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

3

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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: Glycerldehyde-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: Metallopeptidase inhibitor 4; LOAEL: Lowest observed adverse effect level; IPA: Igenuity 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

24

## 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<sup>®</sup> 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<sup>®</sup>- 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.

41

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

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44

## 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].

74 Cell transformation depends on a cascade of complex events that occur within a few hours or weeks  
75 [17]. To decipher these processes, the transcriptomic approach allows access to a global gene  
76 signature of cellular function after cell exposure to chemicals [18]. The aim of this study was thus to  
77 use transcriptomic analysis to identify an early gene signature for Bhas 42 cell transformation that is  
78 shared by an SAS, a crystalline silica and the tumor promoter 12-O-tetradecanoylphorbol-13-acetate  
79 (TPA). Based on previous data [8], the transcriptomic procedure was carried out after Bhas 42 cell  
80 exposure to the SAS NM-203, which in the previous study induced the highest level of cell  
81 transformation. In addition, the crystalline silica Min-U-Sil 5<sup>®</sup> and the amorphous silica DE  
82 (diatomaceous earth) were used as transforming and non-transforming reference particles,  
83 respectively [19].

84

## 85 **2. Materials and methods**

### 86 **2.1 Cell culture**

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

95

### 96 **2.2 Cell transformation and cell survival assays**

97 The CTA in promotion condition, originally proposed by the OECD [16], was modified as described  
98 previously [8] (Supplementary Fig. A.1). Cells were seeded at 1460 cells/cm<sup>2</sup> in a six-well plate on day  
99 0 and the cells were treated once on day 4 (D4). The cells were then post-cultivated in fresh medium  
100 until day 21 (D21), receiving a medium change on days 7 (D7), 10 and 14. Cells were fixed with  
101 ethanol (Fisher, France) and stained with a 5 % (v/v) Giemsa solution (Thermo Fisher Scientific) on  
102 D21. The transformed foci were evaluated according to the morphological criteria recommended by

103 the OECD (2016). For each experiment, the mean number of transformed foci per treatment group  
104 was determined from six replicate wells.  
105 Cell cytotoxicity was assessed by cell survival assay on D7 performed as follows: cells were  
106 suspended by trypsination and stained with a mixture of acridine orange/propidium iodide (Sigma-  
107 Aldrich, Saint Quentin Fallavier, France), and then counted using a Cellometer® (Vision Nexcelom  
108 Bioscience, Lawrence, MA). Cell survival was expressed as the percentage of living cells after cell  
109 exposure to the chemical per living cells counted in the untreated condition.

110

### 111 **2.3 Particles and cells treatments**

112 Silica nanoparticles (NM-200, NM-201, NM-202, and NM-203) were obtained from the Joint Research  
113 Centre nanomaterials repository (Ispra, Italy) (Supplementary Table A.1). Min-U-Sil 5® was obtained  
114 from US Silica Co and DE was obtained from the European Coal and Steel Community (Paris,  
115 France). Thorough physical and chemical characterizations of SAS, Min-U-Sil 5® and DE batches have  
116 been described previously [8]. For cell treatment, vials containing particles suspended in sterile  
117 deionized water (19.2 mg/mL) were sonicated for 5 min at 10 % amplitude in the cup horn of a 400 W  
118 Branson Sonifier S-450D (Branson Ultrasonics Corp, Danbury, CT). Serial dilutions were prepared in  
119 water from the sonicated suspension. Each dilution was added to DF5F to give a final concentration of  
120 water of 2 % (v/v). Final particle concentrations were expressed as mass per cell culture surface area  
121 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  
122 0.05 µg/mL in dimethyl sulfoxide (DMSO, Sigma Aldrich) at a final concentration of 0.1 % (v/v).

123

### 124 **2.4 RNA extraction and quality control**

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

130

131

132

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  $\mu\text{g}/\text{cm}^2$ ), Min-U-Sil 5<sup>®</sup> (20  $\mu\text{g}/\text{cm}^2$ ), DE (5  $\mu\text{g}/\text{cm}^2$ ), TPA (0.05  $\mu\text{g}/\text{mL}$ ) 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  $\mu\text{g}/\text{cm}^2$ ), 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

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

165

## 166 **2.7 Gene expression analysis by RT-qPCR**

167 Total RNA was reverse-transcribed with the iScript™ cDNA synthesis Kit (BioRad, Marnes-la-  
168 Coquette, France) following the manufacturer's protocol. Real-time PCR was performed with CFX96  
169 Touch™ (BioRad) in the presence of iQ™ SYBR® Green Supermix (BioRad) and PrimePCR Assay (or  
170 2.5 µM of each primer (Eurogentec, Anger, France) (Supplementary Table A.2).

171 Amplification was performed following the manufacturer's protocol: the annealing temperature was 60  
172 °C for all pairs of primers. PCR results were analyzed using the SQ method. The results were  
173 expressed as relative to those of control cells treated with H<sub>2</sub>O or DMSO, arbitrarily set to 1. *ACTB*  
174 (beta-Actin), *TUBA* (alpha-Tubulin), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were  
175 used as housekeeping genes whose values have been averaged (geometric mean).

176

## 177 **2.8 Protein analysis**

178 Bhas42 cells were treated as stated before. On D6, cell medium supernatants were harvested and  
179 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>  
180 containing 0.148 M NaCl, 2 mM KCl) and scraped with a rubber policeman with ice-cold RIPA buffer  
181 (50 mM Tris-HCl, pH 8, containing 150 mM NaCl, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium  
182 deoxycholate, 0.1 % (w/v) SDS and 1 % (v/v) protease and phosphatase inhibitor mixture (Thermo  
183 Scientific). The homogenates obtained were placed under agitation for 30 min at 4 °C, then  
184 centrifuged at 16000 g for 20 min at 4 °C. Supernatants were collected and stored at -80 °C. Protein  
185 concentration was measured in each sample and corresponding cell medium supernatant, using  
186 bovine serum albumin as a standard (Protein quantification kit, Sigma-Aldrich).

