The role of Id1 and FOXM1 in the response of various cancer cell lines to cannabinoid treatment

submitted at the

Austrian Marshall Plan Foundation

by

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Submitted on: 05.03.2013

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Abbreviations

bHLH	basic Helix loop Helix
Id	Inhibitor of DNA binding
CBD	Cannabidiol
THC	Tetrahydrocannabinol
FoxM1	Forkhead Box M1
FBS	Fetal Bovine Serum
BCA	bicinchoninic acid
BSA	bovine serum albumin
EDTA	Ethylendiamintetraacetat
TBS (T)	Tris buffered Saline (Tween)
TG(S)	Tris Glycine (SDS)
SDS	sodium dodecyl sulfate
PAGE	poly acryl amid gel electrophoresis
DMSO	dimethylsulfoxid

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1 Introduction:

Cancer nowadays is described as a broad group of diseases with over 200 different known types of cancers in humans. While in the beginning researchers thought that each type of cancer has to be treated like an individual disease – more recent publications suggest that the development of these different cancers is based on 6 underlying principles. These 6 mechanisms – also known as the hallmarks of cancer – may be sufficient to describe the development of most or even all types of human cancer. This is supported by the fact that the molecular mechanisms that drive proliferation, differentiation and cell death are the same in virtually all mammalian cells.[1]

The research done by Dr. Pierre Desprez points in the same direction – he is trying to find characteristics that are shared by a variety of different cancers rather than researching on only one individual type of cancer. His primary focus lies on tumors in an aggressive and invasive stage where the cancer already starts to spread and form metastasis. The treatment options that are available for such late stage cancer patients are still limited.

Dr. Desprez was the first to describe the importance of Id1 as a key regulator in the aggressiveness of human breast cancer. It was shown that mammary epithelial cells which are expressing Id1 were unable to differentiate but acquired the ability to differentiate and invade the extracellular matrix.[2]

Furthermore it was shown that Cannabidiol, a major constituent of CBD, is very potent in down regulating the expression of Id1 in aggressive breast cancer cells leading to a reduction of their aggressive behavior including a reduction of their invasive potential and their proliferation.[3]

The focus of this research project was to find out if the effect of Id1 down regulation by Id1 can be reproduced in other cancer cell lines as well as to check for the change in expression of FOXM1 – another gene playing an important role in the progression of cancer.

The Id protein family

Id – Inhibitor of differentiation/DNA binding – proteins belong to the helix loop helix protein family. The Id protein family consists of four members - Id1,2,3 and 4 and they all carry a well conserved Helix loop Helix domain. This HLH domain enables dimerization with bHLH transcription factors.[4] Dimerization is necessary for bHLH transcription factors to be activated and bind to DNA via the basic domain. The Id proteins however lack this basic domain so that by dimerization with bHLH transcription factors they inhibit DNA binding and therefore prevent the activation or inactivation of downstream events.[4]

The most important member of the Id protein family – concerning cancer development – is Id1. This protein has been identified to play an important role in the development of breast cancer already a decade ago.[5] It has been identified to regulate epithelial cell growth, differentiation and invasion in mammary epithelial cells.[6] Furthermore Id1 increases during the development of prostate cancer and has a significant influence on the expression of kallikrein 3 – a commonly used biomarker in this type of cancer.[7] Increased Id1 expression has been identified in over 20 different types of cancer.[8] In all these cancer types the increased expression of Id1 was correlated with an increased invasive potential, the formation of metastasis and higher proliferation. During earlier stages of cancer progression also the levels of Id1 expression are lower.

