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MOLECULAR MECHANISMS INVOLVED IN THE REGULATION OF CERTAIN miRNAs IN BREAST CANCER CELLS AND CANCER-ASSOCIATED FIBROBLASTS

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Abstract

MicroRNAs (miRNAs) are non-coding small RNA molecules that regulate gene expression by inhibiting the translation of target mRNAs. It has been well demonstrated that miRNAs play a regulatory role in different pathophysiological conditions, including breast cancer. In this regard, a distinct miRNA may be up regulated in certain malignancies and downregulated in others, suggesting both an oncogenic and tumor suppressor role, respectively. It has also reported that in breast cancer estrogens may regulate miRNA expression through the involvement of the classic estrogen receptor (ER) alpha and the G protein coupled receptor, GPER.

By using RT-PCR and western blotting assays, as well as by gene silencing approaches, in the present work we provide novel insights on the involvement of miRNAs in the breast tumor progression. In particular, we ascertained that miR-221 expression is up-regulated in breast cancer cells SkBr3 and MDA-MB 231 and in cancer-associated fibroblasts (CAFs). In addition, we found that the proliferative and migratory effects induced by miR-221 in these cell types are mediated by interfering the A20/c-Rel/CTGF pathway. These stimulatory effects are abolished silencing the expression of the NF-κB component, c-Rel and the Connective Tissue Growth Factor (CTGF), as well as using the specific Locked Nucleic Acid (LNA)-Inhibitor of miR-221 (LNA-i-miR-221).

In a second work, we evaluated the expression of 754 miRNAs by TaqMan Array in ER-negative and GPER-positive SkBr3 breast cancer cells and in CAFs upon 17-β-estradiol (E2) treatment. Among the miRNAs down-regulated by 100nM E2 for 4 hours in these cell types, we identified miR-338-3p that is positively correlated with overall survival in breast cancer patients as ascertained by METABRIC database analysis. According to these data, a miR-338-3p mimic sequence decreased and a miR-338-3p inhibitor sequence rescued the proliferative effects and cell progression induced by 100nM E2 and G-1, selective ligand of GPER in SkBr3 breast cancer cells and CAFs.

Overall, these results provide new insights on the miRNA action in breast cancer, suggesting their use as an innovative therapeutic approach in this malignancy.

CHAPTER 1

Introduction

1.1 Introduction

MicroRNAs are small non-coding RNA sequences of about 20 nucleotides, responsible for the regulation of various biological processes, such as apoptosis, cell growth, neuronal differentiation and response to environmental stress factors [1-5].

Among human diseases, it has been shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes, respectively, depending on the cell context [6].

One of the first solid tumors described for miRNAs expression is breast cancer, the most common female malignancy in western countries [7].

Several are the over-expressed miRNAs in breast cancer: in particular it was discovered that miR-21 is able to mediate cell survival and invasion [6, 8], as well miR-144 is able to induce stimulatory effects in breast cancer cells [9], while miR-103 and miR-107 showed a poor issue in patients with TNBC (Triple Negative Breast Cancer) [10].

Particular attention was paid to the study of miR-221 / miR-222 in different tumor types [11]. For instance, miR-222 is involved in the regulation of EMT, in the progression and the drug-resistance of breast cancer [12-15], while miR-221 causes stimulatory effects in different types of malignancies by downregulating some onco-suppressor genes [16,17].

Moreover, mechanistic studies showed that miR-221 directly targets A20 and reduces its expression levels. The ubiquitin-editing A20 enzyme is responsible for maintaining tissue homeostasis and the prevention of inflammatory disorders [18, 19].

It has been demonstrated that A20 may inhibit NF-κB, a transcription factor widely involved in the development of different types of tumors, reducing the nuclear levels of c-Rel, a member of the nuclear factor kB (NF-κB) transcription factor family [20-23].

In addition to this, it has been reported that NF-κB regulates the expression of the connective tissue growth factor (CTGF), a protein implicated in several cellular processes which has been reported to be involved in various malignancies [24, 25].

On the basis of these data, we tried to provide new indications on the molecular mechanisms by which miR-221 is able to cause oncogenic effects in breast cancer, performing experiments in SkBr3 breast cancer cells, in triple-negative breast cancer cells, MDA-MB-231 and in the tumor microenvironment, using cancer-associated fibroblasts, CAFs [26].

The action performed by miR-221 is able to down-regulate A20 expression and to increase c-Rel and CTGF levels, inducing cell growth and migration. These biological effects were abolished following the use of the specific inhibitor of locked nucleic acid (LNA) of miR-221 (LNA-i-miR-221), a 13-mer oligonucleotide specifically designed to sequester miR-221 [27, 28], able to determine the silencing of the expression of c-Rel and CTGF.

The aforementioned results therefore highlighted the oncogenic action of miR-221, thus indicating its potential therapeutic use or for preventive purposes in breast cancer.

Subsequent studies have been undertaken with the aim of providing further information on the role of miRNAs in breast tumor progression, analyzing the role of estrogens in the regulation of their expression.

In fact, the involvement of the classical ER estrogenic receptor in the regulation of miRNAs by estrogens has been demonstrated. Equally, also the G protein estrogen receptor (GPER) is able to regulate the expression of some miRNAs in normal and cancer cell contexts characterized by the presence or absence of ERs [29-32, 9].

To this end we have identified by microarray that the treatment with estradiol of breast cancer cells SkBr3 and in associated fibroblasts (CAFs), is able to reduce the expression of miR-338-3p, which has been reported to have an oncosuppressive action in different cancers [33-35].

1.2 Breast cancer

Breast cancer is the commonest cause of cancer death among women worldwide. Incidence rates are high in more developed countries, whereas rates in less developed countries are low but increasing [36]. The high possibility of early diagnosis of the neoplasm, adherence to screening programs and taking care of the patient throughout her diagnostic-therapeutic course account for the excellent prognosis of this neoplasm. Also the metastatic forms, once considered to have a poor prognosis, are today treatable with innovative and increasingly targeted clinical treatments that have improved their prognosis [37].

Breast cancer is an extremely heterogeneous neoplasm originating from the epithelial component of the mammary gland, with etiopathogenesis, pathophysiology and different clinical consequences in various types.

The breast is an equal gland, located at the thoracic level and intended for breastfeeding.

It consists of a glandular component and fibroadipose support tissue. The gland has 15-20 lobes which represent its functional unit. Each lobe consists of a lobule, a glandular portion used to produce milk and a galactophore duct that leads milk to the nipple (Figure 1.2.1).

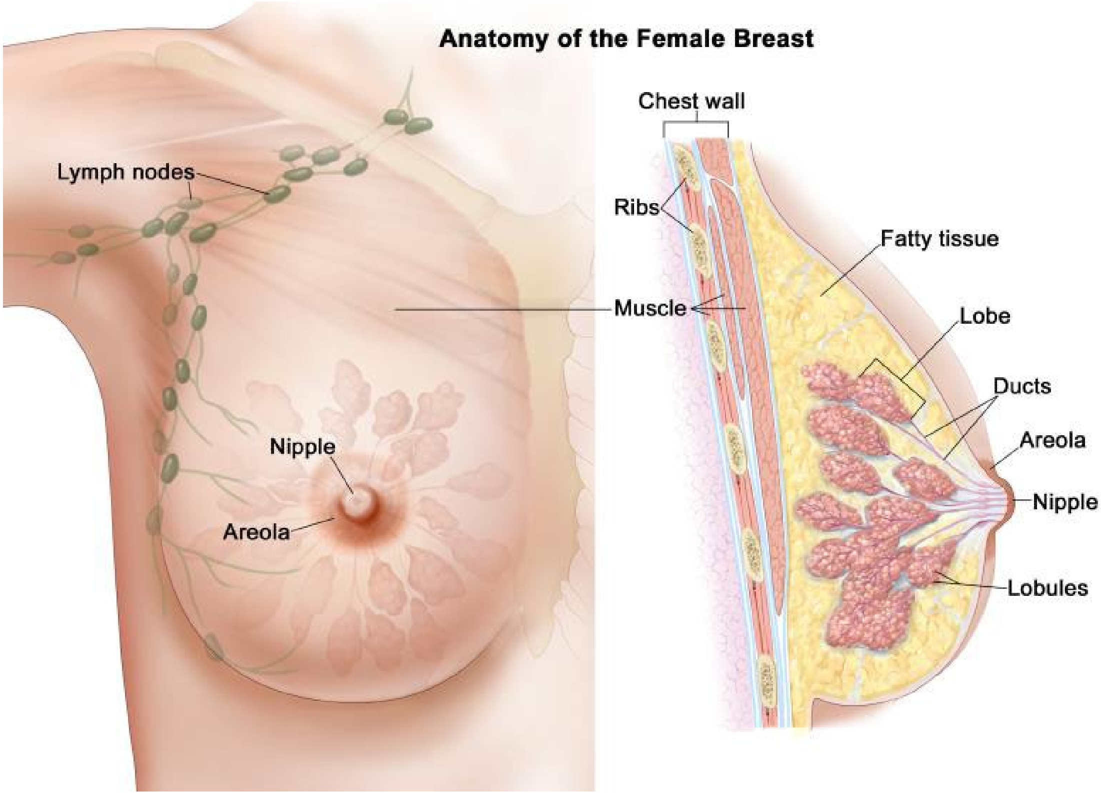


Figure 1.2.1 | Anatomy of the mammary gland *(PubMed Health PDQ Cancer Information Summaries).*

Depending on whether the progenitor cell is ductal, it is possible to distinguish two types of carcinomas:

- ***Non-infiltrating carcinoma (or in situ):*** characterized by a proliferation of malignant epithelial cells that do not pass the basement membrane and therefore do not reach the lymph nodes. This event can occur at the *lobule* or *duct* of the gland, lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS), respectively;
- ***Infiltrative carcinoma:*** characterized by exceeding the basement membrane and therefore by the presence of stromal invasion. The infiltrating carcinoma is therefore invasive and spreads to the lymphatic level. Also in this case we can distinguish infiltrating ductal carcinoma and infiltrating lobular carcinoma, depending on whether the primary pathology is borne by the duct or lobule (Figure 1.2.2).

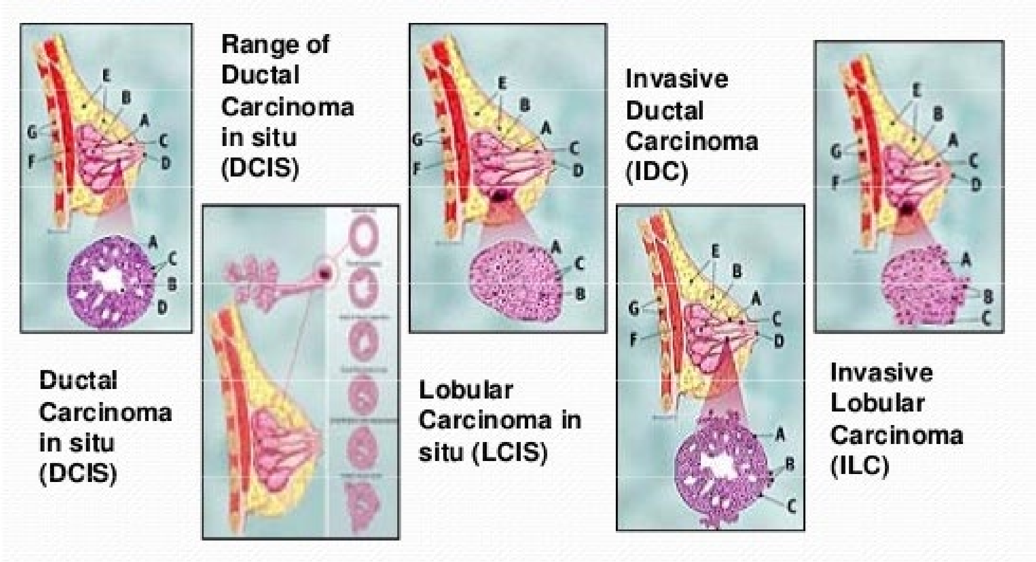


Figure 1.2.2 | Representation of the anatomy of the Lobular Carcinoma and Mammary Ductal Carcinoma.

The reference staging system for breast cancer is the Tumor, Node, Metastasis (TNM), reviewed by the American Joint Committee on Cancer (AJCC), which includes an anatomical classification: the extension of the primitivov tumor [T], the absence or presence and extension of metastases to regional lymph nodes [N], absence or presence of distant metastases [M]) and a prognostic classification (Prognostic Stage Group) which also includes tumor grade, hormone receptor status and the status of HER2 [38].

Based on the above, we can classify carcinomas in 5 stages:

- Stage 0 and DCIS. Lumpectomy plus radiation or mastectomy is the standard treatment. There is some controversy on how best to treat DCIS.
- Stage I: the tumor mass is small (less than 2 cm), is removable and confined, without involvement of the lymph nodes.
- Stage II: the tumor is smaller than 5 cm, but is extended to the axillary lymph nodes. Lumpectomy plus radiation or mastectomy with some sort of lymphnode removal is the standard treatment. Hormone therapy, chemotherapy, and biologic therapy may also be recommended following surgery.
- Stage III: the neoplastic mass completely involves the axillary lymph nodes and also begins to affect the chest muscles. Treatment involves surgery, possibly followed by chemotherapy, hormone therapy, and biologic therapy.
- Stage IV: metastases are present. Treatment may involve surgery, radiation, chemotherapy, hormonal therapy or a combination of these treatments.

The immunophenotypic groups of clinical relevance and with important therapeutic implications, also for adjuvant therapy, are:

- **Luminal A:** hormone-receptor positive, HER2 negative with low proliferative activity (frequently including special histotypes such as tubular carcinoma, classic lobular carcinoma). Luminal A breast tumors are tumors positive to estrogen receptors, positive to progestin receptors with positive values higher than 20%, HER2 negative and with low Ki67.
- **Luminal B/HER2 negative:** hormone-receptor positive, HER2 negative and high proliferative activity;
- **Luminal B/HER2 positive:** hormone-receptor positive, over-expressed or amplified HER2, any value of proliferative activity.
- **HER2 positive (non-luminal):** overexpressed or amplified HER2 and hormone receptors both negative.
- **Triple-negative:** no hormone receptor expression and HER2 negative. The correspondence between the “triple negative” phenotype identified on an immunohistochemical basis and the intrinsic “basal like” subgroup identified on a gene basis only exist in about 80% of cases, further demonstrating the extreme heterogeneity within these subgroups [39, 40].

The risk factors that can induce the development of breast cancer are different, among these there are:

- *Age and gender:* The risk of getting breast cancer increases with increasing age. This correlation could be linked to the continuous and progressive endocrine proliferative stimulus that the mammary epithelium undergoes over the years, together with the progressive damage to DNA and the accumulation of epigenetic alterations with loss of balance of expression between oncogenes and suppressor genes [41].
  The risk of breast cancer is 100 times greater in women than in men [42].
- *Familiarity and inheritance:* The risk of developing breast cancer increases in women with a positive family history for this cancer. An analysis including 52 epidemiological studies with 58.209 women diagnosed with breast cancer and 101,986 healthy women showed an increase in the relative risk of 1.80, 2.93 for women with one, two, three or more affected first-degree relatives and 3.90 respectively [43].
  Although most breast cancers are sporadic forms, 5-7% are related to hereditary factors, 1/4 of which are determined by the mutation of two genes: BRCA-1 and BRCA-2. In

women carrying mutations of the BRCA-1 gene the risk of developing breast cancer during life is 65% and in women with BRCA-2 mutations equal to 40% [44].

Other hereditary factors are represented by:

- mutations of the ATM gene (Ataxia Telangiectasia Mutated) [45] or of the CHEK2 gene [46, 47];
- mutation of the PALB2 gene [48];
- Li-Fraumeni syndrome (p53 mutation);
- Cowden syndrome (PTEN gene mutation);
- ataxia-telangiectasia, Peutz-Jeghers syndrome.

- *Reproductive factors:* A long duration of the fertile period, with an early menarche (<12 years) and a late menopause (> 55 years) represents a factor of increased risk due to a longer exposure of the glandular epithelium to proliferative stimuli of ovarian estrogens. Women with children have a 10-30% lower risk compared to nulliparas and the risk changes according to the number of children and the age at the first full-term pregnancy: women with a first full-term pregnancy over 29 have a 40% higher risk than women with a child before the age of 25 [49]. Breastfeeding seems to play a protective role with a 10-20% less risk for women who breastfeed a total of at least three years compared to women who do not breastfeed [50].
- *Exogenous hormonal factors:* Recent studies have confirmed an increased risk of breast cancer during and immediately after taking modern oral contraceptives, the greater the longer the duration of the intake. An increased risk has also been demonstrated in women taking HRT during menopause, especially if based on synthetic androgenic activity [51].
- *Dietary and metabolic factors:* The high consumption of alcohol and animal fats and the low consumption of vegetable fibers would seem to be associated with an increased risk of breast cancer. Obesity is a recognized risk factor in post-menopause, probably related to the excess of adipose tissue that in this phase represents the main source of synthesis of circulating estrogens, with consequent excessive hormonal stimulation on the mammary gland. Metabolic syndrome increases the risk of cardiovascular disease but also of breast cancer: it is assumed that in subjects with metabolic syndrome there is a resistance to insulin to which the body reacts by increasing insulin levels. Insulin acts on the membrane receptor of insulin-like growth factor 1 (IGF-1R), activating the intracellular signal pathways essential for neoplastic growth. By acting on these risk factors, which can be modified through regular daily physical activity combined with a

balanced diet (for example, the Mediterranean diet), the risk of developing breast cancer could be reduced by improving the metabolic and hormonal structure of the woman [52].

- *Environmental exposure:* A link between exposure to high doses of ionizing radiation and the increased risk of breast cancer has been shown, while there appears to be no correlation with exposure to low doses such as those arising from instrumental investigations.

1.2.1 Estrogens and the classical estrogen receptor (ER)

Estrogen sex steroid hormones exhibit a broad spectrum of physiological functions ranging from regulation of the menstrual cycle and reproduction to modulation of bone density, brain function and cholesterol mobilization [53-55].

Despite the normal and beneficial physiological actions of endogenous estrogen in women, abnormally high estrogen levels are associated with the increased incidence of certain types of cancer, especially those of the breast and endometrium.

The predominant intracellular estrogen is 17β-estradiol (E2). Other types of estrogen include estrone (E1) and estriol (E3) (*Figure 1.2.3*)

Figure 1.2.3 | Biosynthesis of Estrogens *(Prossnitz E.R. and Barton M., 2011).*

In premenopausal women, E1 and E2 are secreted primarily by the ovaries during the menstrual cycle, with minor levels derived from adipose tissue and the adrenal glands. The placenta also produces E3 during pregnancy. In the ovaries, granulosa cells synthesize estrogen from androgen [56]. Ovarian production of estrogen is regulated by the hypothalamic-pituitary-ovarian (HPO) axis and begins by anterior pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in response to the hypothalamic peptide gonadotropin-releasing hormone (GnRH). Acting in concert, LH stimulates androgen production, whereas

FSH upregulates aromatase, which catalyzes the rate-limiting and final step of estrogen biosynthesis: the aromatization of androgen to estrogen. During ovulation, E2 production rises dramatically by eight- to tenfold. High levels of estrogen in turn act via negative feedback to dampen estrogen production to inhibit the release of GnRH, LH, and FSH [57].

The primary mediator of estrogen biosynthesis in postmenopausal women is aromatase, which is found in adipose tissue as well as in the ovaries, placenta, bone, skin, and brain [58, 59]. After menopause, ovarian estrogen biosynthesis is minimal, and circulating estrogen is derived principally from peripheral aromatization of adrenal androgen. As such, for obese postmenopausal women, adipose tissue becomes the main source of estrogen biosynthesis; this biosynthetic route is far less significant for nonobese postmenopausal women [60].

Numerous studies have demonstrated the association of estrogen with the development and/or progression of various types of cancer, including breast, endometrium, ovary, prostate, lung, and colon cancers [55, 61].

Estrogen mediates its biological effects in target tissues primarily by binding to specific intracellular receptors, estrogen receptor [62]. ERα and ERß are encoded by ESR1 and ESR2, respectively; each gene is located on a different chromosome. Like all the members of the nuclear receptor super-family, ERα and ERβ are modular proteins sharing common regions, named A/B, C, D, and E/F, as well as a high sequence homology (*Figure 1.2.4*).

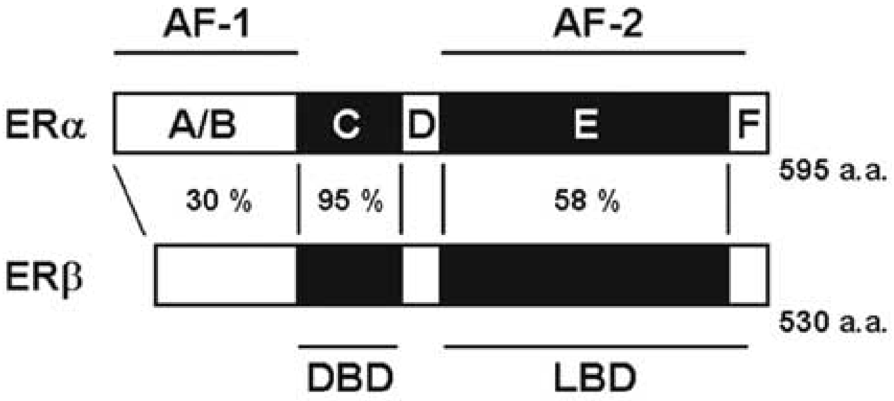


Figure 1.2.4 | Domain organization of human ERαand ERβ. ERs consist of the N- terminal region involved in transactivation (A/B domains, AF-1), the DNA binding domain (DBD, C domain), the hinge region involved in dimerization (D domain), the C-terminal region containing ligand binding domain (LBD, E/F domain, AF-2) and transactivation function-2 (AF-2). *(Marino et al., 2006).*

The ERs are modular proteins consist of four functional domains:

- The N-terminal domain (A/B region) is involved in both inter-molecular and intra-molecular interactions as well as in the activation of gene transcription.

- The DNA binding domain (DBD, C region) allows ER to dimerize and to bind to the specific ERE sequence on DNA through its two "zinc finger" structures.
- The hinge domain (D region) has a role in receptor dimerization and in binding to chaperone heat-shock proteins (Hsp).
- The ligand binding domain (LBD, E/F region, C-terminal) comprises the E2-binding domain and works, synergistically with the N-terminal domain in the regulation of gene transcription [63-66].

There are two distinct types of signaling can be mediated, often referred to as genomic and non-genomic pathways: in the classical genomic mechanism, ligand-activated ER dimerizes and translocates in the nucleus where it recognizes specific hormone response elements located in or near promoter DNA regions of target genes while in a second indirect mechanism, E2 also modulates gene expression through that involves the interaction of ER with other transcription factors that, in turn, bind their elements of cognitive DNA. In this case, ER modulates the activities of transcription factors such as the activating protein (AP) -1, the nuclear factor - κB (NF-κB) and the stimulation of protein-1 (Sp-1), stabilizing the protein complexes DNA and / or recruiting co-activators [67].

In addition to hormone-mediated activation, it is now well accepted that ER function can be modulated by extracellular signals in the absence of E2 (Figure 1.2.5). These findings focus primarily on the ability of polypeptide growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) as well as the intracellular effector analog 8-bromo-cyclic adenosine monophosphate to activate ER and increase the expression of ER target genes [68]. Moreover, E2 exerts its non-genomic actions, which are too rapid to be accounted for by the activation of RNA and protein synthesis, through the activation of four main signaling cascade: phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/AKT, and cAMP/ protein kinase A (PKA).

The E2-induced rapid signals indicate its localization at the plasma membrane. Some authors have suggested that the non genomic actions of estrogen are mediated through a subpopulation of ERα and ERβ located to the plasma membrane. However, in the last few years, a member of the 7-transmembrane G protein-coupled receptor family, GPR30/GPER, has been implicated in mediating both rapid and transcriptional events in response to estrogen under certain circumstances.

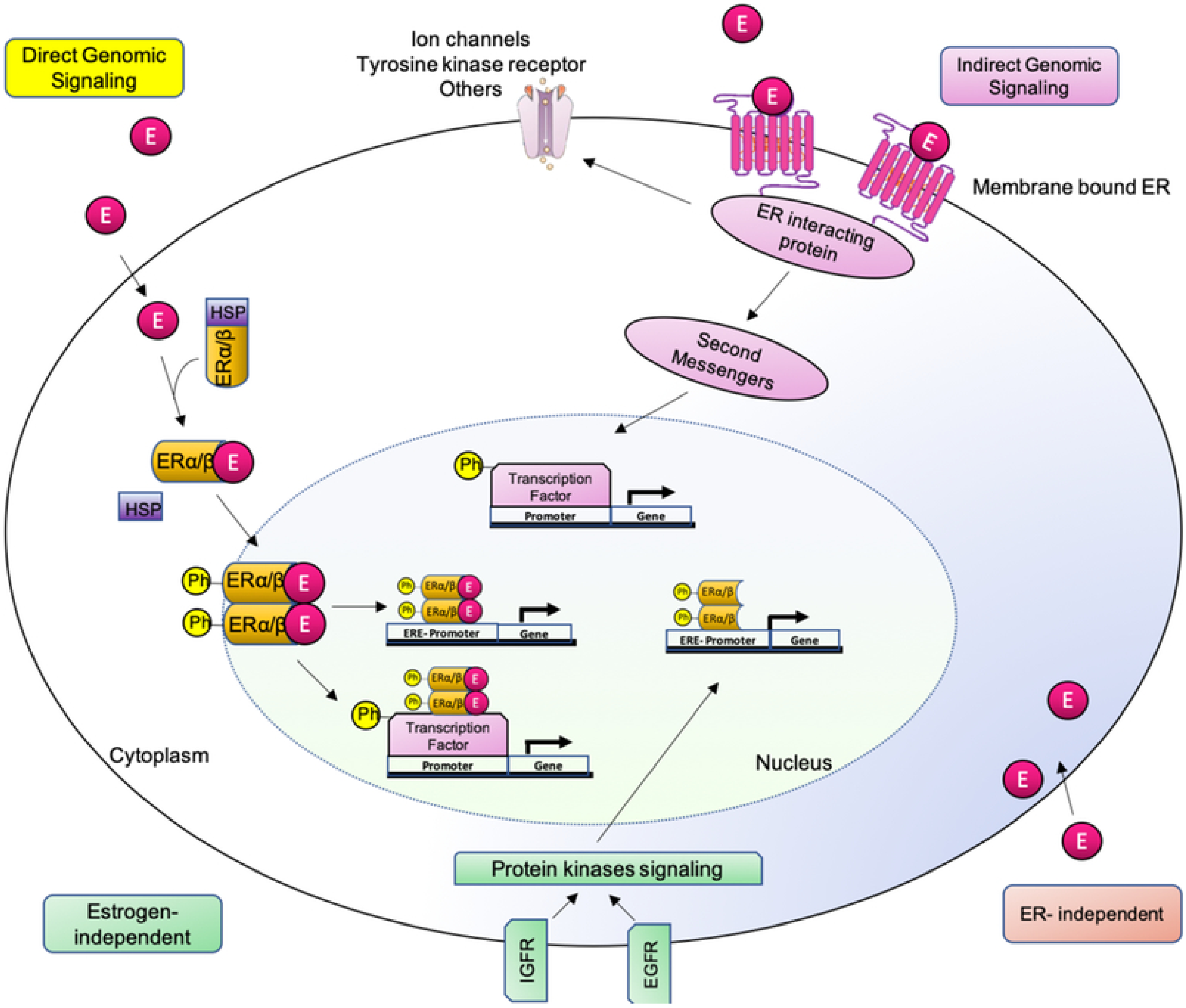


Figure 1.2.5 | Genomic and non-genomic estrogen signaling pathways.
There are different estrogen-mediated signaling mechanisms. 1) Direct genomic signaling: estrogen binds to ERs. The complex dimerizes and translocate to the nucleus inducing transcriptional changes in estrogen-responsive genes with or without EREs. 2) Indirect genomic signaling: the membrane bound receptor induces cytoplasmic events such as modulation of membrane-based ion channels, second-messenger cascades and transcription factors. 3) ER-independent: estrogen exerts antioxidant effects in an ER-independent manner. 4) Estrogen independent: ligand-independent genomic events. *(Fuentes N and Silveyra P, 2019).*

1.2.2 The G protein-coupled estrogen receptor (GPER)

Estrogens are sexual steroid hormones capable of performing various physiological functions such as regulation of the menstrual cycle and reproduction, bone density, brain function, cholesterol mobilization, development of breast tissue and sexual organs and control of the inflammation [69].

Traditionally, the actions of 17β-estradiol are ascribed to two nuclear estrogen receptors (ERs), ERα and ERβ, which function as ligand-activated transcription factors. However, 17β-estradiol also mediates rapid signaling events via pathways that involve transmembrane ERs, such as G-protein-coupled ER (GPER) known as GPER [70].

GPER was first identified as an orphan member of the 7-transmembrane receptor family by multiple groups in the late 1990s [71-73]. It belongs to the rhodopsin-like receptor superfamily and its gene is mapped to chromosome 7p22.3 [71].

Although GPER is a seven-transmembrane receptor, its subcellular localization remains to be fully elucidated. Indeed, several studies have reported the presence of GPER at the plasma membrane, in the endoplasmic reticulum, in the Golgi apparatum as well as in the nucleus of CAFs extracted from mammary biopsies [74-76].

Several studies demonstrated that the ligand dependent activation of GPER trigger the activation of the heterotrimeric G proteins and subsequently Src and adenylyl cyclase (AC), resulting in intracellular cAMP production. Src is involved in matrix metalloproteinases (MMP) activation, which cleave pro-heparan-bound epidermal growth factor (pro-HB-EGF) releasing free HB-EGF. The latter activates EGF receptor (EGFR), leading to multiple downstream events, for example the activation of phospholipase C (PLC), PI3K and MAPK [71]. Activated PLC produces inositol triphosphate (IP3), which further binds to IP3 receptor and leads to intracellular calcium mobilization [77].

