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Identification of vitamin D target genes in human keratinocytes by subtractive screening☆

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Abstract

1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2$ D_3) imposes cell cycle block in late G1 phase in cultured human keratinocytes. We wanted to identify early vitamin D target genes using a subtractive screening approach. Human foreskin keratinocytes were grown to about 70% confluence, treated with 2×10^{-7} M 1α,25$(OH)_2$ D_3 or left untreated and RNA from both populations were isolated after 22 h of incubation. cDNA was synthesised and cloned into plasmid vectors. For screening of the libraries, cDNA was amplified in vitro using T7 RNA polymerase and then the amplified RNA (driver, control population) and single stranded cDNA (tester) were used for subtractive hybridisation. Heterohybrids were then separated from single stranded nucleotides using a hydroxyapatite column. The radiolabeled single stranded cDNA was used for screening a colony blot. Positive clones were rescreened, plasmid DNA was isolated and used for verifying the results by Northern blot analysis, using RNA isolated from untreated keratinocytes, as well as RNA isolated after 6 h, 12 h and 24 h of vitamin D treatment.

Keywords: Subtractive screening; Skin keratinocytes; Vitamin D target genes; Differential expression

1. Introduction

Vitamin D is rather a hormone than a vitamin. It can be synthesised in the skin from 7-dehydrocholesterol by irradiation with ultraviolet light and it has to be turned into its actual active form, which is 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol) by two successive steps of hydroxylations, one takes place in the liver and the second takes place in the kidney (for review, see [1]). The most prominent physiological role of the hormonally active form of vitamin D_3, $1,25(OH)_2D_3$ is the regulation of calcium and phosphorous homeostasis and bone metabolism via actions in intestine, bone, kidney and parathyroid gland. Besides the classical vitamin D targets involved in calcium and phosphorus household, a number of non-classical vitamin D targets have been identified like the islet cells of the pankreas, ovarian cells, the immune system and keratinocytes of the skin. 1α,25$(OH)_2$ D_3 inhibits proliferation of keratinocytes and induces differentiation in vitro and in psoriatic epithelial cells in vivo. Gniadecki [2] showed that 1α,25$(OH)_2$ D_3, depending on the culture conditions, can either stimulate or inhibit DNA synthesis in human keratinocytes. In cells cultured with 0.15 mM calcium in the absence or with low levels (0.1 ng/ml) of epidermal growth factor, exposure to 10^{-11} to 10^{-6} M 1α,25$(OH)_2$ D_3 imposes cell cycle block in late G1 phase. In order to be able to respond to calcitriol, all vitamin D target tissues require a specific nuclear receptor protein, called the vitamin D receptor (VDR). The VDR belongs to the superfamily of steriod nuclear receptors. Upon binding of the ligand (1α,25$(OH)_2$ D_3), the VDR heterodimerises with a co-factor, the retinoid X receptor (RXR). The heterodimer then binds to specific regions located in the promoter regions of vitamin D target genes, called the vitamin D response elements (VDRE). Most VDREs consist of two half sites of imperfect repeats of the sequence AGG TGA, separated by three nucleotides. Binding of the heterodimer to the VDRE permits interaction of the VDR with several cofactors and transcriptional integrators, which in turn activates the transcriptional machinery (reviewed in [1]). Although VDREs have been described for genes like, CYP24, parathyroid hormone related peptide, integrin B3, c-*fos*, the insulin gene [3], the involucrin gene [4], we are still far

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from knowing even the majority of vitamin D responsive genes. To improve our understanding of the biological activity of vitamin D we used a subtractive screening approach to search for genes upregulated by vitamin D.

2. Materials and methods

All methods were performed according to standard procedures as described in Sambrook J. and Russel D.W., Molecular Cloning, A laboratory manual, third edition, 2001, if not otherwise stated.

