

Title: OSTEOGLYCIN AS REGENERATIVE AGENT FOR LUNG TISSUE

5 The invention relates to the field of medicine and tissue engineering. More specifically, it relates to means and methods to boost the repair of soft tissues, such as damaged lung tissue as is observed in diseases with defective lung repair such as chronic obstructive pulmonary disease (COPD).

10 COPD is one of the most common lung diseases in the world, characterized by a progressive decline of lung function and airflow limitation that is not fully reversible. The key problem underlying COPD is abnormal tissue repair. This has two seemingly opposite components: on the one hand bronchitis and small airway remodeling with increased connective
15 tissue, and on the other hand emphysema caused by a net destruction of parenchymal lung tissue. The current treatment protocols of COPD are aimed mostly at alleviating symptoms and have little to no effect on the decline in lung function that COPD patients experience over time. Standard of care includes bronchodilator drugs and anti-inflammatory drugs and
20 bronchoscopic/surgical interventions (placement of coils, lung volume reduction surgery, transplantation) in more severe disease. None of these treatments support regeneration of lung tissue.

 Since current disease therapies can, at best, only relieve symptoms but they do not modify or positively influence the course of the disease, the
25 present inventors aimed at developing new therapies for epithelial tissue repair. In particular, they sought to identify factors that reactivate endogenous repair mechanisms to stimulate regeneration of damaged cells in epithelial tissue, e.g. parenchymal lung tissue, and that can normalize an aberrant tissue repair response.

30 It is known that the application of mesenchymal stromal cells (MCSs) as a regenerative agent may have therapeutic effects in the treatment of emphysema in COPD. *In vitro* and *in vivo* studies have

demonstrated that MSCs have beneficial properties in COPD, including reduced emphysematous changes, attenuated alveolar damage and differentiation of alveolar type II-like cells (Cappetta et al., Stem Cells Int. 2018 Mar 14;2018:9492038). It is also known that these effects are mediated
5 by soluble factors secreted by the cells as well as by extracellular vesicles (EVs) produced by mesenchymal lung fibroblasts (Van Der Koog *et al.*, ERJ Open Research 2022 8: 2; DOI: 10.1183/23120541.LSC-2022.2; Eur. J. Pharm.Vol. 974, 2024, 17661; DOI: 10.1016/j.ejphar.2024.176612) These secreted soluble factors and extracellular vesicles contain >1000 different
10 proteins. However, the identity of the key factor(s) responsible for regeneration are unknown.

The present inventors surprisingly found that, among the thousands of proteins in the secreted soluble factors and in the EVs, there is
15 one that appears to induce an exceptionally strong effect on regenerative responses in soft tissue. This protein is called osteoglycin (OGN), also known as mimecan. OGN is an endogenous small leucine-rich proteoglycan known to have several regulatory functions in the human body. Among these functions are the regulation of fibrosis, tumorigenesis and inflammation.
20 See for example Nulali *et al.* (Biomolecules. 2022 Nov 11;12(11):1674) and Deckx *et al.* (FASEB J. 2016 Aug; 30(8):2651-61).

As is demonstrated herein below, using an organoid model wherein organoid growth is a marker for soft tissue regeneration, it was observed that osteoglycin was able to induce ~75% more organoid formation
25 compared to control whereas other proteins tested did not induce growth with more than 30%.

Accordingly, the invention relates to osteoglycin (OGN) or a functional fragment thereof, for use as an agent to promote the regeneration
30 of epithelial tissue. In a specific aspect, it provides the use of OGN or a functional fragment or derivative thereof to promote the regeneration of epithelial cells in lung tissue, intestinal tissue or liver tissue.

In other words, the invention relates to the use of OGN or a functional fragment thereof, to enhance epithelial tissue repair and/or regeneration.

- 5 The therapeutic use of OGN in epithelial tissue regeneration is not disclosed or suggested in the art. Osteoglycin has been reported as a therapeutic agent for heart disease, retinal disease and osteoporosis. See for example Deckx *et al.* (Matrix Biol. 2018 Mar; 66:110-124); Tasheva *et al.*, (Mol Vis 2002;9) and Chen *et al.* (BMC Musculoskeletal Disorders 2017 18:423). Shi
- 10 *et al.* (Am J Physiol Cell Physiol. 2020 Nov 1;319(5):C895-C905) reported that overexpressed microRNA-140 inhibits pulmonary fibrosis in interstitial lung disease via the Wnt signaling pathway by downregulating osteoglycin. KR2015/0059491A discloses the use of a substance that increases expression of osteoglycin for the treatment or prevention of senile dry skin disease. It
- 15 also relates to screening methods, kits and markers for measuring the amount of OGN after treating epidermal cells with a specific substance and use this to select the specific substance as a substance for improving senile dry skin disease.
- 20 Notably however, each of these known applications relates to (regenerative) effects on fibroblasts and/or osteoblasts, and not on epithelial cells or epithelial tissues. More specifically, OGN has never been proposed as therapeutic agent in the treatment of lung, intestinal or liver diseases.
- 25 As used herein, the term "OGN or a functional fragment thereof" encompasses any of the 3 known transcripts of the human OGN gene and N- and/or C-terminal truncated variants and homologs thereof showing a regenerative effect on epithelial cells/epithelial tissue. Regenerative effects are suitably determined in one or more *in vitro* assays known in the art for
- 30 example using a (murine) organoid model system, precision-cut lung slices or murine models as exemplified herein below. Exemplary OGN homologs for use in the present invention comprise mammalian homologs such as

OGN from bovine, mouse, rat, rabbit or chicken (see Decks et al., FASEB J., 2016 Aug;30(8):2651-610.

In a preferred aspect, the human OGN protein or a functional fragment thereof is used. The human OGN polypeptide sequence is accessible in GenBank under the number AAH37273.1 (Osteoglycin [Homo sapiens]) or UniProtKB/Swiss-Prot P20774, Q9UNK5, or UniProtKB/TrEMBL: A8K0R3, B4DI63, Q7Z532.

Human OGN protein sequence:

MKTLQSTLLLLLVPLIKPAPPTQQDSRIHYDYGTDNFEESIFSQDYEDKY
LDGKNIKEKETVIIPNEKSLQLQKDEAITPLPPKKENDEMPTCLLCVCLS
GSVYCEEVDIDAVPPLPKESAYLYARFNKIKKLTAKDFADIPNLRRLDFT
GNLIEDIEDGTFSKLSLLEELSLAENQLLKLPVLPPKLTLFNAKYNKIKS
RGIKANAFKKLNNLTFLYLDHNALESVPLNLPESLRVIHLQFNNIASITD
DTFCKANDTSYIRDRIEEIRLEGNPIVLGKHPNSFICLKRLPIGSYF

Residues 1-20 (underlined) form a signal sequence. Residues 21-298 represent the OGN polypeptide sequence. An internal disulfide bond may be formed between Cys residues at positions 255 and 288. OGN possesses a core protein comprising of leucine-rich repeats (LRRs) and an N-terminal cysteine-rich cluster that binds specific glycosaminoglycans (GAGs). The full-length protein features a lengthy tail and a sequence of seven LRRs (LRR1-LLR7). The positively charged residues within the LRRs, particularly in LRR4, expose regions suitable for interactions with other proteins, suggesting a role in biological activity/signaling. LRR4 exhibits significant conservation between species, hinting at functional importance.

Encompassed are OGN polypeptides carrying one or more post-translational modifications, such as *N*- and/or *O*-linked glycans. Known sites of glycan modification *O*-linked (GalNAc) at Thr80, and *N*-linked (GlcNAc)(keratan sulfate) at Asn80, Asn214 and Asn258.

The term "functional fragment" of OGN may mean that part of the OGN polypeptide which retains functional activity. It may refer to any polypeptide or oligopeptide comprising or consisting of a stretch of OGN that is capable of exerting a desired regenerative effect on epithelial cells or epithelial tissue. The desired regenerative effect of the functional fragment may be at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or at least 100% of the effect observed for full length OGN used at the same molar concentration. Preferably, the functional OGN fragment has retained at least 100% of the functional (regenerative) activity of OGN.

