



Transglutaminase 2: a new player in bronchopulmonary dysplasia?

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ABSTRACT Aberrant remodelling of the extracellular matrix in the developing lung may underlie arrested alveolarisation associated with bronchopulmonary dysplasia (BPD). Transglutaminases are regulators of extracellular matrix remodelling. Therefore, the expression and activity of transglutaminases were assessed in lungs from human neonates with BPD and in a rodent model of BPD.

Transglutaminase expression and localisation were assessed by RT-PCR, immunoblotting, activity assay and immunohistochemical analyses of human and mouse lung tissues. Transglutaminase regulation by transforming growth factor (TGF)- β was investigated in lung cells by luciferase-based reporter assay and RT-PCR. TGF- β signalling was neutralised *in vivo* in an animal model of BPD, to determine whether TGF- β mediated the hyperoxia-induced changes in transglutaminase expression.

Transglutaminase 2 expression was upregulated in the lungs of preterm infants with BPD and in the lungs of hyperoxia-exposed mouse pups, where lung development was arrested. Transglutaminase 2 localised to the developing alveolar septa. TGF- β was identified as a regulator of transglutaminase 2 expression in human and mouse lung epithelial cells. *In vivo* neutralisation of TGF- β signalling partially restored normal lung structure and normalised lung transglutaminase 2 mRNA expression.

Our data point to a role for perturbed transglutaminase 2 activity in the arrested alveolarisation associated with BPD.



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Introduction

Premature infants frequently receive oxygen supplementation and mechanical ventilation as early life-support measures that, while effective, also cause bronchopulmonary dysplasia (BPD), a commonly encountered cause of morbidity and mortality in a neonatal intensive care setting [1]. While BPD is currently diagnosed exclusively by oxygen dependence criteria, the hallmark pathophysiological characteristic of BPD is an arrest of alveolarisation in the developing lung, which has long-term consequences for survivors. The pathophysiological basis of the arrested alveolarisation is not understood; however, perturbations of the remodelling and maturation of the extracellular matrix (ECM) are key features of BPD pathology [2]. Malformed collagen and elastin structures have been reported in the lungs of patients with BPD [3] and in animal models of BPD [4, 5]. How these ECM structures are malformed is not understood; however, one line of thinking is that the ECM cross-linking systems operative in affected lungs are deregulated, generating ECM structures that are consequently aberrantly remodelled, or that cannot be remodelled, thereby impeding the development of the immature lung [5, 6]. Supporting this idea, increased expression and activity of lysyl oxidases, which are key ECM cross-linking enzymes, have been reported in clinical BPD and experimental animal models of BPD [6]. In the present study, we have addressed the expression and activity of a second family of protein cross-linking enzymes, the transglutaminases, in BPD.

Transglutaminases (EC 2.3.2.12) are Ca^{2+} -dependent enzymes that catalyse the formation of isopeptide bonds either within or between polypeptide chains [7]. The transglutaminase family currently consists of nine members, including transglutaminases 1–7 (encoded by the *TGM1–7* genes in humans and *tgm1–7* genes in mice), and the related factor XIIIa and erythrocyte band 4.2 [7, 8]. The predominant transglutaminase catalytic product is a γ -glutamyl- ϵ -lysine cross-link formed by a transamidation reaction between a glutamyl residue γ -carboxamide group and a lysyl residue ϵ -amino group [7]. Transglutaminases are called “nature’s biological glues” as the γ -glutamyl- ϵ -lysine cross-link is stable to proteolysis and mechanical stress [8]. Substrates of transglutaminases include the ECM proteins collagen II, V, VII and XI, the collagen III propeptide, and fibronectin [7, 8]. Given that transglutaminases can modify and cross-link many ECM proteins, and that pulmonary ECM remodelling and maturation are perturbed in clinical BPD and in experimental animal models of BPD, transglutaminases appear to be interesting potential players in BPD pathogenesis. This idea is supported by two recent reports implicating transglutaminase 2 in pulmonary fibrosis [9, 10]. For these reasons, we set out to examine the pulmonary expression, localisation and regulation of transglutaminases in clinical BPD and in an experimental animal model of BPD.

Methods

Human patient material

The use of human tissues in this study was approved by the Ethik-Kommission (the equivalent of an institutional review board in Germany) of the University of Giessen School of Medicine (Giessen, Germany) under approval number 189/09. Tissue harvesting from pre-term and term neonates has been described in detail [6]. Clinical data for patient material are provided in table 1.

Animal model

Animal experiments performed in Germany were approved by the Regierungspräsidium Gießen (the institutional animal care and use committee equivalent in Germany) under approval 22/2000. The hyperoxia-based animal model of BPD has been described by our group previously [6, 11], where hyperoxia-exposed pups develop a pronounced arrest of alveolarisation that was apparent within 5 days. Briefly, mouse pups were randomised to two groups within 12 h of birth (post-natal day (P)0.5), and exposed to 21% oxygen (normoxia) or 85% oxygen (hyperoxia).

Cells and transforming growth factor- β stimulation

Human lung epithelial A549 (CCL-185) and H441 (HTB-174) cells were obtained commercially from the American Type Culture Collection (Manassas, VA, USA). Primary mouse lung fibroblasts and alveolar type II cells were isolated from adult C57Bl/6J mouse lungs [6, 11]. Primary human lung microvascular endothelial cells (C-12281), pulmonary artery smooth muscle cells (C-12521) and lung fibroblasts (C-12360) were purchased from PromoCell (Heidelberg, Germany). For transforming growth factor (TGF)- β stimulation, cells were exposed to TGF- β_1 (2 ng·mL⁻¹ final concentration; R&D Systems, Wiesbaden-Nordenstadt, Germany) for 18 h. This represents a dose well within the standard range (0.2–10 ng·mL⁻¹) for *in vitro* TGF- β_1 stimulation studies [6, 11].

Analysis of gene and protein expression

Real-time RT-PCR was undertaken exactly as described previously [6, 11, 12] using primers listed in online supplementary table S1, all of which generated threshold cycle values <35 (fig. S2) for human (see table 1

for number of subjects per group), mouse (n=5 per group) or cell culture (n=3 per group) material. The cDNA was synthesised as described previously [6, 11, 12] from total RNA pools prepared from lung tissue or cultured cell homogenates. Immunoblotting was performed exactly as previously described [6, 11, 12] using: goat anti-Tgm1 (diluted 1:200; antibody SC-18127; Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-Tgm2 (1:1000; 06-471; Upstate Biotechnology, Lake Placid, NY, USA); and rabbit anti- α -tubulin (1:2500; SC-5286; Santa Cruz Biotechnology). Immune complexes were detected (n=3 per group per time-point) with donkey anti-goat IgG–horseradish peroxidase conjugate (1:1000; SC-2020; Santa Cruz Biotechnology) and goat anti-rabbit IgG–horseradish peroxidase conjugate (1:3000; 31460; Pierce, Bonn, Germany) using chemiluminescence.

