



Published in final edited form as:

Pharmacol Ther. 2022 August ; 236: 108112. doi:10.1016/j.pharmthera.2022.108112.

Emerging role of an immunomodulatory protein Secretoglobin 3A2 in human diseases

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Abstract

Secretoglobin (SCGB) 3A2 was first identified in 2001 as a protein exhibiting similarities in amino acid sequence and gene structure to SCGB1A1, a multi-functional cytokine-like molecule highly expressed in airway epithelial Club cells that was the first identified and extensively studied member of the SCGB gene superfamily. SCGB3A2 is a small secretory protein of ~10 kDa that forms a dimer and a tetramer. SCGB3A2 is predominantly expressed in airway epithelial Club cells, and has anti-inflammatory, growth factor, anti-fibrotic, and anti-cancer activities that influence various lung diseases. This review summarizes the current understanding of SCGB3A2 biological functions and its role in human diseases with emphasis on its mechanisms of actions and signaling pathway.

Keywords

UGRP1; SCGB1A1; CCSP; CC10; SCGB3A1; anti-inflammatory; growth factor; anti-fibrotic; anti-cancer; lung diseases

1. Introduction

Secretoglobin 3A2 (secretoglobin (SCGB) family 3A member 2) was originally identified as a downstream target for the homeodomain transcription factor, NKX2-1 (NK2 Homeobox

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Conflict of interest

ALP is an employee of APCBio Innovations, Inc. and has a >50% interest in APCBio Innovations, Inc., an affiliate of Trove Therapeutics, Inc., both of which have an interest in commercializing the rhSCGB3A2. No conflicts of interest, financial or otherwise, are declared by the other authors.

1) in embryonic lungs of *Nkx2-1*-null vs. wild-type mice using a suppressive subtractive hybridization method (Niimi, et al., 2001). SCGB3A2 was initially named uteroglobin-related protein 1 (UGRP1) because it shared sequence similarity with SCGB1A1, also called uteroglobin, Club cell 10-kDa protein (CC10), Club cell 16-kDa protein (CC16), or Club cell secretory protein (CCSP) (Niimi, et al., 2001). SCGB3A2 has also been called HIN-2 (high in normal-2) (Bin, Nielson, Liu, Mason, & Shu, 2003) or LuLeu1 (Lung Leucine-rich 1) (Reynolds, Reynolds, Pryhuber, Finder, & Stripp, 2002). Both SCGB1A1 and SCGB3A2 have a signal peptide at their N-terminus and have a high degree of similarity in their amino acid sequences, particularly in alpha helix 3 of a secreted monomer from which antinflammin peptides are derived (amino acid residues 60–69 of mouse SCGB1A1 translation product) (Miele, Cordella-Miele, Facchiano, & Mukherjee, 1988; Niimi, et al., 2001), which is responsible for the phospholipase A2 inhibitory activity of SCGB1A1 (Chowdhury, et al., 2000; Mukherjee, Zhang, & Chilton, 2007). The sequence similarities and the subsequently revealed gene structures suggested that SCGB3A2 belongs to the same gene family as SCGB1A1 that has been extensively studied (Mukherjee, et al., 1999). There were many other individually described proteins with similar sequences and gene structures published under different names, which created much confusion (Klug, et al., 2000; Porter, Lahti-Domenici, Torres-Arzayus, Chin, & Polyak, 2002; Reynolds, et al., 2002). In 2000, an international conference on “Uteroglobin/Clara cell (the name previously used for “Club cell”) Protein Family” adopted a nomenclature of “secretoglobin” for this family of proteins with SCGB1A1 as the founding member, in which all have the common characteristics of being secreted, small size (~10 kDa), alpha-helical bundle monomeric structure, and dimeric or tetrameric secondary structure (Durairaj, Pageat, & Bienboire-Frosini, 2018; Klug, et al., 2000; Reynolds, et al., 2002). The Human Gene Nomenclature Committee (<https://www.genenames.org>) then officially approved the generic name “secretoglobin” and its symbol SCGB.

SCGB1A1 is a multifunctional cytokine-like molecule, highly expressed in airway Club cells having anti-inflammatory, anti-allergic, anti-tumorigenic, anti-fibrotic, and embryonic growth-stimulatory activities (Lee, Zhang, & Mukherjee, 2006; Mukherjee, et al., 1999; Mukherjee, et al., 2007). While the mechanisms for biological activities and their pathways remain elusive, its role was established in maintaining airway integrity and as a biomarker for healthy airway epithelial cells and their loss due to the progressive airway remodeling that characterizes chronic lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Almuntashiri, et al., 2020; Broeckaert & Bernard, 2000; Wong, Keating, & Waddell, 2009; Zhai, et al., 2019).

Since its discovery in 2001, a number of studies demonstrated that SCGB3A2 exhibits many characteristics similar to those of SCGB1A1 including anti-inflammatory, growth stimulatory, anti-fibrotic, and anti-tumorigenesis activities; many reviews are available on various activities of SCGB1A1 (Agusti & Sin, 2014; Almuntashiri, et al., 2020; Laucho-Contreras, et al., 2016; Mootz, Jakwerth, Schmidt-Weber, & Zissler, 2021; Mukherjee, et al., 1999; Mukherjee, et al., 2007). In this review, we focus on SCGB3A2 by providing an overview of SCGB3A2 and a comparison with SCGB1A1 and SCGB3A1 wherever appropriate, summarizing the current understanding of its biological functions and their role in human diseases, the mechanisms of its actions, and the SCGB3A2 signaling pathway.

2. Fundamental characteristics of SCGB3A2

2-1. SCGB3A subfamily

SCGB3A2 together with another subfamily member SCGB3A1 (also called HIN-1 [high in normal-1 and UGRP2 [uteroglobin-related protein 2]] (Niimi, Copeland, et al., 2002) constitutes the SCGB3A subfamily within the SCGB gene superfamily (I. E. Krop, et al., 2001). Both genes consist of 3 exons characteristic of all SCGB family genes (Niimi, et al., 2001) (Figure 1). There are several variant transcripts derived from each of the *Scgb3a1* and *Scgb3a2* genes, in addition to the major form that arise through alternative splicing with retention of all or part of the introns, or the presence of additional exon (Niimi, Copeland, et al., 2002; Niimi, et al., 2001). Details on the spatiotemporal expression of these variants and what role they may play, remain elusive. The major forms of SCGB3A1 and SCGB3A2 are composed of 90–100 amino acids, of which the first ~20 residues constituting a signal peptide, producing a mature protein of ~10 kDa after its cleavage. Native human SCGB3A2 immunoreactivity was observed as a monomer (~6 kDa) and dimer (~12 kDa), along with other peptides that may represent alternate conformations (~10 kDa) and degradation products (<3 kDa) in tracheal aspirates (Cai, et al., 2014). Recombinant SCGB3A2 forms a homodimer and higher order multimers (Yokoyama, et al., 2018). Mouse SCGB3A1 and SCGB3A2 share ~30% amino acid identity (Niimi, et al., 2001).

