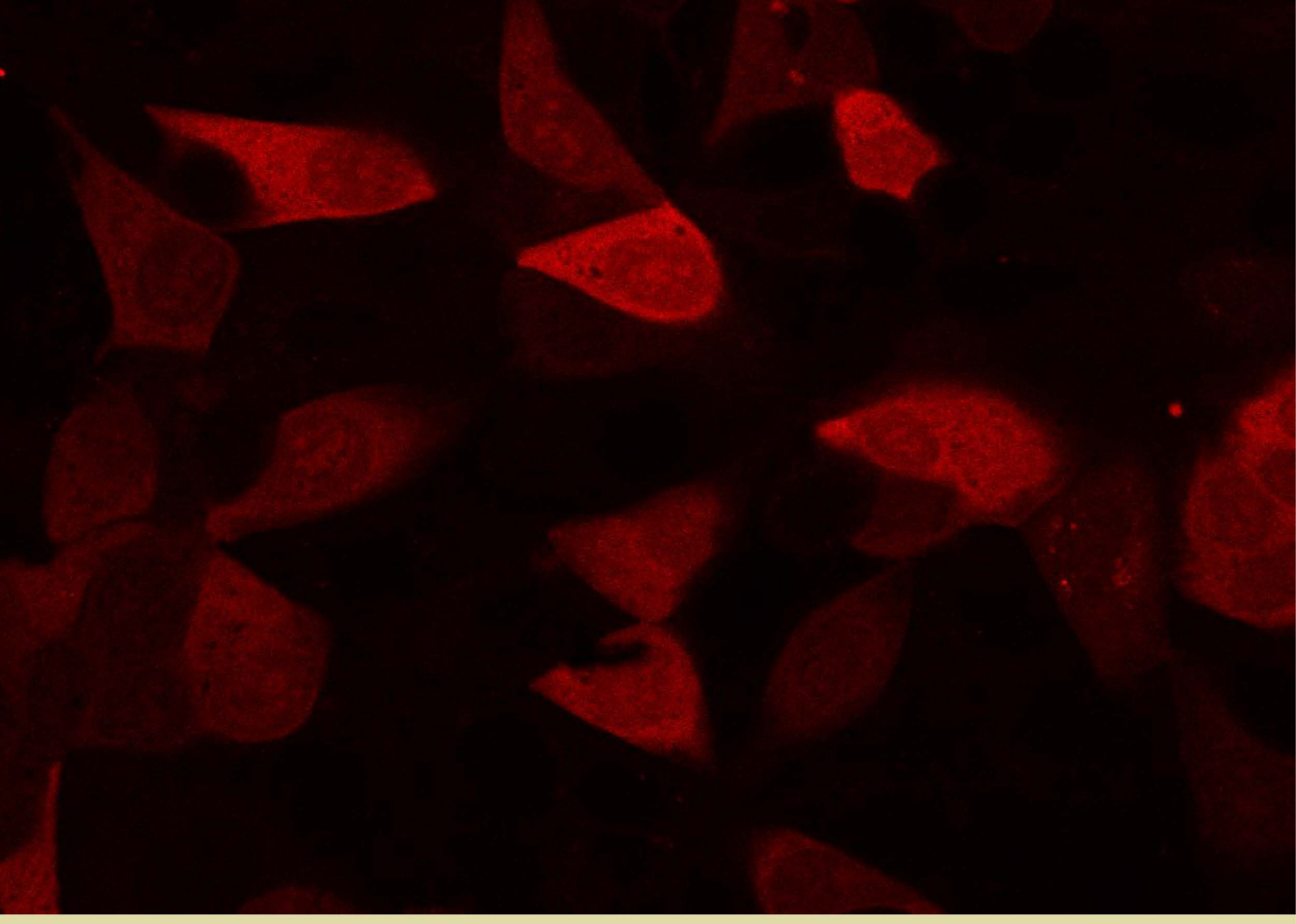

Catarina da Silva Pechincha

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Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

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Resumo

O cancro da mama é um dos tipos de cancro mais diagnosticado em todo o Mundo e a principal causa de morte associada com cancro, em mulheres. Apesar do elevado número de casos diagnosticados devido ao desenvolvimento de técnicas para deteção precoce, mortes de doentes com cancro da mama continuam associadas ao desenvolvimento de resistência à quimioterapia e, consequentemente, formação de metástases. Para combater estas evidências, é importante desvendar os mecanismos moleculares por detrás da iniciação, expansão e metastização do cancro da mama. A nucleolina é uma proteína envolvida na proliferação celular, ligação ao ADN, ARN e proteínas, estando envolvida na regulação genética, replicação e recombinação de ADN, metabolismo de ARN, transcrição e sinalização celular. A nucleolina está sobrexpressa em células cancerígenas, incluindo células estaminais de cancro da mama, e células endoteliais de vasos tumorais angiogénicos. Devido ao elevado reconhecimento da sua importância, a nucleolina à superfície da célula tem sido estudada como um possível alvo para terapias anticancerígenas, com diversas moléculas em desenvolvimento contra células cancerígenas humanas.

De acordo com a literatura, neste trabalho formulou-se a hipótese de que a nucleolina pode funcionar como um proto-oncogene em células de cancro da mama, ao modular propriedades estaminais. Para uma melhor compreensão de como a expressão de nucleolina afeta as células de cancro da mama, foi desenvolvida uma nova ferramenta molecular para modular a expressão de nucleolina em células de mamífero. O silenciamento de nucleolina foi conseguido usando um shRNA num sistema lentiviral e a sua sobrexpressão usando um sistema lentiviral induzível. Transdução dos lentivíruses nas linhas celulares humanas de cancro da mama MCF-7 e MDA-MB-231 permitiu estabelecer linhas celulares estáveis geneticamente modificadas, expressando diferentes níveis de nucleolina. Validação *in vitro* destes sistemas permitiu confirmar o silenciamento e sobrexpressão da nucleolina.

O silenciamento da nucleolina levou a uma diminuição da capacidade migratória de ambas as linhas celulares de cancro da mama, MCF-7 e MDA-MB-231, em aproximadamente 2 vezes e 3,3 vezes relativo aos correspondentes controlos de células não transduzidas, respetivamente. Ensaios i*n vitro* usando estas linhas celulares sugeriram que a nucleolina poderá ter impacto nas características carcinogénicas de células de cancro da mama.

Palavras-chave: nucleolina, células estaminais cancerígenas, cancro da mama, sistema lentiviral.

Abstract

Breast cancer is one of most common cancer diagnosed worldwide and the leading cause of cancer-related deaths among women. Despite the high rate of diagnosis owed to the advancement of techniques for early detection, breast cancer death remains associated with chemotherapy resistance and, hence, development of metastasis. To counteract these facts, it is important to unravel the molecular mechanisms behind breast cancer initiation, expansion and metastasis. Nucleolin plays important roles in cell proliferation, binding to DNA, RNA and proteins, enabling the regulation of gene expression, DNA replication and recombination, RNA metabolism, transcription and signal transduction. Nucleolin is overexpressed in cancer cells, including putative breast CSC, and endothelial cells of angiogenic tumour vessels. Owed to the growing recognized importance, surface nucleolin is being exploited as a target for anti-cancer strategies, with several molecules in development against human cancers.

Accordingly, in this work it is hypothesized that nucleolin may function as an oncogenic driver of breast cancer, upon modulating stemness properties. For a better understanding on how modulation of nucleolin expression affects breast cancer cells feature, a novel molecular tool was developed, aiming at modulating nucleolin expression in mammalian cells. Nucleolin silencing was accomplished using a lentiviral shRNA approach and nucleolin-overexpression using an inducible lentiviral system. Transduction of the lentiviruses in MCF-7 and MDA-MB-231 breast cancer cell lines allowed to establish genetically modified stable cell lines, to expressing different levels of nucleolin. Further *in vitro* validation of the systems was performed, confirming the knockdown and overexpression of nucleolin.

Nucleolin downregulation decreased the migration capacity on both MCF-7 and MDA-MB-231 breast cancer cell lines in approximately 2-fold and 3.3-fold, respectively, relative to the corresponding non-transduced control. *In vitro* assays using this cell lines suggested that nucleolin might have an impact in the carcinogenic feature of breast cancer cells.

Keywords: nucleolin, cancer stem cells, breast cancer, lentiviral system.

List of abbreviations

A

AP-1 – Activator Protein-1

B

Bcl-2 - B-cell lymphoma-2

BCRP - Breast Cancer Resistance Protein

bps - Base pairs

C

Cdc2 - Cell division cycle protein-2

cDNA - Complementary DNA

CDS – Coding DNA Sequence

CIAP - Calf Intestinal Alkaline Phosphatase

CKII - Casein Kinase II

CMV - Cytomegalovirus

cPPT - Central purine tract

CSC - Cancer Stem Cells

CTS - Central Termination Sequence

D

DNA - Deoxyribonucleic Acid

DOX - Doxycycline

DTT - Ditiotreitol

E

EDTA - Ethylenediamine tetraacetic acid

EGTA - Ethylene glycol tetraacetic acid

ER+ - Oestrogen Receptor positive

ESC - Embryonic Stem Cells

F

FBS - Fetal Bovine Serum

G

GAR – Glycine Arginine Region

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

H

HER2 - Human Epidermal growth factor Receptor 2

HIV - Human Immunodeficiency Virus

hNCL - Human Nucleolin

hPGK - human Phosphoglycerate Kinase

HRP - Horseradish Peroxidase

K

KD - Knockdown

L

LTR - Long Terminal Repeats

M

MAPK - Mitogen Activated Protein Kinase

MFE - Mammospheres Formation Efficiency

miRNA - micro RNA

mRNA - messenger RNA

N

NCL - nucleolin

P

PCR - Polymerase Chain Reaction

PI3K - Phosphatidylinositol-3-kinase

PMSF - Phenylmethylsulfonyl Fluoride

PolyA - Polyadenylation signal

PuroR - Puromycin Resistance gene

PPT - Polypurine Tract

Q

qRT-PCR - quantitative Real Time PCR

R

RBL - Rat Basophilic Leukocytes

RBP - RNA Biding Protein

rDNA - ribossomal DNA

RNA - Ribonucleic Acid

RNAPII – RNA Polymerase II

rNcl - rat nucleolin

rRNA - ribossomal RNA

RT - Reverse Transcription

rtTA - reverse Tet-controlled Transactivator

S

shRNA - short hairpin RNA

T

Tet - tetracycline

TetO - Tet operator

TetOn - Tetracycline On

TIC - Tumour Initiating Cells

TPCK - Tosyl Phenylalanyl Chloromethyl Ketone

TRE - Tet Responsive Element

U

UTR - Untranslated Region

V

VSV-G - Vesicular Stomatitis Virus G glycoprotein

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Chapter 1 – Introduction

1.1 Cancer

1.1.1 Breast cancer

In the last decades, cancer prevalence has increased, thus becoming one of the diseases with the highest death rate in the western world. In 2012, breast cancer was the second most common cancer diagnosed, representing 25% of all types of cancer and with 1.7 million new cases (mainly women) worldwide (Ferlay *et al.,* 2015). Among women, breast cancer was the leading cause of cancer-related deaths in developing countries and the second most common in developed countries (Ferlay *et al.,* 2015).

Breast cancer is an extremely complex disease at both the cellular and molecular levels, which depends not only from intrinsic factors (such as, genetic and epigenetic mutations), but also influenced by the tumour microenvironment (Hanahan *et al.,* 2011). Those factors combined, lead to the very diverse gene expression profiles and phenotypes found in breast tumours. Ultimately, they contribute to the different intrinsic subtypes of breast cancer (Perou *et al.,* 2000) and to the heterogeneity identified within the same tumour (Visvader *et al.,* 2012).

Breast cancer was initially characterized based on the morphological differences of the two types of epithelial cells found in the human mammary gland: basal cells and luminal epithelial cells (Altmannsberger *et al.,* 1981). This characterisation was established based on the keratin staining profile observed in immunohistochemistry assays. Several keratin types are exclusively expressed only by one of the two cell lineages (Perou *et al.*, 2000). A more detailed classification was then proposed, dividing breast cancer into different subtypes: Luminal A, Luminal B, Triple-negative, HER2-enriched and normal-like breast cancer. These differ on the expression of hormone (progesterone or oestrogen) or HER2 receptors (reviewed in Sotiriou and Pusztai 2009), such as schematized in Table 1.1.

Table 1.1 – Molecular classification of breast cancer types.

Types	Hormone-receptor (oestrogen and progesterone)	HER2	Growth	Prognosis	Observations
Luminal A	+	-	Slow	The best	
Normal-like	+	-	Slow	Slightly worse than luminal A	
Luminal B	+	+ or -	Faster than Luminal A	Slightly worse than Luminal A	
HER2-enriched	-	+	Faster than Luminal	Slightly worse than Luminal	Often successfully treated with targeted therapies aimed at the HER2 protein.
Triple-negative	-	-	The fastest	The worst	Common in women with *BRCA1* gene mutations

Adapted from Sotiriou *et al.,* 2009.

Among the different subtypes, triple negative tumours are the most aggressive with a high tendency towards metastasis, particularly visceral metastasis (Rodriguez-Pinilla *et al.,* 2006). Despite the current high rate of diagnosis owed to the advancement of techniques for early cancer detection, breast cancer related-deaths remain associated with chemotherapy resistance and, hence, recurrence of the disease (detected in approximately 30% of patients after chemotherapy) (Gonzalez-Angulo *et al.*, 2007). In general, chemotherapy is efficient at the beginning of treatment in 90% of primary breast cancers and 50% of metastases (Gonzalez-Angulo *et al.*, 2007). When therapy is not efficient, the disease progresses due to the development of drug resistance, often associated with the development of metastasis and hormonal-resistant breast cancer relapse (Gonzalez-Angulo *et al.*, 2007). To counteract these statistics, it is important to unravel the molecular mechanisms behind breast cancer initiation, expansion and metastases, which underlie the relapse of the disease.

1.1.2 The involvement of cancer stem cells in tumour initiation, propagation and therapy resistance

Currently, it is accepted that only a small percentage of tumour cells within a solid tumour can form a new tumour when transplanted into immunocompromised mice, recapitulating the heterogeneity of the original tumour. This suggests the existence of a distinct subpopulation of cancer cells with stem cell-like features, named cancer stem cells (CSCs), with the capacity to efficiently proliferate and give rise to a new solid tumour (Visvader *et al.,* 2012).

These cells support the CSCs model (or hierarchic model), which proposes the existence of a hierarchical organisation of cells in a variety of primary tumours (Visvader *et al.,* 2012). This model has been supported by the discovery of CSCs in solid tumours from different origins (Visvader *et al.,* 2008), including in breast cancer tumours (Al-Hajj *et al.,* 2003). Al-Hajj and colleagues reported the existence of a highly tumorigenic cell population in human breast cancer tissues. When isolated from the tumours, these cells were able to give rise to new tumours resembling the phenotype of the original one, *in vivo* (Al-Hajj *et al.*, 2003).

Chemotherapy resistance has been associated with CSCs, since they are naturally resistant to chemotherapy due to the higher expression of P-glycoprotein, the breast cancer resistance protein (BCRP) and Bcl-2, relative to non-stem cancer cells (non-SCC) (Yan Jia et al. 2015).

Accordingly, CSCs may survive to chemotherapy by adopting a quiescent state and eventually regain dividing capacity, which supports disease recurrence (Dean *et al.,* 2005) and metastasis formation (Dontu *et al.,* 2003), as presented in Fig. 1.1.

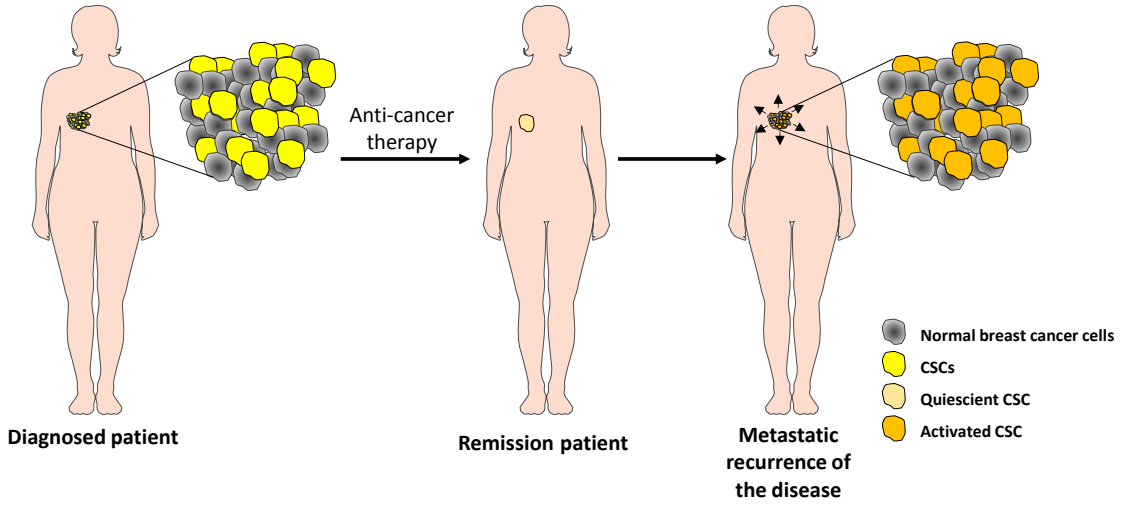


Figure 1.1 - Schematic representation of the possible mechanism by which CSCs are responsible for breast cancer relapse after chemotherapy and appearance of metastasis. After the treatment, resistant CSCs remain in the body in a dormancy state (quiescent CSC) while the patient is in remission. Many years after, these quiescent cells can be re-activated leading to the formation of a whole new tumour.

The finding of these CSC unravelled the question about the existence of tumour heterogeneity, posing an enormous challenge from a therapeutic standpoint. Thus, identification and study of the origin of CSCs and their biological properties within a tumour would lead to new, more efficient therapeutic strategies (Alison *et al.,* 2011).

1.2 Nucleolin, a pleiotropic protein

Nucleolin, a 100-110 kDa protein, is one of the major and most extensively studied nucleolar proteins, firstly identified by Orrick *et al.* and Archie W. Prestayko *et al.*, as C23 by two-dimensional gel electrophoresis of ribosomal subunits of rat hepatoma ascites. Among other eukaryotic organisms, an homologous protein was found in humans (Srivastava *et al.,* 1989).

1.2.1 Nucleolin structure and localisation in cells

The amino-acid sequence analysis of nucleolin and other nucleolar proteins (nucleolin-like proteins) of different eukaryotic species allowed the confirmation of a structural similarity between nucleolin from different species (Ginisty *et al.,* 1999). The sequencing of human nucleolin also enabled the first associations between structure and functions (Srivastava *et al.*, 1989). In the human nucleolin nucleotide sequence, different regions have been identified (Fig. 1.2): seventeen dibasic cleavage sites/proteolytic processing signalling sequences that give rise to the other nucleolar proteins (Bugler *et al.,* 1982; Lischwe *et al.,* 1981); karyophilic sequence signals responsible for the transport of ribosomal components to the nucleolus (Borer *et al.,* 1989); six nucleolar localisation signals, conserved sequences with a hydroxy amino acid, a proline and paired dibasic residues; acidic regions, rich in aspartate and glutamate, involved in histone binding (Erard *et al.,* 1988); seven well-conserved phosphorylation sites (Krebs *et al.*, 1986; Schneider *et al.,* 1989); four conserved RNA-binding domains (RBD) (Bugler *et al.*, 1987); conserved glycine-rich C-terminal involved in protein-protein and protein-nucleic acid interaction; and five conserved glycosylation sites (Lapeyre *et al.*, 1987).

Secondary structure prediction enabled to confirm the existence of possible interaction sites with proteins, RNA and DNA on nucleolin (Srivastava *et al.*, 1989).