Immunohistochemistry

Mouse lungs were pressure-fixed at 20 cmH₂O pressure and embedded in paraffin. Paraffin-embedded human and mouse lung tissue was sectioned at 3 μ m. Transglutaminases or the γ -glutamyl- ϵ -lysine cross-link were detected in 3- μ m sections as previously described [6, 11, 12] with goat anti-Tgm1, goat anti-Tgm2 or mouse anti-N- γ -glutamyl- ϵ -lysine (1:100; ab424; Abcam, Cambridge, UK) primary antibodies. Staining specificity was confirmed by the pre-adsorption of primary antibodies with a 100-fold molar excess of a competing peptide (SC-50062P; Santa Cruz Biotechnology) for Tgm1 and H- γ -glutamyl- ϵ -lysyl-OH (G-1970; Bachem, Bubendorf, Switzerland) for the γ -glutamyl- ϵ -lysine cross-link. No pre-adsorption agent was available for Tgm2 detection, where isotype-matched, nonimmune antibodies substituted the specific antibody. Target antigen localisation was visualised with biotinylated rabbit anti-mouse (95-6543B; Invitrogen, Carlsbad, CA, USA) and anti-goat (A10518; Invitrogen) antibodies coupled with a streptavidin–horseradish peroxidase complex colorimetric detection system. Histological sections presented are representative of the staining patterns seen in at least three samples per group.

Dual luciferase reporter assay

The dual luciferase reporter assay to assess transglutaminase promoter induction by the TGF- β signalling pathway was performed exactly as described previously [6, 11], using either the 2.2-kb human *TGM1* [13] or the 4-kb mouse *tgm2* [14] promoter to drive firefly luciferase expression from a pGL3 backbone in A549 or H441 cells (n=5 per group). *Renilla* luciferase activity, driven by the herpes simplex virus thymidine kinase promoter in pRLTK (Promega, Madison, WI, USA), yielded low to moderate levels of constitutive expression in cotransfected cells and was used to normalise firefly luciferase activity in a dual luciferase ratio.

In vivo TGF- β neutralisation experiments

The inhibition of TGF- β signalling in mouse pups in the hyperoxia-based animal model of BPD has shown by our own [6, 15] and other [16, 17] groups to normalise TGF- β signalling, and to partially restore normal alveolar development. Neutralisation of TGF- β signalling in mouse pup lungs (n=6 per group) was undertaken exactly as described previously using pan-TGF- β neutralising IgG (1D11; R&D Systems) and an isotype-matched nonimmune IgG (MOPC21; Sigma, St Louis, MO, USA) [6, 15].

Transglutaminase 2 assay

Transglutaminase 2 activity was assessed with a commercially available kit (CS1070; Sigma) with 100 μ g protein (n=7 per group) over a 60-min period. This assay determines transglutaminase 2 activity by measuring the covalent incorporation of the transglutaminase 2-specific substrate biotin-TVQQEL-OH into poly-L-lysine that has been immobilised on an ELISA plate. The biotin-TVQQEL-OH serves as the acyl donor, where conjugation occurs *via* the γ -carboxamide group of biotin-TVQQEL-OH. The incorporated biotin-TVQQEL-OH was assessed *via* biotin recognition of a streptavidin–peroxidase conjugate, detected using a spectrophotometer at 450 nm with 3,3',5,5'-tetramethylbenzidine as a substrate.

Statistics

Data from patient samples were compared using one-way ANOVA with a Newman–Keuls *post hoc* test. For cell-culture studies, data were compared between two groups using an unpaired t-test, while groups containing more than two samples were compared using a one-way ANOVA with Tukey's *post hoc* test. Statistical outliers were determined using Grubbs' test for outliers.

Results

Transglutaminase expression is altered in the lungs of patients with or at risk for BPD

The expression of seven transglutaminases (*TGM1–TGM7*) was assessed in the lungs of seven patients with or at risk of BPD. Five patients had clinically defined BPD under current definition criteria. BPD groups are difficult to control for, as neonates are born prematurely and prematurely born neonates breathe gaseous air over a period where controls age-matched for gestational age continue to develop *in utero* with fluid-filled

TABLE 1 Clinical characteristics of control patients, and patients with bronchopulmonary dysplasia (BPD) or at risk of developing BPD

Patient	Birth weight g	Sex	Gestational age weeks	Chronological age at death days	Duration F_{iO_2} >0.50 days	Mechanical ventilation days	Cause of death/autopsy diagnosis	Medication
Group 1								
1	1625	M	33	3	0	0	Congenital heart malformation	Atropine Prostaglandin A ₁ Atropine Adrenaline
2	2350	M	35	<1	0	<1 h	Perinatal asphyxia	
3	1740	F	34	<1	0	0	Intrauterine death (ventriculomegaly)	
4	1800	M	32	5	0	5	Meningoencephalitis	
5	1190	M	31	<1	0	0	Placental abruption	
Median	1740		33					
Mean \pm SE	1741 \pm 186		33 \pm 0.7					
Group 2								
6	954	M	26	<1	0	0	Intracranial haemorrhage	
7	1210	M	28	<1	0	0	Hypoxic–ischaemic encephalopathy	
8	826	M	29	<1	0	0	Hydrocephalus	
9	852	M	27	<1	0	0	Arnold–Chiari malformation	
10	995	M	27	<1	0	0	Hypoxic–ischaemic encephalopathy	
Median	954		27					
Mean \pm SE	967 \pm 68		27 \pm 0.5					
Group 3								
11#	720	M	29	62	13	62	BPD IRDS	Surfactant Inotropes ^s Tobramycin
12	825	F	27	6	6	6	Staphylococcus aureus sepsis	Flucloxacillin Cortisone Surfactant Inotropes ^s Tobramycin
13#	835	M	26	65	27	65	Bronchopneumonia Intracranial haemorrhage	Penicillin Surfactant Inotropes ^s Dexamethasone
14#	930	M	26	99	98	99	Cerebral bleeding Ductus arteriosus BPD IRDS	Theophylline Surfactant Inotropes ^s Dexamethasone
15#	1250	F	28	34	34	34	Pneumothorax Subependymal haemorrhage BPD IRDS	Tobramycin Penicillin Amphotericin Surfactant Furosemide Amoxicillin Erythromycin

TABLE 1 Continued

Patient	Birth weight g	Sex	Gestational age weeks	Chronological age at death days	Duration FiO_2 >0.50 days	Mechanical ventilation days	Cause of death/autopsy diagnosis	Medication
16 [#]	1220	M	31	35	35	35	BPD IRDS Right ventricular hypertrophy Anaemia Rickets	Furosemide Amoxicillin Vancomycin
Median	882		26	49	31	49		
Mean \pm SE	963 \pm 90		28 \pm 0.8	50 \pm 13	36 \pm 13	50 \pm 13		
p-value versus group 1[§]	0.0032		0.0004	0.229 ⁺				
p-value versus group 2[§]	0.987 ⁺		0.672 ⁺	<0.0001				

Group 1: Control group age matched to the BPD group (group 3) for chronological age at death; group 2: control group matched to the BPD group (Group 3) for birth weight and gestational age at birth; group 3: BPD group, including patients with BPD or at risk of BPD. FiO_2 : inspiratory oxygen fraction; M: male; F: female; IRDS: infant respiratory distress syndrome. [#]: patient had clinically defined BPD. [†]: unpaired t-test; in the case of chronological age at death, the post-menstrual ages at death, rounded to the nearest full week, were compared. ⁺: nonsignificant; [§]: including dopamine, dobutamine and adrenaline.

