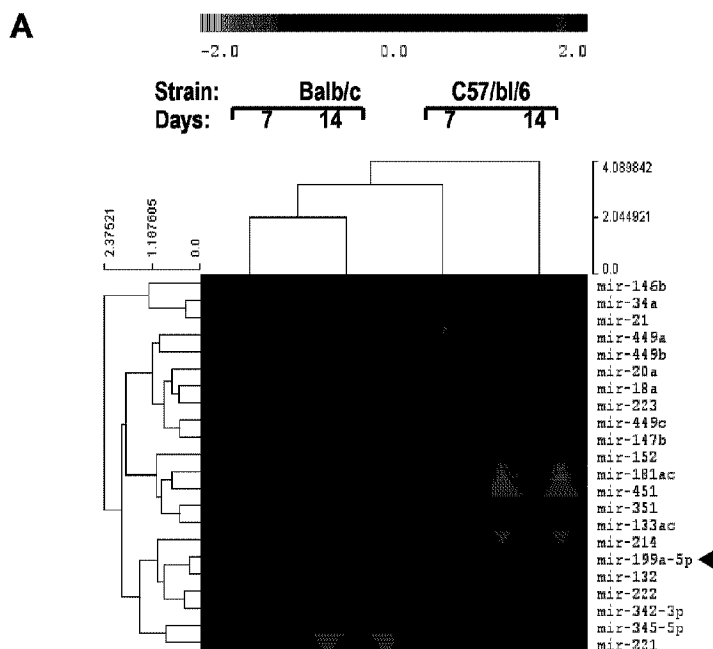




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(54) **Titre : UTILISATION DE L'EXPRESSION MIR-199A-5P, CIBLES ET/OU INHIBITEURS DE CELLE-CI POUR LE DIAGNOSTIC, LE PRONOSTIC ET LE TRAITEMENT DE TROUBLES FIBROPROLIFERATEURS**  
(54) **Title: USE OF MIR-199A-5P, TARGETS AND/OR INHIBITORS THEREOF FOR THE DIAGNOSIS, PROGNOSIS AND TREATMENT OF FIBROPROLIFERATIVE DISORDERS**



(57) **Abrégé/Abstract:**

The present invention relates to the use of the miRNA expression profile, particularly of miR-199a-5p, and the target genes regulated thereby for the diagnosis, prognosis and use of miR-199a-5p inhibitors for treating fibroproliferative disorders.



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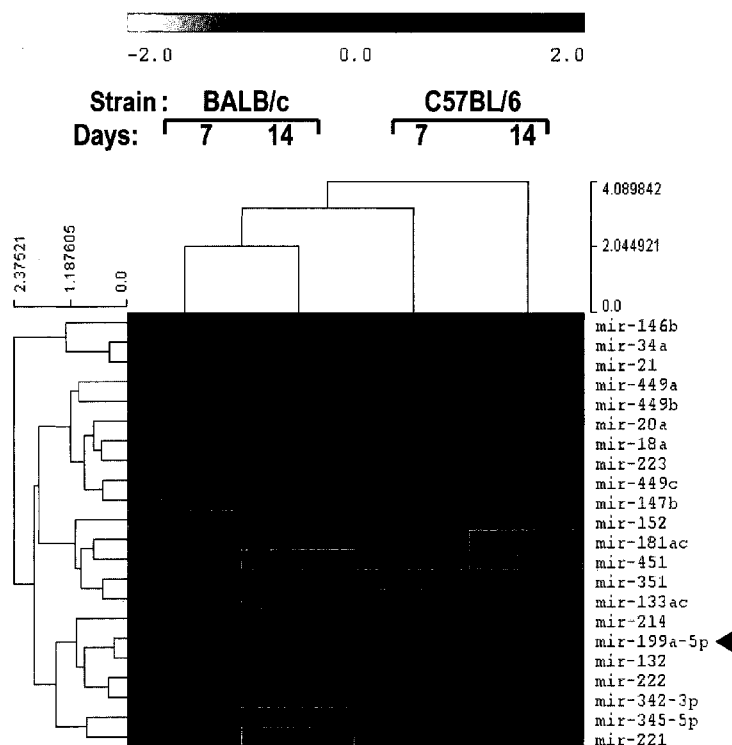
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(54) Title: USE OF miR-199a-5p, TARGETS AND/OR INHIBITORS THEREOF FOR THE DIAGNOSIS, PROGNOSIS AND TREATMENT OF FIBROPROLIFERATIVE DISORDERS



(57) Abstract: The present invention relates to the use of the miRNA expression profile, particularly of miR-199a-5p, and the target genes regulated thereby for the diagnosis, prognosis and use of miR-199a-5p inhibitors for treating fibroproliferative disorders.

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**USE OF miR-199a-5p, TARGETS AND/OR INHIBITORS THEREOF  
FOR THE DIAGNOSIS, PROGNOSIS AND TREATMENT OF  
FIBROPROLIFERATIVE DISORDERS**

**Field of the invention**

The present invention relates to the field of fibroproliferative disorders, particularly idiopathic pulmonary fibrosis (IPF), and the role of microRNA (miRNA) in the tissue fibrosis process.

5       The present invention relates more specifically to the use of microRNA (miRNA), particularly miR-199a-5p, targets and/or inhibitors thereof for the diagnosis, the prognosis and treatment of fibroproliferative disorders, namely idiopathic pulmonary fibrosis.

10       In the description hereinafter, the references between square brackets ([ ]) refer to the list of references given at the end of the text.

**State of the related art**

15       Tissue fibrosis, defined as the excessive persistent formation of non-functional connective scar tissue in response to a chronic tissue lesion, is a major cause of morbidity and mortality associated with loss of function of the damaged organ in various  
20 disorders such as those affecting the pulmonary interstitium [Wynn, J. Clin. Invest., 117: 524-529, 2007] [1]. Fibroproliferative disorders are a major public health problem. Indeed, in the United States alone, 45% of deaths are attributed to  
25 fibroproliferative disorders and the prevalence thereof

is constantly increasing [Wynn, Nat. Rev. Immunol., 4: 583-594, 2004] [2].

Of the pulmonary interstitial disorders of unknown origin, idiopathic pulmonary fibrosis (IPF) is the most frequent and lethal form with an average survival rate of 3 to 5 years post-diagnosis [Wilson and Wynn, Mucosal. Immunol., 2: 103-121, 2009] [3]. IPF is a chronic and frequently fatal lung disease characterised by fibroblast proliferation and excessive extracellular matrix protein deposition. IPF is a rare disorder with a prevalence of 13 to 20 cases per 100,000 inhabitants and whose causes remain poorly understood, and for which no effective treatment is currently available.

Observations based on animal pulmonary fibrosis models and lung sections of patients with IPF suggest a dynamic biopathological process involving excessive scar tissue formation with chronic inflammation, epithelial and endothelial cell apoptosis, mesenchymatous cell proliferation and activation with fibroblast/myofibroblast site formation, and finally excessive extracellular matrix deposition resulting in the destruction of the lung structure and loss of lung function. In physiopathological terms, at the present time, repeated alveolar epithelium damage is considered to be responsible for lesions on the pulmonary epithelium promoting alveolar plasma exudate formation and clotting process activation, secretion of growth factor such as TGF $\beta$  by pneumocytes enabling pulmonary fibroblast recruitment, proliferation and activation and abnormal and excessive extracellular matrix deposition [Wilson and Wynn, 2009, cited above] [3].

During the fibrosis process, the fibroblasts have a myofibroblast phenotype and are organised into fibroblastic foci. Other mechanisms may also be involved in the fibrosis process such as mesenchymal  
5 epithelial transition of epithelial, endothelial or mesothelial cells and pulmonary medullary circulating fibrocyte recruitment [Wilson and Wynn, 2009, cited above] [3].

MicroRNAs (miRNAs) are a class of small non-coding  
10 RNA with approximately 22 bases and having a key role in a wide range of cell phenomena such as development, differentiation, survival, response to stress, apoptosis, proliferation, homeostasis or differentiation [Ambros, Nature, 431: 350-355, 2004]  
15 [4]. Recent studies have identified specific miRNA expression profiles associated with the initiation and progression of various disorders including cancer and inflammatory and autoimmune disorders. Moreover, miRNA function gain and loss studies have revealed pathogenic  
20 miRNA functions accentuating the major role thereof *in vivo*. Preferably, the term "microRNA" or "miRNA", in the context of the present invention, means an RNA oligonucleotide consisting of between 18 to 25 nucleotides in length. In functional terms miRNAs are  
25 typically regulatory endogenous RNA molecules.

The mechanism of action thereof involves the formation of a complex between a plurality of miRNA bases and the 3'-non-coding part of the target mRNA [Brennecke et al., PLoS. Biol., 3: e85, 2005] [5]. This  
30 interaction gives rise to destabilisation of the target mRNA and/or protein synthesis inhibition [Brennecke et

al., 2005, cited above] [5]. Recognition between miRNA and the target thereof is essentially controlled by a sequence of approximately 7 bases, situated in the 5' part of the miRNA (recognition sequence or seed)  
5 [Brennecke et al., 2005, cited above] [5]. For this reason, each miRNA would appear to be capable of regulating the stability of a broad range of distinct mRNAs. The terms "target gene" or "target mRNA" refer to regulatory mRNA targets of microRNAs, in which said  
10 "target gene" or "target mRNA" is regulated post-transcriptionally by the microRNA based on near-perfect or perfect complementarity between the miRNA and its target site resulting in target mRNA cleavage; or limited complementarity, often conferred to  
15 complementarity between the so-called seed sequence (nucleotides 2-7 of the miRNA) and the target site resulting in translational inhibition of the target mRNA.

About 2000 miRNAs have been identified in humans  
20 to date (miRBase release 19 <http://www.mirbase.org>) where they would appear to regulate more than 30% of transcripts. MiRNA regulation is thus considered as a major form of gene expression regulation, the impact of which has been largely underestimated until now [Xie et  
25 al., nature, 434: 338-345, 2005; Berezikov et al., Cell, 120: 21-24, 2005] [6, 7].

MiRNAs are transcribed in the nucleus in the form of long precursors (pri-miRNA) and undergo a first maturation step in the nucleus to produce pre-miRNA  
30 having a smaller hairpin structure. This miRNA precursor is thus exported from the nucleus to the

cytoplasm where it undergoes a final maturation step with Dicer enzyme generating two single-stranded miRNAs (one 5p strand and one 3p strand): the "mature" strand is taken on by a multi-protein complex (RNA Induced Silencing Complex or RISC) interacting with the 3'-non-coding part of the target mRNA, whereas the "star" complementary strand undergoes degradation (annotation: miR-xx\*).

In clinical terms, numerous studies suggest possible use of miRNAs as a diagnostic tool. Indeed, miRNAs display good stability in biological fluids such as serum [Mitchell et al., Proc. Natl. Acad. Sci. U.S.A., 105: 10513-10518, 2008] [8] or urine [Weber et al., Clin. Chem., 56: 1733-1741, 2010] [9]. For this reason, studying the expression thereof in these biological media offers new non-invasive prospects for the development of new diagnostic or prognostic biomarkers. Moreover, miRNA tissue expression profiles could also offer new prognostic or diagnostic tools as already demonstrated in cancer treatment [Lu et al., Nature, 435: 834-838, 2005] [10]. For example, miR-199a-5p, one of the two mature miRNA species derived from the miR-199a precursor, has been associated with malignancy not only in hepatocellular carcinoma [Jiang et al., Clin. Cancer Res., 14(2): 419-427, 2008] [11] but also in bronchial tumours [Mascaux et al., Eur. Respir. J., 33(2): 352-359 (Epub 2008), 2009; Puisségur et al., Cell death Differ., 18(3): 465-478, 2011] [12, 13].

In therapeutic terms, miRNA expression modulation could also enable the development of new treatments

[Krutzfeldt et al., Nature, 438: 685-689, 2005] [14]. For example, the use of miR-122 inhibitor in the development of new hepatitis C treatments has made it possible to obtain a significant reduction in the viral  
5 load of this virus [Lanford et al., Science, 327: 198-201, 2010] [15].

Despite increasing evidence of the involvement of miRNAs in the tissue fibrosis process, the precise role thereof and mechanism(s) of action thereof have yet to  
10 be explored extensively [Jiang et al., FEBS J., 277: 2015-2021, 2010] [16]. Although the cause of IPF is unknown, a central role of miRNAs was recently suggested in the pathogenesis thereof. However, the role of miRNAs in fibrotic disorders, particularly in  
15 pulmonary fibrosis, is poorly documented and only a few miRNAs such as miR-21 or let7-d have been studied to date [Liu et al., J. Exp. Med., 20: 1589-1597, 2010; Pandit et al., Am. J. Respir. Crit. Care Med., 182: 220-229, 2010] [17, 18]. Therefore, miRNAs for which  
20 the expression is associated with pulmonary fibrosis could offer particularly promising tools for the development of new diagnostic and prognostic markers of IPF and new therapeutic strategies for this disorder which continues to be incurable and have a poor  
25 prognosis [Pandit et al., Transl. Res., 157: 191-199, 2011] [19].

### **Description of the invention**

The inventors demonstrated, for the first time and  
30 completely unexpectedly, the role of miRNAs,

particularly miR-199a-5p, and the targets thereof in pulmonary, hepatic and renal fibrosis.

The inventors more specifically used an experimental pulmonary fibrosis model to identify, in the lung, i) miRNAs differentially expressed only in C57BL/6 mice sensitive to bleomycin-induced pulmonary fibrosis and ii) correlated miRNAs during the progression of the fibrotic process. For this purpose, the inventors used various experimental approaches combining miRNA biochips, *in situ* hybridisation, and quantitative RT-PCR.

In this way, they identified an expression profile of 22 specific miRNAs with respect to the pulmonary response of C57BL/6 mice sensitive to bleomycin-induced pulmonary fibrosis.

Among the 22 miRNAs of the identified expression profile, they demonstrated, for the first time, the unique role of miR-199a-5p in the pulmonary fibrosis process. In this way, they identified significant miR-199a-5p up-regulation in the lungs of mice with bleomycin-induced fibrosis. Indeed, miR-199a-5p would appear to be the most discriminatory miRNA between sensitive C57BL/6 mouse strains and resistant Balb/c mouse strains in respect of bleomycin-induced pulmonary fibrosis, thus enabling a distinction between pathological and normal cases.

Significant miR-199a-5p up-regulation was also identified in the lungs of patients suffering from IPF. More specifically, the miR-199a-5p levels increased selectively in the myofibroblasts of damaged lungs.

Consequently, the profibrotic effects of miR-199a-5p were studied further in pulmonary fibroblasts. In this way, they demonstrated pulmonary fibroblast activation to a profibrotic phenotype after miR-199a-5p overexpression. Finally, the inventors demonstrated that miR-199a-5p overexpression partially mimicked the transcriptional signature and cellular effects of TGF $\beta$ , one of the main factors involved in fibrotic mechanisms, which is also capable of increasing miR-199a-5p expression.

They also demonstrated that the role of miR-199a-5p can be extrapolated to other fibrotic disorders in mammals since this miRNA is common to the various forms of pulmonary, hepatic and renal fibrosis; and could thus become a universal marker thereof. Indeed, they demonstrated abnormal miR-199a-5p expression in mouse renal and hepatic fibrosis models, demonstrating that miR-199a-5p deregulation represents a general mechanism contributing to the fibrosis process.

Furthermore, the inventors combined *in silico* approaches (target prediction bioinformatics software) and experimental approaches (transcriptome chips, ectopic miRNA expression and reporter vectors containing the 3'-UTR part of a gene of interest fused with luciferase) in order to determine and characterise the target genes specifically regulated by miR-199a-5p. In particular, variation in expression of the gene coding for caveolin-1 (CAV1), a critical mediator in pulmonary fibrosis, according to the level of miR-199a-5p expression, was observed. In this way, they identified CAV1 as a genuine target of miR-199a-5p.

The present invention thus relates to an *in vitro* diagnostic method for a fibroproliferative disorder in a subject characterised in that it comprises the following steps:

- 5 (i) quantitative measurement of the level of miRNA expression in a biological sample from said subject;
- (ii) optionally determining the miRNA expression profile of said biological sample from said subject;
- (iii) comparing the miRNA expression profile of
- 10 the biological sample from said subject with the same miRNA expression profile of a reference biological sample, said expression profile consisting of mir-146b, miR-34a, miR-21, miR-449a, miR-449b, miR-20a, miR-18a, miR-223, miR-449c, miR-147b, miR-152, miR-181ac, miR-
- 15 451, miR-351, miR-133ac, miR-214, miR-199a-5p, miR-132, miR-222, miR-342-3p, miR-345-5p and miR-221;
- (iv) identifying at least one miRNA for which the level of expression by said biological sample from said subject differs with respect to the level of expression
- 20 of the same miRNA by said reference sample.

According to one particular embodiment, the 22 miRNAs of the specific expression profile for the pulmonary response to bleomycin-induced pulmonary fibrosis are represented by the following accession

25 numbers:

| miRNA name | miRBase accession number |
|------------|--------------------------|
| mir-146b   | MIMAT0003475             |
| mir-34a    | MIMAT0000542             |
| mir-21     | MIMAT0000530             |
| mir-449a   | MIMAT0001542             |
| mir-449b   | MIMAT0005447             |

|             |              |
|-------------|--------------|
| mir-20a     | MIMAT0000529 |
| mir-18a     | MIMAT0000528 |
| mir-223     | MIMAT0000665 |
| mir-449c    | MIMAT0003460 |
| mir-147b    | MIMAT0004857 |
| mir-152     | MIMAT0000154 |
| mir-181ac   | MIMAT0000210 |
| mir-451     | MIMAT0001632 |
| mir-351     | MIMAT0000609 |
| mir-133ac   | MIMAT0000145 |
| mir-214     | MIMAT0000661 |
| mir-199a-5p | MIMAT0000229 |
| mir-132     | MIMAT0000144 |
| mir-222     | MIMAT0000670 |
| mir-342-3p  | MIMAT0000590 |
| mir-345-5p  | MIMAT0000595 |
| mir-221     | MIMAT0000669 |

The term "fibroproliferative disorder" according to the present invention refers to disorders characterised by a parenchymal organ lesion and fibrosis. For example, it refers to pulmonary, hepatic and renal fibrosis, particularly idiopathic pulmonary fibrosis (IPF).

The term "subject" according to the present invention refers to a vertebrate, particularly a mammal, more particularly a human.

The term "biological sample" according to the present invention refers to a bronchial, liver or kidney biopsy. For example, it refers to epithelial tissue, particularly from the respiratory, hepatic or renal epithelium.

The term "reference biological sample" according to the present invention refers to a biological sample as defined above obtained from a healthy subject, i.e. not presenting fibrosis, or a subject in whom the miR-199a-5p expression is known and associated with a particular clinical stage. For example, it refers to the level of miR-199a-5p expression obtained after analysing human biopsy samples (see table 1 hereinafter) or mouse lung samples (see table 2 hereinafter) with "miRNA" biochips (Agilent technology) in healthy subjects (control) or subjects suffering from idiopathic pulmonary fibrosis (IPF).

Table 1

| Probe (ID <sup>a</sup> ) | miRNA (name)    | Control <sup>b</sup> | IPF <sup>b</sup> | Ratio <sup>c</sup> | P-value <sup>d</sup> |
|--------------------------|-----------------|----------------------|------------------|--------------------|----------------------|
| A_25_P00010700           | Hsa-miR-199a-5p | 8.38                 | 8.69             | 1.24               | 0.006                |
| A_25_P00010701           | Hsa-miR-199a-5p | 6.31                 | 6.69             | 1.30               | 0.005                |
| A_25_P00010069           | Hsa-miR-199a-3p | 8.97                 | 9.47             | 1.41               | 0.015                |
| A_25_P00010068           | Hsa-miR-199a-3p | 7.89                 | 8.19             | 1.23               | 0.045                |

<sup>a</sup>ID Agilent SurePrint G3 hsa chip probe <sup>b</sup>log2 median expression <sup>c</sup>IPF vs control

<sup>d</sup>Wilcoxon test

Table 2

| Experimental mouse model | Probe (ID <sup>a</sup> ) | MiRNA (name)    | Control <sup>b</sup> | Fibrosis <sup>b</sup> | Ratio <sup>c</sup> | P-value <sup>d</sup> |
|--------------------------|--------------------------|-----------------|----------------------|-----------------------|--------------------|----------------------|
| Pulmonary fibrosis       | MIMAT0000229             | mmu-miR-199a-5p | 3.23                 | 4.61                  | 2.89               | 4.10E-03             |
| Renal fibrosis           | MIMAT0000229             | mmu-miR-199a-5p | 2.61                 | 4.52                  | 3.71               | 4.12E-07             |

|                     |              |                         |      |      |      |          |
|---------------------|--------------|-------------------------|------|------|------|----------|
| Hepatic<br>fibrosis | MIMAT0000229 | mmu-<br>miR-<br>199a-5p | 2.60 | 4.10 | 2.82 | 4.00E-03 |
|---------------------|--------------|-------------------------|------|------|------|----------|

<sup>a</sup>miRBase ID (<http://www.mirbase.org>) <sup>b</sup>log2 median expression <sup>c</sup>bleomycin vs PBS control <sup>d</sup>Wilcoxon test

The level of miRNA expression in a cell or tissue is determined by measuring the miRNAs present in said cell or said tissue. The level of miRNA expression may be measured using any technique known to those skilled in the art. For example, after RNA extraction, high-speed miRNA sequencing, NASBA (Nucleic Acid Strand Based Amplification) sequencing, primer extension sequencing, "miRNA" DNA chips, quantitative RT-PCR methods applied to miRNA or *in situ* hybridisation.

The *in vitro* diagnostic method according to the invention may comprise the additional step for (v) identifying at least one target gene for which the level of expression is regulated by the level of miR-199a-5p expression identified in step (iii).

The level of expression of a target gene in a cell or a tissue is determined by measuring the transcript expressed in said cell or said tissue. The level of target gene expression may be measured using any technique known to those skilled in the art. For example, it may be carried out after quantitative RT-PCR RNA extraction, or on tissue sections by means of immunocytochemistry or immunocytology.

Preferably, said at least one miRNA identified in step (iv) is miR-199a-5p.

Preferably, said at least one target gene identified in step (v) codes for caveolin-1 (Gene ID: 857 / <http://www.ncbi.nlm.nih.gov/gene/857>).

The present invention also relates to a method for identifying a subject liable to develop a fibroproliferative disorder characterised in that it comprises or consists of the following steps:

5 (i) quantitative measurement of the level of miR-199a-5p expression in a biological sample from said subject;

(ii) comparing the level of miR-199a-5p expression in said biological sample from said subject with the  
10 level of miR-199a-5p expression in a reference biological sample;

(iii) detecting an increase in the level of miR-199a-5p expression.

The present invention also relates to the use of a  
15 miR-199a-5p inhibitor for preventing and/or treating a fibroproliferative disorder, preferably idiopathic pulmonary fibrosis.

The present invention also relates to the use of a miR-199a-5p inhibitor for stimulating wound repair.

20 The term "miRNA inhibitor" according to the present invention refers to DNA analogues or "oligomer", consisting of a contiguous sequence of from 7 to at least 22 nucleotides in length. The term "nucleotide" as used herein, refers to a glycoside comprising a  
25 sugar moiety, a base moiety and a covalently linked group (linkage group), such as a phosphate or phosphorothioate internucleotide linkage group. It covers both naturally occurring nucleotides and non-naturally occurring nucleotides comprising modified  
30 sugar and/or base moieties, which are also referred to as "nucleotide analogues" herein. Non-naturally

occurring nucleotides include nucleotides which have sugar moieties, such as bicyclic nucleotides or 2' modified nucleotides or 2' modified nucleotides such as 2' substituted nucleotides. "Nucleotide analogues" are variants of natural oligonucleotides by virtue of modifications in the sugar and/or base moieties. Preferably, without being limited by this explanation, the analogues will have a functional effect on the way in which the oligomer works to bind to its target; for example by producing increased binding affinity to the target and/or increased resistance to nucleases and/or increased ease of transport into the cell. Specific examples of nucleoside analogues are described by Freier and Altman (Nucl. Acid Res., 25: 4429-4443, 1997) [35] and Uhlmann (Curr. Opinion in Drug Development, 3: 293-213, 2000) [36]. Incorporation of affinity-enhancing analogues in the oligomer, including Locked Nucleic Acid (LNA<sup>TM</sup>), can allow the size of the specifically binding oligomer to be reduced and may also reduce the upper limit to the size of the oligomer before non-specific or aberrant binding takes place. The term "LNA<sup>TM</sup>" refers to a bicyclic nucleoside analogue, known as "Locked Nucleic Acid" (Rajwanshi et al., Angew Chem. Int. Ed. Engl., 39(9): 1656-1659, 2000) [37]. It may refer to an LNA<sup>TM</sup> monomer, or, when used in the context of an "LNA<sup>TM</sup> oligonucleotide" to an oligonucleotide containing one or more such bicyclic analogues.

Suitably, the oligomer of the invention is capable of specifically inhibiting (silencing) the activity of miR-199a-5p ("anti-miR-199-5p") or preventing the

binding of miR-199a-5p to specific miR-199a-5p binding sites in target RNAs ("miR-199a-5p Target Site Blocker"). Preferably, a "anti-miR-199a-5p" refers to antisense oligonucleotides with sequence complementary to miR-199a-5p (e.g., anti-miR-199a-5p LNA<sup>TM</sup> miRNA inhibitor; Product No.: 426918-00, Product name: hsa-miR-199a-5p, Description: miRCURY LNA<sup>TM</sup> microRNA Power Inhibitor, EXIQON). These oligomers may comprise or consist of a contiguous nucleotide sequence of a total of 7 to at least 22 contiguous nucleotides in length, up to 70% nucleotide analogues (LNA<sup>TM</sup>). The shortest oligomer (7 nucleotides) will likely correspond to an antisense oligonucleotide with perfect sequence complementarity matching to the first 7 nucleotides located at the 5' end of mature miR-199a-5p, and comprising the 7 nucleotide sequence at position 2-8 from 5' end called the "seed" sequence (i.e., "ccagugu" for miR-199a-5p, see Seq ID N°13) involved in miRNA target specificity (Lewis et al., Cell. 2005 Jan 14;120(1):15-20) [38]. A "miR-199a-5p Target Site Blocker" refers to antisense oligonucleotides with sequence complementary to a miR-199a-5p binding site located on a specific mRNA. These oligomers may be designed according to the teaching of US 20090137504. These oligomers may comprise or consist of a contiguous nucleotide sequence of a total of 8 to 23 contiguous nucleotides in length. These sequences may span from 20 nucleotides in the 5' or the 3' direction from the sequence corresponding to the reverse complement of miR-199a-5p "seed" sequence. For example, "CAV1 Target Site Blockers" comprise or consist of a contiguous

sequence of a total of 8 to 23 contiguous nucleotides in length which corresponds to the reverse complement of a specific nucleotide sequence present in the CAV1 mRNA (NM\_001753.4, NM\_001172895.1, NM\_001172896.1, 5 NM\_001172897.1 or naturally occurring variants thereof and RNA nucleic acids derived therefrom, preferably mRNA) and defined as miR-199a-5p site (Fig.5A: SEQ ID N°10). The nucleotide sequence of the oligomers of invention will bind with high affinity to the miR-199a- 10 5p site on CAV1 mRNA, preventing the binding of miR-199a-5p on the 3'UTR of CAV1 mRNA) at this particular target site.

