Comparing the cellular distribution of myosin VI in MCF10a and MCF7 cell lines

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1 Declaration

No part of this thesis has been submitted in support of an application for any degree of qualification of the University of Kent or any other University or institute of learning.

Laura Medda

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2 Acknowledgement

I would like to express my special thanks of gratitude to my supervisor Christopher P. Toseland who gave me the opportunity to do this project, which also help me in doing a lot of research and I came to know about so many new things, I’m really thankful to them.

I would like to thank Yukti Hari-Gupta for all assistance and for her patience to teach me always.

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Secondly, I would also like to thank you my sister that gave me the strength to go through this year; and my family for providing me with unfailing support and continuous encouragement.

This accomplishment would not have possible without them.

3 Abbreviations

MVI= myosin VI

MCF7=tumorigenic breast cell line

MCF10a=epithelial breast cell line

TIP=2,4,6-triiodophenol

ATP=adenosine triphosphate

ADP=adenosine diphosphate

P_i=phosphate inorganic

IQ=isoleucine

CBD=cargo binding domain

NDP52=nuclear dot protein 52

T6BP=Traf6-binding protein

GIPc=Gaip interacting protein C-terminus

TOM1=Tom like 1

Dab2=Disabled-2

LMTK2=Lemur tyrosine kinase 2

SAP97=Synapse associated protein 97

cδ3=phospholipase c delta 3

Dock 7=dedicator of cytokinesis

PIP_2=phosphatidylinositol 4,5-biphosphate

LI=Large insert

NI=Non insert

GFP=green fluorescent protein

RNA Pol II=RNA polymerase II

DNA=Deoxyribonucleic acid

RNA=Ribonucleic acid

CFTR=Cystic fibrosis transmembrane conductance regulator

PCR=Polymerase chain reaction

RT-PCR=Reverse transcription polymerase chain reaction

HeLa=ovarian cancer cells

HER-2=Epidermal growth factor receptor

NHR=Nuclear hormone receptor superfamily

ERs=Estrogen receptors

mERS=Estrogen receptor plasma membranes

mtDNA=mitochondrial DNA

ERE=Estrogen response element

HSP=Heat shock proteins

HAT=Acetyl transferase

HDAC=Deacetylase activity

4 Abstract

Myosin VI is a motor protein involved in different cellular processes. The role of myosin VI in cell migration has been linked to ovarian, prostate and breast cancer, in which myosin VI has been reported to be positively correlated with tumor grade and metastatic potential.

However, its role in tumorigenesis of breast cancer and its pathological function remain unknown. This study is focused on the characterization of myosin VI and compares the protein expression and distribution using a reliable model of non-tumorigenic cell line, MCF10a cells and in parallel the tumorigenic breast cell line MCF7.

Immunofluorescence and western blot analysis demonstrate overexpression of myosin VI and estrogen receptor in the tumorigenic cells MCF7. Conversely, MCF10a showed low expression of myosin VI and no expression of estrogen receptor.

To investigate the role of myosin in breast cancer cells were subjected to hormone treatment where a hormone-dependent distribution for myosin VI inside the nucleus in MCF7 cells was observed. To investigate this in more detail, the endogenous expression of myosin VI and estrogen receptor were inhibited in MCF7 and in MCF10a cells using small RNA interference. Moreover, inhibition of the myosin VI ATPase was performed using the small inhibitor 2,4,6-triidrophenol (TIP). Taken together, this impacted on the cellular distribution of myosin VI. This adds support the role of myosin VI in hormone-dependent transcription with may be useful in future therapy and diagnosis.

5 Contents

6 Table of Figures

7 Table of Tables

8 Introduction

8.1 The myosin superfamily

Myosins are a large and diverse superfamily of molecular motors that perform vital roles in diverse cellular processes. They are expressed in all eukaryotic species and can be classifies into more than 20 different classes. In humans, the myosin superfamily comprises 40 known genes which can be grouped into 12 subfamilies [1]. They are further divided in to two major groups, called conventional myosin-II and unconventional myosins [2].

Conventional Myosin II is the largest (13 genes), expressed in muscle cells and in contractile rings of non-muscle cells, contains a long coiled-coil dimerization domain at their C-terminal and forms bipolar filaments that pull actin filaments together to produce contractions.

The rest of myosin motors are overall unconventional myosins (myosin I, V, VI and X) and they are very divergent in the region outside their motor domains and consequently they play different cellular functions [3]. They do not form bipolar thick filaments, and all those identified to move toward the plus (+) end of actin filaments except for myosin VI, which moves in the opposite direction [4]. This work is focussed on understanding the roles that myosin VI plays in the cellular events and will first examine its structure and properties.

8.2 Myosin VI

8.2.1 Structure and properties

Myosin VI was initially identified and partially characterized in *Drosophila melanogaster* [5], Myosin VI is an actin-based motor protein of 150 kDa, that uses the energy derived from the ATP hydrolysis to perform mechanical work and move cargos along actin tracks [6].

Structurally, myosin VI consists of the following basic myosin domains: an N-terminal highly conserved motor domain, following by a neck region consisting of a unique insert and an IQ domain, a tail with a helical region and a C-terminal globular domain (Figure 8.1).

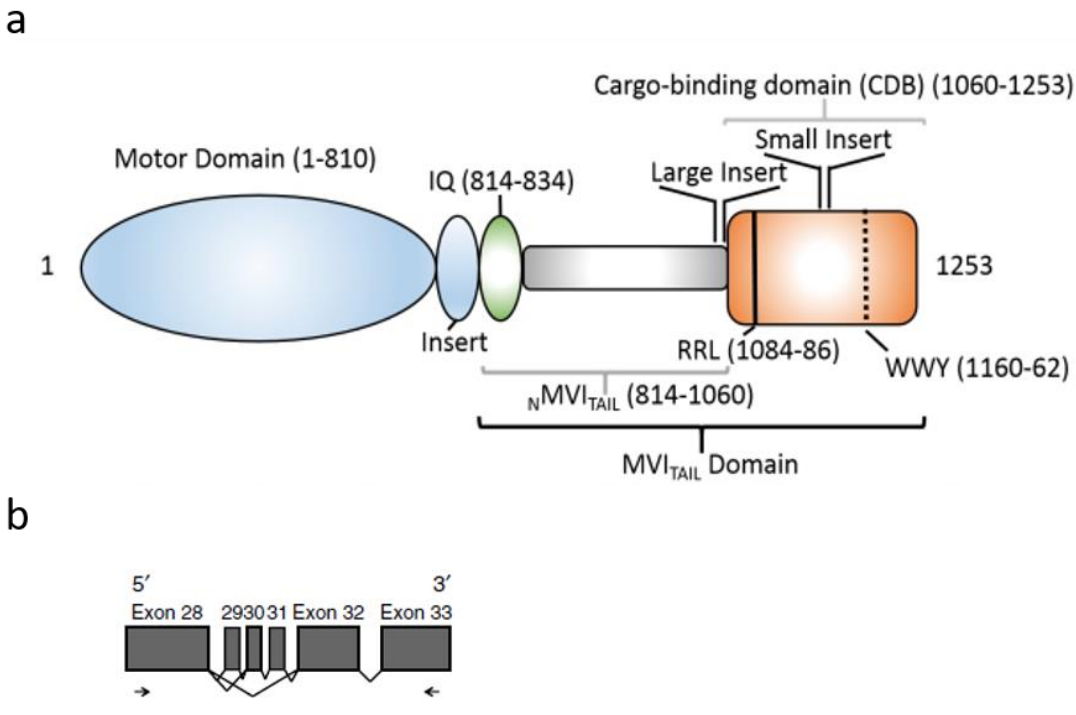

Figure 8.1: Structure of myosin VI.

a) Cartoon illustrating domain organization of myosin VI with the motor domain, neck region with a unique insert, an IQ domain, a tail and a cargo-binding domain. (Fili *et al*, Nat. Com. 2017). b) Schematic presentation of the region coding the myosin VI isoforms. ((Wollsheid *et al*, Nat. Struct. & Mol. Biol., 2016).

The ~80-kDa catalytic motor domain contains the ATP binding pocket and an actin binding interface that can bind filaments of actin. The ATPase activity of myosin VI is coupled to the binding and release of actin such that the motor is able to do mechanical work. Each cycle of ATP hydrolysis causes conformational changes in the motor domain that are transmitted through a converter region into a large movement of the adjacent lever arm and then the motor protein takes a step along actin.

Next to the motor domain, there is also a unique insert, which reverses the direction of the motor, allowing myosin VI to move in the opposite direction to all other myosins.

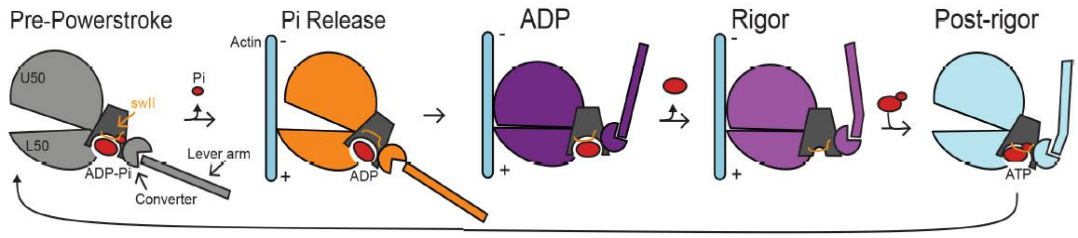

Figure 8.2: Myosin VI states in the force generation cycle.

Filament polarity is indicated with pointed end as (-) and barbed end as (+). The picture shows the motor domain binds and hydrolyzes ATP, which causes conformational changes (pre-power stroke state). The transient opening of the small switch II loop adjacent to the nucleotide binding cleft releases phosphate (P_i release state), the next state in the middle shows a transition to a moderate F-actin binding affinity state (ADP state) accompanied by big rearrangement in the converter region, which is propagated to lever arm to generate power stroke. The release of ADP is converted in high affinity actin-myosin VI interaction (rigor state). Re-binding of ATP in the nucleotide binding site promote myosin VI dissociation from the filament (post-rigor). (Gurel *et al*, eLIFE 2017).

The C-terminal globular region, known as cargo-binding domain (CBD) contains two myosin VI ligand interaction surfaces: the RRL and WWY amino acid motifs. Numerous adaptors proteins compete to interact with these two motifs.

The region containing the RRL motif interacts with nuclear dot protein 52 (NDP52), Traf6-binding protein (T6BP), optineurin and GAIP-interacting protein C-terminus (GIPC), and the region include the WWY motif is important for binding to Tom1,

Disable-2 (Dab2) and Lemur tyrosine kinase-2 (LMTK2). For the other myosin VI adaptor molecules, such as SAP97, otoferlin, phospholipase Cɗ3 and Dock7 the exact binding domain has not yet been determined [7].

Myosin VI can exist in a number of different isoform, two regions within the tail can be alternatively spliced in a 31 residue insertion (large Insert, LI) between the helical and the CBD, and/or an 8 residue insertion in the CBD (small insert, SI). This led to four splice isoforms express from one gene: the non-insert (NI), small insert (SI), large insert (LI) and one with both insert (LI+SI). The gene of myosin VI consists of 36 coding exons, three of wich (exons 29, 30 and 31) generate LI. The myosin VI isoforms expressed in human cell lines are also namely: myosin 1 and 3 (or LI) which have different lengths (23- or 32-residues long link to the presence or absence of exon 29) and isoform 2 short insert (or NI) which lacks the LI enterely exon 29, 30 and 31 are absent (Figure **8.1**) Two variations of the large insert (LI), the 31 aa insert is expressed in gut and a shorter 23 amino acid insert is expressed in brain. The tissue distribution of these isoforms show that the large insert is expressed in polarized epithelial cells containing apical microvilli such as liver, kidney and small intestine whereas the isoforms with the small or no insert are expressed in unpolarized tissue culture cell lines and in tissue such a testis. The tissue-specific distribution of these isoforms variants is most likely to be a mechanism to control motor-cargo interactions and their subcellular localisation and function.

8.2.2 Intracellular functions of myosin VI in mammalian cells.

One of the basic cellular functions of myosins VI is the sorting, transport and distribution of vesicles, protein complexes, membranes and other specific cargoes along the dynamic actin cytoskeleton to maintain the health and integrity of the cell.

Myosin VI exerts these different functions in mammalian cells through its ability to bind specific cargo adaptors proteins in a number of specialized cell types: polarized epithelial cell containing microvilli, hair cells of the inner ear with stereocilia, dividing cells undergoing cytokinesis, migratory cells and neuronal cells;

and the differential variety of isoforms of myosin VI has been suggested to regulate the motor's role.

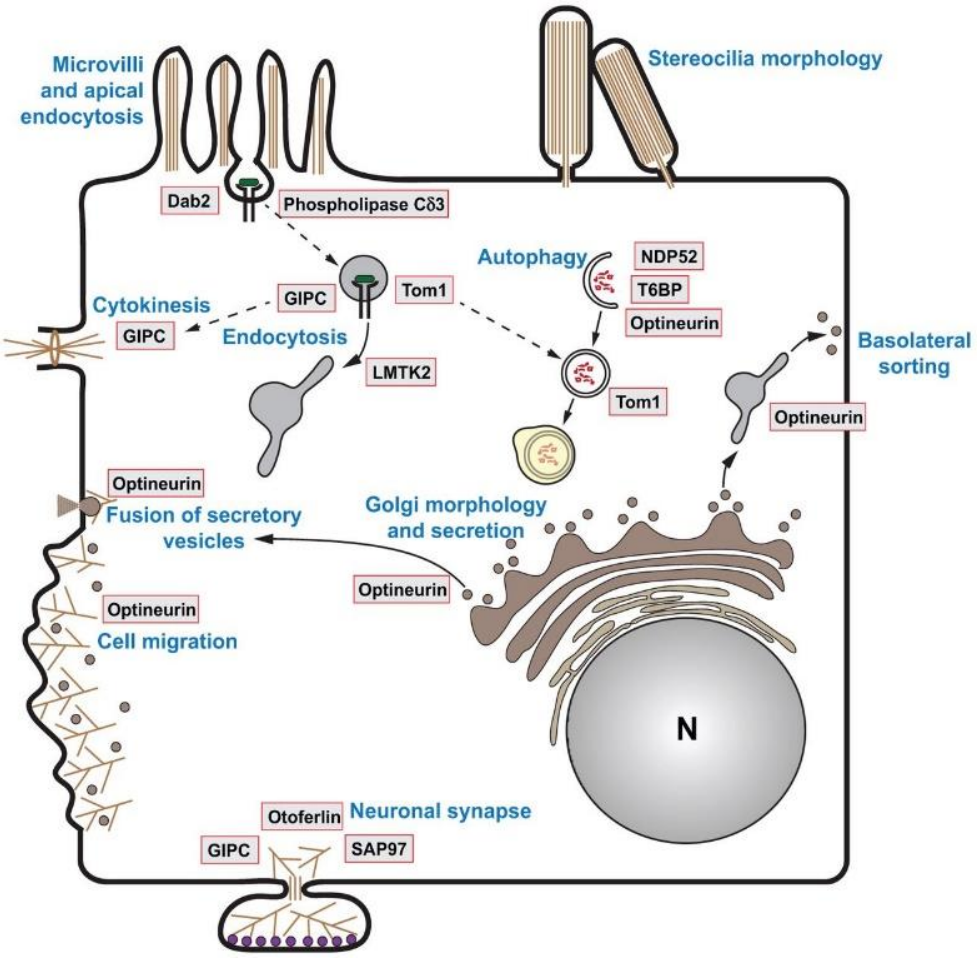

Figure 8.3: Cellular functions and various membrane trafficking of myosin VI

Directed by different cargo binding proteins in different cell types. (Tumbarello *et al*. Journal of Science. 2013).

The isoform expressing the large insert (~32 amino acid) in tail region is specifically expressed in polarized cells with apical microvilli and might play a role in enhancing the targeting of myosin VI to clathrin-coated structures in these cells [8]. This isoform of myosin VI colocalized with the endocytotic adaptor protein, the tumour suppressor Dab-2 (Disabled-2), in clathrin-coated pits and vesicles [9].