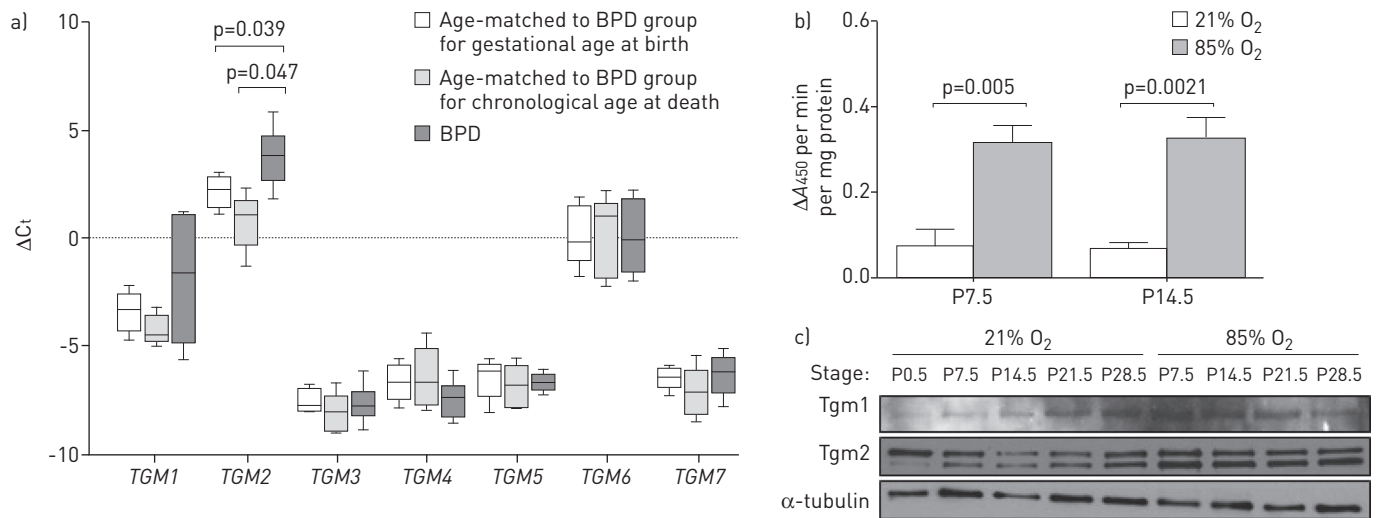


FIGURE 1 Lung transglutaminase expression human neonates and mouse pups with normal and aberrant late lung development. a) The mRNA expression levels of all seven classical members (*TGM1–TGM7*) of the transglutaminase family were assessed by real-time RT-PCR with the primers listed in table S1, using mRNA pools from patients afflicted with or at risk of bronchopulmonary dysplasia (BPD), as well as mRNA pools from control patients matched to the BPD group either for gestational age at birth or for chronological age at death. Clinical characteristics of the patients are provided in table 1. The *HPRT* gene (hypoxanthine-guanine phosphoribosyltransferase) was used as a reference. The whiskers in the graph represent the range of the data points, the boxes represent the lower and upper quartiles, and the solid line within the quartile box indicates the median. All data sets were screened by Grubbs' test for significant outliers but none were found. p-values were assessed by one-way ANOVA with the Newman-Keuls *post hoc* test. b) Transglutaminase 2 activity was assessed by the incorporation of biotin-TVQQL-OH into immobilised poly-L-lysine *via* the γ -carboxamide group of biotin-TVQQL-OH. Data are presented as mean \pm SD Tgm2 conjugating activity in mouse lung extracts. p-values were assessed by unpaired t-test (n=7 per group). c) Protein expression of Tgm1 and Tgm2 was assessed in protein extracts from whole-lung homogenates from mouse pups over the course of late lung development, during exposure to 21% or 85% oxygen from post-natal day (P)0.5, by immunoblotting. A single representative series is illustrated that is representative of at least two other series, which are quantified by densitometry in figure S1. Δ : change in; C: threshold cycle; A450: absorbance at 450 nm.

lungs. Therefore, transglutaminase gene expression was assessed in two groups of control patients, one group matched to the BPD group for chronological age at death, while the other control group was matched to the BPD group for gestational age at birth. Only *TGM2* expression was affected in the lungs of neonates with or at risk of BPD, compared with infants age-matched for both gestational age at birth and chronological age at death (fig. 1a). Infants with or at risk of BPD also exhibited an increased range of mRNA expression levels for *TGM1*, compared with both control groups. However, the expression levels of *TGM1* mRNA were not altered compared to either control group, taking $p=0.05$ as a threshold value (fig. 1a).

Lung transglutaminase expression is altered in an experimental animal model of BPD

The expression of all seven members of the transglutaminases was assessed in normally developing mouse pups over the first month of post-natal lung development, as well as in the lungs of mouse pups exposed to normobaric hyperoxia (85% oxygen), where a pronounced arrest of alveolarisation has been noted [6, 11, 17, 18]. In line with the findings in human BPD patients, transglutaminase 2 activity was also increased in aberrantly developing lungs from hyperoxia-exposed mouse pups at P7.5 and P14.5 (fig. 1b). Consistent with this observation, immunoblot analysis of Tgm1 and Tgm2 expression in the lungs of mouse pups over the first month of post-natal life, by which time lung development is complete, revealed a pronounced increase in protein abundance of Tgm1 and Tgm2 in hyperoxia-exposed mouse pups at P7.5 and P14.5 (fig. 1c, quantified in fig. S1). Particularly noteworthy was a sustained increase in protein abundance of Tgm2, which was correctly detected as a doublet, in the lungs of hyperoxia-exposed mouse pups at P7.5, P14.5 and P21.5, compared with littermates exposed to 21% oxygen (fig. 1c, quantified in fig. S1b). These data indicate that, similar to human patients with or at risk for BPD, the lung expression of Tgm2 is elevated in mouse pups in an experimental animal model of BPD. These observations prompted a screen of transglutaminase gene expression over the first 2 weeks of life, which is the critical secondary septation period. As early as day P2.5 (after 2 days of exposure to 85% oxygen), elevated expression of *tgm1* was observed (fig. 2). Over the first 14 days of life, hyperoxia-exposed mouse pups exhibited stably elevated *tgm1* expression, in contrast to normoxia-exposed pups, where a dynamic expression pattern was evident, reaching a trough at P9.5, perhaps reflecting the normal developmental expression of (and thus changing need for) Tgm1 (fig. 2a). By P9.5, by which time alveolarisation is well underway, the expression of *tgm1*

