

**1 S100A8/A9 mediate the reprogramming of normal mammary epithelial cells**  
**2 induced by dynamic cell-cell interactions with adjacent breast cancer cells.**

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4 Seol Hwa Jo (cho5761@hanmail.net)<sup>1</sup>, Woo Hang Heo (woohang92@gmail.com)<sup>1</sup>,  
 5 Bok Sil Hong (boksil427@gmail.com)<sup>2</sup>, Ju Hee Kim (jhsbzero@naver.com)<sup>2</sup>, Jiwoo  
 6 Lee (leehy69@snu.ac.kr)<sup>2</sup>, Eun-Shin Lee (silvershoe99@gmail.com)<sup>3</sup>, Han-Byoel Lee  
 7 (hblee80@gmail.com)<sup>3</sup>, Wonshik Han (hanw@snu.ac.kr)<sup>2,3,4</sup>, Yeonju Park  
 8 (pyj11@snu.ac.kr)<sup>5</sup>, Dong-Sup Lee (dlee5522@snu.ac.kr)<sup>5</sup>, Nam Hoon Kwon  
 9 (nanarom1@snu.ac.kr)<sup>6</sup>, Min Chul Park (minchul.park@neomics.com)<sup>6</sup>, Jeessoo Chae  
 10 (moverm0210@gmail.com)<sup>4,7</sup>, Jong-Il Kim (jongil@snu.ac.kr)<sup>4,7</sup>, Dong-Young Noh  
 11 (dynoh@snu.ac.kr)<sup>3,4,8</sup>, Hyeong-Gon Moon (moonhg74@snu.ac.kr)<sup>2,3,4</sup>.

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13 <sup>1</sup>Interdisciplinary Graduate Program in Cancer Biology, Seoul National University  
 14 College of Medicine, Seoul, Korea,

15 <sup>2</sup>Center for Medical Innovation, Biomedical Research Institute, Seoul National  
 16 University Hospital, Seoul, Korea,

17 <sup>3</sup>Department of Surgery, Seoul National University College of Medicine, Seoul, Korea,

18 <sup>4</sup>Genomic Medicine Institute, Medical Research Center, Seoul National University  
 19 College of Medicine, Seoul, Korea,

20 <sup>5</sup>Department of Biomedical Sciences, Seoul National University College of Medicine,  
 21 Seoul, Korea,

22 <sup>6</sup>Medicinal Bioconvergence Research Center, Seoul National University, Suwon, Korea,

23 <sup>7</sup>Department of Biochemistry, Seoul National University College of Medicine, Seoul,  
 24 Korea

25 <sup>8</sup>Cancer Research Institute, Seoul National University College of Medicine, Seoul,  
 26 Korea

27

28 **Address correspondence to:**

29 Dong-Young Noh, Seoul National University College of Medicine, 103 Daehak-ro,  
30 Jongno-gu, Seoul, 03080, South Korea. Phone: 82-2-2072-2772; Fax: 82-2-766-3975;  
31 E-mail: [dynoh@snu.ac.kr](mailto:dynoh@snu.ac.kr)

32

33 Hyeong-Gon Moon, Seoul National University College of Medicine, 103 Daehak-ro,  
34 Jongno-gu, Seoul, 03080, South Korea. Phone: 82-2-2072-2634; Fax: 82-2-766-3975;  
35 E-mail: [mooonhg74@snu.ac.kr](mailto:mooonhg74@snu.ac.kr)

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## 54 **Abstract**

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56 **Background:** Tumor microenvironment drive the progression of solid tumors. Among  
57 the various cell types in the breast cancer microenvironments, non-tumorigenic  
58 mammary epithelial cells are often located in the vicinity of malignant cancer cells.  
59 However, the biologic characteristics of non-tumorigenic mammary epithelial cells  
60 adjacent to breast cancer cells are poorly understood.

61 **Methods:** Non-tumorigenic mammary epithelial MCF10A cells were co-cultured with  
62 various breast cancer cells and the extent of cell-cell interactions was evaluated by  
63 time-lapse imaging. The molecular and functional traits of the co-cultured MCF10A  
64 cells were investigated by using RNA sequencing, phosphor-protein arrays, pathway  
65 analysis, and various *in vitro* assays. The molecular changes observed in vitro were  
66 validated using breast cancer xenograft mouse models. The phenotype changes in the  
67 S100A8/A9-overexpressing MCF10A cells were investigated to determine the causal  
68 relationship of S100A8/A9 upregulation observed in co-cultured MCF10A cells.

69 **Results:** When the cells were co-cultured directly, there were dynamic cell-cell  
70 interactions between the MCF10A cells and breast cancer cells including lamellipodia-  
71 or nanotube-like contacts and transfer of extracellular vesicles. MCF10A cells exhibited  
72 features of epithelial-mesenchymal transition, and showed increased capacity of cell  
73 proliferation, migration, colony formation, and 3-dimensional sphere formation upon the  
74 direct co-culture with breast cancer cells. Transcriptome analysis and phosphor-protein  
75 array suggested that several cancer-related pathways are significantly dysregulated in  
76 MCF10A cells after the direct co-culture with breast cancer cells. S100A8/A9 showed  
77 distinct up-regulation in the co-cultured MCF10A cells and orthotopic xenograft of  
78 syngeneic mouse mammary tumors. When S100A8/A9 overexpression was induced in

79 MCF10A cells, the cells showed phenotypic features of directly co-cultured MCF10A  
80 cells in terms of *in vitro* cell behaviors and signaling activities.

81 **Conclusions:** This study suggests the possibility of dynamic cell-cell interactions  
82 between non-tumorigenic mammary epithelial cells and breast cancer cells that could  
83 lead to a substantial transition of molecular profiles and functional characteristics in  
84 mammary epithelial cells. S100A8/A9, along with other dysregulations in cancer-  
85 related signaling pathways, may mediate this phenotype transition of non-tumorigenic  
86 mammary epithelial cells.

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88 Keywords: Breast cancer, Mammary epithelial cells, Microenvironment, Cell-cell  
89 interaction, S100A8/A9, mTOR

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## 106 **Backgrounds**

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108 In solid tumors, the complex tumor microenvironment controls all steps of tumor  
109 progression and metastasis [1, 2]. The tumor microenvironment is comprised of various  
110 endogenous and recruited cells that undergo dynamic cell-cell interactions with  
111 malignant epithelial cells and contribute to the tumor cell's behaviors [3, 4]. For  
112 example, cancer-associated fibroblasts actively remodel extracellular matrix and  
113 immune microenvironment, and cancer-associated adipocytes provide inflammatory  
114 milieu that support tumor growth [5, 6]. Moreover, recent efforts to target the immune  
115 microenvironment have shown promising therapeutic responses in selected solid  
116 tumors [7]. Therefore, understanding the molecular mechanisms of the tumor-  
117 microenvironment interactions can provide scientific basis for developing novel  
118 therapeutic strategies that target the tumor microenvironment [3, 8, 9].