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A regenerative effect is readily determined using functional assays and methods known in the art and herein disclosed in detail in the Examples. Suitable functional assays may involve a mammalian organoid system or precision-cut lung slices (PCLS). In some embodiments, the regenerative effect is assessed by determining the colony forming efficiency (CFE) in murine or human lung organoids, optionally including a test set-up involving exposure to cigarette smoke. In other embodiments, the regenerative effect is assessed by evaluating gene expression of alveolar type (AT) I and/or II cell markers in elastase-treated PCLS. Still further, (candidate) functional fragments can be evaluated in a (murine) *in vivo* model such as described herein below.

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Preferred functional fragments comprise up to 200 amino acids, up to 180 amino acids, up to 170 amino acids or up to 160 amino acids. In one embodiment, the OGN functional fragment is a N-terminal truncated variant lacking at least residues 1-10, preferably at least residues 1-20. However, larger parts of the N-terminus may be deleted without compromising the therapeutic effect of OGN. This may be done for example with the aim of limiting the effects of OGN on collagen cross-linking, whilst preserving the regenerating/signaling properties of OGN. For example, the functional fragment may lack N-terminal residues 1-50, residues 1-100, residues 1-150, residues 1-160, residues 1-170 or residues 1-179. Good

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results were obtained with a fragment of about 15 kDa consisting of residues 180-298. In some embodiments, the fragment comprises or consists of one or more of the leucine-rich repeat (LRR) motifs as found in OGN. Preferably, the fragment comprises or consists of one or more LRR4 (residues 180-199), LRR5 (residues 200-225), LRR6 (residues 226-246) and LRR7 (residues 247-277). For example, at least motif LRR4, LRR5, LRR6 or LRR7 is present. The functional fragment may comprise one or more copies of a given LRR, e.g. of LRR4, LRR5, LRR6 and/or LRR7. In one aspect, the fragment comprises or consists of LRR4-LRR7 (residues 180-277). In a preferred embodiment, the functional OGN fragment comprises or consists of the amino acid sequence of motif LRR4 (LKLPVLPPKLTFLNAKYNKI). Also encompassed are variant OGN (fragments) comprising one or more (conserved) amino acid substitutions as compared to the wild-type OGN sequence. For example, one or more Lys residues can be replaced with Arg or His.

In a specific aspect, the functional fragment comprises at least residues Leu180-Phe298. In another embodiment, the OGN functional fragment is a C-terminal truncated variant lacking at least residue Phe298, or residues Tyr297 and Phe298, or residues Ser296-Tyr297-Phe298. In a specific aspect, the functional fragment comprises at least residues Leu180-Gly295.

According to the present invention, OGN or a functional fragment thereof is used as regenerative agent for epithelial cells or in epithelial tissue.

Epithelial cells may be squamous, cuboidal, or columnar in shape and may be arranged in single or multiple layers. Simple cuboidal epithelium is found in glandular tissue and in the kidney tubules. Simple columnar epithelium lines the stomach and intestines. Pseudostratified columnar epithelium lines portions of the respiratory tract and some of the tubes of the male reproductive tract. Most epithelia replenish themselves through a process called tissue homeostasis, in which the number of cell divisions within a tissue compensates for the number of cells lost. Tissue

homeostasis is ensured by the existence of stem cells (SCs) located within specialized microenvironments, referred to as niches. Upon wounding, epithelial tissues undergo different phases of hemostasis, inflammation, proliferation and remodeling, often resulting in fibrosis and scarring.

5 Epithelial cells of the lung are located at the interface between the environment and the organism and serve many important functions including barrier protection, fluid balance, gas exchange, clearance of particulate, initiation of immune responses, mucus and surfactant production, and repair following injury.

10 The epithelial cells of the liver are grouped together to form functional units referred to as liver lobules. Major functions of hepatocytes are to synthesize and secrete bile, form and store proteins, remove toxins, and store and release carbohydrates.

 The intestinal epithelial cells (IECs) form a selective permeability
15 barrier separating luminal content from underlying tissues. Upon injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the balance of three cellular events; restitution, proliferation, and differentiation of epithelial cells adjacent to the wounded area. Episodes of tissue injury and incomplete healing of the
20 intestinal epithelium are a prerequisite for immune reactivation and account for recurrent, chronically progressing phenotypes of inflammatory bowel diseases (IBD).

 In a preferred embodiment, the invention provides the use of OGN or a
25 functional fragment or derivative thereof to promote the regeneration of epithelial cells in lung tissue, (gastro)intestinal tissue or liver tissue.

 As is exemplified herein below, the invention provides the use of OGN to enhance expansion of (distal) lung stem cells into airway and/or alveolar
30 epithelial cells.

In a preferred aspect, the invention provides OGN or a functional fragment thereof for use in a method for treatment of a disease involving defective lung tissue repair, defective intestinal tissue repair and/or defective liver tissue repair. Also provided is a method for treatment of a disease involving defective lung tissue repair, defective intestinal tissue repair and/or defective liver tissue repair in a (human) subject, comprising administering to the subject a therapeutically effective amount of OGN or a functional fragment thereof. Preferably, the method comprises administering OGN or a functional fragment thereof by pulmonary delivery.

Preferably, OGN or a functional fragment thereof is used in a disease or disorder involving defective lung tissue repair and/or lung function decline. For example, the disease is selected from the group consisting of COPD, lung fibrosis, cystic fibrosis, lung repair post-pneumonia, acute lung injury/ARDS, long-COVID and sarcoidosis.

According to the invention, OGN or a functional OGN fragment may be used (administered) as protein, or as DNA or mRNA encoding the protein.

In one embodiment, OGN or a functional fragment thereof is administered as protein. The OGN protein may be recombinantly produced e.g. using eukaryotic, e.g. yeast or mammalian host cells. In some embodiments, OGN or functional OGN fragment is produced synthetically. Synthetic OGN products may comprise proteinogenic and/or non-proteinogenic amino acids. The OGN may be used as regenerative agent either its glycosylated or non-glycosylated form. In one embodiment, OGN is glycosylated, preferably N- and O-glycosylated.

A further aspect relates to a pharmaceutical composition comprising OGN polypeptide or a functional fragment thereof, or OGN mRNA, or a functional fragment thereof, and a pharmaceutically acceptable carrier, vehicle or diluent, which preferably would stabilize the protein or mRNA, or enhance its therapeutic activity. In a further embodiment, the OGN polypeptide or a

functional fragment thereof or OGN mRNA, or a functional fragment thereof, is formulated in a suitable dosage form for administration via the desired route of administration, for example in a liquid formulation for injection or a liquid suitable for nasal administration. In a preferred
5 embodiment, the composition is formulated for pulmonary delivery. Pulmonary drug delivery is the inhalation of drug formulation through mouth e.g. wherein patients use an inhaler to inhale their medication. The further deposition of inhaled pharmacological agent in lower airways wherein drugs are absorbed into the bloodstream via the lung mucous
10 membrane is the main purpose of this drug delivery route. In some embodiments, OGN polypeptide or a functional fragment thereof is in the form of a powder for inhalation, or a liquid for nebulization.

OGN polypeptide or a functional fragment thereof may be formulated in solutions containing functional excipients such as, but not
15 limited to; buffers like phosphate or acetate buffers, stabilizers such as sugars, polyols or amino acids, surfactants such as polysorbate 80 and antioxidants such as ascorbic acid.

In the dry form, OGN polypeptide or a functional fragment thereof may be stabilized by incorporation of the protein in a matrix containing
20 sugars, such as trehalose, and/or polysaccharides such as inulin or pullulan, and/or polyols such as mannitol, and/or buffers, and/or amino acids.

Dry powder medicaments may be in a pure formulation consisting of only OGN as active pharmaceutical ingredient (API), or the formulation may
25 comprise other substances for different purposes, e.g., excipients for improving the product's stability, increasing the bio-availability and/ or bio-activity of the API. The API may also be incorporated in a matrix of excipients, before it is formulated into the final dosage form for which again one or more excipients may be used. Pharmacologically inert excipients may
30 be included for diluting a potent API, in order to act as carrier of the API or to improve the flowability of the formulation to enhance metering and filling properties of the powder.

Powders with a particle size suitable for inhalation, i.e., particles having an aerodynamic diameter (AD) in a range 0.5 - 5 μm , have a tendency of aggregating, in other words to form smaller or larger aggregates, which then have to be de-aggregated before the particles enter into the airways of the user. De-aggregation is defined as breaking up aggregated powder by introducing energy e.g. electrical, mechanical, pneumatic or aerodynamic energy. The aerodynamic diameter of a particle of any shape is defined as the diameter of a spherical particle having a density of 1 g/cm³ that has the same inertial properties in air as the particle of interest.

