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## Predictive early gene signature during mouse Bhas 42 cell transformation induced by synthetic amorphous silica nanoparticles

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## Abbreviations

SAS: Synthetic amorphous silica nanoparticles; TPA: 12-O-tetradecanoylphorbol-13-acetate; DE: Diatomaceous earth; CTA: Cell transformation assays; OECD: Organization for economic cooperation and development; FBS: Fetal bovine serum; EDTA: Ethylenediaminetetraacetic acid; D21: Day 21; DMSO: Dimethyl sulfoxide; RINs: RNA integrity number; FC: Fold change; GSEA: Gene set enrichment analysis; ES: Enrichment score; MSigDB: Molecular signatures database; ACTB:  $\beta$ -Actin; TUBA:  $\alpha$ -Tubulin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PBS: Phosphate buffer saline; RIPA: Radioimmunoprecipitation assay buffer; SDS PAGE: Sodium dodecyl sulphate poly-acrylamide gel electrophoresis; PVDF: Polyvinylidene difluoride; BSA: Bovin serum albumin; HRP: Horseradish peroxidase; AUNIP: Aurora kinase A and Ninein interacting protein; LGALS7: Galectin-7; LUM: Lumican; IL1RL1: Interleukin1 receptor-like 1; MCM10: Mini Chromosome Maintenance deficient 10; PRL2C: Proliferin; TIMP4: Metalloproteinase inhibitor 4; LOAEL: Lowest observed adverse effect level; IPA: Ingenuity pathway analysis; ELISA: Enzyme-linked immunosorbent assay

## 19    **Highlights**

- 20        • Synthetic amorphous silica nanomaterials induce transformation of Bhas 42 cells
- 21        • Early modulated signaling pathways are linked to cell adhesion and proliferation
- 22        • Twelve genes were selected based on their potential role in cell transformation
- 23        • These genes may serve as early markers of Bhas 42 cell transformation induced by SAS

## 25    **Abstract**

26    Synthetic amorphous silica nanoparticles (SAS) are used widely in industrial applications. These  
27    nanoparticles are not classified for their carcinogenicity in humans. However, some data still  
28    demonstrate a potential carcinogenic risk of these compounds in humans. The Bhas 42 cell line was  
29    developed to screen chemicals, as tumor-initiators or -promoters according to their ability to trigger  
30    cell-to-cell transformation, in a cell transformation assay. In the present study, we performed  
31    unsupervised transcriptomic analysis after exposure of Bhas 42 cells to NM-203 SAS as well as to  
32    positive (Min-U-Sil 5® crystalline silica microparticles, and 12-O-tetradecanoylphorbol-13-acetate) and  
33    negative (diatomaceous earth) control compounds. We identified a common gene signature for 21  
34    genes involved in the early stage of the SAS- Min-U-Sil 5®- or TPA-induced cell transformation. These  
35    genes were related to cell proliferation (over expression) and cell adhesion (under expression). Among  
36    them, 12 were selected on the basis of their potential impact on cell transformation. RT-qPCR and  
37    western blotting were used to confirm the transcriptomic data. Moreover, similar gene alterations were  
38    found when Bhas 42 cells were treated with two other transforming SAS. In conclusion, the results  
39    obtained in the current study highlight a 12-gene signature that could be considered as a potential  
40    early "bio-marker" of cell transformation induced by SAS and perhaps other chemicals.

42    **Keywords:** silica particles, nanomaterials, Bhas 42 cells, cell transformation, transcriptomic profile

## 45        1. Introduction

46        Synthetic amorphous silica (SAS) is one of the nanomaterials produced and used most widely in  
47        industrial applications, with worldwide production estimated at 1.5 million tons. SAS are mainly  
48        produced in industry by either a wet process, to yield precipitated SAS, or a thermal process, to form  
49        pyrogenic SAS [1]. Being a nanostructured material, SAS possesses appealing properties such as a  
50        large specific surface area, and it has been used for decades in various industrial applications such as  
51        chemistry, cosmetics, pharmaceuticals and food [2].

52        Several studies have reported toxicological effects of SAS, including oxidative stress, cytotoxicity,  
53        inflammation and genotoxicity [1, 3]. Precipitated and pyrogenic SAS have also been found to cause  
54        increased dose-dependent changes in lung inflammation after repeated intratracheal instillations in  
55        rats [4]. However, little is known about the carcinogenic potential of SAS. Unlike crystalline silicas,  
56        which are classified as carcinogenic in humans by the International Agency for Research of Cancer,  
57        SAS are not classified as carcinogenic due to a lack of data [5]. Two long-term carcinogenesis studies  
58        with SAS have been published to date, showing that repeated intratracheal instillations of pyrogenic  
59        SAS in rats increased the incidence of lung tumors [6, 7]. Recently, we showed that precipitated and  
60        pyrogenic SAS (NM-200, NM-201 NM-202 and NM-203) induced the transformation of Bhas 42 cells in  
61        cell transformation assays (CTA) [8].

62        The Bhas 42 CTA is one of the existing *in vitro* models for cell transformation [9]. Cell transformation is  
63        an early event of *in vivo* multistep carcinogenesis, involving changes in the morphological properties of  
64        normal to neoplastic cells. Cell transformation assays simulate *in vivo* initiation and promotion stages  
65        of carcinogenesis and can be used to distinguish tumor initiators from tumor promoters [9-11].

66        The transgenic Bhas 42 cell line, established from Balb/c 3T3 cells [12], contains several copies of the  
67        v-Ha-ras gene. Similar to the parental Balb/c 3T3 cells, untransformed Bhas 42 cells grow to  
68        confluence, forming a cell-density-dependent monolayer. However, after exposure to a transforming  
69        chemical, cells become morphologically altered and form discrete foci on the confluent monolayer.  
70        Bhas 42 cells can respond to a tumor-promoter without undergoing preliminary treatment with an  
71        initiator, in contrast with the two-stage Balb/c 3T3 CTA [13, 14]. The Bhas 42 CTA was validated with  
72        a number of known carcinogens and non-carcinogens [15] and is now the subject of a guidance  
73        document produced by the Organization for Economic Cooperation and Development [16].

Cell transformation depends on a cascade of complex events that occur within a few hours or weeks [17]. To decipher these processes, the transcriptomic approach allows access to a global gene signature of cellular function after cell exposure to chemicals [18]. The aim of this study was thus to use transcriptomic analysis to identify an early gene signature for Bhas 42 cell transformation that is shared by an SAS, a crystalline silica and the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Based on previous data [8], the transcriptomic procedure was carried out after Bhas 42 cell exposure to the SAS NM-203, which in the previous study induced the highest level of cell transformation. In addition, the crystalline silica Min-U-Sil 5<sup>®</sup> and the amorphous silica DE (diatomaceous earth) were used as transforming and non-transforming reference particles, respectively [19].

## **2. Materials and methods**

### **2.1 Cell culture**

Bhas 42 cells were obtained from Harlan Laboratories (Rossdorf, Germany). Cells were grown in Eagle's minimum essential medium (Gibco™, Thermo Fisher Scientific, Villebon sur Yvette, France), supplemented with 1 % (v/v) antibiotic (Streptomycin and Penicillin, Gibco™) and 10 % (v/v) fetal bovine serum (FBS, Dutscher, Brumath, France) (M10F) in a humidified 5 % CO<sub>2</sub> incubator at 37 °C up to about 70 % confluence. Cells were recovered using a 0.25 % (v/v) Trypsin/EDTA solution (Invitrogen) and sub cultured in Dulbecco's modified Eagle's medium/Ham's F12 (Gibco™) supplemented with 1 % (v/v) antibiotic and 5 % (v/v) FBS (DF5F) at about 70 % of the confluence to be used in the CTA.

### **2.2 Cell transformation and cell survival assays**

The CTA in promotion condition, originally proposed by the OECD [16], was modified as described previously [8] (Supplementary Fig. A.1). Cells were seeded at 1460 cells/cm<sup>2</sup> in a six-well plate on day 0 and the cells were treated once on day 4 (D4). The cells were then post-cultivated in fresh medium until day 21 (D21), receiving a medium change on days 7 (D7), 10 and 14. Cells were fixed with ethanol (Fisher, France) and stained with a 5 % (v/v) Giemsa solution (Thermo Fisher Scientific) on D21. The transformed foci were evaluated according to the morphological criteria recommended by

the OECD (2016). For each experiment, the mean number of transformed foci per treatment group was determined from six replicate wells.

Cell cytotoxicity was assessed by cell survival assay on D7 performed as follows: cells were suspended by trypsination and stained with a mixture of acridine orange/propidium iodide (Sigma-Aldrich, Saint Quentin Fallavier, France), and then counted using a Cellometer® (Vision Nexcelom Bioscience, Lawrence, MA). Cell survival was expressed as the percentage of living cells after cell exposure to the chemical per living cells counted in the untreated condition.