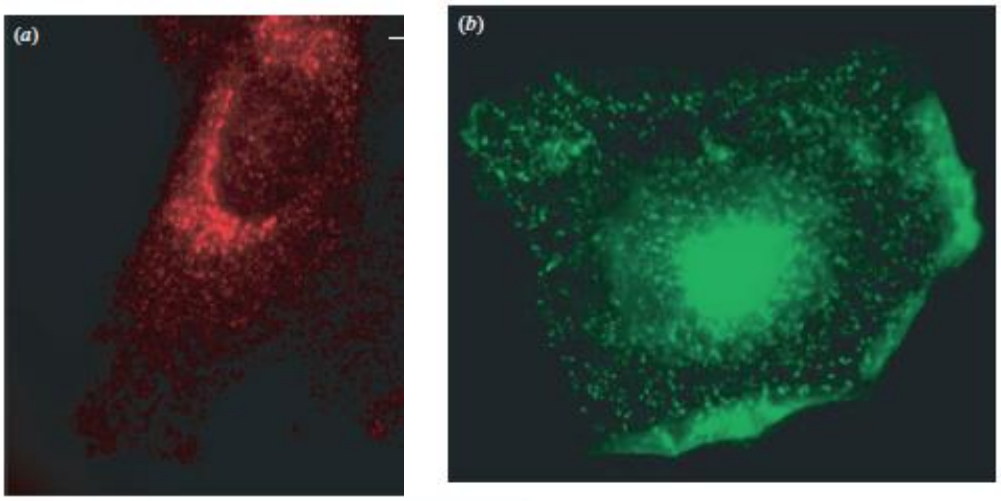

Figure 8.4: Localisation of myosin VI in fibroblasts.

a) endogenous myosin VI localised by immunofluorescence microscopy usin a polyclonal antibody. b) the expression of GFP-tagged myosin VI shows the localization of myosin VI in the Golgi Complex, in the perinuclear region of the cell, in membrane ruffles at the leading edge the cell, and in vescicles distributed throughout the cell. (Roberts *et al*. The Royal society 2004).

The isoform expressing non-insert (NI) or small insert of myosin VI are expressed in non-polarized cells and they localize to the uncoated vesicles. These isoforms are involved in the early step of the endocytic pathway [10].

Recently, it was revealed that myosin VI is recruited to the nucleus in an isoform specific manner. The large insert isoform has been shown to encode an amphipathic α-helix (α_2 linker) that block the RRL motif and this isoform is not recruited to the nucleus [11,12].The non-insert (NI) isoform of myosin VI is recruited to the nucleus and therefore it is believed that the RRL motif, and associated binding partner, is linked to nuclear recruitment [12].

8.2.3 Myosin VI and diseases.

A breakdown in the regulation mechanism of myosin VI, mutation, or its loss are associated with a number of pathological disorders including hypertrophic cardiomyopathy [13], hereditary hearing loss [14,15], neurodegeneration, defects in neuronal AMPA receptor [16] and cystic fibrosis CFTR receptor endocytosis. An interesting connection between Myosin VI a human cancer was recently reported. [17]. Several studies also indicate that myosin VI expression is extensively up-regulated in human ovarian cancer [17], prostate [18] and breast cancer cells, which is positively correlates with tumour grade and metastatic potential. In prostate cancer, the inhibition of myosin VI expression impeded cell migration *in vitro.*

Dunn et al. confirmed by immunohistochemical analysis of myosin VI expression an antibody specificity for the major band (150 kDa) of myosin VI in the majority of cancerous epithelial cells and negative or weak staining in normal epithelium as show the array spot (**Figure** 8.5).

Further comparative analysis of myosin VI expression level was performed between normal tissue and cancerous tissue using pathological Gleason scores (

Figure 8.5). The majority of cancerous tissues demonstrate high level expression of myosin VI in the early stadium of cancer at the Gleason score (grade 3) respect at the high-grade prostate cancer (Gleason score 8 to 10) [19].

Similarly, the level of expression of myosin VI has been shown to correlate with the aggressiveness of human ovarian cancers, while an inhibition of myosin VI expression in metastatic ovarian cancer cells decreased both their migration *in vitro* and spreading and metastasis throughout the abdominal cavity *in vivo* in a mouse model [17].

Several studies have reported the relevant differential expression of myosin VI isoform in tumours. To understand the possible differential expression of myosin VI

isoforms in tumours Wollsheid *et al*. analysed expression by reverse transcription PCR (RT-PCR). The RT-PCR was performed with RNA extracted from high-grade primary ovarian cancer cells cultured *in vitro*. In contrast to their control, ten out of ten tumours expressed the myosin NI isoform, suggesting a positive selection for this isoform in tumour progression and metastasis.

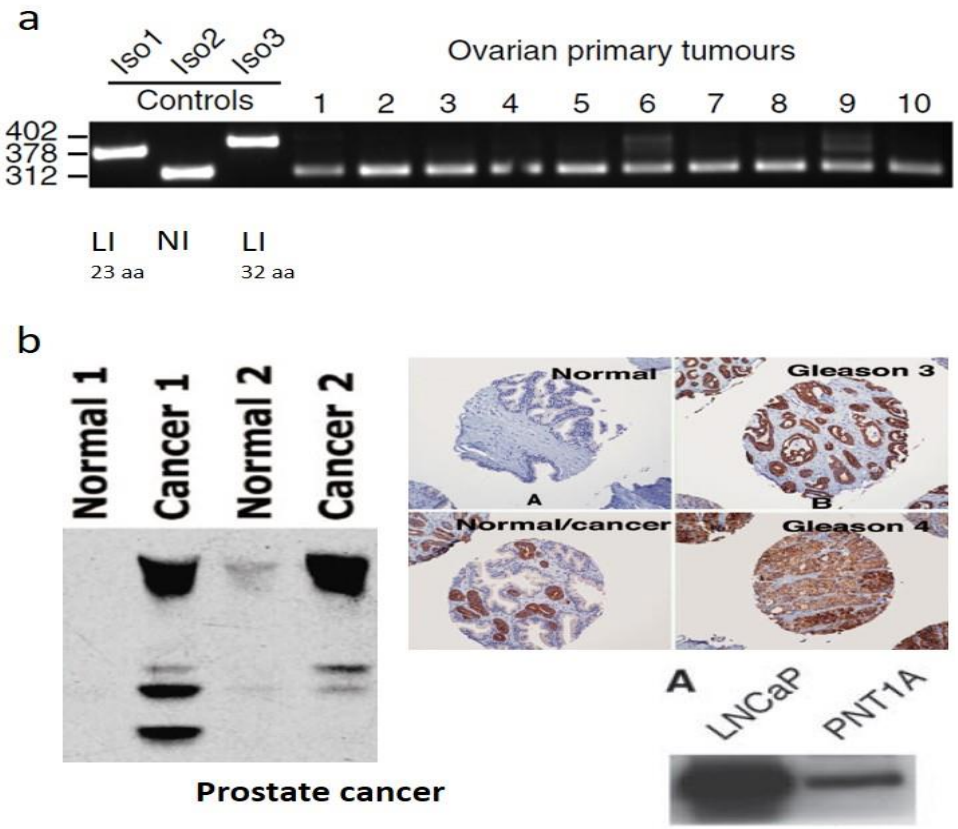

Figure 8.5: TMA analysis of myosin VI and RT-PCR.

a) RT-PCR from cDNA prepared from primary tumour cells and cell lines on the left (Wollsheid *et al*, Nat. Struct. & Mol. Biol., 2016). b) Confirmation antibody specify in cancer tissue on the right (Dunn *et al.* Am. Jour. Path, 2006)

8.2.4 The nuclear role of myosin VI

Recently, myosin VI has been discovered in the nucleus, the genetic storage compartment of the cell, where it has been shown to play an important role through interactions with nuclear receptors. This allows myosin VI to drive expression of target genes.

Normally myosin VI is in a back-folded state with the CBD folded upon the motor domain. The association of a binding partner releases the auto-inhibited backfolded state. This exposes DNA binding sites in the CBD and subsequently allows association with RNA Polymerase II. The RNA Polymerase II interactions are not limited to the C-terminus of MVI. The motor domain also associates with RNA Polymerase II through short polymers of actin [20]. This means could be a bipartite association between myosin VI and RNAPII complex, in an unfolded state MVI utilises its mechanical ability to anchor the complex in situ, or act as an auxiliary motor driving RNA Polymerase II.

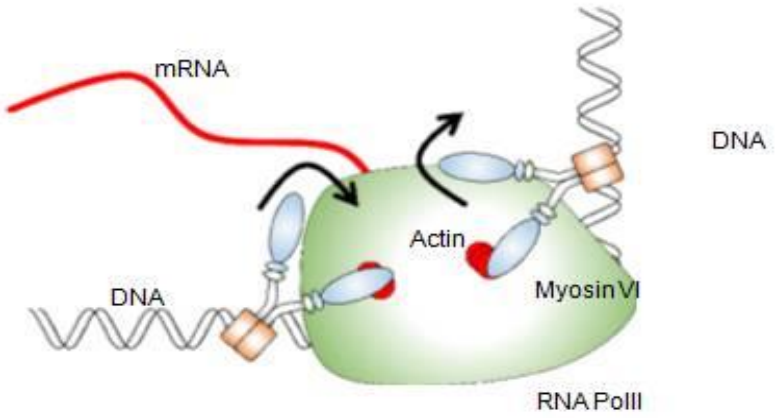

Figure 8.6: Model of MVI in transcription.

MVI bound a binding partner and DNA at the C-terminus and RNAPII trough actin at the N terminus (Fili *et al*. Nature Communication 2017)

8.2.5 Myosin VI, estrogen receptor and breast cancer.

Breast cancer (BC) is an example of a hormone-dependent neoplasm, that originates from the galactophore ducts (ductal form) or from terminal small ducts (lobular form) of the mammary gland. Development and growth of the tumour mass is stimulated by feminine sexual steroids that exercise a daily mitogenic action on mammary gland cells [21].

The carcinogenetic action of estrogens, during the tumour progression, is due to their ability to promote morphologies changes that enhanced migratory and invasive capabilities [22]. However, the mechanisms of estrogens that determine survival and proliferation of breast cancer cells are still not entirely clear; this explains the difficulties that still exist in the prognostic evaluation and in the therapeutic management of the women suffering from this disease.

Pleiotropic and tissue-specific effects of estrogens are mediated by different nuclear receptors. The different forms of breast cancer are distinguished, on the basis of immunohistochemical analysis, by the presence or absence of three receptors: the estrogen receptor ERα (Estrogen Receptor α), the progesterone receptor PR (Progesterone Receptor) and the HER-2 epidermal growth factor receptor (Human Epidermal Growth Factor Receptor-2).

More than 60% of breast cancers are positive for these receptors. However, a lower percentage, around 10-17%, does not express any of these receptors and therefore is called triple negative.

Recently, it was reported that a series of molecular motors were implicated in the development of breast cancer. Myosin X mediate breast tumour cell invasion by transporting integrins to filopodian tips in breast cancer [23].

Others studies reported that elevated expression of myosin X and myosin II in tumours contribute to breast cancer aggressiveness and metastasis [24,25].

As is known, myosin VI plays a role as oncogenic promoter in different human cancers, a new study of myosin VI examined the biological functions in human breast cancer cell line MCF7. Estrogen receptor activation therefore cell growth appears to be dependent upon myosin VI based upon research from Toseland Lab [31].

8.2.6 Functions of myosin VI in MCF7 cancer cells

To explore the cellular function of myosin VI they focus in specific to understand the nuclear role of myosin VI in MCF7 cell line. MCF7 cells are luminal cancer breast cell line. These cells are characterized by estrogen receptor and/or progesterone receptor expression.

MCF7 breast cancer cells are estrogen sensitive cells and they depend on estradiol in order to proliferate [26]. They express high levels of ERα but low levels of ERβ [27]. These cells have the characteristic of being very differentiated and to have low capacity to migrate due to tight cell-cell junctions, consistent with that at the tumour level [26]. They are poorly aggressive and non-invasive cell line [28] normally they are considered to have low metastatic potential [29,30].

In these cells myosin VI has shown to be important for the expression of genes responsive to estrogen receptor (ER) signalling *PS2* and *GREB1* [31]. Knockdown of myosin VI resulted in a decrease of 70-80 percent *PS2* and 30-40 percent for *GREB1* (Figure 8.7).

This suggest a link between receptor binding and the nuclear role of myosin VI in transcription.

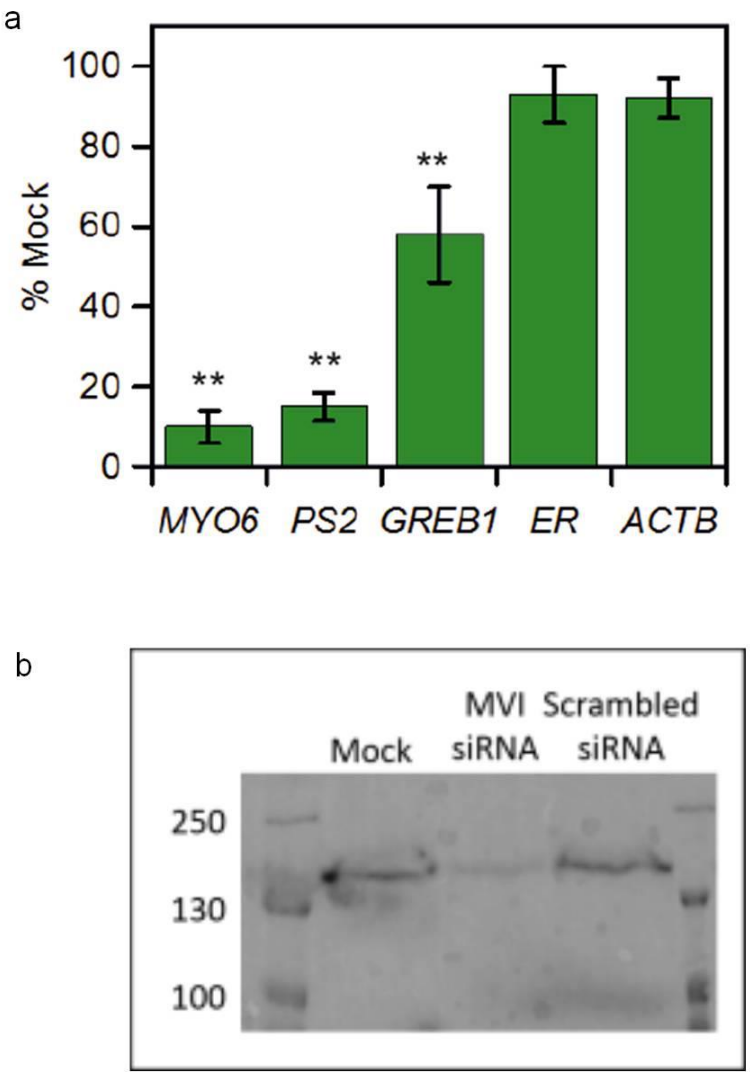

Figure 8.7: Expression of ER target genes following siRNA in MCF7 cells.

a) The expression is reported as a percentage of expression in mock cells. ER and ACTB were used to show the global change in transcription (Fili *et al*. Nature Communication 2017). b) Western Blot analysis showed no expression for myosin VI knockdown.

To elucidate if there is a generic role for myosin VI in the expression of estrogen receptor targets genes, a luciferase reporter assay was used. A generic ERE promoter drove expression of the luciferase in MCF7 cells but this was inhibited when myosin VI was knocked-down [31]. This supports a generic role for myosin VI in the estrogen receptor signalling pathway.

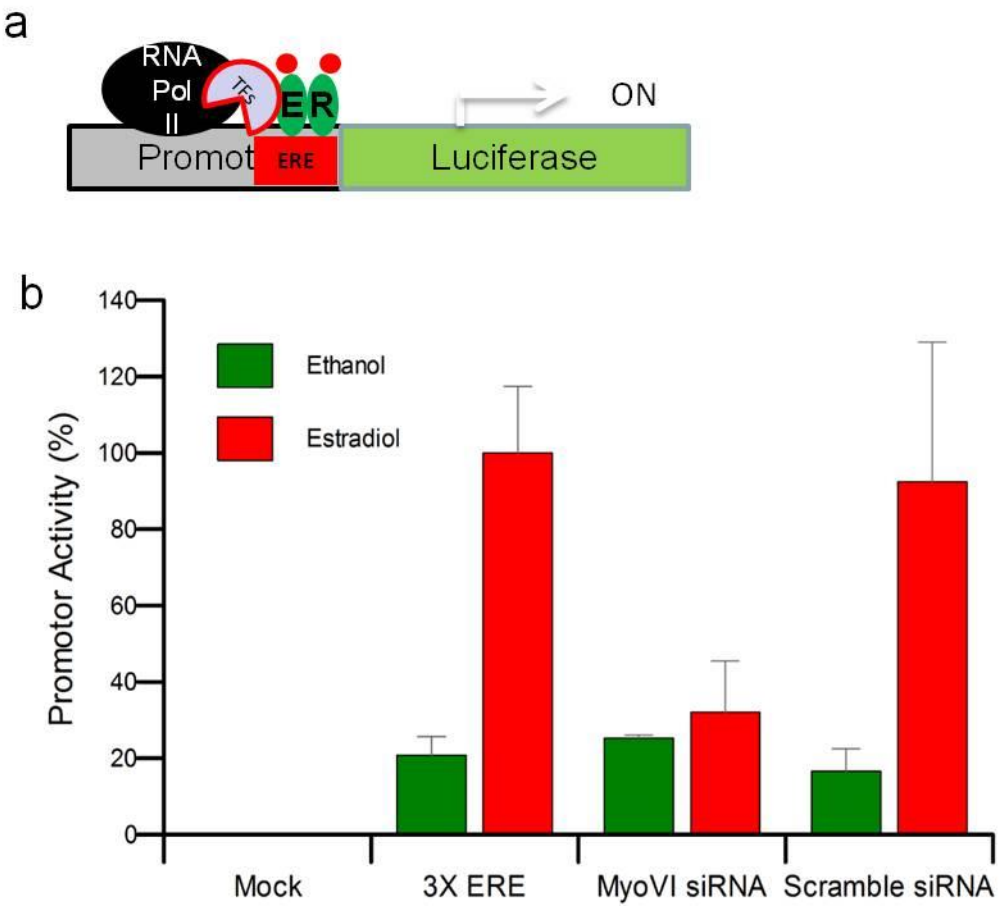

Figure 8.8: Luciferase assay driven by ERE promoter in MCF7cells.

a) Luciferase assay driven by ERE promoter in tumour breast cell line, MCF7. b) Estradiol increase 5-fold the promoter activity. SiRNA knockdown of MVI reduce 3-fold the promoter activity. Ethanol was used as a carrier control for the experiments.(Fili *et al*. Nature Communication 2017).