To confirm the oncogenic potential of Id1 a mammary epithelial cell line was transfected with a constitutively expressed Id1 gene. The cells than failed to differentiate, started to proliferate and became invasive.[2] In an in vivo experiment nude mice were infected with the MDA-MB231 breast cancer cell line – in one group the 231 cells expressed antisense Id1 and this group developed significantly fewer lung tumors compared to the group infected with the parent 231 cells.[2]

Id1 proteins are also expressed in healthy cells during development and embryogenesis but their expression in healthy adult cells is significantly lower and we couldn't detect it by Western Blotting.[9] The fact that Id1 is only weakly expressed in healthy adult cells makes this protein a promising target for anti

cancer therapies. Furthermore Id1 can be used as a molecular marker in various cancer types including breast and prostate cancer.[10]

CBD as an anticancer agent

CBD is a compound found in cannabis that already has proven medical effects. Although its structure is very similar to THC (Fig.1) - unlike THC it has no psychoactive effects when consumed and actually counter acts some of the effects of THC. The amount of CBD and THC in individual cannabis strains is inversed proportional so that CBD rich strains for medical use are grown today.[11]

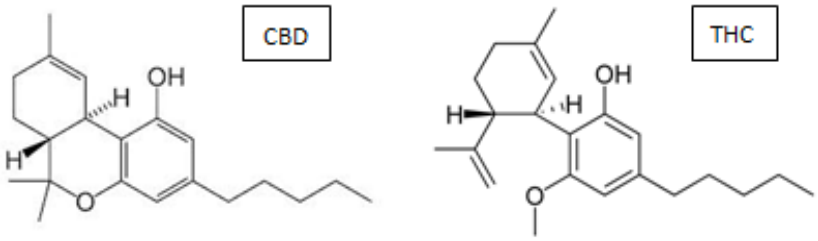

Figure 1 Comparison of the molecular structure of CBD and THC

CBD is currently used to treat inflammation, anxiety, nausea and chemotherapeutic side effects. While the anticancer effects of THC are already known – the clinical applications of this compound are limited due to its psychotropic activity. More recently also CBD has been shown to have anti cancerous effects being a potent inhibitor of cancer cell growth.[12] The role of CBD has been extensively studied in human breast cancer. Treating breast cancer cells in vitro lead to a significant reduction of their invasive potential and their proliferative abilities.[3] In fact the CBD treatment lead to the same effects as the knockdown of Id1 in those cells.[3] A microarray experiment – to this date unpublished – confirmed that Id1 expression in MDA-MB231 cells is strongly influenced by CBD treatment. Comparing untreated cells with CBD treated cells on an RNA level showed that Id1 is the second most influenced transcriptional

regulator out of the approximately 2600 transcriptional regulators in the human genome. This experiment was repeated twice with different hardware and the result was still consistent – however as mentioned the result was not published yet.

In addition to in vitro experiments the effect of CBD on breast cancer has also been shown with in vivo experiments. Nude mice have been infected with MDA-MB231 cancer and then treated with CBD – compared to the untreated control group the CBD treated mice showed a significant reduction in tumor volume and a reduced formation of metastasis.[12]

Foxm1 and its role in tumor Metastasis

Another important player in tumor progression is the protein FoxM1. It is over expressed in various human malignancies and has been identified to be over expressed in all human carcinomas.[13] In glioblastomas the high expression of FoxM1 correlates with the tumorigenicity of the glioma cells and in breast cancer an over expression of FoxM1 correlates strongly with a poor prognosis.[14]

FoxM1 expression is associated with proliferating cells only and in adult mammals it can only be found in progenitor and regenerating tissues.[15] Thus – similar to Id1 – FoxM1 is a promising target for cancer treatments as its expression is strongly correlated to proliferating tumors.

The microarray performed by Dr. Pierre Desprez laboratory showed that the expression of FoxM1 is strongly down regulated by CBD on an RNA level. In fact FoxM1 was the first transcriptional regulator to show up on the list of influenced genes – followed by Id1.

2 Aim of my studies

The goal of my work in the laboratory of Dr. Desprez was to show the effect of CBD on Id1 and Foxm1 at the protein level to confirm the results of the performed microarray. The effect has already been shown in breast cancer cell lines – I had to find out if this result can also be reproduced in other cancer cell lines.

