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Differences in transcriptional effects of 1α,25 dihydroxyvitamin D3 on fibroblasts associated to breast carcinomas and from paired normal breast tissues

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ABSTRACT

The effects of 1α,25 dihydroxyvitamin D3 (1,25D) on breast carcinoma associated fibroblasts (CAFs) are still unknown. This study aimed to identify genes whose expression was altered after 1,25D treatment in CAFs and matched adjacent normal mammary associated fibroblasts (NAFs). CAFs and NAFs (from 5 patients) were cultured with or without (control) 1,25D 100 nM. Both CAF and NAF expressed vitamin D receptor (VDR) and 1,25D induction of the genomic pathway was detected through up-regulation of the target gene CYP24A1. Microarray analysis showed that despite presenting 50% of overlapping genes, CAFs and NAFs exhibited distinct transcriptional profiles after 1,25D treatment (FDR < 0.05). Functional analysis revealed that in CAFs, genes associated with proliferation (*NRG1*, *WNT5A*, *PDGFC*) were down regulated and those involved in immune modulation (*NFKBIA*, *TREM-1*) were up regulated, consistent with anti tumor activities of 1,25D in breast cancer. In NAFs, a distinct subset of genes was induced by 1,25D, involved in anti apoptosis, detoxification, antibacterial defense system and protection against oxidative stress, which may limit carcinogenesis. Co-expression network and interactome analysis of genes commonly regulated by 1,25D in NAFs and CAFs revealed differences in their co-expression values, suggesting that 1,25D effects in NAFs are distinct from those triggered in CAFs.

1. Introduction

Breast carcinomas are complex tissues in which epithelial cells interact with their surrounding stromal compartment, which consists of extracellular matrix (ECM) molecules, resident fibroblasts, inflammatory, immune and endothelial cells [1]. Cancer associated fibroblasts (CAFs) are the most abundant cell type in breast tumor stroma. CAFs possess an activated phenotype and α-smooth muscle actin (αSMA) is the most commonly used marker for identifying cancer-associated fibroblasts but the tumor microenvironment also contains αSMA negative fibroblasts. CAFs are also characterized by increased expression of S100A4, that marks a population of fibroblasts distinct from those αSMA positive [2]. CAFs secrete growth and angiogenic factors as well as a range of chemokines and ECM proteins [3]. They differ from normal fibroblasts by displaying a distinct gene expression pattern [4]. Importantly, tumor promoting properties of CAFs appear to be partially independent of the presence of tumor cells and are maintained in vitro even in the absence of the epithelial cells [5]. In addition, interactions between breast cancer associated fibroblasts and mammary epithelial cells result in alterations in the transcriptional profiles of both cell types [6].

Numerous studies have established that 1α,25 dihydroxyvitamin D3 (1,25D) modulates cell cycle, progression, differentiation and apoptosis in breast carcinomas via interaction with the vitamin D receptor (VDR), but the major focus has been the epithelial compartment (reviewed in [7]). Although various studies of gene profiling have been conducted to elucidate the molecular mechanisms underlying vitamin D3 effects in breast carcinoma epithelial cells [8], there are few studies addressing the effects of 1,25D in stromal cells. Response of fibroblasts to the hormone has been carried out in mesenchymal multipotent cells in culture [9–11], lung fibroblasts [12] and human primary prostatic stromal cells [13,14].

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In the current study we have established primary fibroblast cultures isolated from breast carcinomas (CAFs) and paired adjacent normal tissue (normal tissue associated fibroblasts, NAFs) and used microarrays to investigate the differential effects of 1,25D on gene expression profiles of each group of fibroblasts. Identification of 1,25D target genes in these cells may provide a starting point for a better understanding of the molecular mechanisms involved in the 1,25D effects on breast cancer microenvironment.

2. Patients, materials and methods

2.1. Patients

Breast tissue specimens were obtained from five patients undergoing surgery for breast carcinoma. Mean age of the patients was 53.3 ± 10.7 years, all were post-menopausal. Patients were diagnosed with invasive ductal carcinoma (IDC) confirmed histopathologically, clinically staged as II or III. Tumors were classified as ER and PR negative, HER-2 positive. None of the patients had received preoperative chemotherapy. All patients were operated on Instituto Brasileiro de Controle do Câncer, IBCC, São Paulo, Brazil. This study was approved by the Institutional Ethics Committee and written informed consent was obtained from all participants, after full explanation of the purpose and nature of all procedures used.

2.2. Tissue samples and primary cell culture

Fibroblasts associated to human breast carcinoma were obtained from tissue samples from patients diagnosed with primary ductal invasive breast cancer tumors (CAF) or isolated from normal areas of the same carcinomas (NAF). H&E-stained, frozen histological sections were prepared from each tissue sample to confirm malignancy or morphologically normal epithelial tissue. After adipose tissue removal, tissue was minced (1–4 mm^3) into pieces in PBS (Na_2HPO_4 10 mM, NaCl 1.37 mM, KCl 27 mM, KH_2PO_4 2 mM; Life Technologies, Grand Island, NY, USA), under sterile conditions. A total of 10–15 fragments were obtained for each group of fibroblasts and transferred to 25 cm^2 culture flasks and covered with Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), 20% FBS (Life Technologies), 100 μg/ml ampicillin, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Outgrowth of cells was recorded after 10–20 days and medium was renewed once or twice a week thereafter. After fibroblast migration, tissue fragments were removed and cells were passaged by mild trypsinization with trypsin 0.5% (Life Technologies), and fibroblastoid cells were seeded into new culture flasks containing the same medium. After three cell passages, morphologically homogeneous cultures containing only fibroblastoid cells were obtained. At this stage no macrophages were left. Absence of macrophages and endothelial cells was confirmed using human anti-CD31 and CD163 [6,15]. All CAFs and NAFs used were from passages 4 to 6.

2.3. Characterization of fibroblasts

Primary cultures of breast cancer associated fibroblasts and normal adjacent fibroblasts were characterized by immunofluorescence. Briefly, cells in early passages (passage 3) were plated in circular slides (Ø13 mm, Glasscyto, Bioslide Technology, Walnut, CA, USA) and incubated with human anti-vimentin (clone Vim 3b4), human anti-smooth muscle actin (clone M0635), human anti-S100A4 (clone A5114), human anti-pancytokeratin (clones AE1/AE3) and human anti-CD31 (clone JC70A), all antibodies from Dako Corporation (Carpinteria, CA, USA) and human anti-CD163 (clone 10D6) from Vector Laboratories (Burlingame, CA, USA). After that, cells were incubated with the secondary antibody (Alexa Fluor 488 anti-rabbit or mouse IgG (Life Technologies) diluted in PBS. Immunofluorescence was also performed to verify the presence of vitamin D receptors. Anti-VDR (clone 9A7, Affinity Bioreagents, Rockford, IL, USA) was used as primary antibody and FITC-conjugated anti-mouse IgG (Sigma–Aldrich, St. Louis, MO, USA) as second antibody. The cell analysis was performed using a Zeiss Axioplan microscope (Carl Zeiss; Jena, Thüringen, Germany).

2.4. Cell treatment

CAFs and NAFs were plated in 75 cm^2 culture flasks (5×10^4 cells per flask, TPP – Techno Plastic Products AG, Trasadigen, Switzerland) and grown until cells reached 70% confluence. At this point, cells were treated with DMEM containing 0.5 or 100 nM 1,25D (cat. no. 679101, Merck, Darmstadt, Germany) or vehicle (0.1% ethanol) for 24 h.

