Universitat de Girona

Faculty of Sciences

Final Degree Project Report

Development and analysis of response biomarkers for miR-323a-5p therapy in Neuroblastoma

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RESUM

El neuroblastoma (NB) és el càncer embrionari més comú entre nens. S'origina al sistema nerviós simpàtic perifèric i pot metastatitzar a diverses parts el cos. Malauradament, els tractaments actuals no eviten la recaiguda dels pacients d'alt risc, suggerint la necessitat de noves teràpies. Les teràpies basades en ARN, especialment aquelles que involucren microRNAs (miRNAs), mostren potencial en el tractament del NB, sent el miR-323a-5p un dels miRNAs que exhibeix més potencial com a supressor tumoral, convertint-lo en un candidat terapèutic prometedor. Tot i això, l'administració efectiva del miR-323a-5p és un repte degut a la degradació d'aquest en el torrent sanguini i les dificultats a ser captat per les cèl·lules. Les nanopartícules ofereixen una potencial solució, permetent l'administració *in vivo* del miR-323a-5p, demostrant eficàcia en les dianes moleculars conegudes. Tot i que aquests gens diana reflecteixen fenotípicament els efectes del miR-323a-5p, podrien no detectar òptimament l'eficàcia d'aquesta nanomedicina, suggerint la necessitat de treballar en l'estudi d'un biomarcador de resposta que presenti major modulació.

En aquest estudi, mitjançant una anàlisi d'RNA-seq de mostres de NB tractades amb el miR-323a-5p, s'ha identificat i analitzat potencials biomarcadors de resposta que podrien actuar com a dianes per determinar l'eficàcia antitumoral d'aquest miRNA *in vivo*. Aquests resultats van ser validats mitjançant qPCR, considerant dos sistemes d'entrega diferents: Lipofectamina 2000 (LF 2000), utilitzat en estudis *in vitro* i Quatsomes (QS), un sistema d'entrega basat en nanopartícules.

L'anàlisi d'RNA-seq ha identificat 8 gens modulats pel miR-323a-5p amb potencial com a biomarcadors de resposta. Tot i que dos dels gens no van poder ser amplificats, aquests resultats van ser en gran part confirmats per la validació mitjançant qPCR. Malgrat haver detectat modulació, els resultats *in vitro* no revelen un clar candidat que millori significativament els gens utilitzats actualment com a biomarcadors de resposta. L'estudi també revela una variació significativa en la modulació gènica entre els sistemes d'entrega, amb QS mostrant una menor eficiència en comparació amb LF 2000. Aquests resultats suggereixen la necessitat d'una futura validació *in vivo*, investigacions en diferents línies cel·lulars de NB i la consideració d'aproximacions multi-òmiques per comprendre millor l'impacte terapèutic del miR-323a-5p en el NB.

RESUMEN

El neuroblastoma (NB) es el cáncer embrionario más común entre niños. Se origina en el sistema nervioso simpático periférico y puede metastatizar a diversas partes del cuerpo. Lamentablemente, los tratamientos actuales no previenen la recaída en pacientes de alto riesgo, lo que sugiere la necesidad de nuevas terapias. Las terapias basadas en ARN, especialmente aquellas que involucran microRNAs (miRNAs), muestran potencial en el tratamiento del NB, siendo miR-323a-5p uno de los miRNAs que exhibe mayor potencial como supresor tumoral, convirtiéndolo en un candidato terapéutico prometedor. Sin embargo, la administración efectiva de miR-323a-5p es un reto debido a su degradación en el torrente sanguíneo y a las dificultades para ser captado por las células. Las nanopartículas ofrecen una solución potencial, permitiendo la administración *in vivo* de miR-323a-5p y demostrando eficacia en las dianas moleculares conocidas. Aunque estos genes diana reflejan fenotípicamente los efectos de miR-323a-5p, podrían no detectar óptimamente la eficacia de esta nanomedicina, sugiriendo la necesidad de estudiar un biomarcador de respuesta con mayor modulación.

En este estudio, mediante un análisis de RNA-seq de muestras de NB tratadas con miR-323a-5p, se identificaron y analizaron potenciales biomarcadores de respuesta que podrían actuar como dianas para determinar la eficacia antitumoral de este miRNA *in vivo*. Estos resultados fueron validados mediante qPCR, considerando dos sistemas de entrega diferentes: Lipofectamina 2000 (LF 2000), utilizado en estudios *in vitro* y Quatsomes (QS), un sistema de entrega basado en nanopartículas.

A través del análisis e RNA-seq se han identificado 8 genes modulados por el miR-323a-5p que potencialmente podrían actuar como biomarcadores de respuesta. A pesar de que dos de los genes no pudieron ser amplificados, estos resultados fueron en gran parte confirmados mediante qPCR. Aunque se detectó modulación, los resultados in vitro no revelan un claro candidato que mejore significativamente los biomarcadores utilizados actualmente. El estudio también revela una variación significativa en la modulación génica entre los sistemas de entrega, siendo QS menos eficiente en comparación con LF 2000. Estos hallazgos sugieren la necesidad de una futura validación in vivo, investigaciones en diferentes líneas celulares de NB y la consideración de enfoques multiómicos para comprender mejor el impacto terapéutico de miR-323a-5p en el NB.