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The composition for pulmonary delivery may further comprise one or more additional therapeutically active agents selected from the group consisting of bronchodilator drugs and anti-inflammatory compounds. Exemplary further agents include Saba's (e.g. salbutamol), Laba's (e.g. formoterol), Lama's (tiotropium) and corticosteroids (e.g. budesonide).

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In another embodiment, the pharmaceutical formulation is in the form of a formulation which can deliver OGN (protein) intact at the site of intestinal damage. This can be achieved either via the oral route, exploiting the possibilities of site-specific drug delivery or prodrug approaches, or via the parenteral route using specific drug-targeting technologies.

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In yet another embodiment, e.g. when OGN is used for liver repair, the composition makes use of technologies that allow for site specific delivery of the therapeutic protein to the liver. See for example described by Böttger *et al.* Advanced Drug Delivery Reviews 154–155 (2020) 79–101 or by Poelstra *et al.* Journal of Controlled Release 161 (2012) 188–197.

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OGN polypeptide may be present in the composition in a dissolved or dry state. Preferably, it is stabilized by one or more excipient(s), for example selected from the group consisting of extremolytes, surfactants, sugars, sugar alcohols, polysaccharides and buffers, or by incorporation into

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amorphous sugar glasses containing mono-, di-, or polysaccharides, buffers, amino acids, or inert peptides or proteins.

Other exemplary pharmaceutical compositions according to the invention
5 comprise OGN mRNA. For example, use is made of a non-viral delivery
system for RNA therapeutics known in the art. See for example Paunovska
et al. (Nature Reviews Genetics volume 23, pg. 265–280 (2022)). These
involve synthetic materials that encapsulate RNA, such as polymers, lipids
and lipid nanoparticles (LNPs). Typically, a LNP formulation consists of a
10 cationic lipid, a neutral lipid and/or cholesterol and a PEG-lipid. Of
particular interest for the present invention are LNPs that have a
preferential uptake by alveolar epithelial cells, such as LNPs comprised of
DOTAP, DPPC, cholesterol, DLin-MC3-DMA, and DSPE-PEG. See also
Lokugamage *et al.* (Nature Biomedical Eng. Volume 5, pg.1059–1068 (2021))
15 reporting the design of LNPs for the efficient delivery of therapeutic RNAs
to the lung via nebulization. The liquid formulation of the nanoparticles
may also contain sugar(s) like sucrose.

In another embodiment, OGN mRNA is administered using LNPs that have
20 a preferential uptake by the liver, such as LNPs incorporating the lipid
containing tris(2-aminoethyl)amine (TREN) and 3 linoleyl chain, termed
TREN₃, optionally further containing unsaturated fatty acid (Yu *et al.*,
Biomaterials. 2012 Sep; 33(25): 5924–5934).

25 Also provided is a medical device comprising a pharmaceutical composition
according to the invention. For example, the device is an autoinjector
comprising a stabilized (aqueous) solution of OGN. As another example, the
medical device is a nebulizer, a metered-dose inhaler (MDI), or a dry powder
inhaler (DPI) comprising OGN formulated for pulmonary delivery.

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DPI devices have become accepted in the medical service, because they
deliver an effective dose in a single inhalation, they are reliable, often quite

small in size and easy to operate for a user. Two types are common, multi-dose dry powder inhalers and single dose dry powder inhalers. Multi-dose devices have the advantage that a quantity of medicament powder, enough for a large number of doses, is stored inside the inhaler and a dose is
5 metered from the store shortly before it is supposed to be inhaled. Single dose inhalers use pre-metered doses and such inhalers are loaded with a limited number of individually packaged pre-metered doses, where each dose package or container is opened shortly before inhalation of the enclosed dose is supposed to take place.

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Other aspects relate to individually packaged (sealed) pre-metered OGN doses for use in combination with the inhalation device, where each dose package or container is opened shortly before inhalation of the enclosed dose is supposed to take place.

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LEGEND TO THE FIGURES

Figure 1: Representative bright field images of murine organoid cultures at day 14 after treatment with different recombinant growth factors.

20 Scale=500 μ m

Figure 2: **(A)** Colony forming efficiency of murine organoids in drug screening on day 14 (mean $\pm$ SEM, N=3-5, paired Friedman test). **(B)** Log of murine organoid diameter in drug screening on day 14 (median is shown, N=3-5, Kolmogorov-Smirnov test (after Bonferroni correction: $\alpha = 0.00417$)).
25 Statistically significant comparisons with control are represented by *p < 0.05 and **p < 0.01.

Figure 3: **(A)** Representative brightfield images of murine lung organoids
30 **(B)** Colony forming efficiency (mean $\pm$ SEM, N=8, paired Friedman test) and log diameter (median is shown, N=8, Kolmogorov-Smirnov test (after

Bonferroni correction: $\alpha = 0.0017$) of murine organoids treated with increasing concentrations of OGN or fragment on day 14.

Figure 4: **(A)** Representative brightfield images of CSE-exposed murine lung organoids. **(B)** Colony forming efficiency (mean $\pm$ SEM, N=8-11, paired Friedman test) and log diameter (median is shown, N=8-11, Kolmogorov-Smirnov test (after Bonferroni correction: $\alpha = 0.0013$) of CSE-exposed murine organoids treated with increasing concentration of OGN or fragment on day 14. **(C)** Representative immunofluorescence images of stained organoids for alveolar-type (surfactant protein C, green channel) and nuclei (Dapi, blue channel). **(D)** Quantification of immunohistochemistry for pro-surfactant protein C in CSE-exposed murine organoids on day 14, to identify alveolar-type organoids (mean $\pm$ SEM, N=7-8, paired Friedman test).

Figure 5: **(A)** Representative brightfield images of TGF-beta-exposed murine lung organoids. **(B)** Colony forming efficiency (mean $\pm$ SEM, N=8, paired Friedman test) and log diameter (median is shown, N=8, Kolmogorov-Smirnov test (after Bonferroni correction: $\alpha = 0.0013$) of TGF-beta-exposed murine organoids treated with increasing concentration of OGN or fragment on day 14.

Figure 6: **(A)** Representative brightfield images of murine lung organoids treated with denatured OGN. **(B)** Normalized organoid counts (mean $\pm$ SEM, N=5, paired T-test) and size (median is shown, N=8-11, Kolmogorov-Smirnov test) of murine organoids treated with denatured OGN.

Figure 7: Colony forming efficiency (mean $\pm$ SEM, N=5, paired Friedman test) and log diameter (median is shown, N=5, Kolmogorov-Smirnov test (after Bonferroni correction: $\alpha = 0.025$) of human COPD IV lung organoids treated with OGN (300 nm) or fragment (300 nm) on day 14.

Figure 8: **(A)** Gene expression levels of alveolar epithelial cell markers (Aqp5, Rage, Con43, Sftpc) in precision-cut lungs slices treated with elastase (16h) and/or OGN (40h) (N=2). **(B)** Gene expression levels of Ogn in precision-cut lung slices treated with elastase (16h) (mean $\pm$ SEM, N=5, paired T-test on log-transformed data).

Figure 9: **(A)** Example images of PCLS stained for F-actin filaments (green) and Dapi (blue) (scale = 100 μ m) after treatment with vehicle control, elastase, or elastase + OGN or fragment. **(B)** Mean linear intercept measurements following treatments are shown as μ m (mean $\pm$ SEM, N=6, One-Way ANOVA followed by Sidak's multiple comparison). Statistically significant comparisons with control are represented by **p < 0.01 and ****p < 0.0001, and with elastase by ## p < 0.01 and ### p < 0.001.