Human *SCGB3A1* and *SCGB3A2*, and mouse *Scgb3a1* and *Scgb3a2* genes are localized on human chromosome 5q35.3 and 5q32, and on mouse chromosome 11B1.2 and 18B3, which are the syntenic regions to each corresponding human gene, respectively (Niimi, Copeland, et al., 2002; Niimi, et al., 2001). The human *SCGB3A2* gene resides within the 5q32 chromosomal loci known to be associated with asthma susceptibility and where many genes related to inflammation, including interleukin (IL)-3, -4, -5, -9, -13, and -17B, CD14, and colony stimulation factor 2 are located (Mathias, 2014; Niimi, Copeland, et al., 2002). This proximity led to the discovery of anti-inflammatory activity of SCGB3A2 as described below.

2-2. Unique spatiotemporal expression of SCGB3A2 in lung

Both SCGB3A1 and SCGB3A2 are predominantly expressed in the same airway Club cells as SCGB1A1 (Naizhen, Kido, Yokoyama, Linnoila, & Kimura, 2019; Niimi, Copeland, et al., 2002; Niimi, et al., 2001; Reynolds, et al., 2002). The expression of SCGB3A2 and SCGB1A1 were detected in both large (bronchus) and small (bronchioles) airways, while SCGB3A2 expression was observed at embryonic day (E) 14.5 during mouse gestation, which preceded SCGB1A1 expression detectable at E16.5. Historically, SCGB1A1 has been used as a lung epithelial Club cell marker (Broeckeaert & Bernard, 2000). The two embryonic day difference in the detection of expression between SCGB1A1 (E16.5) and SCGB3A2 (E14.5) may be useful to distinguish the events occurring between E14.5 and 16.5 during lung development. The expression of SCGB3A2 peaked around birth, whereas that of SCGB1A1 continuously increased for at least 3 weeks after birth (Naizhen, et al., 2019; Reynolds, et al., 2002; Tomita, et al., 2008). As a downstream target of NKX2-1 that is expressed in thyroid, lung epithelium, and ventral forebrain during early embryogenesis, and in accordance with the fact that *Nkx2-1*-null mice lack the thyroid and pituitary (Kimura, et

al., 1996), the expression of SCGB3A2 was also found in thyroid albeit at low levels (Niimi, et al., 2001) and pituitary (see section 3-8) and thus might contribute to the development/function of these organs (Miyano, et al., 2014).

The expression of SCGB3A1 was found only in large, but not small airways at E16.5, and was increased for at least 3 weeks after birth (Naizhen, et al., 2019; Reynolds, et al., 2002). Low level expression of *Scgb3a1* was detected in many other adult and developing organs (Niimi, Copeland, et al., 2002; Porter, et al., 2002). SCGB3A1 expression levels are high in normal but low in cancerous tissues, thus accounting for the original nomenclature of high in normal-1, HIN-1 (I. E. Krop, et al., 2001). SCGB3A1 is known as a tumor suppressor in various cancers including those derived from mammary gland, prostate, lung, and pancreas, due to methylation of the *SCGB3A1* gene promoter (I. Krop, et al., 2004; I. E. Krop, et al., 2001); however, little is known about SCGB3A1 expression and function beyond the tumor suppressor activity.

Scgb3a1- and *Scgb3a2*-null mice show no changes in the expression levels of *Scgb3a2* and *Scgb3a1*, respectively, suggesting no compensatory mechanisms in the expression of these two SCGBs (Naizhen, et al., 2019). This is in contrast to the *Ccsp* (*Scgb1a1*)-null mice that have increased *Scgb3a2* expression (Watson, et al., 2001) and *Scgb3a1* (Reynolds, et al., 2002). How the functions of these three SCGBs expressed in airway Club cells relate to each other, and in particular whether there is any compensatory function shared among the three SCGBs remain to be understood.

2-3. Transcriptional regulation of *Scgb3a2*

In vitro promoter assays, electrophoretic mobility shift analysis, and chromatin immunoprecipitation analysis demonstrated that NKX2-1 binds to specific sites in the *Scgb3a2* promoter synergistically with C/EBP (CCAAT/enhancer-binding protein) α and δ and activates transcription (Niimi, et al., 2001; Tomita, et al., 2008). NKX2-1 also regulates *Scgb3a2* gene transcription through binding in the *Scgb3a2* gene promoter in conjunction with RBPJ (recombination signal binding protein for immunoglobulin kappa J region; a transcriptional regulator of Notch signaling), which contributes to secretory cell fate determination in developing murine airways (Guha, et al., 2012). Further, in human lung epithelial cells, *SCGB3A2* gene expression was induced by IL-10 in an NKX2-1-dependent manner at the transcriptional level (Srisodsai, et al., 2004).

2-4. SCGB3A2 receptors and binding proteins

The macrophage scavenger receptor with collagenous structure (MARCO) was initially identified using expression cloning techniques as a SCGB3A2 receptor expressed on the surface of alveolar macrophages (Bin, et al., 2003). The same study also demonstrated that SCGB3A2 binds to apolipoprotein A-1, a major protein component of high density lipoprotein, LPS (as described in 3-6 below), and Gram-positive, Gram-negative bacteria, and yeast (Bin, et al., 2003). MARCO also binds to Gram-positive and Gram-negative bacteria, and unopsonized environmental dusts, and is critically involved in their clearance (Arredouani, et al., 2005; Elomaa, et al., 1995). It was proposed that SCGB3A2 acts as an opsonin, and the SCGB3A2-MARCO ligand-receptor pair is likely involved in inflammation

and pathogen clearance in lung (Bin, et al., 2003). Another study suggested the presence of another SCGB3A2 receptor different from MARCO on the surface of mesenchymal cells of fetal mouse lungs in primary culture (Kurotani, et al., 2008). Recently, syndecan-1 (SDC1), a transmembrane heparan sulfate (HS) proteoglycan and a member of the syndecan proteoglycan family, was identified as a receptor for SCGB3A2 using a protein-protein interaction array (Yokoyama, et al., 2018). Binding of SCGB3A2 to SDC1 was abrogated by the addition of heparin, suggesting the importance of heparan sulfate (HS) moiety of SDC1 for the binding of SCGB3A2 (see below 3-6) (Yokoyama, et al., 2018).

