

Pro-inflammatory mediators increase levels of the noncoding RNA GAS5 in airway smooth muscle and epithelial cells¹

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Abstract: The long noncoding RNA (lncRNA) GAS5 has been found to act as a decoy for the glucocorticoid receptor (GR), thus implicating GAS5 as a potential regulator of glucocorticoid sensitivity and resistance. Airway smooth muscle (ASM) cells and airway epithelial cells (AEC) play an important role in the pathogenesis and persistence of asthma and other chronic airways diseases. These airway structural cell types are also important cellular targets of the anti-inflammatory actions of glucocorticoids. In this study, we sought to examine the relevance of GAS5 to glucocorticoid sensitivity and resistance in ASM and AEC. We provide the first evidence that pro-inflammatory mediators up-regulate GAS5 levels in both airway epithelial and smooth muscle cells, and that decreasing GAS5 levels can enhance glucocorticoid action in AEC.

Key words: glucocorticoid, steroid resistance, non-coding RNA, lncRNA, airway inflammation.

Résumé : Le long ARN non codant (ARNnc) GAS5 s'est avéré agir comme leurre du récepteur des glucocorticoïdes (GR), l'impliquant ainsi comme régulateur potentiel de la sensibilité et de la résistance aux glucocorticoïdes. Les cellules de muscle lisse respiratoire (MLR) et les cellules épithéliales respiratoires (CER) jouent un rôle important dans la pathogenèse et la persistance de l'asthme et d'autres maladies respiratoires chroniques. Les types de cellules structurales des voies respiratoires constituent aussi des cibles importantes de l'action anti-inflammatoire des glucocorticoïdes. Dans cette étude, les auteurs cherchaient à examiner la pertinence de GAS5 dans la sensibilité et la résistance aux glucocorticoïdes chez les MLR et les CER. Ils fournissent les premières indications que des médiateurs pro-inflammatoires régulent à la hausse les niveaux de GAS5 tant dans les cellules épithéliales respiratoires que dans les cellules de muscle lisse, et que la diminution des niveaux de GAS5 peut accroître l'action des glucocorticoïdes dans les CER. [Traduit par la Rédaction]

Mots-clés : glucocorticoïde, résistance aux stéroïdes, ARN non codant, ARNnc, inflammatoire des voies respiratoires.

Introduction

Long noncoding RNAs (lncRNAs) are now known to be important regulators of gene expression and cellular function and can perform diverse functions, from tethering transcription factors to DNA, to sequestering micro-RNA (miRNA) or destabilizing mRNA (see recent review by Turner et al. (2014)). Despite the large number of lncRNAs identified, relatively few lncRNAs have been functionally characterized. One of the few lncRNAs to have an identified gene regulatory function is growth arrest-specific transcript 5 (GAS5), originally identified in 1988 in growth-arrested mouse fibroblast cells (Schneider et al. 1988), and since found to have important pathophysiological roles in breast cancer (Mourtada-Maarabouni et al. 2009), prostate cancer (Pickard et al. 2013), malignant pleural mesothelioma (Renganathan et al. 2014), and non-small lung cancer (NSCLC) (Shi et al. 2013). Importantly, GAS5 has been found to act as a decoy for the glucocorticoid receptor (GR), thus implicating GAS5 in regulating glucocorticoid sensitivity (Kino et al. 2010).

Glucocorticoids remain the most effective prophylactic agents available for the treatment of asthma and other chronic inflammatory diseases, and are thus one of the most highly prescribed drugs in the world. At a molecular level, glucocorticoids exert

their effects by binding to GR, which can interact directly with DNA to increase (transactivate) or decrease (nGRE-transrepress) gene transcription, and can interact with other transcription factors (e.g., NF- κ B, AP-1) to modulate their transcriptional activity (tethered transrepression). Further actions involve signaling through a variety of non-genomic pathways, including PI3K and MAPK (Oakley and Cidlowski 2013). Dose-limiting adverse effects and insensitivity to the anti-inflammatory effects of glucocorticoids are well known to limit their clinical utility. However, despite extensive investigations into mechanisms of glucocorticoid resistance in airways, attempts to restore glucocorticoid sensitivity have so far shown limited promise (see recent review by Barnes (2013)).

