

***Id-1 Gene and Protein as Novel Therapeutic Targets for
Metastatic Cancer***

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by
Yacine BENHAMOU

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Practical Training Semester at the California Pacific Medical Center

Degree program: Medical and Pharmaceutical Biotechnology, IMC FH Krems

Internal Supervisor: Professor Christoph Wiesner

External Supervisor: Dr. Pierre-Yves Desprez, Dr. Ryuichi Murase

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List of Abbreviations Used

APS	Ammonium persulfate
BCA	Bicinchoninic acid
BSA	Bovine serum
CV	Cristal violet
CBD	Cannabidiol
ID1	Inhibitor of DNA-binding 1
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
HRP	<i>horseradish peroxidase</i>
MMP	Matrix Metalloproteinase
M-PER	Mammalian Protein Extraction Reagent
MW	Molecular weight
PAGE	Polyacrylamide gel
Ponceau	Ponceau staining
PVDF	Polyvinylidene fluoride
RT	Room temperature
SAS	Squamous cell carcinoma
SDS	Sodium Dodecyl Sulfate
ShRNA	small hairpin RNA
TBS	Tris- <i>buffered</i> saline
TBST	Tris- <i>buffered</i> saline transfer Tween 20
TEMED	Tetramethylethylenediamine
TG	Tris-Glycine
TGS	Tris-Glycine SDS

1. Abstract

Cancer progression is a multi-step process, and the invasiveness properties and metastasis of cancer cells during the final steps of tumor progression have been the least understood genetically [1]. Recent studies show that a specific protein, ID1, may represent a molecular target for aggressive cancer and metastasis therapy. Interestingly, the gene encoding for ID1 is expressed during embryogenesis where it induces proliferation, migration and invasion, but remains silent in normal adult tissues [2]. However, recent studies showed that the re-expression of ID1 gene contributes to cancer aggressiveness and metastasis. Ectopic expression of ID1 or reduction of its expression influences tumor behavior considerably. Indeed, ectopic expression in non-aggressive cancer cells rendered them highly proliferative and invasive due to an increase in the levels of matrix metalloproteinase (MMP). On the contrary, a decrease in ID1 gene expression in highly metastatic cells using shRNA re-differentiated the cells and rendered them much less aggressive [3]. Moreover, according to a recent study, a nontoxic compound had the capacity to target the ID1 gene and turn off its expression. This compound, called cannabidiol (CBD), down regulates ID1 gene expression in all aggressive human cancer cells tested, i.e., from breast, brain, prostate or head and neck origin. As a result, CBD could inhibit tumor cell proliferation and invasion in cell culture, and metastasis in animal models [1]. The goal of this research is to identify the mechanisms by which ID1 acts as a key gene for the regulation of tumor cell aggressiveness.

2. Introduction:

Metastatic cancer:

Metastasis occurs when cancer cells spread from one part of the body to another part of the body [4]. In order to spread, cells travel through two possible paths via the blood stream or the lymph system [5]. Few cells will survive during this travel. The cells that survive settle in another part of the body, and proliferate and form secondary tumors [5]. Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumor [6].

Often, the primary tumors are located in a non-vital organ and thus surgical removal is sufficient to eliminate the cancer. However when metastasis occurs, the tumor cells tend to migrate to parts of the body that cannot simply be removed, such as the brain or the spine [6].

Recent studies showed that genetic alteration such as evasion of growth suppression or of DNA-damage checkpoints contributes to metastasis. As mentioned before, few cells survive and successfully colonize the distant organ. Healthy tissues provide many barriers. Therefore, in order to achieve metastasis, cancer cells must surpass these barriers, which have been established through millions of years of organismal evolution. Cancer cells evolve to overcome the body's natural barriers. Metastasis is thus similar to an evolutionary process [6].

Other studies showed that it is rare to find all the characteristics of a metastatic cell within malignant cell populations. Therefore, in order to successfully execute the metastasis cascade, many genes involved in the regulation of cell-cell interaction, migration, invasion or proliferation are necessary [6].

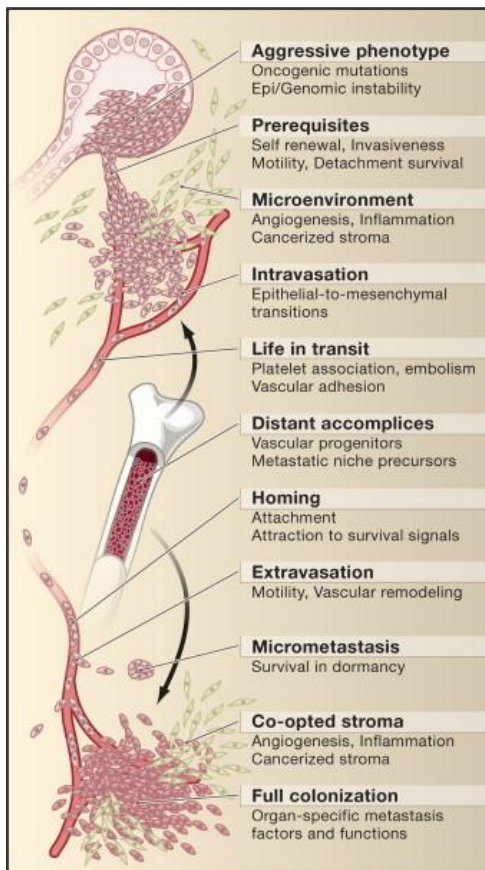


Fig 1: Stages of Metastatic Progression

ID gene and protein expression:

Inhibitor of DNA binding, also known as ID protein, is composed of four known members, ID1, ID2, ID3 and ID4. They belong to the helix-loop-helix group of transcriptional regulators. Most of this family contains DNA binding domain, but ID members do not. In order to bind, the DNA domain activates the promoter and thus the transcriptional process starts. The presence of two bHLH is necessary [7]. It is composed of two helices linked by a loop, containing two homodimers or heterodimers that bind to the DNA domain. When ID is expressed, it will bind to one member of the bHLH dimer and thus will prevent the binding to DNA. Promoters therefore cannot be activated [6].

ID proteins act as key regulators for developmental and cellular processes.

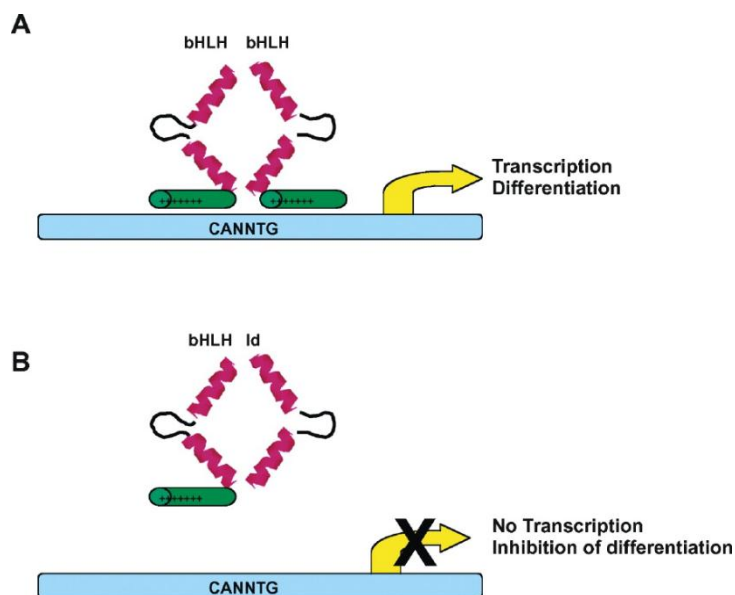


Fig 2: Model for ID effects on transcription

It has been found that the overexpression of ID genes affects key oncogenic pathways [7].

Matrix metalloproteinases (MMPs):

MMPs are considered to be major enzymes that degrade the extracellular matrix. The timely breakdown of ECM is essential for several processes like embryogenesis, angiogenesis and metastasis [8]. Recent studies showed that MMPs are involved in the release of several biological fragment such as growth factor and therefore regulates cell behavior, such as apoptosis, cell differentiation or cell migration. Usually expressed at low levels in a normal cell, MMPs play a critical role in cancer cells where they are usually over expressed [9].

Epithelial to mesenchymal transition (EMT):

Several oncogenic pathways may induce an EMT. However, the concept of EMT is not completely accepted among researchers. If a tumor cell undergoes an EMT, it could explain the detachment from the neighboring cells, and the entrance to the bloodstream to seed secondary tumors [10].

One of the characteristics of EMT is the high level of invasiveness. Tumors derived from epithelial cells can become more motile and invasive by acquiring characteristics of mesenchymal cells [11]. EMT facilitates intravasation of tumor cells into blood or lymph vessels and subsequent formation of distant metastases [10].

HYPOTHESIS UNDER FIRE

Reactivation of a key developmental pathway could explain how tumour cells invade distant tissues.

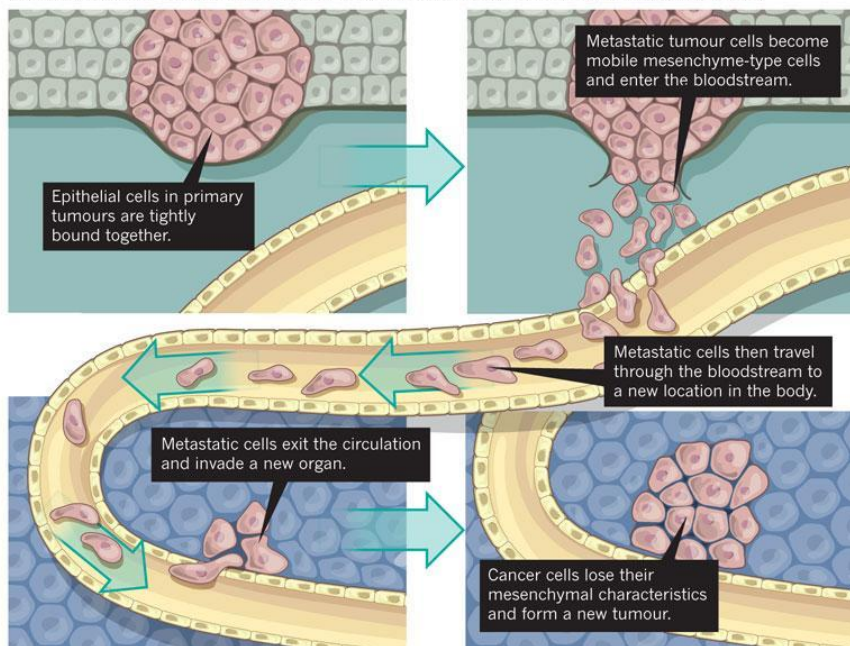


Fig 3: EMT pathway

The role of ID 1 gene and protein in metastatic cancer:

As mentioned earlier, ID1 inhibits the basic HLH transcription factors and renders epithelial cells unable to differentiate. These cells also acquire the ability to proliferate and to invade the extracellular matrix due to an increase in MMPs expression. Moreover, a high level of expression of ID1 in the majority of infiltrating grade III breast carcinomas of ductal origin suggested that ID1 might serve as a reliable marker for breast cancer progression, invasion and metastasis [3].

In recent studies, the knockdown of ID1 expression rendered metastatic breast cancer cells less invasive *in vitro* and less metastatic *in vivo*. In addition the expression of MMPs decreased in proportion to the decrease in ID1 protein levels, which implies potential therapeutic approaches to tumor metastasis. ID proteins play an important role for the spreading of the cancer [3].

ShRNA –SiRNA:

The function of small hairpin or short RNA is to alter the expression of a gene in a living cell or organism by inducing sequence specific gene silencing [12]. It is encoded by a plasmid and then inserted into a lentivirus. This lentivirus is then able

to enter inside the cell where the H6 promoter is activated, so shRNAs are expressed.

Neoangiogenesis:

The formation of new blood vessels is crucial for the development of a cancer. Here, we will focus on one type of pro-angiogenic factor which is the Vascular Endothelial Growth Factor (VEGF).

Cannabidiol (CBD):

One of the pharmacologically active compounds that bind to specific G protein coupled receptor is cannabidiol. Cannabis sativa is the origin of the plant's phytocannabinoids. Endogenous cannabinoids are made naturally by humans and animals, while synthetic cannabinoids are produced in the laboratory. Two types of cannabinoid receptors are found in the body. The first type is found in the brain (CB1), and immune cells express the second type (CB2). The modulation of signaling pathways central to the growth and spread of cancer can be triggered by cannabinoids [12]. As mentioned previously, ID1 expression has a high influence on cell proliferation, invasiveness and also metastasis. CBD down regulates the expression of ID1, hence providing a rational therapeutic strategy for the treatment of aggressive human cancer [1].

Cell line used:

SAS is a cell line derived from squamous cell carcinoma, which is one of the most devastating and fatal forms of nonmelanoma skin cancer. More than 67% of the head and neck cancers are squamous cell carcinoma. ACCM is a cell line derived from salivary gland cancer.

3. METHODS:

a. Western blot:

Introduced in 1979 western blotting is a common technique used for protein analysis. It uses specific antibodies to detect proteins which have been separated according to their size via gel electrophoresis. This detection is based on antibody antigen interaction, which enables a target protein to be identified producing a qualitative and semi-quantitative data about the protein [13].

Procedure:

Mammalian Protein Extraction Reagent is used. Cytoplasmic and nuclear protein from cultured mammalian cells is extracted using MPER. The samples are kept at 4°C and the fresh inhibitors are added to the lysis buffer. Halt Protease and Phosphatase Inhibitor Cocktail is used.

The protein assay used is the Bicinchoninic Acid (BCA) assay. Once the protein concentration is known, it is frozen at -80°C or used for loading onto a gel. The sample is taken out from freezer and melted at RT. Usually 9 standards in duplicates and a define number of sample in duplicates are used. An amount of 10 µl of standards and 10 µl of protein sample is applied in the corresponding well of the 96 well plates. Then 200 µl of Reagent A and B is applied in each well with a ratio of 50:1 of A to B. It is then incubated for at least 30 min at 37°C in the incubator.

The preparation of sample is then done: 44 µl is the total amount prepared. It is composed of 22 µl protein sample and 22 µl loading sample. Then the samples are put on heating plate during 10min at 65°C.

A precast of 4-15% is used for the electrophoresis. Criterion™ precast gels are used. The apparatus is prepared for the electrophoresis. SDS PAGE is the type of electrophoresis used. The marker used here is the novex sharp pre-stained. Each slot contains a maximum of 40 µl of sample otherwise the sample can spill into adjacent slot and give wrong data. 38 µl is usually loaded into the slot.

When all the samples are loaded in the gel, the gel starts to run until the bands reach the bottom of the gel. The power is then turned off. The transfer has to be done immediately so that the proteins do not elute.

The transfer of proteins from the gel to a membrane can be done under two conditions: the wet or semi-dry transfer. As the wet transfer is less likely to fail, it is the one used in the experiment.

The membrane and the gel are placed together with filter paper between the two electrodes. The proteins possess a negative electric charge which was added by SDS. The membrane has the function of blotting the protein. The PVDF membrane is hydrophobic, therefore before the transfer the membrane has to be soaked with methanol.

