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# *In-Vitro* Effect of Steroids on Melanoma Cell Growth—A Prelude to Melanoma Treatment?

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## Abstract

Skin is not only a target organ for various sex steroids and hormones, but also an endocrine organ, which produces sex steroids. It has been suggested by Nikolakis *et al.* that impairment in skin steroidogenesis may result in inflammatory or autoimmune or other skin disorders. Melanoma is one such skin disease or disorder, which is believed to be caused by UV rays. But, epidemiological, clinical, *in-vivo* and *in-vitro* studies suggested the involvement of steroids in the regulation of melanoma growth. However, these studies either did not identify the steroid involved or did not relate to the protective function of the steroid in menstruating females in melanoma, as reported by the clinical studies. In this context, our studies with mouse and human melanoma cell lines showed that female sex steroid progesterone not only inhibited melanoma cell growth, but also affected adhesion and migration functions. In addition, our studies also showed that the effect of progesterone was not a toxic or spurious, but a specific effect on melanoma cells. Hence, our *in-vitro* studies along with previous other studies subscribed to the idea proposed earlier by Slominski *et al.* that modulation of local steroids could be a new therapeutic approach for treatment of skin disease or disorder, melanoma.

## Keywords

Steroids in Skin, Steroids Action on Melanoma Cells, Progesterone Treatment, Melanoma Cell Growth

## 1. Introduction

Skin is not only a target organ for various hormones [1], but also an endocrine organ which produces sex steroids with intracrine and paracrine functions [2] [3]. Skin has the biochemical apparatus to produce androgens, estrogens or glucocorticoids [4]. Locally produced glucocorticoid, estrogens and androgens not

only affect the function of epidermis, but also immune activity [5]. It has been suggested that impairment in these steroidogenic activities can lead to inflammatory or autoimmune or other skin disorders such as rosacea, atopic dermatitis, psoriasis [5] [6]. Melanoma, is one such fatal disorder or disease of the skin [7], which is believed to be caused by UV rays [8]. According to the Cancer Society Report, melanoma is on the rise. In 2018 alone 91,720 new cases will be diagnosed with an estimated 9000 deaths [9]. Several studies showed the involvement of steroids in the regulation of melanoma growth. First the epidemiological study known as SEER (Surveillance, Epidemiological, End Research program) data [10], showed an increase in mortality rate in males than females, suggesting a sex difference. Second, clinical studies showed that menstruating females were better protected (delayed metastasis and increased survival) in melanoma than post-menopausal women and men of any age [11], suggesting the involvement of sex steroids in the protection function. But, clinical studies did not correlate with steroids status of females. Third, previous animal studies also pointed to the involvement of steroids in the regulation of melanoma growth [12] [13] [14]. Fourth, various *in-vitro* studies also showed the inhibitory effect of steroids and other hormones on a variety of melanoma cell lines [15] [16] [17]. But, these studies either did not identify the sex steroid involved in the protection or lacked information on the effect of steroid on normal or on other cancer cell lines. However, our systematic study not only checked the effect of steroids on melanoma cells, but also on normal cells as well as on other cancer cell lines [18] [19]. Moreover, our studies also showed the inhibitory effect of steroid on adhesion and migration functions (essential for metastasis) of melanoma cells [20]. Hence, our *in-vitro* studies along with other previous studies, subscribed to the idea proposed by Slominski *et al.* [5] that modulation of steroids could be a new therapeutic approach for melanoma treatment.

## 2. Experiments on Mouse Melanoma (B16F10) Cell Line [18]

### 2.1. Effect of Steroids on Mouse Melanoma Cell Growth

In the initial study, 3 androgens and 1 female sex steroid hormones were used to check their effect on mouse melanoma cell growth. The three androgens were dehydroepiandrosterone (DHEA), androstenedione (AD) and testosterone (T) and the one female sex steroid was progesterone (P4). Each hormone was incubated separately with B16F10 cells and the cell growth was monitored by MTT assay. Melanoma cell growth was inhibited by all the steroids tested in a dose-dependent manner (**Figure 1**). But, the decrease (87%) in cell growth was significant with progesterone at 200 μM concentration.

### 2.2. Effect of Progesterone on Mouse Melanoma Cell Growth

As the initial experiment was carried out at high concentrations (100, 150 and 200 μM) of steroids, the inhibition could be due to toxic effect of the steroids. So, a dose-response study was carried out with progesterone starting from 1 μM up