2.5. Microarray analysis

Total RNA was isolated and purified using TRIzol® reagent (Life Technologies) and RNeasy Mini Kit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturers' protocols. RNA integrity was verified in a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and only samples with RNA integrity number >7 were considered. Beginning with 5 μg total RNA, a one-round linear amplification was carried out according to an Affymetrix protocol (One Cycle Target Labeling Kit, Affymetrix, Santa Clara, USA). Afterwards, biotin-labeled cRNA was reverse transcribed using IVT labeling kit (Affymetrix) and 15 μg of biotinylated fragmented aRNA was hybridized onto the Human Genome U133 Plus 2.0 GeneChip. The transcript levels in each sample were determined by using the Affymetrix HU133 plus 2.0 GeneChip (Affymetrix, 54,210 probe sets covering over 47,000 transcripts and splice variants). Hybridized GeneChips were scanned using Affymetrix GeneChip Scanner 3000 and after visual inspection, raw data were saved into CEL files.

The quality of microarray images from individual GeneChips and the expression distribution at the probe set level between GeneChips were inspected using Affymetrix GeneChip Operating Software (GCOS). All of the GeneChips presented high quality and were used for subsequent analysis.

Background correction, normalization and summarization of raw data (CEL files) were performed using the Robust Multi-Array Average (RMA) method available on R package (http://www.R-project.org), with the "Affy" library of Bioconductor Package (http://www.bioconductor.org).

2.6. Statistical analysis of microarray data

First of all, filtering was set to select 30% of genes with the highest standard deviation. Comparisons of expression levels were performed using MeV (MultiExperiment Viewer, version 4.5.1) software. Differentially expressed transcripts after 1,25D treatment (control vs 1,25D treated) were identified using the Significance Analysis of Microarray program (SAM, version 3.02). Data for each comparison were analyzed using a two class, paired analysis with 1000 permutations. For each transcript SAM uses permutation of the data to identify a False Discovery Rate (FDR) that balances type I and type II statistical error rates. Significance for differential expression due to 1,25D treatment was determined at the 5% FDR. To identify and visualize biological processes that were enriched due to $1,25(OH)_2D$ treatment, differentially expressed genes were subjected to subsequent Gene Ontology (GO) analysis using ToppGene software (http://toppgene.cchmc.org/).

2.7. Quantitative real time RT-PCR

Reverse transcription was performed using 3 μg of total RNA, Oligo $dT_{(12-18)}$ and Superscript™ III Reverse Transcriptase (Life Technologies). Primers were designed for different exons to avoid amplification of genomic DNA using Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA).

Quantitative RT-PCRs were performed using the ABI Prism™ 7500 Sequence Detection System. RT-PCRs were carried out using SYBR® Green PCR Power MasterMix using the following program: 10 min at 95 °C for the initial denaturing, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The efficiency of each pair of primers was calculated using standard curve dilutions (as described in the Life Technologies protocols). The duplicate average values were used for quantification and the relative expression of genes of interest was normalized to that of *GAPDH*. Gene expression in each treated sample was then compared with expression in fibroblasts from control samples. Relative gene expression between sample groups was calculated employing the efficiency-corrected equation. We generated changes in gene expression values of each treated sample by comparing it with the respective control sample. Statistical analyses were carried out using *t*-test.

2.8. Co-expression network

We searched for pairs of genes whose co-expression had changed and the network was constructed based on all possible combinations starting from the 69 commonly altered genes by 1,25D. Pearson correlation coefficient (PCC) between pairs of genes was calculated independently for CAF and NAF [16]. Differences between the correlations in the CAF and NAF groups were ranked for gene pairs. Pairs with absolute differences greater than 1.5 were selected. The idea was to select pairs of genes that have changed their co-expression between CAFs and NAFs. Data analysis and visualization were conducted by Cytoscape software (version 2.8.0; www.cytoscape.org).

2.9. Network construction

Interaction network analysis was carried out for the 69 genes commonly altered in both normal and tumor associated fibroblasts after 1,25D treatment by querying three human interactome databases (HPRD, MINT and IntAct) [17–19]. We overlaid the expression values of each gene on its corresponding protein and we constructed a protein–protein interaction network with those genes that were mapped in the databases and their direct partners. To identify broker and bridge genes in network, a previously published algorithm [20] was implemented using the site http://bioinfo.lbhc.hcancer.org.br/interactomegraph/, a free web tool to improve the interactome database analysis. Brokers are genes that connect many proteins that would not be connected otherwise. Bridges are nodes that have few links but connect communities, i.e., very connected groups. We considered as bridge genes the 2% ones with higher bridging centrality values and the 2% ones with higher brokering values were considered broker genes. We used the top 2% more connected genes to select the hubs.

After construction of the interactome using as our "seed" the aforementioned 69 genes, we searched for pairs of connected genes present in the microarray. Using the expression values of the microarray, the Pearson correlation coefficient (PCC) between pairs of genes was calculated independently for CAFs and NAFs [16]. Differences between the correlations in the CAF and NAF groups were ranked for gene pairs. We selected ranked pairs with difference greater then 1.5.

3. Results

At first, to characterize the primary culture cell population, immunofluorescence assays to recognize specific cell antigens were performed using cell culture at passage 4. Cell monolayers were negative for pan-cytokeratin, CD31 (Fig. 1A) and CD163 (data not shown) and stained uniformly for vimentin (Fig. 1B) confirming the stromal origin of cells and absence of contaminating endothelial, epithelial or macrophage cells. Expression of α-SMA was detected in both CAFs and NAFs, however, with some variability among individual donors and the percentage of positive cells was generally higher in CAFs, which often take on the appearance of an activated or myofibroblastic phenotype (Fig. 1A). The α-SMA expression by NAFs may arise during cell culture conditions as previously suggested [21 and references therein]. S100A4, which marks a distinct population of fibroblasts than α-SMA positive fibroblasts [2], was detected in CAFs as well as in NAFs, but with a higher proportion of positive cells in CAFs (Fig. 1A).

We next determined the presence of vitamin D receptor in CAFs and NAFs. Immunofluorescence analysis through confocal microscopy revealed the presence of VDR in all five CAF population, with predominant localization in the nucleus rather than in the cytoplasm. Similar results were found in NAFs (Fig. 1C).

The starting point of this work was the comparison of gene expression patterns from CAF-untreated controls and NAF-untreated controls, as there are reports indicating differences between them [22]. Supplementary Table 1 summarizes the expression differences, assuming a 1.4-fold difference cutoff between CAFs and matched NAFs (CAF/NAF ratio). Gene ontology categories (biological process) enriched in genes more expressed in CAFs were cell cycle, protein transport, apoptosis, cell proliferation and carbohydrate metabolic processes and in NAFs were immune response, lipid metabolic processes, cell differentiation, cell adhesion and cell proliferation (Supplementary Fig. 1).

We next investigated the ability of 1,25D to differentially regulate gene expression of CAFs and NAFs, and for this analysis two hormone concentrations were used, 0.5 nM, which can be safely attained in vivo [23] and 100 nM, a supra physiological concentration, commonly utilized in experiments in vitro, to investigate global gene expression in cancer epithelial or mesenchymal cell lines [7,11].

In CAFs, 1,25D 0.5 nM responsiveness was modest, but it was detected as an induction of the hormone target CYP24A1, whose expression increased 6.7-fold at 24 h as compared to counterpart controls in the absence of 1,25D. Another 274 genes were induced, with fold changes ranging from 1.13 to 2.46, including 11 genes, whose expression was further analyzed by RT-PCR, in a technical validation assay. In general there was qualitative agreement between the two techniques with all genes showing similar trends, including two (TGFβ2 and CYP24) which were confirmed as more expressed in 1,25D treated fibroblasts. A possible explanation for the lack of statistical significance for the other PCR determinations might be the small fold changes in gene expression (data not shown).