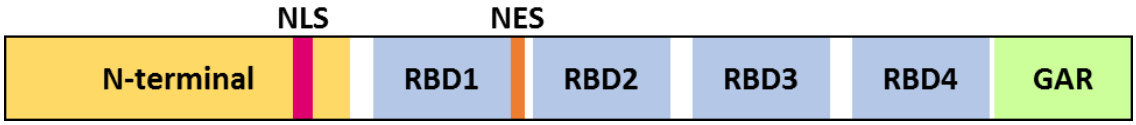


Figure 1.2- Schematic Nucleolin structure representing the different domains: N-terminal (in yellow) which has acidic regions and the Nuclear Localisation Signal (NLS, in pink); four conserved RNA Binding Domains (in blue); a Nuclear Export Signal (NES, in orange); Glycine-Arginine Region (in green). Adapted from (Ginisty *et al*., 1999 and Xiao *et al*., 2014).

After its discovery, many studies focused on the different localisation of nucleolin inside the cell (Fig. 1.3). Many studies reported nucleolin as part of the fibrillar component of nucleolus, either in normal or tumour cells (Lischwe *et al.*, 1981), thus linking it with the nucleolar organisation (Spector *et al.,* 1984). For example, the different domains that constitute nucleolin are responsible for different interactions with nucleolar factors (such as rDNA and rRNA) leading to its nucleolar accumulation (Ginisty *et al.*, 1999).

Furthermore, nucleolin translocates back and forth from the nucleolus to the cytoplasm, constantly transporting preribosomal particles (Borer *et al.*, 1989). The translocation depends on the nucleolin phosphorylation state: when dephosphorylated, nucleolin presents a nucleolar location; whereas phosphorylation enables cytoplasmic localisation (Schwab, M. S. *et al.,* 1997). As nucleolin phosphorylation on serine residues by a casein kinase II (CKII) protein (Caizergues-Ferrer *et al.*, 1987) and nucleolin maturation (Bugler *et al.*, 1982) have been associated with high proliferating cells (Bouche *et al.*, 1984), it is possible that nucleolin translocation to the cytoplasm during cell growth presents itself as an advantage. Indeed, in animals, nucleolin is required for the transcription, processing and assembly of rRNA into ribosomal particles in the nucleolus (Bouche *et al.*, 1984), which are then transported to the cytoplasm.

Despite the absence of a transmembrane hydrophobic or plasma membrane domains, nucleolin was also identified in a phosphorylated conformation at the cell surface by several research groups (Ginisty *et al.*, 1999), where it is associated with the intracellular actin filaments as well as acting as a surface antigen (Hovanessian *et al.*, 2000). N-glycosylation was described as being responsible for nucleolin translocation to the cell surface (Losfeld *et al.,* 2011; Watanabe *et al.*, 2010) as well as its association with membrane proteins (Alete *et al.,* 2006).

Interesting, nucleolar, cytosolic and cell-surface nucleolin have completely different functions and can act independently. In fact, a decrease in nucleolin mRNA levels by transcription blocking or in nucleolin protein levels by translation blocking, reduces cytoplasmic and surface nucleolin without affecting nucleolar nucleolin (Hovanessian *et al.*, 2010). These findings suggested that nucleolin mRNA is continually produced and translated in cancer cells, leading to nucleolin translocation to the cell surface (Hovanessian *et al.*, 2010). Hence, cytoplasmic and cell-surface nucleolin represent the majority of *de novo* synthesized protein, the proportion of which in different types of cells is less than 10% of nucleolin located in the nucleus (Carpentier *et al.*, 2005; Hovanessian *et al.*, 2000). Furthermore, induction of surface nucleolin is linked to the proliferative capacity of cancer cells (Hovanessian *et al.*, 2010).

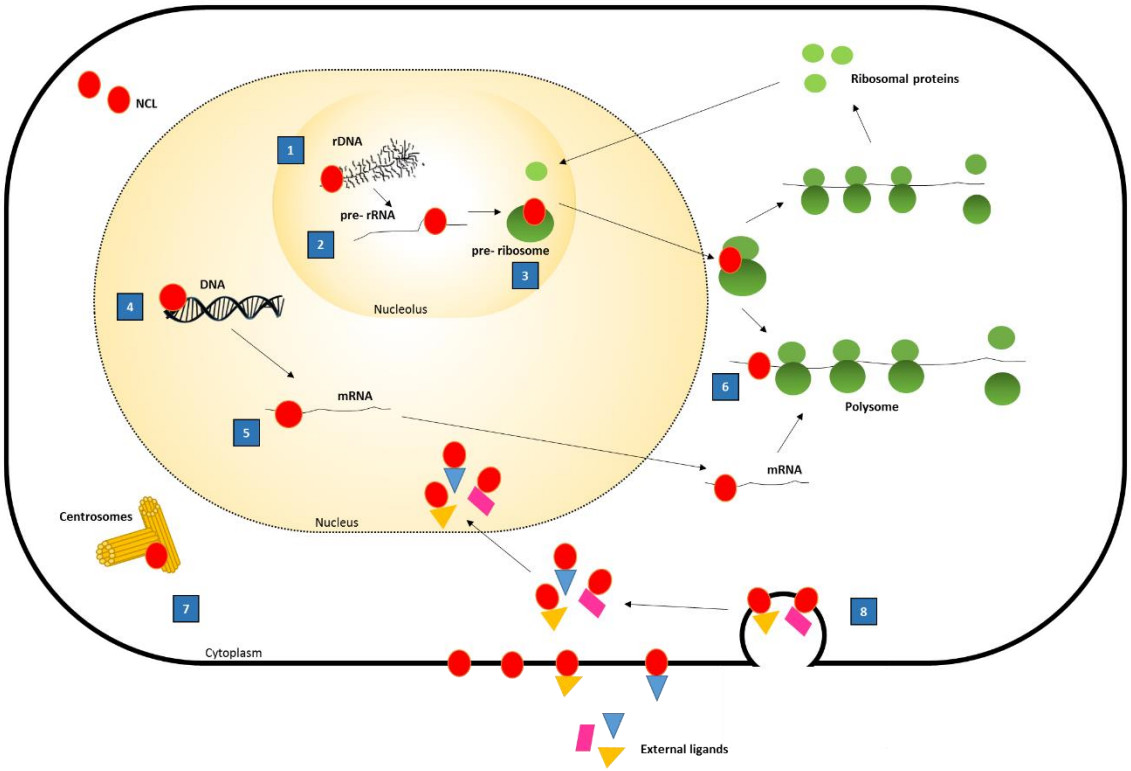


Figure 1.3 - Schematic representation of nucleolin multifunction. In the nucleolus, nucleolin associates with nucleolar chromatin/rDNA where it regulates RNA polymerase I transcription (1), interacts with pre-rRNA (2) helping in its folding and maturation as well as ribosomal protein assembly with the rRNA (3). In the nucleus, nucleolin regulates gene transcription by interaction with chromatin, gene promoters, regulates RNA polymerase II transcription (4) and binds mRNAs (5). In the cytoplasm, nucleolin interacts with mRNAs regulating its translation (6) and with centrosomes (7). On the cell surface, nucleolin bind external ligands inducing their endocytosis and translocation to the nucleus (8).

1.2.2 Involvement in cell cycle regulation

During mitosis, nucleolin localisation inside the cell and even inside the nucleolus is altered, suggesting an important role in regulating mitosis. In fact, the localisation of nucleolin along cell cycle phase was analysed in Chinese Hamster Ovary (CHO) dividing cells: in interphase, nucleolin is associated with the preribossomal ribonucleoprotein components (Escande *et al.*, 1985); in metaphase, it is located in the nucleolus organizing region in chromosome periphery (Lischwe *et al.*, 1981; Weisenberger *et al.,* 1995); from late prophase to telophase, it is located in the nucleolar remnants (Gas N. *et al.,* 1985); and in telophase, it is located in prenucleolar bodies (Gas N. *et al.,* 1985). The results showing that nucleolin is present along all the cell cycle phases, suggested that this protein is highly involved in cells organisation and growth.

In human HeLa cells, and in line with previous data in rodent cells, it was found that nucleolin is important in several steps along the mitotic process. Nucleolin inactivation resulted in the accumulation of cells with delayed prometaphase (Ma *et al.*, 2007). This was owed to the activation of the spindle checkpoint in response to an improper kinetochore-microtubule attachment, from which resulted misalign and disoriented chromosomes (Ma *et al.*, 2007). Even

the chromosomes that were able to align at the spindle equator, exhibited reduced centromere stretching and reduced kinetochore-microtubule fibers (Ma *et al.*, 2007). The influence of nucleolin in microtubules organisation is also evident since shutdown of nucleolin contributed to the existence of multipolar spindles resulting from the multi centrosomes found inside a cell (Ma *et al.*, 2007). Depletion of nucleolin was also associated with cell cycle blockage in G2 phase, resulting in an increase of multinuclear cells and cells without nucleus (Ugrinova *et al.*, 2007).

Phosphorylation of mitosis-associated proteins is important to control the initiation and progression of mitosis (Vandre *et al.,* 1984). Likewise, nucleolin phosphorylation is also critical. During mitosis, this protein is phosphorylated by Cdc2 kinase (Belenguer *et al.,* 1990), being found in in the periphery of spindle poles at the phosphorylated state during mitosis (Ma *et al.*, 2007).

1.2.3 Regulation of ribosome biogenesis

As described above, nucleolin localises in transcriptionally active nucleolar regions, suggesting that it is involved in rDNA transcription, pre-rRNA synthesis and processing, pre-ribosomal processing, ribosomal assembly and maturation (Fig. 1.3). In fact, Bugler *et al.* and Orrick *et al.*, demonstrated, in eukaryotic cells, that nucleolin was imported to the nucleolus where it was responsible for the control of rDNA transcription/pre-rRNA synthesis and ribosome assembly (Orrick *et al.,* 1973; Bugler *et al.,* 1982).

Nucleolin depleted cells exhibited lower levels of rDNA transcription, when compared with normal cells (Ugrinova *et al.*, 2007). This was supported by the regulatory functions of nucleolin over RNA polymerase I (RNAPI) activity and chromatin condensation (Erard *et al.*, 1988) as well as its interaction with the nascent transcripts (Bourbon *et al.,* 1983), as represented in Fig. 1.3.

The interaction of nucleolin with pre-rRNA, through its RNA binding domains and carboxyl terminal domains, helped maturation of rRNA and assembly of ribosomes (H Ginisty *et al.*, 1999). Furthermore, it was reported in murine cells that maturation of nucleolin was positively correlated with pre-rRNA synthesis (Bouche *et al.*, 1984) and occurred simultaneously with pre-ribosome biogenesis (Bourbon *et al.*, 1983; Bugler *et al.*, 1982). Moreover, phosphorylation of nucleolin, referred above to be implicated in nucleolus-cytoplasm exchange (Borer *et al.*, 1989), was also reported to be involved in nucleolin maturation (Bourbon *et al.*, 1983; Schneider *et al.,* 1989). Accordingly, its involvement in ribosome biogenesis revealed that nucleolin was critical for nucleolus formation/nucleogenesis (Medina *et al.,* 1995; Ugrinova *et al.,* 2007; Ma *et al.,* 2007).

1.2.4 Regulation of gene transcription

Nevertheless, nucleolin is also essential for processes occurring in the nucleoplasm (Dickinson *et al.,* 1995). Nucleolin has an important role in gene transcription by RNA polymerase II (RNAPII) (Storck *et al.* 2007), as represented in Fig. 1.3. Nucleolin binds RNAPII, being necessary for its transcriptional activity (Rickards *et al.,* 2007). Another mechanism of transcription regulation mediated by nucleolin might rely on its ability to promote chromatin remodeling and histones dimerisation, facilitating RNAPII-mediated transcription (Angelov *et al.*, 2006).

Furthermore, nucleolin binds to transcriptional promoter motifs resulting in the regulation of gene expression (Huddleson *et al.*, 2006; Masumi *et al.*, 2006), either to increase or decrease transcription (Fig. 1.3). In the case of the activator protein-1 (AP-1) transcription factor, nucleolin binding to its promoter resulted in the decrease of AP-1-dependent gene transcription (Samuel *et al.,* 2008). AP-1 is a major regulator of several cellular processes, such as cell proliferation, survival, oncogenic transformation, death and differentiation (Shaulian *et al.,* 2002).

1.2.5 Regulation of translation by mRNA interaction

As a RNA-binding protein (RBP), nucleolin is able to regulate gene expression by controlling post-translational processes (Abdelmohsen *et al.*, 2011), as represented in Fig. 1.3. Nucleolin can interact with the 3'untranslated region (UTR) of numerous mRNAs enhancing their stability and with the 5'UTR of other mRNAs repressing their translation (Abdelmohsen *et al.*, 2011; Abdelmohsen *et al.*, 2012). Hence, by modulating mRNAs stability and mediating post-transcriptional modifications, nucleolin has as impact on protein synthesis. This however, is not a ubiquitous process but rather protein-specific (Fähling *et al.*, 2005; Pichiorri *et al.*, 2013).

Nucleolin target mRNAs largely encode proteins involved in cancer, genetic disorders and cell growth and proliferation, suggesting nucleolin is involved in important cellular processes (Abdelmohsen *et al.*, 2011).

Knockdown of nucleolin in both HeLa cells and embryonic stem cells (ESC) induces an increase of p53 protein, enhancing its activity due to increased translation/stabilisation of p53 mRNA (Ugrinova *et al.*, 2007). In ESC, nucleolin knockdown revealed a higher activation of p53 pathway concomitantly with increased ESC apoptosis, decreased cell proliferation and ESC differentiation (Yang *et al.*, 2011). Not only p53 activity was affected, but also the levels of p53 protein increased gradually with nucleolin levels decrease along time (Yang *et al.*, 2011). Contrary to p53, nucleolin increases Bcl-2 (an anti-apoptotic protein) expression by enhancing its mRNA stability (Sengupta *et al.,* 2004; Ishimaru *et al.,* 2010). Nucleolin binds to the 3'UTR of Bcl-2 mRNA preventing its degradation by inhibition of the deadenylation process (Ishimaru *et al.*, 2010). Specifically in breast cancer cells, both nucleolin and Bcl-2 are expressed in high levels

in the cytoplasm of MCF-7 and MDA-MB-231 cells when compared with the MCF-10A cell line (Soundararajan *et al.,* 2008).

1.2.6 Development and differentiation

Nucleolin also present potential roles in development and differentiation of tissues in different organisms. During chicken development, the levels of nucleolin vary temporally and spatially. From the diverse tissues studied, across development stages, different amounts of nucleolin were reported. For example, in early embryonic tissues, nucleolin was found in higher amount as compared to adult tissues (Maridor *et al.,* 1990). In adult tissues, nucleolin protein levels were lower but nucleolin mRNA is present in high amounts, suggesting that regulation of nucleolin expression was likely dependent on post-transcriptional modifications, during development (Maridor *et al.*, 1990).

Nonetheless, during differentiation of HL-60 cells (leukaemia cells), a constant decrease in nucleolin levels was observed (Méhes *et al.,* 1995). The same decrease in nucleolin levels was observed upon differentiation of mouse ESC, suggesting that nucleolin was a marker of undifferentiated cells and responsible for ESC self-renewal (Yang *et al.*, 2011; Fonseca *et al.*, 2015).

1.2.7 Cell proliferation and survival

Conjugating all the functional roles of nucleolin, aforementioned, it is intuitive that nucleolin might have a fundamental role in cell division and thus necessary for cell proliferation, at least in physiological conditions.

Indeed, nucleolin might regulate, in part, cellular growth, as nucleolin levels are increased in cells stimulated to proliferate and diminished in non-dividing cells (such as differentiated or starving cells) (Chen *et al.*, 1991; Derenzini *et al.*, 1995). The modulation of cell proliferation by nucleolin is reinforced by the fact that nucleolin also becomes more stable in proliferating cells (Fang *et al.,* 1993). For example, upon depletion of nucleolin in HeLa cells, their growth rate diminished, leading to a decrease in cell proliferation and increase in apoptosis (Ugrinova *et al.*, 2007). This was also supported by the reduction of cell growth and increased apoptosis in ESC upon nucleolin knockdown, which was linked to upregulation of p53 pathway (Yang *et al.*, 2011).

Thus, nucleolin has been associated with cellular self-renewal capacity, proliferation and survival (Yang *et al.*, 2011).

1.2.8 Interactions with external ligands

As already described, nucleolin was not only located inside the cell, but also on the surface of cancer cells and tumour endothelial cells, where it acts as an anchor for several ligands (reviewed in Fujiki *et al.*, 2014), as represented in Fig. 1.3. Among others, nucleolin interacts

with hepatitis delta antigens, modulating the virus replication (Lee *et al.,* 1998), and urokinase, implicated in cell-surface adhesion and mitogenesis (Dumler *et al.*, 1999). It has been described the interaction of surface nucleolin with anti-HIV particles, which compete with HIV-1 at the same binding site on nucleolin (Hovanessian *et al.,* 2006; Nisole *et al.*, 2002). Lactoferrin, a protein important in host defence against HIV and CMV and for anti-cancer effect, is internalised with nucleolin, inducing its internalisation and recycling/degradation or its translocation to the nucleus (Legrand *et al.*, 2004). Furthermore, P-selectin has been described as inducing nucleolin tyrosine phosphorylation enabling the formation of a signalling complex with PI3K and p38 MAPK, involved in tumour development (Reyes-Reyes *et al.,* 2008). Finally, Tipα, the carcinogenic factor of *Helicobacter pillory*, interacts with nucleolin promoting their concomitant internalisation (Watanabe *et al.*, 2010).

Overall, nucleolin itself or its putative proteolytic products were proposed to function as surface receptors mediating extracellular regulation of nuclear events (Srivastava *et al.,* 1999). This property is now being studied with the goal to use nucleolin in anti-cancer therapies, a subject discussed ahead in Chapter 1.4.

1.3 Nucleolin's contribution to tumorigenesis and cancer progression

Besides the role of nucleolin in cell proliferation and survival, which are considered parameters to predict the tumour growth rate (Srivastava *et al.,* 1999), many of the ligands interacting with surface nucleolin are involved in tumorigenic and angiogenic processes, reinforcing the importance of nucleolin in tumour biology.

Indeed, it was found that nucleolin was overexpressed in several types of human cancer samples, including B-cell chronic lymphocytic leukaemia (Otake *et al.*, 2009), melanoma (Hoja-Łukowicz *et al.*, 2009), glioblastoma (Galzio *et al.*, 2012), colorectal (Hammoudi *et al.*, 2013), gastric (Wensheng Qiu *et al.*, 2013), lung (Zhao *et al.*, 2013), breast (Berger *et al.,* 2015), hepatocellular carcinoma (Wei Qiu *et al.,* 2015), ependymoma (Chen *et al.,* 2016) and retinoblastoma (Subramanian *et al.*, 2016). In fact, nucleolin overexpression was associated with decreased survival rates on breast cancer patients (Wolfson *et al.,* 2016).

In addition to the interactions of surface nucleolin mentioned above (Chapter 1.2.8), nucleolin also interacts with important ligands involved in signal transduction and malignant transformation (examples presented in Fig. 1.4). The interaction between nucleolin and the cytoplasmic tail of ErbB receptor is one of the most studied. Nucleolin induces ErbB dimerisation, phosphorylation and activation (Di Segni *et al.,* 2008; Wolfson *et al.*, 2016). Nucleolin C-terminal amino acids interact with the nuclear localisation sequence (NLS) of ErbB receptor, resulting in the activation of the signalling cascade by phosphorylation of the tyrosine kinase domain (Farin *et al.,* 2009), as represented in Fig. 1.4.