3. Biological properties of SCGB3A2 and their implication in human diseases/conditions

3-1. Anti-inflammatory activity

As mentioned above, the chromosomal localization of the human *SCGB3A2* gene suggested the possibility that SCGB3A2 is involved in regulating inflammation. The initial experiments showed that reduced *Scgb3a2* mRNA expression levels in allergen-exposed mouse lungs was reversed after dexamethasone treatment (Niimi, et al., 2001). Similarly, the levels of *Scgb3a1* mRNA slightly reduced in allergen-exposed mouse lungs, also returned to normal after dexamethasone treatment (Niimi, et al., 2001). When mice were subjected to an ovalbumin (OVA)-induced airway inflammation model, their lungs had lower SCGB3A2 protein as well as mRNA expression, that was inversely correlated with the increased *Ii5* and *Ii9* mRNA levels in bronchoalveolar lavage fluid (BALF) (Chiba, Kusakabe, & Kimura, 2004; Chiba, Srisodsai, Supavilai, & Kimura, 2005). On the other hand, intranasal administration of IL-5 or IL-9 to naïve mice decreased *Scgb3a2* expression in lungs (Chiba, et al., 2004; Chiba, et al., 2005). Further, IL-10, an anti-inflammatory cytokine, induced *Scgb3a2* mRNA and/or protein expression in lung epithelial cells of naïve mouse when intranasally administered IL-10, and E15.5 embryonic mouse lungs in culture (Srisodsai, et al., 2004). When OVA-treated model mice were given an intranasally administered recombinant adenovirus expressing SCGB3A2, they presented reduced levels of pro-inflammatory TH2 cytokines such as IL-4, 5, and 13, and inflammatory cell numbers in BALF, and lung histopathology similar to control lungs (Chiba, et al., 2006) (Figure 2). Further, when *Scgb3a2*-null mice were subjected to either OVA- or house dust mite (HDM)-induced allergic airway inflammation models, they exhibited exacerbated inflammation as compared with wild-type mice (albeit the effect was small but significant), confirming the anti-inflammatory activity of SCGB3A2 (Kido, et al., 2014; Yoneda, et al., 2016). Since CCR4 (C-C chemokine receptor type 4) and CCL17 (chemokine (C-C) motif ligand 17), but not CCR3 or CCL11 mRNA expression were both elevated in lungs of HDM-challenged *Scgb3a2*-null mice compared to wild-type mice, SCGB3A2 appears to modulate the CCR4-CCL17 but not CCR3- CCL11 pathway (Yoneda, et al., 2016). The biological role of this pathway remains unknown.

The amino acid residues 42–51 of mature (secreted form) murine SCGB3A2 has high similarity to the amino acid residues 39–48 of mature murine SCGB1A1, the latter referred to as an “antiflammin peptide” located in the alpha helix 3 region of SCGB1A1 (Niimi, et al., 2001). It is responsible for phospholipase A2 inhibitory activity and at least part of the

anti-inflammatory activity of SCGB1A1 (Facchiano, Cordella-Miele, Miele, & Mukherjee, 1991; Mukherjee, et al., 1999). SCGB3A2 was also shown to exhibit phospholipase A2 inhibitory activity (Cai, et al., 2014). The mechanism for the anti-inflammatory activity of SCGB3A2, and whether and/or how SCGB3A2's anti-inflammatory activity is related to its phospholipase A2 inhibitory activity, has not been elucidated.

Altered levels of SCGB3A2 were reported in inflammatory diseases; down-regulation of SCGB3A2 protein in the upper airway of chronic rhinosinusitis patients (Lu, et al., 2011), induced protein levels in the sputum of patients with asthma and rhinitis (de Burbure, et al., 2007), and lower plasma SCGB3A2 levels in asthmatics vs. mild/moderate asthmatic patients or controls (Inoue, et al., 2008). Pre-incubation of SCGB3A2 with BEAS-2B cells for 3 hours before addition of lipopolysaccharide (LPS) inhibited upregulation of tumor necrosis factor (TNF)- α and C-X-C motif chemokine ligand 8 (CXCL8) mRNA expression through the JNK and ERK pathways (Wang, et al., 2015). Thus, all available results suggest the possibility that administration of SCGB3A2 protein may alleviate the symptoms of allergic airway inflammation including asthma and rhinosinusitis/rhinitis.

3-2. SCGB3A2 as a biomarker for Club cells and airway integrity

Naphthalene exposure has been used as a model to study lung injury in mice. Naphthalene specifically ablates Club cells in the airways. *Scgb3a2* mRNA was found co-expressed with SCGB1A1 protein in cells that were initially thought to be nascently regenerated Club cells at regenerating bronchiolar epithelium, thus indicating that SCGB3A2 is an early differentiation marker for Club cell lineage (Reynolds, et al., 2002). Several years after this paper was published, a minor population of Club cells located around the bronchioalveolar duct junction (BADJ) were characterized as bronchioalveolar stem cells (BASC) that are resistant to the naphthalene-induced damage and are involved in the repair and regeneration of lung epithelial cells (C. F. Kim, et al., 2005). These cells express SCGB1A1 and the alveolar type II cell marker surfactant protein C (SP-C). Thus, SCGB1A1+ Club cells located at BADJ have been a focus for understanding the role of these cells in the maintenance of lung homeostasis, repair, and carcinogenesis (Jones-Freeman & Starkey, 2020; Liu, et al., 2019) (C. F. Kim, et al., 2005; Rawlins, et al., 2009). It was suggested that SCGB3A2 may also be expressed in BASC (Reynolds, et al., 2002).

The earlier onset of SCGB3A2 expression was used as an early lung epithelial marker to facilitate the purification and characterization of human airway lineages during pluripotent stem cell differentiation (McCauley, et al., 2018) and to distinguish from Krt17(keratin 17)+ basal cell progenitors (Kiyokawa, et al., 2021). SCGB3A2 has been used as a marker, together with SCGB1A1, in identification of Club cells during development of human induced pluripotent stem cells (hiPSCs) (Tsuji, Yamada, Hirai, Asakura, & Kanda, 2021) and in studies on the role of Club cells in pathogenesis of idiopathic pulmonary fibrosis (Zuo, et al., 2020) (Figure 2).