119 Normal epithelial cells are closest neighbors to the malignant transformed cells in  
120 human epithelial tumors arising from solid organs. During the early steps of  
121 carcinogenesis, the normal epithelial cells may exert tumor-suppressive effects by  
122 promoting protrusion of transformed epithelial cells from the epithelial layers [10-12].  
123 However, the tumor-suppressive effects of normal epithelial cells may not last  
124 throughout the solid tumor progression. While the normal myoepithelial cells obtained  
125 from healthy human breast tissues contribute to the maintaining polarity of mammary  
126 epithelial cells and suppress aberrant growth, the myoepithelial cells derived from  
127 breast cancer tissues failed to restore physiologic polarity in mammary epithelial cells  
128 and showed increased expression of various chemokines such as CXCL12 [13, 14].  
129 These reports suggest a potential functional transition of normal epithelial cells caused  
130 by adjacent malignant epithelial cells which may contribute the progression of solid

131 tumors.

132 In this study, we show that breast cancer cells and non-tumorigenic mammary epithelial  
 133 cells undergo dynamic cell-cell interactions that lead to a substantial reprogramming of  
 134 molecular characteristics of the mammary epithelial cells. The reprogramming of normal  
 135 mammary epithelial cells includes phenotypes changes as well as dysregulations of  
 136 mRNA expression and cell signaling activities. Our data suggests that S100A8/A9  
 137 upregulation in non-tumorigenic mammary epithelial cells may play a critical role in the  
 138 phenotype shifting induced by adjacent cancer cells.

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## 141 **Methods**

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### 143 **Cell culture**

144 Cells were purchased from Korean Cell Line Bank (Seoul, Korea). Non-tumorigenic  
145 mammary epithelial MCF10A cells were maintained in a 1:1 mixture of Dulbecco's  
146 Modified Eagle's Medium (DMEM) and Ham's F12 medium (F12) with 5% horse serum,  
147 20 ng/mL epidermal growth factor (EGF), 100 ng/mL cholera toxin, 10 µg/mL insulin,  
148 and 500 ng/mL hydrocortisone. MCF7 and MDA-MB-231 breast cancer cells were  
149 cultured in DMEM with 10% FBS, 1% penicillin, and 1% streptomycin. SK-BR3 breast  
150 cancer cells were cultured in RPMI 1640 with 10% FBS, 1% penicillin, and 1%  
151 streptomycin. For fluorescence tagged cells, puromycin was added.

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### 153 **Co-culture of MCF10A cells and breast cancer cells**

154 To optimize the culture medium for co-culture, we tested the effect of various mixture of  
155 MDA-MB-231 culture media and MCF10A culture media on cell survival. Based on the  
156 effect on cell proliferation, we chose 7:3 ratio mixture of MDA-MB-231 media and  
157 MCF10A media for the direct co-culture of the cells (Additional File 1). For effective  
158 separation of MCF10A cells and MDA-MB-231 cells after the direct co-culture, MCF10A  
159 cells and MDA-MB-231 cells were transfected with GFP and RFP using lentiviral  
160 vectors, respectively. After the direct co-culture, cells were isolated using a FACS Aria II  
161 cell sorter (Becton Dickinson, NJ) as shown in the Additional File 2.

162 Indirect co-culture was performed by using transwell inserts (pore size 0.4µm). Cancer  
163 cells were seeded on the membrane of the insert and MCF10A cells were seeded on  
164 the bottom six well plates. For controls, same cells were seeded in the bottom wells  
165 and on the transwell inserts.

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## 167 ***In vitro* assays measuring cell phenotypes**

168 Proliferation assays were conducted using CellTiter-Glo Luminescent Cell Viability  
169 Assay (Promega, Madison, USA) following the manufacturer's protocol. Cells were  
170 seeded in triplicate into 96-well plates at a density of 2,000 cells per well. For migration  
171 assay,  $2 \times 10^4$  cells were seeded in an insert (8µm pore size) with serum free media and  
172 media with 10% FBS was added in lower chambers. Cells were incubated for 20hrs  
173 and fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. For colony  
174 formation assay,  $2 \times 10^3$  cells were seeded into 6-well plates. After 2 weeks, colonies  
175 were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet.

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## 177 **RNA Sequencing and qPCR**

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179 RNA sequencing libraries were prepared using TruSeq RNA Access library kit (Illumina,  
180 Inc., San Diego, CA, USA) according to the manufacturer's protocol. After validation of  
181 the libraries, using Agilent DNA screentape D1000 kit on a TapeStation (Agilent  
182 Technologies, Santa Clara, CA, USA), the hybridization steps were performed using  
183 exome capture probes and streptavidin coated beads. RNA sequencing was performed  
184 by HiSeq 2000 (Illumina, San Diego, USA by the Macrogen Incorporated). We  
185 processed reads from the sequencer and aligned them to the Homo sapiens (hg19).  
186 After aligning reads to genome, Cufflinks v2.2.1 was used to assemble aligned reads  
187 into transcripts and to estimate their abundance. The transcript counts in isoform and  
188 gene level were calculated, and the relative transcript abundances were measured in  
189 FPKM (Fragments Per Kilobase of exon per Million fragments mapped) from Cufflinks.  
190 RNA sequencing data of breast cancer was generated through a separate project to  
191 characterize the genomic profiles of the primary breast tumor and patient-derived  
192 xenograft tumors which will be presented in other reports (IRB No. 1402-054-555).



193 For qPCR, total RNA was extracted from isolated cells with TRIzol (Favorgen, Taiwan).  
194 PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) were used for reverse  
195 transcription of RNA, and resulting cDNA was amplified using Power SYBR® Green  
196 PCR Master Mix (Applied Biosystems, CA). The information on the primer sequences  
197 used in this study is listed in the Additional File 3.

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### 199 **Phosphorylation array and western blotting**

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201 For phosphorylation array, protein extraction was done with buffer following the  
202 manufacturer's protocol. Protein concentration was measured by BCA assay kit  
203 (Thermo scientific, Palm Springs, CA, USA). Phosphorylation array was performed by  
204 using the human phosphor-kinase array kit (R&D systems, USA).