Figure 10: **(A)** Schematic of *in vivo* murine experimental elastase-induced lung injury model. **(B to D)** Lung function parameters: forced expiratory flow at 0.2 second (FEF0.2), forced vital capacity (FVC), and forced expiratory flow at 50% of forced vital capacity (FEF_50%FVC) as measured with the FlexiVent (median $\pm$ minimum and maximum data point, N=7-8, One-Way ANOVA followed by Sidak's multiple comparison). **(E)** Pressure-volume loops to assess the distensibility of the lungs (N=7-8, Two-way ANOVA-analysis, Dunnett's multiple comparison). Statistically significant comparisons are represented by *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 11: **(A-C)** Example images of OGN staining in whole lung tissue of never smoker, current smoker, and ex-smoker donors (scale = 4000 μ m). **(D-E)** Positively stained area percentage (%) for OGN in whole lung tissue and parenchyma in never, current, and ex-smokers. **(F-G)** Intensity of staining for OGN in whole lung tissue and parenchyma in never, current, and ex-smokers. **(H)** Example image of OGN staining in whole lung tissue in moderate-severe-COPD (COPD II/III) (scale = 4000 μ m). **(I)** Example image

of OGN staining in whole lung tissue in severe-early onset (SEO)-COPD (scale = 4000 μm). **(J-K)** Positively stained area percentage (%) for OGN in whole lung tissue and parenchyma in COPD II/III patients. **(L-M)** Intensity of staining for OGN in whole lung tissue and parenchyma in COPD II/III patients. **(N-O)** Positively stained area percentage (%) for OGN in whole lung tissue and parenchyma in SEO-COPD patients. **(P-Q)** Intensity of staining for OGN in whole lung tissue and parenchyma in SEO-COPD patients. Statistically significant comparisons are represented by * $p < 0.05$ and ** $p < 0.01$.

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Figure 12: **(A-D)** Gene expression of alpha-smooth muscle actin, collagen1a1, fibronectin, and TGF-beta measured by RT qPCR in human lung fibroblasts (MRC5). MRC5 fibroblasts were treated with TGF-beta, OGN (10 $\mu\text{g/mL}$) and/or fragment (4.5 $\mu\text{g/mL}$). Values are displayed as 2- $\Delta\Delta\text{Ct}$ values relative to the average of the vehicle control values (mean $\pm$ standard error of the mean, N=5-6, One-Way ANOVA followed by Sidak's multiple comparison. Statistically significant comparisons with are represented by * $p < 0.05$ and ** $p < 0.01$.

20 Figure 13: Fibroblast and myofibroblast cell signature based on transcriptomics of murine fibroblasts treated with vehicle or OGN resorted from organoids and subjected to bulk RNA sequencing (mean $\pm$ minimum and maximum value, N=4, One-Way ANOVA followed by Dunnett's comparison).

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Figure 14: Normalized organoid count after 14 days upon treatment with (fresh) OGN fragment or spray-dried fragment with inulin or mannitol as an excipient (mean $\pm$ SEM, N=6, paired Friedman test) and organoid size (median is shown, N=6, Kolmogorov-Smirnov test (after Bonferroni correction: $\alpha = 0.017$). Spray-dried fragment was added immediately after spray-drying to the organoid culture to test whether the organoid supporting potential was preserved upon drying.

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Figure 15: Normalized organoid count after 14 days upon treatment with (fresh) OGN fragment or spray-dried fragment with inulin or mannitol as an excipient (mean $\pm$ SEM, N=6, paired Friedman test) and organoid size (median is shown, N=6, Kolmogorov-Smirnov test (after Bonferroni correction: $\alpha = 0.017$). Spray-dried fragments were subjected to storage for one week under two distinct conditions: 60°C with 0% relative humidity and 30°C with 43% relative humidity. This experiment aimed to assess the resilience of organoid supporting potential of the spray-dried fragment after extreme storage conditions.

Figure 16: Proteomics-guided drug target strategy.

EXPERIMENTAL SECTION

Materials and methods

Animals

Mouse experiments for organoid studies and PCLS were performed at the Central Animal Facility (CDP) of the University Medical Center Groningen (UMCG) in accordance with the national guidelines and upon approval of the experimental procedures by CDP and the Institutional Animal Care and Use Committee (IACUC) of the University of Groningen. Animals were housed conventionally under a 12-h light-dark cycle and received food and water ad libitum.

Human material

Human lung tissue was obtained from lung transplant donors in strict adherence to the Research Code of the UMCG, as stated on <https://umcgresearch.org/w/research-code-umcg> as well as national ethical and professional guidelines Code of Conduct for Health Research (<https://www.coreon.org/wp-content/uploads/2023/06/Code-of-Conduct-for->

Health-Research-2022.pdf). The use of left-over lung tissue in this study was not subject to Medical Research Human Subjects Act in the Netherlands, as

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confirmed by a statement of the Medical Ethical Committee of the UMCG and therefore exempt from consent according to national laws (Dutch laws: Medical Treatment Agreement Act (WGBO) art 458 / GDPR art 9/ UAVG art 24). Human lung tissue was acquired from extra tissue left over after lung
5 surgeries, such as lung resections and transplants, which exceed the amount needed for clinical care purposes. All samples and clinical information were coded before experiments were performed, blinding any identifiable information to the investigators.

10 *Cell culture*

Mouse fibroblasts, CCL206 cells (ATCC, Mlg2908), were cultured in DMEM/F12 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma Aldrich, 12103C), 100 U/ml penicillin/streptomycin (Gibco, 15070-063), 2 mM L-glutamine (Gibco, 25030-024), and 1%
15 amphotericin B (Gibco, 15290026) within a humidified atmosphere under 5% CO₂/95% air at 37 °C. The human fetal mesenchymal lung fibroblast MRC-5 cell line (Sigma Aldrich, 05081101) was cultured in Ham's F12 medium (Thermo Fisher, 11320033) supplemented with 10% (v/v) FBS, 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine within a humidified
20 atmosphere under 5% CO₂/95% air at 37 °C.

For organoid experiments, before both fibroblast types (CCL206 and MRC5) were co-cultured with primary epithelial cells, proliferation of fibroblasts was inactivated by incubation with mitomycin C (10 µg/mL) in growth
25 medium (Sigma Aldrich, M4287-5X2MG) for 2 h. After incubation with mitomycin C, the fibroblasts were washed with warm PBS and then allowed to recover for 1 h in mitomycin C-free growth medium.

Organoid culture

30 Isolation of murine primary alveolar epithelial cells, in brief Epcam⁺ cells (CD31-/CD45-/CD326⁺), was based on previously published protocols (Wu *et al.*, Front Pharmacol. 2021 Jan 20;11:609509). In brief, the lungs of mice

were flushed through the heart with PBS, instilled with dispase (Corning, 354235), and incubated at room temperature for 45 mins. To obtain a single cell suspension, lung tissue was then digested with DNase1 (VWR, A3778.0500). Using the QuadroMACS™ Separator (Miltenyi Biotec, 130-091-051) and a mix of antibody-bound magnetic microbeads, the cell suspension was negatively selected for CD31 (Miltenyi Biotec, 130-097-418) and CD45 (Miltenyi Biotec, 130-052-301). Subsequently, to obtain Epcam⁺ cells, CD31⁻/CD45⁻ cells were positively selected with anti-mouse CD326 microbeads (Miltenyi Biotec, 130-105-958).

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For human organoids, adult human donor tissue was isolated from histologically normal regions of lung tissue specimens obtained at the UMCG from N = 5 patients with GOLD stage IV COPD. Human lung tissue was stored in MACS® Tissue Storage Buffer (Miltenyi Biotec, 130-100-008) until further processing. Lung tissue was cut in small pieces (~ 1 mm³) and transferred to a dissociation mixture containing 1% penicillin/streptomycin, 1 mg/mL collagenase/dispase (Roche, 11097113001), and 1.8 µg/mL DNase 1 in PBS. The tissue was further dissociated using a gentleMACS™ Octo Dissociator with heaters (130-096-427, Miltenyi Biotec) for 20 mins at 37 °C. The obtained single cell suspension was washed and red blood cells were lysed using lysis buffer (ammonium chloride (155 mM), potassium bicarbonate (1 mM), titriplex III (0.001 mM), and 10 µg/mL DNase 1 in ultra-pure water) for 10 mins at 4 °C. Selection for EpCAM⁺ cells was similar to that described above for murine lung tissue.

25

Epithelial organoid culture

For murine organoids, freshly isolated Epcam⁺ cells were combined with CCL206 murine lung fibroblasts at a 1:1 ratio (10,000 cells each) in DMEM/F12 containing 10% (v/v) FBS. The cell suspension was then diluted 1:1 (v/v) with Corning® Matrigel® Membrane Matrix (Corning, 356234), and were then seeded into transwell inserts (Greiner, 662641) in 24-well plates (100 µl/insert). Similarly, human organoids were generated by co-

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culturing freshly isolated EpCAM⁺ cells with proliferation-inactivated MRC-5 lung fibroblasts. The Matrigel™ was allowed to solidify at 37 °C for 30 mins. Upon solidification, 410 µl of organoid medium (DMEM/Ham's F12 supplemented with 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 5 1% amphotericin B, 0.025 % epidermal growth factor (EGF) (Sigma Aldrich, SRP3196-500UG), 1% insulin-transferrin-selenium (Gibco, 51300044), and 1.75 % bovine pituitary extract (Thermo Fisher, 11568866)) was added underneath the insert. On the day of seeding, 10 µM Y-27632 dihydrochloride (Axon, 1683) was added to selectively inhibit Rho-Kinase.