2.1. Cellculture and treatments

Human foreskin keratinocytes were cultured in serum-free medium with 0.12 mM Ca^{2+} and 0.1 ng/ml hEGF and grown to about 70% confluence, 20 flasks were treated with 2×10^{-7} M 1α,25(OH)$_2$ D_3 dissolved in ethanol and 20 flasks were treated with ethanol only as a control. RNA from both populations was isolated after 22 h of incubation. For Northern blot analysis RNA was isolated from untreated keratinocytes and after 6 h, 12 h and 24 h of incubation with vitamin D.

2.2. RNA Isolation and cDNA synthesis

RNA was isolated using the acidic Phenol–Guanidinium–Thiocyanate–Chloroform extraction. cDNA was synthesised from 6 μg poly *A*+RNA using an oligo dT Primer containing a T7 RNA polymerase promoter and cloned into plasmid vectors (modified pT7/T3-A18).

2.3. Colony lift

Filter replicas of 140,000 cDNA clones from the vitamin D treated library were produced on nitrocellulose membranes (Schleicher & Schuell BA135) and baked at 80 °C for 2 h before hybridisation.

2.4. Subtractive hybridisation

Six micrograms of poly A + RNA isolated from vitamin D treated and control keratinocytes, respectively, were transcribed into cDNA using an oligo dT primer containing a T7 promotor and M-MLV reverse transcriptase (BRL Superscript II) for first strand, and *Bst* DNA polymerase (NEB) for second strand synthesis. cDNA was then amplified by in vitro transcription using the AmpliScribe™ T7 transcription kit, according to the manufacturer's protocol. 10 μg of the in vitro amplified RNA (originating from vitamin D treated cells) were transcribed into radiolabeled first strand cDNA. Single stranded radiolabeled cDNA (referred to as the tester cDNA) was mixed together with in vitro amplified RNA from the control population (referred to as the driver RNA) at a 1:10 ratio. Hybridisation was carried out at 65 °C for 48 h. Double stranded heterohybrids were separated from ss nucleic acids on a hydroxyapatite column at 65 °C and the fractions containing ss cDNA were added to the prehybridised colony filters. Hybridisation was carried out over night and the washed filters were exposed to X-ray films (Kodak X-omatic). Positive clones were picked and rescreened once.

2.5. Sequencing of cDNA clones

The cDNA sequence was determined using an ABI PRISM™ 310 genetic analyzer (Perkin Elmer). The obtained sequences were compared with the databases by BLAST analysis. Results of a BLAST search are automatically linked to the NCBI LocusLink and UniGene databases (http://www.ncbi.nlm.nih.gov).

2.6. Northern blots

10 μg of total RNA prepared from untreated keratinocytes as well as vitamin D treated cells were separated on an 1,2% agarosegel (6,5% formaldehyde) and transferred on nylonmembranes (GeneScreen, NEN). RNA was covalently cross-linked to the membrane by UV irradiation. Random primed radioactive (α^{32}PdCTP, Amersham Pharmacia) labelling reactions were prepared from positive cDNA clones as described by Feinberg and Vogelstein [5]. After hybridisation with specific probes membranes were exposed to X-ray films (Kodak X-omatic) and analysed at the phosphoimager (BAS Reader 3.1 and AIDA analyser 2.11 software) to determine the intensities of the probes.

3. Results

After screening of about 140.000 cDNA clones with a subtracted probe we picked 428 positive clones. They were rescreened, sequenced and blasted with GenBank/EMBL databases. Ninety-nine clones were coding for keratins (keratin 6 and keratin 16). Cyp24, the 1α,25 dihydroxyvitamin D_3-24-hydroxylase, the degradation enzyme of vitamin D, was among the positive clones, which can be regarded as a positive control (Fig. 1A). A few clones were picked and analysed on Northern blots. For data normalisation the expression of Large Ribosomal Protein 0 (LRP0) was measured as a reference. LRP0 was shown to be expressed at comparable amounts in a large number of tissues and under different environmental/physiological parameters [6]. Northern blot analysis confirmed the following clones to be upregulated by 1α,25-dihydroxyvitamin D_3: CD9 antigen (Fig. 1B) was upregulated 2.6-fold after 24 h of exposure, as well as membrane-bound transcription factor protease, site 1 (Fig. 1C), Image clone 4762400 was upregulated 2,3-fold (Fig. 1D), p63 2.04-fold (Fig. 1E), retinoblastoma

