Glucocorticoid signalling drives reduced versican levels in the fetal mouse lung

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Abstract

Glucocorticoid (GC) signaling via the glucocorticoid receptor (GR) is essential for lung maturation in mammals. Previous studies using global or conditional mouse model knockouts of the GR gene have established that GR-mediated signaling in the interstitial mesenchyme of the fetal lung is critical for normal lung development. Screens for downstream GC-targets in conditional mesenchymal GR deficient mouse lung (GRmesKO) identified Versican (Vcan), an important extracellular matrix component and cell proliferation regulator, as a potential GR-regulated target. We show that, of the five major VCAN isoforms, the VCAN-V1 isoform containing the GAGβ domain is the predominant VCAN isoform in the fetal mouse lung distal mesenchyme at both E16.5 and E18.5, whereas the GAGα-specific VCAN-V2 isoform was only localized to the smooth muscle surrounding proximal airways. Both Vcan-V1 mRNA and protein levels were strongly overexpressed in the GRmesKO lung at E18.5. Finally, we investigated the GC regulation of the ECM protease ADAMTS 12 and showed that Adamts 12 mRNA levels were markedly reduced at E18.5 in GRmesKO fetal mouse lung and were strongly induced by both cortisol and betamethasone in cultures of primary rat fetal lung fibroblasts. ADAMTS12 protein immunoreactivity was also strongly increased in the distal lung at E18.5, after dexamethasone treatment in utero. In summary, glucocorticoid signaling via GR represses GAGβ domain-containing VCAN isoforms in distal lung mesenchyme in vivo by repressing Vcan gene expression and, in part, by inducing the ECM protease ADAMTS12, thereby contributing to the control of ECM remodelling and lung cell proliferation prior to birth.

Key Words
- glucocorticoids
- glucocorticoid receptors
- versican
- lung development

Introduction

The final stages of mammalian lung development are characterised by a dramatic reduction in and remodelling of the mesenchymal compartment to provide a thinning of the distal airways. This process is, in part, regulated via the action of the glucocorticoid (GC) steroids acting via the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily (Oshika et al. 1998, Cole et al. 2019). The importance of GCs in human lung development is highlighted by the now routine clinical administration of strong synthetic GCs, such as betamethasone, to mothers who are at risk of preterm birth to greatly reduce the morbidity and mortality of preterm infants at risk of developing respiratory distress syndrome (Liggins & Howie 1972, Roberts & Dalziel 2006).
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Materials and methods

Animals

The care and use of all animals was approved by the Monash Animal Research Platform-1 Animal Ethics Committee at Monash University. Time-mated pregnant Sprague–Dawley rats were provided by Monash Animal Research Platform. Global GR-null fetal mice and mesenchymal-specific GR-null fetal mice (Mus musculus) were all of an isogenic C57BL/6 genetic background and generated using the Cre/LoxP gene recombination system and, as previously described (Cole et al. 1995, Bird et al. 2003, Bird et al. 2007, 2014) briefly, mice expressing Cre under the control of the Dermo1 promoter were crossed with GRloxP/loxP mice to generate GRloxP/loxP, Dermo1cre/+ mice. These mice were then time-mated with GRloxP/loxP to generate GRloxP/loxP, Dermo1cre/+ fetal mice. Pregnant dams were treated with dexamethasone (0.1 mg/kg) at E14.5 and E15.5, and fetal lung tissue were removed from pups at E18.5. The morning of a positive plug mating was designated as embryonic (E) day 0.5. Pregnant mice were killed according to approved guidelines of the Animal Ethics Committee at Monash University at E14.5, E15.5, E18.5, and PN 0.5, and the pups were killed by decapitation. The lungs of fetal pups (at least n=4 for each genotype collected from 2–3 litters) were isolated and either snap frozen in liquid N2 or fixed in 4% paraformaldehyde for further analysis as described subsequently. Tail snips from pups were collected at dissection for genotyping by PCR and gel electrophoresis as previously described (Bird et al. 2014).

