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Abstract

The 16-kDa N-terminal fragment of human prolactin (16K hPRL) is a potent angiostatic factor that inhibits tumor growth in mouse models. Using microarray experiments, we have dissected how the endothelial-cell genome responds to 16K hPRL treatment. We found 216 genes that show regulation by 16K hPRL, of which a large proportion turned out to be associated with the process of immunity. 16K hPRL induces expression of various chemokines and endothelial adhesion molecules. These expressions, under the control of NF-κB, result in an enhanced leukocyte-endothelial cell interaction. Furthermore, analysis of B16-F10 tumor tissues reveals a higher expression of adhesion molecules (ICAM-1, VCAM-1 or E-selectin) in endothelial cells and a significantly higher number of infiltrated leukocytes within the tumor treated with 16K hPRL compare to the untreated ones. In conclusion, this study describes a new anti-tumor mechanism of 16K hPRL. Since cellular immunity against tumor cells is a crucial step in therapy, the discovery that treatment with 16K hPRL overcomes tumor-induced anergy may become important for therapeutic perspectives.

Introduction

Angiogenesis is a pivotal process in the outgrowth and metastasis of tumors (16). We have shown previously that recombinant 16-kDa N-terminal fragment of human prolactin (16K hPRL) has angiostatic properties both *in vitro* and *in vivo* (8). In addition, its ability to prevent angiogenesis in tumor and retinopathy mouse models has raised interest in its potential therapeutic use (14, 17, 22).

Angiostatic factors act on endothelial cells at various biological levels (survival, proliferation, migration, organization) and target different molecular pathways. Better understanding of the underlying mechanisms will allow the development of effective angiostatic therapy. So far the mechanisms by which 16K hPRL inhibits angiogenesis have only been partially elucidated. The angiostatic effect appears to be mediated by an as yet unknown saturable high-affinity 16K PRL binding site distinct from the PRL receptor (2). Endothelial cell migration requires activation of proteases, including urokinase. It has been shown that 16K hPRL inhibits urokinase activation by increasing the expression of type 1 plasminogen activator inhibitor (7). 16K hPRL has also been shown to induce endothelial cell cycle arrest at both the G_0–G_1 and G_2–M phases through combined effects on positive and negative regulators of cell cycle progression (down-regulation of cyclin-D1 and cyclin-B1 and up-regulation of p21cip1 and p27kip1) (25). This cell-cycle arrest correlates with inhibition by 16K hPRL of vascular endothelial growth factor (VEGF)-induced Ras activation causing MAPK activation to be blocked (9). Finally, it has been demonstrated that 16K hPRL induces endothelial cell apoptosis. Signaling events associated with 16K hPRL-induced apoptosis include increased DNA fragmentation and activation of caspase-1 and caspase-3 (12). Furthermore, Nuclear Factor κB (NF-

κB) activation is required for 16K hPRL-induced apoptosis. It is necessary for activation of caspase-8 and -9, which in turn trigger caspase-3 activation and DNA fragmentation (18). Given this broad range of effects, the use of genome-wide analysis techniques such as microarray analysis appears to be a necessary tool to elucidate the mechanism of action of 16K hPRL. The identification of novel 16K hPRL-regulated biological processes and target genes should shed new light on the molecular mechanisms triggered by angiostatic compounds, thus contributing towards improved therapeutic strategies.

This study presents a genome-wide picture of how gene expression in endothelial cells responds to 16K hPRL treatment. This large-scale analysis identified a central role of NF-κB in the angiostatic action of 16K hPRL. Furthermore, our data support the idea that 16K hPRL affects not only survival and proliferation, but also inflammation, causing enhanced leukocyte-endothelial cell interaction by increasing levels of inflammatory and endothelial adhesion molecules. This is the first report to suggest a role of 16K hPRL in overcoming angiogenesis-associated endothelial cell anergy.

Results

Overview of the microarray data

Miocroarray analysis was used to investigate the response of endothelial cells to 16K hPRL. Adult bovine aortic endothelial (ABAE) cells were treated with 10 nM 16K hPRL prior to RNA isolation. In order to focus mainly on the primary response induced by 16K hPRL, endothelial cells were treated for a short period (2 h). Three separate experiments were performed and only genes showing significant (at least 2-fold) regulation untreated vs 16K hPRL-treated in all three experiments were considered for further analysis. Of all transcripts represented on the chips, 55% showed significant expression in endothelial cells. According to our criteria, 216 transcripts (1.7%) appeared to be regulated by 16K hPRL. Among these, 165 transcripts displayed induction (76%) and 51 repression (24%) after treatment. Based on public databases, 126 of the 216 transcripts are known genes and are listed in supplemental material 1.