The genes, that are under control of the estrogen receptor, regulates the cell growth.

To understand the role of myosin VI in the expression of these genes Toseland Lab studied the effect of myosin VI knockdown in the cell growth in different cell lines of breast cancer: MCF7, T47D and CAMA-1.

T47D is a human breast epithelial cancer cell line derived from the pleural effusion of a ductal carcinoma found in the mammary gland of an elderly human patient. T47D cell are shown to have a progesterone receptor not regulated by estradiol

[32]. Cam-1 is a luminal type of human breast cancer cell line that present rounded morphology in adherent tissue culture. These cells are considered HER-2 negative and estrogen receptor/progesterone receptor positive [33].

Cell proliferation was monitored following myosin VI siRNA Knockdown in these different cell lines (Figure 8.9). As expected myosin VI knockdown cause decrease of cell growth in all cell lines.

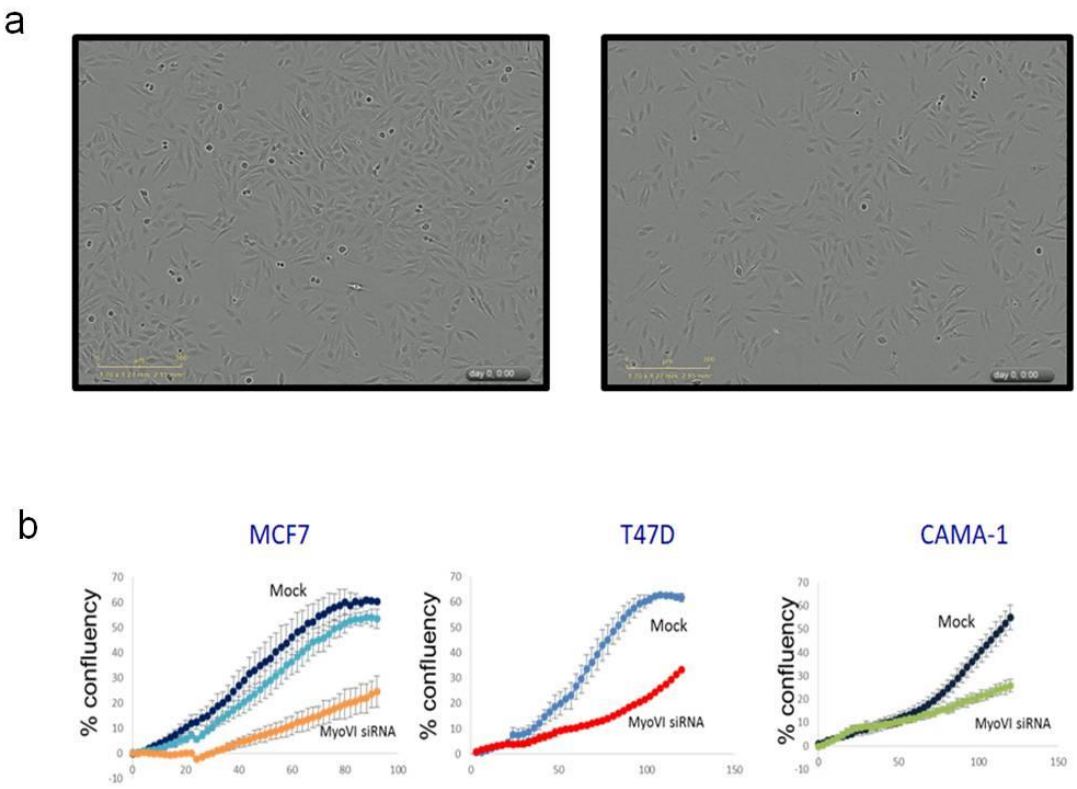

Figure 8.9: Percentage in different tumour breast cell lines was detected in real time by proliferation assays in normal condition and following myosin VI knockdown.

It is believed that the importance of myosin VI in these cells is related to a direct interaction between myosin VI and the estrogen receptor (Figure 8.10). The C-terminus of myosin VI interacts with a nuclear receptor binding motif LxxLL in the ER, as shown by pull-down assay with recombinant proteins. Mutation within the nuclear receptor binding motif prevented the pull-down [31].

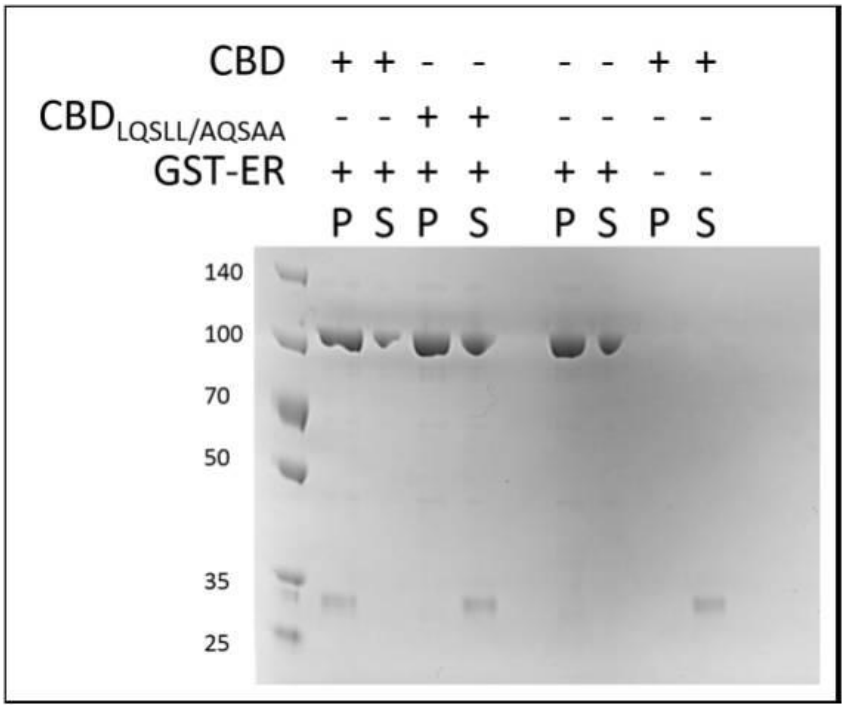

Figure 8.10: Pull down of CBD by GST-ER.

P and S represent pellet and supernatant fractions. (Fili *et al*. Nat. Com. 2017)

8.2.7 Estrogen receptors: structure and functional domain

The estrogens receptors are modular proteins that act as transcription factors and they belong to the nuclear hormone receptors (NHR) superfamily. The first estrogen receptor discovered was the estrogen receptor α (ERα) in 1950 by Elwood Jensen [34] and soon after the characterization of the ERα knockout mouse it was discovered Estrogen receptor β [35]. Both receptors are encoded by two distinct genes and are expressed in the tissues at varying levels. The ERα is protein of 66 kDa that is composed of 595 amino-acids [36,37]. The estrogen receptor beta (ERβ) consist of 530 amino acids with molecular mass 60 kDa [38]. The ERα predominates, whereas ERβ plays a minor role, in the uterus, mammary glands, pituitary gland, skeletal muscle, adipose tissue, and bone. ERβ in contrast is found in mediating signalling in the ovary, prostate, lung, cardiovascular and central nervous system.

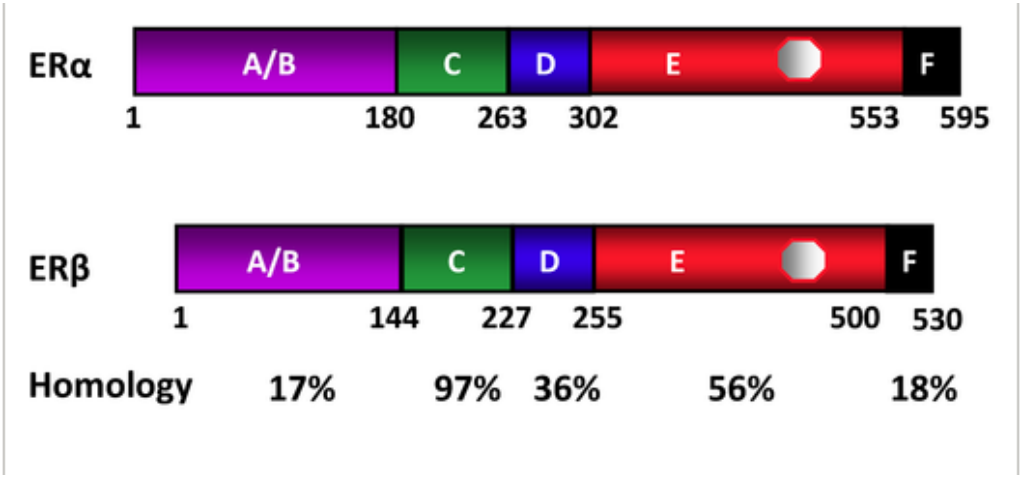

Figure 8.11: Illustration of the estrogen receptor α and estrogen receptor β structural regions.

(Y. Pelin *et al*, Reproductive Medicine and Biology 2017).

The estrogen receptors are subdivided into six functionally distinct domains [39]. The amino-terminal A/B domains share a 17% amino-acid identity between the ERs. The near identical C region is the DNA binding domain (DBD). The flexible hinge, or D, domain (36%) contain a nuclear localization signal (NSL) and links the C domain to the multifunctional carboxyl-terminal (E) domain. This domain is also called ligand binding domain (LBD) and it shows 56% amino-acid homology between the ERs. The LBD is a globular region that contains a hormone-binding site, a dimerization interface (homo and hetero-dimerization), and a ligand dependent co-regulator interaction function (activation function, AF-2). The F domain shares 18% amino-acid identity and it is located at the extreme carboxyl-terminus of the receptors (Figure 8.11). Both receptors interacts with the same DNA response Elements and exhibit similar ligand binding characteristic. Where ERs are co-expressed, Erβ works as a trans-dominant inhibitor of Erα transcriptional activity at sub-saturating hormone levels and ERβ decrease cellular sensitivity to estradiol.

8.2.8 Estrogen signalling pathways

The Estrogen signalling is generated from various cellular locations and this appears to be critical in the regulation of cellular proliferation, differentiation, motility, and death dependent on the estrogen target tissue.

There are different mechanism of activation of estrogens signalling.

Estrogen receptor mediated membrane signalling. This mechanism is activated through some receptors for estrogens located on the plasma membrane (mERs) that transduce the signal after a few seconds or some minutes from the hormone binding. This pathway determines the increase of the ion calcium ions, cAMP and Inositol phosphate that activates different kinases. These pathways can interact with each other and converge in the regulation of the expression of specific genes and in protein synthesis through rapid activation of the transcription factors CREBP (CRE Binding Protein) or NF-kB (Nuclear Factor) [40].

Estrogen receptor-mediated mitochondrial events. The mitochondrion is important for the action of estradiol, which inhibit the early stage of apoptosis [41]. In mitochondrial the bound of estradiol alters the mitochondrial functions and mediate the expression of target genes through a direct interaction with mtDNA with the binding of the mitochondrial RNA polymerase and the mitochondrial transcription factors. The mitochondrial functions are also modulated by nuclear estrogen receptor through expression of genes that cause production of protein involved in mitochondrial functions.

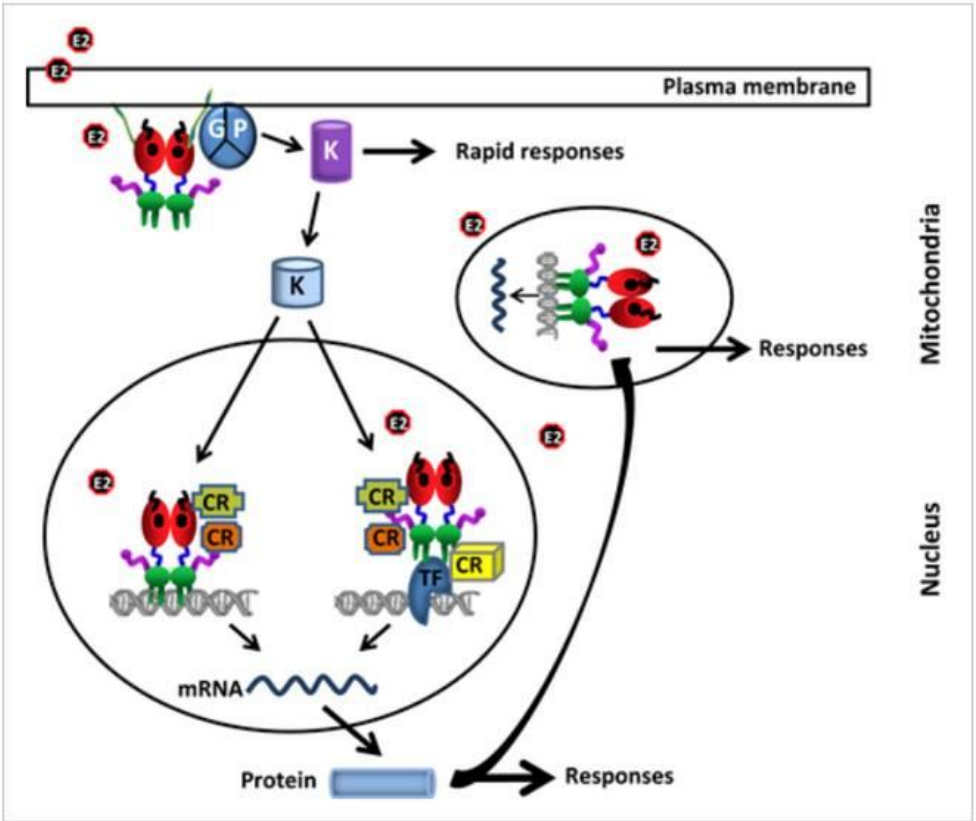

Figure 8.12: Illustration shows the multifaceted mechanisms of estrogen receptors and signalling.

(Y. Pelin *et al*, Reproductive Medicine and Biology 2017).

8.2.9 Estrogen response element signalling pathway-Intracellular estrogen receptor.

The 17-β-estradiol regulates cellular response through its ability to bind the intracellular ERα.

In the absence of the ligand, the estrogen receptor α is located at the cytoplasmic and nuclear level, where it is associated with HSP proteins (Heat Shock Proteins).

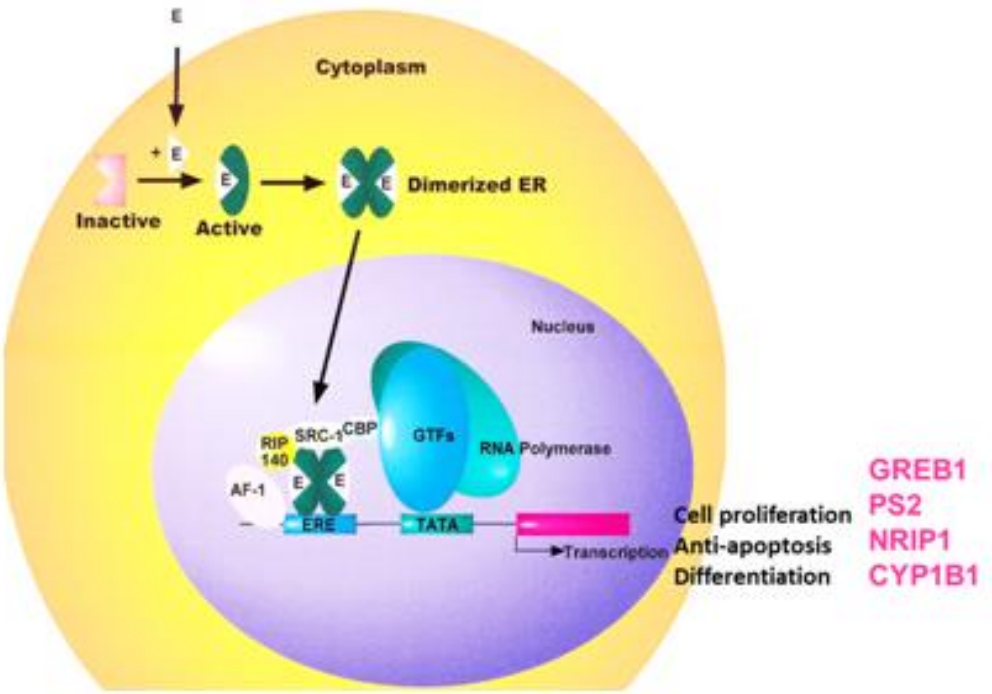

Figure 8.13: Estrogen dependent signalling pathway.