The downstream signal of PI3K is the AKT pathway, closely related to the growth of tumor cells involved in cell survival and proliferation [78]. The activation of MAPK and PI3K involves the activation of numerous cytosolic and nuclear protein pathways, which in turn regulate transcription factors such as SRF, CREB and members of the E26 family of specific transformation (ETS) by direct phosphorylation [79]. These promote the expression of a second wave of transcription factors such as FOS, JUN, EGR1, ATF3, C/EBPδ and NR4A2. The cells are reprogrammed under the influence of this network of transcription factors and a series of GPER target genes such as CTGF are up-regulated [80]. *(Figure 1.2.6).*

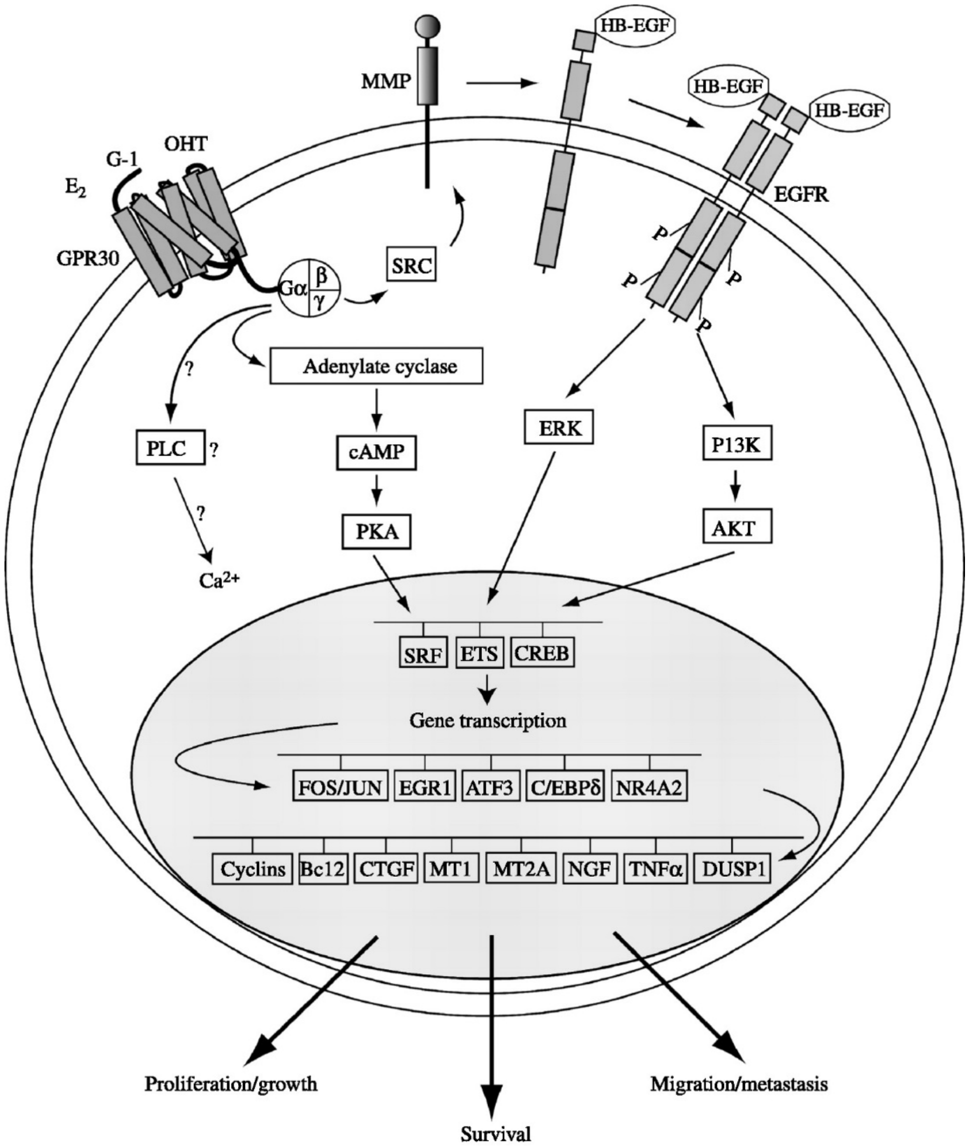


Figure 1.2.6 | Schematic representation of the GPR30 signaling network. *(Maggiolini M and Picard D, 2010).*

In addition, there may be a variety of signaling crosstalk pathways and both negative and positive feedback loops. For example, it has been demonstrated that EGF up-regulates GPER expression through the EGFR/MAPK pathway in ER-negative breast cancer cells, most likely by promoting the recruitment of the c-FOS-containing transcription factor AP-1 to the GPER promoter [81].

Considering that GPER signaling uses the EGFR/MAPK pathway, a positive feedback loop is conceivable. This mechanism is also operational for EGF and the related growth factor TGFα in ERα-positive breast cancer cells [82]. GPER gene expression has been detected in at least four kinds of human tumor specimens or cell lines, including breast cancer [72, 82-84], endometrial cancer [83-85], ovarian cancer [86-88], thyroid cancer [89], and a rat pheochromocytoma cell line PC-12 [90].

Furthermore, there is a growing number of evidence to support the fact that GPER is strongly associated with cancer proliferation [91], migration [92], invasion [93], metastasis [94, 95], differentiation [96] and drug resistance [97, 98]. In fact, since estrogens stimulate the progression of breast cancer in about two thirds of patients expressing ER [99, 100], some selective estrogen receptor modulators (SERMs), such as tamoxifen, have been clinically used to antagonize the binding of 'estrogen with its classic ER, which is an effective therapeutic strategy to attenuate the growth of ER-positive breast tumors. However, there are approximately 25% of patients with ER-positive breast cancer who do not respond to anti-estrogen therapy. This implies that the blocking of classical ER alone may not be sufficient to completely abolish the growth of estrogen-induced breast cancer cells, since estrogens can promote it through other receptors besides the classic ERs. This hypothesis is further supported by the discovery of GPER in a third specific ER with different structure and function with respect to ERα and ERβ. GPER has a high binding affinity not only for estrogens, but also for some ER antagonists, such as tamoxifen and ICI 182.780. In particular, estrogens and the aforementioned antiestrogens stimulate GPER signaling [101]. These results provide an additional possible mechanism for the progression of estrogen-related tumors and raise a new potential target for anti-estrogen therapy. With regard to clinical results, GPER overexpression has been associated with lower survival rates in patients with endometrial and ovarian cancer [102, 103], as well as a higher risk of developing metastatic diseases in breast cancer patients [104]. Furthermore, in a previous large survey, GPER was highly expressed and significantly associated with tumor size (> 2 cm), with the presence of distant metastases and an increase in human expression of EGFR-2 (HER-2) / neu [105]. In recent years particular attention has been paid to the identification of synthetic GPER ligands that act as agonists or antagonists. In particular, several compounds called G-1 [105] G-15 [106], GPER-L1 and GPER L2 [107] and MIBE [108], have been identified by virtual and biomolecular screening and are used to evaluate mediated signaling from GPER and functions. Furthermore, several studies show that ICI 182.780 [109] and 4-hydroxyamoxifen (OHT) [108, 109] are also able to bind GPER and mimic the effects of estrogen.

1.3 Tumor Microenviroment

The breast cancer microenvironment is a complex network of several different cell types and molecules and it is a key contributor to malignant progression [110]. The role of tumor microenvironment is becoming more and more important in breast cancer. Several stromal cell types are implicated in promoting 'hallmarks' of cancer cells [111].

The microenvironment includes fibroblasts, macrophages, immune cells, adipocytes, endothelial cells, and antigenic vascular cells. Stromal cells surround and interact with tumor cells. Tumor microenvironment has been shown to play a crucial role in tumorigenesis, from initiation to progression. Stromal cells promote cancer growth and invasion through the chemokine–chemokine receptor axis [112, 113]. Infiltrating immune cells energize the immune effectors and vascular cells permit nutrients and oxygen uptake by tumors. In a normal mammary duct, there are luminal epithelial cells internally and myoepithelial cells externally delimited by a basement membrane, which maintains the luminal cell polarity [114]. The extracellular matrix (ECM) allows communication with the surrounding stroma. The presence of genetic and epigenetic alterations leads to the proliferation of luminal cells, the loss of epithelial polarity and the decrease of myoepithelial cells and changes in the ECM / basal membrane, eventually leading to the development of mammary tumors [115]. Unlike normal fibroblasts, cancer-associated fibroblasts (CAF) [116] facilitate and promote tumor growth and metastasis by producing growth factors and ECM proteins and modulating immune polarization [117]. Furthermore, the number of CAFs is increased during tumor progression [118]. Consequently, growth factors, cytokines, chemokines and matrix metalloproteinases secreted by stromal cells lead to the recruitment of macrophages, endothelial precursor cells and regulatory lymphocytes, which support tumor progression [119]. It is worth noting that stroma has been correlated with clinical outcomes and response to therapy in breast cancer [120]. The expression of ECM genes, uniformly expressed in both neoplastic and adjacent stromal cells, may divide breast cancers into different subgroups with different clinical outcomes [121, 122]. A study performing hierarchical clustering of the gene-expression profile of ECM-related genes classified breast cancer samples into four groups associated with different clinical outcomes [123]. The tumor microenvironment influences patient outcomes and the signatures of stromal gene expression represent a strong prognostic value that recapitulates immune, angiogenic and hypoxic responses [124]. Stromal cells can be divided into three general classes (*Figure 1.3.1*):

- Infiltrating immune cells
- Angiogenic vascular cells

- Cancer-associated fibroblast cells.

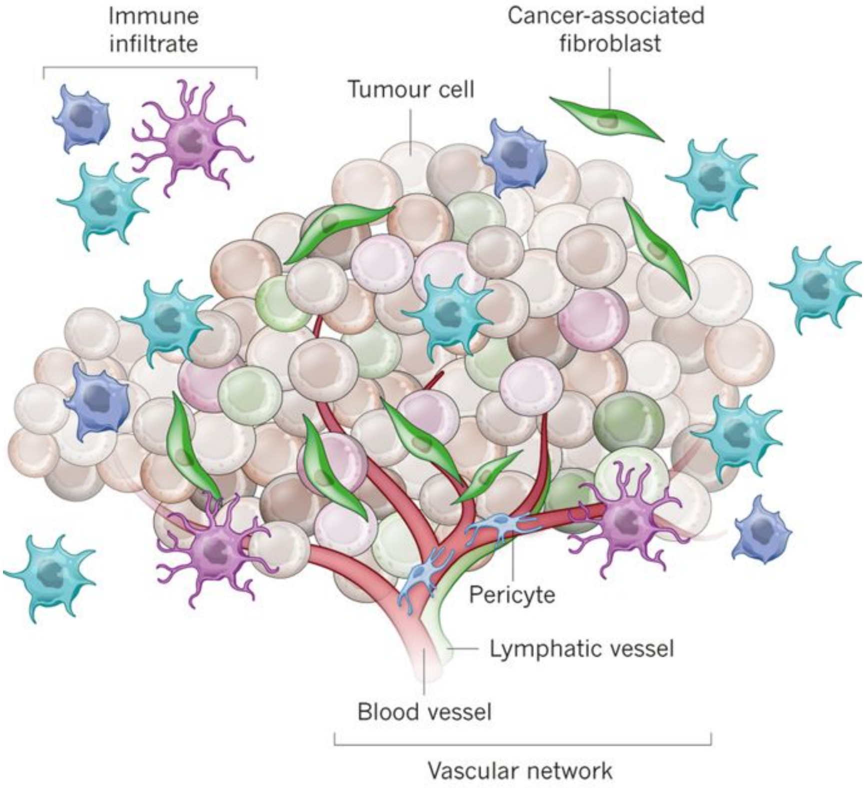


Figure 1.3.1 | The tumor microenvironment (TME). Tumour formation involves the co-evolution of neoplastic cells together with extracellular matrix and vascular endothelial, stromal and immune cells. The tumour niche is a dynamic physical topography in which structural support, access to growth factors, vascular supply and immune cell interactions can vary drastically even within the same lesion. The immune infiltrate can include multiple cell types, these cell populations can have both pro- and anti-tumour functions and can vary in their activation status and their localization within the tumour. The vascular network can differ in regard to the vessel's tissue of origin, maturity (extent of pericyte coverage), interstitial pressure and functionality. Cancer-associated fibroblasts can have significant plasticity and diverge with regard to activation status, localization within the tissue, stress response and origin. *(Junttila MR and De Sauvage FJ, 2013).*

1.3.1 Cancer-associated fibroblasts (CAFs)

During tumorigenesis, the microenvironment turns into an altered stroma (ie reactive or desmoplastic) composed of non-malignant support cells (ie blood vessels, infiltrating inflammatory cells and blast-like cells) [125, 126].

This altered microenvironment works by influencing tumor cell homeostasis via paracrine regulators (eg, growth factors, cytokines and chemokines) and exosomes containing nucleic acids [125, 127, 128]. Cancer-associated fibroblasts (CAF), prominent stromal elements in most types of human carcinomas, are smooth, spindle-shaped, α-muscular cells similar to bursts. The differentiation of CAF from other cell types, such as local fibroblasts, hepatic stellate cells, mesenchymal stem cells, endothelial cells and epithelial cells, is mainly mediated by the transformation of growth factor-β1 (TGF-β1), but also other factors, such as growth hormones (ie, epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet growth factor (PDGF), chemokines, epigenetic regulators and oxidative stress perform a role in the differentiation of CAF [128-130] (*Figure 1.3.2*).

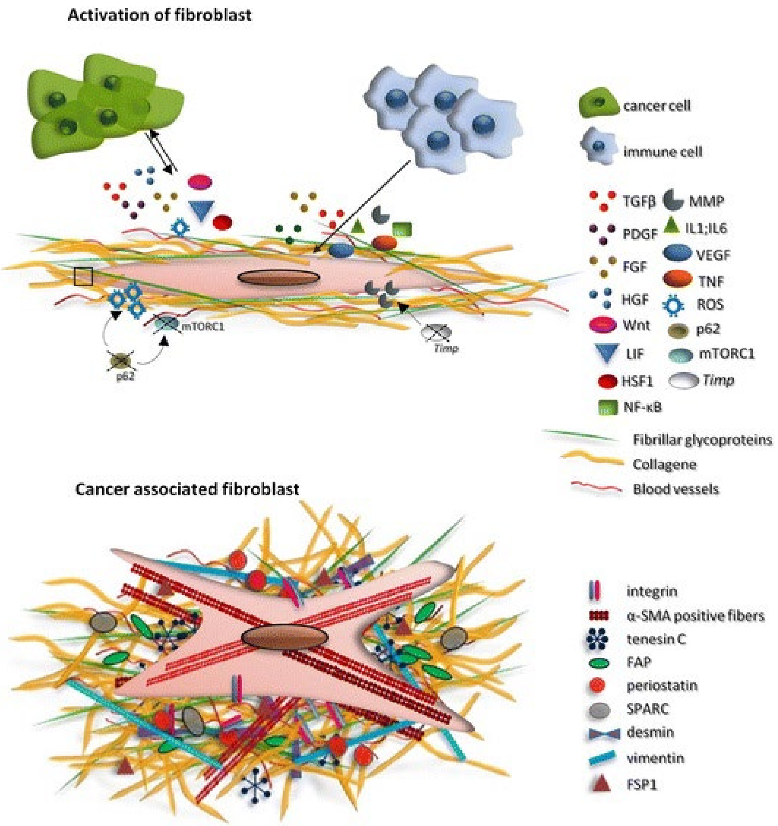


Figure 1.3.2 | Molecular mechanism of fibroblast activation. Normal fibroblasts are the most common cell type in the extracellular matrix and are responsible for the synthesis of collagens and fibrilar proteins. Under normal conditions, fibroblasts maintain tissue homeostasis and contribute to proper cell communication and function. Fibroblasts can be activated by a diverse set of factors secreted from cancer or immune cells such as growth factors (TGF-β, PDGF, HGF and FGF) and also interleukins, metalloproteinases and reactive oxygen species can promote activation. Once activated, fibroblasts undergo a phenotype switch and become cancer-associated fibroblasts (CAFs) expressing various markers such as α-SMA, FSP1, vimentin and periostatin**.** *(Kuzet SE and Gaggioli C, 2016)*.

CAFs, phenotypically, resemble the normal myofibroblasts, but express specific markers (ie fibroblast activation protein (FAP), fibroblast-specific protein 1, neuronglial-2 antigen, vimentin, T-1, tenascin (TN) -C, periostin (POSTN), palladin or podoplanin (PDPN) and show an increase in proliferation and in vitro migration behavior [131, 132]. CAF produce and secrete various proteins of the extracellular matrix (ECM) (eg Collagens I, III , IV), proteoglycans (eg, fibronectin, laminin, TN), chemokines (eg CXCL and CCL), cytokines (eg interleukin (IL) -6 and IL-8) and other tumor promoting factors that affect vascularization (eg, PDGF, vascular endothelial growth factor (VEGF), stroma-derived factor 1 (SDF-1), matrix metalloproteinases (MMPs), tumor cell proliferation, invasiveness, and survival (ie TGF-β, EGF, hepatocyte growth factor (HGF) or FGF) [125, 133-136].

With regards to anticancer therapy, the frequency of genetic mutations in CAF is of fundamental importance. Cells with genetic stability may be less prone to escape or chemotherapy resistance than those with genomic instability [137]. Several studies have shown that a high percentage of CAF undergoes genetic alterations, such as loss of heterozygosity or mutation of tumor suppressor genes (ie homologous phosphatase and tensine and P53) [138-141]. The theory of genetic coevolution of CAF and neighboring cells (ie, random mutation of CAF generated independently of neoplastic epithelial cells that can support tumor progression) is under discussion, due to the potential artifacts caused by the analytical methods used for identification of these genetic alterations [141].

It is interesting to note that proteins derived from CAF that [125] can play an important role in the development of environment-mediated drug resistance, [126] can act as powerful prognostic markers and [127] can be promising targets for therapy antitumor [141].

1.4 miRNAs

MicroRNAs (miRNAs) are small single-stranded non-coding small molecules of about 20-25 nt, able to regulate the expression of target genes by binding to specific complementary sequences in the 3'-UTR region of mRNA in diverse eukaryotic lineages [142].

They are small regulatory RNAs that are processed from stem-loop regions of longer RNA transcripts. Hundreds of different miRNAs have been identified in humans, many of which are conserved in other animals, and these conserved miRNAs have preferentially conserved interactions with most human mRNAs [143].

This inferred regulation of most human mRNAs suggests that miRNAs influence essentially all developmental process and diseases.

As with many discoveries of fundamental importance to mammalian development, physiology, and disease, the first known miRNA was not found in humans or other mammals but was instead found in an invertebrate model organism. Molecular geneticists studying the lin-4 and let-7 genes, which are each required for the proper timing of C. elegans development, found that instead of producing mRNAs, these genes produce noncoding RNAs, including short

RNAs ~22 nt in length [144, 145]. The lin-4 and let-7 RNAs both had imperfect complementarity to conserved sites within the 3′ UTRs of genetically identified regulatory targets, which led to a model in which these small RNAs mediate translational repression through antisense interactions [144-148]. The let-7 RNA was subsequently recognized in humans and other bilaterian animals, with temporal expression resembling that observed in C. elegans. This discovery showed that these regulatory RNAs were not mere curiosities of worms and led to the idea that additional "small temporal RNAs" might exist to regulate the timing of other developmental transitions [149]. Soon thereafter, molecular searches for endogenous small RNAs refined the identities of the lin-4 and let-7 RNAs and revealed that these RNAs were actually part of a much larger class of small RNAs. Members of this class all resembled lin-4 and let-7 RNAs in their small size and potential to be processed from hairpin precursors, but most were not expressed in a temporal manner. Because they were identified without the help of genetics, their functions were not known-what was known is that they were small, and so they were called "microRNAs" [150-152].

MicroRNAs are grouped into families based on their targeting properties, which depend primarily on the identity of their extended seed region (miRNA nucleotides 2–8) [1]. For example, mice and humans each have three members of the miR-1/206 seed family (miR-1-1, miR-1-2, and miR-206), which are paralogous miRNAs that arose through duplication of an ancestral gene inherited from a common ancestor of all bilaterian animals. Indeed, members of the same seed family are usually evolutionarily related, and evolutionarily related miRNAs are usually members of the same seed family. However, the use of the term "family" does not strictly denote common ancestry. For example, miR-32, which is not related to other members of the miR-25/32/92/363/367 seed family, is nonetheless an adopted member because it has converged on the same extended seed and thus has the same targeting preferences. Other miRNAs, such as miR-200a and miR-200b, which are clearly related, have a single-nucleotide difference in their seed regions that place them into different families because of their divergent targeting preferences.

1.4.1 Biogenesis and maturation

MiRNA biogenesis begins in the nucleus with transcription by RNA Polymerase II [142]. The primary transcript (pri-miRNA) presents the cap of 7-methyl-Guanosine at 5 ', polyA tail at 3' (*Figure 1.4.1*), it can also be several kb long and assume very complex structures (*Figure 1.4.2*)

[153, 154]. MiRNAs of viral origin, deriving from tRNA or associated with Alu repeats are transcribed instead by RNA polymerase III [142, 153].

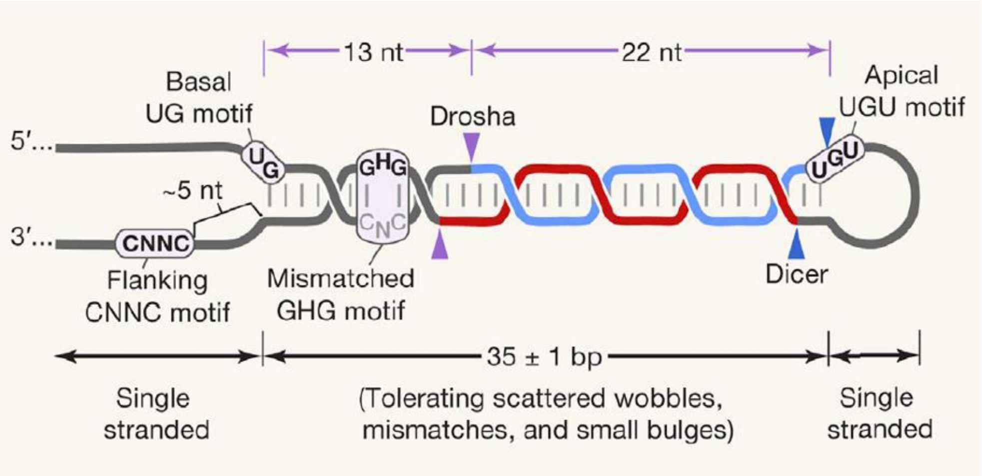
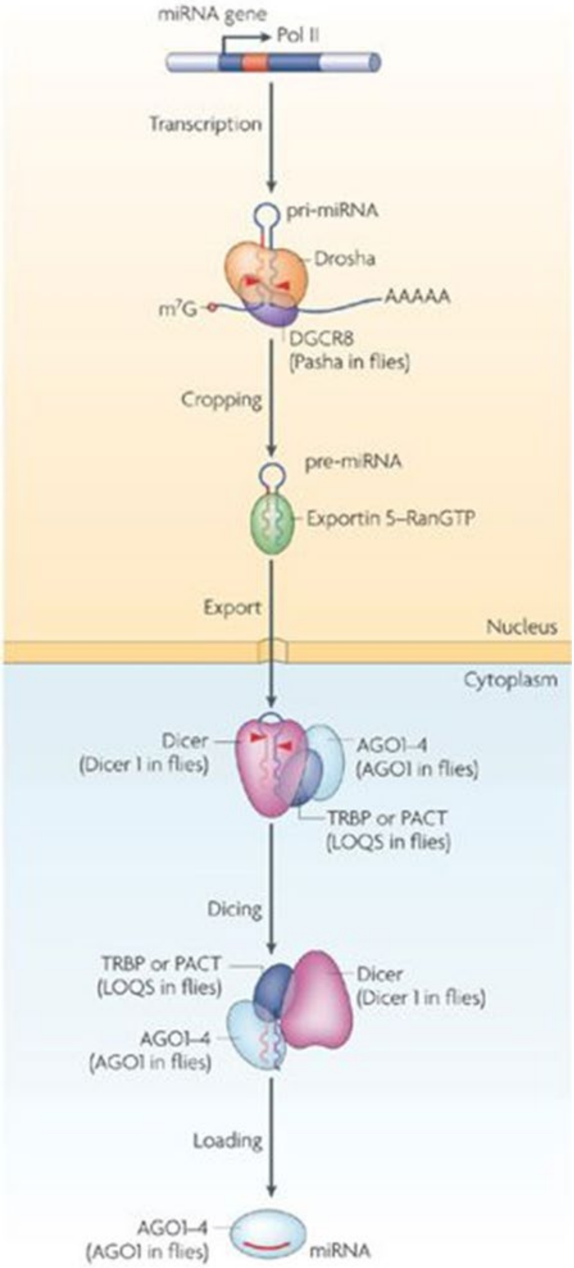


Figure 1.4.1 | Structural and primary sequence characteristics of a pri miRNA. *(Bartel DP, 2018)*



1.4.2 | Biogenesis and maturation of miRNAs (*Kim VN et al, 2009*).

Some sequences are essential for processing: in Homo sapiens there is a UGUG motif in the terminal loop and two elements in the basal region, UG and CNNC (*Figure 1.4.1*). The latter is recognized by SRp20, a splicing factor and p72, a DEAD-box helicase whose activity increases Drosha processing [153].

The structure is very important because it determines recognition and processing. It consists of a stem-loop of approximately 33 nt, substrate of a large enzymatic complex called Microprocessor. The latter consists of two main components: Drosha, a type III RNase and

DGCR8, its essential cofactor. DGCR8 is a dsRNA Binding Protein responsible for the recognition of the substrate and the catalysis, it interacts with the stem and the single-stranded flanking sequences, while Drosha, cuts to 11 bp from the single filament RNA junction (ssRNA) -RNA with double strand (dsRNA) , operating a staggered cut typical of this class of enzymes (*Figure 1.4.3 A*).

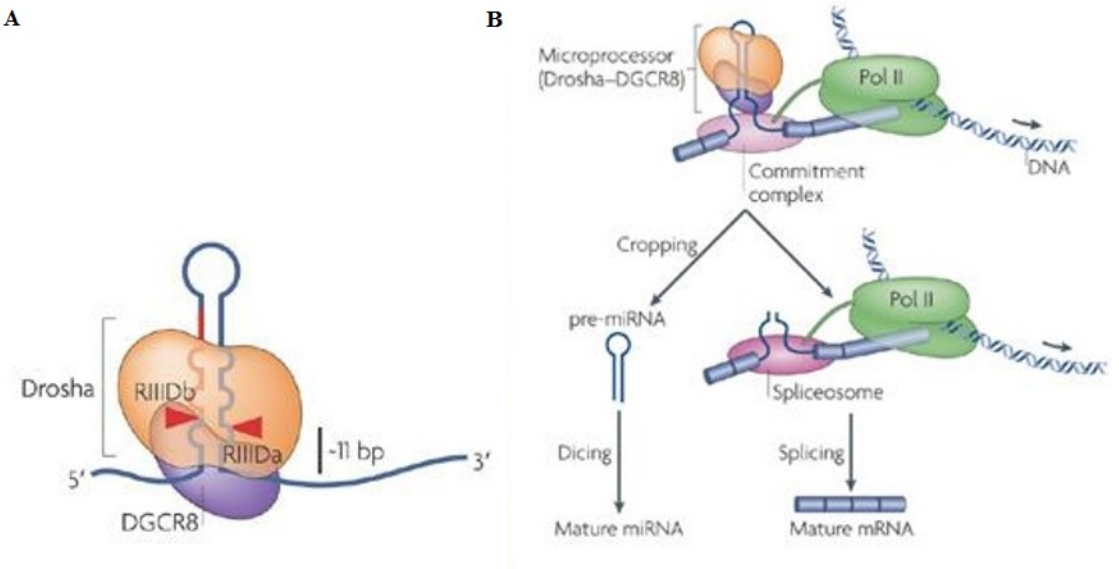


Figure 1.4.3 | (A) Recognition and cutting of a pri miRNA by Drosha / DGCR8. **(B)** Drosha /DGCR8 processing of an intronic pri miRNA. (*Kim VN et al., 2009*).

The catalytic site consists of two domains, RIIIDa and RIIIDb, which dimerize in tertiary structure. Without this final conformation the cut cannot take place and the 2nts at 3 'are due to the way in which RIIIDa and RIIIDb are associated. The result is a pre-miRNA, 70-100 nt long with a secondary hairpin structure consisting of a stem-loop of about 30 nt and a 3 'single-strand 2 nt [142, 153].

In the case of non-independent promoters, the pri-miRNAs will be processed in a co-transcriptional manner. In particular, if the precursor is intronic it will be cut before splicing, but it will not affect the maturation of the mRNA (*Figure 1.4.3 B*) [155, 156]. The fragments resulting from the cut are degraded by the nuclear exosome (exonuclease 3 '→ 5') and by XRN2 (exonuclease 5 '→ 3'), associated with the processing region of the intronic pri-miRNAs. If miRNA is encoded in an exon, binding and cutting by Drosha-DGCR8 will result in destabilization of the transcript and reduction in protein levels. The Microprocessor complex has no affinity for the pri-miRNAs as such, but for the nature and structure of the substrate: it is in fact capable of cutting mRNA still in the nucleus, provided they have the same conformation [155].

Following the cutting of Drosha, the precursor-miRNA (pre-miRNA) must be exported to the cytoplasm using Esportin 5 (EXP5) [157, 154].

EXP5 has affinity for dsRNA over 14 bp long and with a single-strand tract of 1-8 nt at 3 'and binds the pre-miRNA at stem level, for a double-stranded stretch of 14 base pairs just above the junction dsRNA-ssRNA, and 2nt at the 3 'end [153, 154].

EXP5 is a member of the Kariopherine β nuclear transport receptor family, and uses the Ran-GDP / Ran-GTP system to catalyze the export reaction and ensure its unidirectionality. In the nucleus, EXP5 binds the cargo and the Ran-GTP with high affinity and passes through the nucleopore complex (NPC). Once in the cytoplasm, GTP is hydrolyzed to GDP by a Ran-GTP Activated Protein (Ran-GAP), located near the cytoplasmic fibrils of the NPC. The hydrolysis of guanosine triphosphate causes a conformational change in the ternary complex, which causes the transition to a low-affinity state necessary for the release of the cargo (*Figure 1.4.4*).