Validation of the microarray data

For independent validation of the microarray results, ten 16K hPRL-regulated genes were chosen for. For these genes, a time course (from 1 h to 6 h) of mRNA expression was monitored after treatment with 10 nM 16K hPRL. All of the tested genes showed a similar regulation in qRT-PCR analysis. The time course analysis of induced genes revealed a maximum up-regulation between 2 and 4 hours after treatment, and showed normalization after 6 hours of treatment (Fig. 1A). For Sox18, down-regulation was maximal after 4 hours of treatment with 16K hPRL (Fig. 1B). To check the gene regulation at the protein level, we measured the levels of caspase-3,

ATF3 in EC before and after treatment with 10 nM 16K hPRL. The levels of both proteins were found to increase after the start of treatment and to reach a maximum after 4 to 6 h (Fig. 1C).

Regulatory network analysis

To identify new molecular networks or pathways involved in the action of 16K hPRL, we used Ingenuity Systems Pathways (Ingenuity® Systems, www.ingenuity.com) to analyze the regulated gene list. Networks significantly regulated by 16K hPRL were identified (See Supplemental material 2). Interestingly, the network with the highest score turned out to be involved in the immune response, motility and functioning of the haematological system. The expression levels of all the 35 genes in this network were significantly altered by 16K hPRL treatment. This computational analysis points out a central role of the transcription factor NF-κB, as can be seen in Figure 2A. To confirm the major role of NF-κB in the 16K hPRL response, we used the oPossum software (26) to analyze the over-representation of transcription factor binding sites in the 16K hPRL-induced genes with respect to a precompiled background set. Statistical analysis revealed that NF-κB family members (p65, p50 and c-Rel) are the transcription factors most significantly involved in the 16K hPRL response (p<0.0001, data not shown). These two independent bioinformatics analyses strongly suggest a key role for NF-κB in the primary molecular response induced by 16K hPRL in endothelial cells.

Activation of NF-κB in endothelial cells treated with 16K hPRL was confirmed by EMSA analysis on nuclear extracts from ABAE (Fig. 2B) and Human Umbilical Vein Endothelial Cells (HUVEC, Fig. 2C). Nuclear extracts of EC either or not stimulated with 16K hPRL (10 nM) for 45 min were incubated with a ^{32}P-labeled

κB DNA sequence before gel electrophoresis. Indeed, the band corresponding to the κB/NF-κB complex was more intense after treatment with 16K hPRL (compare lane 1 with lane 2). The specificity of this band was demonstrated by its reduced intensity in the presence of excess of an unlabeled κB probe (lane 5) and its unchanged intensity in the presence of an unlabeled mutated κB probe (lane 6). Furthermore, the p50/p65 heterodimer was identified by its immunoreactivity towards p50- and p65-specific antibodies (lanes 3–4). These experiments prove that 16K hPRL activates NF-κB in endothelial cells.

Functional analysis

Besides genes involved in functions already known to be affected by 16K hPRL, such as apoptosis or proliferation, many genes involved in inflammatory regulation have been found to be significantly induced in endothelial cells. 49 genes out of 126 could be connected with immunity or adhesion, such as pro-inflammatory cytokines belonging to the CXC family (*CXCL1*, *2*, *6*, and *8*) and the CC family (*CCL2*, *5*, *8*, *20* and *26*). In addition, 16K hPRL also up-regulates several genes involved in the adhesion of leukocytes to endothelial cells (*ICAM-1*, *VCAM-1*, *E-selectin*, and *P-selectin*). This functional analysis suggests that 16K hPRL could overcome angiogenesis-induced endothelial cell anergy.

16K hPRL increases leukocyte-endothelial cell adhesion via NF-κB.

