Cellular Retinol Binding Protein I transfection in H460 non-small lung carcinoma cells reduces proliferation and AKT-related gene expression

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Research article

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Abstract

Background: In recent years, new treatments with novel action mechanisms have been explored for advancer lung cancer. Retinoids were shown to promote cancer cell differentiation and death of and their action is mediated from specific cytoplasmic and nuclear receptors. The purpose of this study was to investigate the effect of Cellular retinol binding Protein I (CRBPI) transfection in H460 human non-small cell lung cancer cell line normally not expressing that receptor.

Methods: H460 cells were transfected by using a vector pTargeT Mammalian expression system carrying the whole sequence of CRBPI gene. For proliferation and apoptosis studies, cells were treated with different concentrations of all-trans retinoic acid (at RA) and retinol. AKT-related gene expression was analyzed by using western blot and Signosis array. Results were analysed using d by one-way analysis of variance (ANOVA) followed from a Bonferroni post hoc test or with t-student test.

Results: CRBPI + showed a reduced proliferation and viability in basal condition and after at RA treatment when compared to empty-transfected H460 cells. Reduced proliferation in CRBPI + H460 cells associated to the down-regulation of pAKT/pERK/pEGFR genes. In particular, gene array documented in CRBPI + H460 cells the down-regulation of AKT and Stat-3-related genes, including M-Tor, Akt1, Akt2, Akt3, Foxo1, Foxo3, p21, p27, pTen, Jun.

Conclusion: Restoration of CRBPI expression in H460 cells reduced proliferation and viability in both basal condition and after at RA treatment, likely by down-regulating AKT-related gene level. Further studies are needed to better clarify how those CRBPI-related intracellular pathways contribute to counteract non-small cell lung carcinoma progression in order to suggest a potential tool to improve efficacy of retioid anti lung cancer adjuvant therapy.

Background

Lung cancer is the first cause of neoplastic death worldwide both in man and women [1]. Nevertheless the recent innovative treatment for non-small lung cancer (NSLCC), the prognosis remains very poor. Lung cancer are largely classified into non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma, with NSCLC accounting for about 80% of all cases and only 18% all patients are alive for 5 or more years after diagnosis [2]. In lung cancer, Vitamin A deficiency has been associated with bronchial metaplasia and increased lung cancer development with many other factors [3–5]. Retinoids were shown to promote differentiation and cell death of cancer cells in a number of experimental systems, including lung [5–7]. In recent years, new treatments with novel action mechanisms have been explored for advancer lung cancer, including retinoids administration [3, 7, 8]. Biological activity of retinoids, in particular *all-trans* retinoic acid (*at*RA) is normally mediated by specific cytoplasmatic and nuclear receptors [8–10]. Cellular retinol binding protein type I (CRBPI) is a 15 kDa cytosolic binding protein crucial for uptake and subsequent esterification of retinol, by regulating its bioavailability [8, 11]. CRBPI is indispensable for embryonic development and growth of vertebrates and, in the lung parenchyma, CRBPI expressing cells

observed during development and pre-natal alveolus formation [12]. Defects in CRBPI gene expression has been linked to oncogenic process in breast, prostatic, renal, lung and endometrial cancer [13–15]. Retinoids exert their pleiotropic and transcriptional effects through the binding to nuclear receptors, namely the retinoic acid receptors RARα, β, and γ, and retinoid X receptors RXRα, β, and γ [5, 8, 10, 16]. The latter may form omo/heterodimers among them [17, 18] or with other receptors as thyroid hormone receptors, vitamin D3 receptors, peroxisome proliferator active receptors (PPARs) and several orphan receptors, contributing to starting alternative signalling pathways [19]. For example, RARβ and RXRα, in normal respiratory epithelium, binding PPARγ with other cofactors, ensures cyclin D1 mediated cell cycle inhibition hence favouring apoptosis or differentiation [5, 18]. Down regulation of RARβ combined with AP-1 up-regulation triggers tumor progression and proliferation of NSCL cells [20]. Cuncurrently, the inability of RXRα to form heretodimers with PPARγ enables an AP-1/CRB-dependent up regulation of Cox2, resulting in inhibition of apoptosis [16, 21]. The loss of RARβ mRNA expression has been observed in many lung cancer cells line and its expression is contingent on intracellular concentration of retinoids, mediated by CRBPI and II [20]. Moreover, retinoids can activate several pathway, including AKT/ERK signalling in lung cancer cells through a transcritional independent-mechanism [3, 22]. Retinol can induce cytokine-dependent activation of JAk2 and subsequently STATs transcription factor, while the exchange from RBP to intracellular CRBPI is mediated by Stra6 receptor [23].