The first step was the optimization of the drug treatment. I used 8 different cancer cell lines – each of them requires slightly different treatment conditions. The indicator for a successful treatment was a good down regulation of Id1 – shown on the Western Blots. Together with the drug treatment I had to optimize the Western Blot procedure, and the conditions to use the Id1 Antibody.

After the treatment conditions were optimized I was able to use the lysates and blot for FoxM1 – again this procedure had to be adjusted and the right conditions for the FoxM1 antibody had to be found.

3 Materials and Methods

Used cell lines

The following adherent cancer cell lines have been used to study the effect of CBD on the expression of Id1 and FoxM1:

AccM	derived from human adenoid cystic carcinoma, Metastatic form of Acc2,
DU145	prostate cancer cell line derived from brain metastasis, moderate Metastatic potential,
MDA-MB-231	breast cancer cell line isolated from pleural effusions of a Caucasian patient, highly invasive and proliferative potential
MDA-MB-436	breast cancer cell line derived from pleural fluid of a breast cancer Patient in 1976, high metastatic potential
PC3	prostate cancer cell line derived from bone metastasis, high Metastatic potential,
SAS	head and neck cancer cell line – derived from human tongue Carcinoma, high invasive potential
SF126	glioblastoma cell line derived from brain carcinoma,
U251	glioblastoma cell line derived from astrocytoma,

Western Blot

Western Blotting is laboratory technique developed in the late 70's. Many researchers at that time had to struggle with the problem that probing antigens while the proteins are still trapped in electrophoresis gels did not lead to satisfying results[16]. In different laboratories at that time a similar technique was found to solve this problem and make the proteins better accessible for immunological probing procedures. Similar to Southern Blotting - where Nucleic acids are

transferred to Nitrocellulose Membranes – they applied an electric field perpendicular to the gel thus transferring the proteins from the gel onto a nitrocellulose (or similar) membrane[17]. This revolutionized the way that proteins could be analyzed for many reasons: while the membranes are not only a lot easier to handle compared to gels, the proteins are also better accessible for ligand binding after immobilization. The gels can be used for more than one transfer and the membranes can be stripped and reprobed with different antibodies. Once immobilized on a membrane the protein pattern from the gel is conserved and can be stored for later probing[18].

While the basic idea of Western Blotting is always the same – many different ways of transferring proteins have evolved. Also the final detection and amplification of the target band on the membrane can be carried out in different ways. In this thesis I will summarize the most common used techniques in today's laboratories (MMIMB3).

The sample for the Western Blot is a protein lysate which can be derived from cells or tissues. This step involves the lysis of the cell. Among other techniques the use of an extraction regent which lysis the cells and releases their proteins is the easiest. To prevent degradation the lysis reagent contains Protease and Phosphatase inhibitors and a chelating agent.[19,20] The Western Blot itself basically consists of three parts: **Separation** of the Proteins in the lysate using a gel electrophoresis like SDS PAGE, Native PAGE, isoelectric focusing or 2D gel electrophoresis. During this step the proteins are focused in bands in the gel according to their size/structure, pI, electric charge or a combination of those features. During the **Blotting** the Proteins are transferred from the gel onto a membrane (either nitrocellulose or PVDF). Transferring the proteins out of the gel onto a membrane is the crucial step during a Western Blot and allows for a wide variety of analytical methods to be applied. During the **Detection** antibodies specifically bind to the protein of interest. These antibodies carry enzymes, radioactive labels or fluorescent dyes which enable to detect the location of the target protein on the membrane and also a quantification of the amount of protein detected.