10 Organoid cultures were cultured at 37 °C with 5% CO₂. Medium was refreshed every 2-3 days. Treatment agents were added to the organoid medium underneath the culturing insert. Full length recombinant human osteoglycin (OGN) produced in yeast (Orbyt, ORB383003) or a His-tagged functional fragment (aa 180-298) produced in *E. coli* (LSBio, LS-G15022)

15 was used at the concentration indicated. The total number of organoids per well was counted manually 14 days after seeding using light microscopy at 20x magnification. Organoid diameter was measured at the same day using NIS-Elements software. The organoid number represents the ability of alveolar epithelial progenitor cells to be activated and form organoids,

20 whereas organoid size is a measure for organoid swelling or proliferation.

Cigarette smoke extract

To generate 100% cigarette smoke extract (CSE), the smoke from two 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, 25 Lexington, KY) without a filter was introduced into 25 mL of warm fibroblast culture medium. The smoke was delivered into the medium using a peristaltic pump (Watson Marlow 323 E/D, Rotterdam, The Netherlands) at a speed of 45 rpm. CSE was freshly prepared before each set of experiments. For organoid experiments, we have used 5% CSE in organoid

30 growth medium.

Organoid resorting to regain fibroblasts and epithelial cells

For organoid resorting, a mixture of 300,000 Epcam⁺ cells and 300.000 CCL206 fibroblasts was seeded in a 1 mL solution of Matrigel diluted 1:1 (v/v) with DMEM/F12 (supplemented with 10% FBS) and added to one well of a 6-well plate. After the Matrigel solidified for an hour, 2 mL of organoid culture medium was added on top of the Matrigel, including OGN (10 µg/mL). After three days, dispase (Corning, 354235) was added to each well for 30 mins at 37 °C to dissociate the Matrigel. MACS buffer (MACS rinsing solution (Milteny Biotec, 130-091-222) premixed with BSA (Milteny Biotec, 130-091-376)) was added to stop the dispase activity. Organoids were collected and centrifuged at 300 g for 5 mins. The pellets were resuspended in 5 mL diluted trypsin (1:5 in PBS, v/v) (T7409, Sigma-Aldrich) for 5 mins at 37 °C, after which 9 mL DMEM/F-12 supplemented with 10% FBS was added to neutralize trypsin action. The cell pellets were incubated with CD326 microbeads for 20 mins and resuspended in MACS buffer. The cell suspensions were introduced to the QuadroMACS™ Separator system to obtain CD326⁻ fibroblasts and CD326⁺ (Epcam⁺) epithelial cells derived from organoids, which were then used for further experimentation.

Bulk RNA sequencing analysis

Total RNA was extracted from cells resorted from organoids using the NucleoSpin RNA isolation kit (Bioké, 740955.50) according to the manufacturer's instructions. Bulk RNA sequencing (RNAseq) on resorted Epcam⁺ and fibroblasts from organoids was performed by GenomeScan (www.genomescan.nl) using an Illumina NovaSeq 6000 sequencer. The analysis procedure comprised several steps, including data quality control, adapter trimming, alignment of short reads, and feature counting. To ensure the integrity of the library preparation, calculations were performed to assess the ribosomal (and globin) content. Additionally, checks were conducted to identify potential samples and barcode contaminations. Quality control tools, such as FastQC v0.34 and FastQA, were employed to establish a set of standard quality metrics for the raw dataset. Prior to

alignment, Trimmomatic v0.30 was utilized to remove adapter sequences from the reads. The reads of each sample were aligned against the ensemble mouse reference GRCm38 (patch 6). Principal component analyses were performed in R using the R package DESeq2 in order to visualize the overall effects of experimental covariates as well as batch effects. The same R packages were used to identify differentially expressed genes (DEGs) between control and treated samples following standard normalization procedures. Gene set enrichment analysis (GSEA) of the top 50 differentially regulated genes was performed with ShinyGO 0.77 (www.bioinformatics.sdstate.edu/go/). Kyoto Encyclopedia of Genes and Genomes (KEGG) was used as reference database, and the statistically significant pathway enrichment with FDR q value < 0.05 are reported.

Immunohistochemical staining on human lung slides

Control and COPD human lung tissue was obtained from leftover material at the UMCg and St. Mary's Hospital, Mayo Clinic Rochester, MN. This staining was part of the HOLLAND (HistopathOlogy of Lung Aging aNd COPD) cohort. Lung tissue was embedded in paraffin and cut into 6 μ m thick sections. These sections were deparaffinized and rehydrated, after which antigen retrieval was performed with 10 mM citrate buffer (pH 6). Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxidase (H_2O_2), followed by overnight incubation at 4 °C with a primary OGN antibody (1:400) (Abcam, ab168348) in 1% BSA-PBS. Subsequently, sections were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:100 in 1% BSA-PBS containing 2% normal human serum. Staining was then visualized upon 5 mins incubation with Vector NovaRED Substrate (Vector Laboratories, SK-4800). Sections were then counterstained with hematoxylin, mounted and scanned using a Hamamatsu NanoZoomer 2.0HT digital slide scanner at 40x magnification.

30

OGN expression analysis in human lung slides

Briefly, Aperio ImageScope software V.12.4.3 (Leica Biosystems) was used to extract images from the scans. For whole lung tissue analysis, scanned images were used after removal of artefacts. This step was followed by
 5 extracting specific areas, including airway wall, bronchial epithelium, and blood vessels, using Adobe Photoshop software (Adobe Inc. CA), to analyze OGN expression in the parenchyma. Fiji/ImageJ software was used to quantify the intensity and area of positive staining of OGN in whole tissue and parenchyma. Analysis of the stained human lung slides was performed
 10 according to Ngassie *et al.* (Am J Physiol Lung Cell Mol Physiol. 2023 June 1;324(6):L799-L814). The formula used to calculate the percentage of area stained positive for the protein is as follows:

$$Area (\%) = \frac{Area (Nova Red)}{Area (Total)} \times 100\%$$

Precision-cut lung slices

PCLS were obtained from naive C57BL/6J mice (eight to fourteen weeks old). A total of twelve mice (male and female, ratio 1:1) were used for PCLS experiments. Murine lungs were inflated with 1.5 mL 1.5% (w/v) low melting agarose solution (Gerbü Biotechnik). After inflation, the agarose
 20 was allowed to solidify at 4 °C for 15 mins before the lungs were harvested. A tissue slicer (Leica VT 1000 S Vibratome line) was used to cut lung slices with a thickness of 250 µm. The lung slices were extensively washed and subsequently cultured in DMEM (Gibco, 42430-025) supplemented with sodium pyruvate (1 mM), MEM non-essential amino acids mixture (1:100,
 25 Gibco, 11140-050), gentamycin (45 µg/mL, Merck, G1397), penicillin/streptomycin (100 U/mL), and amphotericin B (1.5 µg/mL, Gibco, 15290-026) in 12-well culture plates, using three slices per well. Slices from the same mouse were matched based on lung region (middle region of the left lung lobe or middle region of superior right lung lobe). To induce
 30 emphysematous changes, matched slices were treated with 2.5 µg/mL

elastase for 16h. Slices were treated with 10 µg/mL OGN or 4.48 µg/mL OGN active fragment for 40h, overlapping with the 16h elastase treatment.