A sensitive sandwich ELISA assay was developed to determine the level of SCGB3A2 in human biological fluids, including serum, urine, nasal lavage fluid, BALF, induced sputum, and amniotic fluid (Van De Velde, Courtens, & Bernard, 2010). The levels of SCGB3A2 present in serum and urine in healthy subjects were similar to those of SCGB1A1

(~5–10 µg/L (Van De Velde, et al., 2010)). This expression level was much higher than the plasma SCGB3A2 levels of ~0.12 µg/L reported by another study (Inoue, et al., 2008). SCGB3A2 was not detected in nasal lavage fluid while SCGB1A1 was present at similar levels to serum. SCGB1A1 was found at much higher level than SCGB3A2 in BALF (~710 vs. ~180 µg/L) and induced sputum (9,400 vs. ~370), while amniotic fluid showed lower levels of SCGB1A1 expression than SCGB3A2 (~50 vs. ~410). In a clinical trial of recombinant human (rh) SCGB1A1 in severely premature infants (24–29 weeks post-menstrual age), tracheal aspirate fluids (TAF) from placebo controls at 24 hours after delivery had SCGB3A2 concentrations that ranged from 395–804 µg/L while SCGB1A1 concentrations ranged from 85–1,669 µg/L (Cai, et al., 2014; Levine, et al., 2005). That both SCGB proteins are found in amniotic fluid and the TAF of premature infants is in agreement with the fact that both play a role in lung development (see section 3-4 for SCGB3A2) (Kurotani, et al., 2008; Mukherjee, et al., 2007). It should be noted that these studies used different ELISAs for SCGB3A2 and comparisons of concentrations studies may not be ideal. Nevertheless, SCGB3A2 in different fluids may serve as a surrogate marker that is less variable than SCGB1A1, depending on individual situation, to assess airway epithelium integrity and/or a risk factor in development of asthma/respiratory allergies (Van De Velde, et al., 2010).

3-3. Polymorphisms associated with human diseases

A polymorphism of G→A conversion located at –112 bp from the transcription start site of the human *SCGB3A2* gene was initially identified in association with increased risk of developing asthma in Japanese populations (Niimi, Munakata, et al., 2002). The homozygous AA variant had reduced transcriptional activity of the *SCGB3A2* gene (Niimi, Munakata, et al., 2002; Song, et al., 2009) and significantly decreased serum SCGB3A2 levels (Chistiakov, Voronova, Turakulov, & Savost' anov, 2011). The reduced plasma SCGB3A2 levels were associated with the G-112A polymorphism and the severity of asthma in Japanese populations (Inoue, et al., 2008). Studies supporting the association of the G-112A (rs1368408) polymorphism to asthma include those of Korean populations (S. K. Kim, et al., 2017) and meta-analysis in Asian populations but not Caucasian populations (Xie, et al., 2014). Other studies did not find an association to asthma, including studies of an Indian population (Batra, Niphadkar, Sharma, & Ghosh, 2005), Caucasian population (Heinzmann, Dietrich, & Deichmann, 2003), Sicilian population (Rigoli, et al., 2007), in childhood asthma in Japanese population (Jian, Nakayama, Noguchi, Shibasaki, & Arinami, 2003). It is possible that even though the G-112A polymorphism is associated with the susceptibility to asthma, the association may be weak and could be masked by other stronger association(s), depending on study design and/or sample cohorts. Detection of SCGB3A2-associated susceptibility to asthma may also depend on environmental and lifestyle factors such as exposure to pollutants, cleaning chemicals, and cigarette smoke in the targeted population. Thus, additional studies using increased sample numbers are warranted. *SCGB3A2* is one of many genetic loci identified using candidate gene and/or genome-wide association approaches that are associated with the phenotypes of asthma (Mathias, 2014). Other single nucleotide polymorphisms in the *SCGB3A2* gene were reported, such as G-659A (rs6882292) for increased risk of asthma (S. K. Kim, et al.,

2017), and rs7726552 for allergic rhinitis without any association to asthma (Andiappan, et al., 2011).

Genome-wide analysis demonstrated a susceptibility locus for autoimmune thyroid disease on Chromosome 5q31-q33 (Sakai, et al., 2001) and Graves' disease on 5q31 (Jin, et al., 2003), the region in which *SCGB3A2* gene is located. An association of the G-112A polymorphism to the increased risk of developing thyroid Graves' disease was demonstrated by genome-wide association studies, linkage analysis, and meta-analysis both in Chinese and Caucasian populations (Chistiakov, et al., 2011; Kus, et al., 2019; Simmonds, et al., 2010; Song, et al., 2009; Xue, Han, Pan, & Song, 2014). On the other hand, a few studies of Chinese populations demonstrated no association (Kus, et al., 2019; Yang, et al., 2005), however, these studies used much smaller sample sizes than those having the association, suggesting that the G-112A polymorphism may likely be one of the susceptible loci to thyroid Graves' disease. Another study suggested that *SCGB3A2* might be used as a marker to predict Graves' disease patients who develop hypothyroidism (Zhou, et al., 2019).

3-4. Growth factor activity

Based on the fact that the lungs of *Nkx2-1*-null mouse embryos are severely hypoplastic, and *SCGB3A2* is highly expressed in airway epithelial cells, it was hypothesized that the *NKX2-1* downstream target *SCGB3A2* may play a role in embryonic lung development (Kurotani, et al., 2008). Careful examination found low expression of *SCGB3A2* by immunohistochemistry in the epithelial cells of embryonic day (E) 11.5 and around the growing tips of bronchi of E13.5 mouse fetal lungs, the latter particularly suggesting a possible role for *SCGB3A2* in lung branching morphogenesis (Kurotani, et al., 2008). An *ex vivo* mouse lung organ culture study using E11.5 embryonic lungs demonstrated that the addition of recombinant mouse (rm) *SCGB3A2* in the culture media promoted branching morphogenesis, while it was suppressed by the addition of anti-*SCGB3A2* antibody or *SCGB3A2* siRNA (Kurotani, et al., 2008) (Figure 2). Further, the embryonic lung development was promoted by the intravenous administration of rm*SCGB3A2* to the pregnant female mice from E13.5 to E16.5, followed by examination of the embryos at E17.5 (1 day before natural birth) by lung histology and breathing scores of newborns (Kurotani, et al., 2008). Embryos administered *SCGB3A2* and examined at E17.5 had the same breathing score as normal E18.5 newborns (initially labored breathing, followed by unlabored breathing once fluid was cleared from the lung). E17.5 embryos without *SCGB3A2* administration exhibited no breathing or gasping, indicating that *SCGB3A2* promoted lung development *in vivo* (Kurotani, et al., 2008). When *Nkx2-1*-null lungs were cultured *ex vivo* in the presence of rm*SCGB3A2*, the epithelia showed drastic morphological changes such as appearance of pleated and/or dentate epithelia and duct-like structures, and increased layers of mesenchyme. Further, increased Ki-67 and phosphorylated histone H3 positive signals as proliferation markers were observed (Kurotani, et al., 2008). Similarly, the lungs of *Nkx2-1*-null mice administered rm*SCGB3A2* had increased numbers of cells positive for Ki-67 and phosphorylated histone H3 upon administration of rm*SCGB3A2*. Thus, *SCGB3A2* is a growth factor accelerating embryonic lung development. Recombinant human (rh) *SCGB3A2*, when used in *ex vivo* and *in vivo* studies, promoted murine lung branching morphogenesis and lung development, respectively, to a similar degree to those

obtained with rmSCGB3A2 protein (Cai, et al., 2014). The results suggest that rhSCGB3A2 may be used to treat severely premature babies with respiratory distress to stimulate lung development. The mechanism for the growth factor activity of SCGB3A2 remains to be understood.