205 Proteins were harvested with RIPA buffer (Thermo scientific, Palm Springs, CA, USA),  
206 protease & phosphatase inhibitor and 0.5M EDTA solution. Protein concentration was  
207 measured by BCA assay kit (Thermo scientific, Palm Springs, CA, USA). Cell lysates  
208 were loaded onto 10% gels and transferred to a PVDF membrane. The membrane was  
209 blocked with 5% skim milk and incubated with primary antibody overnight at 4°C. The  
210 peroxidase-conjugated secondary antibody was used for detection. Bands were  
211 detected by LAS. The information on the antibodies used in this study is listed in the  
212 Additional File 4.

213

### 214 **Cell imaging**

215

216 For time-lapse imaging of cells during the direct co-culture,  $9 \times 10^3$  GFP-transfected  
217 MCF10A cells and  $2 \times 10^4$  RFP-transfected MDA-MB-231 cells were seeded in 8-well

218 chambers and cultured for 24 hours. After 24 hours culture, live cells images were  
219 taken every 10 minutes with fixed position for 16 hours by using Leica confocal  
220 microscope (Leica TCS SP8, Leica Microsystems Ltd, Korea), and the obtained images  
221 were analyzed by Leica LAS X program.

222 For *in vivo* imaging, the experiments were performed in accordance with the Animal  
223 Care and Use Committee guidelines of Woosung BSC (Suwon, Korea). Hair-removed  
224 ear skin of C57BL/6 mouse (10 weeks old) was injected with  $4.6 \times 10^4$  cells of the  
225 mixture MCF10A-GFP ( $2.3 \times 10^4$ ) and MDA-MB-231-RFP ( $2.3 \times 10^4$ ) cell lines. The  
226 mice were anesthetized with an intraperitoneal injection of Zoletil (30 mg/kg, Virbac,  
227 Carros, France) and Rompun (10 mg/kg, Bayer-Korea, Seoul, Korea) before imaging.  
228 The mice were placed on the heated plate of a motorized XYZ translational stage. The  
229 *in vivo* movement of MCF10A and MDA-MB-231 was monitored by modified custom-  
230 built laser-scanning confocal microscopy {Choe, 2015 #3}. GFP-expressing cells were  
231 visualized at an excitation wavelength of 491 nm and detected through a bandpass  
232 filter of 502 nm to 537 nm (Semrock Inc, Rochester, NY, USA). RFP-expressing cells  
233 were imaged at an excitation wavelength of 532 nm and detected using a bandpass  
234 filter of 562 nm to 596 nm (Semrock Inc). Cell movement was visualized at 1 min  
235 interval for 2 hr. After acquired from the imaging system, 512 x 512 pixel images were  
236 then compensated with Matlab (Mathworks, Natick, MA, USA) and reconstructed by  
237 ImageJ software.

238

### 239 **S100A8/A9 expression in xenograft tumor-bearing mouse fatpad**

240

241 5-week-old BALB/c mice were purchased from KOATECH (Seoul, Korea) and housed  
242 in Seoul National University Hospital Clinical Research Institute's Specific Pathogen  
243 Free zone. All experiments were approved by the Institutional Animal Care and Use

244 Committee in Seoul National University Hospital (SNUH-IACUC, 17-0165-S1A0 (1)). To  
245 obtain control mouse fatpads, eight-week-old non-tumor-bearing BALB/c mouse was  
246 sacrificed and mammary fat pad was resected. To obtain tumor-bearing mouse fat pad,  
247  $2 \times 10^5$  4T1 mouse mammary epithelial cancer cells were injected in 6-week-old  
248 BALB/c mouse's mammary fat pad. At two weeks after the tumor injection, the mouse  
249 was sacrificed and tumor-bearing mammary fatpad was resected. The resected fatpads  
250 were fixed with 4% PFA and embedded in paraffin.

251

## 252 **S100A8/A9-overexpressing MCF10A cell line**

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254 The coding sequences of S100A8 and S100A9 were acquired by RT-PCR and cloned  
255 into pCDH-GFP and pCDH-RFP vectors. MCF10A cells were transfected with lentivirus  
256 S100A8-GFP and S100A9-RFP construct. Transfected cells were selected by  
257 puromycin.

258

## 259 **Statistical analysis**

260

261 In general, most data represent the mean  $\pm$  S.D and are representative of 3  
262 independent experiments, except for RNA sequencing and phospho-protein arrays  
263 which are two independent experiments. Graph Pad Prism (ver. 7.01) was used for  
264 generating graphs and heatmaps and performing statistical tests. P values were  
265 calculated from unpaired two-tailed Student's t tests or Mann-Whitney test as  
266 appropriate.

267 To identify differentially expressed genes, we filtered genes with one more than zeroed  
268 FPKM values and the data were log2-transformed and subjected to quantile  
269 normalization. Statistical significance of the differential expression data was determined

270 using fold change in which the null hypothesis was that no difference exists among  
271 samples. Gene pathway analysis for the DEG was done based on KEGG pathway  
272 (<http://www.genome.jp/kegg/pathway.html>).

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## 278     **Results**

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### 280     **Breast cancer cells show dynamic cell-cell interactions with non-transformed** 281     **mammary epithelial cells *in vitro* and *in vivo*.**

282

283     First, we determined the presence and the extent of cell-cell interactions *in vitro*  
 284     between the breast cancer cells and mammary epithelial cells. We co-cultured the  
 285     RFP-transfected breast cancer cells (MDA-MB-231) with GFP-transfected non-  
 286     transformed mammary epithelial cells (MCF10A) using *in vitro* direct co-culture method.  
 287     While the majority of MCF10A cells maintained the clusters of adherent cells, MDA-MB-  
 288     231 cells showed spreading patterns of cell growth and the cells infiltrated between the  
 289     MCF10A cell clusters (Additional File 5). The time-lapse imaging of the cells showed  
 290     that MDA-MB-231 cells had more frequent cell movements than the MCF10A cells and  
 291     the cells showed various dynamic cell-cell interaction patterns (Additional File 6 and 7).  
 292     MDA-MB-231 cells formed both lamellipodia-like structures for adjacent cells and  
 293     nanotube-like projections for long-range cell-cell interactions (Figure 1a and 1b,  
 294     Additional File 5) [15, 16]. The lamellipodia-like structures of MDA-MB-231 cells  
 295     actively contacted the MCF10A cells and a portion of extended lamellipodia could  
 296     remain as extracellular vesicles which were then engulfed by adjacent MCF10A cells  
 297     (Figure 1c, Additional File 7).  
 298     Among the various cell-cell interactions, exchanges of extracellular vesicles were  
 299     frequently seen between the cells. MCF10A cells engulfed extracellular vesicles  
 300     originated from MDA-MB-231 cells, and the vesicles were often transferred to the  
 301     nucleus while some stayed at cytoplasm (Figure 1d and 1e). Additionally, a small  
 302     proportion of co-cultured cells (1~2%) exhibited mixed fluorescence (Figure 1f,  
 303     Additional File 2).