The data above urged us to investigate the ability of 16K hPRL to regulate leukocyte-endothelial cell interactions. Leukocyte adhesion on endothelial cells was significantly higher after a 4-hour treatment with 16K hPRL (p<0.01, Figure 3). To assess the importance of NF-κB in the 16K hPRL-induced leukocyte adhesion, we

blocked NF-κB activation with BAY1170-82, a chemical inhibitor known to interfere specifically with IKK activation (23). A luciferase reporter assay (pElam-Luc plasmid coding for a luciferase reporter gene under the control of the Elam-1 promoter (3)) was first used to test the ability of BAY1170-82 to inhibit 16K hPRL-induced NF-κB activation in endothelial cells (Fig. 4A). 16K hPRL was found to induce luciferase activity in the transfected cells by >15-fold and this induction was inhibited in a concentration-dependent manner by pretreatment with BAY1170-82. Induction with 10 nM 16K hPRL was nearly completely abolished by BAY1170-82 at 5 µM (to only 2-fold).

Pretreatment of endothelial cells with this concentration of BAY1170-82 significantly reduces the expression of ICAM-1 and E-Selectin (Fig. 4B). The figure 4C shows that BAY1170-82 also significantly inhibited the effect of 16K hPRL on leukocyte adhesion both with ABAE and HUVEC endothelial cells ($p<0.05$). These results strongly suggest that leukocyte-endothelial cell adhesion induced by 16K hPRL is predominantly dependent of NF-κB activation.

16K hPRL increases leukocyte infiltration in tumors mice

To further investigate the effect of 16K hPRL on leukocyte infiltration in tumors *in vivo*, we used the B16-F10 mouse melanoma model. As recently demonstrated, 16K hPRL-treated mice showed reduced tumor incidence and tumor outgrowth, as demonstrated to be caused by angiogenesis inhibition (Nguyen *et al*. see footnotes). In order to analyze the expression of adhesion molecules in tumor treated or not with 16K hPRL, RNA were extracted and subject to qRT-PCR to measure level of ICAM-1, VCAM-1 and E-Selectin (Fig. 5A). These experiments reveal a

significant higher expression (p<0.05) of the three adhesion molecules after treatment with 16K hPRL.

To further characterize this response, immunostaining for ICAM-1 has been performed and confirms a higher expression in tumor vessels of 16K hPRL-treated mice. Interestingly, this regulation of adhesion molecules by 16K hPRL caused a significant 7-fold increase (p<0.05) in the number of infiltrated leukocytes (CD-45 positive cells) as compared to infiltration in untreated mice (Fig. 5B and C). Taken together, these results demonstrate that both at the mRNA and protein level, 16K hPRL induces over-expression of endothelial adhesion molecules in tumors *in vivo*. Furthermore, this expression leads to an enhanced infiltration of leukocytes into the tumor.

Discussion

Here we have employed genomic array analysis to examine how the endothelial transcriptome responds to treatment with 16K hPRL. Our study reveals for the first time the complexity of the biological effect of 16K hPRL in endothelial cells. In addition to provide new clues about the molecular pathways that mediate the proapoptotic and antiproliferative action of 16K hPRL, our microarray analyses reveal a new and unexpected function of 16K hPRL, namely the up-regulation of numerous inflammatory molecules. Chemokines are multifunctional mediators that can promote immune responses (29). 16K hPRL induces up-regulation of various CXC chemokines (CXCL1, 2, 6, 8) and CC chemokines (CCL2, 5, 8, 20, 26). It also induces up-regulation of adhesion molecules (ICAM-1, E-selectin, VCAM-1) known to play a role in leukocyte recruitment. Leukocyte rolling on, adhesion to, and diapedesis through the tumor vessel wall are processes very important in both immune surveillance and anticancer immunotherapy (19, 27). Leukocyte-vessel wall interaction requires expression on the endothelial cell surface of adhesion molecules such as ICAM-1, ICAM-2, VCAM-1, E-selectin, P-Selectin and CD34 (28). Down-regulation of these molecules is one of the escape mechanisms used by the tumor to protect itself against the immune system (20) and many studies show that angiogenic factors produced by the tumor down-regulate endothelial adhesion molecule synthesis (4, 5). This down-regulation results in a diminished leukocyte-vessel wall interaction and a reduced inflammatory signal. In this study we show that 16K hPRL treatment results in increased leukocyte-endothelial cell adhesion *in vitro*, and that this process requires expression of adhesion molecules and NF-κB activation. In addition, our studies reveal a higher expression of adhesion molecules *in vivo* as well as strong