(R.D Brinton, Learning & memory 2019).

Due their hydrophobic nature, the estrogens diffuses across the plasma membrane, following the binding of the hormone (E2), these receptors undergo a conformational change, which triggers dimerization of the receptor.

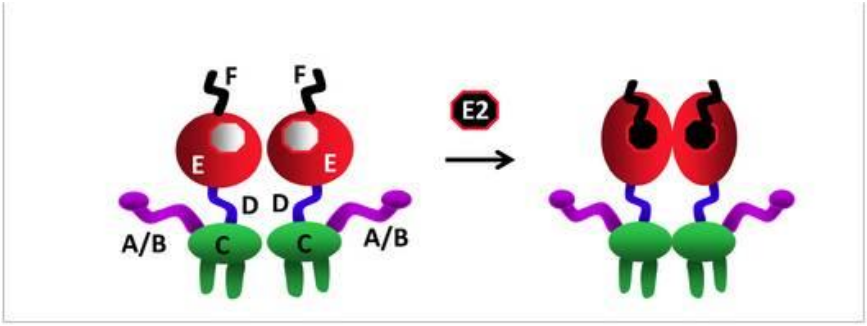

Figure 8.14: The 17-β-estradiol bound induces conformational changes in the receptors.

(Y. Pelin *et al*, Reproductive Medicine and Biology 2017).

The nuclear localisation signal (NLS), located in the D region of the estrogen receptor, is required for the translocation of the estrogen receptor to the nucleus but the mechanism by which the estrogen receptor is translocated into the nucleus remains unclear.

However, microtubule-associated motor proteins have been implicated [42].

Inside the nucleus the estrogen receptor interacts with a consensus estrogen responsive element (ERE), DNA palindrome sequence (5^{I}-GGTCAnnnTGACC-3^{I}) separate by three nucleotides and localized at the level of genes subjected to estrogenic control [43,44]. ERE's act in regulating the expression of target genes (*PS2, GREB, NR1B1* and *CYP1B1*) implicate in numerous cellular processes: regulation of cellular proliferation, differentiation, motility and anti-apoptosis.

The interaction between receptors and co-regulators is based on the presence of conserved hydrophobic α helix, rich in leucine that contain the sequence LXXLL. The LXXLL determines specificity to the ER-coregulator interaction and serve as signal input domains by anchoring the members to the promoter (ERE) [45].

Several co-regulators are involved in chromatin remodelling: many coactivators present activity histone acetyl-transferase (HAT), or are associated with HAT, facilitating the activation of transcription relaxation. Similarly, the corepressors have histone deacetylase activity (HDAC) favouring the chromatin compaction, and therefore gene silencing one interacts with the major groove of the double helix and the other stabilizes the complex [45,46].

8.2.10 MCF10a, a reliable model of non-cancerous human mammary epithelial cells.

Various cell models *in vitro* and *in vivo* have been used to study breast cancer tumorigenesis, metastasis and drug sensitivity.Different mouse models have been used to study breast tumorigenesis, but these models shown limitations.

The properties of breast cancer have been studied in different number of breast epithelial tumour cell lines. Primary cultures of normal breast epithelium have showed difficulty to study long term due the senescence and established normal immortalized non- tumorigenic breast cell lines have been difficult to establish without chemical or viral intervention.

Recently it was found the spontaneously development of immortalized but non transformed breast epithelial cells called MCF10 cells [47]. These cells are a valuable cell model in which to study transformation and immortalisation of breast epithelial cells. The MCF10 cells derived spontaneously from mortal cells cultured from breast tissue of a woman with fibrocystic disease.

The MCF10a cell line is pseudodiploid with minimal chromosome rearrangements and exhibits the inability to induce tumour in mice after exposure of estrogen [47]. They are non-tumorigenic and do not express ER, mRNA or protein that have unaltered gene.

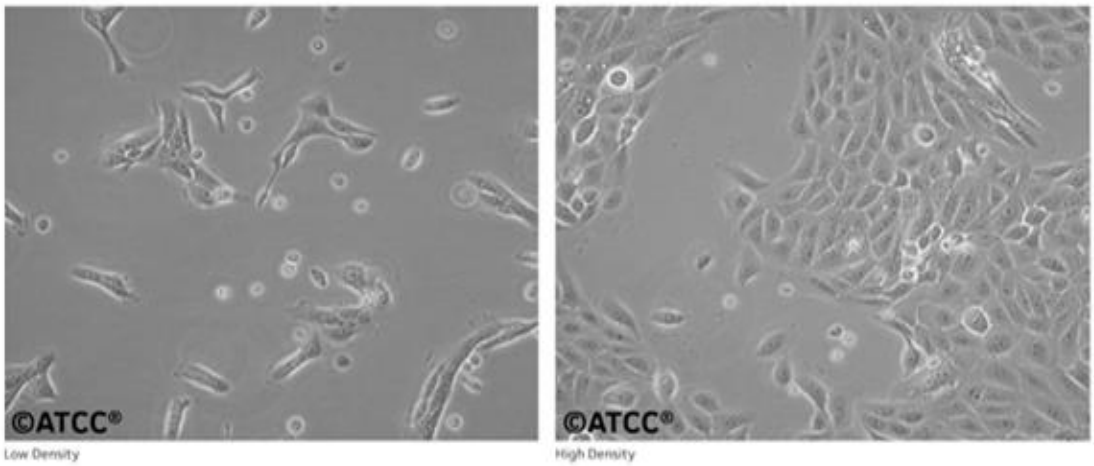

Figure 8.15: shows the low density and high density of MCF10a cells.

They present specific characteristics include depletion of the chromosomal locus containing the genes critical for the regulation of senescence and they share some feature of mesenchymal epithelial cancer cell lines [48].

8.3 Project outline

This project is focused in characterising myosin VI in presence of estrogen receptor positive breast cancer.

We try to investigate if myosin VI localisation is altered in response to ER activity or if the activity of myosin VI in normal and different conditions altered the distribution and localisation of the estrogen receptor.

We try to understand the behaviour of estrogen receptor in different cell lines, if the localisation is always dependent in response to estradiol, or there is something inside the tumour cells that determine the subcellular localisation.

The experiments have been conducted using an evaluable model of a mammary epithelial non-tumorigenic cell line, MCF10a, and using in parallel the tumorigenic cell line, MCF7.

This study explores the mechanism of myosin VI translocation into the nucleus, its distribution within the nucleus, and its the functions related to other endogenous proteins, RNA polymerase II and estrogen receptor, with regard to the regulation of transcription and expression of hormone-dependent target genes.

This project also aims to understand how the cells respond to hormones and how dependent these responses are on myosin VI and in specific describe the behaviour of myosin VI.

9 Materials and method

9.1 Cell culture

MCF10A cells

MCF-10a cells were maintained in PHR-free Dulbecco's Modified Eagle's Medium/Nutrient F12 (DMEM/F12) optimal cell culture medium (1:1; Gibco, Thermofisher) supplemented with 5% Horse serum (H1138; Gibco, Thermofisher, no charcoal stripped), 10 ng/ml cholera enterotoxin (C8052; Sigma-Aldrich), 10 ng/ml insulin (i9278; Sigma-Aldrich), 20 ng/ml human epidermal growth factor (HEGF; E9644 Sigma-Aldrich), Hydrocortisone 0,5 µg/ml (H6909 Sigma-Aldrich), 100 units per ml penicillin and 100 $\mu g/ml^{-1}$ streptomicin (Gibco, Thermofisher; 15140122).

Cell were cultured at 37 C° and 5% CO_2 and grown to confluency before splitting with passaging which occurred every four days.

MCF7 cells

MCF7 cells were maintained in Gibco MEM media Alpha medium with GlutaMAX (no nucleosides), supplemented with 10% Fetal Bovine Serum (10082147; Gibco, Thermofisher, no charcoal stripped), 100 units per ml penicillin and 100 $\mu g/ml^{-1}$ streptomycin (Gibco, Thermofisher; 15140122).

Cells were cultured at 37 C° and 5% CO_2 and grown to confluency before splitting with passaging which occurred every four days MCF7 cells.

9.2 Hormone starvation and stimulation

MCF7 cells and MCF10a cells were seeded at the density of 1.2 x 10^5 cell per well, MCF7 cells were growth in a 24-wellplate and MCF10a cells were growth on glass coverslips treated with Poly-D-lysine solution in a 24-wellplate.

When the cells reached 100% of confluence were starved with half normal MEM αlpha medium and half starved-medium (MEM 1x Minimum essential media, Gibco Thermofisher) supplemented with 2% FBS, Charcoal stripped (12676029; Gibco, Thermofisher).

The following day the medium was replaced with starved media.

After 72 hours of starvation the cells were stimulated with the hormone, the media was replaced with fresh media with 100 µM of estradiol (Sigma; E8875-250MG) and after 8 hours the cells have been a second stimulation of hormone.

Then the mixture was replaced with fresh media.

9.3 Inhibition with TIP (2,4,6-triiodophenol)

MCF7 cells and MCF10a cells were seeded at the density of 1.2 x 10^5 cell per well, MCF7 cells were growth in a 24-wellplate and MCF10a cells were growth on glass coverslips treated with Poly-D-lysine solution.

When MCF7 and MCF10a reached 100% of confluence were inhibited with 1 µM of 2,4,6-triiodophenol (TIP) (Stock solution of TIP in DMSO 100 mM, final solution 25 µM, Alfa Aesar, A17145) and 1 µM of DMSO (Stock solution of DMSO 100 mM, final solution 25 µM Sigma-Aldrich, D2650).

In MCF7 cells the mixture with the inhibitor was replaced after 1 hour with the fresh media. In MCF10a cells the inhibition was performed in different times: 1 hour, 3 hours and 24 hours.

For the 24 hours inhibition the mixture with TIP was replaced with fresh mixture with the inhibitor TIP every 4 hours. After 24 hours the mixture was replaced with fresh media.

A biological repeat was performed with 100 µM of TIP in MCF10a cells in different times: after 1 hours and 2 hours. Then the mixture with the inhibitor was replaced with fresh media.

9.4 Transfection

For transient expression of myosin VI isoforms the MCF7 cells were growth on glass coverslips were transfected with EGF-LI-MVI and EGFP-NI-MVI constructs using the Lipofectamine 2000 (Invitrogen).

Two tubes have been made: one tube contained the DNA stock required to have 500 ng of DNA, the DNA was diluted in 50 µl of Optimem (Reduced serum media, Gibco) and mixed gently (Tube I), then the lipofectamine 2000 was diluited in 50 µl Optimem (Reduced serum media, Gibco) and mixed gently tube II). The tube I and tube II were mixed and incubated for 5-10 minutes at room temperature.

Meanwhile the cells have been washed once with 250 µl Optimem (Reduced serum media, Gibco) and replaced with medium 10% FBS without antibiotics and put them into incubator. After that the mixture was added onto the cells, put in the incubator at 37 C° for 4-6 h. After this time, fresh media was added. At 72h post transfection, cells were subjected to nuclear staining, fixed and analysed or subjected to indirect immunofluorescence.

For myosin VI knock-down experiments, MCF7 and MCF10A cells monolayer, seeded to 30-50% confluency, were transfected with human myosin VI siRNA duplex (GUUAAUGAAGtt-3^{l}) (Ambion) at a concentration of 50 nM and for ER knock-down cell were transfected with human ER siRNA (humann estrogen receptor siRNA Ambion AM16708) at the concentration of 50 nM of siRNA using Lipofectamine invitrogen 2000 (Invitrogen).

Two tubes have been made: one tube contained the DNA, which was diluted in 50 µl of Optimem and mixed gently (Tube I), then the lipofectamine 2000 was diluited in 50 µl Optimem and mixed gently tube II). The tube I and tube II were mixed by pipetting up-down and incubated for 5-20 minutes at room temperature.

Meanwhile the cells have been washed once with 250 µl Optimem (Reduced serum media, Gibco) and replaced with medium 10% FBS without antibiotics and put them into incubator. After that the mixture was added onto the cells, put in the incubator at 37 C°. The day after the mixture was replaced with fresh media.

9.5 Nuclear staining and immunofluorescence

Glass coverslip (Thermofisher) were prepared by incubating them on Poly-D-lysine solution in H_2O, by 30 minutes, at room temperature, washed 3 times with PBS.

Immobilised nuclei were stained with Hoeschst 33342 (Thermofisher): prepare a 1712300 dilution of 12.3 mg/ml (20mM) Hoechst stock in normal medium for MCF10a cells (DMEM/F12/5%FBS/PenStrep) and for MCF7 (10%FBS/EMEM-PenStrep) to make 1mg/ml final solution: dilute 1 µl into 12.3 ml of medium in each well were added 0.5 ml/well (24-well plate) and incubate for 10 minutes at the room temperature.

After 10 minutes the media was removed and the cells were washed twice with PBS and then the cells were fixed in 4%(w/v) paraformaldehyde/PBS (PFA) for 10 minutes (outside the hoot).

Following the fixation cells were washes three times with PBS and residual PFA was quenched by incubating the cells with 50mM ammonium chloride (NH_4Cl) for 15 minutes.

Then the cells were washed three times with PBS. All steps were performed at room temperature.

After that the cells were permealized and simultaneously blocked for 15-30 minutes with 2% (w/s) BSA, 0,1% Triton (v/v) X-100 in PBS.

Cells were than immune-stained against by the endogenous proteins by 1 h incubation with the indicate primary antibody and then coverslips were washed three times with PBS and subsequently the cells were immuno-stained with the appropriate fluorophore-conjugated secondary antibody for 1h. Both antibodies were diluted in 2% (w/s) BSA, 0,1% Triton (v/v) X-100 in PBS.

The antibodies were used at the indicate dilutions:

Rabbit anti-MVI (1:200 Atlas sigma HPA035483-100UL), Mouse anti-Erα (1:100 SC-8002 Santa Cruz Biotechnology), mouse anti-PolII (1:500, Abcam, ab5401) and donkey anti-rabbit Alexa Fluor 555-conjugated antibody (1:500, Abcam, ab150074), anti-mouse 488-cojugated antibody (1:500 Abcam, ab181289), anti-rabbit-conjugated (1:500 Abcam, ab181346).

Then the coverslips were washed one by one with sterilized water (in a beaker) and than were mounted on microscope slides with Mowiol (10% (w/v) Mowiol 4-88, 25% (w/v) glycerol, 0,2 M Tris-Hcl, pH 8.5), supplemented with 2.5% (w/v) of the anti-fading reagent DABCO.

The coverslips were stored overnight in the dark at room temperature until the Mowiol sets, sometimes it helps incubate at 37 C° and the after day the coverslips were stored at 4 C° in the fridge.

9.6 Buffers and reagents

Table 9-1: Buffers Table

Buffer	Components
PBS 1x (Phosphate buffered saline)	NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4, 10 mM, KH_2PO_4 1.8 mM
TBS 1x (Tris buffer saline)	Tris-HCl 50 mM pH 7.5, NaCl 150 mM
4% PFA (Paraformaldehyde/PBS)	Paraformaldehyde 4%, Phosphate buffered saline 1x (PBS)
Mowiol mounting medium	Glycerol 24 % (w/v), mowiol 4-88 96%, H_2O, 0.2 M Tris (pH 8.5)
Ammonium chloride (NH_4Cl)	50 mM NH_4Cl, 1xPBS filtered
SDS-PAGE Running buffer	25mM Tris, 192 mM glycine, 0,1% SDS pH 8.3.
Blocking Buffer	5%semi-skinned powdered milk (m/v),Tris buffer saline (TBS), 0,1% tween-20.
Washing Buffer	1%semi-skinned powdered (m/v),Tris buffer saline (TBS), 0,1% tween-20.

9.7 Wide field fluorescence microscopy

The imaging was conducted using an Olympus B61 epifluorescence microscope equipped with a cooled CCD camera and with DAPI, FITc, Tritc filters and the analysis by Image J.