Isolation of primary rat fetal lung fibroblasts

At E20, pregnant Sprague–Dawley rats were killed by CO2 according to approved Animal Ethics Committee guidelines. Fetal lung fibroblasts (99% fibroblast cultures) were then isolated from pups via differential attachment as previously described (McDougall et al. 2011, 2013). Fibroblast cells from a single litter were pooled together and treated as one biological replicate (n=1). Cells were seeded onto six well dishes for RNA isolation in late stages of lung development. This was assessed using isoform-specific qPCR, immunohistochemistry, and Western blot analysis in the GRmesKO and the normal fetal mouse lung. Finally, we assessed the expression levels and mode of GC regulation of three members of the ADAMTS ECM protease family.

There is, however, limited understanding of the important GC-regulated molecular mechanisms and gene networks in the developing lung required for normal development. Studies using global or conditional mouse knockouts of the GR gene (Bird et al. 2015) have established that GR signaling in the mesenchymal compartment of the lung is particularly crucial for normal lung maturation (Bird et al. 2014). Both global and mesenchymal conditional GR knockout (GRmesKO) mice die shortly after birth due to respiratory failure, as a result of lung mesenchymal cell hyperplasia which thickens the gas exchange barrier (Cole et al. 1995, Bird et al. 2014). Previous screens for differentially expressed genes in GRmesKO lungs have identified the mesenchyme-localised extracellular matrix (ECM) lectican protein Versican (VCAN) as strongly overexpressed in distal lung compartments (Bird et al. 2014).

VCAN has a core protein structure consisting of an N-terminal G1 domain and a carboxy-terminal G3 domain as well as two central chondroitin sulfate glycosaminoglycan (GAG) attachment domains, termed the GAGα and GAGβ domains (Wight 2002). The Vcan gene can be alternatively spliced into five isoforms V0, V1, V2, V3, and V4, and the encoded proteins have molecular weights of approximately 370, 263, 180, 74, and 115 kDa, respectively (Zimmermann & Ruoslahti 1989, Ricciardelli et al. 2009, Kischel et al. 2010). The VCAN-V1 isoform is expressed in the developing lung, heart, and limbs, while VCAN-V2 is the predominant VCAN isoform in the nervous system (Milev et al. 1998, Snyder et al. 2015). VCAN-V1 is known to promote cell proliferation and mesenchymal-epithelial transitions, whereas VCAN-V2 is known to inhibit cell proliferation (Sheng et al. 2005, 2006).

VCAN is an important ECM proteoglycan that, in the embryo, together with hyaluronan and fibronectin form a loose hydrated matrix to allow tissue hydration, solute permeability, appropriate cell proliferation, cell migration, and ECM assembly. This promotes cell growth and differentiation and tissue remodelling in a range of fetal tissues (Wight 2002, Ricciardelli et al. 2009, Nandadasa et al. 2014). Toward the end of embryogenesis there is a tightly regulated degradation and remodelling of ECM components in many tissues, including the fetal lung. The breakdown and clearance of VCAN involves a number of members of the A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) proteinases family (Stanton et al. 2011). We have investigated the isoform-specific expression of Vcan in the mouse fetal lung and the GC/GR-mediated repression of VCAN during the
Waymouth’s MB 752/1 medium containing 10% heat-inactivated fetal calf serum (ThermoFisher), 50 U/mL penicillin, and 50 µg/mL streptomycin and were cultured for 6 h at 37°C in 5% CO₂.

**Treatment of fetal lung fibroblasts**

E20 primary rat lung fibroblasts were maintained in Waymouth’s MB media containing 10% charcoal-stripped FBS, 50 U/mL Penicillin, and 50 µg/mL Streptomycin. The fibroblasts were treated with either corticosterone (1 µM in ethanol, Sigma-Aldrich), betamethasone (1 µM Celestone Chronodose, Schering-Plough, Australia), or 100% ethanol (vehicle control) for 6 h at 37°C at 5% CO₂.