leukocyte recruitment into mouse tumors treated with 16K hPRL. Taken together, theses results show for the first time, the role of 16K hPRL in mediating immune response. Our results suggest that 16K hPRL could be very useful in cancer therapy by its multiple simultaneous actions on endothelial cells: proliferation arrest, apoptosis, enhanced synthesis of endothelial adhesion molecules and chemokines. Here we show that an angiostatic factor upregulates immune molecules. Since others have shown that immune cells may contribute to angiogenesis (10), the beneficial effects of the 16K hPRL-induced immune response could be debatable. Yet because 16K hPRL induces tumor growth arrest and since leukocyte infiltration into tumors is often associated with better prognosis and survival (13, 21, 24), we favor the view that leukocyte recruitment is an important step in anti-tumor immunity. 16K hPRL might thus be expected to inhibit tumor growth both by preventing angiogenesis and by increasing the immune response.

Very recently, Kisseleva *et al.* reported on the role of NF-κB signaling in endothelial cell function *in vivo* (30). In this study Tie2 promoter/enhancer-IκBα$^{S32A-S36A}$ transgenic mice were generated. In these mice, endothelial specific promoter drives the expression of a dominant interfering IκB-α. Mutation of two critical serine-32 and -36 blocks IκB-α degradation and renders NF-κB inactive (11). Despite endothelial inhibition of NF-κB signaling, these mice developed and reproduced normally and they exhibited a normal pattern of vascular development. However, inoculated tumors grew faster in these mice and histological analysis revealed a striking increase in tumor vascularization in transgenic mice. This study highlights for the first time, the *in vivo* role of NF-κB in tumor angiogenesis. From this report, one can suggest that activation of NF-κB in endothelial cells appears as a promising way to block angiogenesis. Nevertheless, this report also begs several questions: The first

is how NF-κB activation could interfere with tumor angiogenesis and the second is how to specifically activate NF-κB in endothelial cells. In this paper, we describe that NF-κB activation is a key process for the angiostatic property of 16K hPRL. We have previously demonstrated that NF-κB activation is required to 16K hPRL-induced apoptosis, here our results support a new role of NF-κB in endothelial cells treated by angiostatic agent that could explain the anti-tumor activity of NF-κB. Our results highlight the crucial and global role of NF-κB in the action of an angiostatic agent. In addition, based on these results it could be argued that treatment with NF-κB inhibitors, which was frequently proposed to improve the apoptotic response of tumor cells to radiotherapy or chemotherapy (6, 15, 31), involves also mechanisms beneficial for tumor growth on the vascular compartment, suggesting a more careful use of this strategy.

In conclusion, we have described the full angiostatic response induced in endothelial cells by 16K hPRL. Next to 16K hPRL-regulated genes that could lead to understand how 16K hPRL induces proliferation arrest and apoptosis, we have discovered unsuspected links between 16K hPRL and the immune system. This paper shows that 16K hPRL induces leukocytes adhesion to endothelial cells by activation of NF-κB. The therapeutic potential of this discovery is substantial but further studies will be required to evaluate the precise contribution of this immune response to the 16K hPRL anti-tumor action. This study provides new road maps to understand the mechanisms whereby 16K hPRL, but also other angiostatic factors act on endothelial cells.

Materials and Methods

Production of recombinant protein and chemical compounds

Recombinant 16K hPRL was produced and purified from *E. coli* as previously described (18). The purity of the recombinant protein exceeded 95% (as estimated by Coomassie Blue staining) and the endotoxin level was 0.5 pg/ng recombinant protein, as quantified with "the rapid endo test" of the European endotoxin testing service (Cambrex Bioproducts Europe). The quantity of endotoxin present in 10nM of 16K hPRL has been found to have no effect on the biological effects observed in this paper. BAY1170-82 and cycloheximide (CHX) were purchased from Calbiochem.

Cell cultures

ABAE cells were isolated and cultured as previously described (1). Confluent cells corresponding to passages 8 to 13 were used in the experiments. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described (20). Only cells from passage 1 to 3 were used for experiments.