9.8 SDS-PAGE and Western blot analysis

The MCF10a cells at $1x10^6$ of density and MCF7 cells at $1x10^5$ of density were spin at 1000 rpm for 5 minutes, briefly for the isolation protein the extracs were prepared with: sodium dodecyl sulphate (SDS) sample buffer [4x] Novex with 1 mM DL-Dithiothreiol (DTT) and Phosphate buffered saline (PBS), and the pellet was boiled for 10 minutes at 95 C°.

Proteins were resolved by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) with 1x SDS-page Running Buffer (Tris-base, glycine and SDS) until dye front runs towards the end of glass plates.

Electrophoresis was performed at a constant voltage 166 mV for 90 minutes. After Electrophoresis the proteins were transferred to a Polyvinylidene difluoride membranes (PVDF).

The PVDF membrane was then blocked in 5% semi-skinned powdered milk (m/v) in Tris buffer saline (TBS) and 0,1% tween-20 (Sigma-Aldrich) (Blocking Buffer) for 45 minutes. After the blocking step the membranes PVDF were incubated with Rabbit anti-MVI (1:500 Atlas sigma HPA035483-100UL), Mouse anti-Erα (1:1000 SC-8002 Santa Cruz Biotechnology) and Rabbit anti-actin (1:5000 Abcam, ab8227) overnight at 4 C° with shaking.

Following the membranes were washed three times with washing buffer solution (1% semi-skinned powdered milk (m/v), in Tris buffer saline (TBS) and 0,1% tween-20 (Sigma-Aldrich) for 10 minutes each, then incubated with Goat pAb to Rabbit IgG (Hrp) conjugated secondary antibody (1:15.000 Abcam, ab6721) and Goat pAb to

mouse IgG (Hrp) (1:10.000 Abcam ab97023) conjugated secondary antibody, raised against the primary antibodies, for 1 hour at room temperature with agitation.

Finally the membranes were washes three times: two with washing buffer solution (1% semi.skinned powdered milk (m/v), in Tris buffer saline (TBS) and 0,1% tween-20 (Sigma-Aldrich) for 10 minutes each and once with Tris buffer saline (TBS) at room temperature with agitation and incubated with enhanced luminol-based detection (ECL) reagent and then expose on a Syngene G:Box-Fluorescence imager.

10 Results

10.1 MVI expression and distribution in MCF7 and in MCF10a cells.

Immunofluorescence was performed to investigate the differences in localization and levels of MVI in breast cancer cell line, MCF7, and non-cancerous breast cell line, MCF10a. In both cell lines, it was possible to see staining in the nucleus and the cytoplasm (Figure **10.1).** In fact, myosin VI was evenly distributed between both regions in MCF10a cells; in MCF7 cells seems to be more protein in the cytoplasm and potentially in the Golgi apparatus than in the nucleus. MCF7 cells, as expected, showed high levels of ER localised specifically to the nucleus, but mostly absent from the nucleolus. Conversely, MCF10a cells were essentially negative for ER staining. Signal was detected within the cytoplasm suggesting low levels of cytoplasmic protein, or background antibody staining.

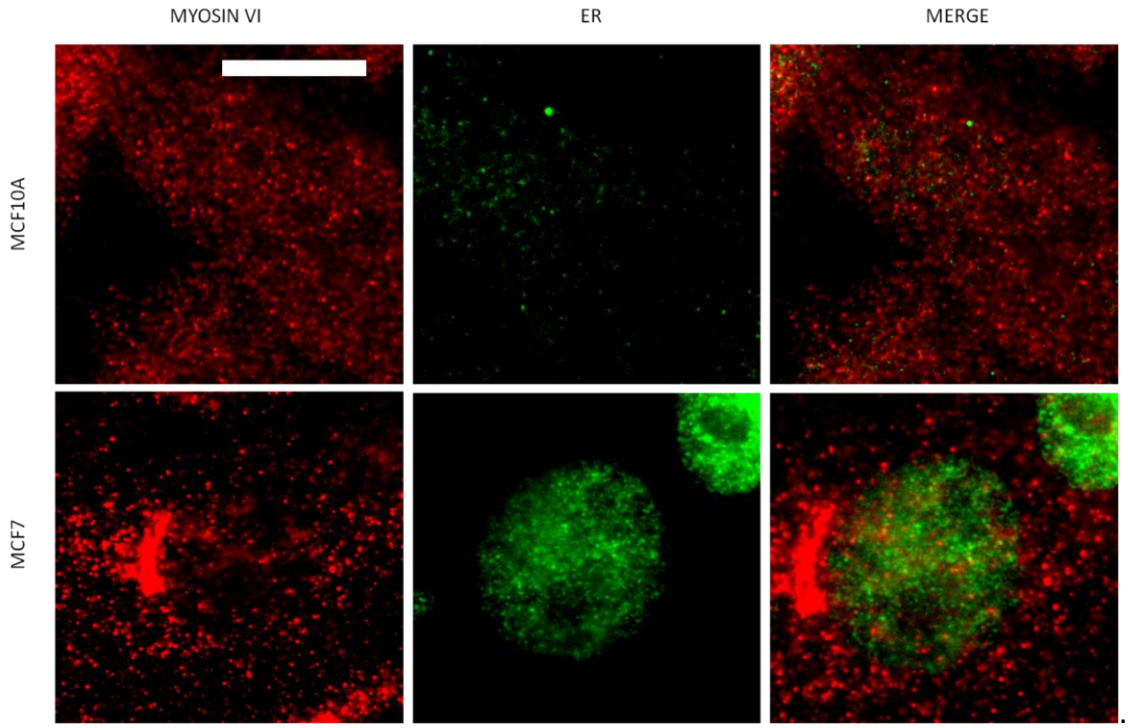

Figure 10.1: Immunofluorescence showed localisation of myosin VI and estrogen receptor in MCF10a and MCF7 cells.

MCF10a cells and MCF7 shows immunofluorescence of myosin VI and the estrogen receptor. **Scale bar is equal to 10 µm**.

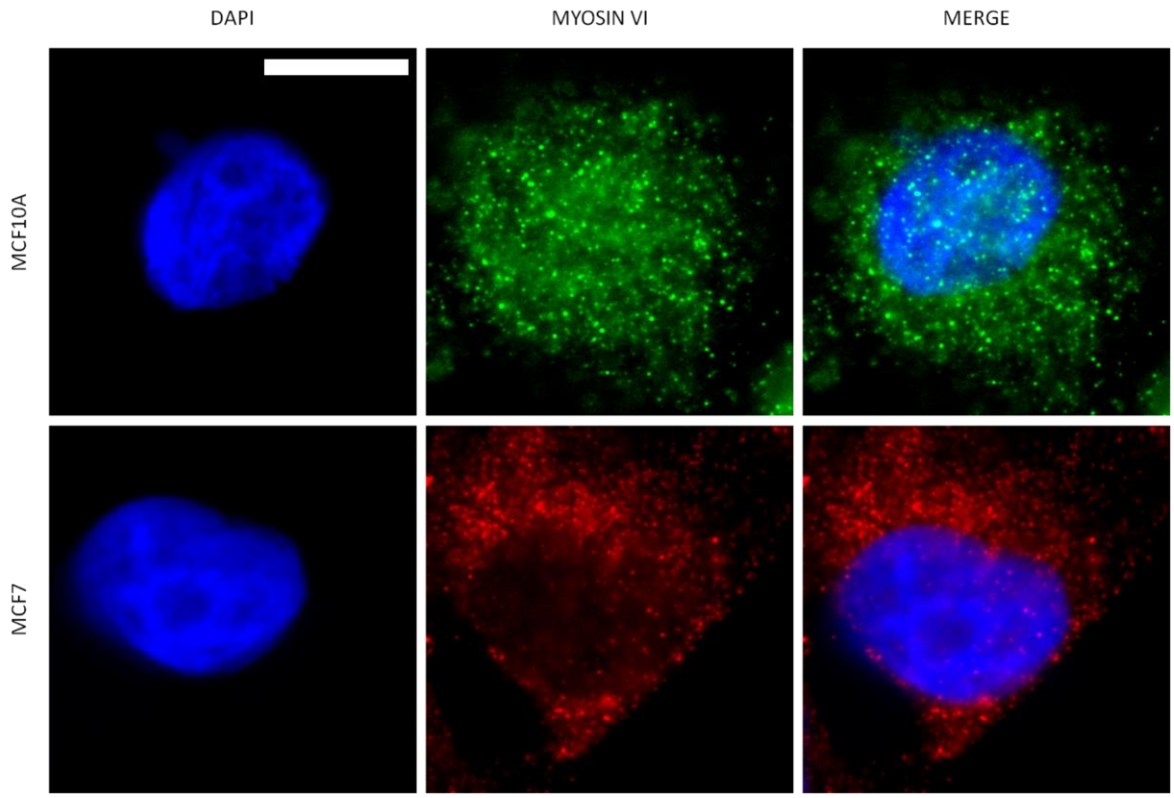

Figure 10.2: Immunofluorescence showed localisation of myosin VI in MCF10A and in MCF7 cells. Scale bar is equal to 10 µm

To confirm the distribution of Myosin VI in Figure **10.1**, immunofluorescence was performed to show the localisation of Myosin VI in MCF10a and MCF7 cells, both cell line were stained with DAPI to visualise the nucleus Figure **10.2**.

The staining of myosin VI in MCF10a cells was more viariable, the protein was distribuited in both regions, in MCF7 cells myosin VI was more concentrate in the cytoplasm with some dots in the nucleus.

Therefore to complement the imaging western blot analysis was performed to access the level of myosin VI (Figure **10.3**).

The western blot shows a band at 150 kDa for myosin VI in both cell lines. Consistent with the images, MCF7 cells express a higher level of myosin VI compared to MCF10a. It was also possible to test for the estrogen receptor. As expected, a strong band of 67 kDa was seen in MCF7 cells. However, no protein was present in MCF10a cells which suggests the signal in the staining relates to background signal during the immunofluorescence protocol.

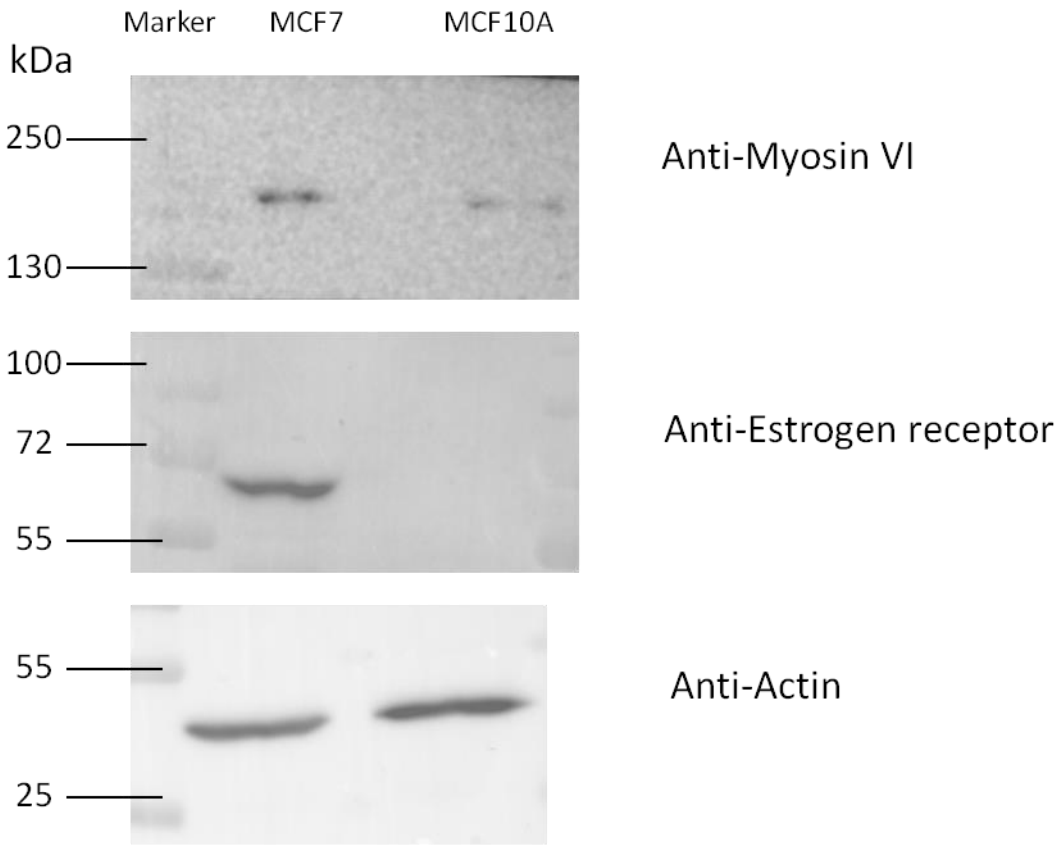

Figure 10.3: Western blot analysis of myosin VI and estrogen receptor expressions in MCF7 and MCF10a cells, without treatment. Anti-Actin was used as loading control.

10.2 Cellular localization isoforms

Previously it was reported that MVI localization is isoform dependent [32]. To address this issue in these cell lines, the cellular localization of myosin VI isoforms (LI+NI) was studied. The cells were transfected with GFP-LI-MVI or GFP-NI-MVI. Figure 10.4 shows MCF7 cells expressing myosin VI large insert (top row) and myosin VI non-insert isoforms (bottom row). The GFP-LI-MVI shows a homogeneous staining throughout the cytosol but is essentially absent from the nucleus.The GFP-NI-MVI isoform showed the same distribution to the endogenous myosin VI where it is present in the cytoplasm as well as inside the nucleus Figure **10.2**. This is in agreement with the previous study focused on HeLa cells [31] which suggests there is a general model for how myosin VI is recruited to the nucleus. Moreover, this result is consisted with the NI isoform being associated to primary tumours [11] where the function of nuclear myosin VI may drive disease.

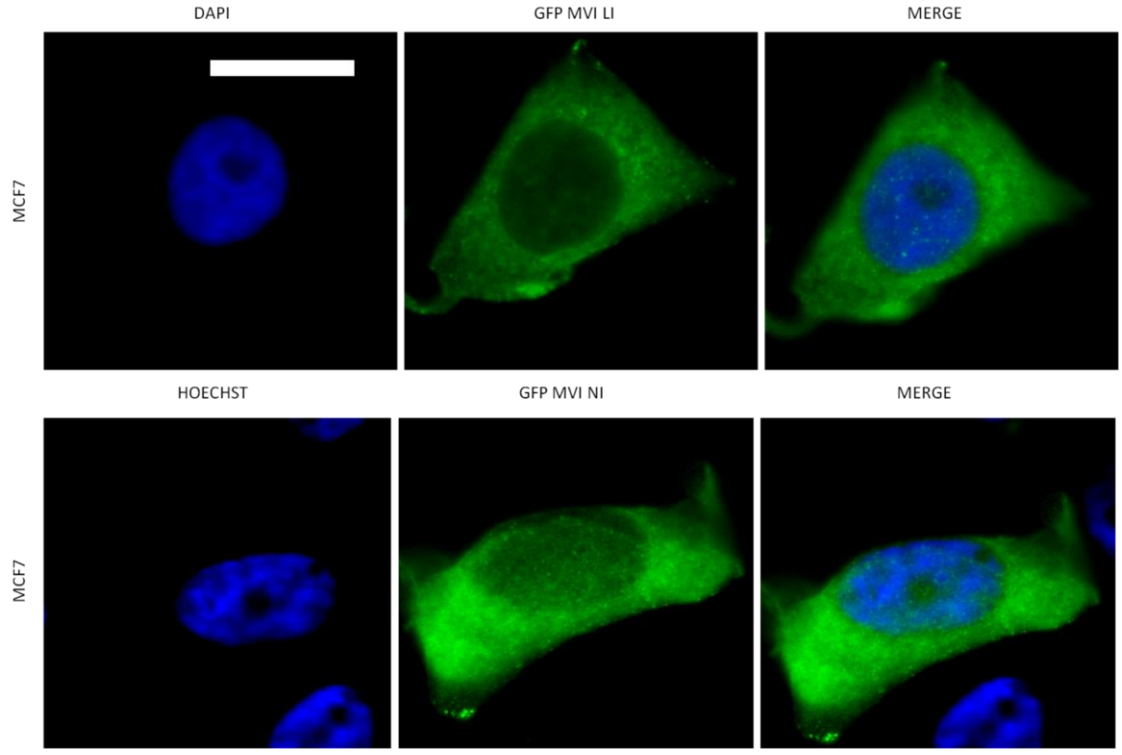

Figure 10.4: Representative images transiently expression of LI- and NI-GFP-MVI cells combined with DNA staining.