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

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

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

206

## 207 **2.9 Statistics**

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

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

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

214

## 215 **3. Results**

### 216 **3.1 Cytotoxic and transforming effects of NM-203, Min-U-Sil 5<sup>®</sup>, DE and TPA.**

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

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

226

### 227 **3.2 NM-203, Min-U-Sil 5<sup>®</sup> and TPA induce alteration of genes involved in cell**

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

229 After normalization and filtering of the raw transcriptomic data using the Genespring software, 19,569  
230 probes were listed. Non supervised hierarchical clustering (Fig. 2A) of these 19,569 remaining probes,  
231 showed two main branches, dividing the samples into two groups with regards to the transforming  
232 potential: a first group with control conditions (ctrl = 2 % (v/v) H<sub>2</sub>O or 0.1 % (v/v) DMSO) and non-  
233 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-  
234 203 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  
235 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  
236 TPA (0.05 µg/ml) group. The NM-203 (2 µg/cm<sup>2</sup>) group, which was a transforming condition in the  
237 Bhas 42 CTA performed with three treatments but not with a single treatment [8], seems to induce  
238 gene expression modulation even with a single treatment. To study the dose-effect of NM-203, an  
239 ANOVA test was performed with a *p*-value lower than 0.005 selected. Genes that were expressed  
240 differently in at least one condition (vs. control) with a fold change (FC) greater than 4 were selected  
241 (supplementary Table A.4). Hierarchical clustering of the 290 differentially expressed genes showed  
242 that increasing the concentration of NM-203 led to a higher increase or decrease response at the  
243 mRNA level (Fig. 2B). Treeview analysis highlighted co-regulated groups of genes. Interestingly, the  
244 upper part of the dendrogram grouped clusters of genes down-regulated after NM-203 treatment (124  
245 genes) and involved in matrix remodeling and cell adhesion, and the lower part of the dendrogram  
246 highlighted genes up-regulated after NM-203 treatment (166 genes) and involved in cell proliferation.  
247 Gene set enrichment analysis (GSEA) identified significant enrichments (*p* < 0.01) of signatures for  
248 each treatment (Supplementary Table A.5). Comparison of the top 50 signatures highlighted for each  
249 treatment showed that 24 % of signatures were common to the three conditions (Fig. 3A). These  
250 twelve common signatures are related mainly to proliferation and cancer features (Fig. 3B-C) [20, 21].  
251 To identify genes significantly deregulated by each treatment, an unpaired t-test (Bonferroni FWER  
252 correction) with a *p*-value < 0.05, FC > 2 was used. RNA levels of 1,580 genes were modified after cell  
253 treatment by NM203 (5 µg/cm<sup>2</sup>) (Supplementary Table A.6), 1,044 genes with Min-U-Sil 5<sup>®</sup>

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

259

### 260 **3.3 Comparison of early molecular signature and selection of common genes**

261 Genes significantly highly regulated by NM-203 (5 µg/cm<sup>2</sup>), Min-U-Sil 5<sup>®</sup> and TPA were compared (p <  
262 0.005 and FC > 4) (Fig. 4A). Of the 309 genes altered by treatments, a large majority (83 %) were  
263 affected by NM-203. Among these, 142 (46 %) were affected solely by NM-203 treatment and 23 (7  
264 %) and 29 genes (9 %) were deregulated by Min-U-Sil 5<sup>®</sup> and TPA, respectively. Interestingly, 21  
265 common genes were regulated significantly by NM-203 (5 µg/cm<sup>2</sup>), Min-U-Sil 5<sup>®</sup> and TPA (p < 0.005,  
266 FC > 4). Twelve genes, *CACNA1G*, *CMBL*, *ENPP2*, *GDPD2*, *HMGCS2*, *LGALS7*, *LUM*, *POSTN*,  
267 *SEMA3G*, *SPARCL1*, *TIMP4*, and A230065H16Rik, were underexpressed whereas 9 genes,  
268 *RAD51AP1*, *NLRP2*, *SGOL1*, *IL1RL1*, *PRL2C3*, *PRL2C5*, *PRL2C1*, *AUNIP* and *MCM10* were  
269 overexpressed (Fig. 4B). We selected 6 genes known to be involved in cell proliferation (*AUNIP*,  
270 *IL1RL1*, *MCM10*, *PRL2C1*, *PRL2C3* and *PRL2C5*) and 6 genes involved in cell-cell adhesion  
271 (*LGALS7*, *LUM*, *POSTN*, *SEMA3G*, *SPARCL1*, and *TIMP4*) (Table 1). For all these genes, RT-qPCR  
272 results were broadly in line with transcriptomic results and confirmed the dose-effect produced by NM-  
273 203 (Table 2).

274

### 275 **3.4 Transforming silica nanoparticles NM-200 and NM-202 induce similar changes at** 276 **the RNA level of the selected genes.**

277 We then assessed whether Bhas 42 cell exposure to other SAS, namely NM-200, NM 201 and  
278 NM202, led to cell transformation and involved any changes at the RNA level of the selected genes.  
279 NM-200 were slightly cytotoxic at the highest concentration (40 µg/cm<sup>2</sup>) while NM-202 decreased cell  
280 survival by approximately 50 % at 40 µg/cm<sup>2</sup>. NM-201 treatment had no cytotoxic or transforming  
281 effect (Fig.1B and supplementary Table A3). Bhas 42 cell exposure to NM-200 (20 µg/cm<sup>2</sup>) or NM-202  
282 (20 µg/cm<sup>2</sup>) led to similar expression patterns as those obtained when cells were treated with NM203  
283 (5 µg/cm<sup>2</sup>), *i.e.* an increase in RNA levels of *AUNIP*, *IL1RL1*, *MCM10*, *PRL2C1*, *PRL2C3* and