ABSTRACT

Neuroblastoma (NB) is the most common embryonal cancer among children. It originates in the peripheral sympathetic nervous system and can metastasize to various parts of the body. Unfortunately, current treatments do not prevent relapse in high-risk patients, suggesting the need for new therapies. RNA-based therapies, especially those involving microRNAs (miRNAs), show potential in the treatment of NB, with miR-323a-5p being one of the miRNAs that exhibits more potential as a tumor suppressor, making it a promising therapeutic candidate. However, effective delivery of miR-323a-5p is challenging due to its degradation in the bloodstream and cellular uptake. Nanoparticles offer a potential solution and enabled the *in vivo* delivery of miR-323a-5p, demonstrating efficacy in known molecular targets. Although these target genes phenotypically reflect the effects of miR-323a-5p, they may not optimally detect this nanomedicine efficacy, suggesting the need of working on the study of a response biomarker that is further modulated.

In this study, by RNA-seq analysis of NB samples treated with miR-323a-5p, potential biomarkers of response that could act as targets to determine the antitumor efficacy of this miRNA in vivo were identified and analyzed. These results were validated by qPCR, considering two different delivery systems: Lipofectamine 2000 (LF 2000), commonly used in *in vitro* studies and Quatsomes (QS), a nanoparticle-based delivery system.

RNA-seq analysis identified 8 genes that were modulated by miR-323a-5p. Although two of the genes could not be amplified, the RNA-seq results were largely confirmed by qPCR. Although modulation was detected, the *in vitro* results do not reveal a clear candidate response biomarker that significantly improves upon those currently used. The study also reveals significant variation in gene modulation between delivery systems, with QS being less efficient compared to LF 2000. These findings suggest the need for future *in vivo* validation, studies using different NB cell lines and consideration of multi-omics approaches to better understand the therapeutic impact of miR-323a-5p in NB.

REFLECTION ON ETHICS

During the development of this project, we worked with samples from mice. Therefore, when working with live animals, it is crucial to avoid malpractice. Thus, all experimental procedures involving animals have been previously approved by the Animal Experimentation Ethical Committee from VHIR and carried out in accordance with the Catalan, Spanish and European Union regulations, considering the current legislation (Real Decreto 53/2014, February 1st), which establishes the basic applicable standards for the protection of animals used in experimentation.

REFLECTION ON SUSTAINABILITY

In this project, we have worked with nanomolecules as the main subject of study. The manufacturing practices of these nanomolecules can be inefficient, generating waste and emissions that can have significant impacts on environmental sustainability. The methodology used for the production of the nanomedicine used in this study is a scalable manufacturing process that can accomplish Good Manufacturing Practices (GMP) requirements, which are aligned with several United Nations Sustainable Development Goals (SDGs). This approach not only aims to provide accessible treatments but also has a direct impact on the sustainability of public health systems.

REFLECTION ON GENDER PERSPECTIVE

Recent advancements in gender equality within the scientific field are notable, yet significant challenges persist. Women constitute only one-third of students globally in science, with this disparity increasing in advanced academic positions (Codeço & Dias, 2018). According to Generalitat data, 59.5% of research and development personnel are men. In higher positions, this bias is more evident where women occupy only 27% of group leader roles and less than 23% of full professor positions, highlighting the "glass ceiling" effect. This male dominance in science has historical roots dating back to the 17th century (Jones & Hawkins, 2015).The question arises of how this trend could be changed. Addressing this issue requires institutional policies with a gender perspective and early educational interventions to tackle deep-seated social biases in order to continue advancing and, who knows, at some point eradicate these differences in any field, including the world of science

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1. Introduction

1.1 Neuroblastoma

Annually in Europe, about 15,000 children under the age of 15 and 20,000 adolescents and young adults between the ages of 15-24 receive a cancer diagnosis (Ferlay et al., 2010). Despite youth malignancies are really uncommon, pediatric cancer remains as the leading cause of mortality among children and adolescents (Cunningham et al., 2018) causing approximately a quarter part of all children deaths (Amalia et al., 2023). Moreover, as survivors of childhood cancer reach old age, the side effects and long-term disabilities become apparent. Improved access to new therapeutic opportunities designed particularly for pediatric cancer is an urgent need, however, the number of therapies approved for pediatric cancer remains significantly lower compared to those for adults (Kattner et al., 2019).

Among childhood malignancies, neuroblastoma (NB) is the most common type of embryonal cancer and ranks as the second most prevalent solid tumor in children aged under 15 years globally (Gatta et al., 2012; Ward et al., 2014). Approximately 10% of diagnosed pediatric cancers correspond to this type of tumor, exhibiting an incidence of 10 cases per million inhabitants and contributing to 15% of total childhood cancer deaths (Matthay et al., 2016). NB is a tumor of the peripheral sympathetic nervous system, originating from embryonic neural crest cells (Brodeur, 2003). Any region of the sympathetic nerve system can give rise to these tumors, although the most often reported primary site is the adrenal gland. Other sympathetic nervous system locations include the thoracic, cervical, abdominal, and pelvic regions. NB can metastasize to lymph nodes, bone marrow, bones, liver, skin and with decreased frequency, to the lungs or brain (Malis, 2013).