Immunofluorescence staining PCLS

- 5 PCLS were fixed for 15 mins at 4 °C with 4% paraformaldehyde (Sigma Aldrich, P6148) and then washed with PBS. To visualize the parenchyma of PCLS, actin filaments were stained with Alexa Fluor™ 488 Phalloidin (Thermo Fisher, A12379) for 15 mins at room temperature. After incubation, the slices were washed with PBS and transferred onto a glass slide with two
10 drops of mounting medium containing DAPI (Abcam, 104139). Fluorescence imaging was performed using a confocal laser scanning microscope equipped with a true confocal scanner (SP8 Leica) using a 20x lens. All images were acquired within the linear range, with an image resolution of 1024 x 1024 pixels, and a pinhole size of 1 Airy unit to avoid local saturation. The
15 presented images represent a single z-scan.

mRNA isolation and real-time PCR

- Total RNA was isolated from PCLS using the Maxwell 16 instrument and corresponding Maxwell 16 LEV simply RNA tissue kit, suitable for
20 automated purification according to manufacturer's instructions. Total RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer. Equal amounts of total mRNA were then reverse transcribed (Promega, Madison, USA0), and cDNA was subjected to real-time qPCR. Real time PCR was performed with SYBR green as the DNA
25 binding dye (Roche Applied Science, Mannheim, Germany) on a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems), with denaturation at 94 °C for 30 seconds, annealing at 59 °C for 30 seconds and extension at 72 °C for 30 seconds for 45 cycles followed by 10 minutes at 72 °C. Real-time qPCR data were analysed using the Ct method. The amount of
30 target gene was normalized to the endogenous reference genes *B2M* and *18S*. The nucleotide sequences of the forward and reverse primers used are listed in Table 1.

Table 1: primer sequences

Target Gene	Forward primer	Reverse primer
<i>B2M</i>	5'-ATGGGAAGCCGAACATACTG-3'	5'-CAGTCTCAGTGGGGGTGAAT-3'
<i>18S</i>	5'-AAACGGCTACCACATCCAAG-3'	5'-CCTCCAATGGATCCTCGTTA-3'
<i>Aqp5</i>	5'-CTCACTGGGTCTTCTGGGTAG-3'	5'-TGCCGGTCAGTGTGCC-3'
<i>Rage</i>	5'-CACAGGCTCTGTGGGTGAG-3'	5'-TTCAGCTCTGCACGTTCCCTC-3'
<i>Con43</i>	5'-TCCTTTTCGTTTGACTTCAGCCTC-3'	5'-TCTGAAAATGAAGAGCACCGACA-3'
<i>Sftpc</i>	5'-GGAGCACCGGAAACTCAGAA-3'	5'-GGAGCCGCTGGTAGTCATAC-3'

In vivo Elastase mouse model

- 5 For this study C57BL/6J male and female mice (ratio 1:1) were included and randomly allocated into six experimental groups. Pulmonary emphysematous changes were induced by intratracheal instillation of pancreatic porcine elastase (40 U/kg body weight) in 40 µL sterile PBS on day zero. Animals were treated every other day from day zero till day nine (5 treatments in total) with OGN fragment (6.75 µg or 20.25 µg). After ten days, mice were sacrificed by exsanguination under anaesthesia, after which the therapeutic effects were examined. To ensure objective analysis, samples for *in vivo* experiments were blinded before analysis.

15 *Lung function measurements*

Respiratory function was measured using a FlexiVent system module 2 (Scireq). Mice were anesthetized with Dexdomitor® and Ketamine® and a muscle relaxant, rocuronium bromide (Fresenius Kabi, 10 mg/mL) was

administered. Mice were ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/min in order to reach a mean lung volume similar to that of spontaneous breathing. Lung function parameters were assessed using pre-installed protocols for SnapShot, Primewave perturbation, and forced expired volume maneuver using the Flexiware V8.3.0 software. Three recordings per animal were taken.

Fibrotic effects of OGN and its active fragment in human fibroblasts

The human fetal mesenchymal lung fibroblast MRC-5 cell line (Sigma Aldrich, 05081101) was cultured in Ham’s F12 medium (Thermo Fisher, 11320033) supplemented with 10% (v/v) FBS, 100 U/mL penicillin/streptomycin (P/S), and 2 mM L-glutamine within a humidified atmosphere under 5% CO₂/95% air at 37 °C. Once the cells grew confluent, 300,000 fibroblasts were seeded into 6-well plates (Greiner Bio-one, 657160). After 72 hours, the MRC-5 cells were starved in DMEM/F12 containing 0.5% HI-FBS, 2% P/S, and 1% L-glutamine for 24 hours. The next day, cells were treated with the following treatments for 48 hours:

Condition	Concentration
Control	-
TGF-beta	2 ng/mL
Osteoglycin	10 µg/mL
Osteoglycin fragment	4.5 µg/mL
TGF-beta + osteoglycin	2 ng/mL + 10 µg/mL
TGF-beta + osteoglycin fragment	2 ng/mL + 4.5 µg/mL

RT qPCR in MRC5 cells

TRIzol (Invitrogen, Waltham, MA, USA, 15596018) was added into each well, leading to cell lysis. mRNA was extracted into the TRIzol, pipetted into
5 an Eppendorf cup and centrifuged for 10 minutes at 4 °C and 12,000 rcf using the 5427R centrifuge (Eppendorf SE, Hamburg, Germany). The supernatant was poured into a new cup and 1-bromo-3-chloropropane (Sigma-Aldrich, B9673) was added to each cup. After vortexing, the cups were left on room temperature for 10 minutes followed by 15 minutes
10 centrifuging at 4 °C and 12,000 rcf. Next, the aqueous phase was transferred to a new cup and ice-cold isopropanol (Biosolve, Valkenswaard, The Netherlands, 162606) was added. Again, the cups were vortexed and put on ice for 10 minutes followed by 8 minutes of centrifuging at 4 °C and 12,000 rcf. Isopropanol was carefully aspirated, leaving a transparent pellet
15 behind. To wash the mRNA-pellet, 80% ice-cold RNase free ethanol (VWR international B.V., Leuven, Belgium, 8025.2500) was added initially, subsequently centrifuged for 5 minutes at 4 °C and 7,500 rcf and finally removed. These washing steps were repeated one more time. After the ethanol was evaporated, the mRNA pellet was resuspended in 30 µL RNase
20 free water (Macherey-Nagel, Dueren, Germany, 740378.1000).

Equal amounts of total mRNA (1 µg) were then reverse transcribed, and the obtained cDNA was subjected to real time (RT) qPCR. QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems), using RealQ Plux 2x Master
25 Mix Green as DNA binding dye (Ampliqon, 5000840-1250), was used for RT qPCR, with denaturation at 94 °C for 30 seconds, annealing at 64 °C for 30 seconds and extension at 72 °C for 30 seconds for 45 cycles followed by 10 mins at 72 °C. RT qPCR data were analyzed using the Ct method and normalized to the endogenous references genes *B2M* and *18S*. The specific
30 forward and reverse primers (1 µM) used are listed in Table 2 below.

Table 2 : PCR primer sequences

Name	Species		Sequence
Alpha-smooth actin	Homo Sapiens	Forward	GACCCTGAAGTACCCGATAGAAC
		Reverse	GGGCAACACGAAGCTCATTG
Collagen 1a1	Homo Sapiens	Forward	AGCCAGCAGATCGAGAACAT
		Reverse	TCTTGCCTTGGGGTTCTTG
Fibronectin	Homo Sapiens	Forward	TCGAGGAGGAAATTCCAATG
		Reverse	ACACAGGTGCACCTCATCAT
TGF-beta	Homo Sapiens	Forward	TACCTGAACCCGTGTTGCTCTC
		Reverse	GTTGCTGAGGTATCGCCAGGAA
18S	Homo Sapiens	Forward	CGCCGCTAGAGGTGAAATTC
		Reverse	TTGGCAAATGCTTTCGCTC
B2M	Homo Sapiens	Forward	CCACTGAAAAAGATGAGTATGCCT
		Reverse	CCAATCCAAATGCGGCATCTTCA

Statistical analysis

5 All data is presented as mean ± SEM. All data was assessed for statistical significance using one-way analysis of variance (ANOVA) with Geisser Greenhouse correction. Statistical analyses were performed with GraphPad Prism 10 software.

10

EXAMPLE 1: Ligand screening in murine organoids

Using proteomics analysis, we identified a wealth of different growth factors and cytokines present in the secretome of alveolar lung fibroblasts. Based on
15 a proteomics-guided ligand target strategy and a literature search, we identified potential therapeutic drug targets to be studied further in our epithelial organoid model. Figure 16 represents the ligand target strategy that was performed to identify potential therapeutic targets. Since the

corresponding receptor of osteoglycin is currently unknown, we included this protein in our drug screening.

Figure 1 shows the bright field pictures of the organoid culture at day 14.