Procedure

Cell lysis

After the drug treatment the media from the plates was aspirated and the plates were washed with 5mL plain RPMI (Invitrogen). The Mammalian Protein Extraction Reagent (Thermo Scientific) is than prepared by addition of Protease and Nuclease Inhibitors as well as EDTA (Thermo Scientific). Up to 400 µL of Extraction Reagent are applied to each plate and incubated at 4°C for 5 min. After Incubation a cell scraper is used to detach the cells from the surface. The lysate is transferred to a micro centrifugation tube and re suspended through a syringe for homogenization. The micro centrifugation tube is then spun down at 4°C and 12.000 rpm for 30 minutes. The supernatant is transferred to a fresh tube and used for Western Blot analysis.

BCA assay

For the determination of the protein concentration the BCA assay is used. As a standard a dilution series of BSA is taken with a range from 25 to 2000 mg/mL. 10µL of each of the standard dilutions as well as the samples from the cell lysis were pipetted in duplicate into a 96 well plate. The BCA reagent (Thermo Scientific) is prepared by mixing solution A and Bat a ratio of 50:1. 200µL of the prepared BCA reagent were added to each of the wells and mixed by pipetting. The plate was than incubated at 37°C for 30 minutes. Afterwards the absorption at 568 nm was read. The standards were used to determine a linear relationship between concentration and absorption to calculate the concentration of the unknown samples

Sample Preparation

The determined concentrations were used to calculate the amount of lysate needed to load a certain amount of protein on the gel. Before loading the sample onto the gel it had to be denatured. The needed amount of lysate was mixed with 12.5 µL of 4x Laemmli Buffer (Bio-Rad) and dH_2O was used to fill the volume up to

50 µL. The sample was then heated up to 95°C for 5 minutes in micro centrifugation tubes.

Gel Loading

45 µL of the denaturized samples were loaded onto a 4 – 15 % Tris-HCl SDS PAGE gel (Bio-Rad). TGS was used as a running buffer. The gels were run at 120 V for approximately 2 hours.

Transfer

Once the SDS PAGE was finished the gels were removed from the chambers and transferred into ice cold TG Buffer. The transfer chamber for a wet transfer was assembled. The gel was put onto a PVDF membrane (Bio-Rad) and a wet filter paper on top and beneath to form the transfer stack. TG buffer was used as a transfer buffer. The transfer was performed at 4°C and 85V for 2 hours.

Immunoblotting

After the transfer the PVDF membrane was blocked in 5 % skim milk solution for 1 hour and then incubated with the primary antibody (Id1 – BioCheck, FoxM1 – SantaCruz). All the used primary antibodies were diluted in 5 % skim milk solution and the membranes were incubated over night at 4°C. After the primary antibody incubation the membranes were washed three times with TBST for 10 minutes each. Then they were incubated in the proper secondary antibody diluted in 5 % skim milk for 2 hours at room temperature. After the incubation the membranes were again washed three times with TBST for 10 minutes each. To develop the blots the Chemiluminescent Substrate was prepared by mixing both reagents 1:1 (Superisgnal Femto, ThermoScientific). The substrate was applied directly to the film and the produced light was captured on an autoradiography film (Dutcher) and developed.

Cell culture

Culture conditions

All the cell lines were grown in regular RPMI medium supplemented with 10% FBS. 10 cm culture dishes were used and the culture conditions were 37°C and 5% CO_2.

Passaging

Once the plates reached 80% confluence they were passaged. The media was aspirated and the cells were washed with 5 mL plain RPMI medium. 1mL Trypsin was added to the plates and after the cells were completely detached 9mL RPMI (containing 10% FBS) were used to re suspend the cells. 1 mL of this cell suspension was transferred to a new 10cm cell culture dish and 9 mL RPMI (containing 10 % FBS) was added. The dishes were then put back into the incubator.