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

286

### 287 **3.5 Changes at the protein level**

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

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

306 Among the genes showing decreased expression after cell exposure to SAS, periostin, involved in cell  
307 matrix adhesion and epithelial mesenchymal transition [25], was measured by ELISA in cell medium  
308 supernatants. Protein levels were lowered when Bhas 42 cells were treated with 20 µg/cm<sup>2</sup> of NM-200,  
309 NM-202 and Min-U-Sil 5<sup>®</sup>, 5 µg/cm<sup>2</sup> NM-203 and 0.05 µg/ml TPA, in agreement with the observed  
310 decrease in mRNA levels. However, protein levels were also low when cells were exposed to NM-201  
311 (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  
312 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]  
313 was also detected in whole cell homogenate as a band estimated at 100 kDa. We did not observe any

314 significant changes at the protein level whatever the treatment tested. These results could be related  
315 to the experimental design of the CTA, since, from the beginning of cell treatment on D4, Bhas 42 cells  
316 continue to undergo cell division until D6, maintaining a high level of MCM10, a protein necessary for  
317 DNA synthesis [26]. Finally, galectin 7, involved in cell-cell or cell-matrix interaction [27], was detected  
318 in whole cell homogenates with a band estimated at 37 kDa (Fig. 6). Galectin 7 levels were lowered  
319 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  
320  $\mu\text{g}/\text{cm}^2$ ), whereas no change was found in the other conditions tested.

321

## 322 4. Discussion

323

### 324 4.1 Selection of genes as possible markers of SAS-induced Bhas42 cell

#### 325 transformation

326 Although SAS are extensively produced and used, few studies have focused on their transforming or  
327 carcinogenic potential [1, 3]. In a previous study, the transforming properties of the NM-200, NM-201,  
328 NM-202 and NM-203 SAS were revealed in the Bhas 42 CTA using the promotion assay. In order to  
329 decipher the molecular changes during the Bhas 42 CTA, an unsupervised transcriptomic analysis  
330 was performed following exposure of cells to SAS and control chemicals for 48h. In this treatment  
331 time, NM-203 and Min-U-Sil 5<sup>®</sup> have been shown to induce epigenetic effects in Bhas 42 cells in the  
332 promotion assay [28]. We therefore hypothesized that this treatment time could highlight earlier gene  
333 changes involved in Bhas 42 cell transformation. The lowest concentrations of chemicals able to  
334 induce cell transformation without inducing cytotoxicity were selected in transcriptomic and RT-qPCR  
335 assays. The expression of 21 genes was altered by the three treatments, NM-203, Min-U-Sil 5<sup>®</sup> and  
336 TPA. Among them, twelve genes, whose involvement in cell proliferation and adhesion has been  
337 proven (Table 1), were selected as putative markers of the transforming properties of SAS  
338 nanomaterials. To confirm this hypothesis, we tested the NM-200, NM201 and NM-202 SAS (Fig. 1B  
339 and 5). Similar changes in the RNA levels of each selected gene were obtained with NM-200 and NM-  
340 202, supporting the hypothesis that the genes in this set are potential markers of SAS-induced cell  
341 transformation. However, NM-201 treatment induced no change in the expression of these 12 genes  
342 (Fig. 5). This could be explained by the fact that, unlike the three repeated treatments of NM-201 [8]),  
343 a single treatment did not cause Bhas 42 cell transformation. One reason for this could be that NM-

344 201 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  
345 nanomaterials is often associated with their high specific surface area, which results in a high reactivity  
346 with the biological environment. In addition, we demonstrated concomitant changes in the mRNA and  
347 protein levels of several genes, such as galectin 7, periostin or proliferin. However, a concordance  
348 between mRNA and protein levels was not systematically found. It is possible that regulation  
349 mechanisms of protein synthesis and processing may occur.

350 Interestingly, the increase in *PRL2Ci* mRNA levels, encoding proliferin, was observed in CTA  
351 performed with BALB c/3T3 cells [13, 14] and C3H/10T1/2 cells [29, 30] treated with various chemicals  
352 including amorphous silica particles (silica gel of 5  $\mu$ m). Proliferin expression was considered as a  
353 predictive biomarker of cell transformation [13, 14]. However, even though the expression of three  
354 members of the proliferin gene family, *PRC12C2,3,5*, was upregulated, proliferin was revealed as a  
355 single band by western blotting. On the other hand, c-myc upregulation was also linked to C3H/10T1/2  
356 [29] and Bhas 42 cell transformation [28]. In the latter, *C-MYC* mRNA and protein levels increased  
357 after cell treatment with NM-203 and Min-U-Sil 5<sup>®</sup> over 2 days. Similar changes in mRNA level were  
358 also found in the present study with  $p < 0.005$  and  $FC > 4$  (Supplementary Tables A10-A11).  
359 Accordingly, Bhas 42 cell transformation is associated with early increased *C-MYC* expression, but not  
360 in the late phase of SAS-induced cell transformation as shown previously [28]. These results, among  
361 others [14, 31], confirm that markers of transformation must be identified in the early steps of the  
362 processes. Finally, epigenetic modifications have also been related to cell transformation as recently  
363 reviewed [32]: it has been shown that cadmium chloride, methyl carbamate and lithocholic acid (three  
364 transforming compounds) can induce genome-wide DNA methylation changes in Bhas 42 cells [33].  
365 Moreover, Seidel *et al.* proposed that the transforming activity of the crystalline silica Min-U-Sil 5<sup>®</sup> in  
366 Bhas 42 cells was related to early epigenetic DNA methylation [28].