We recently documented that high expression of CRBPI in lung adenocarcinoma in vivo was associated to a lower overall survival [24]. Napoli et al. proposed that CRBPI expression in NSLCC should be considered as marker of RARβ down regulation [8]. In fact, authors suggested that CRBPI cytoplasmic accumulation represent a block of nuclear disponibility of retinoids.

In the present study, we investigated the effect of *at*RA in native and CRBP1-transfected H460 lung cancer cells with particular reference also to the modulation of RAR/RXRs and pAKT/pERK/pEGFR gene signaling.

Methods

Cell transfection

Human non-small cell lung cancer (H460) cell line (kindly provided by Dr. Carlo Leonetti, Regina Elena National Cancer Institute, Rome, Italy) was maintained in RPMI 1640 (Lonza Bio Pharma AG, Switzerland) and transfected by using a vector pTargeT Mammalian expression system carrying the whole sequence of CRBPI gene (NM_002899) and the gene for the resistance to G418 (Promega, Italy), or the G418-resistance gene alone, as reported [25]. After 20 days, stable transfected clones were collected in G418-containing medium and tested by PCR and western blot. The correct plasmid sequence was confirmed by Sanger sequencing. Experimental procedures were repeated by using different transfected clones, which gave similar results (dat not shown).

Cell growth, viability and clonogenic assay

For proliferation studies, native and transfected H460 cells were treated with different concentrations of *all-trans* retinoic acid (*at*RA) and retinol (ROL; Sigma-Aldrich, St. Louis, USA) in 0.1% FBS up to 3 days. For cell viability, 3-(4,5 dimethylthiazol-2-yl)-2,5diphenyl-tetrazolimbromide assay (MTT, Sigma-Aldrich) was performed [26]. For the clonogenic assay, cells were seeded and treated with 5 μM *at*RA. Colonies arising from survival cells were fixed and stained with 1% methylene blue (Sigma-Aldrich) in 0.1% methanol and their percentages as plating efficiency (PE) calculated [24].

Gene expression analysis

Gene expression analysis was performed using Signosis array (Signosis, Inc. Santa Clara, CA, USA). Briefly, total RNA was extracted [27, 28], reverse-transcribed into cDNA in the presence of biotin-dUTP and a profile of 24 genes for human AKT and Stat-3 pathway cDNA plate array (Signosis). Luminescence relative light units (RLUs) was evaluated on a microplate luminometer, according to the manufacturer' instruction. Real-time PCR for RxR, RARs and cytokeratins was also performed in triplicate [24] with primers listed in Supplemental Table 1, using ß2-microglobulin, ß-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping genes.

Western blot analysis

After isolation, content determination and electrophoresis, proteins were elettroblotted [29] and incubated with a polyclonal rabbit anti-CRBP-1, anti-RXRα, anti-RARα (1:500, Santa Cruz Biotechnology, USA), anti-RARβ, anti-RARγ, anti-cytokeratin-5/6, anti-cytokeratin-10 (1:500, Abcam, Cambridge, UK), anti-phosphorylated-AKT (pAKT Ser^{473}), anti-AKT, anti-phosphorylated ERK1/2, anti-phosphorylated EGFR (Thr669) and mouse anti-total tubulin antibody (Sigma-Aldrich), followed from horseradish peroxidase conjugate goat anti-rabbit or anti-mouse IgGs (Pierce, Rockford, USA). Specific complexes were revealed and quantified as reported [29] in three independent experiments. AKT and EGFR activity was expressed as phospho/total protein ratio [30].