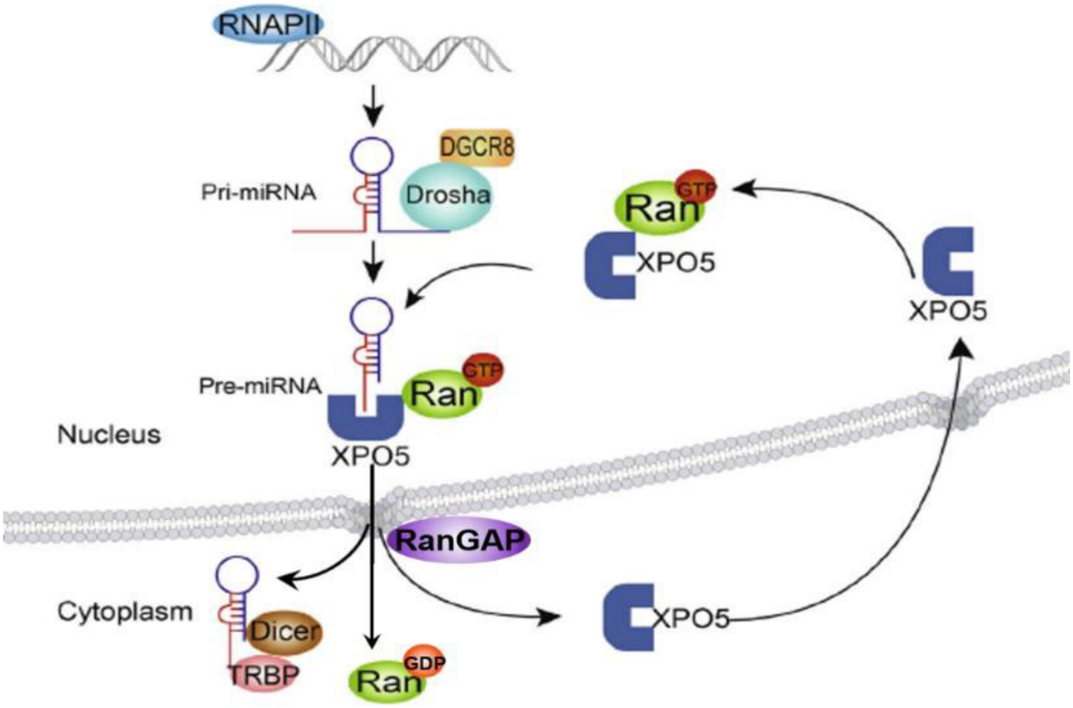


Figure 1.4.4 | Representation of export of miRNAs by EXP 5. (*Singh SK et al., 2008*).

Following the hydrolysis of GTP to GDP, the pre-miRNA is released into the cytoplasm, where it will be a Dicer substrate, a type III RNase discovered thanks to its role in RNA interference (RNAi). Dicer is a multidomained, ATP-dependent enzyme that associates with pre-miRNA thanks to the PAZ domain (Piwi / Argonauta / Zwille), which recognizes the 2nt at the end 3'. Dicer also needs association with a dsRNA Binding Protein, TRPB, which has three dsRNA binding domains and binds the pre-miRNA at the 3' protruding end. The first contact between

Dicer and the substrate, mediated by the PAZ domain, is unstable and not sufficient to block it. Other interactions are created between the enzyme and the stem, which will "flow" until it reaches the catalytic site [142, 158]. Dicer has affinity for this structure because in the PAZ domain there are two basic pockets, simultaneously binding the transcript terminations, and the association is stable only if the 3 'is 2nt longer than the 5' [153] . TRBP and PACT will then be recalled contributing to stabilization. Once the exact position is reached, the cut will occur at the base of the loop, defining the second end of the miRNA and giving rise to a dsRNA approximately 22 nt long. The double filament is not perfectly matched, contrary to what is observed in RNAi, and wobble G:U bonds and small bulges due to insertions of a nucleotide are frequent. These imperfections and the nucleotide composition of the extremities are decisive for the choice of the guiding filament, which will act as miRNA, and its complementary, the passenger filament or miRNA *. The most thermostable end is bound by a protein with two dsRBD, R2D2, which steadily dimerizes with Dicer. An Argonauta (Ago) protein is recalled and RISC loading complex (RLC) is formed, which guarantees the directionality of the assembly of the RISC complex (RNA Induced Silencing Complex), in which only the filament will be loaded with the 'extremity 5' less thermodynamically stable [142, 153, 159].

Ago proteins adopt bilobed architecture, composed of an amino-terminal PAZ domain (Piwi / Argonauta / Zwille) and PIWI and MID (middle) carboxy-terminal domains (*Figure 1.4.5*) that bind the 3 'ssRNA, recognizing the 2nt single-stranded, via the PAZ domain, also present in the Dicer structure [153, 155, 156]. In humans there are four Argonauta proteins (Ago1-4), but only Ago2 has the cutting activity, called "slicing". All proteins of the RLC complex are detached except Ago. The mature RISC complex will be a ribonucleoprotein, consisting of the miRNA and the associated Argonauta protein. The passing strand will be cut by Ago2, thanks to the complementarity of the central zone, and degraded by the C3PO nuclease, in the case of perfectly matched siRNA or miRNA [155-158]. This second hypothesis is more rare: commonly, there is the intervention of helicases, the breaking of hydrogen bridges and the consequent degradation by cytoplasmic exonucleases [153, 158].

The loading of dsmiRNA on the RISC complex is a highly endoergonic process, guaranteed by the energy deriving from the hydrolysis of ATP by the cytoplasmic chaperones HSC70 / HSP90 [142, 153]. There are cases in which both subunits will be associated with the RISC complex and in this case they will be called 3-p and 5-p, depending on whether they are the 3’ or 5’ filament [158].

1.4.2 Mechanism of action

The RISC complex is ready to perform its function of translational repression by recognizing complementary sites in the messenger 3'-UTR region. The area of pairing essential for repression is the sequence from the 2nd to the 7th nucleotide at 5 'of the guide miRNA, called "seed" [1, 142, 160-162; 156].

The "seed" will form a small propeller predisposed to pairing, while the remaining part will be structured in such a way as not to appear on the target, but eventually bind itself later, otherwise the nucleotide in position number 1 remains locked in a conformation unfavorable [1]. In particular, Ago α-helix 7 leaves only the nucleotides 2-5 available for pairing, which is why this portion of the seed sequence is the most important for recognition. When the pairing propagates to nucleotides 6 and 7, the propeller is displaced, so as to consolidate it. As for extensive pairing, there is the formation of a second nucleation center at 3 'rather than the extension of the bonds established by the seed (*Figure 1.4.5*) [142].

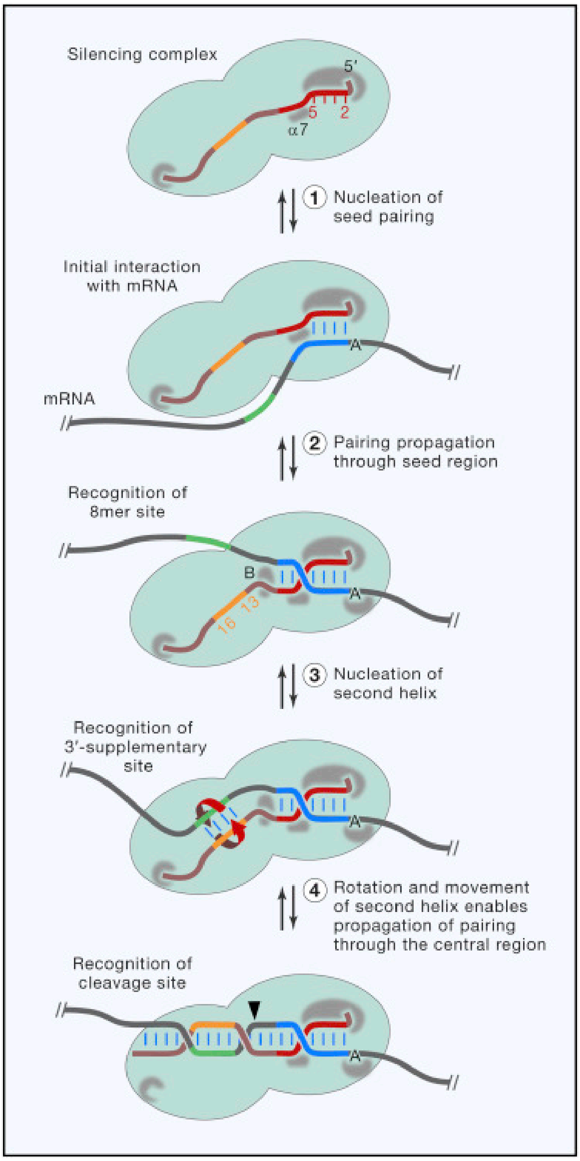


Figure 1.4.5 | Unified model for miRNA target recognition and basic pairing propagation. *(Bartel DP, 2018).*

The link determines the reduction of the translational rate in different ways. The proposed model involves the recruitment of GW182 (TNRC6 in humans) which is physically associated with PABPC (PolyA Binding Protein), linked to the 3' tail of mRNA. TNRC6 then recalls the complexes assigned to the Ccr4 / Pop2 / Not1 and PAN2/3 deadenylation that displace PABPC and begin to degrade the messenger. Ccr4/Pop2/Not1 in turn recruits a helicase, DDX6, associated with the decapping complex. DDX6 also interacts with eIF4E-T (eIF4E-Transporter), which competes with eIF4G for binding to eIF4E by inhibiting translation, both increasing degradation of the transcript but also directly affecting the initiation phase of protein synthesis (*Figure 1.4.6)* [142, 159, 160].

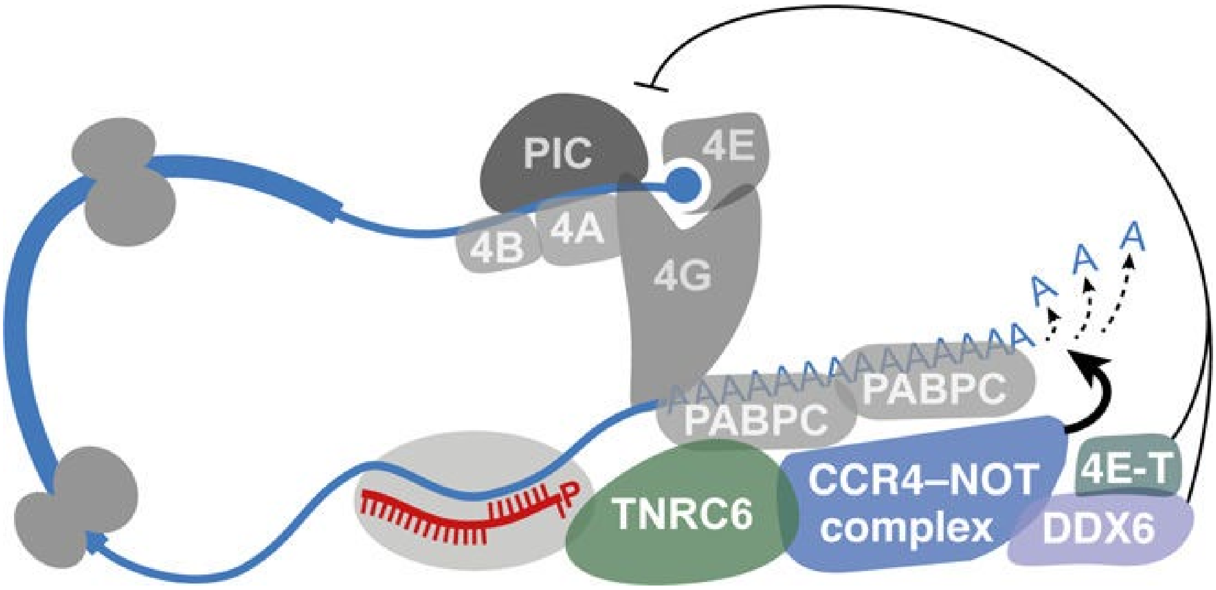


Figure 1.4.6 | The Mechanisms of miRNA-guided Repression. (*Bartel DP, 2018*).

The enzymes that take part in the messenger degradation machinery often colocalize, together with the transcripts, in phylogenetically conserved cytoplasmic foci, from yeasts to mammals, called P-bodies. In fact we find Dcp1 / Dcp2, the enzymes responsible for decapping, Dhh1/Me31B/RCK/p54, helicase complex activator of Dcp1 / 2, Lsm1-7 and Pat1, also involved in decapping, XRN1, the major exonuclease 5 '→ 3' , and Ccr4 / Pop2 / Not1, the most important cytoplasmic deadenylase complex. Colocalize also eIF4E and CPEB in the P-bodies which bind respectively the cap and the polyA tail of the messengers, as well as the mRNAs themselves and other proteins with different functions [156, 159-162]. However, targeting to P-bodies is not always a sign of degradation: they can also act as translation modulators, temporarily seizing the transcript and then releasing it with consequent recruitment of polysomes and resumption of protein synthesis [162], as in the case of CAT1 (cationic aminoacid transporter 1) which is downregulated in mir-122 hepatocytes. It is sequestered in the P-bodies but not degraded and the starvation of amino acids induces its release and translation [159, 160]. The sequestration of the ribonucleoprotein RISC/mRNA complex in P-bodies is therefore a very common pathway [142, 156, 159-162].

Another modality is the blocking of the translation by steric hindrance which causes the dissociation of the ribosome. The interaction between proteins associated with miRISC and the starting factors of translation explains why cases of mRNA with IRES (Internal Ribosome Entry

Sites) have been found whose repression was not observed [160]. It has also been shown that if there is a perfect pairing for the whole sequence or with the nucleotides in position 9-12, the central zone, the transcript is cut, similarly to what happens in the processing of siRNAs, as long as the Ago protein and Slicer (Ago2), the only one with endonuclease activity [142 156, 159-162]. This mode, although very common in plants, is quite rare in other kingdoms. In particular, the only class among all the bilateral symmetry animals in which cases of translational repression with direct cutting of the transcript have been reported is that of mammals, but it is a fairly rare process, found only in 20 mRNA, few viral transcripts and only one circular RNA [142].

Extensive analyzes carried out on 87 classes of highly conserved miRNAs in the vertebrate subphylum have found different types of seeds in addition to the canonical ones, based on which the more or less effective translation rate is reduced (*Figure 1.4.7*). In particular, we have three classes: canonical sites, marginal sites and atypical sites [1, 142, 143]. To influence the effectiveness of the process we also find characteristics of the 3'UTR, such as the positioning within 15 nt of the stop codon, over half in the very long 3'UTRs, the percentage of AU, the presence of secondary structures and other sites miRNA target near the seed recognition site [1]. MiRNAs are very stable and may have half-lives even days [142, 153, 158, 159]. The dissociation of the RISC complex and the degradation of miRNA are processes much less known than in biogenesis. The nucleases involved have not yet been identified and although adenylation and uridylation have been associated with the process, the pathways that bind these two types of tailing to decay are still not known [142, 153, 159, 163]. Only one modality has been delineated, miRNA degradation directed by the target RNA (TDMD), in which the extensive 3’ miRNA pairing pushes this terminal out of the confines of Ago, available for cutting by exonucleases [142, 159]. Other examples of direct degradation are the exonucleases 5 ’→ 3’ XRN1 and XRN2 in Caenorhabditis elegans, and the exonucleases 3 ’→ 5’ PNPT1 and ERI1 in human melanoma cells and murine lymphocytes respectively [153].

To contribute to the regulation of expression we have U-tailing, by the TUT 4 and 7 both on pre-miRNAs and on mature miRNAs, A-tailing, after cutting Dicer, editing, by the ADARs on the pre-miRNA, and O-methylation on 5'-P, by the RNA methyl transferase BCDIN3. All these modifications, often very rare, have the effect of lowering the maturation rate of miRNAs [142, 153, 159]. Single nucleotide polymorphisms (SNPs) have also been found, affecting both biogenesis and target specificity, with pathogenetic results [142, 153, 159, 163].

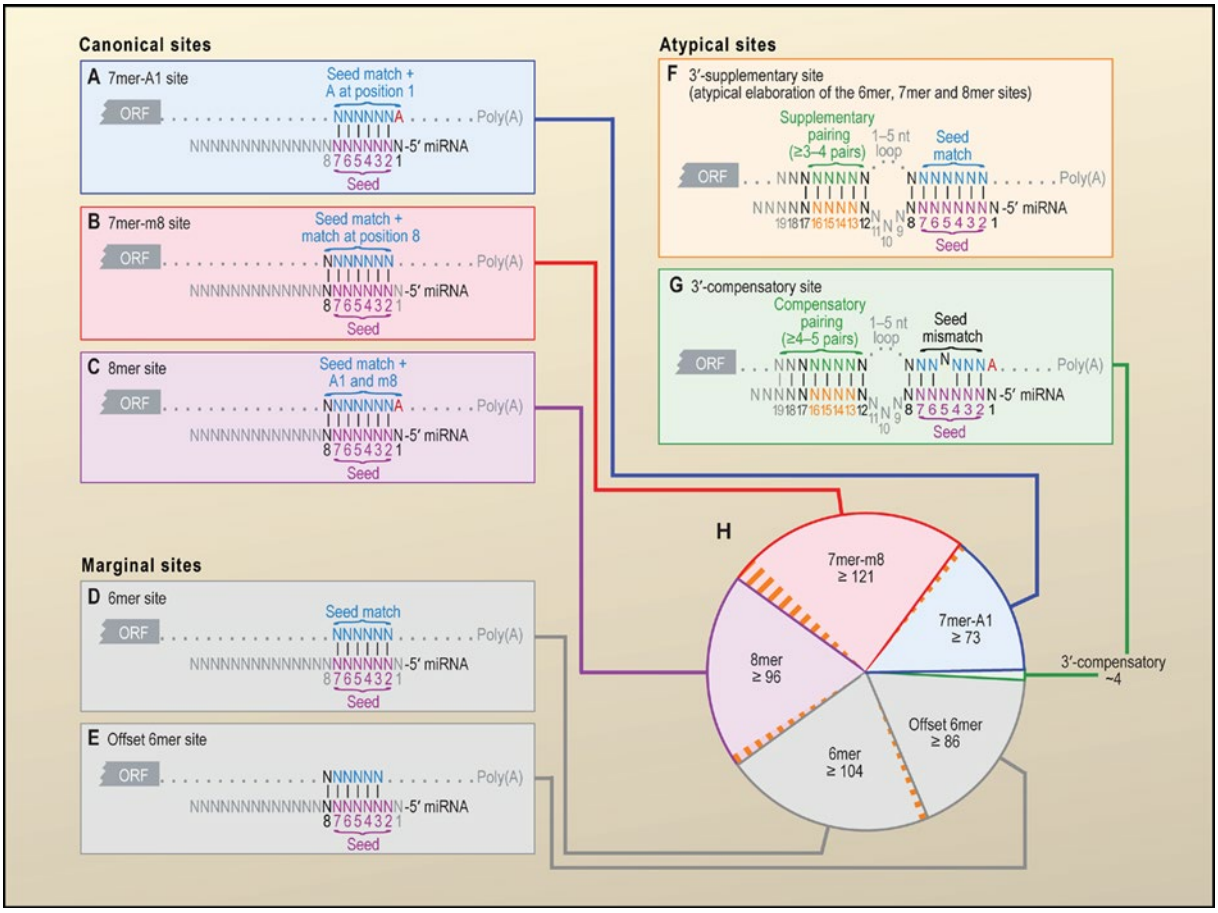


Figure 1.4.7 | Seed classification. A, B, C | Canonical sites, with 7/8 pairing. D, E | Marginal sites, with a 6 nt match They are inefficient. F, G | Atypical sites, with productive pairing at 3’. F | We have a 6mer seed, but perfectly matched, with an additional site on the 3rd. They have a good effectiveness. G | The mismatch in the seed needs compensatory interactions at 3 '. The interactions are still productive. H | Percentages of seed types found in 87 highly conserved miR families in vertebrates. *(Bartel DP, 2009)*.

For example, a C → T transition in the CNNC sequence in the pri-miR-15a~16-1 loop reduces the cutting by the Microprocessor complex and SNPs in miR-196a-2-3p and miR-146a-3p were associated with neoplastic manifestations [153]. Editing has been associated with a double meaning: degradation, recalling the Tudor-SN endonuclease, which has affinity for I: U pairs, or redirection of mature miRNA, loaded again into RISC [159].

Several biochemical pathways have been outlined regarding the control of the Microprocessor, Dicer, Argonauta, GW182 complex and related cofactors [142, 153, 159] and in particular was found an interaction between ERα and the Microprocessor complex, in which the nuclear receptor was found to be responsible for inhibiting the processing of several pri-miRNAs [159].

1.4.3 miRNAs and cancer

The emergence of microRNAs has been one of the defining developments in cancer biology over the past decade, and the explosion of knowledge in this area has brought forward new diagnostic and therapeutic opportunities. The importance of microRNAs in cancer has been underlined by the identification of alterations in microRNA target binding sites and the microRNA processing machinery in tumor cells [164].

MicroRNA dysregulation in cancer was first reported in 2002, when a cluster of two microRNAs, miR-15 and miR-16, was identified at 13q14.3, a frequently deleted region in chronic lymphocytic leukemia (CLL) [165]. This microRNA deletion was shown to act at least in part through allowing higher expression of the miR-15/16 anti-apoptotic target B-cell lymphoma 2 (BCL2). Since then it has been documented that microRNAs have roles in all of the cancer hallmarks and are implicated in the clinical management of cancers at every stage [166].

Each tumor type has a distinct microRNA signature that distinguishes it from normal tissues and other cancer types.

Aberrant expression of miRNAs can arise through a number of different mechanisms (*Figure 1.4.8*):

- *Genomic abnormality*. As exemplified by miR-15a and -16-1, chromosomal abnormality is one reason for miRNA deregulation in cancers. Tumorigenesis is often accompanied by chromosomal aberration such as deletion, amplification, translocation, etc. In silico analysis revealed that a significant fraction of miRNAs are mapped to these cancer-associated genomic regions or fragile sites in human [167] and mouse [168].
- *Epigenetic factors*. Epigenetic factors could also affect miRNA expression. In many cancers, hypermethylation of CpG islands in promoter regions results in heritable transcriptional silencing of tumor suppressor genes. Gene silencing by DNA methylation is closely related with histone modification. In silico analyses indicated CpG islands near dozens of miRNAs. In addition, some miRNAs were up-regulated upon exposure of cells to the demethylating agent 5-aza-2′-deoxycytidine [169], upon mutation of DNMTs (DNA methyltransferases) [170], or upon HDAC (histone deacetylase) inhibitor treatment [171]. These studies identified some miRNAs which are repressed by CpG hypermethylation in cancers relative to normal tissue. Representative examples are miR-9-1 in breast cancer [169] and miR-124a in colorectal tumors [170]. In case of miR-124a, hypermethylation is tumor-type specific, as no

methylation was seen in neuroblastoma. Epigenetic silencing of a miRNA may be a reflection of tissue specificity. For example, miR-124a is normally highly expressed in neuronal tissues, so that its epigenetic repression in colorectal tumors is not surprising. miRNAs may counteract CpG methylation. For example, miR-29 directly targets DNA methyltransferases Dnmt3A and -3B. In agreement with this, ectopic expression of miR-29 resulted in a global reduction of DNA methylation, subsequently leading to a de-repression of some tumor suppressor genes which had been silenced by promoter methylation in cancer cells [172].

- *Transcriptional regulation.* MiRNA expression is tightly controlled by different transcription factors, so abnormal expression of miRNA in cancer could be due to dysregulation of some key transcription factors, such as p53 and c-Myc.

  The p53-miR-34 regulatory axis is an example of how transcriptional factor regulates miRNA expression to mediate tumor suppressive function [173]. The p53 is a tumor suppressor encoded by the gene TP53, one of the most commonly mutated genes in human cancers. p53-regulated expression of many genes, including miRNA genes, forming a complex p53 network to regulate cell-cycle progression and apoptosis. Similar to p53-mediated phenotypes, miR-34 family including miR-34a/b/c promotes cell-cycle arrest, cell senescence and apoptosis in cancer [174], implying p53 and miR-34 are in the same regulatory pathway. p53 can induce the expression of miR-34a to trigger apoptosis through direct binding to the promoter of mir-34a gene [175, 176]. In turn, miR-34a promotes p53 expression by targeting SIRT1, a negative regulator of p53 via deacetylation [177].

  C-Myc, frequently upregulated in many malignancies to regulate cell proliferation and apoptosis, activates the transcription of oncogenic miR-17–92 cluster through binding to E-box elements in miR-17–92 promoter [178] and also represses transcriptional activity of tumor suppressive miRNAs such as mir-15a, miR-26, miR-29, mir-30 and let-7 families [179]. Ghoshal's group found the reciprocal regulation of c-Myc and tumor suppressor miR-122 in hepatocellular cancer. C-Myc represses miR-122 expression by associating with its promoter and miR-122 indirectly inhibits c-Myc transcription by targeting Tfdp2 and E2f1. Therefore, the disruption of this feedback loop between miR-122 and c-Myc is essential for hepatocellular cancer development [180]. Another functional c-Myc-miRNA feedback loop is also dysregulated in hepatocellular cancer. C-Myc directly binds to the promoters of miR-148a-5p/miR-363-3p genes and represses their expression, inducing hepatocellular tumorigenesis by promoting G1 to S phase progression. In turn, miR-148a-5p directly targets and inhibits c-Myc expression, whereas miR-363-3p destabilizes c-Myc by directly targeting ubiquitin-specific protease 28 [181].

- *Defects in miRNA biogenesis machinery*. MiRNAs biogenesis is controlled by several enzymes and regulatory proteins, such as Drosha, Dicer, DGCR8, Argonaute proteins and exprotin 5, allowing correct miRNA maturation from primary miRNA precursors. Therefore, mutation or aberrant expression of any component of the miRNA biogenesis machinery could lead to abnormal expression of miRNAs. Drosha and Dicer are dysregulated in certain tumors [182]. DGCR8 and Drosha have single-nucleotide substitution/deletion mutations in 15% of 534 Wilms' tumors, leading to significantly decreased expression of mature Let-7a and miR-200 family [183]. Moreover, it was observed that Dicer1 impairment in colorectal cancer (CRC) cells induces the acquisition of a greater capacity for tumor initiation and metastasis [184] and that high Dicer and Drosha mRNA levels in ovarian cancer are associated with increased median survival [185] and reversely, decreased Dicer expression significantly correlates with reduced patient survival [186-187].

  Similar to Dicer and Drosha, dysregulation of Argonaute proteins also occurs in cancer. For example, human EIF2C1/hAgo1 gene is often lost in Wilms' tumors of the kidney [188]. The expression of human argonaute proteins (AGO) is regulated in a cell-dependent manner. For instance, AGO2 expression levels in primary gastric cancer and corresponding lymph node metastases are significantly higher than that in healthy controls [189] whereas AGO2 expression is lower, corresponding reduced RNAi efficiency, in melanoma compared with primary melanocytes [190].

  Exportin 5 (XPO5) is a double-stranded RNA-binding protein that mediates nuclear export of pre-miRNA into the cytoplasm. It has been found that XPO5 gene has inactivating mutations in a subset of human tumors with microsatellite instability. In CRC cells HCT-15 and DLD-1, the insertion of an ''A'' in exon 32 generates a premature termination codon, resulting in frameshift mutation and production of truncated version of the protein. This truncated XPO5 loses the function to export pre-miRNAs. Pre-miRNAs are therefore trapped in the nucleus, resulting in reduced miRNA processing. Most importantly, the restoration of XPO5 functions reverses the impaired export of pre-miRNAs and has tumor suppressor features [191].
- *Sequence variation in miRNA target sites.* Caused by SNP and mutations/deletions at the 3' UTR of the target [192].

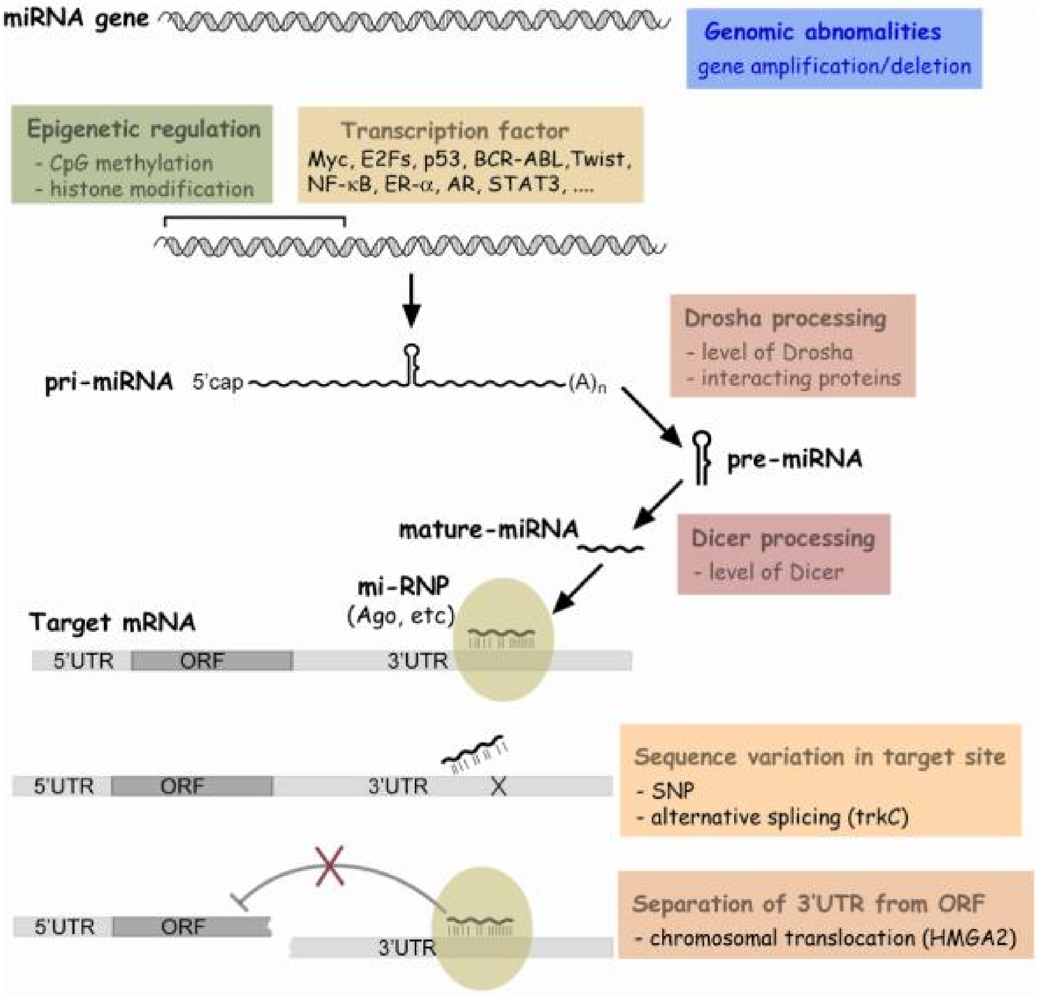


Figure1.4.8 | Various mechanisms for deregulation of miRNA expression or function. (*Lee YS and Dutta A, 2009*).

Given that abnormal miRNA expression in tumors, it is believed that the dysregulated miRNAs could affect one or several of the cancer hallmarks for tumor initiation and progression.

In cancer there are both over-expressed miRNAs, therefore with oncogenic function (oncomiRNA) and downregulated with oncoprotective function, defined as tumor suppressor miRNA (tsmiRNA) [193-195].

1.4.4 miR-221

The transcriptional unit of miR-221 is localized on the X chromosome, in a cluster with miR-222, its paralogue. They have been discovered through bionformatic analysis of the transcriptome, by aligning phylogenetically conserved sequences between *Fugu rubripes* and *Mus musculus*. It is widely recognized as an oncogenic miRNA or "oncomiRNA" because it is upregulated in numerous malignant neoplasms and has seeds in the 3'UTR of several mRNAs encoding tumor suppressors and factors that contrast the epithelial-mesenchymal transition (EMT) [196, 197].