367

#### 368 **4.2 Gene expression changes after SAS exposure**

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

374 example, *IL1RL1* has been found to be involved in inflammasome activation induced by crystalline  
375 silica in human lung epithelial cells (BEAS2B) and in primary human bronchial epithelial cells (NHBE)  
376 [34, 35]. An increase in the expression of *LUM*, *MCM10*, *POSTN*, *SPARCL1* and *IL1RL1* has been  
377 observed in RAW 264, 7 cells (murine macrophages cells) treated with SAS (10 or 300 nm) [34].  
378 Pisani et al. showed overexpression of *LUM*, *LGALS7* and *MCM10* in FE1 cells treated with AEROSIL  
379 200® (NM-203) [36]. In addition, variations in gene expression related to the cell cycle or cell adhesion  
380 were found after treatment of the lung epithelial cell line FE1 exposed for 24h to 12, 15 and 20 nm  
381 SAS [37]. Furthermore, pathway analysis has revealed that protein contents in the adenocarcinomic  
382 human alveolar basal epithelial cell line A549 treated with Min-U-Sil 5® for 24h are associated with cell  
383 proliferation [38].

384

### 385 **4.3 Gene expression changes related to cancer progression**

386 All of the genes selected are known to be involved in cancer progression. Six of the genes were  
387 overexpressed by the three treatments. In previous studies, for example, overexpression of *IL1RL1*,  
388 has been observed in tumor cells from human colon cancer [39], the respective expression of the  
389 *PRL2C* gene family was increased in fallopian tube epithelium derived ovarian cancer cells [40],  
390 *MCM10* was increased in medulloblastoma, urothelial carcinomas, prostate cancer and breast cancer  
391 [41-44] and finally, *AUNIP* was overexpressed in various brain tumors [45]. Conversely, respective  
392 expression of the 6 selected genes was downregulated by the three treatments in this study. In  
393 previous studies, expression of *LUM*, *POSTN*, *SPARCL1*, *LGALS7*, and *TIMP4*, all involved in cell-cell  
394 adhesion, was found to be lowered in various cancers: *LGALS7* expression was decreased in cervical  
395 cancer, colon cancer, gastric cancer and in malignant melanoma [27, 46-48]; *LUM* was downregulated  
396 in various human cancer cell lines [48,49,50], *POSTN* expression was likewise downregulated in  
397 bladder and lung cancer [49]; low expression of *SPARCL1* has been shown to be associated with  
398 tumor progression in human gastric, breast and prostate cancers [50-52], and expression of *TIMP4*  
399 has been found to be low in malignant peripheral nerve sheath tumors associated with  
400 neurofibromatosis type 1 [53]. Moreover, some gene expression changes observed in this study are  
401 related to cancer progression. For example, *SEMA3G* expression decreased under our experimental  
402 conditions. In contrast, overexpression of *SEMA3G* limits the progression of breast cancer or  
403 melanoma cells or the migration and invasion of glioma cells [54, 55]. GSEA analysis also revealed an

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

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411

## 412 **5 Conclusion**

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

419

420

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428

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

<b>Gene symbol</b>	<b>Gene title</b>	<b>Gene ontology</b>	<b>Protein localisation</b>	<b>Expression level</b>	<b>References</b>
<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 <sup>®</sup> (20 µg/cm <sup>2</sup> )	DE (5 µg/cm <sup>2</sup> )	TPA (0.05 µg/ml)	<i>r</i>
<i>AUNIP</i>	Microarray	1.87	5.90	6.22	5.02	1.37	4.14	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	4.33	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	4.14	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>SFMD3G</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	