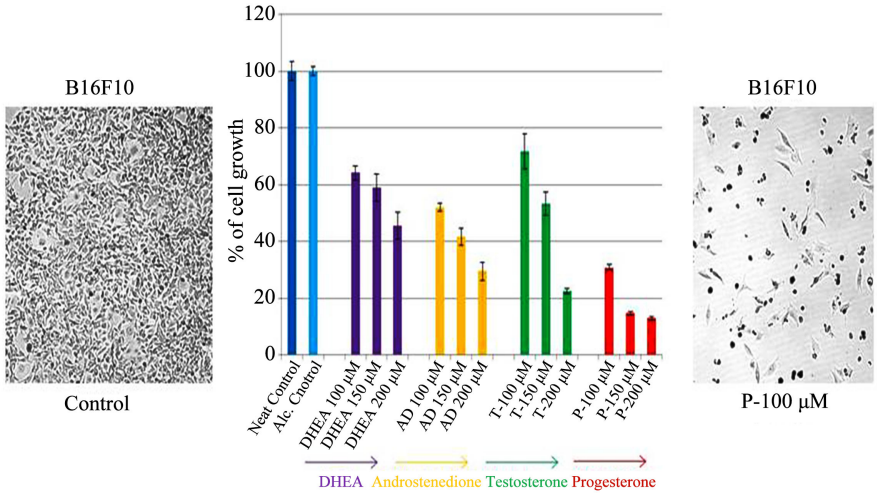


**Figure 1.** Effect of various steroids on B16F10 melanoma cell growth: Three androgens, dehydroepiandrostrone (DHEA), androstenedione (AD), testoserone (T) and one female sex steroid progesterone (P4), were incubated separetely at 100, 150 and 200 µM concentrations on B16F10 cells for 48 hrs. After 48 hrs, cell growth was quantitated using MTT assay. There was a dose-dependent decrease in cell growth with all the steroids, but the inhibition (87%) of cell growth was significant with progesterone at 200 µM concentration.

to 200 µM. The sigmoidal dose-response curve suggested that the inhibition was not a toxic effect, but a biological effect of progesterone on mouse melanoma cells (**Figure 2**).

### 2.3. Effect of Related Steroids on Mouse Melanoma Cell Growth

In order to check the effect of other steroids on B16F10 cell line, dose-response studies were carried out with related steroids such as estradiol, RU-486, DHEA and dexamethasone. Only RU-486 showed a dose-response curve pattern similar to that of progesterone (**Figure 3**). Though estradiol showed inhibition of melanoma cell growth, it was not as significant inhibition as that of progesterone and RU-486.

### 2.4. Effect of Cholesterol on Mouse Melanoma Cell Growth

Since, a synthetic steroid (RU-486) also showed inhibition, it was decided to rule out the possibility of spurious effect of steroid on cell growth inhibition. So, dose-response study was carried out with cholesterol (parent compound of all steroids). Though cholesterol initially showed a mild inhibition at 1 µM, yet it did not show a dose-response pattern (**Figure 4**). It maintained almost a flat line up to 200 µM, indicating no spurious effect was involved in the inhibition of cell growth by progesterone and RU-486 on mouse melanoma cells.

### 2.5. Effect of Progesterone and RU-486 on Human Gastric Cancer (NUGC3) Cell Line

In order to rule out a non-specific effect of progesterone and RU-486 on any

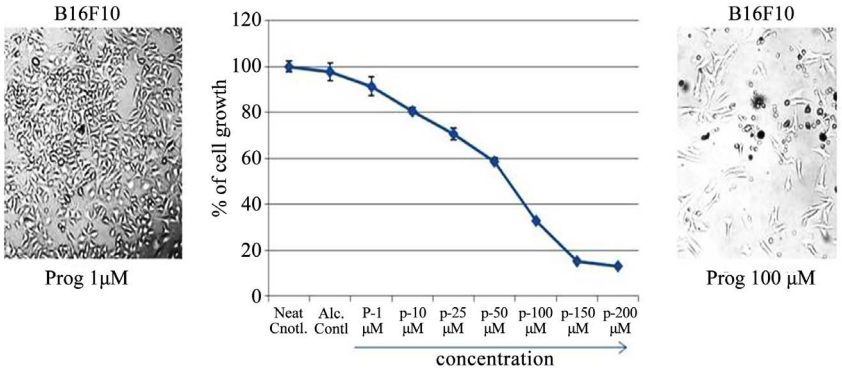


**Figure 2.** Dose-dependent effect of progesterone on mouse melanoma cell growth: A dose-response study was carried out with progesterone starting from 1 μM up to 200 μM. Cell growth was assessed as usual by MTT assay after 48 hrs of incubation with progesterone. There was a dose-dependent decrease in cell growth suggesting the decrease in cell growth was not due to toxic effect, but due to biological action of progesterone.

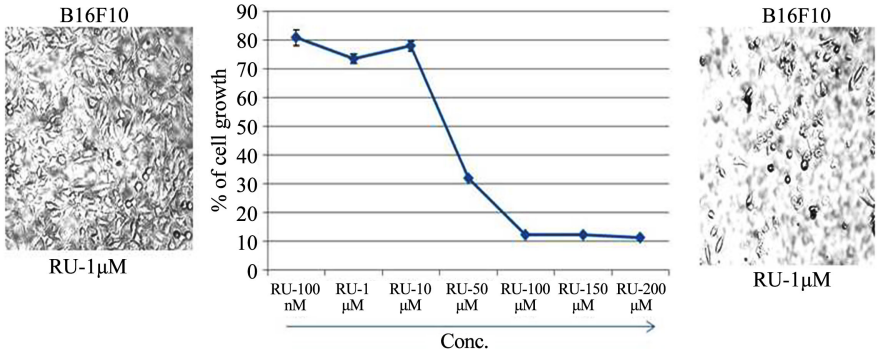


**Figure 3.** Dose-response study with RU-486: The inhibitory effect of other related steroids such as estradiol, DHEA, dexamethasone (water soluble) and RU-486 were checked. Only RU-486, a progesterone receptor antagonist and a synthetic steroid showed a significant dose-dependent decrease in cell growth comparable to that of progesterone dose-response curve on B16F10 cells [**Figure 2**].