Biotinylated probe preparation and microarray hybridization

Total RNA was extracted using the Rneasy kit (Qiagen) according to the manufacturer's instructions. The quality and integrity of each RNA preparation was first verified on an Agilent platform (Agilent 2100 Bioanalyser). 5 µg total RNA was reverse-transcribed and biotin-labeled. Subsequent hybridization and scanning were performed according to the manufacturer's instructions (Affimetrix). "GeneChip bovine genome arrays" were used to analyze the expression level of 23,000 bovine

transcripts. The biotinylated probe was produced and microarray hybridization performed by the GIGA Genomics Facility (Liège, Belgium)

Microarray data analysis

Gescop software (Affymetrix) was used to analyze the chip data. The list was reduced by removing any genes identified as 'absent' under both untreated and 16K hPRL-treated conditions. Only transcripts with a fold change of more than 2 or less than -2 were considered to be respectively up-regulated or down-regulated. Genes were annotated using the NETAFFX definition file (release December 21, 2005) or by homology with human sequences. Gene symbols were fetched from OMIM (http://www.ncbi.nlm.nih.gov). The classification of the genes of interest was based on the functional information available from GenAtlas (http://www.dsi.univ-paris5.fr/genatlas) and PubMed (http://www.ncbi.nlm.nih.gov). Significant networks modified by 16K hPRL treatment were determined with the "Ingenuity Systems Pathways Knowledge Base" (Winter '05 release) and transcription factor representation was analyzed with the oPossum software (version 1.2) (http://sonoma.cmmt.ubc.ca/oPosssum).

qRT-PCR (Real-Time quantitative RT-PCR)

Total RNA was extracted using the Rneasy kit (Qiagen) according to the manufacturer's instructions. Synthesis of cDNA was performed starting with 1 µg total RNA which was reverse transcribed with the Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer's instructions. The resulting cDNA (300 pg) was used for Real-time PCR with the one-step 2X Mastermix (Diagenode, Liege, Belgium) containing SYBR green. Thermal cycling was

performed on an Applied Biosystems 7000 detection system. For all reactions, negative controls were run with no template present and random RNA preparations were also subjected to sham qRT-PCR (no reverse transcription) to verify lack of genomic DNA amplification. Relative transcript levels for each gene were obtained using the relative standard curve method and normalized with respect to the housekeeping gene GAPDH for in vitro assays and cyclophilinB for mice assays. Primers, whose sequences are available upon request, were designed using the Primer Express software and selected to span exon-exon junctions to avoid detection of genomic DNA.

Preparation of cell extracts

Total and nuclear protein cell extracts were obtained as previously described (18, 25). Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay reagent.

Western blot analysis

Western blot analyses were performed using conventional procedures with cell extracts (30 µg total protein) as described previously (25). Anti-caspase-3 and anti-ATF3 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Electrophoretic Mobility Shift Assays (EMSAs)

EMSAs were performed as previously described (18).

Luciferase reporter assays

NF-κB luciferase reporter assays were performed as previously described (18). Luciferase activity was normalized using the β-galactosidase activity with the the β-gal Reporter Gene Assay Kit (Roche).

Leukocyte adhesion assay

Human blood leukocytes were isolated by Ficoll gradient centrifugation (Amersham) and labeled with 10 µM 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probe) for 30 min at 37°C. Cells were washed twice with PBS and subsequently allowed to adhere to confluent endothelial cells for 1 h at room temperature. After washing, the cells were harvested and fixed for 30 min with 1% paraformaldehyde at room temperature. Leukocyte-endothelial cell adhesion was quantified by flow cytometry analysis by detecting the number of CSFE-labeled leukocytes *vs* endothelial cells (32).

B16-F10 Tumor and immunostaining

Primary B16-F10 tumors used in this study are described in Nguyen et al. (See footnotes). Tumors tissues were frozen at –70°C or fixed in 4% PFA for 3-4 h, dehydrated, and embedded in paraffin (Labonord, Templemars, France). The tissues were then sectioned (6 µm thick). For assessment of leukocyte recruitment and ICAM-1 expresion, tumor tissues were fixed in 80% MetOH (VWR, Leuven, Belgium) for 10 min at -20°C. Endogenous peroxidase was subsequently blocked with 3% H_2O_2/H2O (Sigma-Aldrich, Steinheim, Germany) for 20 min, and non-specific binding was prevented in normal rabbit serum for 1 h at room temperature. Sections were then incubated first with a rat monoclonal anti-ICAM-1 or CD45 (for 1 h at room temperature), and then with a biotinylated secondary antibody (1/400, DAKO,

Heverlee, Belgium) for 30 min at room temperature. This was followed by incubation with streptavidin/HRP complex (1/500, DAKO, Heverlee, Belgium). CD45 or ICAM-1 positive cells were visualized after coloring sections for 3 min with 3-amino-9-ethylcarbazole (AEC+) (DAKO, Heverlee, Belgium). The sections were finally counterstained with hematoxylin and mounted for microscopy.