Freeze Down

For freezing down cells the plates had to reach a confluence of above 90 %. The media was aspirated and the cells washed with 5mL plain RPMI. 1 mL of Trypsin was added to the plates and then incubated at 37°Cuntil the cells detached from the dish. 9 mL of RPMI (containing 10% FBS) were added to the plates and the cell suspension was then transferred to a 10 mL Falcon tube and centrifuged at 12.000 rpm for 10 minutes. As a freeze down media FBS (containing 10% DMSO) was used. After centrifugation the supernatant was aspirated and the pellet was re suspended in 3 mL of freeze down media. The cell suspension was then transferred into 3 cryogenic vials – 1mL each. The cryogenic vials were stored at -80°C until the cells had to be used again.

Drug treatment

A very crucial factor for the success of the CBD drug treatment is the initial plating density of the cells. Prior to the drug treatment a certain amount of cells had to be seeded on each of the plates. To determine the amount of cells a Hematocytometer was used. CBD was reconstituted in Ethanol and therefore Ethanol was used as a vehicle control on the untreated plates. The drug treatment was carried out in 4 days.

Day 1:

A nearly confluent plate of the desired cell line was trypsinized to detach the cells and 10µL of the cell suspension was transferred to the Hematocytometer to determine the amount of cells per volume. The desired plating confluence can be found in Table 1 for each of the used cell lines. The amount of cell suspension needed per plate was calculated from the cell count and transferred to a fresh plate. RPMI (containing 10% FBS) was used to fill up the volume to 10mL. The plates were then put into the incubator over night.

Day 2:

The media was aspirated and the plates were washed with plain RPMI. Afterwards 10 mL of RPMI (containing 0.1% FBS) were added to each of the plates. The drug stock (concentration 50 mM) was diluted down to a concentration of 1.5 mM and 2.0 mM. 10µL of the diluted drug stock were added to the plates to reach a final concentration of 1.5 µM and 2.0 µM. For the vehicle control plates 10 µL of Ethanol were added. Afterwards the plates were pit back into the incubator.

Day 3:

The media was replaced with fresh RPMI (containing 0.1% FBS) on all of the plates and fresh drug was added as describe in day 2. The plates were then put back into the incubator.

Day 4:

On day 4 the cells were lysed and the proteins were collected as described in the Western Blot procedure.

4 Results

Id1 blots

Breast Cancer

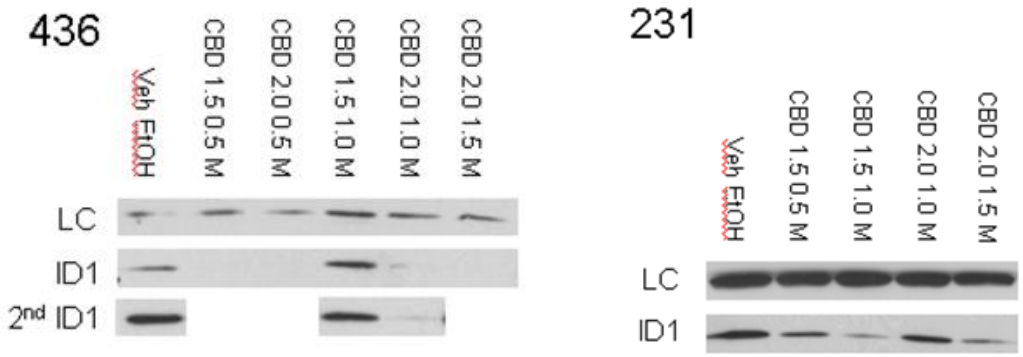

Figure 2 - Down regulation of Id1 expression upon CBD treatment in breast cancer cell lines MDA-MB-436 and MDA-MB-231. The used drug concentrations were 1.5 µM (CBD 1.5) and 2.0 µM (CBD 2.0). The cells were plated at different initial plating densities ranging from 500k cells (0.5M) to 1.5 Mio cells (1.5M).