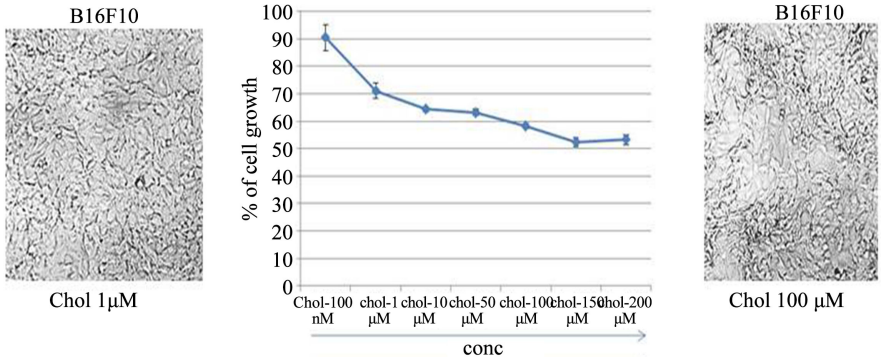


**Figure 4.** Dose-response study with cholesterol: This study would not only rule out non-specific effect, but also any spurious effect of steroids. Cholesterol showed a mild inhibition starting from 1 μM, but then remained almost a flat line up to 200 μM, indicating there was no non-specific or spurious effect (such as requirement of any specific functional group or steric hindrance) of steroids on mouse melanoma cell growth inhibition.

cancer cell line, dose-response study was carried out with progesterone and RU-486 on human gastric cancer (NUGC3) cell line. Dose-response curves of progesterone and RU-486 did not show any appreciable inhibition of cell growth (Figure 5), suggesting the significant inhibition seen with mouse melanoma cell line was a specific effect of progesterone and RU-486.

### 2.6. Effect of Progesterone and RU-486 on Sub-Cultured Normal Rat Aortic Vascular Smooth Muscle Cells

So far the effect of progesterone and RU-486 were studied on transformed cell lines, but their effects on normal cell were not known. Moreover, this would also serve as a non-specific control. Since mouse and human cell lines were already used, normal rat vascular smooth muscle cells were used as non-specific control cells in this experiment. Again, progesterone and RU-486 did not show any appreciable change in inhibition (Figure 6), suggesting the effect of progesterone and RU-486 on melanoma cell line was not a non-specific effect.

### 2.7. Progesterone and RU-486 Co-Incubation with Mouse Melanoma Cell Line

Since, progesterone and its receptor antagonist RU-486 separately showed inhibition of mouse melanoma cell growth, it was decided to find out whether their actions were mediated through progesterone receptor. So, a fixed concentration of progesterone (50 μM) was co-incubated with varying concentrations of RU-486 (50, 100 and 150 μM) on mouse melanoma cell line. If the actions were mediated through progesterone receptor, co-incubation study should show an increase in cell growth due to competition between progesterone and RU-486 for a limited number of progesterone receptors. But, there was an additive effect on the inhibition of cell growth with co-incubation of progesterone and RU-486

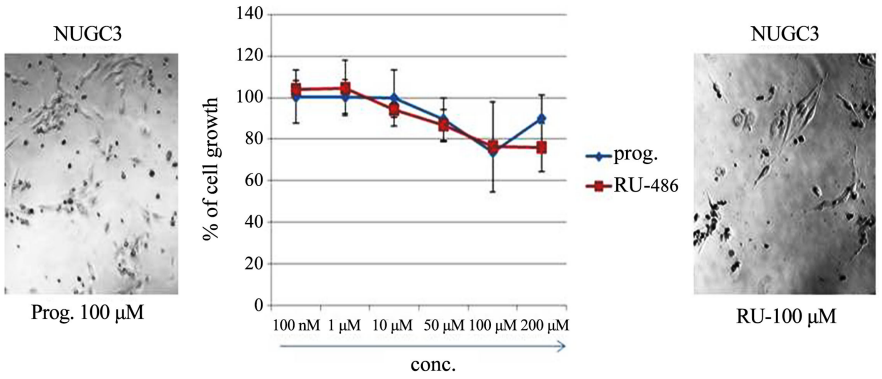


Figure 5. Dose-response curves of progesterone and RU-486 on human gastric cancer cell line (NUGC3): In order to answer the question whether the inhibition by progesterone and RU-486 was a universal inhibition on any other cancer cells or was it a specific effect on melanoma cells, dose-response study of progesterone and RU-486 were carried out on human gastric cancer cell line (NUGC3). Dose-response curves showed only mild inhibition with NUGC3 cell line, it was not as significant as that of progesterone and RU-486 on B16F10 mouse melanoma cell line, indicating that the effect of progesterone and RU-486 were specific to melanoma cell line.