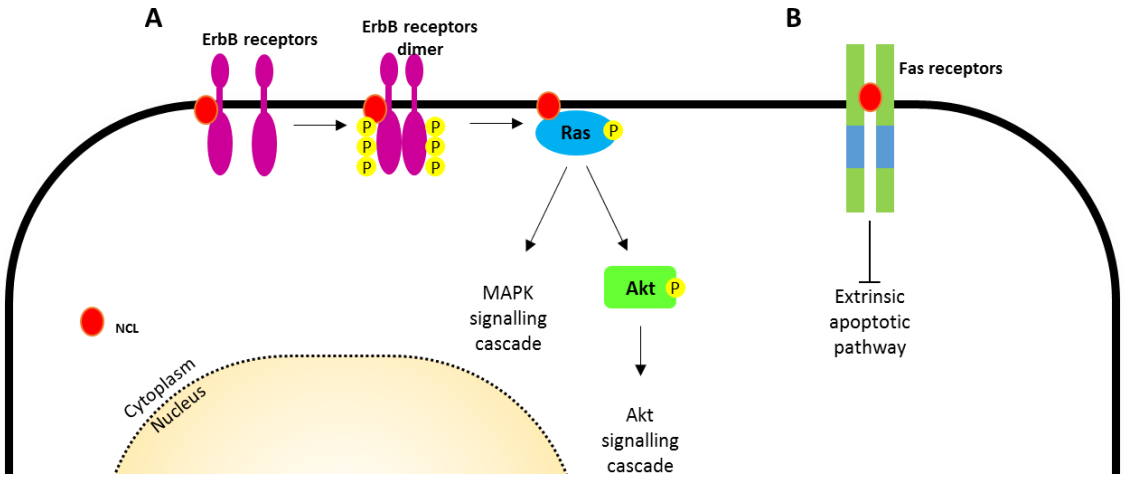


Figure 1.4 - Schematic representation of the Ras signaling and extrinsic apoptotic pathways modulation by nucleolin. (A) Cross-talk between surface nucleolin, ErbB receptors and signal transduction. Nucleolin interacted with ErbB receptors leading to its dimerisation and phosphorylation. Hence, ErbB receptors become active resulting in activation of Ras protein and following activation of MAPK and Akt signaling cascades. (B) Surface nucleolin interacted with Fas receptor, which blocks its interaction with Fas ligand, inhibiting the extrinsic apoptotic pathway activation.

Besides this direct interaction, a functional cross-talk between nucleolin, ErbB receptors and Ras proteins has been reported. Nucleolin binding to Ras, independently of its GTP/GDP conformation, promoted its clustering on the plasma membrane (Farin *et al.*, 2011), interfering with the MAPK signal transduction (Inder *et al.*, 2009). This mechanism has been reported as responsible for malignant cell transformation and migration induced by epidermal growth factor receptor (EGFR), a member of ErbB family (Q. Xie *et al.*, 2016). Within the EGF/Nucleolin/Akt axis, Sp1 is considered crucial for its tumorigenic effects. Upon Akt activation, nucleolin is phosphorylated leading to the increased translation of Sp1 transcription factor, involved in cell cycle, apoptosis, differentiation, tumorigenesis and angiogenesis (Hung *et al.*, 2014) through the control of Bcl-2, Bax, Bak, Fas, VEGF, FGF and p21 expression, among other proteins (Black *et al.,* 2001). Cell surface nucleolin is also known to bind Fas receptor blocking its interaction with Fas ligand, which is associated with the anti-apoptotic and initiation of carcinogenesis roles of nucleolin (Wise *et al.* 2013).

Moreover, nucleolin is involved in the maturation of miRNAs known to be upregulated and directly related with breast cancer tumorigenesis and therapy resistance (Avino *et al.*, 2016).

Surface nucleolin also interacts with endostatin, critical in cancer development. Nucleolin mediates endostatin internalisation and translocation to the nucleus, where it inhibits nucleolin phosphorylation and arrest proliferation (Christian *et al.,* 2003). As such, nucleolin contributes to the anti-angiogenic and anti-tumoural activity of endostatin (Shi *et al.*, 2007).

1.4 Nucleolin as a target for anti-cancer therapy

Owed to the growingly recognized importance of surface nucleolin in cell proliferation and tumorigenesis, nucleolin is being exploited as a target for anti-cancer strategies, with several molecules in development against a variety of human cancers, either to directly tackle tumour cells or angiogenesis (reviewed in Fujiki *et al.,* 2014).

For example, the aptamer AS1411 (Mogelard *et al.*, 2010) which binds to cell surface nucleolin, has been described to internalize together with this protein, inhibiting nucleolin binding to target mRNAs and reduce levels of nucleolin-dependent miRNA and target genes, increasing apoptosis and decreasing cancer aggressiveness (Pichiorri *et al.*, 2013).

Moreover, pseudo-peptide HB-19 binds to surface nucleolin on the GAR domain, acting as its antagonist, which has effects on cell proliferation, adhesion and cell death (Hovanessian *et al.,* 2010; Krust *et al.,* 2011; Birmpas *et al.,* 2012).

Nucleolin is also being studied as a possible target for immunotherapy. The immunoagent 4LB5, a single-chain fragment variable antibody against a nucleolin-RBD, was able to decrease specifically, *in vitro*, cell viability and proliferation, as well as reduce tumour size in a breast cancer animal model (Palmieri *et al.,* 2015; Avino *et al.,* 2016).

In a different context, nucleolin is being explored as a target for nanotechnology-based targeted delivery. In fact, a liposomal nanoparticle delivery system functionalized with F3 peptide (Moura *et al.*, 2012), known to recognize nucleolin, is being developed to target nucleolin in order to promote the intracellular delivery of encapsulated single drugs (Gomes-Da-Silva et al., 2012; Moura et al., 2012) or drug combinations (Fonseca *et al.*, 2015).

Chapter 2 – Objectives

Bearing in mind, nucleolin has been described as a possible proto-oncogene, it is in fact overexpressed in cancer cells from different histological origins and it is being studied as a target for anti-cancer therapies, it is important to get additional insights on the role of nucleolin in cancer.

The working hypothesis of the present work postulates that nucleolin may be an oncogenic driver of breast cancer by modulating stemness properties in breast cancer cells.

A better understanding on how nucleolin gain or loss of expression in cancer cells affect their phenotype, is vital to better understand this protein function in carcinogenesis. To induce these alterations in nucleolin expression, it is necessary to development molecular tools enabling the control of nucleolin expression.

Accordingly, the main objectives of this project were:

- to develop a molecular system aiming to modify nucleolin expression;
- validate this system in human breast cancer cell lines;
- unravel the impact of the modulation of nucleolin expression in some of the phenotypic features of breast cancer cells.

Chapter 3 - Materials and Methods

3.1 Lentiviral system

Lentiviruses are a unique subtype of Retrovirus owing to their ability to infect both dividing and non-dividing cells and integrate a gene of interest within the genome of the target cells (Pluta *et al.,* 2009). Among other types of lentiviral vectors for gene delivery, HIV-1-based lentiviral vectors have been more extensively used in research for delivery of transgenes and gene-silencing or overexpression tools, cellular reprograming, *in vivo* and *in vitro* tracking, production of transgenic animals and immortalisation of cell lines (Sakuma *et al.,* 2012). This system started to be engineered by Helseth and colleagues, in 1990, who originally described a replication-defective HIV-1 virus. From that moment forward, many modifications to the HIV-1 provirus were developed to originate a safe and optimized lentiviral vector. Briefly, HIV proteins associated with virulence were removed (Nef, Vif, Vpr and Vpu) and external elements were introduced (cPPT, CTS, PCE, poly A and chromatin insulators) to increase transgene expression, virus titre and storage time (Pluta *et al.,* 2009).

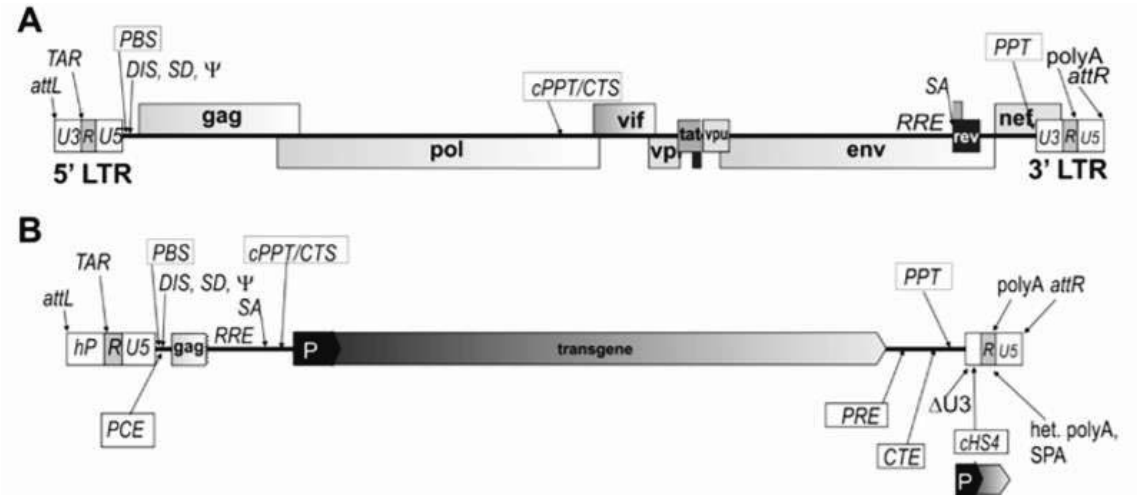


Figure 3.1- Comparison of wild-type HIV-1 provirus (A) and optimized lentiviral vector (B). Adapted from Pluta *et al.*, 2009.

Besides these alterations, the lentiviral system was divided in three cassettes: packaging expression vector, transfer vector and envelope expressing vector. This strategy was adopted to separate *cis*-acting sequences (responsible for RNA synthesis, packaging, reverse transcription of viral RNA and cDNA integration) from *trans* elements (encoding accessory proteins and viral enzymes) (Pluta *et al.,* 2009).

The envelope vector encodes for an envelope glycoprotein, usually VSV-G, which allows infection of mammalian cells by enabling virus endocytosis (Pluta *et al.,* 2009).

The transfer vector expresses the gene of interest and the *cis* elements to increase transcription in both producer and target cells, virus packaging and titre, RT, nuclear import and genome integration in the target cell (Follenzi *et al.,* 2000; Pluta *et al.,* 2009; Zufferey *et al.,* 1999).

Importantly, the packaging vector has evolved to increase biosafety of lentiviral usage, giving rise to three generations of these vectors, constituted by viral structural proteins and enzymes essential for virus formation. The 1st generation lentiviral packaging vector still presented cytotoxicity owed to virulence, despite the partial removal of HIV-1 virulent sequences. Further removal of non-essential sequences for lentiviruses activity, it was introduced in the 2nd generation lentiviral packaging vector, expressing only Gag, Pol, Rev and Tat proteins. The 3rd generation lentiviral system differs from the previous one by the removal of *tat* from the system and division of the plasmid into two separated plasmids: one coding the gag and pol and the other coding Rev (Pluta *et al.,* 2009).

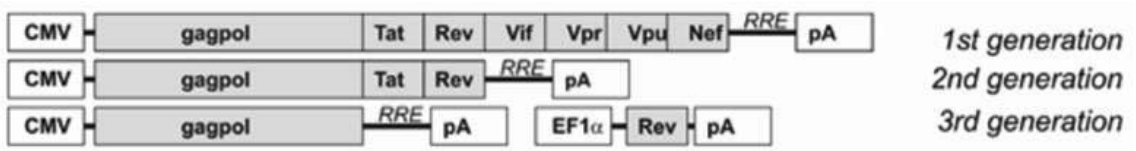


Figure 3.2 – Comparison between the three generations of lentiviral packaging vectors. First generation coding all regulatory and accessory genes downstream the CMV promoter. Second generation encoding only for Tat and Rev proteins. Third generation coding Gag and Pol from CMV promoter and Rev from EF1α in a separate plasmid. Adapted from (Pluta *et al.,* 2009).

An advantage of the lentiviruses rests on their capacity to integrate the genome of host cells. These viruses interact with the host cell chromatin and integrate their genome in chromosomal regions rich in actively transcribed genes, close to introns (Pluta *et al.,* 2009). The integration is facilitated by LTRs, flanking the gene of interest in the lentiviral transfer vector. The *att* repeats, there localised, recombine with the integration sites in the host cell DNA. For further specification of the integration process, lentiviruses with site-directed integration into genome are being developed aiming at decreasing possible DNA damage induced by lentiviruses integration (Cornu *et al.,* 2007; Lombardo *et al.*, 2007; Moldt *et al.,* 2008).

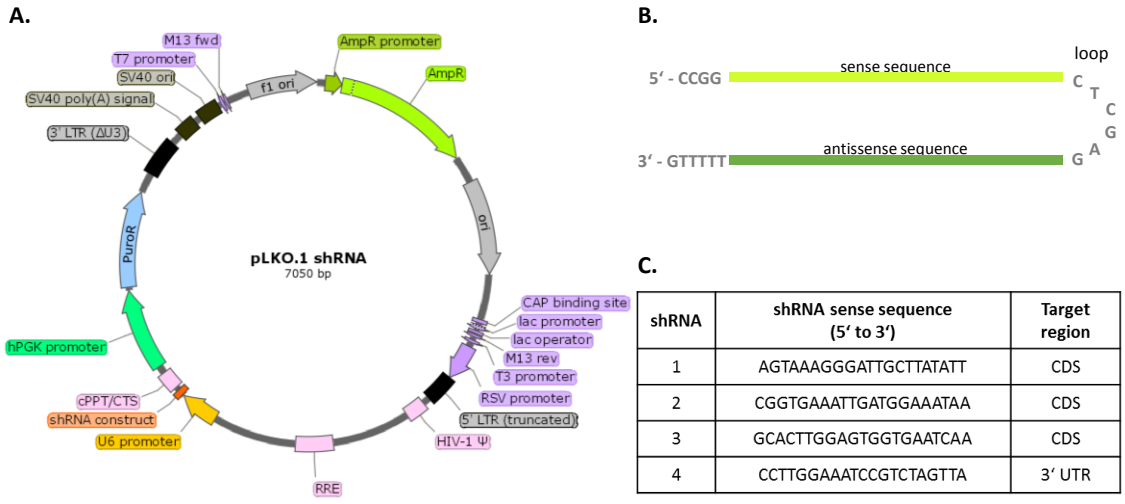
A. Nucleolin downregulation-encoding plasmids

The lentiviral plasmids used to produce lentiviral particles aiming to knockdown of human nucleolin were pLKO.1 vectors (chosen by the The RNAi Consortium for expression of short-hairpins RNA (shRNA)) containing different shRNAs against human nucleolin sequence (Sigma-Aldrich, USA). This vector contains a human U6 promoter driving the constitutive transcription of the shRNA sequence and a hPGK (human phosphorglycerate kinase gene) promoter driving the expression of PuroR (Puromycin resistance gene), which enables the selection of transduced cells.

shRNAs is a type of RNAi (interference RNA) which consists in two complementary RNA sequences, of 19-22 bps (base pairs), linked by a short loop, of 4-11 base pairs, creating a hairpin similar to miRNA structures found naturally in cells. shRNAs can be delivered and integrate into the DNA of mammalian cells. Through infection with lentiviral vectors, stable cell lines with a long-term knockdown of the target gene may be generated. Inside the mammalian cells transduced with the Lentivirus, shRNA sequences are transcribed and exported to the cytosol binding to the target mRNA and priming its degradation (Moore *et al.*, 2010).

Each shRNA used is constituted by a sense sequence of 21 bps targeting human nucleolin sequence (Fig. 3.3 C), a loop (CTCGAG) and an antisense sequence, complementary to the sense sequence (Fig. 3.3 B).

As a control for pLKO.1 hNCL shRNA plasmids, it was used a pLKO.1 plasmid with a scramble shRNA, with limited homology to any known human genomic sequence.



C.

shRNA	shRNA sense sequence (5' to 3')	Target region
1	AGTAAAGGGATTGCTTATATT	CDS
2	CGGTGAAATTGATGGAAATAA	CDS
3	GCACTTGGAGTGGTGAATCAA	CDS
4	CCTTGGAAATCCGTCTAGTTA	3' UTR

Figure 3.3 - pLKO.1 shRNA lentiviral vector and anti-NCL shRNAs structure. (A) Schematic representation of pLKO.1 vector evidencing the expression cassette flanked by 5' LTR and 3' LTR: HIV elements essential for lentiviral particles production (in light pink - HIV-1 ψ, RRE, cPPT/CTS), U6 promoter (in yellow) for shRNA expression (in orange) and the hPGK promoter (in blue-green) upstream PuroR (in light blue). (B) Representation of the hairpin structure formed by anti-NCL shRNA sequence cloned in the pLK0.1 vector. (C) Table summarizing the anti-NCL shRNA sequences tested for nucleolin knockdown.

B. Nucleolin overexpression-encoding plasmids

Two plasmids were constructed to produce lentiviral particles aiming to overexpress nucleolin *in vitro*: one coding for human nucleolin (pCW-hNCL-T2A-mCherry) to increase nucleolin expression on human breast cancer cell lines and another coding for rat nucleolin (pCW-rNcl-T2A-mCherry) to promote the knockdown rescue of nucleolin introduced by the pLKO.1 hNCL shRNA.

These Lentiviral plasmids were designed and constructed in accordance with Tetracycline On (TetOn) system, or rtTA-dependent system, which allows controlling the expression of our gene of interest (nucleolin). TetOn is an inducible system dependent of the presence of three elements: rtTA, a promoter responsive to rtTA and a tetracycline. rtTA is a recombinant tetracycline controlled transcription factor, which binds to the added tetracycline, working as a transactivator of a rtTA responsive promoter by interacting with it. When bound to the tetracycline, rtTA interacts with DNA, inducing transcription. The promoter used in this system consists in a RNA polymerase II promoter (transcriptionally silent in the absence of additional transcription factor binding sites) fused to multimerized tetracycline operator (*tetO)* sequences making it responsive to rtTA. Tetracycline is the system's inducer, being responsible for the activation of transcription. Typically, the tetracycline used is doxycycline (DOX).

The two plasmids aforementioned (pCW-hNCL-T2A-mCherry, pCW-rNcl-T2A-mCherry) contain a TRE (Tet Responsive Element) promoter, which is composed by a cytomegalovirus (CMV) promoter fused to seven *tetO* sequences. This promoter drives the tetracycline-dependent expression of human nucleolin (hNCL) and rat nucleolin (rNcl), cloned downstream, and linked to mCherry, used as a reporter gene, by the T2A (self-cleaving peptide) coding sequence. These plasmids also have a hPGK promoter constitutively driving the expression of PuroR linked to rtTA3 (reverse tetracycline-controlled transactivator) by the T2A coding sequence.

Aiming at future *in vivo* studies, two plasmids for the overexpression of human and rat nucleolin were further constructed using a similar strategy as above, but with luciferase replacing the mCherry reporter gene (pCW-hNCL-T2A-Luciferase and pCW-rNcl-T2A-Luciferase, respectively).

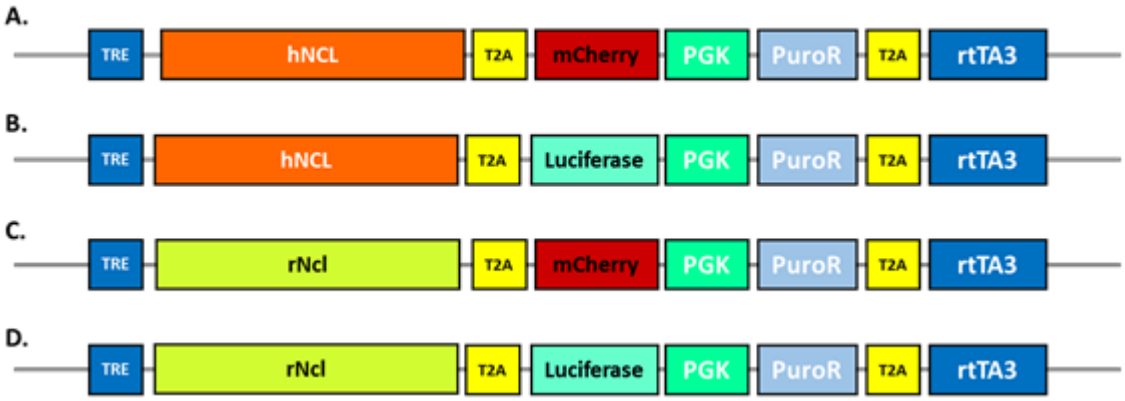


Figure 3.4- Schematic representation of the lentiviral vectors constructions for in vitro and in vivo usage. (A) pCW-hNCL-T2A-mCherry (B) pCW-hNCL-T2A-Luciferase (C) pCW-rNcl-T2A-mCherry (D) pCW-rNcl-T2A-Luciferase. Representation of the human and rat nucleolin sequences cloned downstream TRE promoter, linked to mCherry and Luciferase by T2A sequence, and PuroR, linked to rtTA3 by T2A sequence, cloned downstream PGK promoter.