Among its targets we find PTEN, as reported in stomach cancer [198] and in multiple myeloma [199], cell cycle regulators such as p27 and p57, both targets of miRNA in glioblastoma [200], hepatocarcinoma [201] and multiple myeloma [199], as well as the tissue inhibitor of metalloprotease-2 (TIMP2) in lung cancer non-small cell (NSCLC), where it favors the digestion of the extracellular matrix [202].

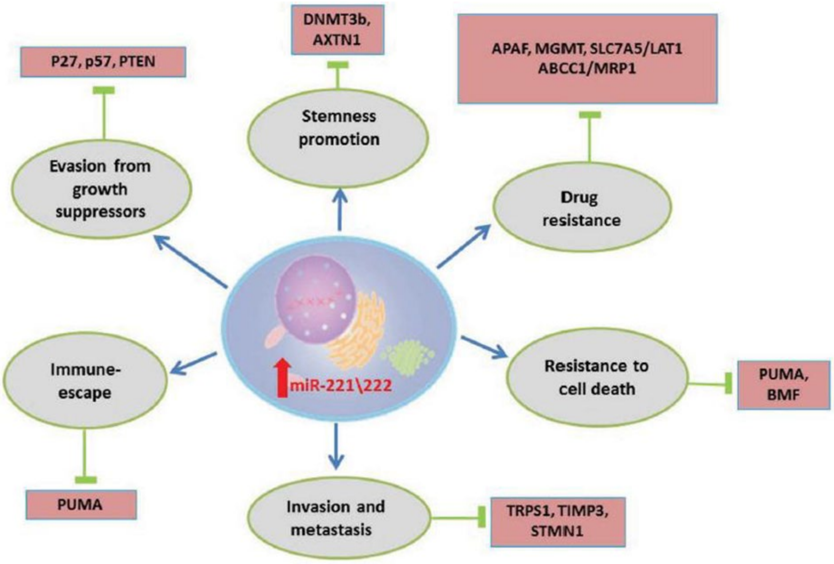


Figure 1.4.9 | Main targets involved in oncogenic activity of miR-221/222 cluster. *(Di Martino MT et al., 2016).*

In negative ERα breast cancer, upregulation of miR-221 transcription promotes insensitivity to cell cycle control by p27 downregulation, as demonstrated in over-expression experiments [203], facilitates EMT by promoting the extracellular matrix digestion and by inhibiting the synthesis of E-cadherins. Indeed, miR-221 targets TIMP3, a tissue inhibitor of metalloproteinases-3, TRPS1 [204] and AXTN1, both transcription factors that promote transcription of integrin [205]. In particular, TRPS1 inhibits the synthesis of ZEB2, which negatively regulates E-cad [204]. MiR-221 also promotes the undifferentiated state, repressing DNA methyl transferase 3b (DNMT3b), which methylates promoters of genes associated with staminality [206]. The transcription of miR-221 is under the control of a transcription factor of the Fos family, FOSL1 or Fra-1, which heterodimerizes with Jun and binds the AP-1 sites in the promoter sequences, including that present 12 kb upstream the cluster miR-221/222 [204]. In the promoter of the locus there is also an E-box, specifically recognized by Slug, a master-regulator of the EMT that represses the transcription of E-cadherins (*Figure 1.4.10*) [207], in support of its promoter role of a malignant and highly metastatizing phenotype.

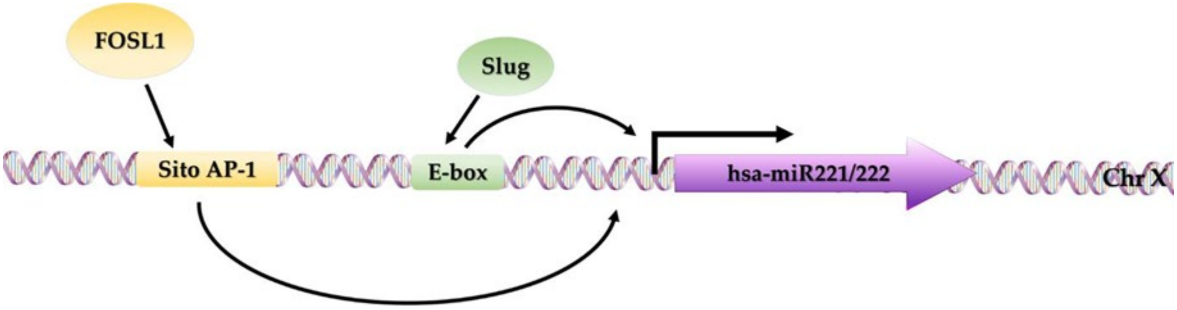


Figure 1.4.10 | Transcriptional elements that underlie the regulation of miR 221.

In fact it is very expressed in MDA-MB 231, triple negative and extremely aggressive, but not in MCF7, little invasive and expressing all three receptors [207, 204]. The motivation lies in the different cell physiology of the two lines: Fra-1 is activated by the MAP kinase cascade (*Figure 1.4.11*) and the MDA-MB 231 express a constitutionally active Ras mutant [204], with Slug levels significantly higher in MDA-MB 231 than in MCF-7 [207].

Microarray analysis showed that between breast cancer MCF-7 tamoxifen-resistant lines miR-221 and miR 222 are up-regulated, similarly to what was found in HER2-positive and HER2-negative biopsy samples [203]. In fact, it promotes drug resistance to both anti-oestrogens such as Fulvestran [208] and Tamoxifen [209], by downregulating ER-α [208, 209] than to Trastuzumab, through the post-transcriptional repression of PTEN [210]. Carcinomas expressing miR-221 therefore have a high propensity to metastatization and insensitivity to cell cycle control, as well as a low degree of differentiation [196] and poor prognosis, therefore considered a good biomarker [211].

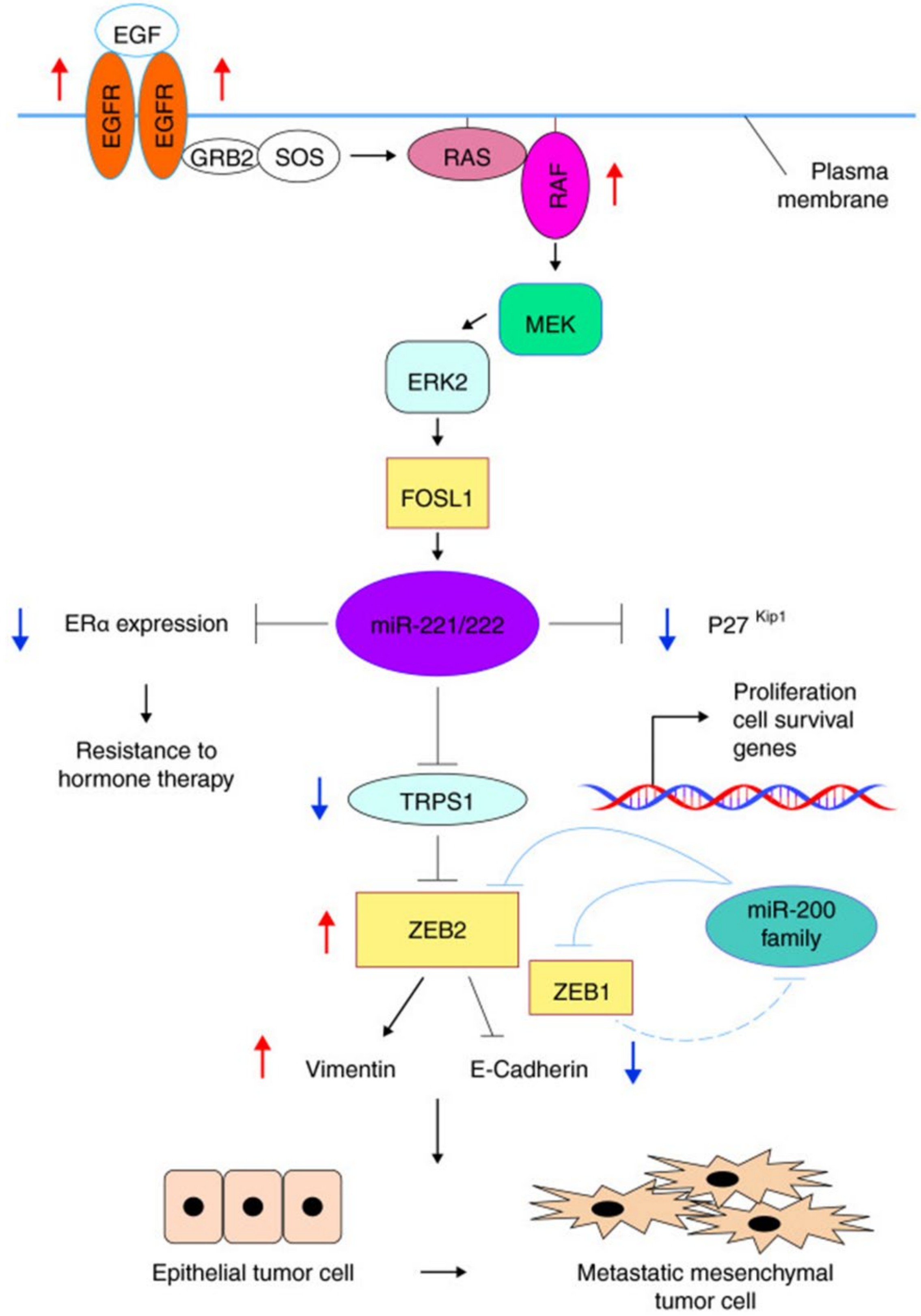


Figure 1.4.11 | Involvement of miR-221/222 as downstream effectors of the EGFR-RAS-RAF-MEK pathway in progression of metastatic transformation of breast cancer tumors. (*Shah MY and Calin GA., 2011*).

1.4.5 miR-338-3p

The miR-338 gene is located on chromosome 17 and produces two mature forms: miR-338-3p and miR-338-5p. miR-338 is located within the 7th intron of the apoptosis-associated tyrosine kinase (AATK) gene [212], and AATK kinase activity plays an essential role in promoting neurite extension in developing neurons [213] (*Figure 1.4.12*).

Previous miRNA array studies indicated that miR-338 was up-regulated in squamous cell carcinoma of the human tongue [214], as well as in 7,12-dimethyl-benz(a)anthrance-induced oral cancer tissues in hamster [215].

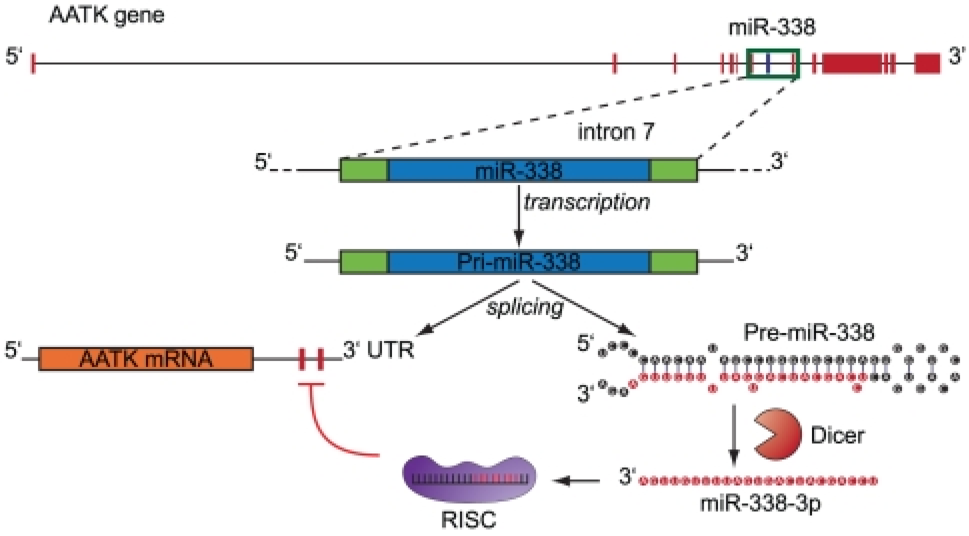


Figure 1.4.12 | Proposed model of AATK regulation by its intronic miR-338. *(Kos A et al., 2012).*

Recently, the expression pattern and function of the miR-338-3p was widely studied in various cancers, and reported to function as a tumor suppressor gene in various cancer, including hepatocellular carcinoma [216], neuroblastoma [217], ovarian cancer [218], malignant melanoma [219], gastric cancer [220, 221] and colorectal cancer [222, 223].

In particular, miR-338-3p was found to inhibit growth, metastasis, and invasion of non-small cell lung cancer (NSCLC) cells [224, 222], while in gastric cancer cells, miR-338-3p suppresses the epithelial–mesenchymal transition, proliferation, and migration [224]. Indeed, a recent study demonstrated that miR-338-3p suppresses the expression of PREX2a by binding to its 3′-UTR, leading to inhibition of neuroblastoma cell growth [217]. Other studies indicated that miR-338-3p targets PREX2a and SSX2IP in gastric cancer cells [222, 223] and smoothened, cyclinD1 and hypoxia inducible factor-1 in liver cancer cells [216, 225].

In the field of breast cancer, it has been observed that miR-338-3p was downregulated in breast tumor samples from patients compared with adjacent normal breast tissues and that the expression of miR-338-3p was inversely correlated with the clinical phases and the metastatic state of breast cancer. Moreover, overexpression of miR-338-3p in breast cancer cells inhibited cell proliferation, migration and invasion in vitro and suppressed tumor growth in vivo [226]. Emphasizing the importance of miR-338-3p, the molecular mechanisms underlying the biological behavior of mammary carcinoma cells by regulating miR-338-3p were further analyzed. The TargetScan and miRanda algorithm was selected to search for putative genetic targets of miR-338-3p protein coding, in particular those that have the ability to promote tumor cell proliferation, migration and invasion. Based on this logic, SOX4 was selected as a potential target for further validation, since it was shown that deregulated expression of SOX4 is related to the increase in tumor cell proliferation, cell survival, apoptosis inhibition and of tumor progression [227, 228]. Sex-determining region Y-box 4 (SOX4) is a member of the highly conserved SoxC (SRY-related high motility group box) transcription factor family, which contains two other members, SOX11 and SOX12 [229]. It is overexpressed in several types of cancer including breast cancer [230]. Moreover, genome-wide chromatin immunoprecipitation studies have uncovered that SOX4 regulates the transcription of genes involved in TGF-β, Wnt, Hedgehog, and Notch pathways and components of miRNA processing machinery including Dicer, Argonaute 1 and RNA Helicase A [231, 232]. It was therefore confirmed that SOX4 is a target of miR-338-3p and that the upregulation of miR-338-3p reduced the expression of SOX4 at the level of mRNA and protein level [226].

Further studies allowed us to analyze the function of miR-338-3p on ovarian cancer cells by several in vitro approaches and in a nude mouse model, we demonstrated that overexpression of miR-338-3p impaired proliferation, colony formation, invasion and migration, and induced apoptosis of various ovarian cancer cells, as well as suppressed tumor growth in a nude mouse model, which is in accordance with previous studies that demonstrated miR-338-3-mediated suppression of cell growth in different types of cancer [216, 220-223]. It was observed the Runx2 oncogene, which was reportedly overexpressed in ovarian cancer tissue [233], as a potential target of miR-338-3p. Runx2, an important member of runt-related transcription factor (Runx) gene family, is a key regulator of normal bone development, homeostasis and remodeling [234] and is aberrantly expressed in several cancer types [235-238], and play a role in invasive breast [235], prostate [236], bone [238], thyroid [239] and pancreatic cancer [240]. miR-338-3p functions as a tumor suppressor in ovarian cancer and directly targets Runx2, and that overexpression of miR-338-3p can reduce the expression of Runx2 protein and the phosphorylation of PI3K (Tyr458) phosphorylation of p-AKT (Serine473, Thr308), suggesting that miR-338-3p inhibits ovarian cancer cell proliferation, migration and invasion through PI3K/AKT signaling pathways by targeting Runx2.

These findings suggest that miR-338-3p mimic is a promising therapeutic strategy for this malignancy.

1.4.6 Regulation of miRNAs by estrogen

Estradiol (E2) and other estrogen receptor (ER) ligands suppress or stimulate miRNA expression in human breast cancer cells, endometrial cells, rat mammary gland, and mouse uterus and post-translationally regulate protein expression. Aberrant miRNA expression is implicated in estrogen-related breast and endometrial cancers and a number of miRNAs downregulate ERα [241].

The first report correlating miRNA expression with estrogen receptor α (ERα) in breast tumors was published in 2005 [6]. The first report on 17β-estradiol (E2) regulation of miRNAs, found that E2 repressed miR-206 which, in turn, repressed ERα protein expression in MCF-7 human breast cancer cells [242].

MiRNAs whose expression is increased in tumor cells, often as a result of chromosomal or molecular genomic aberrations, and which inhibit the translational expression of tumor suppressor genes, are called *oncomiRNA*s or *oncomiRs*. One well-characterized oncomiR in breast, and many other cancer types, is miR-21 which suppresses the expression of the tumor suppressors PTEN and PDCD4 [243, 244]. MiR-21 expression is increased in breast tumors and antisense to miR-21 (AS-miR-21) suppresses MCF-7 cell growth in vitro and in tumor xenografts in mice, by down-regulating the apoptosis regulator BCL-2 [51]. It has also been reported that overexpression of miR-21 in MCF-7 cells increases soft agar colony formation, reflecting increased tumorigenicity of these cells [245]. It was recently demonstrated that miR-21 binds to a seed element in the 3′-UTR of the PDCD4 gene and reduces PDCD4 protein expression [246]. E2 regulates miR-21, although whether it stimulates or represses its transcription varies depending on experimental conditions, cell line, and control genes used in miRNA analysis.

Conversely, tumor suppressor miRNAs (*tsmiRNAs* or *tsmiRs*) show lower expression in tumors and the reduction in their expression allows translation of mRNAs encoding oncogenes and other genes resulting in increased tumor cell proliferation, invasion, and angiogenesis and an inhibition of apoptosis [247].

Because of its role in breast cancer, much of what we know about estrogenic regulation of miRNA expression comes from studies of how E2 treatment of breast cancer cell lines affects mature miRNA expression. Although it is likely that estrogens regulate miRNAs by both genomic (transcriptional) and 'non-genomic' mechanisms of action, e.g., plasma membrane ERα or GPR30- associated signaling cascades, investigators are only beginning to examine these pathways.

The breast cancer oncogene/coactivator AIB1/SRC-3/NCOA3 is regulated by miR-17-5p and there is a reciprocal relationship between reduced miR-17-5p and increased AIB1 in breast cancer cells. Overexpression of miR-17-5p reduced E2-stimulated proliferation of MCF-7 breast cancer cells, indicating a role for deregulation of miR-17-5p in breast cancer [248]. Overexpression of miR-125a and miR-125b decreased ERBB2 and ERBB3 mRNA and protein levels, inhibited phosphorylation of ERK1/2 and AKT, and inhibited the anchorage-independent growth of ERα-negative/ErbB2-overexpressing SKBR3 breast cancer cells [249]. ERα mRNA stability is negatively regulated by miR-206 in MCF-7 cells and miR-206 expression is higher in ERα negative MDA-MB-231 cells [242].

Estrogen signaling plays a critical role in regulating reproduction, lactation, bone density, cardiovascular function, neuronal signaling, immune function, and homeostasis in a wide variety of tissues. The reduction in serum E2 in postmenopausal women is involved in a number of age-associated disorders. Research on the mechanisms by which E2 and other estrogens regulate diverse physiological effects has established both genomic and nongenomic mechanisms involving ERα, ERβ, and GPR30 in signal transduction (*Figure 1.4.13*).

MiRNAs are small, non-coding RNAs that bind to the 3' UTR of target mRNAs and either block the translation of the message or bind the ORF and target the mRNA transcript to be degraded. Although there are a number of studies identifying miRNA changes in breast tumors and comparing ERα-positive versus ERα-negative miRNA signatures for their potential use as biomarkers, there are few studies identifying E2-responsive miRNAs in any normal or neoplastic tissues or cell models. In those few studies that have identified E2-induced alterations in miRNA expression, there is little, if any, mechanistic detail elaborated for the E2 effect (s) on miRNA expression. Further, it appears that E2 regulates miRNA expression in a cell-type-dependent manner. Thus, identification of E2-regulated miRNAs and the function of miRNAs within specific tissues and cells still remains to be determined.

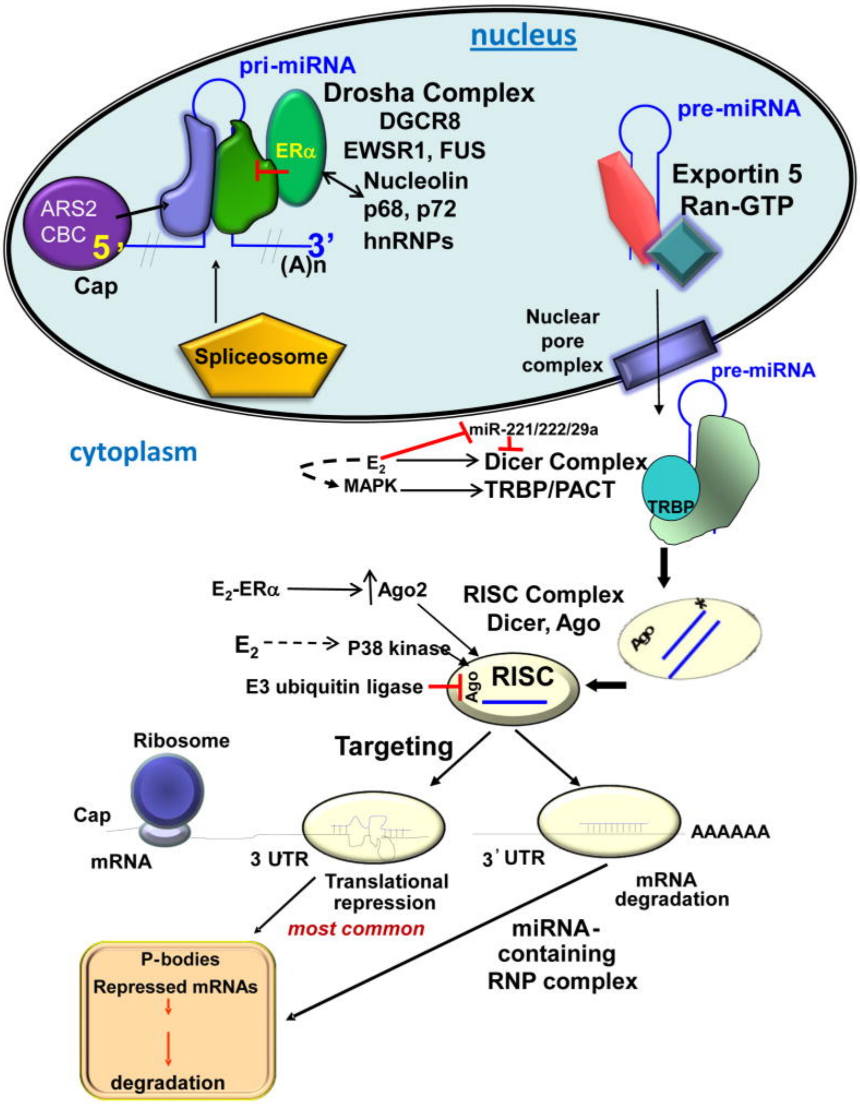


Figure 1.4.13 | miRNA regulation of ERα and subsequent ERα transcriptional activity. *(Klinge CM, 2012).*

1.5 Target genes of miRNAs

1.5.1 A20

TNFα induced protein 3 (TNFAIP3) or A20 is an ubiquitin-editing protein discovered as a gene responsive to tissue necrosis factor α (TNFα) [250]. The first function attributed was that of protection against TNFα-induced cytotoxicity [251]. A20 has a double enzymatic function, with an aminoterminal deubiquitinase OTU (DUB) domain and E3-ligase activity in the carboxyterminal domain, consisting of seven C2-C2 zinc-fingers of which the central is essential for catalysis [252, 253] (*Figure 1.5.1*).

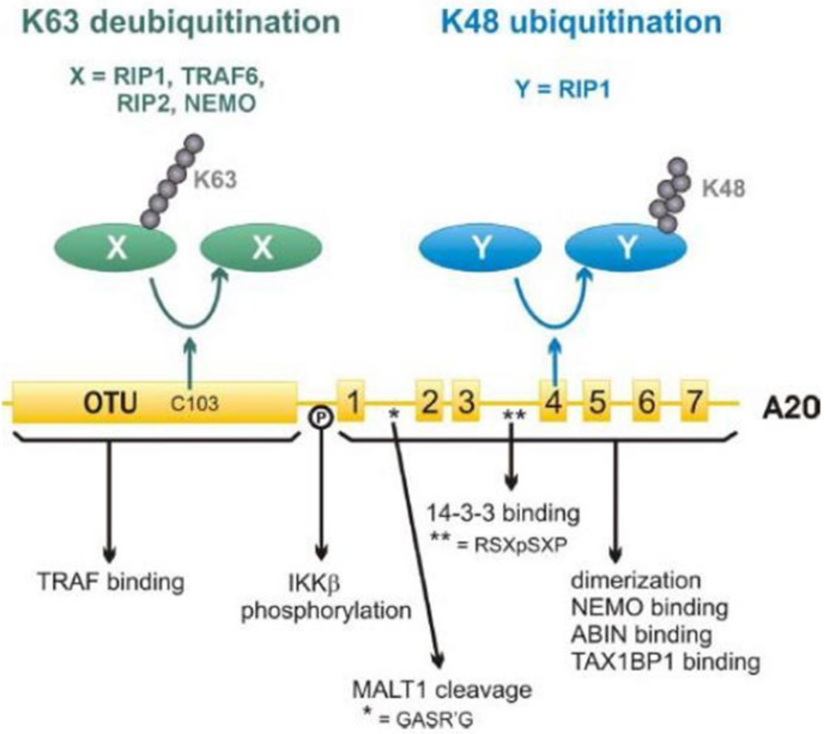


Figure 1.5.1 | Schematic representation of the structural domains of human A20 involved in its ubiquitin-editing function and interaction with regulatory proteins. *(Coorneart B et al., 2009).*

An example of the activity of A20 is RIP1, transfucer of TNFα-activating NF-κB signal. TNFAIP3 deubiquitin RIP1 at the level of K63 chains preventing their interaction with NEMO and therefore PC activation [252-254], while adding chains to the activator of ubiquitin K48, addressing it to the proteasome [253, 255]. A20 does not exert its negative modulation only through RIP1, but it has a role in every pathway that leads to the activation of NF-κB. Downregulation of TNFAIP3 causes aberrant activation, an important contribution to the transformed phenotype. Numerous mutations and variants in A20 have been related to inherited autoimmune diseases [252, 256-259]. In particular, A20 regulates the c-Rel subunit by activating the MALT1 paracaspase, which induces degradation by ubiquitination and addressing to the proteasome [260]. The regulation of A20 is under the control of several factors, including NF-κB, as two κB sequences are present in the promoter region. It is rapidly induced by a vast number of stimuli, not being constitutively expressed by the great majority of tissues [261]. At post-translational level, its activity is finely regulated by a wide range of proteins, with a very different mechanism of action. Positive regulators are ABIN and TAX1BP1, which address it to the substrate, bound by the K63 polyubiquitin chains, recruiting it to NEMO [262], RIP1 and TRAF6 respectively [263, 264]. It is also positively regulated by IKKβ, by phosphorylation on serine 381[265]. As far as downregulation is concerned, we have the main role covered by the MALT1 paracaspase, which operates a site-specific cut, and by degradation via proteasome, establishing a negative feedback circuit with the protease. MiRNAs capable of downregulating their expression were identified and the most relevant to date are miR-125a/b [266], miR-19b [267] and miR-29c [268].

1.5.2 c-Rel

C-Rel is a 587 amino acid protein identified for the first time as a homolog of the v-Rel of the avian Rev-T virus [269]. It belongs to the family of transcription factors Rel together with p105/50, p100/52, RelA (p65) and RelB (*Figure 1.5.2 A*), which dimerizing together constitute the nuclear factor κB (NF-κB). NF-κB has a transcription factor function and is able to induce the expression of approximately 500 target genes by recognizing the κB consensus sequence in the promoter region upstream of the transcripts.

The domain common to all five members is the amino-terminal Rel Homology Domain (RHD). RHD is made up of about 300 amino acids and has three functions: DNA binding, dimerization with components of the same family and binding to proteins with inhibitory function, IκB (*Figure 1.5.2 B*) [259; 270, 271]. IκB inhibit NF-κB activity by binding NF-κB dimers by ankirina repeats, thereby masking the nuclear localization signal (NLS), with subsequent seizure in the cytoplasm [259, 270, 272].

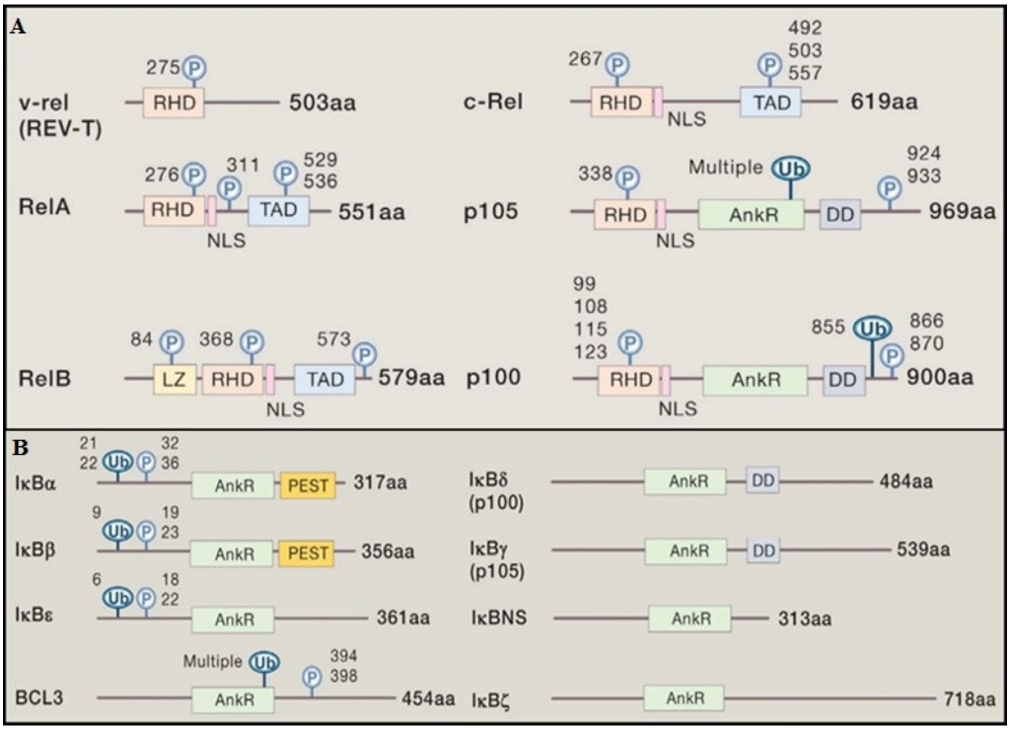


Figure 1.5.2 | (A) Schematic structure of v-Rel and of the five components of the NF-κB family. (B) Schematic structure of the eight components of the IκB family. *(Zhang Q et al., 2017).*

The activation of NF-κB passes through two main pathways: the canonical pathway (CP) and the alternative pathway (AP) (*Figure 1.5.3*).