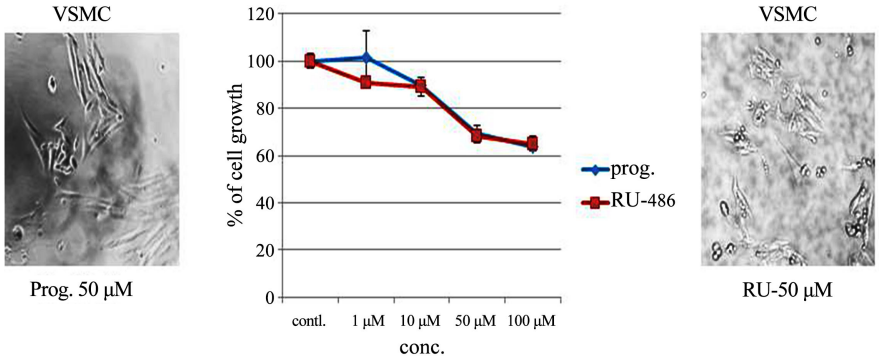


**Figure 6.** Dose-response curves of progesterone and RU-486 on sub-cultured normal rat aortic vascular smooth muscle cells: Dose-response study was carried out with sub-cultured normal rat vascular smooth muscle cells. There was a moderate inhibition, but it was not as significant as that of the mouse melanoma cell growth inhibition. This experiment also served as a non-specific control experiment. So, there was no non-specific effect of progesterone and RU-486 on mouse melanoma cell line.

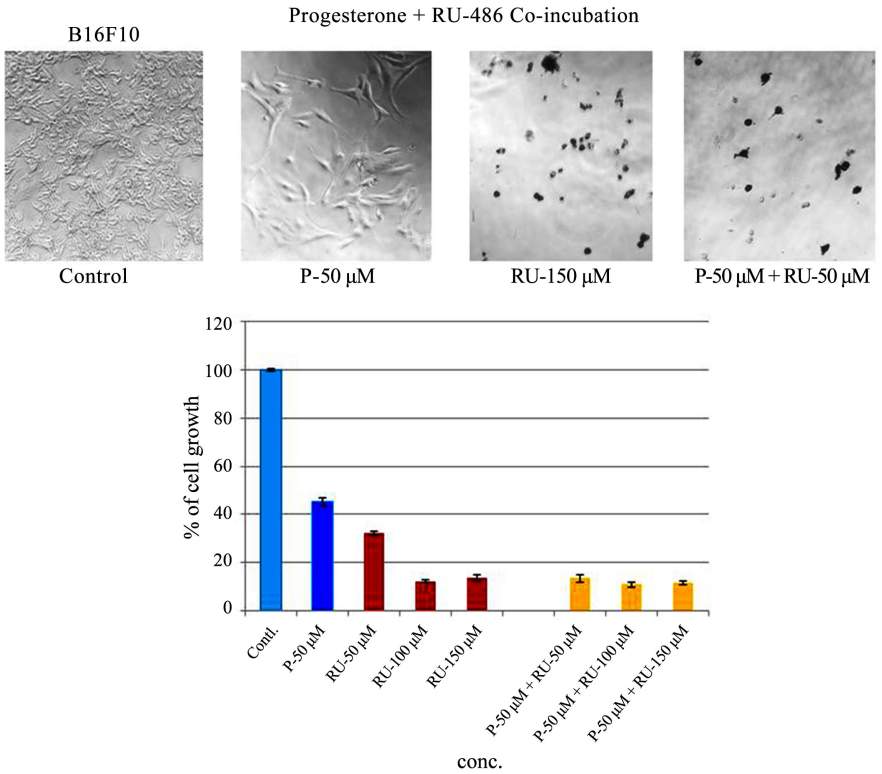


**Figure 7.** Co-incubation of progesterone and RU-486 on mouse melanoma cells:A fixed concentration of progesterone (50 µM) was co-incubated separately with 50, 100 and 150 µM concentrations of RU-486 for 48 hrs. After 48 hrs, cell growth was assessed by MTT assay. Progesterone 50 µM + Ru-486 50 µM co-incubation showed a significant inhibition of cell growth, comparable to the inhibition by RU-486 alone at 150 µM concentration. Co-incubation experiment showed that the action was not mediated through progesterone receptor, as co-incubation of progesterone and RU-486 would set-up competition for a limited number of progesterone receptors and should result in an increase in cell growth. The experiment suggested that both progesterone and RU-486 actions might be mediated through two different mechanisms resulting in an additive effect on cell growth inhibition.

(Figure 7), suggesting the actions were not mediated through progesterone receptor. Moreover, the experiment also suggested the actions could be mediated through two different mechanisms resulting in an additive effect.

## 3. Experiments on Human Melanoma (BLM) Cell Line [19] [20]

### 3.1. Effect of Progesterone on Human Melanoma Cell Line

Since, the study with mouse melanoma cell line showed a significant inhibition of cell growth, the study was extended to human melanoma (BLM) cell line to check the effect of progesterone on human melanoma cell growth. So, progesterone dose-response study was carried out on human melanoma cell line. There was a dose-dependent decrease of human melanoma cell growth by progesterone, comparable to that of progesterone effect on mouse melanoma cell line (Figure 8).

### 3.2. Co-Incubation of Progesterone and RU-486 with Human Melanoma Cell Line

Since, progesterone showed a significant inhibition of human melanoma cell growth, it was decided to check whether the action was mediated through progesterone receptor. So, co-incubation study was carried out with human melanoma cell line, just like the one on mouse melanoma cell line [18]. Fixed concentration of progesterone (10 μM) was co-incubated with varying concentrations of RU-486 (10, 50 and 100 μM). Co-incubation study showed an additive effect on the inhibition of human melanoma cell growth (Figure 9), suggesting the actions were not mediated through progesterone receptor. Since, co-incubation experiment showed an additive effect, the actions of the 2 steroids could be mediated through two different mechanisms.