It is also referred to as an “enigmatic tumor” due to its varied biological features, vast variety of clinical manifestations and unpredictable prognoses, which can vary from benign to incurable or metastatic illness with very bad outcomes. NB patients are classified into different risk groups based on clinical and molecular factors, such as the stage of the disease (Table 1), the age of the individual at diagnosis (Jereb et al., 1984), chromosomal alterations and MYCN amplification (Newman et al., 2019). According to Tonini et al. (2012), low risk NB category includes stage 1, stage 2 and stage 4S patients with non-amplified MYCN. Intermediate risk includes stage 3 patients older than 2 years and stage 4 patients younger than 18 months of age. The high-risk NB group includes patients in any stage of the INSS classification with MYCN amplification and patients older than 18 months with metastatic NB in stage 4.

The clinical behavior of NB tumors is notably variable, exhibiting a wide range from spontaneous remission to an aggressive course and eventual disease-related death (Stallings, 2009). Overall, NB has the worst outcome among the embryonal tumors, being 68% the 5-year survival rate for this disease (Gatta et al., 2012); however, the prognosis of patients depends on the stage of the disease (Table 1). According to the International Neuroblastoma Staging System (INSS), the 3-year event-free survival rates (EFS) vary as follows: patients in stages 1, 2 and 4S show EFS rates between 75% and 90%. Infants with stage 3 tumors show rates of 80-90% while those with stage 4 tumors have EFS rates of 60-75%. Children with stage 3 and 4 tumors show rates of 50% and 15% respectively (Castleberry, 1997).

Table 1. *International staging system for neuroblastoma.*

Stage 1	Localized tumor confined to the area of origin; complete gross resection, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph node negative for tumor.
Stage 2A	Unilateral with incomplete gross resection; identifiable ipsilateral and contralateral lymph node negative for tumor.
Stage 2B	Unilateral with complete or incomplete gross resection; with ipsilateral lymph node positive for tumor; identifiable contralateral lymph node negative for tumor.
Stage 3	Tumor infiltrating across midline with or without regional lymph node involvement; or unilateral tumor with contralateral lymph node involvement; or midline tumor with bilateral lymph node involvement.
Stage 4	Dissemination of tumor to distant lymph nodes, bone marrow, liver, or other organs except as defined in 4s.
Stage 4S	Localized primary tumor as defined for stage 1 or 2 with dissemination limited to liver, skin or bone marrow.

Note: From R.P. Castleberry (1997)

Although approximately 1-2% of case are reported as familial occurrences, NB typically presents as a sporadic condition (Aygun, 2018). Many chromosomal abnormalities and its consequences in cellular signaling pathways have been linked to NB development. MYCN amplification is one of the most highlighted abnormalities as its oncogenic role it's related to tumor aggressiveness being a strong indicator of poor prognosis (Louis & Shohet, 2015). Other genomic changes linked to tumorigenesis are 1p36 deletion, the loss of heterogeneity of 11q or the 17q gain (Aygun, 2018).

1.2 Current treatments and challenges

Currently, the main therapeutic options for NB are surgical resection, chemotherapy, radiotherapy and immunotherapy (Bhoopathi et al., 2021). The treatment given to each patient is based on the degree of risk. The main treatment for low risk tumors is surgical resection as these tumors are characterized by exhibiting spontaneous regression potential, while for intermediate risk tumors, patients also receive chemotherapy at different intensities according to the type of patient (Tonini et al., 2012). In high-risk patients, the treatment consists of different phases which include chemotherapy, surgery, radiotherapy, stem cell transplant, immunotherapy and retinoid therapy (Figure 1) (Krystal & Foster, 2023). But even with those multimodal treatments nearly half of high-risk patients relapse (Simon et al., 2011) and the survival rate for high risk NB patients does not reach 50% (Smith & Foster, 2018). This is a consequence of the ability of NB cells to undergo genetic and molecular changes, which are being studied with the aim of designing new targeted therapies to control the progression of the disease more effectively (Aravindan et al., 2019).