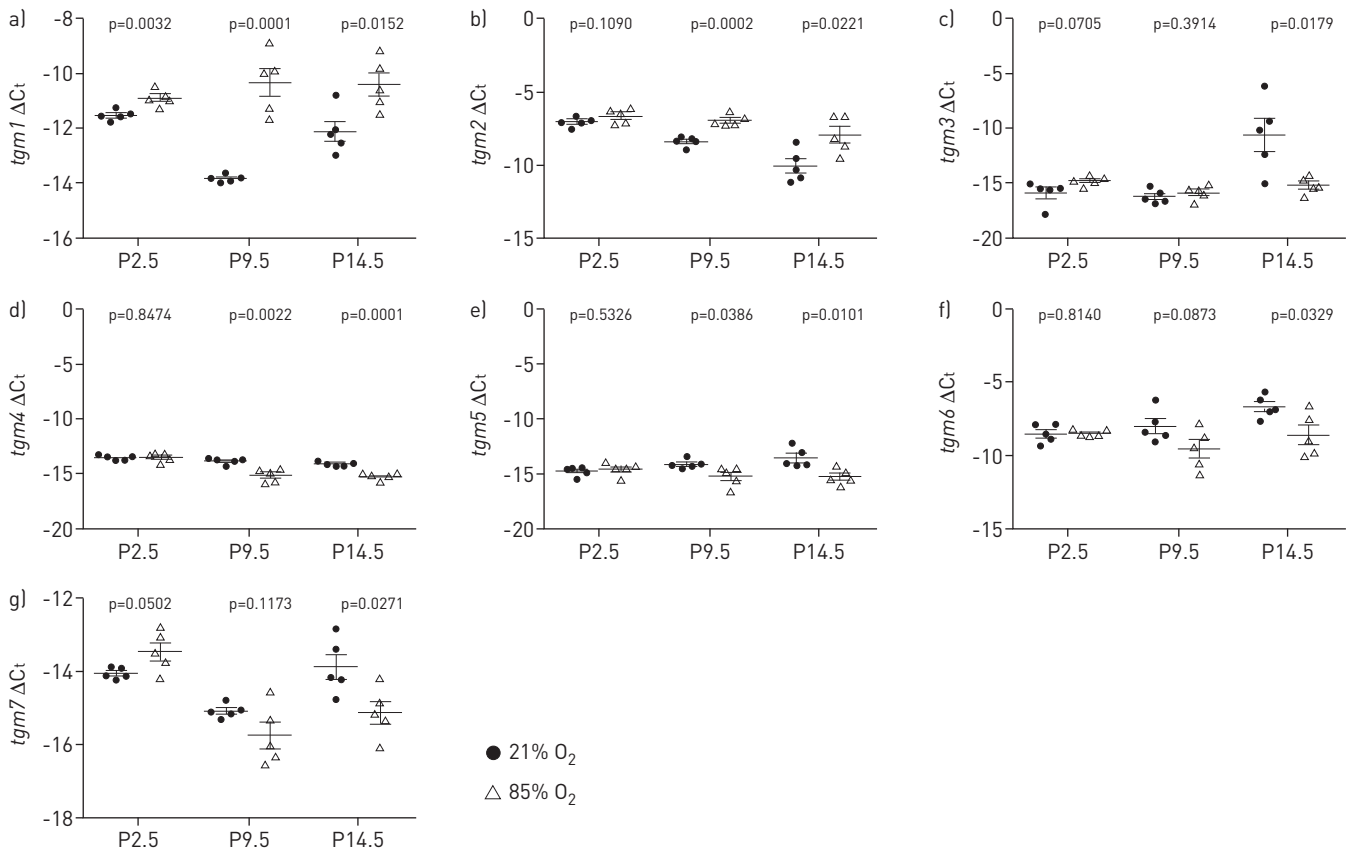


FIGURE 2 Expression of transglutaminase genes in the lungs neonatal mouse pups with normal or aberrant late lung development. The mRNA expression levels of all seven classical members of the transglutaminase family of protein cross-linking enzymes were assessed by real-time RT-PCR with the primers listed in table S1, using mRNA pools from lung homogenates from mouse pups at post-natal day (P)2.5, P9.5 and P14.5, after exposure to 21% or 85% oxygen from P0.5. a) *tgm1*; b) *tgm2*; c) *tgm3*; d) *tgm4*; e) *tgm5*; f) *tgm6*; g) *tgm7*. The 18S ribosomal RNA was used as a reference. Data are presented as mean \pm SE (n=5). p-values compared the 21% and 85% oxygen groups, and were assessed by one-way ANOVA with Tukey's *post hoc* test. Δ : change in; Ct: threshold cycle.

and *tgm2* was elevated in the lungs of hyperoxia-exposed mouse pups, while expression of *tgm4* and *tgm5* was down-regulated (fig. 2).

Transglutaminases and transglutaminase activity localise to regions of intense remodelling during alveolarisation

Transglutaminase activity, assessed by the presence of the transglutaminase-generated γ -glutamyl- ϵ -lysine cross-link, was detected in the developing mouse lungs (fig. 3), where staining was noted in the developing septa and epithelial structures of the parenchyma. A parallel pattern of expression was also noted for both Tgm1 (fig. 3f-i) and Tgm2 (fig. 3k-n). Furthermore, Tgm2 staining could be seen in the endothelial lining of large vessels and in regions of the vessel wall (fig. 3h). While the authors do not consider the relative intensity of immunohistochemical staining to be quantitative, the apparent increase in staining intensity for Tgm2 in the parenchymal regions of lungs from mouse pups exposed to 85% oxygen (fig. 3mn), compared with comparatively less Tgm2 staining in the lungs of 21% oxygen-exposed littermates (fig. 3k-l), was consistent with the Tgm2 immunoblot data (fig. 1c), which revealed an increased lung Tgm2 expression after exposure to hyperoxia.