304 To observe the cell-cell interactions *in vivo*, we inoculated a mixture of MDA-MB-231  
305 cells and MCF10A cells in the earlobes of nude mouse and obtained the time-lapse  
306 imaging data. After one hour of the inoculation, the movements of the MDA-MB-231  
307 cells around the MCF10A cells were detectable (Additional File 8 and 9). After 24 hours,  
308 the cell-cell interactions and exchange of vesicles were more frequently seen between  
309 the cells (Figure 1g, Additional File 9). These data suggest that the cancer cells and  
310 epithelial cells can undergo a dynamic range of physical contacts and cell-cell  
311 interactions both *in vitro* and *in vivo*.

312

### 313 **Direct co-culture with breast cancer cells induce phenotypic changes in MCF10A** 314 **cells**

315

316 We then determined whether the cell-cell interactions with breast cancer cells induce  
317 phenotype changes in MCF10A cells. First, we co-cultured MCF10A cells with breast  
318 cancer cell lines using in-direct co-culture system. MCF10A cells co-cultured indirectly  
319 with cancer cells showed no significant changes in cell morphology or the cell growth  
320 patterns in 2D and 3D cultures (Additional File 10). However, when the MCF10A cells  
321 were cultured in the direct co-culture system with breast cancer cells, the MCF10A cells  
322 showed substantial phenotypic changes. Directly co-cultured MCF10A cells, sorted by  
323 GFP-expression, showed changes in cell morphology such as transition into spindle-  
324 shaped cells and loss of cell-cell adhesions (Figure 2a).

325 Additionally, the MCF10A cells showed marked decrease in E-cadherin expression  
326 when they were directly co-cultured with MDA-MB-231 cells (Figure 2b). When the  
327 isolated MCF10A cells were grown *in vitro*, the proliferation rate, colony formation  
328 capacity, and the cell migration ability were significantly enhanced in cells directly co-  
329 cultured with MDA-MB-231 cells (Figure 2c-2e). MCF10A cells also showed higher

330 numbers and larger sizes of cell spheres when they were grown in 3D matrigels  
331 (Figure 2f). Collectively, our data show that direct co-culture with breast cancer cells  
332 induce substantial phenotype changes in non-tumorigenic mammary epithelial cells.

333

### 334 **Transcriptome changes and pathway dysregulations in MCF10A cells when** 335 **directly co-cultured with MDA-MD-231 cells**

336

337 To understand the molecular mechanisms underlying the phenotype changes induced  
338 by direct co-culture, we analyzed the transcriptome profiles of MCF10A cells when they  
339 were directly co-cultured with MDA-MB-231 cells. A total of 151 genes were  
340 dysregulated in directly co-cultured MCF10A cells more than two-fold in expression  
341 (Figure 3a, Additional File 11). Again, we observed the decreased expression of  
342 mammary epithelial differentiation markers such as CDH1 or CD24. Based on the gene  
343 expression profiles, we identified several cancer-related pathways, including metabolic  
344 pathway, cell adhesion, growth factor signaling, and TP53 pathway, that were  
345 significantly dysregulated in MCF10A cells after the direct co-culture (Figure 3b).

346 To determine the changes in the cell signaling in MCF10A cells when the cells were  
347 directly co-cultured with MDA-MB-231 cells, we used the phosphorylation antibody  
348 array to profile the phosphor-protein signaling pathways. Many of the candidate  
349 phosphor-proteins showed differential levels of expression in the directly co-cultured  
350 MCF10A cells. PRAS40, STAT6, ERK1/2 levels were substantially increased while  
351 HSP60,  $\beta$ -catenin, RSK1/2/3, STAT3, and p53 levels were downregulated (Figure 3c).  
352 These results suggest that non-tumorigenic mammary epithelial cells undergo a  
353 significant shift in transcriptomic and proteomic characteristics.

354

### 355 **S100A8/A9 induction in peritumoral mammary epithelial cells and stromal**

## 356 **tissues**

357

358 Among the differentially expressed genes, S100A8 and S100A9 genes were highly  
359 upregulated in directly co-cultured MCF10A cells and their increased expression was  
360 validated in mRNA and protein levels (Figure 3a and 4a). When S100A8 and S100A9  
361 mRNA levels in normal breast and breast cancer tissues were examined using RNA  
362 sequencing data comprised of 65 normal and 68 cancer tissues, both genes were  
363 significantly upregulated in breast cancer tissues when compared to normal breast  
364 tissues (Figure 4b). Furthermore, the mRNA dataset from Finak et al [17] showed that  
365 S100A8 and S100A9 genes were significantly upregulated in the adjacent stroma  
366 tissues in human breast cancer (Figure 4c).

367 We then used mouse mammary carcinoma xenograft model to determine whether the  
368 induction of S100A8/A9 expression by breast cancer cells also occurs *in vivo*. BALB/c  
369 mouse fatpads were injected with 4T1 murine mammary carcinoma cells and were  
370 subsequently harvested. Compared to non-tumor bearing fatpads, mouse mammary  
371 epithelial cells and peritumoral stromal cells showed increased expression of both  
372 S100A8 and S100A9 (Figure 4d and 4e). These findings in addition to the above  
373 transcriptome data indicate that breast cancer cells induce S100A8/9 expression in  
374 tumor microenvironment especially in non-tumorigenic mammary epithelial cells.

375

## 376 **S100A8/A9 upregulation contribute to the phenotypic and molecular changes in** 377 **the co-cultured MCF10A cells**

378

379 To determine the functional importance of S100A8 and S100A9 gene expression in  
380 mammary epithelial cells, we established a stable MCF10A cells that overexpress  
381 S100A8 and S100A9 genes. Transduction of S100A8 overexpression vector resulted in



382 upregulation of both S100A8 and S100A9 proteins (Additional File 12). S100A8-  
383 overexpressing MCF10A cells showed higher rate of cell proliferation when compared  
384 to the control cells (Figure 5a). Furthermore, S100A8-overexpressing MCF10A cells  
385 showed increased cell migration, invasion, colony formation, and 3-dimensional cell  
386 growth which are characteristics of the MCF10A cells directly co-cultured with breast  
387 cancer cells. (Figure 5b-5d).