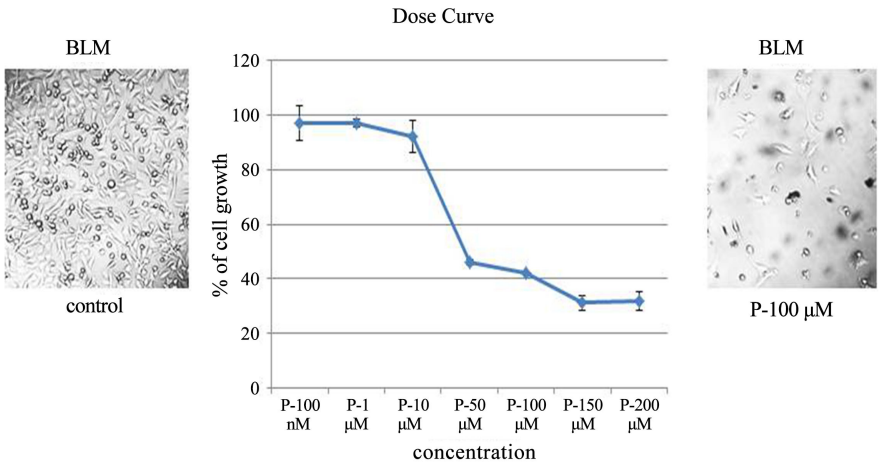


Figure 8. Dose-response study of progesterone with human melanoma (BLM) cell line: Progesterone ranging in concentrations from 100 nM up to 200 μM were incubated for 48 hrs with human melanoma cells. After 48 hrs, MTT assay was used to assess cell growth, which showed a dose-dependent decrease of human melanoma cell growth.

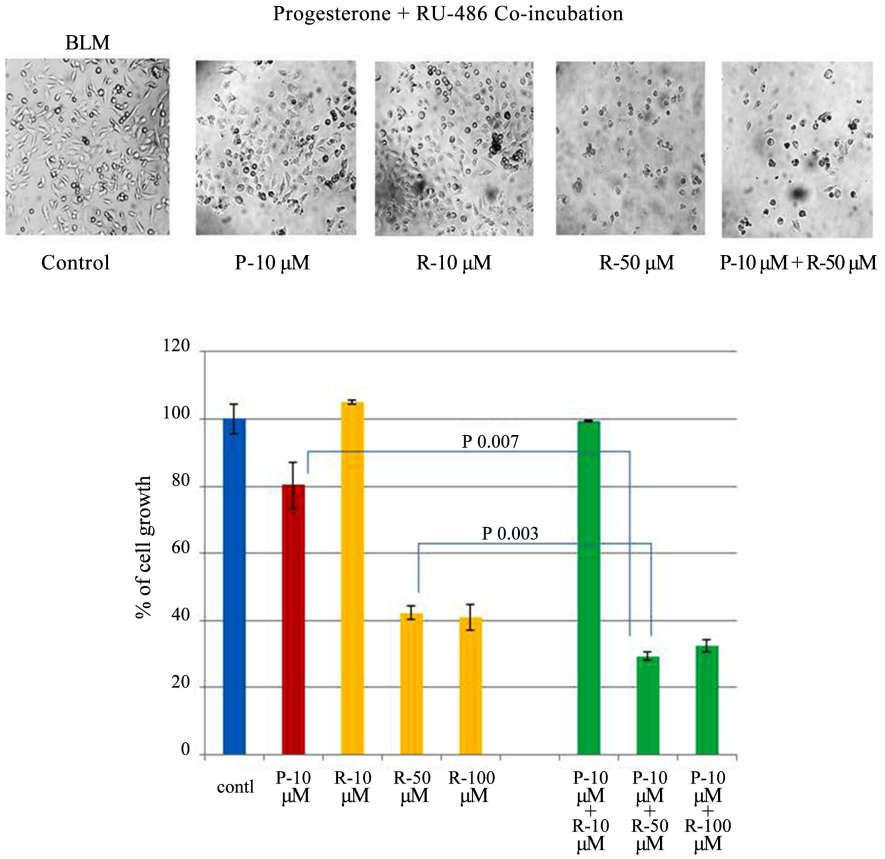


**Figure 9.** Co-incubation of progesterone and RU-486 with human melanoma (BLM) cell line: A fixed concentration of progesterone 10 μM was co-incubated with varying concentrations of RU-486 (10, 50 and 100 μM). Cells were co-incubated for 48 hrs. At the end of 48 hrs, MTT assay was used to assess cell growth. Co-incubation experiment showed an additive effect on the inhibition of cell growth, suggesting that both progesterone and RU-486 actions were not mediated through progesterone receptor and the actions of progesterone and RU-486 could be mediated through two different mechanisms.