Transglutaminase expression is regulated by TGF- β_1

The regulation of transglutaminase expression by TGF- β_1 was assessed in the constituent cell types of the developing alveolus. While TGF- β_1 did not impact *TGM1* expression in the human A549 cell-line (fig. 4a), TGF- β_1 did upregulate mRNA levels of *tgm1* in primary mouse alveolar type II cells (fig. 4b) and upregulated expression of *TGM2* in A549 cells (fig. 4e) and *tgm2* in primary mouse alveolar type II cells (fig. 4f). TGF- β_1 was without effect on *TGM1* (fig. 4c) or *TGM2* (fig. 4g) expression in primary human lung fibroblasts, or on *tgm1* (fig. 4d) or *tgm2* (fig. 4h) expression in primary mouse lung fibroblasts. The impact of TGF- β_1 was also assessed for all other transglutaminases, where the only notable effects of TGF- β_1 were the downregulation of *TGM3* mRNA levels in A549 cells (fig. S3) and a downregulation of *TGM7*

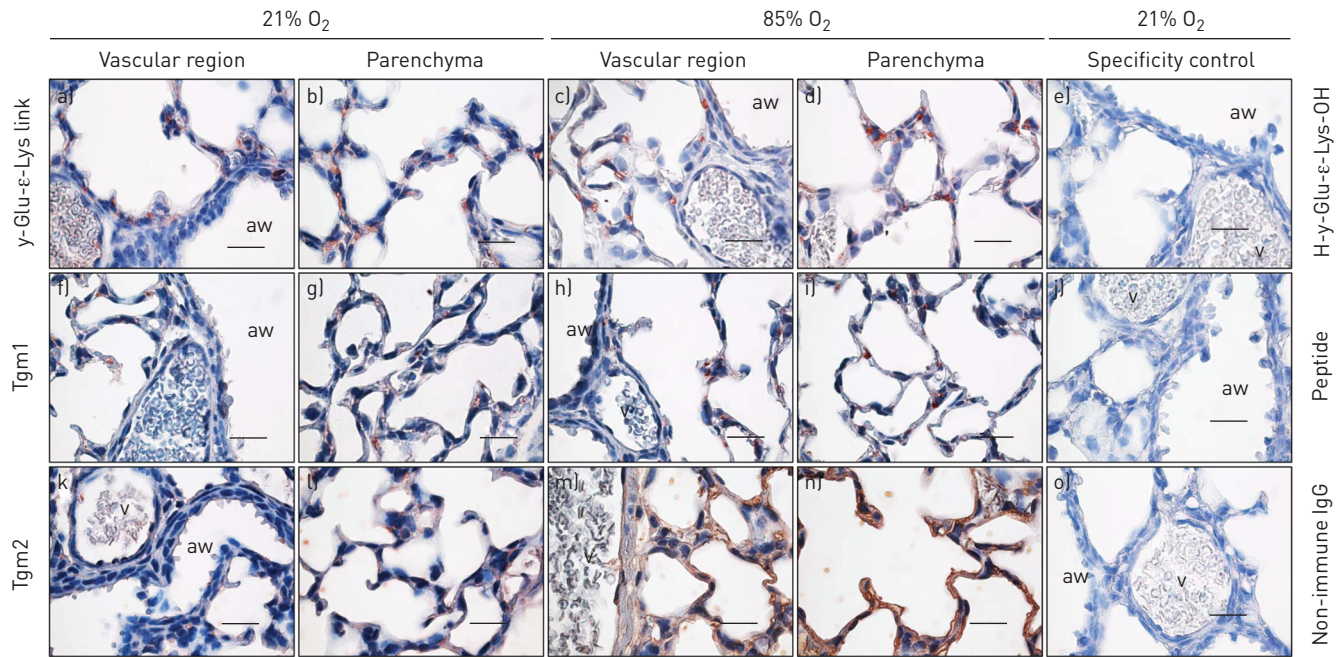


FIGURE 3 Localization of transglutaminase expression in the parenchyma and vascular regions of the lungs of mouse pups at post natal day (P)7.5, after exposure to 21% or 85% oxygen from P0.5. e, j, o) The specificity control column of photomicrographs indicates staining of the parenchyma and vascular regions of the lungs of mouse pups at P7.5, after exposure to 21% oxygen from P0.5. For specificity control, primary antibodies against the γ -glutamyl- ϵ -lysine cross-link and Tgm1 were pre-adsorbed with the H- γ -glutamyl- ϵ -lysyl-OH dipeptide or the Tgm1 immunogenic peptide, respectively. In the case of Tgm2, primary antibodies were replaced with nonimmune, isotype-matched control IgG, as no pre-adsorption agent is available for anti-Tgm2 antibodies. The airways (aw) and vessels (v) are indicated. Scale bars=20 μ m.

mRNA levels in primary human lung microvascular endothelial cells (fig.S4a). Interestingly, the downregulation of *TGM3* and *TGM7* expression by TGF- β ₁ correlated with the downregulation of *tgm3* and *tgm7* in the mouse model of BPD (fig. 2), suggesting that TGF- β ₁ may mediate this phenomenon in hyperoxia-exposed mouse pups. There was no impact of TGF- β ₁ on the expression levels of other *tgm* genes in primary mouse alveolar type II cells (fig. S5a) or in primary mouse lung fibroblasts (fig. S5b).

TGF- β signalling drives transglutaminase promoter activation in lung epithelial cells

The human *TGM1* promoter was not activated by stimulation of A549 cells with TGF- β ₁ (fig. 5a); however, TGF- β ₁ stimulation did activate the *TGM1* promoter in H441 cells (fig. 5a). Furthermore, the mouse *tgm2* promoter was activated by TGF- β ₁ stimulation of both A549 (fig. 5c) and H441 (fig. 5d) cells. These data indicate that TGF- β ₁ can be a regulator of transglutaminase expression in the lung epithelium and are supported by the mRNA expression data in A549 cells and primary mouse alveolar type II cells (fig. 4).

Hyperoxia-induced changes in lung Tgm2 expression are mediated by TGF- β ₁

Neutralisation of TGF- β signalling in animal models of arrested lung development both normalises lung TGF- β signalling and partially restores normal alveolarisation [17–19]. In the present study, it was revealed that *in vivo* neutralisation of TGF- β signalling has no impact on hyperoxia-induced increases in *tgm1* expression (fig. 6a). However, neutralisation of TGF- β signalling with the 1D11 antibody did normalise *tgm2* expression in the lungs of hyperoxia-exposed mouse pups. While the authors do not consider the intensity of immunohistochemical staining to be quantitative, the apparent increase in staining intensity for Tgm2 in the developing septa of lungs from mouse pups exposed to 85% oxygen (fig. S6c), contrasted with comparatively less Tgm2 staining in the lungs of 21% oxygen-exposed littermates (fig. S6a), appeared to be diminished when compared with developing septa of lungs from mouse pups exposed to 85% oxygen that received a TGF- β -neutralising antibody (fig. S6d *versus* S6c).