The figure shows a field view of MCF7 cells stained with Hoechst to visualize the nuclei (left column), transfected with LI- and NI-GFP-MVI (middle column) and merged. Hoechst/LI- and NI-GFP-MVI (right column). **Scale bar is equal to 10 µm**

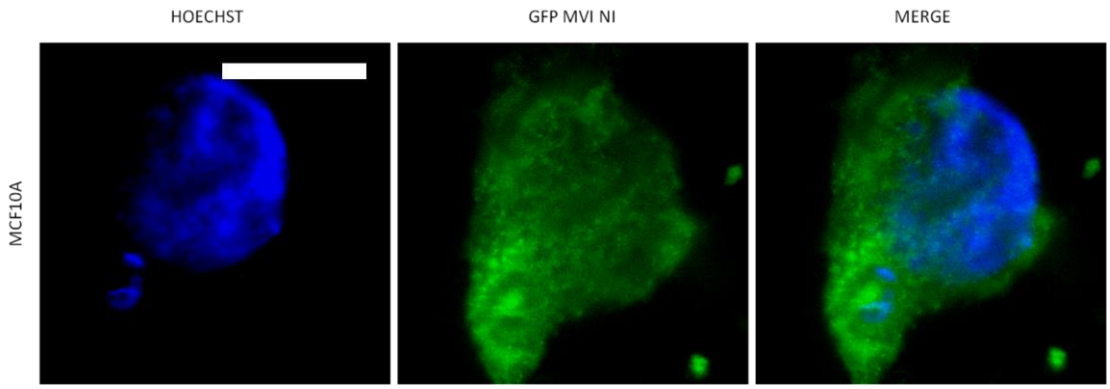

Figure 10.5: Representative images of transiently expression of GFP-NI-MVI in MCF10a cells combined with DNA staining. Scale bar is equal to 10 µm

To compare the distribution of MVI isoforms in non-tumorigenic MCF10a cells, the GFP-LI-MVI and GFP-NI-MVI plasmids were also transfected in these cells. The transfection efficiency in MCF10a cells was very low with GFP-NI myosin VI and the transfected cells started dying.

Figure 10.5 show an example of a cell expressing the GFP-NI-MVI. The cells displays cytoplasmic and nuclear localisation, similar MCF7. However, due to the low success rate, it is not possible to draw conclusions from this result. The transfection with GFP-LI isoform was unsuccessful after several attempts. This suggests overexpression of myosin VI may be toxic to the non-tumorigenic cell line, or it may be a specific issue relating to MCF10a cells.

10.3 Optimization of methods to inhibit the motor activity of myosin

Myosin VI is an ATPase protein and several inhibitors have been developed to target this activity. TIP (2,4,6-triiodophenol) is a such an inhibitor and has been shown to perturbs the myosin VI motor activity [50].

Figure 10.6 shows an example of myosin VI distribution in MCF7 cells treated 25 µM of TIP for 1h.

As seen in the image, myosin VI is restricted to the cytoplasm and in there is very little, or no, myosin VI staining in the nucleus during TIP treatment. Qualitatively,

there also appears to be less myosin VI present within the cells.

Overall, this suggests nuclear localisation is coupled to the motor activity of myosin VI. Moreover, the retention of nuclear myosin VI requires motor activity whereby lack of ATPase causes either a degradation of the nuclear protein, or its rapid export. Either way, treating cells with TIP is an ideal methodology to perturb the activity of nuclear myosin VI by causing its depletion.

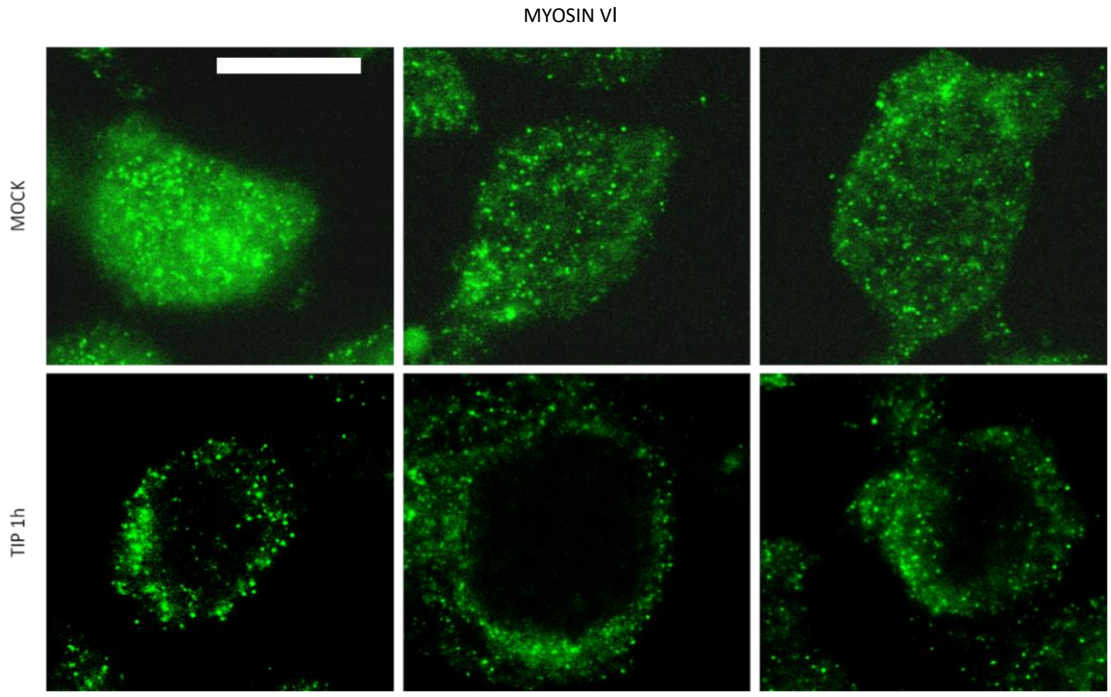

Figure 10.6: Immunofluorescence showed colocalization of myosin VI without and with treatment with TIP in MCF7 cells Scale bar is equal to 10 µm

First line show the normal distribution of myosin VI in untreated cells and the second line shows MCF7 cells treated with the inhibitor TIP

MCF10a cells were treated with 25 µM of TIP for 1h, 3h and 24h (Figure 10.7); No discernible change in the localisation of myosin VI could be record for measurements performed at 1 and 3hr. Four additions of 25 µM TIP was added during the 24h treatment period to account for its breakdown during this time-course. 24h treatment did influence myosin VI, however, this was in respect to the total protein labelling where the amount of myosin VI appeared to decreased.

There was not clear change in cellular localisation.

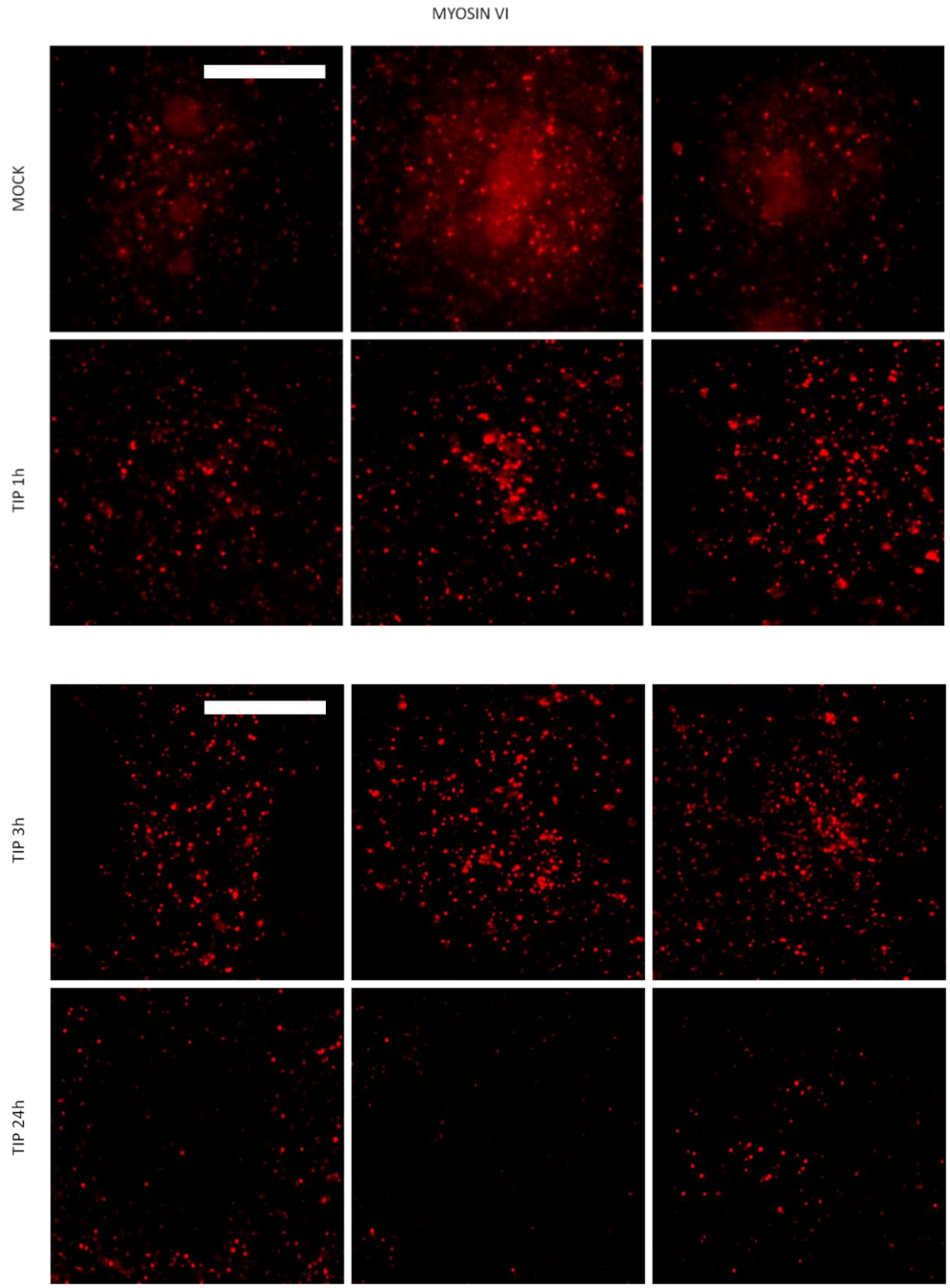

Figure 10.7: Immunofluorescence of myosin VI in non-treated cells and after treatment with TIP 1 hour; 3 hours and 24 hours in MCF10a cells. Scale bar is equal to 10 µm

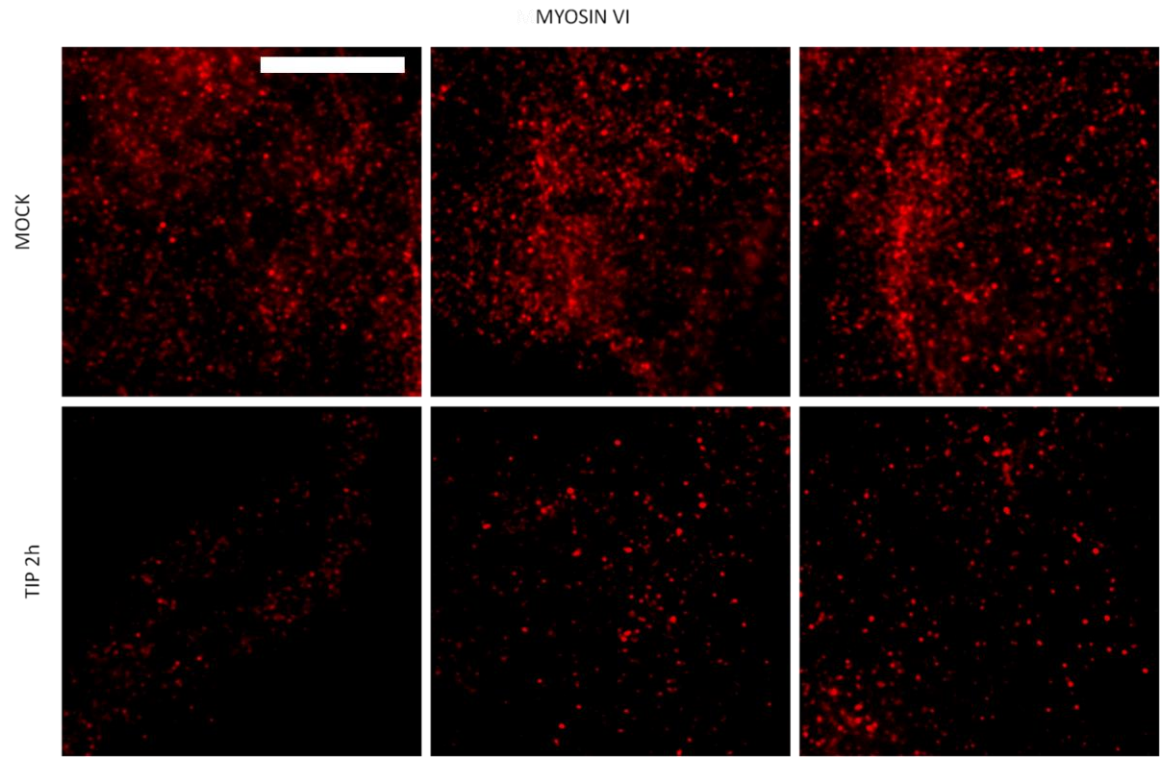

Figure 10.8: Immunofluorescence localisation of myosin VI without and with treatment with TIP 2 hours in MC10a cells. Scale bar is equal to 10 µm

The experiment was repeated with a higher concentration of TIP (100 µM) for 2 h consistent which is equal to the 24h dosage (4x 25 µM). (Figure **10.8**)

Consistent with the previous experiments, the treatment did not changed the localization of myosin VI but the total amount of protein appeared to decrease. Overall, this suggest there are different processes regulating myosin VI nuclear recruitment in each cell line. However, the decrease of myosin VI may correlate with a degradation of nuclear myosin VI in MCF7 cells.

10.4 Effect of TIP on ER distribution in MCF7 cells

Having established that TIP alters the cellular distribution of myosin VI in MCF7 cells, we wanted to explore how this influences the localisation of the estrogen receptor. In Figure 10.9, the effect of TIP upon myosin VI is once again observed. Here, along with a depletion of nuclear protein, there is also an apparent decrease in total protein. However, there is no discernible effect upon the localisation of the

nuclear receptor. The nuclear estrogen receptor is a long-lived protein therefore the short-term TIP treatment would be unlikely to change the distribution. However, this assay cannot determine if the receptor is active under these conditions. Indeed, it has been previously shown that TIP treatment prevents gene expression [12].

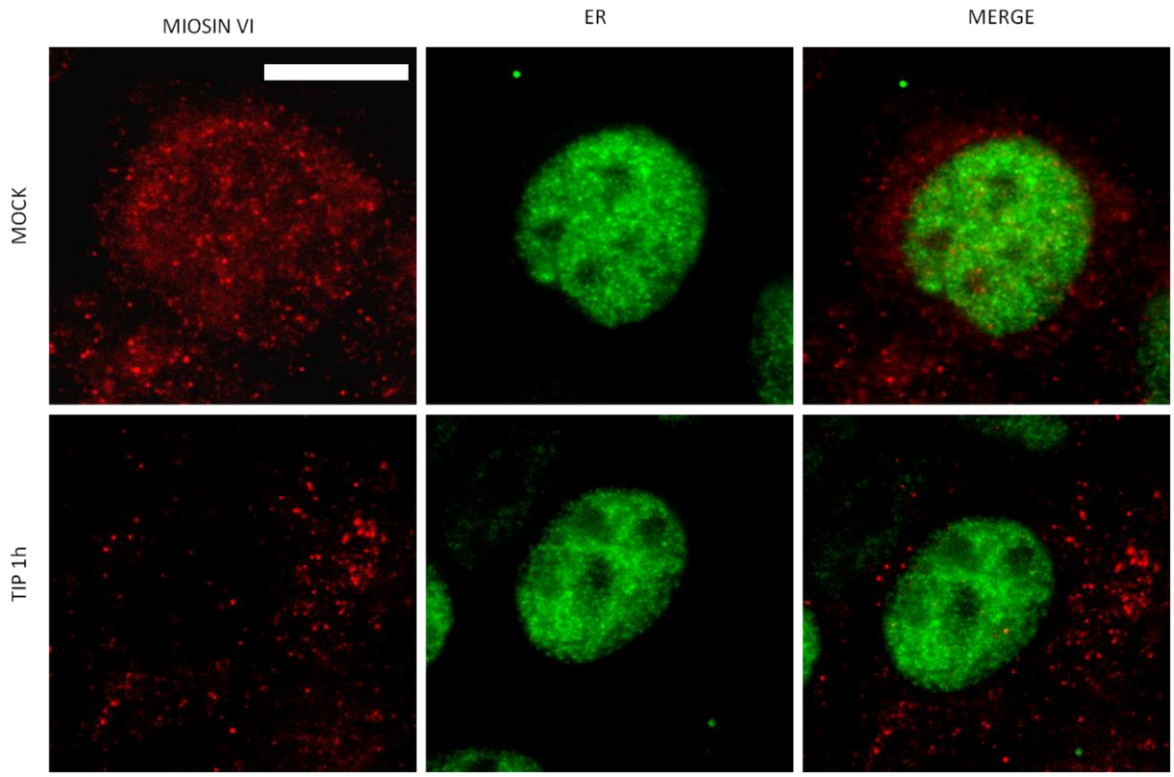

Figure 10.9: Immunofluorescence shows localisation of myosin VI and estrogen receptor without and with TIP in MCF7 cells. Scale bar is equal to 10 µm

Top row shows the normal distribution of myosin VI and estrogen receptor in untreated cells and the second line shows MCF7 cells treated with 25 µM of TIP.