### 3.3. Mechanism of Inhibition of Human Melanoma Cell Growth by Progesterone

Since, co-incubation study suggested that progesterone and RU-486 could have inhibited melanoma cell growth through two different mechanisms, it was necessary to find out the mechanism of inhibition of cell growth by progesterone. After having ruled out necrosis and apoptosis, it was found out that the mechanism of inhibition of cell growth was due to autophagy by co-incubating cells with progesterone and 3-methyladenine (3-MA). Previous studies showed that 3-methyladenine (3-MA) was used either to check or to inhibit autophagy [21] [22] [23]. Progesterone was co-incubated with 3-MA for 48 hrs. In cells where 3-MA was co-incubated with progesterone, there was a partial increase in cell growth (**Figure 10**), suggesting there was a rescue of cell growth, as 3-MA suppressed autophagosome formation in those cells. So, mechanism of inhibition of cell growth by progesterone was due to autophagy.

### 3.4. Effect of Progesterone on Adhesion and Migration Functions of Human Melanoma Cells (20)

Previous clinical studies reported delayed metastasis and increased survival (protection) in menstruating females in melanoma. Since, progesterone level vary between 1000 - 1500 ng/dL in menstruating females and our *in-vitro* studies showed inhibitory effect of progesterone on melanoma cell growth, it was decided to check progesterone effect on adhesion and migration functions (essential for metastasis) of human melanoma cells. Effect on adhesion and migration were checked after 48 hrs of incubation of human cells with progesterone. Progesterone at 100 μM concentration, partially inhibited adhesion function (Figure 11). Similarly, progesterone (50 μM) treatment significantly decreased migration function of human melanoma cells (Figure 11). This study indicated that progesterone treatment decreased adhesion and migration functions (essential for metastasis), implying a delayed metastasis and a significant inhibition of human melanoma cell growth resulting in increased survival. So, progesterone could be playing an important role (protection) in menstruating females in melanoma.

## 4. Summary

Previous *in-vitro* studies showed the inhibitory effect of steroids on various melanoma cell lines growth. But these studies either did not tie the steroid to protection function or did not rule out toxic or spurious or non-specific effect of steroids or lacked experiments on normal or control cells. But, our studies on mouse melanoma cell line showed not only the inhibitory effect of progesterone

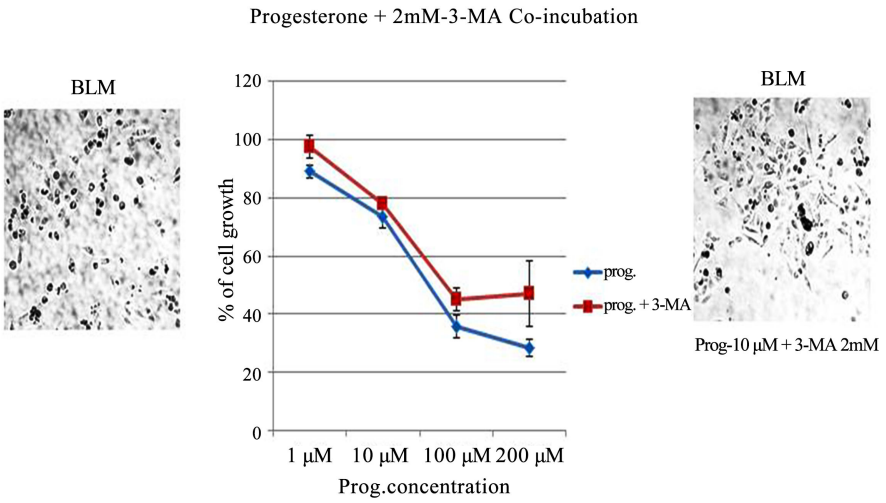


**Figure 10.** Partial rescue of human melanoma cell growth by co-incubation of progesterone with 3-MA: After having ruled out necrosis and apoptosis, it was decided to check autophagy. So BLM cells were co-incubated with progesterone at various concentrations and 2 mM of 3-methyladenine for 48 hrs. Co-incubation experiment with 2 mM of 3-MA showed partial increase in cell growth at all the concentrations of progesterone checked, suggesting there was a partial increase in cell growth by suppression of autophagy by 3-MA.