Transglutaminase 2 is widely expressed in the lungs of human neonates

Given the increased mRNA expression of *TGM2* in the lungs of human patients with BPD, lung tissues from normally developing human lungs as well as lung tissues from BPD patients were examined for TGM2 localisation by immunohistochemistry (fig. 7). TGM2 immunoreactivity was observed in the alveolar septa, airway epithelium, and airway and vascular smooth muscle walls, as well as the endothelium.

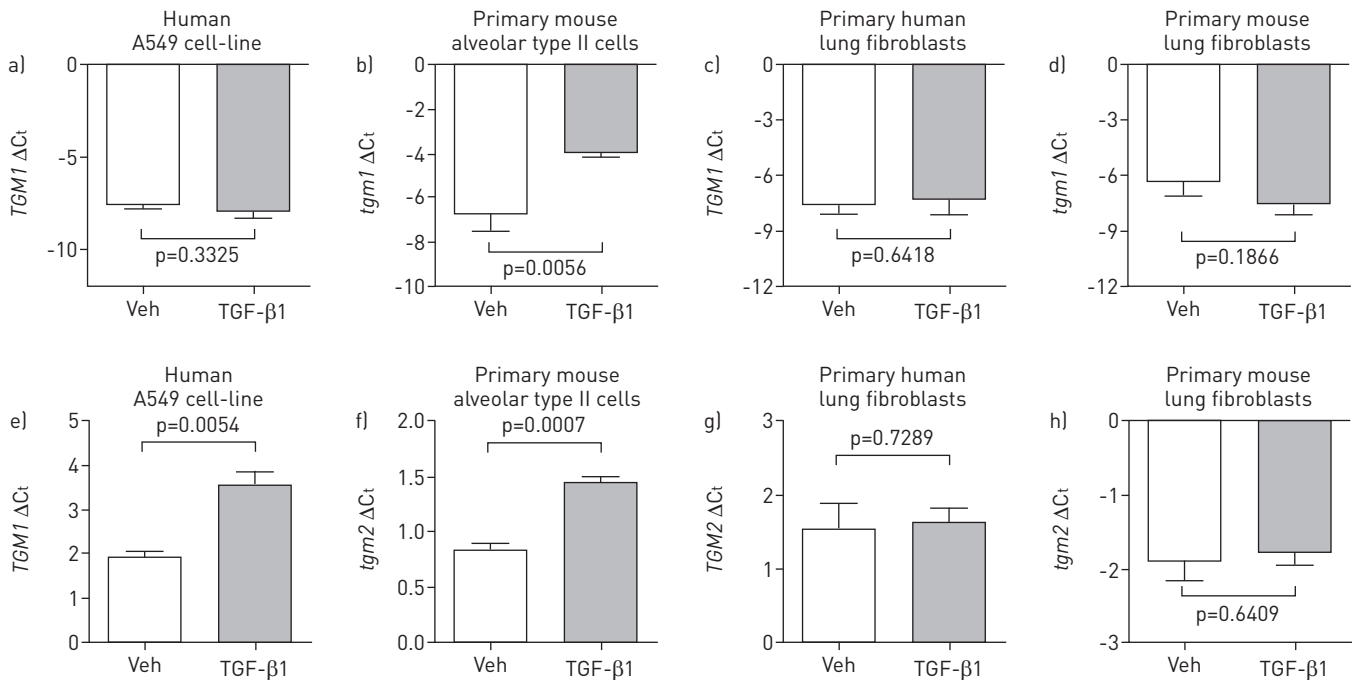


FIGURE 4 Regulation of transglutaminase mRNA levels by transforming growth factor (TGF)- β 1. The influence of TGF- β 1 on a–d) transglutaminase 1 and e–h) transglutaminase 2 gene expression was assessed in a, e) the human A549 lung epithelial cell-line, b, f) primary mouse alveolar type II cells, c, g) primary human lung fibroblasts and d, h) primary mouse lung fibroblasts. Cells were treated with vehicle (Veh) alone (PBS) or TGF- β 1 (2 ng·mL⁻¹ in PBS). The *HPRT* and *hrpt* genes (hypoxanthine–guanine phosphoribosyltransferase) were employed as reference genes for human and mouse cells, respectively. Primer sequences are provided in table S1. The impact of TGF- β 1 on transglutaminase gene expression (*TGM1–TGM7*) in primary human lung vascular endothelial cells and primary human pulmonary artery smooth muscle cells, and for the remaining transglutaminase genes (*tgm3–tgm7*) in primary mouse alveolar type II cells are provided in the online supplement in figures S1 and S2, respectively. p-values were assessed by unpaired t-test (n=3 per group) and compare Veh *versus* TGF- β 1-treated groups. Δ : change in; C: threshold cycle.

Discussion

Although transglutaminases have been widely studied in neurodegenerative and autoimmune diseases, and genetic diseases including lamellar ichthyosis and factor XIII deficiency [7], transglutaminases have received scant attention in the lung. Two recent reports have implicated Tgm2 in pulmonary fibrosis [9, 10], while one earlier study detected Tgm2 in the normally developing rat lung, where externalisation of Tgm2 from intracellular to extracellular pools was thought to delay basement membrane remodelling and to stabilise extracellular components such as microfibrils [20]. Our data reveal pronounced deregulation of transglutaminase 2 in clinical BPD and in an experimental animal model of BPD. The localisation studies reported here place transglutaminase 2 in the parenchyma of the developing mouse lung, a region of intense remodelling during alveolarisation. TGF- β was identified as a regulator of epithelial *tgm2* expression and as the factor that mediated hyperoxia-induced changes in *tgm2* expression in an experimental animal model of BPD. While the bulk of these data point to perturbed TGM2 expression in clinical BPD, altered Tgm1 expression was also noted in the mouse BPD model. This is interesting, as Tgm1 has been reported to be expressed in the normal respiratory epithelium, as well as in bronchial pre-invasive lesions and lung cancer [21], and increased Tgm1 expression is associated with squamous metaplasia of the respiratory epithelium [22], which is in line with the squamous metaplasia noted in BPD.

Abnormally elevated transglutaminase 1 and 2 expression could promote aberrant late lung development. It has been proposed that ECM cross-linking enzymes, such as the lysyl oxidases, are abnormally active in clinical BPD and an experimental animal model of BPD [6]. The same appears to be true for transglutaminases, which may then drive excessive cross-linking (and, hence, excessive stabilisation) of the lung ECM, rendering the ECM resistant to proteolysis, thus “locking” the lung structure and preventing the remodelling processes that normally facilitate alveolarisation. Transglutaminases are particularly active in basement membrane remodelling and the final phase of late lung development is achieved by fusion of epithelial and endothelial basement membranes [20]. Improperly cross-linked basement membrane collagens and other structural molecules may hinder the prerequisite remodelling of basement membranes to promote basement membrane fusion.