### **2.3 Particles and cells treatments**

Silica nanoparticles (NM-200, NM-201, NM-202, and NM-203) were obtained from the Joint Research Centre nanomaterials repository (Ispra, Italy) (Supplementary Table A.1). Min-U-Sil 5® was obtained from US Silica Co and DE was obtained from the European Coal and Steel Community (Paris, France). Thorough physical and chemical characterizations of SAS, Min-U-Sil 5® and DE batches have been described previously [8]. For cell treatment, vials containing particles suspended in sterile deionized water (19.2 mg/mL) were sonicated for 5 min at 10 % amplitude in the cup horn of a 400 W Branson Sonifier S-450D (Branson Ultrasonics Corp, Danbury, CT). Serial dilutions were prepared in water from the sonicated suspension. Each dilution was added to DF5F to give a final concentration of water of 2 % (v/v). Final particle concentrations were expressed as mass per cell culture surface area and ranged from 1 µg/cm<sup>2</sup> to 40 µg/cm<sup>2</sup> depending on the assay. TPA (Sigma-Aldrich) was diluted at 0.05 µg/mL in dimethyl sulfoxide (DMSO, Sigma Aldrich) at a final concentration of 0.1 % (v/v).

### **2.4 RNA extraction and quality control**

For both transcriptomic analysis and RT-qPCR, RNA samples were obtained from four independent experiments. In both cases, total RNA was extracted from treated cells on D6 using the RNeasy Plus Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. RNA purity and integrity were evaluated with an Agilent Bioanalyser (Agilent Technologies). All RINs (RNA integrity numbers) were greater than 8.

133

134

## 135 **2.5 Transcriptomic analysis**

136 Transcriptomic analysis was carried out with total RNA extracted from cells exposed from D4 to D6 to  
137 NM-203 (1, 2 and 5 µg/cm<sup>2</sup>), Min-U-Sil 5<sup>®</sup> (20 µg/cm<sup>2</sup>), DE (5 µg/cm<sup>2</sup>), TPA (0.05 µg/mL) and their  
138 associated vehicle control: 2% (v/v) H<sub>2</sub>O for NM-203, Min-U-Sil 5<sup>®</sup> and DE and 0.1% (v/v) DMSO for  
139 TPA. One hundred nanograms of total RNA were used to synthesize double-stranded cDNA and  
140 cyanine labeled cRNA using the one-color low-input QuickAmp labeling kit (Agilent Technologies)  
141 according to the manufacturer's instructions. cRNA were purified using the RNeasy Plus Mini Kit  
142 (Qiagen). cRNA were hybridized to an Agilent Sureprint G3 mouse gene expression v2 8x60k  
143 pangenomic microarray. Gene expression data were extracted using Feature Extraction 11.0.1.1 and  
144 analysed using GeneSpring 14.9-GX-PA software (Agilent Technologies).

145 The microarray data obtained in this study were deposited in the NCBI Gene Expression Omnibus and  
146 were accessible through GEO (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133279>).

147 To determine the dose effect of NM-203 (at 1, 2 and 5 µg/cm<sup>2</sup>), a one-way ANOVA, followed by a  
148 Tukey post-hoc test and a Benjamini-Hochberg multiple correction test, was performed with a p-value  
149 < 0.005 and a Fold Change (FC) > 4. To determine genes deregulated significantly between two  
150 conditions (treatment vs control (H<sub>2</sub>O or DMSO)), an unpaired t-test (Benjamini-Hochberg correction)  
151 with a p-value < 0.05, FC > 2 or with a p-value < 0.005, FC > 4 was used.

152

## 153 **2.6 Bioinformatic analysis**

154 Microarray data were clustered with Gene Cluster 3.0 software and the tree was visualized using  
155 TreeView 1.6 software. An uncentred correlation was applied for the measure of similarity and gene  
156 clustering was based on complete linkage. Biological functions corresponding to the main clusters  
157 were evaluated using the FuncAssociate 2.0 web-based tool.

158 For treatment signature analyses, top canonical pathways were identified with ingenuity pathway  
159 analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA) and signature enrichment  
160 analysis was conducted with Gene Set Enrichment Analysis (GSEA) software by using the Java-tool  
161 developed at the Broad Institute (Cambridge, MA, USA) on a normalized and filtered list of genes.  
162 Unsupervised GSEA was performed on the whole C2 collection of curated gene sets from the

molecular signatures database (MSigDB). Enrichment score (ES) was determined after 1000 permutations.

## **2.7 Gene expression analysis by RT-qPCR**

Total RNA was reverse-transcribed with the iScript™ cDNA synthesis Kit (BioRad, Marnes-la-Coquette, France) following the manufacturer's protocol. Real-time PCR was performed with CFX96 Touch™ (BioRad) in the presence of iQ™ SYBR® Green Supermix (BioRad) and PrimePCR Assay (or 2.5 µM of each primer (Eurogentec, Anger, France) (Supplementary Table A.2). Amplification was performed following the manufacturer's protocol: the annealing temperature was 60 °C for all pairs of primers. PCR results were analyzed using the SQ method. The results were expressed as relative to those of control cells treated with H<sub>2</sub>O or DMSO, arbitrarily set to 1. *ACTB* (beta-Actin), *TUBA* (alpha-Tubulin), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were used as housekeeping genes whose values have been averaged (geometric mean).

## **2.8 Protein analysis**

Bhas42 cells were treated as stated before. On D6, cell medium supernatants were harvested and stored at -80 °C. Cell layers were washed 3 times with cold PBS (1.44 mM Na<sub>2</sub>HPO<sub>4</sub>, 0,2 mM KH<sub>2</sub>PO<sub>4</sub> containing 0.148 M NaCl, 2 mM KCl) and scraped with a rubber policeman with ice-cold RIPA buffer (50 mM Tris-HCl, pH 8, containing 150 mM NaCl, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS and 1 % (v/v) protease and phosphatase inhibitor mixture (Thermo Scientific). The homogenates obtained were placed under agitation for 30 min at 4 °C, then centrifuged at 16000 g for 20 min at 4 °C. Supernatants were collected and stored at -80 °C. Protein concentration was measured in each sample and corresponding cell medium supernatant, using bovine serum albumin as a standard (Protein quantification kit, Sigma-Aldrich).

Proteins (50 µg) of whole cell homogenates or 20 µl of corresponding cell medium supernatants were resolved in 4–20 % SDS PAGE (Criterion™ Tris-Glycine eXtended Stain-Free Protein Gel, BioRad) and transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad), using semi-dry transfert (BioRad). The saturation step was performed in PBS-T buffer (PBS supplemented with 0.1 % (v/v) Tween 20) containing 5 % (w/v) non-fat milk for 1 h at room temperature. Antibody solutions were prepared in PBS-T containing 5 % (w/v) BSA) and membranes were incubated overnight at 4 °C with



gentle agitation. Primary antibodies were directed against Aurora kinase A and Ninein interacting protein (AUNIP, Cell Signaling Technology, Ozyme, Saint-Cyr-l'École, France; 1: 500), Galectin-7 (ab206435; Abcam, Paris, France; 1: 1000), Lumican (LUM, 7076P2, Cell Signaling Technology; 1: 250), Interleukin1 receptor-like 1 (IL1RL1, Cell Signaling Technology, 1: 1000), Mini Chromosome Maintenance deficient 10 (MCM10, Cell Signaling Technology, 1:500), Proliferin (AF1623; R&D System; 1: 1000), Metalloproteinase inhibitor 4 (TIMP4, Abcam; 1: 5000), and  $\beta$ -Actin (4970; Cell Signaling Technology; 1: 10000). Membranes were washed 3 times with PBST, then incubated for 1h at room temperature with either HRP-conjugated anti-goat IgG (1: 5000) or HRP-conjugated anti-rabbit IgG (1: 2000) in PBST containing 5 % (w/v) non-fat milk. Finally, membranes were washed 3 times with PBST before chemiluminescence detection (Clarity™ Western ECL Substrate, BioRad) using ChemiDoc™ (ChemiDoc™ System, BioRad).

Secreted periostin concentration was estimated using a Mouse Periostin/OSF-2 DuoSet ELISA kit (R&D Systems, Lille, France). Sample concentration was expressed as ng of periostin/mg of protein.

## 2.9 Statistics

Statistical analyses were performed using Graph Pad Prism software (Version 8.0.1; Graphpad, San Diego, CA). For CTA and cell survival assays logarithmic transformation was applied to the data. For multiple comparisons, the significance was evaluated by a one-way ANOVA followed by a Dunnett's multiple comparisons test.

An unpaired t-test was used for two by two comparison (treatments vs. control (H<sub>2</sub>O or DMSO)).

