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Impact of sex hormones in gene and protein expression of PTGS2/Cox-2, RNASEL/RNase-L and miR-146a in skin cells

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ABSTRACT

Immunity and inflammatory pathways are important in the genesis of cancers, including skin cancer. Functional genetic variation in immune and inflammatory modulators has the potential to affect disease predisposition.

Amongst the different mediators of inflammation, the cyclooxygenases (COXs) evidently appear to be implicated in cancer. Scientific data suggested that COX-2 may play a role in different steps of cancer progression, by increasing proliferation of mutated cells, thus favoring tumor promotion as well as by affecting programmed cell death. COX-2 is a protein encoded by PTGS2 gene.

Another gene that contributes to innate immunity and cell metabolism including inflammation, cell proliferation and migration, differentiation, apoptosis is the RNASEL which encodes a component of the interferon-regulated 2-5A system that functions in the antiviral and anti-proliferative roles of interferons. Several studies have identified variations in this gene associated with risk of different malignancies.

Epigenetics also plays an important role in melanoma development and progression. Changes in methylation patterns of genomic DNA, histone modifications and microRNA expression can alter important cellular pathways. In particular, high expression of miR-146a has been correlated with various cancers, including melanoma. Polymorphisms in the PTGS2 and RNASEL genes and in miR-146a associated with risk of various cancers, have been described. Previously, in our laboratory, the impact of PTGS2 gene polymorphism rs20417, RNASEL gene polymorphism rs486907 and miR-146a polymorphism rs2910164 was investigated in relation to the risk of predisposition to melanoma skin cancer. Combined genotypes of rs2910164C allele (miR-146a) together with rs486907A allele (RNASEL) and rs2910164C allele (miR-146a) together with rs20417GG genotype (PTGS2) showed a higher risk of melanoma only in the male population suggesting a sex-specific interaction between miR-146a and PTGS2 or RNASEL in their association with melanoma risk.

Sex is a genetic factor involved in melanoma skin cancer, in particularly sex hormones can influence in development, survival, progression of this type of cancer.

In the present study, we investigate if gene and protein expression of PTGS2/COX-2, RNASEL/RNase-L and miR-146a expression are differently affected by sex hormones

in skin cells. The effect of 17β-estradiol or testosterone in melanoma cells, keratinocyte cells and primary fibroblasts was analyzed. The eventuality miR-146a regulation upon PTGS2 or RNASEL 3'UTR genes expression was tested in keratinocyte cells.

SUMMARY/RIASSUNTO

La pelle o cute è il rivestimento più esterno del corpo e l'organo più esteso dell'apparato tegumentario. La cute svolge diverse funzioni; quella più importante è la protezione del nostro organismo da ogni tipo di agente esterno. Le altre funzioni svolte dalla cute sono: la funzione sensoriale, la secrezione del sudore, la produzione di cheratina e di pigmenti come la melanina che ci protegge dai raggi ultravioletti, la regolazione della temperatura dell'organismo, la permeabilità che garantisce il bilancio idroelettrolitico e la funzione immunitaria. La pelle è costituita da una componente superficiale detta epidermide, e una più profondo detto derma, una sorta di impalcatura di sostegno irrorata da una fitta rete di vasi sanguigni. A separare queste due componenti vi è una sottile struttura detta membrana basale o giunzione dermo-epidermica. Nell'epidermide sono presenti diversi tipi di cellule: i cheratinociti, i melanociti, le cellule di Langerhans, le cellule di Merkel e rari linfociti T. In particolare, i cheratinociti e i melanociti sono le cellule della cute da cui possono derivare i tumori cutanei più frequenti. Nel derma e nell'ipodema sono presenti i fibroblasti. Sotto il derma, uno strato di grasso sottocutaneo e tessuto connettivo di vario spessore crea il terzo strato di pelle. Il melanoma maligno cutaneo (MM) è un tumore derivato dai melanociti epidermici attivati o geneticamente modificati; è quindi il risultato di complesse interazioni tra fattori genetici, costituzionali e ambientali. Associati alla predisposizione e alla progressione del melanoma vi sono numerosi geni ed interazioni geniche. In particolare, la partecipazione di geni che contribuiscono ai processi infiammatori è ampiamente riconosciuta in diversi tumori; incluso melanoma. Tra i differenti mediatori dell'infiammazione, le cicloossigenasi (COXs) sembrano chiaramente implicate nel cancro. Dati scientifici suggeriscono che la proteina COX-2 può svolgere un ruolo in diverse fasi della progressione del cancro, aumentando la proliferazione delle cellule mutate, favorendo la promozione del tumore e influenzando la morte cellulare programmata. COX-2 è una proteina codificata dal gene PTGS2. Un altro gene che contribuisce all'immunità innata e al metabolismo cellulare tra cui infiammazione, proliferazione e migrazione cellulare, differenziazione e apoptosi, è il gene RNASEL che codifica un componente del sistema 2-5A regolato dall'interferone, il quale sistema ha funzioni antivirali e antiproliferative.

Anche l'epigenetica svolge un ruolo importante nello sviluppo e nella progressione del melanoma. I cambiamenti nei modelli di metilazione del DNA genomico, le modificazioni istoniche e l'espressione del microRNA possono alterare importanti vie cellulari. In particolare, un over-espressione di miR-146a è stata correlata a vari tipi di cancro, incluso il melanoma.

Polimorfismi nei geni PTGS2 e RNASEL e nel miR-146a sono stati associati ad un maggiore rischio in vari tipi di cancro. Precedentemente, nel nostro laboratorio, è stata studiata l'influenza del polimorfismo rs20417 nel gene PTGS2, del polimorfismo rs486907 nel gene RNASEL e del polimorfismo rs2910164 del miR-146a associata al rischio alla predisposizione al melanoma. Più precisamente l'allele C dell'rs20417 nel gene PTGS2 e l'allele C dell'rs2910164 nel miR-146a possono rappresentare un fattore di rischio con un effetto additivo nella predisposizione al melanoma, ma solo nella popolazione maschile. Successivamente è stato eseguito uno studio di associazione tra genotipi dello SNP rs2910164 (miR-146a) combinato con lo SNP rs486907 (RNASEL), e lo SNP rs2910164 (miR-146a) combinato con lo SNP rs20417 (PTGS2). Ambedue le associazioni di polimorfismi hanno mostrato presentare un maggior rischio alla predisposizione del melanoma, (rs2910164 allele C vs rs20417 genotipo GG e rs2910164 allele C vs rs486907 allele A) ma solo nella popolazione maschile. Tale dato suggerisce un'interazione sessuale specifica tra miR-146a e PTGS2 o RNASEL nella loro associazione con il rischio di melanoma.

Il sesso è un fattore genetico coinvolto nel melanoma del cancro della pelle, in particolare gli ormoni sessuali possono influenzare lo sviluppo, la sopravvivenza, la progressione di questo tipo di cancro.

Nel presente studio, verifichiamo se l'espressione genica e proteica di PTGS2/COX-2, RNASEL/RNase-L e dell'espressione del miR-146a, sono influenzate in modo differente dagli ormoni sessuali. L'effetto del 17β-estradiolo e del testosterone è stato analizzato in cellule di melanoma, in cheratinociti e in cellule di fibroblasti primari. Successivamente, l'eventuale regolazione del miR-146a sull'espressione dei geni PTGS2 o RNASEL è stata valutata nei cheratinociti.

INDEX

FIGURES LIST

Figure 16. Luciferase activity after different plasmid transfection in HaCaT cells.

TABLES LIST

ABBREVIATIONS

ABCE1: ATP Binding Cassette Subfamily E Member 1

AGO: Argonaute

AICD: Activation-induced cell death

AKT: Serine/Threonine Kinase

AP: Activator protein

AR: Androgen Receptor

ARD: Ankyrin repeat domain

AREBPs: ARE-binding proteins

AREs: Adenylate-uridylate-rich elements

ATP: Adenosine triphosphate

BCC: Basal cell carcinoma

BRAF: B-Raf Proto-Oncogene

CDK4: Cyclin Dependent Kinase

CDKN2A: Cyclin Dependent Kinase Inhibitor 2A

cDNA: Complementary DNA

CM: Cutaneous melanoma

c-MYB: MYB Proto-Oncogene

COX-1: Cyclooxygenase-1

COX-2: Cyclooxygenase-2

COXIBS: COXs inhibitors

CPSF: Cleavage polyadenylation specificity factor

CRE: Cyclic monophosphate response element

DCs: Dendritic cells

DGCR8: DiGeorge Syndrome Critical Region 8

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic Acid

dsRNA: Double-stranded RNA

EGFR: Epidermal growth factor receptor

EP1: Prostaglandin receptor 1

EP2: Prostaglandin receptor 2

EP3: Prostaglandin receptor 3

EP4: Prostaglandin receptor 4

eRF3: Eukaryotic release factor 3

ERs: Estrogen receptors

ERα: Estrogen receptor alfa

ERβ: Estrogen receptor beta

FADD: Fas-associated death domain

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GOI: Gene of interest

HaCaT: Human keratinocyte

HKG: Housekeeping gene

HPRT1: Hypoxanthine Phosphoribosyl transferase 1

HRP: Horseradish peroxidase

HSD11B1: Hydroxysteroid 11-Beta Dehydrogenase 1

IFN: Interferon

IFN-γ: Interferon gamma

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL-1: Interleukin-1

IL-12: Interleukin-12

IL1R1: Interleukin 1 receptor

IL-1β Interleukin-1 beta

IL-2: Interleukin-2

IL-6: Interleukin-6

IQGAP1: IQ Motif Containing GTPase Activating Protein 1

IRAK: Receptor Associated Kinase

KO: Knock-out

LCs: Langerhans cells

MAPK: Mitogen-activated protein kinase

miRISC: miRNA-induced silencing complex

miRNA: microRNA

MM: Malignant melanoma

MMP-2: Matrix metalloproteinase-2

MMP-9: Matrix metalloproteinase-9

MREs: microRNA recognition elements

mRNA: Messenger ribonucleic acid

NF-IL6: Nuclear factor interleukin-6

NF-κB: Nuclear Factor Kappa B

NK Cells: Natural killer Cells

NMSC: Non-melanoma skin cancer

NRAS: NRAS Proto-Oncogene

NSAIDS: Nonsteroidal anti-inflammatory drugs

OAS: Oligoadenylate synthetase

PABP: Poly (A)-binding protein

PBS: Phosphate buffered saline

Pca: Prostate carcinogenesis

PEA3: Polyoma enhancer activator 3

PGE2: Prostaglandin E2

PGs: Prostaglandins

PI3K: Phosphoinositide 3-kinase

POLR2K: RNA Polymerase II Subunit K

PPIA: Peptidylprolyl Isomerase A

pre-miRNAs: Precursor miRNAs

pri-miRNAs: Primary miRNAs

PTGS2: Prostaglandin-endoperoxide synthase 2

PVDF: Polyvinylidene Difluoride

qRT-PCR: Quantitative real-time PCR

RanGTP: RAs-related NuclearGTP

RG: Reference Gene

RLI: RNase-L inhibitor

RNA: RiboNucleic Acid

RNase III: Ribonuclease III

RNASEL / RNase-L: Ribonuclease L

RNI: Reactive Nitrogen Intermediates

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute

SCC: Squamous cell carcinoma

SDS: Sodium dodecyl sulfate

shRNA: Short hairpin RNA

SNP: Single nucleotide polymorphism

STAT1: Signal transducer and activator of transcription 1

STAT3 Signal transducer and activator of transcription 3

STDEV: Standard deviation

TBP: TATA-Box Binding Protein

TBS: TRIS buffered saline

TCR: T cell receptor

TGFβ: Transforming growth factor β

Th1: T helper cells 1

Th2: T helper cells 2

TLR2: Toll Like Receptor 2

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor-alfa

Treg cells: Regulatory T cells

USEs: Upstream enhancer elements

UTR: Untranslated region

UV: Ultraviolet

VEGF: Vascular endothelial growth factor

XPO5: Exportin-5

YES1: YES Proto-Oncogene 1

1 INTRODUCTION

1.1 Cutaneous melanoma

Cutaneous malignant melanoma (MM) is a tumor derived from activated or genetically altered epidermal melanocytes, the result of complex interactions between genetic, constitutional, and environmental factors.[1]

Melanocytes arise from the neural crest (pluripotent cells that give rise to neurons, glial cells, adrenal medulla, cardiac cells and craniofacial tissue) during embryonic development, and throughout their maturation migrate widely and proliferate extensively prior to their terminal differentiation and entry into the epidermis and hair follicle. In addition, melanocytes undergo multiple cycles of regeneration, in which melanocyte stem cells give rise to new populations of melanocytes.[2]

In the skin, melanocytes are located in the basal layer of the epidermis. Melanocytes have a round shape with extensions named dendrites. Within the melanocytes are unique melanosomes organelles, which produce melanin. The melanosomes are transferred by the dendrites to the keratin layer where they are taken in by receptor- mediated endocytosis and deposited over keratin nucleus to protect the DNA from UV light. Keratinocytes secrete factors that regulate melanocytes survival, differentiation, proliferation and motility, stimulating the melanocytes to produce melanin and resulting in the tanning response.[3] Also fibroblasts have a role in melanogenesis, and participate actively in the signal cross-talk between melanocytes and keratinocytes.[4]

1.1.1 Type of melanoma/histology

There are two main categories of skin cancer: non-melanoma skin cancer (NMSC) which originates in keratinocytes and pluripotent skin cells and cutaneous melanoma (CM) which originates from the transformation of melanocytes. Depending on the epidermal layer keratinocytes originate from, NMSC can be subdivided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC).[5]

1.1.2 Non melanoma skin cancer

Basal Cell Carcinoma (BCC), also called basal cell epithelioma or rodent ulcer is the least aggressive type of skin cancer. This cancer develops from basal cells which line the deepest layer of the epidermis. An abnormal growth a tumor of this layer is what is referred to as BCC. It typically occurs in areas of chronic sun exposure such as the face, ears, neck, scalp and shoulders but can also develop on the back or lower legs. BCC is usually slow growing and very rarely metastasizes.

Squamous Cell Carcinoma (SCC) begins in cells called keratinocytes, which are in the upper level of the epidermis. SCC most often develops in areas that have been exposed to the sun but can also develop in scars, areas of skin that have been burnt in the past and areas of skin that have been ulcerated for a long time. SCC is more aggressive than BCC as it has a faster growth rate, less well demarcated margins and greater metastatic potential.[6]

1.1.3 Cutaneous Melanoma

Cutaneous melanomas (CM) are the least common but most serious type of skin cancer. Although CM can occur anywhere in the body, including in the internal organs, this thesis only focuses on the experiences of those who have developed melanoma of the skin often referred to as cutaneous malignant melanoma. There are four basic types of melanoma which differ in frequency and location on the body.

1.1.4 Staging Melanoma

Accurate staging is vital for managing patients with cancer effectively. There were several methods for staging MM used in the 1980s and 1990s all of which have now been replaced by a single system developed from the existing American Joint Committee on Cancer (AJCC) staging system.[7] This system was developed from data collected on more than 17,000 patients and provides clearer, more accurate prognostic categories.[8] The system is based on information about the primary tumor (T), the regional lymph node status (N) and the presence of distant metastases (M). Primary melanoma is divided into four groups, according to histological thickness: under one millimeter in thickness (Tl), between one and two millimeters (T2), between two and four millimeters (T3) and above

four millimeters (T4). Normal regional lymph nodes are designated as N0, whereas one involved node is designated as N1, two to four nodes as N2 and more than four nodes as N3. Metastases found in between the primary site and the regional nodes local or in transit metastases are included in the N category.
Although individuals who develop distant metastases are likely to die from their disease, rates of progression vary between better prognosis skin and distant lymph node metastases (M1), and increasingly worse prognosis lung (M2), and liver and brain (M3) secondary.[8]

1.1.5 Etiology

As in most types of cancers, there are two sets of factors that present significant risk for melanoma in humans: endogenous and exogenous factors.
The single most important exogenous factor is exposure to ultraviolet (UV) radiation[9/10], traditionally equated with exposure to the sun but more recently also with artificial sources of UV radiation, such as sunbeds and sunlamps. The endogenous factors which include skin phototype (the amount of melanin pigment in the skin), number of moles, having atypical melanocytic naevi (unusual moles) and having a family history of skin cancer, are also important predictors of melanoma risk.[11/12]

1.1.6 Exogenous factor

Sun exposure is the main risk factor for malignant melanoma (MM). However, the UV-related pathogenetic mechanisms leading to MM are far to be fully elucidated.[13]
The risk of melanoma appears to depend on the interaction between the nature of the sun exposure and the skin type. There is less evidence that sun exposure during adulthood contributes to the risk of MM. However, most of the evidence that the pattern of exposure is important relates to exposure in adulthood. Although different patterns of sun exposure are associated with different levels of risk for melanoma among individuals, it seems that intermittent sun exposure is associated with greater risk than total lifetime exposure.[14/15]
Many clinical studies have reported that exposure to sunbeds or sunlamps has adverse effects on the skin and that their use might increase the risk of developing MM.