3.1.1 Cloning techniques

These plasmids were assembled using sequential cloning strategies. Briefly, all the cloning was executed as follows: PCR amplification (from cDNA or an intermediary cloning plasmid), enzymatic restriction digestions, ligation reaction of the insert with the vector, transformation of competent cells with the resulting plasmid, screening colony PCR, plasmid DNA isolation and purification, confirmation of the construct by digestion with restriction enzymes and sequencing of the final plasmid.

a. Total RNA isolation

RBL (rat basophilic leukocytes) and MDA-MB-231 (human breast cancer cell line) total RNA was isolated using Qiazol buffer (Qiagen, USA) according to the manufacturer instruction. RNA was subsequently purified using RNAeasy mini Kit (Qiagen, USA).

b. Reverse transcription reaction

cDNA from RBL and MDA-MB-231 cells was produced by RT reaction using total RNA, isolated as described above, as a template. RT reaction was performed using 1-5 µg of total RNA template and SuperScript® II Reverse Transcriptase (Invitrogen, USA), as described by the manufacturer. Briefly, samples were denatured by heat-shock (10 min at 70 °C followed by 2 min on ice), annealing with Oligo(dN) primer (Invitrogen, USA) was performed at 42 °C during 3 min and the reverse transcription reaction was completed at 42 °C during 50 min.

c. Human and rat nucleolin coding sequence amplification by PCR

Human nucleolin and rat nucleolin coding sequences were amplified by PCR from MDA-MB-231 cDNA library (mentioned above) with the primers PR1/PR2 (described in Table S1, in Appendix) and from RBL cDNA library (mentioned above) with the primers PR3/PR4 (Table S1), respectively, using Thermo Scientific Phusion High-Fidelity DNA Polymerase. Amplification was performed on the conditions showed in Fig. 3.5: initial denaturation (2 min at 95 °C), followed by 32 cycles of denaturation (20 s at 95 °C), primers annealing (20 s at 58 °C), extension (variable time, depending on the fragment size, at 72 °C) and final elongation (10 min at 72 °C).

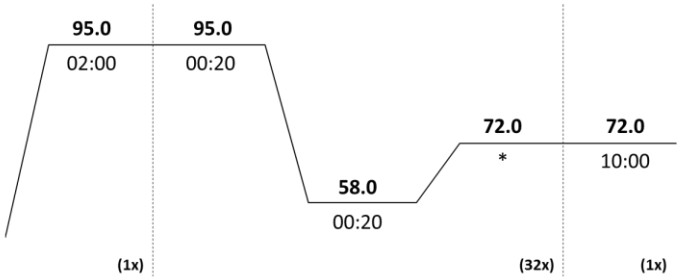


Figure 3.5– Diagram representing the settings used for PCR amplifications with Thermo Scientific Phusion High-Fidelity DNA Polymerase and Promega GoTaq DNA Polymerase. Initial denaturation (2 min at 95 °C), followed by 32 cycles of denaturation (20 s at 95 °C), primers annealing (20 s at 58 °C) and extension (at 72 °C) and a final elongation (10 min at 72 °C). * indicates variable time: 1 min *per* 3000 bps (Phusion High Fidelity DNA Polymerase) or 1 min *per* 1000 bps (GoTaq DNA Polymerase).

d. Horizontal agarose gel electrophoresis

To visualize the PCR amplification products and plasmid enzymatic restriction digestions, the samples were applied in a 0.8% agarose gel stained with RedSafe™ Nucleic Acid Staining Solution (INtRON Biotechnology, South Korea) and run in parallel with *GeneRuler 1kb* DNA Ladder (*Thermo* Fisher Scientific, USA) as a reference.

e. Enzymatic restriction digestion

Vectors and inserts were digested with restriction enzymes to prepare them to ligation reaction. Digestions with BamHI, BglII, EcoRV, NheI, NotI and SmaI (Takara Bio, USA) were performed at 37 ^{o}C during 1.5 h, as described by the manufacturer. Moreover, double restriction digestions of the plasmid DNAs of interest was used to confirm the constructions through horizontal agarose gel electrophoresis.

f. Plasmid dephosphorylation

To avoid self-ligation of the linear vector and prepare it to ligate the insert, vectors were dephosphorylated with CIAP (Calf Intestinal Alkaline Phosphatase) (Takara Bio, USA), during 15 min at 37 ^{o}C. Following dephosphorylation, CIAP was completely inactivated by heating at 70 ^{o}C followed by phenol/chloroform extraction and DNA precipitation.

g. Ligation reaction

The ligation reactions were performed, overnight at 22 ^{o}C using T4 (Thermo Fisher Scientific, USA) with 50-100 ng of the vector and 3-fold excess of the respective insert. Prior to ligation, vectors and inserts were purified by DNA precipitation and semiquantified on horizontal agarose gel electrophoresis.

h. Transformation of competent cells

After ligation, HB101 (Promega, USA) competent cells (prepared in our lab) were transformed with the produced plasmids through heat-shock. Briefly, plasmid DNA was placed in contact with the HB101 cells during 30 min on ice, followed by 1 min at 42 ^{o}C and 3-4 min at 0 ^{o}C. Afterwards, LB medium was added to the cells which were incubated for 1.5 h at 37 ^{o}C while shaking, allowing cells with acquired antibiotic resistance (enabled by the plasmids) to grow. Transformed cells were then incubated for 24 h at 30 ^{o}C in LB agar plates supplemented with Ampicilin at the concentration of 215 mM (in the case of pCW vectors) or with Spectinomycin at the concentration of 301 mM (in the case of pcDNA ENTR BP). These competent cells are *recA*- which minimizes recombination events, also reduced by culture the bacteria at 30 ^{o}C.

i. Colony PCR screening

To select the bacteria colony containing the vector of interest, a screening colony PCR was performed with GoTaq DNA Polymerase (Promega, USA; conditions showed in Fig. 3.5) using

primers specific for each construction (mentioned hereafter) enabling to infer the successful cloning. From this colony PCR screening, a single positive colony of each construction, was grown for 24 h at 30 °C in liquid LB medium supplemented with Ampicilin at the concentration of 215 mM (in the case of pCW vectors) or with Spectinomycin at the concentration of 301 mM (in the case of pcDNA ENTR BP).

j. Plasmids isolation and purification

Plasmid DNA was isolated and purified with the E.Z.N.A.® Plasmid DNA Mini Kit (Omega Bio-Tek, USA), or E.Z.N.A.® Plasmid DNA Midi Kit (Omega Bio-Tek, USA) when higher amounts of plasmid DNA were required, as described by the manufacturer. Both techniques rely on the same principle: centrifugation to obtain a pellet of the bacteria followed by alkaline lysis to release genetic material and proteins; removal of proteins and RNA from the solution; retain DNA in a specific column; wash of plasmid DNA; elution of the plasmid DNA from the column.

3.1.2 Cloning strategy

a. Cloning lentiviral vector preparation

The lentiviral plasmid engineering started with a preparation of the cloning vector (pCW-X) where the different inserts were cloned (Fig. S1). The pCW-X was a gift from Eric Lander & David Sabatini (Addgene plasmid #50661). This vector was digested with the restriction enzymes NheI and BamHI which released X cloning DNA sequence. After digestion with these two restriction enzymes, pCW vector was dephosphorylated.

b. Subcloning of mCherry and Luciferase into pcDNA ENTR BP mammalian expression vector

mCherry and luciferase coding sequences were amplified with Thermo Scientific Phusion High-Fidelity DNA Polymerase, from plasmids available in the laboratory, using the primers PR10/PR11 and PR12/PR13 (Table S1), respectively. PR10/PR11 introduce NotI and EcoRV restriction sites on the 5'-end and 3'-end of mCherry, respectively. PR12/PR13 introduce NotI and SmaII restriction sites on the 5'-end and 3'-end of Luciferase, respectively.

Using NotI/EcoRV and NotI/SmaII restriction enzymes, mCherry and Luciferase were respectively subcloned into pcDNA ENTR BP vector, previously digested with NotI and EcoRV restriction enzymes and dephosphorylated (Fig. S2). EcoRV and SmaII restriction enzymes cut symmetrically, leaving blunt ends and no single strand DNA overhangs. This characteristic, in this specific case, allowed to ligate Luciferase 3’-end digested with SmaI to the vector digested with EcoRV.

To confirm the constructions, PCR colony screening were performed with primers PR14/PR15 (Table S1) which amplify a product about 750 bps or 1750 bps, only present in bacterial colonies

transformed with the right construction, confirming the presence of mCherry or Luciferase, respectively.

c. **Subcloning of T2A into pcDNA ENTR BP-mCherry and pcDNA ENTR BP-Luciferase**

The T2A coding sequence was synthetized by GeneCust with BamHI and NotI restriction sites on the 5'-end and 3'-end, respectively. Using these two restriction enzymes, T2A sequence was subcloned upstream the reporter gene into pcDNA ENTR BP mCherry and pcDNA ENTR BP Luciferase vectors, mentioned above (Fig. S2). Construction confirmation was performed by PCR colony screening with primers PR16/PR15 (Table S1). Only positive bacterial colonies with the correct construction amplify a product of about 830 bps or 1830 bps, confirming the presence of T2A, respectively cloned upstream mCherry or luciferase, with the right orientation.

From these plasmids, T2A-mCherry and T2A-Luciferase fragments were amplified with introduction of new restriction sites. For the first, primers PR16 and PR17 (Table S1), which introduces BglII restriction site on mCherry 3'-end, were used. The latter was amplified using primers PR16 and PR18 (Table S1), which introduce restriction site for BglII enzyme on Luciferase 3'-end. Afterwards, the PCR products of these amplifications (about 720 bps or 1720 bps, respectively) were digested with the restriction enzymes BamHI and BglII to prepare the following cloning into the final lentiviral plasmid.

d. Human and rat nucleolin cloning into pCW

Human NCL and rat Ncl coding sequences, previously amplified as mentioned, were digested with NheI and BamHI and ligated to the pCW lentiviral vector previously prepared, as described above (Fig. S3). The constructions (pCW-hNCL and pCW-rNcl) were confirmed by PCR colony screening with primers PR1(for hNCL)/PR3(for rNcl) and PR9 (Table S1). Only bacterial colonies successfully transformed with the right constructions amplify a product of about 2662 bps, confirming the cloning of hNCL or rNcl with the right orientation downstream TRE promoter and upstream hPGK promoter. Further confirmation was obtained by enzymatic restriction through digestion with NheI and BamHI of plasmid DNA isolated from the positive bacterium, leading to the release of hNCL/rNcl from the plasmid.

e. T2A-mCherry and T2A-Luciferase cloning into pCW-hNCL or pCW-rNcl

To obtain the final constructions, T2A-mCherry, from pcDNA ENTR BP-T2A-mCherry, digested with BamHI and BglII as mentioned, was cloned into the pCW-hNCL or pCW-rNcl, previously digested with BamHI. Likewise, T2A-Luciferase, from pcDNA ENTR BP-T2A-Luciferase, digested with BamHI and BglII, was cloned into pCW-hNCL or pCW-rNcl (Fig. S3).

These pair of enzymes (BamHI and BglII), among others, presented a challenge in terms of cloning strategy as their restriction sites were compatible cohesive ends. Thus, there was a 50%

probability of wrong orientation of the insert upon ligation, therefore increasing the need for orientation tests. Accordingly, the constructions (Fig. 3.4) and orientation of T2A-mCherry/T2A-Luciferase were confirmed by PCR colony screenings with primers PR16/PR9 (Table S1). With this strategy, only bacterial colonies transformed with the proper construction (adequate orientation of T2A-mCherry/T2A-Luciferase between hNCL/rNcl and hPGK promoter) amplified a PCR product of about 1300 bps or 2300 bps, confirming the cloning of T2A-mCherry or T2A-Luciferase, respectively.

3.1.3 Lentiviruses production

Lentiviral plasmids (pLKO.1 anti-NCL shRNA, pLKO.1 control shRNA, pCW-hNCL-T2A-mCherry, pCW-rNcl-T2A-mCherry, pCW-hNCL-T2A-Luciferase and pCW-rNcl-T2A-Luciferase) were purified as described in Chapter 3.1.1 j). Lentiviruses production was performed by transient co-transfection. STAR-RDpro-HV#1 cells (ECACC 04072114), cultured with DMEM High Glucose (BioWest, France) at 50-80% confluence in 25 cm^2 polylisine-coated flask, were transfected with the lentiviral plasmid constructions combined with pMD2.G and psPAX2, at a molar proportion of 1:1:1, using jetPrime transfection reagent (Polyplus transfection, France). pMD2.G and psPAX2 (schematic representation in Fig. S4, in Appendix), gifts from Didier Trono (Addgene plasmid #12259 and #12269, respectively), are essential accessory lentiviral plasmids as they are VSV-G envelope expressing and packaging plasmids, respectively.

Two days after co-transfection, STAR-RDpro-HV#1 cell line released the produced lentiviral particles by exocytosis. Supernatants containing the lentivirus were collected and centrifuged to clean debris. Lentiviral production was evaluated by transduction and selection in easily transducible 293A cells (Invitrogen, USA).

3.2 Cell culture

MDA-MB-231 and MCF-7 breast cancer cell lines were acquired from ATCC (Virginia, USA) and cultured in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% (v/v) of heat-inactivated FBS (BioWest, France), 100 U/mL penicillin (BioWest, France), 100 µg/mL streptomycin (BioWest, France) and maintained at 37°C in a 5% CO_2 atmosphere. MDA-MB-231 cell line is a triple negative breast cancer cell line (not presenting progesterone and oestrogen receptors as well as HER2 expression) with a claudin-like phenotype (Prat & Perou, 2010). MCF-7 cell line represents the less aggressive luminal A epithelial phenotype of breast cancer and is oestrogen receptor positive (Soule *et al.*, 1973).

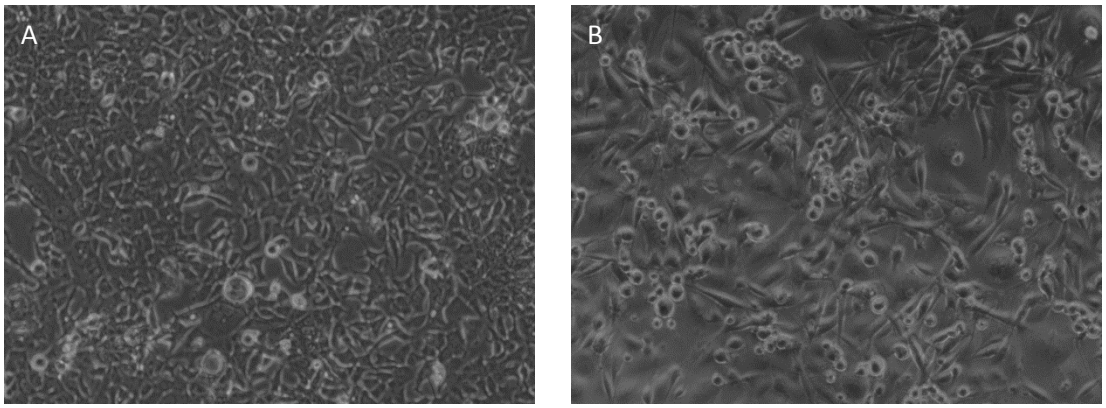


Figure 3.6- Bright field microscopic images of MCF-7 (A) and MDA-MB-231 (B) cell lines acquired at the magnification 10X on an inverted microscope.

STAR-RDpro-HV#1 cell line (ECACC 04072114) and 293A from ATCC (Virginia, USA) were cultured in DMEM High Glucose (BioWest, France) supplemented with 10% (v/v) of heat-inactivated Fetal Bovine Serum (FBS) (BioWest, France), 100 U/mL penicillin (BioWest, France), 100 µg/mL streptomycin (BioWest, France), non-essential aminoacids (BioWest, France) and maintained at 37°C in a 5% CO_2 atmosphere.

Routine cell culture manipulations were made on Class II Biosafety Cell Culture Flow Chambers, using sterile techniques. Cell stocks were maintained in cryotubes at -80 ^{o}C or in a liquid nitrogen tank, resuspended in medium supplemented with 10% sterile DMSO.

Transduction of breast cancer cell lines and selection

For gene transduction, MDA-MB-231 and MCF-7 breast cancer cell lines were incubated during 3 days, at 37°C in a 5% CO_2 atmosphere, with the lentiviral supernatants containing the lentiviruses, in RPMI medium (see Chapter 3.2) supplemented with 16 µM of polybrene to increase the efficiency of transduction.

All lentiviral plasmids used to produce the lentiviruses had a PuroR, as mentioned above, enabling the selection of transduced cells by adding puromycin (toxic to non-transduced cells) to the culture medium. Aiming at obtaining highly homogeneous cell cultures, three days after

transduction, puromycin was added to the cell culture medium in a final concentration of 18.4 µM for, at least, two weeks and then every three splits.

An additional selection was performed in cells transduced with hNCL-T2A-mCherry-enconding lentiviruses (overexpression lentiviruses). Upon puromycin selection, as described above, the cell cultures were seeded, at single cell, in a 15 cm culture dish (Corning, USA) for one week until visible at naked eye colonies were formed. Afterwards, the inducible system was activated upon culturing those cells in RPMI medium (see Chapter 3.2) supplemented with doxycycline (at the concentrations of 1.125 µM and 2.25 µM) until 6 days. Colonies expressing mCherry were identified by live cell imaging using an inverted microscope (See Chapter 3.3) and collected for further seeding in 24-well plates. These isolated cells were kept in culture with puromycin and doxycycline for further cell selection and mCherry expressing cells visualisation, respectively.

After cell expansion, cellular homogeneity in terms of mCherry expression (number of cells and intensity of expression) was assessed. Colonies with approximately 100% cells expressing mCherry were selected and expanded.

During all the experiments, control condition represents non-transduced cell lines, or *wild type*.

3.2.1 Cell culture Assays

a) Assessment of cell migration capacity

Cells grew in monolayer in 12-well plates until 90% confluence. Afterwards, a scratch was made in the monolayer, cells were washed with PBS to remove the detached cells and subsequently incubated with fresh culture medium at 37 ^{o}C with 5% CO_2 during the experiment time. Image acquisition of each wound overtime was performed using an inverted microscope (See Chapter 3.3). The first image was acquired immediately after scratch (T_0) followed by periodic acquisitions, until 12 h after T_0, within the same area of scratch. Migration area (in μm^2) was calculated for each wound by subtraction of the scratched area at the different time points to the scratched area at T_0.

b) Mammosphere formation assay

Mammosphere formation assay was used as a measure of stemness capability of the different conditions in each cell lines. Briefly, 5000 single cells were seeded in 2 mL Mammocult® Medium supplemented with 4 µg/mL of heparin and 0.5 µg/mL of hydrocortisone (StemCell Technologies, Canada) *per* well, in low-adhesion 6-well plates (Greiner, Austria). For 1st generation sphere formation, cells were maintained for 7 days. To assess self-renewal, 1st generation spheres were collected by centrifugation at 115 g for 5 min, and then dissociated

with 0.5% Trypsin (Sigma-Aldrich, USA). Five thousand mammosphere-derived single cells were then seeded as described above for 15 days. Mammosphere formation efficiency was assessed upon image acquisition (9 random images per well) using an inverted microscope (See Chapter 3.3). Mammosphere formation efficiency (%) was calculated by the formula:

$$\frac{\text{number of spheres}}{\text{number of total events}} \text{x } 100$$

*where *total events* are a sum of the number of mammospheres and single cells (5000 cells).