10.5 Myosin VI KD using siRNA in MCF7 cells

Along with inhibiting myosin VI using TIP, it is also possible to suppress the expression of myosin VI. Here, MCF7 cells were transfected with human myosin VI siRNA duplex for 48 h. Staining for myosin VI shows that the knock-down was successful in MCF7 cells (Figure 10.10), whereby only a background level of

signal remained. Overall, the expression of myosin VI appeared to decrease respectively compared the control groups. Interestingly, the Hoechst staining of the chromatin is altered upon myosin VI knockdown. This is represented in the example image in Figure **10.10** where nuclear structures such as the nucleolus are less visible. This suggests myosin VI may be coupled to organisation of the chromatin, or nuclear sub-structure in MCF7 cells. To investigate this process further, the knock-down was repeated in MCF10a cells (Figure **10.11**). As with MCF7 cells, the knockdown was successful. However, there was no measurable difference on the chromatine staining pattern. This is consistent with a lower level of myosin VI within these cells whereby there is likely to be less functions dependent upon the protein.

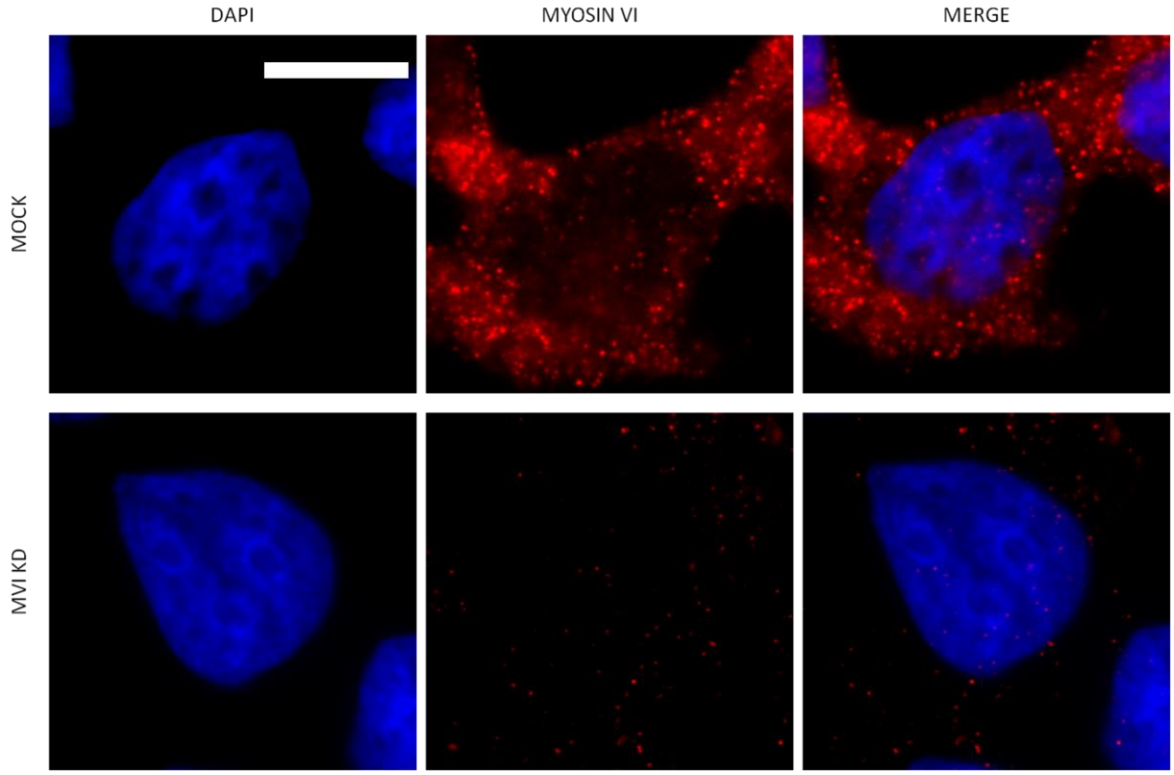

Figure 10.10: Myosin VI knockdown in MCF7 cells using siRNA technology. Top row showed normal distribution on myosin VI in MCF7 cells. Scale bar is equal to 10 µm

MCF7 cells were stained with Hoechst to visualize the nuclei (left column) with anti-myosin VI antibody (middle column) and merged Hoechst/myosin VI (right column)

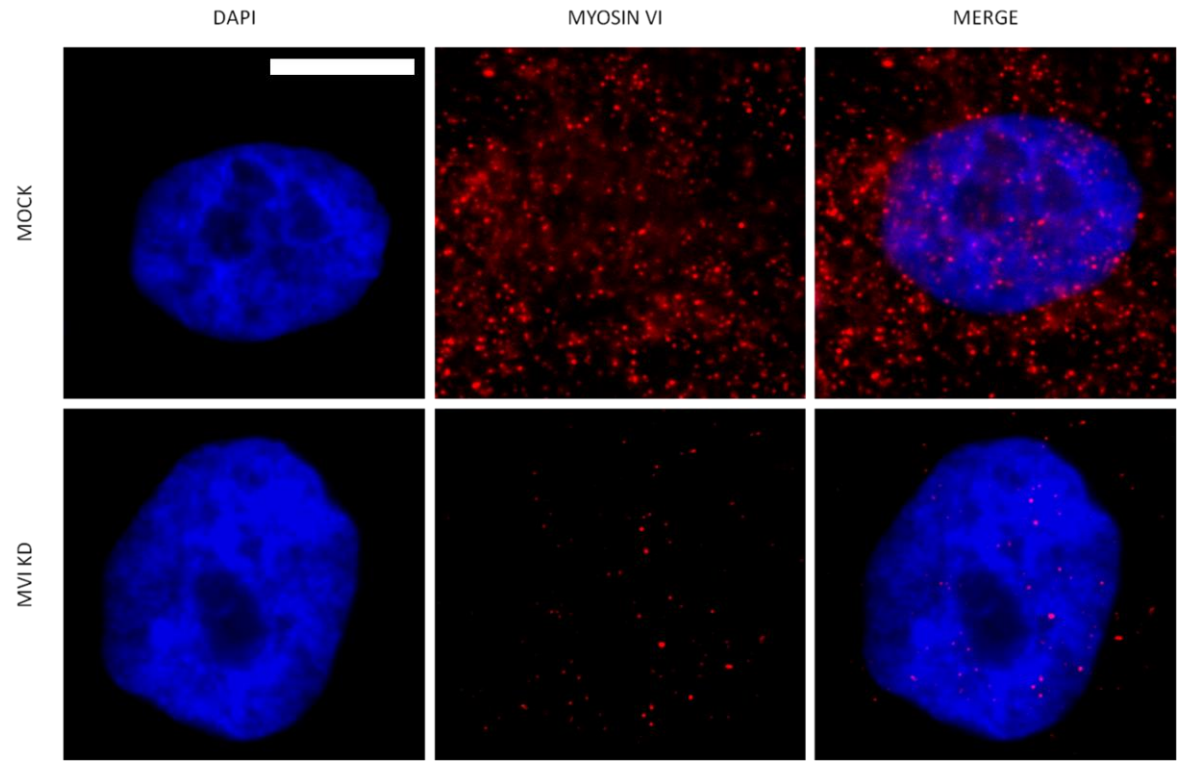

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Figure 10.11: Myosin VI knockdown in MCF10a cells using siRNA technology.

Top row showed normal distribution on myosin VI in MCF10A cells. MCF10A cells were stained with Hoechst to visualize the nuclei (left column) with anti-myosin VI antibody (middle column) and merged Hoechst/myosin VI (right column**). Scale bar is equal to 10 µm**

Having established a succesful knock-down procedure, it was then possible to see how this treatment impacts the localisation of the estrogen receptor. As can be seen in Figure **10.12**, the estrogen receptor localisation remained normal 48h following transfection with siRNA. As with the TIP treatment, knock-down of myosin VI is known to impact the activity of the estrogen receptor in gene expression (Fili *et al.* Nature Communication 2017). Therefore, we can conclude that the impact of myosin VI on the estrogen receptor activity is not linked to controlling nuclear localisation. The impact of myosin VI must therefore occur on the genome level at sites of transcription.

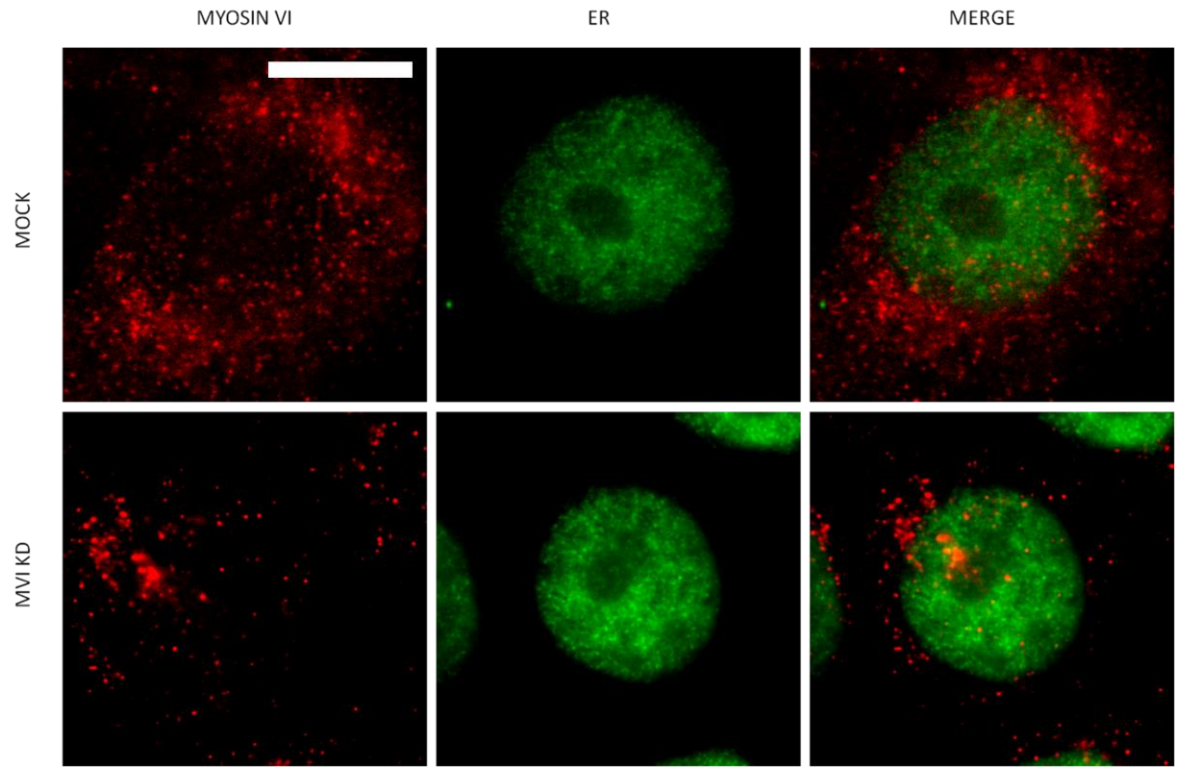

Figure 10.12: Myosin VI knockdown in MCF7 cells after transfection by human myosin VI siRNA and impact in the estrogen receptor. Scale bar is equal to 10 µm

10.6 Effect of estrogen receptor knockdown in myosin VI levels

Having explored the impact of myosin VI upon the estrogen receptor, it is now possible investigate what is the impact of the estrogen receptor upon myosin VI? For this approach, the estrogen receptor was knocked-down in MCF7 cells using siRNA treatment (Figure **10.13**). The expression of estrogen receptor appeared to decrease, compared the control cells. As already mentioned, the estrogen receptor is a long-lived protein therefore protein degradation is not complete during a transient knockdown.

Following knockdown, the total staining of myosin VI was reduced, suggesting expression of the protein, or its stability is reduced.The myosin VI gene contains a putative ERE promoter so a reduction in expression is possible. The nuclear localization was also reduced with myosin VI being more restricted to the cell cytoplasm. Overall, this suggests myosin VI localisation is coupled to the estrogen

receptor.

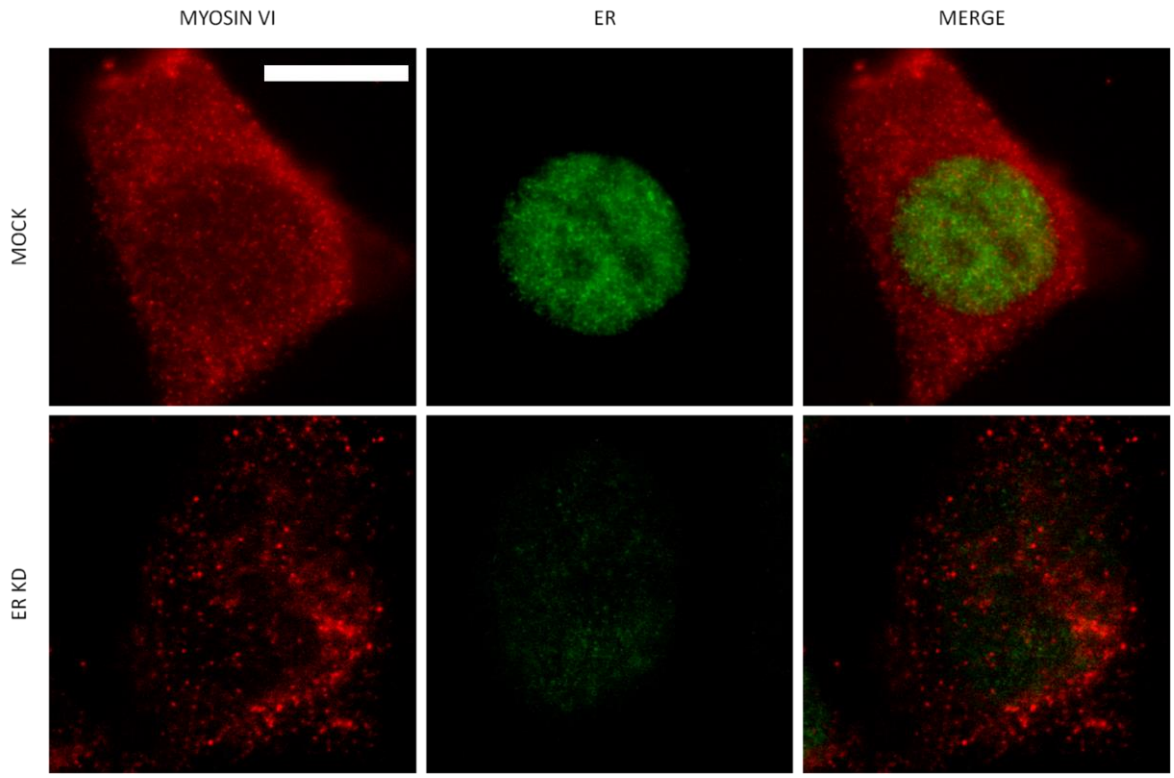

Figure 10.13: ER Knock-down in MCF-7. Scale bar is equal to 10 µm

Immunofluorescence showed localization of myosin VI before and after estrogen receptor knockdown.

10.7 Effect of hormone starvation and stimulation on distribution levels of myosin VI and estrogen receptor

Having found that estrogen receptor knockdown effects the localisation of myosin VI, we then wanted to explore how hormone treatment may perturb myosin VI. MCF7 cells were starved for 48h in minimal media. Staining for myosin VI was then repeated as before (Figure **10.14**). Compared to control measurements, and those shown above, myosin VI was found to be mostly restricted to the cytoplasm. This is in a similar manner to the estrogen receptor knockdown. This implies that an active estrogen receptor was not greatly affected by the removal of hormone, which is consistent with the long-lived protein. However, the receptor did become more diffuse which caused the nucleolus to not be visible. This may be related dissociating from the genome which would lead to the lack of activity in gene

expression.