Data are given as a mean  $\pm$  standard deviation (mean  $\pm$  SD).

## 3. Results

### 3.1 Cytotoxic and transforming effects of NM-203, Min-U-Sil 5®, DE and TPA.

CTAs in promotion condition were performed according to the OECD guidance document [16], except that instead of receiving three treatments during the assay, cells were treated once on Day 4. Cytotoxicity assays were performed on D7 applying the dose range of particles used in the previous study [8]. The cytotoxic effect of NM-203, Min-U-Sil 5® and DE was moderate; the decrease in cell survival was less than 30 % (Supplementary data Table A.3). NM-203 and Min-U-Sil 5® induced a significant dose-response effect on cell transformation (Fig. 1A). The lowest observed adverse effect level (LOAEL) was observed at 5  $\mu$ g/cm<sup>2</sup> and 20  $\mu$ g/cm<sup>2</sup> for NM-203 and Min-U-Sil 5®, respectively.

TPA at 0.05 µg/ml, used as a positive control, induced cell transformation. In contrast, DE did not show any transforming activity.

### **3.2 NM-203, Min-U-Sil 5<sup>®</sup> and TPA induce alteration of genes involved in cell**

#### **proliferation and cell adhesion**

After normalization and filtering of the raw transcriptomic data using the Genespring software, 19,569 probes were listed. Non supervised hierarchical clustering (Fig. 2A) of these 19,569 remaining probes, showed two main branches, dividing the samples into two groups with regards to the transforming potential: a first group with control conditions (ctrl = 2 % (v/v) H<sub>2</sub>O or 0.1 % (v/v) DMSO) and non-transforming conditions (DE at 5 µg/cm<sup>2</sup> and NM-203 at 1 µg/cm<sup>2</sup>) and a second group including NM-203 at 2 µg/cm<sup>2</sup> and transforming conditions (NM-203 at 5 µg/cm<sup>2</sup>, Min-U-Sil<sup>®</sup> (5 µg/cm<sup>2</sup>), TPA (0.05 µg/ml)). The Min-U-Sil 5<sup>®</sup> (20 µg/cm<sup>2</sup>) group was closer to the NM-203 (5 µg/cm<sup>2</sup>) group than to the TPA (0.05 µg/ml) group. The NM-203 (2 µg/cm<sup>2</sup>) group, which was a transforming condition in the Bhas 42 CTA performed with three treatments but not with a single treatment [8], seems to induce gene expression modulation even with a single treatment. To study the dose-effect of NM-203, an ANOVA test was performed with a *p*-value lower than 0.005 selected. Genes that were expressed differently in at least one condition (vs. control) with a fold change (FC) greater than 4 were selected (supplementary Table A.4). Hierarchical clustering of the 290 differentially expressed genes showed that increasing the concentration of NM-203 led to a higher increase or decrease response at the mRNA level (Fig. 2B). Treeview analysis highlighted co-regulated groups of genes. Interestingly, the upper part of the dendrogram grouped clusters of genes down-regulated after NM-203 treatment (124 genes) and involved in matrix remodeling and cell adhesion, and the lower part of the dendrogram highlighted genes up-regulated after NM-203 treatment (166 genes) and involved in cell proliferation. Gene set enrichment analysis (GSEA) identified significant enrichments (*p* < 0.01) of signatures for each treatment (Supplementary Table A.5). Comparison of the top 50 signatures highlighted for each treatment showed that 24 % of signatures were common to the three conditions (Fig. 3A). These twelve common signatures are related mainly to proliferation and cancer features (Fig. 3B-C) [20, 21]. To identify genes significantly deregulated by each treatment, an unpaired t-test (Bonferroni FWER correction) with a *p*-value < 0.05, FC > 2 was used. RNA levels of 1,580 genes were modified after cell treatment by NM203 (5 µg/cm<sup>2</sup>) (Supplementary Table A.6), 1,044 genes with Min-U-Sil 5<sup>®</sup>

(Supplementary Table A.7), and 383 genes by TPA (Supplementary Table A.8). Interestingly, NM-203 at 2  $\mu\text{g}/\text{cm}^2$  induced significant changes in the expression of 512 genes (Supplementary Table A.9). IPA analysis on a smaller set of genes (selected with an unpaired t-test with a p-value < 0.005, FC > 4; Supplementary Table A.10-A.13) showed that the former three treatments regulated a number of common pathways involved mainly in cell proliferation (Supplementary Table A.14).

### 3.3 Comparison of early molecular signature and selection of common genes

Genes significantly highly regulated by NM-203 (5  $\mu\text{g}/\text{cm}^2$ ), Min-U-Sil 5<sup>®</sup> and TPA were compared (p < 0.005 and FC > 4) (Fig. 4A). Of the 309 genes altered by treatments, a large majority (83 %) were affected by NM-203. Among these, 142 (46 %) were affected solely by NM-203 treatment and 23 (7 %) and 29 genes (9 %) were deregulated by Min-U-Sil 5<sup>®</sup> and TPA, respectively. Interestingly, 21 common genes were regulated significantly by NM-203 (5  $\mu\text{g}/\text{cm}^2$ ), Min-U-Sil 5<sup>®</sup> and TPA (p < 0.005, FC > 4). Twelve genes, *CACNA1G*, *CMBL*, *ENPP2*, *GDPD2*, *HMGCS2*, *LGALS7*, *LUM*, *POSTN*, *SEMA3G*, *SPARCL1*, *TIMP4*, and A230065H16Rik, were underexpressed whereas 9 genes, *RAD51AP1*, *NLRP2*, *SGOL1*, *IL1RL1*, *PRL2C3*, *PRL2C5*, *PRL2C1*, *AUNIP* and *MCM10* were overexpressed (Fig. 4B). We selected 6 genes known to be involved in cell proliferation (*AUNIP*, *IL1RL1*, *MCM10*, *PRL2C1*, *PRL2C3* and *PRL2C5*) and 6 genes involved in cell-cell adhesion (*LGALS7*, *LUM*, *POSTN*, *SEMA3G*, *SPARCL1*, and *TIMP4*) (Table 1). For all these genes, RT-qPCR results were broadly in line with transcriptomic results and confirmed the dose-effect produced by NM-203 (Table 2).

### 3.4 Transforming silica nanoparticles NM-200 and NM-202 induce similar changes at the RNA level of the selected genes.

We then assessed whether Bhas 42 cell exposure to other SAS, namely NM-200, NM 201 and NM202, led to cell transformation and involved any changes at the RNA level of the selected genes. NM-200 were slightly cytotoxic at the highest concentration (40  $\mu\text{g}/\text{cm}^2$ ) while NM-202 decreased cell survival by approximately 50 % at 40  $\mu\text{g}/\text{cm}^2$ . NM-201 treatment had no cytotoxic or transforming effect (Fig.1B and supplementary Table A3). Bhas 42 cell exposure to NM-200 (20  $\mu\text{g}/\text{cm}^2$ ) or NM-202 (20  $\mu\text{g}/\text{cm}^2$ ) led to similar expression patterns as those obtained when cells were treated with NM203 (5  $\mu\text{g}/\text{cm}^2$ ), i.e. an increase in RNA levels of *AUNIP*, *IL1RL1*, *MCM10*, *PRL2C1*, *PRL2C3* and

*PRL2C5*, and a decrease for *LGALS7*, *LUM*, *POSTN*, *SEMA3G*, *SPARCL1*, and *TIMP4*. In contrast, cells exposed to NM-201 showed no change in each gene expression (Fig. 5).

### 3.5 Changes at the protein level

We assessed whether the increase at the mRNA level of selected genes was related to corresponding changes at the protein level. We performed Western blotting procedure or ELISA with available commercial antibodies (Fig. 6). Among the antibodies tested, we could not estimate the respective protein expression levels of AUNIP, LUM, TIMP4, due to a lack of a specific antibody (presence of a non-specific band) or due to low immune signals.

Among the list of genes with increased expression after Bhas42 cell treatment, IL1RL1, as the receptor of IL33, was detected with an estimated band at 37 kDa, in all conditions tested, with slight changes between control and treated cell homogenates. Moreover, glycosylated IL1R1 could be secreted as a response to reduce receptor signaling by IL33 [22]. Glycosylated IL1R1 appears as a broad band around 50 kDa, but slight changes in the secreted receptor levels were observed. Proliferin, as a growth hormone /prolactin related secreted glycoprotein [23, 24], encoded by *PRL2C1*, *PRL2C3*, *PRL2C5* genes, was detected in whole cell homogenate and in cell medium supernatant as a single band at 60 KDa. As shown in Figure 6, the protein was secreted in all conditions tested, but we failed to detect it in homogenates prepared from cells treated with H<sub>2</sub>O, DMSO, NM-201, low concentrations of NM-203 (1 and 2 µg/cm<sup>2</sup>), and DE, supporting the concept that proliferin is continuously secreted. In contrast, the protein was detected in cell homogenates prepared from cells exposed to NM-200, NM-202, NM-203 (2 and 5 µg/cm<sup>2</sup>), Min-U-Sil 5®, and TPA, suggesting that the synthesis rate of the protein is enhanced under these experimental conditions.

