

# HEALTH BEHAVIORS AND BREAST CANCER RISK IN NON-HISPANIC WHITE & HISPANIC WOMEN

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## Purpose

Approximately a third of breast cancer incidence is related to lifestyle choices, and the risk for developing the disease varies among ethnicities [1]. Cigarette smoking, alcohol consumption, body size and shape, physical activity, and diet are all modifiable factors associated with breast cancer risk [1-3]. However, not much is known of the combined effects of the risk factors. Some epidemiological studies suggest that a combination of unhealthy risk factors is associated with increased cancer risk [4-6]. There is also little information on minority populations. This study developed a healthy behavior index (HBI) and compared its association with breast cancer risk in non-Hispanic white (NHW) and Hispanic (H) women.

The main objective was to discover if the healthy behavior index is associated with breast cancer risk by case-control status and race. It has not been discovered yet why there is a difference between NHW and H women and their risks for developing breast cancer.

## Methods {Study Population}

### New Mexico Site of 4-Corners Women's Health Study (1999-2005)

Study Objective	■ To evaluate the association between combined modifiable factors and breast cancer risk in non-Hispanic white and Hispanic women.
Case Eligibility Criteria and Ascertainment	■ Hispanic, Native American or NHW ethnicity (self-reported) ■ resident of New Mexico ■ age 25-79 years ■ diagnosed with a 1st primary breast cancer between 10/1999 and 05/2004 ■ ascertained from state cancer registry (SEER)
Control Eligibility Criteria and Ascertainment	■ women <65 years randomly selected from driver's license lists ■ women ≥65 years selected from Center for Medicare Services list ■ frequency-matched to cases (5-year age distribution & ethnicity)
HBI Sample Size	■ Cases = 982 (NHW = 635; H = 347) ■ Controls = 919 (NHW=604; H = 315)

## Methods {HBI Construction}

### Construction of the Healthy Behavior Index

HBI Variables	Definitions
Smoking	0 = never smoker; 1 = former; 2 = current
Alcohol Consumption*	0 ≤ ½ standard drink/day; 1 = ≤ 1 standard drink/day; 2 = ≥ standard drink/day
Body Mass Index (BMI)*	0 = normal (<25 kg/m <sup>2</sup> ); 1 = overweight (25-30 kg/m <sup>2</sup> ); 2 = obese (>30 kg/m <sup>2</sup> )
Waist-Hip Ratio (WHR)	0 = T1; 1 = T2; 2 = T3
Diet Pattern***	0 = Q1; 1 = Q2; 2 = Q3; 3 = Q4
Vigorous Physical activity	0 = >75 min/wk; 1 = ≤75 min/wk; 2 = no PA
Healthy Behavior Index***	Range 0-13; Q1 = 0-3; Q2 = 4-5; Q3 = 6-7; Q4 = 8-13

\*based on American Cancer Society cancer prevention guidelines

\*\*Based on Murtaugh et al. (2007). Diet pattern reflects a diet high in eggs, cheese, corn, fresh tomato-based products, beans, chicken, and low in refined grains, snacks, beef, and fast food.

\*\*\*Categorization of the HBI is based on distribution of controls.

T = Tertiles; Q = Quartiles

Data on modifiable behavioral factors were collected via in-person interviews for the year prior to

## Methods {Statistical Analysis}

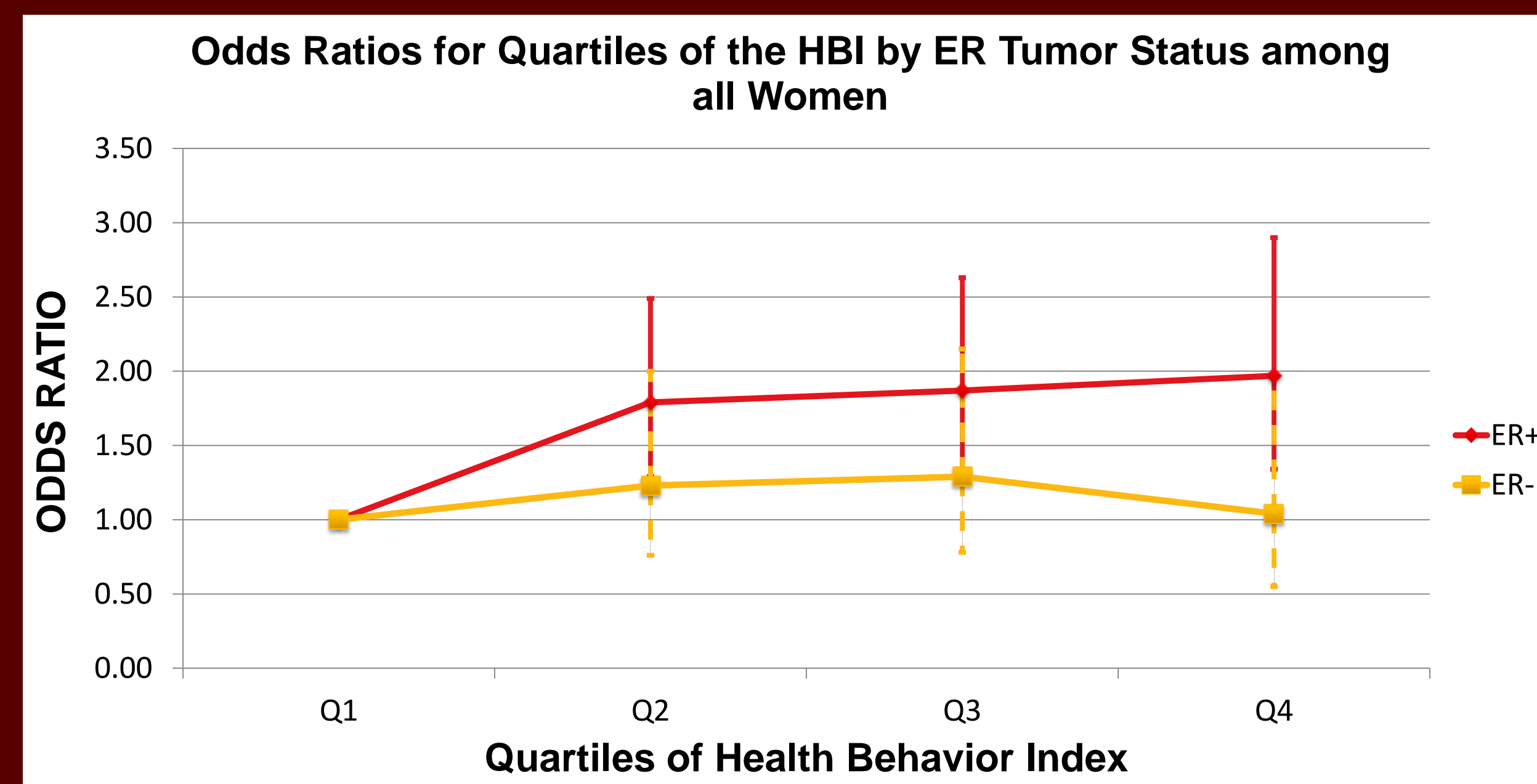
Using SAS Version 9.4 (Cary, NC):

Descriptive characteristics were compared using chi-square (X<sup>2</sup>) for significance stratified by case-control status and ethnicity. Associations between healthy behavior index scores and breast cancer risk were calculated with multivariable logistic regression to estimate odds ratios (ORs) with 95% confidence intervals between cases and controls. Multinomial logistic regression determined associations between HBI and risk of breast cancer defined by ER status, compared to controls.

## Results

Table 1. Description and Prevalences of the Healthy Behavior Index Factors (n=1901)

	All women			Non-Hispanic White			Hispanic		
	Case (n=982)	Control (n=919)	p-value <sup>1</sup>	Case (n=635)	Control (n=604)	p-value <sup>2</sup>	Case (n=347)	Control (n=315)	p-value <sup>3</sup>
Age (Mean ± SD)	55.4 ±11.5 n (%)	55.4 ±11.0 n (%)	0.93	56.7 ±11.6 n (%)	56.5 ±11.1 n (%)	0.20	53.0 ±10.9 n (%)	53.2 ±10.6 n (%)	0.56
Menopausal Status			0.46			0.67			0.53
Premenopausal	345 (35.1)	308 (33.5)		209 (32.9)	192 (31.8)		136 (39.2)	116 (36.8)	
Postmenopausal	637 (64.9)	611 (66.5)		426 (67.1)	412 (68.2)		211 (60.8)	199 (63.2)	
Education			0.16			0.93			0.09
<Highschool	642 (65.4)	611 (66.5)		486 (76.5)	459 (76.0)		156 (45.0)	152 (48.3)	
Highschool	226 (23.0)	231 (25.1)		124 (19.5)	126 (20.9)		102 (29.4)	105 (33.3)	
>Highschool	114 (11.6)	77 (8.4)		25 (3.9)	19 (3.2)		89 (25.7)	58 (18.4)	
Cigarette Smoking Status			0.97			0.47			0.31
Never	547 (55.7)	522 (56.8)		322 (50.7)	324 (53.6)		225 (64.8)	198 (62.9)	
Current	288 (29.3)	248 (27.0)		207 (32.6)	180 (29.8)		180 (51.8)	68 (21.6)	
Former	147 (15.0)	149 (16.2)		106 (16.7)	100 (16.6)		41 (11.8)	49 (15.6)	
Alcohol Consumption			0.32			0.06			0.25
Non-drinker	803 (81.8)	775 (84.3)		488 (76.9)	495 (81.6)		315 (90.8)	280 (88.9)	
1 Drink/day	90 (9.2)	63 (6.9)		75 (11.8)	52 (8.6)		15 (4.3)	11 (3.5)	
1+ Drink/day	89 (9.1)	81 (8.8)		72 (11.3)	57 (9.4)		17 (4.9)	24 (7.6)	
Body Mass Index (kg/m <sup>2</sup> )			0.23			0.60			0.15
<25	432 (44.0)	392 (42.7)		304 (47.9)	295 (48.8)		128 (36.9)	97 (30.8)	
25.0-29.99	329 (33.5)	292 (31.8)		210 (33.1)	174 (28.8)		119 (34.3)	118 (37.5)	
30+	221 (22.5)	235 (25.6)		121 (19.1)	135 (23.4)		100 (28.8)	100 (31.8)	
Waist-Hip Circumference (cm)			0.04			0.07			0.36
<0.775	273 (27.8)	301 (32.8)		202 (31.8)	234 (38.7)		71 (20.5)	67 (21.3)	
0.775-0.84	352 (35.9)	309 (33.6)		241 (38.0)	196 (32.5)		111 (32.0)	113 (35.9)	
0.84+	357 (36.4)	309 (33.6)		192 (30.2)	174 (28.8)		165 (47.6)	135 (42.9)	
Vigorous Physical Activity			0.16			0.14			0.79
No Activity	250 (25.5)	253 (27.5)		173 (27.2)	180 (29.8)		77 (22.2)	73 (23.2)	
≤75 min/week	305 (31.1)	295 (32.1)		207 (32.6)	207 (34.3)		98 (28.2)	88 (27.9)	
>75 min/week	427 (43.5)	371 (40.4)		255 (40.2)	217 (35.9)		172 (49.6)	154 (48.9)	
Diet Pattern			0.01			0.002			0.24
Q1	224 (22.8)	246 (26.8)		56 (8.8)	85 (14.1)		168 (48.4)	161 (51.1)	
Q2	241 (24.5)	236 (25.7)		134 (21.1)	136 (22.5)		107 (30.8)	100 (31.8)	
Q3	256 (26.1)	230 (25.0)		202 (31.8)	187 (31.0)		54 (15.6)	43 (13.7)	
Q4	261 (26.6)	207 (22.5)		243 (38.3)	196 (32.5)		18 (5.2)	11 (3.5)	
Estrogen Receptor Status									0.01
ER+	497 (77.7)	--		334 (80.7)	--		163 (72.1)	--	
ER-	143 (22.3)	--		80 (19.3)	--		63 (27.9)	--	



## Results

Table 2. Associations between individual HBI components, the HBI, and breast cancer risk stratified by ethnicity

HBI Components	All Women		Non-Hispanic White Women				Hispanic Women					
	Crude OR	95% CI	Adjusted* OR	95% CI	Crude OR	95% CI	Adjusted* OR	95% CI	Crude OR	95% CI	Adjusted* OR	95% CI
<b>Cigarette Smoking Status</b>												
Never	1.00		1.00		1.00		1.00		1.00		1.00	
Former	1.11	0.90-1.36	1.13	0.92-1.39	1.16	0.90-1.49	1.16	0.90-1.49	1.05	0.72-1.52	1.08	0.74-1.58
Current	0.94	0.73-1.22	0.95	0.73-1.23	1.07	0.78-1.46	1.06	0.77-1.46	0.74	0.47-1.16	0.76	0.48-1.20
<b>Alcohol Consumption</b>												
Non-drinker	1.00		1.00		1.00		1.00		1.00		1.00	
1 Drink/day	1.38	0.99-1.93	<b>1.41</b>	<b>1.01-1.98</b>	<b>1.46</b>	<b>1.01-2.13</b>	<b>1.47</b>	<b>1.01-2.14</b>	1.21	0.54-2.68	1.25	0.56-2.78
1+ Drink/day	1.06	0.77-1.46	1.08	0.78-1.48	1.28	0.89-1.85	1.28	0.88-1.85	0.63	0.33-1.20	0.66	0.34-1.25
<b>Body Mass Index (kg/m<sup>2</sup>)</b>												
<25	1.00		1.00		1.00		1.00		1.00		1.00	
25.0-29.99	1.02	0.83-1.26	1.02	0.82-1.26	1.17	0.91-1.51	1.18	0.91-1.52	0.76	0.53-1.10	0.76	0.52-1.10
30+	0.85	0.68-1.07	0.83	0.66-1.05	0.87	0.65-1.17	0.87	0.65-1.18	0.76	0.52-1.11	0.72	0.49-1.06
<b>Waist-Hip Circumference (cm)</b>												
<0.775	1.00		1.00		1.00		1.00		1.00		1.00	
0.775-0.84	<b>1.26</b>	<b>1.00-1.57</b>	<b>1.27</b>	<b>1.01-1.60</b>	<b>1.42</b>	<b>1.09-1.86</b>	<b>1.46</b>	<b>1.12-1.92</b>	0.927	0.61-1.42	0.92	0.60-1.40
0.84+	<b>1.27</b>	<b>1.02-1.59</b>	<b>1.27</b>	<b>1.01-1.59</b>	1.28	0.97-1.69	1.322	0.99-1.76	1.15	0.77-1.73	1.09	0.72-1.65
<b>Vigorous Physical Activity</b>												
No Activity	1.00		1.00		1.00		1.00		1.00		1.00	
≤75 min/week	1.05	0.83-1.33	1.05	0.83-1.33	1.04	0.78-1.38	1.05	0.79-1.39	1.06	0.69-1.62	1.06	0.68-1.63
>75 min/week	1.17	0.93-1.46	1.16	0.92-1.45	1.22	0.93-1.61	1.25	0.94-1.65	1.06	0.72-1.56	1.01	0.68-1.50
<b>Diet Pattern</b>												
Q1	1.00		1.00		1.00		1.00		1.00		1.00	
Q2	1.12	0.87-1.45	1.22	0.94-1.58	1.50	0.99-2.26	1.49	0.98-2.25	1.03	0.72-1.45	1.14	0.80-1.64
Q3	1.22	0.95-1.58	<b>1.35</b>	<b>1.04-1.76</b>	<b>1.64</b>	<b>1.11-2.43</b>	<b>1.64</b>	<b>1.11-2.43</b>	1.20	0.76-1.90	1.36	0.85-2.17
Q4	<b>1.39</b>	<b>1.07-1.79</b>	<b>1.56</b>	<b>1.19-2.22</b>	<b>1.88</b>	<b>1.28-2.77</b>	<b>1.90</b>	<b>1.29-2.80</b>	1.57	0.72-3.42	1.81	0.82-4.01
<b>HBI</b>												
Q1	1.00		1.00		1.00		1.00		1.00		1.00	
Q2	<b>1.47</b>	<b>1.14-1.90</b>	<b>1.48</b>	<b>1.14-1.91</b>	<b>1.96</b>	<b>1.40-2.75</b>	<b>2.00</b>	<b>1.42-2.81</b>	1.00	0.70-1.48	0.98	0.65-1.46
Q3	<b>1.55</b>	<b>1.19-2.02</b>	<b>1.58</b>	<b>1.21-2.06</b>	<b>2.01</b>	<b>1.42-2.84</b>	<b>2.10</b>	<b>1.47-2.98</b>	1.10	0.73-1.68	1.07	0.70-1.64
Q4	<b>1.52</b>	<b>1.12-2.06</b>	<b>1.59</b>	<b>1.17-2.17</b>	<b>2.04</b>	<b>1.41-2.96</b>	<b>2.16</b>	<b>1.47-3.16</b>	0.93	0.51-1.69	0.96	0.52-1.76
p-trend		<b>0.0055</b>		<b>0.003</b>		<b>0.0008</b>		<b>0.0004</b>		<b>0.88</b>		<b>0.88</b>

\*Adjusted for education and menopausal status

P-interaction is significant P=0.0376

## Conclusions

- The HBI was associated with breast cancer risk.
- The association is stronger in non-Hispanic white women, and in women with estrogen receptor positive tumor phenotypes.
- The direction of the association suggests that women with multiple unhealthy behaviors, including drinking, smoking, a poor diet, a high BMI and WHR, are at greater risk for developing breast cancer.
- Future research may be directed at refining the HBI in replicating these findings in a larger study.
- A policy implication is that a reduction of multiple rather than single risk factors should be addressed in primary interventions.
- Inclusion of risk biomarkers in conjunction with HBI could provide a more sensitive tool for cancer prevention.