3-5. Anti-fibrotic activity

A bleomycin (BLM)-induced pulmonary fibrosis model mouse was used to examine the effect of SCGB3A2 in the development of pulmonary fibrosis (Kurotani, Okumura, et al., 2011). Mice were intratracheally intubated with BLM and treated either with SCGB3A2 or PBS daily for a week starting 2 weeks after intubation, followed by lung examination on day 21 (Figure 2). Mice treated with SCGB3A2 presented no fibrosis in contrast to BLM+PBS group, which had grade 1 (fibrosis found at 0–25% of a whole lung) or 2 (25–50%) fibrosis with strong Masson trichrome staining that detects collagen fibers. Those treated with SCGB3A2 also exhibited very low macrophage and neutrophil numbers in contrast to massive increase of their numbers in bronchoalveolar lavage fluid (BALF) of BLM+PBS group of mice. Similar levels of anti-fibrotic activity were demonstrated using the bleomycin model with the administration of rhSCGB3A2 instead of rmSCGB3A2 (Cai, et al., 2014). These results suggest the possible use of SCGB3A2 as a therapeutic to treat pulmonary fibrosis.

In the murine BLM model, pulmonary fibrosis typically resolves over time without treatment and *Scgb3a2*-null mice exposed to BLM exhibited exacerbated pulmonary fibrosis (Cai & Kimura, 2015), while transgenic mice specifically overexpressing *Scgb3a2* in the lungs exhibited accelerated resolution of pulmonary fibrosis (Cai, et al., 2015). The results confirmed the earlier findings that SCGB3A2 possesses anti-fibrotic activity.

The partial mechanism for the anti-fibrotic activity of SCGB3A2 was demonstrated by *in vitro* studies of culturing mouse primary lung fibroblasts in the presence of transforming growth factor (TGF)- β with and without SCGB3A2 (Kurotani, Okumura, et al., 2011) (Figure 3). TGF- β increased phosphorylation of SMAD2 and SMAD3, and the expression levels of α -smooth muscle actin protein and collagen genes as expected, however, these increases were all suppressed by the addition of SCGB3A2. At the same time, SCGB3A2 administration increased phosphorylation of STAT1 and expression of SMAD7, the inhibitory SMAD, thus suppressing TGF- β signaling. The addition of neutralizing antibody to the receptor for Interferon (IFN)- γ , a prototypical cytokine known to activate STAT1 phosphorylation, did not interfere SCGB3A2-induced STAT1 phosphorylation and SMAD7 elevation. Further, the addition of cycloheximide suppressed SCGB3A2-induced STAT1 phosphorylation, suggesting that SCGB3A2-induced STAT1 phosphorylation is unrelated to IFN- γ signaling, and that a cycloheximide-sensitive intermediate molecule is involved in SCGB3A2 signaling, leading to anti-fibrotic activity. However, the receptor to which SCGB3A2 binds and initiates the anti-fibrotic signaling was not determined in this study.

3-6. Anti-cancer activity

SCGB3A2 suppressed the growth of Lewis lung carcinoma (LLC) cells *in vitro*. In an *in vivo* model of LLC intravenous metastasis, *Scgb3a2*-null mice had increased number of lung

tumors as compared with wild-type mice and the administration of rhSCGB3A2 suppressed development of lung tumors in comparison to mice without SCGB3A2 administration (Figure 2). When *Scgb3a2*-null mice were given rhSCGB3A2, the number of tumors was similar to those of wild-type controls (Yokoyama, et al., 2018). These results suggested that SCGB3A2 has anti-cancer activity and detailed studies were carried out to understand the mechanism for SCGB3A2 anti-cancer activity. It was observed that SCGB3A2 is a lipopolysaccharide (LPS) binding protein and that it chaperones LPS into cell cytosol through binding of an SCGB3A2-LPS complex to a cell surface receptor, syndecan-1 (SDC1). Internalized LPS binds to and activates caspase-11 (in mice) or caspase-4 (in human), which further activates the non-canonical NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome pathway, leading to pyroptosis, a type of inflammatory cell death (Yokoyama, et al., 2018) (Figure 4). Involvement of internalized LPS in the activation of the non-canonical inflammasome pathway was well documented (Rathinam, Zhao, & Shao, 2019; Skirecki & Cavaillon, 2019), however, how extracellular LPS enters cells remained elusive. This study clearly demonstrated a SCGB3A2-dependent route for LPS entry to cells through SDC1. Moreover, SDC1 is expressed on the surface of LLC cells as well as on macrophage-derived RAW264.7 cells. Both cells were susceptible to SCGB3A2-LPS-induced pyroptosis, suggesting this evolutionarily conserved mechanism in cancer cells that was initially evolved in immune cells to fight against infection (Yokoyama, et al., 2018). SCGB3A2-LPS-induced pyroptosis was inhibited in the presence of heparin, suggesting that the heparan sulfate (HS) moiety of SDC1 is critical for SCGB3A2-LPS-induced pyroptosis.

In order to determine whether pyroptosis can be utilized as a way to kill cancer cells, a panel of 20 human cancer-derived cell lines from different origins (17 lung, 2 colon, and 1 cervix) were analyzed for the susceptibility to SCGB3A2 (Yokoyama, Nakayama, Xu, Pilon, & Kimura, 2021). Five out of 11 NSCLC (non-small cell lung cancer) cells (H596, H358, H322, A549, and H157) showed SCGB3A2-induced growth inhibition in the presence of LPS *in vitro* and reduced tumor sizes *in vivo* in xenografts of SCGB3A2-treated mice. All susceptible NSCLC cells expressed SDC1 on their cell surface with abundant HS and caspase-4 (*CASP4*), the critical molecule in the non-canonical inflammasome pathway. In contrast, none of 6 SCLC cells (H1688, H146, H526, H417, H446, H82) examined showed growth inhibition, and none expressed SDC1 and/or *CASP4* (Yokoyama, et al., 2021). Two epithelial-derived colorectal cancer cell lines (HCT116 and SW620), that abundantly expressed SDC1 on the cell surface and *CASP4*, also underwent SCGB3A2-induced pyroptosis. These results suggest that SCGB3A2 may serve as a novel therapeutic to treat cancers regardless of tissue types, as long as cells express SDC1 with abundant HS and *CASP4* (Yokoyama, et al., 2021). Pyroptosis has been gaining attention in recent years as a tool to treat cancer (Fang, et al., 2020; Nagarajan, Soundarapandian, Thorne, Li, & Li, 2019).