We then decided to use a pharmacological concentration (100 nM), commonly used to investigate 1,25D effects in cell culture assays. Adopting a 5% FDR cut-off we identified 126 and 123 genes differentially expressed in NAFs and CAFs 1,25D treated, respectively, as compared with their untreated controls. A Venn diagram detected 69 overlapped genes regulated by 1,25D 100 nM in both CAFs and NAFs, among which, only 3 were down regulated (Fig. 2, Supplementary Tables 2–4). Fifty four genes were exclusively

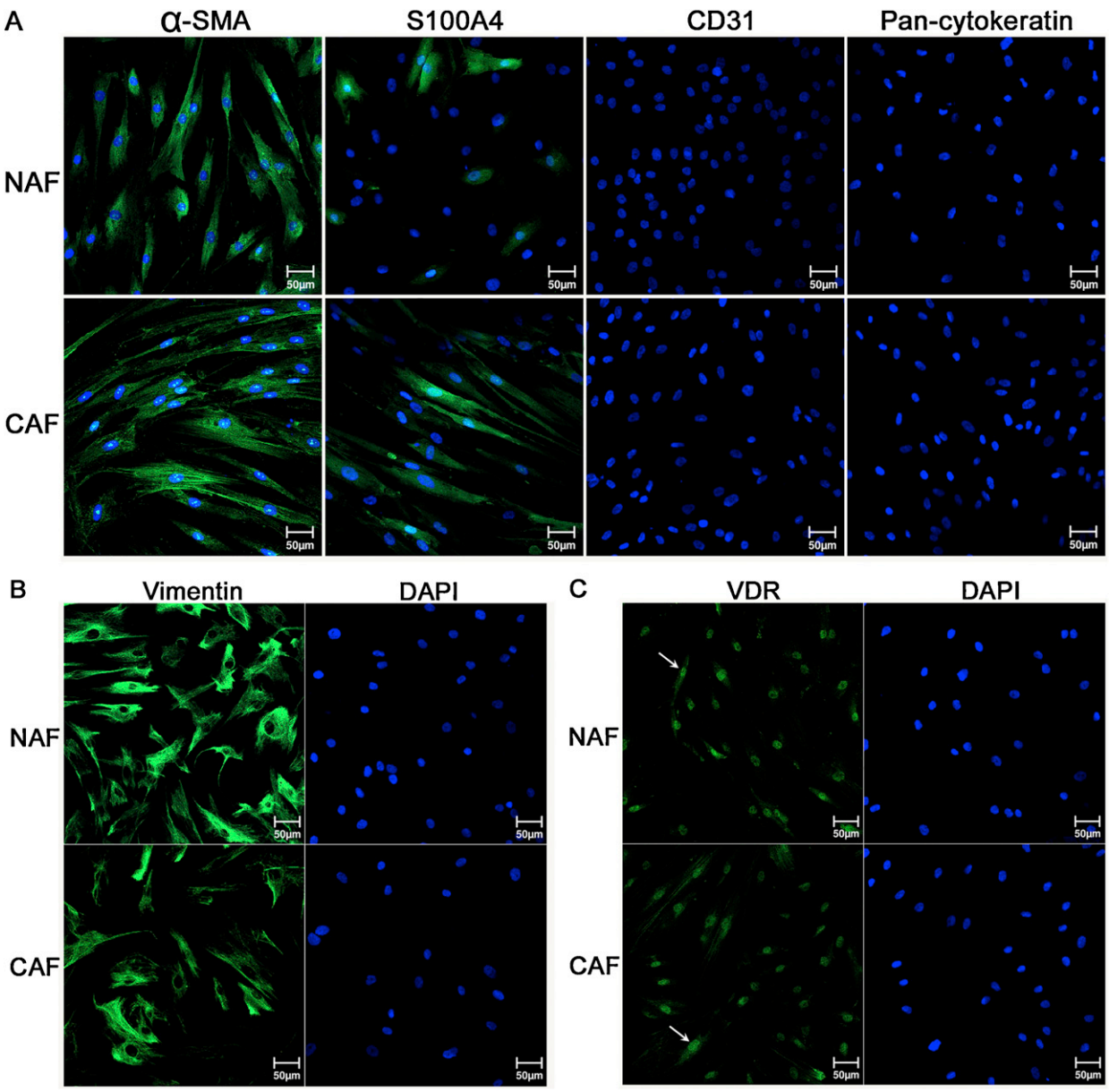

Fig. 1. Characterization of fibroblast primary culture isolated from breast carcinoma tissue sample (CAF) and from paired normal adjacent breast tissue (NAF). Immunofluorescence analysis followed by confocal microscopy in representative cases (A) showed uniformly positive staining for α-SMA and negative staining for pan-cytokeratin and CD31, while S100A4 positive stained cell frequency was higher in CAFs than in NAFs. (B) Vimentin expression was restricted to cytoplasm and (C) VDR expression in both cells was more concentrated in the nucleus than in the cytoplasm (arrows indicate in one representative cell VDR expression in nuclei and cytoplasm): DAPI was used as nuclear marker (blue fluorescence) in all experiments. Scale bar: 50 μm.

modulated in CAFs (but not in NAFs): 27 up- and 27 down-regulated. Specifically in NAFs (but not in CAFs) 1,25D3 treatment up regulated 55 and down regulated other two genes.

Among genes specifically regulated in CAFs, proliferation/apoptosis related processes were over represented. In these fibroblasts, out of 27 genes down regulated by 1,25D, several were related to this particular function (*ADK*, *SOX9*, *WNT5A*, *PDGFC*, *NRG1*, *F2R*) except for *NFKappaBIA* (an inhibitor of NF-KappaB), which was up-regulated. Another functional categories enriched after 1,25D treatment included genes associated either with response to wounding, like *TREM1*, *WNT5A*, *F2RL2* (*PAR*-3), *F2R* (*PAR*-1), or with extracellular matrix and intracellular transport (Table 1).

In NAFs, genes exclusively modulated by 1,25D were involved in multiple cellular processes. Several up-regulated genes were involved in negative regulation of proliferation or in anti-apoptosis (out of 11 genes, 7 were anti-apoptotic) such as *VEGFA*, *TCF7L2*, *GCLC*, *IL7R1*, *NCAM1*, *SERPINB9* and *CNKSR2*. A number of up regulated genes fall in the category of immune response (*TLR4*, *PRDM1*, *IL7R*, *PEL12*, *LRIG1*, *BMP6*). However, genes associated with other biologic processes such as transmembrane transport and regulation of transport (*KCNK3*, *SLC4A7*, *RAB8B1*, *ATP2B1*), xenobiotic metabolic process (*GLYAT*, *GCLC* and *CYP3A5*), and ATP catabolic process (*ABCB4*, *ABCA6*, *ABCA8*) were also up regulated by 1,25D in NAFs (Table 2).