388 To investigate the effect of S100A8 and S100A9 expression on the signaling pathway  
389 activities, we repeated the phosphorylation antibody array in S100A8-overexpressed  
390 MCF10A cells (Figure 5e-5f). More than half of the signaling proteins observed in  
391 S100A8-overexpressed MCF10A cells showed concordant expression patterns with  
392 directly co-cultured MCF10A cells and only one protein showed opposite expression  
393 pattern between the two cells (Figure 5g). The dysregulation of signaling pathways in  
394 both S100A8 overexpressing MCF10A cells and the MCF10A cells directly co-cultured  
395 with MDA-MB-231 cells were further validated using western blotting as shown in  
396 Figure 5h. Taken together, our data demonstrate that, at least in part, the phenotypic  
397 and molecular transitions seen in the mammary epithelial cells when co-cultured with  
398 breast cancer cells can be explained by the upregulation of S100A8/A9.

399

400

## 401 Discussion

402

403 Normal mammary epithelial cells are closest neighboring cells to malignant epithelial  
 404 cells during the initial process of carcinogenesis. Also, cancer cells often contact  
 405 adjacent normal epithelial cells during the invasion process. The underlying hypothesis  
 406 of our work was that normal epithelial cells adjacent to cancer cells may undergo  
 407 molecular and functional transition despite the normal microscopic appearance. Indeed,  
 408 we observed that non-tumorigenic epithelial cells undergo a substantial molecular and  
 409 phenotypic changes when the cells were directly exposed to the breast cancer cells.  
 410 Non-tumorigenic mammary epithelial MCF10A cells showed significant increase in cell  
 411 proliferation and migration when they were directly co-cultured with breast cancer cells.  
 412 Along with the changes in cell behavior, directly co-cultured MCF10A cells also  
 413 exhibited spindle-shaped morphologies and significant downregulation of epithelial  
 414 adhesion markers such as E-cadherin and Zo-1. Previous studies have shown that E-  
 415 cadherin expression in normal epithelial cells plays an important role during the  
 416 process initial carcinogenesis by regulating cell protrusion formation of neighboring  
 417 transformed cells [12, 18]. Additionally, three-dimensional direct co-culture with breast  
 418 cancer cells induced loss of epithelial differentiation features in non-tumorigenic MDCK  
 419 cells [19]. Finally, Trujillo et al [20] have shown that normal epithelial cells adjacent  
 420 breast tumors show increased expression of EMT markers such as  $\alpha$ -SMA and  
 421 S100A4. Our data, along with these previous reports, suggests that breast cancer cells  
 422 may induce epithelial mesenchymal transition in adjacent normal mammary epithelial  
 423 cells via direct cell-cell contacts.

424 Using the *in vitro* direct co-culture approach, we observed a dynamic and complex cell-  
 425 cell interactions between breast cancer cells and non-tumorigenic mammary epithelial  
 426 cells that include diverse physical contacts and vesicle transfers. We were also able to

demonstrate that the tumor cells and normal epithelial cells exhibit various physical interactions *in vivo* by showing live images taken from the mouse earlobe model. Such dynamic interactions have also been reported in a three-dimensional co-culture experiment [20] and between various stromal cell types in microenvironment [21]. Recently, Roh-Johnson et al [22] have shown that the cytoplasmic transfer during the cell-cell contacts between melanoma cells and macrophages is critical in cancer cell invasion *in vivo*. The clinical and biologic consequences of this cell-cell interactions between malignant cancer cells and adjacent normal epithelial cells are unclear. Normal epithelial cells exert tumor-suppressive effects during the initial phase of carcinogenesis by cell competition and protrusion of neighboring transformed cells [18, 23, 24]. Alternatively, tumor cells can reprogram the adjacent normal epithelial cells to form a pro-tumorigenic microenvironment at the late stages of carcinogenesis [13]. This phenomenon of tumor-driven reprogramming of microenvironment has been shown for other cell types such as fibroblast or adipocytes in breast cancer [5, 6]. Further research is needed to clarify the role of molecular transformation of normal epithelial cells during the breast cancer progression.

In molecular levels, we observed that S100A8/A9 expression levels are significantly up-regulated when non-tumorigenic MCF10A epithelial cells were co-cultured with MDA-MB-231 cells. The up-regulation of S100A8/A9 was also observed in a syngeneic mouse xenograft model of 4T1 murine mammary carcinoma cells. S100A8/A9 act as alarmins that trigger damage-associated molecular patterns molecules and modulate the immune response [25]. S100A8/A9 overexpression in MCF10A cells resulted in a similar cell phenotype seen in directly co-cultured MCF10A cells in terms of *in vitro* cell behaviors and cell signaling activities. Moon A and the colleagues have previously shown that S100A8/A9 overexpression can induce phenotype transformation in MCF10AHRAS cells by . Our results show that S100A8/A9, potentially critical

453 regulators of cell behavior, could be induced in non-tumorigenic mammary epithelial  
 454 cells during the dynamic cell-cell interactions with adjacent breast cancer cells.  
 455 Additionally, the directly co-cultured and S100A8/A9-overexpressing cells both showed  
 456 increased phosphorylation of PRAS40-Thr<sup>246</sup> and its upstream Akt-Ser<sup>473</sup>.  
 457 Phosphorylation of Akt-Ser<sup>473</sup> and PRAS40-Thr<sup>246</sup> activate mTOR signaling that affects  
 458 diverse biologic aspects of the epithelial cells and other cells of tumor  
 459 microenvironment [26-28]. The biologic implications and therapeutic potentials of the  
 460 observed mTOR signaling dysregulation in mammary epithelial cells in breast cancer  
 461 microenvironment should be further explored.

462 Our study has several limitations. First, we have used a limited number of cell lines  
 463 during the present study. The molecular transitions in non-tumorigenic mammary  
 464 epithelial cells might vary along the different subtypes of breast cancers. Second, the  
 465 effect of this phenotype changes in mammary epithelial cells on the breast cancer cells  
 466 in terms of breast cancer growth and metastasis has not been investigated using *in*  
 467 *vivo* breast cancer models. Finally, although we've demonstrated the up-regulation of  
 468 S100A8/A9 expression in mouse fatpad xenograft tumors, we were not able to  
 469 examine the S100A8/A9 up-regulation in human breast cancer tissues. Further studies  
 470 investigating the biologic consequence and clinical implications of the molecular  
 471 changes in the non-tumorigenic mammary epithelial cells in breast cancer  
 472 microenvironment are needed.

473

474

## 475 **Conclusion**

476

477 In conclusion, our study demonstrate that breast cancer cells may induce substantial  
 478 molecular changes in non-tumorigenic mammary epithelial cells via dynamic cell-cell  
 479 interactions. As the results, mammary epithelial cells undergo a phenotype transition  
 480 which involves more active proliferation and migration. S100A8/A9 may play a pivotal  
 481 role during this phenotype transition of mammary epithelial cells. Our study provides  
 482 scientific basis for pursuing a novel therapeutic strategy that targets the non-  
 483 tumorigenic mammary epithelial cells in tumor microenvironments.