- 5 The control panel shows an organoid culture that did not receive any treatment. The panels show organoid cultures that received a treatment with several identified recombinant growth factors with every medium change. On day 14, the colony forming efficiency of organoids was quantified and the size of the obtained organoids was determined (Figure 2).
- 10 Surprisingly, it was observed that among all proteins tested in the secretome of lung fibroblasts, there is one that appears to induce an exceptionally strong effect on regenerative responses in our organoid culture. The name of this protein is osteoglycin (OGN).
- 15 These data confirmed an exceptional effect of treatment with OGN (10.0 µg/mL), with a significant increase in the number of organoids by $73.00 \pm 12.75\%$ ($p = 0.018$). As compared to control organoids, organoid size of organoids treated with OGN were significantly smaller ($189.80 \pm 9.63 \mu\text{m}$ versus $157.50 \pm 6.20 \mu\text{m}$, $p = 0.0021$).

20

EXAMPLE 2: OGN and fragment dose response curve in murine organoids.

- This example shows that OGN and an active fragment thereof induce its
- 25 supportive effect in a concentration-dependent manner. A C-terminal 15.1

kDa fragment of OGN comprising residues 180-298 including LRR4-7, was commercially obtained from LS Bio, Shirley, USA. Three different equimolar concentrations of OGN and fragment were used: 3, 30, and 300 nM)

- 5 With increasing doses of OGN or OGN fragment, an enhanced number of organoids that were formed were observed (see Figure 3A and B). Treatment with the highest concentration of OGN or fragment significantly increased the CFE of organoids (Figure 3A).

10 **EXAMPLE 3: OGN and fragment dose response curve in presence of cigarette smoke extract and TGF-beta in murine organoids.**

This example demonstrates that both the full length OGN protein or a C-terminal 15.1kDa fragment thereof (residues 180-298) have regenerative
15 effects in a murine organoid model, which effect is maintained in the presence of CSE (Figure 4). Three different equimolar concentrations of OGN and fragment were used: 3, 30, and 300 nM). As shown in Figure 4, increasing concentrations of OGN protein or OGN fragment led to a significant increase in the CFE of organoids generated in the presence of
20 CSE. Additionally, staining for pro-surfactant protein C, an alveolar marker, revealed that OGN and its active fragment enhance the proportion of organoids expressing this marker. This implies increased numbers of alveolar organoids within the treated organoids.

- 25 In addition to CSE, the organoid culture was exposed to the COPD-related cytokine transforming growth factor β (TGF-beta), which yielded significantly less organoids (Figure 5). Increasing concentrations of OGN counteracted the adverse effects of TGF- β on CFE, without affecting organoid diameter (Figure 6). Treatment with OGN increased the
30 proportion of SPC⁺ organoids in the presence of TGF- β (Figure 6).

EXAMPLE 4: Denatured OGN does not increase organoid formation

As a negative control, OGN (300 nM) was denatured by heating the protein for 10 minutes at 95 °C. Treating organoids with denatured OGN resulted in no significant changes in the number and size of the organoids that were
5 formed (Figure 6)

EXAMPLE 5: Regenerative effect of OGN and fragment on human organoids.

10 Next, the supportive effect of OGN and fragment in the formation of organoids that are derived from human lung tissue from COPD IV patients was investigated. As shown in Figure 7, OGN and its active fragment supported the formation of human alveolar epithelial organoids from 5 donors, which is a rather extraordinary effect in this human organoid
15 model. There does not seem to be an effect on organoid size in the human organoid model.

EXAMPLE 6: Effect of OGN and its fragment on gene expression in precision-cut lung slices (PCLS).

20 Whereas organoids represent a valuable and well-controlled model, its complexity and translational value have some limitations compared to tissue explants or animal models. Therefore, we also applied precision-cut lung slices (PCLS) from murine origin to demonstrate the regenerative
25 potential of OGN. The advantage of PCLS is that all lung cell types are present in their natural architecture, and cell-cell contacts and cell-matrix interactions are preserved. Our previous experience with PCLS demonstrated that exposure to elastase induces emphysema and alterations in gene expression of alveolar epithelial markers (Van Dijk *et al.*, Front
30 Physiol. 2017 Jan 4;7:657).

Lung slices were treated with elastase for 16h to induce emphysema. OGN (300 nM) was added from the start for a total treatment

time of 40h. Figure 8A shows the gene expression levels for alveolar type (AT) I (Aqp5, Rage, and Con43) and ATII (Stfpc) cell markers. Elastase treatment decreased gene expression of ATI and ATII cell markers, whereas the presence of OGN during enzyme treatment could prevent this effect. As these genes are markers for alveolar epithelial cells, a decreased expression of these markers due to treatment with elastase indicates parenchymal damage. Treatment with OGN increases expression of the alveolar epithelial cell markers, which indicates a protective effect on parenchymal damage.

Furthermore, we determined the expression of OGN in lung slices that were treated with elastase versus control (Figure 8B). Interestingly, elastase-treated slices that were emphysematous showed a decreased expression of osteoglycin.

EXAMPLE 7: Effect of OGN and its active fragment on elastase-induced injury in PCLS

After 16h incubation with elastase, parenchymal tissue damage indicative of emphysema was induced, as evidenced by a significant increase in LMI (Figure 9). Interestingly, treatment with OGN or active fragment for 40h, during which the first 16h were concomitant with elastase exposure, effectively prevented the induction of lung tissue injury (Figure 9).

EXAMPLE 8: Effect of OGN fragment on elastase induced injury in mice.

We evaluated whether a fragment of OGN could improve lung function parameters in a murine model of elastase-induced lung injury (Eur. J. Pharm.Vol. 974, 2024, 17661). Upon inducing lung injury with elastase, mice received low (4.5 µg) or high (20.5 µg) doses of functional OGN fragment (Figure 10A). We assessed several lung function parameters. The forced expiratory volume at 0.2 second (FEF0.2) significantly increased upon

elastase exposure, while the forced vital capacity (FVC) and forced expiratory flow at 50% of forced vital capacity (FEF_{50%}FVC) were significantly decreased (Figure 10B-D). Treatment with high doses of OGN fragment reversed the negative effects of elastase on lung function significantly (Figure 10B-D). In addition, elastase treatment induced a decrease in elastic recoil, shown by the pressure-volume loops of emphysematous mice displaying a typical significant upward shift (Figure 10E), suggesting that elastase diminished the distensibility of the lungs. This upward shift of the pressure-volume loop observed in mice with emphysema was absent in mice treated with high doses of OGN fragment. Taken together, the active fragment of OGN improves the lung function of mice with elastase-induced lung injury.

EXAMPLE 9: OGN expression in human lung sections.

To study the impact of tobacco smoking on OGN expression, OGN expression in lung tissue of never-, current, and ex-smoker donors was determined (Figure 11A-C). Image analysis of whole lung tissue and parenchyma sections from never, current, and ex-smoker donors revealed proportionally lower percentage of OGN-positive area in tissue from current smoker donors compared to those from never smoker controls (Figure 11D-E).

Interestingly, the average OGN expression in the lung tissue of ex-smokers was lower compared to that of never smokers (not statistically significant), implying a persistent impact of cigarette smoke on OGN expression. Quantitative analysis of the average intensity of OGN-staining indicated that there were no overall differences between never, current, and ex-smoker lung tissue in whole lung tissue or parenchyma (Figure 11F-G). Next, we assessed whether OGN expression changed in patients with moderate-severe COPD or severe-early onset (SEO-)COPD compared to ex-smoker control lung tissue (Figure 11H-I). In patients with moderate-severe

COPD or SEO-COPD, no significant differences were observed in OGN expression in both whole lung and parenchyma (Figure 11J-M and 11N-Q). However, there was a tendency toward lower average expression in the parenchyma ($p=0.09$) of SEO-COPD patients (Figure 11O). Interestingly, there was a tendency for increased intensity in the whole lung tissue ($p=0.09$), whereas the average intensity of the staining was significantly enhanced in the parenchyma (Figure 11P-Q).

Taken together, this indicates that there may be a lack of OGN in emphysematous lungs, which may reduce the regenerative capacities of the human lung in COPD.

EXAMPLE 10: Lack of fibrotic effects of OGN.