Among overlapping genes altered by 1,25D in CAFs as well in NAFs ($n = 69$), several functional groups were identified: genes with roles in proliferation and apoptosis, immune response and inflammation, lipid metabolism, ras family, cell adhesion and regulation of cell transduction were the most represented. The range of fold regulation of gene expression varied from 1.71 to 5.54, with small differences in fold change values between NAFs and CAFs, exception

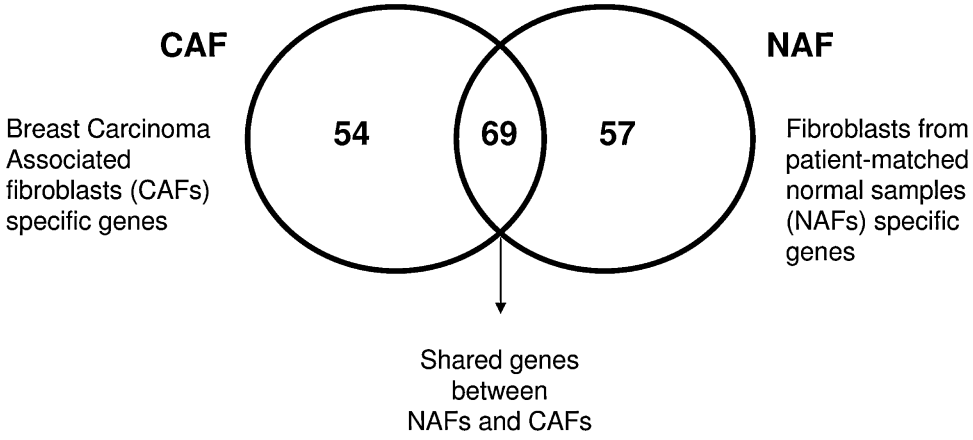

Fig. 2. Venn diagram showing the number of genes differentially expressed in CAFs and NAFs after 1,25D treatment. Using microarray analysis we determinate genes differentially expressed in CAFs and NAFs modulated by 1,25D 100 nM vs controls. Among these genes, 69 were commonly regulated by 1,25D in both CAFs and NAFs.

made for *CYP24A1*, which exhibited by far the highest up-regulation of all (516-fold in CAFs and 299-fold in NAFs) (Table 3).

Although the gene expression profiles varied considerably when comparing control CAFs and NAFs (Supplementary Table 1), this set of genes did not overlap significantly with the set of differentially expressed genes following 1,25D treatment, except for a small number of genes. However, comparing the functional categories of these two gene sets we could observe that some similar functions were present in both of them (Supplementary Fig. 1 and Tables 1–3).

Comparing 1,25D transcriptional effects specifically in NAFs and genes differentially expressed in NAFs vs CAFs, only 5 genes were found, most of them ($n = 4$) more expressed in NAFs and up-regulated by the hormone: ACSL3, ATP2B1, FHOD3, VEGFA, and one, less expressed in NAFs (vs CAFs) and up-regulated by 1,25D.

Among overlapping genes regulated by1,25D in CAFs and genes differentially expressed between CAFs vs NAFs the scenario was more complex: 7 genes more expressed in CAFs (than NAFs) were down-regulated by 1,25D in CAFs (ADK, AMIGO2, CCDC99, E2F7, HAS2, MYBL1, PDGFC); 5 genes less expressed in CAFs (vs NAF) were up-regulated by 1,25D in CAFs (CCDC85B, CSF1, MAN1C1, MTSS1, SULF2). Another two genes were regulated by 1,25D in CAFs: GALNT12 (less expressed in CAFs and down regulated by 1,25D) and SOD2 (more expressed in CAFs and up-regulated by 1,25D). In addition, six genes were up-regulated by 1,25D in both CAFs and NAFs, all of them considered less expressed in CAFs (vs NAFs): MALL, NLRP1, OSR1, OSR2, TGFB2, TMEM119. Hence, from 20 genes differentially expressed in CAFs (vs NAFs) and modulated by 1,25D, 18 were regulated by the hormone toward a closer similarity with NAFs. An example is HAS2 (hyaluronan synthase 2) which plays a critical role in the development of a pro metastatic microenvironment [24]. HAS2 expression was 6.97-fold higher in CAFs (vs NAFs) and was reduced in CAFs after 1,25D treatment. On the other hand, IL8 which was more expressed in CAFs than in NAFs presenting a fold of 37.4 as compared to the values found in NAFs (Supplementary Table 1) did not appear as differentially expressed in the list of CAFs treated with 1,25D vs respective in treated control. Our interpretation of the above comparison is that the expression levels of the genes differentially expressed between control CAFs and NAFs may be differentially affected after 1,25D treatment but the resulting expression levels did not reach significant differences. Contrariwise, other genes whose basal levels did not significantly differ between the two control cell types have been changed greatly after 1,25D treatment.

Nineteen genes (with the highest fold changes induced by 1,25D) were selected for technical validation assays through quantitative real time RT-PCR. These experiments confirmed 84% of the gene expression changes were induced in CAFs or NAFs by 1,25D. The results are visualized in Supplementary Table 5 and indicate that 1,25D modulates the expression of genes involved in diverse biological processes, such as metabolism (*CYP24A1*, *CYP3A5*, *IDH2*, *GJA1*), steroidal metabolism (*SULT1C2*), cell proliferation, cell cycle and transduction signals (*G0S2*, *IGF1*, *MAPK13*, *FGF9*, *NRG1*, *TGFB2*) as well as inflammatory and immune responses (*CD14*, *CSF2RB*, *IL7R*, *TLR4*) and *ATP* catabolic process (*ABCB4*).

As a biological validation procedure we compared 1,25D effects shared by fibroblasts (CAFs and NAFs) with breast cancer fresh tissue slices exposed in vitro to the hormone. All samples were from post-menopausal patients with invasive ductal carcinoma (2 of them ER positive and 2 HER-2 positive) and tumor slices were cultured in the presence or absence of 1,25D 100 nM for 24 h. We have used this tissue model before and shown that VDR is present and that the genomic pathway is active, detected as CYP24A1 induction [25]. Comparing 1,25D effects in CAFs and NAFs with breast cancer slices we identified 24 genes commonly regulated, 22 of them up-regulated by 1,25D. Genes up-regulated in both CAFs and NAFs and breast cancer tissue slices included: APBB1IP, CD14, CILP, CLMN, CYP24A1, DPP4, EFTUD1, FAM20C, FOXF1, G0S2, GRK5, IL1RL1, RGNEF, SERPINB1, SLC1A1, THBD [26].

To better investigate findings related to the genes commonly altered by 1,25D in both NAFs and CAFs, we decided to use two approaches.

We first constructed a gene–gene network (co-expression network) using the values of Pearson correlation between pairs of genes, based on all possible combinations of the 69 commonly altered (in both CAFs and NAFs) genes by 1,25D. Differences between the correlations in either NAF or CAF groups were ranked for gene pairs. Pairs with high absolute differences indicated that the two correlations were in opposite sides of zero and closer to 1 and −1 or vice versa respectively. By restricting the analysis to those with absolute differences greater than 1.5 we selected a group of 38 pairs of genes (Supplementary Table 6). It is important to note we found changes in the correlations of the expression levels of genes between NAF and CAF groups.

Using the dynamic structure of the human protein interaction network (interactome) it has recently been shown that genes that are not differently expressed may indicate relevant differences between tumor samples and controls [27].

Using the set of genes commonly differentially expressed in 1,25D treated CAFs and NAFs as compared with respective controls ($n = 69$) we next searched for interactions among those genes and their neighbors in the human interactome [28] (Fig. 3). To explore

Table 1
Genes with expression exclusively regulated in CAFs by 1,25D.