1.1.7 Endogenous factor

The most important endogenous etiological factor of MM is the presence of both common acquired and atypical (dysplastic) melanocytic naevi (moles) and risk increases with the number of naevi.[11]

People with very high numbers (100+) of common moles on their bodies have nearly seven times the risk compared to people with very few (0-15 moles).[16] Furthermore, it has been seen that patients with a family history of melanoma are at increased risk. Around 5-12% of patients with melanoma have a family history of MM in one or more first-degree relative.[17] Some of these patients have inherited genes which are associated with a significantly increased risk of melanoma. To date, the major high-penetrance susceptibility genes that have been identified are CDKN2A (pi6), and CDK4. Overall, approximately 20%-40% of tested melanoma families showed inheritance of mutations in CDKN2A[18], so far seventeen families have been found to have mutations in CDK4.

The color of unexposed skin and the ability to tan, known as skin photo type are both factors linked to melanoma risk.

1.1.8 Incidence

The incidence of cutaneous melanoma continues to increase in the Caucasian population in the United States. In 2014, women only accounted for 42% of the 76,100 new melanoma cases and only 33% of the 9,710 deaths associated with CM in the United States.[19] These trends are consistently observed in populations around the world. Indeed, sex disparity in melanoma outcome is so repeatedly observed that gender has been suggested as an important prognostic factor, despite not being previously incorporated in staging algorithms.[20] The source of this gender disparity in melanoma remains unclear, but likely represents both biological and behavioral etiologies.

1.1.9 Sex-linked physiologic differences in skin

Skin is a dynamic, complex, integrated arrangement of cells, tissues, and matrix elements that mediates a diverse array of functions, including physical permeability barrier, protection from infectious agents, thermoregulation, sensation, ultraviolet (UV) protection, wound repair and regeneration. These various functions of skin are mediated by its major layers: the epidermis, dermis, and subcutaneous fat.

The thickness of the skin is greater in men than in women at all ages.[21/22] Skin thickness decreases in men and women starting at the age of 45, and women's skin gets 10% thinner after menopause.[23/24] These differences are likely modulated, in part, by hormones, given that they manifest during puberty and increase with age.[25]

Women and men differ in the metabolism of and response to androgens and estrogens.[26] Estrogens are known to accelerate wound healing, improve inflammatory disorders, increase epidermal thickness, and protect against photo aging of the.[27] The cellular effects of estrogens are mediated by estrogen receptors (ERs), ERα and ERβ, which belong to the nuclear steroid hormone receptor superfamily. ERα and ERβ are widely expressed in human tissue, but have differential distributions in various tissues, including the skin. ERα is primarily expressed in the uterus, liver, kidneys, breasts, and heart, whereas ERβ is primarily detected in what are known as non-classical estrogen- responsive tissues: the ovaries, colon, lungs, adipose tissue, prostate, bladder, and skin.[28] Cutaneous ER levels are generally known to be higher in women as compared with men. However, relative levels of ERα and ERβ in men and women are not well understood. In women, the amount of ERs declines after menopause with declining levels of estradiol.[28] In contrast to estrogens[29], androgens such as testosterone and 5α- dihydroxy testosterone may be able to promote melanoma tumorigenesis. There are also several baseline differences in the immune systems of men and women. On average, women have higher measured IgG and IgM levels, as well as a greater percentage of CD3 + T lymphocytes, as compared with men, suggesting that men have a relative attenuation of the adaptive immune response compared with women.[30] This is further evidenced by observations that men are more susceptible to bacterial and viral infections[31], while women are more prone to autoimmune and inflammatory diseases.[32] Men are also more prone to skin cancer; this increased risk may be partly explained by their heightened susceptibility to ultraviolet-induced immunosuppression compared with women.[33] Ultraviolet (UV) irradiation is known to inhibit contact hypersensitivity or delayed-type hypersensitivity. Also, sex

hormones may have an additional differential effect on immune cells.[34]

1.1.10 Sex differences in melanoma

Gender-specific differences in melanoma epidemiology are well established. The probability of developing melanoma during one's lifetime is 1.72% in males and 1.22% in females.[35] In the Netherlands, a large population-based cohort study including 10,538 melanoma patients from 1993 to 2004 analyzed the gender difference in melanoma survival. The relative mortality risk excess was 2.70 (95% CI [2.38, 3.06]) in males versus females.

The natural history of melanoma in women parallels the physiologic hormonal changes they undergo. The incidence of melanoma is rare before puberty, rises abruptly through the reproductive ages until approximately 50 years of age, and then diminishes after menopause.[36] In agreement with the aforementioned survival advantages of young women and women with localized melanoma, women who present with stage IV disease also show higher survival rates compared to men, who have a two-fold greater death rate from melanoma.[37/38] It also reported that premenopausal women show a higher survival rate compared to postmenopausal women, which was more pronounced in women with advanced disease. Although the survival advantage decreases with age, postmenopausal women still have better rates of survival compared with men.

1.2 The skin

The skin forms a physical, chemical and immunological barrier of the organism towards the environment, preventing the invasion of pathogens, protecting from trans-epithelial water loss and regulating body temperature. Human skin consists of three major layers, the epidermis, the dermis and the underlying subcutaneous fat tissue (Figure 1).

The epidermis consists mainly of keratinocytes which create the outermost barrier of the skin in a structure of multiple layers (Figure 1). From bottom to top, these histologically distinct epidermal layers are termed *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* and *stratum corneum*. Keratinocytes in the stratum basale proliferate constantly and migrate upwards, thus forming the different epidermal layers. During this process, the keratinocytes differentiate in various stages until they finally die and shed off. Keratinocytes are thus forming the physical barrier of the skin, but more

than that they are also important players in the immune surveillance of the skin by actively recognizing invading pathogens.[39]

Besides keratinocytes, the epidermis also contains Langerhans cells, which are epidermis specific antigen-presenting dendritic cells that capture and present foreign antigens towards cells of the adaptive immune system.[40] The basal layer of the epidermis also contains a population of melanocytes that produce melanin, a pigment that protects the organism from damages by UV irradiation [41], as well as Merkel cells, which sense tactile sensation.[42]

Below the epidermis is the dermis, which is characterized by collagen- and elastin-rich connective tissue. The extensive extracellular matrix in the dermis creates stability and flexibility of the skin and is majorly produced by fibroblasts. The dermis contains blood vessels, hair follicles, sweat glands and sebaceous glands. The hair follicle does not only produce hair, but serves also as a niche for epidermal stem cells.[43] The dermis hosts a large number and variety of immune cells, among them macrophages, dendritic cells, T and B cells, and NK cells, which generally provide additional immune surveillance, memory of previous infections and a quick primary immune response against invading pathogens. It has been estimated that skin resident T cells outnumber T cells in the circulation by two to one[44], highlighting the importance of immune cells within the skin. Under the dermis, a layer of subcutaneous fat and connective tissue in varying thickness creates the third layer of the skin.

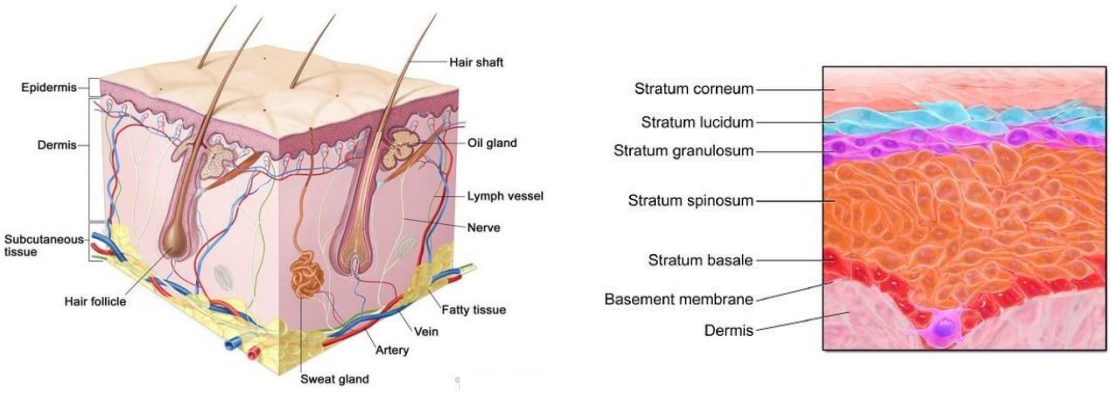


Figure 1: Structure of the skin (left) and the epidermis (right). Illustration from the National Cancer Institute and the Wikiversity Journal of Medicine.

1.3 Inflammation and cancer

Cancer results from the outgrowth of a clonal population of cells from tissue. The development of cancer, called to as carcinogenesis, requires the acquisition of six crucial properties: self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, the maintenance of vascularization, and, for malignancy, tissue invasion and metastasis.[45] Cancer can also be considered with regard to a step-wise development functionally grouped into three phases: initiation, promotion, and progression.[46]

1.3.1 Inflammatory and tumor initiation

Tumor initiation is a process in which normal cells acquire the first mutational hit that sends them on the tumorigenic track by providing growth and survival advantages over their neighbors. In most cases, a single mutation is insufficient and many cancers require at least four or five mutations.[47/45] It is also certain that each mutation be transmitted to the cell's progeny, and in cancers that arise within rapidly renewed epithelia (intestinal and skin cancers), oncogenic mutations must occur in either long- lived stem cells or transient amplifying cells rather than within differentiated cells, which are quickly eliminated before the next mutation can strike. Alternatively, oncogenic mutations can occur within differentiated epithelial cells, such as hepatocytes, which are capable of proliferation and are fairly long lived to allow subsequent mutational hits.

It has been seen that an inflammatory microenvironment can increase mutation rates, in addition to enhancing the proliferation of mutated cells. In fact, Reactive Oxygen Species (ROS) and Reactive Nitrogen Intermediates (RNI) produced by inflammatory cells may cause mutations in neighboring epithelial cells. Also, cytokines produced by inflammatory cells can elevate intracellular ROS and RNI in premalignant cells. In addition, inflammation can result in epigenetic changes that help tumor initiation. Tumor-associated inflammation contributes to further ROS, RNI, and cytokine production.[48]

1.3.2 Inflammatory and tumor promotion

Tumor promotion is the action of tumor growth from a single initiated cell into a completely developed primary tumor. Initial tumor growth depends on increased cell proliferation and reduced cell death, both of which are stimulated by inflammation- driven

mechanisms. Indeed, many of the enhancing effects of inflammation on cancer are exerted at the level of tumor promotion, and most known tumor promoters, for instance phorbol esters, are potent inducers of inflammation.[49] Inflammation-induced tumor promotion may occur early or late in tumor development and can lead to activation of premalignant lesions that were dormant for many years. The mechanisms through which inflammation affects tumor promotion are numerous and, in addition to increased proliferation and enhanced survival, can also involve the so-called angiogenic switch, which allows a small dormant tumor to receive the blood necessary for the next growth phase.[50]

For example, the cytokines produced by tumor-infiltrating immune cells activate key transcription factors, such as NF-κB or STAT3, in premalignant cells to control numerous pro-tumorigenic processes, including survival, proliferation, growth, angiogenesis, and invasion. As elements of positive feed-forward loops, NF-κB and STAT3 induce production of chemokines that attract further immune/inflammatory cells to assist tumor-associated inflammation.[48]

1.3.3 Inflammatory and tumor progression

Metastasis is the most critical aspect of tumorigenesis, because over 90% of cancer mortality is caused by metastasis. Recent studies unambiguously show that metastasis requires close collaboration between cancer cells, immune and inflammatory cells, and stromal elements. The process of metastasis can be grossly divided into four major steps. In the first step, cancer cells acquire fibroblastoid characteristics that increase their motility and allow them to invade epithelial linings/basal membranes and reach efferent blood vessels or lymphatics.[51] Loss of E-cadherin expression is envisioned as a key event in the epithelial-mesenchymal transition. In the second phases, cancer cells intravasate into blood vessels and lymphatics.[48] Inflammation may promote this through production of mediators that increase vascular permeability. This is followed by the third phases, in which metastasis-initiating cells survive and travel throughout the circulation.[48] Next, integrin-mediated arrest allows the extravasation of circulating cancer cells. Finally, single metastatic progenitors interact with immune, inflammatory, and stromal cells and start to proliferate.[52] One of these inflammatory signals is the extracellular matrix component versican, which leads to macrophage activation and production of the metastasis-promoting cytokine TNF-α.[53]

1.4 Cyclooxygenases (COXs family)

Cyclooxygenases are enzymes necessary for the metabolic conversion of arachidonic acid to prostaglandins, including PGE2, a major mediator of inflammation and angiogenesis (Figure 2). PGE2 signals through four pharmacologically distinct G- protein coupled receptors, EP1, EP2, EP3, and EP4, which each activate different downstream signaling pathways.[54] In turn, PGE2 is catabolized to the inactive 15-keto- PGE2 by the enzyme 15-hydroxyprostaglandin dehydrogenase.[55/56] There are two isoforms of cyclooxygenase: COX-1 and COX-2. Both exist as integral, membrane- bound proteins, located primarily on the luminal side of the endoplasmic reticulum and nuclear envelope.[55] COX-1 is characterized as a housekeeping enzyme required for the maintenance of basal level prostaglandins[57] and is expressed constitutively in most tissues. It is responsible for the maintenance of internal homeostasis by participating in processes such as platelet aggregation, cytoprotection of the gastric mucosa, vascular smooth muscle functioning, and renal function. In contraposition, COX-2 usually remains undetected in healthy tissues and organs. In adults, it is found only in the central nervous system, kidneys, vesicles, and placenta, whereas in the fetus, it occurs in the heart, kidneys, lungs, and skin.[55/58] COX-2 is highly inducible and can be rapidly upregulated in response to various proinflammatory agents, including cytokines, mitogens, and tumor promoters, especially in cells involved in inflammation, pain, fever, Alzheimer's disease, osteoarthritis, or tumor formation.[55/59] Under normal conditions, acute inflammation is a tightly controlled self-limiting response, where upon abatement of the inflammatory stimulus, specific cytokines, including interleukin-1 (IL-1) and interleukin-6 (IL-6), exert feedback inhibition causing COX-2 expression and PGE2 production to cease and the inflammatory response to subside.[60] However, with sustained exposure to proinflammatory stimuli, continued expression of COX-2 leads to the transition from acute to chronic inflammation.[55/60] In recent decades, COX-2 overexpression has been reported in several human cancers including breast[61/62], lung[61/63], skin[64], colon[63/65/66], bone[67/68/69], cervical[70], esophageal[71], pancreatic[72], prostate[73] and bladder cancer.[74] Constitutive expression of COX-2 and sustained biogenesis of PGE2 appear to play predominant roles in the initiation and promotion of cancer progression. PGE2 can mediate these effects through numerous signaling pathways including activation of vascular endothelial growth factor (VEGF) leading to increased cell proliferation, metastatic and invasive potential, and angiogenesis[75/76]; increased expression of the protooncogenes, and the epidermal growth

factor receptor (EGFR), through the activation of the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3- kinase (PI3K)/AKT pathway, respectively[77/78]; increased transcriptional activity of the antiapoptotic mediator nuclear factor κB (NF-κB)[79]; enhanced metastasis and invasion by activation of matrix metalloproteases (MMP-2 and MMP-9)[80] and suppression of the production of IL-12, leading to immunosuppression.[81] Therefore, COX-2 and the prostaglandin cascade play important roles in the "inflammogenesis of cancer".[82]

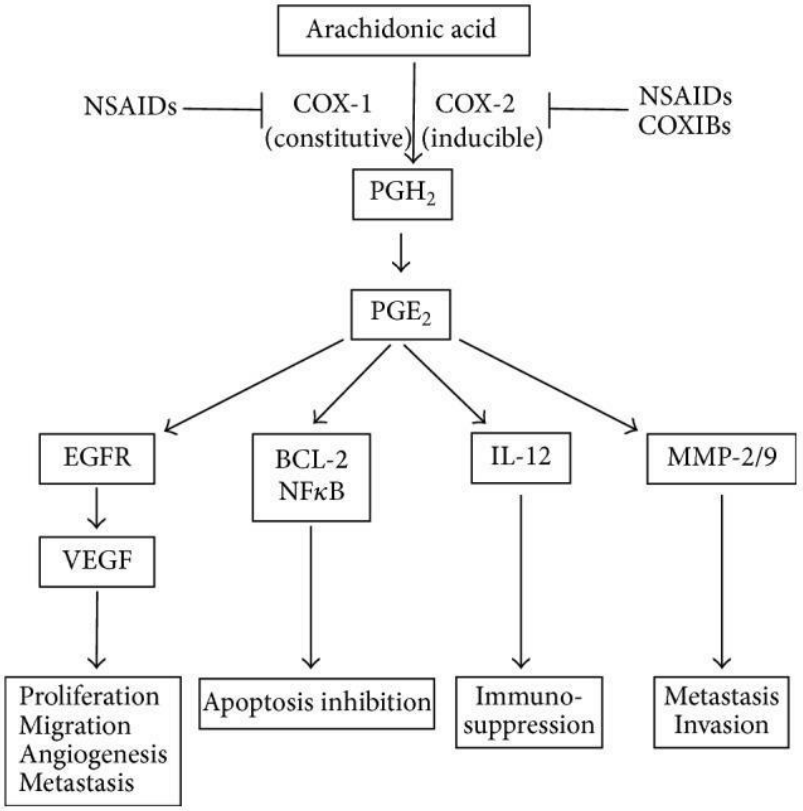


Figure 2: Prostaglandin E2 biosynthesis and downstream cellular effects. Arachidonic acid is released from cellular membranes and converted to PGH2 through the activity of the COX enzymes. COX-1 is constitutively expressed in many cells, generating low levels of prostaglandins that are cytoprotective and maintain homeostasis. In contrast, COX-2 is absent from most cells and is induced by a number of inflammatory stimuli. PGH2 is rapidly converted to PGE2, which plays a predominant role in cancer progression by stimulating tumor cell proliferation, migration, angiogenesis, apoptosis resistance, invasion, and metastasis. NSAIDS and COXIBS can pharmacologically block the activity of the COX enzymes.