Statistical analysis

Results were analyzed as the arithmetical mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed from a Bonferroni post hoc test and using the Student t-test. The differences were considered statistically significant for p values < 0.05. All the statistical analyses were performed with SSPS V20 (Stat Corp, College Station, Texas, USA).

Results

Restoration of CRBPI expression reduces survival and clonogenicity of H460 cells

As reported in Fig. 1A and B, empty-transfected H460 cells did not express CRBPI, similarly to native cells. To investigate the effect of CRBPI transfection on H460 cell survival, cell counting and MTT assay were performed. Our results showed that 10% FBS-cultured CRBPI$^+$ grew less than empty-transfected H460 cells starting from second day ($p < 0.05$) (Fig. 1C). CRBPI$^+$ viability was also reduced more markedly in the presence of different *at*RA concentrations (1–20 ∝M; IC_{50} = 5∝M; Fig. 1D) when compared to empty-transfected H460 cells. Finally, CRBPI$^+$ both in basal condition and after 48 h of *at*RA treatment showed a poor ability to form colonies compared to empty-transfected H460 cells (Fig. 1E and F).

CRBPI transfection influences proliferative and transcriptional gene levels of H460 cells

In order to investigate the CRBPI-regulated intracellular signalling, gene expression and western blotting analysis were performed. A series of genes involved in AKT and Stat-3 related proliferation pathways were down-regulated in CRBPI-transfected compared to empty-transfected H460 cells: 14-3-3-sigma, 4E-BP, Enos, M-Tor, Akt1, Akt2, Akt3, Foxo1, Foxo3, p21, p27, p70, PDK1, Bad, pTen, Casp9, A2M, Jun; Mdm2, Gsk-3a, IGF-1R, PI3K, C-myc, Cycline-E, MCL1, DNMT1, Bcl-2, GP130, Bcl-xl, GSK-3B, C-fos genes were up-regulated (Fig. 2A and B). Complessively, CRBPI expression seems to reduce the expression of proliferative proteins and to increase that of differentiation markers. As concerning cytokeratins (CK), we observed the upregulation of CK1 and 10 ($p < 0.02$ and $p < 0.01$, respectively) and the down-regulation of CK5 expression ($p < 0.01$) in CRBPI$^+$ compared to empty-transfected H460 cells (Fig. 3A and B). In CRBPI$^+$ H460 cells maintained with 10% of FBS, only RARα protein expression resulted down-regulated compared to empty-transfected cells, whereas CRBPI signaling did not significally influences other RAR/RXR receptor expression (Fig. 3C). As reported in Fig. 2D, gene assay documented in serum-cultured CRBPI$^+$ H460 cells the reduction of pAKT expression ($p < 0.05$), but no difference in pERK and pEGFR expression compared to empty-tranfected cells. After 48 h of 5∝M *at*RA treatment, we observed the downregulation of pAKT, pERK and pEGFR expression. Instead, ROL treatment after 48 h did not influence those protein expression (2E-G).

CRBPI expression influences AKT signaling pathway

To further investigate CRBPI-related gene signalling in patients' tissue, we analyzed changes of AKT signaling in a cohort of patients with high or low CRBPI immunoexpression and in a cohort of control lung tissues. As reported in Fig. 4, patients with high CRBPI expression showed down regulation of the most genes implicated in AKT and the up-regulation of Pten.

Discussion

The H460 cell line represent a suitable experimental model of highly proliferative, drug resistant, metastatic NSCLC cell line [7]. Recently, it has been demonstrated the presence of cancer stem cells in H460 line [31] thus explaining at least in part the aggressiveness of those cells.