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## Abstract

Prostate cancer is the most prevalent form of cancer in men with over 3.3 million existing cases in the U.S. Cadmium is a toxic heavy metal with widespread use in industry, making it a common environmental pollutant. Cadmium exposure induces prostate cancer in humans, as well as *in vitro* human cell lines and *in vivo* mouse xenograft models. In previous studies, the natural compound 3, 9-dihydroxy-2-prenylcoumestan [psoralidin (pso)] induced reactive oxygen species in cadmium-transformed prostate epithelial cells (CTPE) to inhibit cancer cell growth and prevent metastasis. It has also been shown to induce both autophagy and apoptosis in these cells. Although these relationships have been established, the molecular processes by which cadmium transformation occurs and pso inhibits cell growth and metastasis are not well defined. To understand these processes, studies on the *in vitro* transformation of normal human prostate epithelial cells (RWPE-1) into CTPE cells, as well as the molecular effect of pso on CTPE cells, were conducted.

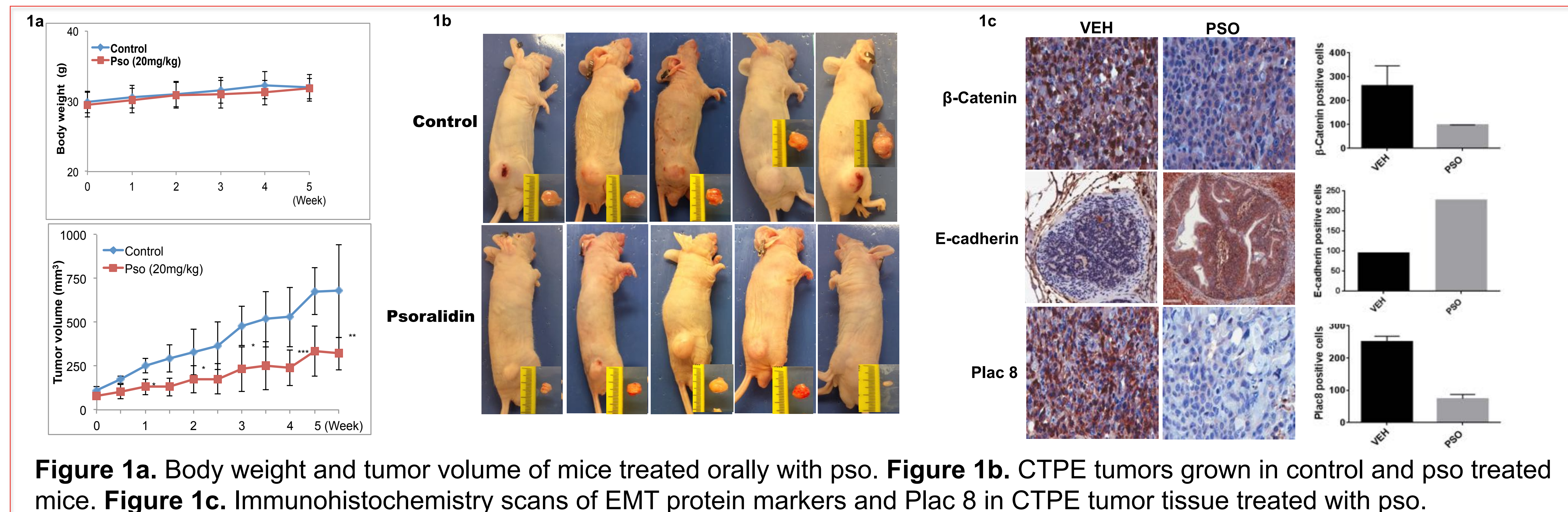
**Methods:** In this study, we evaluated the change in mRNA expression of epithelial mesenchymal transition (EMT) markers. CTPE cells were exposed to cadmium or pso for 0, 12, 24, 48, and 72 hours, then RNA was extracted and steady state mRNA levels of the EMT markers determined using qRT-PCR.

**Hypothesis:** We hypothesized that cadmium would induce metastasis by affecting the EMT. Conversely, Pso will prevent metastasis in cadmium-transformed cells by inhibiting EMT and lower proliferation by inhibiting autophagy.

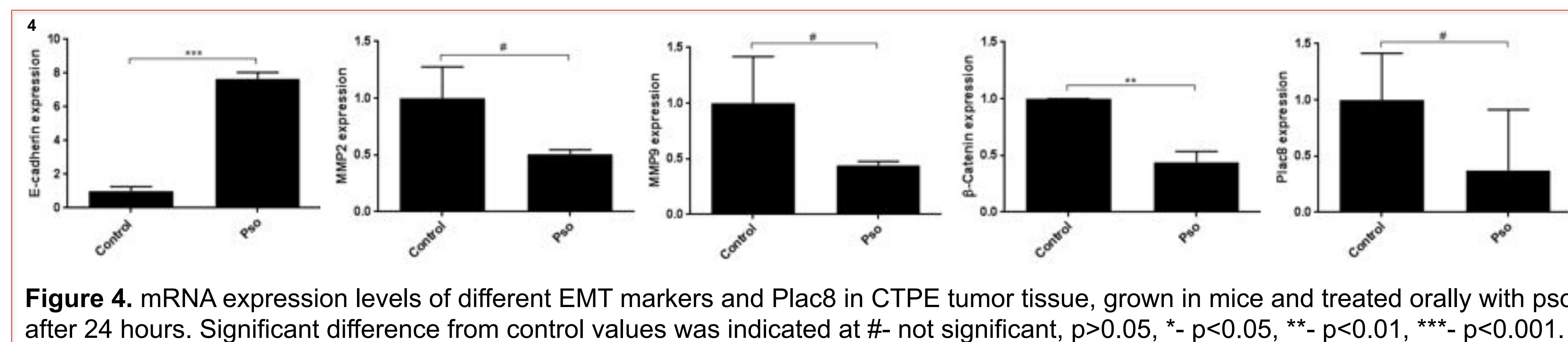
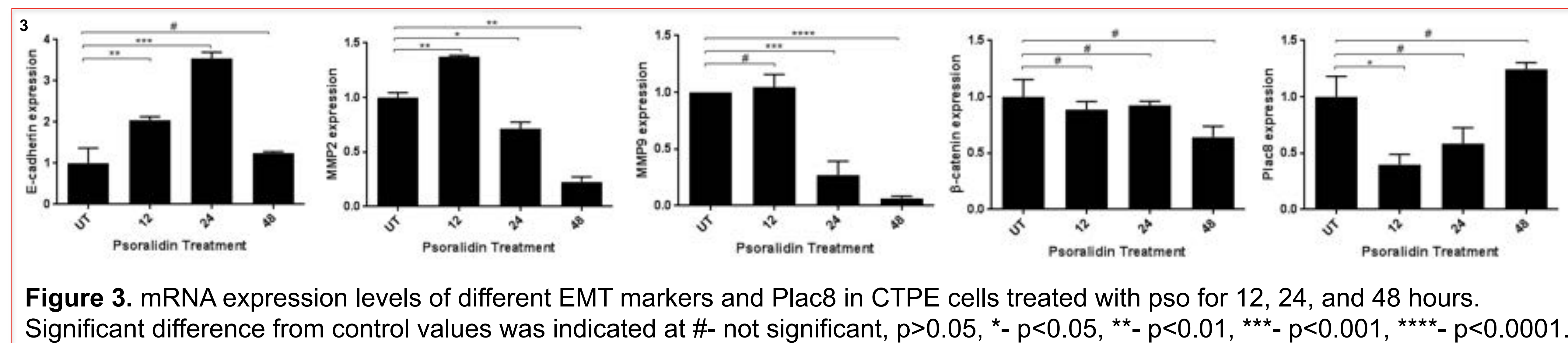
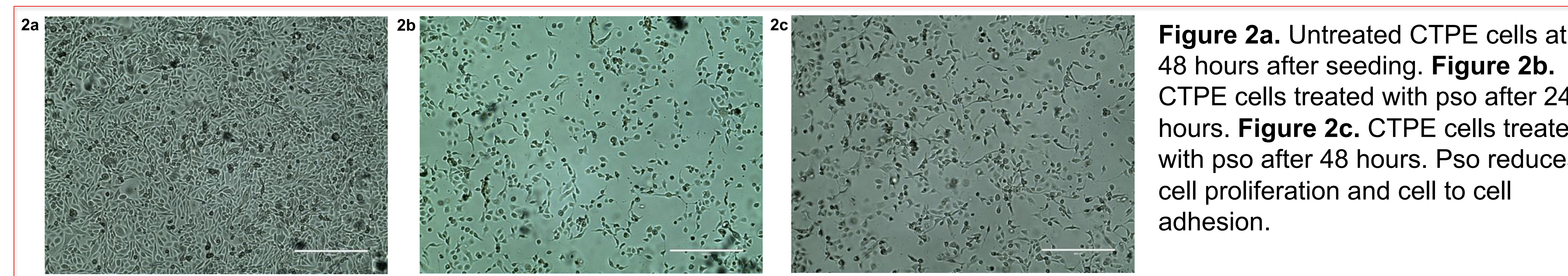
**Results:** In CTPE cell lines, we found two EMT promoting markers, MMP2 and MMP9, showed significantly decreased expression after 24 and 48 hours of pso treatment,  $p < 0.05$ . Plac8, a marker for autophagy, also showed a significant decrease in expression after 12 hours,  $p < 0.05$ , but returned to normal levels after 24 hours. Pso-treatment significantly increased expression of E-cadherin, an EMT inhibition marker, at 12 and 24 hours,  $p < 0.01$ . E-cadherin expression also significantly increased in CTPE xenograft tumor tissue grown in mice treated orally with pso at 24 hours,  $p < 0.001$ . CTPE tumor tissue also showed a significant decrease in  $\beta$ -catenin expression, an EMT promoter, at 24 hours pso treatment,  $p < 0.01$ . When CTPE was treated with cadmium,  $\beta$ -catenin and Plac8 expression showed significant increase by 72 hours,  $p < 0.01$ . Not all markers tested are shown.

**Conclusions:** Although not all EMT markers tested responded to pso treatment, these results suggest that pso has an inhibitory effect on the EMT and autophagy in cancer cells at the RNA level. This effect prevents metastasis and decreases proliferation by lowering the cancer cell's protective capacity. Alternatively, cadmium may cause an increase in certain EMT and autophagy markers, leading to cancer cell metastasis and survival.

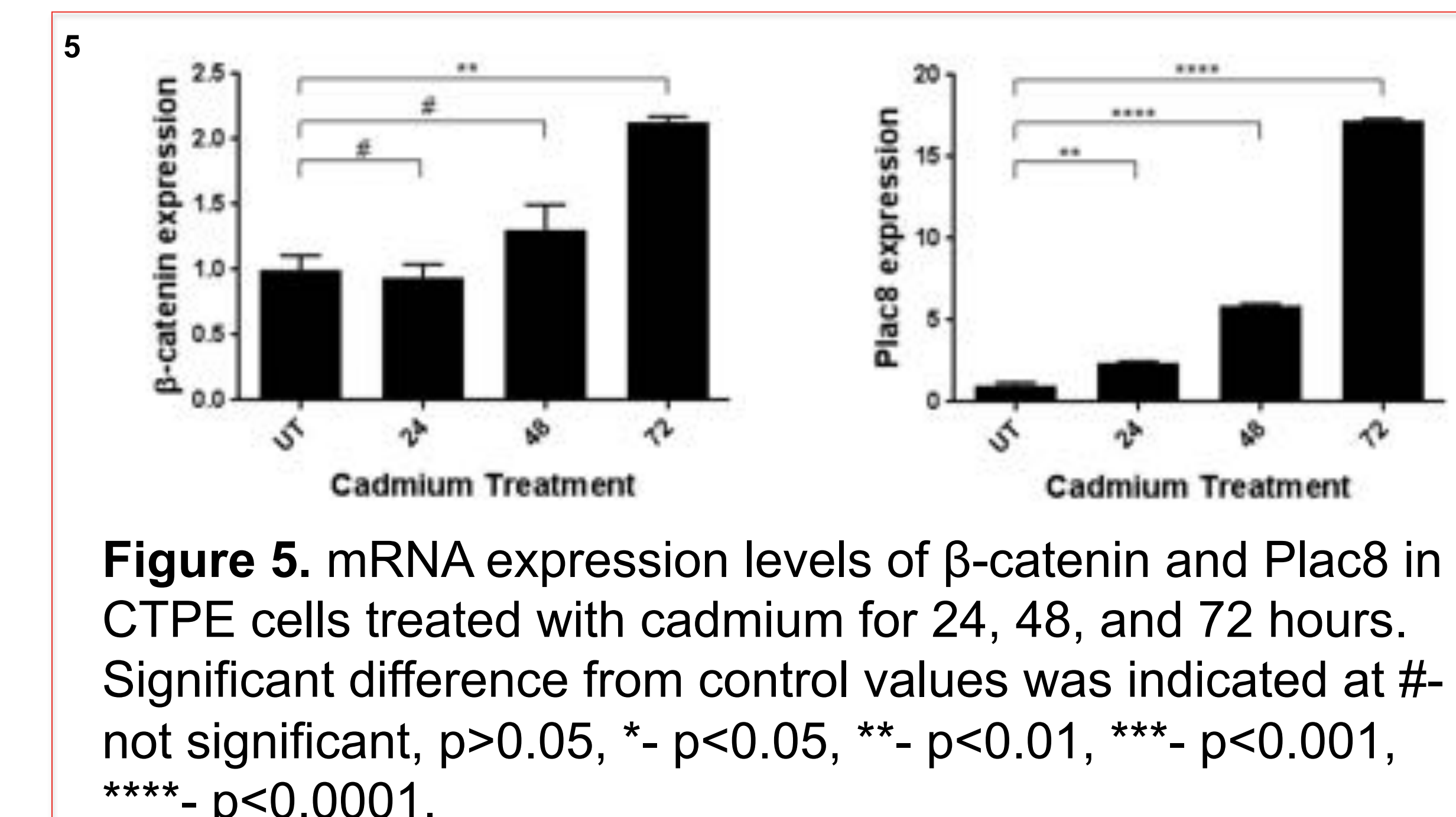
## Preliminary Findings



## Results



## Results



## Conclusions

- Pso inhibits EMT by effecting expression levels of MMP2, MMP9, and E-cadherin.
- Pso inhibits autophagy by decreasing expression levels of Plac8.
- The molecular changes induced by pso suggests it will inhibit metastasis and proliferation in cadmium-induced prostate cancer.
- Cadmium promotes EMT by effecting expression levels of  $\beta$ -catenin.
- Cadmium promotes autophagy by increasing expression levels of Plac8.
- The molecular changes induced by cadmium suggests it will promote metastasis and cancer cell survival.

## Future Directions

- Evaluate mRNA expression levels of EMT markers and Plac8 in RWPE-1 cells treated with cadmium.
- Perform Western immunoblots to determine change in protein expression of EMT markers and Plac8 after pso and cadmium treatment.
- Confocal microscopy is needed to evaluate pso and cadmium effect on protein localization, especially for markers which did not significantly inhibit EMT. A change in localization could lead to inactivity without changing expression levels.

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## Abstract

**Introduction:** The novel small molecule XB05 (1-bromo-1,1-difluoro-non-2-yn-4-ol) has been previously demonstrated to selectively target malignant, but not non-malignant cell lines *in vitro* by disrupting cellular redox homeostasis<sup>1</sup>. The mechanism of action for XB05 is unknown, but *in silico* and *in vitro* studies have identified possible roles for SOX9<sup>2</sup> and glutathione reductase (GR)<sup>3</sup>. SOX9 is of particular interest because it is a marker for tumor initiating cells (TICs), a subpopulation of tumor cells identified to drive metastasis and recurrence of cancers<sup>4,5,6</sup>. The purpose of this study was to investigate the effect of XB05 treatment on levels of SOX9 and GR proteins in two cancer cell lines. Additionally, two candidate inhibitors of SOX9 (JT1 and JT2) that were designed to directly target SOX9 activity were examined.