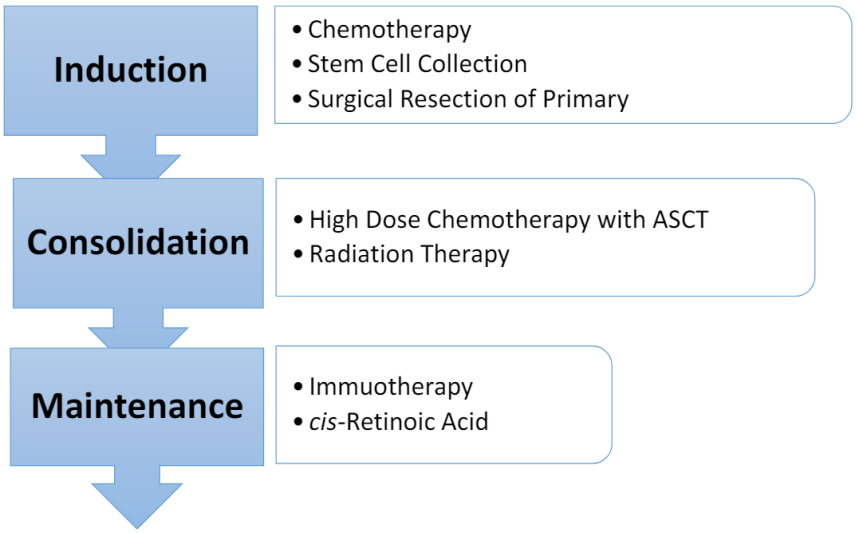

Figure 1. *Standard treatment for high-risk NB consists of 3 phases and lasts about 18 months. From Smith & Foster (2018)*

Some of the therapies currently in development are based on the inhibition of different targets involved in the progression of the disease. One example is the use of MDM2 inhibitors. This type of drug acts targeting the p53-MDM2 pathway by inhibiting MDM2 gene and restoring p53 levels which is a tumor-suppressor protein (W. Wang et al., 2020). Although, several challenges are reported as toxicity and side effects, the development of resistance by NB cells and the need for more effective MDM2 inhibitors (Zafar, Wang, Liu, Xian, et al., 2021). Other drugs that have been demonstrated to exhibit

therapeutic potential are inhibitors of PARP-1, an enzyme involved in DNA damage response (King et al., 2020), inhibitors of MEK, a kinase of the Raf-MEK-ERK Signaling Cascade involved in cell growth (Chilamakuri & Agarwal, 2022) and ALK inhibitors, that target a tyrosine kinase receptor reported to be an oncogenic driver of NB (Pacenta & Macy, 2018). Nevertheless, for these drugs, some drawbacks have been reported, these include the development of resistance to inhibitors by cancer cells, toxicity and side effects, the need to be combined with other treatments, dependence on the mutational context or the requirement for personalized approaches (Eleveld et al., 2023; Makvandi et al., 2019; Zafar, Wang, Liu, Wang, et al., 2021). Therefore, to solve outstanding issues and improve NB patients' treatment outcomes, continued research and the creation of innovative treatments are still crucial.

1.3 RNA-based therapies

RNA-based therapies are new pharmacological strategies that act against specific targets and provide an alternative for conventional treatments. Specially, the use of post-transcriptional regulators like noncoding RNAs is emerging as a promising therapeutic alternative, with microRNAs (miRNAs) being among the most interesting candidates. By targeting the transcriptome, miRNAs offer the possibility of acting on more undruggable molecules than conventional treatments do. The number of preclinical trials using these molecules is growing and showing similar effects or even improvements compared to conventional therapies (Boloix et al., 2016; Zhu et al., 2022).

MiRNAs are small RNA molecules between 19-25 nucleotides that play an important role in post-transcriptional gene regulation by binding to the 3' UTR region of the target mRNAs, thereby inhibiting their translation or inducing their degradation (Lu & Rothenberg, 2018). These molecules show different patterns of expression that can be correlated with cancer and can act as oncogenes or tumor suppressors (Lee & Dutta, 2009). It has been demonstrated that many miRNA are differently expressed in some genetic subtypes of NB tumors and their deregulation is involved in cancer tumorigenesis (Chen & Stallings, 2007). In human malignancies as NB, this abnormal miRNA expression is associated to chromosomal abnormalities, changes in the transcriptional control, epigenetic changes and malfunctions in the machinery of miRNA biogenesis (Peng & Croce, 2016). Therefore, using therapies based on restoring these miRNA levels could be beneficial for treating this disease.

In Soriano et al. (2019), a high-throughput functional screening of 2048 miRNA mimics was performed to evaluate their antiproliferative effect in SK-N-BE(2) NB cells. They

examined the genomic distribution of the miRNAs that exhibited more reduction on cell proliferation and found that many of them were located in the 14q32 loci, a region that is frequently lost or silenced in NB. MiR-323a-5p is reported to be downregulated in MYCN amplified NB patients (Schulte et al., 2010). This molecule was one of the miRNAs with higher therapeutic potential exhibiting tumor-suppressor role in a representative panel of NB cell lines and its overexpression in NB *in vivo* models reduced tumor growth by targeting cell cycle and inducing apoptosis (Soriano et al., 2019).