Prostate Cancer

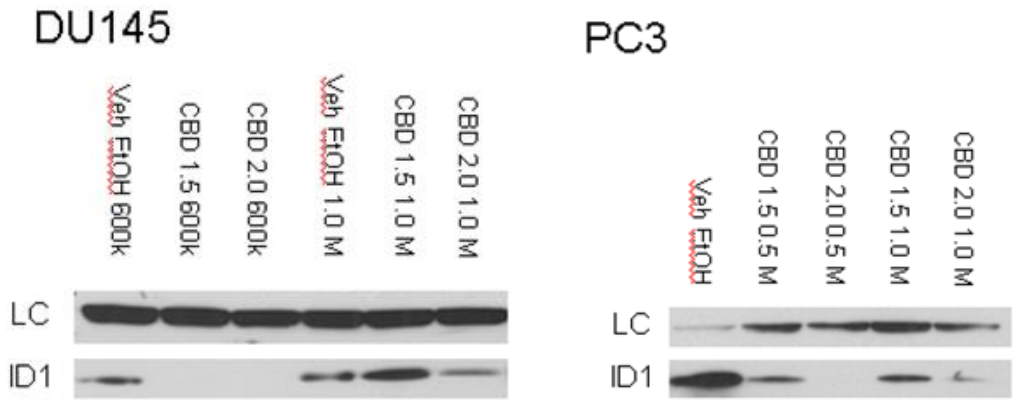

Figure 3 - Down regulation of Id1 expression upon CBD treatment in prostate cancer cell lines DU145 and PC3. The used drug concentrations were 1.5 µM (CBD 1.5) and 2.0 µM (CBD 2.0). The cells were plated at different initial plating densities ranging from 500k cells (0.5M) to 1.5 Mio cells (1.5M).

Glioblastoma:

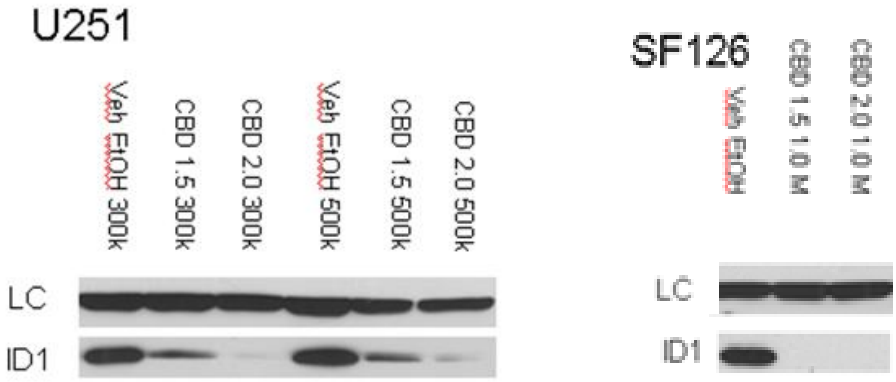

Figure 4 - Down regulation of Id1 expression upon CBD treatment in glioblastoma cell lines U251 and SF126. The used drug concentrations were 1.5 µM (CBD 1.5) and 2.0 µM (CBD 2.0). The cells were plated at different initial plating densities ranging from 300k cells (300k) to 1 Mio cells (1.0M).

Head and Neck Cancer

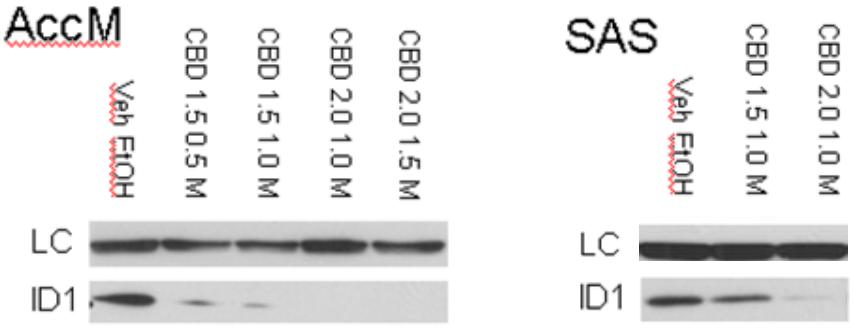

Figure 5 - Down regulation of Id1 expression upon CBD treatment head and neck cancer cell lines AccM and SAS. The used drug concentrations were 1.5 µM (CBD 1.5) and 2.0 µM (CBD 2.0). The cells were plated at different initial plating densities ranging from 500k cells (0.5 M) to 1.5 Mio. Cells (1.5 M)