Gene symbol	Gene description	Fold change
Cell proliferation		
SOD2[a]	Superoxide dismutase 2, mitochondrial	9.52
FGF9	Fibroblast growth factor 9 (glia-activating factor)	2.77
EGFR	Epidermal growth factor receptor	1.96
CSF1	Colony stimulating factor 1 (macrophage)	1.84
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.82
PTCH1	Patched 1	1.61
PDGFC[a]	Platelet derived growth factor C	−1.91
WNT5A[a]	Wingless-type MMTV integration site family, member 5A	−2.22
SOX9	SRY (sex determining region Y)-box 9	−2.23
E2F7	E2F transcription factor 7	−2.33
ADK	Adenosine kinase	−2.35
F2R	Coagulation factor II (thrombin) receptor	−2.80
NRG1	Neuregulin 1	−3.23
MAPK cascade		
FGF9	Fibroblast growth factor 9 (glia-activating factor)	2.77
EGFR	Epidermal growth factor receptor	1.96
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.22
OXTR	Oxytocin receptor	−2.36
RGS4	Regulator of G-protein signaling 4	−2.38
F2R	Coagulation factor II (thrombin) receptor	−2.80
NRG1	Neuregulin 1	−3.23
Apoptosis		
SOD2[b]	Superoxide dismutase 2, mitochondrial	9.52
EGFR	Epidermal growth factor receptor	1.96
DUSP1[b]	Dual specificity phosphatase 1	1.84
NFKBIA[b]	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.82
TWIST2[b]	Twist homolog 2 (Drosophila)	1.75
AMIGO2[b]	Adhesion molecule with Ig-like domain 2	−1.88
PHF17	PHD finger protein 17	−2.05
SERPINB2[b]	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	−2.06
FAM176A	Family with sequence similarity 176, member A	−2.12
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.22
SOX9	SRY (sex determining region Y)-box 9	−2.23
F2R	Coagulation factor II (thrombin) receptor	−2.80
NRG1[b]	Neuregulin 1	−3.23
Response to vitamin		
DUSP1	Dual specificity phosphatase 1	1.84
PTCH1	Patched 1	1.61
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.22
SOX9	SRY (sex determining region Y)-box 9	−2.23
Extracellular structure organization		
OXTR	Oxytocin receptor	2.36
LOX	Lysyl oxidase	1.85
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.22
F2R	Coagulation factor II (thrombin) receptor	−2.80
NRG1	Neuregulin 1	−3.23
Regulation of cell communication		
FGF9	Fibroblast growth factor 9 (glia-activating factor)	2.77
OXTR	Oxytocin receptor	2.36
EGFR	Epidermal growth factor receptor	1.96
CSF1	Colony stimulating factor 1 (macrophage)	1.84
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.82
RGS4	Regulator of G-protein signaling 4	−2.06
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.22
RIMS1	Regulating synaptic membrane exocytosis 1	−2.45
F2R	Coagulation factor II (thrombin) receptor	−2.80
NRG1	Neuregulin 1	−3.23
Intracellular transport		
FGF9	Fibroblast growth factor 9 (glia-activating factor)	2.77
EGFR	Epidermal growth factor receptor	1.96
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.82
PPP1R3C	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	−2.19
RIMS1	Regulating synaptic membrane exocytosis 1	−2.45
F2R	Coagulation factor II (thrombin) receptor	−2.80
Response to wounding		
SOD2	Superoxide dismutase 2, mitochondrial	9.52
TREM1	Triggering receptor expressed on myeloid cells 1	4.11
LOX	Lysyl oxidase	1.85
SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	−2.06
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.22
F2RL2	Coagulation factor II (thrombin) receptor-like 2	−2.34
F2R	Coagulation factor II (thrombin) receptor	−2.80
NRG1	Neuregulin 1	−3.23

[a] Genes involved with regulation of fibroblast proliferation.
[b] Genes involved with anti-apoptosis.

Table 2
Genes with expression exclusively regulated in NAFs by 1,25D.

Gene symbol	Gene description	Fold change
Cell proliferation		
AKR1B1[a]	ATPase, Ca^{2+} transporting, plasma membrane 1	1.60
ATP2B1[b]	Aldo–keto reductase family 1, member B1 (aldose reductase)	1.94
BCAT1	Branched chain amino-acid transaminase 1, cytosolic	2.50
CRIP1	Cysteine-rich protein 1 (intestinal)	3.44
FIGF[a]	c-fos induced growth factor (vascular endothelial growth factor D)	1.90
GJA1[b]	Gap junction protein, alpha 1, 43 kDa	1.71
LRRC17	Leucine rich repeat containing 17	1.53
NFIB[b]	Nuclear factor I/B	2.93
PRDM1[b]	PR domain containing 1, with ZNF domain	2.00
Apoptosis		
ABCB1[b]	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1.90
CNKSR2[b]	Connector enhancer of kinase suppressor of Ras 2	1.94
FEM1B[a]	fem-1 homolog b (*C. elegans*)	2.30
GCLC[b]	Glutamate-cysteine ligase, catalytic subunit	2.20
IL7R[a]	Interleukin 7 receptor	2.60
NCAM1[b]	Neural cell adhesion molecule 1	1.85
SEMA3B	Semaphorin 3B	1.82
SERPINB9[b]	Serpin peptidase inhibitor, clade B (ovalbumin), member 9	1.80
TCF7L2[b]	Toll-like receptor 4	2.20
TLR4[a]	Transcription factor 7-like 2 (T-cell specific, HMG-box)	2.90
VEGFA[b]	Vascular endothelial growth factor A	1.92
Immune system process		
BMP6	Bone morphogenetic protein 6	1.70
FZD8	Frizzled homolog 8 (*Drosophila*)	1.60
IL7R	Interleukin 7 receptor	2.60
LRIG1	Leucine rich repeat containing 17	2.22
LRRC17	Leucine-rich repeats and immunoglobulin-like domains 1	1.58
PELI2	Pellino homolog 2 (*Drosophila*)	1.80
PODXL	Podocalyxin-like	2.94
PRDM1	PR domain containing 1, with ZNF domain	1.99
TLR4	Toll-like receptor 4	1.70
Cellular transport		
Transmembrane transport		
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1.90
ABCB4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	2.20
ATP2B1	ATPase, Ca^{2+} transporting, plasma membrane 1	2.00
KCNK3	Potassium channel, subfamily K, member 3	5.60
SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	1.65
STEAP1	Six transmembrane epithelial antigen of the prostate 1	1.70
TMEM16D	Anoctamin 4	1.90
Regulation of transport		
GJA1	Gap junction protein, alpha 1, 43 kDa	1.70
KCNK3	Potassium channel, subfamily K, member 3	5.60
RAB8B	RAB8B, member RAS oncogene family	2.06
TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	1.60
TLR4	Toll-like receptor 4	1.70
Cell–cell adhesion		
CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	1.70
NCAM1	Neural cell adhesion molecule 1	1.85
PLEKHA7	Pleckstrin homology domain containing, family A member 7	3.35
PODXL	Podocalyxin-like	2.94
Regulation of metabolic process		
BMP6	Bone morphogenetic protein 6	1.70
FZD8	Frizzled homolog 8 (*Drosophila*)	1.60
GCLC	Gap junction protein, alpha 1, 43 kDa	2.20
GJA1	Glutamate-cysteine ligase, catalytic subunit	1.70
NFIB	Nuclear factor I/B	2.98
PRDM1	PR domain containing 1, with ZNF domain	3.00
TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	2.20
TLR4	Toll-like receptor 4	2.90
VEGFA	Vascular endothelial growth factor A	1.92
ATP catabolic process		
ABCB4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	2.20
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	1.94
ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	2.40
Xenobiotic metabolic process		
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	4.20
GCLC	Glutamate-cysteine ligase, catalytic subunit	2.20
GLYAT	Glycine-N-acyltransferase	2.15

[a] Genes involved with positive regulation of cell proliferation.
[b] Genes involved with negative regulation of cell proliferation.