**Methods:** MDA-MB-231 (breast cancer) and U937 (myeloid leukemia) cell lines were plated at a density of  $8.0 \times 10^5$  cells/T-150 flask and allowed to adhere for 24 hours before treatment with 2  $\mu$ M XB05, JT1, JT2, or dimethyl sulfoxide (DMSO) as a vehicle control for untreated samples. After treatment for 24 hours, the cell lysates were collected with RIPA buffer containing protease and phosphatase inhibitors and protein concentration was determined. Gel electrophoresis and a subsequent transfer onto a polyvinylidene fluoride membrane were performed on the samples for western blotting using antibodies against SOX9, glutathione reductase and GAPDH (loading control).

**Results:** Triplicate western blot analyses revealed that SOX9 was expressed in the MDA-MB 231 line, but not in the U937 line and that SOX9 protein levels remained similar to the vehicle controls after 24 hour treatment with 2  $\mu$ M XB05, JT1, or JT2. Both the MDA-MB-231 and U937 lines expressed GR and GR levels remained similar to the vehicle controls after 24 hour treatment with 2  $\mu$ M XB05.

**Conclusion:** It appears that although XB05 shows a general preference for SOX9-high cancer cells<sup>2</sup>, there are some exceptions because the U937 cell line (which has high sensitivity to XB05) has very low SOX9 levels. Possibly, XB05 preferentially targets tumor initiating cells (TICs) and SOX9 is a marker for TICs in some cancer types but not in others. Our data do not rule out a role for SOX9 in MDA-MB-231 cells or for GR, but they indicate that XB05 does not induce changes in protein levels. Further research is required to investigate if the **activity** of SOX9 or GR is affected by XB05, JT1 and JT2 treatment.

## Background

Sex determining region Y box 9 (SOX9) is a transcription factor that appears to induce cell proliferation in some types of cancers (colon, prostate, bladder). Studies suggest that tumors contain a subpopulation of tumor initiating cells (TICs) that express SOX9 and drive cancer progression and metastasis<sup>4,5,6</sup>. Previous research has revealed that SOX9-high cancer cells are more susceptible to the cytotoxic effects of XB05 than non malignant cells or SOX9-low cancer cells<sup>1,2</sup>. Therefore, we hypothesized that the previously studied molecule, XB05, may induce cytotoxic effects on malignant cells expressing SOX9 via an unknown direct or indirect mechanism.

The cytotoxicity of XB05 *in vitro* was previously evaluated and described, highlighting the potential application as a synthetic chemotherapeutic drug<sup>1,2</sup>. The results of these studies identify the ability of XB05 to induce cell death through apoptotic and non-apoptotic mechanisms in malignant cells. In summary, this molecule interrupts homeostatic defense mechanisms resulting in cytotoxic levels of reactive oxygen species (ROS) and damages in DNA (DSB). Interestingly, a positive correlation exists between SOX9 expression levels and response to XB05<sup>1,2</sup>. It has been concluded that SOX9 is expressed in the TICs (cancer stem cells), and that targeting these cells via SOX9 may prevent metastasis and recurrence.

Although the mechanism of XB05 action is unknown, *in silico* and *in vitro* studies have identified a possible role for glutathione reductase. A virtual screen of proteins that directly bind to XB05 was performed<sup>3</sup>. The results of the virtual screen identified glutathione reductase as the top protein that is capable of directly binding to XB05. This finding led us to investigate the role of glutathione reductase *in vitro* after XB05 treatment.

## Methods

**Cell culture:** MDA-MB-231 (breast adenocarcinoma) and U937 (myeloid leukemia) cell lines were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) was used for the MDA-MB 231 cells, and RPMI 1640 media (Life Technologies) was used for the U937 cells. Media was prepared with 10% fetal bovine serum (Life Technologies) and 1% Penicillin/Streptomycin (Life Technologies). XB05, JT1, JT2 compounds were synthesized in the UofL Medicinal Chemistry facility. Cells were plated at a density of  $8.0 \times 10^5$  cells in a T-150 flask (20 ml total volume) and allowed to adhere overnight.

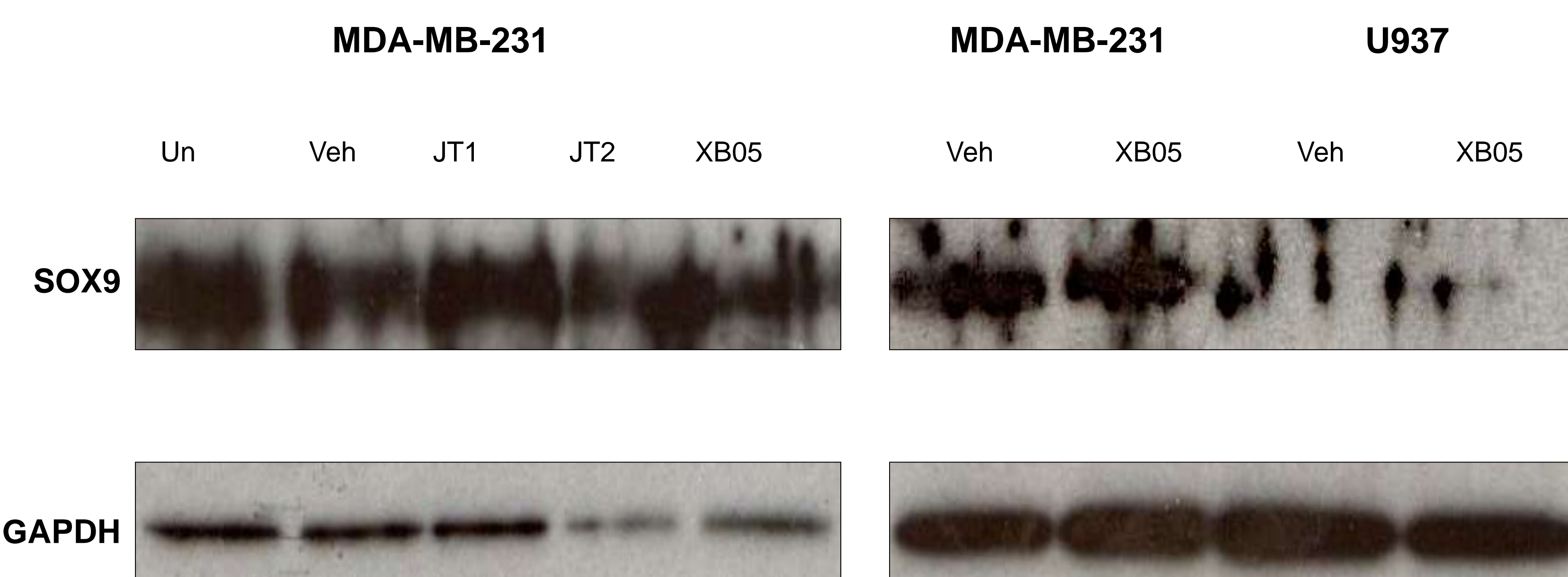
**Cell treatments:** XB05, JT1, JT2 and vehicle control (DMSO) treatments were initially diluted into media and added to cells to a final concentration of 2  $\mu$ M for 24 hours.

**Preparation of cell lysates:** MDA-MB 231 cells were washed with 10 ml ice cold PBS (Life Technologies). Lysates were prepared with RIPA buffer containing phosphatase and protease inhibitors (Millipore). U937 (suspension) cells were pelleted by centrifugation at 1000 x g for 5 min. RIPA buffer containing phosphatase and protease inhibitors was added to the pellet. Cell extracts from both lines were centrifuged at 14,000 x g at 4°C for 15 min and supernatant was collected. Pierce BCA Protein Assay (Thermo Fisher Scientific) was used according to manufacturer's directions to determine protein concentration.

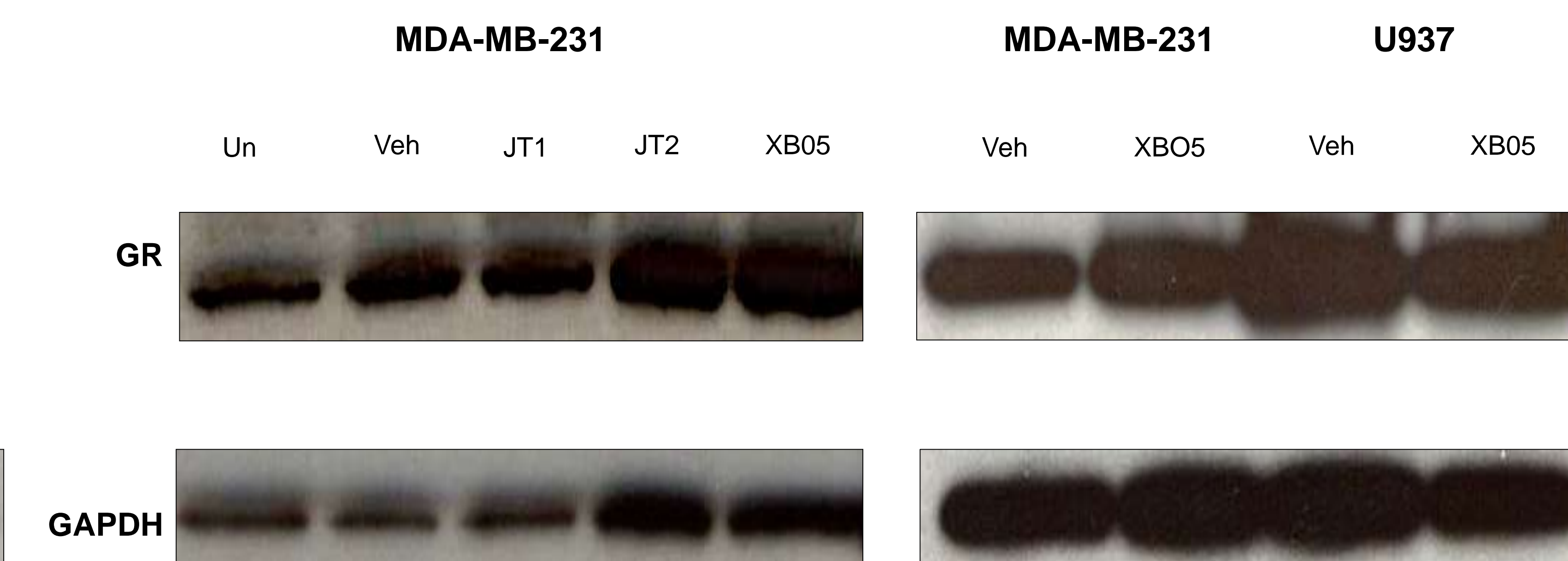
**Western blot:** Samples were separated by SDS-PAGE using 25  $\mu$ g of protein per well using a 4-20% Tris-Glycine gel (Invitrogen). Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% milk in TBS-T. Membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies (Santa Cruz Biotechnology) were utilized at the following dilutions: GAPDH Ms 1:1000, SOX9 Rb 1:1000, GR Rb 1:1000. Secondary antibodies (Santa Cruz Biotechnology) were utilized at the following dilutions: Goat anti-mouse 1: 25,000, Goat anti-rabbit 1:10,000. Bands were visualized using Pierce ECL Western Blotting Substrate (Fisher) and Amersham Hyperfilm (GE Healthcare).

## Results

Western blot analysis of SOX9 protein levels and the loading control GAPDH in MDA-MB-231 and U937 cell lines.



Western blot analysis of glutathione reductase protein levels and the loading control GAPDH in MDA-MB-231 and U937 cell lines.



## Conclusions

- Although XB05 shows a general preference for SOX9-high cancer cells<sup>2</sup> (such as MDA-MB-231), there are some exceptions because the U937 cell line (which has high sensitivity to XB05) has very low SOX9 protein levels.
- Possibly, XB05 preferentially targets tumor initiating cells (TICs) and SOX9 is a marker for TICs in some cancer types but not in others.
- Our data do not rule out a role for SOX9 as a mechanism for XB05's activities in MDA-MB-231 cells or for GR, but they indicate that XB05 does not induce changes in protein levels.
- Further research is required to investigate if the **activity** of SOX9 or GR is affected by XB05, JT1 and JT2 treatments.

## Acknowledgments

This research was supported in part by a R25 grant from the National Cancer Institute (R25 CA134283).

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# Paracrine Induction of Macrophages by Melanoma Exosomes

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Department of Pharmacology and Toxicology and the James Graham Brown Cancer Center

## Introduction

Macrophages are key participants in tumor pathogenesis. They can be divided into two general classes (M1 and M2) based on function. M1 polarized macrophages mediate effective anti-tumor immunity. M2's promote tumor growth via immune suppression.

Melanoma tumors and derived factors have been shown to suppress the anti-tumor immune response. Our previous investigations demonstrated that melanoma exosomes prepare lymph nodes for tumor metastasis by creating a pro-angiogenic cancer friendly microenvironment (1, 2). Melanoma exosomes can also inhibit cytotoxic anti-tumor T cells, NK cells and induce MDSCs (3). However, to date, there have been minimal investigations into the direct influence of melanoma exosomes on macrophage function.

In this study, we hypothesized that melanoma exosomes might directly induce macrophage M2 polarization.

## Significance & Innovation

The role of melanoma exosomes in directly influencing macrophage function is poorly understood.

Understanding the ability of melanoma exosomes to influence macrophage mediated pro-tumor processes will further our basic understanding of melanoma pathogenesis.

These investigations provide a foundation for the development of novel exosome based therapeutics to antagonize melanoma exosome mediated induction of tumor supportive macrophage functions.

## Methods

•Cell Culture: B16F10 melanoma and Raw 264.7 macrophage cell lines were cultured in DMEM with 10% FBS media at 37°C and 5% CO<sub>2</sub>.

•Exosome Isolation: Exosomes were isolated from B16F10 cell culture media via differential centrifugation. Exosome quantities were measured using a BCA (bicinchoninic acid) assay (Thermo Scientific) to determine protein concentration.

•Treatment: Raw 264.7 cells were added to 96 well plates. After 24 hours, culture media was aspirated and replaced with one of six treatments: Non-treated (Exosome Free Media), Exosome Treated, LPS treated, IL4 Treated, LPS + Exosome Treated, and IL4 + Exosome Treated. After 24 hours, supernatants were transferred to be analyzed by ELISA or RT<sup>2</sup> PCR.

•Cell Viability Assay: PrestoBlue Cell Viability Reagent (Invitrogen) was used to measure the viability and proliferation of non-treated and treated cells.

•Macrophage Response: ELISArray (Affymetrix) was used to test cytokine production in treated and non-treated cell supernatants. RT-RT PCR arrays (Qiagen) were used to confirm and extend ELISA results.

## Results

### M1 Polarization

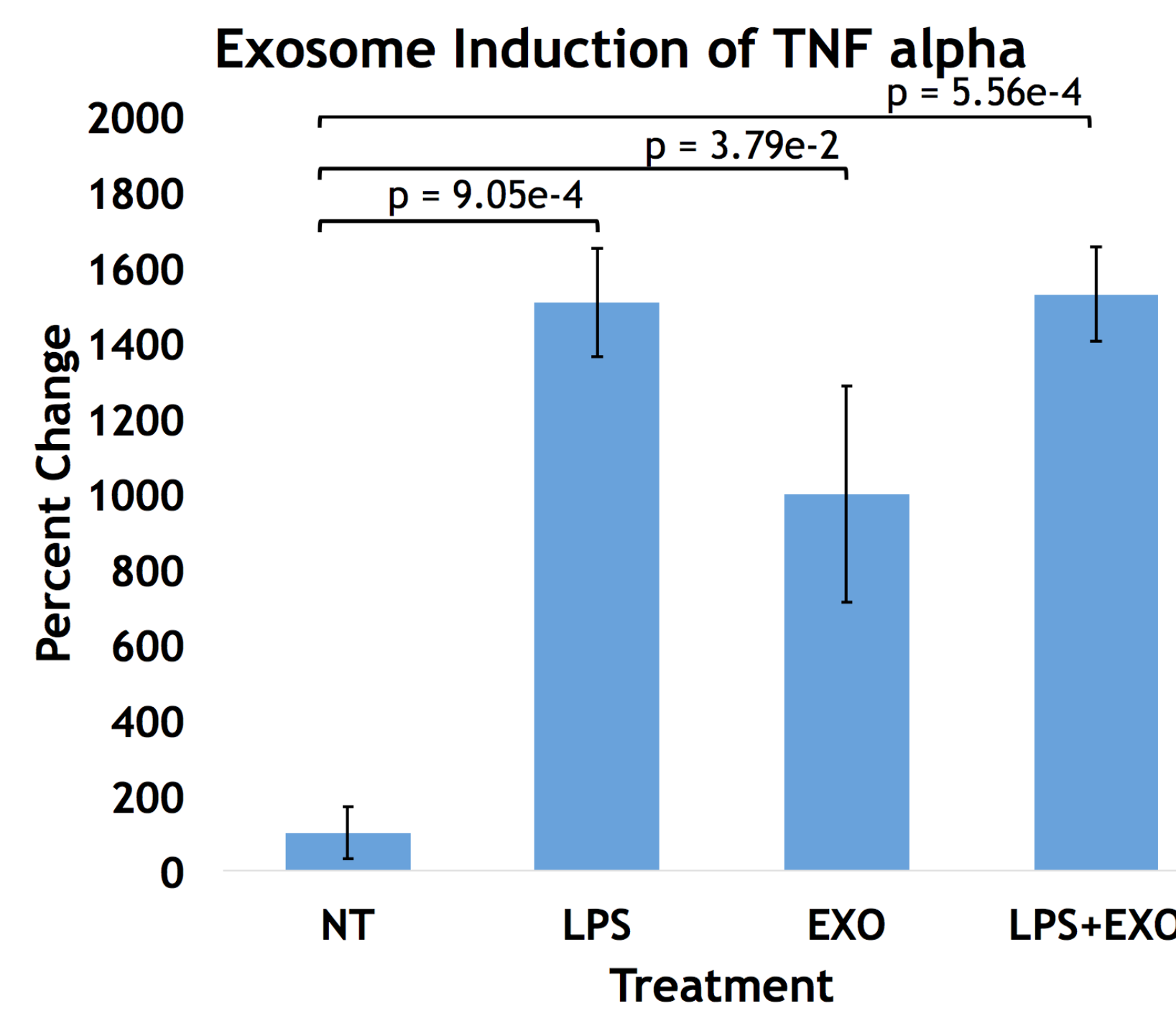


Figure 1 - TNF- $\alpha$  production in cells treated with LPS, exosomes, and LPS+exosomes. LPS increases the production of TNF- $\alpha$ . Exosome treated cells produced significantly more TNF- $\alpha$  than non-treated cells ( $p = 3.79e-2$ ). However, LPS + exosome treated cells performed similarly to cells treated with LPS alone.