433

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

434

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

435

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

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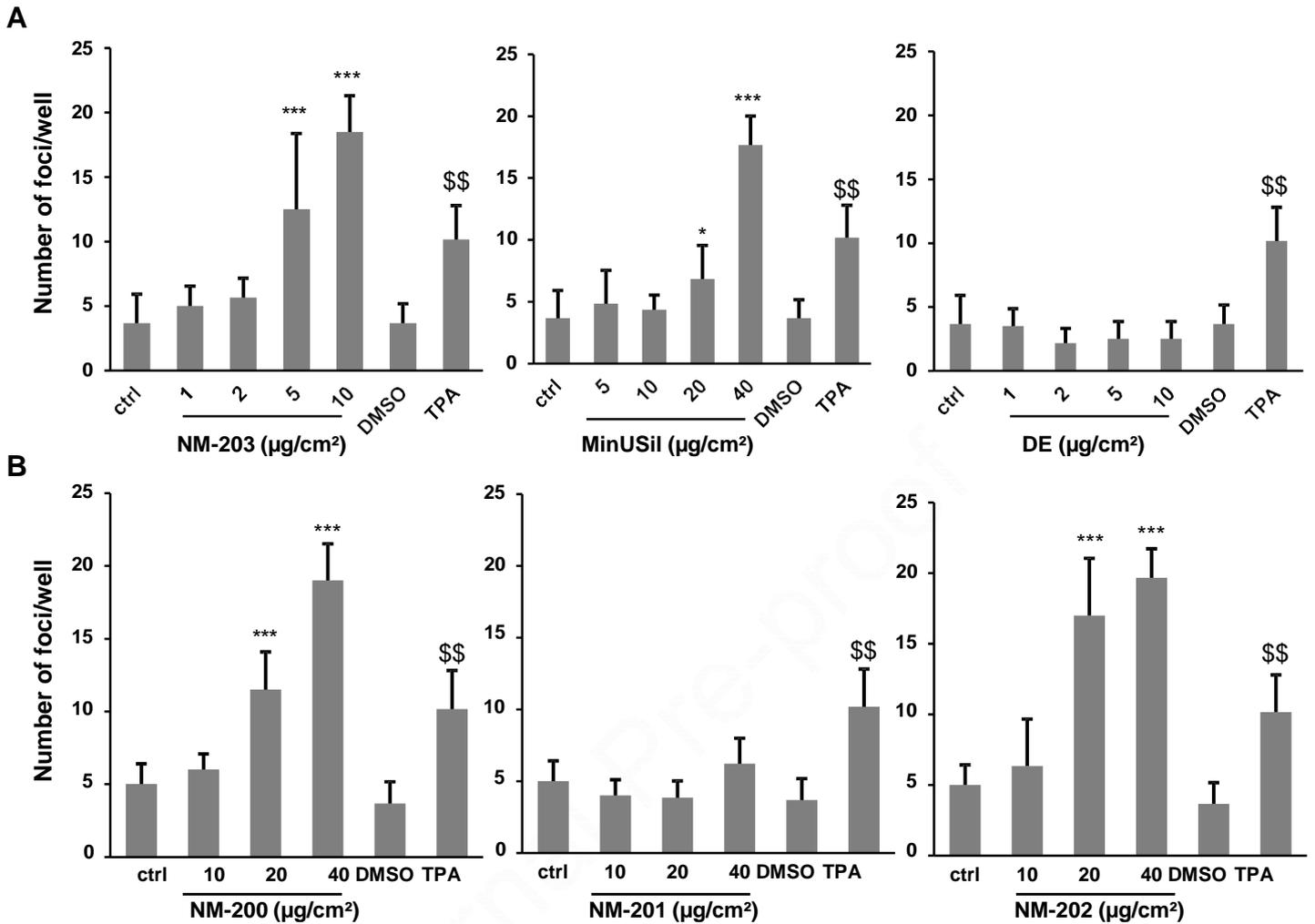
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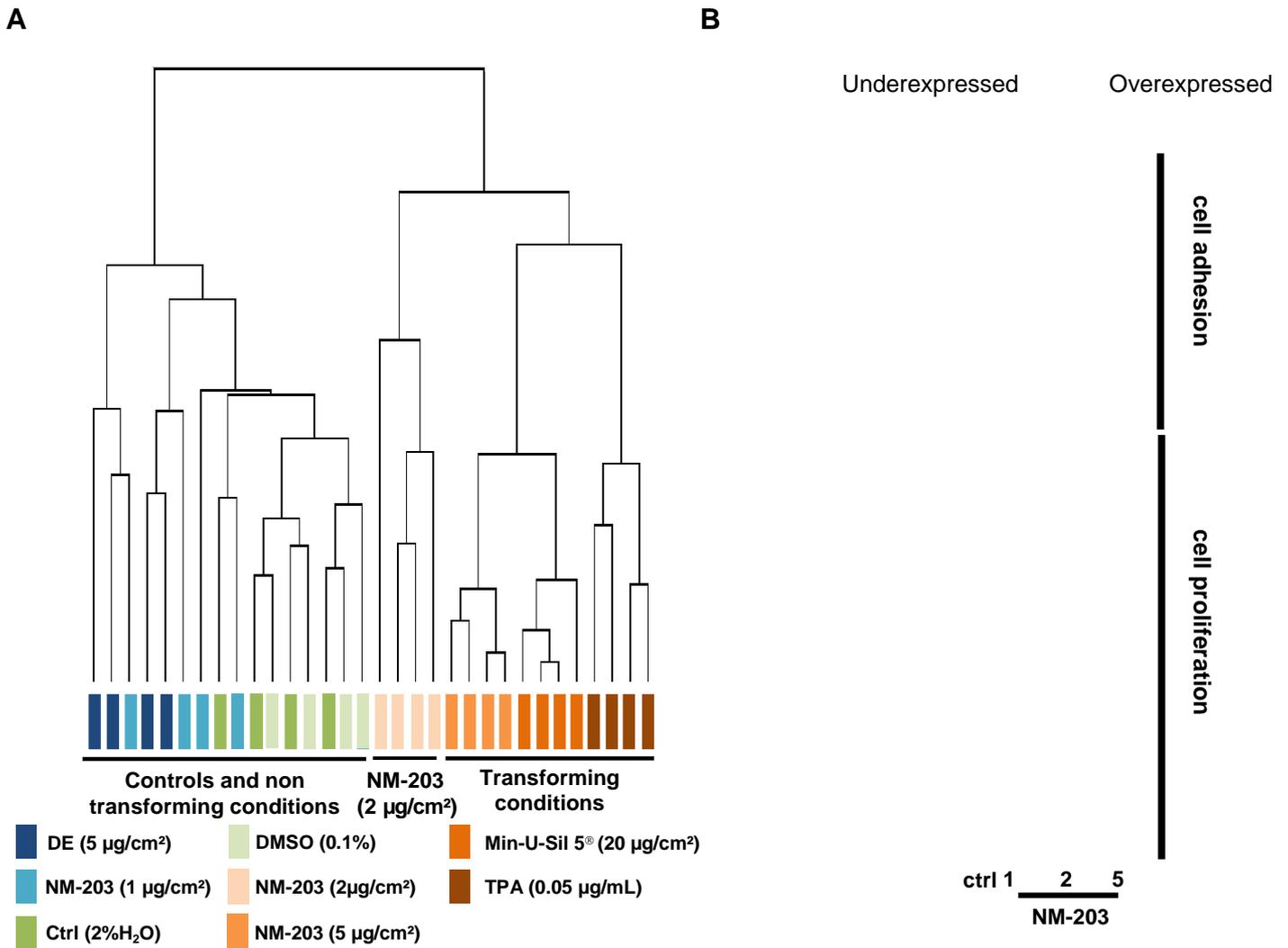
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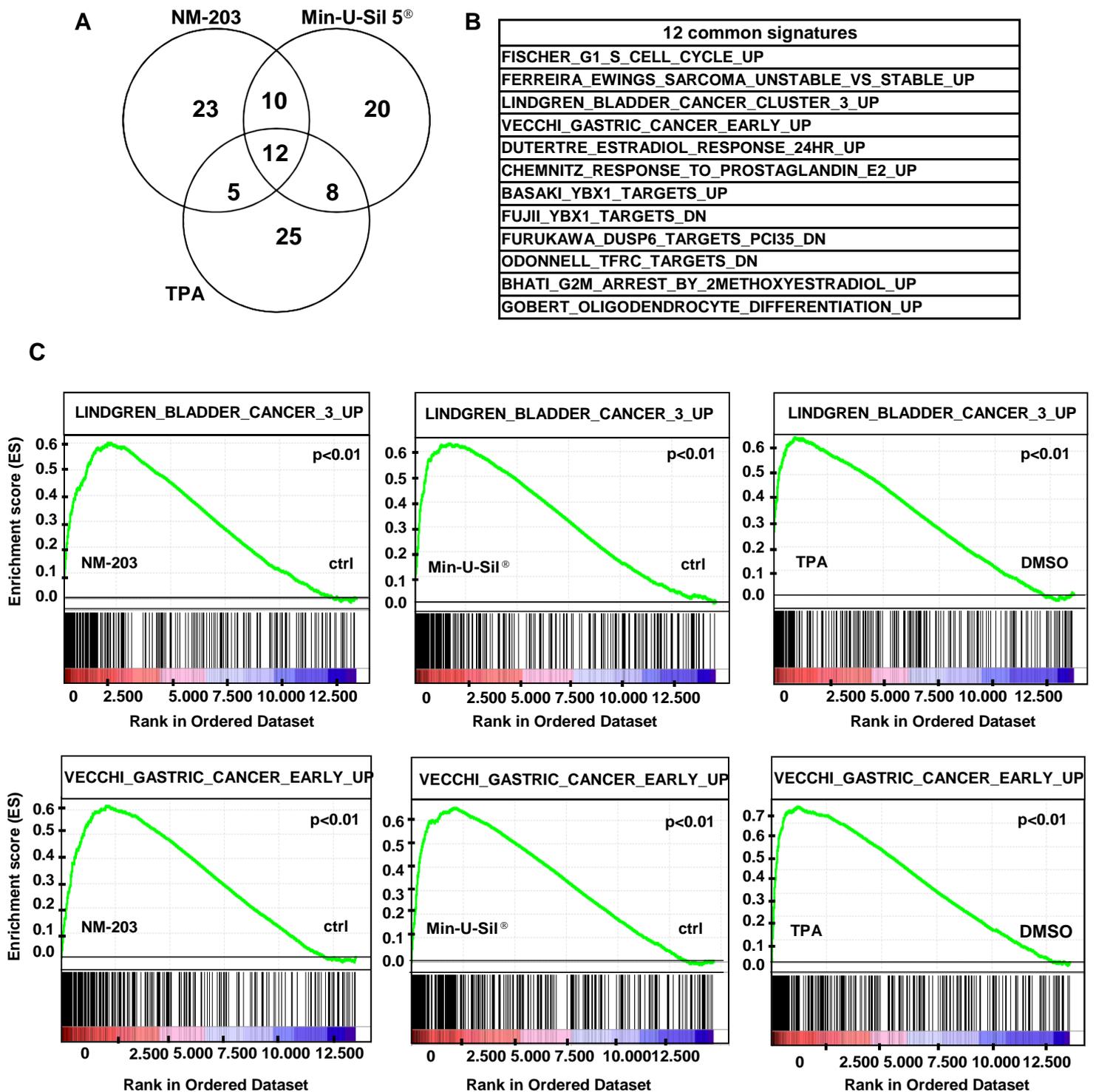
**Figure 1: Bhas 42 cell transformation assay.**