3-7. SCGB3A2 as a cancer marker

The prevalence of expression of SCGB3A2 in lung cancer was investigated by immunohistochemistry using mice as well as human lung cancer tissues. In mouse studies, the expression of SCGB3A2, which is normally found in Club cells, was observed in

alveolar Type II cell carcinomas and Club cell adenocarcinomas using 28 lung tumors from aging B6;129 mice and 9 lung adenocarcinoma from CC10 (SCGB1A1) transgenic mice that express SV40 large T antigen under the mouse *Scgb1a1* gene promoter (Kurotani, Kumaki, et al., 2011) (Figure 2). No expression of SCGB3A2 was found in Type II cell hyperplasias or adenomas. Of interest was that SCGB3A2 expression in carcinomas was observed in the portion of the tumor where NKX2-1 expression was reduced or nearly absent, suggesting that *Scgb3a2* gene expression is no longer under the regulation of NKX2-1. When 23 human non-small cell lung carcinoma (NSCLC) specimens were examined, SCGB3A2 expression was observed in papillary adenocarcinomas and atypical adenomatous hyperplasia, a premalignant lesion (Kurotani, Kumaki, et al., 2011). A similar correlation was also observed between high SCGB3A2 expression and low/no NKX2-1 expression in these specimens. Another human study using 156 primary human lung cancer specimens, demonstrated that SCGB3A2 is highly expressed in adenocarcinomas (87%), particularly papillary adenocarcinomas, however this study found no significant relationship between SCGB3A2 expression and tumor differentiation, pathological stage, or survival (Tachihara-Yoshikawa, et al., 2008). Another study showed that plasma levels of SCGB3A2 in patients increased in the order of pneumonia, stage I-II adenocarcinoma, and stage III-IV adenocarcinoma relative to normal controls (Li, et al., 2018). These results indicate a potential use of plasma SCGB3A2 and/or SCGB3A2 in lung cancer biopsies by immunohistochemistry as a specific marker for primary pulmonary adenocarcinomas both in mice and humans.

3-8. Other biological roles of SCGB3A2

Acrolein, an abundant component in cigarette smoke, acts on lung epithelial cells and reacts with macromolecules, leading to alteration of lung pathogenesis, cell survival, and cell signaling (Bein & Leikauf, 2011). Acrolein is thought to contribute to the development of various lung diseases including bronchitis and emphysema in chronic obstructive pulmonary disease. Acrolein increases reactive oxygen species (ROS) and phosphorylation of p53 (Ser18), resulting in increased apoptosis in mouse lung fibroblast derived MLg cells (Kurotani, et al., 2015). SCGB3A2 suppressed the acrolein-induced apoptosis through decreased phosphorylation of p53 without altering ROS levels. The results suggest that SCGB3A2 may play a role in the development of cigarette smoke-related emphysema.

SCGB3A2 immunopositive protein was found in the posterior as well as the anterior lobes of pituitary of the adult mouse pituitary gland (Miyano, et al., 2014). Because NKX2-1 is expressed only in the posterior lobe of the pituitary (Miyano, et al., 2014) and SCGB3A2 is a direct target of NKX2-1 (Niimi, et al., 2001; Tomita, et al., 2008), the involvement of transcription factors other than NKX2-1, such as C/EBPs was suggested in the regulation of the *Scgb3a2* gene in the anterior pituitary (Miyano, et al., 2014). SCGB3A2 was co-localized with gonadotrophs expressing luteinizing hormone (LH) and follicular stimulating hormone (FSH) in adult mouse pituitary by immunohistochemistry (Miyano, et al., 2014). SCGB3A2 suppressed the mRNA levels of LH and FSH in rat primary anterior pituitary cells in culture (Miyano, et al., 2014). These results suggest that SCGB3A2 may regulate LH/FSH production in the anterior pituitary.

4. Conclusions and future prospects

Two decades have passed since SCGB3A2 was first identified (Niimi, et al., 2001), however, the function of this protein remains poorly understood. During this period, a number of studies have revealed multiple immunomodulatory functions of SCGB3A2, particularly in the lung, including anti-inflammatory, growth factor, anti-fibrotic, and anti-cancer activities (Figure 2). The fetal growth factor activity of SCGB3A2 could be applied to treat or prevent chronic lung disease and prevent arrested lung development in premature babies, as well as *in vitro* applications to culture artificial lungs for transplant. Inflammation and fibrosis are the two most prevalent conditions in the pathophysiology of most lung diseases, suggesting a wide range of applications for SCGB3A2 in the treatment and/or prevention of lung diseases. The association of the SCGB3A2 G-112A allele with Grave's disease suggests a potential application for SCGB3A2 augmentation therapy in this disease. Finally, the anti-cancer activity of SCGB3A2 may be maximized perhaps in combination with immuno- and/or targeted therapies. Major advances will be expected in the next decade.

Acknowledgment

This work was supported by the National Cancer Institute Intramural Research Program (ZIA BC 010449).

Abbreviations

| | |
|---------------|---|
| BALF | bronchoalveolar lavage fluid |
| BLM | bleomycin |
| CASP | caspase |
| CC10 | Club cell 10-kDa protein (also known as CCSP, CC16, uteroglobin, urine protein-1) |
| CCSP | Club cell secretory protein |
| C/EBP | CCAAT/enhancer-binding protein |
| HS | heparan sulfate |
| IL | interleukin |
| LLC | Lewis lung carcinoma |
| LPS | lipopolysaccharide |
| rh | recombinant human |
| rm | recombinant mouse |
| NKX2-1 | homeodomain transcription factor NK2 homeobox 1 |
| NSCLC | non-small cell lung cancer |
| SCGB | secretoglobin |

| | |
|-------------|--------------------------|
| SCLC | small cell lung cancer |
| SDC1 | syndecan-1 |
| TAF | tracheal aspirate fluids |

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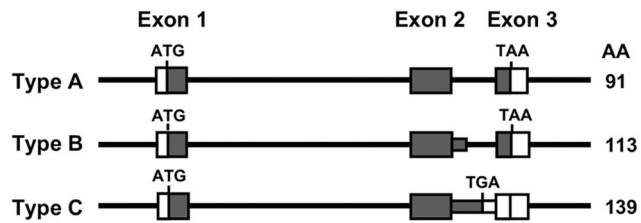
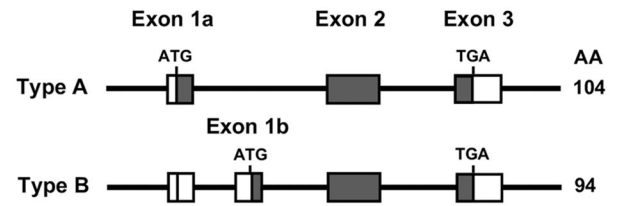
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A *Scgb3a2***B *Scgb3a1*****Figure 1. Structure of the SCGB family genes.**