It is well documented that chemio and radiotherapy resistance is mainly due to so called cancer stem cells selection, epigenetic mechanisms silencing physiological pathway (for example hypo or hypermethilation of genes) and finally acquisition of other mutations [32]. In fact, also with the promising tyrosine kinase inhibitors or the immunotherapy patients develop inevitably progression [28, 33, 34]. *at*RA is well known to have a dramatic effect on M3 subtype of acute myeloid leukemia [34]. However, in most other cancers this effect is not observed because of epigenetic silencing of retinoid pathway [35]. Low doses of *at*RA (20 mg/m2/day) in combination with chemotherapy showed remarkable activity as demonstrated in a randomized phase II trial of patients with advanced NSCLC [6]. Our results showed a reduced viability and the down-regulation of several genes and proteins involved in proliferative and transcriptional pathways in CRBPI^{+} compared to empty-transfected H460 cells in both basal condition and after atRA treatment. AtRA was used in clinical trials to suppress the growth and progression of different cancer types [18, 36]. However, its effectiveness is limited in some cancer, including lung cancer [6, 37, 38]. *At*RA is an active metabolite of Vitamin A that regulates diverse cellular functions such differentiation, proliferation and apoptosis by binding with RAR/RXR receptors [8]. RARβ is a tumor suppressor gene whose expression is significantly decrease in human cancers and increase with atRA treatment [39]. The loss of RARβ mRNA expression has been observed in many lung cancer cells line and its expression is contingent on intracellular concentration of retinoids, mediated by CRBPI [20]. CRBPI loss may be responsible for intracellular retinoid deficiency, since CRBPI is required for retinol bioconversion [40–42]. Epidemiological studies suggest that the addition of atRA or synthetic retinoids to human cancer cell lines or human tumor xenografts in nude mice result in growth arrest, apoptosis or differentiation [43]. Expression of CRBPI may help to switch between proliferation and differentiation in response to oncogenetic stimuli [44].

It has been reported that in human mammary tumor cells the reintroduction of CRBPI reduces tumorigenicity in athymic mice [45]. Moreover, the inhibition of PI3K/Akt pathway was involved in the antitumor effect of CRBPI mediated from p85 regulatory and p110 catalytic subunit heterodimerization [46]. It is possible to hypothesize that derepression of PI3K/Akt signaling mediates CRBPI loss-induced cancer progression and down-regulation of RARβ in a trascription-indipendent mechanism of atRA. Several in vitro studies showed that atRA induces transcription-indipendent activation of the PI3K/Akt pathway [18, 24]. For these reasons, we transfected H460 lung cancer cell line with CRBPI and compared the effect of atRA in Akt signalling and RAR expression. We observed that restoration of CRBPI expression reduces survival and clonogenicity compared to empty-transfected H460 cells. Moreover, CRBPI^{+} H460 cells showed a poor ability to form colonies compared to CRBPI^{-} cells, both in basal condition and after 48 h of atRA treatment.

A series of genes involved in Akt and Stat-3 related proliferation pathways were down-regulated in CRBPI$^+$ H460 cells as Akt1, Akt2, Akt3, Foxo3, p21, p27, p70, Casp9, Gsk-3a, IGF-1R, PI3K, C-myc, Cycline-E, whereas, Bcl-2, GP130, Bcl-xl, GSK-3B, C-fos genes were up-regulated. Those evidences confirmed the hypothesis that CRBPI expression in H460 cells reduces proliferative and increase differentiative markers. As concerning CK expression, we observed the upregulation of CK1 and 10 and a down-regulation in CK5 expression in CRBPI$^+$ compared to empty-transfected H460 cells. In basal condition, CRBPI$^+$ H460 cells showed a significant reduction of pAKT protein expression, but no significant difference in pERK and pEGFR expression. A greater and significant downregulation of pAKT, pERK and pEGFR in CRBP$^+$ compared to empty-transfected H460 cells was documented after 48 h of 5 ∝M *at*RA treatment. Finally, we showed that RARα expression was decreased and RARβ increased in CRBP$^+$ where compared to empty-transfected H460 cells. Therefore, the restoration of CRBPI influenced the proliferation and AKT signalling pathway in H460 cell line also in the presence of *at*RA treatment.

Conclusion

Further studied are needed to clarify if those CRBP-1-related pathways involved in NSCLC carcinogenesis may be modulated, with the possible beneficial opportunity for a more personalized chemotherapeutic regimens employing adjuvant retinoid therapy.

Abbreviations

NSLCC:Non-small lung cancer; atRA:all-trans retinoic acid; CRBPI:Cellular retinol binding protein type I; PPARs:Peroxisome proliferator active receptors; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; CK:Cytokeratins.

Declarations

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Authors' contributions

ED performed the experiments. AF and AO conceived the research. GC analyzed the data.