The present invention also relates to the use of a miR-199a-5p inhibitor for obtaining a medicinal product. 15 For example, it consists of the use of a miR-199a-5p inhibitor by the aerosol route for inhibiting fibrogenesis in the pathological respiratory epithelium in subjects suffering from pulmonary fibrosis and thus restoring the integrity of the pathological tissue so 20 as to restore full functionality.

According to one particular embodiment of the invention, said medicinal product is intended to prevent and/or treat a fibroproliferative disorder, preferably idiopathic pulmonary fibrosis.

25 The examples of embodiments of the present invention hereinafter, along with the appended figures, illustrate the invention and are given as non-limiting examples.

In accordance with one aspect, there is provided a miR-199a-5p inhibitor for use in the prevention and/or treatment of a fibroproliferative disorder.

In accordance with another aspect there is provided use of a miR-199a-5p inhibitor in the manufacture of a medicament for treating a fibroproliferative disorder or stimulating a wound repair.

In accordance with a further aspect, there is provided an in vitro diagnostic method for a fibroproliferative disorder in a subject characterised in that it comprises the following steps :

(i) quantitative measurement of the level of miR-199a-5p expression in a biological sample from the subject;

(ii) comparing the miR-199a-5p expression of the biological sample from the subject with the same miR-199a-5p expression of a reference biological sample;

(iii) identifying and measuring in the biological sample from the subject the expression of a target gene for which the level of expression is regulated by the level of miR-199a-5p identified in step (ii); wherein the target gene codes for caveolin-1;

(iv) comparing the caveolin-1 expression in the biological sample from the subject with the caveolin-1 expression of a reference biological sample; and

(v) determining whether the levels of the miR-199a-5p expression and of the caveolin-1 expression are associated with a fibroproliferative disorder.

In accordance with yet another aspect, there is provided a method for identifying a subject liable to develop a fibroproliferative disorder characterised in that it comprises the following steps :

(i) quantitative measurement of the level of miR-199a-5p expression in a biological sample from the subject;

(ii) comparing the level of miR-199a-5p expression in the biological sample from the subject with the level of miR-199a-5p expression in a reference biological sample;

(iii) identifying and measuring in the biological sample from the subject the expression level of a target gene for which the level of expression is regulated by the level of miR-199a-5p expression identified in step (iii); wherein the target gene codes for caveolin-1;

(iv) comparing the level of caveolin-1 expression in the biological sample from the subject with the level of caveolin-1 expression in a reference biological sample ; and

(v) detecting an increase in the level of miR-199a-5p expression and a decrease in the level of caveolin-1 expression.

In accordance with yet a further aspect, there is provided a miR-199a-5p inhibitor for use in stimulating wound repair.

In accordance with one embodiment, there is provided a method for monitoring progression of a fibroproliferative disorder in a subject, characterized in that it comprises the following steps :

i) quantitative measurement of the level of miR-199a-5p expression and of the level of expression of a target gene for which said expression level is regulated by the level of miR-199a-5p expression and wherein the target gene codes for caveolin-1, in biological samples from the subject taken at different time intervals; and

(ii) comparing the level of miR-199a-5p expression and of the level of expression of the target gene in each of said samples from said subject, and determining whether there is progression of the fibroproliferative disorder or not.

**Brief description of the figures**

- Figure 1 represents miR-199a-5p expression during bleomycin-induced pulmonary fibrosis (A) Hierarchical clustering (or tree) representing differential statistically significantly modulated miRNA expression (adjusted p-value <0.05) in the lungs of BALB/c and C57BL/6 mice in response to bleomycin at the indicated time points. Up-regulated miRNAs are shown in progressively brighter shades of red, and down-regulated miRNAs are shown in progressively brighter shades of green based on the log2 value thereof (Bleomycin / PBS). MiR-199a-5p is shown with an arrow. N=3 mice in each group. (B) MiR-199a-5p in the lungs of BALB/c and C57BL/6 mice in response to bleomycin at the specified times. n=3 mice in each group. The data are expressed as the mean  $\pm$  standard error of the mean. \*\*p<0.01. (C) Paraffin sections prepared from C57BL/6 mice collected 14 days after intratracheal bleomycin instillation. *In situ* hybridisation and immunohistochemistry tests were carried out to determine the colocation of miR-199a-5p and  $\alpha$ -SMA. The results represent one out of three tests conducted independently.

- Figure 2 represents miR-199a-5p and pri-miR-199a expression in C57BL/6 mice 14 days after exposure to bleomycin (A) Real-time PCR conducted to confirm the increase in mature miR-199a-5p expression in the lungs of C57BL/6 and BALB/c mice 14 days after administering bleomycin; n=5 mice in each group, the data are expressed as the mean  $\pm$  standard error of the mean.\*p<0.05 (B) Expression of pri-miR-199a-1 and pri-

miR-199a-2 genes in the lungs of C57BL/6 mice 14 days after bleomycin installation. N=5 mice in each group, the data are expressed as the mean  $\pm$  standard error of the mean. \* $p < 0.05$  and \*\* $p < 0.01$ .

5           - Figure 3 represents the identification of miR-199a-5p candidate targets using a transcriptomic approach. Normal hFL1 human lung fibroblasts were transfected with pre-miR-Neg, pre-miR-199-5p or pre-miR21 (n=2). RNA samples were collected 48 hours after  
10   transfection and the expression profiles were determined with genomic chips (A) Hierarchical tree comparing the standardised log2 of the ratios between the signal under the various conditions and the pre-miR-neg signal (B) Overexpression of specific predicted  
15   targets in the set of down-regulated transcripts following transfection with miR-199a-5p and miR-21. The representation of the predicted miRNA targets in the set of down-regulated genes was compared with the set of all the expressed genes using the miRonTop tool:  
20   <http://www.microarray.fr:8080/miRonTop/index>. The graphs show the extent of the enrichment, represented in -log10 form (adjusted p-value), based on the enrichment level using three different prediction tools for all known miRNAs: miR-199a-5p and miR-21 are  
25   represented as  $\diamond$  and  $\Delta$ , respectively. The threshold values used to define the set of up-regulated and down-regulated genes: AveExp=7.0; log FC=0,7; adjusted p-value=0.05. (C) Venn diagram comparing the number of miR-199a-5p targets among the set of significantly  
30   down-regulated genes following pre-miR-199a-5p transfection based on three separate prediction tools

(targetScan, Pictar and miRanda). Selection thresholds are equal to 7.0 for log2 (signal), -1.5 for log2 (ratio), and 0.01 for the adjusted p-value.

- Figure 4 represents the list of themes  
5 corresponding to the "canonical pathway" annotations identified by Ingenuity Pathway Analysis software in response to miR-199a-5p and miR-21 overexpression in hFL1 human lung fibroblasts. hFL1 human lung fibroblasts were transfected with pre-miR-Neg, pre-miR-  
10 199a-5p or pre-miR-21 (n=2). RNA samples were collected 48 hours after transfection and the expression profiles were determined with whole genome biochips. The probability of obtaining the number of genes in a specific pathway in the list of differentially  
15 expressed genes between miR-199a-5p or miR-21 vs miR-Neg was compared with the same representation of the same pathway among all the genes in the biochip; -log10 of the Fisher exact probability is shown. While both miR-199a-5p and miR-21 signatures were associated with  
20 "TGF $\beta$  signaling", several signatures were specifically associated with miR-199a-5p, including "Integrin signaling" and "Caveolae-mediated endocytosis signaling".

- Figure 5 represents CAV1 as the direct target of  
25 miR-199a-5p. (A) Co-transfection of pre-miR-199a-5p or pre-miR-Neg and human CAV1 3'UTR-derived psiCHECK™-2 construct (wild type or mutated in the putative miR-199a-5p seed region) in HEK293 cells demonstrates a significant decrease in standardised luciferase  
30 activity 48 hours after transfection for the wild type construct only. \*p<0.05. The position of the miR-199a-

5p target site in CAV1 3'UTR and the sequence alignment of miR-199a-5p and CAV1 3'UTR using various species are shown. The representation is limited to the region around the complementary miR-199a-5p site. The miR-199a-5p binding site retained in CAV1 3'UTR is shown in bold. (B) Relative CAV1 expression determined by real-time PCR in hFL1 lung fibroblasts after transfection with pre-miR-199a-5p, pre-miR-21 or si-CAV1. The data represent three independent tests. The data are expressed as the mean  $\pm$  standard error of the mean.\* $p < 0.01$  (C) Standardised fluorescence expression values of CAV1 in hFL1 lung fibroblasts after transfection with pre-miR-199a-5p, pre-miR-21 or siCAV1 based on biochip tests. The data represent two independent tests. The data are expressed as the mean  $\pm$  standard error of the mean.\* $p < 0.01$  (D) Western blot analysis demonstrating the down-regulation of CAV1 protein expression after hFL1 lung fibroblast transfection with pre-miR-199a-5p, pre-miR-21 or siCAV1. One representative test out of two is shown.

- Figure 6 represents the decrease in CAV1 expression after MRC-5 lung fibroblast transfection with pre-miR-199a-5p. (A) MRC-5 lung fibroblasts transfected with 10 nM of pre-miR-199a-5p for 24 hours display a significant decrease in CAV1 expression as determined by means of real-time PCR. The data are expressed as the mean  $\pm$  standard error of the mean.\*\* $p < 0.01$ . (B) Western blot analysis demonstrating the decrease in CAV1 expression after MRC-5 lung fibroblast transfection with pre-miR-199a-5p. The data represent two independent tests.

- Figure 7 represents CAV1 and miR-199a-5p expression regulation with TGF $\beta$ . MRC-5 lung fibroblasts were treated with 10ng/ml of TGF $\beta$  for 24 hours and 48 hours. The levels of expression of miR-199a-5p (A) and CAV1 (B) were determined by Taqman<sup>TM</sup> PCR. The data are expressed as the mean  $\pm$  standard error of the mean.\*p<0.01. (C) The CAV1 protein levels were determined by immunoblot analysis. (D) MRC5 cells were transfected with LNA-miR-199a-5p, a CAV1 LNA-Target Site Blocker (CAV1 protector) or LNA-control then incubated with or without 10 ng/ml TGF $\beta$  for 24h and CAV1 protein levels were determined by immunoblot analysis. The data represent three independent tests.

- Figure 8 represents altered CAV1 expression in a mouse bleomycin-induced pulmonary fibrosis model. (A) Biochip analysis demonstrates a significant reduction in CAV1 expression in C57BL/6 mice treated with bleomycin for 14 days (n=5) compared to the control mice (n=5). The data are expressed in the form of normalized fluorescence values  $\pm$  standard error of the mean.\*p<0.01 (B) Real-time PCR conducted to confirm the decrease in CAV1 expression in the lungs of C57BL/6 mice 14 days after administering bleomycin. N=5 mice in each group. The data are expressed as the mean  $\pm$  standard error of the mean.\*p<0.05 (C) CAV1 expression as detected by immunoblot analysis in lung tissue samples from C57BL/6 mice treated with bleomycin for 14 days (n=3) and control mice (n=3) (D) Immunohistochemical analysis of CAV1 expression in sections of lung tissue from C57BL/6 mice 14 days after

intratracheal bleomycin instillation. One representative section out of three is shown.

- Figure 9 represents pulmonary CAV1 expression in "lung fibrosis-resistant" BALB/c mice 14 days after injecting bleomycin. Real-time PCR was conducted to evaluate CAV1 expression in the lungs of BALB/c mice 14 days after exposure to bleomycin. n=5 mice in each group, the data are expressed as the mean  $\pm$  standard error of the mean. n.s.= non-significant.

10 - Figure 10 represents the list of the 133 genes modulated by miR-199a-5p in lung fibroblasts which are also deregulated in the lungs of C57BL/6 mice 14 days after bleomycin treatment [Average intensity: log2 average intensity; LogRatio: log2 (Bleo / PBS); PValue: adjusted p-value for a large number of events according to the Benjamini Hochberg corrected test]. Both CAV1 and CAV2 are significantly down-regulated in the 2 models.

- Figure 11 represents the deregulation of miR-199a-5p and the CAV1 target thereof in IPF. (A and B) Box plots showing the normalized expression of log2-transformed CAV1 and miR-199a-5p in both IPF (n=94) and control (n=83) lungs. The box represents the 25-75% quartiles, the line in the box represents the median and whiskers represent the range. (\*\*p<0.001) (C) Immunohistochemical analysis of CAV1 expression in sections of lung tissue from patients suffering from IPF. One representative section is shown (D) *In situ* hybridisation demonstrating miR-199a-5p expression (i) or a control probe (ii) in sections of lung tissue from patients suffering from IPF.

- Figure 12 represents the functional impact of miR-199a-5p on lung fibroblasts. The increase in miR-199a-5p expression in the lung fibroblasts results in an increase in the ability of the fibroblasts to migrate, proliferate, invade matrigel and differentiate into myofibroblasts. (A and B) *In vitro* healing test conducted to evaluate the migration rate of lung fibroblasts transfected with 10 nM of miR-199a-5p or control 0, 6.5 and 9.5 hours after scratch injury. A significant increase ( $p < 0.01$ ) in the migration rate was observed in lung fibroblasts transfected with miR-199a-5p compared to the control. The data represent two independent tests. (C) Invasion assay on matrigel showing that overexpression of miR-199a-5p increases MRC5 lung fibroblast invasiveness. Data are representative of two independent experiments (D) Effects of miR-199a-5p on lung fibroblast proliferation determined by bivariate flow cytometry analysis EdU/DNA stained cells. The x-axis represents the linear intensity obtained using propidium iodide (total DNA content), and the y-axis represents the logarithmic intensity obtained using Alexa Fluor647. The cells were separated into G0/G1 phase and G2/M phase based on the DNA content thereof, and into labelled undivided and labelled divided subgroups based on the DNA content of the EdU-labelled cells. One representative test out of three is shown. (E) Confocal microscopy of MRC5 cells overexpressing miR-199a-5p revealed that increasing miR-199a-5p levels in lung fibroblasts promotes their differentiation into myofibroblasts. Cells were stained with an antibody against  $\alpha$ -SMA (green) and phalloidin

(red). Experiments were performed twice. (F) Relative expression of COL1A1 was assessed by Taqman PCR in MRC5 cells overexpressing miR-199a-5p and exposed or not to TGF $\beta$ . Data are expressed as mean  $\pm$  SEM and derived from  
5 2 independent experiments. \*p<0.05.

- Figure 13 represents the comparison of transcriptome changes induced by miR-199a-5p and siRNA targeted against CAV1. Normal hFL1 human lung fibroblasts were transfected with pre-miR-Neg, pre-miR-  
10 199a-5p and with siCAV1 or control siRNA (n=2). RNA samples were collected 48 hours after transfection and the expression profiles were determined with a set of genomic biochips. (A) Decision tree comparing the standardised log2 of the ratios between the pre-miR-  
15 199a-5p signals vs pre-miR-Neg or the siCAV1 signals vs si-Neg (B) Venn diagram comparing the set of down-regulated transcripts followed induction with miR-199a-5p and siCAV1. The selection thresholds are equal to 7.0 for log2 (signal), 0.7 for log2 (ratio), and 0.05  
20 for the adjusted p-value.

- Figure 14 represents miR-199a-5p as a TGF $\beta$  pathway effector. (A) Decision tree of the significant canonical pathways associated with miR-199a-5p, miR-21 or siCAV1 overexpression contexts identified using  
25 Ingenuity Pathway Analysis<sup>TM</sup> software. (B) GSEA graphs for an experimental TGF $\beta$  signature showing a significative enrichment for genes up-regulated and down-regulated by miR-199a-5p. The data were processed using GSEA software, with transcripts ordered based on  
30 the miR-199a-5p vs miR-Neg contexts measured using the standard deviation thereof. Up- and down-regulated

genes were handled separately with respect to an experimental TGF $\beta$  signature obtained in the same cell models (hFL1 fibroblasts treated with 10 ng/ml of TGF $\beta$ 1 for 48 hours): two sets of 134 and 113 genes corresponding to sets of genes up-regulated and down-regulated by TGF $\beta$  were selected as described in Example 1 ( $p < 0.05$ ). In each case, the graphs show enrichment, the peak score corresponding to the SE and the position of the set of TGF $\beta$  genes in the list of classified genes (each transcript is shown with a vertical line).

(C) MRC5 cells transfected with LNA-miR-199a-5p, CAV1 Target Site Blocker (CAV1 protector) or LNA-control, then incubated with or without 10 ng/ml TGF $\beta$  for 24h, and cells stained with an antibody against  $\alpha$ -SMA (green), phalloidin (red) and DAPI (blue). Experiments were performed twice. (D) Cells co-transfected with SMAD-luciferase reporter plasmid, luciferase activities analyzed 48h after transfection. All firefly luciferase activities were normalized with renilla luciferase activity. \* $p < 0.05$ . Two independent experiments. (E) and (F) *In vitro* healing (or scratch) test conducted to evaluate the migration rate of TGF $\beta$ -stimulated hFL1 lung fibroblasts treated with 25 nM of LNA-anti-miR-199a-5p, LNA-control inhibitor and CAV1 protector. A significant decrease ( $p < 0.01$ ) in the migration rate was observed in lung fibroblasts transfected with LNA-anti-miR-199a-5p and CAV1 protector compared to the control. The data represent two independent tests.

- Figure 15 represents the comparison of the change of gene expression between genes regulated by miR-199a-5p in lung fibroblasts and lungs of C57BL/6

mice 14 days after injecting bleomycin. (A) Venn diagram showing the correlations of the changes in gene expression between lung fibroblasts transfected with miR-199a-5p (two independent tests) and the lungs of C57BL/6 mice 14 days after bleomycin treatment (n=5 mice). The numbers of genes for which expression was detected differentially under each condition at  $p < 0.05$  are shown. The biochip analysis demonstrates a significant reduction in the expression of CAV1 (B), TGFBR1 (C), CCL2 (D), ACTA2 (E) and MMP3 (F) in C57BL/6 mice treated with bleomycin for 14 days (n=5) compared to control mice (n=5). The data are expressed as the mean  $\pm$  standard error of the mean.  $**p < 0.01$ .

- Figure 16 represents the profibrotic genes significantly modulated in lung fibroblasts by miR-199a-5p regardless of CAV1 regulation. Lung fibroblasts were transfected with miR-199a-5p, siCAV1 or negative controls. The biochip analysis shows the expression of known fibrotic genes: CAV1 (A), TGFBR1 (B), MMP3 (C), PLAU (D) and CCL2 (E) 48 hours after transfection. The data are expressed as the mean  $\pm$  standard error of the mean.

- Figure 17 represents miR-199a-5p deregulation in three experimental mouse hepatic, pulmonary and renal fibrosis models. Venn diagrams showing the correlations of changes of miRNA expression in lungs of C57BL/6 mice 14 days after bleomycin treatment (n=4 mice), livers of BALB/c mice 6 weeks after administering CCl<sub>4</sub> (n=5 mice) and kidneys of C57BL/6 mice 28 days after unilateral ureteral obstruction (n=4 mice). The number of miRNAs for which expression was detected differentially in

each mouse model at  $p < 0.01$  are shown. The hepatic fibrosis data are taken from [24].

- Figure 18 represents enhanced expression of miR-199a-5p in clinical samples from patients with liver fibrosis. *In situ* hybridization assay was performed to determine the localization of miR-199a-5p in normal and fibrotic human livers. Results represent one out of three independent experiments.

- Figure 19 represents the alteration of miR-199a-5p expression in a mouse CCl<sub>4</sub>-induced hepatic fibrosis model. (A) MiR-199a-5p expression in the livers of BALB/c mice treated with oil or CCl<sub>4</sub> for 6 weeks analysed by qPCR;  $n=5$  per group. The data are expressed as the mean  $\pm$  standard error of the mean.  $**p < 0.01$ . (B) MiR-199a-5p expression in the livers of C57BL/6 mice treated with oil or CCl<sub>4</sub> for 6 weeks analysed by qPCR;  $n=5$  per group. The data are expressed as the mean  $\pm$  standard error of the mean.  $*p < 0.05$ . (C) MiR-199a-5p expression in the livers of C57BL/6 mice 21 days after bile duct ligation or the control procedure analysed by qPCR;  $n=4$  mice per group. The data are expressed as the mean  $\pm$  standard error of the mean.  $**p < 0.05$ . (D) MiR-199a-5p expression in the liver of C57BL/6 mice during fibrosis regression. Hepatic fibrosis was induced by injecting CCl<sub>4</sub> and the miR-199a-5p expression was evaluated 6 weeks after the CCl<sub>4</sub> treatment and 2 and 4 weeks after the final injection. The data are expressed as the mean  $\pm$  standard error of the mean.  $**p < 0.05$ . (E) MiR-199a-5p and CAV1 expression in primary stellate cells isolated from C57BL/6 mice and stimulated with 20 ng/ml of TGF $\beta$  for 48 hours.

- Figure 20 represents the alteration of miR-199a-5p and CAV1 expression in a mouse unilateral ureteral obstruction (UUO) renal fibrosis model. (A) MiR-199a-5p expression in the kidney of C57BL/6 mice after UUO at the indicated time points. n=5 to 7 mice in each group. The data are expressed as the mean  $\pm$  standard error of the mean. \*\*p<0.01. (B) Paraffin sections prepared from kidneys of C57BL/6 mice collected 28 days after UUO. The *in situ* hybridisation test was conducted to determine the location of miR-199a-5p in the kidney. The results represent one out of three independent tests. (C) Immunohistochemical analysis of CAV1 expression in kidney tissue sections from C57BL/6 mice 28 days after UUO. One representative section out of two is shown.

## **EXAMPLES**

### **EXAMPLE 1: MATERIALS AND METHODS**

#### **5 Cell lines, reagents and antibodies**

MRC-5 and hFL1 5CCL-153) normal human lung fibroblasts and the A549 human lung cancer cell line were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM medium  
10 containing 10% foetal calf serum, at 37°C with 5% CO<sub>2</sub> v/v.

Recombinant TGF $\beta$  was acquired from Sigma-Aldrich.

The following monoclonal and polyclonal antibodies were used for the immunohistochemistry and Western blot  
15 analyses: rabbit anti-CAV1 polyclonal antibody (sc-894, Santa Cruz Biotechnology Inc.), rabbit anti- $\beta$ -actin monoclonal antibody (13E5, cell signaling), mouse anti- $\alpha$ -SMA monoclonal antibody (1A4, Dako).

#### **20 Experimental pulmonary, renal and hepatic fibrosis models**

##### **Mouse pulmonary fibrosis model**

Male C57BL/6 and BALB/c strain mice, aged from 9  
25 to 12 weeks, were acquired from Charles River, France. To induce the fibrotic changes, the mice were instilled by the intratracheal route with bleomycin or PBS for the controls. For this purpose, the mice were anaesthetised by sevoflurane (Abbott) inhalation and  
30 placed in the supine position. Transtracheal insertion of a 24-G supply needle was used for instilling

bleomycin (0.75 units/ml) or excipient (PBS), in an 80 µl volume. The mice were sacrificed 7 and 14 days after instillation and the lungs were removed for subsequent analysis.

5

#### Mouse renal fibrosis model

Male C57BL/6 and BALB/c strain mice, aged from 9 to 12 weeks, were acquired from Charles River, France. The mice were anaesthetised by means of intraperitoneal injection of pentobarbital (50 mg/kg of body weight). After standard laparotomy, the left proximal ureter was exposed and ligated at two points with 4-0 silk. The "simulation or control" procedure consisted of a similar identification of the left ureter, but the ureter was not ligated.

15

#### Mouse hepatic fibrosis model

Male C57BL/6 and BALB/c strain mice, aged from 6 to 8 weeks, were acquired from Jackson Laboratory (Bar Harbor). To induce hepatic fibrosis, the mice received 0.6 ml/kg of body weight of CCl<sub>4</sub> (Merck) mixed with corn oil (Sigma life science) by the intraperitoneal route as described above [Roderbrug et al., Hepatol., 53: 209-218, 2011] [24]. The bile duct was ligated by exposing the common hepatic duct and conducting a double-ligation thereof, and subsequently cutting between the ligations as described by Roderburg et al. [2011, cited above] [24]. To induce fibrosis regression, the mice were treated for 6 weeks with CCl<sub>4</sub> as described above and sacrificed 2 or 4 weeks, respectively, after the end of the treatment.

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30

### **Primary stellate cell isolation**

Primary stellate cells were isolated from 40-55 week old C57BL/6 mice and stimulated with 20 ng/ml of  
5 TGF- $\beta$ 1 (Sigma Aldrich) for 48 hours as previously described by Roderburg et al. [2011, cited above] [24].

### **Human lung tissue**

Frozen lung tissues from subjects with IPF and  
10 free from chronic lung disease were obtained from the "Lung Tissue Research Consortium (LTRC)". The diagnoses were based on ATS/ERS guidelines [Demedts and Costabel, Eur. Respir. J., 19: 794-796, 2002; Steele et al., Am. J. Respir. Crit. Care Med., 172: 1146-1152, 2005] [32,  
15 33] based on the clinical history, pathology and radiology. All the tests were approved by the local Institutional Review Board at the University of Pittsburgh. The clinical data were made fully available to the investigators for examination.

20 Paraffin-treated lung sections from patients with IPF were obtained from Lille hospital. The tests were approved by the Lille hospital institutional research commission.

### **25 Histopathology**

Mouse kidneys and lungs were fixed overnight with neutral buffered formalin and then included in paraffin. 5  $\mu$ m thick tissue sections were mounted and stained with haematoxylin and eosin and with Masson's trichrome  
30 to evaluate the degree of fibrosis. The histological sections were examined by an experienced pathologist.

### **Total RNA extraction and quality control**

The total RNA was extracted from lung cell and tissue samples using Trizol Reagent (Invitrogen) according to the manufacturer's recommendations. The purity and concentration of the total RNA samples were first evaluated using the Nanodrop spectrophotometer. The 260/280 and 260/230 ratios were checked and should have a value close to 2. The RNA was then loaded onto a chi nano chip and the quality thereof (RNA integrity and degradation level) was analysed using the Bioanalyzer System (Agilent Technologies, France).

### **Biochips**

#### Mouse lung miRNA biochips

The oligonucleotide sequences corresponding 2054 mature miRNAs found in the miRNA registry are available at <http://www.microarray.fr:8080/merge/index> (follow the link to "microARN": platform references on the NCBI GEO database in GPL4718). Three biological reproductions were produced for each comparison. The experimental data and the biochip design were registered on the NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under series GSE34812. The experimental design used a "dye-swap" approach, such that each mouse probe, imprinted 8 times on the biochip was measured 16 times independently for each sample. The target preparation and biochip hybridisation were carried out as previously described [Pottier et al., PLoS. One, 4: e6718, 2009; Triboulet

et al., Science, 315: 1579-1582, 2007; Puisségur et al., 2011, cited above] [25, 26, 13].