Among the genes showing decreased expression after cell exposure to SAS, periostin, involved in cell matrix adhesion and epithelial mesenchymal transition [25], was measured by ELISA in cell medium supernatants. Protein levels were lowered when Bhas 42 cells were treated with 20 µg/cm<sup>2</sup> of NM-200, NM-202 and Min-U-Sil 5®, 5 µg/cm<sup>2</sup> NM-203 and 0.05 µg/ml TPA, in agreement with the observed decrease in mRNA levels. However, protein levels were also low when cells were exposed to NM-201 (20 µg/cm<sup>2</sup>) or NM-203 (2 µg/cm<sup>2</sup>), whereas no change was found when cells were treated with 1 µg/cm<sup>2</sup> NM-203 or 5 µg/cm<sup>2</sup> DE (Fig. 6). MCM10, involved in DNA recombination and elongation [26] was also detected in whole cell homogenate as a band estimated at 100 kDa. We did not observe any

significant changes at the protein level whatever the treatment tested. These results could be related to the experimental design of the CTA, since, from the beginning of cell treatment on D4, Bhas 42 cells continue to undergo cell division until D6, maintaining a high level of MCM10, a protein necessary for DNA synthesis [26]. Finally, galectin 7, involved in cell-cell or cell-matrix interaction [27], was detected in whole cell homogenates with a band estimated at 37 kDa (Fig. 6). Galectin 7 levels were lowered when cells were treated with NM-200, NM-202, NM-203 (2 and 5  $\mu\text{g}/\text{cm}^2$ ), and Min-U-Sil 5<sup>®</sup> (20  $\mu\text{g}/\text{cm}^2$ ), whereas no change was found in the other conditions tested.

## **4. Discussion**

### **4.1 Selection of genes as possible markers of SAS-induced Bhas42 cell**

#### **transformation**

Although SAS are extensively produced and used, few studies have focused on their transforming or carcinogenic potential [1, 3]. In a previous study, the transforming properties of the NM-200, NM-201, NM-202 and NM-203 SAS were revealed in the Bhas 42 CTA using the promotion assay. In order to decipher the molecular changes during the Bhas 42 CTA, an unsupervised transcriptomic analysis was performed following exposure of cells to SAS and control chemicals for 48h. In this treatment time, NM-203 and Min-U-Sil 5<sup>®</sup> have been shown to induce epigenetic effects in Bhas 42 cells in the promotion assay [28]. We therefore hypothesized that this treatment time could highlight earlier gene changes involved in Bhas 42 cell transformation. The lowest concentrations of chemicals able to induce cell transformation without inducing cytotoxicity were selected in transcriptomic and RT-qPCR assays. The expression of 21 genes was altered by the three treatments, NM-203, Min-U-Sil 5<sup>®</sup> and TPA. Among them, twelve genes, whose involvement in cell proliferation and adhesion has been proven (Table 1), were selected as putative markers of the transforming properties of SAS nanomaterials. To confirm this hypothesis, we tested the NM-200, NM201 and NM-202 SAS (Fig. 1B and 5). Similar changes in the RNA levels of each selected gene were obtained with NM-200 and NM-202, supporting the hypothesis that the genes in this set are potential markers of SAS-induced cell transformation. However, NM-201 treatment induced no change in the expression of these 12 genes (Fig. 5). This could be explained by the fact that, unlike the three repeated treatments of NM-201 [8]), a single treatment did not cause Bhas 42 cell transformation. One reason for this could be that NM-

201 has a lower BET-SSA than the other SAS (140 vs  $\approx$  200 g/cm<sup>2</sup>) (Table A.1). Indeed, the toxicity of nanomaterials is often associated with their high specific surface area, which results in a high reactivity with the biological environment. In addition, we demonstrated concomitant changes in the mRNA and protein levels of several genes, such as galectin 7, periostin or proliferin. However, a concordance between mRNA and protein levels was not systematically found. It is possible that regulation mechanisms of protein synthesis and processing may occur.

Interestingly, the increase in *PRL2Ci* mRNA levels, encoding proliferin, was observed in CTA performed with BALB c/3T3 cells [13, 14] and C3H/10T1/2 cells [29, 30] treated with various chemicals including amorphous silica particles (silica gel of 5  $\mu$ m). Proliferin expression was considered as a predictive biomarker of cell transformation [13, 14]. However, even though the expression of three members of the proliferin gene family, *PRC/2C2,3,5*, was upregulated, proliferin was revealed as a single band by western blotting. On the other hand, c-myc upregulation was also linked to C3H/10T1/2 [29] and Bhas 42 cell transformation [28]. In the latter, *C-MYC* mRNA and protein levels increased after cell treatment with NM-203 and Min-U-Sil 5<sup>®</sup> over 2 days. Similar changes in mRNA level were also found in the present study with  $p < 0.005$  and  $FC > 4$  (Supplementary Tables A10-A11). Accordingly, Bhas 42 cell transformation is associated with early increased *C-MYC* expression, but not in the late phase of SAS-induced cell transformation as shown previously [28]. These results, among others [14, 31], confirm that markers of transformation must be identified in the early steps of the processes. Finally, epigenetic modifications have also been related to cell transformation as recently reviewed [32]: it has been shown that cadmium chloride, methyl carbamate and lithocholic acid (three transforming compounds) can induce genome-wide DNA methylation changes in Bhas 42 cells [33]. Moreover, Seidel *et al.* proposed that the transforming activity of the crystalline silica Min-U-Sil 5<sup>®</sup> in Bhas 42 cells was related to early epigenetic DNA methylation [28].

#### 4.2 Gene expression changes after SAS exposure

The transcriptomic analyses showed that NM-203, Min-U-Sil 5<sup>®</sup> and TPA are related to increases in the expression of genes involved in cell cycle progression, to DNA recombination, to decreased matrix and remodeling functions, and to cell-cell adhesion. This is in accordance with different transcriptomic studies that have used the BALB c/3T3 cell line in the context of CTA [14, 31]. Moreover, *IL1RL1*, *LGALS7*, *LUM*, *MCM10*, *POSTN* and *SPARCL1* have all been associated with SAS toxicity. For

example, *IL1RL1* has been found to be involved in inflammasome activation induced by crystalline silica in human lung epithelial cells (BEAS2B) and in primary human bronchial epithelial cells (NHBE) [34, 35]. An increase in the expression of *LUM*, *MCM10*, *POSTN*, *SPARCL1* and *IL1RL1* has been observed in RAW 264, 7 cells (murine macrophages cells) treated with SAS (10 or 300 nm) [34]. Pisani et al. showed overexpression of *LUM*, *LGALS7* and *MCM10* in FE1 cells treated with AEROSIL 200® (NM-203) [36]. In addition, variations in gene expression related to the cell cycle or cell adhesion were found after treatment of the lung epithelial cell line FE1 exposed for 24h to 12, 15 and 20 nm SAS [37]. Furthermore, pathway analysis has revealed that protein contents in the adenocarcinomic human alveolar basal epithelial cell line A549 treated with Min-U-Sil 5® for 24h are associated with cell proliferation [38].

#### 4.3 Gene expression changes related to cancer progression

All of the genes selected are known to be involved in cancer progression. Six of the genes were overexpressed by the three treatments. In previous studies, for example, overexpression of *IL1RL1*, has been observed in tumor cells from human colon cancer [39], the respective expression of the *PRL2C* gene family was increased in fallopian tube epithelium derived ovarian cancer cells [40], *MCM10* was increased in medulloblastoma, urothelial carcinomas, prostate cancer and breast cancer [41-44] and finally, *AUNIP* was overexpressed in various brain tumors [45]. Conversely, respective expression of the 6 selected genes was downregulated by the three treatments in this study. In previous studies, expression of *LUM*, *POSTN*, *SPARCL1*, *LGALS7*, and *TIMP4*, all involved in cell-cell adhesion, was found to be lowered in various cancers: *LGALS7* expression was decreased in cervical cancer, colon cancer, gastric cancer and in malignant melanoma [27, 46-48]; *LUM* was downregulated in various human cancer cell lines [48,49,50], *POSTN* expression was likewise downregulated in bladder and lung cancer [49]; low expression of *SPARCL1* has been shown to be associated with tumor progression in human gastric, breast and prostate cancers [50-52], and expression of *TIMP4* has been found to be low in malignant peripheral nerve sheath tumors associated with neurofibromatosis type 1 [53]. Moreover, some gene expression changes observed in this study are related to cancer progression. For example, *SEMA3G* expression decreased under our experimental conditions. In contrast, overexpression of *SEMA3G* limits the progression of breast cancer or melanoma cells or the migration and invasion of glioma cells [54, 55]. GSEA analysis also revealed an

overall enrichment of gene signatures in response to treatment with NM-203, Min-U-Sil 5<sup>®</sup> and TPA. Among the signatures found, SAS exposure was related to the response to estradiol and prostaglandin and to G2/M arrest by methoxyestradiol and oligodendrocyte differentiation. Estrogen is an important causal factor in breast cancer [56].