Table 3
Gene expression commonly regulated in CAFs and NAFs by 1,25D.

Gene symbol	Gene description	Fold change NAF	Fold change CAF
Cell proliferation			
BCL2	B-cell CLL/lymphoma 2	1.91	2.34
DPP4	Dipeptidyl-peptidase 4	1.77	1.74
EDNRB	Endothelin receptor type B	1.97	5.34
FOXF1	Forkhead box F1	3.02	2.33
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.10	3.81
NAMPT	Nicotinamide phosphoribosyltransferase	3.15	1.79
OSR1	Odd-skipped related 1 (*Drosophila*)	3.91	3.63
PDPN	Podoplanin	2.72	2.86
TACSTD2	Tumor-associated calcium signal transducer 2	2.37	1.69
TGFB2	Transforming growth factor, beta 2	4.74	5.38
Apoptosis			
BCL2	B-cell CLL/lymphoma 2	1.91	2.34
CD14	CD14 molecule	5.54	3.02
DDIT4	DNA-damage-inducible transcript 4	1.77	1.70
EDNRB	Endothelin receptor type B	1.97	5.34
IER3	Immediate early response 3	−2.42	−2.36
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.10	3.81
NLRP1	NLR family, pyrin domain containing 1	2.80	2.62
OSR1	Odd-skipped related 1 (*Drosophila*)	3.91	3.63
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	2.59	2.04
SIX1	SIX homeobox 1	2.02	1.99
SPP1	Secreted phosphoprotein 1	2.26	2.21
TGFB2	Transforming growth factor, beta 2	4.74	5.38
Immune system process			
BCL2	B-cell CLL/lymphoma 2	1.91	2.34
CD14	CD14 molecule	5.54	3.02
CD97	CD97 molecule	1.93	1.98
DPP4	Dipeptidyl-peptidase 4	1.77	1.71
DUSP10	Dual specificity phosphatase 10	2.91	1.71
EDNRB	Endothelin receptor type B	1.97	5.34
FOXF1	Forkhead box F1	3.02	2.33
GEM	GTP binding protein overexpressed in skeletal muscle	1.92	2.25
IL1RL1	Interleukin 1 receptor-like 1	3.48	3.85
PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1	1.55	3.83
SIX1	SIX homeobox 1	2.02	1.99
TGFB2	Transforming growth factor, beta 2	4.74	5.38
THBD	Thrombomodulin	4.74	5.38
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)	2.60	2.05
Response to wounding			
APBB1IP	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	1.95	2.01
BCL2	B-cell CLL/lymphoma 2	1.91	2.34
CD14	CD14 molecule	5.54	3.02
CD97	CD97 molecule	1.93	1.98
DUSP10	Dual specificity phosphatase 10	2.91	1.71
FOXF1	Forkhead box F1	3.02	2.33
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.10	3.81
IL1RL1	Interleukin 1 receptor-like 1	3.48	3.85
NINJ1	Ninjurin 1	2.05	2.02
PDPN	Podoplanin	2.72	2.86
SPP1	Secreted phosphoprotein 1	2.26	2.21
TGFB2	Transforming growth factor, beta 2	4.74	5.38
THBD	Thrombomodulin	5.61	3.41
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)	2.6	2.05
Actin cytoskeleton organization			
AVIL	Advillin	3.82	2.78
BCL2	B-cell CLL/lymphoma 2	1.91	2.34
RHOJ	Ras homolog gene family, member J	1.95	1.96
RHOU	Ras homolog gene family, member U	3.02	4.80
Cell adhesion			
BCL2	B-cell CLL/lymphoma 2	1.91	2.34
CD97	CD97 molecule	1.93	1.98
DPP4	Dipeptidyl-peptidase 4	1.77	1.71
FOXF1	Forkhead box F1	3.02	2.33
LGALS9	Lectin, galactoside-binding, soluble, 9	2.72	1.98
NID2	Nidogen 2 (osteonidogen)	3.29	3.13
NINJ1	Ninjurin 1	2.05	2.02
PDPN	Podoplanin	2.72	2.86
SPP1	Secreted phosphoprotein 1	2.26	2.21
TGFB2	Transforming growth factor, beta 2	4.74	5.38
Ras protein signal transduction			
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.10	3.81
MAPK13	MITOGEN-activated protein kinase 13	4.53	3.52
PLD1	phospholipase D1, phosphatidylcholine-specific	1.61	2.66
RGNEF	190 kDa guanine nucleotide exchange factor	2.25	2.16
RHOJ	Ras homolog gene family, member J	1.95	1.96

Table 3 (*Continued*)

Gene symbol	Gene description	Fold change NAF	Fold change CAF
RHOU	Ras homolog gene family, member U	3.02	4.80
Regulation of cell transduction			
CILP	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	5.51	5.39
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	4.21	2.41
DDIT4	DNA-damage-inducible transcript 4	1.77	1.70
DUSP10	Dual specificity phosphatase 10	2.91	1.71
GRK5	G protein-coupled receptor kinase 5	1.95	2.14
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.10	3.81
IL1RL1	Interleukin 1 receptor-like 1	3.48	3.85
LGALS9	Lectin, galactoside-binding, soluble, 9	2.72	1.98
RGNEF	190 kDa guanine nucleotide exchange factor	2.25	2.16
RHOJ	Ras homolog gene family, member J	1.95	1.96
RHOU	Ras homolog gene family, member U	3.02	4.8
TGFB2	Transforming growth factor, beta 2	4.74	5.38
Lipid metabolic process			
AKR1C1	Aldo–keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	1.84	2.00
AKR1C2	Aldo–keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	1.98	1.79
AKR1C3	Aldo–keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	1.39	1.66
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	512.61	298.69
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	4.21	2.41
GDPD5	Glycerophosphodiester phosphodiesterase domain containing 5	1.88	2.12
NPC1	Niemann–Pick disease, type C1	3.27	2.21
PDPN	Podoplanin	2.72	2.86
PLD1	Phospholipase D1, phosphatidylcholine-specific	1.61	2.66
SERINC2	Serine incorporator 2	2.80	2.93

the most important genes we searched for hubs, broker and bridge genes in the network. We have considered bridge genes the 2% ones with high bridging centrality values and broker genes the 2% ones with high brokering values. Hubs were the 2% nodes more connected with ≥54 partners. The best 2% broker genes were *MAPK13*, *GRB2* and *BCL2* which were also hubs. The breast bridge genes were *RSEA*, *ADA*, *GLGC*. We also searched for genes connected by the interactome (presented in Fig. 3) in the microarray platform and we found 381 genes. We finally constructed the interactions using the connection values as the Pearson correlation between gene pairs in each NAF and CAF groups treated with 1,25D. Differences between the correlations in the CAF and NAF groups were ranked for gene pairs. By restricting gene pairs in which the absolute correlation differences were greater than 1.5 we selected a group of 73 gene pairs (Supplementary Table 7) (0.3% of all pairs selected). Fig. 4 shows the changes in the correlation (positive or negative vice versa) of the expression level of genes coding for interacting proteins between the NAF and CAF groups.