#### Human lung miRNA biochip analysis

5        MiRNA biochip analysis was conducted as previously described [Pandit et al., 2010, cited above] [18]. In brief, 100 ng of total RNA was labelled and hybridised on Agilent microRNA Microarray Release 16.0, 8x60K. After washing, the chips were scanned using an Agilent  
10   Microarray scanner. The scanned images were processed with Agilent's Feature Extraction software version 9.5.3. The miRNA biochip data were analysed using GeneSpring v11.5 and BRB-ArrayTools v.1 developed by Dr. Richard Simon and the BRB-ArrayTools development team.  
15   The data were standardised per quantile. The miRNA biochip data are available to the public via the Lung Genomics Research Consortium (LGRC) website (lung-genomics.org).

#### 20   Expression biochips

      The RNA samples were labelled with Cy3 stain using the low RNA input QuickAmp kit (Agilent) according to the manufacturer's recommendations. 825 ng of labelled cRNA probe was hybridised on mouse or human Agilent  
25   8x60K high density SurePrint G3 gene expression biochips. Two (*in vitro* human tests) or five (*in vivo*-derived samples) biological reproductions were produced for each comparison. The experimental data were registered on the NCBI GEO database  
30   (<http://www.ncbi.nlm.nih.gov/geo/>) under SuperSeries GSE34818 (series GSE34812 and GSE34814 for the miRNA

and mRNA responses in the mouse bleomycin model, respectively; series GSE34815 for the miRNA/siRNA transfection tests in hFL1 human fibroblasts). For the human gene expression chips, the data were converted  
5 into log2 and standardised using a cyclic Loess algorithm in the R programming environment as previously described [Wu et al., BMC. Bioinformatics, 6: 309, 2005] [34]. The human biochip data have been made  
10 available to the public on the LTRC (ltrcpublic.org) and LGRC websites as part of the LTRC protocol.

#### Statistical analysis and biological thematic analysis

The standardisation was carried out using the Limma program available from Bioconductor  
15 (<http://www.bioconductor.org>). The within-chip (two-colour dye-swap tests only) and between-chip standardisation was carried out using the "PrintTip Loess" method and the quantile method, respectively. The mean ratios of all the comparisons were calculated  
20 and a B test analysis was conducted. Differentially expressed genes were selected using the Benjamini-Hochberg p-value correction for multiple tests, based on a p-value less than 0.05. The expression biochip data were analysed for biological theme enrichment  
25 (canonical pathways and genetic ontology molecular function) and to construct biological networks using Ingenuity Pathway Analysis software (<http://www.ingenuity.com/>) and Mediente  
(<http://www.microarray.fr:8080/merge/index>) [Le and  
30 Barbry, Bioinformatics, 23: 1304-1306, 2007] [27], an information system containing various information on

probes and data sets. Gene Set Enrichment Analysis (GSEA) software was used to determine whether a gene set defined in principle can characterise differences between two biological states [Subramanian et al., Proc. Natl. Acad. Sci. U.S.A., 102: 15545-15550, 2005] [28]. Hierarchical clusters were produced with MultiExperiment Viewer (MeV) version 4.3, using the Manhattan distance and the mean link.

#### 10 MiRNA target analysis

MiRonTop [Le et al., Bioinformatics, 26: 3131-3132, 2010] [29] is an online Java network tool (available at <http://www.microarray.fr:8080/miRonTop/index>) which integrates DNA biochip data to identify the potential involvement of miRNAs in a specific biological system. In brief, MiRonTop classifies transcripts into two categories ("up-regulated" and "down-regulated"), based on expression level and differential expression thresholds. It then calculates the number of predicted targets for each miRNA, based on the selected prediction software (Targetscan, MiRBase, PicTar, exact progeny search: 2-7 or 1-8 first nucleotides of miRNA, TarBase v1), in each gene set. MiRNA target enrichment in each category is then tested using the hypergeometric function. The absence of a non-siRNA target effect was verified in si-CAV1 transcriptome tests using the Sylamer tool [Van et al., Nat. Methods, 5: 1023-1025, 2008] [30].

#### 30 **Transfection and luciferase assays**

Pre-miRNA, LNA-based miRNA inhibitors, target site blocker and siRNA transfection in lung fibroblasts

Pre-miR-199-5p, pre-miR-21 and control miRNA (miR-Neg#1) were acquired from Ambion. For the miR-199-5p knock-down tests, anti-miR-199-5p LNA and the anti-miR-159s LNA negative control (miRCURY LNA knock-down probe) were ordered from Exiqon. SiRNA targeted against CAV1 and control siRNA (Silencer Select validated siRNAs) were acquired from Applied Biosystems.

MRC5/hFL1 cells were cultured in DMEM medium containing 10% FCS and transfected to 30-40% confluence in 6- or 12-well plates using Lipofectamine RNAi MAX<sup>TM</sup> (Invitrogen) with pre-miRNA, siRNA or LNA inhibitors at a final concentration of 10 nM unless indicated.

Pre-miRNA and psiCHECK<sup>TM</sup>-2 plasmid construction cotransfection

Molecular constructions were produced in psiCHECK<sup>TM</sup>-2 vector (Promega) by cloning behind Renilla luciferase in the XhoI and NotI restriction sites, CAV1 3'UTR derived hybrid oligonucleotides described hereinafter. HEK293 cells were cultured in DMEM medium containing 10% FCS to confluence. The cells were then distributed into 96-well plates and cotransfected using lipofectamine 2000<sup>TM</sup> (Invitrogen) with 0.2 µg of psiCHECK<sup>TM</sup>-2 plasmid construction or pre-miR-199-5p or control miRNA at a final concentration of 10, 30 and 50 nM. 48 hours after transfection, the renilla and firefly luciferase activities were evaluated with the Dual Glo Luciferase Assay System kit (Promega) and

measured using a luminometer (Luminoskan Ascent, Thermolab system).

hsa-CAV1: WT (sense):

TCGAGGACACTTTAATTACCAACCTGTTACCTACTTTGACTTTTTGCATT  
 5 TAAACAGACACTGGCATGGATATAGTTTTACTTTTAAACTGTGTACGC (SEQ  
 ID NO: 1)

hsa-CAV1: WT (anti-sense):

GGCCGCGTACACAGTTTAAAAGTAAACTATATCCATGCCAGTGTCTGTT  
 TTAAATGCAAAAAGTCAAAGTAGGTAACAGGTTGGTAATTAAAGTGTCC (SEQ  
 10 ID NO: 2)

hsa-CAV1: MUT (sense):

TCGAGGACACTTTAATTACCAACCTGTTACCTACTTTGACTTTTTGCATT  
 TAAACAGAGAGTCGCATGGATATAGTTTTACTTTTAAACTGTGTACGC (SEQ  
 ID NO: 3)

15 hsa-CAV1: MUT (anti-sense):

GGCCGCGTACACAGTTTAAAAGTAAACTATATCCATGCGACTCTCTGTT  
 TTAAATGCAAAAAGTCAAAGTAGGTAACAGGTTGGTAATTAAAGTGTCC (SEQ  
 ID NO: 4)

## 20 **SMAD reported assay**

MRC5 cells were seeded in 96 well plate and cotransfected 24h later at 4% confluency using RNAi MAX lipofectamine reagent with 100 ng SMAD reported vector (Signal Smad Reporter, Qiagen) and 10 nM LNA-control,  
 25 LNA-199a-5p or CAV1 protector. 24h after transfection, cells were serum starved 3h before adding 10 ng/ml TGFβ. Cells were lyzed and Glo luciferase assay (Promega) was performed 24h following TGFβ exposure.

## 30 **Quantitative RT-PCR**

#### Mature miRNA expression

The miR-199a-5p expression was evaluated using the TaqMan MicroRNA Assay kit (Applied Biosystems) as specified in the protocol. Real-time PCR was conducted  
5 using the GeneAmp Fast PCR Master Mix product (Applied Biosystems) and the ABI 7900HT real-time PCR machine. The levels of mature miRNA expression were evaluated using the comparative CT method ( $2^{-\Delta\text{CT}}$ ).

#### 10 Pri-miRNA expression

The pri-miR-199a-1 and pri-miR-199a-2 expressions were evaluated using the TaqMan pri-microRNA Assay system (Applied Biosystems) according to the manufacturer's recommendations. Real-time PCR was  
15 conducted using the TaqMan<sup>TM</sup> Gene Expression Master Mix product (Applied Biosystems) and the ABI 7900HT real-time PCR machine. The levels of pri-miRNA expression were evaluated using the comparative CT method ( $2^{-\Delta\text{CT}}$ ).

20

#### Genetic expression

The levels of human and mouse CAV1 expression were analysed using the TaqMan MicroRNA Assay system (Applied Biosystems) according to the manufacturer's  
25 recommendations. Real-time PCR was conducted using the TaqMan<sup>TM</sup> Gene Expression Master Mix product (Applied Biosystems) and the ABI 7900HT real-time PCR machine. The levels of CAV1 were evaluated using the comparative CT method ( $2^{-\Delta\text{CT}}$ ).

30

#### **Protein extraction and immunoblot**

The cells or tissues were lysed in a lysis buffer (M-PER protein extraction buffer for cells, T-PER protein extraction reagent for tissues) and a mixture of protease inhibitors (Pierce). The lysates were  
5 assayed for protein concentrations using the Bradford assay (Biorad). The proteins (10 µg per sample) underwent electrophoresis in SDS-polyacrylamide gel and transferred into liquid medium on a nitrocellulose membrane (Hybond™ C Extra, Amersham Bioscience) for 1  
10 hr 30 in a transfer buffer (50 mM Tris base, 40 mM Glycine, 1.3 mM SDS and 10% ethanol). After transfer, the membrane was blocked in a 5% milk 0.1% TBS-Tween™ solution for 1 hour at ambient temperature under stirring. It was then incubated with anti-CAV1 primary  
15 antibodies (Santa Cruz) diluted to 1:400, anti-beta actin antibodies (Cell Signaling Technology) to 1:1000 overnight at 4°C on a rotating plate. After washing 3 times in 0.1% TBS-Tween for 30 minutes at ambient temperature, the membrane was incubated for 1 hour in  
20 the presence of mouse anti-IgG secondary antibody coupled with HRP (Horse Radish Peroxidase, Cell Signaling) diluted to 1:5000 5% milk TBS-0.1% Tween under stirring. After washing several times, in 0.1% TBS-Tween for 30 minutes, the proteins of interest were  
25 detected by chemoluminescence (ECL substrates, Amersham).

#### **Immuno-histochemistry**

5 µm thick mouse lung sections included in  
30 paraffin were deparaffinised in xylene (2 x 5 minutes) and rehydrated by incubating 2 x 5 minutes in 100%

ethanol, 2 x 5 minutes in 95% ethanol, and 2 x 5 minutes in 80% ethanol. After washing with water, the antigen was unmasked by thermal denaturation using a citrate buffer (pH=6.0, DAKO) for 20 minutes. The sections were cooled to ambient temperature and then washed in 0.1% TBS-Tween, and the endogenous peroxidases were inactivated with 3% hydrogen peroxide in TBS for 10 minutes. The sections were also blocked for avidin/biotin activity, blocked with serum-free blocking reagents, and incubated with anti-CAV1 or anti alpha-SMA primary antibody for 1 hour at ambient temperature and overnight at 4°C, respectively. The proteins of interest were then detected with diaminobenzidine (DAB, DAKO) after incubating the secondary antibody.

### ***In situ hybridisation***

The tissue location of miR-199a-5p was detected using the double DIG-labeled LNA probes system (Exiqon, Woburn, MA). Mouse tissues included in paraffin were deparaffinised in xylene (2 x 5 minutes) and rehydrated by incubating 2 x 5 minutes in 100% ethanol, 2 x 5 minutes in 95% ethanol, and 2 x 5 minutes in 80% ethanol. The slides were then washed in PBS (pH 7.5) and permeabilised by incubating for 15 minutes at 37°C in proteinase K (Ambion). The slides were washed again in PBS, and pre-hybridised in a hybridisation buffer (50% formamide, 5x SSC, 0.1% Tween 20, 9.2 mM citric acid, 50 µg/ml heparin, and 500 µg/ml yeast RNA, pH 6) in a wet chamber. The double DIG-labelled LNA probes were then added at a concentration of 80 nM and

incubated for 2 hours at 50°C in a wet chamber. The slides were rinsed in 5xSSC, 1x SSC and 0.2x SSC solutions at the same hybridisation temperature. This was followed by blocking with 2% sheep serum, 2 mg/ml BSA in PBS + 0.1% Tween 20 (PBST) and incubation with anti-DIG-AP Fab fragments (1:800) (Roche Applied Sciences) for 2 hours at ambient temperature. After washing in PBST, the stained reaction was produced by incubating in a 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) solution (Roche Applied Sciences) with 1 mM levamisole overnight at ambient temperature. The stained reaction was stopped after observing sufficient blue precipitate development by washing with PBST. The slides were then counterstained with Fastred, mounted and topped with a slide cover.

#### **Immunofluorescence analysis**

MRC5 cells were grown on a Round Glass Coverslips Ø 16 mm (Thermo scientific) placed inside a 12 Multiwell Plate. Coverslips slides were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde for 15 min, cells were then permeated using 0.1% Triton<sup>TM</sup> X-102 (Agilent Technologies) for 10 min and blocked with PBS solution containing BSA (3%) for 30 min. Incubation with primary antibodies was performed in a blocking solution BSA (1%) at 37°C for 1h at the following dilutions: α-SMA (1:1000), CAV1 (1:50). After three washes with PBS, cells were incubated with secondary Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) (1:500), Alexa Fluor 647 goat

anti-rabbit IgG (Invitrogen) (1:500) and Alexa Fluor®  
647 phalloidin (A22287, Life Technologies)  
(1 unit/slide). 45 min later, Coverslips slides were  
fixed on microscope slides using ProLong® Gold Antifade  
5 Reagent with DAPI (Invitrogen). Fluorescence was viewed  
with an FV10i Olympus confocal scanning microscope.

### **Cell proliferation study**

The human lung fibroblasts (MRC-5 or hFL-1) were  
10 distributed into 6-well plates (150,000 cells per well)  
in DMEM medium supplemented with 10% FCS. The following  
day, the culture medium was replaced with serum-free  
medium and the cells were transfected with pre-miR-  
199a-5p. The cell proliferation was then evaluated 48  
15 hours after transfection by flow cytometry using the  
click-iT™ EdU Cell Proliferation Assay kit (Invitrogen)  
according to the manufacturer's recommendations.

### **Cell migration study: *In vitro* healing test**

20 The human lung fibroblasts (MRC-5 or hFL-1) were  
placed in 12-well plates (500,000 cells per well). At  
confluence, the culture medium was replaced with serum-  
free medium and the cells were transfected with pre-  
miR-199a-5p or an anti-miR-199-5p inhibitor (LNA anti-  
25 miR-199-5p, Exiqon). 24 hours after transfection, the  
cells were scratched with a pipette tip and treated or  
untreated with TGFβ (20 ng/ml). The "*in vitro*" healing  
mechanism was then filmed for 24 hours after scratching  
by video microscopy using an Axiovert 200 M inverted  
30 microscope (Carl Zeiss) equipped with a regulation  
insert at 5% CO<sub>2</sub> and 37°C (Pecon GmbH). Images with a

light background were taken every 30 minutes through a factor 10 phase contrast lens with a CoolSNAPHQ CCD camera managed using Metamorph software (Roper Scientific). The cell motility was calculated by evaluating the repaired area percentage using ImageJ image analysis software.

### **Invasion assay**

Invasion of MRC5 fibroblast overexpressing mR-199a-5p was assessed using commercially available 24-well BioCoat Matrigel Invasion Chamber (BD Biosciences). In brief, pulmonary fibroblasts were transfected either with pre-miR-199a-5p or negative control as described above. 24h after transfection, cells were harvested with trypsin-EDTA, centrifuged, and resuspended in DMEM medium. Cell suspensions ( $1 \times 10^5$  cells/well) were added to the upper chamber. Bottom wells of the chamber were filled with DMEM medium containing 10% FBS as chemoattractant, whereas the upper chamber was filled with DMEM only. After incubation for 48h at 37°C, the non-invading cells on the top of the membrane were removed with a cotton swab. Membrane containing invading-cells were fixed with methanol, washed three times with PBS and mounted with DAPI hard set (Vector Laboratories) onto glass slides for fluorescent microscopy.

### **Statistical analysis**

The results were given as the mean  $\pm$  standard error of the mean. The statistical analyses were

carried out using the Student test as provided by Microsoft Excel™.

## **EXAMPLE 2: RESULTS**

5

### **Pulmonary fibrosis-resistant and sensitive mice display a distinct miRNA expression profile in response to bleomycin**

Bleomycin is the experimental tool of choice for  
10 inducing pulmonary fibrosis on various animal models, including mice. In this way, C57BL/6 type mice are considered to be sensitive to bleomycin-induced pulmonary fibrosis whereas BALB/c type mice are resistant. To identify the miRNAs potentially involved  
15 in the pulmonary fibrosis process, the pulmonary miRNA expression profile in response to bleomycin in bleomycin-induced pulmonary fibrosis-sensitive and resistant mice (n=3 mice per group) was studied using a biochip-based platform (data set 1, accession number  
20 GSE34812) described elsewhere [25, 26, 13]. In particular, the expression profile study focused on the fibrotic phase of the fibrosis process, i.e. 7 days and 14 days after administering bleomycin. The expression profile identified consists of 22 significantly  
25 differentially expressed miRNAs between the lungs of control and bleomycin-treated animals in at least one type, the majority being up-regulated in lungs instilled with bleomycin (figure 1A). Interestingly, miR-199a-5p displayed increased expression in response  
30 to bleomycin during the progression of pulmonary fibrosis only in C57BL/6 type mice (figure 1B). The

increase in the level of miR-199a-5p expression 14 days after administering bleomycin was confirmed independently by real-time PCR on a larger number of animals (n=5 mice per group) (figure 2A). Consequently, these results demonstrated that, in principle, miR-199a-5p plays an important role during the pulmonary fibrosis process.

To study the regulation mechanisms underlying miR-199a-5p production, the expression status of two mouse genes was evaluated, miR-199a-1 (on chromosome 9) and miR-199a-2 (on chromosome 1), in response to bleomycin using the Taqman assay designed to discriminate between pri-miR-199a-1 and pri-miR-199a-2. The results demonstrated that, 14 days after instilling bleomycin, the two pri-miR-199a transcripts were up-regulated in the lungs of C57BL/6J mice (figure 2B) and thus contribute to miR-199a-5p production.

Moreover, *in situ* hybridisation tests conducted on histological lung sections obtained from C57BL/6 mice, 14 days after administering bleomycin (fibrotic lungs) or PBS (healthy lungs), revealed selective miR-199a-5p expression in myofibroblasts in the damaged areas of the diseased lungs corresponding to the fibroblastic sites (figure 1C).

It is important to note that, in line with previous studies, miR-21 up-regulation was detected in response to bleomycin (figures 1A). Nonetheless, miR-21 expression increased significantly in both types of mouse after administering bleomycin. Given that miR-21 was previously clearly associated with the development of fibrotic disorders including pulmonary fibrosis,

these results suggest that BALB/c could have compensatory mechanisms which could counteract the adverse effects of miR-21.

## 5 **Identification of specific miR-199a-5p targets in lung fibroblasts**

One of the major stakes in miRNA biology is that of being able to identify and characterise regulated target mRNA experimentally. In this context, *in silico*  
10 approaches (target prediction bioinformatics software) and experimental approaches (transcriptome chips), ectopic miRNA expression and reporter vectors containing the 3'-UTR part of a gene of interest fused with luciferase were combined, as previously described  
15 [25, 13]. A number of target prediction algorithms have been proposed. They are generally based on i) the complementarity between miRNA and target mRNA 3'-UTR in the 5' region of miRNA (referred to as the "seed"), and ii) the phylogenetic conservation of this sequence in  
20 the target mRNA 3'-UTR.

To determine the target genes regulated by miR-199a-5p, Agilent® expression chip (Agilent Technology) mRNA profiling was carried out after manipulating the miR-199a-5p expression level by transfection in lung  
25 fibroblasts. The influence of miR-199a-5p on the transcriptome of hFL1 human lung fibroblasts was compared to that of miR-21 (data set 2, accession number GEO GSE34815). The ectopic expression of each miRNA induced major modifications in the transcriptome  
30 48 hours after transfection with modulated genes 1261 and 753 in miR-199a-5p and miR-21 contexts,

respectively. Whereas these two miRNAs induced very different modulation profiles (figure 3A), a functional annotation of these two signature profiles with signature with Ingenuity Pathway<sup>TM</sup> software showed an overlap for the "canonical pathways" including "Cell cycle regulation" and "TGF $\beta$  signaling" (figure 4). In line with previous studies, very significant pathways associated with miR-21 were associated with "Cell cycle and cyclin control" and "Chromosomal cell cycle control", "Mismatch repair in eukaryotes" and "ATM signaling". Whereas the highest scoring pathway for miR-199a-5p consisted of the "Steroid biosynthesis" metabolic pathways, enrichment was also observed for the pathways associated with "Integrin signaling" and "Caveolin-mediated endocytosis signaling".

Direct putative targets were then searched in the down-regulated transcript population using the MiRonTop tool. This indicated specific over-representation of predicted targets in the down-regulated transcript population after heterologous miR-199a-5p or miR-21 expression, using a number of prediction tools including an additional direct progeny search or TargetScan (figure 3B).

The analysis then focused on a subset of transcripts containing complementary miR-199a-5p hexamers in the 3'UTR thereof displaying the most expression inhibition. Three different prediction tools, TargetScan, PicTar and miRanda, produced contrasting results, as shown in the Venn diagrams represented in figure 3C. While a total of 92 transcripts were predicted by at least one of these algorithms, only 21

transcripts were predicted by the three different algorithms (TargetScan, Pictar, MiRanda) using the MiRonTop tool (<http://www.microarray.fr:8080/miRonTop/index>) (see table 3 hereinafter).

Table 3

| GeneName | Accession | Description                                                                                                              | AveExpr <sup>1</sup> | logFC <sup>2</sup> |
|----------|-----------|--------------------------------------------------------------------------------------------------------------------------|----------------------|--------------------|
| ACSL4    | NM_004458 | Homo sapiens acyl-CoA synthetase long-chain family member 4 (ACSL4), transcript variant 1, mRNA                          | 10.56                | -1.97              |
| AKAP7    | NM_016377 | Homo sapiens A kinase (PRKA) anchor protein 7 (AKAP7), transcript variant gamma, mRNA                                    | 9.25                 | -1.51              |
| AP1S2    | NM_003916 | Homo sapiens adaptor-related protein complex 1, sigma 2 subunit (AP1S2), mRNA                                            | 13.01                | -1.37              |
| ARCN1    | NM_001655 | Homo sapiens archain 1 (ARCN1), transcript variant 1, mRNA                                                               | 11.99                | -1.60              |
| ARHGAP12 | NM_018287 | Homo sapiens Rho GTPase activating protein 12 (ARHGAP12), mRNA                                                           | 11.12                | -1.96              |
| ATP6V1H  | NM_015941 | Homo sapiens ATPase, H <sup>+</sup> transporting, lysosomal 50/57kDa, V1 subunit H (ATP6V1H), transcript variant 1, mRNA | 10.14                | -1.86              |
| BNC1     | NM_001717 | Homo sapiens basonuclin 1 (BNC1), mRNA                                                                                   | 8.87                 | -1.08              |
| BTRC     | NM_033637 | Homo sapiens beta-transducin repeat containing (BTRC), transcript variant 1, mRNA                                        | 9.37                 | -1.33              |
| C17orf63 | NM_018182 | Homo sapiens chromosome 17 open reading frame 63 (C17orf63), transcript variant 2, mRNA                                  | 11.90                | -1.58              |
| C18orf25 | NM_145055 | Homo sapiens chromosome 18 open reading frame 25 (C18orf25), transcript variant 1, mRNA                                  | 9.74                 | -1.56              |
| C1GALT1  | NM_020156 | Homo sapiens core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1 (C1GALT1), mRNA         | 9.19                 | -1.68              |
| C1orf213 | NR_033690 | Homo sapiens chromosome 1 open reading frame 213 (C1orf213), transcript variant 1, non-coding RNA                        | 9.29                 | -1.69              |

|         |              |                                                                                                                                                        |       |       |
|---------|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|-------|-------|
| C5orf13 | NM_004772    | Homo sapiens chromosome 5 open reading frame 13 (C5orf13), transcript variant 1, mRNA                                                                  | 15.85 | -1.03 |
| C5orf24 | NM_001135586 | Homo sapiens chromosome 5 open reading frame 24 (C5orf24), transcript variant 1, mRNA                                                                  | 11.26 | -1.43 |
| C9orf5  | NM_032012    | Homo sapiens chromosome 9 open reading frame 5 (C9orf5), mRNA                                                                                          | 12.19 | -1.27 |
| CAV1    | NM_001753    | Homo sapiens caveolin 1, caveolae protein, 22kDa (CAV1), transcript variant 1, mRNA                                                                    | 15.28 | -1.78 |
| CDCA7L  | NM_018719    | Homo sapiens cell division cycle associated 7-like (CDCA7L), transcript variant 1, mRNA                                                                | 11.03 | -1.55 |
| CDH2    | NM_001792    | Homo sapiens cadherin 2, type 1, N-cadherin (neuronal) (CDH2), mRNA                                                                                    | 13.27 | -1.97 |
| CHCHD4  | NM_144636    | Homo sapiens coiled-coil-helix-coiled-coil-helix domain containing 4 (CHCHD4), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA | 11.56 | -1.35 |
| CNOT6   | NM_015455    | Homo sapiens CCR4-NOT transcription complex, subunit 6 (CNOT6), mRNA                                                                                   | 9.25  | -1.36 |
| CNPY2   | NM_014255    | Homo sapiens canopy 2 homolog (zebrafish) (CNPY2), transcript variant 1, mRNA                                                                          | 15.41 | -1.50 |
| DCBLD2  | NM_080927    | Homo sapiens discoidin, CUB and LCCL domain containing 2 (DCBLD2), mRNA                                                                                | 13.37 | -2.87 |
| DDR1    | NM_013993    | Homo sapiens discoidin domain receptor tyrosine kinase 1 (DDR1), transcript variant 2, mRNA                                                            | 9.57  | -2.31 |
| DISC1   | NM_018662    | Homo sapiens disrupted in schizophrenia 1 (DISC1), transcript variant L, mRNA                                                                          | 10.00 | -1.07 |
| ELP2    | NM_018255    | Homo sapiens elongation protein 2 homolog (S. cerevisiae) (ELP2), mRNA                                                                                 | 14.00 | -1.54 |
| EPAS1   | NM_001430    | Homo sapiens endothelial PAS domain protein 1 (EPAS1), mRNA                                                                                            | 14.79 | -2.61 |
| EPB41L1 | NM_012156    | Homo sapiens erythrocyte membrane protein band 4.1-like 1 (EPB41L1), transcript variant 1, mRNA                                                        | 12.88 | -1.94 |
| EXTL3   | NM_001440    | Homo sapiens exostoses (multiple)-like 3 (EXTL3), mRNA                                                                                                 | 13.66 | -1.20 |
| FAM188A | NM_024948    | Homo sapiens family with sequence similarity 188, member A (FAM188A), mRNA                                                                             | 11.55 | -1.33 |