## **5 Conclusion**

Using an unsupervised transcriptomic approach, we have identified 12 genes of Bhas 42 cells that are potentially involved in the transforming effect of SAS. The selected genes were mainly related to positive regulation of cell proliferation and negative regulation of cell adhesion. For the first time, this set of genes can be proposed as an *in vitro* RT-qPCR screening assay for assessment of the early stages of SAS-induced Bhas 42 cell transformation. Our analysis will be extended to other nanomaterials and chemicals in order to strengthen the predictivity of this early gene signature.

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429 **Table 1:** Characteristics of the selected genes

Gene symbol	Gene title	Gene ontology	Protein localisation	Expression level	References
<i>AUNIP</i>	Aurora Kinase A And Ninein Interacting Protein	Aurk-A interaction during cell cycle	Cytoplasm	Up	[45]
<i>IL1RL1</i>	Interleukin-1 receptor-like 1	Immune response	Plasma membrane	Up	[22]
<i>LGALS7</i>	Galectin-7	Cell-cell and/or cell-matrix interactions	Extracellular space	Down	[57]
<i>LUM</i>	Lumican	Extracellular matrix organization	Extracellular space	Down	[58]
<i>MCM10</i>	Mini-chromosome Maintenance 10	DNA replication	Nucleus	Up	[59]
<i>POSTN</i>	Periostin	Cell adhesion	Extracellular space	Down	[60]
<i>PRL2C1</i>	Prolactin family 2, subfamily c, member 1	Positive regulation of cell proliferation	Extracellular space	Up	[24], [23]
<i>PRL2C3</i>	Prolactin family 2, subfamily c, member 3	Positive regulation of cell proliferation	Extracellular space	Up	[24], [23]
<i>PRL2C5</i>	Prolactin family 2, subfamily c, member 5	Positive regulation of cell proliferation	Extracellular space	Up	[24], [23]
<i>SEMA3G</i>	Semaphorin-3G	Endothelial cell migration	Cytoplasm	Down	[61]
<i>SPARCL1</i>	Secreted protein acidic and rich in cysteine like protein 1	Cell adhesion	Extracellular space	Down	[62]
<i>TIMP4</i>	Metalloproteinase inhibitor 4	Inhibitor of metalloproteinase	Extracellular space	Down	[63]

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**Table 2:** Fold change value\* and correlation between microarray data and RT-qPCR results

Gene symbol		NM-203 (1 µg/cm <sup>2</sup> )	NM-203 (2 µg/cm <sup>2</sup> )	NM-203 (5 µg/cm <sup>2</sup> )	Min-U-Sil 5® (20 µg/cm <sup>2</sup> )	DE (5 µg/cm <sup>2</sup> )	TPA (0.05 µg/ml)	r
<i>AUNIP</i>	Microarray	1.87	5.90	6.22	5.02	1.37	4.23	0.742
	RT-qPCR	1.10	1.70	7.06	2.94	1.38	4.23	
<i>IL1RI1R</i>	Microarray	1.64	3.74	4.60	5.06	1.66	4.35	0.844
	RT-qPCR	1.30	1.69	5.88	3.78	1.83	4.76	
<i>ICAM1</i>	Microarray	0.81	0.68	0.10	0.16	0.63	0.25	0.961
	RT-qPCR	0.69	0.42	0.02	0.06	0.75	0.09	
<i>IL1M</i>	Microarray	0.64	0.70	0.11	0.23	0.55	0.25	0.928
	RT-qPCR	0.49	0.33	0.05	0.06	0.67	0.16	
<i>MCM10</i>	Microarray	1.64	5.26	7.29	6.27	1.25	4.74	0.846
	RT-qPCR	1.27	2.08	8.50	3.94	1.48	3.82	
<i>POSTN</i>	Microarray	0.53	0.35	0.09	0.10	0.47	0.08	0.969
	RT-qPCR	0.66	0.31	0.04	0.04	0.71	0.03	
<i>PRI2C1</i>	Microarray	1.49	3.94	18.95	17.46	1.60	13.12	0.967
	RT-qPCR	2.10	4.17	54.40	53.26	3.44	20.00	
<i>PRI2C2</i>	Microarray	1.63	5.42	22.82	22.73	1.61	20.26	0.954
	RT-qPCR	1.45	3.10	29.11	29.64	2.30	14.75	
<i>PRI2C5</i>	Microarray	1.64	4.81	17.51	17.35	1.61	13.22	0.980
	RT-qPCR	1.97	4.25	18.55	8.44	4.10	15.00	
<i>SEMA3G</i>	Microarray	0.60	0.50	0.14	0.27	0.46	0.21	0.889
	RT-qPCR	0.73	0.38	0.13	0.05	0.87	0.09	
<i>SPARC1</i>	Microarray	0.61	0.65	0.04	0.10	0.44	0.21	0.929
	RT-qPCR	0.59	0.32	0.02	0.03	0.64	0.12	
<i>TIMP1</i>	Microarray	0.57	0.52	0.11	0.22	0.54	0.12	0.960
	RT-qPCR	0.67	0.45	0.02	0.06	0.79	0.03	

\*, fold change values were calculated from results obtained from either microarray or RT-qPCR.

Results are expressed with respect to control cells, taken as 1.00. Control cells were treated with H<sub>2</sub>O

used as the vehicle for respective SAS, DE or Min-U-Sil 5®, and with DMSO as the vehicle for TPA.