**Isolation of RNA, cDNA synthesis and qRT-PCR**

Total RNA was obtained from embryonic mice and embryonic fetal rat lung fibroblasts using TRIzol (Invitrogen), and cDNA was prepared with a QuantiTech RT kit (Qiagen) (Moore et al. 2000). To measure mRNA levels of the four Vcan isoforms quantitative real-time PCR using SyBr green was performed following a previous protocol (Wong et al. 2007). Vcan DNA PCR primers were designed to overlap specific exon-exon junctions to amplify fragments representing the isoform mRNAs (exon 7–9), and Rps29 mRNA levels were used as a reference gene for normalisation of gene expression between samples. All qPCR products were verified by DNA sequencing. PCR DNA sequences (5′ to 3′) were: mouse Vcan-V0 GAAGCAGGTCGATTGAGTG (forward) and GCAACAGATCATGCAGTGG (reverse); mouse Vcan-V1 TGCTTTAAACGTCGATTGAGTG (forward) and CGACAAGGGTTAGTGACA (reverse); mouse Vcan-V2 TGCTTTAAACGTCGATTGAGTG (forward) and CCGACAAGGTTAGATGGA (reverse); and mouse Rps29 (forward) and TGCACACAGACAGAGGTAGAGT (reverse).

**Immunohistochemistry analysis**

Stained sections were imaged by light microscopy using Eclipse E400, Nikon. Three sections per animal were stained and the three photos per section were analysed using the image analysis software, Image-pro plus, Version 6.2, media cybernetics.
Western blot

Proteins were isolated from mouse lung tissue in urea buffer (4 M urea, 50 mM sodium acetate, 0.2 M NaCl, and 0.5% Triton×× in H₂O). Briefly, 500 µL of urea buffer was added to each lung sample, and the samples were then homogenised and incubated on ice for 15 min. Lung samples were centrifuged for 20 min at 4°C and 14,000 g. supernatants collected, and protein concentrations determined using a DC assay (BioRad). 40 µg of protein was pre-treated with 0.5 U of Chondroitinase ABC (Sigma-Aldrich) in 50 mM Tris pH 8.0 and 60 mM sodium acetate for 2 h before separation by electrophoresis on a TGX sain-
free 7.5% fastcast SDS-PAGE polyacrylamide gel (BioRad). Following a standard protocol, the protein was transferred onto a nylon membrane and incubated with either VCAN GAG-β specific antibody (0.5 µg/mL; Millipore, AB1033), VCAN GAG alpha specific antibody (0.5 µg/mL; Millipore, AB1032), or VIMENTIN (1:200 dilution; Cell signalling, D21H3). After X-ray imaging, the membranes were stripped with 0.5 M NaOH and incubated with a BETA ACTIN antibody (1:100,000; Sigma-Aldrich) to control protein loading.

Statistical analysis

GraphPad Prism software was used for all statistical analysis, with statistical significance nominally set at P<0.05. Two groups were compared using an unpaired t-test, and multiple groups were compared by either a 1-way or 2-way ANOVA with a Tukey’s post hoc test.

Results

Expression of Vcan isoforms in the developing lung during late gestation

The mRNA levels of specific Vcan isoforms were measured with isoform-specific primer pairs (Fig. 1) in total RNA samples from the fetal lung at various times during fetal lung development using qPCR (n=4/group). mRNA levels were measured from E14.5 to P0.5 and showed a large 11-, 8.5-, 6.6-, and 6.1-fold decrease of V0, V1, V2, and V3 isoform mRNA levels from E14.5 to P0.5 (P≤0.05) respectively (Fig. 2A, B, C and D). We also investigated VCAN protein levels with a VCAN GAG-beta-specific and a GAG-alpha-specific antibody at E16.5 (n=3) and E18.5 (n=3) in the mouse fetal lung via Western blot analysis (Fig. 2E, F and G) and detected a significant decrease in VCAN protein level from E16.5 to E18.5. These results show that Vcan expression at both the RNA and protein levels strongly decrease in the fetal lung shortly before birth.