$n = 3$  independent experiments using pooled batches of exosomes. Error bars = S.E.M.  $p < 0.05$  was considered significant

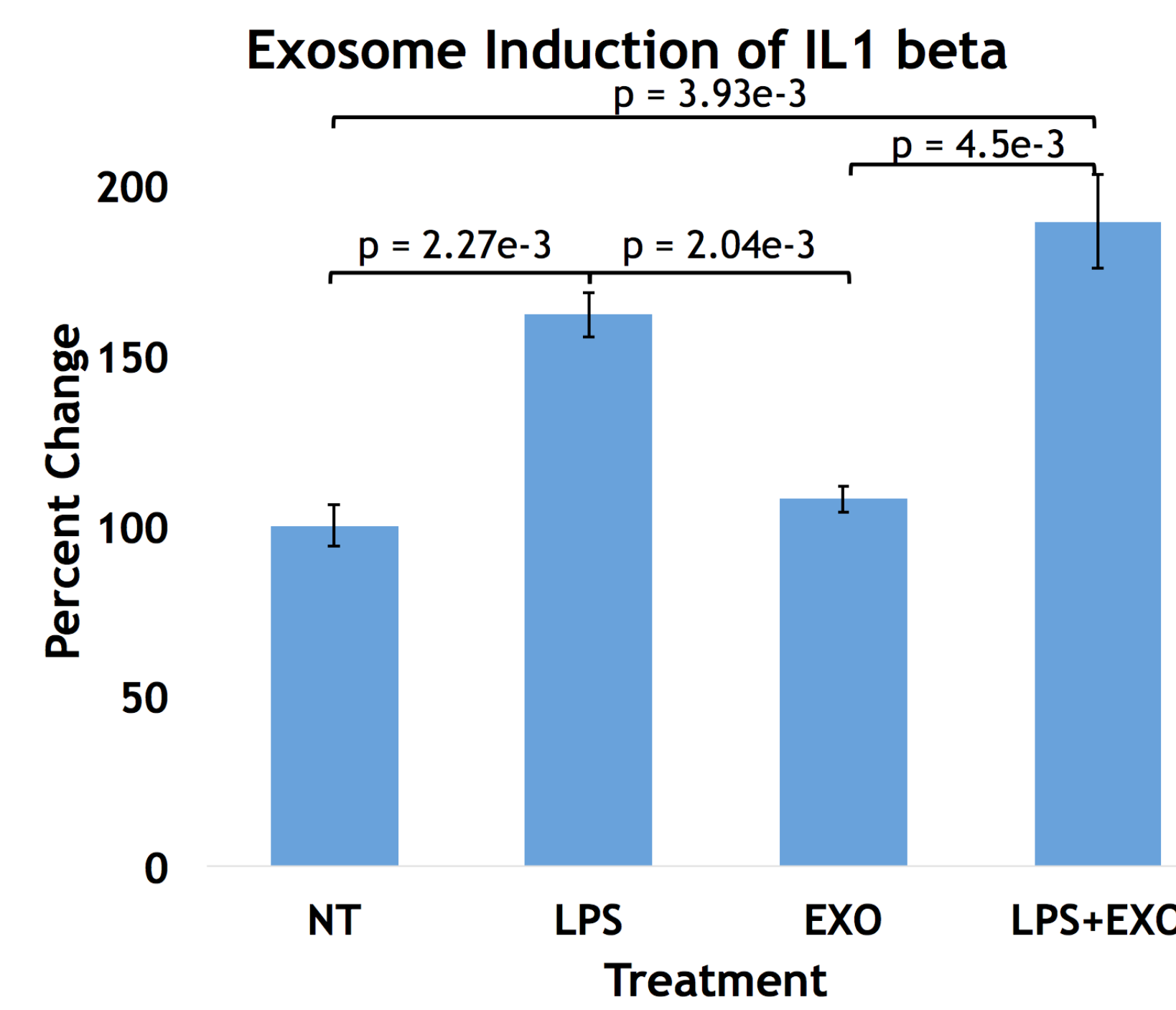


Figure 2 - IL-1 $\beta$  production in cells treated with LPS, exosomes, and LPS+exosomes. In cells treated with melanoma exosomes, there was no increase in IL-1 $\beta$  production over non-treated cells. Cells treated with LPS and cells treated with LPS + exosomes both increased IL-1 $\beta$ .

$n = 3$  independent experiments using pooled batches of exosomes. Error bars = S.E.M.  $p < 0.05$  was considered significant.

### M2 Polarization

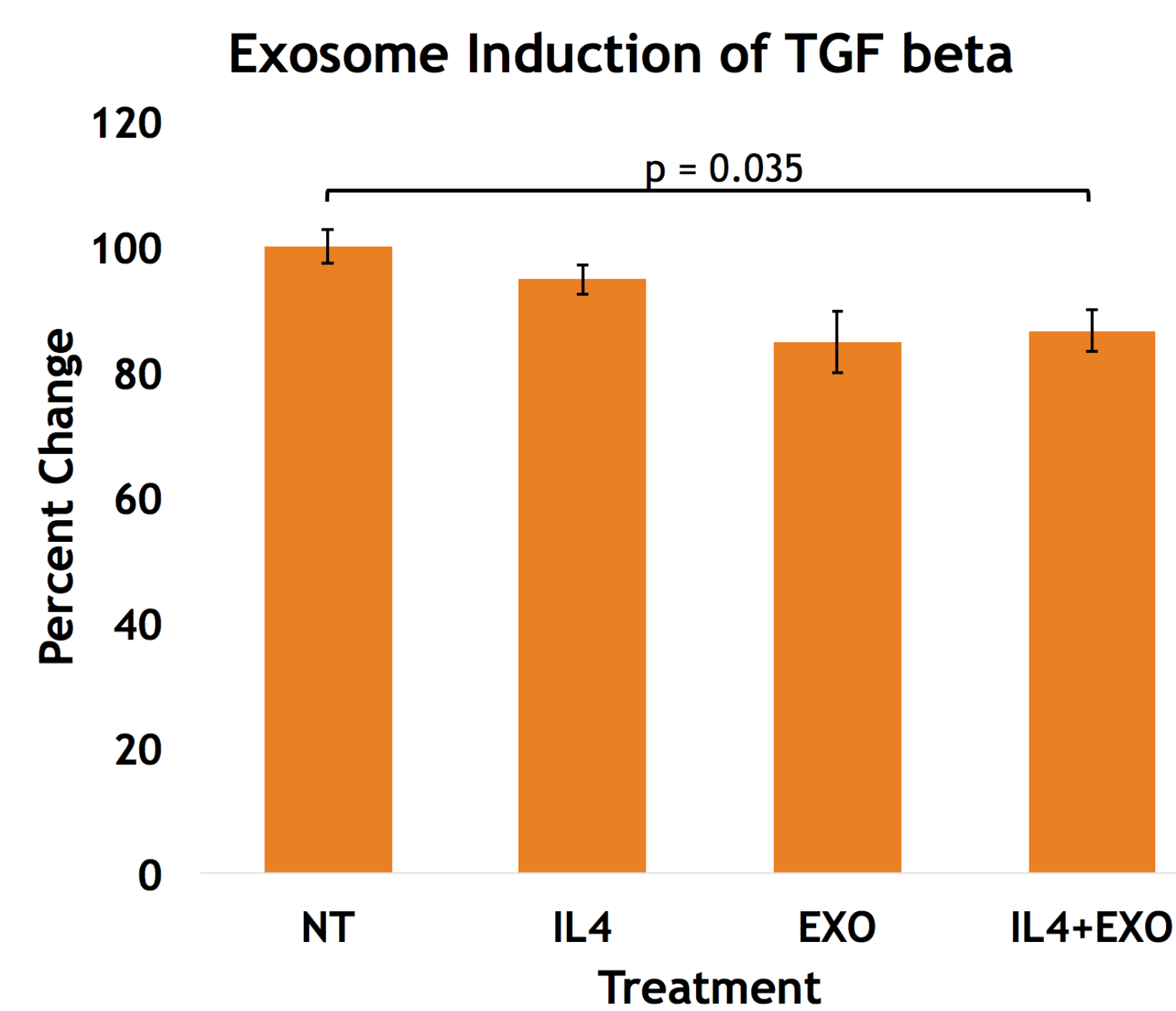


Figure 4 - TGF- $\beta$  production in cells treated with IL4, exosomes, and IL4+exosomes. Treatment with IL4 + melanoma exosomes significantly lowers TGF- $\beta$  production compared to non-treated cells. IL4 and exosomes alone did not lower TGF- $\beta$  production.

$n = 3$  independent experiments using pooled batches of exosomes. Error bars = S.E.M.  $p < 0.05$  was considered significant

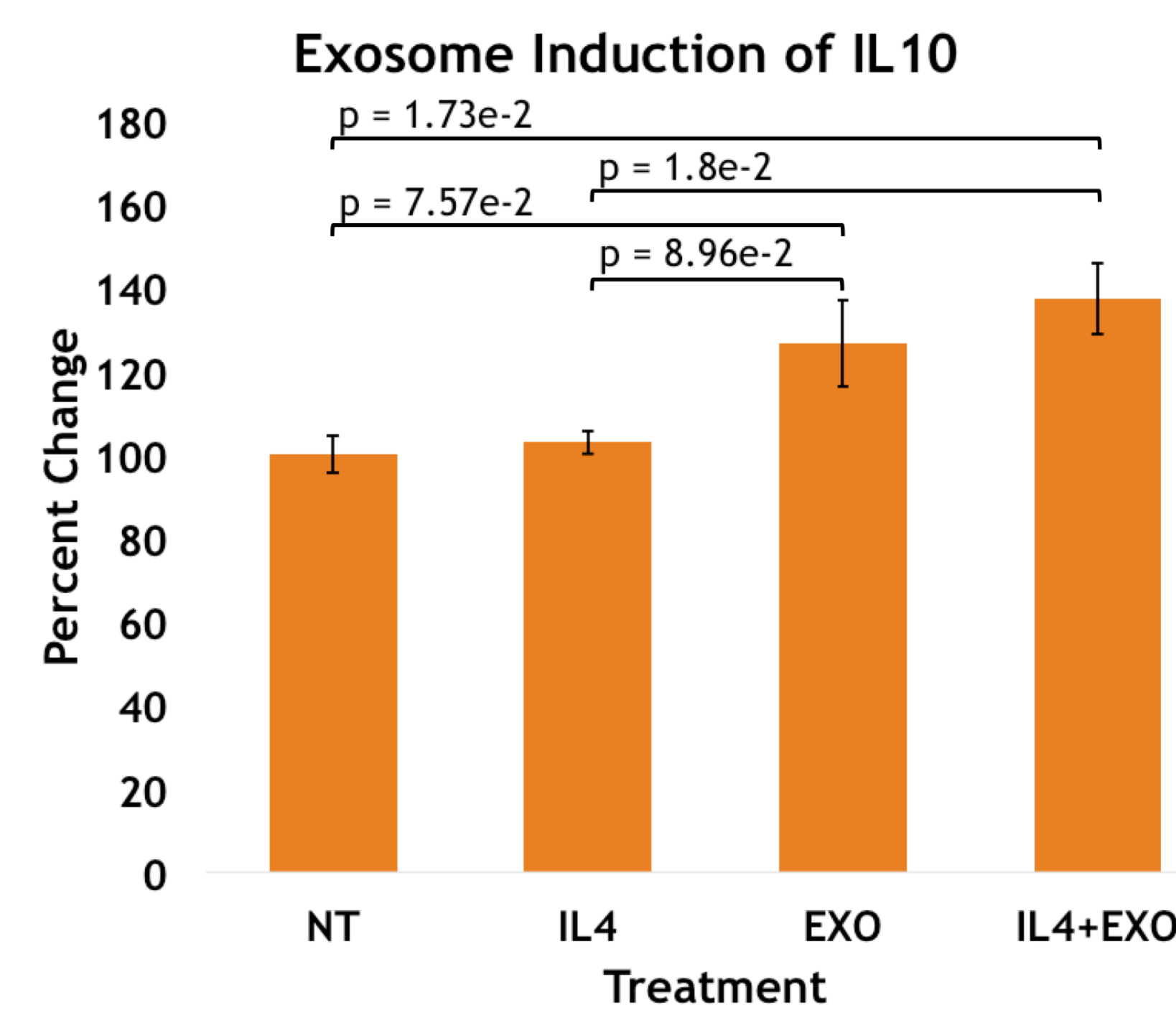


Figure 5 - IL10 production in cells treated with IL4, exosomes, and IL4+exosomes. Treatment with IL4 alone did not increase the amount of IL10. Exosome treated and IL4+exosome treated cells produced significantly more IL10 than non-treated and IL4 treated cells.

$n = 3$  independent experiments using pooled batches of exosomes. Error bars = S.E.M.  $p < 0.05$  was considered significant

## Conclusions

Here, we hypothesized that melanoma exosomes would directly induce macrophage M2 polarization. Our ELISA findings revealed that melanoma exosomes do not polarize macrophages exclusively in the M1 or M2 direction. Assessment of M1 cytokines revealed that melanoma exosomes significantly increased the production of TNF- $\alpha$ . TNF- $\alpha$  is known to participate in M1 macrophage mediated anti-tumor immunity. However, TNF- $\alpha$  also plays a role in promoting tumor angiogenesis (4). In contrast, IL-1 $\beta$ , another M1 derived pro-inflammatory cytokine, was not induced by melanoma exosomes.

Induction of standard M2 cytokines revealed similar findings via ELISA. TGF- $\beta$  was not induced by melanoma exosomes. However, IL-10, the chief M2 cytokine, was significantly expressed.

Additionally, combining exosomes with LPS treatments trended toward increasing TNF- $\alpha$  and IL-1 $\beta$ , while combining exosomes with IL-4 decreased TGF- $\beta$  and significantly increased IL-10 over IL-4 alone.

Results from the RT-RT PCR arrays largely corroborated our ELISA data. However, in contrast to the ELISA results, the PCR data shows that exosomes significantly increased IL-1 $\beta$  mRNA synthesis. Analysis of M2 results revealed, similar to the IL-1 $\beta$  findings, that exosome treated cells contained more TGF- $\beta$  mRNA despite no difference observed by ELISA. The discrepancy could be a result of assay sensitivity with RT-RT pcr being more sensitive or may reflect undefined post-transcriptional regulation mechanisms requiring more investigation.

Collectively, these findings suggest that melanoma exosomes induce a mixed macrophage phenotype. For M1, exosomes increase TNF- $\alpha$ . For M2, exosomes increase IL-10. A number of previous studies suggest a complicated relationship between TNF- $\alpha$  and IL-10. TNF- $\alpha$  can induce IL-10 expression (5) or alternatively, IL-10 can suppress TNF- $\alpha$  (6).

Overall, the pattern of macrophage cytokines induced by melanoma exosomes best describes M2b polarization, which is characterized by IL10 expression in the context of typical M1 representative cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL6 and iNOS (7). The M2b response is traditionally associated with humoral immunity, allergic and anti-parasitic immune functions (6). Induction of VEGF-A and Stat3 by melanoma exosomes further supports polarization toward an M2-like phenotype. This finding is of great importance to our understanding of melanoma exosome mediated tumorigenesis. The results demonstrate that melanoma exosomes can directly polarize macrophages toward a phenotype capable of facilitating pro-tumor supportive angiogenic (TNF- $\alpha$ , VEGF-A) and immunosuppressive (IL-10) functions.