binding protein 1 (Fig. 1F) and c-yes 1 (Fig. 1G) were 1.7-fold upregulated, whereas S100 Calcium binding protein A2 (Fig. 1H), phosphatidylinositol-3-kinase (Fig. 1I) and Annexin II (Fig. 1J) showed 1.7-, 1.4- and 1.3-fold induction, respectively. The quantitation by Northern blot seems to give lower yields compared to real-time PCR. For a particular clone the result was a four-fold induction estimated by Northern blot compared to a seven-fold induction by real-time PCR (data not shown). Some of the clones used for Northern blot analysis have been described in the context with vitamin D and Keratinocytes before, like phosphoinositol-3-kinase [7] and Annexin II [8,9] and of course CYP 24, the 25-hydroxyvitamin D_3-24-hydroxylase, the degradation enzyme of vitamin D_3. Keratin 1 and 10, typical keratins expressed upon keratinocyte differentiation, were not found in our screen, due to the short exposure to vitamin D. Northern blot analysis of calcium treated human keratinocytes revealed detectable mRNA of Keratin 1 only

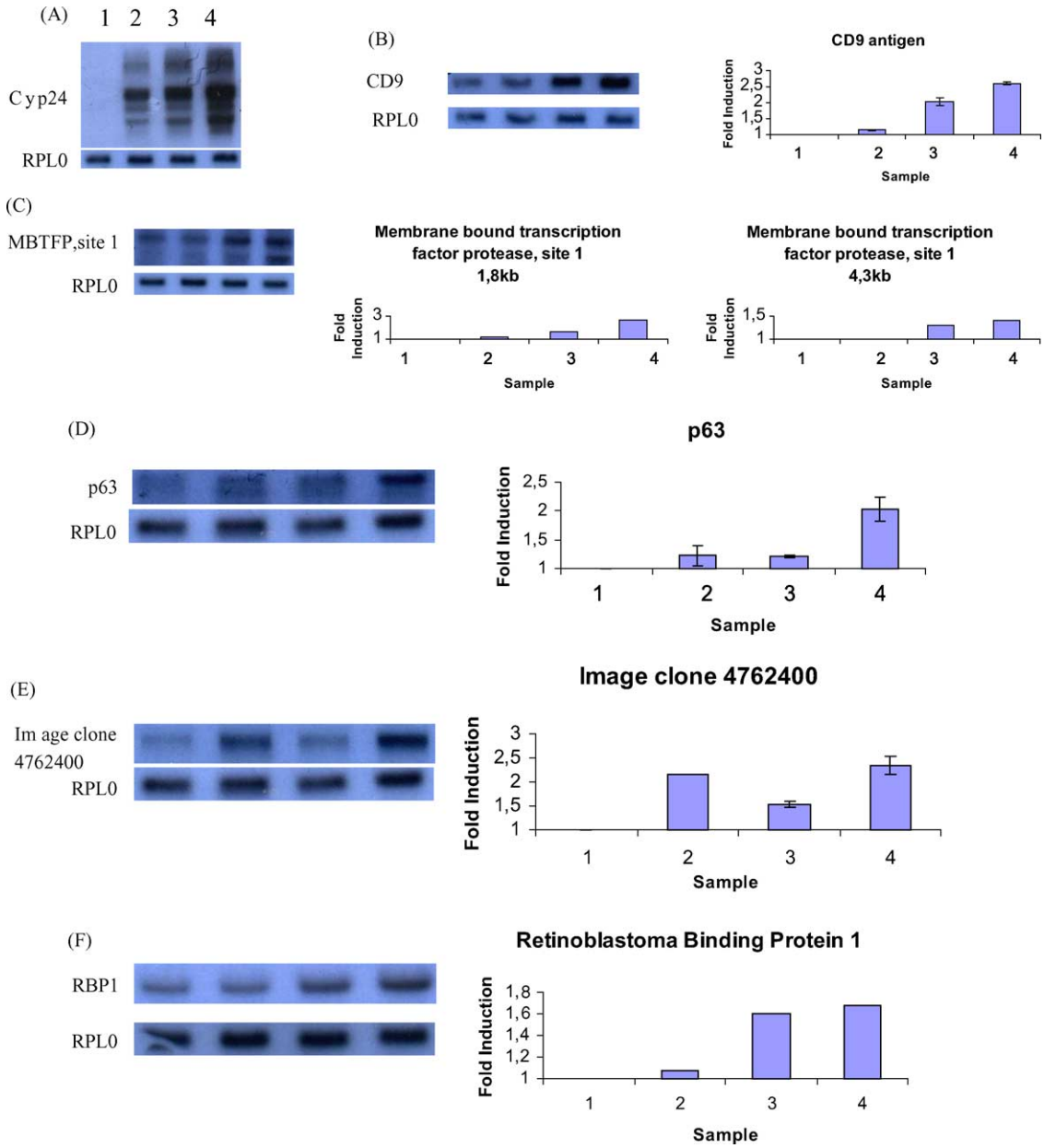

Fig. 1. Increased gene expression after admission of 1α,25 dihydroxyvitamin D_3 to primary human keratinocyte culture shown on Northern blot. Differential expression of cDNA clones identified by subtractive screening: Cyp24 (A), CD9 (B), membrane bound transcription factor protease, site 1 (C), p63 (D), Image clone 4762400 (E), retinoblastoma binding protein 1 (F), c-yes 1 (G), S100Ca binding protein A2 (H), PI-3K (I), Annexin II (J). Expression was confirmed by Northern analysis using total RNA isolated from human primary foreskin keratinocytes harvested at 70% confluence as control (lane 1) and after admission of 2×10^{-7} M 1α,25 dihydroxyvitamin D_3 and culture for 6 h (lane 2), 12 h (lane 3) and 24 h (lane 4) (left panels: autoradiographs of mRNA levels, right panels: the relative amount of mRNA as estimated by an imaging analyser). The values were normalised to ribosomal protein, large, P0 mRNA content (shown underneath). The results are representative of two independent experiments.