FoxM1 Blots

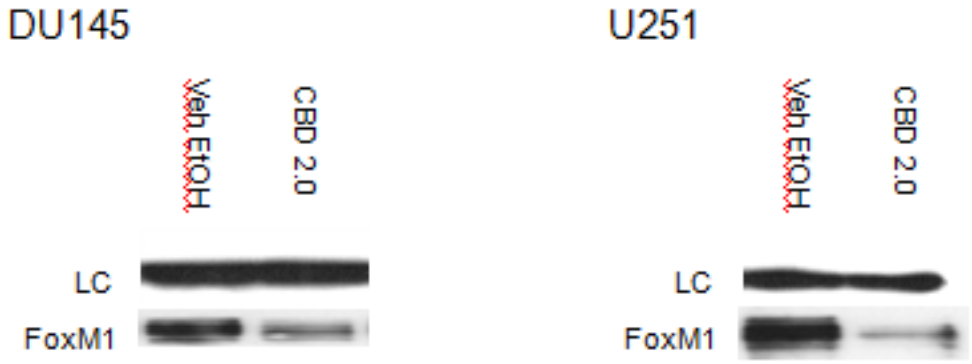

Figure 6 - Down regulation of FoxM1 expression upon CBD treatment in prostate cancer (DU145) and a glioblastoma cell line (U251). The used CBD concentration was 2.0 µM (CBD 2.0). Actin was used as a Loading Control (LC)

Ideal Plating Confluence

Cell Line	Ideal plating confluences
AccM	1.0 – 1.5 Mio
DU145	600 k – 1.0 Mio
MDA-MB-231	600 k – 1.0 Mio
MDA-MB-436	500 k – 1.0 Mio
PC3	500 k – 1.0 Mio
SAS	800 k – 1.0 Mio
SF126	600 k
U251	300 k – 500 k

Table 1 - Ideal plating confluences for the used cancer cell lines

5 Discussion

As seen on Fig.2 – 5 it was possible to successfully down regulate Id1 expression in all the used cancer cell lines. As expected the higher concentration of 2.0 µM CBD had a stronger effect on the expression of Id1. In the MDA-MB-436 cells it was possible to get a down regulation of nearly 100 % - this effect is similar or even stronger than the knockdown which can be achieved by using siRNA (Fig 2). The MDA-MB-231 cancer cell line – which is more aggressive – was also sensitive to the CBD treatment – but at a plating confluence beyond 1.0 million cells the down regulation became less than 50% (Fig 2). Both Prostate cancer cell lines showed a complete down regulation of Id1 at around 500 k cells and a CBD concentration of 2.0 µM – a higher initial plating density leads to an unsatisfying down regulation (Fig 3). The same result can be observed on both of the glioblastoma cell lines (Fig. 4) and the two head and neck cancer cell lines (Fig 5). This data shows that a higher number of cells decreases the effect of CBD on the individual down regulation of Id1. The data was used to compile Table 1 – showing the ideal plating confluences for a successful or complete down regulation of Id1. This shows that the result of the microarray is also consistent on the protein level and that CBD is effective in all the used cancer cell lines.

The lysates which had a nearly complete down regulation of Id1 were used to blot for another important transcriptional regulator – FoxM1. It was able to optimize the Western Blot conditions in one glioblastoma (U251) and one prostate cancer cell line (DU145). The Blots show a result which is consistent with the microarray – once Id1 is down regulated in the cells (CBD 2.0) the FoxM1 expression goes down as well (Fig. 6)

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