In order to have an efficient transfer, an adequate balance of SDS methanol in the transfer buffer, the protein size and gel percentage has to be done. Then it is run at 75V for 3hrs or 85V for 2hrs. After the transfer, the total protein on the membrane is often stained with a dye, such as Ponceau S to check the transfer efficiency.

The primary antibody (usually ID1 protein or actin) binds directly to the protein which is on the membrane. The secondary antibody (anti rabbit or anti mouse) is labeled with a molecule of HRP and binds to the first antibody. Dilution factors for the primary and secondary are between 1/1000 and 1/3000. In order to control the incubation time in the developing agent and fixation agent, manual film development is used.

Washing buffer TBST:

- TBS

TBS 10x (concentrated TBS):

- 24.23 g Trizma HCl
- 80.06 g NaCl
- Mix in 800 ml ultra-pure water.
- pH to 7.6 with pure HCl

TBST:

For 1 L: 100 ml of TBS 10x +
900 ml ultra-pure water + 1ml
Tween20

Leammli 2x buffer:

- 4% SDS
- 10% 2-mercaptoethanol
- 20% glycerol

- 0.004% bromophenolblue
- 0.125 M TrisHCl

Loading buffer:

- 450ml H₂O
- 50ml TGS 10x

Transfer buffer:

- 150ml TG10x
- 300ml Methanol
- 1050ml H₂O

Preparing Blocking Buffer: Skim milk:

- Pour 30ml of TBS
- Heat for 12sec
- Add 5% of Dry milk => 1.5g
- Stirring for at least 5 min
- After that the protein are transferred to the membrane it is blocked for 1 hour with skim milk

b. Zymogram:

In order to test for proteolytic activity, zymogram gels are used. The gel contains gelatin that acts as a substrate for proteases that are separated on the gel. The renaturing solution contains renaturing enzymes which detect the proteases. The development period starts when the protease breaks down the substrate. The coomassie Blue R 250 stains the substrate and leaves white areas around the active proteases [14].

Procedure:

The ready gel zymogram is used with gelatin 10%. The sample preparation consists of a ratio 1:1 of 22ul of sample + 22ul of sample buffer. The running conditions need a voltage of 100V during 90 min.

Then, the renaturing process has to be done. The gel is put into the renaturing solution. The total volume used for the renaturing solution is 200ml. 5ml of triton and 195ml H₂O. After 40 min, the membrane is then put into the development solution. The total volume used is 200ml including 2.34g of NaCl, 0.14g of CaCl₂ and the rest of H₂O. The staining solution is then prepared. It is composed of 40% of methanol, 10% acetic acid and 0.5% coomassie blue and the rest of H₂O. Finally the de-staining solution is prepared. It is composed of 40% methanol and 10% acetic acid.

c. Invasion Assay:

It is a very common technique used to determine the degree of invasiveness of different type of cells. The invasion is one of the characteristics of angiogenesis, embryonic development, immune response, and metastasis of cancer cells [15]. We used 24 well plates consisting of 2 chambers separated by Matrigel. Cells are suspended at the top of the chamber and, at the bottom chamber, the filter and the test media are settled. Cells migrate from top to bottom of the filter. The detection of cell invasion is then quantified [15].

Procedure:

Staining and counting part: 2.5% of glutaraldehyde in PBS and 33% of crystal violet (CV) in PBS are prepared. Then, we aspirate media from the wells. 320µl of 2.5% glutaraldehyde is pipetted into each well. Filter wells are fixed in the 2.5% glutaraldehyde solution for 15 min.

320µl PBS is added in to each well after removal of the fixing solution. Then, after washing, 300µl 33%CV staining buffer is added to each well for 20-30min at RT. After staining, using tweezers and a twisted kimwipe, we gently remove the Matrigel from the filter wells. We finally add 200µl of PBS to each of the filter well, and use the light microscope to count the total number of cell attached to the bottom of each filter well.

d. MTT Assay:

MTT assay is one among a number of chemosensitive assays which is based on cell viability. This test show that in order to obtain a colored formazan product a reduced form of salt: [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide] (MTT) is used [16].

Each cell type can only be applied to a limited number of cells because this test is based on metabolic rate (taking place in mitochondria and based on dehydrogenases activity [17].

Procedure:

The suspension cells are harvested by centrifugation. Adherent cells are trypsinized in order to be released from their substrate.

The cells are resuspended at 1×10^6 per ml.

Serial dilutions of cells are prepared in culture medium from 1×10^6 to 1×10^3 cells per ml.

100 μ l of the dilutions is plated out, in triplicate, into wells of a microtiter plate.

Three control wells of medium are included alone to provide the blanks for absorbance readings.

The cells are incubated under appropriate conditions for the cell line for 6 to 48 hours. Overnight is sufficient for most cell types.

10 μ l of MTT Reagent is added to each well, including controls.

Plate is returned to cell culture incubator for 2 to 4 hours.

The cells are checked periodically under an inverted microscope for presence of intracellular punctate purple precipitate.

100 μ l of Detergent Reagent to all wells, including controls is added to the purple precipitate when it is clearly visible under the microscope. It is important to not shake but to swirl gently.