MiR-323a-5p plays a direct or indirect regulatory role in many genes involved in cell cycle as CHAF1A, CCND1 and p27 (Figure 2). CHAF1A (Chromatin Assembly Factor-1) is involved in chromatin assembly. It facilitates the deposition of histone proteins H3 and H4 onto DNA and promotes cell differentiation by inhibiting cellular differentiation and suppressing apoptosis by maintaining the heterochromatin (Shen et al., 2020). MiR-323a-5p inhibits CHAF1A, this translates to an induction of cellular differentiation and a decrease of cell proliferation. CCND1 is also inhibited by miR-323a-5p. This cyclin is a key allosteric activator of the associated cyclin-dependent kinases 4/6 (CDK 4/6) throughout the cell cycle, essential for the initiation of the DNA replication process (Nie et al., 2020). This activation promotes the phosphorylation of the Rb protein, resulting in an active protein that induces cellular division. By targeting CCND1, miR-323a-5p reduces the levels of this it leading to a reduction in Rb phosphorylation causing a stop in the progression of the cell cycle. Due to antiproliferative and differentiation signals p27 is increased, which inhibits the complex cyclin-CDK resulting in the cessation of cell cycle (Razavipour et al., 2020). Despite the antiproliferative potential of miR-323a-5p in NB, the administration of this RNA-based therapy still faces several challenges that need to be addressed before its clinical application.

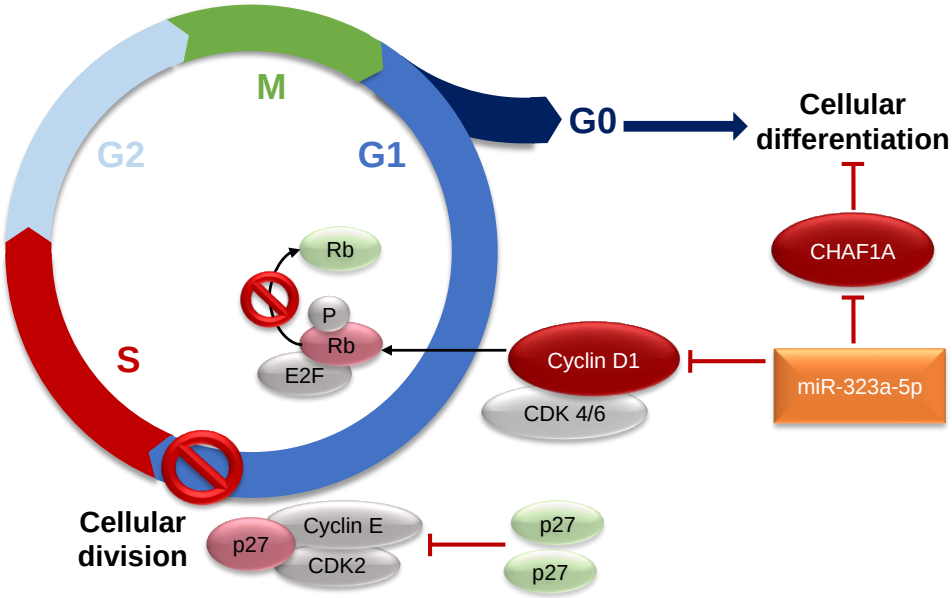

Figure 2. *Schematic representation of miR-323a-5p regulatory role in NB cells. MiR-323a-5p inhibits CHAF1A, CCND1 and increase p27 levels promoting cell cycle arrest and inducing cell differentiation.*

The degradation in blood by the action of nucleases and the difficulties of this molecule entering to the target cells are some of the main challenges of using these small RNAs as a drug, suggesting that the use of nanoparticles (NP) could be an interesting vehicle to deliver these types of molecules into target cells. In a study of Boloix et al., (2022), miR-323a-5p conjugated with NP was administered *in vivo*. The results showed that these complexes could effectively deliver this miRNA to both the primary tumor and NB metastatic sites.

We designed NPs that enabled the *in* vivo administration of miR-323a-5p, demonstrating efficacy in known molecular targets. However, the effects observed in the selected genes like CCND1, CHAF1A or p27 appeared to be modest. Although the silencing of these genes reflects phenotypically the effects of miR-323a-5p in NB cells, they may not be the optimal response biomarkers to test this nanomedicine efficacy. This suggests the need of more sensitive response biomarkers to monitor the efficacy of this nanomedicine. Therefore, in this study, RNA-seq results were analyzed to identify potential response biomarkers exhibiting higher modulation that could act as targets to determine the antitumor effectiveness of this miRNA *in vivo.*

2. Objectives

We hypothesize that there might be other genes that exhibit higher modulation than those previously identified, which could facilitate the determination of miR-323a-5p efficacy *in vivo*.

In consequence, the main objective of this work is to:

- Identify more sensitive response biomarkers for miR-323a-5p after their administration *in vivo*.

This main objective is subdivided in two secondary objectives:

- Validate by qPCR the modulated genes identified by RNA-seq.
- Analyze the differences between the identified target genes depending on the delivery systems used for miR-323a-5p transfection *in vitro*.

3. Methodology

3.1 RNA-seq analysis

Bioinformatic analysis of RNA-seq samples was carried out at Dreamgenics. The raw FASTQ files were evaluated using FAastQC quality controls, and sequence *trimming* was performed to remove bases, adapters, and other low-quality sequences using fastp software.

Pseudo-alignment of the sequences was performed against the reference transcriptome for Homo sapiens GRCh38.p14 (Gencode:release 44) and direct quantification of transcripts was conducted using Salmon (v1.10.0).