Since the discovery of GAS5 as a novel repressor of GR activity, it has been proposed that elevated GAS5 levels may contribute to clinical glucocorticoid insensitivity (Kino et al. 2010). GAS5 has been found to be ubiquitously expressed during development and in most adult tissues, including the lung (Coccia et al. 1992). Therefore, GAS5 has the potential to interfere with glucocorticoid efficacy in chronic inflammatory diseases of the airways. However, this mechanism is yet to be proven. Existing evidence of a role of GAS5 in regulating glucocorticoid action has all been derived from

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Abbreviations: ASM, airway smooth muscle; AEC, airway epithelial cells; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; GAS5, growth arrest-specific transcript 5; GR, glucocorticoid receptor; GRE, glucocorticoid response element; lncRNA, long non-coding RNA; nGRE, negative glucocorticoid response element; IL-1 α , interleukin-1 alpha; NMD, nonsense-mediated RNA decay; TGF- β 1, transforming growth factor beta-1; TNF α , tumor necrosis factor alpha.

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non-airway derived immortalized cell lines (HeLa and HepG2) representing phenotypes that are not involved in the induction, persistence, or resolution of airway inflammation (Kino et al. 2010).

Airway smooth muscle cells and epithelial cells play an important role in the pathogenesis and persistence of asthma and other chronic airways diseases (Holgate 2011; Doeing and Solway 2013). They are also important cellular targets of the anti-inflammatory actions of glucocorticoids (Keenan et al. 2012). Whole transcriptome sequencing has revealed that pro-inflammatory stimuli can up-regulate hundreds of lncRNA species in mouse embryonic fibroblasts, with distinct but overlapping sets of lncRNAs induced in response to different stimuli (Rapicavoli et al. 2013). We therefore sought to examine the influences of a host of inflammatory mediators, either known mediators of glucocorticoid resistance or known to have other effects in these cell types, on GAS5 expression levels. Different cell types respond to glucocorticoids in different ways (So et al. 2007). Therefore, we further sought to examine the relevance of GAS5 on glucocorticoid sensitivity in airway epithelial cells.

Materials and methods

Cell culture

Primary human airway smooth muscle (ASM) cultures were established from bronchi obtained from macroscopically normal resections of lung transplant patients (Alfred Hospital, Melbourne, Australia) as described previously (Fernandes et al. 1999). Tissue specimens were obtained with approval from the University of Melbourne's ethics committee (HREC980168X). For each experiment, separate primary cultures established from a minimum of 4 different donors ($n = 4$) were used with cells between the 4th and 13th passage. For experiments, ASM cells were seeded onto 24-well plates (0.6×10^4 cells/cm²) in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2.0 mmol/L L-glutamine, 1.0 mmol/L sodium pyruvate, 1% v/v non-essential amino acids (Sigma), 5% v/v heat-inactivated fetal bovine serum, and 2.5 µg/mL amphotericin. BEAS-2B bronchial epithelial cells were cultured in LHC-9 medium as described previously (Keenan et al. 2014). Cells were serum-starved for 24 h prior to the addition of 100 nmol/L dexamethasone (Sigma), 300.0 pmol/L bFGF (Promega, Madison, Wisconsin, USA), 100.0 nmol/L TGF-β1 (R&D Systems, Minneapolis, Minnesota, USA), RU486 (Sigma-Aldrich), 10.0 ng/mL TNFα (BD Biosciences, San Diego, California, USA), and 1.0 ng/mL IL-1α (Sigma-Aldrich).

siRNA and reporter gene transfection

The levels of RNA for GAS5 were reduced by transfection of cells with siRNA using RNAiMax Lipofectamine (Invitrogen, Mulgrave, Victoria, Australia) as described previously (Schuliga et al. 2010). The 2 GAS5-targeting siRNA sequences were used in this study were as follows: (A) 5'-GCU CUG GAU AGC ACC UUA UTT-3', and (B) 5'-GGA CCA GCU UAA UGG UUC UTT-3' (GenePharma, Shanghai, China). A negative control, siRNA, was used (sequence 5'-UUC UCC GAA CGU GUC ACG UTT-3'), which has no homologous sequence with the GAS5 gene of interest (GenePharma). Glucocorticoid response element (GRE) activity was assessed by transient transfection of a GRE-SEAP reporter construct, as described previously (Keenan et al. 2014).

RNA extraction and reverse-transcription quantitative PCR

Cells were lysed and RNA purified using RNeasy miniprep kits (QIAGEN, Doncaster, Vic.) or TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse-transcription of total RNA and the subsequent real-time PCR using an ABI Prism 7900HT sequence detection system (Applied Biosystems) with the relevant forward and reverse primers were conducted as previously described (Schuliga et al. 2010). GAS5-specific primers were as follows: forward primer, 5'-AGC TGG AAG TTG AAA TGG-3'; reverse primer, 5'-CAA GCC CGA CTC TCC ATA CC-3'.