Plate with cover is left in the dark for 3 hours at room temperature.

Plate cover is removed and the absorbance in each well is measured, including the blanks, at 570 nm in a microtiter plate reader. [Absorbances read in the wavelength of 570nm. The reference wavelength is 700 nm. The blanks should give values close to zero (+/- 0.1).]

The average values are determined from triplicate readings and the average value is subtracted to obtain the blank. Plots consist of absorbance in the Y axis and the number of cells/ml in the X axis. The number of cells to use in your assay should lie within the linear portion of the plot and yield an absorbance of 0.75[18].

4. RESULTS & DISCUSSION

In order to determine whether ID1 knockdown has an influence on the regulation on proliferation and invasion, several experiments were performed. Different methods were used: the technique of shRNA or using the compound CBD to reduce ID1 gene expression. The method of western blotting was applied, but we also used zymography, invasion assay and the MTT assay.

Quantitative Western Analysis:

The protein were separated by SDS PAGE then blotted on Immunobilon membrane and probed with the appropriate primary and secondary antibodies. Band intensity values were obtained from the blot. [1]

Invasion Assay:

Invasion assay was performed as described in the methods. Filters were coated with Matrigel. Cells were added to the upper chamber in serum free medium.[1] After incubation, the bottoms of the filter were fixed and cells counted by using light microscopy. Cell were assayed in triplicate or quadruplicate.[2]

MTT Assay:

In order to quantify cell proliferation, the MTT assay was used. Cells were seeded in 96 well plates. Cells, which were previously treated with the compounds, were incubated and then isopropanol with HCl were added. Absorbance was read and the percentage control was calculated. [1]

Zymogram:

The samples were loaded on the gels. The gels were run and then treated by the renaturing solution and the development solution, where the MMPs degrade the gelatin by showing a white band.

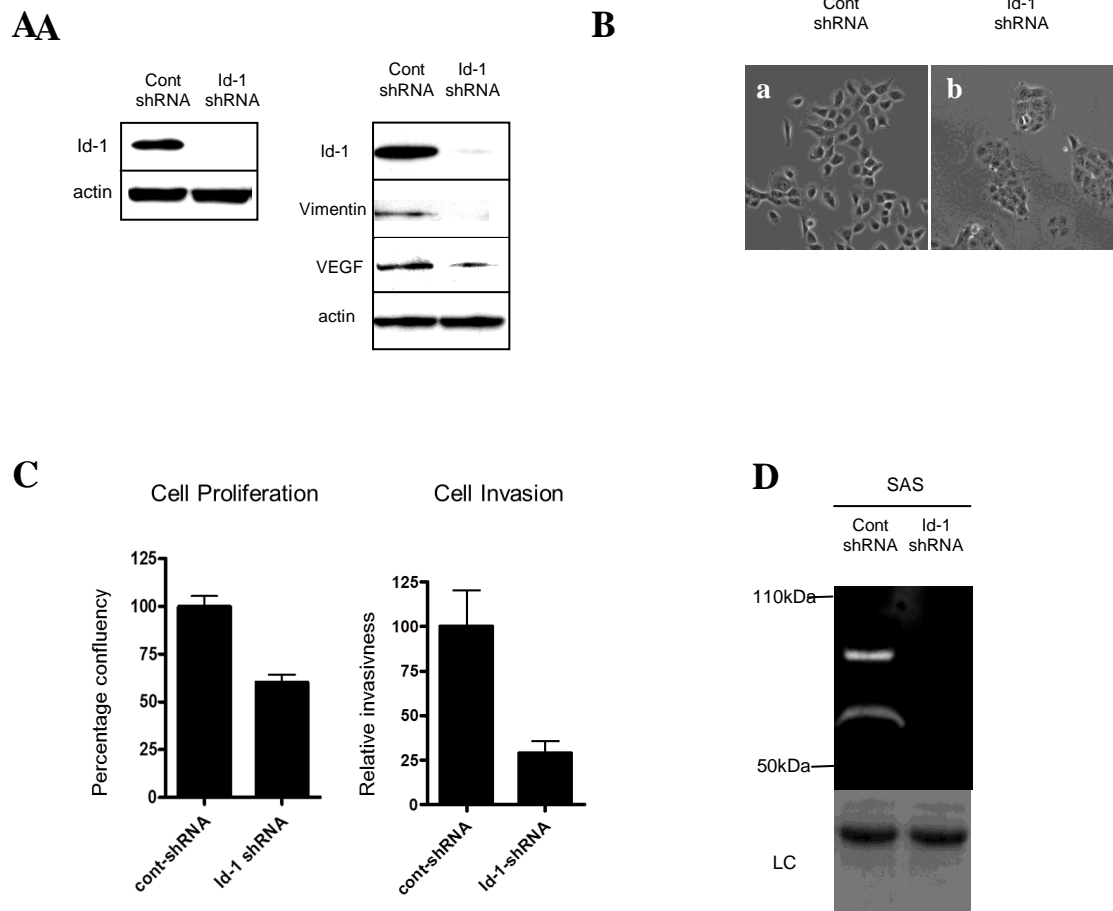


Figure 4: Analysis of the effects of ID1 knockdown in the SAS cell line.