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## 488 **Declarations**

489

### 490 *Ethics approval and consent to participate*

491 This study does not include data on the human subjects. For animal experiments, the  
492 fatpad xenograft tumor experiment was approved by the Institutional Animal Care and  
493 Use Committee in Seoul National University Hospital (SNUH-IACUC, 17-0165-S1A0  
494 (1)), and the *in vivo* imaging experiments were performed in accordance with the  
495 Animal Care and Use Committee guidelines of Woosung BSC (Suwon, Korea).

496

### 497 *Consent for publication*

498 This manuscript does not contain any individual person's data in any form (including  
499 individual details, images or videos).

500

### 501 *Availability of data and materials*

502 Data supporting these results are available from the authors upon request.

503

### 504 *Competing interests*

505 The authors declare that they have no competing interests.

506

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514

515 *Authors' contributions*

516 SHJ performed the bulk of the work reported, including writing the initial draft,  
 517 generating cell lines, and phospho-protein arrays. WHH performed fatpad tumor  
 518 xenograft studies, PCR, western blotting and writing the initial draft. BSH, JHK  
 519 performed YP and DSL assisted and provide guidance to cell sorting and animal  
 520 experiments. NHK and MCP performed in vivo cell imaging studies. ESL, HBL, and WH  
 521 analyzed the transcriptome data and pathway analysis. JC and JIK provided RNA  
 522 sequencing data of breast cancer and analyzed the target gene expression levels.  
 523 DYN and HGM initiated the study, provided overall direction and oversight to the  
 524 project, and finalized the manuscript. All authors read and approved the final  
 525 manuscript.

526

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## 623 **Figure legends**

624

### 625 **Figure 1. Dynamic cell-cell interactions between breast cancer cells and non-** 626 **tumorigenic mammary epithelial cells.**

627

628 Representative images of cell-cell interactions between RFP-transfected MDA-MB-231  
629 breast cancer cells and GFP-transfected MCF10A non-tumorigenic mammary epithelial  
630 cells were captured using time-lapse confocal microscopy (a-d). **a** MDA-MB-231 cells  
631 use lamellipodia-like structures to contacting MCF10A cells (yellow arrow) or distant  
632 MCF10A cells (white arrow). **b** Nanotube-like structures extending from MDA-MB-231  
633 cells are seen (white arrows). **c** RFP-expressing vesicles from MDA-MB-231 cells are  
634 transferred to MCF10A cells (white arrows). **d-e** The area within the white rectangle in  
635 Figure 1d is shown in 1e. Transferred vesicles from MDA-MB-231 cells are located in  
636 both nucleus (white arrow) and cytoplasm (yellow arrow) of MCF10A cells. **f** A minority  
637 of co-cultured cells show dual fluorescence. **g** The representative images of the *in vivo*  
638 behavior of MDA-MB-231 cells and MCF10A cells co-injected in the earlobe of mouse  
639 are shown with migrating extracellular vesicles (white arrows).

640

### 641 **Figure 2. Phenotype transition of non-tumorigenic mammary epithelial cells** 642 **when directly co-cultured with breast cancer cells.**

643

644 **a** MCF10A cells were directly co-cultured with MDA-MB-231, SK-BR3, and MCF7  
645 breast cancer cells. After sorting out the MCF10A cells, the cellular morphologies are  
646 shown. **b** The expression levels of epithelial-mesenchymal transition markers were  
647 measured by PCR in MCF10A cells after co-culture with MDA-MB-231 cells (upper)  
648 and quantified (lower). **c** Relative growth rate of co-cultured MCF10A cells was

649 measured based on ATP level with CellTiter-Glo reagent. **d** The representative images  
650 and quantified results of colony-formation assay for co-cultured MCF10A cells are  
651 shown. **e** The representative images and quantified results of transwell migration assay  
652 for co-cultured MCF10A cells are shown. **f** The representative images and quantified  
653 results of 3-dimensional Matrigel culture assay for co-cultured MCF10A cells are shown.  
654 Error bars denote mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. P values are  
655 determined by the Mann-Whitney test.

656

657 **Figure 3. Transcriptomic and proteomic profiles of non-tumorigenic mammary**  
658 **epithelial cells co-cultured with breast cancer cells.**

659

660 **a** Heatmap shows the top 60 genes that were up- or down-regulated in MCF10A cells  
661 co-cultured with MDA-MB-231 cells. **b** Heatmap shows the significant enrichment of  
662 KEGG pathways based on the RNA sequencing data. Pathways significantly  
663 dysregulated in both datasets are listed on the right side. **c** Results of phosphor-protein  
664 array experiments are shown. Spots with more than two-fold changes are marked with  
665 numbered rectangles and listed below (upper). The overall quantification results of  
666 phospho-protein array are shown (lower).

667

668 **Figure 4. S100A8/A9 up-regulation in breast cancer tissues and**  
669 **microenvironments.**

670

671 **a** S100A8/A9 mRNA levels and protein levels in MCF10A cells co-cultured with MDA-  
672 MB-231 cells are shown. **b** The S100A8/A9 mRNA levels extracted from human breast  
673 cancer RNA sequencing data are shown. **c** S100A8/A9 mRNA levels in breast cancer  
674 stromal tissues are shown (Finak et al. GSE9014). **d** Mouse mammary fatpad tissues

675 bearing 4T1 breast cancer cells were stained against S100A8 and S100A9. Lower  
676 panels show magnified images of the upper insets. **e** Non-tumor-bearing mouse fatpad  
677 were stained with S100A8 and S100A9. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. P values are  
678 determined by the unpaired Student t-test.