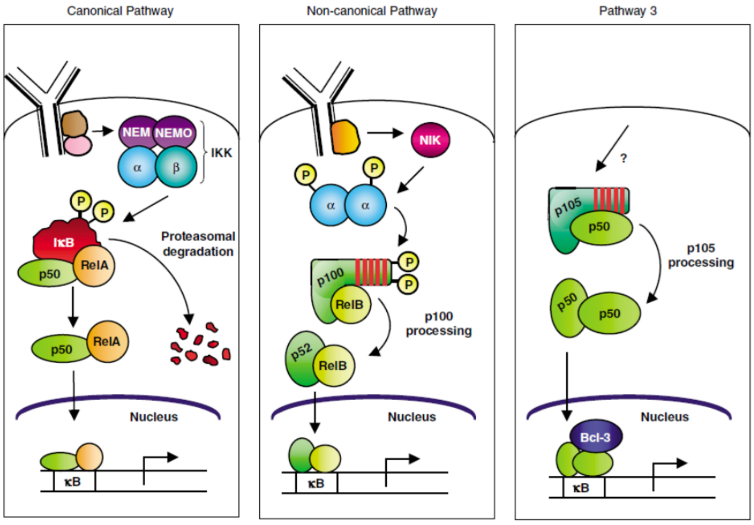


Figure 1.5.3 | NF-κB activation pathways. *(Gilmore TD, 2006).*

The final outcome of both pathways is the phosphorylation and ubiquitination of IκB with consequent degradation of cytoplasmic anchors and detection of NLS. The CP is induced by numerous factors, such as TNFα, cytokines, chemokines, mitogens, various growth factors and bacterial and viral components and leads mostly to activation of the p50 dimer: p65. The transduction of these numerous signals leads to the formation of the IKK complex, consisting of IKKα, IKKβ and NEMO or IKKγ (*Figure 1.5.4*). While the α and β subunits have a kinase catalytic function, NEMO has a regulatory function: it is in fact responsible for the tathering of IKKα and IKKβ on IκB, with consequent phosphorylation. This modification in turn triggers the ubiquitination and addressing to the IκB proteasome. The NLS can then be recognized and NF-κB, the κB sequences and regulate the transcription of target genes [259]. In the alternative pathway, which leads to the activation of p52: RelB, we have instead that the trigger signal leads to the phosphorylation of an IκBα:IκBα homodimer by the NF-κB inducing kinase (NIK). The homodimer then phosphorylates p100, associated with RelB, inducing its maturation through proteolytic shear of the ankirina repeats, which masked the recognition sequence for importin α/β1 (*Figure 1.5.3*) [259; 272, 273].

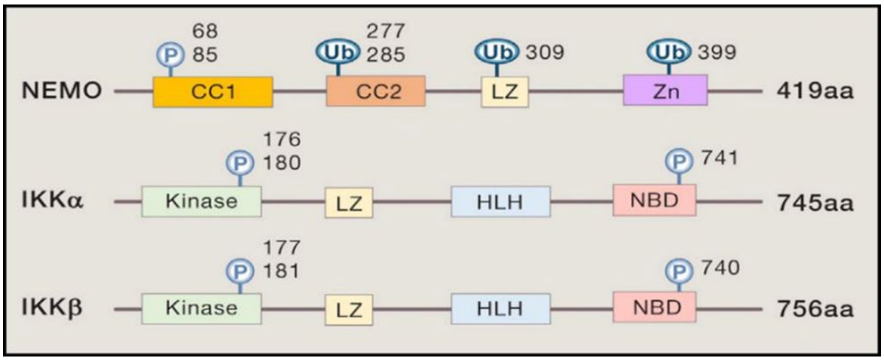


Figure 1.5.4 | Schematic structure of the three components of the IKK complex. *(Zhang Q et al., 2017).*

C-Rel possesses a high degree of homology to RelA and RelB due to the carboxyterminal transactivation domain (TAD), which confers the ability to bind recognized sequences in promoters to induce transcription of valley genes [274]. C-Rel mainly regulates antiapoptotic genes, such as Bcl-x, forming heterodimers preferentially with RelA and p50, although other combinations have been described [269].

NF-κB is a key transcription factor in phlogistic processes, and these are strongly related to carcinogenesis. In fact, inflammation involves the generation of reactive oxygen species (ROS) and nitrogen (RNS), which induce genotoxic effects and recall lymphocytes and macrophages in phlogistic foci with consequent tissue damage and fibrosis [273, 274]. This last process in particular has been correlated with the oncogenic action of c-Rel, identified as a promoter of the deposition of collagen fibers in different neoplasms. Numerous studies have shown how mutations in c-Rel are the cause of solid and hematological tumors. Amplifications of the c-Rel coding gene were found in 50% of Hodgkin's lymphomas and in many diffuse large B-cell lymphomas [274] as well as in pancreatic, stomach, head and breast tumors. In breast cancer, the expression of c-Rel is very high both in cell lines and in murine models from the earliest stages of carcinogenesis. In particular, in Mus musculus its over-expression leads to the synthesis of Cyc D1, c-Myc, and BCL-xL [275] with consequent development of adenocarcinoma [276].

1.5.3 Connective Tissue Growth Factor (CTGF)

Connective tissue growth factor (CTGF), also known as CCN2, is a member of the CCN family, including cysteine-rich protein 61 (Cyr61), also known as CCN1, and nephroblastoma-overexpressed gene (Nov), also known as CCN3, as well as Wisp-1/elm1 (CCN4), Wisp-

2/rCop1 (CCN5) and Wisp-3 (CCN6) [277, 278]. All members of the CCN gene family possess a secretory signal peptide at the NH2 terminus, indicating that they are secreted proteins.

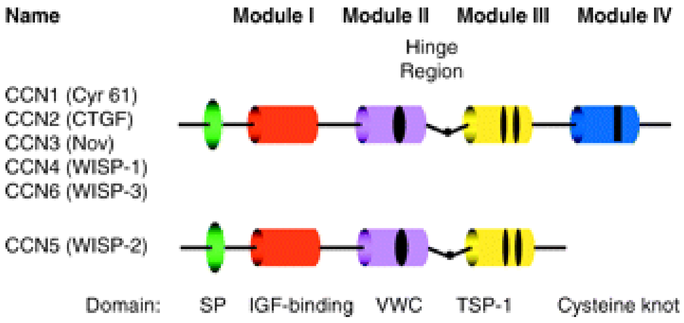


Figure 1.5.5 | Structure of CCN family members. The CCN family members, CCN1 (Cyr61), CCN2 (CTGF), CCN3 (nov), CCN4 (WISP-1), CCN5 (WISP-2) and CCN6 (WISP-3). *(Leask A and Abraham DJ, 2006).*

CTGF is a potent angiogenic factor [279, 280], which was first identified as a mitogen, detected in conditioned medium from human umbilical vein endothelial cells [281].

CTGF can bind to integrins on cell surface [277], and is a potent stimulator of endothelial cell adhesion, proliferation, migration and angiogenesis in vivo [279,280, 282]. Integrin is an important receptor for CCN proteins, and receptor activation may produce a variety of effects. CTGF protein can bind directly to integrins αvβ3 and αIIbβ3 [280, 282]. It has been reported that CTGF mediates endothelial cell adhesion and migration through binding to integrin αvβ3, prolong endothelial cell survival, and induce angiogenesis in vivo. Furthermore, CTGF is a downstream mediator of TGF-β1 action in cancer-associated reactive stroma, and one of the key promoters of angiogenesis in tumor-reactive stromal microenvironment, and plays an important role in prostate carcinogenesis [283]. It was reported that CTGF plays an important role in the progression of several types of cancer. Elevated CTGF levels have been detected in a number of cancers including pancreatic cancer [284, 285], breast cancer [286, 287] prostate cancer [283], esophageal adenocarcinoma [288], glioma [289] and melanoma [290].

In particular, breast cancer stage is positively associated with tumor size, lymph node metastasis status and over-expression of CTGF [287]. Furthermore, apoptosis of MCF-7 cells induced by TGF-β appears to be mediated by CTGF, suggesting that CTGF may play an important role in human breast cancer cell growth [291]. Elevated level of CTGF is significantly correlated with a good prognosis of colorectal cancer [292] and lung adenocarcinoma [293], suggesting that the role of CTGF in different types of cancer may vary considerably, depending on the tissue involved.

1.5.4 c-Fos

The Fos family of transcription factors includes c-Fos (the human homolog of the retroviral oncogene v-Fos), FosB, Fra-1 and Fra-2 as well as smaller FosB splice variants FosB2 and deltaFosB2. Together with Jun family members (c-Jun, JunB and JunD) they form the group of AP-1 proteins which, after dimerisation, bind to so-called TPA-responsive elements (TRE's; TGAC/GTCA) in the promoter and enhancer regions of target genes. Since TRE-containing promoter constructs are strongly activated by the tumour promoter TPA [294] and the first AP-1 proteins (c-jun and c-Fos) to be discovered were found to be transforming in NIH3T3 rat fibroblasts [295], the AP-1 complex was implicated in carcinogenesis soon after discovery.

In contrast to Jun proteins, Fos family members are not able to form homodimers, but heterodimerise with Jun partners, giving rise to various trans-activating or trans-repressing complexes with different biochemical properties. In vitro studies have shown that Jun–Fos heterodimers are more stable and have stronger DNA-binding activity than Jun–Jun homodimers [296, 297]. In F9 teratocarcinoma cells, c-Fos enhanced the trans-activating and transforming properties of c-Jun and JunB [298]. Thus, the expression of Fos proteins might be crucial for the activity of AP-1-regulated genes.

All AP-1 proteins are characterised by a basic leucine-zipper region for dimerisation and DNA-binding. Yet, while c-Fos and FosB proteins harbor a C-terminal transactivation domain, Fra-1, Fra-2 and FosB2 lack this region (*Figure 1.5.6*).

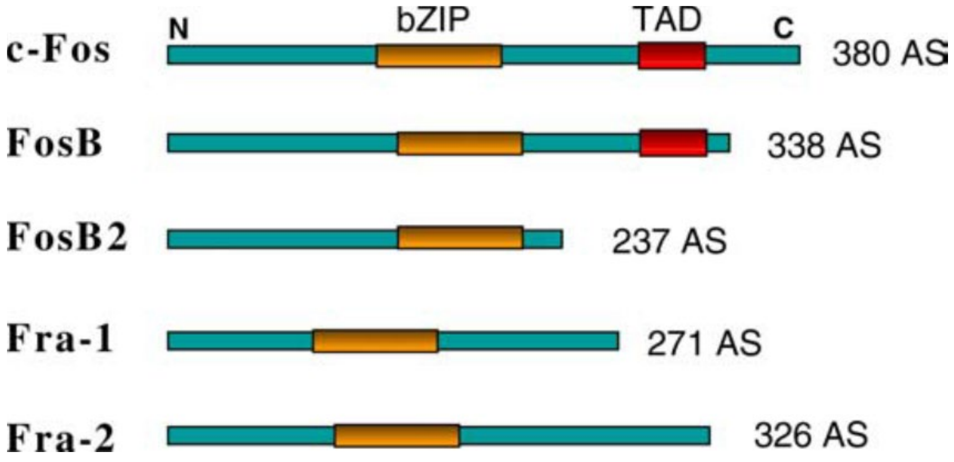


Figure 1.5.6 | Schematical presentation of the structure of Fos proteins. AS, amino acids; bZIP, basic leucine zipper region for dimerisation and DNA-binding; TAD, C-terminal transactivating domain. *(Tulchinsky E, 2000).*

Accordingly, these proteins are not transforming in rat fibroblasts, and an inhibitory function of these factors on AP-1 activity has been proposed [299]. In many tumours, these non-

transforming Fos proteins, especially Fra-1 and Fra-2, might be involved in the progression of many tumour types. Upon stimulation of fibroblasts by serum, c-Fos and FosB are rapidly and transiently induced, whereas Fra-1 and Fra-2 expression is delayed and more stable. This is probably achieved by activation of the Fra-1 and Fra-2 promoters by Jun/Fos dimers [300].
The activity of all Fos family members is also modulated by posttranslational modification: phosphorylation by different kinases, i.e., MAPK, cdc2, PKA or PKC influences protein stability, DNA-binding activity and the trans-activating potential of the transcription factors [301-303]. AP-1-regulated genes include important regulators of invasion and metastasis; proliferation, differentiation and survival; genes associated with hypoxia; and angiogenesis (*Figure 1.5.7*).

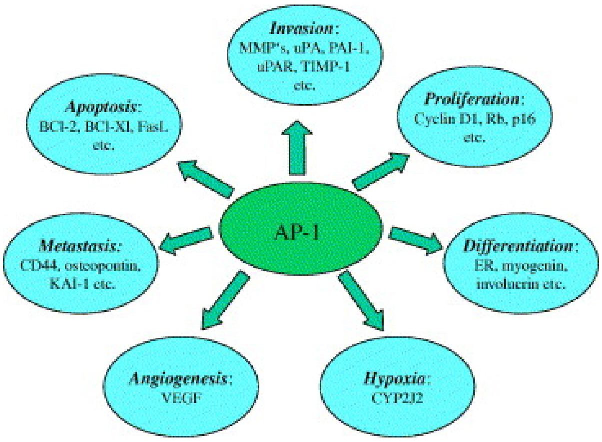


Figure 1.5.7 | AP-1-responsive genes in cancer. *(Milde-Langosch K, 2005).*

Many oncogenic signalling pathways converge at the AP-1 transcription factor complex. The specific influence of a specific AP-1 protein on a promoter depends on the dimer partners, the promoter architecture as well as other transcription factors and co-activators acting on the promoter Therefore, one AP-1-regulated gene might be preferentially induced by Jun–c-Fos, while another gene is mainly induced by JunD/Fra-1 dimers. Experimental data have also shown that single characteristics of the transformed phenotype (anchorage independence, serum independent growth and others) are triggered by specific Jun–Fos or Jun–ATF dimers [304]. Generally, Fos family members have both overlapping as well as unique roles, and function in a tissue-specific way. More recent studies have included the analysis of expression and/or activity of all Jun and Fos family members. Using this approach, it was demonstrated in several experimental systems that malignant transformation and progression is accompanied by a cell-type specific shift in AP-1 dimer composition.

1.5.5 Cyclin D1

The cell cycle is divided into four consecutive phases: G1 phase, during which cells accumulate mass and metabolites necessary for DNA replication; S phase, when DNA is replicated; G2, a gap phase that is essential to ensure accurate DNA replication; and M phase, DNA segregation and cell division. While the primary phases of cell division define states of proliferation and division, the majority of adult cells are maintained in a quiescent state, known as G0 phase, a resting state cells often enter post-mitotically or prior to terminal differentiation [305]. Unlike cells many terminally differentiate cells however, quiescent cells can renter the cell cycle in G1 phase when exposed to appropriate mitogenic stimuli [306]. Transitions through the cell cycle are driven by cyclins and cyclin-dependent kinases (CDKs) [305]. Cyclins are the allosteric activators of cognate CDKs; their levels typically oscillate across the cell cycle, hence gaining the name cyclins. The cyclin family shares a homologous N-terminal 100-amino acid motif referred as the cyclin box that has a highly conserved three-dimensional structure and provides the binding interface for the appropriate CDKs [307]. CDKs define the partner kinases that can be activated only when they bind to their cognate cyclins. Due to their biological significance, CDK activity is stringently regulated by the following mechanisms: the levels of cyclin partners; phosphorylation status; and the abundance of CDK inhibitory proteins, such as, the INK4 family: $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ and the CIP and KIP family: $p21^{CDKN1A}$, $p27^{CDKN1B}$ and $p57^{CDKN1C}$ [308] (*Figure 1.5.8*).

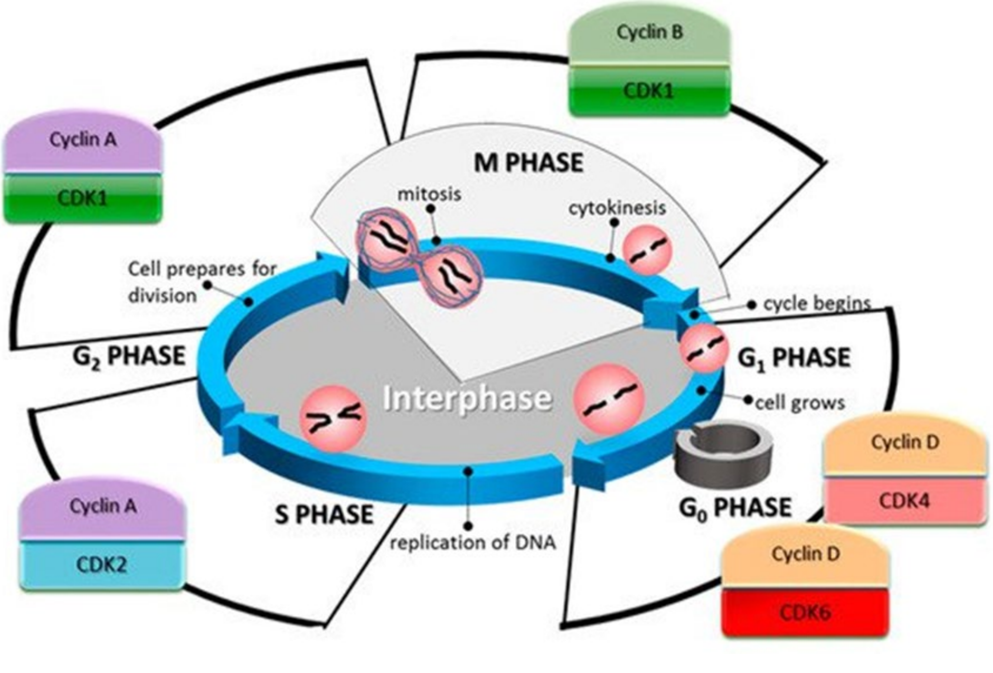


Figure 1.5.8 | The cell cycle phases and their associated cyclin-dependent kinases (CDK)/cyclin complexes. *(García-Reyes B et al., 2018).*

D cyclins, including cyclins D1, D2 and D3, form active complexes with either CDK4 or CDK6, which in turn phosphorylate the retinoblastoma protein (Rb) and drive G1 to S phase progression [309]. D cyclins coordinate cell cycle progression with the extracellular stimulation (e.g. growth factor availability, nutrient availability and integrin-derived adhesion signaling) [310]. Given the role of D cyclins in mediating extracellular cues with cell proliferation, it is not surprising that overexpression of D cyclins or hyperactivation of their cognate CDK directly contributes to neoplastic growth. In particular, cyclin D1 has attracted widespread attention due to the prevalence of its dysregulation in human cancers [311].

Cyclin D1, with its partner CDKs, regulates G1/S transition through Rb phosphorylation [312, 313]. Small polypeptide inhibitors of CDK4/CDK6 efficiently block Rb phosphorylation in vivo. Moreover, Rb is also phosphorylated by cyclin E-CDK2 in the late G1 phase. The hyperphosphorylation of Rb triggers reduced affinity for E2F, thereby permitting E2F activation and transcription of client genes required for cell division [305]. In human tumors, the cyclin D1-CDK4 axis shows a high frequency of alterations, highlighting the importance of this pathway for tumor progression.

In addition to CDK regulation, cyclin D1 has also been implicated in the regulation of gene transcription. Its function as a transcriptional regulator invokes both CDK -independent and -dependent mechanisms. Cyclin D1 can associate with a variety of transcriptional regulators including chromatin modifying enzymes such as histone acetyl transferases P/CAF, NcoA/SRC1a, AIB-1, GRIP-1, TFIID, and TAFII250 [96] and can also function as a co-repressor through recruitment of Histone Deacetylases (HDAC) 3; this repression can be alleviated by trichostatin A treatment [314]. Cyclin D1 also interacts with sequence specific DNA binding proteins such as the estrogen receptor, the androgen receptor and the myb like protein, DMP1 [315-317]. It is interesting to note that such association is generally correlated with transcriptional repression.

Cyclin D1 expression is responsive to a variety of growth factors [318], among which EGF is a classic mediator [319]. EGFR overexpression and/or hyperactivation correlates with poor prognosis in human cancers, including breast cancer, non-small cell lung carcinoma, and colon carcinoma [320]. As a mitogenic growth factor, EGF regulates prostate cancer cell proliferation at least partially through regulating cyclin D1 expression [321], and it regulates cyclin D1 accumulation at both mRNA and protein levels. ErbB2, also known as Neu or Her2, is implicated in 20-30% of human breast cancer [322]. Here again, cyclin D1 expression is induced by Her2/Neu, Ras, Rac, Rho, c-Jun N-terminal kinase and p38 [323]; it is of equal importance that cyclin D1-CDK4 function is required for Her2-driven mammary carcinoma

[323-325]. This contributed directly to the use and therefore to the success of CDK4/6 inhibitors in patients with HER2 positive breast cancer [326].

Cyclin D1 is overexpressed and/or amplified in a large fraction of human cancers [327]. Cancers that frequently harbor cyclin D1 genomic alterations include pancreatic cancer [328], non-small cell lung carcinoma [327, 329, 330], breast cancer [331], head and neck squamous cell carcinoma (HNSCC) [332, 333], melanoma [334], endometrial cancer [335, 336], and colorectal carcinoma [311]. Cyclin D1 mutations can directly perturb its degradation. In esophageal and uterine cancers, mutations that directly target the GSK-3β phosphorylation site, or disruption of the adjacent nuclear export signal are frequently observed [337, 338]. In addition to mutations in cyclin D1, Fbxo4 also undergoes hemizygous missense mutations (S8R, S12L, P13S, L23Q, G30N and P76T), accounting for 14% of the primary esophageal tumors [339]. Such mutations result in cyclin D1 overexpression, being consistent with Fbxo4 mediated cyclin D1 degradation. Mutations in cyclin D1 (P287S, P287T and delta289-292) have also been reported in endometrial cancers [340]. Other dimensions of cyclin D1 upregulation depend on the activation of mitogenic signaling pathways, such as, Ras-MEK-Erk, PI3K-Akt, ErbB2 oncogenic pathways [318, 341-347], and loss of microRNAs (miRNAs) that control cyclin D1 mRNA stability, for example, miR-15a and miR-16 in prostate cancers [348].

1.6 Aim of study

Based on previous studies showing the oncogenic action of miR-221 in hematological and solid tumors, in this work we aimed to provide new knowledge on the molecular mechanisms involved in miR-221-induced progression in breast tumor, by overexpressing in CAFs and in breast cancer cells, MDA-MB-231 and SkBr3, a miR-221 mimic sequence alone or in combination with a specific inhibitory construct, namely LNA-i-miR-221. Furthermore, we have ascertained the involvement of estrogens in miRNA expression, identifying in SkBr3 breast cancer cells and in CAFs a profiling pattern of miRNAs modulated by 100nM E2. In particular, we have elucidated the molecular mechanism involved in the estrogenic down-regulation of miR-338-3p through GPER in these cell types.

CHAPTER 2

Materials and Methods

2.1 Bionformatic tools

The putative promoter sequences of CTGF (− 625 bp/+ 62 bp) and A20 3'-UTR were retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Prediction of transcription factors within CTGF promoter was performed using TransFac (http://www.generegulation.com) site and Promo 3.0.2 (http://alggen.lsi.upc.es/).

miR-221 and miR-338-3p target genes were identified using miRNAbase (http://www.miRNAbase.org), Targetscan (http://www.targetscan.org) and miRDip (http://ophid.utoronto.ca/mirDIP/) sites.

2.2 Cell cultures

The breast cancer cell lines triple-negative, MDA-MB 231, overexpressing epidermal growth factor receptor 2, ER-negative and GPER-positive, SkBr3 and non-malignant breast epithelial cell, MCF10A, were obtained from the ATCC (Manassas, USA) used less than six months after revival and routinely tested and authenticated according to the ATCC suggestions.

SkBr3 and MDA-MB 231 breast cancer cells were maintained, respectively, in RPMI-1640 without phenol red and DMEM/F12 media (Life Technologies, Milan, Italy) with a supplement of 10% fetal bovine serum (FBS) and 100 µg/ml of penicillin/streptomycin (Life Technologies, Milan, Italy).

MCF10A cell line were cultured in DMEM/F12 media (Life Technologies, Milan, Italy).

CAFs (ER-negative and GPER-positive) were extracted from invasive mammary ductal carcinomas obtained from mastectomies. Briefly, samples were cut into smaller pieces (1–2 mm diameter), placed in digestion solution (400 IU collagenase I, 100 IU hyaluronidase, and 10% FBS, containing antibiotic and antimycotic solution) and incubated overnight at 37° C. The cells were then separated by differential centrifugation at 90xg for 2 min. Supernatant containing fibroblasts was centrifuged at 485xg for 8 min; the pellet obtained was suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1 and supplemented with

10% FBS) and cultured at 37° C in 5% CO2. Primary cells cultures of breast fibroblasts were characterized by immunofluorescence. Cells were incubated with human anti-vimentin (V9, sc-6260) and human anti-cytokeratin 14 (LL001 sc 53253), both from Santa Cruz Biotechnology (DBA, Milan, Italy). To characterize fibroblasts activation, we used anti-fibroblast activated protein α (FAPα) antibody (SS-13, sc-100528; Santa Cruz Biotechnology, DBA, Milan, Italy). Signed informed consent from all the patients was obtained and samples were collected, identified and used in accordance with approval by the Institutional Ethical Committee Board (Regional Hospital, Cosenza, Italy).

CAFs were cultured in a mixture of MEDIUM 199 and HAM'S F-12 (1:1) supplemented with 10% FBS and 100 μg/mL of penicillin/streptomycin (Gibco, Life Technologies, Milan, Italy). All cell lines were grown in a 37 °C incubator with 5% CO2.

Cells were switched to medium without serum the day before experimental analysis.

2.3 Reagents

17 β-estradiol (E2) was purchased from Sigma-Aldrich Corp. (Milan, Italy); rel-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone (G-1) was obtained from Tocris Bioscience (Space, Milan, Italy). All compounds were solubilized in dimethyl sulfoxide (DMSO).

2.4 Plasmid and transfection

The mimic miR-221 (miR-221) and negative control (miR-Ctrl) sequences were purchased from Ambion (Life Technologies, Milan, Italy). Custom LNA oligonucleotides were provided by Exiqon (Vedbaek, DenMark).

LNA-i-miR-221 is a 13-mer DNA/LNA oligonucleotide whose sequence is CAGACAATGTAGC, with a fully PS-modified backbone. It was purified by HPLC followed by Na + salt exchange and lyophilisation. The CTGF luciferase reporter plasmid p(− 1999/+ 36)-Luc (CTGF-luc), based on the backbone of vector pGL3-basic (Promega), was a gift from Dr. B. Chaqour. c-Rel shRNA sequence (shRel) was obtained from TRC consortium (TRCN0000039986) and cloned, as described previously, in the piggyback transposon vector (PB-H1-shRNA-GFP), kindly provided by Dr. W.T. Khaled, University of Cambridge, UK.

The shRNA construct for CTGF, obtained from the Open Biosystems (www.Biocat.de), contains the clone ID TRCN0000061950 and is based on the lentiviral expression vector pLKO.1.

The negative control (miR-Ctrl), the miR-338-3p mimic (miR-338-3p m) (ID MC10716) and miR-338-3p inhibitor (miR-338-3p i) (ID MH10716) sequences were purchased from Ambion (Life Technologies, Milan, Italy) and transfected into the cells 48 h before the treatments, using X-treme GENE 9 DNA Transfection Reagent (Roche Diagnostics, Sigma-Adrich, Milan, Italy).

Silencing of GPER expression was obtained by using the construct previously described [83].

The plasmid DN-Fos, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA binding, was a kind gift from Dr. C. Vinson (NIH, Bethesda, MD, USA).

2.5 RNA extraction and real time-PCR

Cells were maintained in regular growth medium and then switched to medium lacking serum before the transfection with 25 nM miR-Ctrl, miR-221, miR-338-3p, miR-338-3p inhibitor, 100 nM LNA-i-miR-221 and 5 µg short hairpin plasmids, as indicated.

Total RNA were extracted from cultured cells using miRVana Isolation Kit (Ambion, Life Technologies, Milan, Italy) according to the manufacturer's recommendations.

The RNA concentrations were determined using Gene5 2.01 Software in Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, AHSI, Milan Italy).

cDNA for miRNA expression was synthesized from 100 ng of total RNA using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Milan, Italy) and the expression levels of miR-221 and miR-338-3p were quantified by TaqMan microRNA Assay Kit (Applied Biosystems, Life Technologies, Milan, Italy).

The expression levels of miR-221 and miR-338-3p were quantified by TaqMan microRNA Assay Kit (Applied Biosystems, Milan, Italy), using the primers for the internal control RNU6B (assay ID 001093), miR-221 (assay ID 000524) and miR-338-3p (assay ID 002252).

In order to measure the mRNA levels of A20, CTGF, c-Fos and Cyclin D1, 3 µg of total RNA were reversely transcribed using the murine leukaemia virus reverse transcriptase (Life Technologies, Milan, Italy), as indicated by the manufacturer. The quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Milan, Italy).

Specific primers for Actin, which was used as internal control, A20, CTGF, c-Fos and Cyclin D1 genes were designed using Primer Express version 2.0 software (Applied Biosystems Inc., Milano, Italy).

The sequences were as follows:

Actin Fwd: 5’-AAGCCAACCCCACTTCTCTCTAA-3′ and Rev.: 5’-CA CCTCCCCTGTGTGGACTT-3′; A20 Fwd: 5’-CTTGTG GCGCTGAAAACGAA-3′ and Rev.: 5’-CTGAACGCCCCACATGTACT- 3′; CTGF Fwd: 5’-GGCCCAGACCCAACTATGATT-3′ and Rev.: 5’-GAACAGGCGCTCCA CTCTGT-3′; c-Fos Fwd: 5’-CGAGCCCTTTGATGACTTCCT-3’ and Rev: 5’-GGAGCGGGCTGTCTCAGA-3’; Cyclin D1 Fwd: 5’-CCGTCCATGCGGAAGATC-3’ and Rev: 5’-ATGGCCAGCGGGAAGAC-3’.

All experiments were performed in triplicate using QuantStudio 6&7 Flex Real Time PCR System (Applied Biosystems, Life Technologies, Milan, Italy). The data were normalized to the geometric mean of housekeeping gene to control the variability into expression levels and fold changes were calculated by relative quantification compared to respective scrambled controls.