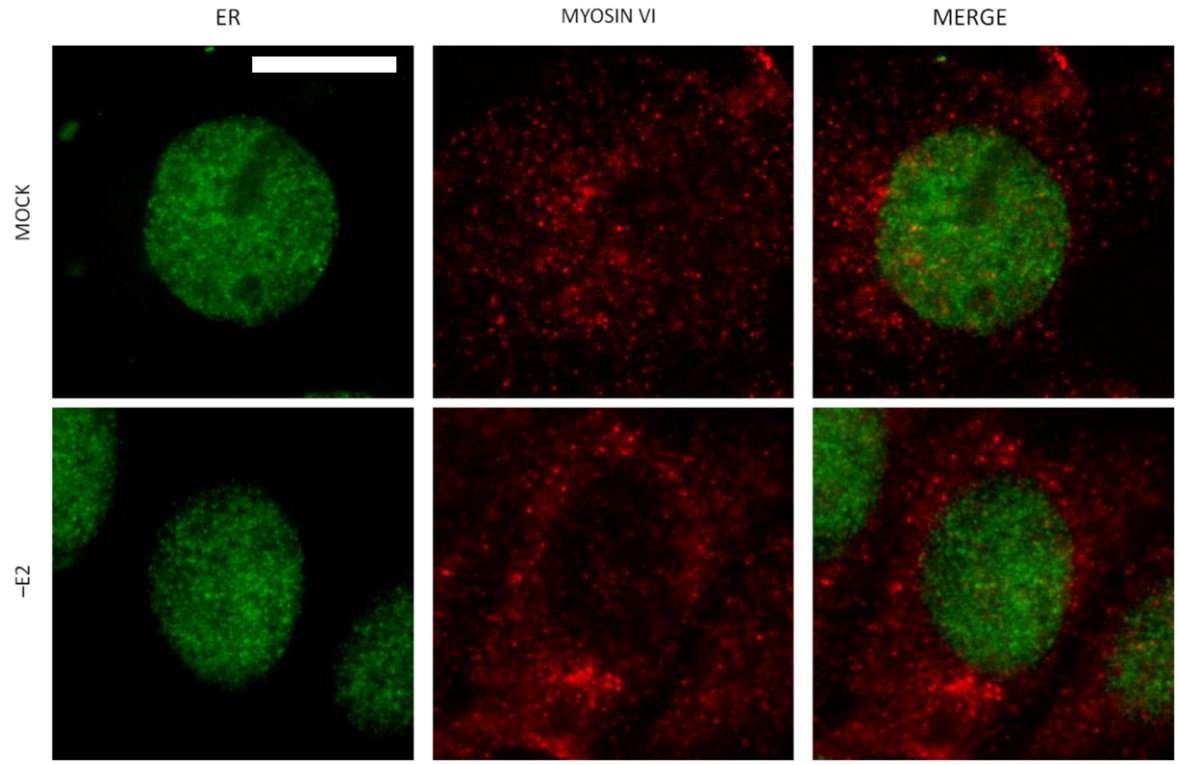

Figure 10.14: Hormone starvation in MCF-7.

Immunofluorescence of estrogen receptor (left column), myosin VI in the middle column and merge myosin VI/estrogen receptor on the right column. **Scale bar is equal to 10 µm**

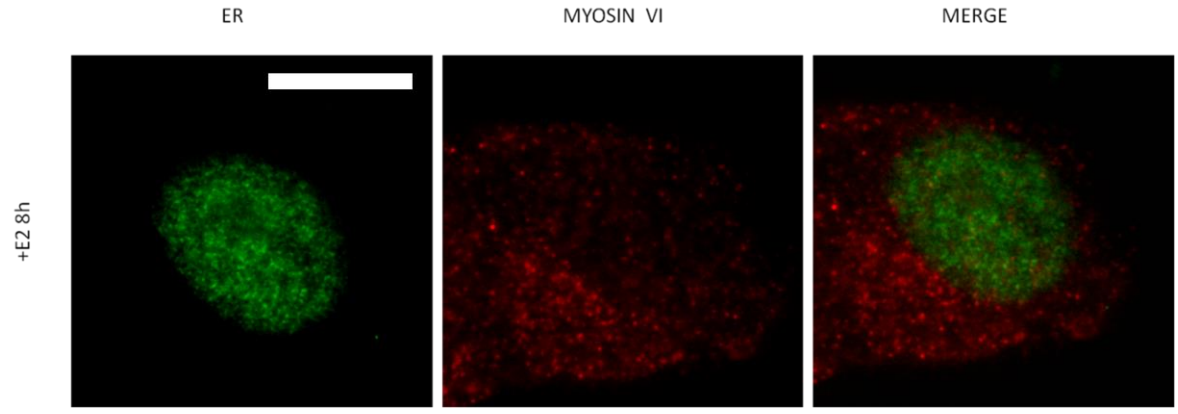

Figure 10.15: Hormone stimulation in MCF-7.

Immunofluorescence of estrogen receptor (left column), myosin VI (middle column) and myosin VI/estrogen receptor (right column) after stimulation with estradiol after 8 hours. **Scale bar is equal to 10 µm**

Hormone starvation of the cells led to a decrease in the nuclear pool of myosin VI therefore it would be expected for hormone stimulation to rescue this phenotype.

For this experiment, the MCF7 cells were first starved for 48h then the cells were treated with fresh media supplemented with 100 µM of estradiol (Figure **10.15**).

After stimulation the estrogen receptor was localise inside the nucleus but displayed the same diffuse distribution, as seen in the starvation condiction. Therefore, it is not possible to determine if the cells responded to the hormone stimulation. Based on the phenotype of the estrogen receptor, it is not surprising that myosin VI remained mostly localised to the cytoplasm.

Control hormone starvation and stimulation experiments were planned to be performed in MCF10a cells. This would reveal if the impact of hormone starvation is linked to the presence of the estrogen receptor. However, as shown in Figure **10.16**, the cell line displayed aberrant staining of myosin VI in the mock condiction therefore experiments could not be performed. In this experiment myosin VI was shown to be strongly localised to the nucleolus which may be a form a stress response within in these cells. It was not possible to repeat these experiments under the given time-frame.

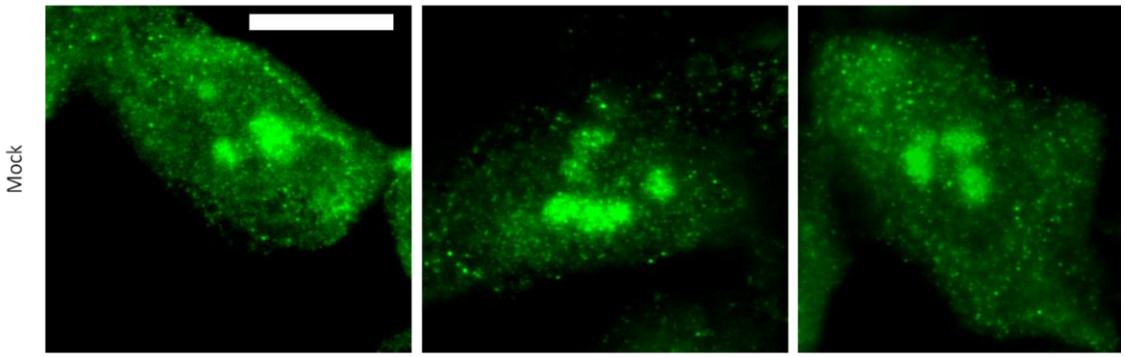

Figure 10.16: Distribution of myosin VI in MCF10a cells. Scale bar is equal to 10 µm

11 Discussion

Myosin VI is involved in a variety of cellular processes, from intracellular trafficking, cell motility and function in different human disease. Increasing numbers of studies reported a cancer specific overexpression of myosin VI in ovarian [17], prostate [18] and breast cancer tissues. Breast cancer is the most frequent malignancy in Western countries and the molecular mechanism interested in the progression of this cancer need to be investigate [49]. This study was focus on characterising myosin VI using a model of mammary epithelial non-tumorigenic cell line MCF10A and the corresponding tumorigenic breast cell line MCF7. In particular, there was interest to understand if a nuclear function of myosin VI could be described.

11.1 MCF7 versus MCF10a

Both cells lines were implemented in this study as a tumour and non-tumorigenic model, respectively. MCF7 cells, like many cancer cell lines, are relatively rapid growing and easy to manipulate. However, MCF10a cells behave in the opposite manner which is also typical of non-tumorigenic cell lines. Throughout this study, this property, led to several difficulties when looking to perform comparison experiments between both cell lines. More time is required to fully study the behaviour of myosin VI within this cell line so that assays can be better established.

11.2 Cellular localisation of ER in MCF7 cells

To address the localisation of estrogen receptor we investigate the distribution in different conditions in MCF7 cells. Under normal conditions the ER was mostly localised in the nuclear region.The treatment with the inhibitor and the impact of myosin VI knockdown didn’t change the localisation of estrogen receptor, but the receptor did become more diffuse. In all conditions the estrogen receptor shown to be localised inside the nucleus, this distribution suggests that many components of the cell culture medium including phenol red, growth factors and various steroids present in the serum have a weak estrogenic activity, especially with human breast cancer cells in absence of stimulation with estradiol.

In breast cancer cells the ERα localization is not static but dynamic with continuous shuttling between the nucleus and the cytoplasm. The nuclear import and export of ERα are regulated by several stimuli that include estradiol, antiestrogens and growth factors. As ERα nuclear accumulation is critical to the regulation of gene expression nuclear export of this receptor modulate the intensity and duration of its concentration in cellular compartment as well as of its nuclear and extranuclear functions. Growth factors such as the epidermal growth factor (EGF) and the insulin-like growth factor 1 (IGF1), can activate the transcriptional activity of ERα through phosphorylation, independently of E_2. Signalling pathway of these growth factors are also involved in ERα subcellular distribution. It has been proposed that growth factors signalling cause posttranslational modification of ERα to promote its nuclear localization in breast cancer cells. Similar to E_2, both EGF and IGF1 promote nuclear localization of ERα. The EGF signalling induces maximal nuclear accumulation of ERα at 60 minutes after MAPK cascade and IGF1 activates the phosphatidylinositol 3-kinase (PI3K) cascade to enrich receptor levels in the nucleus after 90 minutes [50].

To see the effect of hormone starvation and stimulation on distribution levels upon the estrogen receptor, we remove all factors can interfere during the treatment with the estrogens and we starved the cells with minimum essential media supplemented with charcoal stripped serum to reduce the level of the estrogens, the remaining amounts of estrogens that is present in the serum takes time to reduce to the basal level this can explain the nuclear localisation of estrogen receptor in starved condictions.

The estrogen receptor get down during prolong starvation of cell without the hormone but after starvation the estrogen receptor seem not to be affected by the removal of hormone, the receptor did become more diffuse which caused the nucleolus not to be visible, therefore we expect when cells will be treated with estradiol the effect would be noticeable. After stimulation the estrogen receptor was localise inside the nucleus but shown the same diffuse distribution as seen in starved conditions and it wasn’t possible to determine if the cells respond to the hormone stimulation.

11.3 Cellular Localisation of myosin VI

Both cell lines were visualized under different conditions to understand the cellular distribution of myosin VI. Under normal conditions, myosin VI was shown to be distributed throughout the cell body (cytoplasm and nucleus) in both MCF10A and MCF7, but myosin VI more abundant in the latter.

To address if the isoform of myosin VI impacted the cellular localisation, Non-insert (NI) and Large-insert (LI) isoforms were transiently transfected in both cell lines. In MCF7 cells, the LI isoform showed a distribution throughout the cytosol but it was absent from the nucleus. Conversely, the NI isoform was present throughout the entire cell body, including the nucleus. This localisation is consistent with previous studies in HeLa cells and it fits with the model relating to the RRL binding partner motif being related to nuclear localisation. Lastly, based on the isoform dependent localisation, we can conclude that endogenous myosin VI in MCF7 is consistent with being the NI isoform.

Transfections of the isoforms was essentially unsuccessful in the MCF10a cells. The slow growing nature of these cells may make them harder to manipulate through transfection. However, it is also possible that over-expression of myosin VI is detrimental to these cells, hence the cell death following transfection. This could relate to interference with biological functions within these cells.

The ATPase kinetic properties of myosin VI are reduced when the protein is under the effect of the poly-iodinated phenol derivate 2,4,6-triiodophenol (TIP) [51]. Treatments of the cells with this inhibitor led to a rapid nuclear depletion of myosin VI in MCF7 cells. This suggests that the ATPase activity of the protein is required for nuclear localisation and its maintenance within the nucleus. However, the MCF10a cell line behaved very differently. The cellular localisation of myosin VI was not affected by TIP, however long-term, or high-dose exposure caused a decrease in the overall level of myosin VI. Such a treatment may in fact perturb cell viability which indirectly changes the myosin VI expression level. Nevertheless, it is interesting to observe two different responses between these cell lines. Overall,

it suggests that the processes controlling the nuclear localisation in MCF10a are different to MCF7, which links in to the role of the estrogen receptor in this process.

The estrogen receptor play an important role on myosin VI distribution and localisation in MCF7 cells. After ER knockdown, the expression of myosin VI was reduced. This can be attributed to the presence of an ERE promoter element upstream of the myosin VI gene. Interestingly, the nuclear localisation of myosin VI is linked to the presence of the estrogen receptor. In this manner, association with the estrogen receptor may target myosin VI to the nucleus. However, the TIP measurements reveal the ATPase activity, therefore the estrogen receptor only provides targeting.

This is further supported by hormone starvation experiments. Following hormone starvation treatment, myosin VI was shown to be restricted to the cytoplasm. As with the estrogen receptor knockdown experiments, this suggests that activated (by hormone) estrogen receptor is required for the targeting of myosin VI to the nucleus.

Hormone starvation did impact the estrogen receptor. However, it did not lead to noticeable depletion from the nucleus. Longer term treatment is required for the protein to be fully absent from the nucleus, but this greatly perturbs cell growth and viability at S and G2/M stages of the cell cycle, we would expect the starvation to cause delay at this stage [52].

Hormone stimulation experiments were also attempted. However, all evidence suggests the stimulation failed because there was no impact on either the estrogen receptor or myosin VI. This may relate to this batch of cells or degradation of the estradiol. Further repeats would establish where the problem exists.

Overall, these results show that the nuclear localisation of myosin VI is regulated within the cancer cell line and that the phenotypic marker (estrogen receptor) of this cancer is coupled to the nuclear localisation.

11.4 Impact of myosin VI on the estrogen receptor

The immunofluorescence showed that myosin VI does not impact the localisation of the estrogen receptor, as demonstrated in different conditions after treatment with the TIP inhibitor and following the siRNA knockdown. However, it was possible to observe that myosin VI knockdown caused a change in the chromatin staining. This may suggest that myosin VI may perturb the organisation of the genome. Such an impact would need to be followed by investigating the impact of myosin VI on different chromatin markers.

Despite this result, it has been established that both TIP treatment and siRNA knockdown against myosin VI perturbs the estrogen receptor activity during gene expressions. This leads to a decrease in target gene expression [12,31]. This suggests that instead of transporting the estrogen receptor to the nucleus, myosin VI aids stabilisation of the estrogen receptor at sites of transcription, and/or, interacts with RNA polymerase II at these sites to bring about targeted gene expression.

11.5 Future directions

In the future to confirm the association of myosin VI with the estrogen receptor and the results obtained with the immunofluorescence we will study the cellular localisation of myosin VI and receptor ER with cell fractionation and isolate nuclei pellet following by Western blot analysis to determine the relative amount of the proteins in the cytoplasm and the nucleus in starved and stimulated condictions or in presence or absence of inhibitor (TIP).
We will study through gene expression analysis the role of myosin VI in trascription associates with gene regulations and coding sequences and/or with intragenic regions, we will use immunoprecipitations experiments in vivo crosslinked chromatin followed by QT-PCR to determine the relative expression levels of several genes and the localization of myosin VI on the genome in both cell lines MCF10a and MCF7 cells. The same approach can be used to determine wich isoform of myosin VI are expressed in the tissue.
To examine the effects of ER expression could be performed a stable transfection

with a mammalian expression vector containing a human ER WT cDNA in non-cancerous cell line MCF10a and see how is the effect on myosin VI in different condictions hormone starvation and stimulation and determining additional changes on the growth media to see the growth potential after exposure to estradiol and to see the effect upon myosin VI.

12 Conclusions

To recapitulate, this project was focused to understand the distribution and localisation of myosin VI in the tumorigenic breast cell line MCF7 and non-tumorigenic breast cell line MCF10A.

Myosin VI is expressed at low levels in MCF10A cells compared to MCF7 but the protein is present throughout the entire cell body.

In MCF7, the nuclear localisation of myosin VI is dictated by isoform. ATPase activity and estrogen receptor targeting. The processes which determine the cellular localisation in MCF10a remained to be determined. But there is a clear link between the myosin VI nuclear localisation and cancer cell line.

Myosin VI does not impact the nuclear localisation of the estrogen receptor. However, perturbation of myosin VI is known to impact the activity of the receptor in gene expression. Therefore, it is suggested that myosin VI may stabilise the estrogen receptor at sites of transcription and/or couple to RNA polymerase II to drive expression of estrogen receptor target genes.

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