(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 Bhas42 cells after treatments.**

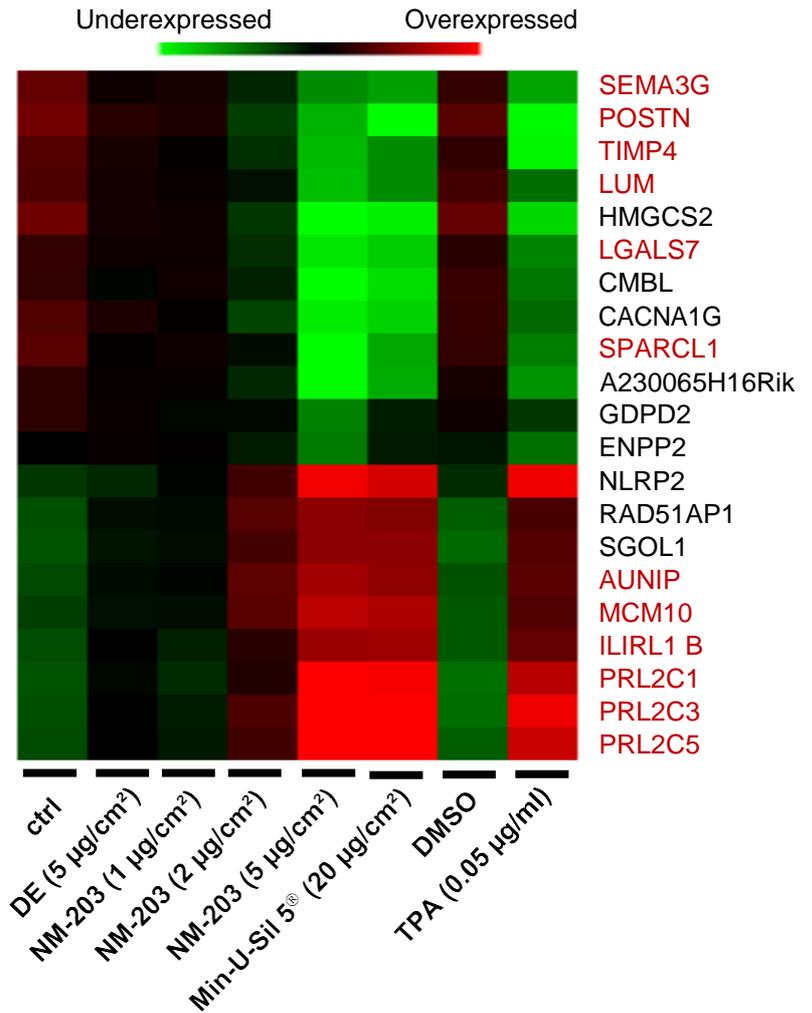
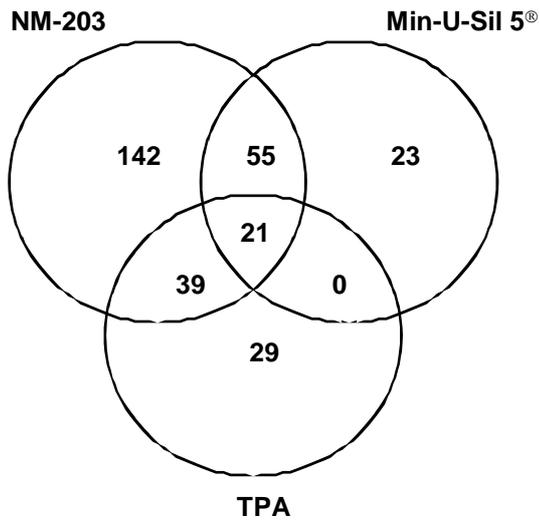
Cells were exposed to NM-203 (1, 2 or 5 µg/cm<sup>2</sup>), Min-U-Sil 5® (20 µg/cm<sup>2</sup>), DE (5 µg/cm<sup>2</sup>) and TPA (0.05 µg/mL). (A) A non supervised hierarchical clustering was obtained after filtering microarray data, using Gene Cluster 3.0 software on 19569 probes. Gene clustering was visualized with Treeview. An uncentred correlation was used as a measure of similarity and complete linkage was used for the clustering method. (B) Direct effect of NM-203 (1-5 µg/cm<sup>2</sup>). 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 µg / cm<sup>2</sup>), NM-203 (2 µg / cm<sup>2</sup>) and NM-203 (5 µg / cm<sup>2</sup>) and control H<sub>2</sub>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 (H<sub>2</sub>O); TPA, 12- O-tetradecanoylphorbol-13-acetate; DE, diatomaceous earth; DMSO, dimethyl sulfoxide).