**Future Directions:** Additional research objectives will include validating these investigations using primary mouse and human macrophages. The long term goal is to develop exosomal therapies for melanoma based on macrophage polarity.

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## Acknowledgements

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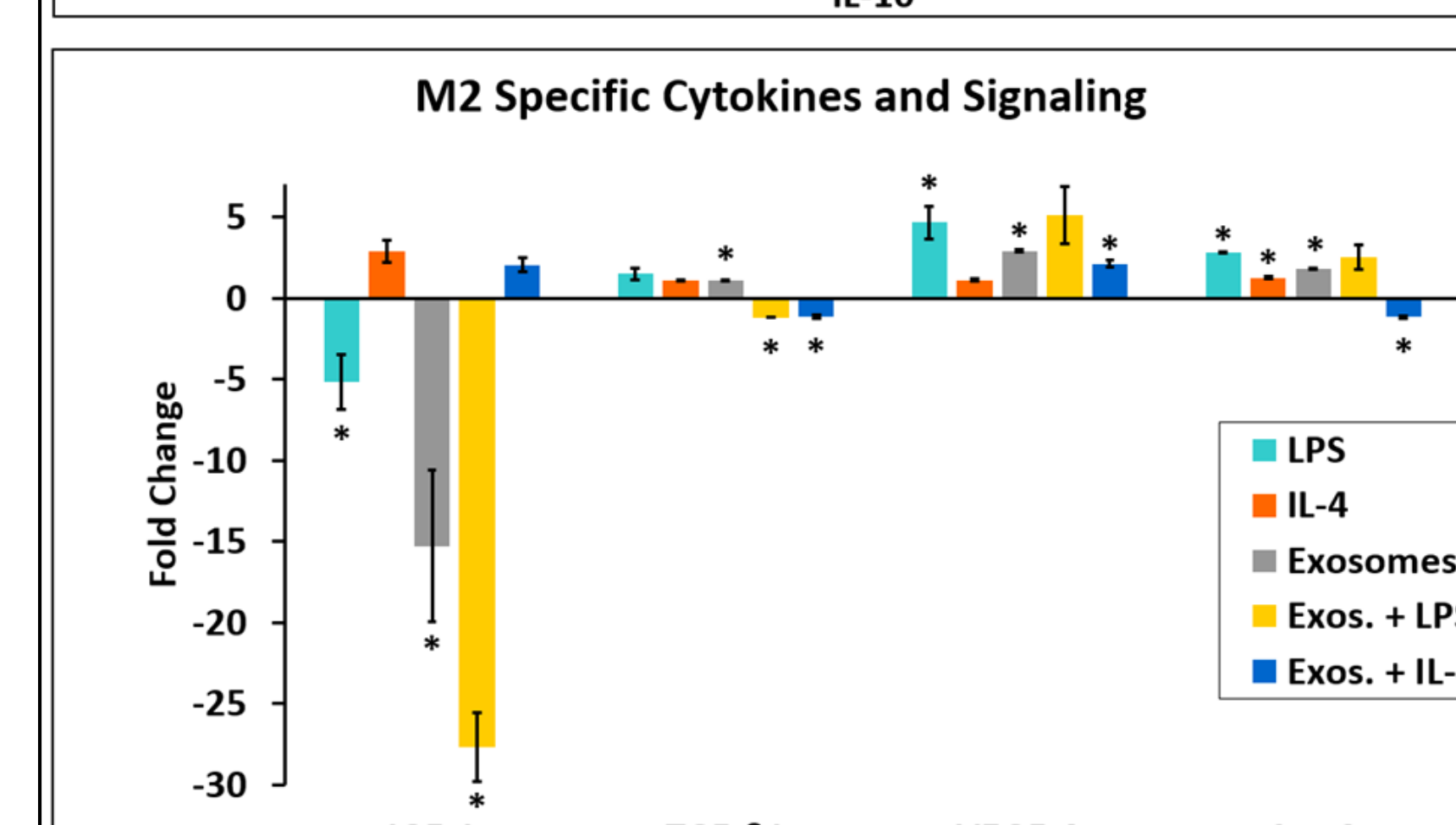
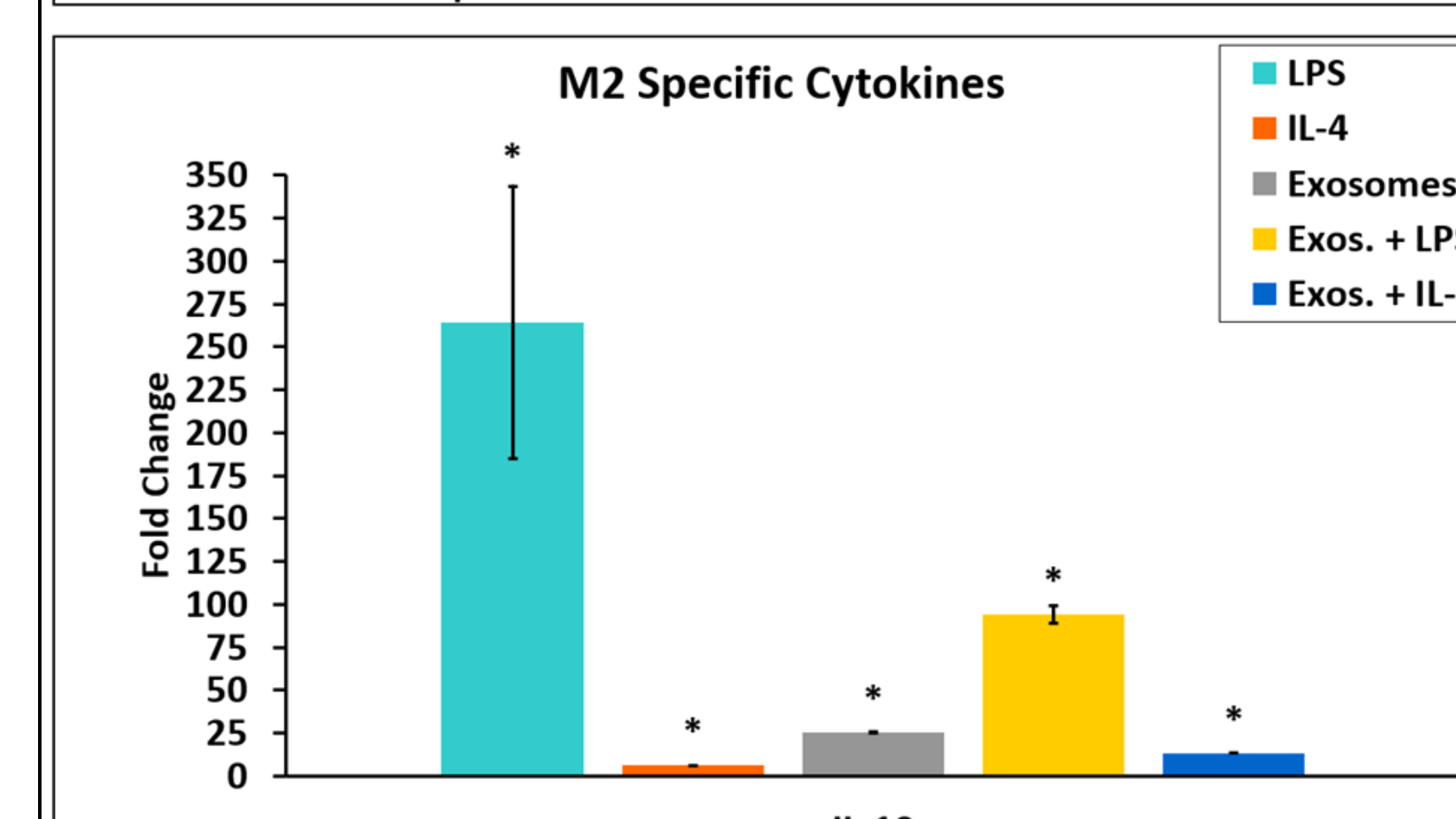
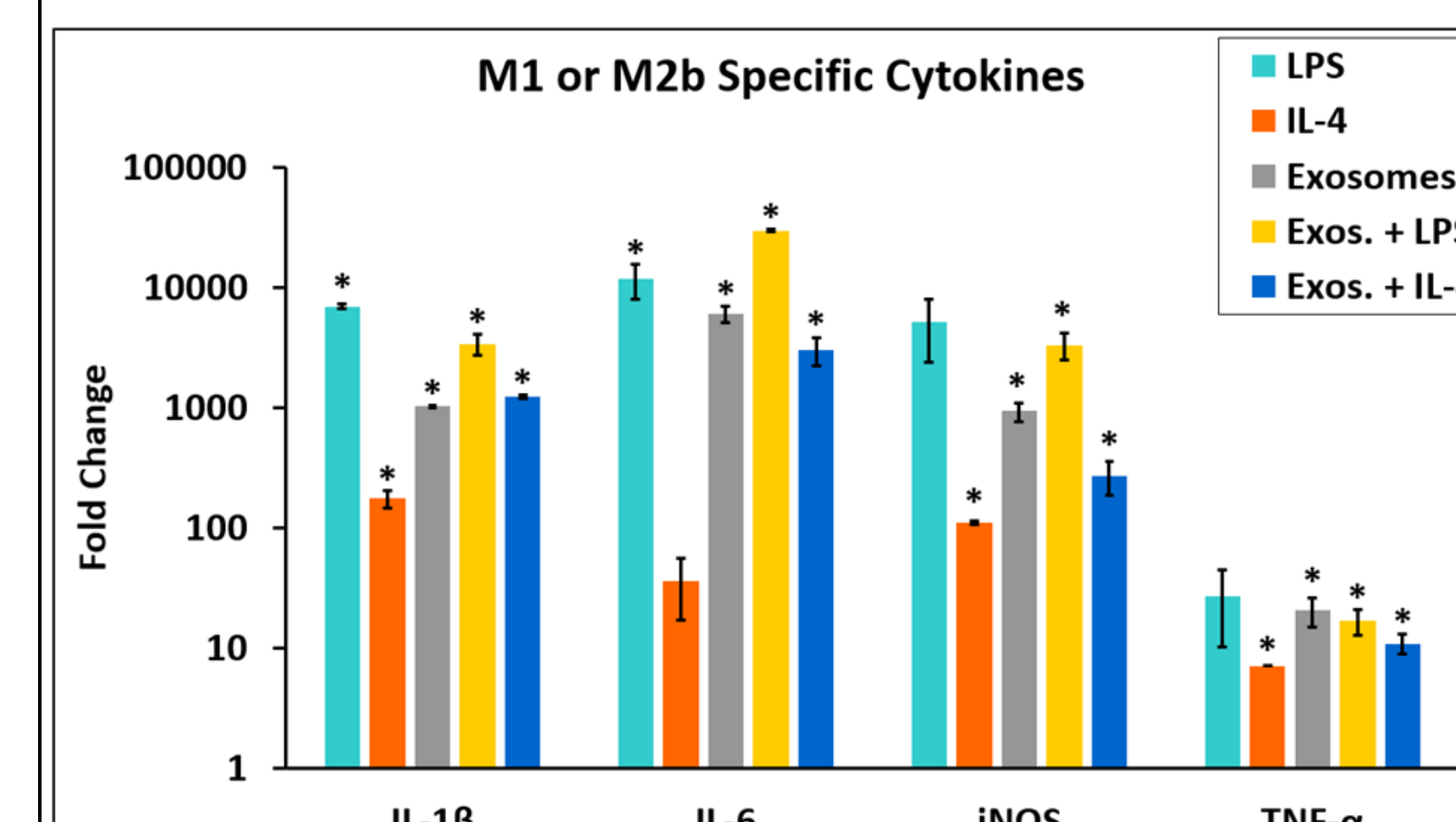
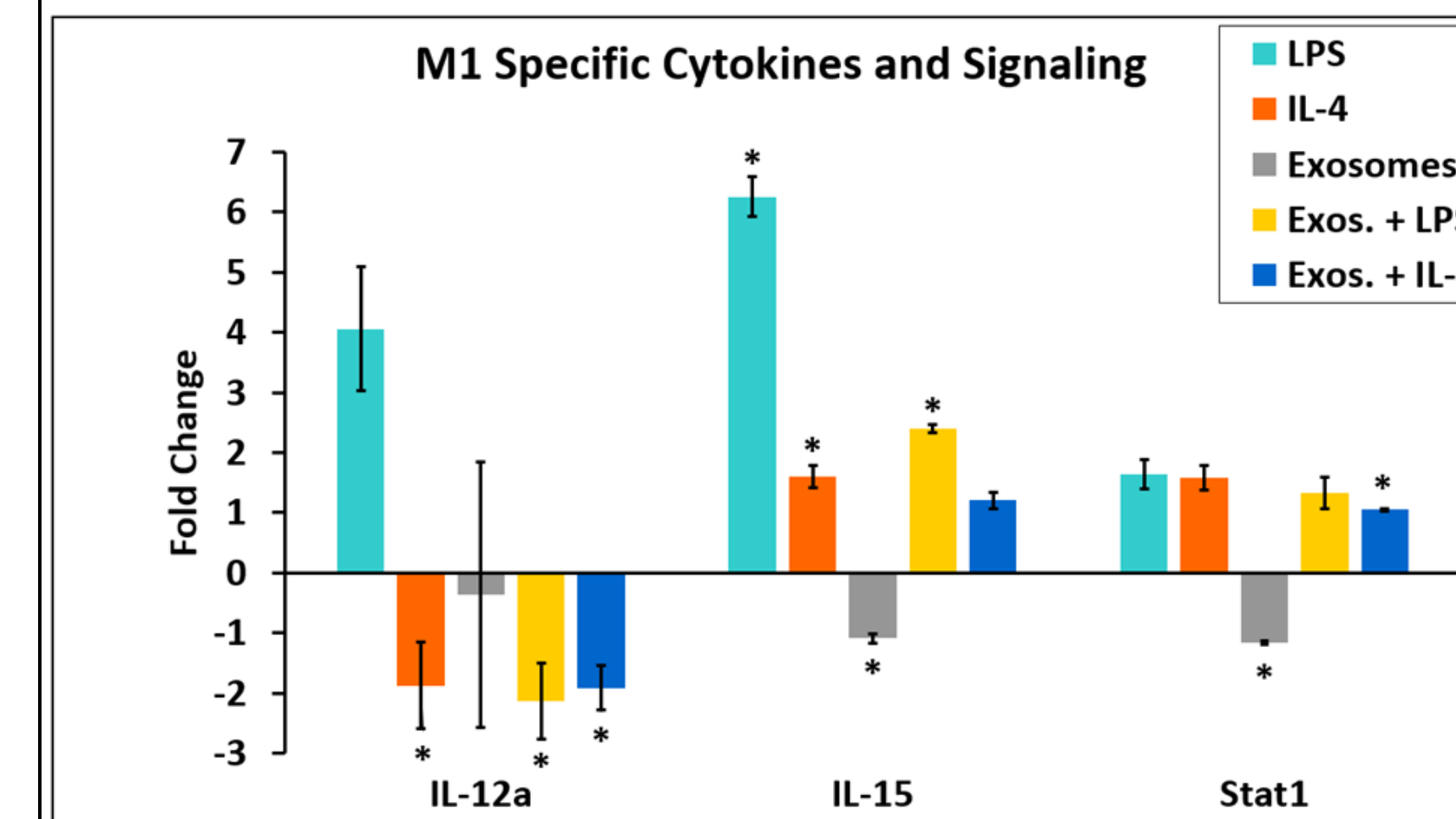
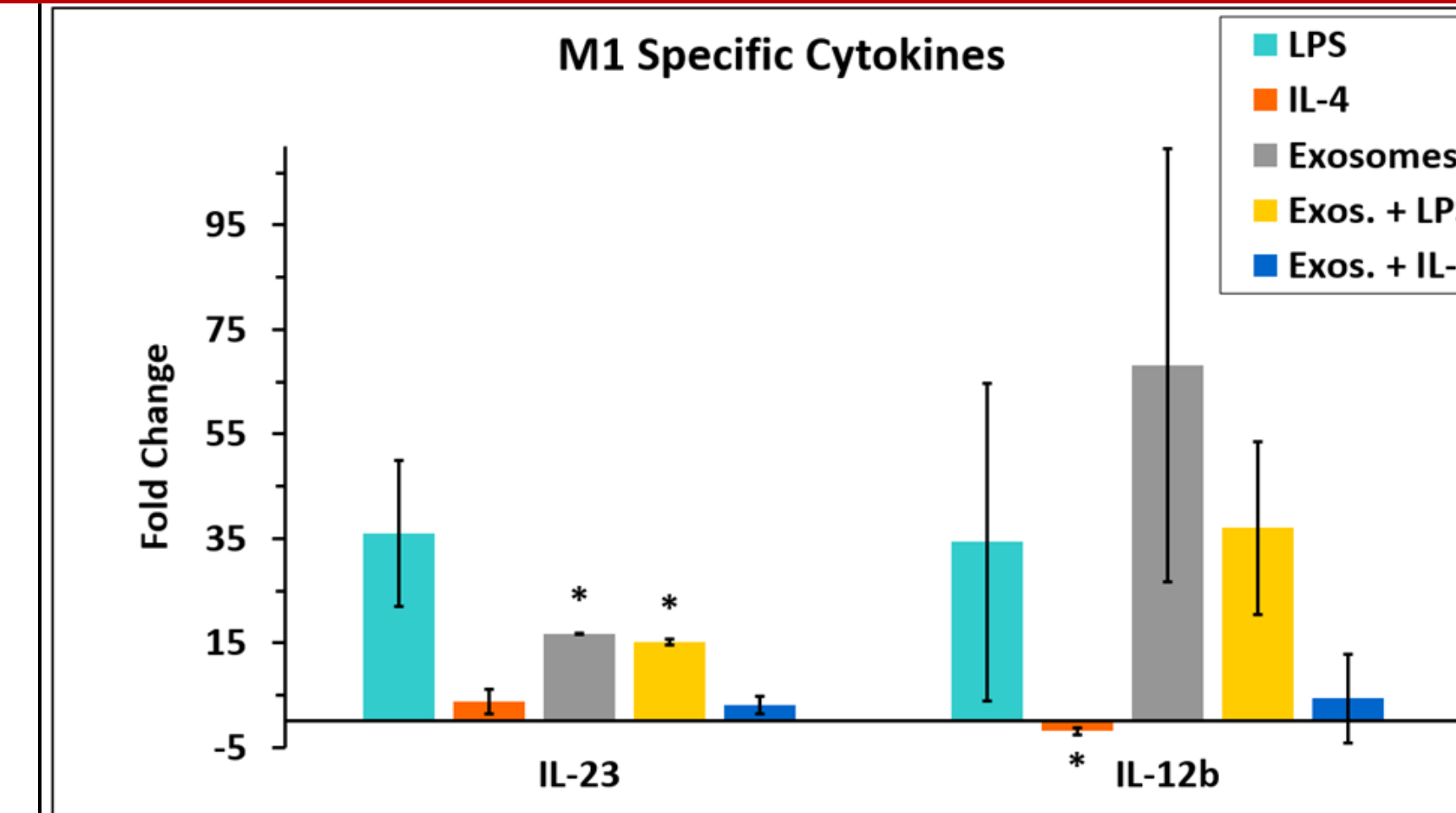