Localization of GAGβ-containing VCAN-V1 and GAGα-containing VCAN-V2 in the mouse lung at E16.5 and E18.5

To determine the localization of GAGα- and GAGβ-containing VCAN proteins at the E16.5 and E18.5 fetal mouse lung, immunohistochemistry was performed using antibodies specific for the GAGβ and GAGα domains of VCAN (Fig. 3). Transverse sections of whole fetal mouse
torsos were used and the left lung lobes were chosen for all imaging. At E16.5, GAGβ-specific VCAN staining was observed in most mesenchymal compartments including the smooth muscle surrounding large proximal airways (Fig. 3A and E) and distal lung mesenchyme (Fig. 3B and F). Interestingly, GAGβ-specific staining appeared to show a gradient of expression. Qualitatively, staining appeared darker toward airways, compared to the distal regions of the lung. Indeed, GAGβ-specific VCAN-V1 staining was often virtually absent in the outermost regions of the lung, adjacent to the mesothelium (Fig. 3B, black arrow, n=3, one representative image is shown). At E18.5, GAGβ-specific VCAN protein staining was barely detectable in the distal lung mesenchyme (Fig. 3F) but instead was primarily restricted to the smooth muscle layer surrounding large proximal airways (Fig. 3E). In contrast GAGα-specific VCAN staining was only reliably detected in the smooth muscle layer surrounding the large proximal airways and was completely absent in the distal lung mesenchyme at both E16.5 and E18.5 (Fig. 3C, D, G and H). The VCAN immunostaining pattern observed in the distal fetal lung mesenchyme can be attributed to the GAGβ-specific VCAN-V1 isoform rather than VCAN-V0, as the latter also contains the GAGα domain that was not stained in this region of the lung by the GAGα-specific antibody. This demonstrates that, at E16.5, the VCAN-V1 isoform is the predominant isoform present in the fetal lung and that the levels of VCAN-V1 regress dramatically over 2 days just before birth.

**Increased Vcan isoform mRNA and VCAN-V1 protein levels in the GRmesKO lung**

We next investigated the influence of glucocorticoid signaling on Vcan-V1 levels in the distal lung mesenchyme using GRmesKO mice, where the GR has been deleted using the Cre/loxP recombination system in the fetal lung mesenchyme ([Bird et al. 2014](https://doi.org/10.1530/JME-14-0031)). We first measured mRNA levels for the specific Vcan isoforms in GRmesKO mouse lungs by qPCR (n=4) (Fig. 4A, B, C and D). We detected a 2.3-, 1.6-, 1.8-, and 1.8-fold increase for Vcan V0, V1, V2, and V3 mRNAs respectively in E18.5 GRmesKO lung relative to control lung (P<0.01) (Fig. 4A, B, C and D). In comparison, we detected no difference in mRNA levels for all isoforms at E16.5 for GRmesKO lung vs controls (Fig. 4A, B, C and D). We then measured protein levels of GAGβ-specific VCAN in whole tissue extracts from E18.5 control and GRmesKO mouse lung (n=7) using Western blot analysis (Fig. 4E and F, 4 representative wells are shown). There was a significant 3-fold increase in GAGβ VCAN protein in the GRmesKO mouse lung. Immunohistochemistry was then used to investigate localisation of VCAN in the GRmesKO mouse lung using the GAGα-specific and GAGβ-specific VCAN antibodies. At E16.5, there appeared to be no difference in the level and localisation of either GAGα- or GAGβ-containing VCAN proteins between the GRmesKO and control lung (Fig. 5A). At E18.5, strong immunostaining of GAGβ-containing VCAN was again evident in the smooth muscle layer surrounding large airways in GRmesKO lungs (Fig. 5A), and in agreement with Fig. 4E, we detected a dramatic increase in GAGβ-specific staining in the distal lung mesenchyme in the E18.5 GRmesKO lung compared to aged-matched controls (Fig. 5A). Similar to Fig. 3, there was virtually no VCAN immunostaining detected in the distal lung with the GAGα-specific antibody in either the GRmesKO lung or controls (Fig. 5A). This indicates that in the absence of GR-mediated signaling there is no normal repression and clearance of VCAN V1 protein from the distal lung mesenchyme close to birth.
Increased GAGβ-specific staining in distal lung areas were also quantitated and compared in GRmesKO and GR-null lung at E18.5 and shown to increase (Fig. 5B). To rule out that this increase did not just reflect on a greatly expanded distal area, Western blot analysis was performed with an antibody to the distal mesenchyme marker VIMENTIN (Fig. 5C). We observed a small but 1.3-fold increase in VIMENTIN protein level that was not unexpected, as GRmesKO mice have more lung mesenchymal tissue; however, we also observed a far greater increase in GAGβ protein levels with an increase of approximately 2-fold (Fig. 5D and E). This confirmed that there was increased GAGβ-specific protein content per mesenchyme area in the distal fetal lung.