4. Discussion

Our microarray data revealed that the genomic pathway is active in CAFs as well as in NAFs, as VDR protein was detected in both of them and the target gene CYP24A1 was highly induced upon 1,25D treatment. In accordance, VDR expression was previously reported in prostate carcinoma associated fibroblasts [14]. CYP24A1 fold induction was higher in CAFs than in NAFs, suggesting a less intensive response to the hormone in the tumor microenvironment as 1,25D may be metabolized to a less active form [29].

Although mammary fibroblasts (CAFs and NAFs) may share a common transcriptional response to 1,25D treatment, an individual response was also characterized. The 1,25D responsive signature specific to CAFs reflected an antiproliferative effect of 1,25D by down regulation of several genes with proliferative functions. One example is neuregulin (NRG1) that represents the largest subclass of ligands of the EGF family that activates HER-2 [30]. Other genes down regulated included *WNT5A*, *SOX9*, *PDGFC*, *F2RL2* (*PAR*-3) and *F2R* (*PAR*-1). Results in pulmonary fibroblasts supported a role for Wnt5a in regulating fibroblast proliferation and survival [31]. *SOX9*, a high mobility group box transcription factor drives growth factor signal transduction [32] and the two genes associated to cellular effects of thrombin (a multifunctional serine protease), *F2RL2* and *F2R* may mediate thrombin induced proliferation, migration and matrix biosynthesis [33], suggesting that the antiproliferative actions of 1,25D are modulated through several different pathways. We also observed modulation of several genes associated with response to wounding, in accordance with the proposed role of 1,25D in wound healing [34], and reinforcing the idea that CAFs may exhibit molecular characteristics similar to that of activated stromal fibroblasts normally associated with wound healing [5].

On the other hand the hormone induced the up-regulation of both *DUSP1* and *NF-KappaBIA*. The former encodes a phosphatase that specifically inactivates MAPKs and *WNT5A* while *NF-KappaBIA*, an interferon related gene codes for an inhibitor of *NF-KappaBIA*. In our comparison of control CAFs vs NAFs we found several genes regulated by *NF-KappaBIA* such as *IL-8*, *COX2* and *IL-1β*, consistent with data showing that fibroblasts may play a major role in inflammatory processes by secretion of pro-inflammatory cytokines in an *NF-kappaB*-dependent manner [35]. 1,25D may exert anti inflammatory effects by decreasing *NF-kappaBIA* activation, an effect previously reported in fibroblasts [9,10].

Addition of 1,25D to NAFs, in contrast with CAFs, induced up regulation of several anti-apoptotic genes that may represent effectors of survival. Our results are in agreement with Artaza et al. [11], who described an anti-apoptotic effect of 1,25D in a multipotent mesenchymal cell model. We also observed in NAFs an up regulation of a number of transcripts for proteins controlling immune functions including toll-like receptor 4 (TLR4) and of Pellino-2, one of the essential components in the TLR4 pathway activations consistent with the anti microbial activity of 1,25D [36]. Some other up regulated genes appear to be involved in ATP catabolic process including ATP binding cassette members and enzymes involved in detoxification (GLYAT, CYP3A5, GCLC). GCLC (γ glutamylcysteine synthesis) is a rate limit enzyme, important for glutathione synthesis, a potent antioxidant protein. The protective role of 1,25D

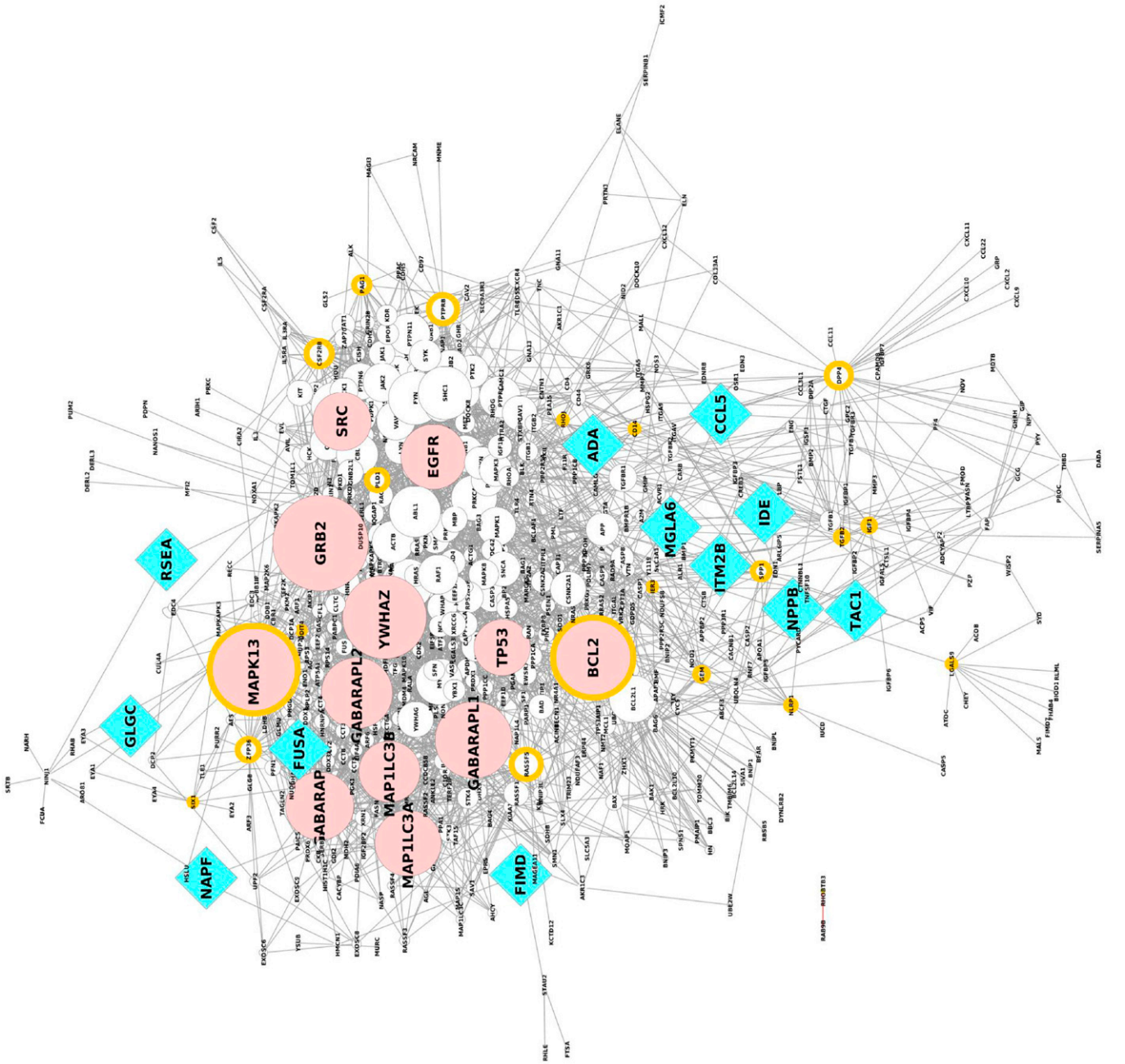

Fig. 3. Interactome network. A protein–protein interaction network was constructed using corresponding protein from each one of the 69 genes commonly altered in both CAFs and NAFs after 1,25D treatment. Bridge genes are represented by blue diamonds and broker genes are represented by pink circles, circles with yellow border represent genes present in the list of 69 genes commonly regulated in NAFs and CAFs. The larger the circle representing a node, the larger the number of connections this node has (hubs).

against cellular stress in the breast epithelial cells was recently discussed [37] and prior observed in non-transformed prostate epithelial cell line [38].