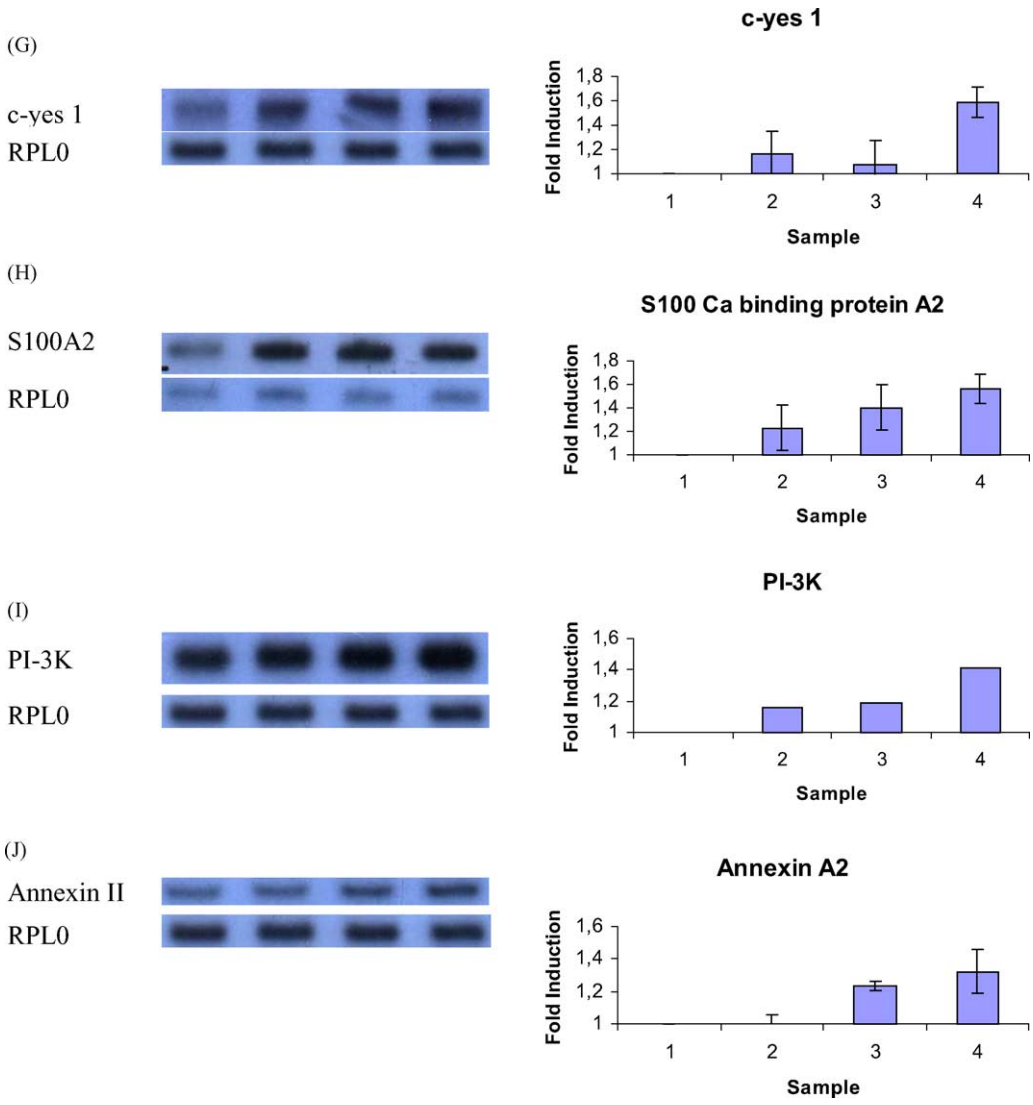

Fig. 1. (*Continued*).

after 5 days [10]. 22 h of treatment had been chosen, because induction of CYP 24 was reported to take place within 16 h of vitamin D exposure [4] in human keratinocytes.

4. Discussion

A few G0/G1 switch genes (data not shown) were found in our subtractive screen as described by Gniadecki [2], but the majority of early vitamin D target genes in human foreskin keratinocytes correspond to a wound healing process (e.g. keratin 6, keratin 16, CD9 [11]).

4.1. CD9

CD9 belongs to the family of tetraspanin proteins, which exhibit four hydrophobic domains and reveal a high tendency to aggregate with other tetraspanins and/or other transmembrane proteins, including the integrins. CD9 is present only at intercellular contacts between keratinocytes in the normal epidermis. Baudoux et al. [11] did not observe any upregulation of CD9 expression upon epidermal differentiation induced by the phorbol ester TPA (a known inducer of protein kinase C signalling). In contrast to that LeNaour et al reported a seven-fold increase in CD9 expression after exposure to TPA in a hematopoetic cell line (K562 cells). The upregulation of CD9 is dependent on protein kinase C activation. CD9 then becomes associated with the integrin β1 [12], and is in that way involved in cell motility and cell adhesion. In the basal layer, stem cells, the least differentiated keratinocytes, have the highest level of β1 integrins (2–3 times more than transit amplifying cells) and β1 integrin expression is lost upon terminal differentiation. The association of CD9 with β1 integrin might be an early step in the loss of β1 integrin expression upon differentiation.