1.4.1 PTGS2 gene regulation

The PTGS2 gene (also named COX2) maps to chromosome 1(1q25.2-25.3), contains 10 exons and codes for a protein of 604 aa: the inducible cyclooxygenase Cox-2 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PTGS2). PTGS2 is an immediate-early response gene whose expression is highly induced in response to hormones, proinflammatory cytokines, growth factors, oncogenes, carcinogens and tumor promoters.[83/84]

PTGS2 expression is regulated at both transcriptional and post transcriptional level, and the deregulation of PTGS2 expression observed in carcinogenesis can in part be due to functional changes affecting these regulatory regions of the gene.

The promoter region of the PTGS2 gene contains several key cis-acting regulatory elements, including a canonical TATA box, and various response elements for nuclear factor kB (NK-kB), PEA3, AP2, nuclear factor interleukin-6 (NF-IL6), Sp1, adenosine 3', 5'-cyclic-monophosphate-response element (CRE), c-MYB, transforming growth factor β (TGFβ), and others. Depending on the stimulus and the cell type, these transcription factors can play a decisive role in the regulation of COX-2 transcription.[85/86/87/88]

Current studies have demonstrated that post-transcriptional regulation plays a central role in the regulatory mechanisms of PTGS2 expression.[89] The PTGS2 3′UTR is larger than average, encompassing ~2.5 kb, and has many interesting features. It has several polyadenylation signals, only two of which are commonly used, resulting in mRNAs of ~2.8 or ~4.6 kb. The proximal polyadenylation signal has a non-consensus CPSF (cleavage polyadenylation specificity factor) binding site (AUUAAA), followed by putative upstream enhancer elements (USEs). It is likely that regulation occurs in part here, resulting in two mRNAs with different RNA metabolism. The significance of the different tissue distributions of these isoforms has not yet been addressed, and tissue specificity might play a role in PTGS2 polyadenylation site choice.[90] The 3′UTR also has 22 repeats of adenylate and uridylate-rich (AU-rich) elements (AREs), composed of the sequence 5'-AUUUA-3. This AU-rich region is highly conserved in both sequence and location among human, mouse, rat, chicken, pig, cow and sheep PTGS2 mRNA transcript, implying that the function of the ARE have been evolutionary conserved. This element, which is present within the 3'UTRs of many proto-oncogene and cytokine mRNAs, confers post-transcriptional control of expression. There are many different polypeptides

that specifically interact with AREs from rapidly degraded mRNAs. These regulatory trans-acting factors include several cytoplasmic mRNA-binding proteins proposed to be involved with the destabilization, stabilization, or mRNA processing and nucleocytoplasmic transport.[90/86/91] Regulation of gene expression at a post-transcriptional level is also mediated by microRNAs, short single-stranded non-coding RNAs, which affect stability and translation of their target mRNAs.[92]
In particular, PTGS2 contains a putative binding for miR-146a and it has been demonstrated that miR-146a directly regulates PTGS2 mRNA expression in lung cancer cells and in gastric epithelial cell lines.[93/94]

1.4.2 PTGS2 polymorphism –765G/C

One of the common polymorphisms present in the promoter region of the PTGS2 gene (-765G/C) has been reported to alter promoter activity.[95/96] Polymorphism -765G/C (rs20417) is located at a putative Sp1 binding site and it has been shown that allele - 765C have significantly lower promoter activity compared with -765G in human lung fibroblasts cells. This polymorphism has been associated to myocardial infarction and stroke, to asthma risk, and to the risk of esophageal cancer. [95/96/97] However, it has also been reported that production of PG by monocytes of asthmatic patients is more than ten-fold higher in –765CC homozygotes, than in –765GG homozygotes.[98] These opposite results could be explained by a tissue specific effect of this polymorphism; - 765G can potentially eliminate an Sp1 binding site, but can also generate a binding site for E2F, a cyclin-dependent regulator of expression of several genes, including PTGS2.[98/99]

A case/control study, by Gomez-Lira M.[100] et al 2014, showed that the -765C allele is more represented in patients than in controls. Frequency of this polymorphism indicated that allele -765C may be associated with a higher risk of MM. Expression analysis of the PTGS2 gene in the same study, showed that allele -765C is associated with a higher gene expression and could represent a risk allele by affecting the functionality of the promoter.

1.5 2’-5’-Oligoadenylate Synthetase and RNase-L System

RNase-L is a cellular endoribonuclease which is involved in degrading viral and cellular RNA as an antiviral mechanism.[101/102] RNase-L together with 2’-5’ oligoadenylate synthetase forms an important component of antiviral innate immune system. Viral dsRNA and interferon induce the expression of 2’-5’-oligoadenylate synthetase (OAS), which converts cellular ATP into a unique oligoadenylate 2’-5’ A, referred to hereafter as 2-5A.[103/104] 2-5A is a unique ligand that binds only RNase-L activating RNase-L. Human RNase-L is a 741 amino acid polypeptide with an N-terminal ankyrin repeat region and a C-terminal endoribonuclease domain. In the absence of 2-5A binding, the N-terminal ankyrin repeat region suppresses the ribonuclease domain. 2-5A binding induces a conformation changes allowing dimerization of inactive RNase-L monomers to form active dimers with potent ribonuclease activity.[105/106] Active RNase-L cleaves both viral and cellular RNA blocking viral replication. RNase-L cleaves within single-stranded regions of RNA, principally on the 3’ sides of UpAp and UpUp dinucleotides, leaving 3’phosphoryl and 5’hydroxyl groups at the termini of the RNA cleavage products.[101]

1.5.1 Biochemical Properties of RNase-L

RNase-L is a regulated endoribonuclease constitutively expressed in every mammalian cell type. RNase-L consists of three major domains; an N-terminal regulatory ankyrin repeat domain (ARD), a middle protein kinase like domain, and a C-terminal ribonuclease domain (RNase).[105/107] Ankyrin repeat domains (ARD) are conserved protein-protein interaction motifs composed of multiple α-helices connected by β- hairpin turn. RNase-L has 9 ankyrin repeats with 2 and 4 responsible for 2-5A binding.[107] Recent crystal structure of RNase-L has further shown that RNase-L recognizes 2-5A through coordinated interactions involving the Ankyrin repeat domain and the pseudo-protein kinase domain, with an unexpected role of nucleotide binding to the pseudo-protein kinase domain, which facilitates the imposition of a specific dimer configuration required for RNase catalytic function.[108] Binding of 2-5A to the ankyrin repeat is a unique feature of RNase-L. In the absence of 2-5A binding, ARD represses the RNase domain.[107/109] 2-5A binding induces a conformation change which releases the RNase domain autoinhibition and unmasks the protein-protein interaction regions. The middle kinase-

like domain provides contact for 2-5A and nucleotide which is responsible for dimerization after 2-5A binding.[107/108] RNase-L lacks kinase activity due to the absence of phosphor acceptor sites required for an active kinase.[102] The kinase- like domain has highly conserved residues responsible for ATP binding and stimulation of enzyme activity and mutations in those regions has been shown to impair RNase-L activity.[110] Two distinct mutants of RNase-L have been described; R667A, which is deficient in the ribonuclease activity, and R462Q, which is defective in dimerization, and has 3-fold decrease in RNase-L activity.[111/112]

1.5.2 Regulation of RNase-L Activity

Activation of RNase-L occurs by specific binding of trimer or higher oligomers of 2-5A to the N-terminal ankyrin repeats. 2-5A is a very unstable molecule and is degraded by a combination of 5'-phosphatase and 2', 5'-phosphodiesterase presents in cells. Activity of RNase is negatively regulated by a cellular protein RNase-L inhibitor (RLI) also known as ABCE1. Overexpression of RLI inhibits 2-5A binding and ribonuclease activity of RNase-L.[113] Expression of RLI is not regulated by interferons but can be induced by certain viruses and dsRNA.[114] RNase-L is expressed in all mammalian cell types and subject to post-transcriptional regulation by the 3'-untranslated region (3' UTR). RNase-L expression is negatively regulated by microRNA miR-29 which has target sites in the 3'-UTR in RNase-L mRNA. RNASEL contains a putative binding for miR-146a, but there are no scientific data concerning their relation.

RNASEL 3'UTR also contains AU-rich elements (AREs) which are cis-acting regulatory regions that modulate mRNA stability through the binding of ARE-binding proteins (AREBPs). HuR is an ARE-binding protein that induces RNase-L expression during stress or myoblast differentiation. HuR and miR-29 therefore mediate antagonistic effects on RNase-L expression.[115] RNase-L activity is also regulated by protein-protein interactions. In addition to RLI, mammalian RNase-L interacts with eukaryotic release factor 3 (eRF3), androgen receptor (AR), IQGAP1 and Filamin A.[113/116] Interaction of RNase-L with eRF3 is 2-5A dependent and modulates translation in cells. In the presence of 2-5A, RNase-L interacts with eRF3 reducing the binding to poly (A)-binding protein (PABP) decreasing translation re-initiation and promoting translation. RNase-L interacts with the androgen receptor in the presence of androgens which is recruited to the promoter of AR responsive genes targeting them for IFN stimulated and specific degradation

downregulating AR signaling.[116] Binding of RNase L to Filamin A forms a barrier in mammalian cells preventing the entry of virus.[116] RNase L is shown to bind several actin-binding proteins and has been shown to localize to the cytoskeletal fraction in cells.[118]

1.5.3 RNASEL polymorphism -1385G/A

A common missense mutation in RNASEL, R462Q (rs486907, G/A), has been studied extensively in genetic epidemiology studies.[119/120/121] The homozygous genotype (AA) has 15% prevalence in Caucasian populations and causes a functionally significant amino acid substitution resulting in a 3-fold decrease in enzymatic activity. This variant could enhance virus susceptibility, decrease control of cellular RNA levels, impair the cellular stress response, or induce apoptosis.[122] While under normal conditions RNASEL has tumor suppressive and anti-proliferative functions, RNASEL variants, including the common rs486907 variant, have been associated with risk of a number of cancers, i.e. prostate, colorectal and pancreatic cancer, and overall risk of cancer in individuals of African descent.[123/122]

A case/control study by Gomez-Lira M[124]. et al 2017, evaluated the impact of RNASEL polymorphism rs486907 on melanoma risk, showing an association of this SNP to melanoma risk.

1.6 MicroRNA

miRNAs are small non-coding RNAs, with an average 22 nucleotides in length.[125] Most miRNAs are transcribed from DNA sequences into primary miRNAs (pri-miRNAs) and processed into precursor miRNAs (pre-miRNAs) and mature miRNAs.[125] In most cases, miRNAs interact with the 3′UTR of target mRNAs to suppress expression.[126] However, interaction of miRNAs with other regions, including the 5′UTR, coding sequence, and

gene promoters, have also been reported.[127] Furthermore, miRNAs have been shown to activate gene expression under certain conditions.[128] Recent studies have suggested that miRNAs are shuttled between different subcellular compartments to control the rate of translation, and even transcription.[129] Aberrant expression of miRNAs is associated with many human diseases.[130/131] In addition, miRNAs are secreted into extracellular fluids. Extracellular miRNAs have been widely reported as potential biomarkers for a variety of diseases and they also serve as signaling molecules to mediate cell-cell communications.[132/133]

1.6.1 Biogenesis

miRNA biogenesis starts with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally.[125/126] About half of all currently identified miRNAs are intragenic and processed mostly from introns and relatively few exons of protein coding genes, while the remaining are intergenic, transcribed independently of a host gene and regulated by their own promoters.[134/135] Sometimes miRNAs are transcribed as one long transcript called clusters, which may have similar seed regions, and in which case they are considered a family.[136] The biogenesis of miRNAs is classified into canonical and non-canonical pathways (Figure 3).

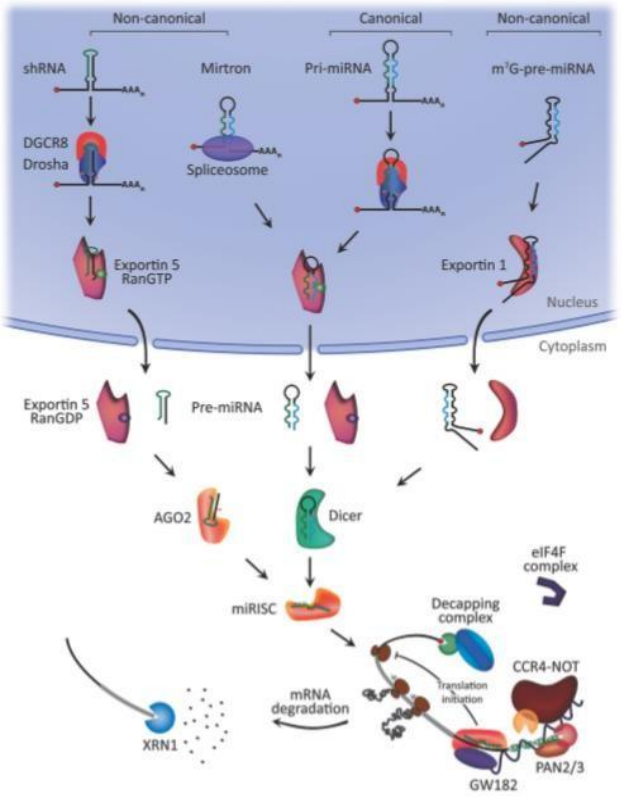


Figure 3: MicroRNA biogenesis and mechanism of action. Modified from Hayder et al.

1.6.2 The canonical pathway

The canonical biogenesis pathway is the dominant pathway by which miRNAs are processed.[125] In this pathway, pri-miRNAs are transcribed from their genes and then processed into pre-miRNAs by the microprocessor complex[125], consisting of an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha.[137] DGCR8 recognizes an N6-methyladenylated GGAC and other motifs within the pri-miRNA[138], while Drosha cleaves the pri-miRNA duplex at the base of the characteristic hairpin structure of pri-miRNA. This results in the formation of a 2 nt 3′ overhang on pre-miRNA. [125/139] Once pre-miRNAs are generated, they are exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP complex and then processed by the RNase III endonuclease Dicer. [125/137/140] This processing involves the removal of the terminal loop, resulting in a mature miRNA duplex.[141] The directionality of the miRNA strand determines the name of the mature miRNA form. The 5p strand arises from the 5′end of the pre-miRNA hairpin while the 3p strand originates from the 3′end. [125] Both strands

derived from the mature miRNA duplex can be loaded into the Argonaute (AGO) family of proteins (AGO1-4 in humans) in an ATP-dependent manner.[141]

1.6.3 Non canonical pathway

Non canonical pathways make use of different combinations of the proteins involved in the canonical pathway, mainly Drosha, Dicer, exportin 5, and AGO2.[125] In general, the non-canonical miRNA biogenesis can be grouped into Drosha/DGCR8-independent and Dicer-independent pathways.[125] Pre-miRNAs produced by the Drosha/DGCR8-independent pathway resemble Dicer substrates.[125] These nascent RNAs are directly exported to the cytoplasm through exportin 1 without the need for Drosha cleavage. There is a strong 3p strand bias most likely due to the m7G cap preventing 5p strand loading into Argonaute.[142] On the other hand, Dicer-independent miRNAs are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts. These pre- miRNAs require AGO2 to complete their maturation within the cytoplasm because they are of insufficient length to be Dicer-substrates.[143] This in turn promotes loading of the entire pre-miRNA into AGO2 and AGO2-dependent slicing of the 3p strand. [125] The 3′- 5′ trimming of the 5p strand completes their maturation.[144]

1.6.4 Mechanism of miRNA mediated gene regulation

Most studies to date have shown that miRNAs bind to a specific sequence at the 3 ′UTR of their target mRNAs to induce translational repression and mRNA deadenylation and decapping.[145/146] miRNA binding sites have also been detected in other mRNA regions including the 5′ UTR and coding region has been reported to induce transcription.[147]

1.6.5 Action of miRNA

MiRNA-mediated gene regulation is dynamic and helps to buffer gene expression to a steady state.[125] It is only recently that a more comprehensive understanding of miRNA dynamics has begun to shed light on the highly robust nature of miRNA-mediated gene

regulation.[125] Factors that may contribute to the robustness of miRNA-mediated gene regulation include the functionalized compartmentalization and shuttling of miRISC within the cells.[125] The availability and abundancy of miRNAs and their target mRNAs are also contributing factors in determining which genes are regulated. Although this is not always the case, miRNA suppression of mRNA targets is not ubiquitous between cell types.[125] Alternative splicing and alternative polyadenylation affecting 3′UTRs, and cell type-specific RNA binding proteins that affect target mRNA secondary structures, change the available pool of MREs.[148/149/150]

This renders subsets of mRNAs sensitive or insensitive to miRNA-mediated gene regulation in a cell type/state-specific manner. [125]

1.6.6 miR-146 family

During the past several years some miRNA functions in the immune system have been unveiled, among which one miRNA has shown important regulatory functions: miR-146. The murine and the human genome contain two miR-146 genes: miR-146a and miR-146b.[151] Mir-146a and miR-146b are both conserved in all vertebrate and are involved in the regulation of inflammation and other processes in innate as well as adaptive immunity. Some miRNAs, like these two miR-146 molecules, are expressed as a family that share the same seed sequence but are encoded by different loci in the genome. MiR-146a and miR-146b are located on chromosome 5 and 10, respectively, and their products differ only by 2nt in the 3' region, while the 'seed' sequence, that is a conserved heptamer situated in the miRNA 5′- end, is identical, implicating that both miR-146a and miR-146b should recognize the same targets (Figure 4).