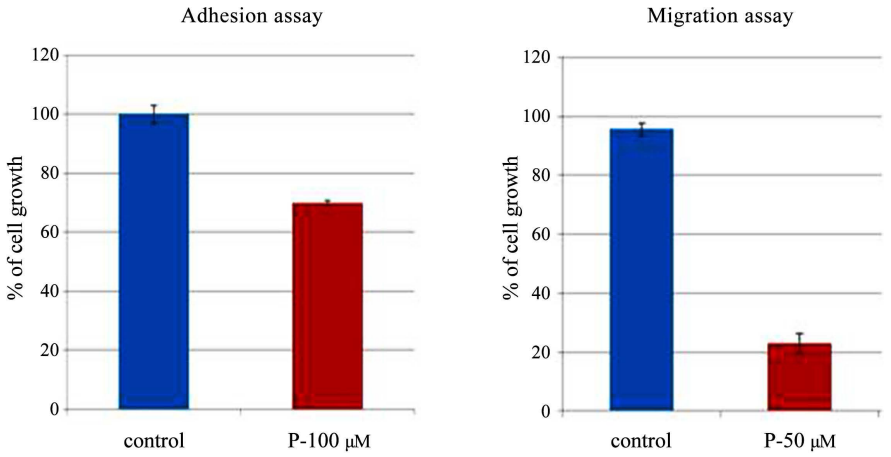


**Figure 11.** *In-vitro* adhesion and migration functions of human melanoma cell line: Human melanoma cells were treated with progesterone at 100 μM for 48 hrs in petri dish.After 48 hrs, both control and treated cells were harvested and plated at 30,000 cells/well in a 96 well plate and allowed to incubate for 60 min at 37˚C. At the end of 60 min, cells were washed 3 times to remove unattached cells and the cells which were attached to the plate were fixed with 2% para-formaldehyde and were stained with 0.2% crystal violet (CV) dye. CV dye was eluted with isopropanol and read at 570 nm in a plate reader. For migration assay, control and progesterone (50 μM) treated cells were harvested after 48 hrs of treatment. Cells were plated in a 24 well plate and allowed to become confluent. A scratch was made at the bottom of the plate with a pipet tip. This was considered as 0 time. After 24 hrs, percentage of cells migrated into the cleared space was calculated with a software program. Adhesion experiment showed partial inhibition in adhesion in treated cells compared to the control cells. Similarly, progesterone treated cells showed a significant decrease in migration in treated cells compared to the control cells.

on mouse melanoma cell growth, but also showed the action was not toxic or spurious or non-specific action of steroid on melanoma cell line. In addition, mouse cell line study also showed that progesterone action was not mediated through progesterone receptor. When the study was extended to human melanoma cell line, progesterone showed inhibition of human melanoma cell growth also and it was found out that the mechanism of inhibition of cell growth was due to autophagy. Again, experiment showed that progesterone action was not mediated through progesterone receptor in human melanoma cell line. A study by Fang *et al.* [24] showed inhibition of two melanoma [A375, A875] cell lines growth by both progesterone and RU-486 and again the actions were not mediated through progesterone receptor. Moroni *et al.* [25] used the same cell lines and showed that progesterone inhibited cell growth by using concentrations up to 1000 μM. Earlier Kanda and Watanbe [26] showed inhibition on another human melanoma cell line by progesterone along with other steroids (estradiol and dihydrotestotorone). Preceding studies and our own studies with progesterone suggested that melanoma cell growth was amenable to steroids action and subscribed to the idea already proposed [5] that modulation of local steroids could be a way to control melanoma cell growth.

## 5. Conclusion

Since, our studies and studies by others showed that progesterone (a female sex steroid) played a major role in melanoma cell growth, adhesion and migration functions, progesterone could be the sex steroid protecting menstruating females (whose progesterone level vary between 1000 - 1500 ng/dL) in melanoma. On the same token progesterone level in post-menopausal women vary between 20 - 100 ng/dL and so they were not protected in melanoma, as per the clinical study [11]. Hence, melanoma cell growth was amenable to steroid hormones action. This finding about progesterone and its ability to protect menstruating females in melanoma, suggested that modulation of local steroids can be a new therapeutic approach for melanoma treatment [5].

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## References

[1] Zouboulis, C.C., Chen, W.C., Thornton, M.J., Qin, K. and Rosenfield, R. (2007) Sexual Hormones in Human Skin. *Hormone and Metabolic Research*, **39**, 85-95. https://doi.org/10.1055/s-2007-961807

[2] Zouboulis, C.C. (2004) The Human Skin as a Hormone Target and an Endocrine Gland. *Hormones*, **3**, 9-26. https://doi.org/10.14310/horm.2002.11109

[3] Zouboulis, C.C. (2000) Human Skin: An Independent Peripheral Endocrine Organ. *Hormone Research*, **54**, 230-242. https://doi.org/10.1159/000053265

[4] Labrie, F. (2001) DHEA and Its Transformation into Androgens and Estrogens in Peripheral Target Tissues: Intracrinology. *Frontieres in Neuroendocrinology*, **22**, 185-212. https://doi.org/10.1006/frne.2001.0216

[5] Slominski, A., Zbytek, B., Nikolakis, G., Manna, P.R., Skobowiat, C., Zmijewski, M., Li, W., Janjetovic, Z., Postlethwaite, A., Zouboulis, C.C. and Tuckey, R.C. (2013) Steroidogenesis in the Skin: Implications for Local Immune Functions. *The Journal of Steroid Biochemistry and Molecular Biology*, **137**, 107-123. https://doi.org/10.1016/j.jsbmb.2013.02.006

[6] Nikolakis, G., Stratakis, C.A., Kanaki, T., Slominski, A. and Zouboulis, C.C. (2016) Skin Steroidogenesis in Health and Disease. *Reviews in Endocrine and Metabolic Disorders*, **17**, 247-258. https://doi.org/10.1007/s11154-016-9390-z

[7] Gray-Schopfer, V., Wellbrock, C. and Marais, R. (2007) Melanoma Biology and New Targeted Therapy. *Nature*, **445**, 851-857. https://doi.org/10.1038/nature05661

[8] Rass, K. and Reicharth, J. (2008) UV Damage and DNA Repair in Malignant Melanoma and Non-Melanoma Skin Cancer. *Advances in Experimental Medicine and Biology*, **624**, 162-178. https://doi.org/10.1007/978-0-387-77574-6_13

[9] Siegel, R.L., Miller, K.D. and Jemal, A. (2018) Cancer Statistics, 2018. *CA*: *A Cancer Journal for Clinicians*. https://doi.org/10.3322/caac.21442