Results

Dexamethasone and RU486 promote higher levels of the lncRNA GAS5 in airway epithelial cells

BEAS-2B bronchial epithelial cells expressed over 4-fold higher levels of the lncRNA GAS5 in response to 4 h treatment with the synthetic glucocorticoid dexamethasone (Dex; 30.0 nmol/L) compared with the vehicle control ($P < 0.05$, $n = 4$) (Fig. 1A), which is consistent with previously published reports (Kino et al. 2010; Mpampakas et al. 2014). BEAS-2B cells treated with the GR trans-activation antagonist and transrepression partial agonist RU486 (10.0 µmol/L) for 4 h also showed elevated GAS5 levels but to the lesser extent of 2.5-fold over the control ($P < 0.05$, $n = 4$) (Fig. 1A).

Pro-inflammatory mediators increase levels of the lncRNA GAS5 in airway epithelial cells

The pro-inflammatory cytokines TNFα (10.0 ng/mL) and IL-1α (1.0 ng/mL) stimulated increases in the levels of GAS5 (2.0–2.5 fold) in BEAS-2B cells after 4 h incubation ($P < 0.05$, $n = 3–4$) (Fig. 1B). The growth factor TGF-β1 (1.0 ng/mL) also elevated GAS5 expression levels (5-fold) following 24 h exposure ($P < 0.05$, $n = 5$) (Fig. 1C), which we have previously shown to induce glucocorticoid insensitivity in this cell type (Keenan et al. 2014). Pre-incubating BEAS-2B cells with TGF-β1 for 20 h prior to the addition of Dex induced an increase in GAS5 lncRNA expression to a level not significantly different from either TGF-β1 or Dex alone ($P > 0.05$, $n = 5$) (Fig. 1C).

Dexamethasone and TGF-β1 increase levels of the lncRNA GAS5 in primary airway smooth muscle cells

Airway smooth muscle cells (ASMs) expressed 3-fold higher levels of the lncRNA GAS5 in response to 6 h treatment with Dex (100.0 nmol/L) compared with the vehicle control ($P < 0.05$, $n = 4$) (Fig. 2A). bFGF (300.0 pmol/L) had no detectable effect on either basal expression of GAS5 lncRNA, or Dex-induced increases in GAS5 levels ($P > 0.05$, $n = 4$) (Fig. 2A). Exposure of ASM cells to TGF-β1 for 24 h induced a 2.5-fold increase in GAS5 levels ($P < 0.05$, $n = 4$) (Fig. 2B).

GAS5-targeting siRNA enhances glucocorticoid response element (GRE) activation, but does not restore airway epithelial glucocorticoid sensitivity in the presence of TGF-β1

GAS5-targeting siRNA was used to knockdown GAS5 expression levels by approximately 50% in BEAS-2B airway epithelial cells ($P < 0.05$, $n = 4$) (Fig. 3A). This level of knockdown enhanced the activation of a GRE-SEAP reporter by 30.0 nmol/L Dex ($P < 0.01$, $n = 4$) (Fig. 3B), a concentration previously shown to induce maximal activation of the GRE-SEAP reporter (Keenan et al. 2014). However, GAS5 siRNA did not affect the degree of impairment of GRE activity by TGF-β1 ($P > 0.05$, $n = 4$) (Fig. 3C). Similar effects on GRE activity were seen for 2 individual GAS5-targeting siRNA sequences (2nd sequence not shown).

Discussion

In this study, we provide the first evidence that pro-inflammatory mediators up-regulate GAS5 levels in airway structural cells (both epithelial and smooth muscle) involved in the induction and persistence of airway inflammation. It is interesting to note that stimuli previously associated with glucocorticoid insensitivity (i.e., TNFα, IL-1α, TGF-β1) caused significant increases in GAS5 transcript levels. However, bFGF, which has not previously been connected with glucocorticoid resistance, did not significantly affect GAS5 expression levels. Furthermore, we provide preliminary evidence of a functional impact of endogenous GAS5 in airway epithelial cells, as decreasing GAS5 levels by siRNA enhanced GRE activity in response to a maximal concentration of dexamethasone.