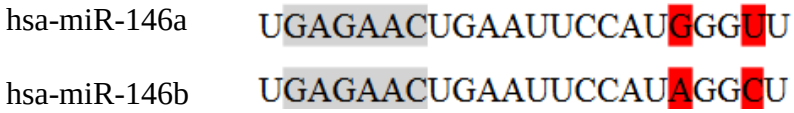


Figure 4: Mature form of miR-146a and miR-146b. The regions highlighted in red display the differences in sequence. Shaded in grey is the seed sequence, important for target recognition and identical in both miRNAs.

Most studies examined primarily miR-146a rather than miR-146b, since these two miRNAs should recognize the same targets, but miR-146a is often more abundantly expressed in the immune system.

1.6.7 miR-146a in innate immunity

The innate immune response is the first line of defense against invading pathogens and the primary initiator of inflammatory responses involving monocytes, macrophages, granulocytes, and various types of DCs (dendritic cells), including LCs (Langerhans cells). The central pathway in innate immunity is the NF-κB pathway, and it is targeted by a number of miRNAs, of which miR-146a was the first discovered to be involved. miR-146a itself is an NF-κB–dependent miRNA, and it targets the TLR4 signaling pathway through a negative feedback regulation loop that involves the downregulation of IL-1 receptor–associated kinase (IRAK) 1 and TNF receptor–associated factor 6.[152] miR-146a expression is higher in LCs compared with that seen in other DCs. It does not influence LC differentiation, but it desensitizes LCs to TLR2-dependent activation.[153] In peripheral blood monocytes in mice and $CD14^+$ monocytes in human subjects, miR- 146a controls monocyte responses during inflammatory challenge through a member of the noncanonical NF-κB/Rel family.[154]

1.6.8 miR-146a in adaptive immunity

Adaptive immunity is mediated mainly by T and B lymphocytes and involves recognition of specific molecules and selection and expansion of antigen-specific T- and B-cell clones. It provides the host with a learned memory that leads to secondary responses, mainly involved in chronic inflammatory processes, including development of autoimmunity and allergic diseases.[155]

As for the role of miR-146a in T cells, by analyzing the expression of miRNAs in subsets of cells of the immune system, it has been show that miR-146a is one of the very few miRNAs differentially expressed between Th1 (T helper cells 1) and Th2 (T helper cells 2) cells in the mouse, suggesting that it might be involved in the fate determination of these cells.[156] In human T cells, miR-146a is expressed at low levels in naïve T lymphocytes while it is abundantly expressed in memory T cells and it is induced upon TCR (T cell receptor) stimulation, consistent with its expression being dependent on NF-

κB induction.[157/158] Treg cells constitute a specialized T-cell subset able to maintain immune homeostasis by limiting the inflammatory responses, and their suppressive function is indispensable for immune homeostasis and survival of higher organisms. Recently, Lu and colleagues[159] reported that miR-146a is among the miRNAs prevalently expressed in Treg cells and showed that it is critical for Treg functions. Indeed, deficiency of miR-146a resulted in increased number but impaired function of Treg cells and as a consequence, breakdown of immunological tolerance.

Overall, these data showed an important role for miR-146a in regulating both innate and adaptive immunity, and a dysregulation of miR-146a expression may lead to a number of diseases, including cancer.

1.6.9 miR-146a polymorphism rs2910164 G/C

An allelic polymorphism, the rs2910164 G/C single-nucleotide polymorphism, altering the amount of mature miR-146a has been described in the gene encoding for pre-miR146a, the precursor molecule of miR-146a,[160/161] but only relatively few studies have investigated its effect on the risk of cancer. Some studies suggested a possible relationship between the pre-miR-146a polymorphism and the risk of different tumor types.[160/162/163] while others have not found such a connection.[163] This polymorphism is located in the seed region of pre-miR-146a-3p sequence and leads to the formation of two different isoforms that can regulate distinct sets of genes.[164]

Gomez-Lira[165] et al 2015 notated that rs2910164 G/C SNP genotype distribution and allele frequency were different between cases (224 patients with MM) and controls (264), indicating an association of the polymorphism with the melanoma risk.

These result are reinforced by a recent study showing that miR-146a is regulated by BRAF and NRAS and plays an essential role in the initiation and progression of melanoma.[166] Stratifying by gender, Gomez Lira[165] et al 2015, observed that the association of the variant with melanoma was restricted to males. Gender-specific association of this polymorphism with carcinogenesis has also been observed in several other studies.[162/167] In 2017, Gomez Lira[124] et al observed a sex-specific effect of the miR-146a rs2910164C allele restricted to individuals carrying the RNASEL rs486907A allele.

Recently, our group, (manuscript in preparation) analyzed the possible effect of the presence of the two polymorphisms (rs2910164G/C in miR-146a and rs20417G/C in the PTGS2 gene) with melanoma predisposition and observed that the risk effect of

rs2910164C allele was restricted to the subgroup of males carrying also the rs20417GG genotype. This is the first study that evaluates the impact of PTGS2 polymorphism rs20417 and miR-146a rs2910164 upon melanoma risk and defines that miR-146a rs2910164C risk allele is restricted to males carry rs20417GG genotype in the PTGS2 gene, suggesting an interaction between the two genes.

In the present study, in order to investigate if gene and/or protein expression of PTGS2/COX-2, RNASEL/RNase-L or miR-146a expression are differently affected by sex hormones in skin cells, we analyzed the effect of 17β-estradiol or testosterone in melanoma cells, keratinocyte cells and primary fibroblast. The eventuality miR-146a regulation upon PTGS2 or RNASEL 3'UTR genes expression was tested in keratinocyte cells.

2 MATERIAL AND METHODS

2.1 Cells culture

A melanoma cell line (LM-20), provided by Dr. Monica Rodolfo (Istituto Nazionale Tumori, Milan) was grown in Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco, Life Technologies). Human keratinocyte cell line (HaCaT) was cultured in the Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies), primary fibroblasts cultures were purchased from Istituto Zooprofilattico Sperimentale, Brescia (Italy) and maintained in Advanced Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies). Culture media were supplemented with 2 mM L-glutamine, penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively) and 10% (v/v) fetal bovine serum (FBS). All cell cultures were maintained in a humidified incubator at 37°C in 5% CO_2.

2.2 Sex hormones treatment for genes expression

Fifty thousand (50.000) HaCaT cells were plated in 24-well plates. Cells were cultured in red phenol free medium supplemented with 10% charcoal treated serum, before and during the treatment. Sex hormones were added to cells at 1 µM, 10 nM and 0.1 nM for testosterone and 500 nM, 100 nM, 1 nM and 0.01 nM for 17β-estradiol, while only ethanol (0.00001% solvent) was added to the control group. After 24 hours, RealTime PCR (qPCR) for PTGS2 and RNASEL genes expression and cells viability were performed to choose the best concentration of both sex hormones. Cells viability was carried out using the method of Trypan blue exclusion.

2.3 Sex hormones treatment

Two hundred seventy thousand (270.000) melanoma, HaCaT and primary fibroblast cells were plated in 6-well plates and grown for 24 h. Testosterone and 17β-estradiol (Sigma Aldrich) were added to the cells at the appropriate concentration, while only pure ethanol (0,00001%) was added to the control group. Cells were grown at 37 °C in 5% CO_2 and 95% air for 24 h. Cells for mRNA expression analysis were lysed directly in the culture

dish adding 1 mL of TRIzol reagent (Invitrogen). For microRNA expression the cells were collected by trypsin digestion followed by centrifugation.

2.4 RNA extraction and cDNA syntheses

Total RNA was isolated with TRIzol reagent, according to the manufacturers' recommendations. Extracted RNAs were quantified by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and the absorbance ratio at 260/280 and 260/230 were measured to assure RNA purity. The integrity of RNAs were controlled by 1% agarose gel electrophoresis.

First strand synthesis of mRNAs was carried out using SensiFAST cDNA Synthesis Kit (Bioline, Trento, Italy), following the manufacturer's protocol. The reactions were incubated for 10 min at 25°C for primer annealing, for 15 min at 42°C to allow the reverse transcription and for 5 min at 85°C to inactivate the reaction.

For cDNA synthesis of miRNAs, cells were resuspended in 1X PBS, lysed by heating at 95°C for 5 min, and immediately chilling on ice before aliquoting 5 µl directly into reverse transcription reactions, as described by Chen *et al.*, 2005. Total RNA was reversed transcribed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific Milan, Italy) according to the manufacturer's protocol. The reactions were incubated at 37°C for 45 min to perform the poly(A) tailing reaction, and at 65°C for 10 min to stop the reaction. Samples were then incubated at 16°C for 60 min to allow the adaptor ligation reaction, for 15 min at 42°C to allow the reverse transcription and 5 min at 85°C to stop the reaction. All cDNAs were stored at -20°C until qRT-PCR assay.

2.5 Quantitative real-time PCR (qRT-PCR)

Following the reverse transcription step, cDNAs were amplified in fluorescence-based real-time polymerase chain reaction (RT-PCR) to determine gene and miRNA expression levels. The qPCR assays were performed in CFX Connect Real-Time System (Bio-Rad), using the SensiFAST SYBR no-rox Kit (Bioline, Trento, Italy) for mRNA expression analyses, and using TaqMan Universal Master Mix II, no UNG, and specific TaqMan probes (Thermo Fisher Scientific Milan, Italy) for miRNA expression analyses. Primers used for mRNA qPCR are shown in Table 1a. The probe used for miRNA are shown in Table 1b. The amplification reactions for mRNAs expression analyses were performed

by an enzyme activation step of 20 sec at 95°C, followed by 40 cycles of amplification which were performed as following: denaturation for 1 sec at 95°C and annealing and extension for 20 sec at 60°C. The amplification reactions for miRNAs expression analyses were performed following the 7500 fast system according to the following thermos cycling profiles: enzyme activation for 20 sec at 95°C, followed by 40 cycles of 3 sec at 95°C for the denaturation step, and 30 sec at 60°C for annealing and extension steps. Each experiment included a no-template control. Each measurement was carried out in triplicate in at least three different experiments.

Normalization of gene and miRNA expression was performed using the most stable reference gene (as described in 3.1.3).

Table 1a: Primers used for the analysis of mRNA expression.

Gene	Forward and reverse primer (5' -> 3')	Product (bp)
PTGS2	TGAAACCCACTCCAAACACA	198
	AGGAGAGGTTAGAGAAGGCT	
RNASEL	CAGGATCTGCAACCACAAAA	82
	CCCACTTGATGCTCTTATCAAA	

Table 1b: Probe used for the analysis of miR expression.

miRNA	Assay ID (Applied Biosystem)	Sequence
hsa-miR-146a-5p	478399_mir	UGAGAACUGAAUUCCAUGGGUU

2.5.1 Reference gene evaluation

Nine candidate reference gene and eight candidate miRs were tested as possible qPCR normalizers. Primers and probes used are described in Table 2a/b.

The stability of each candidate mRNA and miRNA expression was statistically analyzed with three algorithm-based tools: ΔCt method (Silver et al., 2006), Best-Keeper (Pfaffl et al., 2004) and geNorm (Vandesompele et al.,2002). The final ranking was calculated applying the geometric mean of the ranks obtained from the three methods analyzing the expression of each gene.

Table 2a. Primers used for the analysis of candidate mRNA reference genes.

Gene	Forward and reverse primer (5' -> 3')	Product (bp)
GAPDH	TGACACTGGCAAAACAATGCA	103
	GGTCCTTTTCACCAGCAAGCT	
HPRT1	TGACACTGGCAAAACAATGCA	94
	GGTCCTTTTCACCAGCAAGCT	
HSD11B1	AAGCAGAGCAATGGAAGCA	108
	GAAGAACCCATCCAAAGCAA	
IL1R1	AGAGGAAAACAAACCCACAAGG	106
	CTGGCCGGTGACATTACAGAT	
POLR2K	TTTCCGTGCTGTGTAGGG	95
	TGTGACACTCTCCACAGA	
PPIA	CAGGTCCTGGCATCTTGTCC	182
	TTGCTGGTCTTGCCATTCCT	
TBP	TGTATCCACAGTGAATCTTGG	102
	ATGATTACCGCAGCAAACC	
TLR4	AAATTTCCGCTTCCTGG	109
	TCAGCCCATATGTTTCT	
YES1	TGTGAAACCTCAGACTCAAGG	119
	TCCATGTTCCCATCCACA	

Table 2b. Probes used for the analysis of candidate miRNA reference genes.

miRNA	Assay ID (Applied Biosystem)	Sequence
hsa-miR-15b-5p	478313_mir	UAGCAGCACAUCAUGGUUUACA
hsa-miR-20a-5p	478586_mir	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-22-5p	477987_mir	AGUUCUUCAGUGGCAAGCUUUA
hsa-miR-106a-5p	478225_mir	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-128-3p	477892_mir	UCACAGUGAACCGGUCUCUUU
hsa-miR-191-5p	477952_mir	CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-199a-5p	478231_mir	CCCAGUGUUCAGACUACCUGUUC
U6 snRNA	4427975	Not available from the provider

2.6 Statistical analysis

Gene expression was calculated using the method described by Plaffl.[168] Unlike the delta-delta Ct calculation method, which assumes primer efficiencies are similar (usually between 90 – 110%) between the gene of interest (GOI) and the housekeeping gene (HKG), Plaffl calculate relative gene expression data accounting for differences in primer efficiencies for increasing reproducibility.

$$Gene\ expression\ ratio = \frac{(E_{GOI})^{\Delta Ct\ GOI}}{(E_{HKG})^{\Delta Ct\ HKG}}$$

The 'E' in the equation refers to the primer efficiency.

Differences in gene expression were analyzed by Student's t test.

2.7 Plasmid constructs

The pLightSwitch + entire 3'UTR PTGS2 was purchased from SwitchGear Genomics by Istituto Nazionale Tumori, Milan. The entire 3'UTR region of the RNASEL was amplified from genomic DNA using the TransStart FastPfu DNA polymerase (Carlo Erba) with primer Forward and Reverse, which introduce a Xba I restriction site at the 5' end of both primers. The amplified fragment was cloned in PGL3 promoter vector. Through these constructs we were not been able to evaluate if the genes are direct targets of the miR-146a probably because the 3'UTR of both genes inhibits their expression by many different elements. For this reason, we amplified a part of 3'UTR of both genes containing the binding site for miR-146a.

To generate constructs with a reduced 3'UTR region of the PTGS2 and RNASEL, the recombined plasmids, containing the entire 3'UTR were amplified using the TransStart FastPfu DNA polymerase, with primers that, like above, introduce a Xba I restriction site at the 5' end of primer forward and Sla I restriction at the 5'end of the primer reverse for PTGS2, and primers which introduce a Xba I site in both primers for RNASEL. The primers used for amplification and the length of the fragments obtained are reported in Table 3. Plasmid vectors were constructed by ligation of the amplified products to the respective empty plasmids (PLS+3'UTR_PTGS2, figure 5a and PGL3_PV+3'UTR_RNASEL, figure 5b). The correct direction of the insert fragment in PGL3 promoter vector was determined by digestion of the construct with Hind III

restriction enzyme (Neb cutter) which give three bands of 3844, 1949 bp when inserted in the correct direction and fragments of 3585 and 2208 bp when inserted is in the opposite direction. All plasmid constructs were confirmed by sequence analysis (BMR Genomics, Padova, Italy).