3.3 Live cell imaging

Bright field and fluorescence microscopy images of live cells were captured using an inverted Olympus IX51 microscope or Zeiss Axiovert 40 CFL microscope coupled to a High Resolution Color Camera or Zeiss MR3 Color Camera, respectively. Cell expressing mCherry were visualized by excitation of the cell culture using an excitation wavelength range of 530-585 nm. Images were processed and analysed using Fiji-ImageJ.

3.4 Proteins quantification by Western Blotting

Cells were lysed with a lysis buffer (150 mM NaCl, 50 mM Tris pH 7,4, 0,5% NP-40, 0,5% Triton X-100 and 0,5% Sodium deoxycholate) supplemented with proteases inhibitors (EDTA, EGTA, PMSF, Aprotinin, Bestatin, DTT, TPCK). Total protein of each sample was quantified using the Thermo Scientific Pierce BCA Protein Assay Kit, as described by the manufacturer. Protein samples were resolved on a 10% Acrilamide gel and transferred to nitrocellulose membranes. In order to prevent non-specific antibody binding, membranes were blocked in 2% Egg Albumin (MP Biomedicals, USA) during 4 h at room temperature with constant agitation. The membranes were then probed with primary antibodies (Table 3.1) overnight at 4°C, under stirring. Secondary antibodies (Table 3.1) conjugated with HRP (horseradish peroxidase) were incubated at room temperature for 2 h. To detect protein of interest, membranes were incubated with ECL Western Blotting Detecting Reagent (Amersham, UK) and signal resulting from the chemiluminescent reaction was acquired using Chemidoc Touch Imaging System (Bio-Rad Laboratories, USA).

Table 3.1 – List of antibodies used in Western Blotting assays.

Primary antibodies			
Name	**Host**	**Concentration**	**Origin**
Policlonal anti-nucleolin Ab	Goat	1:2500	Sicgene
Monoclonal anti-nucleolin Ab	Mouse	1:500	Invitrogen
Monoclonal anti-nucleolin Ab	Rabbit	1:10000	Abcam
Policlonal anti-mCherry Ab	Goat	1:2500	Sicgene
Policlonal anti-GAPDH Ab	Goat	1:2500	Sicgene
Secondary antibodies			
Anti-goat HRP conjugated	Donkey	1:30000	Bio-Rad
Anti-mouse HRP conjugated	Donkey	1:30000	Bio-Rad
Anti-rabbit HRP conjugated	Donkey	1:30000	Bio-Rad

3.1.1 Cloning techniques

These plasmids were assembled using sequential cloning strategies. Briefly, all the cloning was executed as follows: PCR amplification (from cDNA or an intermediary cloning plasmid), enzymatic restriction digestions, ligation reaction of the insert with the vector, transformation of competent cells with the resulting plasmid, screening colony PCR, plasmid DNA isolation and purification, confirmation of the construct by digestion with restriction enzymes and sequencing of the final plasmid.

a. Total RNA isolation

RBL (rat basophilic leukocytes) and MDA-MB-231 (human breast cancer cell line) total RNA was isolated using Qiazol buffer (Qiagen, USA) according to the manufacturer instruction. RNA was subsequently purified using RNAeasy mini Kit (Qiagen, USA).

b. Reverse transcription reaction

cDNA from RBL and MDA-MB-231 cells was produced by RT reaction using total RNA, isolated as described above, as a template. RT reaction was performed using 1-5 µg of total RNA template and SuperScript® II Reverse Transcriptase (Invitrogen, USA), as described by the manufacturer. Briefly, samples were denatured by heat-shock (10 min at 70 °C followed by 2 min on ice), annealing with Oligo(dN) primer (Invitrogen, USA) was performed at 42 °C during 3 min and the reverse transcription reaction was completed at 42 °C during 50 min.

c. Human and rat nucleolin coding sequence amplification by PCR

Human nucleolin and rat nucleolin coding sequences were amplified by PCR from MDA-MB-231 cDNA library (mentioned above) with the primers PR1/PR2 (described in Table S1, in Appendix) and from RBL cDNA library (mentioned above) with the primers PR3/PR4 (Table S1), respectively, using Thermo Scientific Phusion High-Fidelity DNA Polymerase. Amplification was performed on the conditions showed in Fig. 3.5: initial denaturation (2 min at 95 °C), followed by 32 cycles of denaturation (20 s at 95 °C), primers annealing (20 s at 58 °C), extension (variable time, depending on the fragment size, at 72 °C) and final elongation (10 min at 72 °C).

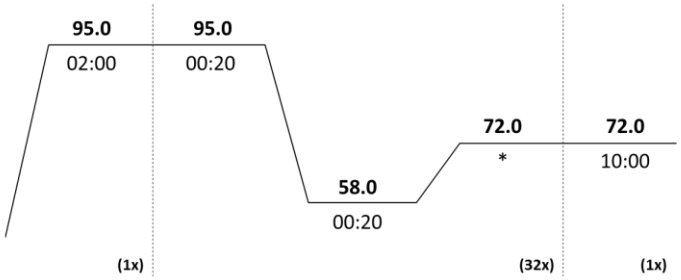


Figure 3.5– Diagram representing the settings used for PCR amplifications with Thermo Scientific Phusion High-Fidelity DNA Polymerase and Promega GoTaq DNA Polymerase. Initial denaturation (2 min at 95 °C), followed by 32 cycles of denaturation (20 s at 95 °C), primers annealing (20 s at 58 °C) and extension (at 72 °C) and a final elongation (10 min at 72 °C). * indicates variable time: 1 min *per* 3000 bps (Phusion High Fidelity DNA Polymerase) or 1 min *per* 1000 bps (GoTaq DNA Polymerase).

Reverse	CTCCTTAATGTCACGCACGAT

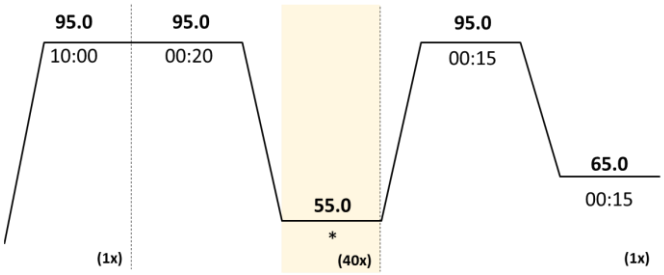


Figure 3.7 - Diagram of the programmable conditions used in RT-qPCR. Initial denaturation (10 min at 95 ºC), followed by 40 cycles of denaturation (20 s at 95 ºC) and primers annealing/extension (* s at 55 ºC) and a dissociation step (15 s at 95 ºC with a gradual decrease until 65 ºC). *indicates variable time from 15 seconds to 30 seconds, depending on the size of the amplicon resulting from each pair of primers.

Chapter 4 – Results and Discussion

4.1 Novel molecular tools to modulate nucleolin expression

To test the working hypothesis, in the context of the current state-of-the art, the development of novel molecular tools to modulate nucleolin expression was carried out.

A short hairpin-RNA (shRNA) lentiviral-based approach was used to knockdown nucleolin, as described in Chapter 3.1A. Anti-NCL shRNA sequences are described in Table 4.1 and Fig. 3.3.

Aiming at overexpressing nucleolin in breast cancer cell lines, hNCL coding sequence was cloned into pCW lentiviral vector, as described in Chapter 3.1.2 and Fig. S5A and B. Two reporter strategies were developed: one relying on the intrinsic mCherry fluorescence (orange/red) upon excitation at 587 nm; and another based on the ability of the luciferase enzyme to act on a substrate (such as luciferin) and generate visible light in the process.

A system enabling the rescue of human nucleolin knockdown, introduced by the expression of anti-NCL shRNA, was prepared by cloning rNcl coding sequence into the same pCW lentiviral vectors, with the two reporter genes, as described in Chapter 3.1B and Fig. S5C and D. BLAST nucleotide analysis of the two transcripts enabled the comparison of the sites targeted by the anti-hNCL shRNA sequences (Table 4.1). ShRNA sequences 1, 2 and 3 targeted both hNCL and rNcl coding sequences and the shRNA sequence 4 is specific for hNCL 3'UTR. As shown in Table 4.1, the target sequences of shRNA 1 and 3 are the same in both species and shRNA target sequence 2, in the rat transcript, only differs from the human transcript in 3 bases (in red in Table 4.1). This is in line with the fact that nucleolin is a protein well conserved among species, especially in eukaryotes (Ginisty *et al.*, 1999). Upon identification of the anti-NCL shRNA efficiently knocking down human nucleolin it would be necessary to perform mutagenesis in the rat nucleolin coding sequences being targeted by the shRNA, to induce resistance to the knockdown induced by the shRNA.

Table 4.1 – Anti-NCL shRNA target sequences in human and rat nucleolin coding sequences.

	shRNA target sequence	Human nucleolin	Rat nucleolin
1	AGTAAAGGGATTGCTTATATT	AGTAAAGGGATTGCTTATATT	AGTAAAGGGATTGCTTATATT
2	CGGTGAAATTGATGGAAATAA	CGGTGAAATTGATGGAAATAA	TGGAGAAATTGATGGAAACAA
3	GCACTTGGAGTGGTGAATCAA	GCACTTGGAGTGGTGAATCAA	GCACTTGGAGTGGTGAATCAA
4	CCTTGGAAATCCGTCTAGTTA (3'UTR)	CCTTGGAAATCCGTCTAGTTA	-

Following the cloning strategies described in detail in Chapter 3.1.2, the lentiviral plasmids coding nucleolin were constructed and plasmid DNA sequenced. pCW lentiviral vector, the

cloning vector for the sequences of interest, was previously sequenced (entire sequence represented in Fig. S6, in Appendix). pCW-hNCL-T2A-mCherry, pCW-rNcl-T2A-mCherry, pCW-hNCL-T2A-Luciferase and pCW-rNcl-T2A-Luciferase plasmids were sequenced with PR19 and PR9 (Table S1, in Appendix), primers annealing in the pCW vector, to confirm the insertion of the nucleolin coding sequences and mCherry/Luciferase coding sequences, respectively. Furthermore, confirming the placement of the correct restriction sites and the inserts, in the correct reading frame.

To exclude any possible mutations in the nucleolin coding sequences, which could lead to the translation into an incorrect form of nucleolin protein, the hNCL coding sequence was sequenced using PR1 and PR2 (Table S1) and rNcl coding sequence with PR3 and PR4 (Table S1). T2A sequence downstream mCherry/Luciferase was likewise confirmed by sequence with PR16. mCherry and Luciferase were sequenced as well, using PR7 and PR8 (mCherry) and PR5 and PR18 (Luciferase), to confirm that the correct coding cDNA sequence was cloned.

Combining the different sequencing results for each plasmid DNA, it was possible to confirm the expression cassette construction in the four plasmids (Fig. 4.1 and complete representation of the plasmids in Fig. S6, in Appendix).

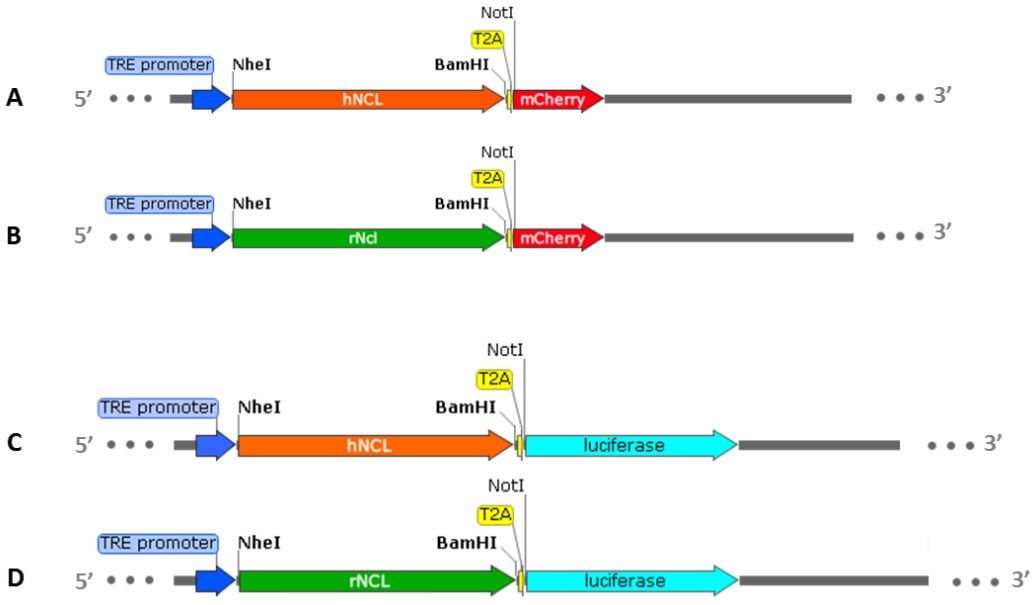


Figure 4.1– Schematic representation of the results obtained by sequencing the lentiviral plasmids aiming at nucleolin overexpression. (A), (B), (C) and (D) represent the sequence of pCW-hNCL-T2A-mCherry, pCW-rNcl-T2A-mCherry, pCW-hNCL-T2A-Luciferase and pCW-rNcl-T2A-Luciferase aiming at overexpression human and rat nucleolin (hNCL and rNcl, respectively) with mCherry and luciferase as reporter genes. According to our predictions, sequencing identified TRE promoter downstream of NheI restriction site in all the plasmids. Following NheI site, hNCL (for A and C) and rNcl (for B and D) coding sequences, with BamHI restriction site at 3'-end, were identified. It was confirmed the presence of T2A sequence, upstream BamHI site and downstream NotI restriction site. mCherry (for A and B) and luciferase (for C and D) sequences were identified following T2A sequence.

Upon confirmation of the constructions, all the four lentiviral vectors were used to produce the respective lentiviruses, as described in Chapter 3.1.3. hNCL-T2A-mCherry-encoding lentiviruses were transduced in MCF-7 and MDA-MB-231 breast cancer cell lines for *in vitro* studies. rNcl-T2A-mCherry-encoding lentiviruses were stored and ready to perform the rescue of hNCL knockdown *in vitro*. hNCL-T2A-Luciferase and rNcl-T2A-Luciferase-encoding lentiviruses were prepared to transduce breast cancer cells aiming at establishing, in the future, the corresponding animal xenografts.

4.2 Validation of the lentiviral systems in breast cancer cell lines

To correctly use the molecular systems described to knockdown and overexpress nucleolin, validation of their function as predicted, was required. Practical questions and experimental issues needed to be answered, to unravel, in the future, functional questions about nucleolin involvement in carcinogenesis as well as in anti-cancer therapies.

4.2.1 Validation of nucleolin knockdown induced by shRNA

Upon transduction of MCF-7 and MDA-MB-231 breast cancer cell lines with the different anti-hNCL shRNAs and antibiotic selection with puromycin, changes in nucleolin expression were assessed. Nucleolin protein quantification by western blot analysis (Fig. 4.2) demonstrated the high knockdown efficiency of shRNA sequence 4 (targeting the 3'UTR), lowering the nucleolin levels to 15.7% and 28.0% in MCF-7 and MDA-MB-231 cell lines, respectively (Fig. 4.2A)

Transient silencing of nucleolin expression using transfection of shRNA or siRNA-expressing plasmids is being used by other research groups to study the effect of nucleolin in breast cancer (Gaume *et al.*, 2011; Pichiorri *et al.*, 2013). Nevertheless, this approach is limited by the short-term expression of silencing tools. Thus, the results presented in Fig. 4.2 supported that long-term nucleolin knockdown can be successfully achieved.

Nucleolin expression levels were quantified relative to GAPDH and normalized to the non-transduced cells (control condition). Importantly, although nucleolin modulates expression of a high range of proteins, GAPDH expression is not altered (Abdelmohsen *et al.*, 2011) and thus fulfilling the requirements as housekeeping gene.

Furthermore, in MCF-7 cell line, the anti-NCL shRNA 1, 2 and 3 increased nucleolin protein levels (Fig. 4.2A, on the left), instead of decreasing them, which was unexpected as the sequences used were previously tested for nucleolin knockdown (Sigma-Aldrich, USA). One possible explanation could rely on a feedback response arising from nucleolin silencing outside 3'UTR, prompting cells to increase the production of nucleolin at a higher rate. Moreover, as lentiviruses have random integration in the genome of host-cells (Pluta *et al.*, 2009), it was possible that those shRNAs were integrated in less transcribed regions, being translated in lower extent. It was also reasonable to attribute this unexpected result to the different responses that each cell type manifests to the same stimuli. In fact, in the MDA-MB-231 cell line, the response to the shRNAs was slightly different (Fig. 4.2A), demonstrating, for example, decreased levels of nucleolin with the Control shRNA and anti-NCL shRNA 2, although without major differences.

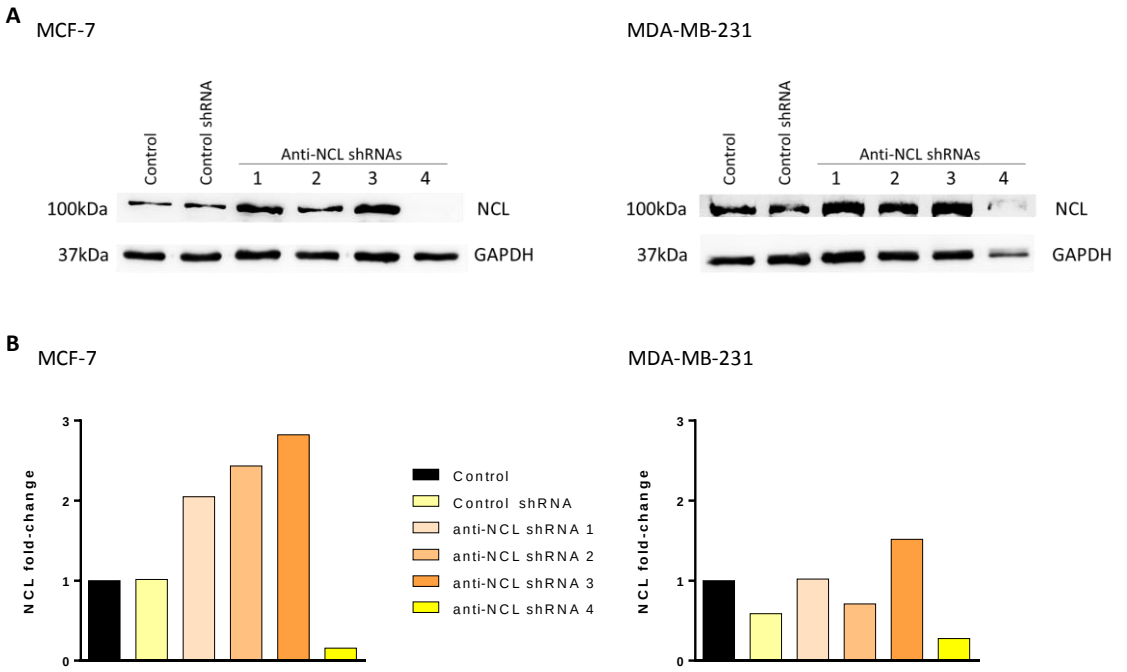


Figure 4.2 – Nucleolin knockdown by anti-NCL shRNA sequence 4 for both MCF-7 and MDA-MB-231 breast cancer cells. (A) Representative nucleolin identification by Western Blot in MCF-7 and MDA-MB-231 cells. Protein extracts were collected after the period of transduction (3 days) and puromycin selection (during 2 weeks). (B) Relative abundance of nucleolin following transduction of cells with anti-NCL shRNAs and respective control shRNA, upon normalisation against the housekeeping gene GAPDH and Control condition.

It was observed (data not shown) that cells maintained in culture without puromycin (antibiotic used for selection) led to a loss of the nucleolin knockdown. Based on this information, the protocol for selection was extended to, at least, 2 weeks (detailed description in Chapter 3.2), to eliminate non-transduced cells. It was hypothesized that the few cells in culture without nucleolin knockdown could overlap the cells with knockdown, as nucleolin is a multifunctional protein with important roles in cell proliferation (reviewed in Chapter 1).