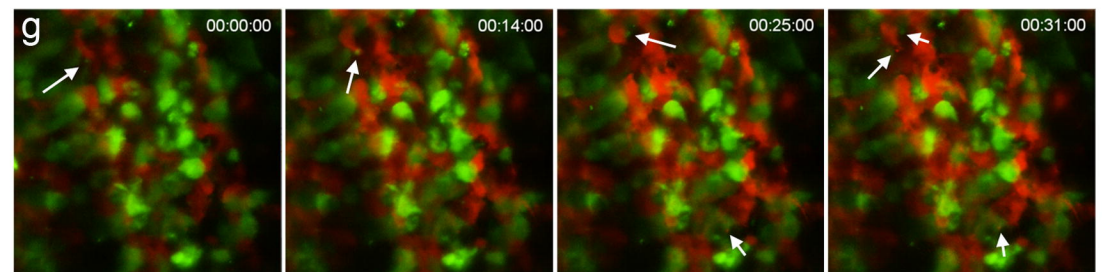
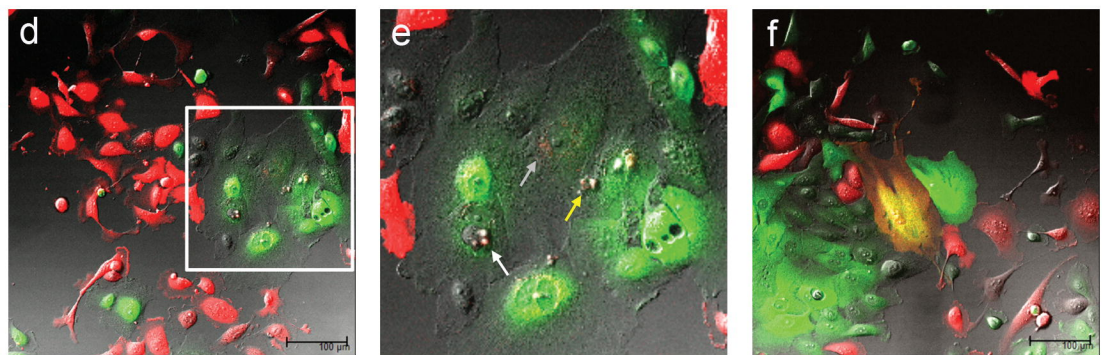
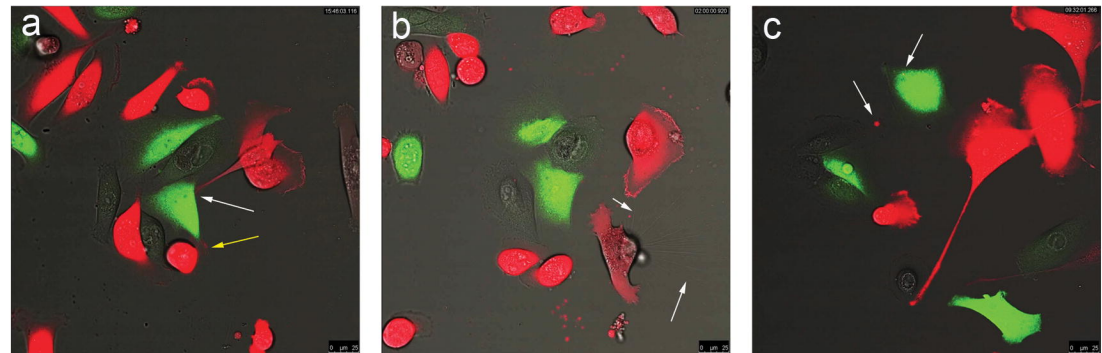
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680 **Figure 5. S100A8/A9-overexpression induce the phenotypes of non-tumorigenic**  
681 **mammary epithelial cells co-cultured with breast cancer cells.**

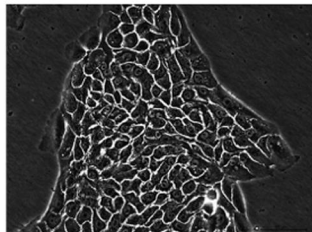
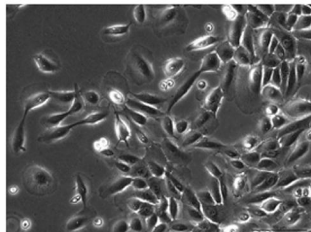
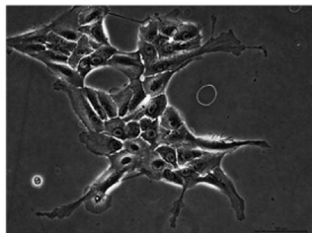
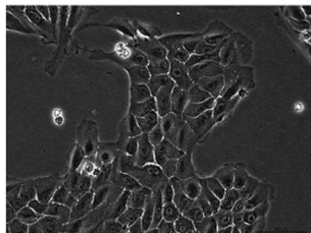
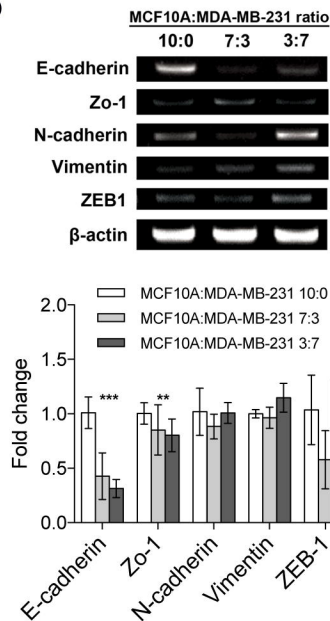
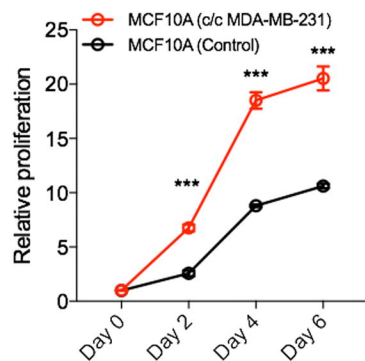
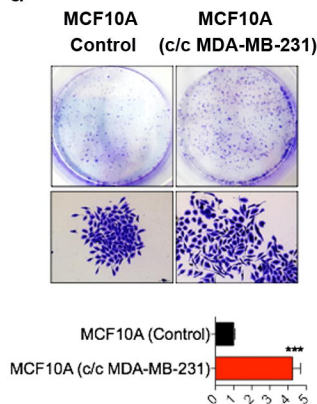
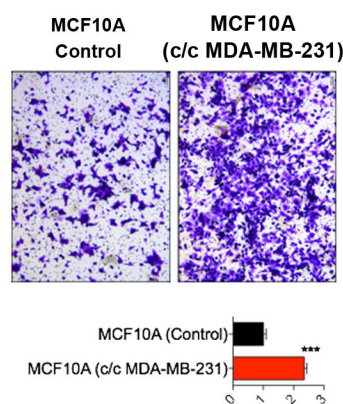
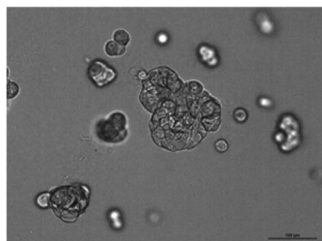
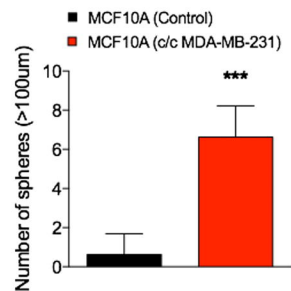
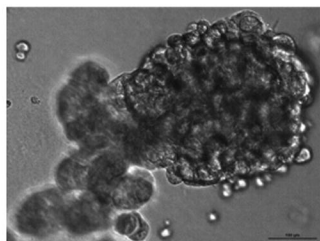
682