Plasmids were mutagenized using primers that deleted 5 nucleotides predicted to bind the seed region (nt2–8) of the miR-146a by QuikChange Site-Directed Mutagenesis Kit (Stratagene) (PLS+3'UTR_PTGS2_MUT and PGL3_PV+3'UTR_RNASEL_MUT).

Primers used for mutagenesis are reported in Table 3.

Table 3. Primer used for the genomic amplification and mutagenesis of plasmids containing the 3'UTR of PTGS2 and RNASEL.

Gene	Forward and reverse primer (5' -> 3')	Product (bp)
PTGS2 (reduced)	AGATCAGAGTTCACTTTCTT	494 bp
	GTGTTTAAGCCTACAATCAT	
PTGS2 (mutagenesis)	CTTTCTTATTTAAAAACAAAACCAAATGATATCTAAGTACAGCAATAATAATAATGACG AT	489 bp
	ATCGTCATTATTATTATTGCTGTACTTAGATATCATTTGGTTTTGTTTTTAAATAAGAAAG	
RNASEL (entire)	CCTTCTAGAAACAAGCCTCAGTGTGATGG	1771 bp
	GGATCTAGACCAGGTGCTCATTACAAATC	
RNASEL (reduced)	CCTTCTAGAAACAAGCCTCAGTGTGATGG	783 bp
	GGATCTAGAACCAAAAACTTCTTCAGACTC	
RNASEL (mutagenesis)	ATGACCTTAAGGCAGTACAACTGGGGGGCAATTT	778 bp
	AAATTGCCCCCCAGTTGTACTGCCTTAAGGTCAT	

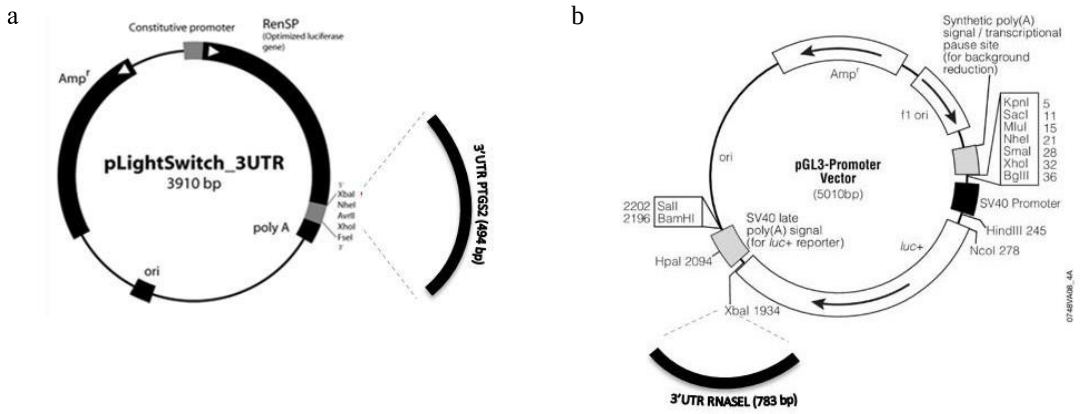


Figure 5a/b: a) pLightSwitch plasmid used for the construction of a plasmid containing a part of the 3'UTR of the PTGS2. b) PGL3 promoter vector used for the construction of a plasmid containing a part the 3'UTR of the RNASEL.

2.8 Transfections

2.8.1 MicroRNAs transfection

Hundred thousand (100.000) HaCaT, primary fibroblast and melanoma cells were seeded, in their appropriate mediums, in a 6 well plate. After 24 hours, 50 nM of miR- 146a mimic were transfected and cells were incubated for another 24 hours. MiR-146a mimic (SIGMA, Aldrich) were transfected into the three skin cultured cells using Metafectene (Biondex). Cells were harvested and resuspended in 1mL TRizol for RNA extraction. PTGS2 and RNASEL genes expression was analyzed as previously described.

2.8.2 Determination of Inhibitor concentration

Hundred thousand (100.000) HaCaT cells in 6 well plate were seeded in the Dulbecco's modified Eagle's medium (DMEM). After 24 hours, different concentrations (1M, 10nM, 50nM, 100nM) of miR-146a inhibitor (antimiR; SIGMA, Aldrich) were transfected. PTGS2 and RNASEL genes expression and miR-146a expression were analyzed as previously described.

2.8.3 Plasmid transfections

Transient transfection of the recombined vectors was performed using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions.
One hundred and thirty thousand (130.000) HaCaT cells were seeded in each well, in 24 well plate. After 24 hours, 5ng of pLightSwitch + reduced 3'UTR_PTGS2 plasmid construct together with 500ng of PGL3 promoter vector (as normalizer), or 1000ng of PGL3 + reduced 3'UTR_RNASEL construct together with 50pg of empty pLightSwitch (as normalizer) were transfected. After 24 hours, the cells were lysed with Passive Lysis Buffer (Promega), and the relative luciferase activity was assessed with the Dual-Luciferase Assay Reporter System (Promega).

2.9 Western blotting

Seven thousand five hundred (750.000) cells were seeded in T25 flasks and incubated with DMEM or RPMI supplemented 10% FBS and 2mM L-glutamine and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively).
After treatments with sex hormones (Testosterone 1 μM, 17β-estradiol 500 nM), HaCaT, primary fibroblast and melanoma cells were washed with ice PBS and then scraped and lysed in RIPA buffer (50mM Tris, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and a mixture of protease inhibitors (Mirus Bio). Cell lysis were carried out on ice for 30 min and then centrifuged for 20 min at 4°C, at 16.000rpm. Total protein concentration in cell lysates was determined by detergent compatible (DC) Bradford Assay analysis (Thermo Fisher Scientific). Cellular proteins were separated in SDS polyacrylamide 10% gel electrophoresis (SDS page) and transferred to PVDF membrane (Thermo Fisher Scientific) by a wet electrophoretic transfer method (Molecular Cloning: A Laboratory Manual, J. Sambrook, E.F. Fritsch, T. Maniatis). Membrane was blocked in TBS solution containing 5% non-fat milk and 0.1% Tween20 (Merck) and then incubated overnight at 4°C with primary antibodies (Rabbit COX-2 polyclonal antibody and Rabbit RNase-L polyclonal antibody (Wuhan Fine Biotech Co) diluted 1:2000 and 1:10.000 in blocking buffer. The next day, membranes were washed three times in 0.1% Tris, 0.2% NaCl, 0.001% Tween 20, and then were incubated with anti-rabbit HRP-conjugated secondary antibody diluted 1:6000 (COX-2) and 1:4000 (RNase-L) in the blocking solution for 1h at room temperature. Signal detection was

accomplished using enhanced chemiluminescence (ECL, Advansta). Images were acquired with Azure C300 Processing machine (Azure Biosystem). Normalization of proteins expression was performed using β-actin. Densitometry analysis of western blot protein bands was carried out using the ImageJ software. Each measurement was carried out in triplicate in at least three different experiments.

3 RESULTS

3.1 Quantitative real-time PCR (qRT-PCR)

3.1.1 Candidate reference genes expression

The expression of nine candidate RGs for mRNA normalization (GAPDH, HPRT1, HSD11B1, IL1R1, POLR2K, PPIA, TBP, TLR4 and YES1) and eight for miRNA normalization (U6 snRNA, miR-15b-5p, miR-20a-5p, miR-22-5p, miR-106a-3p, miR-128-3p, miR-191-5p, and miR-199a-5p) was tested. Specifications of each candidate reference gene are reported in Table 4.

Table 4. Function of the Reference gene analyzed in this study.

Gene symbol	Gene name	Gene function	Gene Bank or miRbase Acc. N°.
Candidate reference genes for mRNA normalization			
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis Organization of the cytoskeleton	NM_002046
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	Generation of purine nucleotides	NM_000194
HSD11B1	Hydroxysteroid 11-Beta Dehydrogenase 1	Conversion of cortisol to cortisone	NM_005525
IL1R1	Interleukin 1 Receptor Type 1	Mediator of cytokine-induced immune and inflammatory responses	NM_000877
POLR2K	RNA Polymerase II Subunit K	Transcription	NM_005034
PPIA	Peptidylprolyl Isomerase A	Folding of proteins	NM_021130
TBP	TATA-Box Binding Protein	Transcription factor	NM_003194
TLR4	Toll Like Receptor 4	Pathogen recognition and activation of innate immunity	NM_138554
YES1	YES Proto-Oncogene 1	Tyrosine Kinase	NM_005433
Candidate reference genes for miRNA normalization			
hsa-miR-15b	MicroRNA 15b (5p)	Apoptosis promotion	MIMAT0000417
hsa-miR-20a	MicroRNA 20a (5p)	RNA polymerase II core binding	MIMAT0000075
hsa-miR-22	MicroRNA 22 (5p)	Cell proliferation and migration impairment *in vitro*	MIMAT0004495
hsa-miR-106a	MicroRNA 106a (3p)	Cell migration and invasion repression	MIMAT0004517
hsa-miR-128	MicroRNA 128 (3p)	Differentiation of hematopoietic stem cells inhibition	MIMAT0000424
hsa-miR-191	MicroRNA 191 (5p)	Cell proliferation and migration promotion	MIMAT0000440
hsa-miR-199a	MicroRNA 199a (5p)	Cell differentiation proliferation	MIMAT0000231
RNU6-1	U6 snRNA	Required to proceed with the process of splicing	NR_002082.1

Ct values of each candidate RG in HaCaT cells, fibroblasts, and melanoma cells in the presence or absence of hormone treatment was determined. The box-plot show the distribution of Ct values of each candidate gene (Figure 6). Across all the cell cultures the

most abundant mRNA resulted the PPIA gene; the least abundant was the HSD11B1 (Figure 6a). MiR-15b-5p was the highest expressed in melanoma cells, U6 snRNA was the highest expressed in fibroblast and in HaCaT cells. MiR-199a-5p was the lowest expressed miRNA in HaCaT cells and fibroblasts, and miR-20a-5p was the lowest expressed in melanoma cells (Figure 6b).

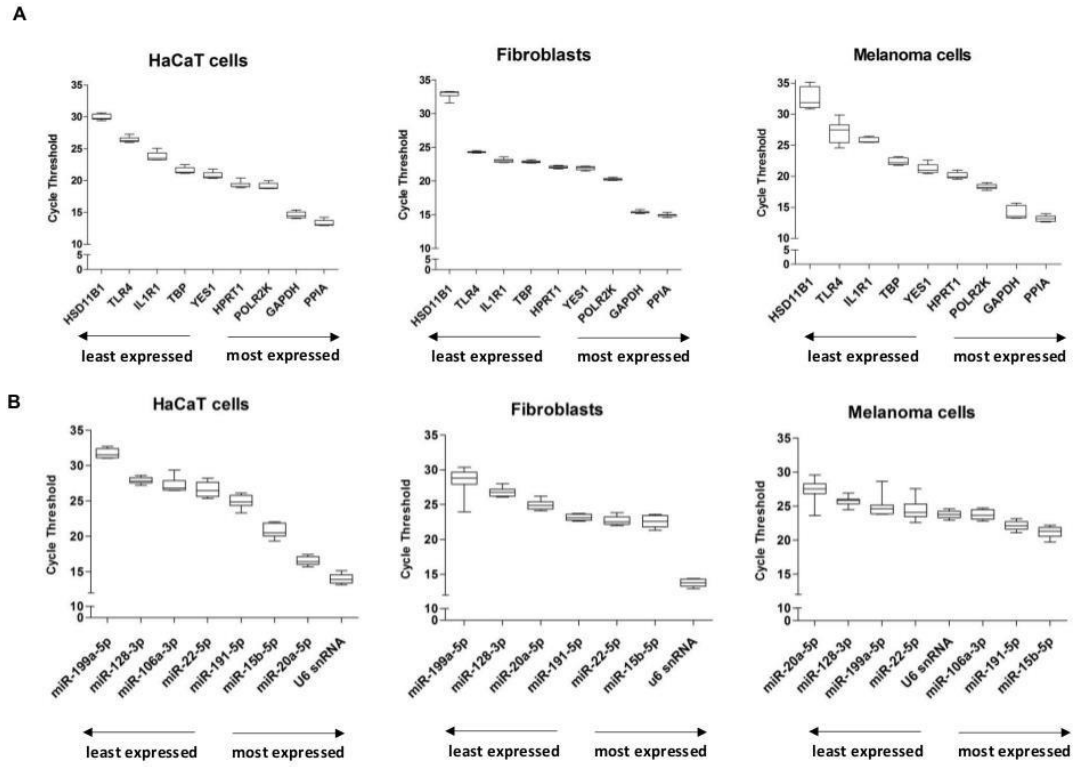


Figure 6: Box-plot of the quantification cycle (Ct) values for each mRNA and miRNA candidate reference gene. The line across the box represents the median. The box specified the 25% and the 75%.

3.1.2 Gene stability

Three algorithm-based tools were applied to establish the stability of coding genes and miRNA: ΔCt method, geNorm, and Best-Keeper. The stability ranking of the mRNA and of miRNA RGs is reported in Table 4. The ΔCt method consists in the quantitative comparison of the ΔCt variability of pairs of reference genes. The stability of each gene is quantified as the average standard deviation over the pairwise comparisons. The values showing an average of the standard deviation STDEV > 1 were excluded from the

analyses. Applying the ΔCt method, in HaCaT cells the analyses showed that PPIA and TBP are the most stable mRNAs (Table 5a). TBP resulted the most stable gene also in fibroblasts and in melanoma cells (Table 5b/c). The analyses of miRNA stability showed that miR-20a-5p and miR-191-5p are the most stable genes in HaCaT cells (Table 4d). The high stability of miR-191-5p expression was also demonstrated in the fibroblasts (Table 5e) and in the melanoma cells (Table 5f).

The second algorithm, the geNorm program, calculates the M stability value, which is an average pairwise variation between all the genes under investigation, providing a ranking of the tested genes. The M values of all the assessed candidate genes included in the analyses showed a value under 1.5 that indicates a stable expression. The most stable mRNA indicated by geNorm was TBP in all the three cultured cells (Table 5a/b/c). In HaCaT cells, PPIA has the same value of TBP (Table 5a). MiR-191-5p resulted the most stable miRNA in both HaCaT cells (Table 5d) and fibroblasts (Table 5e); miR-128-3p was the most stable in melanoma cells (Table 5f).

The third method, the Best-Keeper algorithm, developed by Pfaffl et al, uses an Excel-based application to determine the most stable gene, by calculating the STDEV, from a panel of up to ten candidate genes. The best reference genes have the lowest standard deviation. Reference genes with STDEV > 1 are considered not stable and were excluded from the analyses. Beside Ct standard deviation, the Best-Keeper considers the Best-Keeper index (geometric mean of Ct values for each sample across all genes) in order to calculate the correlation coefficient value (r). Genes showing “r” values close to 1.0 represent the most stable genes. The correlation coefficient showed that TBP is the most stable gene across all the three cultured cells (Table 5a/b/c). The analyses of miRNAs identify miR-15b-5p as the most stable in both HaCaT cells and melanoma cells (Table 5d/f), and miR-128-3p the most stable in fibroblast cells (Table 5e). MiR- 106-3p was excluded from the analyses in fibroblasts due to its low expression.