Most SCGB genes including those of mice and humans consist of 3 exons, typically within 3–4 kbp; the transcription start site is located within exon 1, while the termination codon is located in exon 3. The illustration shows *Scgb3a2* (A) and *Scgb3a1* (B) transcript variants (type B and C for *Scgb3a2* and B for *Scgb3a1*) in addition to the major form (type A) that were identified in mouse adult lung by cDNA library screening. The number on the right in each form indicates the length of amino acid residues (AA). Empty and dark boxes indicate non-coding and coding sequences, respectively. Position and sequence of the initiation and the termination codons are shown.

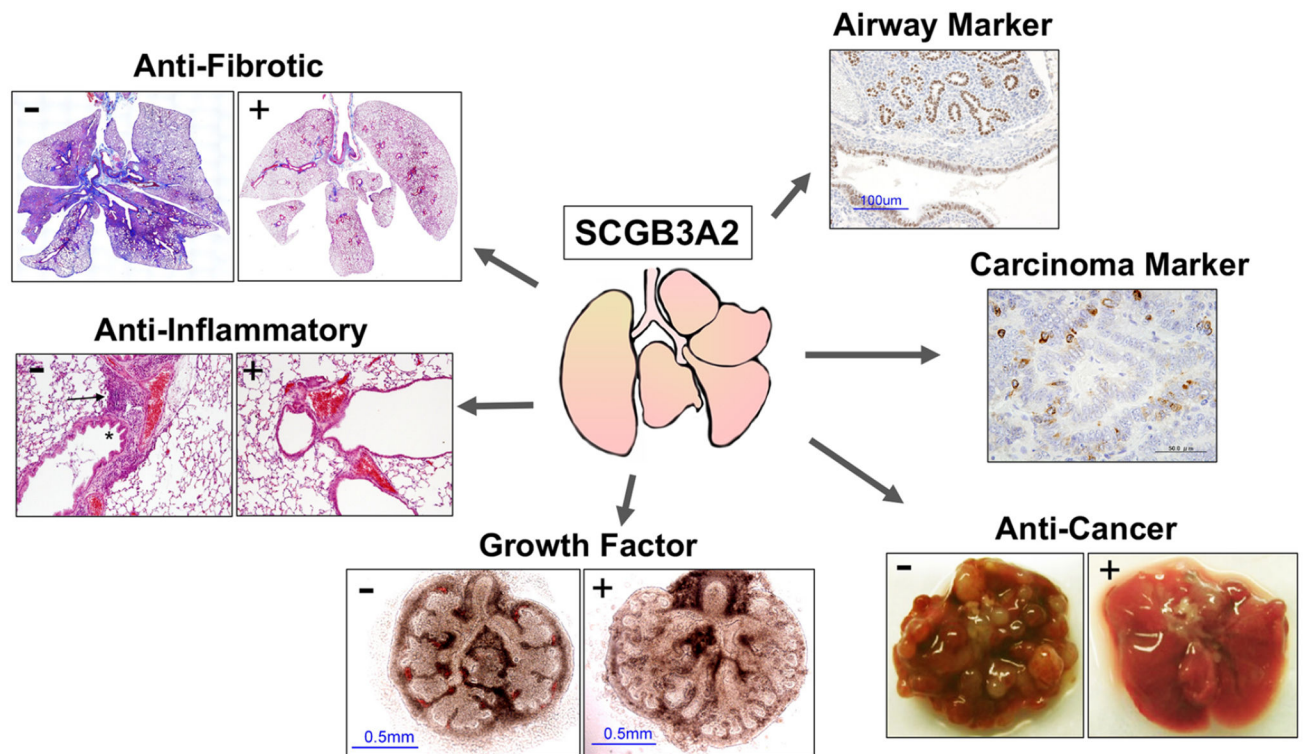


Figure 2. Summary of the major biological functions of SCGB3A2 in lung.

No (-) or addition/administration (+) of SCGB3A2 as indicated. Blue in anti-fibrotic activity is Masson trichrome staining to detect collagen fiber. Airway and carcinoma markers are immunohistochemistry results (brown) for SCGB3A2. Arrow and asterisk in anti-inflammatory activity are infiltrating immune cells and reactive hyperplasia of epithelial cells, respectively.