AF wrote the manuscript. GC helps to write the manuscript.

SA and FC helped to perform experiments. AO revised the manuscript and supervised the study.

All authors read the paper and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The Authors declare no conflicts of interest.

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Figures

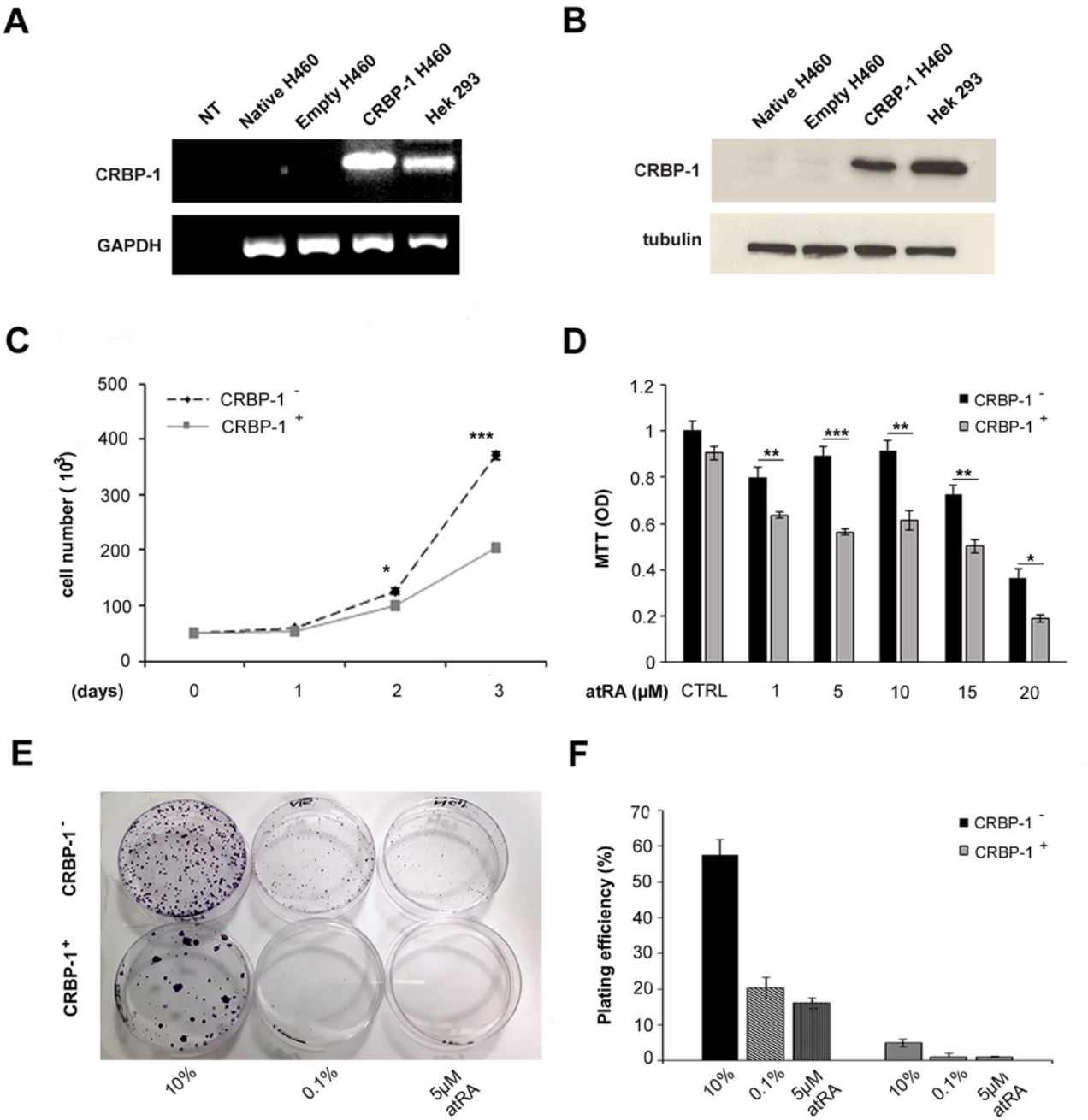

Figure 1

Figure 1

CRBPI transfection reduces viability and retinoid-related survival of H460 cells. A, RT-PCR and B, representative blots of protein expression in CRBPI-transfected in H460 cell line. Hek-293 cells as positive control. C, CRBPI+ H460 cells growth is increased compared to empty-trasfected cells. D, MTT assay shows reduced viability of CRBPI + compared to empty-trasfected H460 cells after 2 days of atRA treatment in the presence of 0.1% FBS. E-F, CRBPI + H460 cells both in basal condition and after 48h of