Table 5a/b/c/d/e/f. Stability ranking of mRNA RGs analyzed by ΔCt, geNorm and Best-Keeper.

a

HaCaT cells (mRNA RGs)					
ΔCt method		geNorm		Best-Keeper	
Gene	Average STDEV	Gene	Stability Value (M)	Gene	r
HPRT1	0,320	HPRT1	0,320	HPRT1	0,871
HSD11B1	0,289	IL1R1	0,297	HSD11B1	0,871
IL1R1	0,277	HSD11B1	0,289	TLR4	0,937
TLR4	0,223	TLR4	0,243	POLR2K	0,956
YES1	0,215	YES1	0,215	IL1R1	0,969
POLR2K	0,214	POLR2K	0,214	YES1	0,969
GAPDH	0,207	GAPDH	0,207	GAPDH	0,975
TBP	0,203	PPIA	0,177	PPIA	0,991
PPIA	0,177	TBP	0,177	TBP	0,996

b

Fibroblasts (mRNA RGs)					
ΔCt method		geNorm		Best-Keeper	
Gene	Average STDEV	Gene	Stability Value (M)	Gene	r
HSD11B1	0,555	HSD11B1	0,555	TLR4	0,417
YES1	0,250	YES1	0,250	HSD11B1	0,602
IL1R1	0,243	IL1R1	0,248	POLR2K	0,765
TLR4	0,239	TLR4	0,244	GAPDH	0,786
GAPDH	0,214	GAPDH	0,214	YES1	0,796
POLR2K	0,210	POLR2K	0,210	HPRT1	0,863
PPIA	0,207	PPIA	0,207	IL1R1	0,876
HPRT1	0,196	HPRT1	0,196	PPIA	0,919
TBP	0,191	TBP	0,183	TBP	0,947

c

Melanoma cells (mRNA RGs)					
ΔCt method		geNorm		Best-Keeper	
Gene	Average STDEV	Gene	Stability Value (M)	Gene	r
TLR4	-	TLR4	-	TLR4	-
HSD11B1	-	HSD11B1	-	HSD11B1	-
GAPDH	0,724	GAPDH	0,658	POLR2K	0,658
POLR2K	0,508	YES1	0,501	PPIA	0,856
YES1	0,501	POLR2K	0,500	HPRT1	0,881
PPIA	0,421	PPIA	0,434	IL1R1	0,891
HPRT1	0,393	HPRT1	0,386	GAPDH	0,912
IL1R1	0,387	IL1R1	0,371	YES1	0,924
TBP	0,374	TBP	0,355	TBP	0,985

d

HaCaT cells (miRNA RGs)					
ΔCt method		geNorm		Best-Keeper	
miRNA	Average STDEV	miRNA	Stability Value (M)	miRNA	r
U6 snRNA	-	U6 snRNA	1,395	U6 snRNA	-0,540
miR-199a-5p	0,908	miR-128-3p	1,197	miR-199a-5p	0,473
miR-106a-p	0,862	miR-106a-3p	1,126	miR-128-3p	0,669
miR-22-5p	0,853	miR-22-5p	1,125	miR-191-5p	0,723
miR-128-3p	0,714	miR-199a-5p	1,002	miR-106a-3p	0,724
miR-15b-5p	0,677	miR-15b-5p	0,850	miR-20a-5p	0,732
miR-191-5p	0,652	miR-20a-5p	0,831	miR-22-5p	0,807
miR-20a-5p	0,580	miR-191-5p	0,819	miR-15b-5p	0,844

e

Fibroblasts (miRNA RGs)					
ΔCt method		geNorm		Best-Keeper	
miRNA	Average STDEV	miRNA	Stability Value(M)	miRNA	r
miR-199a-5p	-	miR-199a-5p	1,061	miR-199a-5p	-
U6 snRNA	0,993	U6 snRNA	1,021	U6 snRNA	-0,140
miR-20a-5p	0,805	miR-15b-5p	0,993	miR-20a-5p	0,536
miR-15b-5p	0,771	miR-20a-5p	0,854	miR-15b-5p	0,738
miR-22-5p	0,636	miR-128-3p	0,770	miR-22-5p	0,780
miR-128-3p	0,608	miR-22-5p	0,770	miR-191-5p	0,891
miR-191-5p	0,570	miR-191-5p	0,684	miR-128-3p	0,915

f

Melanoma cells (miRNA RGs)					
ΔCt method		geNorm		Best-Keeper	
miRNA	Average STDEV	miRNA	Stability Value (M)	miRNA	r
miR-199a-5p	-	miR-199a-5p	-	miR-199a-5p	-
miR-22-5p	-	U6 snRNA	0,963	miR-22-5p	-
miR-20a-5p	-	miR-22-5p	0,796	miR-20a-5p	-
U6 snRNA	0,730	miR-106a-3p	0,739	miR-128-3p	0,892
miR-15b-5p	0,505	miR-20a-5p	0,709	U6 snRNA	0,912
miR-128-3p	0,485	miR-191-5p	0,609	miR-106a-3p	0,912
miR-106a-3p	0,467	miR-15b-5p	0,585	miR-191-5p	0,927
miR-191-5p	0,450	miR-128-3p	0,569	miR-15b-5p	0,965

(-) not included in the analysis

3.1.3 Candidate RGs final ranking

The stability measurements calculated by ΔCt, geNorm and Best-Keeper methods were combined to define the final ranking of each mRNA and miRNA, in the three skin cell types. The final ranking was calculated applying the geometric mean of the ranks obtained by the three methods for each gene (Figure 7). The rank-order of candidates RGs are represented from the least to the most stable. In HaCaT cells, PPIA and miR-191-5p resulted as best candidate references for the normalization of mRNA and miRNA, respectively. The least stable genes were HPRT1 and miR-199a-5p (Figure 7a). In fibroblasts, the most stable genes were represented by TBP, miR-20a-5p, miR-15b-5p and miR-191-5p. The least stable genes were HSD11B1 and U6 snRNA (Figure 7b). These analyses indicate that in melanoma cells, TBP, miR-191-5p and miR-15b-5p can be considered the best reference genes, whereas POLR2K and miR-22-5p may be considered the least stable (Figure 7c). HSD11B1 and TLR4 were excluded from the final ranking because of its high variability across the three tools of analyses.

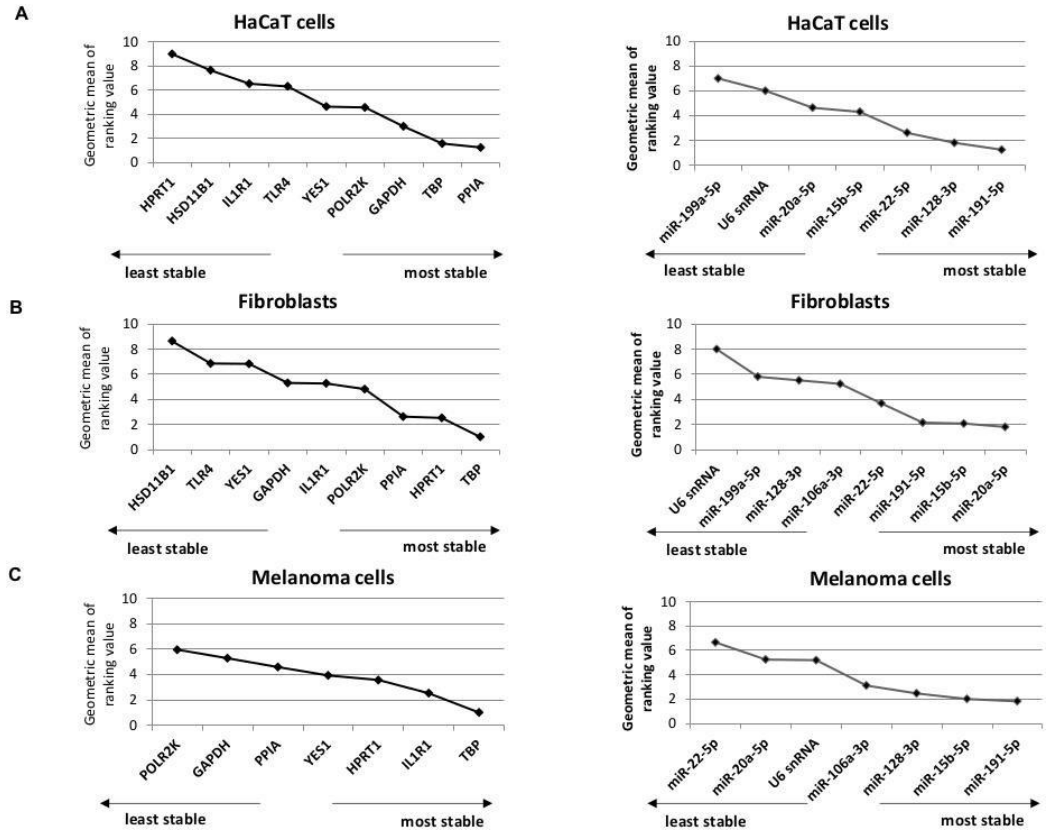


Figure 7a/b/c: Comprehensive geometric mean of the ranking values of mRNA and miRNA candidate reference genes calculating by ΔCt, geNorm and Best-Keeper methods in (a) HaCaT cells, (b) primary fibroblasts, and (c) melanoma cells.

3.2 miR-146a and PTGS2 RNASEL genes expression

PTGS2 and RNASEL genes and miR-146a basal expression was analyzed in primary fibroblasts, HaCaT and melanoma cells. The PTGS2 expression is higher in HaCaT cells compared to melanoma and fibroblast cells. The RNASEL expression is low in fibroblast and HaCaT cells and extremely low in melanoma cells. The highest expression of miR-146a is detected in melanoma cells, much lower in HaCaT and in fibroblast. Figure 8 reports the results.

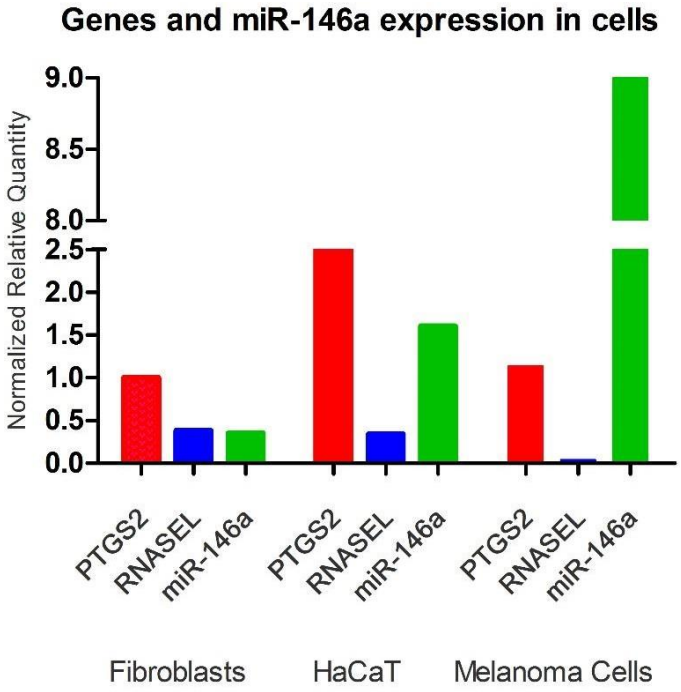


Figure 8: mRNAs from Fibroblasts, HaCaT and Melanoma cells were analyzed by qPCR for PTGS2 (red column), RNASEL (blue column) transcripts and miR-146a (green column). The results were expressed as relative fold change of expression levels.

3.3 Sex hormones treatment and gene expression assay

The dye exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. In this test, a cell suspension is mixed with the dye and then microscope visualize to determine whether cells take up or exclude dye. No effect in cell viability was observed by the different concentration of sex hormones (data not showed).

The effect of different concentration of hormones upon PTGS2 and RNASEL expression in HaCaT cells for 24 hours was evaluated by qPCR (Figure 9). Data analysis of real time PCR showed that the appropriate concentration for subsequent experiment were 1 μM for testosterone and 500 nM for 17β-estradiol.

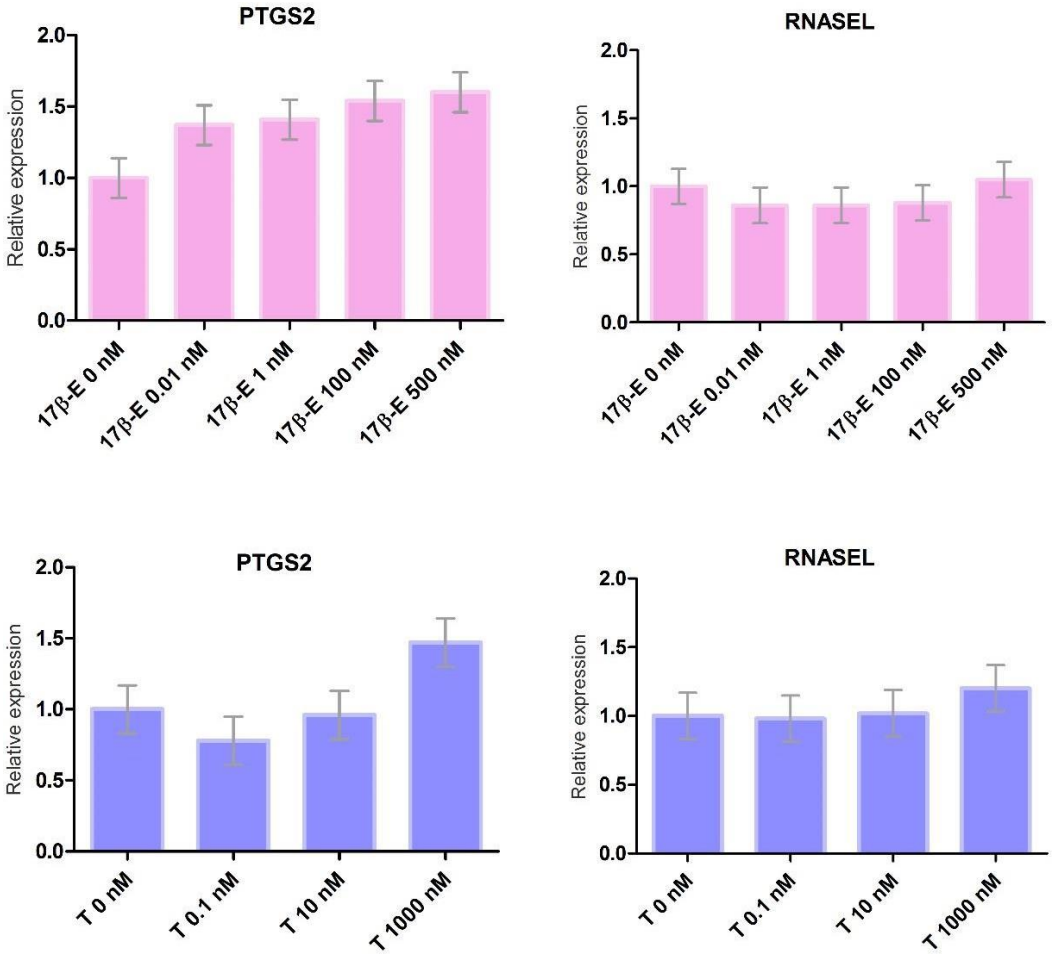


Figure 9: Graphs show the relative genes expression determined by quantitative real-time PCR. The HaCaT cells were treated with different concentration of sexual hormone. After 24 hours the PTGS2 and RNASEL gene expression was evaluated. In the upper graphs (in pink) =17 beta estradiol (17 β-E); in the lower graphs (in blue) =testosterone (T).

Subsequently a time-point experiment was performed (0, 4, 6, 24, 48 hours) treating the cells with the concentration of 1 µM for testosterone and 500 nM for 17β-estradiol. PTGS2 and RNASEL expression was detected by qPCR (Figure 10). The results showed that the best incubation time for the experiments was 24 hours in both genes.

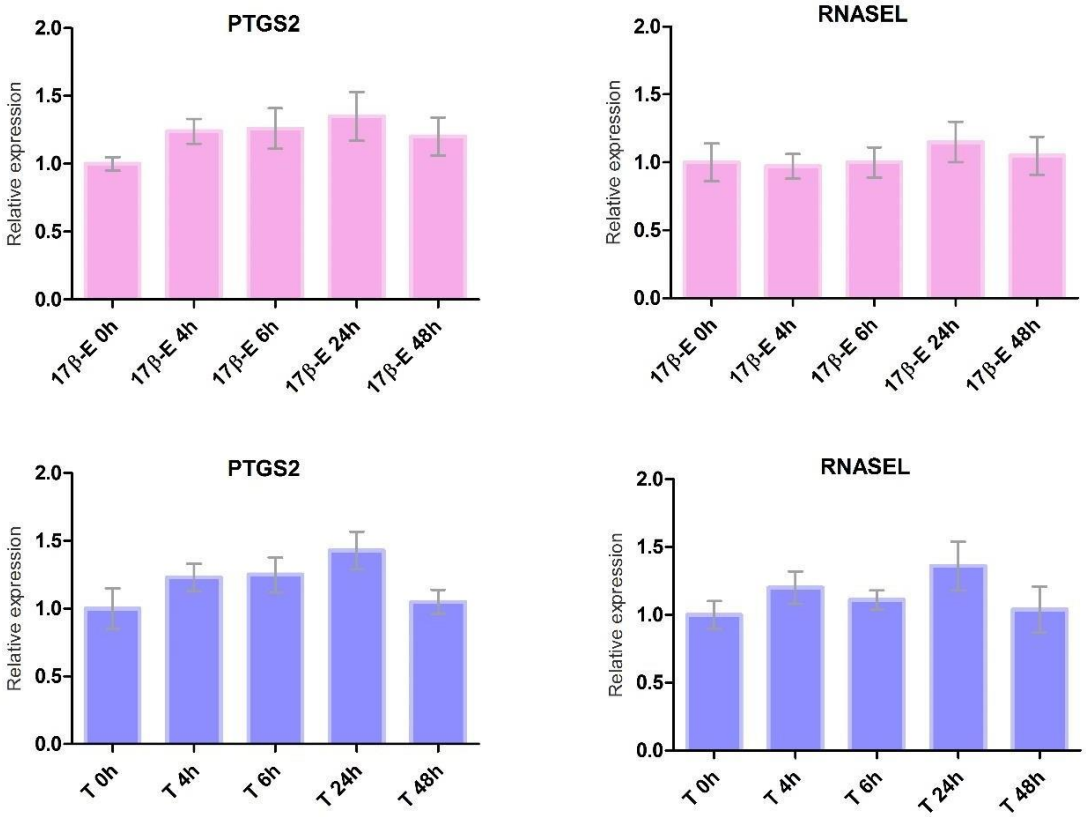


Figure 10: Graphs show the relative genes expression determined by qPCR. The PTGS2 and RNASEL gene expression in HaCaT cells treated with 1 µM of testosterone and 500nM of 17 β-estradiol was evaluated at different times (0h,4h,6h,24h,48h). In the upper graphs (in pink) =17 beta estradiol (17 β-E); in lower graphs (in blue) =testosterone (T).