|          |              |                                                                                                                                                   |       |       |
|----------|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-------|-------|
| FBXO28   | NM_015176    | Homo sapiens F-box protein 28 (FBXO28), transcript variant 1, mRNA                                                                                | 10.39 | -1.73 |
| FZD6     | NM_003506    | Homo sapiens frizzled homolog 6 (Drosophila) (FZD6), transcript variant 1, mRNA                                                                   | 11.62 | -2.07 |
| GPD2     | NM_001083112 | Homo sapiens glycerol-3-phosphate dehydrogenase 2 (mitochondrial) (GPD2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA | 9.81  | -2.15 |
| GPR56    | NM_201525    | Homo sapiens G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA                                                                    | 10.62 | -1.14 |
| GPRC5A   | NM_003979    | Homo sapiens G protein-coupled receptor, family C, group 5, member A (GPRC5A), mRNA                                                               | 8.12  | -2.04 |
| HOXB6    | NM_018952    | Homo sapiens homeobox B6 (HOXB6), mRNA                                                                                                            | 14.50 | -1.10 |
| HVCN1    | NM_001040107 | Homo sapiens hydrogen voltage-gated channel 1 (HVCN1), transcript variant 1, mRNA                                                                 | 7.86  | -1.31 |
| IKBKB    | NM_001556    | Homo sapiens inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB), transcript variant 1, mRNA                       | 10.18 | -1.31 |
| IPO8     | NM_006390    | Homo sapiens importin 8 (IPO8), transcript variant 1, mRNA                                                                                        | 12.00 | -2.09 |
| ITFG3    | NM_032039    | Homo sapiens integrin alpha FG-GAP repeat containing 3 (ITFG3), mRNA                                                                              | 13.18 | -1.22 |
| ITGA3    | NM_002204    | Homo sapiens integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) (ITGA3), transcript variant a, mRNA                             | 10.58 | -2.66 |
| KIF1B    | NM_183416    | Homo sapiens kinesin family member 1B (KIF1B), transcript variant 2, mRNA                                                                         | 12.31 | -2.26 |
| KLHL3    | NM_017415    | Homo sapiens kelch-like 3 (Drosophila) (KLHL3), mRNA                                                                                              | 8.44  | -1.32 |
| KPNA4    | NM_002268    | Homo sapiens karyopherin alpha 4 (importin alpha 3) (KPNA4), mRNA                                                                                 | 13.44 | -1.11 |
| LEPREL1  | NM_018192    | Homo sapiens leprecan-like 1 (LEPREL1), transcript variant 1, mRNA                                                                                | 13.48 | -1.21 |
| MAP3K11  | NM_002419    | Homo sapiens mitogen-activated protein kinase kinase kinase 11 (MAP3K11), mRNA                                                                    | 13.11 | -2.77 |
| MAPKAPK3 | NM_004635    | Homo sapiens mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3), mRNA                                                         | 11.13 | -1.16 |

|          |              |                                                                                                                       |       |       |
|----------|--------------|-----------------------------------------------------------------------------------------------------------------------|-------|-------|
| MAX      | NM_145113    | Homo sapiens MYC associated factor X (MAX), transcript variant 3, mRNA                                                | 13.11 | -1.21 |
| MCFD2    | NM_139279    | Homo sapiens multiple coagulation factor deficiency 2 (MCFD2), transcript variant 1, mRNA                             | 12.48 | -1.21 |
| MPP5     | NM_022474    | Homo sapiens membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5) (MPP5), mRNA                            | 11.76 | -1.99 |
| NAB1     | NM_005966    | Homo sapiens NGFI-A binding protein 1 (EGR1 binding protein 1) (NAB1), mRNA                                           | 9.59  | -1.23 |
| NAV3     | NM_014903    | Homo sapiens neuron navigator 3 (NAV3), mRNA                                                                          | 10.22 | -1.36 |
| NCEH1    | NM_020792    | Homo sapiens neutral cholesterol ester hydrolase 1 (NCEH1), transcript variant 2, mRNA                                | 11.44 | -1.64 |
| NCSTN    | NM_015331    | Homo sapiens nicastrin (NCSTN), mRNA                                                                                  | 13.89 | -1.02 |
| NFKB1    | NM_003998    | Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1), transcript variant 1, mRNA | 11.43 | -1.25 |
| NLK      | NM_016231    | Homo sapiens nemo-like kinase (NLK), mRNA                                                                             | 11.36 | -1.63 |
| NOTCH2NL | NM_203458    | Homo sapiens notch 2 N-terminal like (NOTCH2NL), mRNA                                                                 | 12.92 | -1.74 |
| PCYOX1   | NM_016297    | Homo sapiens prenylcysteine oxidase 1 (PCYOX1), mRNA                                                                  | 12.23 | -1.84 |
| PEX3     | NM_003630    | Homo sapiens peroxisomal biogenesis factor 3 (PEX3), mRNA                                                             | 9.79  | -1.43 |
| PHF6     | NM_032335    | Homo sapiens PHD finger protein 6 (PHF6), transcript variant 3, mRNA                                                  | 8.48  | -1.60 |
| PIK3CD   | NM_005026    | Homo sapiens phosphoinositide-3-kinase, catalytic, delta polypeptide (PIK3CD), mRNA                                   | 9.18  | -1.96 |
| PLAU     | NM_002658    | Homo sapiens plasminogen activator, urokinase (PLAU), transcript variant 1, mRNA                                      | 15.54 | -2.48 |
| PLXND1   | NM_015103    | Homo sapiens plexin D1 (PLXND1), mRNA                                                                                 | 12.63 | -1.40 |
| PODXL    | NM_001018111 | Homo sapiens podocalyxin-like (PODXL), transcript variant 1, mRNA                                                     | 11.21 | -2.59 |
| PPP1R2   | NM_006241    | Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 2 (PPP1R2), mRNA                                   | 11.29 | -2.50 |

|          |              |                                                                                                                                                   |       |       |
|----------|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-------|-------|
| PPP2R3A  | NM_002718    | Homo sapiens protein phosphatase 2, regulatory subunit B", alpha (PPP2R3A), transcript variant 1, mRNA                                            | 11.82 | -1.34 |
| PRKAA1   | NM_206907    | Homo sapiens protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1), transcript variant 2, mRNA                                        | 9.36  | -1.59 |
| PTGS1    | NM_000962    | Homo sapiens prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) (PTGS1), transcript variant 1, mRNA            | 10.61 | -1.67 |
| PXN      | NM_002859    | Homo sapiens paxillin (PXN), transcript variant 2, mRNA                                                                                           | 14.99 | -1.41 |
| R3HDM2   | NM_014925    | Homo sapiens R3H domain containing 2 (R3HDM2), mRNA                                                                                               | 11.95 | -1.32 |
| RBM24    | NM_153020    | Homo sapiens RNA binding motif protein 24 (RBM24), transcript variant 2, mRNA                                                                     | 8.66  | -2.44 |
| RNF11    | NM_014372    | Homo sapiens ring finger protein 11 (RNF11), mRNA                                                                                                 | 14.30 | -1.09 |
| RNF141   | NM_016422    | Homo sapiens ring finger protein 141 (RNF141), mRNA                                                                                               | 11.70 | -1.52 |
| RPS6KA3  | NM_004586    | Homo sapiens ribosomal protein S6 kinase, 90kDa, polypeptide 3 (RPS6KA3), mRNA                                                                    | 10.64 | -1.59 |
| RTN4     | NM_020532    | Homo sapiens reticulon 4 (RTN4), transcript variant 1, mRNA                                                                                       | 17.62 | -1.01 |
| SCCPDH   | NM_016002    | Homo sapiens saccharopine dehydrogenase (putative) (SCCPDH), mRNA                                                                                 | 13.63 | -1.10 |
| SERPINE1 | NM_000602    | Homo sapiens serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1), transcript variant 1, mRNA | 10.72 | -1.98 |
| SLC14A1  | NM_001146037 | Homo sapiens solute carrier family 14 (urea transporter), member 1 (Kidd blood group) (SLC14A1), transcript variant 4, mRNA                       | 11.83 | -1.95 |
| SLC35A2  | NM_001042498 | Homo sapiens solute carrier family 35 (UDP-galactose transporter), member A2 (SLC35A2), transcript variant 3, mRNA                                | 11.12 | -1.58 |
| SRGN     | NM_002727    | Homo sapiens serglycin (SRGN), transcript variant 1, mRNA                                                                                         | 9.68  | -1.40 |
| SSH2     | NM_033389    | Homo sapiens slingshot homolog 2 (Drosophila) (SSH2), mRNA                                                                                        | 10.81 | -1.28 |

|         |                  |                                                                                                                 |       |       |
|---------|------------------|-----------------------------------------------------------------------------------------------------------------|-------|-------|
| ST6GAL1 | NM_173216        | Homo sapiens ST6 beta-galactosamide alpha-2,6-sialyltransferase 1 (ST6GAL1), transcript variant 1, mRNA         | 10.67 | -1.70 |
| TAF9B   | NM_015975        | Homo sapiens TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa (TAF9B), mRNA     | 9.77  | -2.25 |
| TSPAN6  | NM_003270        | Homo sapiens tetraspanin 6 (TSPAN6), mRNA                                                                       | 12.41 | -1.65 |
| TST     | NM_003312        | Homo sapiens thiosulfate sulfurtransferase (rhodanese) (TST), nuclear gene encoding mitochondrial protein, mRNA | 14.03 | -2.02 |
| TXNDC12 | NM_015913        | Homo sapiens thioredoxin domain containing 12 (endoplasmic reticulum) (TXNDC12), mRNA                           | 11.33 | -1.74 |
| UHMK1   | NM_175866        | Homo sapiens U2AF homology motif (UHM) kinase 1 (UHMK1), transcript variant 1, mRNA                             | 10.14 | -2.57 |
| VPS26A  | NM_004896        | Homo sapiens vacuolar protein sorting 26 homolog A (S. pombe) (VPS26A), transcript variant 1, mRNA              | 12.37 | -1.60 |
| ZNF512  | NM_032434        | Homo sapiens zinc finger protein 512 (ZNF512), mRNA                                                             | 10.47 | -1.16 |
| ZNF512B | NM_020713        | Homo sapiens zinc finger protein 512B (ZNF512B), mRNA                                                           | 12.08 | -1.02 |
| ZNF584  | NM_173548        | Homo sapiens zinc finger protein 584 (ZNF584), mRNA                                                             | 9.46  | -1.41 |
| ZNF706  | NM_0010425<br>10 | Homo sapiens zinc finger protein 706 (ZNF706), transcript variant 1, mRNA                                       | 8.15  | -1.10 |
| ZNF776  | NM_173632        | Homo sapiens zinc finger protein 776 (ZNF776), mRNA                                                             | 9.93  | -1.22 |

<sup>1</sup> logarithm (base 2) of the average intensity (AveExpr)

<sup>2</sup> logarithm (base 2) of the ratio of miR-199a-5p / miR-Neg (logFC)

A genetic network analysis was then focused on  
 5 these 21 targets, and a more limited list of candidate  
 genes associated with the most significant canonical  
 pathways described above was identified. Finally, the  
 levels of expression of these candidate genes were  
 compared with the mouse orthologues thereof in a  
 10 bleomycin model using whole genome biochips (C57BL/6J  
 mice 14 days after instilling bleomycin or PBS, data  
 set 3, accession number GEO GSE34814). It was observed  
 that 4 of 21 best putative targets of miR-199a-5p were

also down-regulated in fibrotic lung tissue: ARHGAP12, CAV1, MAP3K11 and MPP5 (table 3). Of these, caveolin-1 (CAV1) would appear to represent a key target of miR-199a-5p, based on previous studies demonstrating a significant link between CAV1 down-regulation in lung fibroblasts and adverse TGF $\beta$ -mediated effects [Wang et al., J. Exp. Med., 203: 2895-2906, 2006; Xia et al., Am. J. Pathol., 176: 2626-2637, 2010] [23, 31].

#### 10 **Validation of caveolin-1 (CAV1) as a target of miR-199a-5p**

Caveolin-1 (CAV1) is a 22 kDa membrane protein essential for the formation of small plasma membrane invaginations referred to as caveolae. Caveolae are found in most cell types, in varying quantities according to the tissue. They are particularly abundant in differentiated cells such as adipocytes, endothelial cells, type I pneumocytes, fibroblasts and smooth and striated muscle cells. Caveolae represent a subcategory of lipid rafts, morphologically identifiable due to the invaginated and circular shape thereof, and characterised by the presence of structuring proteins named caveolin-1, caveolin-2 and caveolin-3. Caveolin-1 and -2 have a relatively ubiquitous distribution in most differentiated cells with the exception of skeletal muscle fibres and cardiac myocytes [Scherer et al., J. Cell Biol., 127: 1233-1243, 1994] [20]. The expression of caveolin-3 is restricted to the skeletal muscles, diaphragm and heart [Tang et al., J. Biol. Chem., 271: 2255-2261, 1996] [21]. Interestingly, studies recently demonstrated that transgenic mice in

which the caveolin-1 gene has been deleted present various anomalies, particularly in the lungs [Park et al., Biochemistry, 42: 15124-15131, 2003] [22]. These mice in particular develop pulmonary fibrosis and endothelial cell proliferation. *In vitro* studies have also demonstrated that decreasing caveolin-1 expression in lung fibroblast cells promotes the pro-fibrotic effects of TGF $\beta$  on this cell type, particularly the activation, proliferation and differentiation of fibroblasts to myofibroblasts [Wang et al., 2006, cited above] [23].

Using the MicroCible algorithm, a potential binding site for miR-199a-5p was identified in the 3'UTR sequence of CAV1 (figure 5A). To evaluate whether miR-199a-5p alters CAV1 expression, the 3'UTR of human CAV1 was cloned in psiCHECK<sup>TM</sup>-2 vector downstream from the sequence coding for luciferase, and cotransfected in HEK293 cells in the presence of miR-199a-5p or a negative control miRNA (figure 5A). As a control, a CAV1 3'UTR construct mutated on the predicted miR-199a-5p site was also used. Human pre-miR-199a-5p induced a significant decrease in the normalized luciferase activity relative to control in the presence of the wild type construction only, confirming that it represents a functional site. Moreover, the transfection of pre-miR-199a-5p in MRC5 and hFL1 lung fibroblasts gave rise to a significant and specific decrease in CAV1 in respect of mRNA and protein levels while miR-21 had no significant effect (figures 5B-5D and 6).

30

**TGF $\beta$  regulates miR-199a-5p and CAV1 expression in lung fibroblasts**

It was studied whether the decrease in CAV1 expression following stimulation with TGF $\beta$  is associated with an increase in miR-199a-5p expression. In order to test this hypothesis, the MRC5 cell line  
5 was exposed to TGF $\beta$ , and the levels of CAV1 and miR-199a-5p expression were analysed. As detected by Taqman RT-PCR, treating human fibroblasts with TGF $\beta$  for 24 to 48 hours gave rise to a marked reduction in CAV1 mRNA, whereas miR-199a-5p expression was significantly up-  
10 regulated (figures 7A and 7B). Finally, a time-dependent decrease in CAV1 protein levels was also observed after TGF $\beta$  treatment (figure 7C). All these results demonstrate that, in principle, miR-199a-5p may contribute to TGF $\beta$ -dependent down-regulation of CAV1  
15 expression in human fibroblasts.

To further investigate whether miR-199-5p is involved in TGF $\beta$ -induced downregulation of CAV1, additional experiments using a LNA-based inhibitor of miR-199a-5p as well as a LNA-based Target Site Blocker  
20 preventing miR-199-5p binding on CAV1 3'UTR mRNA (CAV1 protector) were performed to specifically interfere with miR-199a-5p binding on CAV1 3'UTR. As depicted in Figure 7D, both LNA-mediated silencing of miR-199a-5p and blocking miR-199a-5p binding on CAV1 3'UTR inhibit  
25 TGF $\beta$ -induced downregulation of CAV1. Altogether these results demonstrate that, in lung fibroblasts, induction of miR-199a-5p in response to TGF $\beta$  mediates CAV1 downregulation through binding on a unique site located in CAV1 3'UTR.

### **Altered CAV1 expression in lungs of mice suffering from bleomycin-induced pulmonary fibrosis**

CAV1 expression was studied in fibrotic mouse lungs. In line with previous studies, the data show a  
5 significant decrease in the level of CAV1 protein and  
mRNA expression in C57BL/6J mice, 14 days after  
administering bleomycin (figures 8A, 8B and 8C).  
Moreover, immunohistochemical staining of CAV1 on lung  
tissue sections obtained from C57BL/6J mice 14 days  
10 after bleomycin treatment, demonstrated a marked  
reduction in CAV1 in the fibrotic area of the lungs  
(figure 8D). Collectively, these results demonstrated  
that, in lung fibroblasts, miR-199a-5p expression up-  
regulation in fibrotic mouse lungs correlates with CAV1  
15 down-regulation.

It should be noted that the BALB/c mice, for which  
miR-199a-5p expression is not up-regulated in response  
to bleomycin, did not display a significant decrease in  
the level of CAV1 mRNA expression 14 days after  
20 bleomycin treatment (figure 9).

### **Altered CAV1 and miR-199a-5p expression in lungs of patients suffering from IPF**

It was studied whether miR-199a-5p expression is  
25 also deregulated in lungs of patients suffering from  
IPF, using a recently published data set (accession  
number GEO GSE13316) consisting of 10 IPF samples and  
10 control samples. Interestingly, compared to the  
control, miR-199a-5p expression in the IPF samples  
30 increased very significantly ( $p=0.005$ ,  $p=0.006$ , table  
1).

These results were then studied on a larger cohort of IPF patients (IPF n=94 and control n=83) where the inverse correlation between CAV1 and miR-199a-5p observed in mice was confirmed in humans (figures 11A and 11B). In this way, the lungs of patients with IPF displayed significantly increased miR-199a-5p expression along with significantly decreased CAV1 expression compared to healthy lungs. The linear ratio for CAV1 between IPF and control was 0.54 (FDR<0.05) and the linear ratio for miR-199a-5p for the same subjects was 1.35 (p<0.05). Finally, the examination of lung sections subject to IPF demonstrated the miR-199a-5p expression in lung fibroblasts along with reduced CAV1 expression (figures 11C and 11D).

15

#### **MiR-199a-5p-mediated fibrogenic activation of lung fibroblasts**

Given that the level of CAV1 expression is a critical factor involved in the fibrogenic activation of lung fibroblasts, it was studied whether miR-199a-5p overexpression in lung fibroblasts is sufficient to recapitulate the known profibrotic effects associated with a decrease in CAV1 expression (*i.e.* fibroblast proliferation, migration and differentiation to myofibroblasts). As represented in figure 12, increasing miR-199a-5p levels by transfecting miR-199a-5p precursors gave rise to a significant increase in lung fibroblast migration (figures 12A and 12B), invasion (figure 12C) and proliferation (figure 12D). Moreover, miR-199a-5p overexpression also gave rise to an increase in ACTA2 expression (marking myofibroblast

30

differentiation) (Figure 12E), as well as to a significant potentialization of COL1A1 induction in response to TGF $\beta$  (Figure 12F).

It was then studied whether miR-199a-5p has  
5 further profibrotic effects regardless of CAV1 regulation. For this purpose, the genetic expression profile obtained in lung fibroblasts transfected with miR-199a-5p precursors was compared with that obtained after transfection with a siRNA specifically targeted  
10 against CAV1. Interestingly, some overlap was detected between the two signatures, essentially among the down-regulated transcripts (figure 13A, group 2), with 34% of transcripts down-regulated by miR-199a-5p also repressed by si-CAV1 (figure 13B).

15 For the purposes of familiarisation with the pathways modulated by miR-199a-5p, the Ingenuity Pathways<sup>TM</sup> canonical pathways of miR-199a-5p were analysed and compared to those of miR-21 and siCAV1 contexts. As shown in the decision tree in figure 14A,  
20 hierarchical clusters support the proximity between the pathways regulated by miR-199a-5p and siCAV1. Specific pathways for miR-199a-5p were also detected, particularly some associated with inflammation, such as "IL-1 signaling", "Acute phase response signaling" and  
25 "P38 MAPK signaling", which are all typical of fibrotic processes. Of the genes specifically regulated by miR-199a-5p, a plurality of known profibrotic genes with distinct biological activities was detected, and for which abnormal expression was confirmed *in vivo*  
30 (figures 10 and 15). This confirms that miR-199a-5p regulates multiple different signaling pathways

involved in pulmonary fibrogenesis. In particular, compared to cells transfected with siCAV1, miR-199a-5p overexpression increased CCL2, TGFBR1 and MMP3 expression significantly and decreased CAV1 and PLA2 expression significantly (figure 16). It should be noted that these two down-regulated genes are taken to be direct targets of miR-199a-5p based on the Pictar algorithm.

10 **MiR-199a-5p is an effector of TGF $\beta$  signaling in lung fibroblasts by regulating CAV1**

Finally, it was demonstrated that miR-199a-5p is involved in TGF $\beta$  signaling. For this, a TGF $\beta$  signaling signature was defined experimentally in lung  
15 fibroblasts and compared to the signature of miR-199a-5p using the GSEA method. This analysis showed a significant overlap between these two signatures, evaluated with standardised enrichment scores greater than 1 (1.4 and 2.17 for up- and down-regulated genes,  
20 respectively, with a nominal p-value and an FDR q value <0.05), demonstrating that miR-199a-5p is, in principle, a TGF $\beta$  response mediator in lung fibroblasts (figure 14B).

To further demonstrate the importance of miR-199a-5p in TGF $\beta$  response, silencing of miR-199a-5p was performed in lung fibroblasts using LNA-based inhibitors. In particular, it was shown that LNA-mediated silencing of miR-199a-5p strongly inhibited TGF $\beta$ -induced differentiation of lung fibroblasts into  
30 myofibroblasts (Figures 14C), SMAD signaling (Figure

14D), and stimulation of wound repair by slowing down wound closure significantly (Figures 14E and 14F).

Remarkably, similar results were obtained using a LNA-based Target Site Blocker (CAV1 protector) demonstrating therefore that miR-199a-5p is a key effector of TGF $\beta$  response through CAV1 regulation (Figures 14C, 14D, 14E, 14F).

#### **MiR-199a-5p is deregulated in mouse renal fibrosis and cirrhosis (hepatic fibrosis) models**

Increasing evidence suggests that miRNA takes part in the fibrotic process in various organs such as the heart, kidneys, liver or lungs. For example, previous studies have shown that miR-21 has an important role in pulmonary and cardiac fibrosis. In this way, it was studied whether miR-199a-5p is also deregulated in one forms of tissue fibrosis, i.e. renal and hepatic fibrosis using well-characterised experimental mouse models. For this purpose, the expression profiles of miRNAs obtained in these fibrosis models were compared using the same platform based on the miRNAs. 5 miRNAs routinely deregulated to a p-value <0.01 were identified (figure 17). Of these miRNAs, 3 were down-regulated (miR-193, miR-30b and miR-29c) and 2 were up-regulated (miR-199a-3p and miR-199a-5p) (see table 4 hereinafter).

Table 4

| miRNA       | miRNA sequence                           | Change in expression |
|-------------|------------------------------------------|----------------------|
| mmu-miR-193 | AACUGGCCUACAAAGUCCCAGU<br>(SEQ ID NO: 5) | Down                 |

|                 |                                           |      |
|-----------------|-------------------------------------------|------|
| mmu-miR-29c     | UAGCACCAUUUGAAAUCGGUUA<br>(SEQ ID NO: 6)  | Down |
| mmu-miR-30b     | UGUAAACAUCCUACACUCAGCU<br>(SEQ ID NO: 7)  | Down |
| mmu-miR-199a-3p | ACAGUAGUCUGCACAUUGGUUA<br>(SEQ ID NO: 8)  | Up   |
| mmu-miR-199a-5p | CCCAGUGUUCAGACUACCUGUUC<br>(SEQ ID NO: 9) | Up   |

The increase in miR-199a-5p expression was confirmed in two independent experimental hepatic fibrosis models (figures 19A, 19B and 19C) and was correlated with the severity of the hepatic fibrosis, given that BALB/c mice have more pronounced hepatic fibrosis than C57BL/6 mice, after administering CCl<sub>4</sub> (figures 19A and 19B). Moreover, miR-199a-5p expression decreased significantly during the regression of the experimental hepatic fibrosis induced by CCl<sub>4</sub> (figure 19D). Furthermore, it was demonstrated that the exposure of stellate cells to TGF $\beta$  was associated with an increase in miR-199a-5p expression and a decrease in the level of CAV1 expression (figures 19E and 19F). Interestingly, enhanced expression of miR-199a-5p was also observed in clinical samples from patients with liver fibrosis (Figure 18).

Similarly, the data obtained from the unilateral ureteral obstruction renal fibrosis model demonstrated an increase in miR-199a-5p in damaged kidneys compared to mice undergoing the control procedure (figure 20A). Interestingly, as in the case of pulmonary fibrosis, renal miR-199a-5p expression was correlated with a progression of the disorder (figure 20A). As

represented in figure 20B, *in situ* hybridisation conducted 28 days after the procedure (i.e. when the fibrosis is established) did not display any detectable signal for miR-199a-5p in normal kidneys, whereas the  
5 hybridisation signal increased significantly throughout the damaged kidneys in the area compatible with myofibroblasts. Furthermore, CAV1 immunochemistry conducted on fibrotic mouse kidneys 28 days after the procedure demonstrated a marked reduction in CAV1  
10 expression in the fibrotic area of the kidneys (figure 20C).