A) In order to show that ID1 expression was down regulated by ID1 shRNA, the technique of western blotting was performed. We started the investigation by measuring the protein expression in two samples: control shRNA and ID1 shRNA. ID1 and actin protein expressions were tested in these samples. There was less ID1 protein in ID1 shRNA samples than in the control shRNA samples. Actin blotting was used as a loading control. Less protein expression was also noticed for vimentin and VEGF in ID1 shRNA knockdown.

B) Two microscope images of the control shRNA and the ID1 shRNA are represented. Cells shown in b tend to regroup themselves and become more differentiated whereas the control cells are not differentiated.

C) In order to determine the effects of ID1 knockdown on cancer cell phenotypes, the MTT assay as well as the invasion assay were performed. The ID1 shRNA tested significantly reduced the invasion of SAS cells as well as the proliferation of SAS cells.

The MTT assay indicated a significant inhibition of proliferation upon ID1 knockdown (40% decreases in proliferation compared to controls). The invasion assay was also performed and indicated a decrease of about 80% in the invasive ability compared to control cells.

D) In order to determine whether there was an effect on the level of expression of MMPs, zymogram analysis was performed. Zymography shows a significant decrease in the expression of two MMPs upon ID1 knockdown. These data may explain why these cells are less invasive.

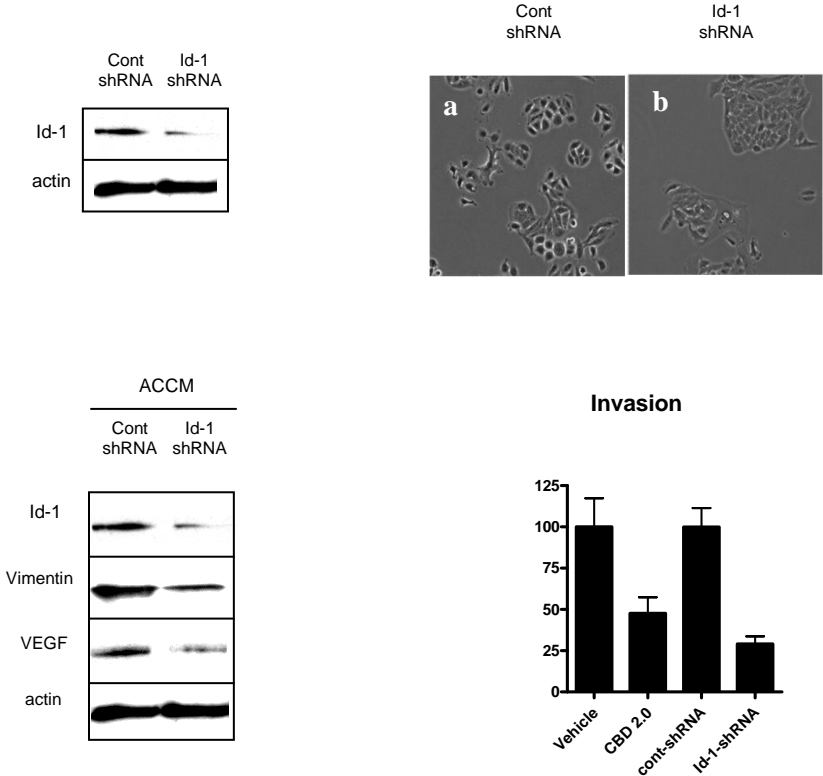


Figure 5: Analysis performed using the ACCM cell line.

The results obtained with the ACCM cell line are similar to the results obtained with SAS cells, i.e., ID1 knockdown triggers morphological changes, a decrease in vimentin and VEGF expression, and a significant reduction in the invasive abilities.