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

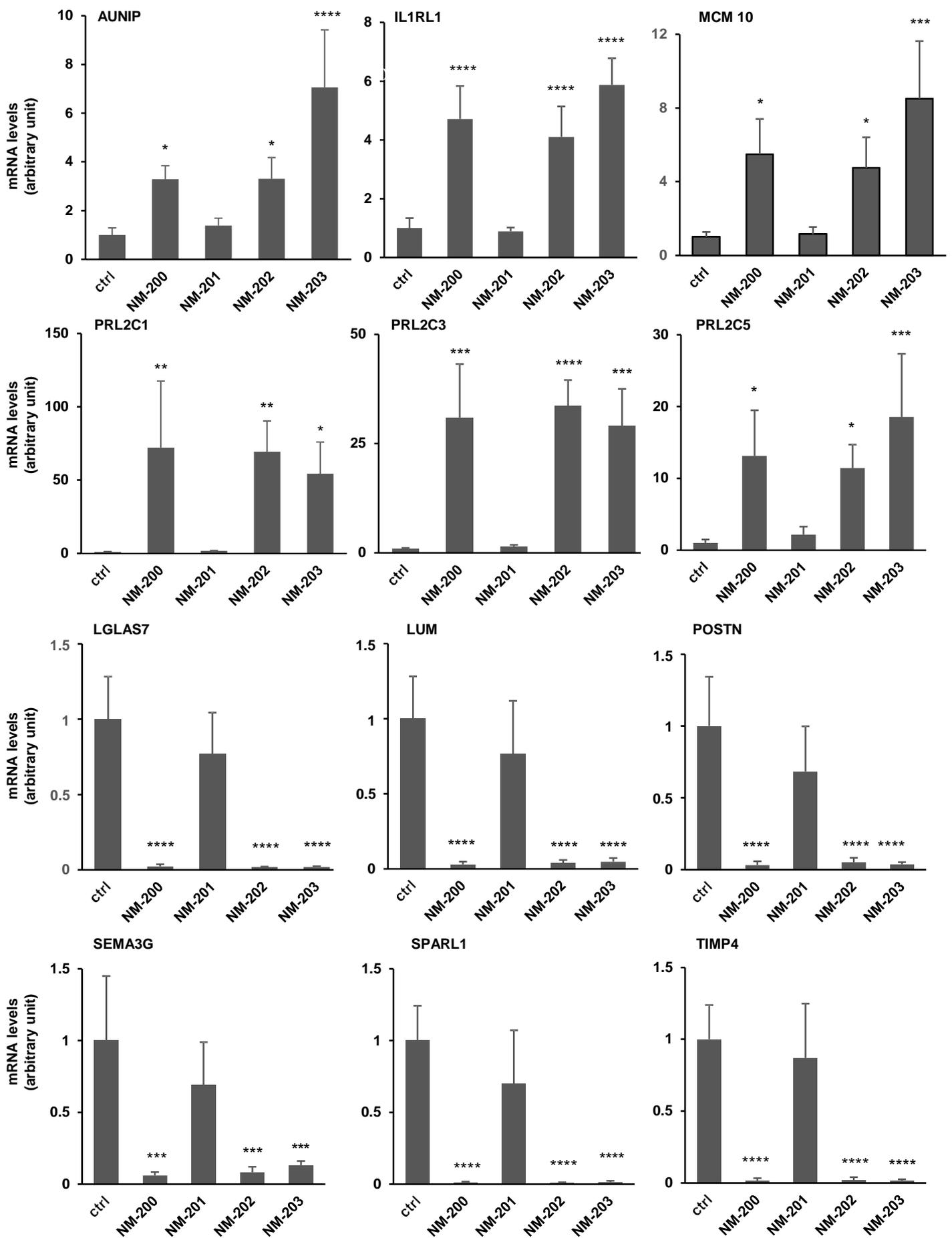
(A) GSEA analysis using the filtered gene expression profiles of Bhas 42 cells 48h after treatment with NM-203 (5  $\mu\text{g}/\text{cm}^2$ ), Min-U-Sil 5<sup>®</sup> (20  $\mu\text{g}/\text{cm}^2$ ) and TPA (0.05  $\mu\text{g}/\text{ml}$ ) over 48 hours. Venn diagram of the top 50 signatures. (B) List 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