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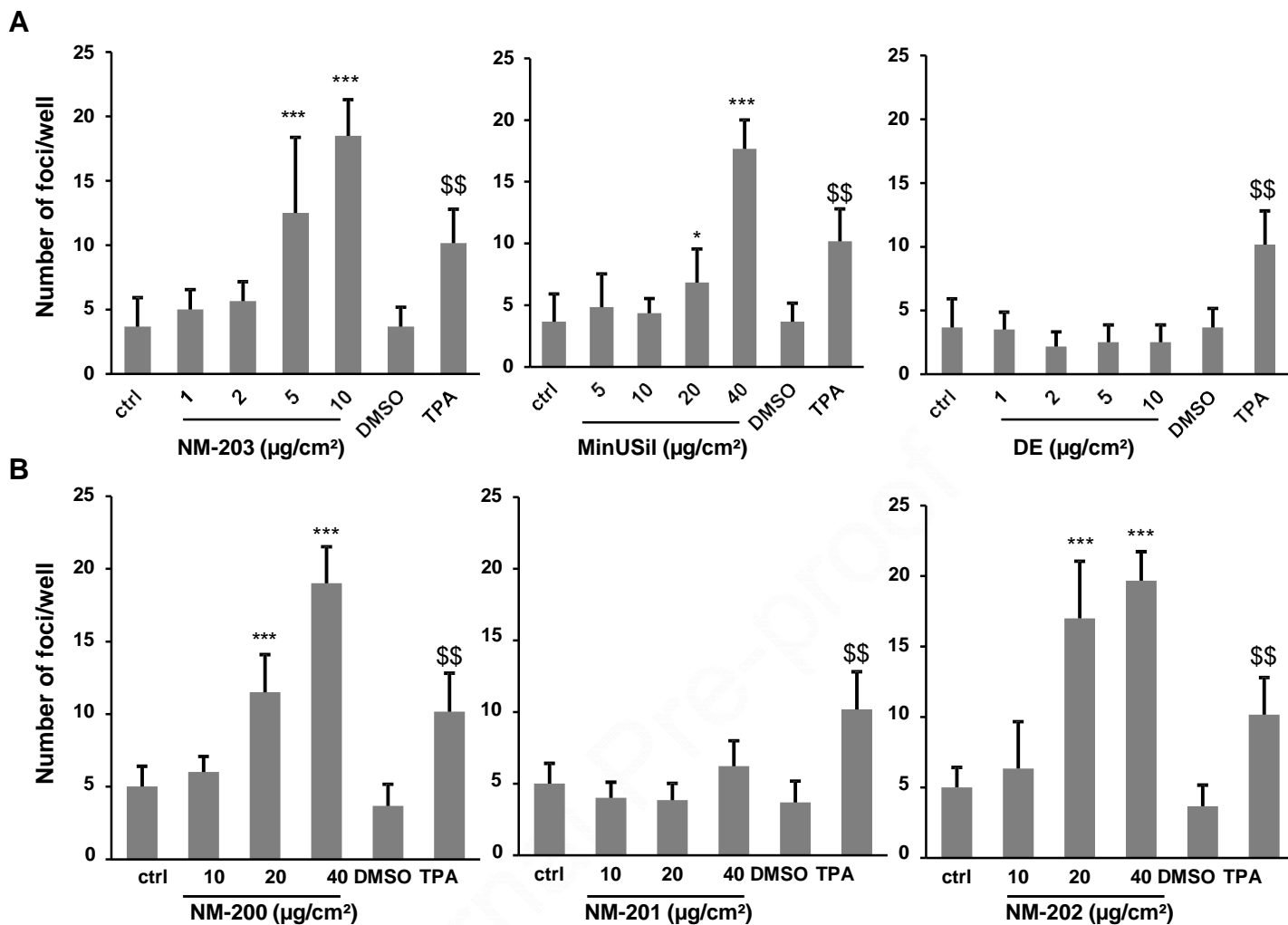
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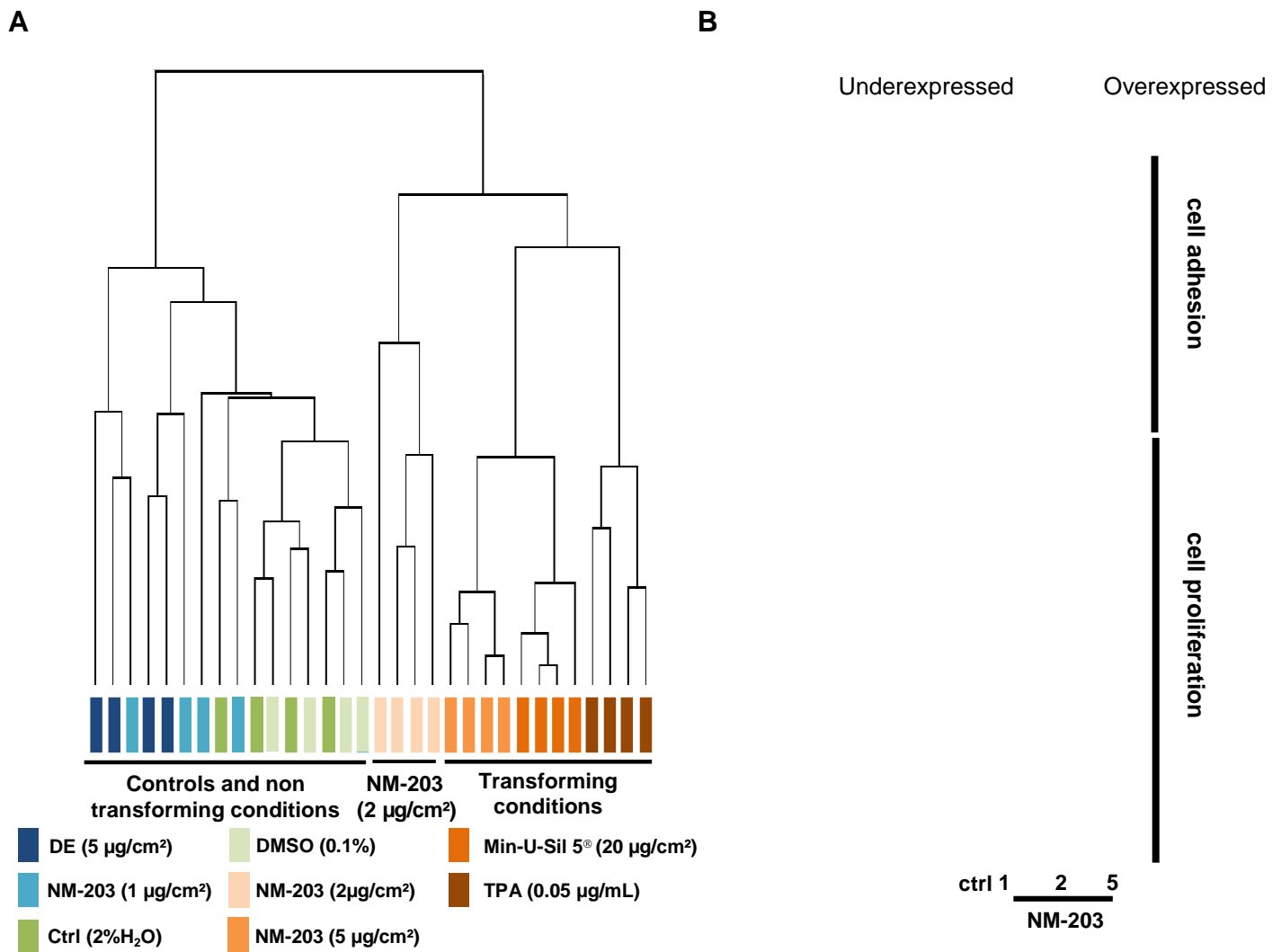
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**Figure 1: Bhas 42 cell transformation assay.**

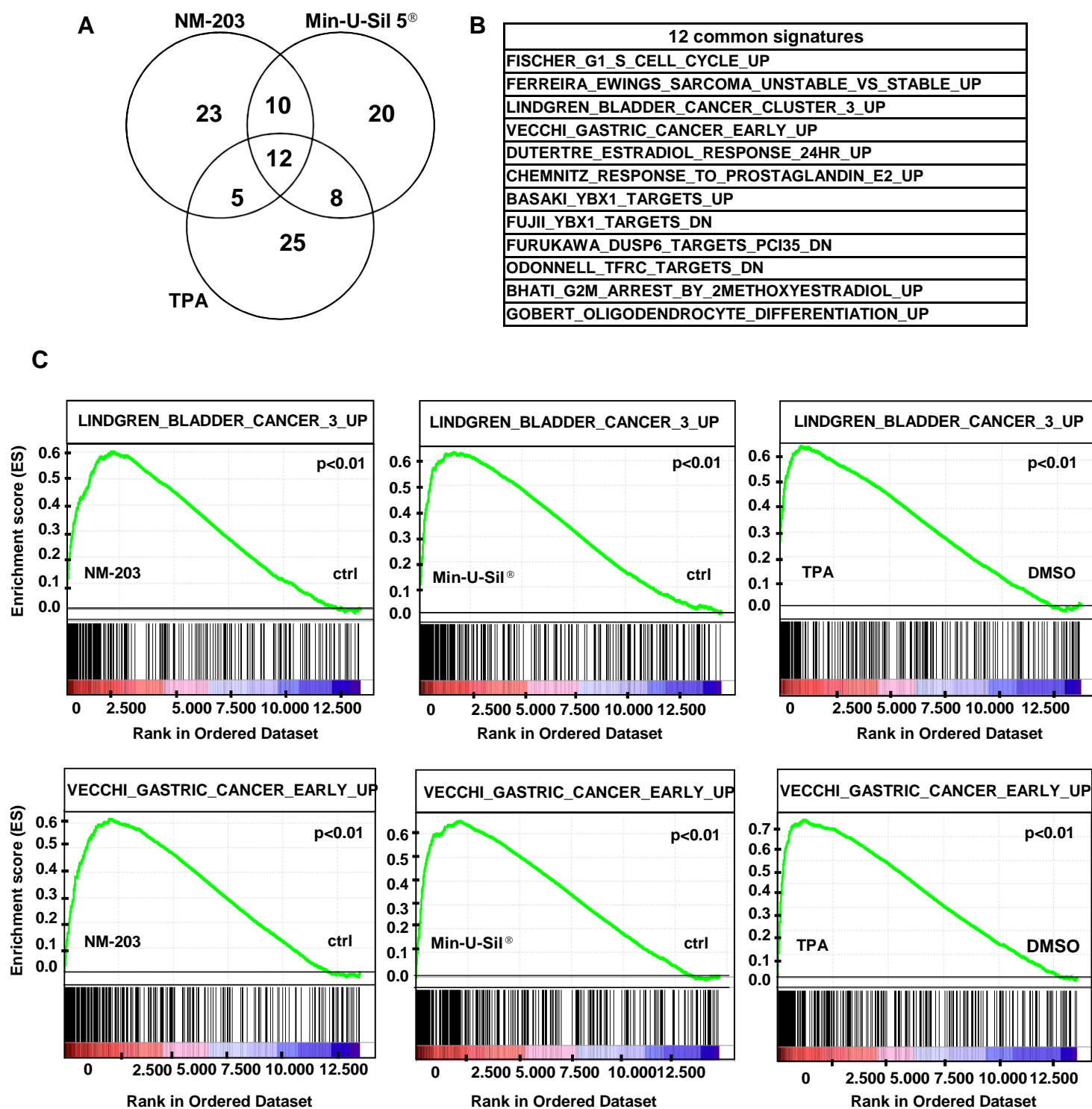
(A). Bhas 42 cells were treated once with increasing concentrations of NM-203, Min-U-Sil 5<sup>®</sup> or DE cultivated over 21 days. Foci, resulting from cell transformation, were counted as described in the *materials and methods* section. (B). Similar experiments were performed with NM-200, NM-201 and NM-202. The values obtained after cell exposure to TPA (0.05  $\mu\text{g}/\text{ml}$ ) or DMSO (used as the vehicle for TPA), were added to each histogram. Results are expressed as the mean  $\pm$  SD ( $n = 6$ ). \*\*\*:  $p < 0.005$ , comparison of results obtained from cells exposed to nanoparticle *versus* cells treated with H<sub>2</sub>O (ctrl). \$\$:  $p < 0.01$ , comparison of results obtained from cells exposed to TPA *versus* cells treated with DMSO after mixed linear regression modelling followed by a Bonferroni correction.