Nucleolin knockdown is expected to reduce cell surface nucleolin, as described before (Hovanessian *et al.*, 2010). Moreover, this can be tested by using an immunocytochemistry protocol for nucleolin and its detection in a high-resolution microscope. Alternatively, a different approach would be to perform cell fractionation and use western blot to quantify nucleolin protein in the different cell fractions (nucleus, cytoplasm and membrane).

Overall, both cell lines transduced with anti-NCL shRNA 4 were chosen to represent nucleolin knockdown, from now on designated as “anti-NCL shRNA” condition.

4.2.2 Validation of nucleolin overexpression using lentiviral TetOn system in 293A cells

The strategy to overexpress hNCL was based on a TetOn-dependent system, induced by the presence of doxycycline. This was first validated with 293A cells, upon transduction with hNCL-T2A-mCherry-encoding lentiviruses. Upon 5 days of selection with puromycin, doxycycline was

added to the culture medium, for 1 week, to activate the TetOn system. Protein extracts from these cells (and non-transduced cells), were collected afterwards. Western blot analysis demonstrated the expression of the reporter gene, mCherry, (Fig. 4.3A) as well as a 4-fold increase in nucleolin protein levels (Fig. 4.3B). Data on mCherry demonstrated a band of 30 kDa corresponding to the intact protein and two fragments with lower molecular weight (Fig. 4.3A), likely corresponding to mCherry degradation products, which may occur after long-term expression.

Overall, these results validated the chosen methodology to enable inducible nucleolin overexpression system with 293A cells.

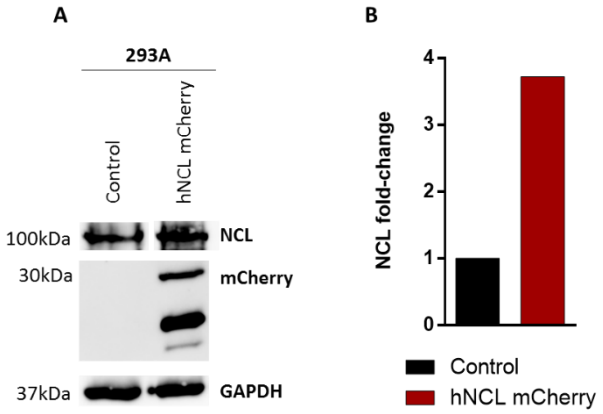


Figure 4.3 – TetOn system validation in 293A cells transduced with hNCL-T2A-mCherry-encoding lentiviruses. (A) Nucleolin, mCherry and GAPDH detection by western blot in 293A control cells (non-transduced cells) and cells transduced with hNCL-T2A-mCherry-encoding lentiviruses upon TetOn activation. Protein extracts of 293A hNCL-mCherry cells were collected after 5 days of antibiotic selection and 1 week of culture in the presence of 2,2 µM of DOX. (B) Quantification of nucleolin expression in 293A cells transduced with hNCL-T2A-mCherry-encoding lentiviruses, relative to the control, upon normalisation against the housekeeping gene GAPDH.

4.2.3 Validation of nucleolin overexpression using lentiviral TetOn system in breast cancer cell lines

Following the previous results, the developed methodology for inducible nucleolin expression was further tested in MCF-7 and MDA-MB-231 breast cancer cell lines.

Following puromycin selection of transduced MCF-7 and MDA-MB-231 cells, evaluation of TetOn system activation by doxycycline was required. It was demonstrated that doxycycline may present a dose-dependent cytotoxic effect in some cell lines (Xie *et al.*, 2008). For those cell lines the use of concentrations below 9 µM (Xie *et al.*, 2008) and with the ability to induce TetOn activation was required, as reported by the supplier of the lentiviral system (Clontech Laboratories, USA). Accordingly, two doxycycline concentrations (1.125 µM and 2.25 µM in the culture medium) were tested.

Live fluorescence microscopy images, enabled the observation of mCherry-expressing cells in both cell lines, confirming TetOn activation (Fig. 4.4). Interestingly, mCherry-expressing cells were observed for the first time at different time-points and doxycycline concentration, for both cell lines (Fig. 4.4). mCherry-expressing MCF-7 cells were evident after 5 and 3 days in culture, in the presence of 1.125 µM and 2.25 µM of DOX, respectively (Fig. 4.4, top panel). mCherry expression by MDA-MB-231 cells started slightly earlier (after 2 days) for both DOX concentrations tested (Fig. 4.4, bottom panel). From these results, the concentration of doxycycline selected for future experiments was 2.25 µM.

Additionally, collected images demonstrated a clear heterogeneity within the cell cultures in terms of expression of the transduced reporter protein. In fact, different cells within the same culture can integrate lentiviral DNA in different regions of the genome, thus contributing to distinct expression efficiencies of the same protein (Pluta *et al.*, 2009).

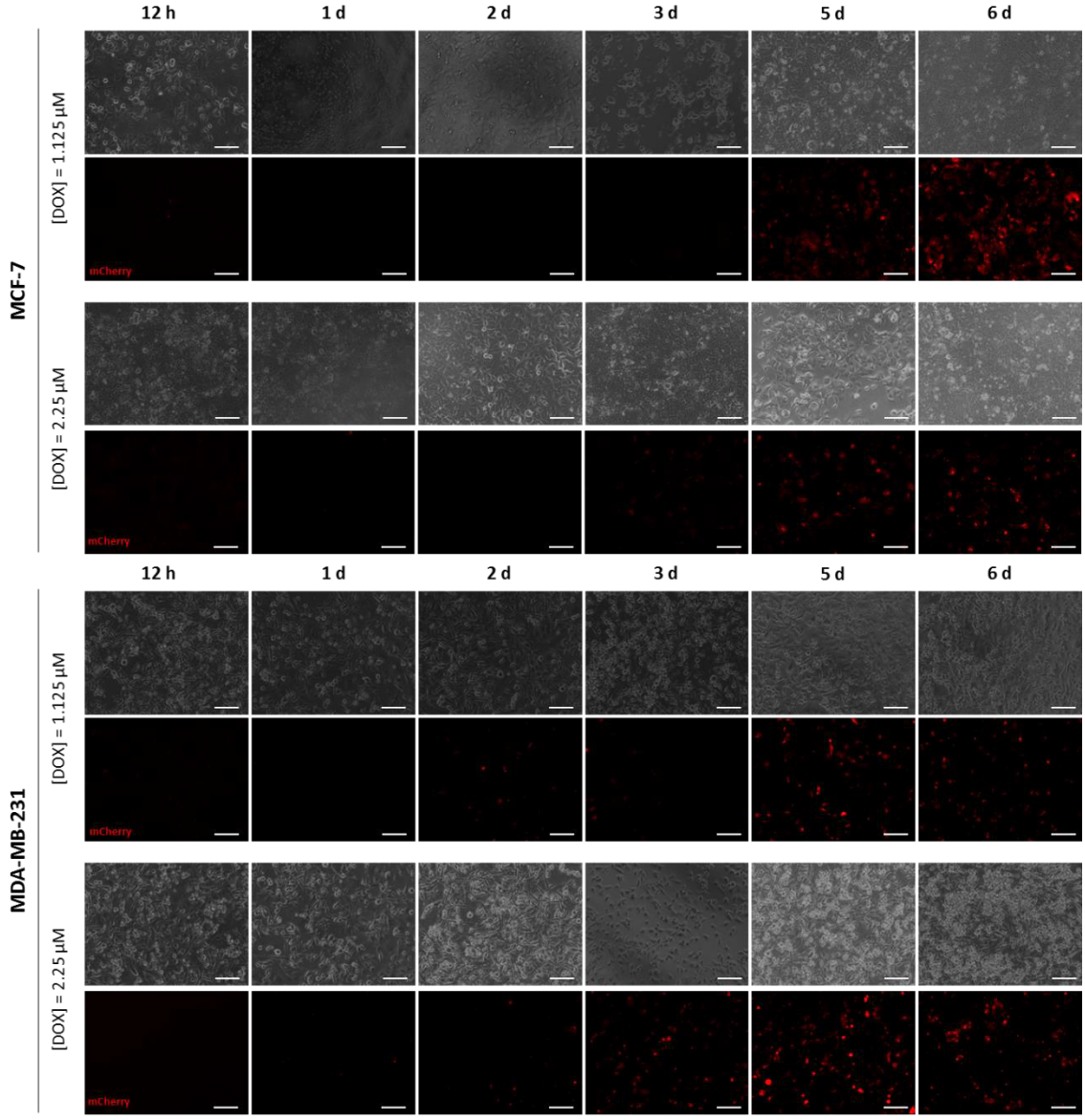


Figure 4.4 – Evaluation of TetOn system activation by doxycycline in MCF-7 and MDA-MB-231 breast cancer cell lines transduced with hNCL-T2A-mCherry-encoding lentiviruses. Bright field and fluorescence microscopy images (mCherry in red) of both cell lines, at different time points, with 1.125 µM and 2.25 µM of DOX concentrations in the culture medium. Doxycycline was added to the culture medium following cells transduction and selection. Scale bar = 100 µm.

Aiming at having a more homogeneous nucleolin-overexpressing breast cancer cell culture, upon TetOn activation, both transduced MCF-7 and MDA-MB-231 cell lines were submitted to a colony selection based on the mCherry expression, as described in Chapter 3.2. Those displaying the highest fluorescence intensity and the highest percentage of cells expressing mCherry were selected for isolation (Fig. 4.5A). From all the isolated colonies, one from each cell line was selected to represent nucleolin overexpression, from now on mentioned only as "hNCL" (Fig. 4.5B).

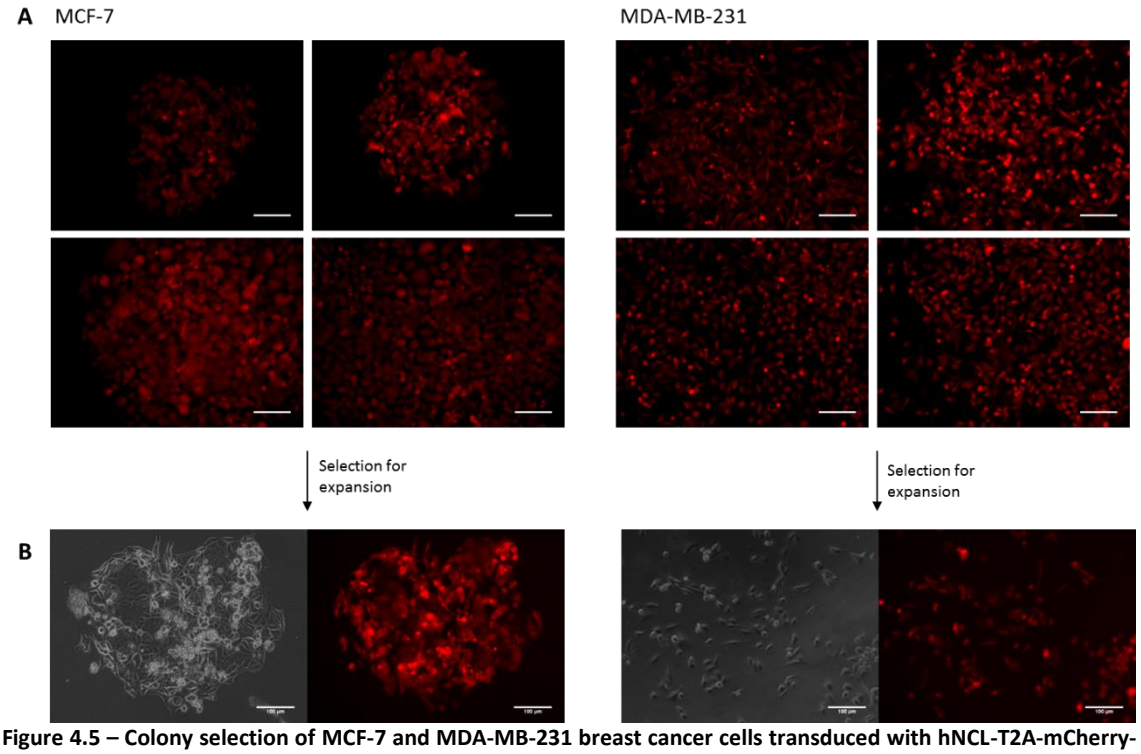


Figure 4.5 – Colony selection of MCF-7 and MDA-MB-231 breast cancer cells transduced with hNCL-T2A-mCherry-encoding lentiviruses. (A) Typical example of live fluorescence microscopy images of isolated colonies showing mCherry expression (in red). Seeded single cells, upon puromycin selection, were incubated in the presence of 2.25 µM of DOX in the culture medium during 6 days, followed by isolation of each colony formed. (B) Bright field and fluorescence microscopy live images of nucleolin-overexpressing MCF-7 and MDA-MB-231 selected colonies. Scale bar = 100 µm.

In the resulting hNCL MCF-7 and hNCL MDA-MB-231 cell cultures, response to the inducible system activation, in the presence of 2.25 µM of DOX in the culture medium, was further tested. In this respect, the percentage of cells expressing the reporter gene, mCherry, was assessed over time (Fig. 4.6). hNCL MCF-7 cell culture exhibited 40% of mCherry-expressing cells on day 1, followed by a gradual increase up to, approximately, 100% on day 5 (Fig. 4.6A). In hNCL MDA-

MB-231 cell culture, this process was slightly faster. Seventy percent of mCherry-expressing cells were already identified on day 1, reaching approximately 100% on day 3 (Fig. 4.6A). The same trend on the pattern of m-Cherry-expressing cells was confirmed by western blot (Fig. 4.7). As expected, hNCL MCF-7 and hNCL MDA-MB-231 cells in the absence of DOX, did not show mCherry expression nor increased nucleolin expression, relative to MCF-7 and MDA-MB-231 control cells, respectively, suggesting a non-leaky inducible system (Fig. 4.7). Upon activation of the TetOn system in the previous cells, nucleolin overexpression was observed by western blot analysis (Fig. 4.7A).

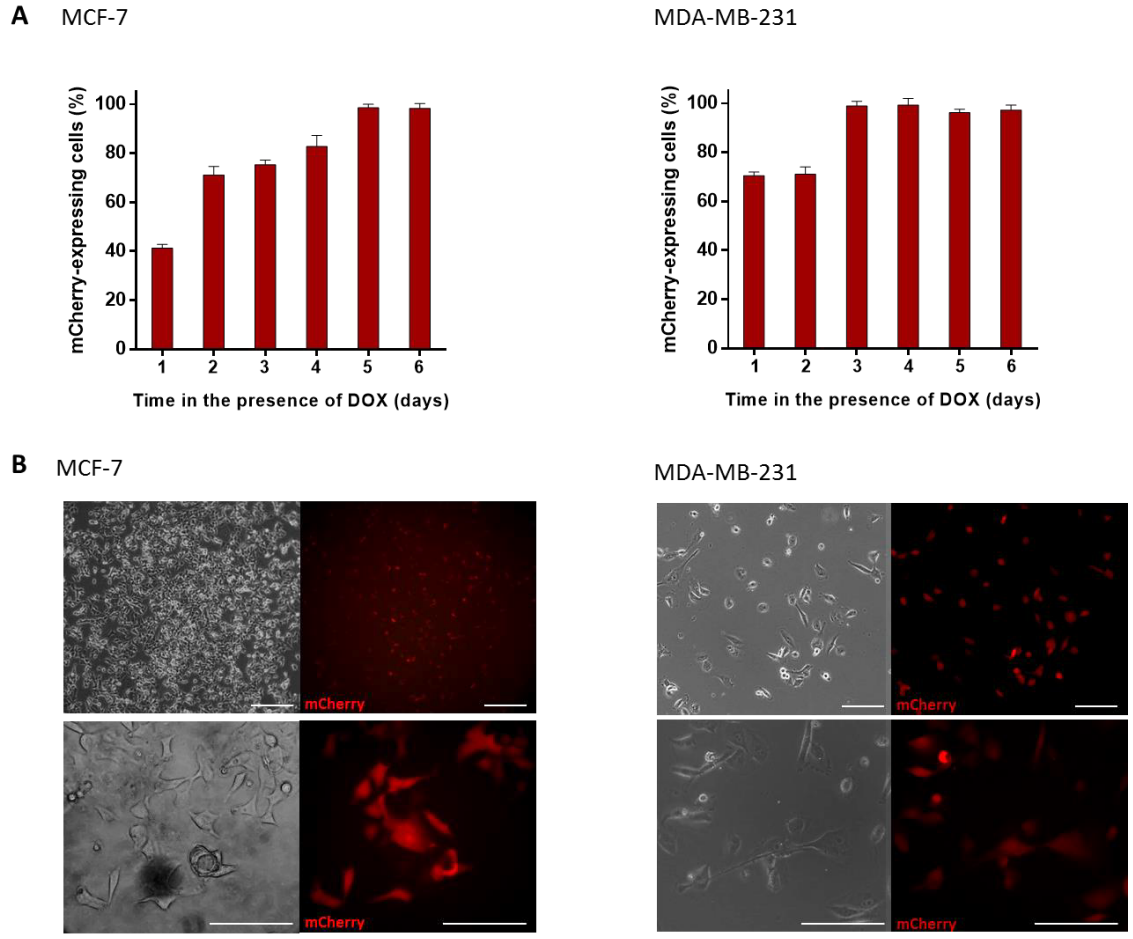


Figure 4.6 – TetOn system activation in hNCL MCF-7 and hNCL MDA-MB-231 selected colonies, following transduction with hNCL-T2A-mCherry-encoding lentivirus. (A) Percentage of mCherry-expressing cells, from the total number of cells, in both cell lines, upon incubation in the presence of 2.25 µM DOX in culture medium, at different time points. Data represent the mean ± SEM (n=2). (B) Typical example of bright field and fluorescence images of both cell lines expressing mCherry, cultured 1 day in the presence of 2.25 µM of DOX in culture medium. Scale bar = 100 µm.

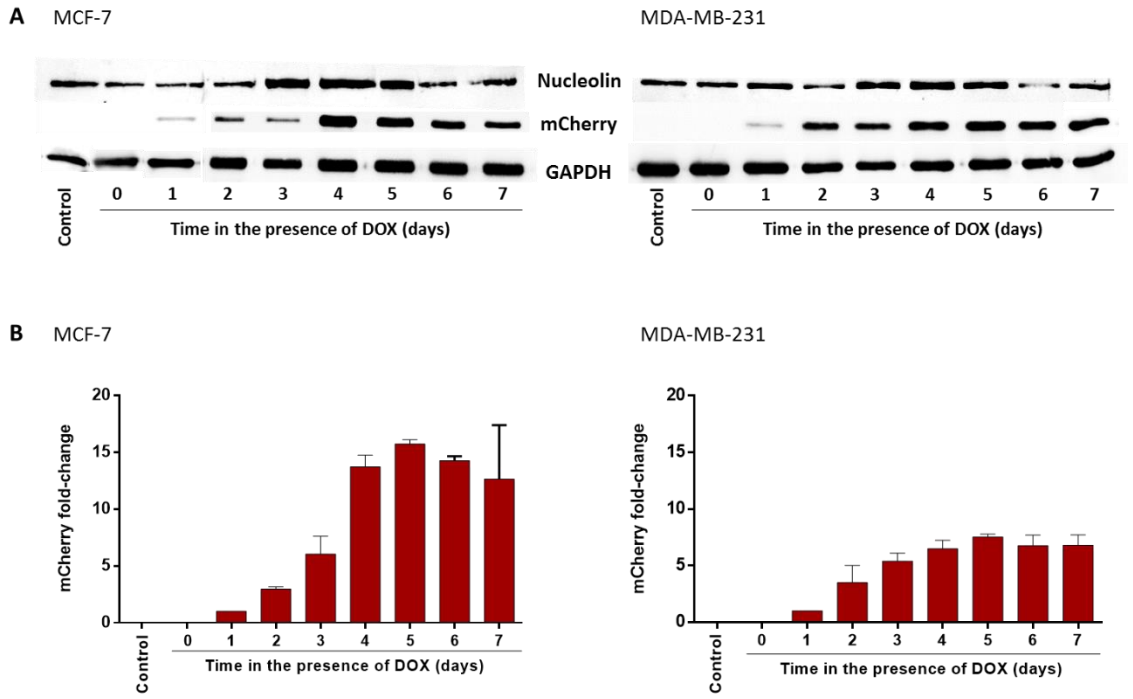


Figure 4.7 – Nucleolin and mCherry expression upon activation of the TetOn system in both hNCL MCF-7 and hNCL MDA-MB-231 selected colonies, following transduction with hNCL-T2A-mCherry-encoding lentiviruses. (A) Nucleolin and mCherry protein detection by western blot in cells transduced with hNCL-T2A-mCherry-encoding lentiviruses, in the absence or presence of 2.25 μM of DOX in the culture medium over time, for both cell lines. (B) mCherry protein expression levels in both cell lines, in non-transduced cells (control condition) or cells transduced with hNCL-T2A-mCherry-encoding lentiviruses, in the absence or presence of 2.25 μM of DOX in the culture medium, at different time points, relative to day 1. Data represent the mean ± SEM (n=3).