**Figure 5**
Localization of the V1 (GAGβ) and V2 (GAGα) isoforms of VCAN at E16.5 and E18.5 in the GRmesKO fetal mouse lung by immunohistochemistry. Immunohistochemistry was performed with antibodies specific for the VCAN GAGβ (V1) and GAGα (V2) domains at E16.5 and E18.5 for control and GRmesKO fetal mice (A). Boxed regions are magnified in the bottom left corner of each image. All images were of the left lobe and are representative of three animals per age group. GAG beta immunohistochemistry analysis (mean ± S.E.M.) on E18.5 control (black bar, n = 8), GRmesKO (light grey bar, n = 6), and GR-null (dark grey bar, n = 7) fetal mice (B). Three sections per an animal were stained with GAG beta, and three images from each section were analyzed using Image-pro plus. Percentage of GAG beta staining is relative to total tissue in the image. (C, D, and E) Western blot analysis of protein levels of GAG beta containing VCAN (V0/V2) and VIMENTIN in E18.5 control and GRmesKO fetal lung (n = 3). Significance between the groups was assessed by 1-way ANOVA with a Tukey’s post hoc test (B: P = 0.0015) or an un-paired t-test (D and E). Significant differences are indicated by *P < 0.05 or individual P value.
ADAMTS protease expression in the fetal mouse lung

Degradation and turnover of VCAN in embryonic tissues is facilitated by the presence and action of specific members of the ADAMTS protease family (Stanton et al. 2011). We assessed the mRNA levels of three Adams proteases, 1, 12, and 15, whose transcripts we detected in a recent RNA-sequencing analysis of GC-treated cultured fetal rat lung fibroblasts (Seow et al. 2019). Adams 1, 12, and 15 mRNA levels were measured by real-time qPCR in total lung RNA at both E16.5 and E18.5 in control and GRmesKO mouse lung. We observed an increase in Adams 1, 12, and 15 mRNA levels from E16.5 to E18.5 with fold changes of 3.3, 6.9, and 2.9, respectively (Fig. 6A, B and C). We also measured the effect of the loss of GR-signalling in the mesenchyme on Adams mRNA levels at E16.5 and E18.5 in GRmesKO mice. At E18.5, we observed a significant loss of the normal increase in mRNA levels for only Adams 12, with no significant effect on Adams 1 and 15 mRNA levels (Fig. 6A, B and C).

Glucocorticoids induce expression of ADAMTS12 in the fetal lung

We detected a rapid reduction in VCAN levels in the fetal lung for over 2 days between E16.5 and E18.5, and this decrease could be facilitated by increased activity of specific ECM proteases such as ADAMTS12. We next assessed direct glucocorticoid induction of Adamts12 mRNA using real-time qPCR with primary cultures of E20 rat fetal lung fibroblasts stimulated with corticosterone and betamethasone for 6 h (Fig. 6D). We observed a strong 4.5-fold increase of Adamts12 mRNA levels in betamethasone treated fetal lung fibroblasts relative to control cultures, clearly implicating Adamts12 protease as a direct GC-induced target in the rodent fetal lung. To confirm induction of ADAMTS12 at the protein level in vivo, we analysed fetal mouse lungs at E18.5 from pups that had been treated in utero with dexamethasone (0.1 mg/kg) at E14.5 and E15.5. Immunohistochemistry using a rabbit anti-ADAMTS12 antibody showed a strong increase in ADAMTS12 immunoreactivity in distal lung regions compared to control treated pups (Fig. 5E). These results suggest that reduced expression and levels of VCAN in the fetal lung is coordinated by glucocorticoid signaling via a repression of Vcan expression and a possible increase in ADAMTS protease activity.
in VCAN degradation and clearance by induction of the ECM protease ADAMTS12.