2.6 miRNA Expression Profiling

TaqMan™ Array Human MicroRNA A+B Cards Set v3.0 was used for global miRNA profiling. The panel includes two 384-well microfluidic cards (human miRNA pool A and pool B) that contain primers and probes for 754 different miRNAs in addition to small nucleolar RNAs that function as endogenous controls for data normalization. Equal quantity (100 ng) of RNA extracted from SkBr3 breast cancer cells and CAFs treated with vehicle or 100 nM E2 for 4 h was reverse-transcribed for cDNA synthesis using the Megaplex RT Primer Pool A or B and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems).in a final volume of 7.5 μL (Applied Biosystems, Milan, Italy).

The reverse transcription reaction was incubated for 2 min at 16 °C, 1 min at 42 °C and 1 s at 50 °C for 40 cycles, followed by 5 min at 85 °C to deactivate the enzyme. The cDNA obtained was pre-amplified using Megaplex Preamp primer pool A or B and TaqMan PreAmp Master Mix 2X in a final volume of 25 μL using the same temperature conditions above described. The product was diluted 1:4 in TE 0.1X, to which were added TaqMan Universal Master Mix no UNG 2X and nuclease free water. 100 μL of the sample/master mix for each multiplex pool were loaded into fill reservoirs on the microfluidic card. The array was then centrifuged, mechanically sealed with the Applied Biosystems sealer device and run on QuantStudio 6&7 Flex Real Time PCR System (Applied Biosystems, Life Technologies, Milan, Italy). The raw array data were analysed by DataAssist^{TM}.

The baseline was set automatically, while the threshold was set manually at 0.2. Samples that had Ct values >32 were removed from the analysis. Each miRNA was normalized against the mean of the four RNU6B and its expression was then assessed in the E2 treated cells against the vehicle treated cells using the $2^{-\Delta\Delta CT}$ method [349]. miRNAs showing an increased value of 2-fold expression and a 50% reduction respect to vehicle-treated cells were selected. Venn diagram was obtained by http://bioinformatics.psb.ugent.be/cgibin/liste/Venn/calculate_venn.htpl.

2.7 Analysis of Public Data Set from METABRIC and Kaplan – Meier Plotter

Prognostic values of miR-338-3p levels, using METABRIC data set, were analyzed by Kaplan - Meier survival curves of breast cancer patients, using Kaplan-Meier Plotter (www.kmplot.com/analysis) [350]. Log-rank test was used for statistical analysis.

2.8 Western blotting

Cells were maintained in complete medium before the transfection assays, which are performed in medium without serum for 48 h and then treated as indicated. Cells were lysed in RIPA buffer containing a mixture of protease inhibitors. Equal amounts of protein extract were resolved on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences, Italy), probed overnight at 4 °C with antibodies against: A20 (A-12, sc-166,692), β-Actin (AC-15, sc-69,879) and c-Fos (E-8, sc-166940) (Santa Cruz Biotechnology, DBA, Italy), CTGF and Cyclin D1 (Origene, DBA, Milan, Italy), c-Rel (Cell Signaling Technology, Milan, Italy), GPER (AB137479) (Abcam, Euroclone, Milan, Italy). Proteins were detected by horseradish peroxidase-linked secondary antibodies (Biorad, Milan, Italy) and revealed using the chemiluminescent substrate for western blottingWestar Nova 2.0 (Cyanagen, Biogenerica, Catania, Italy).

2.9 Chromatin immunoprecipitation (ChIP) assay

Cells were shifted for 24 h to medium lacking serum and then transfected for 48 h with miR-Ctrl and 25 nM miR-221 alone or in presence of 100 nM LNA-i-miR-221. ChIP assay was performed as previously described [24]. In brief, the immune cleared chromatin was immunoprecipitated with anti c-Rel or non specific IgG (Santa Cruz Biotecnology, DBA), used as negative control. A 4 µl volume of each sample and input DNA was used as template to amplify, by real-time PCR a region containing a NF-κB binding site located within the promoter region of CTGF. The primer sequences were: 5'-ACGGAGGAATGCTGAGTGTC-3′ (forward) and 5'- GGCGGCCGAGGCTTTTATAC-3′ (reverse). Real-time PCR data were normalized respect to unprocessed lysates (Input) and the results were reported as fold changes respect to non specific IgG.

2.10 Luciferase assay

Cells were seeded in regular growth medium into 24-well plates. The next day the growth medium was replaced with medium lacking serum and the transfection was performed using X-tremeGene9 reagent, as recommended by the manufacturer (Roche Diagnostics), with a mixture containing CTGF-luc, the internal control pRL-TK, miR-Ctrl or miR-221, alone or in presence of LNA-i-miR-221, shRNA or shRel, alone or in combination with miR-221, Cyclin-D1-luc, the internal control pRL-TK and miR-Ctrl, miR-338-3p m, alone or in presence of miR-338-3p i, shGPER, DN-Fos as indicated.

SkBr3 and CAFs were treated overnight with 100 nM of E2 or G1.

Luciferase activity was measured after 48 h using the Dual Luciferase kit (Promega, Milan, Italy) according to the manufacturer's instructions. Firefly luciferase values were normalized to the internal transfection control provided by the Renilla luciferase activity. The normalized relative light unit (RLU) values obtained from cells transfected with respective scrambled controls were set as 1-fold induction upon which the activity induced by miR-221 and by the treatment was calculated.

2.11 Cell proliferation assay

Cells were seeded in 24-well plates in regular growth medium. After cells had attached, they were washed and then incubated in medium containing 2.5% charcoal stripped fetal bovine serum, before the transfection with 25 nM miR-221, 100 nM LNA-i-miR-221, 500 ng of the indicated short hairpins and before the transfection with 25 nM miR-338-p m and 50 nM miR-338-3p i, as indicated. After 48 h the medium was changed and cell transfection was renewed every 2 days, while the cells were treated every day. Evaluation of cell growth was performed on day 6 using automatic counter (Countess™-Invitrogen).

2.12 Colony formation assay

Cells were transfected as indicated, and then seeded into 6-well plates (2.5 × 104 or 5 × 104 cells per well). The transfections were renewed every 2 days during the assay. After 10 days of incubation, cells were washed with PBS, fixed in acetone:methanol (1:1) for 3 min at room temperature and then stained with 0.5% crystal violet in 20% methanol for 5 min or Giemsa for 10 min. Pictures were captured by using a digital camera. Colonies, with over 50 cells, were counted using the program WCIF ImageJ for Windows.

2.13 Migration assay

Migration assays were performed by using Boyden Chambers (Costar Transwell, 8 mm polycarbonate membrane, Sigma Aldrich, Milan, Italy). Cells were transfected with 25 nM miR-221, 100 nM LNA-i-miR-221 and 500 ng/well shRel or shCTGF as indicated, in medium without serum for 48 h and then seeded in the upper chambers. In the bottom of the chambers was added regular medium. 8 h after seeding the cells on the bottom side of the membrane were fixed, stained with Giemsa and counted by using Cytation 3 Cell Imaging Multimode Reader (BioTek, Winooski, VT).

2.14 Cell cycle analysis

CAFs were cultured in regular medium and shifted in medium containing 2.5% charcoal-stripped FBS at the 70% confluence. Next, miRNA sequences as indicated were added to cells using XtreamGene9 reagent (Roche Diagnostics, Milan, Italy). After 24 h, 100 nM E2 or 100 nM G-1 were put in the medium for additional 24 h. Cells were pelleted, once washed with phosphate buffered saline and stained with a solution containing 50 µg/mL propidium iodide in 1 x PBS (PI), 20 U/mL RNAse-A and 0.1% Triton (Sigma-Aldrich, Milan, Italy). The DNA content was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data acquired using CellQuest software. Cell cycle profiles were determined using ModFit LT. The proportion of the cells in G0/G1, S and G2/M phases was each estimated as a percentage of the total events (10,000 cells).

2.15 Statistical analysis

Data were analysed by one-way ANOVA with Dunnett's multiple comparisons where applicable, using GraphPad Prism version 6.01 (GraphPad Software, Inc., San Diego, CA, USA). $p < 0.05$ (*) and $p < 0.01$ (**) were considered statistically significant.

CHAPTER 3

Results

3.1 miR-221 down-regulates A20 expression in CAFs, MDA-MB 231 and SkBr3 breast cancer cells

On the basis of previous studies showing that miR-221 may act as an oncogenic factor in certain malignancies (reviewed in [11]), we first ascertained that the levels of the endogenous miR-221 are higher in CAFs and in both MDA-MB 231 and SkBr3 breast cancer cells respect to normal fibroblasts and non-transformed MCF10A breast cells (*Figure 3.1 a*).

We then ascertained that the miR-221 inhibitor named LNA-i-miR-221 effectively reduces the levels of miR-221, which was ectopically expressed in CAFs, MDA-MB 231 and SkBr3 cells (*Figure 3.1 b*).

Using available bioinformatics tools (http://www.microrna.org; http://www.targetscan.org; http://ophid.utoronto.ca/mirDIP/), we identified a putative miR-221 binding site in 3'-UTR region of the ubiquitin-editing enzyme TNFAIP3, namely A20 (*Figure 3.1 c*), recently also demonstrated through luciferase assay in macrophages by Zhao et al. [18].

In accordance with these findings, we ascertained that the ectopic expression of miR-221 lowers mRNA and protein levels of A20 in CAFs, MDA-MB 231 and SkBr3 cells (*Figure 3.1 d-g*), however these effects were no longer evident in the presence of LNA-i-miR-221 (*Figure 3.1 d-g).* Altogether, our results suggest the ability of miR-221 to regulate A20 expression in our model system.

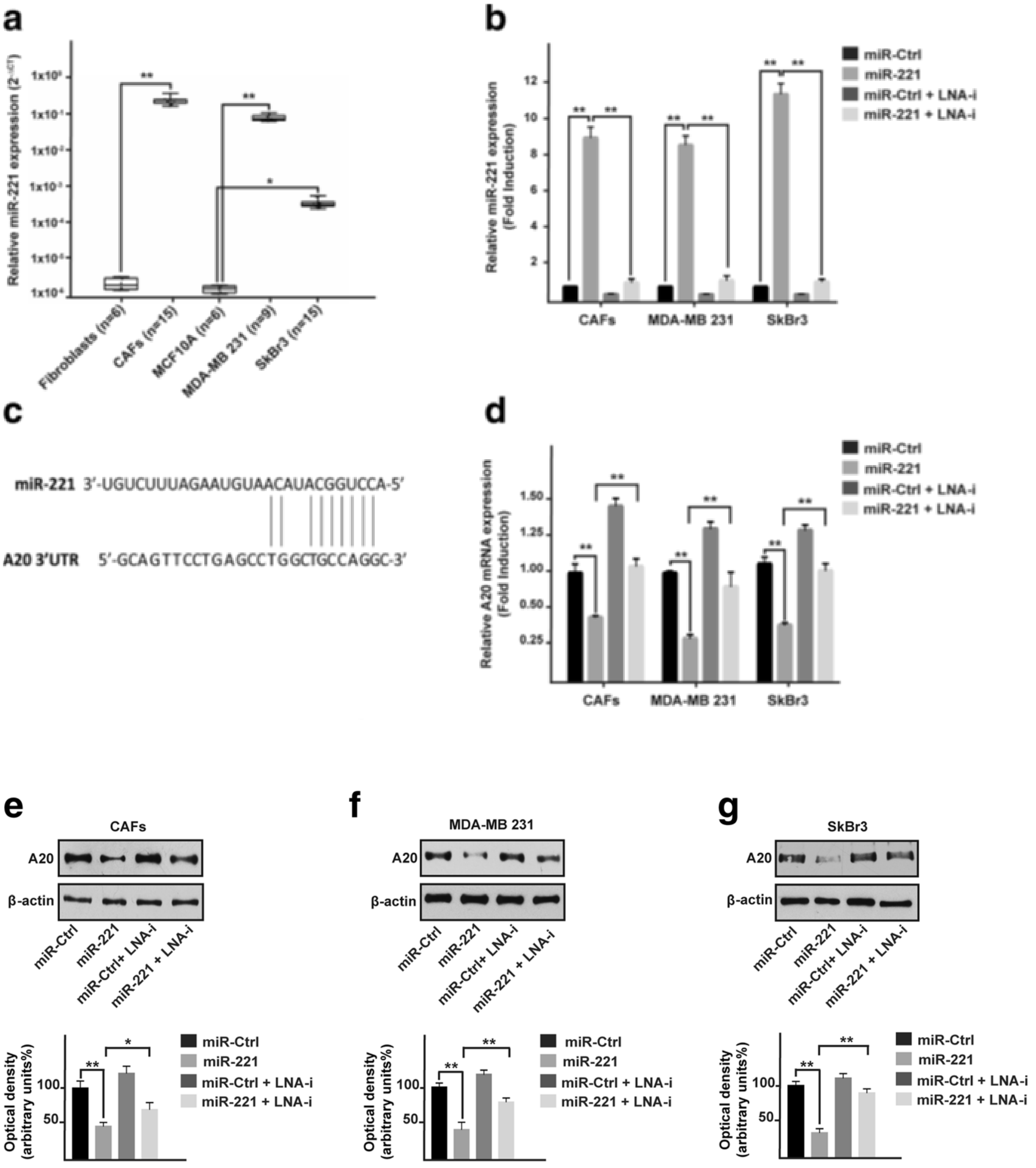


Figure 3.1 | miR-221 regulates A20 expression in CAFs and breast cancer cells. ***a*** Expression of miR-221 in fibroblasts, CAFs, MCF10A, MDA-MB 231 and SkBr3 cells. Raw Ct data were normalized to the housekeeping RNU6 levels and expressed as ΔCt values using the comparative cross threshold (Ct) method. ***b*** miR-221 expression in CAFs, MDA-MB 231 and SkBr3 breast cancer cells after transfection for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). ***c*** Schematic alignment between the miR-221 sequence and the 3'-UTR mRNA region of A20. ***d*** A20 mRNA expression in CAFs, MDA-MB 231 and SkBr3 cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). A20 protein expression in CAFs (***e***), MDA-MB 231 (***f***) and SkBr3 (***g***) cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in in combination with 100 nM LNA-i-miR-221 (LNA-i); β-actin serves as a loading control. Below panels show densitometric analysis of the blots normalized to the loading controls. Each column represents the mean ± SD of three independent experiments performed in triplicate. The data are shown as fold induction respect to cells transfected with miR-Ctrl. (*) indicates $p < 0.05$ and (**) $p < 0.01$.

3.2 miR-221 prompts the expression of c-Rel and its recruitment to the CTGF promoter region in CAFs, MDA-MB 231 and SkBr3 breast cancer cells

Previous studies have shown that A20 may regulate NF-κB. In particular, a negative correlation between the levels of A20 and the expression of the NF-κB component, namely c-Rel has been reported [22]. In this vein, we found that miR-221 dependent c-Rel protein expression was no longer evident in the presence of LNA-i-miR-221 sequence in CAFs, MDA-MB 231 and SkBr3 cells (*Figure 3.2 a-c*).

As NF-κB activity has been demonstrated to regulate CTGF expression [351, 352], we analyzed the CTGF promoter sequence (http://www.ncbi.nlm.nih.gov; http://alggen.lsi.upc.es/; http://www.generegulation.com) identifying a putative NF-κB binding site (*Figure 3.2 d*).

Worthy, performing a ChIP analysis we assessed that the recruitment of c-Rel to the CTGF promoter sequence induced by miR-221 is abrogated in the presence of LNA-i-miR-221 (*Figure 3.2 e-g*). Moreover and in agreement with these results, the transactivation of a CTGF-luc reporter construct by miR-221 was repressed by LNA-i-miR-221 (*Figure 3.2 h*) as well as silencing of c-Rel expression (*Figure 3.2 i*).

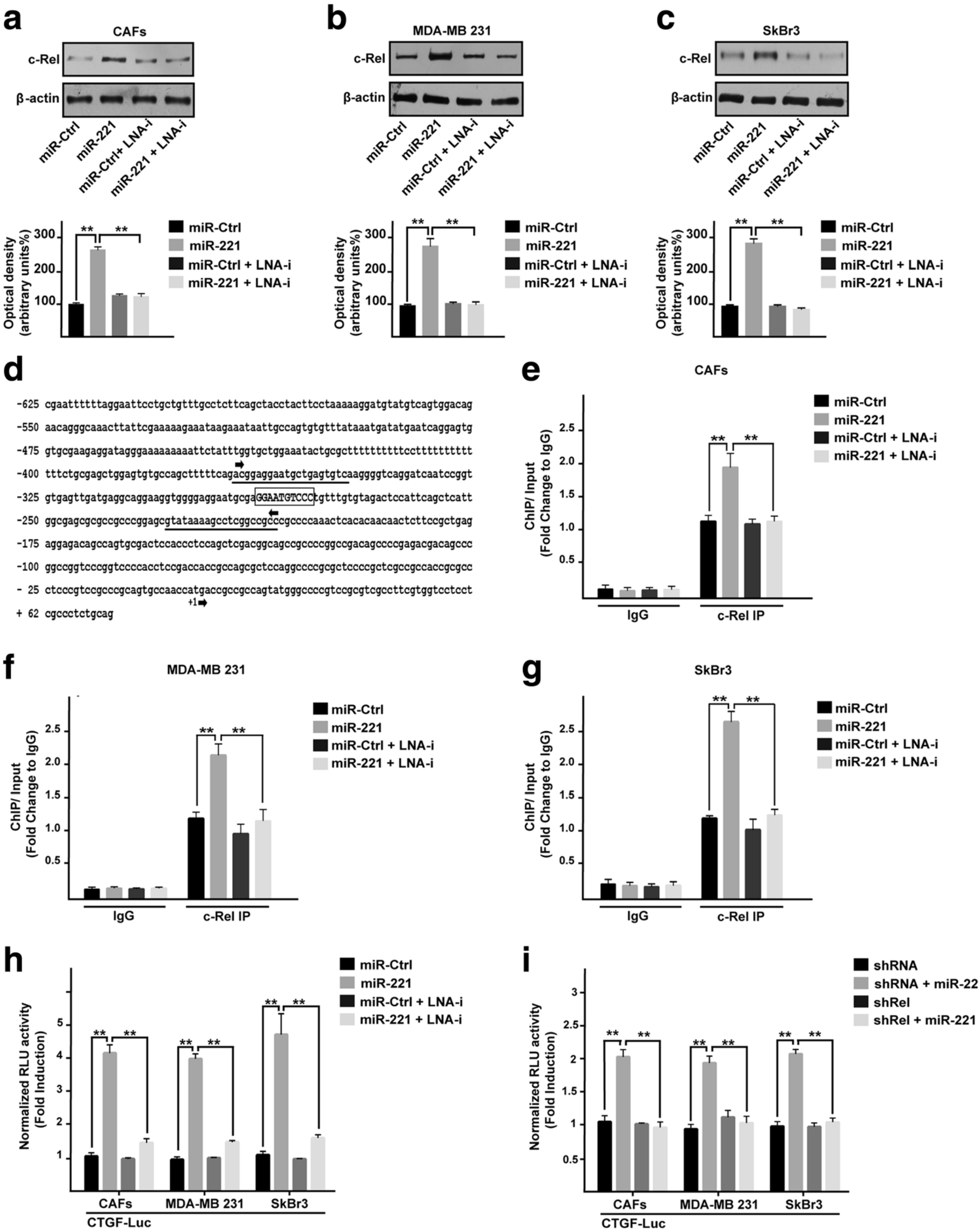


Figure 3.2 | miR-221 prompts the protein expression of c-Rel and its recruitment to the CTGF promoter region. c-Rel protein expression in CAFs (**a**), MDA-MB 231 (**b**) and SkBr3 (**c**) cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). β-actin serves as a loading control. Below panels show densitometric analysis of the blots normalized to the loading controls. **d** Putative NF-κB binding site (capital letters in the rectangle) located within the CTGF promoter sequence (− 625 bp to + 62 bp). The transcriptional start site is indicated as + 1. The position of primers used for ChIP-qPCR analyses is underlined. Recruitment of c-Rel to the NF-κB binding site within the CTGF promoter sequence in CAFs (**e**), MDA-MB 231 (**f**) and SkBr3 (**g**) cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). Data were normalized to the Input and reported as fold changes respect to IgG. **h** Luciferase activity of the CTGF reporter gene in CAFs, MDA-MB 231 and SkBr3 cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). **i** Luciferase activity of the CTGF reporter gene in CAFs, MDA-MB 231 and SkBr3 cells transfected for 8 h with shRNA or shRel and then transfected for 48 h with 25 nM miR-221. The luciferase activity was normalized to the internal transfection control; values of cells receiving scrambled controls were set as 1-fold induction upon which the activity obtained with the indicated effectors was calculated. Each column represents the mean ± SD of three independent experiments performed in triplicate. (**) indicates $p < 0.01$.

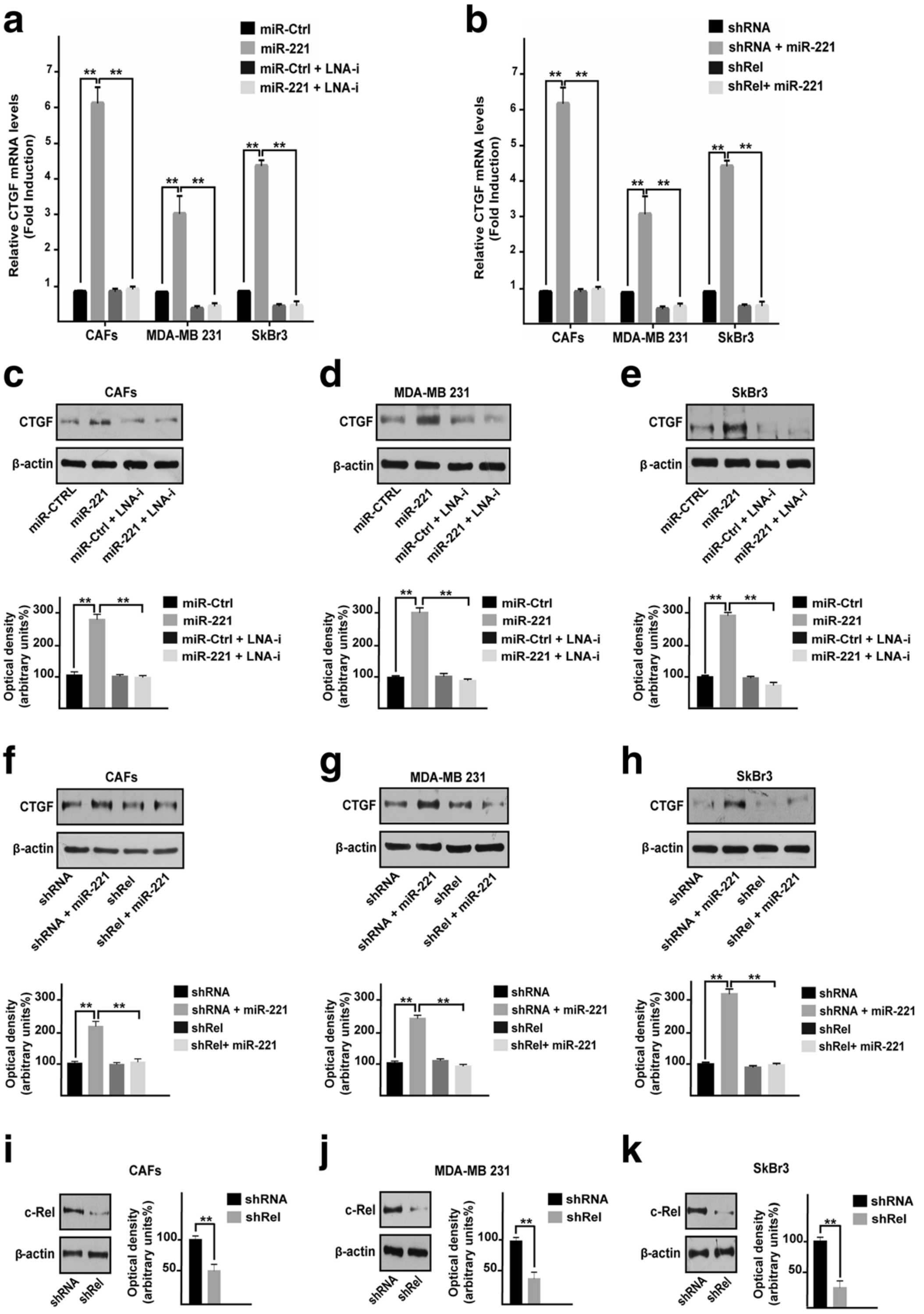


Figure 3.3 | c-Rel is involved in the up-regulation of the CTGF triggered by miR-221. **a** CTGF mRNA levels in CAFs, MDA-MB 231 and SkBr3 cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). Data are shown as fold changes respect to the scrambled controls. **b** CTGF mRNA levels in CAFs, MDA-MB 231 and SkBr3 cells transfected for 8 h with shRNA or shRel and then transfected for 48 h with 25 nM miR-221. Data are shown as fold changes respect to the scrambled controls. CTGF protein levels in CAFs (**c**), MDA-MB 231 (**d**) and SkBr3 (**e**) cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). β-actin serves as a loading control. Below panels show densitometric analysis of the blots normalized to the loading controls. CTGF protein levels in CAFs (**f**), MDA-MB 231 (**g**) and SkBr3 (**h**) cells transfected for 8 h with shRNA or shRel and then transfected for 48 h with 25 nM miR-221. β-actin serves as a loading control. Below panels show densitometric analysis of the blots normalized to the loading controls. Efficacy of c-Rel silencing in CAFs (**i**), MDA-MB 231 (**j**) and SkBr3 (**k**) cells. β-actin serves as a loading control. Side panels show densitometric analysis of the blots normalized to the loading controls. Results shown are representative of at least three independent experiments. (**) indicates $p < 0.01$.

In accordance with these results, the up-regulation of CTGF by miR-221 at both mRNA and protein levels was abolished by LNA-i-miR 221 (*Figure 3.3 a, c-e*) or knock-down c-Rel expression (*Figure 3.3 b, f-k*). Collectively, these data suggest that c-Rel is involved in the up-regulation of CTGF expression by miR-221 in CAFs, MDA-MB 231 and SkBr3 cells.

3.3 miR-221 induces growth and migratory effects through c-Rel and CTGF in CAFs, MDA-MB 231 and SkBr3 breast cancer cells.

As a biological counterpart of the results described above, we ascertained that the proliferative responses elicited by miR-221 are prevented in the presence of LNA-i-miR-221(*Figure 3.4 a, d, g*), or by silencing (*Figure 3.4 b, e, h*) c-Rel and CTGF expression (*Figure 3.4 c, f, i*) in CAFs, MDA-MB 231 and SkBr3 cells.

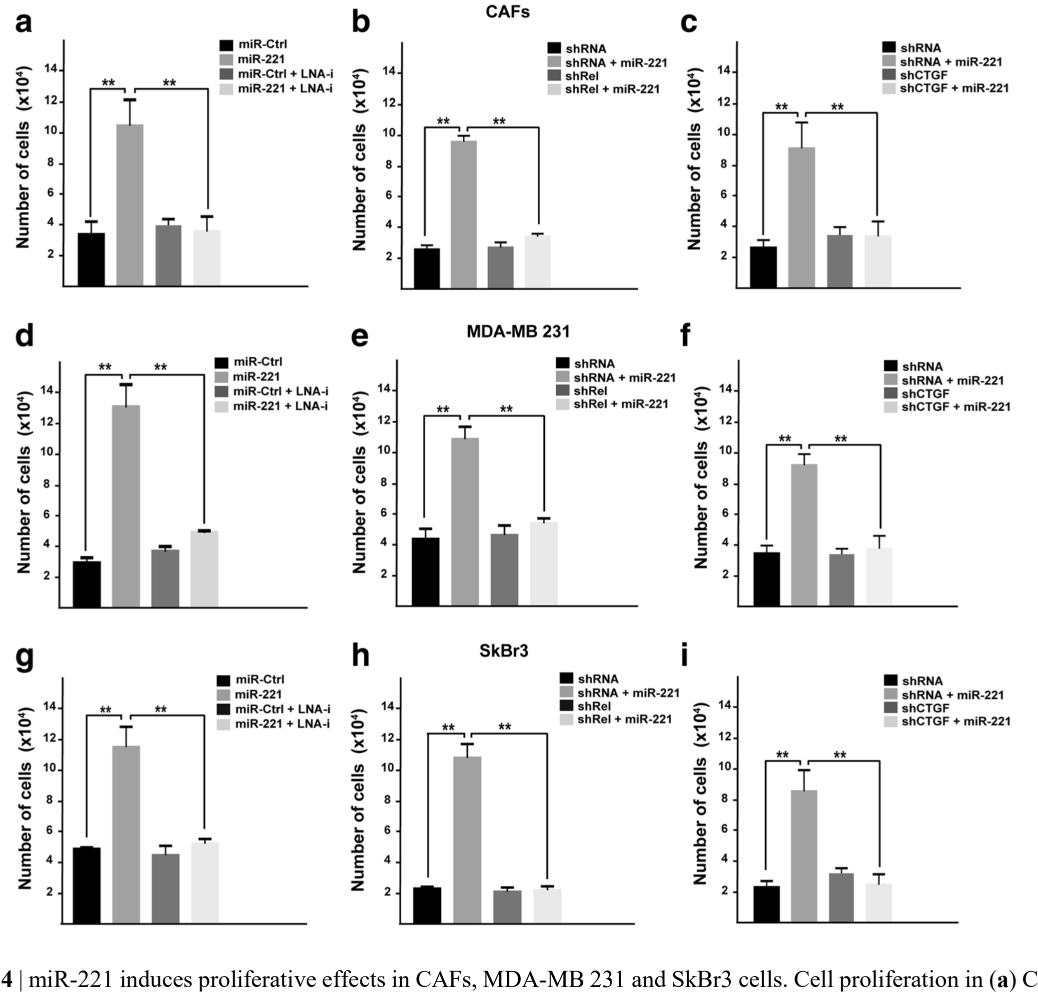


Figure 3.4 | miR-221 induces proliferative effects in CAFs, MDA-MB 231 and SkBr3 cells. Cell proliferation in (**a**) CAFs, (**d**) MDA-MB 231 and (**g**) SkBr3 cells transfected every 2 days with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i) and then counted on day 6. Cell proliferation in CAFs (**b**) MDA-MB 231 (**e**) and SkBr3 (**h**) cells transfected with shRNA or shRel for 8 h and then transfected for 48 h with 25 nM miR-221. Cell proliferation in CAFs (**c**), MDA-MB 231 (**f**) and SkBr3 (**i**) cells transfected with shRNA or shCTGF for 8 h and then transfected for 48 h with 25 nM miR-221. The transfections were renewed every 2 days and cells were counted on day 6. Each data point is the mean ± SD of three independent experiments performed in triplicate. (**) indicates $p < 0.01$.

By colony formation assay, we then assessed that the clonogenic capacity induced by miR-221 in CAFs, MDA-MB 231 and SkBr3 is no longer evident in the presence of LNA-i-miR-221 (*Figure 3.5 a, c, e*), or silencing of c-Rel (*Figure 3.5 b, d, f*) or by CTGF expression (data not shown).