For differential expression analysis between conditions, the DESeq2 algorithm was used, employing the Benjamini-Hochberg method for p-value adjustment (padj). Genes with and adjusted p-value of less than 0.05 and a log2FoldChange greater than 1 or less than -1 were considered differentially expressed.

3.2 Candidate genes compilation

To compile the list of candidate genes, general information of each gene including the main functions and genomic locus was obtained from the UniProt database (UniProt Consortium, 2024). TargetScan (McGeary et al., 2019), miRDB (Chen & Wang, 2020) and Diana Tools (Tastsoglou et al., 2023) were consulted to find predicted direct targets of miR-323a-5p. Relevant pathways for each gene were consulted on Reactome (Milacic et al., 2024) and WikiPathways (Agrawal et al., 2024). Gene expression levels in SK-N-BE(2) NB cell line were obtained from ProteinAtlas (Uhlen et al., 2017).

3.3 Cell culture

SK-N-BE(2), NB cell line from a bone marrow biopsy, was acquired from the Public Health England culture Collection (Salisbury, UK).

Upon resuscitation, cells were cultured in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum, 1% of Insulin-Transferrin-Selenium Supplement, 100 U/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL plasmocin at *37* ºC in a saturated humidified atmosphere of 95% air and 5% CO_2. SK-N-BE(2) cells were tested for mycoplasma contamination periodically.

3.4 miRNA transfection

MiR-323a-5p was overexpressed in SK-N-BE(2) cells via transfection with LF2000® or lipid-based non liposomal particles named Quatsomes (QS). $5.5 \cdot 10^3$ cells from the initial culture were seeded in 3.5mL of IMDM per 60-mm plate to perform the transfection in a total of 6 plates.

The miRNA and LF 2000 conjugates were formed following the manufacturer's protocol. Briefly, 8 µL of LF 2000 (Thermo Fisher,# 11 668 019) were mixed with 250 µL of opti-mem (Thermo Fisher,# 31 985 070) and incubated for 5 min. In parallel, 10 µL of miRNA (0.2 nmols) were mixed with 250 µL of opti-mem. After 5 min, the two solutions were combined mixing them gently and incubated 20 min at room temperature. Next, 500 µL of LF 2000-sRNA conjugates were added to 20 cm2 plates above 3.5 mL of seeded cells without antibiotics.

The next day, the medium was replaced. After 72 hours post-transfection, the cells were harvested. Pellets from the cells were recollected and resuspended in PBS 1x and stored at -80 ºC.

3.5 RNA extraction

Total RNA was extracted from cell pellets using the miRNeasy Mini Kit™ (Qiagen, Las Matas, Spain) according to the manufacturer's instructions.

The concentrations of RNA were determinated with NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific).

3.6 Quantitative RT-PCR

3.6.1 Retrotranscription

Extracted RNA was reverse transcribed (0.5 µg total RNA) by RT-PCR using TaqMan™ RT Kit (#4 366 596, Applied Biosystems, Thermo Fisher Scientific). Master Mix was added above diluted RNA to reach a final RNA concentration of 62.5 ng/µL for primers validation and a concentration of 52.08 ng/µL in 20 µL of final volume.

The reaction tubes were placed into the 2720 Thermal Cycler.

3.6.2 Design of the Primers

Primers from candidate genes were designed with the Primer-BLAST tool (Ye et al., 2012). The primers were considered to have an amplification region between 100-200 bp and spanning an exon-exon junction (Table 2). Primers were reconstituted with water to obtain a stock at 100 µM stored at -20 ºC.

3.6.3 PCR

A Master Mix was prepared for each primer pair using Power SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific). Primers (10 µM) were added in each Master Mix in a final concentration of 0,5 µM and samples were added to each tube to reach a final concentration of 6.25 ng/µL of RNA. The PCR was carried out in the 2720 Thermal Cycler.

Samples were run in 2% agarose gels. Images were acquired using the Gel Doc XR + System (Biorad, Hercules, California, USA).

Table 2. *List of the primers designed for each candidate gene, CCND1, CHAF1A and housekeeping gene L27 using Primer-BLAST designing tool.*