Figure 6 - M1 and M2 cytokine gene expression.  $n = 2$  replicates, error bars = S.D., \* =  $p < 0.05$



## Abstract

I became interested in methylation pattern at differently methylated regions (DMRs) of two paternally imprinted tandem genes H19-IGF2 and DLK1-MEG3 (MEG3 a.k.a. GTL2) in human ovarian cancer cell lines (A2780, CAOV3, and OVCAR4) in comparison to healthy tissue (cord blood used as control). The methylation status of the H19-IGF2 DMR and DLK1-MEG3 DMRs was assessed, and so it was the H19/IGF2 and DLK1/MEG3 ratio of expression. The methods used included combined bisulfite-restriction analysis (COBRA), methylation specific PCR and quantitative real time PCR. In the first part of this study no significant changes in methylation of the IGF2-H19 DMR was observed, but in contrast hypermethylation of the DLK1-MEG3 DMR in A2780 and OVCAR4 has been noticed, and for all tested ovarian carcinoma cell lines loss of imprinting (LOI) in the MEG3 DMR. Moreover, lack of correlation between the IGF2-H19 DMR methylation status and the expression of IGF2 and H19 genes was found in all ovarian carcinoma cell lines. No direct correlation between the methylation status of the DLK1 DMR and the expression of DLK1 has been observed, with the exception of MEG3. The similar methylation status of the cell lines samples with a different ratio between tandem genes expression suggest that the expression is imprinting independent. Since expression of tandem genes from one locus should be balanced from lack of expression in the other, the ratio of IGF2-H19 and of DLK1-MEG3 was analyzed. When a high level of expression of factors that inhibit cell proliferation was observed in one region a high expression of factors that promote proliferation was observed in the other region. This suggest that both tandem regions complement each other in regulation of cell proliferation.

## Objectives

- To examine the methylation status of the Different Methylated Regions (DMRs) in the IGF2-H19 and DLK1-MEG3 tandem gene loci.
- To assess the expression of those genes and the ratio of their expression per tandem gene.

## Background

Genomic imprinting refers to the process that causes genes to be expressed in a monoallelic parental origin-specific manner rather than from both chromosomes homologues. Mammals inherit two complete sets of chromosomes, one from each parent, and most autosomal genes are expressed from both maternal and paternal alleles. Genomic imprinting is related to the methylation of cytosine bases in the CpG dinucleotides in the DNA sequence. Almost all imprinted genes have a CpG-rich differentially methylated region (DMR) found to be a key regulator in imprinted gene's expression. The methylation status of imprinted genes is important in cancer studies because imprinted genes might affect cell proliferation patterns. Paternally expressed genes generally enhance growth, whereas maternally expressed genes appear the opposite effect. It has been hypothesized that this behavior is the result of different evolutionary pressure (parental conflict hypothesis): the mother tends to distribute resources to all offspring equally and to ensure the fetus would not be too big at the moment of delivery. Father drives to maximize growth and resource acquisition for his offspring alone to favor the fitness of his descendants (Ferguson-Smith, *Genomic Imprinting: the emergence of an epigenetic paradigm*, 2011). Of interest for this study are the tandem genes H19 (maternally expressed) and IGF2 (paternally expressed) found on chromosome 11, and the genes DLK1 (paternally expressed) and MEG3 (a.k.a. GTL2, maternally expressed) found on chromosome 14 [see Fig. 1]. These two couplets need to be in balance for effective development of an organism.

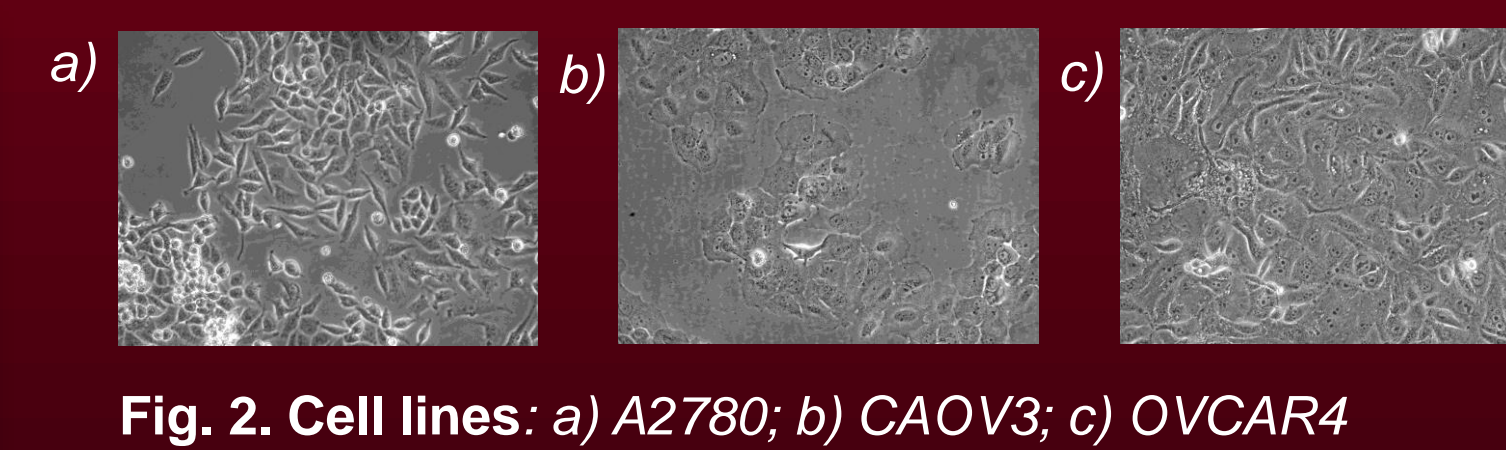
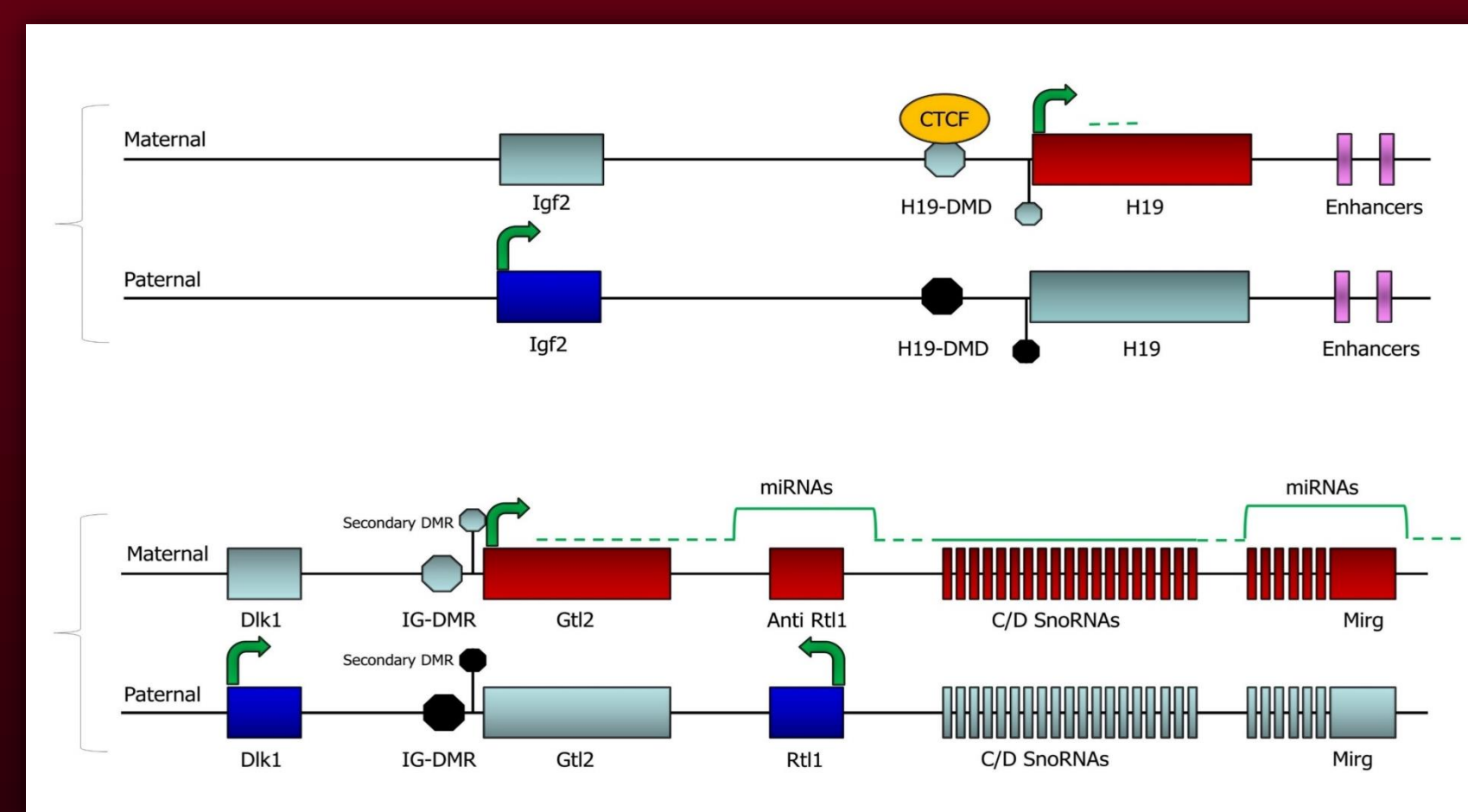


Fig. 2. Cell lines: a) A2780; b) CAOV3; c) OVCAR4

Fig. 1. Diagram of H19-IGF2 and DLK1-MEG3 tandem genes (Ferguson-Smith, *Genomic Imprinting: the emergence of an epigenetic paradigm*, 2011)

## Results

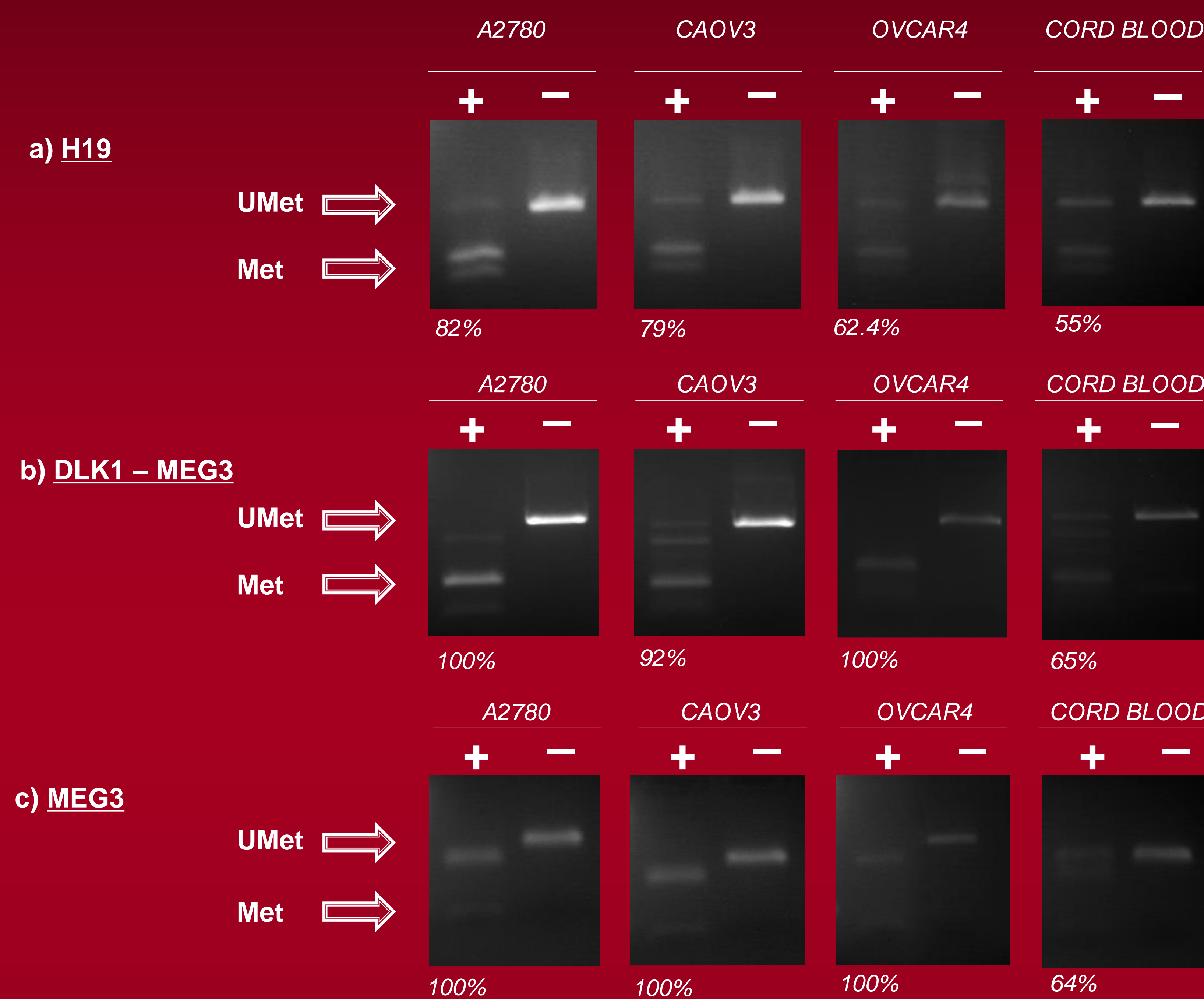


Fig. 3. Methylation pattern of the IG-DMR at the H19-IGF2 and DLK1-MEG3 loci. a) The COBRA assay was used to evaluate the methylation status using BstUI restriction enzyme. b) For DLK1-MEG3 was used BstUI restriction enzyme. c) For ME was used Taq1 restriction enzyme.