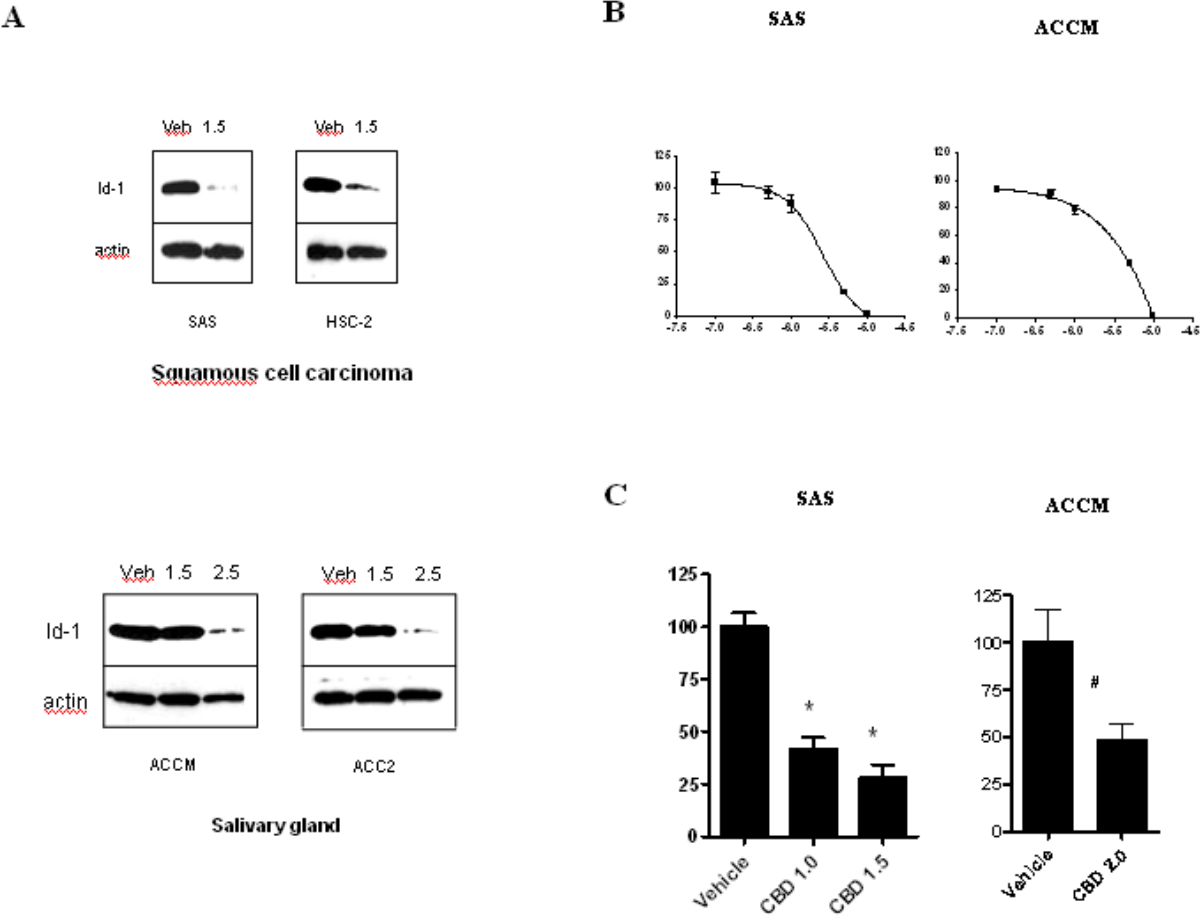


Figure 6: Analyses of cell phenotypes upon CBD treatment.

A) Using the technique of western blotting, we determined that ID1 expression was down-regulated by CBD. We compared ID1 gene expression in vehicle- and CBD1.5-treated SAS, HSC-2, ACCM and ACC2 cells. Actin was used as a loading control. We determined that treatment of all these cells with CBD led to an inhibition in ID1 gene expression.

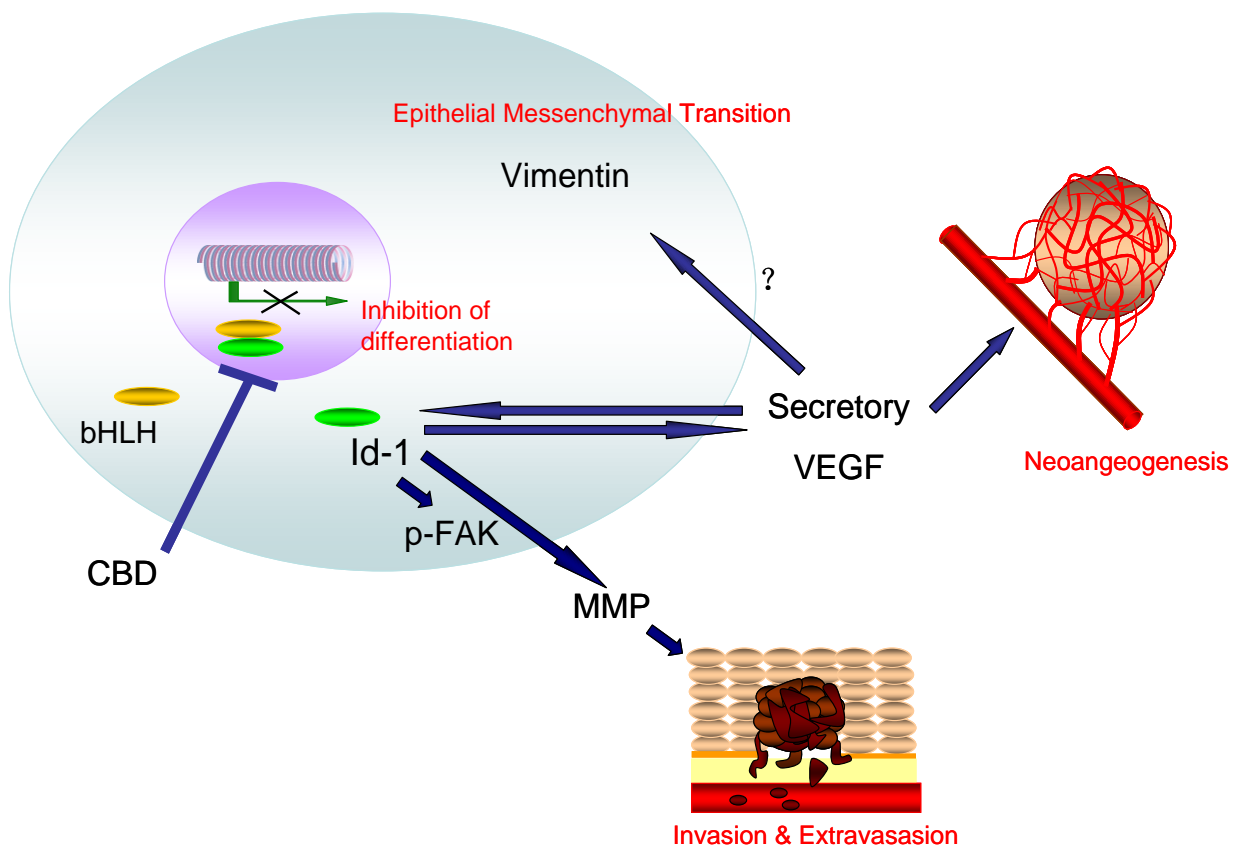
B) This graph shows a decrease in cell proliferation in both SAS and ACCM upon CBD treatment.