3.4 Real Time expression analyses after hormones treatments

To evaluate the effect of sex hormonal (1 µM for testosterone and 500 nM for estradiol for 24 hours) in HaCaT, primary fibroblast and melanoma cells, qPCR for PTGS2, RNASEL gene and miR-146a expression were performed. The results are reported in Figure 11.

In HaCaT cells, RNASEL and PTGS2 gene expressions were significantly increased with testosterone treatments. 17β-estradiol demonstrated no effect in the genes expression in these cells. The expression of miR-146a was increased with 17β-estradiol, while no effect of testosterone was observed.

In melanoma cell, an increased RNASEL expression was observed after the treatment with 17β-estradiol. No significant result was detected with testosterone stimuli. The expression of PTGS2 gene remained unchanged in this cell line with both treatments. The expression of miR-146a was significantly increased with testosterone treatment. 17β-estradiol demonstrate no effect in this cell line.

In primary fibroblasts, no different in RNASEL expression was detected with either hormone. A significant reduction in PTGS2 expression was observed in the case of testosterone treatment. The expression of miR-146a appeared higher, albeit not significant, after 17β-estradiol treatment.

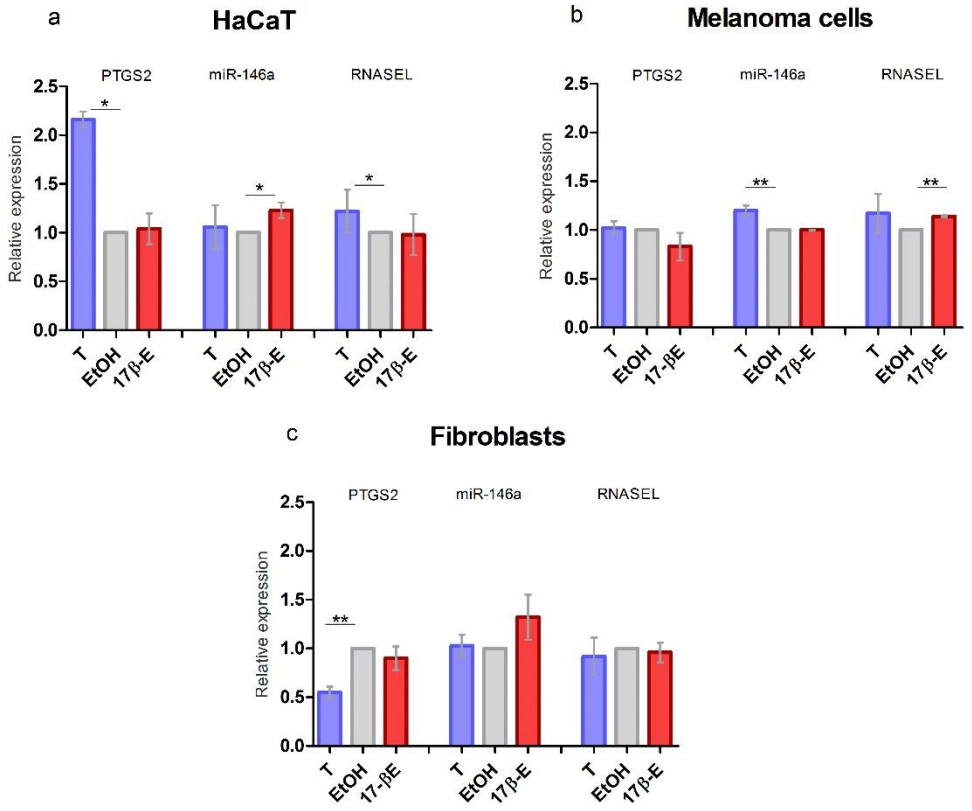


Figure 11: Graphs show the relative gene and miR expression determined by qPCR. Expression of PTGS2, RNASEL and miR-146a after sex hormones treatment for 24 hours was evaluated in the three cultured cells, HaCaT (a), Melanoma cells (b) and Fibroblasts (c). The blue bars indicate the treatment with the 17beta estradiol (17 β-E); the red bars indicate the treatment with testosterone (T); and the grey bars, EtOH, indicate controls with only hormones solvent (ethanol 0,00001%).

3.5 Western blotting analyses after hormones treatments

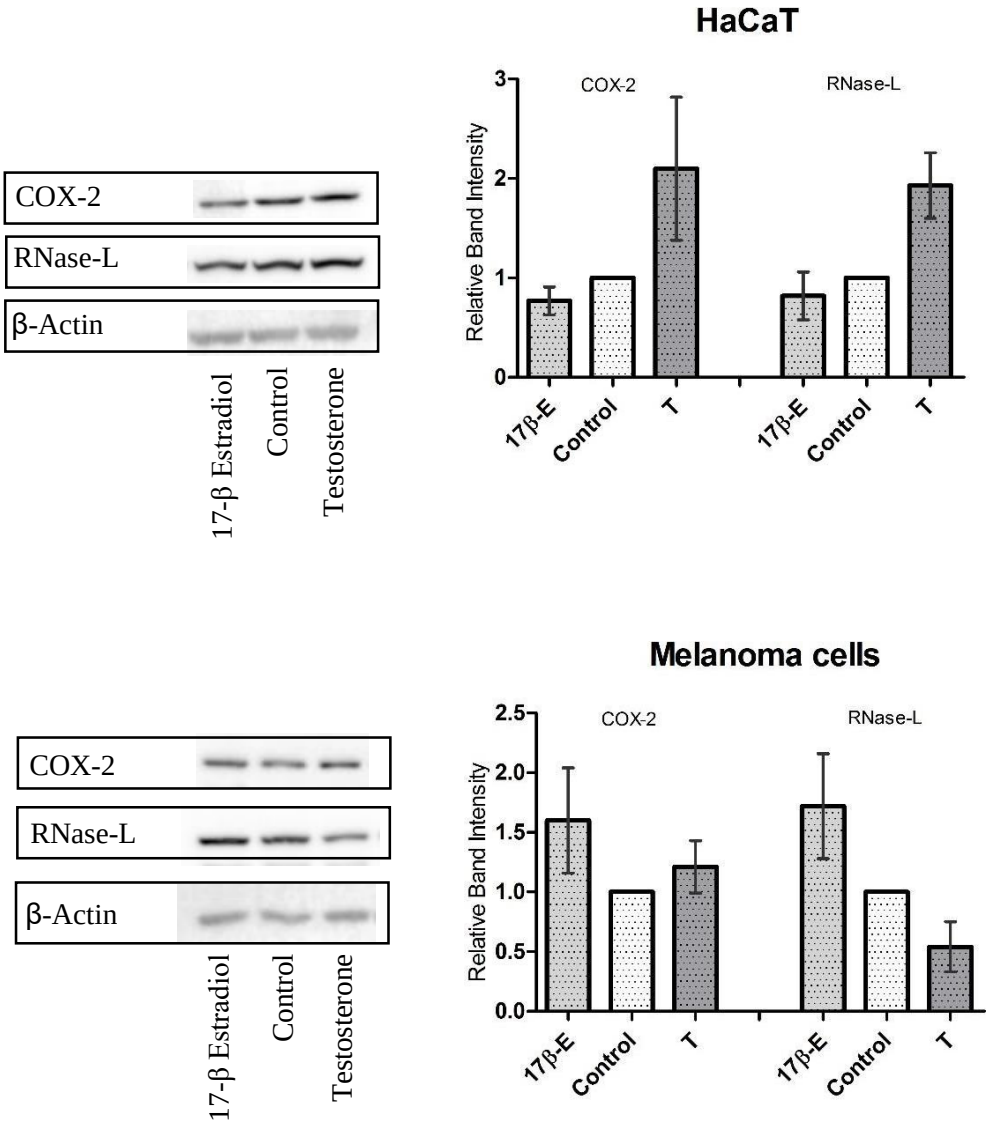
The effect of hormones upon COX-2 and RNase-L proteins expression were evaluated through western blotting analysis. The results are reported in Figure 12.

In HaCaT cells, COX-2 protein expression was increased, although not significant, by testosterone treatment. 17-βestradiol significantly decreases COX-2 protein expression. The expression of RNase-L protein was augmented by testosterone and showed a non-significant decrease by 17β-estradiol treatment.

In melanoma cells, the expression of COX-2 protein resulted up-regulated after 17-βestradiol treatment. Expression of COX-2 remained unchanged with testosterone treatment. An increase of RNase-L protein after the treatment with 17-βestradiol was

observed which correspond to the increased in mRNA expression. Testosterone had an opposite effect, decreasing RNase-L protein expression.

In primary fibroblasts the expression of COX-2 and RNase-L proteins remained unchanged with both treatments.



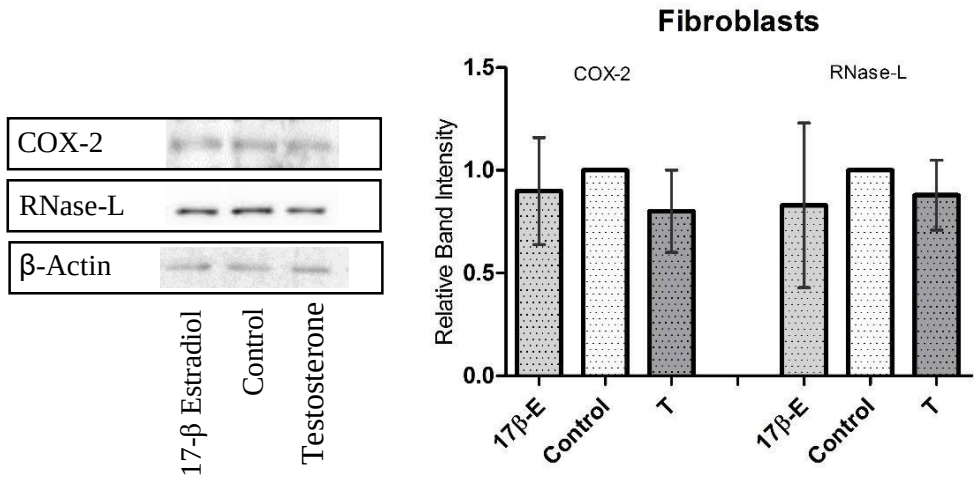


Figure 12: Western blot analysis of COX-2 and RNase-L after 24 hours treatment with 17-beta estradiol (17 β-E) or testosterone (T) in HaCaT, Melanoma and Fibroblast culture cells. The quantification of the amount of COX-2 and RNase-L was calculated relative to the amount of β-actin.

3.6 Effects of miR-146a transfection upon PTGS2 and RNASEL expression.

HaCaT, melanoma and primary fibroblasts cells were transfected with mature synthetic miR-146a at 50 nM concentration for 24 hours. Real Time PCR showed that transfection of mimic-146a showed no significative effect upon the expression of the genes at the used concentration. In figure 13 a/b/c the results are represented.

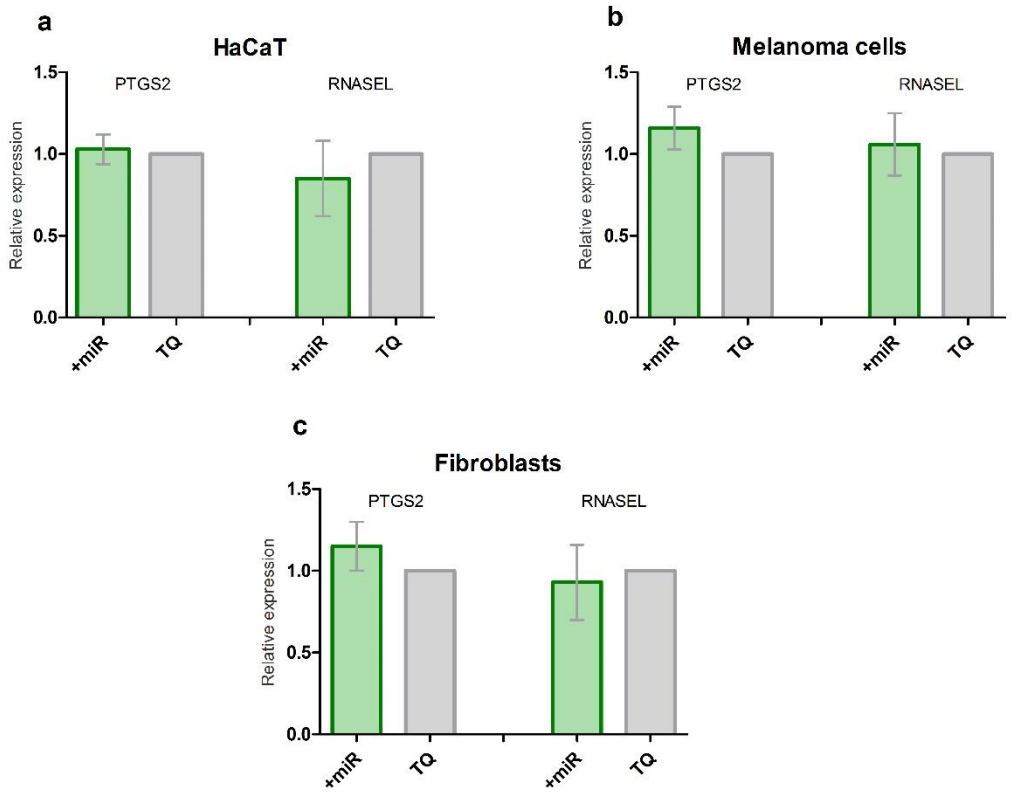


Figure 13: Graphs show the relative gene expression determined by qPCR. Cells were transfected with 50nM miR-146a. After 24 hours cells were harvested and the expression of PTGS2 and RNASEL was assessed in HaCaT, melanoma and fibroblast culture cells. The green bars indicate the gene expression after miR-146a transfection, the grey bar indicate the control.

3.7 Inhibitor concentration effects upon miR-146a expression

The expression of PTGS2, RNASEL and miR-146a was evaluated by real time PCR. Concentrations of 50 nM and 100 nM of inhibitor reduced the expression of miR-146a demonstrating its functionality (Figure 14c). mRNA expression of both genes was not modified by the different inhibitor concentrations indicating that miR-146a is not affecting the genes transcription or stability. (Figure 14 a/b). Further western blot analysis are necessary to determine if miR-146a effects the translation of the coded proteins.

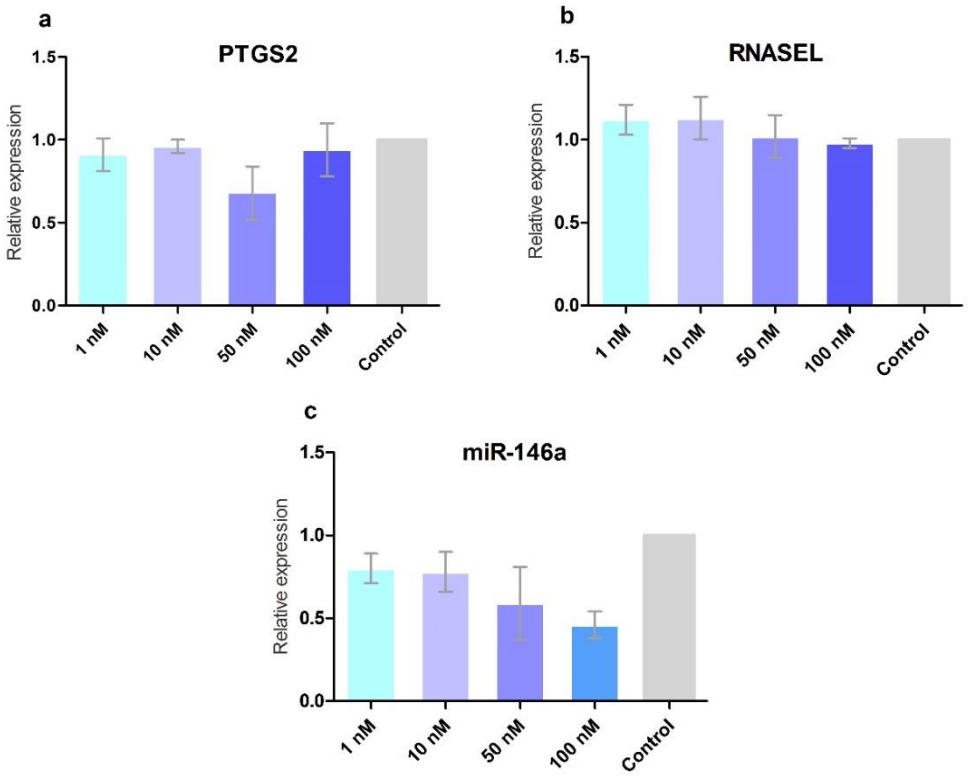


Figure 14 a/b/c: Graphs show the relative gene and miR expression determined by qPCR. Different concentrations of inhibitor of miR-146a (1nM, 10nM, 50nM, 100nM) were transfected in HaCaT cells. After 24 hours, PTGS2 (a), RNASEL (b) and miR-146a (c) expression was determined. Controls were treated only with the transfection reagent.