**Figure 2: Hierarchical clustering of Bhas 42 cells after treatments.**

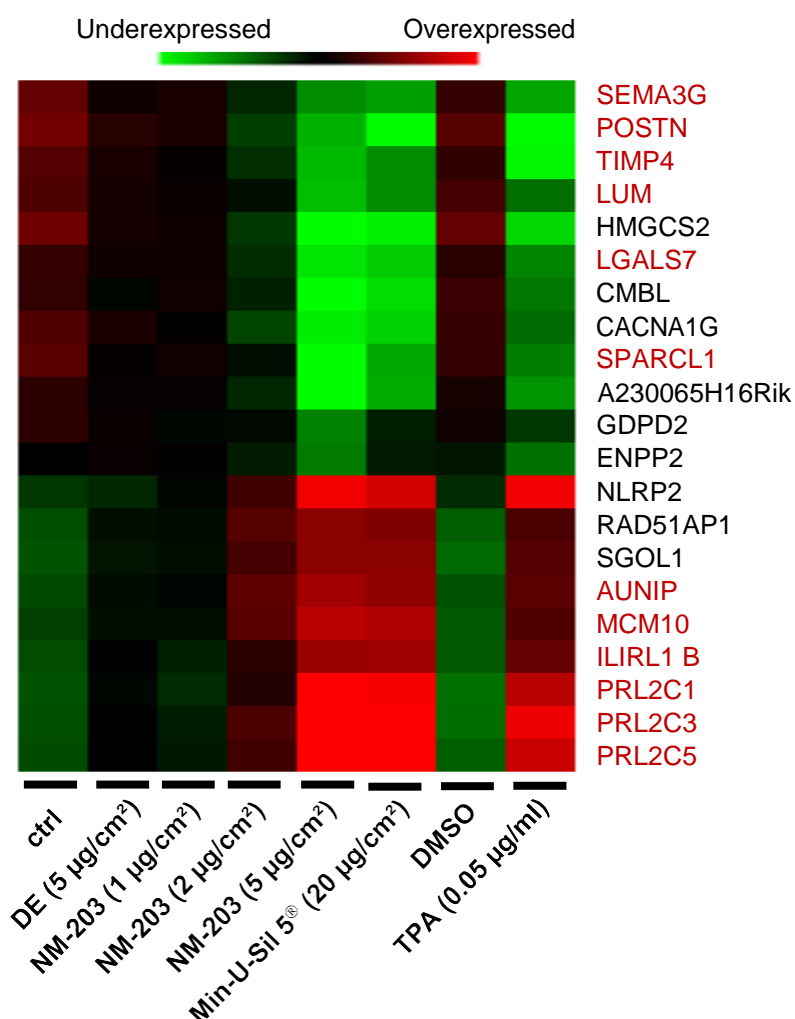
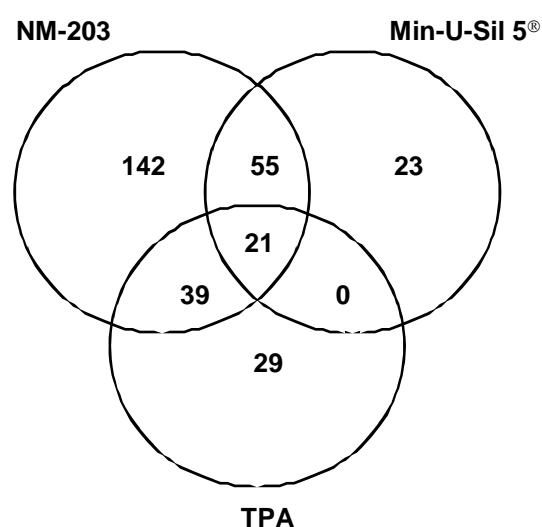
Cells were exposed to NM-203 (1, 2 or 5  $\mu\text{g}/\text{cm}^2$ ), Min-U-Sil 5<sup>®</sup> (20  $\mu\text{g}/\text{cm}^2$ ), DE (5  $\mu\text{g}/\text{cm}^2$ ) and TPA (0.05  $\mu\text{g}/\text{mL}$ ). (A) A non supervised hierarchical clustering was obtained after filtering microarray data, using Gene Cluster 3.0 software on 19569 probes. Gene clustering was visualized with Treeview. An uncentred correlation was used as a measure of similarity and complete linkage was used for the clustering method. (B) Dose effect of NM-203 (1-5  $\mu\text{g}/\text{cm}^2$ ). Supervised hierarchical clustering was performed with Gene Cluster 3.0 software and visualized by Treeview on the 285 probes obtained after filtration and application of the ANOVA statistical test (p-value < 0.005 and FC > 4), by comparing NM-203 (1  $\mu\text{g}/\text{cm}^2$ ), NM-203 (2  $\mu\text{g}/\text{cm}^2$ ) and NM-203 (5  $\mu\text{g}/\text{cm}^2$ ) and control  $\text{H}_2\text{O}$ , followed by a Tukey post-hoc test. An uncentred correlation was used as a measure of similarity and complete linkage was used for the clustering method. Green indicates lower expression; red indicates higher expression. Biological functions related to the main clusters were evaluated using the FuncAssociate 2.0 program. (Abbreviations: Ctrl, control ( $\text{H}_2\text{O}$ ); TPA, 12-O-tetradecanoylphorbol-13-acetate; DE, diatomaceous earth; DMSO, dimethyl sulfoxide).