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## CLAIMS

1. A miR-199a-5p inhibitor for use in the prevention and/or treatment of a fibroproliferative disorder.
2. A miR-199a-5p inhibitor for use according to claim 1, wherein the fibroproliferative disorder is idiopathic pulmonary fibrosis.
3. Use of a miR-199a-5p inhibitor in the manufacture of a medicament for treating a fibroproliferative disorder.
4. *In vitro* diagnostic method for a fibroproliferative disorder in a subject characterised in that it comprises the following steps:
  - (i) quantitative measurement of the level of miR-199a-5p expression in a biological sample from said subject;
  - (ii) comparing the miR-199a-5p expression of the biological sample from said subject with the same miR-199a-5p expression of a reference biological sample;
  - (iii) identifying and measuring in the biological sample from said subject the expression of a target gene for which the level of expression is regulated by the level of miR-199a-5p identified in step (ii); wherein said target gene codes for caveolin-1;
  - (iv) comparing the caveolin-1 expression in the biological sample from said subject with the caveolin-1 expression of a reference biological sample; and
  - (v) determining whether the levels of the miR-199a-5p expression and of the caveolin-1 expression are associated with a fibroproliferative disorder, an increase in the level of miR-199a-5p expression and a decrease in the level of caveolin-1 expression relative to the reference biological sample being associated to a fibroproliferative disorder.
5. Method for identifying a subject liable to develop a fibroproliferative disorder characterised in that it comprises the following steps:
  - (i) quantitative measurement of the level of miR-199a-5p expression in a biological sample from said subject;

(ii) comparing the level of miR-199a-5p expression in said biological sample from said subject with the level of miR-199a-5p expression in a reference biological sample ;

(iii) identifying and measuring in the biological sample from said subject the expression level of a target gene for which the level of expression is regulated by the level of miR-199a-5p expression identified in step (iii) ; wherein said target gene codes for caveolin-1;

(iv) comparing the level of caveolin-1 expression in said biological sample from said subject with the level of caveolin-1 expression in a reference biological sample ; and

(v) determining whether there is an increase in the level of miR-199a-5p expression and a decrease in the level of caveolin-1 expression relative to the reference biological sample, wherein an increase in the level of miR-199a-5p expression and decrease in the level of caveolin-1 expression relative to the reference biological sample identifies said subject as liable to develop a fibroproliferative disorder.

6. A method for monitoring progression of a fibroproliferative disorder in a subject, characterized in that it comprises the following steps:

(i) quantitative measurement of the level of miR-199a-5p expression and of the level of expression of a target gene for which said expression level is regulated by the level of miR-199a-5p expression and wherein said target gene codes for caveolin-1, in biological samples from said subject taken at different time intervals; and

(ii) comparing the level of miR-199a-5p expression and the level of expression of said target gene in each of said samples from said subject, and determining whether there is progression of the fibroproliferative disorder or not,

wherein an increase of the level of miR-199a-5p expression and a decrease in the level of caveolin-1 expression relative to the sample taken at the preceding time interval identify the progression of the fibroproliferative disorder in said subject.

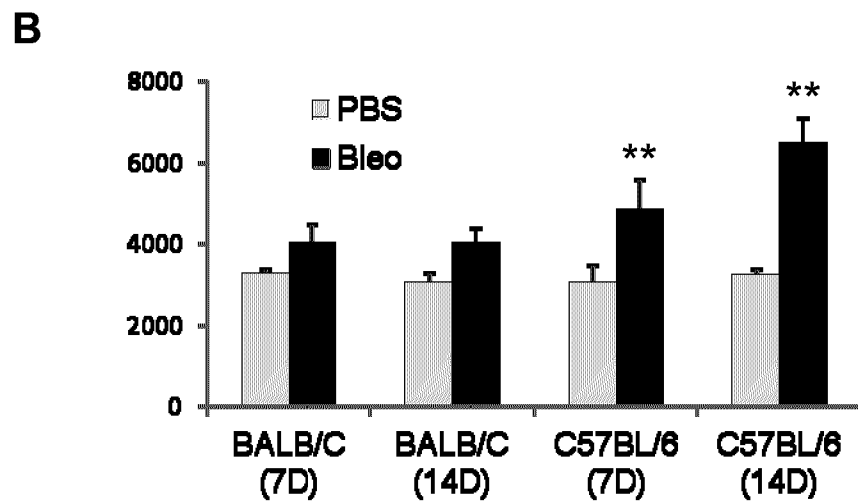
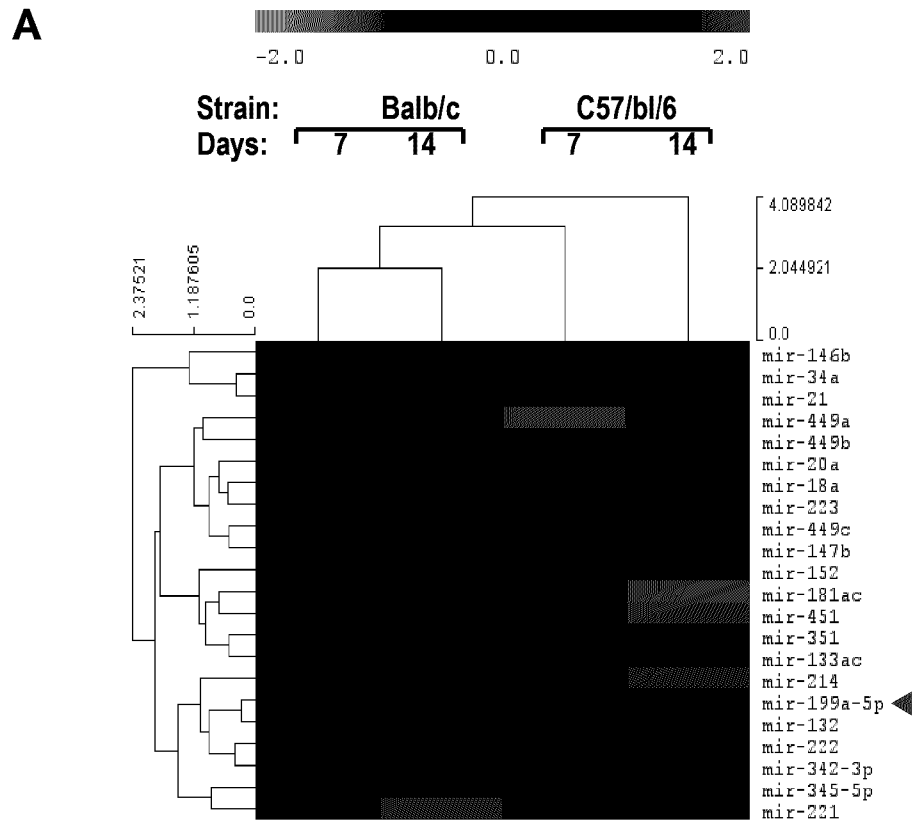


FIGURE 1

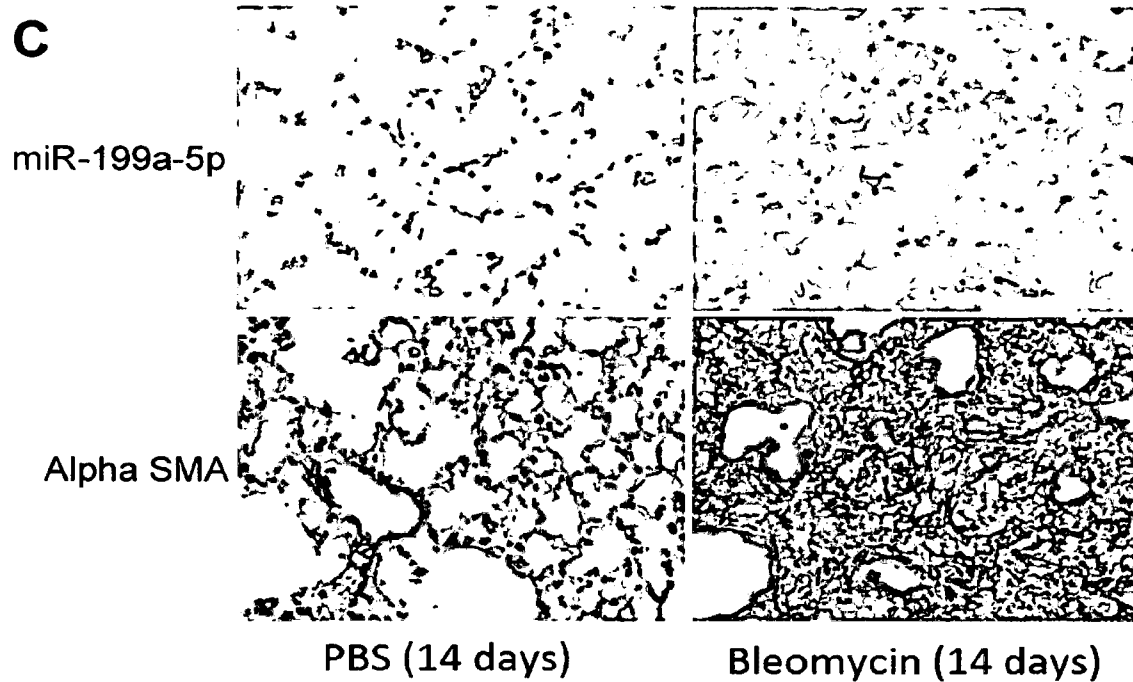


FIGURE 1 (END)

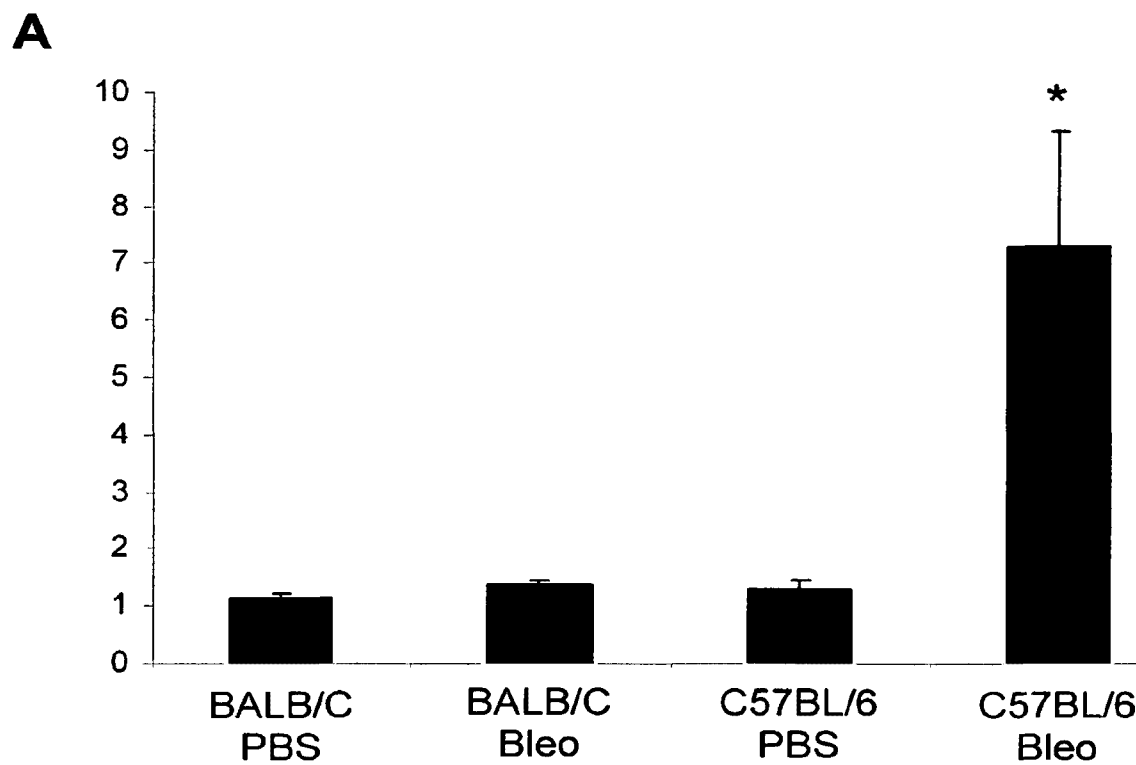


FIGURE 2

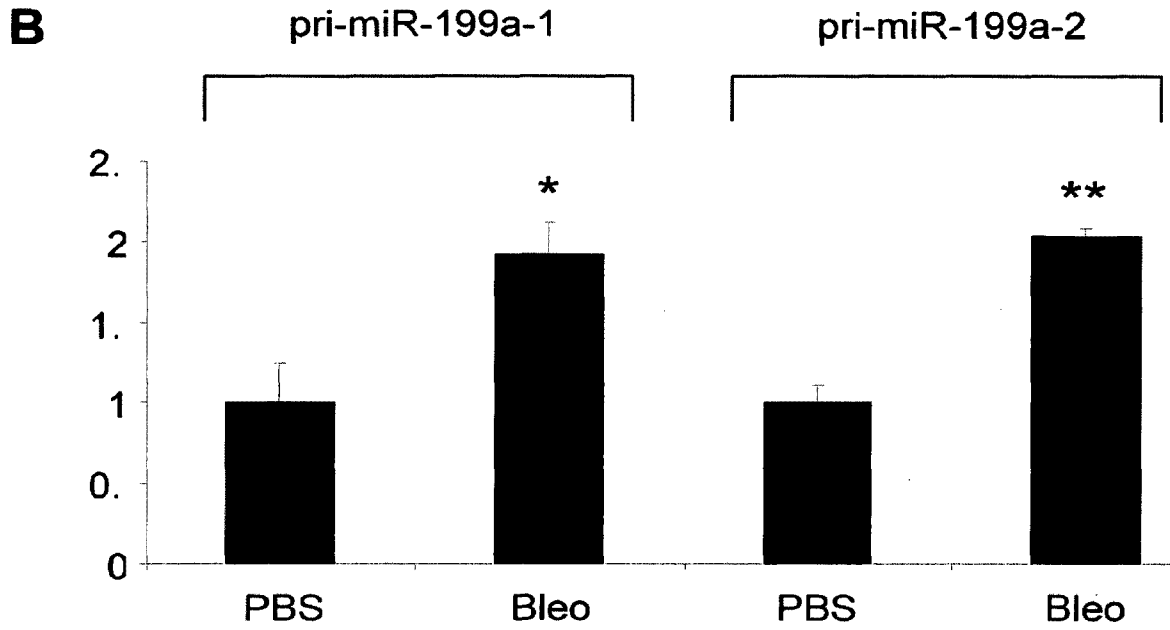


FIGURE 2 (END)

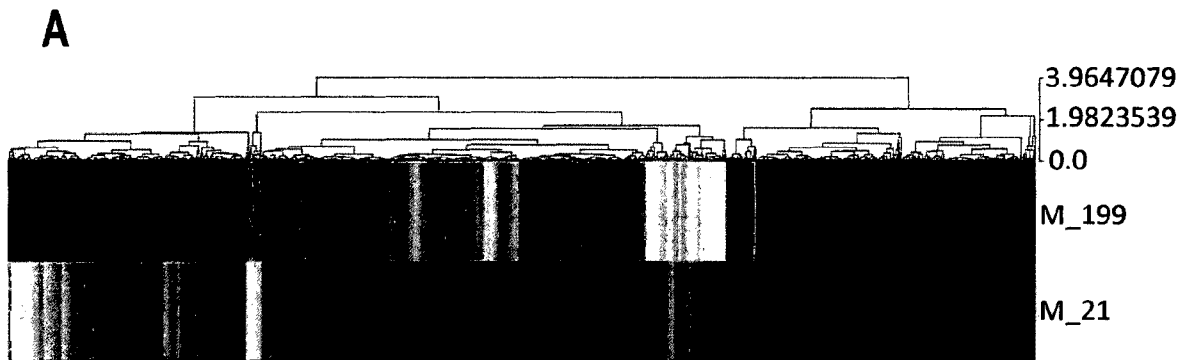
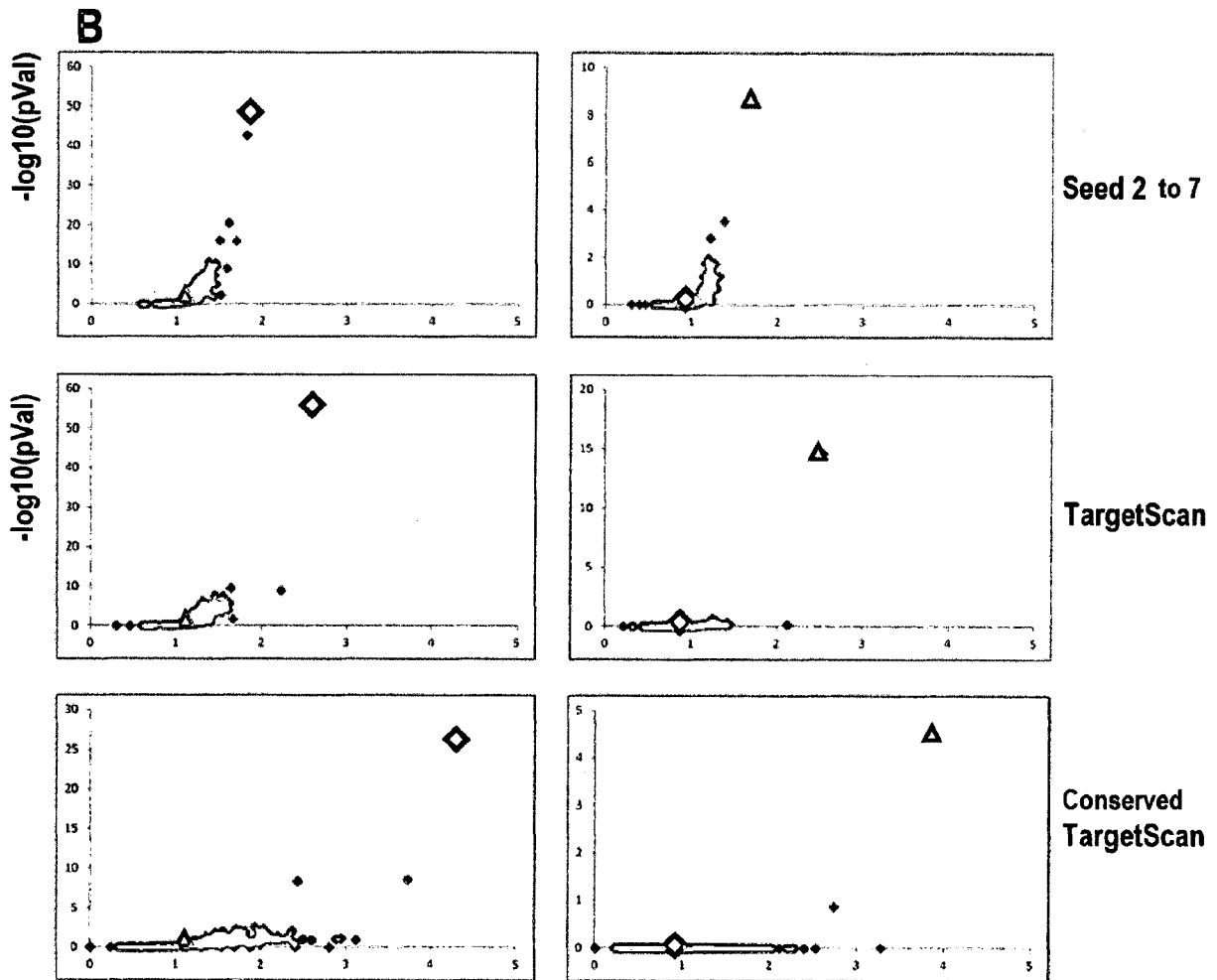


FIGURE 3



**C**

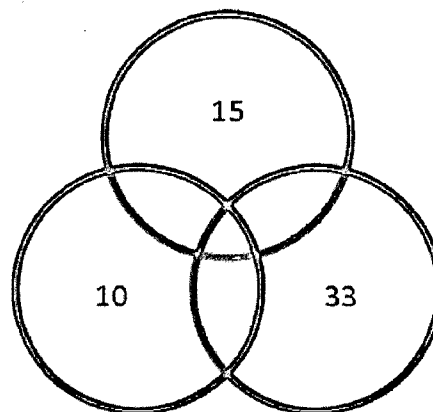


FIGURE 3 (END)

| <b>Ingenuity canonical pathways</b>                   | <b>miR-199-5p</b> | <b>miR-21</b> |
|-------------------------------------------------------|-------------------|---------------|
| Acute phase response signalling                       | 2.39              | ns            |
| April-mediated signalling                             | 2.52              | ns            |
| ATM signalling                                        | ns                | 4.69          |
| Steroid biosynthesis                                  | 6.94              | ns            |
| Caveolae-mediated endocytosis signalling              | 1.46              | ns            |
| Chromosomal replication cell cycle control            | ns                | 6.84          |
| Cell cycle: G1/S checkpoint regulation                | 4.06              | 3.71          |
| Cell cycle: DNA damage G2/M checkpoint regulation     | ns                | 7.71          |
| Cyclin and cell cycle regulation                      | 3.03              | 6.55          |
| Mortality receptor signalling                         | 1.74              | 1.21          |
| Double-strand homologous recombination break repair   | ns                | 2.86          |
| Fatty acid metabolism                                 | 1.31              | ns            |
| GNRH signalling                                       | 1.74              | ns            |
| IL-1 signalling                                       | 2.63              | ns            |
| IL-10 signalling                                      | 2.13              | ns            |
| IL-12 signalling and production in macrophages        | 2.28              | ns            |
| IL-15 signalling                                      | 1.59              | ns            |
| IL-17A signalling in respiratory cells                | 2.16              | ns            |
| IL-17A signalling in fibroblasts                      | ns                | 2.75          |
| IL-6 signalling                                       | 1.89              | ns            |
| Integrin signalling                                   | 1.87              | ns            |
| LPS-stimulated MAPK signalling                        | 2.45              | ns            |
| Mismatch repair in eukaryotes                         | ns                | 3.96          |
| Mitotic roles of Polo type kinase                     | 1.22              | 9.15          |
| Molecular mechanisms of cancer                        | 1.74              | 1.41          |
| NF- $\kappa$ B signalling                             | 3.03              | ns            |
| Nur77 signalling in T lymphocytes                     | 1.57              | ns            |
| MAPK p38 signalling                                   | 2.79              | ns            |
| p53 signalling                                        | 1.78              | ns            |
| Pantothenate and CoA biosynthesis                     | 1.71              | ns            |
| Parkinson's disease signalling                        | 2.04              | ns            |
| Phospholipase C signalling                            | 1.57              | ns            |
| PKC signalling in T lymphocytes                       | 2.12              | ns            |
| PPAR signalling                                       | 1.53              | ns            |
| NO and ROS production in macrophages                  | 1.68              | ns            |
| Protein ubiquitination pathway                        | ns                | 1.50          |
| PTEN signalling                                       | 2.70              | ns            |
| Purine metabolism                                     | 1.69              | ns            |
| Pyrimidine metabolism                                 | 1.52              | 1.19          |
| Role of BRCA1 in DNA damage response                  | ns                | 5.61          |
| Role of CHK proteins in cell cycle checkpoint control | ns                | 6.07          |

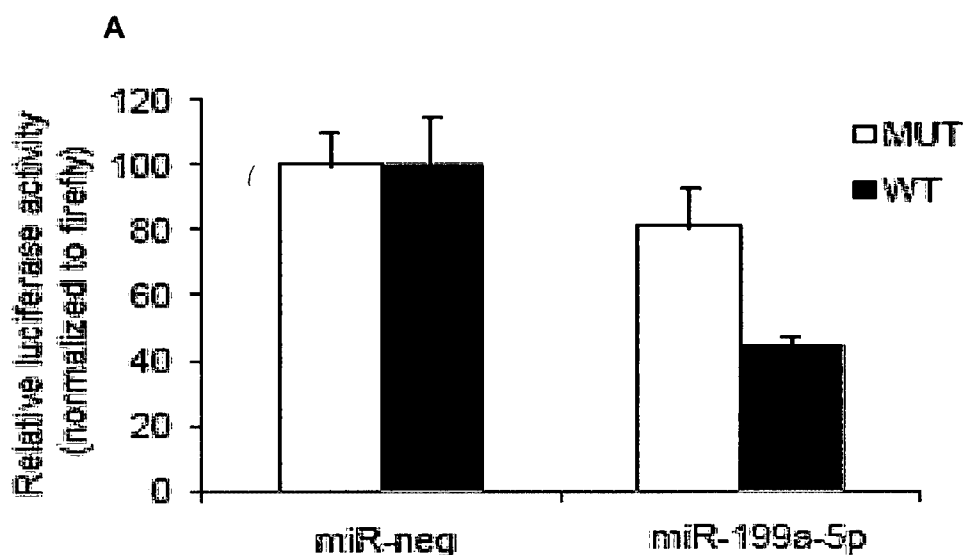
FIGURE 4

**Ingenuity canonical pathways**

Role of JAK family kinases in IL-6 type Cytokine signalling  
 Role of NFAT in cardiac hypertrophy  
 Role of PKR in interferon induction and antiviral response  
 SCLC signalling  
 TGF- $\beta$  signalling  
 TWEAK signalling  
 Diabetes mellitus type I signalling  
 Diabetes mellitus type II signalling  
 Valine, leucine and isoleucine degradation  
 Wnt/ $\beta$ -catenin signalling

| miR-199-5p | miR-21 |
|------------|--------|
| 1.60       | ns     |
| 2.65       | ns     |
| 1.68       | ns     |
| 2.45       | 2.22   |
| 2.22       | 1.41   |
| 2.04       | 1.07   |
| 1.82       | ns     |
| 2.14       | ns     |
| 1.56       | ns     |
| 2.41       | ns     |

FIGURE 4 (END)



|              |                                                                                                                                                                                                                                                        |                |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>Human</b> | 5' ACU UUU UGC AUU UAA AAC AGA <u>CAC</u> <u>UGG</u> CAU 3'                                                                                                                                                                                            | SEQ ID NO : 10 |
| <b>Mouse</b> | 5' AUU GUU UGC AUU UAA AAC AGA <u>CAC</u> <u>UGG</u> CAU 3'                                                                                                                                                                                            | SEQ ID NO : 11 |
| <b>Rat</b>   | 5' AUU GUU CCC AUU UGA AAC AGA <u>CAC</u> <u>UGG</u> CAU 3'                                                                                                                                                                                            | SEQ ID NO : 12 |
|              | <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">      <br/>3' C UUG UCC AUC AGA CUU <u>GUG</u> <u>ACC</u> C 5' </div> <div style="text-align: center;">          <br/> </div> </div> | SEQ ID NO : 13 |