5μM atRA treatment showed a poor ability to form colonies compared to empty-transfected cells. Values expressed as means ± SEM of three different experiments: *p < 0.05, **p < 0.005, ***p <<0.001.

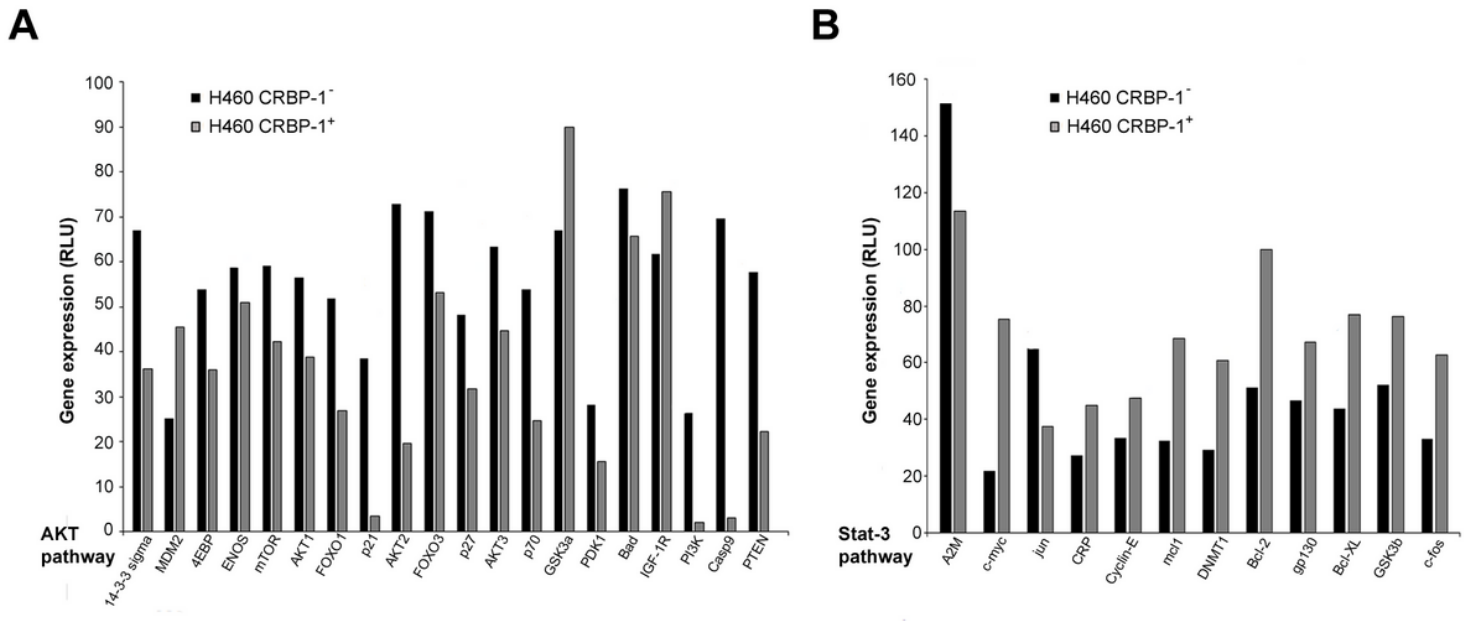

Figure 2

Figure 2

CRBPI transfection influences transcriptional pathways and differentiation of H460 cells. A-B, bar graph of gene array, showing modulation of genes involved in Akt and Stat-3 pathways in CRBPI + compared to empty-trasfected H460 cells.