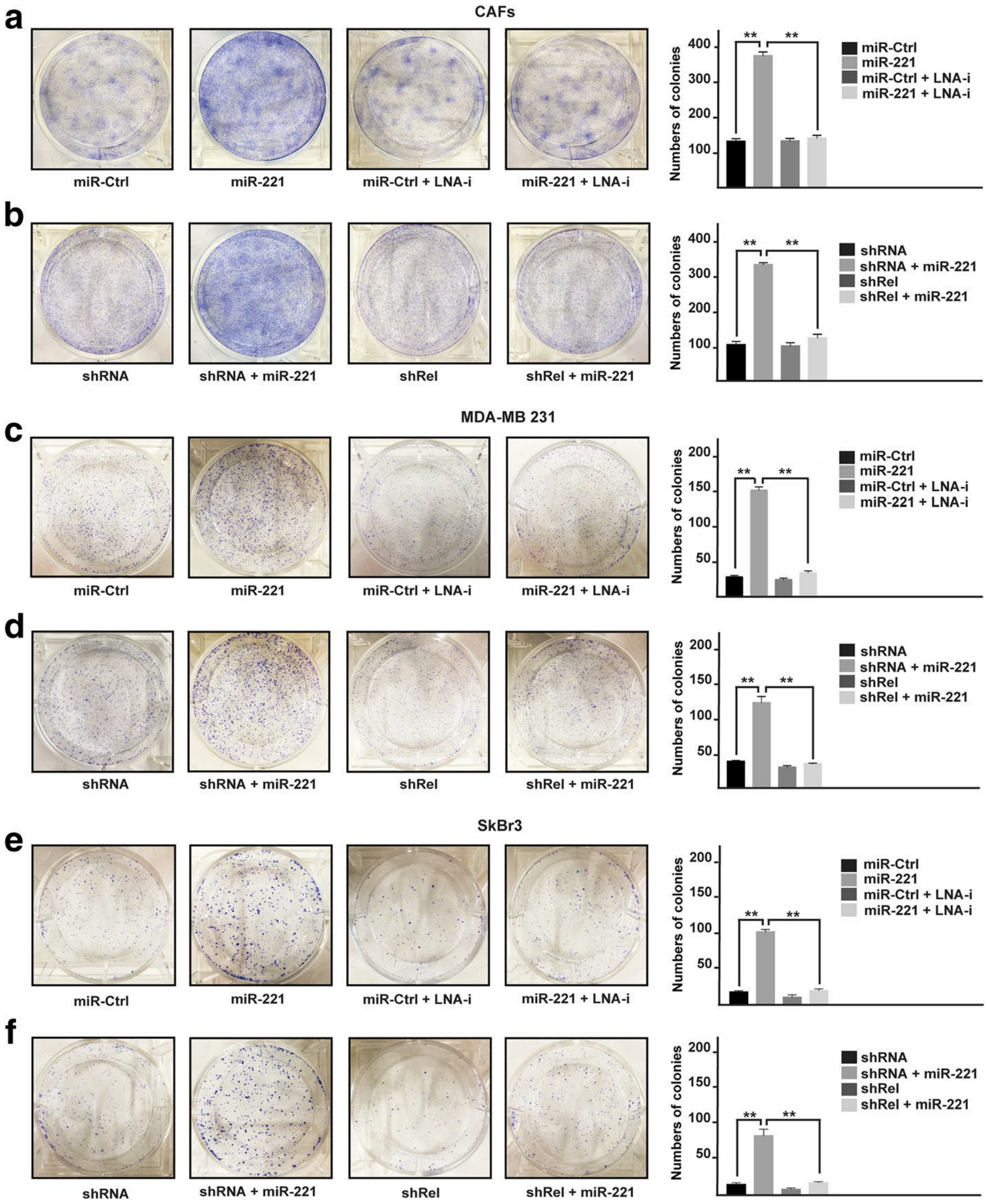


Figure 3.5 | miR-221 promotes colony formation in CAFs, MDA-MB 231 and SkBr3 cells. Colony formation in CAFs (**a**, **b**), MDA-MB 231 (**c**, **d**) and SkBr3 (**e**, **f**) cells transfected every 2 days as indicated, after 10 days of incubation cell colonies were stained and pictures were captured by a digital camera. Colonies were counted using the program WCIF ImageJ for Windows. Each data point is the mean ± SD of three independent experiments performed in triplicate. (**) indicates $p < 0.01$.

Performing Boyden chamber assay in CAFs, MDA-MB-231 and SkBr3 cells, we also determined that the migratory effects stimulated by miR-221 are abolished using LNA-i-miR-221 (*Figure 3.6 a, c, e*), knocking-down c-Rel (*Figure 3.6 b, d, f*) or CTGF expression (data not shown). Overall, these results indicate that both c-Rel and CTGF are involved in the proliferative and migratory effects triggered by miR-221 in our model systems.

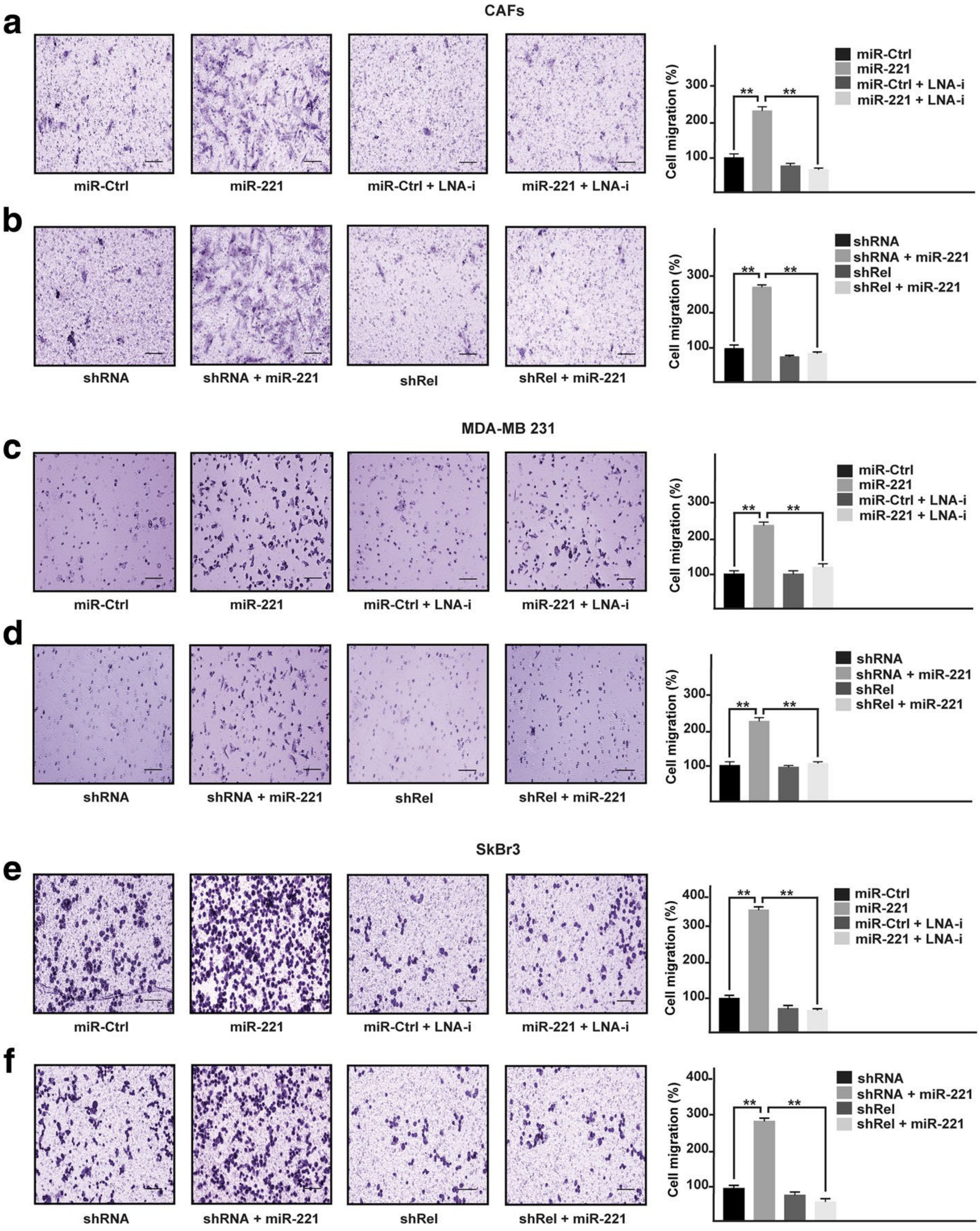


Figure 3.6 | miR-221 triggers migratory effects in CAFs, MDA-MB 231 and SkBr3 cells. Cell migration as evaluated by Boyden Chamber assay in (**a**) CAFs, (**c**) MDA-MB 231 and (**e**) SkBr3 cells transfected for 48 h with 25 nM miR-Ctrl and 25 nM miR-221, alone or in combination with 100 nM LNA-i-miR-221 (LNA-i). Cell migration, evaluated by Boyden Chamber assay, in CAFs (**b**) MDA-MB 231 (**d**) and SkBr3 (**f**) cells transfected with shRNA or shRel for 8 h and then transfected for 48 h with 25 nM miR-221. Cells were counted in at least 10 random fields at 10× magnification (Scale bar =200 μm) in three independent experiments performed in triplicate. (**) indicates $p < 0.01$.

3.4 E2 modulates the expression of certain miRNAs in SkBr3 breast cancer cells and in CAFs

In order to provide novel insights on the involvement of estrogens on miRNA regulation in breast cancer, SkBr3 breast cancer cells and CAFs, both ER-negative and GPER positive were treated with 100 nM E2 for 4 h and then analyzed by TaqMan™ Array Human MicroRNA. A total amount of 754 miRNAs involved in diverse pathophysiological conditions (www.thermofisher.com/order/catalog/product/4444913) were evaluated, thereafter we focused our attention on miRNAs displaying a Ct< 32 along with at least 2 fold increase or 50% reduction upon E2 exposure respect to vehicle-treated cells. On the basis of these criteria, we identified 25 and 29 E2-regulated miRNAs in SkBr3 cancer cells (*Figure 3.7 a*) and CAFs (*Figure 3.8 a*), respectively. In particular, in SkBr3 cancer cells 23 miRNAs were up-regulated and 2 miRNAs were down-regulated by E2 treatment (*Figure 3.7 b*). As it concerns CAFs, among the 29 E2-regulated miRNAs, 7 showed an increase and 22 a reduction upon E2 stimulation (*Figure 3.8 b*).

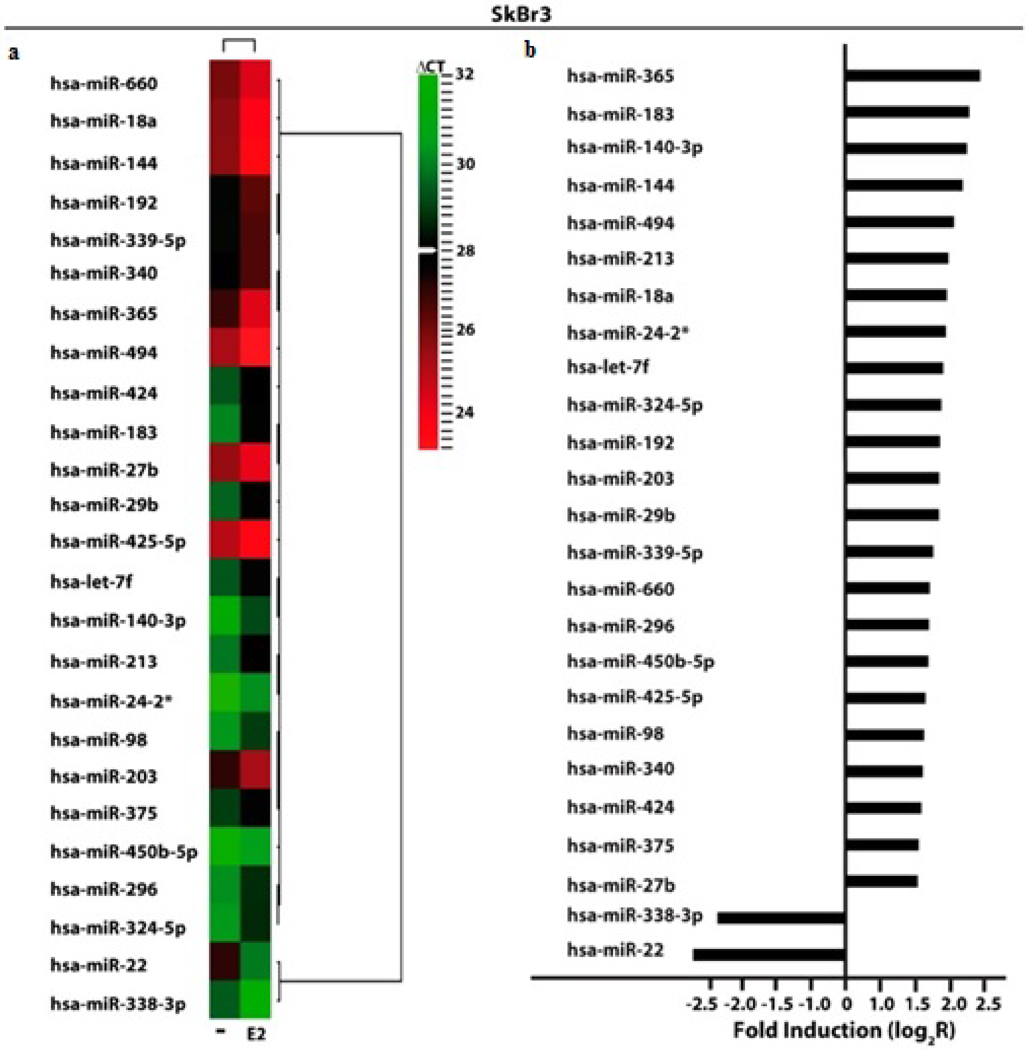


Figure 3.7 | E2-modulated miRNAs expression in SkBr3 breast cancer cells. (**a**) Heat Map representation of E2-modulated miRNAs in SkBr3 cancer cells treated with 100 nM E2 for 4 h and analyzed by TaqMan Low-Density Array Human miRNA. Row represents a miRNA and column represents the treatment used. Each column is illustrated according to a color scale from green (low expression) to red (high expression). The distance measured is Euclidean Distance and the clustering method is complete linkage. Dendrograms of clustering analysis for miRNAs and samples are displayed on the top and right, respectively. (**b**) Up- and down-regulated miRNAs in SkBr3 breast cancer cells upon E2 stimulation. The values are indicated as log2 fold change (R) calculated respect to vehicle (-).

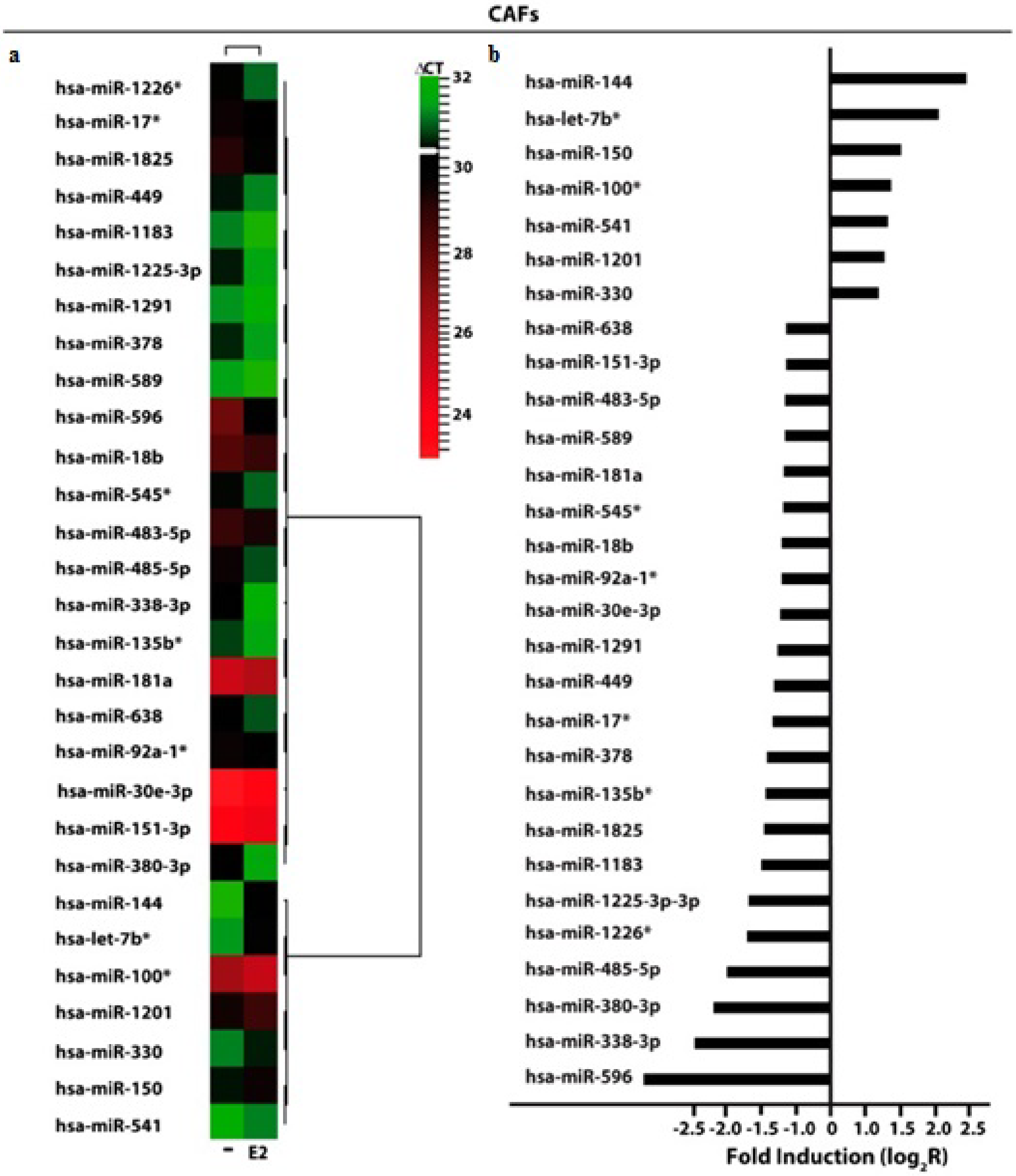
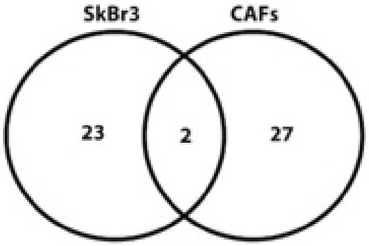
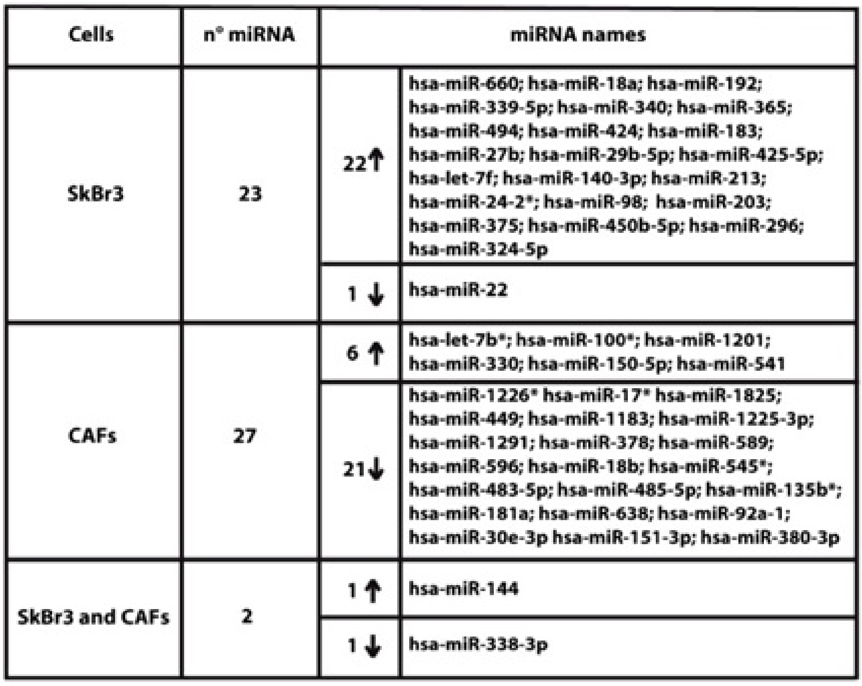


Figure 3.8 | E2-modulated miRNAs expression in CAFs. (**a**) Heat Map representation of E2-modulated miRNAs in CAFs treated with 100 nM E2 for 4 h and analyzed by TaqMan Low-Density Array Human miRNA. Row represents a miRNA and column represents the treatment used. Each column is illustrated according to a color scale from green (low expression) to red (high expression). The distance measured is Euclidean Distance and the clustering method is complete linkage. Dendrograms of clustering analysis for miRNAs and samples are displayed on the top and right, respectively. (**b**) Up- and down-regulated miRNAs in CAFs upon E2 stimulation. The values are indicated as log2 fold change (R) calculated respect to vehicle (-).

To identify unique and shared E2-regulated miRNAs in both cell types, we then calculated a Venn diagram. SkBr3s cancer cells and CAFs shared only the expression of 2 miRNAs (*Figure 3.9 a*), namely miR-144 and miR-338-3p, which exhibited a similar response (*Figure 3.9 b*). Considering that in our previous studies we evaluated the role of miR-144 in tumor cell growth [9], here we aimed to determine the mechanisms leading to the estrogen regulation and function of miR-338-3p in breast cancer. Hence, we began our study ascertaining that miR-338-3p expression correlates positively with the overall survival in 1283 breast tumor patients, as a



b



Cells	n° miRNA	miRNA names	
SkBr3	23	22↑	hsa-miR-660; hsa-miR-18a; hsa-miR-192; hsa-miR-339-5p; hsa-miR-340; hsa-miR-365; hsa-miR-494; hsa-miR-424; hsa-miR-183; hsa-miR-27b; hsa-miR-29b-5p; hsa-miR-425-5p; hsa-let-7f; hsa-miR-140-3p; hsa-miR-213; hsa-miR-24-2*; hsa-miR-98; hsa-miR-203; hsa-miR-375; hsa-miR-450b-5p; hsa-miR-296; hsa-miR-324-5p
		1 ↓	hsa-miR-22
CAFs	27	6 ↑	hsa-let-7b*; hsa-miR-100*; hsa-miR-1201; hsa-miR-330; hsa-miR-150-5p; hsa-miR-541
		21↓	hsa-miR-1226* hsa-miR-17* hsa-miR-1825; hsa-miR-449; hsa-miR-1183; hsa-miR-1225-3p; hsa-miR-1291; hsa-miR-378; hsa-miR-589; hsa-miR-596; hsa-miR-18b; hsa-miR-545*; hsa-miR-483-5p; hsa-miR-485-5p; hsa-miR-135b*; hsa-miR-181a; hsa-miR-638; hsa-miR-92a-1; hsa-miR-30e-3p hsa-miR-151-3p; hsa-miR-380-3p
SkBr3 and CAFs	2	1 ↑	hsa-miR-144
		1 ↓	hsa-miR-338-3p

c

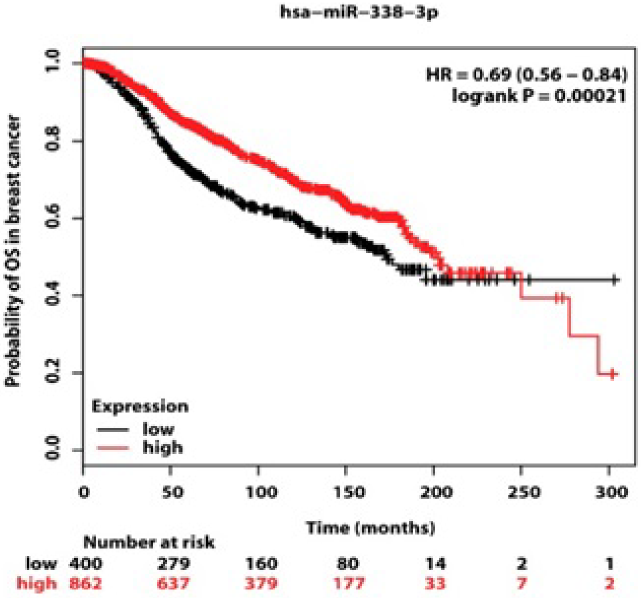


Figure 3.9 | Exclusive and shared expression of miRNAs between SkBr3 and CAFs. (**a**) Venn Diagram of E2-modulated miRNAs in SkBr3 cancer cells and CAFs. (**b**) Up and down-regulated miRNAs by 100 nM E2 treatment for 4 h in SkBr3 cancer cells and CAFs. (**c**) The expression of miR-338-3p is associated with higher overall survival in breast cancer patients. The evaluation was performed by Kaplan–Meier Plotter (http://www.kmplot.com). Statistical analysis was made using the log-rank test.

reported in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) database [353] (*Figure 3.9 c*). Nicely fitting with these findings, previous evidence has suggested that miR-338-3p may function as a tumor suppressor in certain malignancies including breast cancer [33-35, 216, 354].

3.5 GPER is involved in the regulation of miR-338-3p by E2 and G-1 in SkBr3 cancer cells and CAFs

On the basis of the aforementioned results, we then attempted to define the molecular mechanisms involved in the estrogenic regulation of miR-338-3p performing a time-course study upon 100 nM of E2 and 100 nM of the selective GPER ligand G-1. Worthy, the inhibitory effects of E2 and G-1 on miR-338-3p expression were no longer evident silencing GPER in SkBr3 cancer cells (*Figure 3.10 a - c*) and in CAFs (*Figure 3.10 d - f*).

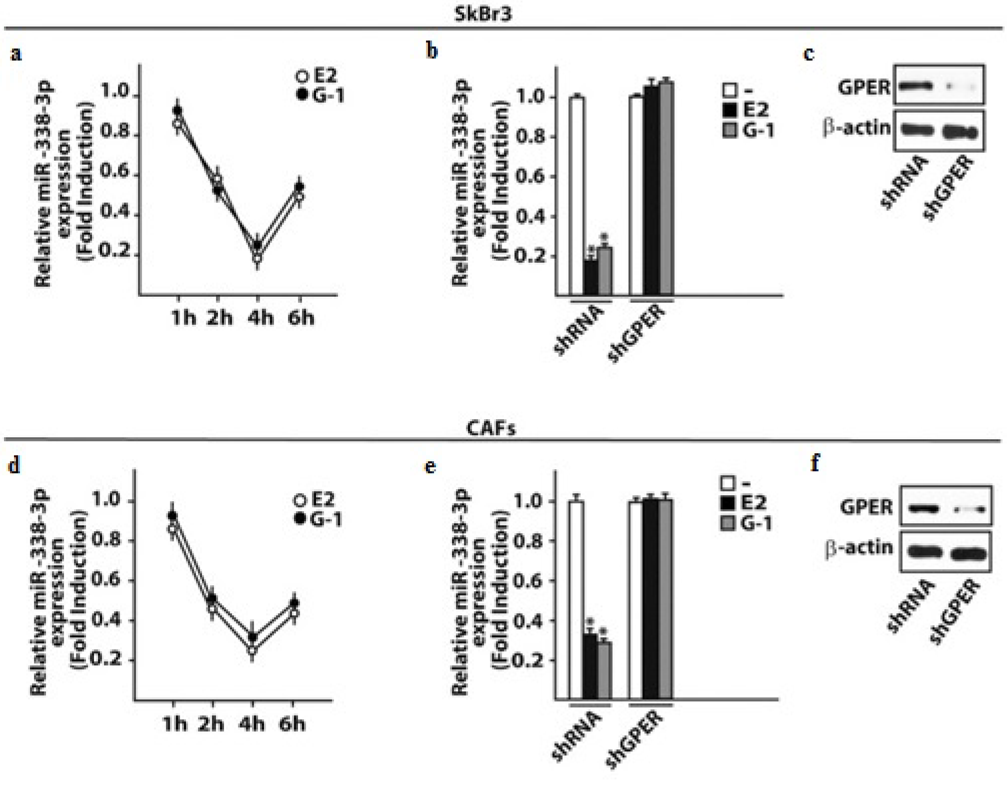


Figure 3.10 | E2 and G-1 down-regulate miR-338-3p levels in SkBr3 cancer cells and CAFs. SkBr3 breast cancer cells (**a**) and CAFs (**d**) were stimulated with 100 nM E2 or 100 nM G-1 as indicated and analyzed by RT-PCR. Each point is plotted as fold changes of cells receiving treatments respect to cells treated with vehicle (-) and represents the mean ± SD of three independent experiments performed in triplicate. MiR-338-3p expression upon a 4 h treatment with 100 nM E2 or 100 nM G-1 in SkBr3 cells (**b**) and CAFs (**e**) previously transfected with shRNA or shGPER for 48 h. Each column represents the mean ± SD of three independent experiments performed in triplicate. Efficacy of GPER silencing in SkBr3 cells (**c**) and CAFs (**f**). β-actin serves as a loading control. (*) indicates $p < 0.05$, for cells receiving treatments vs cells treated with vehicle.

Thereafter, we aimed to identify putative target genes of miR-338-3p by a bioinformatics analysis of available algorithms (http://ophid.utoronto.ca/mirDIP; http://www.microrna.org;http: //www.targetscan.org). Among others, two putative target sequences of miR-338-3p located within the 3’-UTR of the oncogene c-Fos were found (*Figure 3.11 a*). According to our previous studies showing that estrogens regulate c-Fos levels in diverse cancer cell types [85, 87, 89, 99], the induction of c-Fos mRNA and protein expression upon a 4 h treatment with 100 nM E2 and 100 nM G-1 was abolished silencing GPER in SkBr3 cancer cells (*Figure 3.11 b, c*) and CAFs (*Figure 3.11 d, e*).