Overlapping up regulated genes responding similarly to 1,25D in NAFs and CAFs, included those reflecting an antiproliferative/apoptotic/differentiation response such as: *G0S2* (involved in adipocyte differentiation), *IGF1*, *TGFβ2*, *MAPK13* (p38δ) and *DDIT4* (*RDD1*), the latter previously found to be down regulated in cell lines resistant to 1,25D [39]. Our results also identified PAG1 (phosphoprotein associated with glycosphingolipid enriched micro-domains) as a gene induced by 1,25D, which is involved in antimitogenic function, negatively regulating tyrosine kinases [40] and a number of genes of the RAS family (*RhoU*, *RASSF5*, *RGNEF* (*RIP2*), *RAB9B*, *RHOJ*) which were also commonly up regulated. *RASSF2A* and *RASSF5* are considered tumoral suppressors [41], *RhoU*, a Rho family GTPase regulates cell adhesion [42]. Other genes up regulated by 1,25D encompass those coding for hormone metabolizing enzymes such as AKR1C1, AKR1C2 and AKR1C3, involved in progesterone metabolism and polycyclic aromatic detoxification, which were previously described as present in NAFs and CAFs [4]. SULT1C2 has been shown to sulfonate estradiol, inactivating its biological activity, as well as environmental xenobiotics [43]. Enhanced expression of these enzymes by 1,25D may result in diminished effects of the hormonal milieu on breast carcinoma epithelial cells, contributing to the beneficial effect of 1,25D. Consistent with an anti-inflammatory role of 1,25D, immune response genes such as *CD14*, *CD97*, *THBD*, *IL1RL1*, *SPP1*, *DUSP10* (dual specific phosphatase 10) and *GEM* were also up regulated.

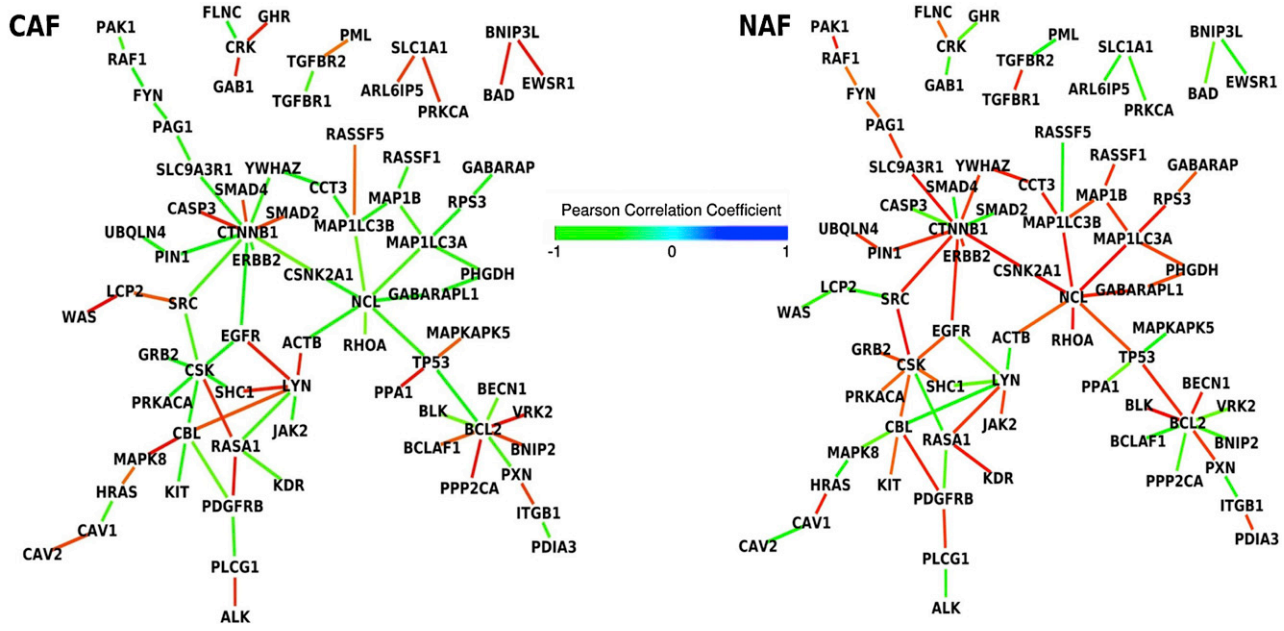

Fig. 4. Genes whose concerted expression levels distinguish CAF and NAF diagrams. Connections between two genes presented in our microarray were established based on the human interactome. Pearson correlation values were calculated for gene pairs. Here we can observe the diagrams of gene pairs that changed their co-expression value more than 1.5-fold comparing NAFs and CAFs treated with 1,25D.

In accordance with other studies investigating 1,25D actions in various cellular models, in CAFs and NAFs our data show modulation of several 1,25D targets already described, suggesting common pathways, irrespective of cellular types [38,39,44–47]. In agreement, we verified that many genes were also modulated in breast cancer tissue slices, containing a mixture of fibroblasts (NAFs and CAFs) and malignant epithelial cells, including CYP24A1, CD14, DPP4, EFTUD1, G0S2, IL1RL1, and THBD.

Although some genes have been commonly differentially expressed in NAFs and CAFs treated with 1,25D we could observe with the analyses of gene–gene co-expression that the co-expression of some of those genes was different in NAFs and CAFs. For instance the gene hNANOS1 repressed by E-cadherin encodes a protein involved in migration and invasive abilities and its pair PLD1 (phospholipase D1) encodes a protein, which is also involved in invasion of breast cancer cells [48,49]. The gene pair *NANOS1* and *PLD1* was positively expressed in CAFs and presented a negative correlation in NAFs.

To further explore the relevance of our set of 69 genes we mapped them into the human interactome and we searched for interactions among these genes. It is important to note that using this network approach, we have observed alterations of coexpression that would not be apparent, looking only to differentially expressed genes, once these connections based on the human interactome, means real interactions by protein–protein. Genes not differentially expressed by the microarray also presented different co-expression in this network. This analysis permitted searching for the most important genes in the network referred as hubs, brokers or bridges genes (Fig. 3). These groups of genes may play an important role in the information flow through the network [50]. Genes from this network that were described as brokers in that they connected many proteins may be important genes that are associated with 1,25D effects on CAFs and NAFs. MAPK13 (p38δ) is one of the p38MAPK isoforms which is involved in response to stress [51] is an example of broker gene.

We finally constructed the interactions based on the human interactome, and we searched pairs present in the microarray. Analyzing the connection values as the Pearson correlation between expressed gene pairs in the CAF and NAF groups, we have identified gene sets whose concerted expression could distinguish 1,25D effects on NAFs and CAFs. One example is *BCL2* that codes for an anti apoptotic protein which was differentially expressed at low levels in the array but was interconnected with other proteins associated to apoptosis such as: *TP53*, *PPP2CA* (protein phosphatase 2, implicated in negative control of growth), *BCLAF1* (*BCL2L* associated transcription factor that induces apoptosis), *BLK* (protects cell from virally induced cell death). Correlation of the expression of BCL2 with the expression of these partners was different in NAFs and CAFs (Fig. 4). These results revealed that measuring the network modularity that indicated altered organization and information flow may improve the response to 1,25D.

In conclusion, CAFs and NAFs displayed differences in their pattern of gene expression in response to 1,25D. Even in the group of overlapped genes we identified gene pairs displaying differential expression between CAFs and NAFs suggesting that the effects of 1,25D on normal fibroblasts are distinct from those triggered in breast cancer associated fibroblasts.

Competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2012.08.002.

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