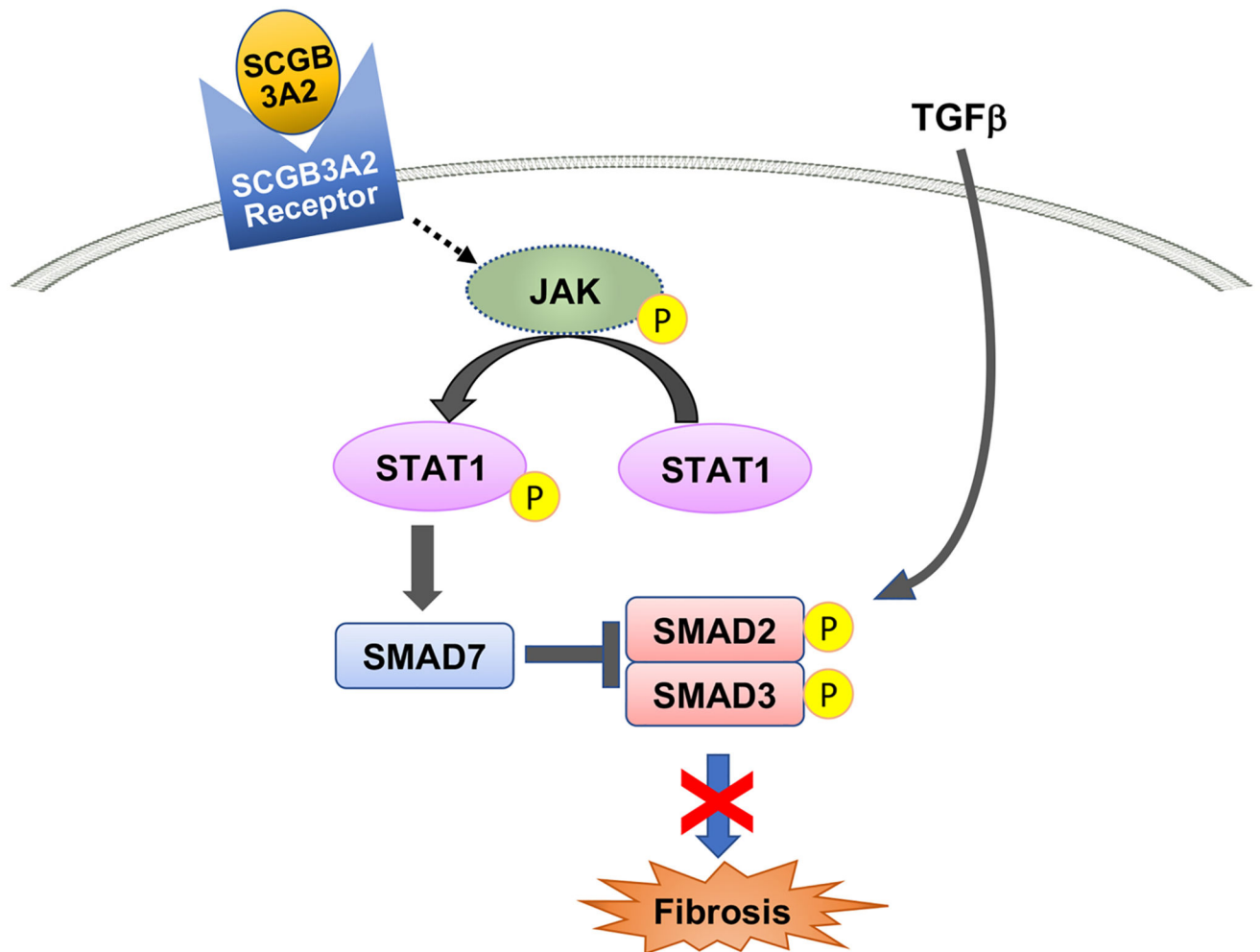


Figure 3. Mechanism for the anti-fibrotic activity of SCGB3A2. SCGB3A2 anti-fibrotic signaling starts upon binding of SCGB3A2 to a SCGB3A2 receptor, which activates STAT1 phosphorylation, increases SMAD7, and suppresses SMAD2/3 phosphorylation, resulting in the suppression of expression of genes involved in fibrosis such as collagens, leading to inhibition of fibrosis.

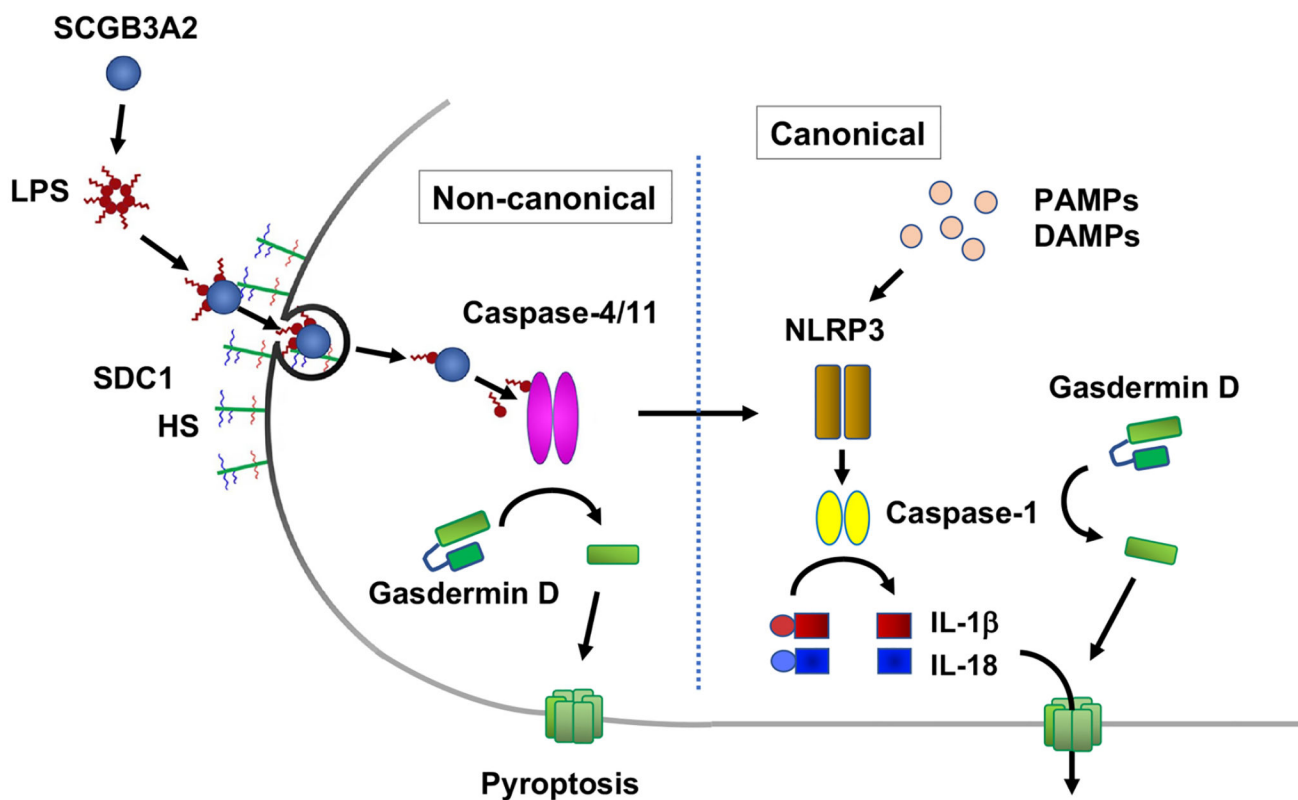


Figure 4. Mechanism for the anti-cancer activity of SCGB3A2 through activation of the non-canonical inflammasome pathway.

SCGB3A2 binds to LPS, and the SCGB3A2-LPS complex enters cell cytosol through binding to syndecan 1 (SDC1) followed by endocytosis. Internalized LPS binds and activates caspase-11 (in mice) and -4 (in human) that activates gasdermin D forming membrane pores, and eventually leading to pyroptosis. The non-canonical inflammasome cross-talks with canonical inflammasome, in which activated caspase-1 through the NLRP3 inflammasome releases IL-1 β and IL-18 through gasdermin D pores. HS: heparan sulfate, PAMP: pathogen-associated molecular patterns such as double-strand RNA, cytosolic DNA, etc. derived from pathogens. DAMP: danger-associated molecular patterns such as host DNA, ATP, etc. NLRP3: NLR family pyrin domain containing 3. Upon entering cells, whether and/or how the SCGB3A2-LPS complex dissociates, and/or releases LPS remain unknown.