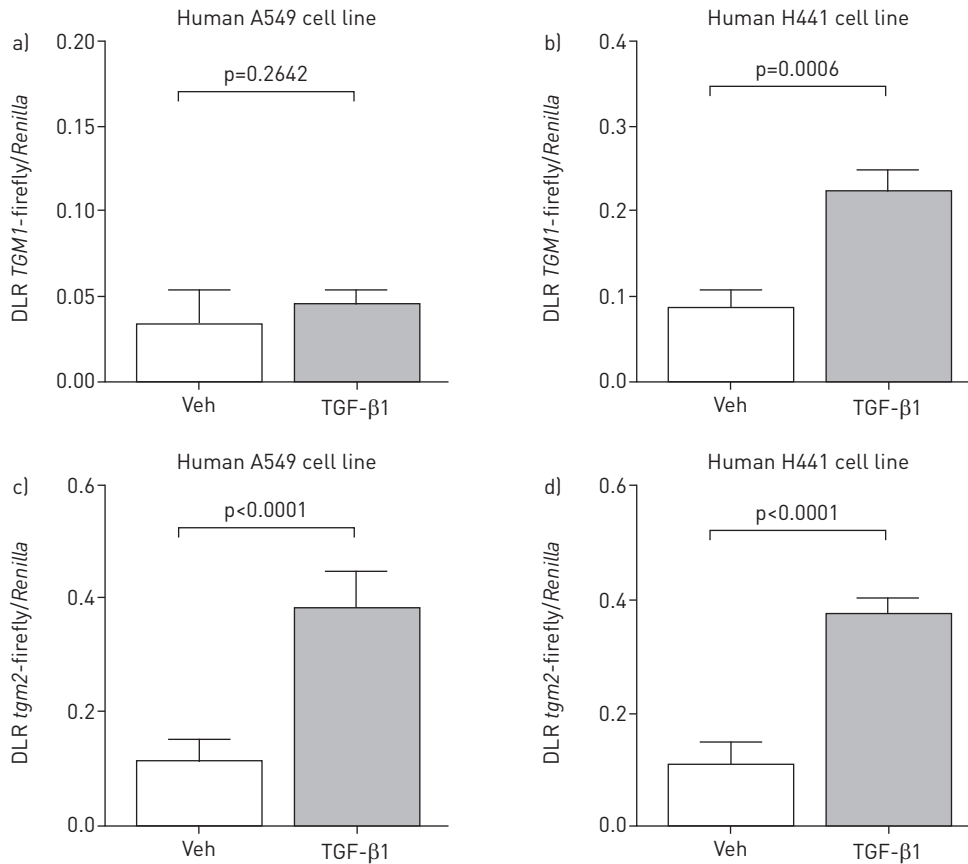


FIGURE 5 Regulation of transglutaminase promoter activity by transforming growth factor (TGF)- β signalling in human epithelial cell lines. Following transfection with plasmid constructs, a, b) the activity of the human *TGM1* and c, d) mouse *tgm2* promoters was assessed by dual luciferase assay a, c) in the human A549 cell-line, as a model of the alveolar epithelium, and b, d) in the human H441 cell-line, as a model of the conducting airway epithelium. Transfected cells were stimulated with vehicle (Veh) (4 mM hydrochloric acid, 1 mg·mL⁻¹ bovine serum albumin; diluted 1:500 in cell-culture medium) or TGF- β ₁ alone (2 ng·mL⁻¹; in cell-culture medium). Data points reflect the dual luciferase ratio (DLR) in which transglutaminase-driven firefly luciferase is normalised to *Renilla* luciferase expression driven by the herpes simplex virus thymidine kinase promoter, providing low to moderate levels of constitutive expression in cotransfected mammalian cells. p-values compare Veh- versus TGF- β -treated groups and were assessed by unpaired t-test (n=5 per group).

In addition to stabilising the ECM, transglutaminases have other notable functions that may be impacted in BPD. The epithelial lining of the lungs of infants with BPD is damaged by mechanical stretch and strain, and TGM2 is known to promote membrane resealing after mechanical damage to human lung A549 cells [23]. Along these lines, Tgm1 has also been shown to be critical for the structural integrity of mouse epithelial cells, by stabilising cadherin-based adherens junctions [24]. Thus, perturbed transglutaminase 1 and 2 expression may impact epithelial cell structural integrity and membrane repair, which are important processes in a premature, inflamed lung that is subjected to the stresses associated with mechanical ventilation and oxygen toxicity, which form part of the medical management of patients with BPD.

TGF- β was identified in this study as a regulator of *tgm2* expression in the mouse lung epithelium and as the mediator of hyperoxia-driven lung *tgm2* expression in the mouse BPD model. These data add to an emerging body of evidence that highlight TGF- β as a mediator of both experimental [12, 17, 18, 25] and clinical [26] BPD, where it has been solidly established by our own and other groups that *in vivo* neutralisation of TGF- β in hypoxia or hyperoxia models of BPD partially restores normal lung architecture. Our findings here demonstrate that *in vivo* TGF- β neutralisation also restored normal *tgm2* expression in the lungs of hyperoxia-treated mouse pups makes a strong case for Tgm2 as a pathogenic ECM remodelling factor in this rodent BPD model. This idea is further strengthened by our findings that TGM2 expression is elevated in human neonates with or at risk for BPD.

TGF- β ₁ did not drive transglutaminase expression in lung fibroblasts, which contrasts with observations made in dermal fibroblasts [27]. However, OLSEN *et al.* [10] also noted that TGF- β ₁ did not drive *TGM2*