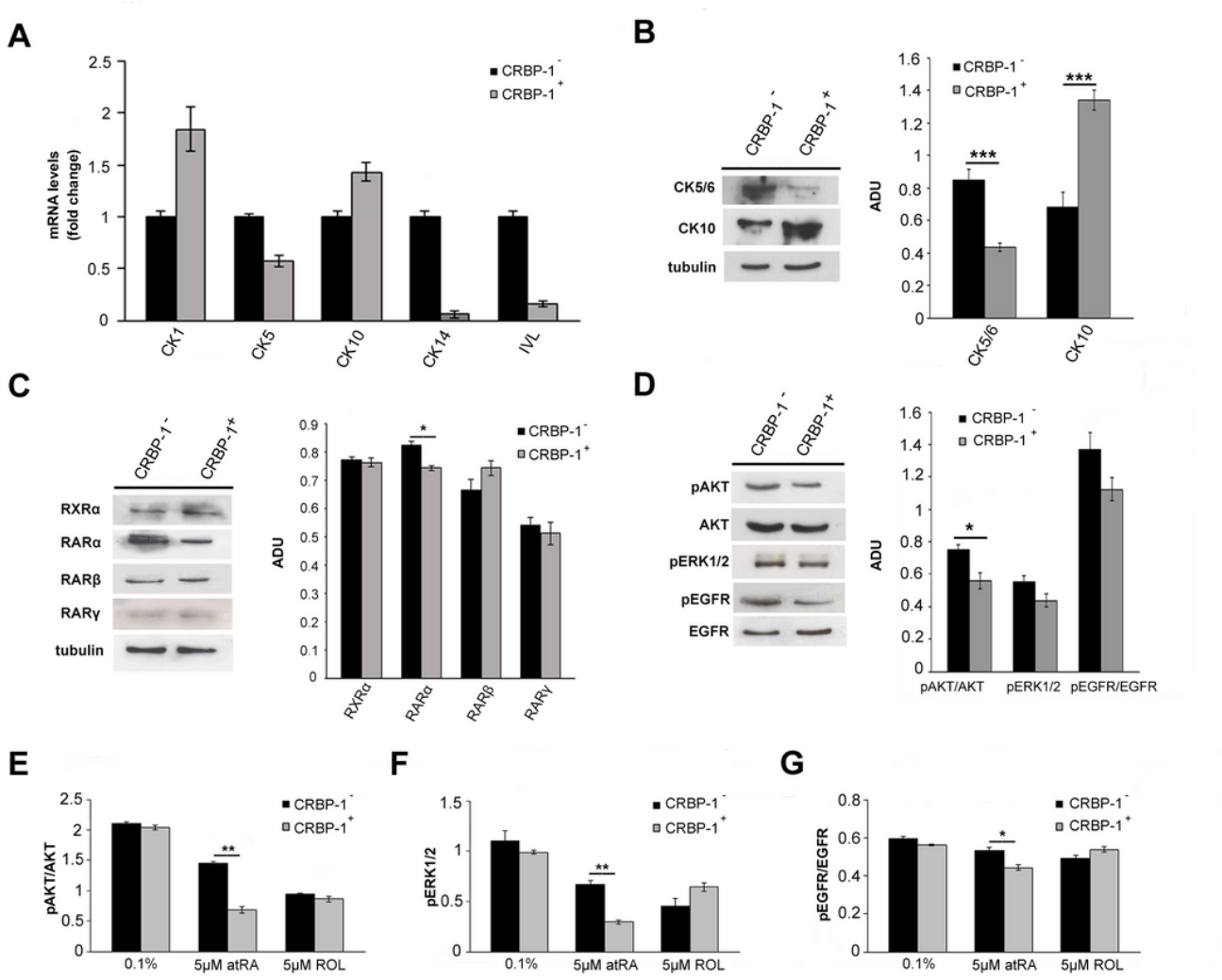

Figure 3

Figure 3

CRBPI transfection modulates cytokeratins and Akt-related gene and protein expression in H460 cells. A, Cytokeratins mRNA levels by Real-Time PCR. Representative blot and bar graph of (B) cytokeratins, (C) RARs/RXR and (D) pAKT/AKT pEGFR/EGFR and pERK1/2 protein expression in basal condition (10% FBS). E-G pAKT/AKT, pEGFR/EGFR and pERK1/2 protein expression in CRBPI + compared to empty-tranfected H460 cells after 48h of treatment with 5μM atRA and ROL. *p<0.05, **p<0.005 and ***p<0.001. Abbreviations: RLU, relative light unit; ADU, arbitrary densitometric units.