The mechanism by which pro-inflammatory mediators increase the levels of GAS5 could be due to the induction of transcription

Fig. 1. Relative expression level of the long noncoding RNA GAS5 in the BEAS-2B bronchial epithelial cell line following (A) 4 h incubation with the vehicle control (0.1% dimethyl sulfoxide (DMSO)), 100.0 nmol/L dexamethasone (Dex), or 10.0 μ mol/L RU486; (B) 4 h incubation with the vehicle control (0.25% bovine serum albumin), 10.0 ng/mL tumor necrosis factor alpha (TNF α), or 1.0 ng/mL interleukin-1 alpha (IL-1 α); and (C) 24 h incubation with transforming growth factor beta 1 (TGF- β 1, 40.0 pmol/L) with a pulse of Dex (100.0 nmol/L) or the vehicle control (0.1% DMSO) added for the last 4 h of incubation; $n = 3-6$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the control.

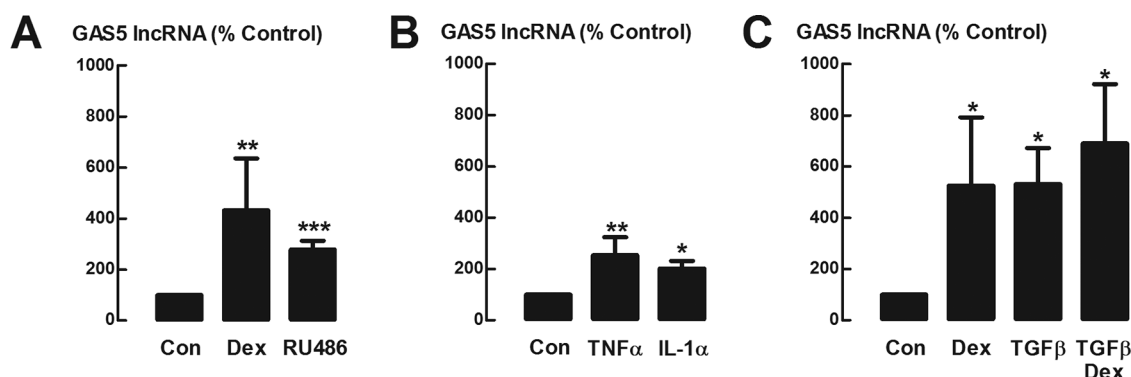


Fig. 2. Relative expression level of the long noncoding RNA GAS5 in primary airway smooth muscle cells following (A) 6 h incubation with vehicle control (0.1% dimethyl sulfoxide) or 100.0 nmol/L dexamethasone (Dex); and (B) 24 h incubation with 100.0 pmol/L transforming growth factor beta 1 (TGF- β 1) or 300.0 pmol/L basic fibroblast growth factor (bFGF) in the presence or absence of Dex; $n = 4$; *, $P < 0.05$ and **, $P < 0.01$ compared with the control.

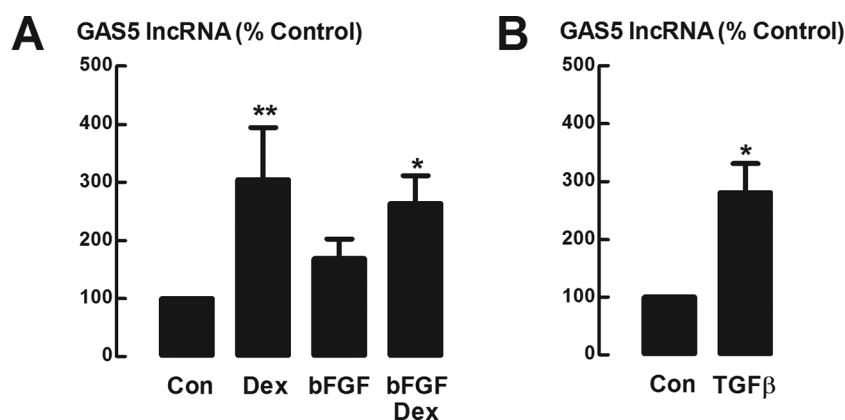
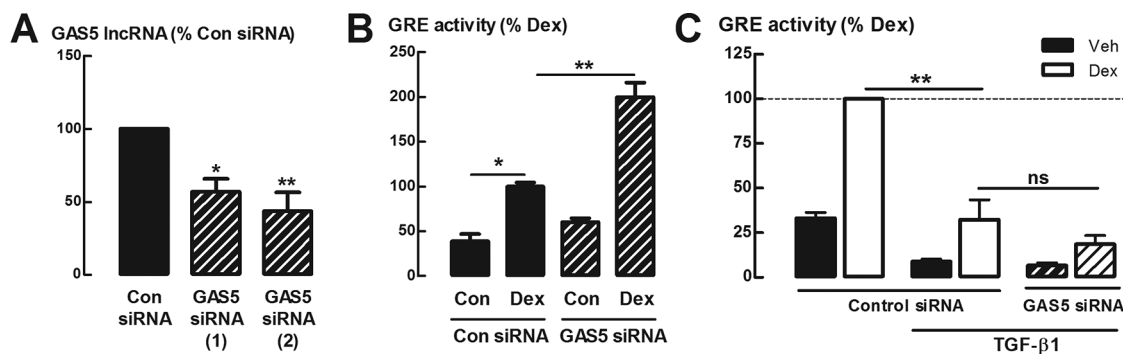