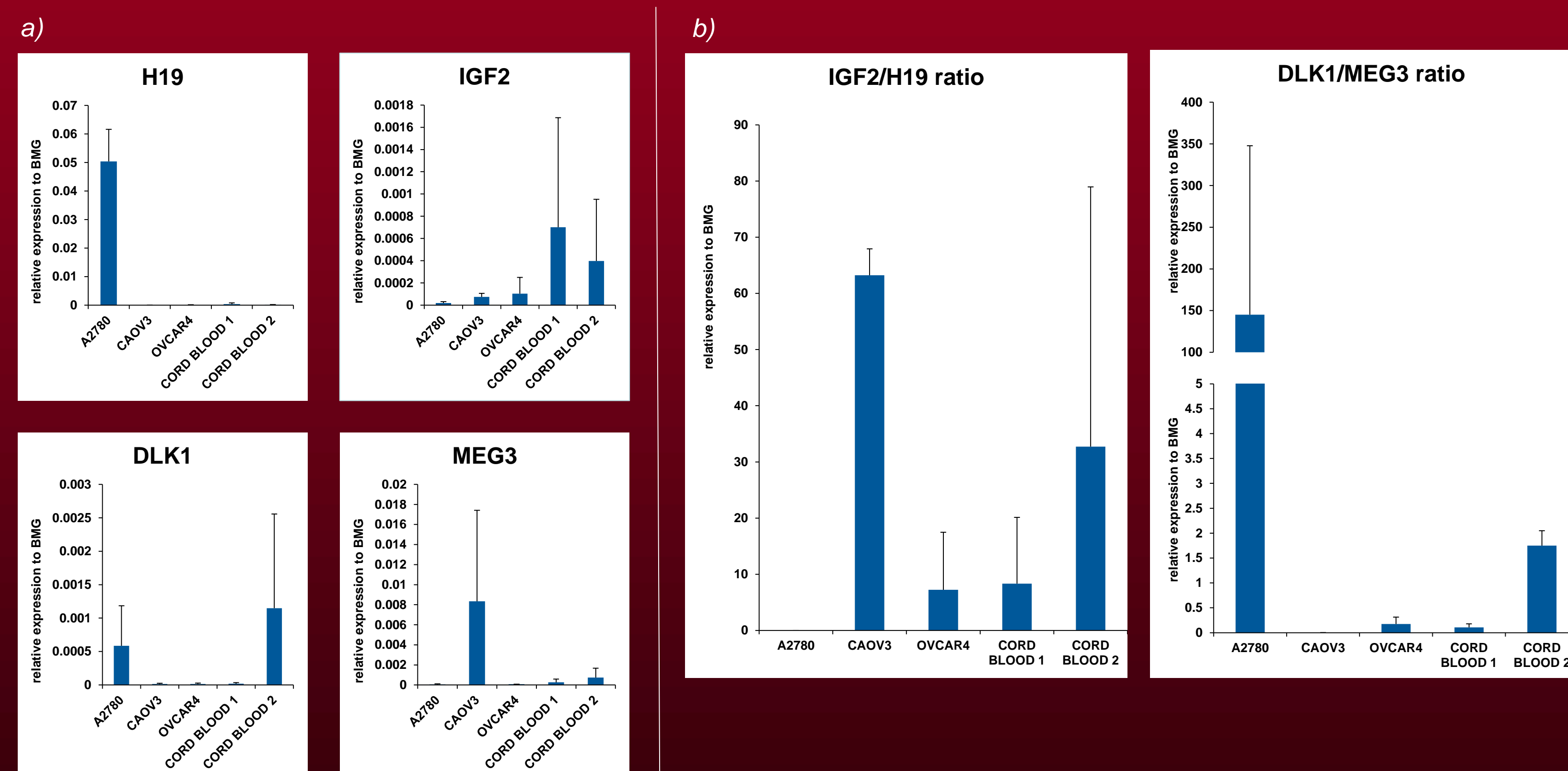


Fig. 4. Expression of the imprinted genes analyzed by qRT-PCR. a) expression of the individual genes. b) Ratio of expression of tandem genes per locus.

## Methods

The methylation analysis was performed on samples from three ovarian carcinoma cell lines – A2780, CAOV3, and OVCAR4 – and on two samples of healthy tissue – cord blood – used as control (see Fig. 2). DNA was isolated from the cells, converted with a bisulfite conversion kit, and cleaned up for methylation analysis. After a methylation specific nested polymerase chain reaction (Meth-PCR) the presence of the desired products was confirmed by agarose gel electrophoresis; then the products were analyzed by combined bisulfite-restriction analysis (COBRA) (see Fig. 3) followed by densitometric analysis. The second part of the study regarded the expression of the genes: RNA was isolated from samples of the cell lines and converted into cDNA by reverse transcription. The obtained cDNA was used as a template for the final step of the analysis of the expression of the genes by quantitative real-time PCR (see Fig. 4).

## Summary of Results

- There are no significant changes in methylation status of the IGF2-H19 DMR.
- DLK1-MEG3 DMR is hypermethylated in A2780 and cell lines.
- Loss of imprinting (LOI) in the MEG3 DMR occurs in all tested ovarian cancer lines.
- Lack of correlation between the IGF2-H19 DMR methylation status and the expression of IGF2 and H19 genes was observed in all ovarian carcinoma cell line samples.
- There is no direct correlation between the methylation status of the DLK1 DMR and the expression of DLK1 gene in all ovarian carcinoma cell line samples
- In contrast MEG3 expression is observed to be the higher in CAOV3 cell line in comparison with A2780 and OVCAR4 cell lines. This correlates with the differences in methylation of DLK1-MEG3 DMR between analyzed ovarian carcinoma cell lines.
- The similar methylation status of the cell lines samples with a different ratio between tandem genes expression suggest that the expression is imprinting independent.

## Conclusions

- Analysis of IGF2/H19 and DLK1/MEG3 ratio of expression indicates a shift toward factors that inhibit cell proliferation in one tandem gene region, compensated by factors that promote proliferation encoded in the other region. This suggest that both tandem regions complement each other in regulation of cell proliferation. Changes in the methylation of DMRs in DLK1-MEG3 cluster suggest that the genes encoded by this specific region might be relevant in the regulation of cell proliferation.

## Future studies

- Future studies on IGF2-H19 and DLK1-MEG3 DMRs methylation will be performed using samples taken from human primary tumors to confirm the results so far obtained.
- Since this study suggest imprinting independent expression of IGF2 and H19, the analysis of the type of expression, monoallelic vs biallelic, will be performed.
- Changes in the methylation of DMRs in DLK1-MEG3 cluster suggest that the genes encoded by this specific region might be relevant in the regulation of cell proliferation. Therefore more detailed studies will be performed by targeting the expression of these genes by transfecting the cells with appropriate plasmids to achieve overexpression of DLK1 or MEG3, and plasmids encoding shRNA to downregulate DLK1 or MEG3 levels.

## Acknowledgments

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# The Effect of G-quadruplex Oligonucleotide Sequences Targeting c-MYC, SOX2 and H-TERT in Melanoma Cell Lines

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## Abstract/Introduction

Malignant melanoma is the deadliest form of skin cancer and it is estimated that 10,310 people will die in the US of melanoma in 2016<sup>1</sup>. SPP1, SOX2, CXCL1, h-TERT, and c-MYC are a few of many genes expressed in melanoma. This study focuses on c-MYC, SOX2, and h-TERT.

- The c-MYC gene is a master regulator that is critically involved in the regulation of many growth promoting signal transduction pathways<sup>2</sup> including proliferation, differentiation, transformation, angiogenesis and apoptosis. c-MYC is overexpressed in 80% of all solid tumors.<sup>3</sup>
- The SOX2 gene plays an essential role in protein synthesis that provide tissues and organs along with promoting cell growth, invasion, migration and metastasis<sup>4</sup>. It has been shown that silencing SOX2 inhibits growth and induces apoptosis in primary melanoma cells when using SOX2 shRNA which resulted in loss of protein.<sup>5</sup>
- The h-TERT (human telomerase reverse transcriptase) is expressed in all cells. h-TERT promoter contains many transcription factor binding site, including c-MYC<sup>6</sup>. It was found that 75% of melanomas have mutations at four specific sites within the G-quadruplex forming sequence of the h-TERT promoter<sup>7</sup>. G → A or C → T mutations of the promoter region of the h-TERT gene can destabilize the quadruplex resulting in over expression of human telomerase which rapidly increases cell division of cancer cells.<sup>7</sup> By attempting to target promoter region of h-TERT in melanoma could allow for an opposite affect on activation of telomerase which is known for cell division of cells<sup>7</sup>.

The G-quadruplex are stable four-stranded G-rich DNA structure that are found preferably in the promoter region of oncogenes. G-quadruplex forming oligonucleotides have been seen as therapeutic agents targeting over-expressed oncogenes. It has recently been found in the Miller Research lab that the Pu27 G-quadruplex inhibits leukemia cell proliferation by silencing the c-MYC gene<sup>8</sup>. This suggests that c-MYC, along with other genes like SOX2 and h-TERT that are overexpressed in melanoma, could be targeted with G-quadruplex forming oligonucleotides.

Targeting G-quadruplexes in oncogenes have been seen as therapeutic agents and can be a possible treatment for melanoma.<sup>5</sup> In this study we evaluate the effect of G-quadruplex forming oligonucleotides in four melanoma cell lines.

## Objectives

In this study we evaluate the efficacy of treatment using G-quadruplex forming oligonucleotides four melanoma cell lines. The oligonucleotides being used specifically target promoter regions of c-MYC, SOX2 and hTERT genes.

## Materials & Methods

**Oligonucleotides:** Pu27 to target the c-MYC gene, Pu3+ to target SOX2 gene, Tert +1, Tert FL, Tert 4/6 to target h-TERT gene.

**Cell Culture:** Four melanoma cell lines and one normal human skin fibroblasts: A375, SK-Mel-2, SK-Mel 3, SK-Mel 28 and HS27, respectively, were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin.

**MTT Assay:** A375, SK-Mel-2, SK-Mel 3, SK-Mel 28 and HS27 were seeded in 96-well flat bottom plates at  $1 \times 10^3$  cells/well in 150  $\mu$ l. Plated cells were treated with doses of 5 and 10  $\mu$ M of oligonucleotides for 6days compared to untreated cells. Cell proliferation was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), DMEM was used as blank and measured using BioTek Microplate Reader and Spectrophotometer to read absorbance at 570nm.

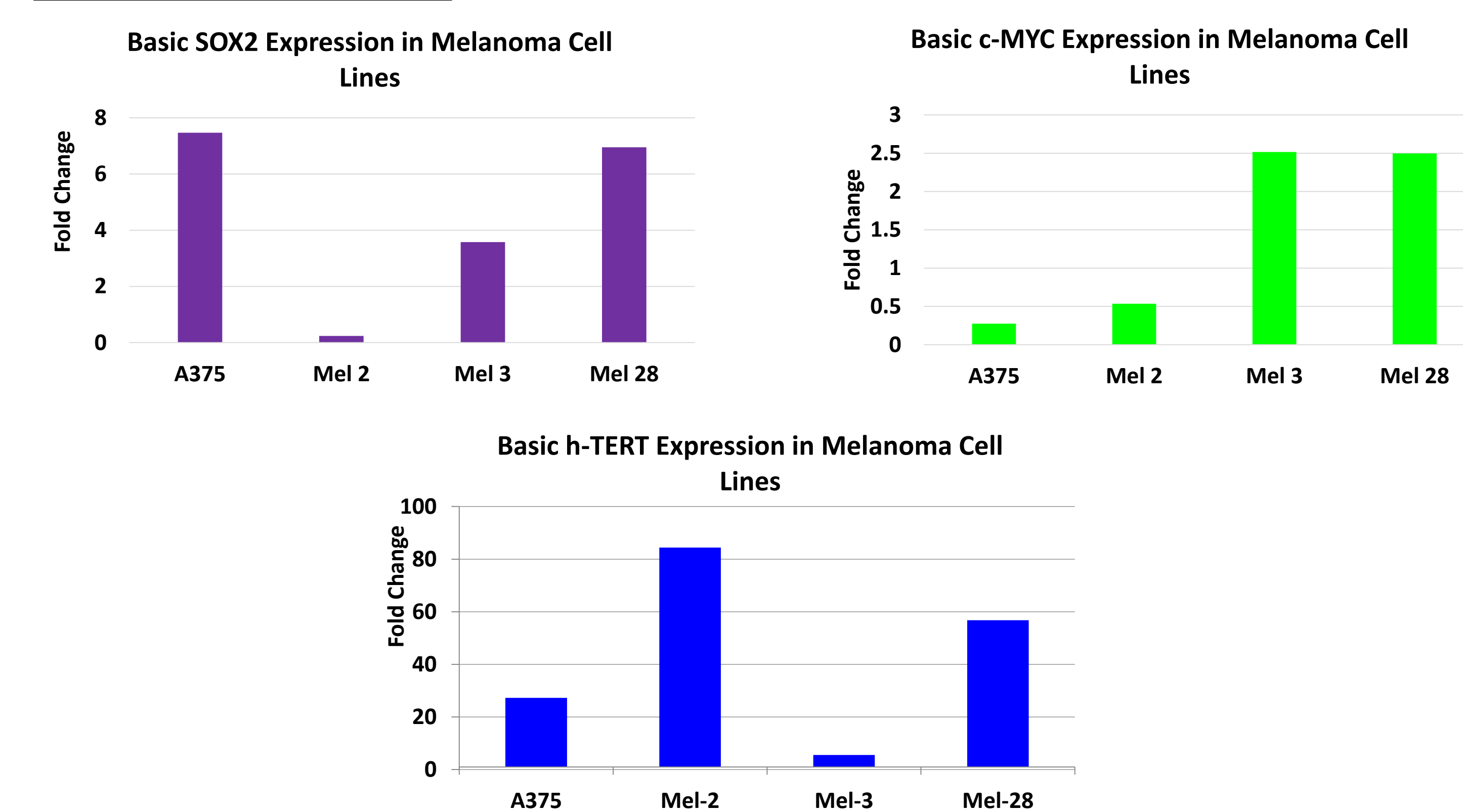
**Cell Treatment for gene expression:** cell line (A375), cell line (SK-Mel-2), and the control cell line (HS27) were plated at  $2 \times 10^5$  cells/well in 6 well plates and treated with 4.3 $\mu$ M of : Pu27, Pu3+ or Tert +1 for 6 days. Cells were collected and gene and protein expressions were evaluated:

**Gene Expression:** The basic gene and protein expression of SOX-2, c-MYC, and h-TERT were determined in in A375, SK-Mel-2, SK-Mel 3, SK-Mel 28 and HS27 cells. The effect of oligonucleotide treatment these genes was evaluated in A375, SK-Mel-2, and HS27 was determined using:

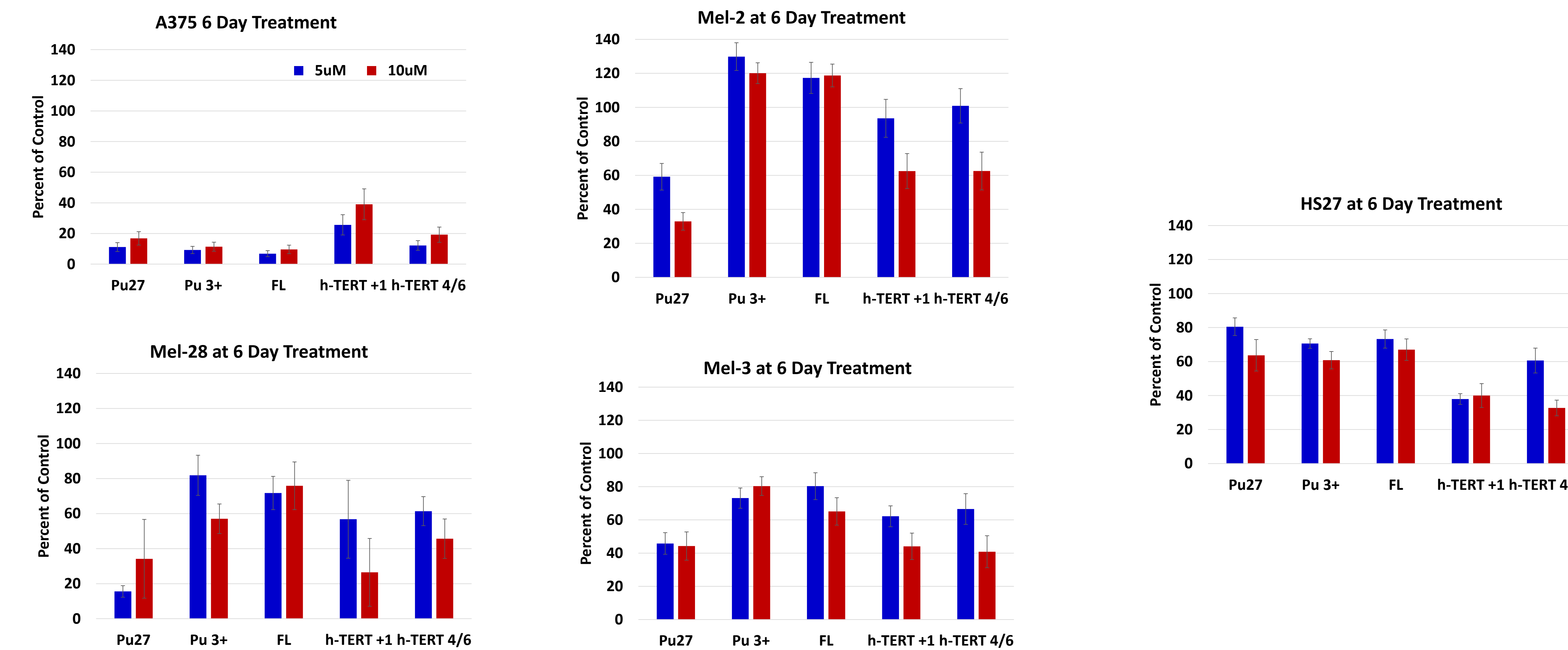
- QRT-PCR:** TRIzol reagent was used to extract RNA. cDNA was synthesized from the extracted RNA using SuperScript VILO cDNA Synthesis Kit. Quantitative Real Time PCR was performed with primers pairs for SOX-2, c-MYC, h-TERT and GAPDH as a housekeeping control.
- Western Blotting:** Protein lysates were extracted using MPER Mammalian Protein Extraction Reagent with Protease Inhibitor. Proteins were separated based on size of protein using SDS-PAGE gel electrophoresis to determine. The following antibodies were used: rabbit anti-SOX-2, followed by anti-rabbit-HRP, mouse anti-c-MYC, followed by anti-mouse-HRP, and rabbit  $\beta$ -actin, followed by anti-rabbit-HRP. The presence of the protein was revealed using chemiluminescence through development on X-ray film.