683 **a** Relative growth rate of S100A8/A9-overexpressing MCF10A cells was determined  
684 based on ATP level with CellTiter-Glo reagent. **b** The representative images and  
685 quantified results of transwell migration assay for S100A8/A9-overexpressing MCF10A  
686 cells are shown. **c** The representative images and quantified results of Matrigel  
687 invasion assay for S100A8/A9-overexpressing MCF10A cells are shown. **d** The  
688 representative images and quantified results of colony-formation assay for S100A8/A9-  
689 overexpressing MCF10A cells are shown. **e-f** Results of phosphor-protein array  
690 experiments with S100A8/A9-overexpressing MCF10A cells (e) and quantified  
691 expression of significantly dysregulated proteins (f) are shown. **g** The comparison of  
692 phospho-protein array patterns for co-cultured MCF10A cells and S100A8/9-  
693 overexpressing MCF10A cells are shown. For example, 'Down/Up' designates spots  
694 that are down-regulated in co-cultured MCF10A cells and up-regulated in S100A8/A9-  
695 overexpressing MCF10A cells, respectively. **h** Western blotting results showing the  
696 protein expression levels of selected dysregulated proteins in both co-cultured  
697 MCF10A cells and S100A8/A9-overexpressing MCF10A cells. Error bars denote mean  
698  $\pm$  SD. \*\*P < 0.01, \*\*\*P < 0.001. P values are determined by the Mann-Whitney test.

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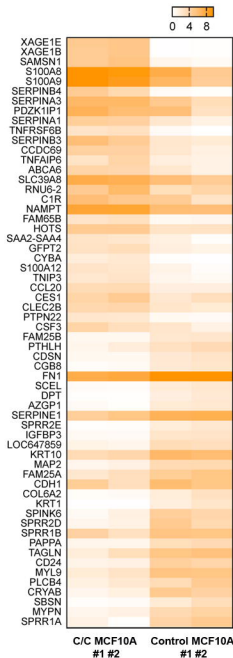




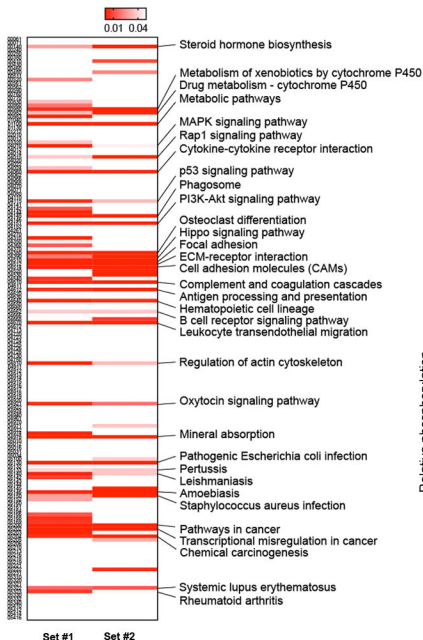
**a****MCF10A (Control)****MCF10A (c/c MDA-MB-231)****MCF10A (c/c SK-BR3)****MCF10A (c/c MCF7)****b****c****d****e****f****MCF10A (control)****MCF10A (c/c MDA-MB-231)**



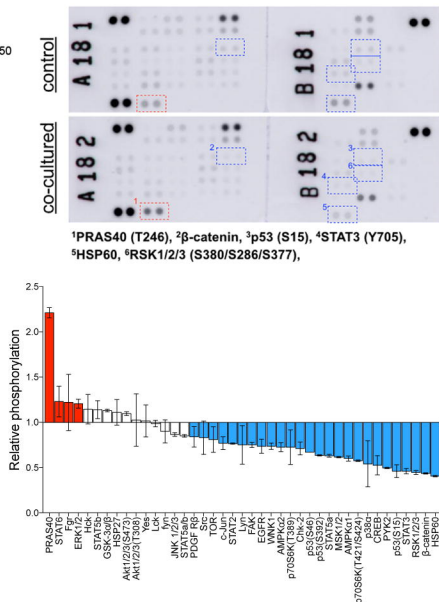
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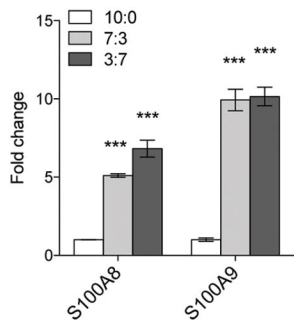
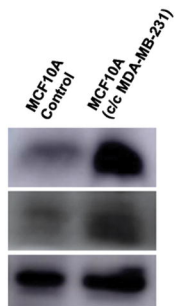
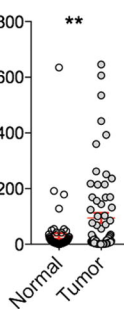
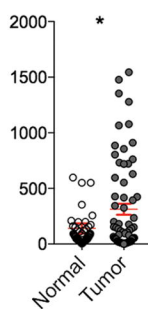
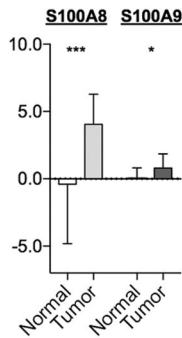
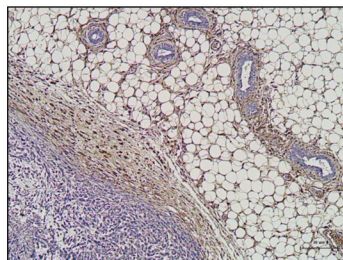
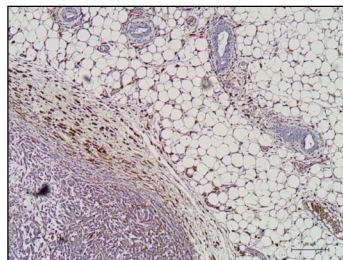
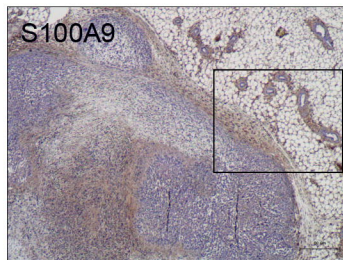
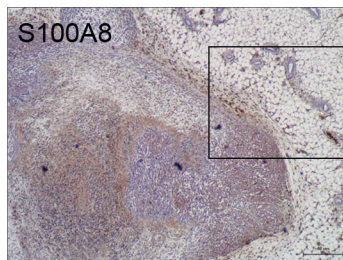


b



c



**a****b****S100A8****S100A9****c****d****e**