Fig. 3. Effect of GAS5 targeting siRNA on (A) GAS5 expression levels, and (B) glucocorticoid response element (GRE) activity in GRE-SEAP transfected BEAS-2B bronchial epithelial cells stimulated with a maximally effective concentration of dexamethasone (Dex; 30.0 nmol/L). (C) GRE activity in BEAS-2B cells pre-incubated with TGF- β 1 (40.0 pmol/L) prior to stimulation with Dex (30.0 nmol/L) stimulation. GRE activity is normalized to that induced by Dex in the presence of control siRNA; $n = 4-7$; *, $P < 0.05$ and **, $P < 0.01$ compared with the control siRNA or as depicted.



or the inhibition of decay. GAS5 is usually degraded by the well-characterized nonsense-mediated RNA decay (NMD) pathway whereby transcripts with premature stop codons, such as GAS5, are degraded during translation. Inhibition of translation can thus result in the accumulation of GAS5 transcript levels, such as

occurs during growth arrest. However, pro-inflammatory stimuli have been shown to directly induce lncRNA expression at a transcriptional level in other contexts (Radicavoli et al. 2013). Therefore, the mechanism by which GAS5 levels are increased is not immediately obvious. It is interesting to note that the induction of

GAS5 expression by different stimuli were not additive, suggesting that they may regulate GAS5 levels through a similar mechanism. It is also interesting to note that although GAS5-targeting siRNA only halved transcript levels in BEAS-2B epithelial cells, this was sufficient to double Dex-induced GRE activity, suggesting a direct inverse relationship between the levels of GAS5 and the transcriptional activity of the GR.

Despite GAS5 knockdown enhancing GRE maximum activity, and TGF- β 1 inducing higher levels of GAS5 transcript, cells pre-incubated with TGF- β 1 prior to the addition of dexamethasone showed negligible GRE activity in BEAS-2B epithelial cells transfected with a GRE reporter. This is not surprising as we have previously shown that TGF- β 1 potentially impairs glucocorticoid activity in epithelial cells (Salem et al. 2012; Keenan et al. 2014). Considering the modest level of knockdown produced by the siRNA sequences used, it is difficult to make definitive conclusions about the potential role of GAS5 in mediating glucocorticoid resistance. However, the TGF- β 1 impairment of GRE activity suggests that the enhancement of GRE maximum activity by GAS5 knockdown is surmountable. The question as to whether further knockdown of GAS5 would increase GR activity to an insurmountable extent remains to be answered.

In this study, both epithelial and smooth muscle cells were serum-starved for 24 h prior to stimulation with exogenous cytokines, growth factors, or glucocorticoids. Since GAS5 is known to accumulate following growth arrest, the levels of GAS5 were presumably increasing over time, and thus the measured induction by exogenous stimuli may have been underestimated. Previous studies in HeLa cells have shown that GAS5 levels are only negligibly increased after 24 h of serum withdrawal, and have barely doubled after 48 h (Kino et al. 2010). Prolonged serum starvation may re-establish GAS5 levels more typical of cells parked in G0 of the cell cycle.

Whether levels of GAS5 are altered in the airways in asthma or other chronic inflammatory diseases is unknown. However, this study has provided preliminary evidence that pro-inflammatory mediators promote increases in GAS5 levels in airway smooth muscle and airway epithelial cells, cells integral to the pathogenesis and persistence of these diseases. Furthermore, we establish that GAS5 levels can modulate glucocorticoid activity in airway epithelial cells, thus providing proof-of-principle evidence that GAS5 levels affect glucocorticoid sensitivity in at least one cell-type of the human airway, and thus may mediate glucocorticoid insensitivity in refractory disease. Whilst lncRNAs are not traditional drug targets, recent work has focused on developing strategies to manipulate lncRNA both in vitro and in vivo (reviewed in Wahlestedt (2013)). Therefore, GAS5 may emerge as a novel therapeutic target for restoring glucocorticoid sensitivity.

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