4.2. Membrane bound transcription factor protease, site 1

The sterol-regulated proteolysis of membrane bound transcription factors (called sterol regulatory element binding proteins, SREBPs) is involved in cholesterol metabolism. SREBPs are cleaved by site 1 protease (S1P), a membrane bound substilisin related serine protease. SREBPs are a family of three proteins and they are inserted into the membranes of the endoplasmic reticulum (ER) and the nuclear envelop in a hairpin orientation. The NH_2-terminal segment of SREBP is a transcription factor of the basic helix-loop-helix-leucin zipper family that projects into the cytoplasm. Two successive cleavages (one by S1P, the second by site-2 protease (S2P)) release the NH_2 terminal fragment of SREBP, allowing it to enter the nucleus where it binds to enhancers and activates transcription of genes encoding the low density lipoprotein (LDL) receptor and multiple enzymes of cholesterol and fatty acid biosynthesis [13]. When hybridised with the specific probe of membrane bound transcription factor protease, site 1, two bands—one at 1,8 kb and a larger one corresponding to the published mRNA of 4338 bases (NM003791) were detected on the Northern blot, both showing an induction. The smaller RNA, which might be a splicing variant, that is produced in human keratinocytes after exposure to calcitriol shows a significantly higher induction (2.6-fold) than the originally described transcript (1.4-fold). Membrane bound transcription factor protease, site 2, the transcript of which is 1759 bases in size, has no significant similarity to site 1 protease, so cross hybridisation can be excluded.

4.3. p63

p63, along with p73 is a member of the p53 family. Members of the p53 family are referred to as the guardians of the genome, since they can induce both, cell cycle arrest (G1 arrest) and apoptosis in response to a wide range of genotoxic and cellular stresses. p53, p63 and p73 posses considerable homology to one another, containing a transactivation domain (TAD), a DNA binding domain (DBD) and an oligomerization domain (OD). The greatest homology among all the p53 family members exists within the DBD suggesting that p63 and p73 may bind to the same DNA sequences as p53 and promote transcriptional activation. Unlike p53, both p63 and p73 share a C-terminal protein-protein interacting motive (sterile alpha-motif, SAM). While p53 is expressed ubiquitously, p63 is specifically expressed in embryonic ectoderm and in basal regenerative layers of epithelial tissues in the adult. p63 knock out mice die at birth due to ectodermal stem cell loss, resulting in truncation of the limbs, as well as absence of the epidermis, prostate, breast and urogenital tissues. p63 has been shown to be upregulated in UV-treated human keratinocytes. The N-terminally truncated form of p63, called ΔNp63, resulting from the usage of a second promoter, has been shown to be upregulated in squamous cell carcinoma [14]. p63 as well as p73 might be useful to complement the function of p53, a molecule that was found to be mutated or lost in 50% of all human tumors [15].

4.4. IMAGE clone 4762400

Sequencing analysis and Blast search identified one of the clones isolated via our subtractive screening approach as Image clone 4762400 (GenBank numbers BC020914 and BC008643). Within 24 h this clone showed a 2.3-fold induction on Northern blot analysis. It is postulated that this clone codes for a putative transmembrane protein (MGC3196), but so far no further information is available. This is presently under further investigation.

4.5. Retinoblastoma binding protein 1 (RBP1)

The retinoblastoma gene product pRB, regulates cell proliferation by binding to and inhibiting the activity of key growth promoting proteins, like the E2F family of transcription factors, via recruitment of retinoblastoma binding protein to its pocket. The “pocket” can interact simultaneously with both E2F and certain cellular Leu-X-Cys-X-Glu (LXCXE) sequences (e.g. in RBP1). Retinoblastoma binding protein 1 (RBP1) is a large nuclear phosphoprotein and a known pRB pocket-binding protein that serves as a bridging molecule to recruit histone deacetylases (HDACs) to turn off target genes. RBP1 has been shown to bind to all three known HDACs (HDAC1, 2 and 3) [16]. In addition to that RBP1 provides a second HDAC-independent repressor function. It possesses transcriptional repression activity and associates with p130-E2F and pRB-E2F complexes specifically during growth arrest. Overexpression of RBP1 both inhibited e2F-dependent gene expression and suppressed cell growth. The repression of E2F-dependent transcription by RBP1 via RB family members may play a central role in inducing growth arrest [17].