**Discussion**

In this study, we have demonstrated that GR-mediated signaling regulates rapid repression and regression of VCAN during the late stages of mouse lung development and that, in the absence of GR-mediated cell signaling in the lung mesenchyme, the Vcan gene is overexpressed and the VCAN-V1 isoform proteoglycan persists in the distal lung prior to birth contributing to a state of ECM dysfunction and cellular over proliferation. We have shown here that the mRNA levels of the Vcan V0, V1, V2, and V3 isoforms all normally decline rapidly during the latter stages of mouse lung development from E14.5 to P0.5, and, using a GAGβ-specific VCAN antibody we also observed a rapid decrease in VCAN-V1 protein levels primarily in the distal regions of the developing mouse fetal lung. This is consistent with previous studies in the developing sheep and mouse lung that showed high levels of Vcan mRNA and VCAN protein at early- to mid-gestation stages and then a rapid decline in the days before birth (Faggian et al. 2007, Snyder et al. 2015). Studies in sheep observed a similar expression pattern and reported that there was an identical reduction in both V0 and V1 isoforms during late lung development (Faggian et al. 2007). We show for the first time that this rapid reduction in V0/V1 VCAN is, in part, driven by glucocorticoid receptor mediated cell signaling in the distal mesenchymal compartment of the fetal lung.

From approximately E16.5 in the mouse, there is extensive remodelling of the distal lung and capillary bed network that involves proteolytic breakdown of matrix proteins (Herriges & Morrisey 2014). At this stage of development, the GAGβ-containing VCAN-V1 isoform was localised to all mesenchymal compartments, yet by E18.5 this was restricted to the smooth muscle layer surrounding the large proximal airways. This coincides with a period of rapid remodelling of the mesenchyme, during which the lung mesenchyme dramatically thins allowing sufficient gas exchange at birth (Herriges & Morrisey 2014). We have previously reported that the reduction in mesenchyme tissue is associated with a decrease of cell proliferation rather than an increase in cell apoptosis (Bird et al. 2014). VCAN is a known promotor of cell proliferation, acting as a cell-surface receptor for growth factors such as the epithelial cell-growth factor midkine (Muramatsu 2014).

In comparison, GAGα-domain containing VCAN isoforms were only detected in the smooth muscle layer surrounding the large airways at both E16.5 and E18.5, and these levels were unchanged during fetal development. At earlier time points in lung development, the localisation of GAGα-containing VCAN to only the smooth muscle in the lung was also described by Snyder et al. (Snyder et al. 2015). The lack of GAGα-staining VCAN in the distal mesenchyme region of the lung suggests that V0 is not a predominant VCAN isoform in the fetal lung, as the V0 isoform contains both the GAGα and GAGβ domains. The V0 isoform could still potentially be expressed in the bronchial smooth muscle, where GAGα and GAGβ-specific antibody staining overlaps. Interestingly, we also observed a consistent proximal to distal gradient of high to low expression of GAGβ at E16.5. This leads us to speculate that the GAGβ domain plays an important role for recruitment of growth factors to stimulate cell proliferation of the more proximal mesenchymal tissue, particularly smooth muscle.