3.8 Effect of miR-146a on reporter protein activity

To determine whether miR-146a directly regulates PTGS2 through its 3′ UTR, luciferase reporter assays were performed in HaCaT cells. The cells were transfected with empty pLightSwitch plasmid (PLS), or plasmid with part of the 3'UTR encompassing the miRNA binding site (PLS+3'UTR_PTGS2); or with the corresponding mutated plasmid (PLS+3'UTR_PTGS2_MUT). Transfections with PLS+3'UTR_PTGS2 down-regulated the relative luciferase reporter activity. The down-regulation of the luciferase activity was unaltered or even lower when transfecting the mutated PLS+3'UTR_PTGS2_MUT, indicating that in keratinocytes, PTGS2 is not a direct target of miR-146a. (Figure 15)

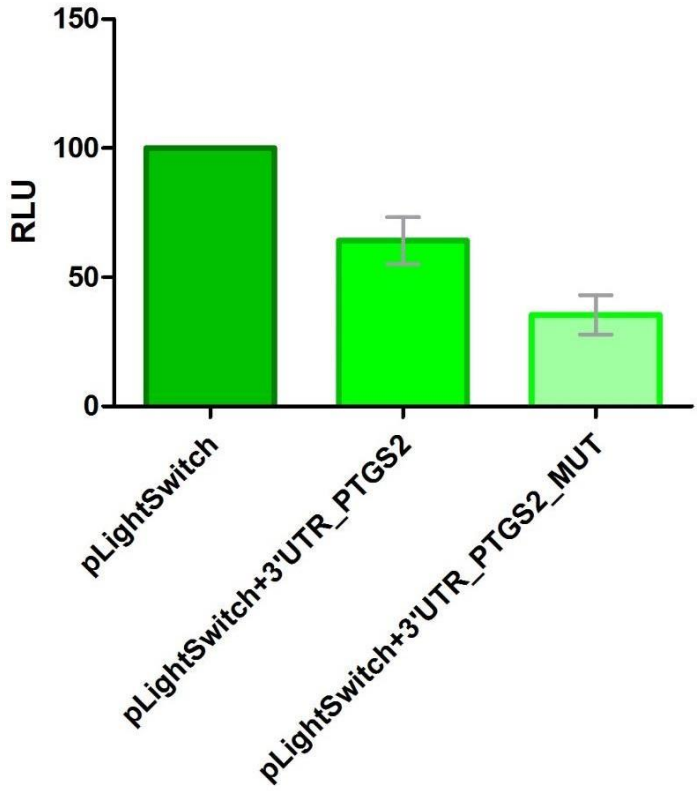
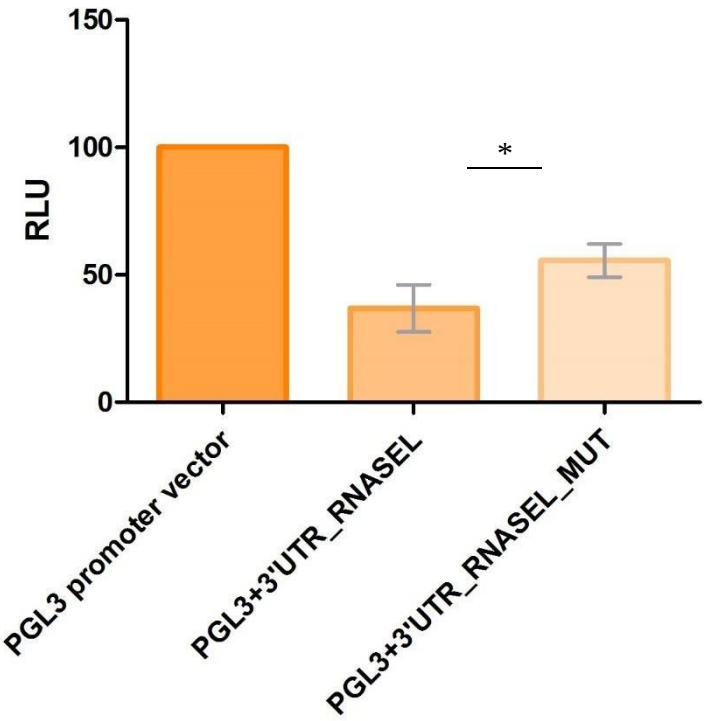


Figure 15: The graph shows a relative activity of luciferase after 24 hours transient transfection in HaCaT cells. The plasmids that were transfected in keratinocyte cells are pLightSwitch (first column), pLightSwitch+3'UTR_PTGS2 (second column) and pLightSwitch+3'UTR_PTGS2_MUT (third column). All plasmids were transfected also with PGL3 promoter vector (as normalized).

3.9 Direct interaction of miR-146a with the RNASEL 3'UTR

To assess the specificity of miR-146a to regulate RNASEL expression through its 3'UTR, luciferase report assays were performed, in figure 16 are reported the results.
HaCaT cells were transfected with the recombinant plasmid containing part of the 3'UTR encompassing the miRNA binding site (PGL3_PV+3'UTR_RNASEL) or with the corresponding mutated plasmid (PGL3_PV+3'UTR_RNASEL_MUT). A Significant down-regulation in relative luciferase reporter activity was observed in response to PGL3_PV+3'UTR_RNASEL construct. The mutated corresponding plasmid restored in part the activity of the luciferase.



*P value = 0,04

Figure 16: The graph shows a relative activity of luciferase after 24 hours transient transfection in HaCaT cells. The plasmids that were transfected in keratinocyte cells are PGL3 promoter vector (first column), PGL3+3'UTR_RNASEL (second column) and PGL3+3'UTR_RNASEL_MUT (third column). All plasmids were transfected also with pLightSwitch (as normalized).

4 DISCUSSION

Growing evidences are showing gender-associated functions playing a role in cancer incidence, progression and response to therapy.[169] Different studies showed the female advantage in several different cancer types.[169] Representative examples of gender disparities have been associated with colorectal cancer, urothelial and kidney cancer[170] as well as melanoma.[171]

Although female sex is associated with a survival advantage in several cancer types, studies in both Europe and United States showed that this advantage was considerably higher in melanoma than in virtually any other type of cancer. Since the late 1960s, Clark observed that cutaneous melanoma was more aggressive in men.[172] More recently, according to EUROCARE4 data, melanoma was reported to display the most significant female advantage in survival of patients.[173]

Nonetheless the protective factors are still unknown. Although the source of gender disparity in melanoma remains unclear, two main hypotheses for female advantage can be offered: 1) differences in behavior and 2) unknown biologic sex differences.[174] Looking for the underlying molecular mechanisms, tumor itself does not appear to be different across gender as confirmed by several studies showing no differences in mutation rates of some important genes in melanoma, most notably in the BRAF gene. However, several host factors, which differ across gender have been linked to melanoma progression and survival and might therefore offer an explanation for the female melanoma advantage. These include hormone levels (estrogens and androgens,), the immune system, autophagy, matrix metalloproteinase-2, skin physiology, vitamin D, obesity and reactive oxygen species. In fact, all these molecules and biological processes have been hypothesized to display sex disparities.[169] The genetics of melanoma is intricate, in addition to the several well documented gene mutations that have been associated with development of melanoma[175], considerable attention is being focused on the participation of epigenetic events. The interplay between epigenetic events affects the regulation of transcriptional and/or translational activities. The epigenetic events involved in initiation and progression of melanoma may be aberrant methylation of the promoter regions, histone modification, chromatin remodeling, and the positioning of nucleosomes.[176] As epigenetics factors, microRNAs (miRs) are considered as important elements regulating gene expression involved in initiation, promotion and progress of melanoma.[177] Gender difference in miRs expression have been described in peripheral blood, brain and colon rectal mucosa[178], in

lung adenocarcinoma, and other cancers.[179]

The interaction of different cells and micro environmental factors are involved in the development of melanoma; cell growth, differentiation, apoptosis and migration are mediated by cell to cell crosstalk. For example, a switch of the developing tumor from the inhibitory interaction with keratinocytes to interact with host fibroblasts results in uncontrolled proliferation and invasion of the malignant cells.[180/181/182/183]

Keratinocytes are important players in melanoma development since they control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact.[182]Among the different functions, these cells protect from the skin by UV rays which are the major risk factor for the development of melanoma. [184] Exposure of the skin to UV produces inflammation characterized by erythema[185] and edema and may promote tumor formation. As well, UVB irradiation has been shown to induce the formation of free radicals and deplete cellular antioxidant stores.[186/187] Data presented by Miller et al.[188] indicate that UVB induces tyrosine kinase activation by oxidative injury, which results in increased prostaglandin synthesis by cyclooxygenase-2 (COX- 2). COX-2 plays a major role in modulating the inflammatory properties observed in UVB-irradiated skin and in UVB-induced experimental tumors.[189/190] COX-2 is strongly expressed in malignant melanoma and may be correlated with the development and progression of disease.[191/192]

Sex differences in prostaglandins production (PGs) have been described in many inflammation-related diseases[193/194/195], such as response to peritonitis, pleurisy, mumps virus infections, autoimmune and chronic diseases. For instance, in cystic fibrosis, severe asthma, or chronic pulmonary obstructive disease females showed a poorer prognosis compared to males.[193]

In the present study, we observed different sex hormone effects upon PTGS2/COX-2 expression depending on the cells analyzed. An augmented expression of COX-2 and its coding gene PTGS2 was observed in keratinocytes upon testosterone treatment, while 17β-estradiol upregulates COX-2 in melanoma cells. Considering the potential role of inflammation in tumor initiation and promotion, our results suggest that PTGS2/COX-2 could be involved in gender bias in the incidence of melanoma through its higher expression and transduction in males. In melanoma cells, the upregulation of COX-2 by 17β-estradiol may play an anti-inflammatory role by generating other set of anti-inflammatory PGs in females, and thus, participating in the better rate of survival.[196/197/198]

The down-regulation of PTGS2 in fibroblasts upon testosterone was not significantly observed in COX-2 protein expression suggesting an effect of testosterone on PTGS2

mRNA stability.[199]

Ribonuclease-L (RNase-L) is known to be a ubiquitous enzyme involved in several cellular functions, especially in innate immunity. It is an established mediator of IFN antiviral and antiproliferative activities that functions as the terminal component of an RNA decay pathway. The cell growth inhibitory and proapoptotic effects of RNase led to the assumption that RNase-L could be a tumor suppressor gene[196/200], by inhibiting oncogenic viruses, but also through its pro-apoptotic and cell growth inhibitory properties. As such, it is possible to speculate that higher expression of RNASEL can have a protective effect upon tumorigenesis.

In the present study, we observed an augmented expression of RNase-L and its coding gene RNASEL in melanoma cells after treatment with 17β-estradiol and a diminished expression of RNase-L protein under testosterone. The cell growth inhibitory and pro-apoptotic effects of RNase-L suggest that higher expression of RNASEL can have a protective effect upon tumor progression and could participate in the survival advantage in females. The results obtained in keratinocytes showed an increase of RNase-L expression as a result of testosterone treatment. This augmented expression of RNASEL could represent an attempt to attenuate androgen signaling which has been demonstrated to promote cancer progression by inhibiting apoptosis or by promoting cell survival or proliferation.[201]

MicroRNAs (miRNAs) can contribute to human cancer predisposition by regulating the genetic expression of proto-oncogenes or tumor suppressor genes. High levels of miR-146a has been reported in melanoma tissue and cell lines[202/203], and its role as oncogene has been discuss in several studies.[204/205] Consistent with these observations, our data showed a very high expression of miR-146a in melanoma cells compared with its expression in HaCaT and fibroblast cells. Testosterone induced an even greater increase expression of miR-146a in melanoma cells. This effect can represent a disadvantage in males since augmented expression of miR-146a can interfere, among others, with the STAT1/ IFN-γ axis which reduced cell-migration, cell cycle activity, and basal oxygen consumption rate in melanoma cells[206/203] and by activating Notch signaling that promoted primary melanoma progression.[207]

On the other hand, 17β-estradiol increases the expression of miR-146a in keratinocytes, suggesting that it could represent a protective factor for females by controlling inflammatory genes in these cells. The dual role of miR-14a have been reported in different diseases. For example, in keratinocytes, miR-146a may contribute to reduce

excess inflammatory responses after exposure to microbial agents[208] and it can direct target members of the NF-kB signaling helping to fine-regulate the immune response.[209] In prostate cancer, miR146a has demonstrated to be a tumor suppressor by targeting regulators of many cellular processes, including the cell cycle, cell-cell adhesion, motility, and of epithelial differentiation.[210]

In summary, the hormonal environment influences miR-146a expression, gene and protein expression of PTGS2 and RNASEL differently depending on the skin cell type. The up-regulation of PTGS2/COX-2 in keratinocytes upon testosterone treatment indicates the possible participation of this gene/protein as a risk factor for males regarding the initiation and promotion of melanoma. On the other hand, the up- regulation of RNASEL/RNase-L in melanoma cells by 17β-estradiol suggest that RNASEL may play a role in the better rate of survival in females. The upregulation of miR-146a expression may represent a risk factor for males in melanoma cells participating in tumor progression whereas it can have a protective role in females by inhibiting inflammatory processes in keratinocytes. In conclusion PTGS2, RNASEL and miR-146a sex hormonal responses, suggest that both genes and miR-146a could participate in the gender bias in melanoma.

The relation between PTGS2, RNASEL and miR-146a

An interaction of RNASEL with miR-146a have been associated to melanoma[211] and to non-melanoma skin cancer risk.[212] In a similar way, a polymorphism in the promoter region of PTGS2 together with a SNP in miR-146a showed an association to melanoma risk (manuscript in preparation). Both genes present binding sites for miR-146a. Based on this information, we investigated the eventuality miR-146a regulation upon PTGS2 or RNASEL 3'UTR genes expression in keratinocyte cells.

The regulation of PTGS2 is complex and many elements have been recognized that modulate mRNA translation, mRNA stability, and translational regulation.[213] Among them, binding site for different miRs are located in the 3'UTR that could regulate translation and stability of the gene.[214]

The direct regulation of PTGS2 by miR-146a has been validated in lung cancer[93] and in human gastric epithelial cells.[215] Nevertheless, if a particular miRNA-target is validated in a specific tissue type, the same interaction may not be true in a different tissue. Indeed, through transfection in keratinocyte cells of a recombinant vector containing part of the 3'UTR of PTGS2, we were not able to demonstrate that PTGS2 is a direct target of miR-

146a as the mutagenized vector showed an even less luciferase activity than the one containing the binding site for the miR.

The expression of RNASEL must be tightly regulated since it mediates critical cellular functions including antiviral, pro-apoptotic, and tumor suppressive activities.[113] The regulatory function of the 3'UTR of the gene has been tested in different cell lines; it contains multiple candidate AREs, and sequential deletion of these elements identified positive and negative regulatory regions.[216] Regulation of RNASEL has been mainly investigated in prostate carcinogenesis (Pca), where several differentially expressed miRNAs between PCa and adjacent benign tissues obtained from PCa patients have been identified.[217]

There is no scientific data demonstrating the interaction of miR-146a with RNASEL. Only one study has shown that RNASEL is a direct target of the miR-146b (miR-146b differs from miR-146a by having two different nucleotides at its 3'-end) in rat cardiomyoblast cell line.[218]

From our expression and transfection data, it can be state, that the putative miR-146a-binding site is a functional site as part of the regulation of RNASEL, although probably its participation in the post-transcriptional regulation of the gene does not play a main role. In fact, in vitro analysis in all three skin cells did not show an effect of miR-146a upon the RNASEL gene or protein expression. Nevertheless, deletion of the miRNA- binding site resulted in a partial restore of luciferase activity demonstrating that RNASEL is a direct target of miR-146a in keratinocytes.

In conclusion in HaCaT cells, miR-146a targets RNASEL with a probably minor effect on its regulation. This direct interaction was not observed in PTGS2 gene.

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