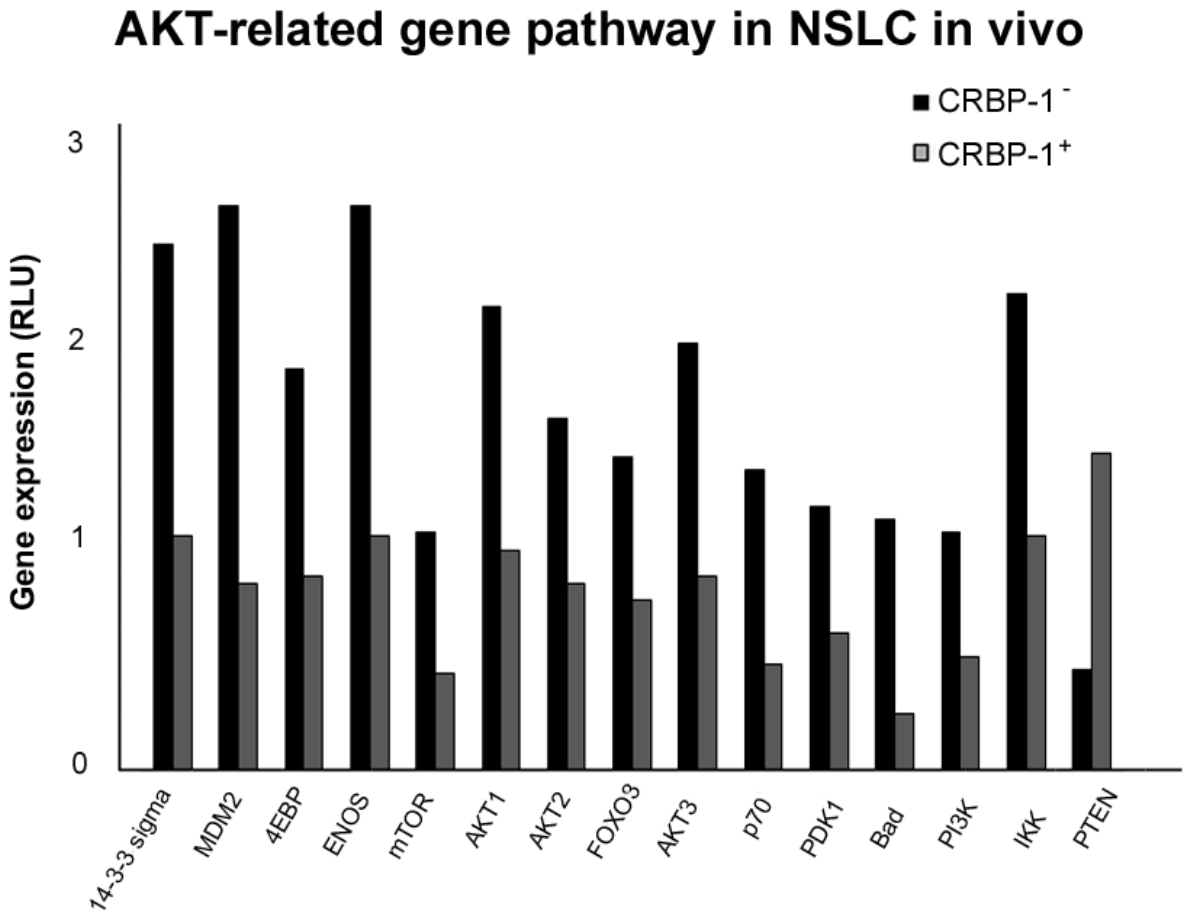

Figure 4

Figure 4

CRBPI transfection associates to modulation of AKT-related gene pathway in H460 cells. Bar graph showing the modulation of Akt-related gene in CRBPI + compared to CRBPI- tissue samples by gene array.