C) This graph shows a reduction in cell invasion upon CBD treatment using two different concentrations in SAS cells and one concentration in ACCM cells.

5. Conclusion:

Considering the past and recent research, it was evident that ID1 had a significant effect on the aggressive behavior of cancer cells such as breast cancer cells. However, no much was known about the role of ID1 in head and neck tumors as well as in salivary gland tumors. Based on the results obtained from this present study using a knockdown approach and a potent drug such as CBD, we could conclude that ID1 also represent a key gene that regulates tumor cell phenotypes in these two types of cancer: ID1 regulates proliferation, invasion, EMT (through the regulation of vimentin) and angiogenesis, all being important part of the metastatic process. Therefore, ID1 appears to represent an ideal target for therapy in patients with metastatic disease (Figure 7).

Figure 7: Pathways for head and neck cancer progression.



References:

[1] Sean D. McAllister, Rigel T. Christian, Maxx P. Horowitz, Amaia Garcia and Pierre-Yves Desprez Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells November 19, 2007

[2] Hashmat A Sikder, Meghann K Devlin, Shariff Dunlap, Byungwoo Ryu and Rhoda M Alani Id proteins in cell growth and tumorigenesis 06.03

[3] Sylvia Fong,* Yoko Itahana,* Tomoki Sumida,* Jarnail Singh, Jean-Philippe Coppe, Yong Liu, Peter C. Richards, James L. Bennington, Nancy M. Lee, Robert J. Debs, and Pierre-Yves Desprez *Id-1* as a molecular target in therapy for breast cancer cell invasion and metastasis January 14, 2003

[4] http://www.ehow.com/about_4567395_metastatic-cancer.html#ixzz1eZCnEloX

[5] <http://www.cancer.org/Cancer/AdvancedCancer/DetailedGuide/advanced-cancer-what-is-metastatic-cancer>

[6] Gupta G, Massagué J. Cancer Metastasis: Building a Framework. *Cell*. 2006 Nov 17;127(4):679-95.

[7] Jonathan Perk; Antonio Iavarone; Robert Benezra ID Family of Helix-Loop-Helix Proteins in Cancer 08.12.05

[8] Mark D. Sternlicht and Zena Werb HOW MATRIX METALLOPROTEINASES REGULATE CELL BEHAVIOR *Annu Rev Cell Dev Biol*. 2001 ; 17: 463–516

[9] Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science*. 2002 Mar 29;295(5564):2387-92.

[10] Heidi Ledford Cancer theory faces doubts 19 April 2011 *Nature* 472, 273

[11] Derek C. Radisky Epithelial-mesenchymal Transition *Journal of Cell Science* 118, 4325-4326 2005 [12] <http://www.cpmc.org/professionals/research/programs/science/sean.html> 09.01.12

[13] <http://www.piercenet.com/browse.cfm?fldID=C946AFF6-CCD2-A65C-8A96-03C4AC3E1E07> 09.01.12

[14] http://www3.biorad.com/B2B/BioRad/product/br_category.jsp?BV_SessionID=@@@@1550593026.1326113281@@@&BV_EngineID=ccceadffjedehgkcfngcfkmdhkkdfm.0&divName=Life+Sci

ence+Research&loggedIn=false&serviceLevel=Lit+Request&lang=French&cse=FR&catLevel=7&catO
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cast+Gel+Systems%2fReady+Gel+Precast+Gels%2fReady+Gel+Selection+Guide%2fReady+Gel+Zy
mogram+Gels

[15] http://www.rndsystems.com/product_detail_objectname_cell_invasion_assays.aspx

[16] Hansen MB 1989, p. 203

[17] Eureka Bioscience collection. 2000-2005. Chapter: Gene Expression

[18] ATCC-MTT Cell Proliferation Assay Instructions Catalog Number 30-1010

Table of figures

Figure 1: www.neurochirurgie-cedres.com/metastases-vertebrales/

Figure 2: Hashmat A Sikder, Meghann K Devlin, Shariff Dunlap, Byungwoo Ryu and Rhoda M Alani Id proteins in cell growth and tumorigenesis 06.03

Figure 3: Heidi Ledford Cancer theory faces doubts 19 April 2011 *Nature* 472, 273

Figure 7: made by Dr Ryuichi MURASE