Although OGN has not previously been associated with COPD, it was suggested in the art to be implicated in pulmonary fibrosis. More in particular, Shi *et al.* (Am J Physiol Cell Physiol. 2020 Nov 1;319(5):C895-C905) reported that microRNA-140 inhibits lung fibroblast proliferation and promotes pulmonary fibroblast apoptosis through direct inhibition of OGN expression. Therefore, the present inventors set out to study potential adverse effect of OGN protein in the induction of pulmonary fibrosis. To that end, gene expression of fibrotic markers upon treatment with OGN or active fragment was evaluated using human lung fibroblasts (MRC5). As expected, treatment with TGF-beta (2 ng/mL), known to induce fibrosis, increased gene expression of alpha-smooth-actin and fibronectin, and showed a tendency towards increased collagen 1a1 expression (Figure 12). TGF-beta gene expression remained unaltered. Notably, treatment with OGN or its active fragment did not change the gene expression of fibrotic markers (Figure 12). Interestingly, co-treatment of TGF-beta and OGN fragment normalized the increase in gene expression of fibrotic markers.

Furthermore, the proportion of fibroblasts and myofibroblasts were predicted based on transcriptomic analysis from fibroblasts resorted from organoids treated with OGN. To this extent, a gene signature based on the top ten genes associated with fibroblasts or myofibroblasts. This analysis
5 shows that the proportion of fibroblasts with a myofibroblast gene signature was lower compared to vehicle (Figure 13). Taken together, these data indicate that, at least on a transcriptomic level, OGN and its active fragment do not alter the fibrotic state of lung fibroblasts.

10 **EXAMPLE 11: Spray drying of active fragment**

Spray drying of proteins using excipients provides a stable formulation that is suitable for respiratory administration. In order to spray dry the active fragment of OGN, the fragment was dissolved in 20 mM HEPES at pH 7.4 containing the stabilizers inulin 4 kDa or 2.5 mM mannitol, and leucine.
15 Spray drying was conducted utilizing a Büchi B-290 mini spray dryer in a closed-loop configuration, equipped with a high-performance cyclone, a B-295 inert loop, and a B-296 dehumidifier.

To investigate whether the therapeutic potential of the active fragment was
20 preserved during spray drying, murine lung organoids were treated and assessed for organoid count. Immediately after spray-drying the fragment with inulin or mannitol, the fragment was added to the organoid medium. Treatment with spray dried OGN fragment increased organoid formation, using either mannitol or inulin as excipient. The number of organoids that
25 were formed were comparable to “fresh” fragment, which was not spray-dried (Figure 14).

To test whether the obtained spray dried formulation was stable, the spray-dried fragments were subjected to storage for one week under two distinct
30 conditions: 60 °C with 0% relative humidity and 30 °C with 43% relative humidity. Upon storage for one week under extreme conditions, the spray-

dried fragment of OGN still induced organoid formation, indicating that the formulation is stable (Figure 15).

EXAMPLE 12: Pharmaceutical compositions comprising OGN.

5

A. Dry powder composition for inhalation of OGN polypeptide

An inulin solution was prepared by dissolving 1.75 g inulin in 28.4 mL, 80 °C Millipore water. The solution was cooled down and 47 mg sodium chloride was added. Subsequently, 700 µL of a 0.1 M HEPES buffer solution (pH 7.5) and 700 µL of a 0.01% (v/v) Tween 80 solution were added. Finally, 10 mL of an aqueous OGN solution (1.77 mg/mL) was added to the solution, resulting in a formulation containing OGN:inulin 1:99 (w/w).

The solution was sprayed into a stainless steel vessel containing liquid nitrogen with the 0.5 mm two-fluid nozzle placed approximately 5 cm above the surface of the liquid nitrogen. The resulting suspension was transferred to a freeze dryer (Christ model Alpha 2–4, Salm & Kipp, Breukelen, The Netherlands), precooled at a shelf temperature of –80 °C. After evaporation of the liquid nitrogen, primary drying was performed for 32 h by gradually increasing the temperature from –40 °C to 5 °C at 0.220 mbar pressure. Secondary drying was done for 12 h at 0.055 mbar pressure with a gradual increase of temperature from 5 °C to 20 °C. The resulting powder was suitable for inhalation using the Twincer® dry powder inhaler, which is a cyclone based dry powder inhaler device.

25

B. Liquid composition formulated for delivery of OGN mRNA

Lipid nanoparticles (LNPs) containing mRNA encoding for OGN were produced with an N/P ratio of 11.8. The lipids 1,2-Distearoyl-*sn*-glycero-3-phosphocholine, (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate, cholesterol and 1,2-distearoyl-*rac*-glycerol-3-methoxypolyethylene glycol-2000, were dissolved in ethanol in a molar ratio of 8:52:39:1. mRNA encoding for OGN was dissolved at 7.5 µg/mL in 20 mM

citric acid buffer (pH 3.2) as the aqueous phase. The two phases were rapidly mixed through Nanoassembler® Benchtop (Precision NanoSystems, Inc.) at a volume ratio of 3: 1 (mRNA: lipid) with a total flow rate of 4 mL/min. After dialysis against phosphate-buffered saline (PBS) pH 7.4
5 overnight, the solutions were finally concentrated through ultrafiltration and re-suspended in PBS.

Claims

1. Osteoglycin (OGN; Osteoinductive factor; mimecan), or a
5 functional fragment thereof, for use as an agent to promote the regeneration
of lung tissue.
2. OGN or a functional fragment thereof for use according to claim 1,
to enhance expansion of lung stem cells into airway and/or alveolar
10 epithelial cells.
3. OGN or a functional fragment thereof for use in a method for treatment
of a disease involving defective lung tissue repair.
- 15 4. OGN or a functional fragment thereof for use according to claim 3,
wherein the disease involves defective lung tissue repair and/or lung
function decline.
5. OGN or a functional fragment thereof for use according to claim 4,
20 wherein the disease is selected from the group consisting of COPD, lung
fibrosis, cystic fibrosis, lung repair post-pneumonia, acute lung
injury/ARDS, long-COVID and sarcoidosis.
6. OGN for use according to any one of the preceding claims, wherein
25 said OGN or functional fragment thereof comprises at least residues
Leu180-Phe298 of the sequence accessible in GenBank under the number
AAH37273.1 (Osteoglycin [Homo sapiens]).
7. OGN for use according to any one of the preceding claims,
30 comprising administering said OGN or a functional fragment thereof as
protein, or as DNA or mRNA encoding the OGN protein or functional
fragment thereof.

8. OGN for use according to claim 7, wherein said OGN or functional fragment thereof is administered as protein.
- 5 9. A pharmaceutical composition comprising OGN polypeptide or a functional fragment thereof, and a pharmaceutically acceptable carrier, vehicle or diluent, wherein said composition is formulated for pulmonary delivery.
- 10 10. Pharmaceutical composition according to claim 9, wherein said composition is formulated in the form of a powder for inhalation, or as a liquid for nebulization.
11. Pharmaceutical composition according to claim 9 or 10,
15 comprising OGN polypeptide or a functional fragment thereof in a dissolved or dry state stabilized by one or more excipient(s), preferably selected from the group consisting of extremolytes, surfactants, sugars, sugar alcohols or buffers, or by incorporation into amorphous sugar glasses containing mono-, di-, or polysaccharides, buffers, amino acids, or inert peptides or proteins.
- 20 12. Pharmaceutical composition according to claim 11, comprising OGN polypeptide or a functional fragment thereof in a dry state stabilized by incorporation in a matrix comprising sugars, such as trehalose, and/or polysaccharides such as inulin or pullulan, and/or polyols such as mannitol,
25 and/or buffers, and/or amino acids.
13. Pharmaceutical composition according to any one of claims 9-12, further comprising one or more additional therapeutically active agent(s), preferably selected from the group consisting of bronchodilator drugs and
30 anti-inflammatory compounds.

14. A medical device comprising a pharmaceutical composition according to any one of claims 9-13.

15. Medical device according to claim 14, being a nebulizer, a
5 metered-dose inhaler (MDI), or a dry powder inhaler (DPI).

16. A packaged pre-metered OGN composition in a dose for use in combination with the medical device according to claim 15.

10 17. A method for treatment of a disease involving defective lung tissue repair, the method comprising administering to a subject in need thereof a therapeutically effective amount of OGN or a functional fragment thereof.

15 18. The method according to claim 17, comprising administering OGN or a functional fragment thereof by pulmonary delivery.

19. The method according to claim 17 or 18, comprising administering a pharmaceutical composition according to any of claims 9-13.

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20. The method according to any one of claims 17-19, wherein the disease is selected from the group consisting of COPD, lung fibrosis, cystic fibrosis, lung repair post-pneumonia, acute lung injury/ARDS, long-COVID and sarcoidosis, preferably wherein the disease is COPD.

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