[10] http://seer.cancer.gov/statfacts/html/melan.html

[11] Miller, J.G. and Neil, S.M. (1997) Gender and Cutaneous Melanoma. *British Journal*

*of Dermatology*, **136**, 657-665. https://doi.org/10.1111/j.1365-2133.1997.tb03648.x

[12] Ladanyi, A., Timar, J., Bocsi, J., Towari, J. and Lapis, K. (1995) Sex-Dependent Liver Metastasis of Human Melanoma Lines in SCID Mice. *Melanoma Research*, **5**, 83-86. https://doi.org/10.1097/00008390-199504000-00002

[13] Feucht, K.A., Walker, M.J., Das Gupta, T.K. and Beattie, C.W. (1998) Effect of 17-Oestradiol on the Growth of Estrogen Receptor Positive Human Melanoma *in Vitro* and in Athymic Mice. *Cancer Research*, **48**, 7093-7101.

[14] Simanainen, U., Ryan, T., Li, D., Suarez, F.G., Gao, Y.R., Watson, G., Wang, Y. and Handelsman, D.J. (2015) Androgen Receptor Actions Modify Skin Structure and Chemical Carcinogen-Induced Skin Cancer Susceptibility in Mice. *Hormones and Cancer*, **6**, 45-53. https://doi.org/10.1007/s12672-014-0210-1

[15] Ghosh, R., Ott, A.M., Seetharam, D., Slaga, T.J. and Kumar, A.P. (2003) Cell Cycle Block and Apoptosis Induction in a Human Melanoma Cell Line Following Treatment with 2-Methoxyostradiol: Therapeutic Implications? *Melanoma Research*, **13**, 119-127. https://doi.org/10.1097/00008390-200304000-00003

[16] Disorbo, D.M., McNulty, B. and Nathanson, L. (1983) *In Vitro* Growth Inhibition of Human Malignant Melanoma Cells by Glucocorticoids. *Cancer Research*, **43**, 2664-2667.

[17] Cos, S., Garcia-Bolado, A. and Sanchez-Barcelo, E. (2001) Direct Antiproliferative Effects of Melatonin on Two Metastatic Cell Sub-Lines of Mouse Melanoma (B16BL6 and PG19). *Melanoma Research*, **11**, 197-201. https://doi.org/10.1097/00008390-200104000-00016

[18] Ramaraj, P. and Cox, J.L. (2014) *In-Vitro* Effect of Sex Steroids on Mouse Melanoma (B16F10) Cell Growth. *CellBio*, **3**, 60-71. https://doi.org/10.4236/cellbio.2014.32007

[19] Ramaraj, P. and Cox, J.L. (2014) *In-Vitro* Effect of Progesterone on Human Melanoma (BLM) Cell Growth. *International Journal of Clinical and Experimental Medicine*, **7**, 3941-3953.

[20] Leder, D.C., Brown, J.R. and Ramaraj, P. (2015) *In-Vitro* Rescue and Recovery Studies of Human Melanoma (BLM) Cell Growth, Adhesion and Migration Functions after Treatment with Progesterone. *International Journal of Clinical and Experimental Medicine*, **8**, 12275-12285.

[21] Seglen, P.O. and Gordon, P.B. (1982) 3-Methyladenine: Specific Inhibitior of Autophagic/Lysosomal Protein Degradation in Isolated Rat Hepatocytes. *Proceedings of the National Academy of Sciences*, **79**, 1889-1892. https://doi.org/10.1073/pnas.79.6.1889

[22] Jagannath, C., Lindsey, D.R., Dhandayuthapani, S., Xu, Y., Hunter, R.L. and Eissa, N.T. (2009) Autophagy Enhances the Efficacy of BCG Vaccine by Increasing Peptide Presentation in Mouse Dendritic Cells. *Nature Medicine*, **15**, 267-276. https://doi.org/10.1038/nm.1928

[23] Vegliante, R., Desideri, E., Di Leo, L. and Ciriolo, R. (2016) Dehydroepiandrosterone Triggers Autophagic Cell Death in Human Hepatoma Cell Line HepG2 via JNK-Mediated p62/SQSTM1 Expression. *Carcinogenesis*, **37**, 233-244. https://doi.org/10.1093/carcin/bgw003

[24] Fang, X., Zhang, X., Zhou, M. and Li, J. (2010) Effects of Progesterone on the Growth Regulation in Classical Progesterone Receptor-Negative Malignant Melanoma Cells. *Journal of Huazhong University of Science and Technology. Medical Science*, **30**, 231-234. https://doi.org/10.1007/s11596-010-0220-3

[25] Moroni, G., Gaziano, R., Bue, C., Agostini, M., Perno, C.F., Sinibaldi-Vallebona, P. and Pica, F. (2015) Progesterone and Melanoma Cells: An Old Story Suspended between Life and Death. *Journal of Steroids & Hormonal Science*, **S13**, 158.

[26] Kanda, N. and Watanbe, S. (2001) 17 Beta-Estradiol, Progesterone and Dihydrotestosterone Suppress the Growth of Human Melanoma by Inhibiting Interleukin-8 Production. *Journal of Investigative Dermatology*, **117**, 274-283. https://doi.org/10.1046/j.1523-1747.2001.01422.x