Genes		Primer sequence (5'→3')		Product length(bp)
FTH1	Primer pair 1	FW	ACTACCACCAGGACTCAGAGG	100
		RV	TCGCGGTCAAAGTAGTAAGACATGG	
	Primer pair 2	FW	ACTACCACCAGGACTCAGAGG	247
		RV	ACTACCACCAGGACTCAGAGG	
FBXL7	Primer pair 1	FW	GGCATGACGGCTACAGGATG	157
		RV	CTCAGGTCGGAGTCTTCGC	
	Primer pair 2	FW	AGTGGAAGAGACCGGGTCAG	182
		RV	TCGAGCTGCCTTTGCATTCT	
CAPN6	Primer pair 1	FW	AAACGTCCCCAGGACATCTG	180
		RV	AGGGTCCCATTCCTGTTCCT	
	Primer pair 2	FW	AGTCCGTTCTCCTGTCCAGA	110
		RV	GGCTGTTCCAGACCTGTACT	
CKAP2L	Primer pair 1	FW	GCTGCCGCTGTCGAAGA	196
		RV	GGACCTTTTAGGTTTGACAGGC	
	Primer pair 2	FW	GCAGCCAAGGGAAAACTGAAG	106
		RV	GAGTTTAATGCTGATGGACAGATTT	
CNGB1	Primer pair 1	FW	CACTGGGGACACAGGGTG	178
		RV	GAGGCAGCACCTGTAGCAA	
	Primer pair 2	FW	ATGTGCAGACCAGGGTGATG	125
		RV	CCCGGGAAGGTCTTCTCTTC	
COL11A2	Primer pair 1	FW	GAGGCTCATAGTCTGCTCCC	189
		RV	GGGTGCACCTGCCCA	
	Primer pair 2	FW	AACCCCAGAGCCAGGACC	134
		RV	TTCCAACACTGCAGGCTCTC	
H2AC6	Primer pair 1	FW	TTTCTCGTGAGCTTAGGCCG	173
		RV	CTGCGTAGTTGCCTTTACGG	
	Primer pair 2	FW	CCTTCCTAACATCCAGGCCG	109
		RV	GGTTTGATAGCGTTTCCGGG	
SNX32	Primer pair 1	FW	CTCAAACACGCCAAGGCCAG	167
		RV	GCACTGCTAGGGAAGGAGAC	
	Primer pair 2	FW	GACTTTGAGGCTTCGAGGGA	114
		RV	GATGGCCAGGTACTCCGCTT	
CCND1	Primer pair 1	FW	GCTGCGAAGTGGAAACCATC	135
		RV	CCTCCTTCTGCACACATTTGAA	
CHAF1A	Primer pair 1	FW	TCACCCAATTCATGAAGAAGC	113
		RV	GATCATACAGTCGCCCTCCT	
L27	Primer pair 1	FW	AGCTGTCATCGTGAAGAA	127
		RV	CTTGGCGATCTTCTTCTTGCC	

3.6.4 Quantitative RT-PCR

For quantitative PCR, a Power SYBR Green Master Mix was prepared, and primers were added to reach a final concentration of 0.5 µM. RNA from the samples was loaded in each tube at a final concentration of 5.2 µM. The qPCR was performed in LightCycler480.

mRNA gene expression of the candidate genes was normalized against housekeeping gene (L27). Quantification of gene expression was performed with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

3.7 Statistical Analysis

Unless otherwise stated, graphs represent the average of three of four independent experiments ± SEM. Statistical significance was determined by unpaired two-tailed Student's t-test (GraphPad Prism Software, USA). * means $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Results

4.1 RNA-seq analysis

Differential analysis of gene expression conducted with DESeq2 is depicted in Figure 3. In Figure 3A, Principal Component Analysis (PCA) is used to explore patterns of variability between samples based on the expression of thousands of genes. PC1 accounts for 63% of the total variance between samples while PC2 accounts for 15%. Results indicate that the expression profile of the miR-323a sample group is distinctly different from that of the control group samples. Figure 3B represents a volcano plot illustrating the relationship between gene differential expression (log_2FC) and the statistical significance ($-log_{10}$ p-value). 591 genes were found to be significantly differentially expressed in response to miR-323a-5p transfection, which are represented in blue. Figure 3C corresponds to an MA plot, another graphical representation used to visualize the change in gene expression between samples transfected with miR-323a-5p and samples transfected with miR-Control. Each gene is represented as a dot where the horizontal axis shows the average expression level and the vertical axis displays the log_2FC, allowing the identification of genes that have differences in their expression between the two conditions independently of the absolute size of the expression, which are represented in blue. In the Figure 3D, we detected that upon miR-323a-5p overexpression, 362 genes were upregulated while 229 were downregulated.

