm (excitation at 561 nm), Alexa Fluor 488 at 502 to 552 nm (excitation at 488 nm), DiD at 645 to 695 nm (excitation at 633 nm). Z stacks were 3D reconstructed, viewed and images recorded using LAS-X version 1.8.1.13759. To compare the immunolabeling range of E-cadherin along the Z axis compared to that of the cell membrane dye (DiD), 2D images were recorded at varying Z distances from the surface of the gel.

Ethics Statements

N/A.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Measured aspect ratios of cells based on microscope images (Original data)

CRediT Author Statement

Gayathri Visakan: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing; **Jingtan Su:** Supervision, Writing – review & editing; **Janet Moradian-Oldak:** Conceptualization, Methodology, Supervision, Investigation, Validation, Writing – review & editing, Project administration, Funding acquisition.

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