4.2.3 Impact of nucleolin modulation on its mRNA levels

The effect of nucleolin overexpression and knockdown systems at the nucleolin mRNA level in MCF-7 and MDA-MB-231 breast cancer cell lines, was further assessed by RT-qPCR (Fig. 4.8), relative to β-actin mRNA expression and non-transduced cells (control condition).

According to the data represented in Fig. 4.8, transduction with the lentiviruses was not interfering with nucleolin mRNA levels by itself. In both cell lines, transduction with control shRNA and hNCL, without activation of the inducible system ("hNCL without DOX"), did not change nucleolin mRNA levels in a significant manner, relative to non-transduced cells (Fig. 4.8).

In MCF-7 breast cancer cells transduced with anti-NCL shRNA, a marked decrease of nucleolin mRNA levels was observed (Fig. 4.8; p value<0.01), confirming the knockdown observed before by western blot in the same cell line (Fig. 4.2). The results for MCF-7 hNCL with TetOn system activation suggested that there was in fact a 2-fold increase of nucleolin mRNA levels, although not statistically significant when compared with non-transduced cells (Fig. 4.8).

Similar experiments performed with the MDA-MB-231 breast cancer cell line, demonstrated a 4-fold increase in nucleolin mRNA expression in MDA-MB-231 hNCL, upon activation of the TetOn system (Fig. 4.8; p<0.001). Transduction of MDA-MB-231 with anti-hNCL shRNA did not

manifest a decrease in nucleolin mRNA levels (Fig. 4.8), notwithstanding the evident downregulation at the protein level (Fig. 4.2). One possible explanation may rely on the possible anti-NCL shRNA blocking of the nucleolin mRNA translation, without degradation of mRNA, thus leading to accumulation of the latter (Holmes *et al.*, 2010). Alternatively, nucleolin knockdown at the protein level could trigger a feedback loop in this cell line, leading to increased transcription of the nucleolin gene, resulting in increased mRNA levels. This incongruent observation between the cell lines might be in line with the previous observations that different breast cancer cell lines have different genomic, transcriptional and biological features (Neve *et al.*, 2009; Sotiriou *et al.*, 2009).

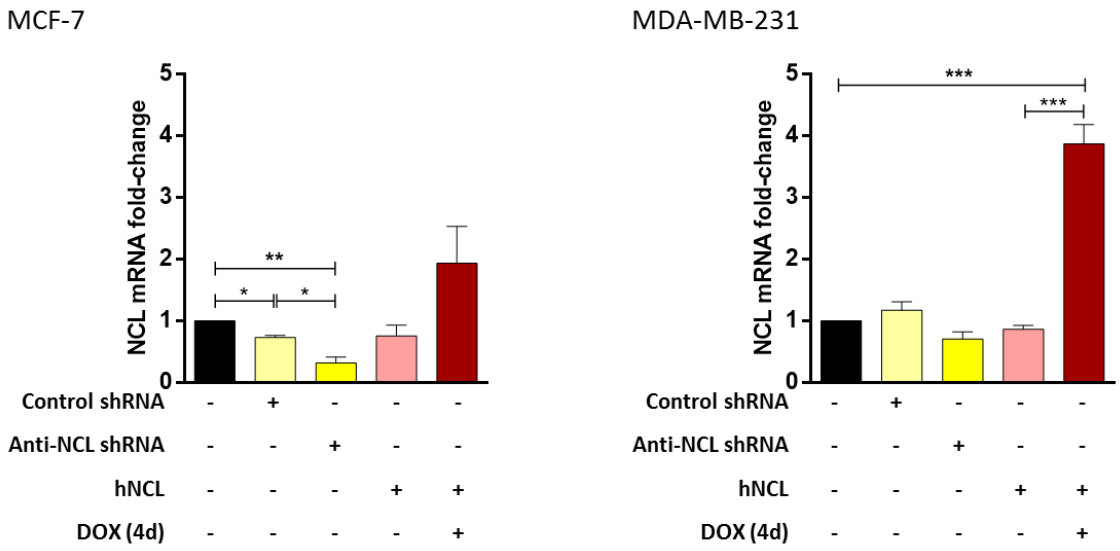


Figure 4.8 – Nucleolin mRNA levels in transduced MCF-7 and MDA-MB-231 breast cancer cells. Total RNA extracts of control shRNA and anti-NCL shRNA were collected, following transduction for 3 days and puromycin selection for 2 weeks. Total RNA extracts from hNCL MCF-7 and hNCL MDA-MB-231 cells were collected upon colony selection (hNCL without DOX). Total RNA extracts from nucleolin-overexpressing cells were collected 4 days upon 2.25 µM of DOX was added to the hNCL MCF-7 and hNCL MDA-MB-231 cells culture. Data represent the mean ± SEM (n=2-3; p value was calculated using *student* t-test relative to non-transduced cells (control condition); * p<0.05, **p<0.01 and ***p<0.001).

4.3 Impact of modulation of nucleolin expression on breast cancer cells phenotype

4.3.1 Stemness properties modulation

In addition to the high nucleolin protein expression in cancer cells, the mRNA levels of nucleolin and pluripotency markers (such as OCT4 and NANOG) tend to be upregulated in the putative CSC subset in both MCF-7 and MDA-MB-231 breast cancer cell lines, which display increased capacity to form mammospheres (Fonseca *et al.*, 2015).

Aiming at starting to address the question on whether nucleolin could be an oncogenic driver of breast cancer by modulating stemness properties, a mammosphere assay was performed using MCF-7 and MDA-MB-231 cells upon modulation of nucleolin expression (Fig. 4.9A and B). In MCF-7 cell line, neither knockdown or overexpression of nucleolin apparently had an impact on mammospheres formation efficiency (MFE) (Fig. 4.9B). In contrast, MDA-MB-231 nucleolin-overexpressing cells presented higher efficiency of mammosphere formation than non-transduced cells (Control) or cells transduced with hNCL-T2A-mCherry-encoding lentiviruses, in the absence of doxycycline (hNCL without DOX), for each mammosphere generation (Fig. 4.9B).

Results suggested important differences on the pattern of MFE among the cell lines tested (Fig. 4.9B). In the case of MCF-7 cells, this could rely, for example, on the loss of their self-renewal capacity after the 1st generation of mammospheres, which was preserved by MDA-MB-231 cells (Fig. 4.9B). Interestingly, these results suggested a different trend from those reported by Wang *et al.*, who observed a higher efficiency of mammosphere formation by MCF-7 cells than MDA-MB-231 cells (Wang *et al.*, 2014).

It has been reported that nucleolin decreased the levels of p53 protein (Ugrinova *et al.*, 2007), while the latter inhibited pluripotency and epithelial-to-mesenchymal transition (EMT) (reviewed by Voutsadakis *et al.*, 2015). In normal mammary and breast cancer cells, it has been reported that CSC express markers of EMT, suggesting that this event drives the generation of CSC (Mani *et al.*, 2008; Morel *et al.*, 2008). The enrichment in CSC within a cell population induces an increase in the mammosphere-forming ability of the population (Mani *et al.*, 2008). Hence, it would be expected that an increase in nucleolin expression level, would result in an enrichment of CSC within the breast cancer cells population and increase in mammospheres formation. The difference on the observed efficiency of mammosphere formation could be related with each of the breast cancer sub-types that each of the cell lines tested represents: the claudin-like triple negative breast cancer, as in the case of MDA-MB-231 cancer cells, and luminal A, in the case of MCF-7 breast cancer cell line (Prat *et al.*, 2010).

Overall, based on the observed differences on the MFE in MDA-MB-231 cells, as a function of the levels of nucleolin expression, it is suggested this protein may have an influence in the stem-like phenotype of the referred breast cancer cells.

To further unveil the possible involvement of nucleolin in the subset of putative CSC, it will be important to characterise this sub-population of cells in nucleolin-overexpressing and nucleolin-downregulated MDA-MB-231 and MCF-7 cells. In line with these experiments, further evaluation of the stem-cell like phenotype should be complemented with *in vivo* tumorigenic assays.

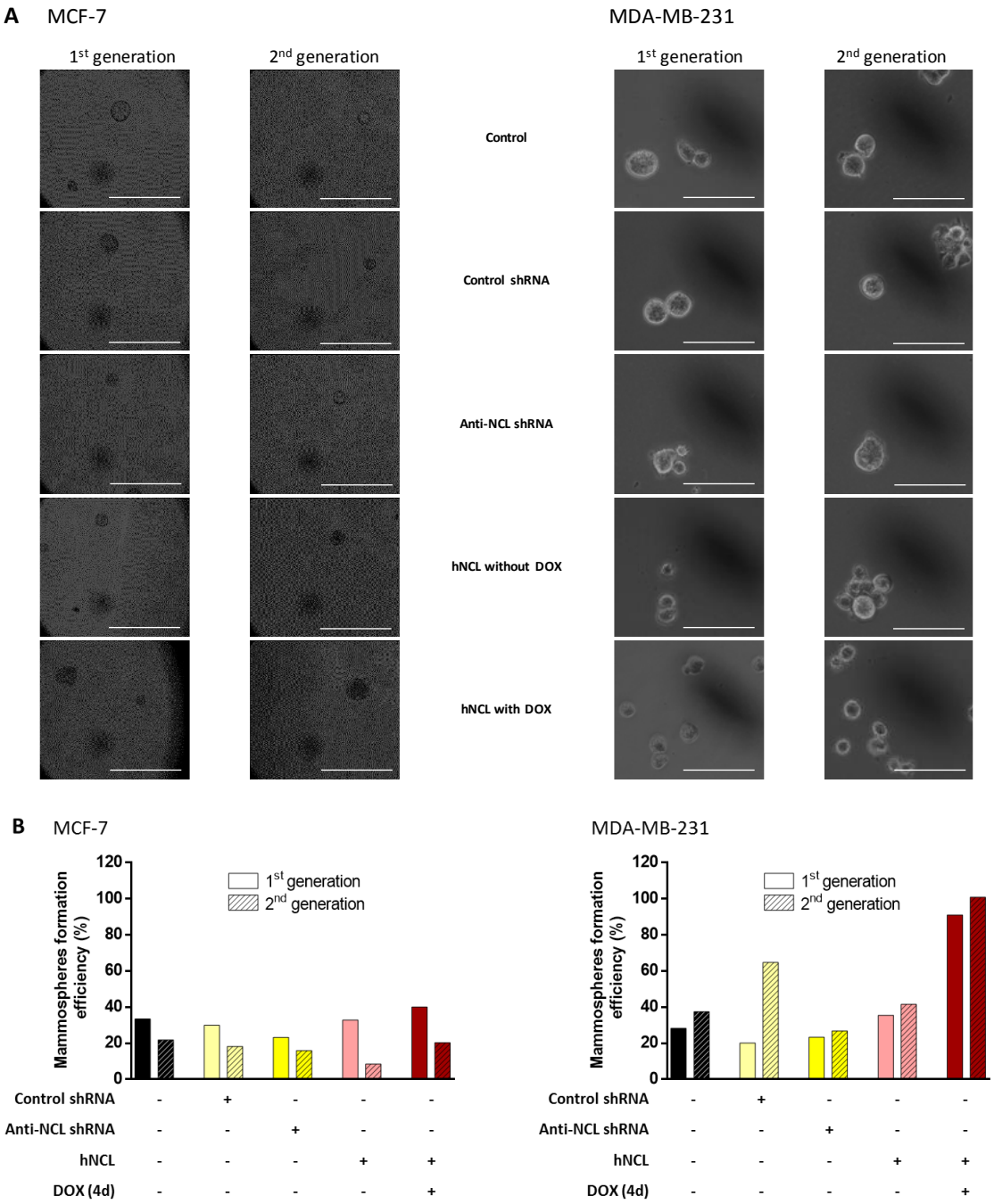


Figure 4.9 – Effect of nucleolin expression modulation on mammospheres formation in both MCF-7 and MDA-MB-231 breast cancer cell lines. Following single cell seeding in low adhesion culture conditions, efficiency (relative to non-transduced cells) of 1st generation mammospheres was assessed after 7 days, for both cell lines. Cells derived from the previous mammospheres were collected and seeded in single cell in the same culture conditions and efficiency of 2nd generation mammospheres was assessed after 14 days. (A) Representative images of 1st and 2nd generation mammospheres from MCF-7 and MDA-MB-231 cell lines are presented (scale bar = 50 µm). (B) Calculated mammospheres formation efficiency of 1st and 2nd generation for both breast cancer cell lines (n=1).

4.3.2 Migration properties of breast cancer cells

Cells with high tumorigenic, proliferating and metastatic potential have increased migration and invasion capacity (Bozzuto *et al.*, 2010). Accordingly, since nucleolin overexpression is associated with those features (reviewed in Chapter 1), it was important to understand whether modulation of nucleolin expression affects migration capacity of breast cancer cells.

The collected data demonstrated that nucleolin knockdown with an anti-NCL shRNA decreases migration capacity of MCF-7 and MDA-MB-231 cells by approximately 2-fold and 3.3-fold relative to non-transduced cells (control condition), respectively, 12 h after wound scratch (Fig. 4.10). These results corroborated experiments performed in other cancer cell types. For example, nucleolin knockdown decreased the migration capacity of the human melanoma A375 cell line (Bi *et al.*, 2013). Furthermore, functional inhibition of surface nucleolin decreased migration capacity of endothelial and glioma cells (Koutsioumpa *et al.*, 2013). These results were in line with the previous observation that nucleolin was involved in microtubules stability, affecting cells migration capacity and cell adhesion (Gaume *et al.*, 2016).

Interestingly, data demonstrated a higher migration capacity of MDA-MB-231 cells compared with MCF-7 cells (Fig. 4.10) confirming the mesenchymal phenotype of MDA-MB-231 cell line, which is characterized by being a high tumorigenic, motile and invasive cell line (Kubaisy *et al.*, 2016).

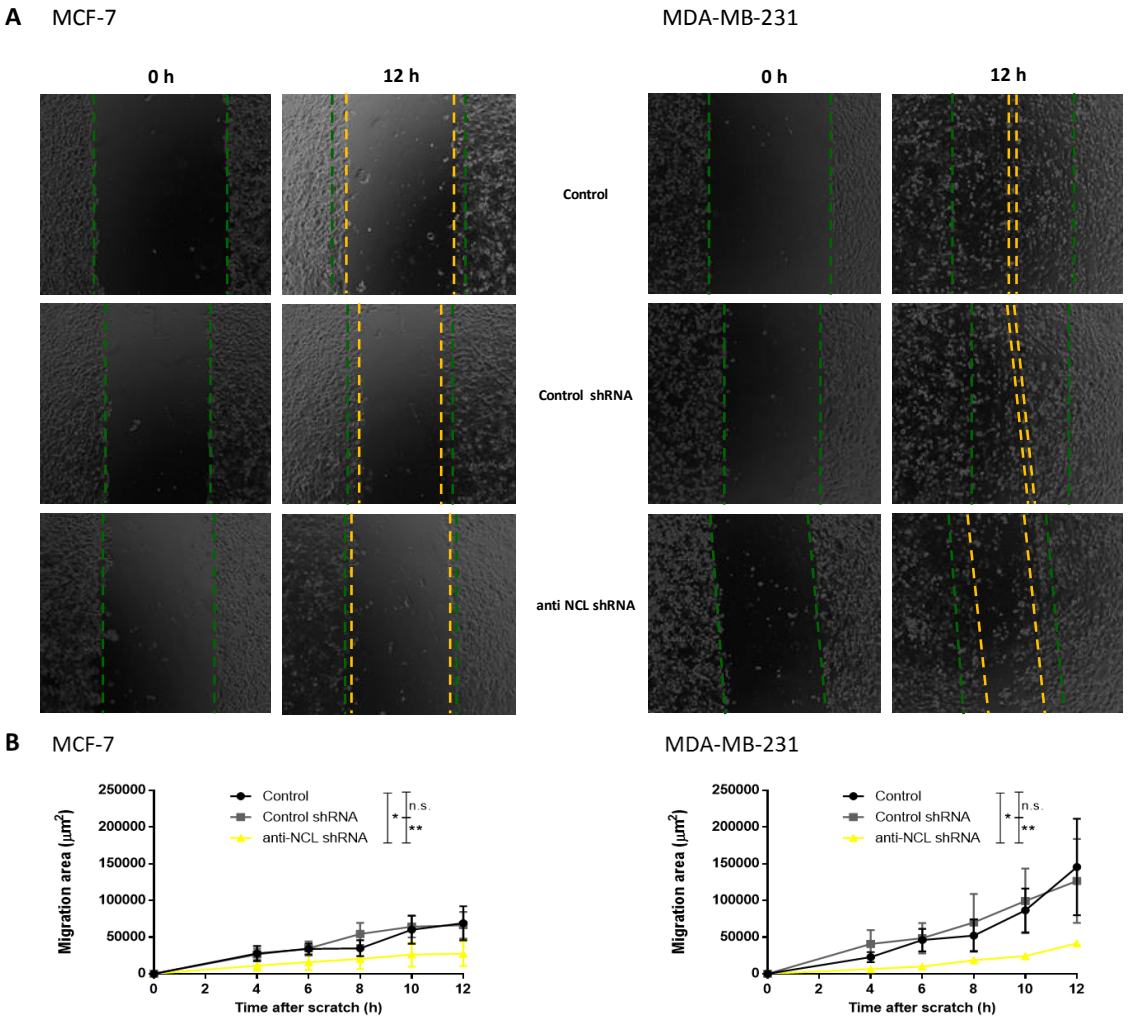


Figure 4.10 – Effect of nucleolin downregulation on the migration capacity of both MCF-7 and MDA-MB-231 breast cancer cell lines. Following a scratch in a monolayer of cells grown until 90% confluence, periodic images were acquired within the same area of scratch, until 12 h after T_0, in each experimental condition tested, with an inverted microscope. (A) Representative images of the wound healing assays in both MCF-7 and MDA-MB-231 breast cancer cell lines, before (0 h) and 12 h after scratch. Wound border was represented by green dashed lines (at time 0 h) or yellow dashed lines (12 h after scratch), for both MCF-7 or MDA-MB-231 cells. (B) Migration area after scratch (in μm^2) was quantified at each time point by subtraction of the scratched area at the different time points to the scratched area at T_0. Data represent the mean ± SEM (n=3-5; p value was calculated using paired *student t*-test; n.s. p>0.05, *p<0,05 and **p<0.01).

In contrast to the results from nucleolin downregulation, its overexpression did not present any impact in terms of migration capacity on both cell lines (Fig. 4.11). This might be due to the intrinsic enhanced migration capacity of breast cancer cell lines, particularly the MDA-MB-231 (Hughes *et al.*, 2008). Alternatively, as nucleolin regulates microtubule stability, it is likely that overexpression of nucleolin in an already saturated system, as cancer cells, has a much lower impact than its downregulation.