4.6. c-yes 1

c-yes 1 is also called Yamaguchi sarcoma viral oncogene homolog 1 and is a nonreceptor tyrosine kinase of the Src family. It functions downstream of GM-CSF (CSF2) and is involved in disrupting cell–cell contacts. c-yes was shown to be distributed diffuse in the cytoplasm of normal epidermal keratinocytes maintained in low calcium-containing medium. Ca^{2+} treatment of normal and tumor derived human keratinocytes resulted in recruitment of c-yes (and Src and Fyn), as well as their putative substrate $p120^{CTN}$ to cell–cell contacts. Src kinase activity is normally required to disassemble cadherin-dependent cell–cell contacts. Tyrosine phosphorylation of E-cadherin correlates with the loss of epithelial differentiation and gain of invasive potential [18].

4.7. S100 calcium binding protein A2

S100 calcium binding protein A2 (also termed CaN19) is a predominantly nuclear, p53-inducible, homodimeric calcium and zinc binding protein of the EF-hand family that is distantly related to calmodulin [19]. S100A2 is the only member of the S100 gene family whose expression is downregulated in cancer cells relative to their normal counterparts and therefore it is considered to be a putative tumor suppressor [20]. The S100A2 gene is located on chromosomal band 1q21, a region termed epidermal differentiation complex, consisting of at least 43 genes that are expressed during keratinocyte differentiation [21]. In squamous cell carcinoma cells S100A2 (as well as Annexin II) is expressed at significantly lower levels than in normal human keratinocytes. Both Annexin A2 and CaN19 are calcium binding proteins that may play a role in normal human keratinocyte differentiation by regulating protein kinase C (PKC) [22]. A recent publication, however, reported downregulation of S100A2 mRNA in human keratinocytes after 48 h of exposure to differentiation promoting agents. The upregulation of S100A2 observed after exposure to $1\alpha,25(OH)_2$ D_3 might be a transient effect. This has to be further investigated.

4.8. Phosphatidylinositol-3-kinase (PI-3K)

Phosphatidylinositol-3-kinase (PI-3K) is a lipid kinase. Addition of $1\alpha,25(OH)_2$ D_3 to cultured human keratinocytes resulted in a rapid but transient phosphorylation of ERK1, ERK2 and JNK1, which was accompanied by increased expression of both mRNA and protein of c-Fos, Fra1 and c-Jun. In addition to that $1\alpha,25(OH)_2$ D_3 increased the AP-1 transcriptional activities in a Ras-dependent manner. Inhibition of both, MEK and PI-3K resulted in reduced activation of ERK1/2 and JNK1 in response to $1\alpha,25(OH)_2$ D_3, demonstrating that the PI-3K/Ras//MEK/ERK1/2 and JNK1 pathway is the principle pathway in the $1\alpha,25(OH)_2$ D_3 – induced activation of AP-1 and in the resulting differentiation [7]. $1\alpha,25(OH)_2$ D_3 induced differentiation of human keratinocytes is mediated via a PKC-independent upregulation of the AP-1 DNA binding activity whereas calcium-induced keratinocyte differentiation is PKC dependent. Johansen et al. proposed a model in which $1\alpha,25(OH)_2$ D_3 binds to the putative membrane receptor Annexin II [8,9], which leads to a PI-3 kinase and/or Ras-dependent activation of the MEK/MAPK pathway. ERK1/2 and JNK1 are rapidly being activated by phosphorylation, leading to increased synthesis of the c-Fos, Fra1 and c-Jun proteins in conjugation with enhanced AP-1 DNA binding activity and increased transcription of specific genes involved in cell differentiation [7].

In conclusion we found some genes to be upregulated after exposure to $1\alpha,25$ –dihydroxyvitamin D_3 that were previously described in erythroid differentiation (e.g. CD9), genes that might play a role in growth arrest (e.g. RBP1, p63), genes described in wound healing processes and cell–cell contacts (CD9 and c-yes), genes with unknown functions (Image clone 4762400) and genes involved in PKC signalling (CD9, S100A2). For further investigation of the function of these clones we want to perform a two-hybrid screen to find partners for these clones, which will help to get a more detailed picture of the molecular mechanisms of actions in response to $1\alpha,25(OH)_2$ D_3 in human keratinocytes.

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