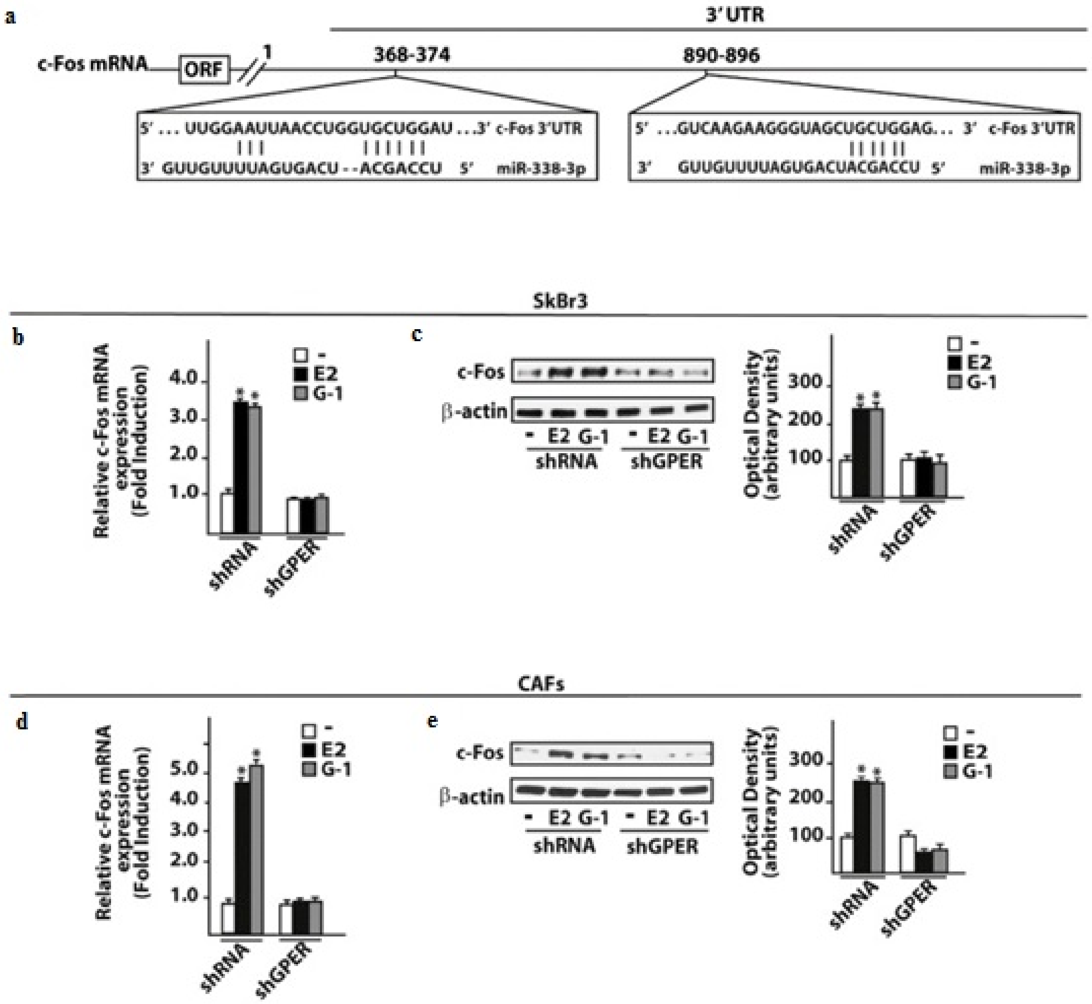


Figure 3.11 | c-Fos is a target gene of miR-338-3p. (**a**) Schematic alignment between the miR-338-3p sequence and the 3’-UTR mRNA region of c-Fos. mRNA expression of c-Fos in SkBr3 cancer cells (**b**) and CAFs (**d**) transfected with shRNA or shGPER for 48 h and then treated for 4 h with 100 nM E2 or 100 nM G-1. Each column represents the mean ± SD of three independent experiments performed in triplicate. c-Fos protein expression in SkBr3 cancer cells (**c**) and CAFs (**e**) transfected with shRNA or shGPER for 48 h and then treated for 4 h with 100 nM E2 or 100 nM G-1. Side panels show densitometry analysis of the blots normalized to the loading control β-actin.

Next, we found that in SkBr3 cells and CAFs transfected for 48 h with a miR-338-3p mimic sequence, the treatment for 4 h with 100 nM E2 and 100 nM G-1 is no longer able to induce c-Fos mRNA and protein levels, a response rescued transfecting the miR-338-3p mimic sequence in combination with a miR-338-3p inhibitor sequence (*Figure 3.12 a - f*).

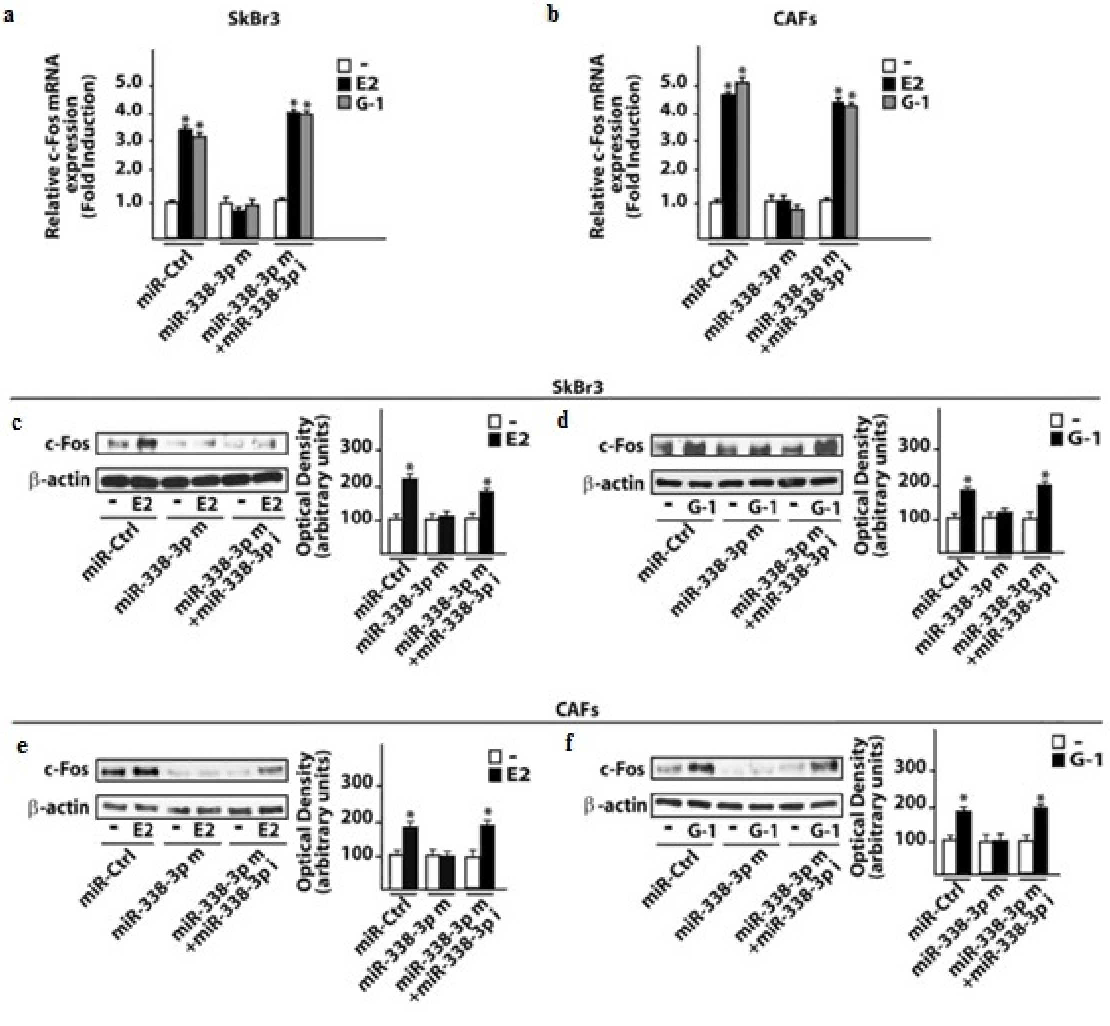


Figure 3.12 | miR-338-3p prevents c-fos induction by E2 and G-1 in SkBr3 cancer cells and CAFs. mRNA levels of c-Fos in SkBr3 cancer cells (**a**) and CAFs (**b**) transfected for 48 h with 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i) and then treated for 4 h with 100 nM E2 or 100 nM G-1. Each column represents the mean ± SD of three independent experiments performed in triplicate. c-Fos protein levels in SkBr3 cancer cells (**c, d**) and CAFs (**e, f**) transfected for 48 h with 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i) and then stimulated for 4 h with 100 nM E2 or 100 nM G-1. Side panels show densitometry analysis of the blots normalized to the loading control β-actin. (*) indicates $p < 0.05$, for cells receiving treatments vs cells treated with vehicle (-).

3.6 miR-338-3p triggers inhibitory effects on the proliferation induced by E2 and G-1

As in our previous investigations c-Fos was involved in the regulation of cyclins [89,76], we assessed that the transactivation of the Cyclin D1 promoter sequence by 100 nM E2 and 100 nM G-1 was prevented co-transfecting a dominant negative c-Fos expression construct (DN-Fos) in SkBr3 and CAFs (*Figure 3.13 a, b*).

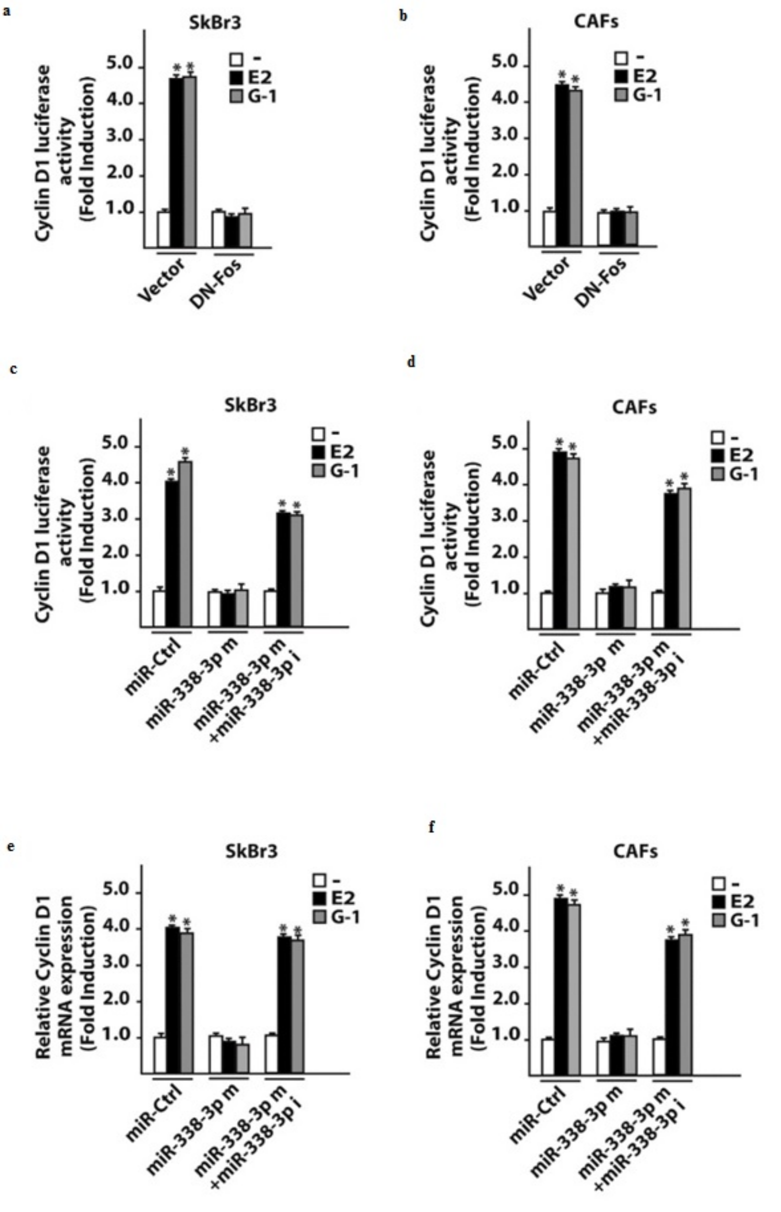


Figure 3.13 | c-Fos and miR-338-3p are involved in Cyclin D1 regulation in SkBr3 cancer cells and CAFs. Luciferase activity of Cyclin D1 reporter gene in SkBr3 cancer cells (**a**) and CAFs (**b**) transfected for 8 h with a vector or a dominant-negative c-Fos construct (DN-Fos) before treatment with 100 nM of E2 and 100 nM G-1 for 18 h. Luciferase activity of Cyclin D1 reporter gene in SkBr3 cancer cells (**c**) and CAFs (**d**) transfected for 24 h with 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i) before treatment for 18 h with 100 nM E2 or 100 nM G-1. The luciferase activity was normalized to the internal transfection control, values of cells receiving vehicle (-) were set as 1-fold induction upon which the activity obtained upon the indicated treatments was calculated. mRNA expression of Cyclin D1 in SkBr3 cells (**e**) and CAFs (**f**) transfected for 48 h with 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i) before treatment for 8 h with 100 nM E2 or 100 nM G-1. Each column represents the mean ± SD of three independent experiments performed in triplicate. (*) indicates $p < 0.05$ for cells receiving treatments vs cells treated with vehicle (-).

Nicely recapitulating the aforementioned results, the Cyclin D1 promoter luciferase activity induced by 100 nM E2 and 100 nM G-1 was inhibited using the miR-338-3p mimic, an effect rescued by the miR-338-3p inhibitor sequence (*Figure 3.13 c, d*). In addition, similar findings were observed evaluating the regulation of Cyclin D1 at both mRNA (*Figure 3.13 e, f*) and protein levels (*Figure 3.14 a - d*).

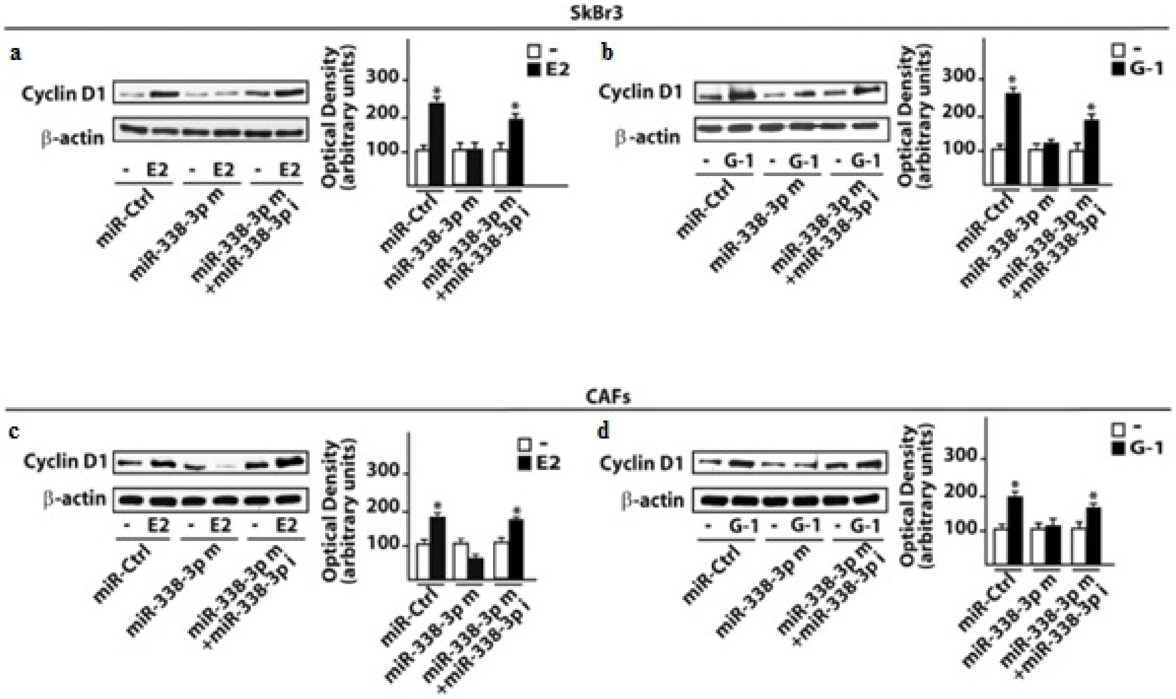
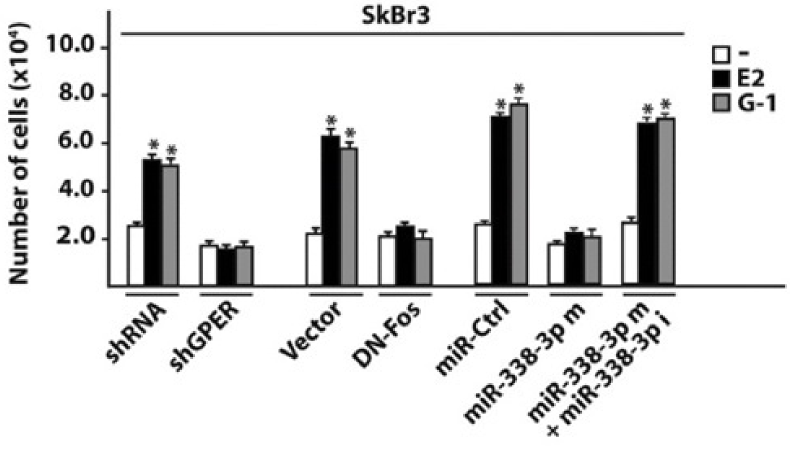


Figure 3.14 | miR-338-3p prevents Cyclin D1 protein induction by E2 and G1 in SkBr3 cancer cells and CAFs. Cyclin D1 protein expression in SkBr3 cancer cells (**A**,**B**) and CAFs (**C**,**D**) transfected for 48 h with 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i) before treatment for 12h with 100 nM E2 or 100 nM G-1. Side panels show densitometry analysis of the blots normalized to the loading control β-actin. (*) indicates $p < 0.05$ for cells receiving treatments vs cells treated with vehicle (-).

As biological counterpart, the proliferative responses elicited by 100 nM E2 and 100 nM G-1 in SkBr3 cancer cells and CAFs were prevented silencing GPER or transfecting the DN-Fos construct (*Figure 3.15 a, b*). Furthermore, the miR-338-3p mimic sequence decreased the proliferation induced by 100 nM E2 and 100 nM G-1 (*Figure 3.15 a, b*), however this effect was rescued co-transfecting the miR-338-3p inhibitor (*Figure 3.15 a, b*). Further supporting the aforementioned findings, the treatment for 24 h with 100 nM E2 and 100 nM G-1 triggered inhibitory effects on cell cycle progression transfecting CAFs with the miR-338-3p mimic sequence, however this response was abolished in the presence of the miR-338-3p inhibitor sequence (*Figure 3.15 c*). Overall, these results suggest that estrogenic GPER signaling regulates miR-338-3p expression and function in SkBr3 cancer cells and CAFs.

a



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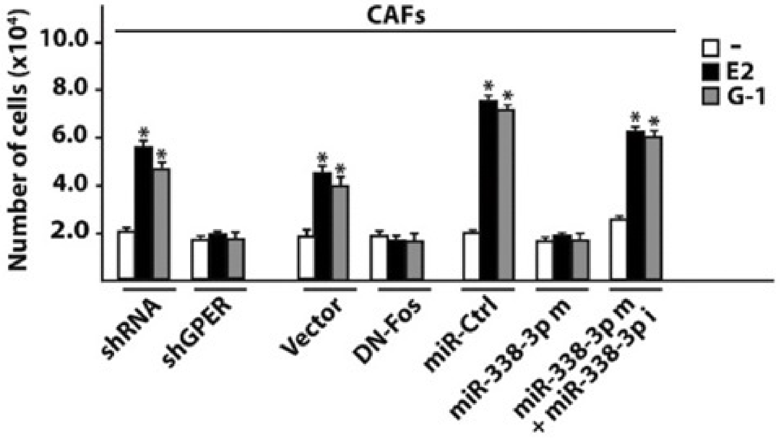

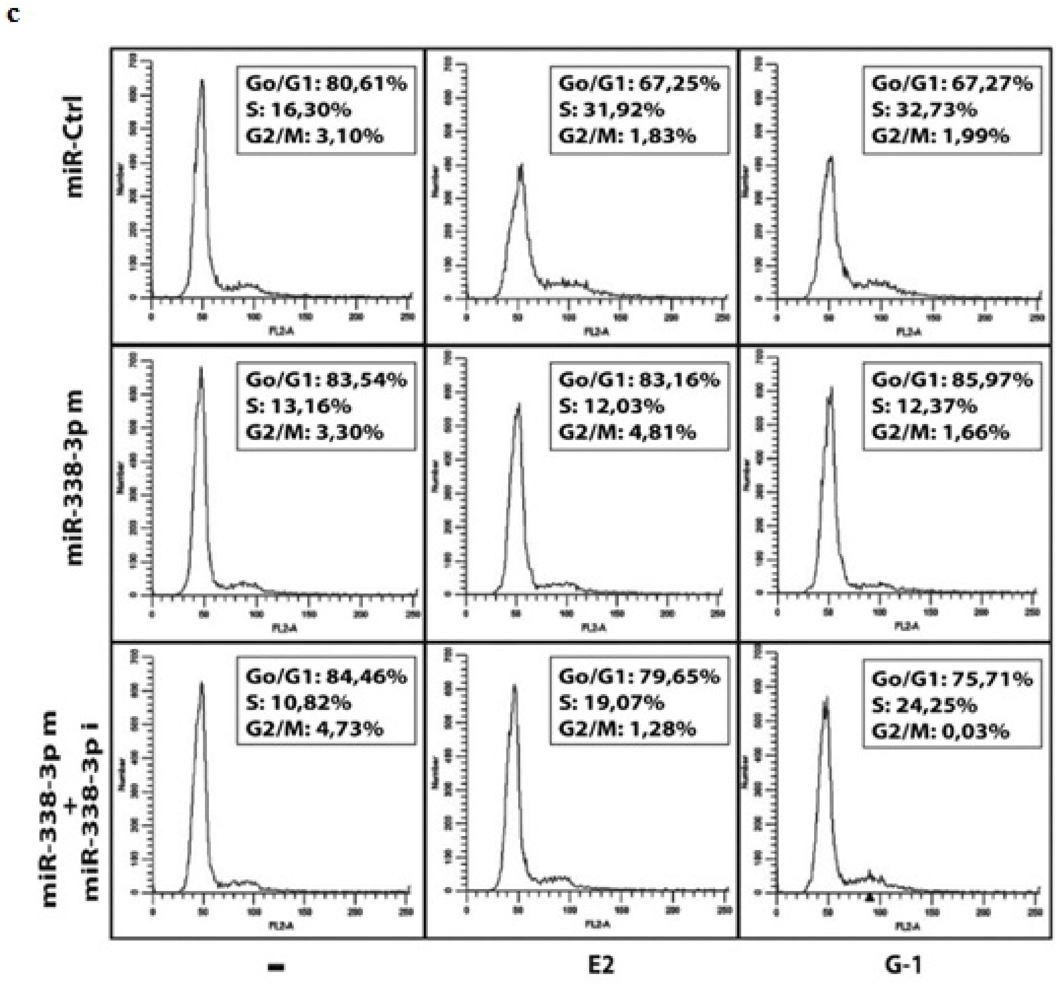

Figure 3.15 | miR-338-3p decreases the proliferation of SkBr3 cancer cells and CAFs induced by E2 and G-1. Cell proliferation in SkBr3 cancer cells (**a**) and CAFs (**b**) transfected every 2 days with 100ng shRNA or shGPER, 100ng vector or a dominant-negative c-Fos construct (DN-Fos) and 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i). Cells were treated every day with 100 nM E2 or 100 nM G-1 and counted on day 6. Each column represents the mean ± SD of three independent experiments performed in triplicate. (*) indicates $p < 0.05$ for cells receiving treatments vs cells treated with vehicle (-). (**c**) Representative pictures of cell cycle analysis in CAFs transfected for 48 h with 25 nM miR-Ctrl or miR-338-3p mimic (miR-338-3p m) in combination or not with 50 nM miR-338-3p inhibitor (miR-338-3p i) before the treatment for 24 h with 100 nM E2 and 100 nM G-1. In each panel, the percentages of cells in G0/G1, S and G2/M phases of the cell cycle are indicated. Values represent the mean ± SD of three independent experiments.

CHAPTER 4

Discussion

In the present study we provide novel knowledge on the role of miRNAs in breast cancer. In particular, we have demonstrated that miR-221 may trigger proliferative and migratory effects through the interference with A20/c-Rel/CTGF signaling in MDA-MB 231 and SkBr3 breast cancer cells, as well as in CAFs, which are crucial players of the tumor microenvironment. Moreover, we elucidate the molecular mechanisms through which E2 induce proliferative effects in SkBr3 breast cancer cells and CAFs, down-regulating the expression of the onco-suppressor miR-338-3p. Overall, our data suggest miRNAs as innovative tools in further preventive and therapeutic strategies against breast tumor.

Breast cancer is a heterogeneous disease that includes various subtypes, with different biological behaviour and clinical outcome [355]. Based on the gene expression profile, three major subtypes of breast cancer have been identified: the luminal (A and B), the basal-like and the epidermal growth factor receptor 2 overexpressing type [355]. The breast cancer subtype expressing hormone receptors, usually indicated as luminal A and B, have a favourable prognosis undergoing anti-hormone treatments [356]. Tumors overexpressing the epidermal growth factor receptor 2 are treated with anti HER2 monoclonal antibodies as trastuzumab and pertuzumab or with immunoconjugates as TDM1 or kinase inhibitors as lapatinib or neratinib, mostly combined [357], whereas the triple-negative breast cancer that is treated with conventional and moderately successful chemotherapies displays a poor prognosis and a high risk of relapse [358, 359]. Currently, there are several confirmations that show that components of the tumor microenvironment, as CAFs, may play a main role in cancer progression and invasiveness [26, 360]. The growth characteristics of CAFs are different from those of fibroblasts associated with normal breast epithelial cells [361]. In particular, CAFs associated with invasive breast carcinoma cells show an abnormal migratory behaviour in vitro [361], altered levels of growth factors like CTGF [362] and insulin-like growth factors I and II [363], increased expression of inflammatory genes [364]. In addition, numerous studies have recently suggested that every cellular process is probably regulated by miRNAs and aberrant miRNAs expression may play a pathogenic role in several diseases, including cancer [6, 365]. Among dysregulated miRNAs, miR-221 is of relevant interest since it is strongly upregulated in a variety of hematologic and solid malignancies, including breast cancer (reviewed in [11]).

Indeed, increased levels of miR-221, which form a cluster together with miR-222 within the human chromosome X, have been related to the invasion of breast cancer cells and advanced clinical stages in breast tumor patients [366]. For this reasons, targeting miR-221 by specific inhibitors like LNA-i-miR-221 may represent a new promising strategy to overcome cancer progression, as in breast tumor [26-28, 367].

In this work, more information was provided in order to better understand the molecular mechanisms by which miR-221 can be involved in the progression of breast cancer. First of all, it has been discovered that in CAFs, in MDA-MB 231 and SkBr3 breast cancer cells, miR-221 down-regulates the expression of the ubiquitin-editing enzyme, A20, at both mRNA and protein levels. These results are consistent with previous data obtained in other cell contexts, showing that miR-221 directly targets the 3'-UTR region of A20 [18]. A20 is a Cys2/Cys2 zinc finger protein, which is induced by a variety of inflammatory stimuli and acts as a negative regulator of NF-κB [18, 260]. Of note, NF-κB signaling is tightly controlled by ubiquitination and A20, through its de-ubiquitinating activity, is one of the proteins that affects this process. For instance, it has been suggested that A20 induces the ubiquitination of the paracaspase MALT1, thus preventing the formation of the MALT1-IkB kinase complex and the consequent activation of the NF-κB signaling that mainly involves the heterodimers RelA, c-Rel, and p50 [22]. In agreement with these observations, it has also been discovered that miR-221 induces c-Rel expression, therefore suggesting that miR-221 may regulate NF-κB action. Interacting with other components of the NF-κB complex, c-Rel forms dimers that binding to specific sequences in the promoter region of target genes modulate gene expression [23]. By bioinformatics analysis, it has been found a putative NF-κB binding site located within the CTGF promoter sequence and we demonstrated that miR-221 induces the recruitment of c-Rel within the CTGF promoter region, toward an increase of CTGF expression. Corroborating these findings, finally it was assessed that the growth and migratory effects induced by miR-221 in CAFs, MDA-MB 231 and SkBr3 cells, are prevented by LNA-i-miR-221 or by silencing of c-Rel and CTGF expression.

Based on previous work, including our, [9], further studies have been undertaken in order to provide further information on the role of miRNAs in mammary tumor progression, analyzing the role of estrogens in the regulation of their expression.

Starting from a microarray analysis of 754 miRNAs involved in different diseases, it was established that several miRNAs are regulated by E2 both in SkBr3 breast cancer cells and in CAFs. In particular, it was found that E2 increases 23 miRNAs and lowers 2 miRNAs in SkBr3 cells, while E2 triggers upregulation of 7 miRNAs and down-regulation of 22 miRNAs in

CAFs. Furthermore, in both cell types E2 induced the expression of miR-144 and repressed miR-338-3p levels, known as an inhibitor of cancer progression [33-35, 354, 216].

A METABRIC analysis revealed a positive correlation of miR-338-3p with overall survival in breast cancer patients, suggesting an onco-suppressor role of this miRNA.

It has been shown that estrogen activation of GPER by E2 stimulates a network of transduction pathways, which triggers the key factors involved in cell growth, differentiation and transformation, such as c-Fos [368, 99, 369, 80]. The proto-oncogene c-Fos represents a prototype "immediate early" gene since its expression is rapidly induced by several extracellular stimuli through the activation of serine-threonine kinase of the mitogenic protein kinase (MAPK) family [370, 371]. The c-Fos encoded nuclear protein interacts with members of the Jun family to form the heterodimer activating protein-1 transcription complex (AP-1), which binds to TGACGTCAA sequences (AP-1 sensitive elements) located within the promoter sequences of target genes [370, 372]. Many studies focusing on the oncogenic functions of c-Fos demonstrated its involvement in tumor growth through the modulation of cyclin D1, which is a nuclear regulatory subunit of the cyclin-dependent kinases (CDK)-4 and CDK-6 [373-375]. Well-fitting with these data, it has been observed that in tumor cells SkBr3 and in CAFs E2 and G-1 induce the expression of c-Fos and Cyclin D1 towards cell proliferation. According to the inhibitory function of miR-338-3p in some types of cancer [33-35, 354, 216], it was also found that miR-338-3p abrogates the above effects triggered by E2 and G-1 in SkBr3 cells and in important components of the tumor microenvironment as CAFs [376, 26].

The aforementioned data highlight additional mechanisms involving miRNAs by which cancer cells and CAFs cooperate to worsen the characteristics of cancer. In accordance with our results, it was established that the development of cancer involves the functional interaction of malignant cells with the tumor microenvironment [377, 378]. For example, stromal cells such as CAFs generate a dynamic signaling network through the secretion of growth factors and cytokines that stimulate the proliferation and spread of tumor cells [379, 360]. In this context, the up-regulation of miR-221 and the down-regulation of miR-338-3p shared by breast cancer cells and CAFs may be a further mechanism towards breast cancer progression.

Altogether, our results provide new insights on the miRNA action in breast cancer, suggesting their use as an innovative prognostic and therapeutic approach in this malignancy.

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