Overall, these results suggested that nucleolin is necessary for cell migration machinery, without being the key factor leading to increased migration capacity of cancer cells.

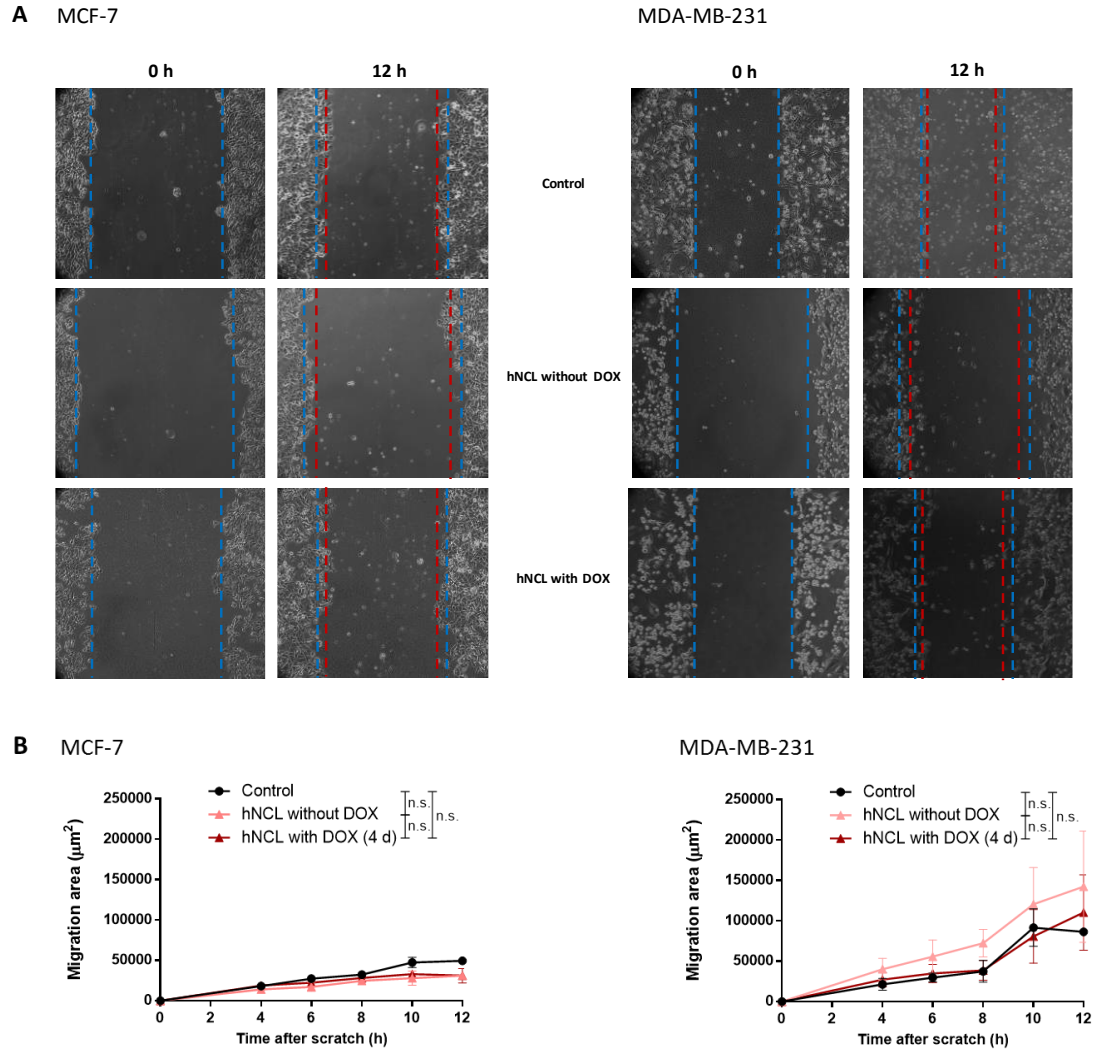


Figure 4.11 - Evaluation of nucleolin overexpression effect in the migration capacity of both MCF-7 and MDA-MB-231 breast cancer cell lines. Following a scratch in a monolayer of cells grown until 90% confluence, periodic images were acquired within the same area of scratch, until 12 h after T_0, in each experimental condition tested, with an inverted microscope. (A) Representative images of the wound healing assays in both MCF-7 and MDA-MB-231 breast cancer cell lines, before (0 h) and 12 h after scratch. Wound border was represented by blue dashed lines (at time 0 h) or red dashed lines (12 h after scratch) for both MCF-7 or MDA-MB-231 cells. (B) Migration area after scratch (in μm^2) was quantified at each time point by subtraction of the scratched area at the different time points to the scratched area at T_0. Data represent the mean ± SEM (n=3-5; p value was calculated using paired *student t*-test; n.s. p>0.05, *p<0,05 and **p<0.01).

Chapter 5 – Final remarks

Nucleolin multifunctionalities are being studied since it has been first discovered, more than 40 years ago (Orrick *et al.*, 1973; Archie *et al.*, 1994). Its involvement in many cellular processes makes it a very complex protein, especially when associated with complex diseases as cancer. In the urgency to counteract statistics of breast cancer diagnose, relapse and death, it is important to unravel the molecular mechanisms behind breast cancer and identify and validate new targets for anti-cancer therapy.

By the construction of stable breast cancer cell lines expressing different levels of nucleolin, this project provided important tools applicable to a myriad of future studies. These tools are expected to contribute to unravel the possible involvement of nucleolin in carcinogenesis and, more importantly, to validate it as a target for anti-cancer therapy.

Validation of the lentiviral-based approach used to knockdown nucleolin enabled to identify a shRNA sequence efficiently down regulating it. Nucleolin overexpression was successfully achieved using a strategy based on a TetOn-dependent lentiviral system, induced by the presence of doxycycline. Fluorescence microscopy and western blot analysis suggested that the previous is a non-leaky inducible system and that four days after system activation overexpression is observed at intense level in both MCF-7 and MDA-MB-231 breast cancer cell lines.

Mammospheres formation assay suggested that nucleolin may have an influence in the stem-like phenotype of MDA-MB-231 breast cancer cells. Moreover, wound healing assay suggested nucleolin is necessary for cell migration machinery.

In the immediate continuation of this work, more experiments need to be done towards a better understanding of the role of nucleolin in carcinogenesis and its validation as a biomarker for cancer cells.

Appendix

Table S 1 – List of primers used for cloning and its description.

	Orientation	Target	Restriction enzyme	Sequence (5' to 3')
PR1	Sense	hNCL	NheI	AAAgctagcATGGTGAAGCTCGCGAAGGCAGGTA
PR2	Antisense	hNCL	BamHI	TATTggatccTTCAAACTTCGTCTTCTTTCCTTGTGGC
PR3	Sense	rNcl	NheI	ATCAgctagcATGGTGAAACTCGCAAAGGC
PR4	Antisense	rNcl	BamHI	GAAGggatccTTCAAACTTCGTGTTCTTT
PR5	Sense	Luciferase	NheI	CTGTTgctagcATGGAAGACGCCAAAAACATAAAGAAA
PR6	Antisense	Luciferase	BamHI	GCTGAggatccTTACATTTTACAATTTGGACTTTCCGC
PR7	Sense	mCherry	BglII	GATCagatctATGGTGAGCAAGGGCGAGGAGGATAAC
PR8	Antisense	mCherry	BglII	TTTGGagatctCTTGTACAGCTCGTCCATGCCGCCG
PR9	Antisense	hPGK	XhoI	GGTACCctcgagCTGGGGAGAGAGGTCGGTG
PR10	Sense	mCherry	NotI	ATTAgcggccgcATGGTGAGCAAGGGCGAGGAGGATAAC
PR11	Antisense	mCherry	EcoRV	AATAgatatcTCACTTGTACAGCTCGTCCATGCCGCCGGTGG
PR12	Sense	Luciferase	NotI	ATTAgcggccgcatgGAAGACGCCAAAAACATAAAGAAA
PR13	Antisense	Luciferase	SmaII	AATAcccgggTTACAATTTGGACTTTCCGCCCTTC
PR14	Sense	pcDNA ENTR BP plasmid	-	ACTTTCCAAAATGTCGTAACAACTCCGCC
PR15	Antisense	pcDNA ENTR BP plasmid	-	CTCTAGATCAACCACTTTGT
PR16	Sense	T2A peptide	BamHI	ggatccTCTAGAGGCAGTGGAGAGGG
PR17	Antisense	mCherry	BglII	TTAGagatctTCACTTGTACAGCTCGTCCATGCCGCC
PR18	Antisense	Luciferase	BglII	TAATTagatctTTACAATTTGGACTTTCCGCCCTT
PR19	Sense	TRE	-	TTATTACAGGGACAGCAGAGATCCAC

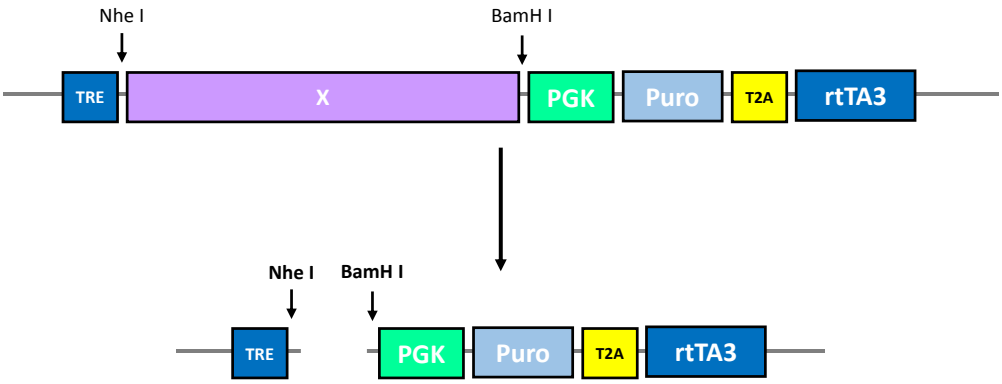


Figure S 1- Diagram of the pCW cloning lentiviral vector preparation. Digestion of pCW-X lentiviral vector with NheI and BamHI restriction enzymes, releasing X sequence and preparing the vector for further cloning.

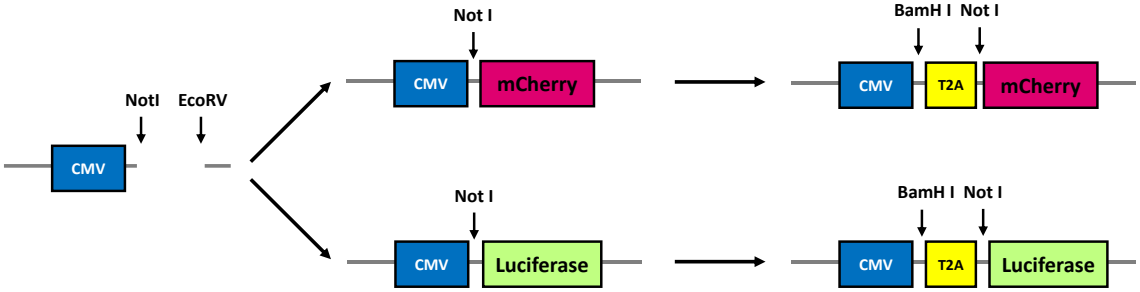


Figure S 2 - Diagram of the construction of intermediary vectors, pcDNA ENTR BP-T2A-mCherry and pcDNA ENTR BP-T2A-Luciferase. Ligation of pcDNA ENTR BP, digested with NotI and EcoRV restriction enzymes, with mCherry or Luciferase, giving rise to pcDNA ENTR BP-mCherry and pcDNA ENTR BP-Luciferase. Further digestion of the resulting vectors for ligation of T2A sequence by the restriction enzymes BamHI and NotI.

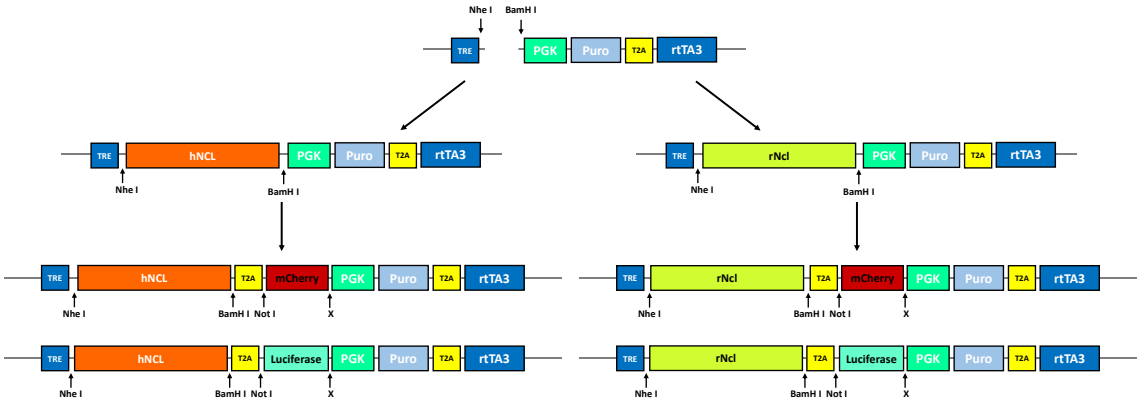


Figure S 3 - Diagram of the construction of the overexpression vectors, pCW-hNCL-T2A-mCherry, pCW rNcl-T2A-mCherry, pCW-hNCL-T2A-Luciferase, pCW rNcl-T2A-Luciferase. Ligation of pCW, digested with NheI and BamHI restriction enzymes, with hNCL or rNcl. Further digestion of the intermediary resulting vectors, pCW-hNCL and pCW-rNCL with BamHI for ligation of T2A-mCherry or T2A-Luciferase, both digested with BamHI and BlgII.

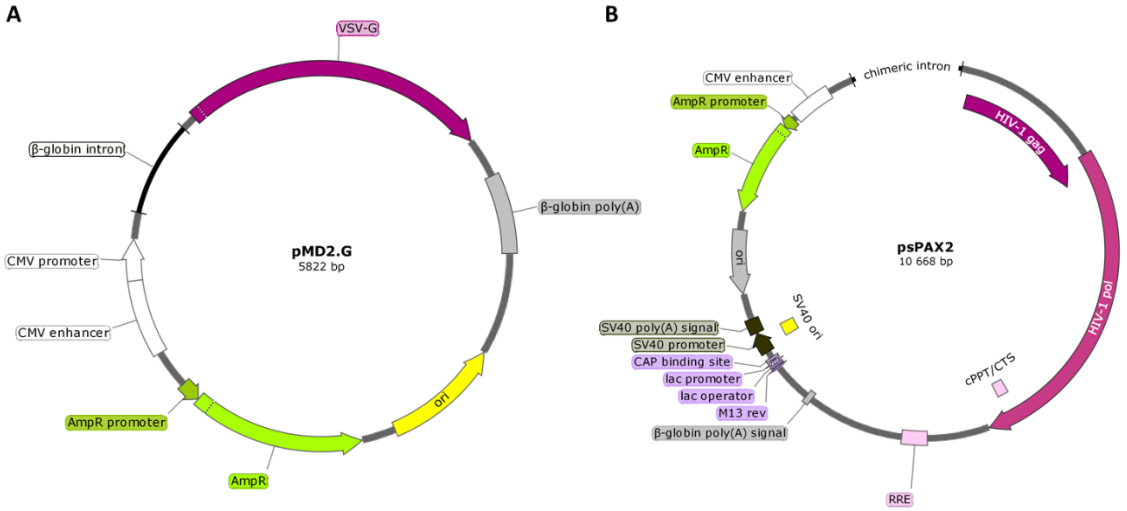


Figure S 4 - Schematic representation of accessory Lentiviral vectors. (A) pMD2.G, the envelope expressing vector with VSV-G (in purple) under CMV promoter (in white) and (B) psPAX2, the packaging vector with gag and pol (in purple) and HIV elements essential for Lentiviral particles production (in light pink - RRE, cPPT/CTS).

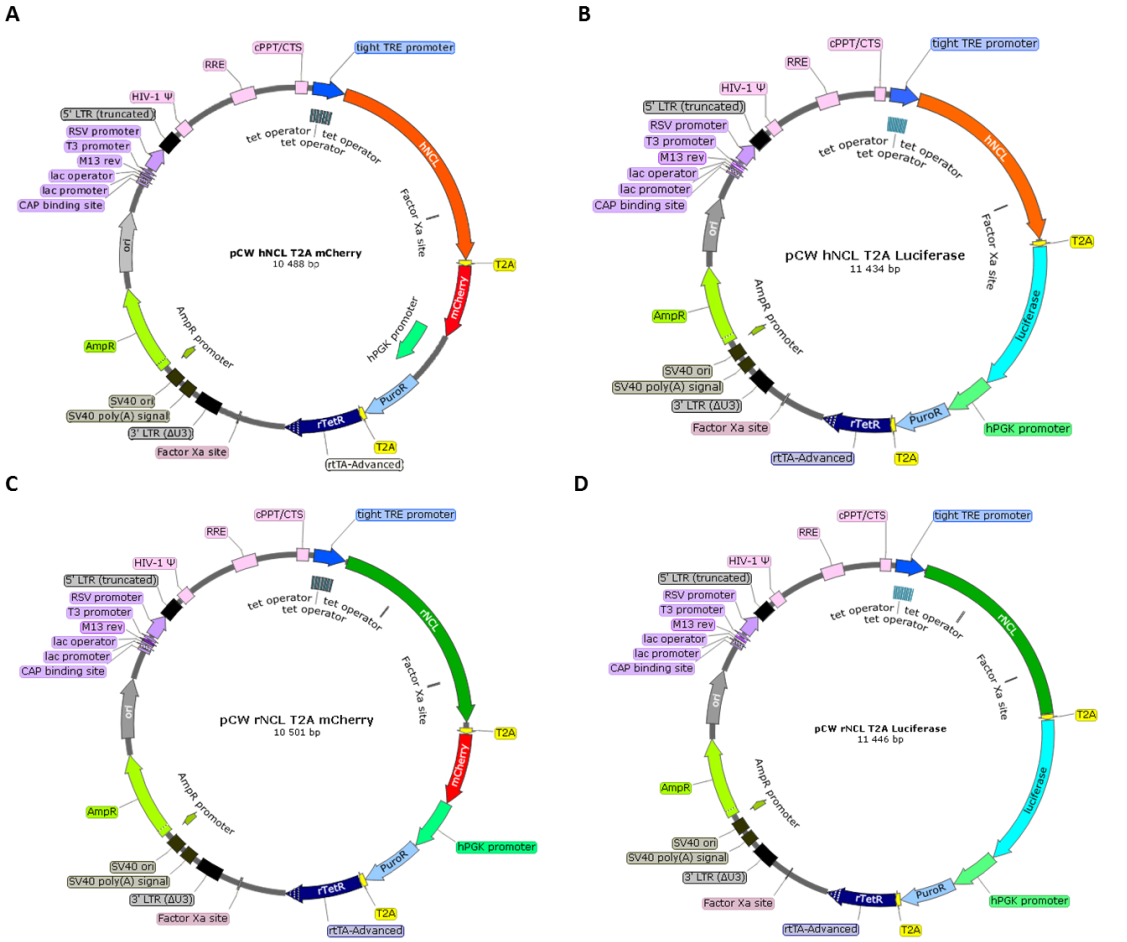


Figure S 5 - Schematic representation of the entire lentiviral plasmids aiming at nucleolin overexpression. Schematic representation of pCW vectors evidencing the expression cassette flanked by 5'LTR and 3'LTR: HIV elements essential for lentiviral particles production (in light pink - HIV-1 ψ, RRE, cPPT/CTS), TRE promoter (in blue) enabling the inducible expression of insert of interest and the hPGK promoter (in blue-green) driving the transient expression of PuroR (in light blue) and rtTA (dark blue). The inserts of interest are hNCL-T2A-mCherry (A), hNCL-T2A-Luciferase (B), rNcl-T2A-mCherry (C) and rNcl-T2A-Luciferase (D).

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