Statistical Analysis

All value are expressed as means ± SD. All experiments were performed in triplicate at least three times. Comparisons between different treatments were assessed with Student's *t* test.

Figure legends

Figure 1: *Validation of selected 16K hPRL-regulated genes by qRT-PCR.*

RNA was extracted from ABAE cells stimulated for various times (from 1 h to 6 h) with 10 nM 16K hPRL and transcript levels were analyzed by qRT-PCR (A and B). Data were normalized by quantification of the GAPDH transcripts and are representative of at least three distinct experiments. Each bar represents the mean ± SD, n = 3. Western blot analysis of the ATF3 and caspase-3 (C) protein levels in extracts of ABAE cells treated for various times with 10 nM 16K hPRL. Protein level of β-tubulin was also measured as an internal control. Data are representative of at least three distinct experiments.

Figure 2: *Role of NF-κB in 16K hPRL signaling.*

(A) Most significant network obtained with "Ingenuity Pathway Analysis". The Network is a graphical representation of the molecular relationships between genes or gene products. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line. All lines are supported by at least 1 reference from the literature. All genes (n=35) in this network are significantly affected by treatment with 16K hPRL. EMSAs were performed on nuclear extracts from ABAE cells (B) or HUVECs (C) untreated (lane 1) or treated for 45 min with 16K hPRL (10 nM) (lane 2) and pre-incubated either without or with addition of p50-specific antibody (lane 3) or p65-specific antibody (lane 4). In competition assays, extracts were pre-incubated with a 50-fold molar excess of unlabeled wild-type (wt) (lane 5) or mutated (mut) probe (lane 6).

Figure 3: *16K hPRL increases leukocyte-vessel wall interactions in endothelial cells*

Human leukocytes were allow to adhere to monolayer of endothelial cells (ABAE cells or HUVECs) treated or not with 10 nM 16K hPRL for 4 hours. CSFE-labeled leukocytes are observed under a fluoresent microscope (A) or counted by flow cytometry (B). Results are presented as mean value ± SD (n=3) of relative leukocyte adhesion compared to untreated cells. (* p<0,01 *vs* Untreated)

Figure 4: *leukocyte-vessel wall interactions is NF-κB dependent.*

(A) ABAE cells transfected with the pElam-Luc reporter gene vector were pretreated or not for 1 h with BAY11-7082 (1 µM) before stimulation for 3 h with 10 nM 16K hPRL. Luciferase activities, normalized with respect to the β-galactosidase activity, are expressed as enhancement factors (treated versus untreated cells). B) RNA was extracted from ABAE cells pretreated or not for 1 h with BAY11-7082 before stimulation for 2 h with 10 nM 16K hPRL. Levels of transcript were analyzed by qRT-PCR. Data were normalized by quantification of the GAPDH transcripts and are representative of at least three distinct experiments. Each bar represents the mean ± SD, n = 3. (* p<0,01 *vs* 16K hPRL). C) Human leukocytes were allow to adhere to monolayer of endothelial cells (ABAE cells or HUVECs) pretreated or not for 1 h with 5 μM BAY11-7082 and then treated or not with 10 nM 16K hPRL for 4h. CSFE-labeled leukocytes are counted by flow cytometry. Results are presented as mean value ± SD (n=3) of relative leukocyte adhesion compared to untreated cells. (* p<0,05 *vs* cells treated with 16K hPRL without BAY1170-82).

Figure 5: *16K hPRL induces leukocyte infiltration in B16-F10 tumors.*

RNA was extracted from tumor treated or not with 16K hPRL and transcript levels were analyzed by qRT-PCR (A). Data were normalized by quantification of the cyclophilinB transcripts and are representative of at least three distinct tumor. Each bar represents the mean ± SD, n = 3,* p<0,05 vs 16K hPRL. Tumors sections stained for ICAM-1 expression (B) or leukocyte infiltration (C) (CD45 + cells) (brown) and conterstain with hematoxylin in B16-F10 tumor control or 16K hPRL treated mice.