FIGURE 5

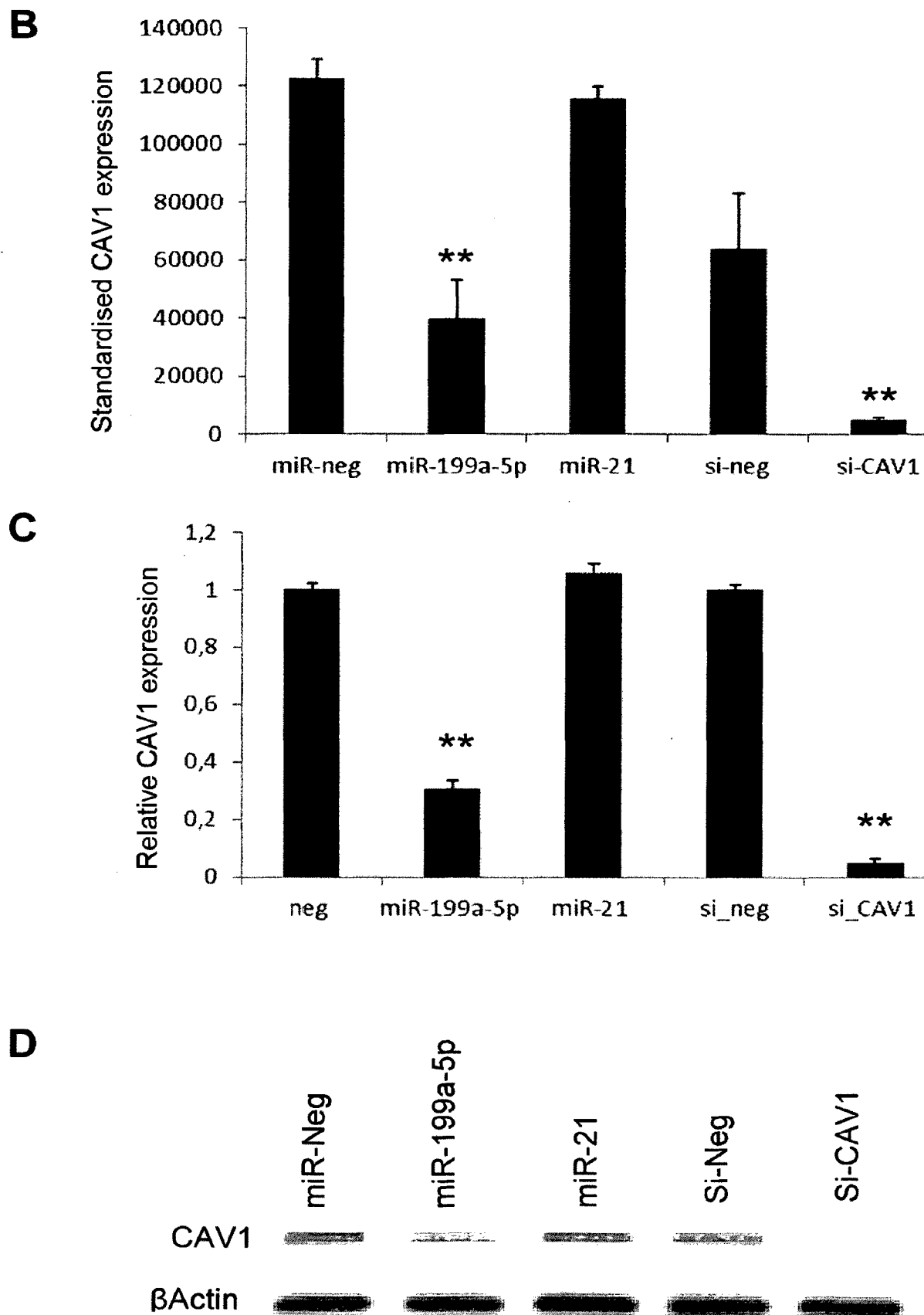


FIGURE 5 (END)

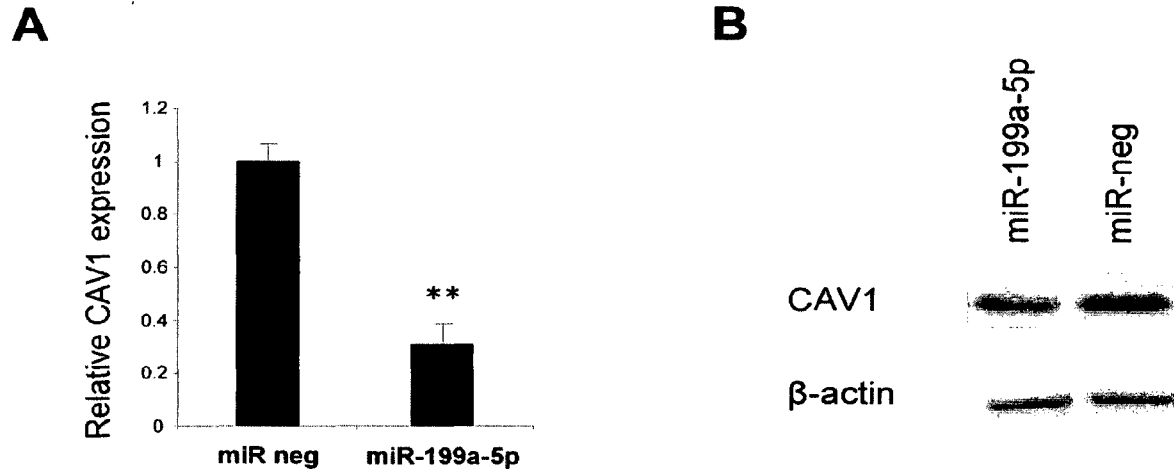


FIGURE 6

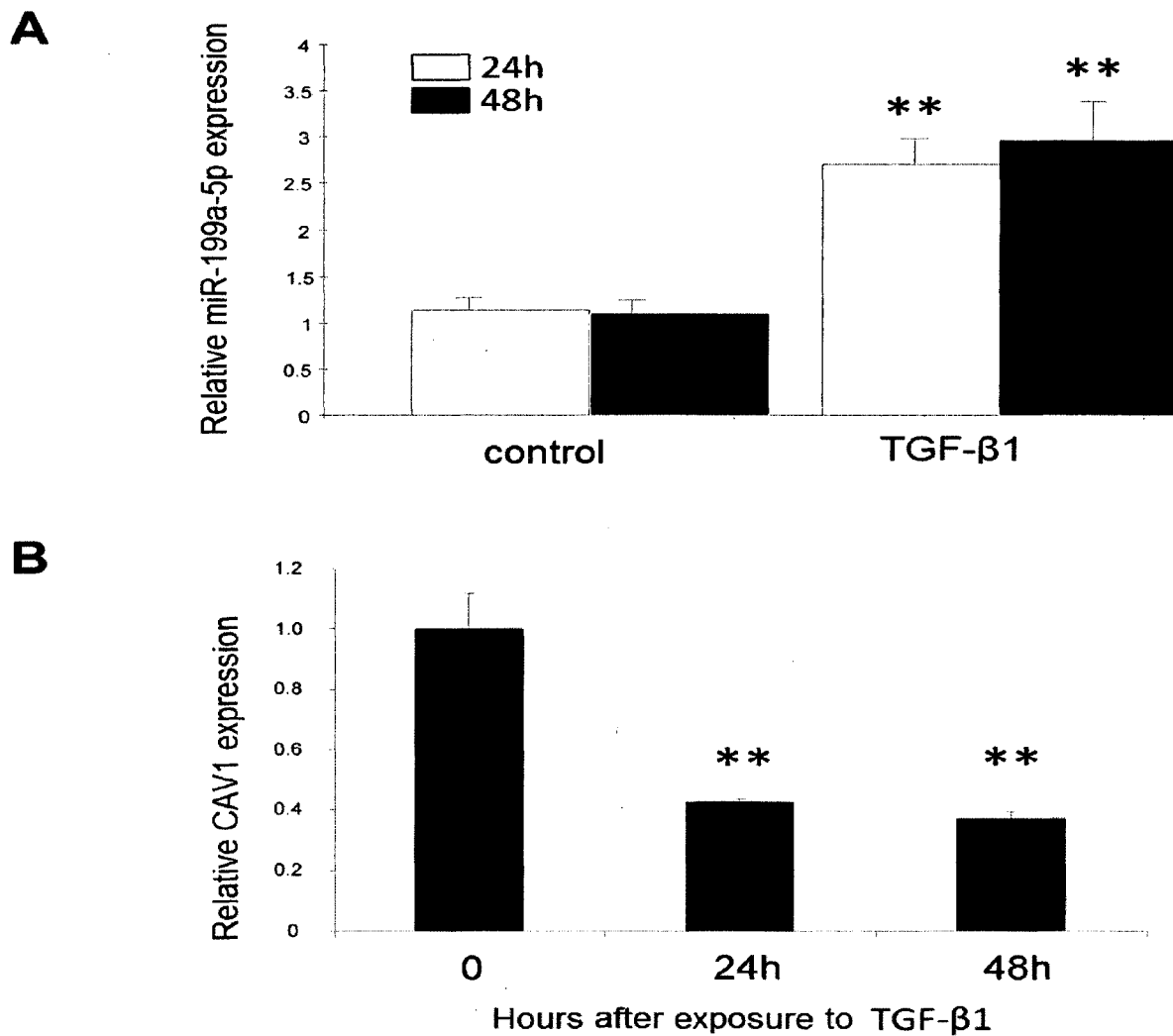


FIGURE 7

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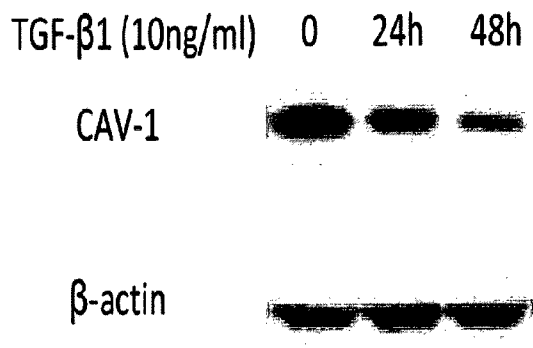
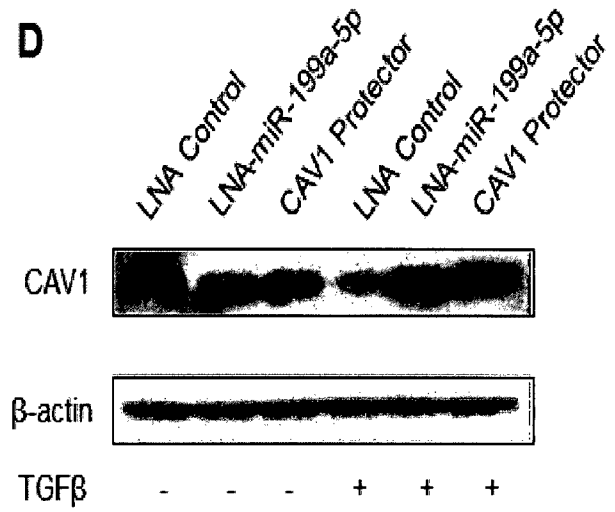
**C****D**

FIGURE 7 (END)

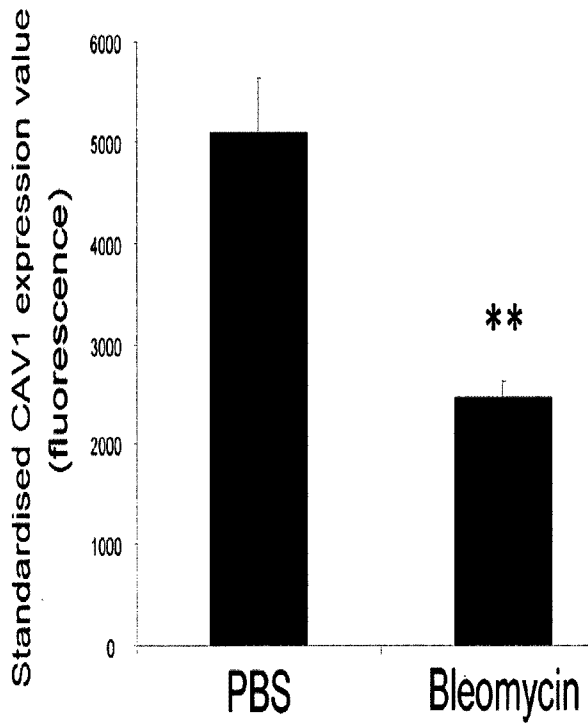
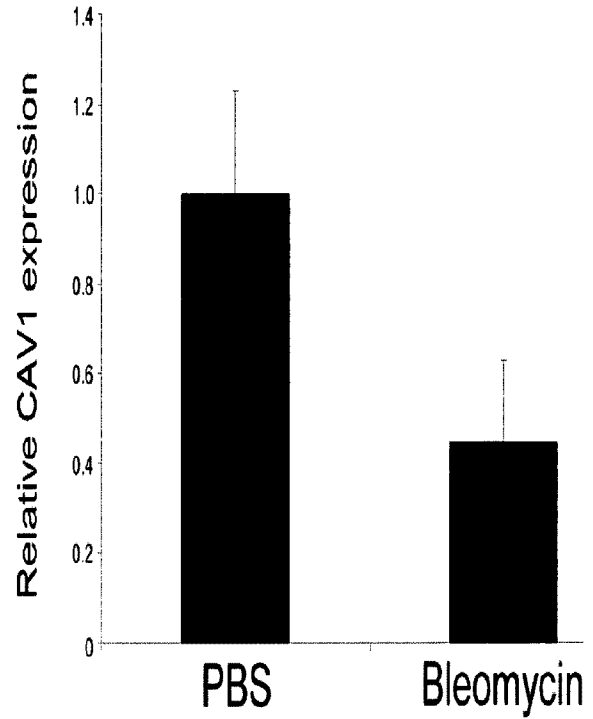
**A****B**

FIGURE 8

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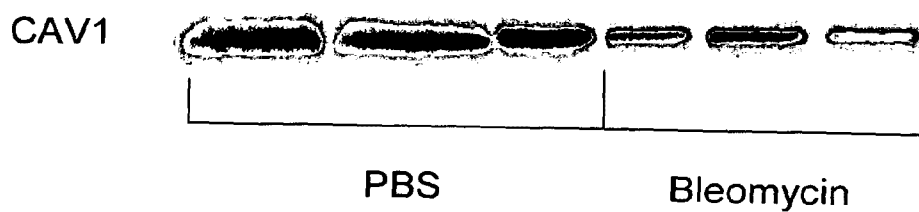
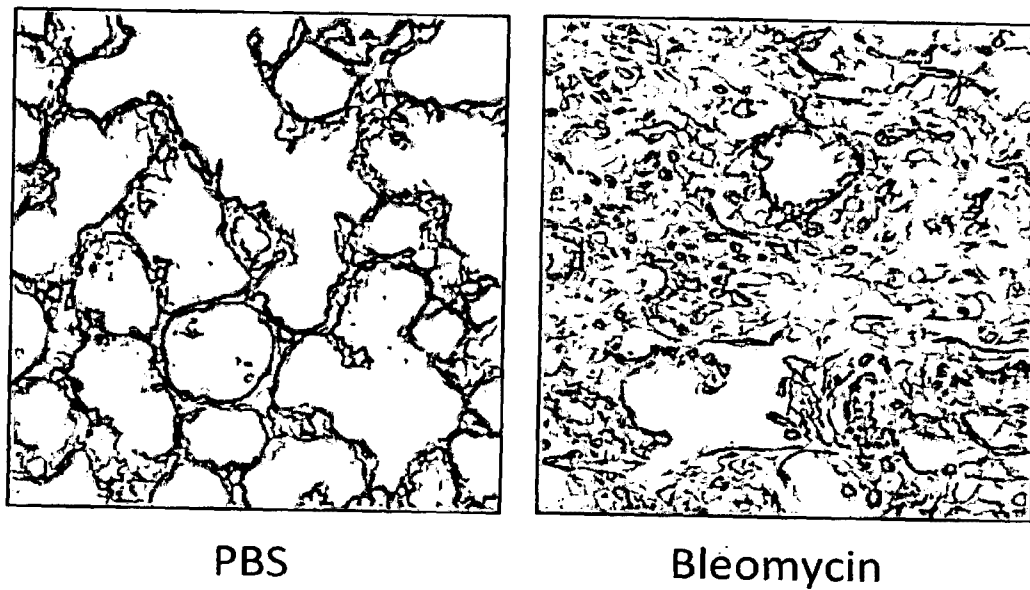
**C****D**

FIGURE 8 (END)

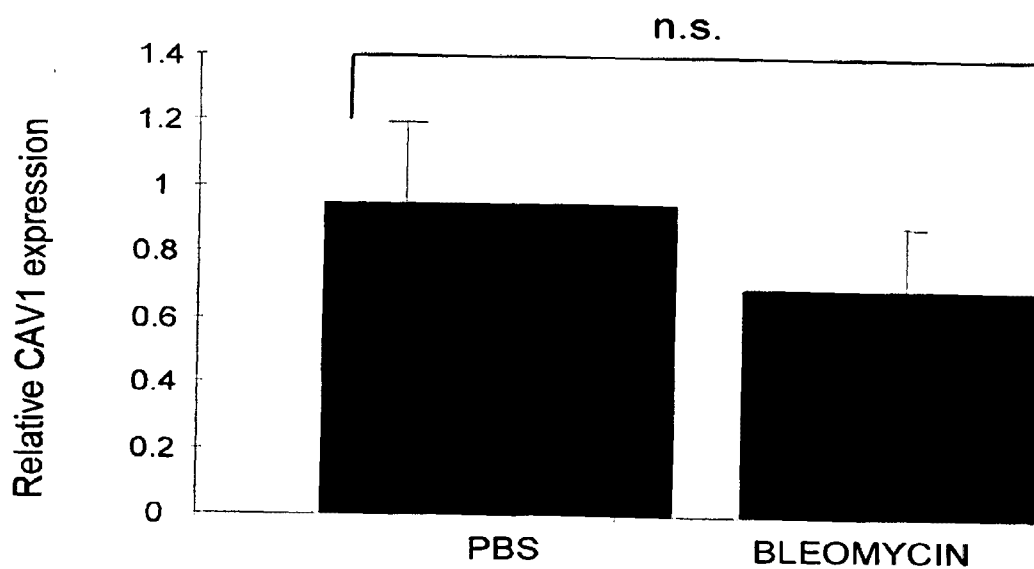


FIGURE 9

| Gene symbol | Lung fibroblasts transfected with miR-199a-5p |           |          | Bleomycin-induced pulmonary fibrosis |           |          |
|-------------|-----------------------------------------------|-----------|----------|--------------------------------------|-----------|----------|
|             | Average intensity                             | Log Ratio | P-value  | Average intensity                    | Log Ratio | P-value  |
| ADAMTS4     | 10.87                                         | 0.77      | 0.037205 | 9.02                                 | 3.17      | 0.000044 |
| AKAP7       | 9.57                                          | -1.59     | 0.002165 | 12.05                                | -1.07     | 0.001094 |
| ANAPC13     | 12.52                                         | -1.22     | 0.001805 | 15.47                                | -0.47     | 0.034385 |
| AOX1        | 11.46                                         | -1.69     | 0.000211 | 12.48                                | -0.98     | 0.001395 |
| ARAP3       | 11.96                                         | -0.70     | 0.027772 | 10.49                                | -1.28     | 0.000042 |
| ARHGAP12    | 11.29                                         | -2.00     | 0.010078 | 10.52                                | -0.85     | 0.000414 |
| ASGR1       | 9.08                                          | -0.99     | 0.012113 | 9.12                                 | -3.12     | 0.000005 |
| ASPH        | 13.01                                         | -1.43     | 0.029179 | 12.21                                | -0.44     | 0.002034 |
| ATP9A       | 13.11                                         | -1.20     | 0.008302 | 16.02                                | -0.36     | 0.049043 |
| BTRC        | 9.16                                          | -1.39     | 0.004120 | 10.11                                | -0.54     | 0.014927 |
| CABYR       | 10.57                                         | 0.94      | 0.041231 | 7.21                                 | 1.32      | 0.003024 |
| CACNB3      | 10.68                                         | 0.72      | 0.048016 | 10.44                                | 0.97      | 0.000449 |
| CAV1        | 15.73                                         | -1.71     | 0.005572 | 11.76                                | -1.03     | 0.002899 |
| CAV2        | 12.60                                         | -0.92     | 0.025258 | 14.01                                | -1.20     | 0.001986 |
| CCNA2       | 11.73                                         | 0.94      | 0.022863 | 10.95                                | 2.20      | 0.000330 |
| CCNB2       | 14.79                                         | 0.89      | 0.015840 | 11.52                                | 1.84      | 0.000200 |
| CDH2        | 14.14                                         | -1.96     | 0.000123 | 10.48                                | -0.54     | 0.027403 |
| CDKN1B      | 11.99                                         | -0.84     | 0.018487 | 13.08                                | -0.90     | 0.017326 |
| CDKN3       | 10.76                                         | 0.90      | 0.026616 | 8.65                                 | 1.88      | 0.000218 |
| CDT1        | 12.14                                         | 1.00      | 0.038265 | 11.65                                | 0.87      | 0.005190 |
| CENPA       | 11.43                                         | 0.83      | 0.045218 | 10.73                                | 1.23      | 0.003830 |
| CENPH       | 10.83                                         | 1.06      | 0.011271 | 8.43                                 | 1.71      | 0.000086 |
| CENPN       | 13.62                                         | 0.76      | 0.023514 | 7.52                                 | 1.06      | 0.015405 |
| CHAF1A      | 13.01                                         | 0.91      | 0.010525 | 9.30                                 | 0.81      | 0.003431 |
| CHCHD10     | 11.89                                         | -1.15     | 0.015618 | 16.97                                | -0.92     | 0.001257 |
| CHTF18      | 11.97                                         | 0.67      | 0.048768 | 9.41                                 | 1.02      | 0.014577 |
| CTF1        | 10.88                                         | -0.69     | 0.039451 | 10.65                                | -0.41     | 0.025602 |
| CTNND1      | 10.78                                         | -0.74     | 0.023539 | 15.01                                | -0.72     | 0.002786 |
| CYB5B       | 8.30                                          | -0.94     | 0.023676 | 13.70                                | -0.72     | 0.009805 |
| CYP2S1      | 7.54                                          | -2.00     | 0.000431 | 15.16                                | -0.79     | 0.002440 |
| CYTL1       | 7.96                                          | -1.23     | 0.008807 | 11.05                                | -1.54     | 0.000494 |
| DALRD3      | 10.58                                         | -0.86     | 0.040702 | 9.06                                 | -0.61     | 0.037489 |
| DDX58       | 9.73                                          | -1.72     | 0.014459 | 9.22                                 | -0.62     | 0.035363 |
| DECR1       | 12.69                                         | -0.79     | 0.042105 | 9.29                                 | -0.66     | 0.015693 |
| EIF2S1      | 11.42                                         | 1.73      | 0.016695 | 12.95                                | 0.53      | 0.029109 |
| ELN         | 10.94                                         | 1.36      | 0.013629 | 13.42                                | 3.08      | 0.000016 |
| ENO3        | 10.06                                         | -2.23     | 0.001120 | 12.35                                | -0.69     | 0.009636 |
| ENPP2       | 9.12                                          | -1.91     | 0.004048 | 12.99                                | -0.41     | 0.016366 |
| EPAS1       | 14.26                                         | -2.59     | 0.000410 | 13.14                                | -0.64     | 0.014242 |

FIGURE 10

| Gene symbol | Lung fibroblasts transfected with miR-199a-5p |           |          | Bleomycin-induced pulmonary fibrosis |           |          |
|-------------|-----------------------------------------------|-----------|----------|--------------------------------------|-----------|----------|
|             | Average intensity                             | Log Ratio | P-value  | Average intensity                    | Log Ratio | P-value  |
| FAM154B     | 7.77                                          | -1.06     | 0.033605 | 10.21                                | -0.36     | 0.035670 |
| FAM20C      | 12.84                                         | 0.87      | 0.022157 | 10.69                                | 1.44      | 0.003179 |
| FSTL1       | 17.14                                         | 1.03      | 0.017730 | 15.16                                | 1.96      | 0.000004 |
| GGCX        | 10.73                                         | -0.73     | 0.029469 | 10.70                                | -0.96     | 0.001969 |
| GGT5        | 7.34                                          | -0.67     | 0.044755 | 8.16                                 | -0.93     | 0.001706 |
| GINS1       | 12.36                                         | 1.10      | 0.013368 | 9.69                                 | 1.32      | 0.000611 |
| GINS2       | 12.70                                         | 0.67      | 0.030172 | 8.98                                 | 1.71      | 0.000083 |
| GPR20       | 9.21                                          | 2.02      | 0.000580 | 7.07                                 | 0.95      | 0.005075 |
| GSTA4       | 12.11                                         | -0.72     | 0.016022 | 11.55                                | -0.61     | 0.017292 |
| HDAC6       | 10.63                                         | 1.53      | 0.000478 | 10.86                                | 0.63      | 0.002000 |
| HES6        | 11.29                                         | 1.53      | 0.016007 | 12.56                                | 0.73      | 0.001583 |
| HIP1R       | 12.29                                         | -1.05     | 0.007774 | 9.86                                 | -0.68     | 0.000640 |
| HMGN3       | 12.82                                         | 0.69      | 0.048957 | 11.26                                | 0.72      | 0.006134 |
| INCENP      | 10.04                                         | 1.31      | 0.036223 | 12.30                                | 1.14      | 0.000272 |
| ING4        | 10.38                                         | -1.08     | 0.008163 | 12.66                                | -0.41     | 0.021327 |
| IQSEC2      | 11.60                                         | -1.04     | 0.009299 | 12.44                                | -0.48     | 0.022345 |
| KCNN2       | 12.06                                         | -1.80     | 0.002929 | 8.47                                 | -1.22     | 0.009402 |
| KCTD14      | 7.07                                          | -0.95     | 0.039884 | 9.80                                 | -0.61     | 0.031442 |
| KIT         | 9.66                                          | -0.99     | 0.027587 | 13.12                                | -0.88     | 0.012905 |
| LBH         | 10.31                                         | 1.56      | 0.002584 | 12.87                                | 0.72      | 0.002351 |
| LPCAT2      | 9.25                                          | 0.98      | 0.037453 | 7.60                                 | 0.90      | 0.008390 |
| LRRFIP1     | 11.97                                         | -0.66     | 0.037696 | 13.27                                | -0.54     | 0.007590 |
| MAGI1       | 7.52                                          | -1.21     | 0.024711 | 9.03                                 | -0.51     | 0.049757 |
| MAP3K11     | 13.29                                         | -2.78     | 0.000346 | 11.58                                | -0.56     | 0.012199 |
| MAP3K5      | 11.98                                         | -1.80     | 0.006828 | 10.00                                | -0.87     | 0.006964 |
| MBOAT1      | 9.75                                          | 1.31      | 0.000828 | 12.08                                | 0.42      | 0.014647 |
| MDGA1       | 9.91                                          | 1.90      | 0.007768 | 8.10                                 | 1.57      | 0.005156 |
| MEGF6       | 9.82                                          | -0.87     | 0.030507 | 12.14                                | -1.07     | 0.000602 |
| NBL1        | 14.18                                         | -0.93     | 0.043617 | 15.72                                | -0.47     | 0.022266 |
| NCBP2       | 11.40                                         | -1.97     | 0.000828 | 10.18                                | -0.45     | 0.014578 |
| NEDD4L      | 10.41                                         | -1.28     | 0.045937 | 9.60                                 | -1.68     | 0.001422 |
| NHLRC3      | 8.90                                          | 1.69      | 0.008096 | 8.34                                 | 0.95      | 0.000194 |
| NIPAL3      | 13.11                                         | -1.34     | 0.001092 | 13.13                                | -1.10     | 0.001203 |
| NUBP1       | 13.19                                         | 0.79      | 0.038434 | 13.00                                | 0.98      | 0.000249 |
| NUSAP1      | 12.38                                         | 0.87      | 0.019359 | 9.80                                 | 1.60      | 0.001794 |
| ODF3B       | 7.27                                          | -0.90     | 0.025859 | 13.09                                | -0.69     | 0.015031 |
| OTUD3       | 9.22                                          | 1.70      | 0.012549 | 11.10                                | 0.72      | 0.009897 |
| PMEPA1      | 10.80                                         | 0.86      | 0.013635 | 13.06                                | 1.49      | 0.000062 |
| PNKD        | 7.12                                          | -1.15     | 0.005612 | 7.96                                 | -1.08     | 0.003491 |
| PNPLA6      | 9.56                                          | -1.63     | 0.009919 | 12.37                                | -0.30     | 0.047030 |