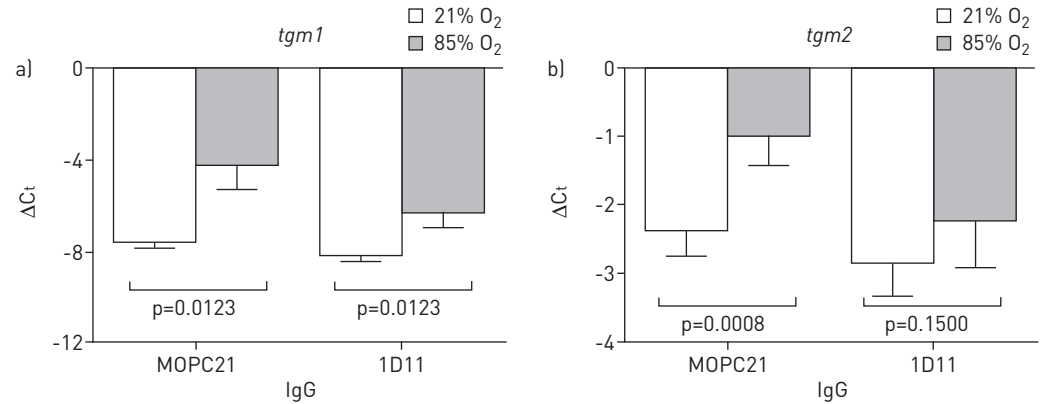


FIGURE 6 Impact of *in vivo* neutralization of transforming growth factor (TGF)- β signalling on hyperoxia-induced transglutaminase gene expression. Gene expression of mRNA encoding a) *tgm1* and b) *tgm2* was assessed in the lungs of mouse pups at post-natal day (P)10.5, which had inspired 21% or 85% oxygen from P0.5, and were treated with a pan-TGF- β_{1-3} -neutralizing IgG (1D11) or with an isotype-matched control (MOPC21) IgG. The mRNA levels were assessed by real-time RT-PCR in mRNA pools from whole-lung homogenates, using the primers in table S1, with the 18S ribosomal RNA serving as a reference. p-values were assessed by unpaired t-test, and were used to compare the 21% and 85% oxygen groups (n=6 per group).

expression in human lung fibroblasts, although TGF- β_1 did rapidly promote the externalisation and secretion of TGM2 by human lung fibroblasts. Thus, in the context of BPD, it is likely that TGF- β drives increased gene expression of transglutaminase II in epithelial cells, and also, increased secretion (and, hence, extracellular activity) of fibroblast-derived transglutaminase 2. In addition to ECM cross-linking functions, intracellular Tgm2 has also been credited with acting: 1) as a rheostat, regulating both apoptosis and autophagy in mouse fibroblasts [28]; and 2) in regulating adhesion [29] and migration [10] of mouse fibroblasts. Thus, perturbations to transglutaminase 2 expression may influence the spatiotemporal

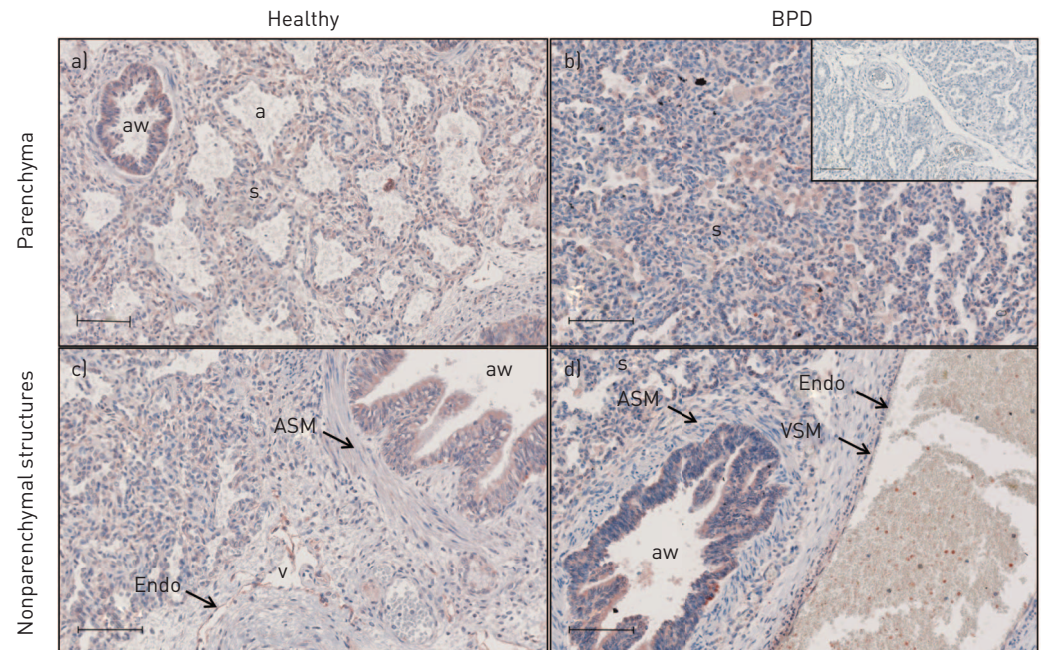


FIGURE 7 Transglutaminase 2 expression in the lung tissues of human neonates with normally or aberrantly developing lungs. The tissue localisation of TGM2 protein was assessed a, c) in the lung tissues of human neonates with normal lung development (patient 2 in table 1) and b, d) in the lung tissue of a patient with BPD (patient 15 in table 1). c) As a specificity control, primary antibodies were replaced with nonimmune isotype-matched control IgG, as no pre-adsorption agent is available for anti-TGM2 antibodies (inset). The airways (aw), airway smooth muscle (ASM), alveolar airspaces (a), endothelium (Endo), vascular smooth muscle (VSM), alveolar septa (s) and vessels (v) are indicated. Scale bars=100 μ m.

organisation of ECM deposition by fibroblasts in the developing alveolar septa. Interestingly, transglutaminase 2 is also known to activate latent TGF- β to active TGF- β [30], suggesting a positive feedback loop, where increased TGF- β activity may drive increased Tgm2 expression and activity, which, in turn, then escalates TGF- β activity in the lung, leading to a vicious circle where Tgm2 and TGF- β drive each other's expression.

BPD is a complicated disease and it should be noted that the animal model employed only mimics the arrested septation component of the alveolar simplification seen in patients with BPD. Furthermore, the mouse model employed in the current study replicates the response of alveolar development to oxygen-induced lung injury, whereas the humans who develop BPD might also have pathological contributions to lung disease due to other factors (such as inflammation and volutrauma) in addition to hyperaemia. This may be regarded as a limitation of the model. However, it is also notable that term mouse pups are born at the same stage of lung development as are pre-term infants who develop BPD and, as such, the mouse model is more suitable for BPD studies than is generally appreciated. Additionally, alveolar simplification due to arrested secondary septation is the key histopathological characteristic of "new" BPD and this was modelled very well in the mouse pup model. Other limitations of this study include the use of cells derived from adult (not neonatal) humans and mice, which may impact the extrapolation of the *in vitro* results to the situation in the neonate. It should also be kept in mind that both control patient groups did not survive post-natally and, as such, no control group that was age-matched for post-natal age has been included. Thus, the impact of post-natal life alone on transglutaminase expression has not been estimated in this study.

In spite of these limitations, our data do make a strong case for a pathogenic role for a second family of protein cross-linking enzymes, the transglutaminases, in the arrested lung development associated with clinical BPD and experimental animal models of BPD. Follow-up studies with transglutaminase inhibitors, currently being initiated by the investigators' laboratories, will assess whether transglutaminases play a causal role or represent therapeutic targets, in experimental animal models of BPD.

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