## Results

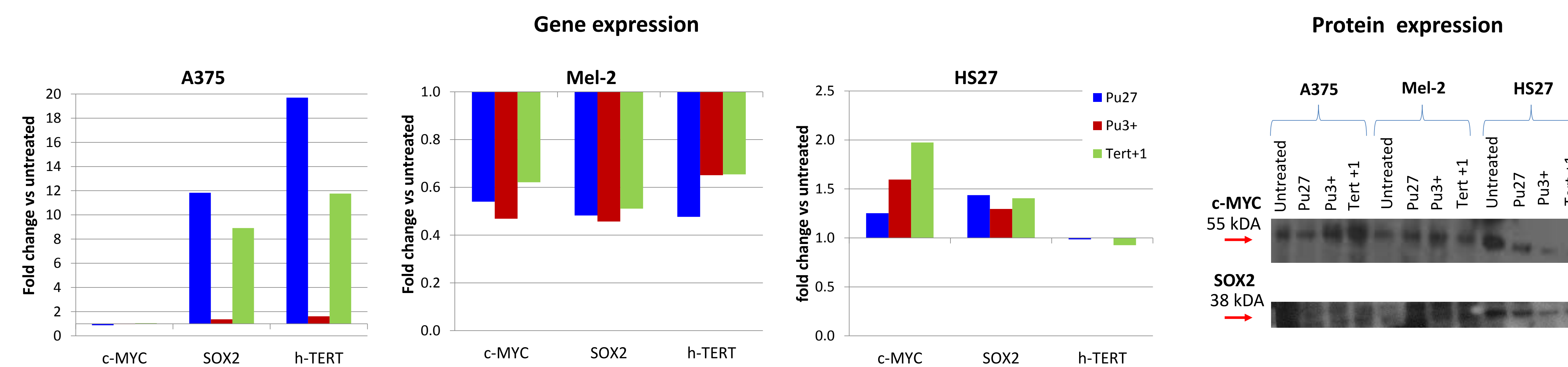
**Figures 1: Evaluation of the basic c-MYC, SOX2, and h-TERT gene expression in melanoma cell line relative to HS27 fibroblast:**



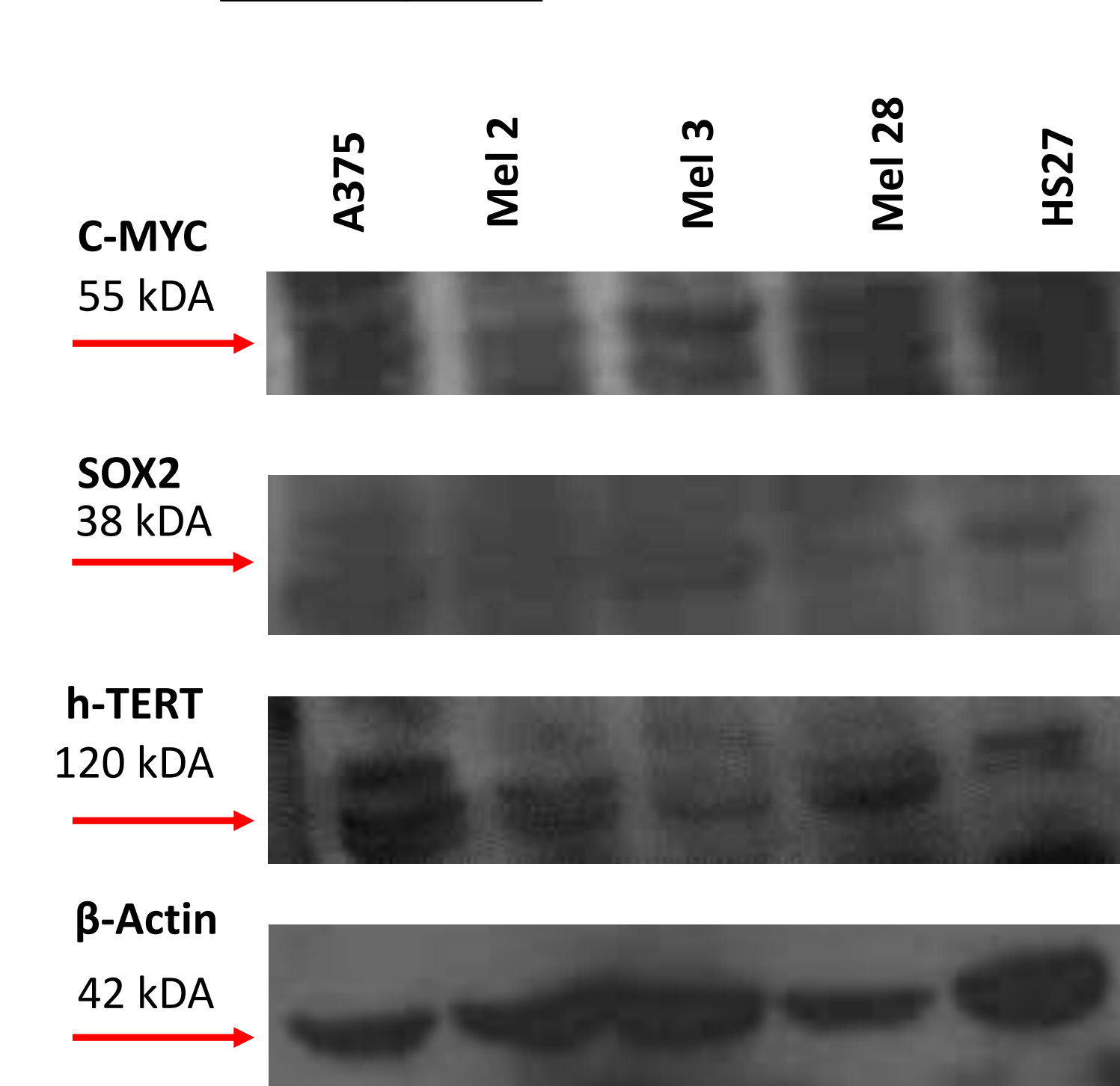
**Figure 3 : Effect of G-quadruplex oligonucleotide treatment for 6 days on cell proliferation**



**Figure 4: SOX2, c-MYC, and h-TERT gene expression on cell lines (A375, Mel 2, and HS27) treated with Pu27, Pu3+ or Tert +1 oligonucleotides for 6 days.**



**Figure 2: Protein Expression of c-MYC, SOX2, h-TERT genes.**



## Results/Discussion

### Basic expression of c-MYC, SOX2, and h-TERT target genes in Melanomas (Fig.1 &2)

- A375 showed up-regulation of c-MYC and h-TERT and down regulation of SOX2 that correlate with the protein expression
- SK-Mel-2 highly over-express h-TERT and had down-regulated c-MYC and SOX2
- SK-Mel3 and SK-Mel28 over-express all 3 genes
- Therefore all these melanoma cell lines were good candidates to evaluate the effect of oligonucleotides targeting c-MYC, SOX2 or h-TERT

### Effect of the oligonucleotides on cell proliferation by MTT assay: (Fig. 3)

- A375 showed the best growth inhibition after treatment with all of the oligonucleotides
- Tert +1 had the best effect on cell inhibition compared to the other two oligonucleotides targeting h-TERT gene.
- All cell lines growth was inhibited by Pu27
- A mild effect on cell growth was observed in HS27 especially with the tert+1 and tert4/6 that will need to be further investigated.
- The response to the oligonucleotide was slightly different for each cell line, we decide to investigate the difference in c-MYC, SOX2 and h-TERT genes expression in 2 melanoma cell lines (A375 that contain a C250T mutation in the h-TERT promoter, and Mel-2) and the normal fibroblast (HS27) after 6 days treatment with Pu27, Pu3+ and tert+1.

### Effect of the oligonucleotides on c-MYC, SOX2 and h-TERT gene and protein expression: (Fig. 4)

- Cells collected after 6 days of treatment were investigated for the gene expression in total RNA for each treatment compared to the untreated.
  - The data show that Pu3+ and tert+1 slightly up-regulate c-MYC but did not affect SOX2 or h-TERT gene expression in HS27
  - While in A375 c-MYC gene was slightly down-regulated by all treatments, however Pu27 and h-TERT up-regulated SOX2 and h-TERT
  - In Mel-2 all genes were down-regulated by all treatments

The protein expression evaluated by western blotting did not support the QRT-PCR

## Conclusion

Most cell lines were sensitive to Pu27 oligonucleotide that target c-MYC and to the different oligonucleotides targeting h-TERT. The use of G-quadruplex forming oligonucleotides to target specific genes such as c-MYC, SOX2 or h-TERT could have therapeutic application in melanoma.

This study suggests that the presence of mutations within h-TERT promoter region of A375 may affect these cells responsiveness to oligonucleotides targeting the same region.

### Future Directions:

Correlation of the sensitivity of the cell lines to the h-TERT oligonucleotides and the presence of mutations in the promoter region of h-TERT. Evaluation of the mechanism of action of h-TERT oligonucleotide and determine molecular pathways in melanoma.

## Acknowledgements

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- Miller Research Lab

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# Decrease of MDSCs by Oral $\beta$ -glucan in Lung Cancer Patients

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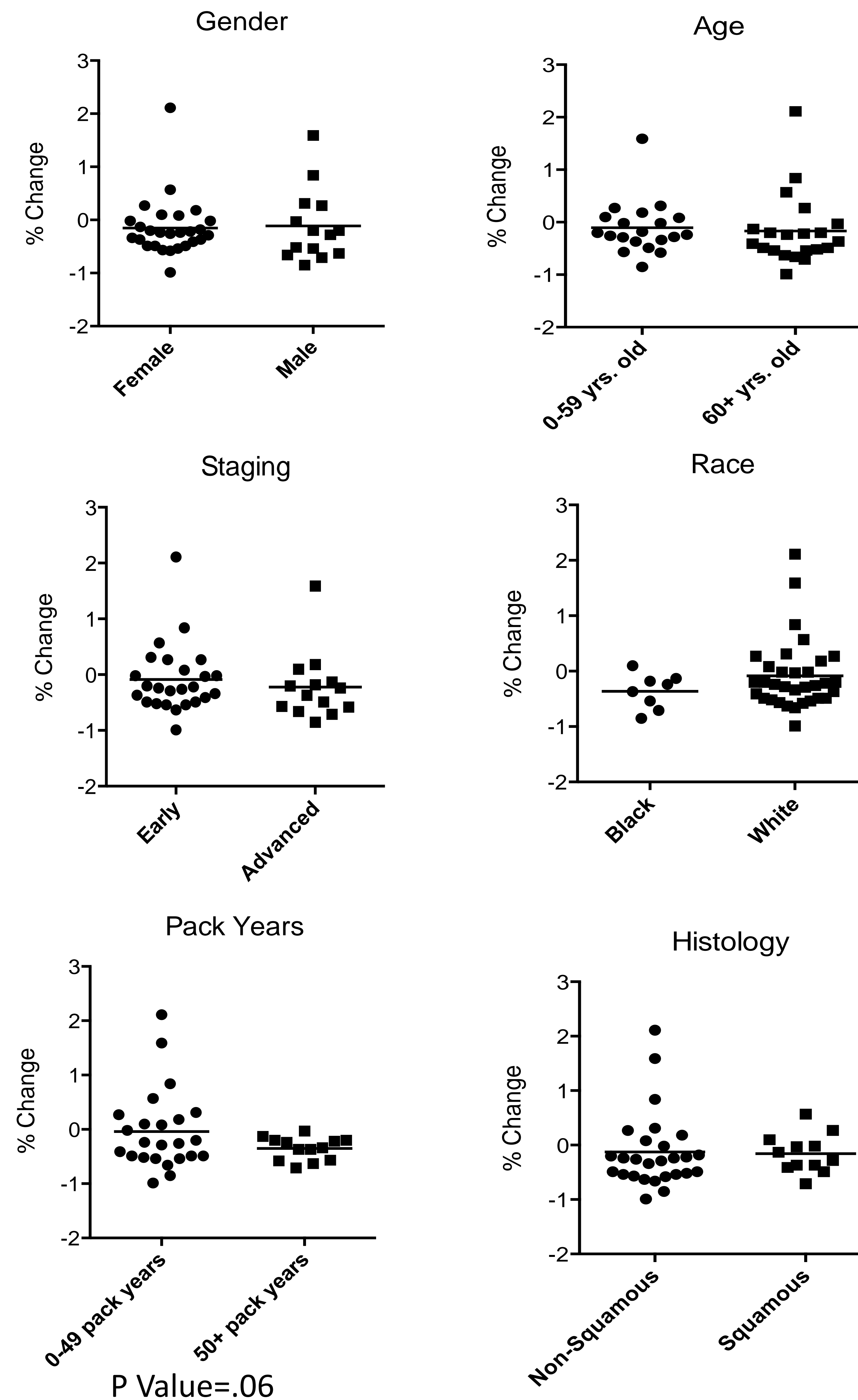
## Introduction

- Current chemotherapeutic treatments for non-small cell lung cancers (NSCLC) can be made more effective through the use of oral supplements.
- $\beta$ -glucan is a naturally occurring product derived from yeast that has been shown to have a substantial effect on reducing tumor progression and inducing a cytotoxic response within the immune system.
- Whole  $\beta$ -glucan particles (WGP) have been shown to reduce the frequency of myeloid derived suppressor cells (MDSC) in peripheral blood through apoptosis.
- MDSCs are undifferentiated myeloid cells that are generated by the secretion of cytokines by cancerous cells. Unlike most myeloid cells, MDSCs have immunosuppressive properties rather than immunosupportive properties.
- The James Graham Brown Cancer Center is conducting an ongoing clinical trial that treats NSCLC patients that haven't received chemotherapy or radiation for at least six months with WGP taken orally for 10-14 days, collecting blood before and after WGP treatment.
- There is currently no data on how demographic variables affect the response of patients to WGP treatment. This study aims to match demographic variables with percent change of MDSC frequency to examine potential trends that statistically show a certain sub-population responds better to WGP treatment.

## Methods

- To obtain demographic information of the patients in this study, past medical records were reviewed and specific information was recorded for six categories: gender, age, race, smoking status (calculated in pack years), staging, and histology.
- Using whole blood samples from patients before and after WGP treatment, MDSC frequency in peripheral blood was analyzed using flow cytometry.
- After compiling the lab and clinical data, a spreadsheet was created and analyzed using the SAS statistical program utilizing a two sample t-test to make a determination on any statistical significance between demographic factors and the percent change of MDSCs before and after WGP treatment.

## Results



These graphs show the clinical variables matched with the percent change of MDSC frequency. A downward shift indicates a positive response, the more downward the shift, the greater the decrease in MDSC percentage.

	Demographic Info	N	Mean % Change
Gender	Male	27	-0.153
	Female	14	-0.115
Age	0-59	20	-0.108
	60+	21	-0.170
Race	Black	8	-0.365
	White	33	-0.085
Stage of Cancer	Early	25	-0.086
	Advanced	14	-0.222
Pack Years	0-40	24	-0.039
	50+	13	-0.353
Histology	Non-Squa.	27	-0.129
	Squamous	13	-0.159

This table summarizes the mean percent change differences of MDSCs among sub-populations. As expected, WGP lowered the MDSC percentage among all groups and sub-populations. Although there is some variation within groups, there was found to be no statistically significant difference in the decrease of MDSCs between any of the sub-populations.

## Conclusions

Despite the fact there were no statistically significant differences found in any of the groups, the data shows some interesting trends between sub-populations when examining mean percentage change. This is especially true when looking at pack years; notice how the difference between the mean percent changes of MDSCs are much greater than any other group analyzed. However, this study simply serves as a pilot analysis that is hypothesis generating to target certain sub-populations for further investigation. More research needs to be conducted to draw any definitive conclusions.

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