FIGURE 10 (CONTINUED)

| Gene symbol | Lung fibroblasts transfected with miR-199a-5p |           |          | Bleomycin-induced pulmonary fibrosis |           |          |
|-------------|-----------------------------------------------|-----------|----------|--------------------------------------|-----------|----------|
|             | Average intensity                             | Log Ratio | P-value  | Average intensity                    | Log Ratio | P-value  |
| POMGNT1     | 13.66                                         | -0.88     | 0.005364 | 12.08                                | -0.32     | 0.023190 |
| PPFIBP2     | 8.82                                          | -0.75     | 0.032615 | 13.05                                | -0.80     | 0.015070 |
| PPP2R3C     | 13.91                                         | 0.86      | 0.033926 | 11.45                                | 0.53      | 0.025985 |
| PRC1        | 14.41                                         | 0.84      | 0.004102 | 11.18                                | 1.78      | 0.000124 |
| PRELID2     | 9.33                                          | 0.99      | 0.042979 | 9.99                                 | 0.56      | 0.005315 |
| PRICKLE1    | 11.41                                         | -1.22     | 0.005224 | 14.07                                | -1.42     | 0.000180 |
| PRMT2       | 10.05                                         | -0.66     | 0.047910 | 14.01                                | -0.67     | 0.030405 |
| PRPS2       | 8.02                                          | -0.95     | 0.041219 | 10.22                                | -0.91     | 0.000555 |
| R3HDM2      | 12.36                                         | -1.23     | 0.019502 | 13.32                                | -0.42     | 0.017910 |
| RALGAPB     | 10.47                                         | -1.18     | 0.023239 | 12.96                                | -0.47     | 0.009014 |
| RASGRP2     | 8.06                                          | -1.37     | 0.009020 | 12.24                                | -0.60     | 0.004782 |
| RBM24       | 9.47                                          | -2.59     | 0.000360 | 9.44                                 | -0.27     | 0.039708 |
| REEP5       | 13.48                                         | -2.17     | 0.002005 | 12.55                                | -0.42     | 0.025985 |
| RGMB        | 9.81                                          | -0.75     | 0.028634 | 11.30                                | -0.87     | 0.005668 |
| RNASEH2A    | 13.07                                         | 0.75      | 0.036633 | 10.81                                | 0.49      | 0.048594 |
| RNF141      | 11.52                                         | -1.54     | 0.015631 | 12.99                                | -0.67     | 0.001122 |
| ROBO4       | 7.54                                          | -1.65     | 0.011311 | 14.51                                | -0.67     | 0.011915 |
| RRM2B       | 9.58                                          | -2.97     | 0.001651 | 8.51                                 | -0.62     | 0.006787 |
| RTCD1       | 14.33                                         | 1.38      | 0.004627 | 12.19                                | 0.52      | 0.007499 |
| SELENBP1    | 12.64                                         | -0.81     | 0.016828 | 17.28                                | -0.45     | 0.040980 |
| SGIP1       | 8.80                                          | -1.31     | 0.012026 | 8.65                                 | -0.62     | 0.019749 |
| SH3D19      | 12.72                                         | -1.29     | 0.008996 | 9.60                                 | -0.91     | 0.013287 |
| SIRT5       | 10.40                                         | -1.13     | 0.011311 | 11.21                                | -0.51     | 0.013278 |
| SKA1        | 9.70                                          | 1.02      | 0.006580 | 8.56                                 | 1.79      | 0.000316 |
| SLC2A6      | 11.76                                         | 0.89      | 0.014990 | 10.92                                | 0.71      | 0.019866 |
| SLC39A14    | 13.51                                         | 0.96      | 0.017472 | 11.15                                | 2.02      | 0.000197 |
| SLC40A1     | 8.90                                          | 1.32      | 0.021215 | 10.64                                | 0.92      | 0.000270 |
| SMARCC2     | 14.23                                         | -0.64     | 0.032899 | 9.83                                 | -0.54     | 0.018426 |
| SMS         | 15.75                                         | -1.76     | 0.017748 | 10.87                                | -0.87     | 0.000163 |
| SMURF2      | 8.88                                          | -1.23     | 0.013799 | 10.89                                | -0.83     | 0.000156 |
| SOCS1       | 13.01                                         | 1.67      | 0.000297 | 10.87                                | 0.60      | 0.009453 |
| SORL1       | 9.72                                          | -1.50     | 0.000718 | 11.34                                | -1.06     | 0.002206 |
| SPC25       | 11.97                                         | 1.35      | 0.015518 | 11.39                                | 2.26      | 0.000033 |
| SPON2       | 15.60                                         | -0.80     | 0.025019 | 12.53                                | -1.24     | 0.011622 |
| SREBF2      | 11.51                                         | 1.25      | 0.001645 | 10.32                                | 1.35      | 0.000207 |
| STX3        | 8.69                                          | -1.21     | 0.024221 | 8.72                                 | -1.13     | 0.000179 |
| SYNGR1      | 9.53                                          | -1.37     | 0.008975 | 7.11                                 | -0.46     | 0.043806 |
| TCEAL1      | 11.86                                         | -0.91     | 0.018668 | 10.23                                | -0.63     | 0.041974 |
| TDG         | 13.68                                         | 0.99      | 0.033435 | 8.30                                 | 0.43      | 0.029690 |
| TGFB3       | 8.06                                          | 1.00      | 0.010858 | 13.52                                | 0.81      | 0.007305 |

FIGURE 10 (CONTINUED)

| Gene symbol | Lung fibroblasts transfected with miR-199a-5p |           |          | Bleomycin-induced pulmonary fibrosis |           |          |
|-------------|-----------------------------------------------|-----------|----------|--------------------------------------|-----------|----------|
|             | Average intensity                             | Log Ratio | P-value  | Average intensity                    | Log Ratio | P-value  |
| TGFB1       | 8.50                                          | 1.74      | 0.007822 | 12.40                                | 0.56      | 0.005692 |
| THBD        | 7.77                                          | -1.61     | 0.012078 | 15.29                                | -1.22     | 0.034879 |
| THBS3       | 10.56                                         | -0.67     | 0.030059 | 13.75                                | -1.70     | 0.000072 |
| TMEM159     | 12.32                                         | -1.33     | 0.001835 | 12.14                                | -0.46     | 0.046713 |
| TMEM164     | 11.32                                         | -1.95     | 0.000495 | 13.69                                | -0.49     | 0.020506 |
| TMEM20      | 7.65                                          | 0.85      | 0.027311 | 9.65                                 | 0.61      | 0.024024 |
| TMEM45A     | 12.57                                         | 1.09      | 0.027911 | 11.66                                | 1.14      | 0.002854 |
| TRAIP       | 11.29                                         | 1.01      | 0.039309 | 8.71                                 | 1.42      | 0.005390 |
| TRAK1       | 11.44                                         | -1.45     | 0.032442 | 7.15                                 | -0.33     | 0.034043 |
| TSPAN13     | 7.88                                          | -1.12     | 0.045464 | 15.27                                | -1.74     | 0.000216 |
| TST         | 14.12                                         | -2.01     | 0.000099 | 12.60                                | -0.86     | 0.001296 |
| TTC39C      | 8.78                                          | 0.82      | 0.025257 | 10.00                                | 1.44      | 0.017367 |
| UBE2V1      | 13.50                                         | 2.19      | 0.000423 | 10.54                                | 0.40      | 0.038237 |
| WASF1       | 12.40                                         | 0.65      | 0.031618 | 8.51                                 | 0.41      | 0.049891 |

FIGURE 10 (END)

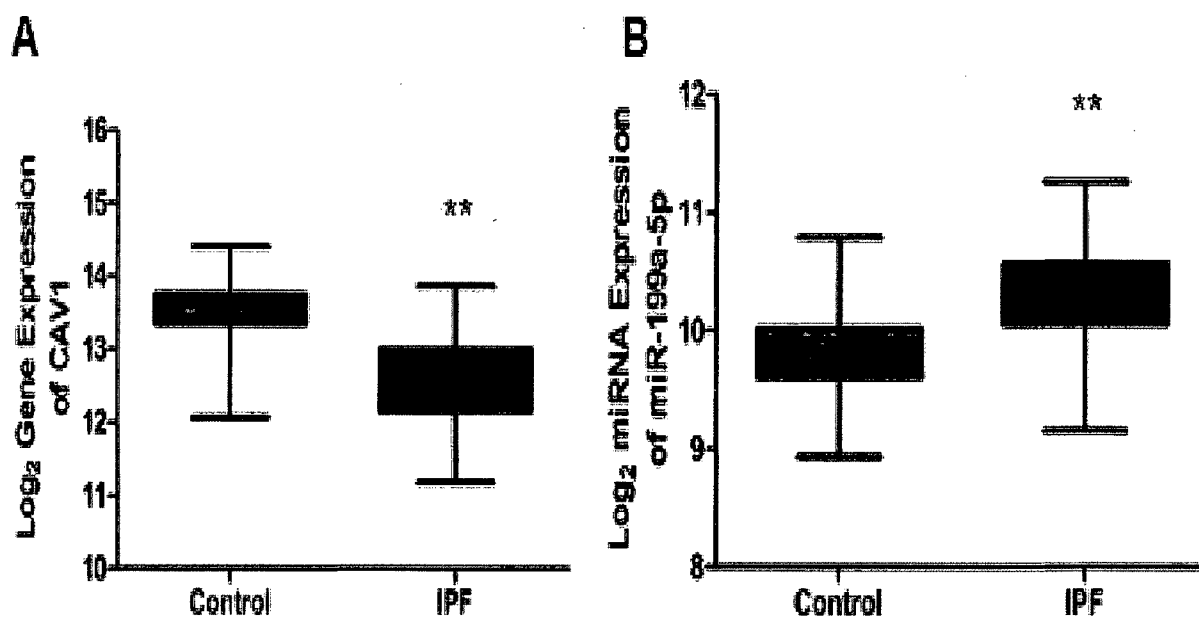


FIGURE 11

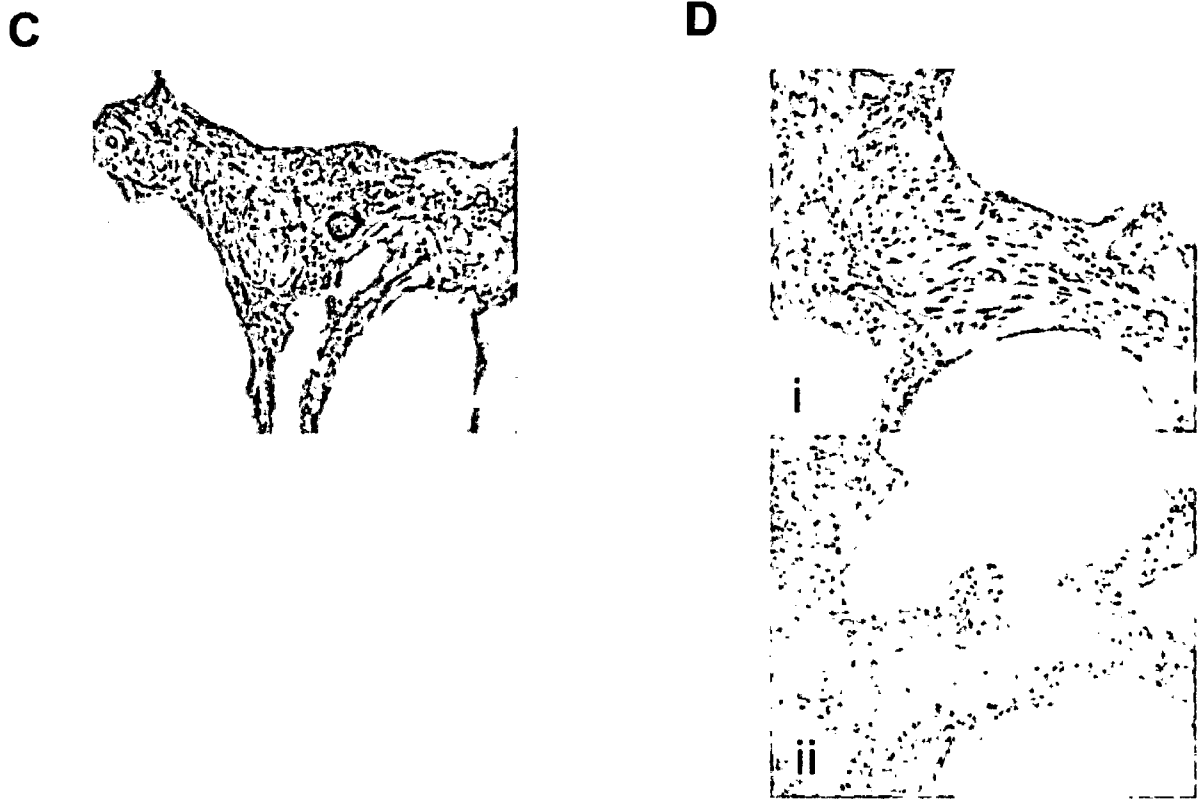


FIGURE 11 (END)

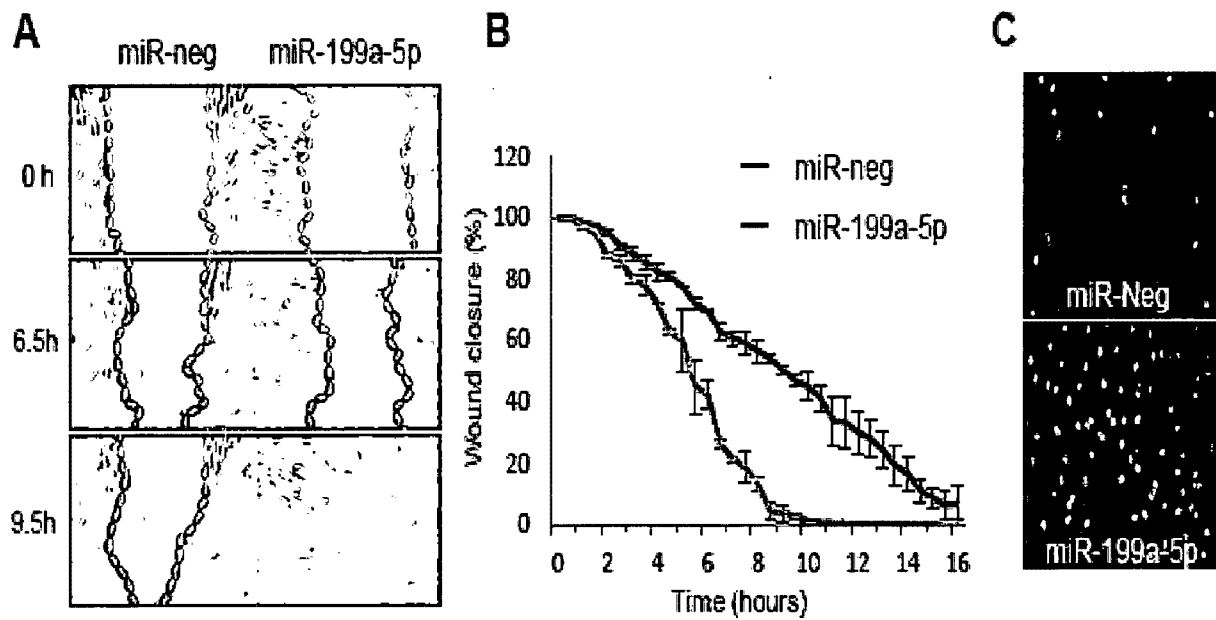
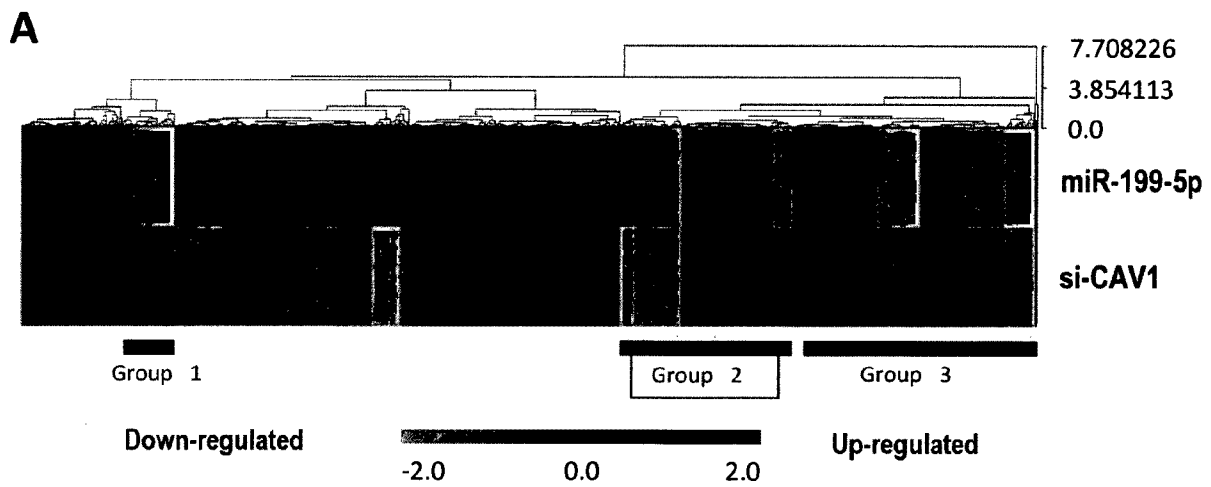
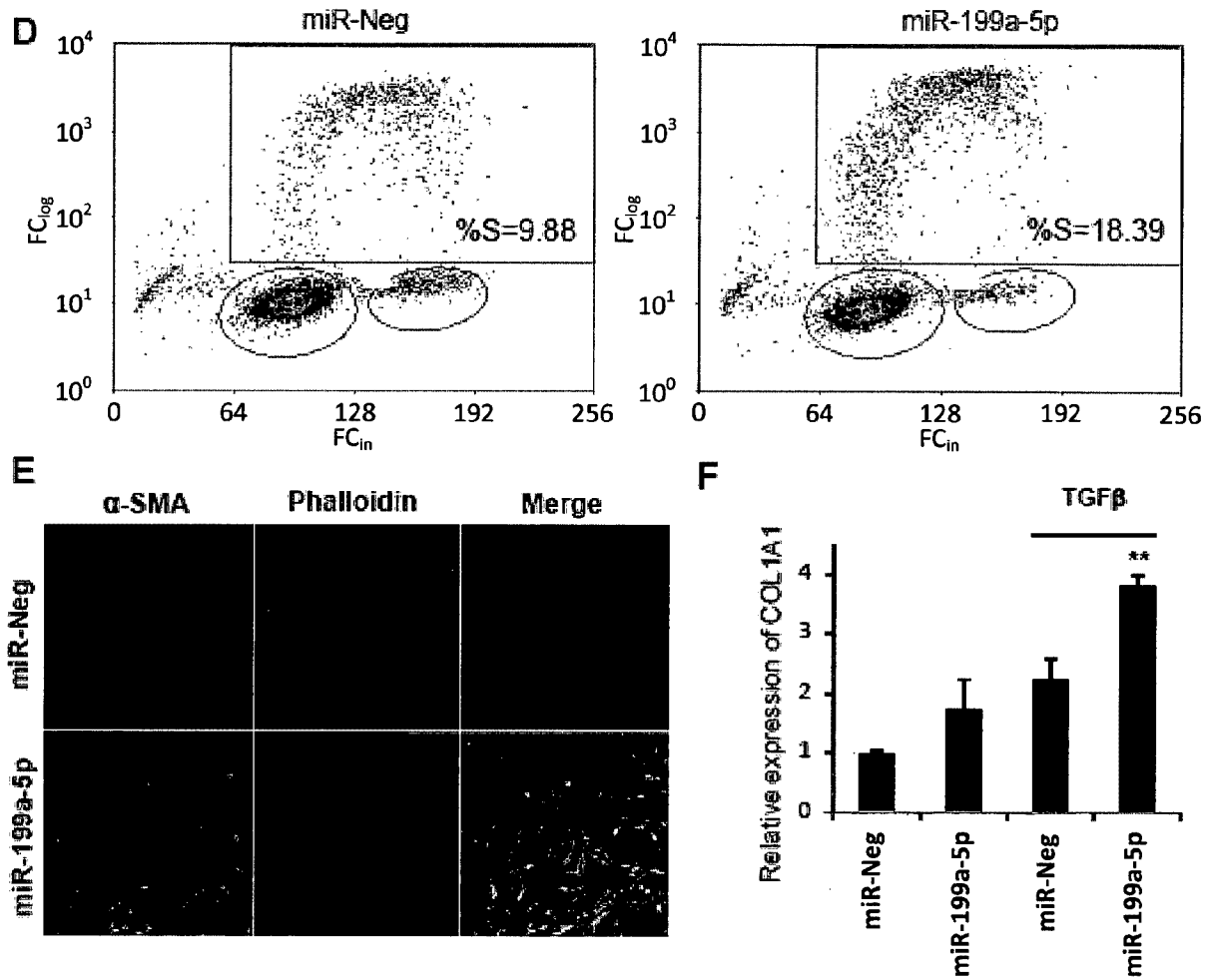


FIGURE 12



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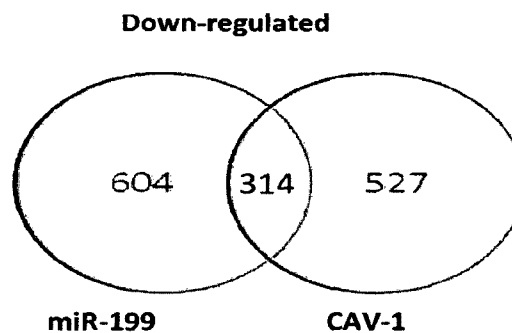
**B**

FIGURE 13 (END)

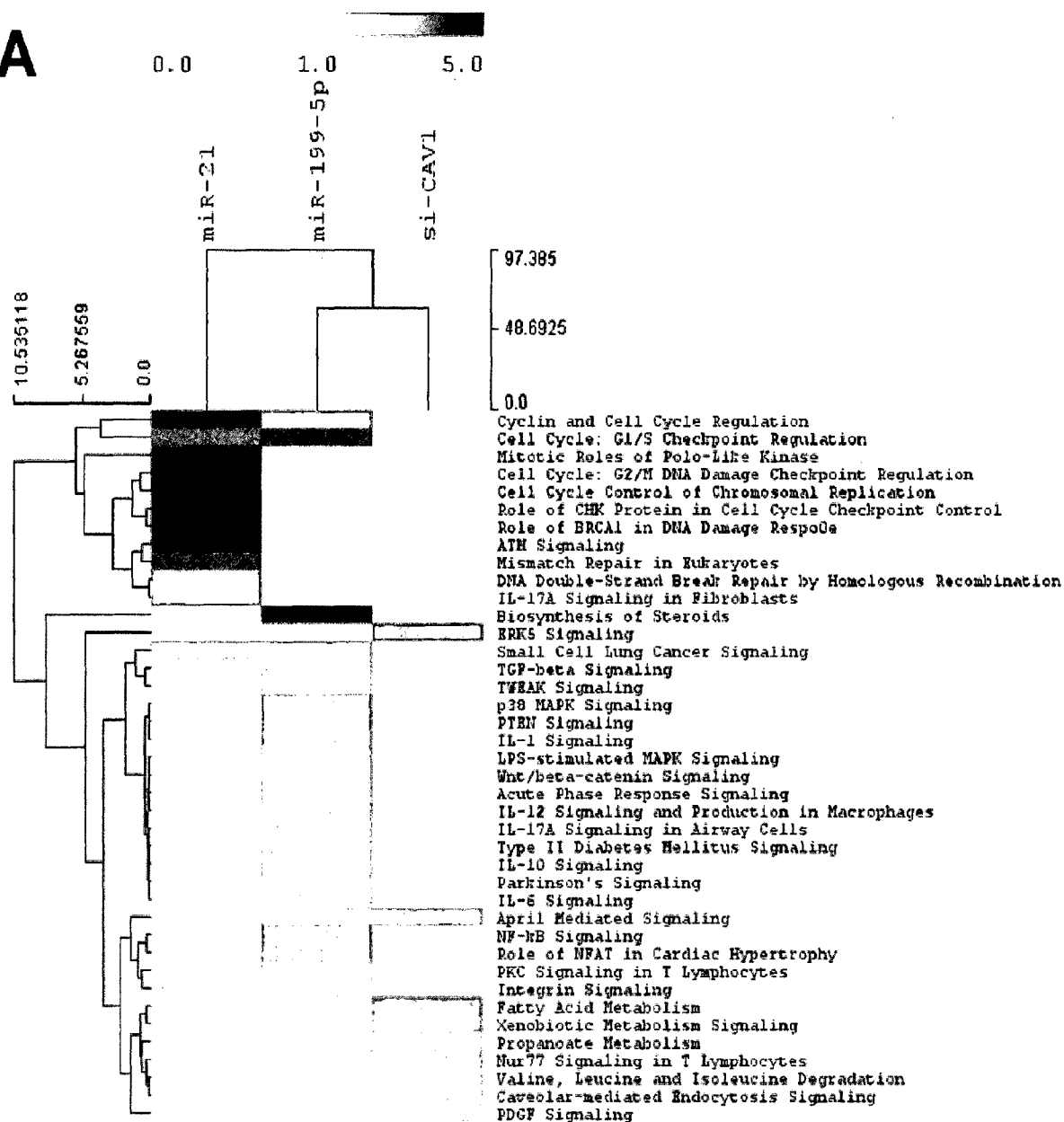
**A**

FIGURE 14

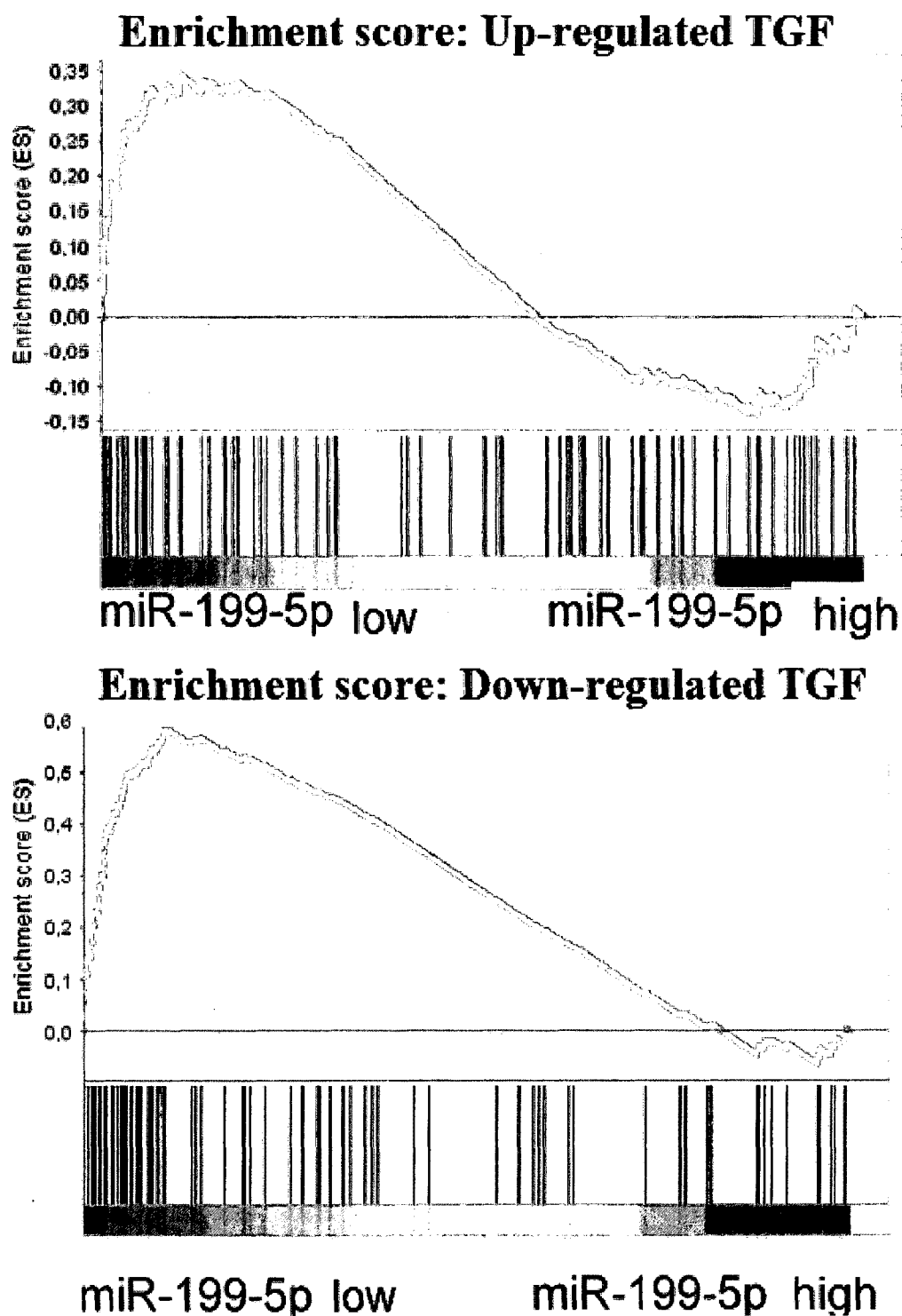
**B**

FIGURE 14 (CONTINUED)