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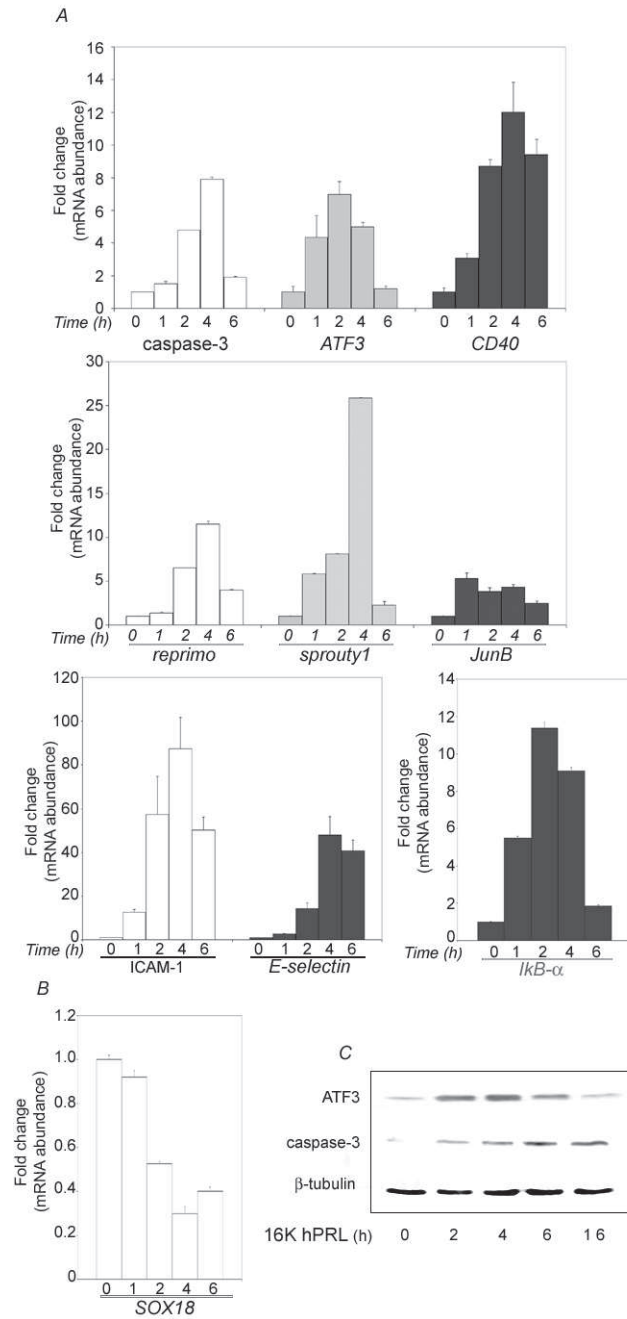
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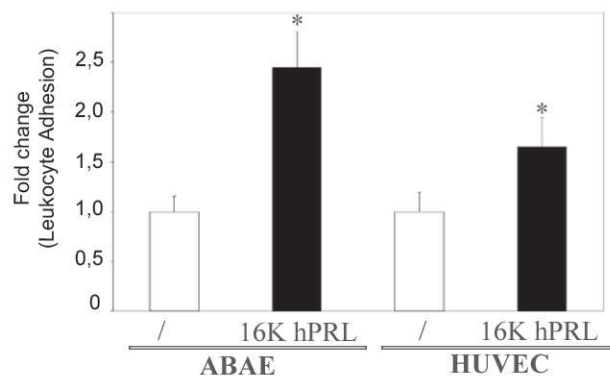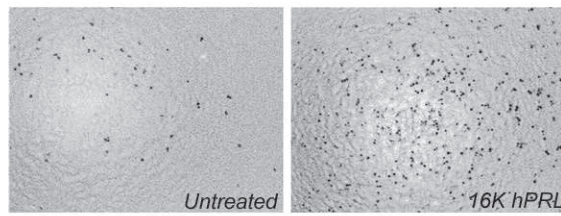
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Figure 2, Tabruyn et al.

Figure 4, Tabruyn et al.