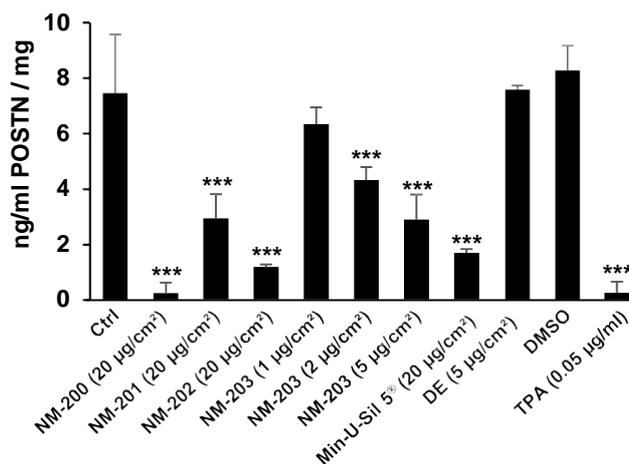
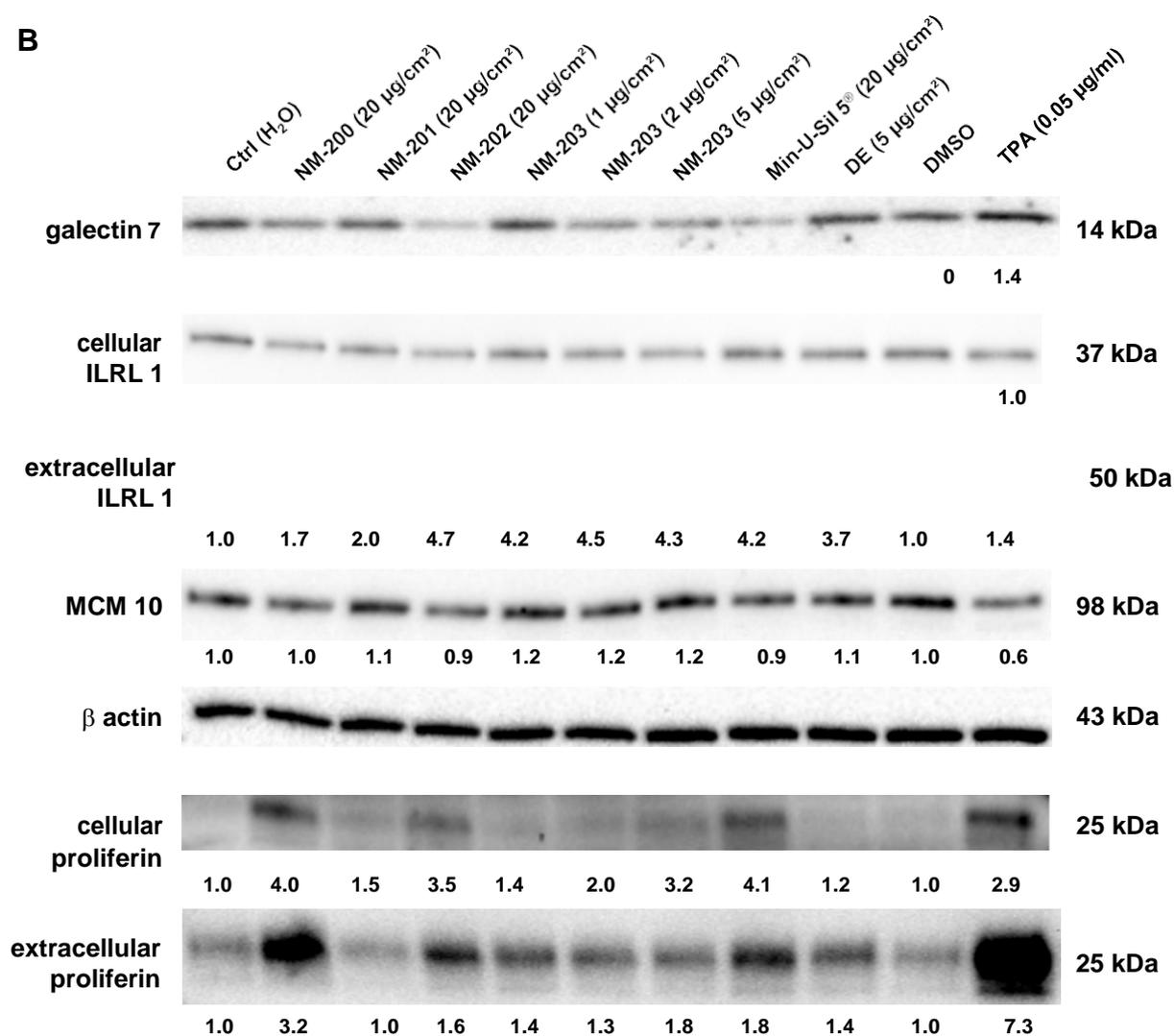
**Figure 4: Molecular signature comparison and selection of common genes.**

(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).



**Figure 5: Expression changes induced by silica nanoparticles NM-200, NM-201 and NM-202.**

Relative expression of the 12 selected genes (*AUNIP*, *IL1RL1* (Isoform B), *MCM10*, *PRL2C1*, *PRL2C3*, *PRL2C5*, *LGLAS7*, *LUM*, *POSTN*, *SEMA3G*, *SPARL1* and *TIMP4*) after 48h-treatment of Bhas 42 cells with NM-200 (20  $\mu\text{g}/\text{cm}^2$ ), NM-201 (20  $\mu\text{g}/\text{cm}^2$ ), NM-202 (20  $\mu\text{g}/\text{cm}^2$ ) and NM203 (5  $\mu\text{g}/\text{cm}^2$ ). The results are presented with respect to those of control cells taken as 1.00. The results are expressed as the mean of four independent experiments. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.005$ , \*\*\*\* indicates  $p < 0.001$  versus control cells after a one-way ANOVA test followed by a Dunnett's multiple comparisons test. (Abbreviations: Ctrl, control ( $\text{H}_2\text{O}$ )).

**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<sub>2</sub>O) 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.