The importance of GC-signalling for lung maturation has been clearly demonstrated in GRnull mice which die shortly after birth due to acute respiratory distress (Cole et al. 1995). It has also been shown that GC-GR signaling in the mesenchymal compartment of the fetal lung is of greatest importance for normal lung development (Bird et al. 2014). Here we demonstrate that the V0, V1, V2, and V3 Vcan isoforms are all overexpressed in the GRmesKO mouse lung at E18.5, indicating a loss on normal GC-GR driven repression of Vcan gene expression. Although there is no significant difference between controls and GRmesKO at E16.5 this is somewhat expected, as the GRnull and GRmesKO lungs appear histologically normal until E15.5 but from E16.5 develop a progressive hypercellularity (Cole et al. 1995). The protein accumulation of GAGα was similar between controls and GRmesKO fetal lungs at both E16.5 and E18.5. However, we did observe variable mesenchymal GAGβ protein staining in E18.5 GRmesKO mouse lung. Furthermore, there was a significant increase in protein levels evident by Western blot. Further analysis of the distal mesenchyme compartment with VIMENTIN showed that there was a significant and higher content of GAG β-VCAN protein per area of distal mesenchyme.

The ADAMTS family of ECM proteases has 19 members, and to date 6 members, ADAMTS1, 4, 5, 9, 15, and 20, have been shown to cleave VCAN (Nandadasa et al. 2014). VCAN has ADAMTS cleavage sites in both the GAG beta and GAG alpha domains, Glu405-Gln406 and Glu1428-Ala1429, respectively (Sandy et al. 2001, Westling et al. 2004). We have demonstrated that Adamts 12 mRNA levels were greatly reduced in the GRmesKO lung, most likely due to a loss of normal GC-induced expression,
and this loss of ADAMTS 12 may contribute to the higher levels of VCAN protein observed in GRmesKO. Multiple ADAMTS proteases have been shown to cleave VCAN, and further studies are required to assess the localisation of specific ADAMTS at the protein level in the fetal lung. We have shown that both corticosterone and betamethasone can rapidly stimulate increased expression of ADAMTS 12 in fetal lung fibroblasts and that dexamethasone treatment in vivo stimulates strong ADAMTS 12 immunostaining in the distal mesenchyme prior to birth. Therefore, loss of normal GC-stimulated induction of ADAMTS 12 may strongly contribute to the lack of normal regression and clearance of VCAN just prior to birth. Respiratory studies in Adams12-deficient mice provide evidence for a protective effect of ADAMTS 12 against bronchial inflammation that is in line with the anti-inflammatory actions of glucocorticoid steroids (Paulissen et al. 2012). Other studies have demonstrated that GCs can induce expression of Adams enzymes, such as Adams1 in C2C12 myoblasts, following treatment with dexamethasone (McRae et al. 2017). We, therefore, suggest that GCs are stimulating increased ADAMTS 12 levels in the lung which, in turn, facilitates a lowering of the levels of VCAN. Further functional studies are required to investigate the direct genomic mechanisms involved between activated GC-GR signaling and regulation of the Adams 12 gene.

VCAN is able to interact with many binding partners (Nandadasa et al. 2014). One interaction, believed to be important, during lung development is of extracellular VCAN with MIDKINE, an epithelial-derived growth factor. MIDKINE is a heparin-binding growth factor, localised to the epithelium with roles in cell survival, migration, cytokine expression, and cell differentiation (Muramatsu 2014). We predict that GC/GR signaling late in lung development represses Vcan expression through either a direct or indirect mechanism, thereby preventing MIDKINE binding to VCAN and promoting cell proliferation. It has been shown that the major form of VCAN that binds to MIDKINE is VCAN-V1 (Zou et al. 2000), and we have demonstrated that VCAN-V1 is the dominant form expressed during fetal lung development.

In conclusion, we have shown that the GAGβ domain-containing VCAN-V1 isoform is the predominant VCAN proteoglycan present during the fetal stages of lung development in rodents. GCs, acting via the GR, drive the repression of Vcan gene expression and also the rapid reduction and potentially the clearance of VCAN from the fetal lung prior to birth. GCs do this, in part, by mediating induction of the ADAMT 12 protease that could then degrade and potentially mediate the clearance of VCAN from distal lung regions. Together, these results demonstrate that GC steroids acting via the GR co-ordinately regulate repression of Vcan gene expression and VCAN protein to contribute to the normal development of the fetal respiratory system in mammals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T J C, A D B, and K L S designed the experiments and wrote the manuscript. K L S, A D B, B K L S, and J N performed the experiments. M W, A M, and S H helped finalise the manuscript.

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