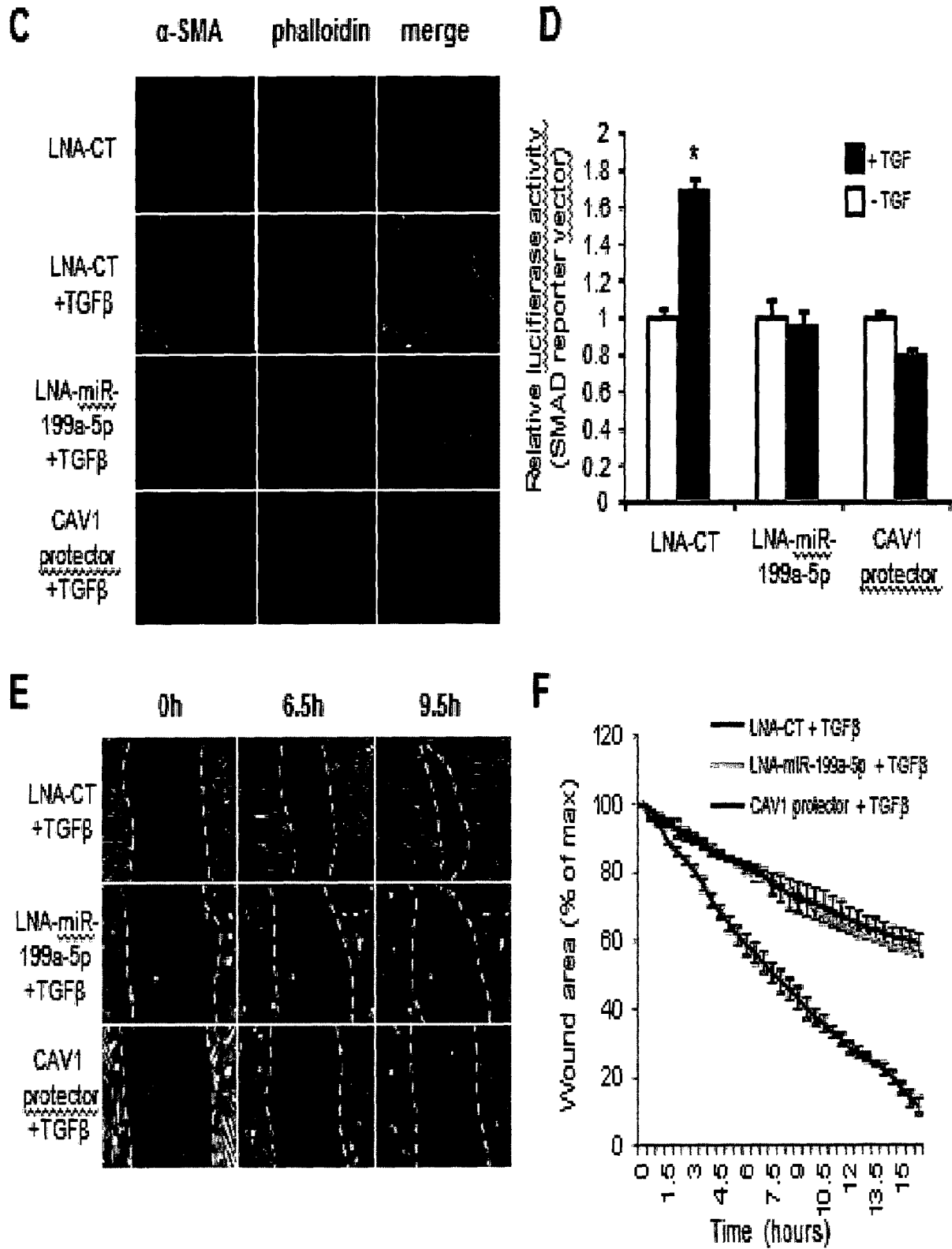


FIGURE 14 (END)

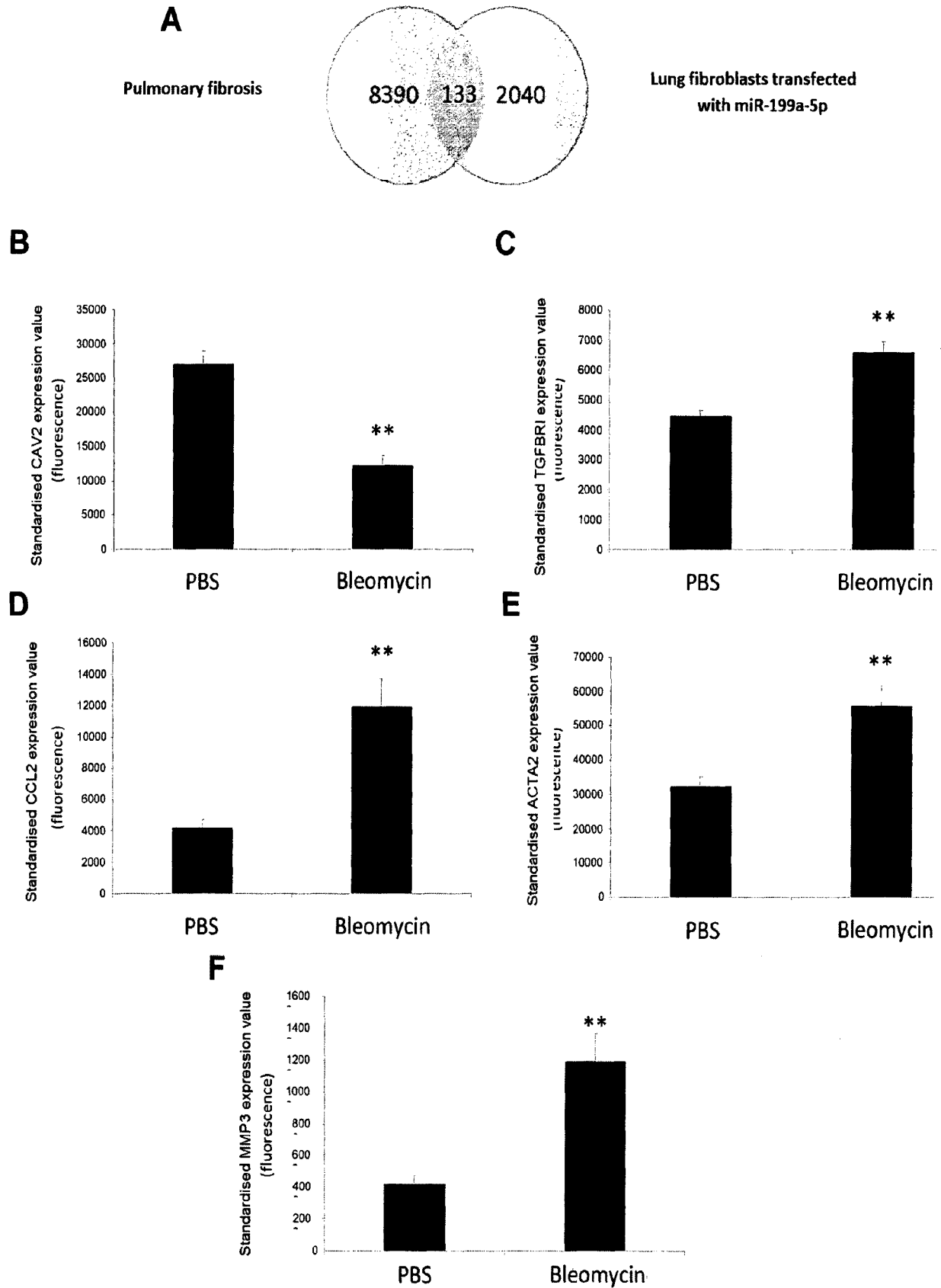


FIGURE 15

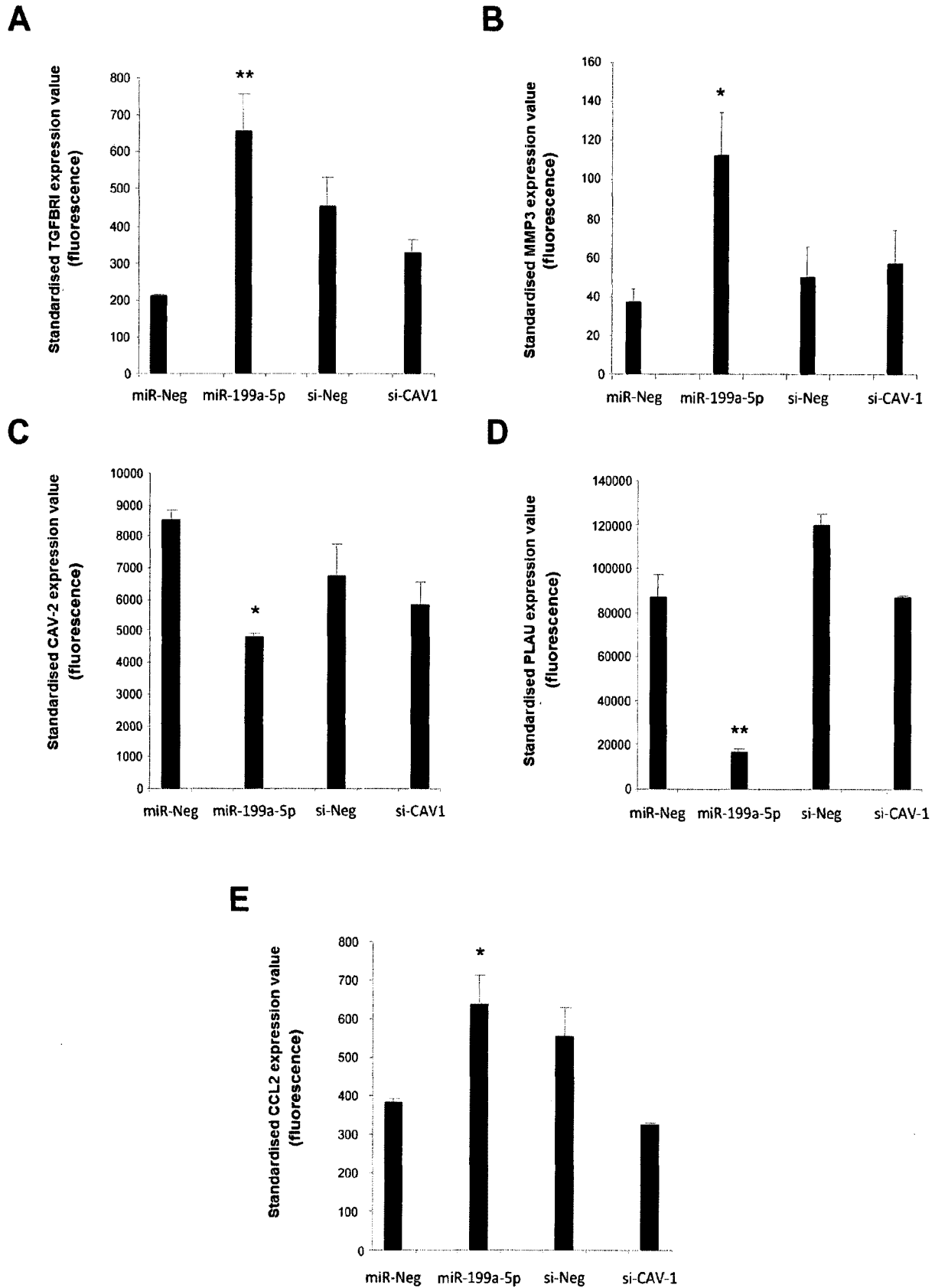


FIGURE 16

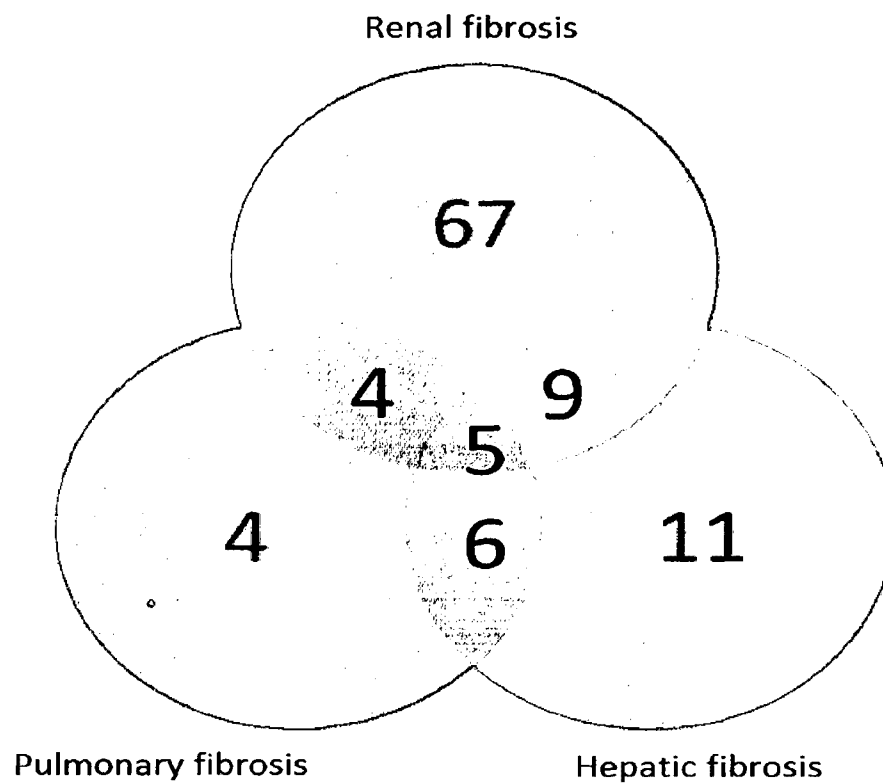


FIGURE 17

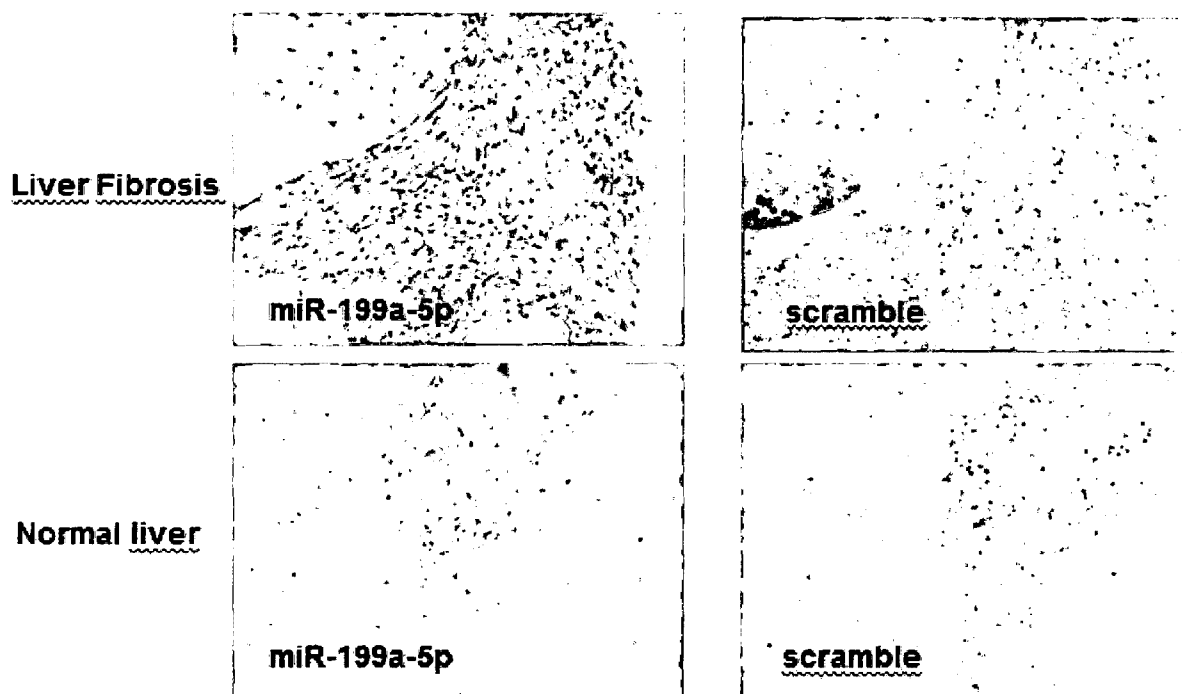


FIGURE 18

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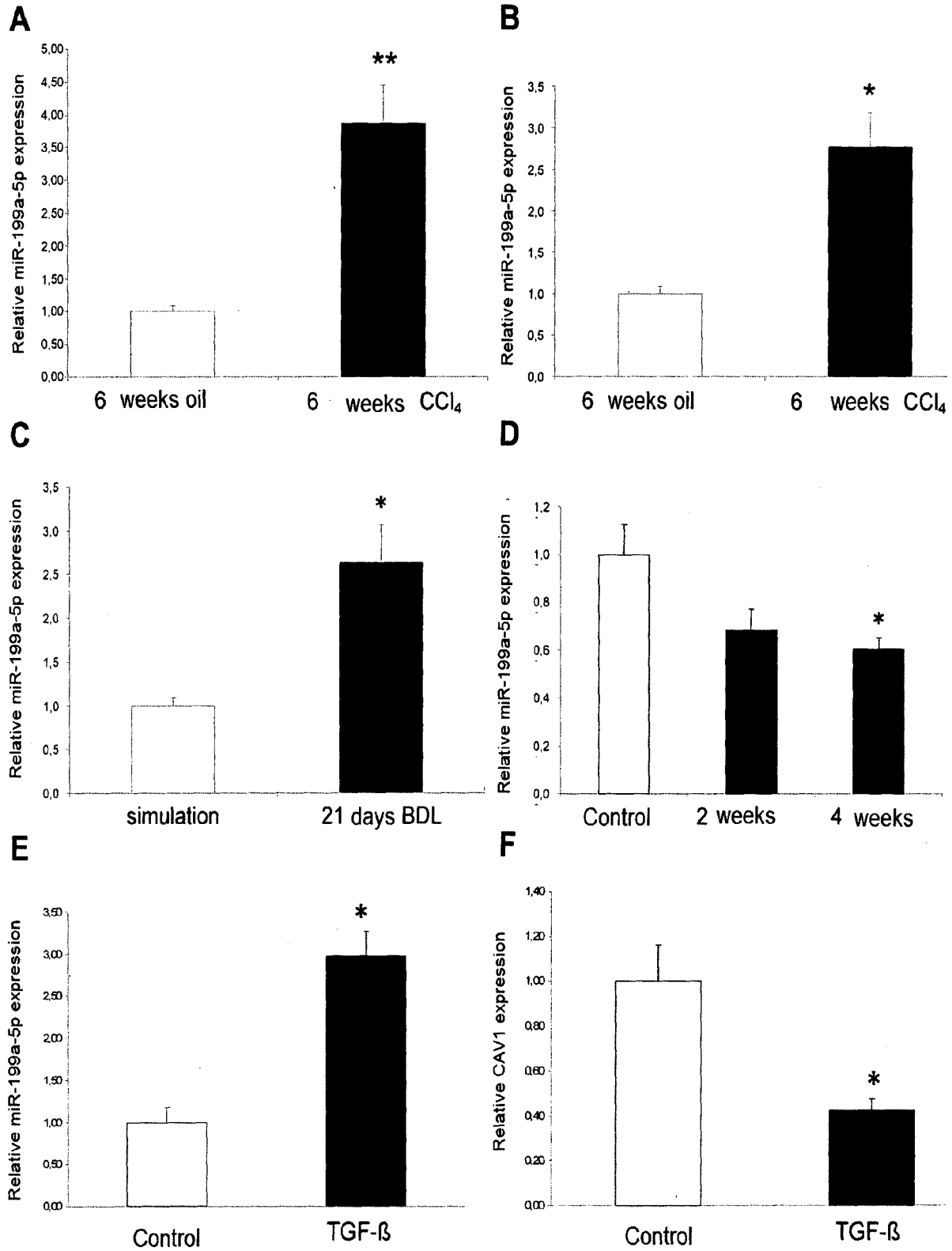


FIGURE 19

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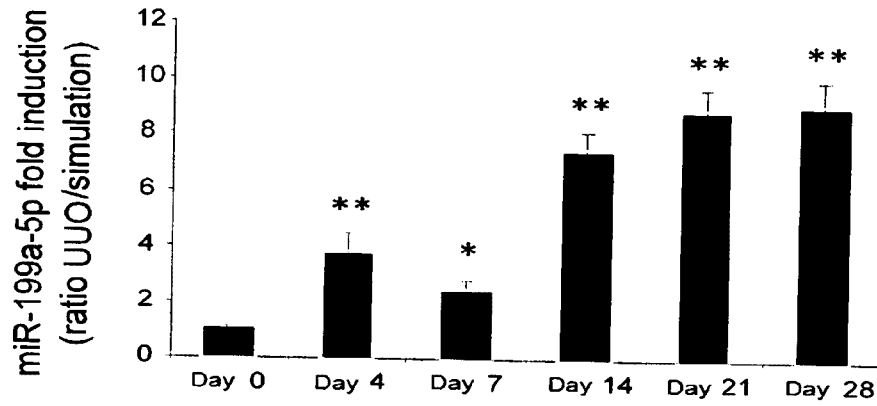
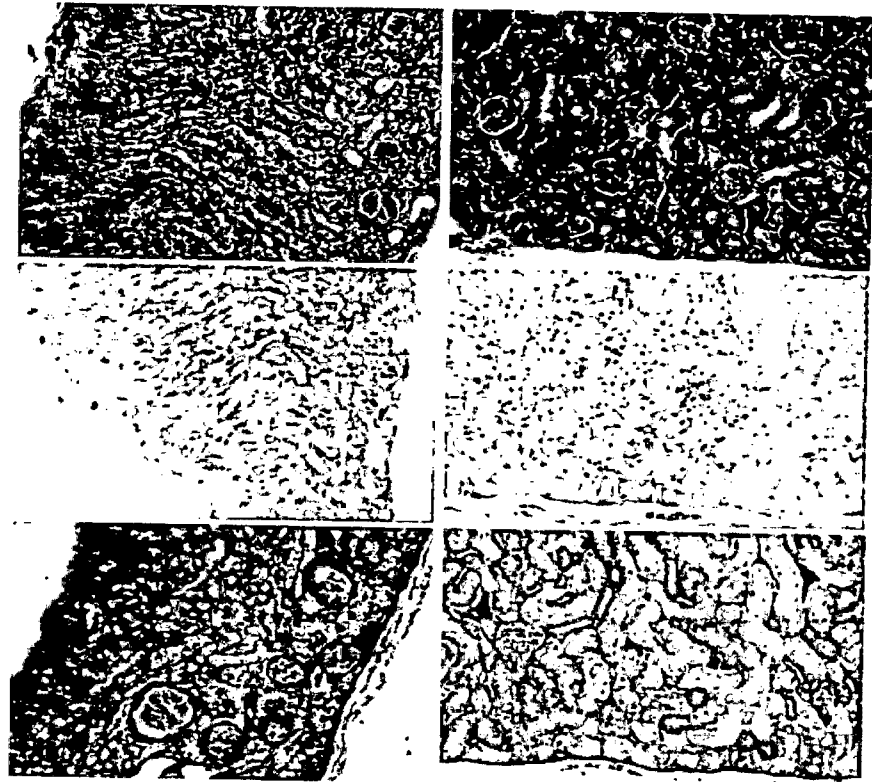
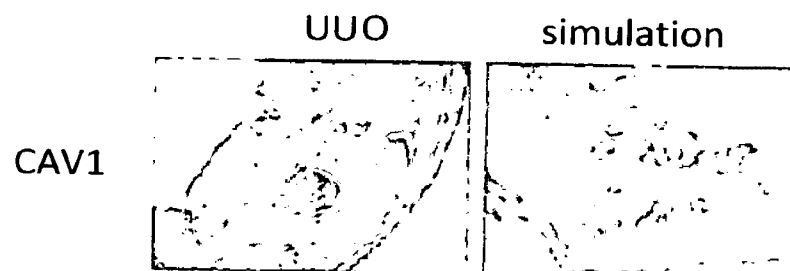
**A****B****C**

FIGURE 20

**A**