**Figure 3: Gene set enrichment analysis.**

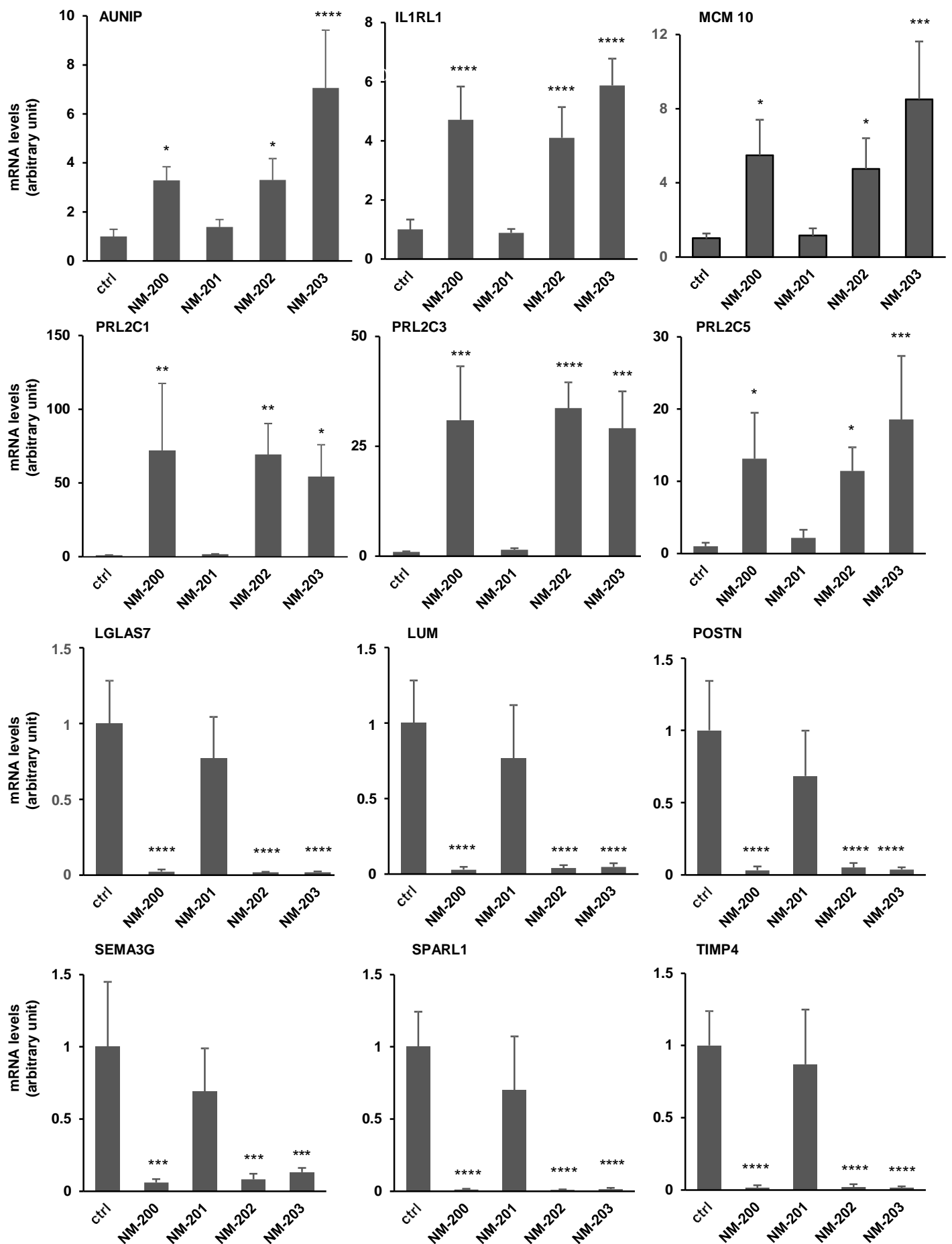
(A) GSEA analysis using the filtered gene expression profiles of Bhas 42 cells 48h after treatment with NM-203 (5  $\mu\text{g}/\text{cm}^2$ ), Min-U-Sil 5<sup>®</sup> (20  $\mu\text{g}/\text{cm}^2$ ) and TPA (0.05  $\mu\text{g}/\text{ml}$ ) over 48 hours. Venn diagram of the top 50 signatures. (B) List of the 12 common signatures. (C) GSEA revealed an enrichment in gene signatures related to cell cycles and cancer that was common to the three conditions, for example the “Lindgren\_bladder\_cancer\_3\_up” signature (upper panels), and the “Vecchi\_gastric\_cancer\_early\_up” signature (lower panels).

A

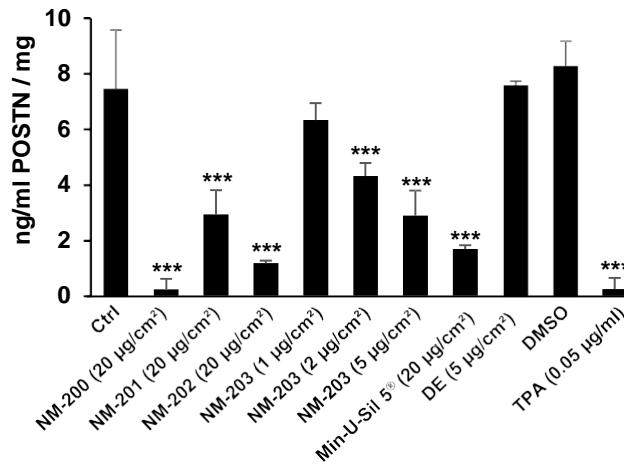


**Figure 4: Molecular signature comparison and selection of common genes.**

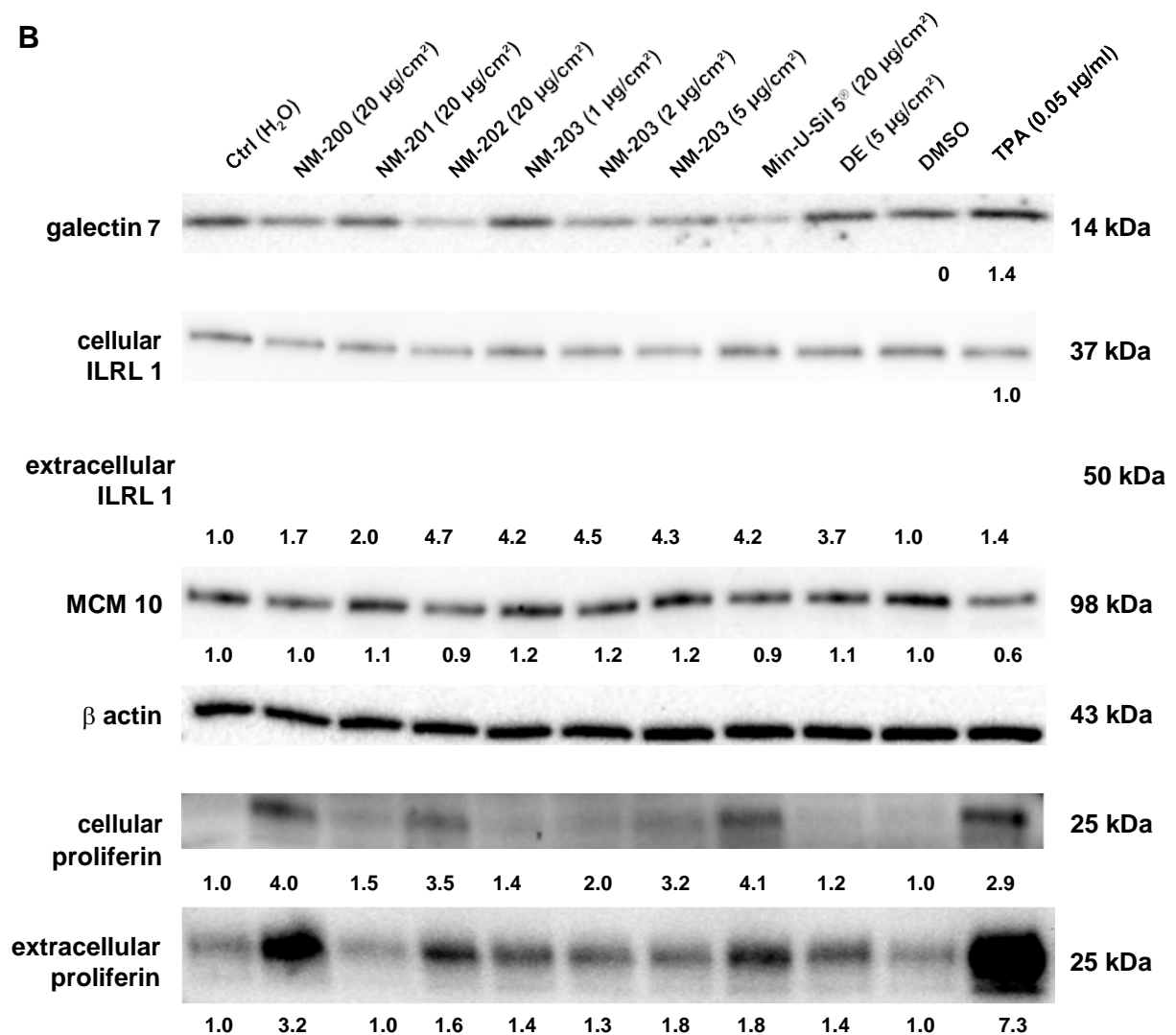
(A) : Venn diagram comparing the molecular signatures of NM-203 (5 µg/cm<sup>2</sup>), Min-U-Sil 5® (20 µg/cm<sup>2</sup>) and TPA (0.05 µg/mL) ( $p < 0.005$  and  $FC > 4$ ). (B): Heatmap of 21 common genes regulated by the three transforming compounds obtained with Gene Cluster 3.0 software and visualized with Treeview. An average of the expression of each gene was obtained for each condition. Green indicates the lower gene expression; red the higher gene expression. The writing in red corresponds to the selected genes of interest. (Abbreviations: ctrl, control H<sub>2</sub>O, TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; DE, diatomaceous earth).



**A**



**B**



**Figure 6: Protein analysis.**

**A)** Periostin secretion (POSTN) was determined by ELISA in supernatants from cell layers exposed to each nanoparticle over 48 h. \*\*\* ,  $p < 0.005$  ( $n = 3$ ) with respect to the control (ctrl =  $H_2O$ ) or DMSO (used as the vehicle for the particles or TPA, respectively), following a mixed linear regression model followed by a Bonferroni multiple comparison test. Results are expressed as ng of POSTN/ ml of supernatant harvested normalized to mg of protein in the respective cell homogenate. **(B)** Protein levels were analysed by Western blotting using cell homogenates. Proliferin and ILR1 were also detected in the cell culture supernatant from treated cells. Results are representative of one out of three independent experiments.  $\beta$ -actin was used as a loading control in each blot, with the exception of extracellular proliferin or IL1R1 detection in cell supernatant.