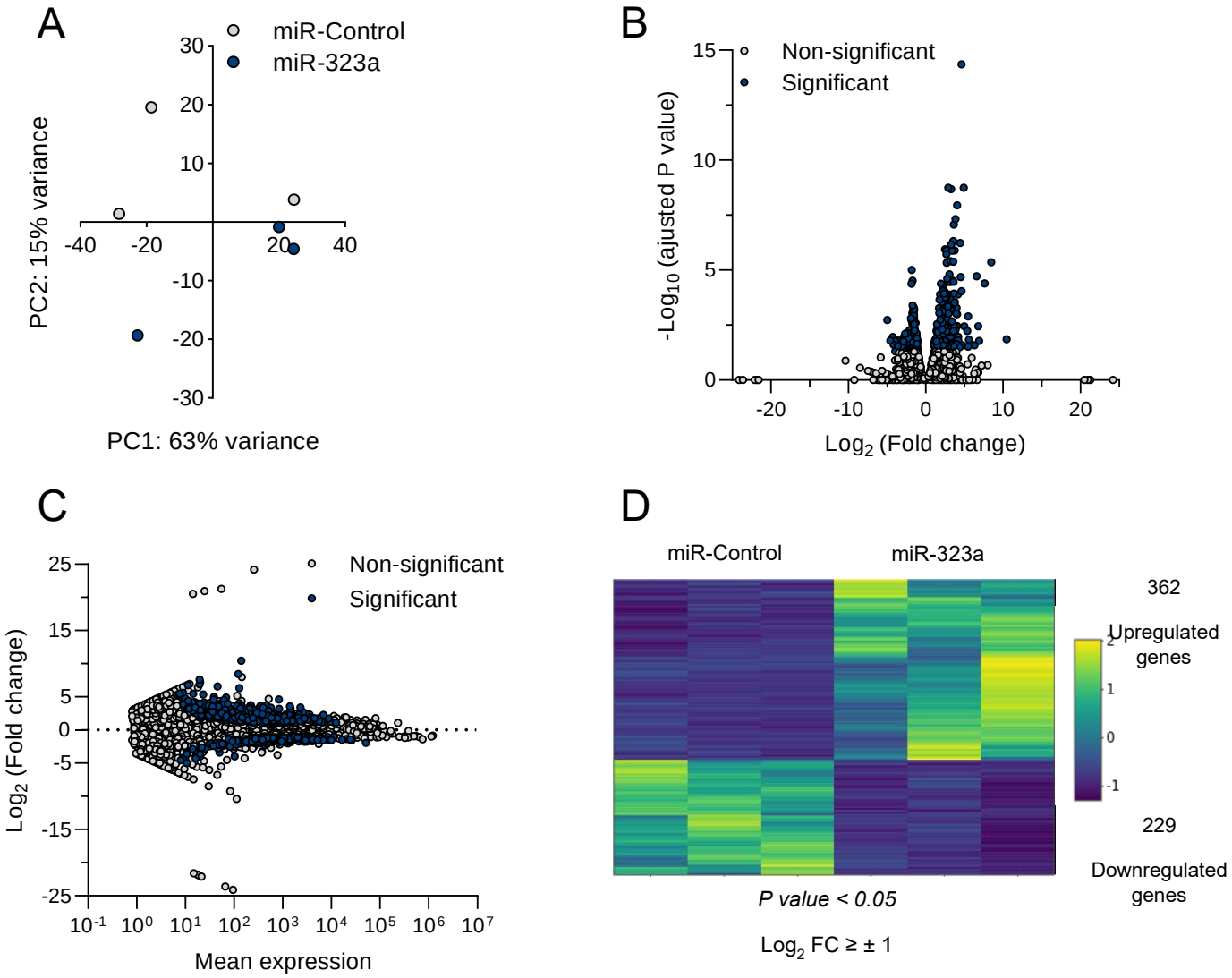

Figure 3. *Differential analysis from RNA-seq data of SK-N-BE(2) cells transfected with miR-323a-5p versus miR-Control. (A) Principal Component Analysis of the samples included in the expression data analysis. (B) Volcano plot representing, for each gene, the log_2FC between samples transfected with miR-323a-5p and miR-Control and the $-log_{10}$ of the comparison p-value. (C) MA plot representing, for each gene, the average expression value across different samples and the log_2FC between samples transfected with miR-323a-5p or miR-Control. (D) Heatmap clustering the simples based on the differentially expressed genes between the two conditions.*

4.2 Identification of miR-323a-5p response biomarkers

Based on the RNA-seq analysis, a list of the most differentially expressed genes was generated, which could serve as potential response biomarker of miR-323a-5p-based therapies. Genes were filtered based on the number of reads per gene in the analysis. A minimum threshold of 500 reads per gene was set to discard genes with low expression. Subsequently, the selected genes were filtered according to significance (p-adjusted-value > 0.05). This list was further sorted by Log2FC. The eight most differentially

expressed genes were selected for validation by qPCR. The list included four upregulated and four downregulated genes (Table 3).

According to TargetScan, *CAPN6* and *SNX32* are predicted to be direct targets of miR-323a-5p, with 4 and 1 binding sites respectively, whereas the rest of the genes do not have miR-323a-5p binding sites in their 3'UTR region. According to the literature (Ali et al., 2021; Di Sanzo et al., 2011, 2020; Q. Li et al., 2022; Y.-F. Li et al., 2020; Liu et al., 2011; Sanchez-Diaz et al., 2017; P. Wang & He, 2020; Y. Wang et al., 2022), some of the genes, primarily the downregulated ones, have implications in cancer.

For each gene, the most relevant pathways are annotated based on the enrichment analysis obtained from RNA-seq. However, there are no clear relationships between the pathways in which each of the genes is involved.

Table 3. *Summary of top differentially expressed genes identified from RNA-seq analysis in SK-N-BE(2) cells transfected with miR-323a-5p versus miR-Control. This table lists the top four upregulated and the top four downregulated genes based on Log2 fold change (Log2FC) and statistical significance (p-adjusted-value > 0.05). Detailed information of each gene is included as their main function, genomic locus, potential as a miR-323a-5p direct target, implication in cancer and some related pathways.*

Gene symbol	Main Function	Genomic locus	Fold change miR-323a vs Control	Predicted as direct target?	Role in cancer	Related Pathways
CAPN6	Microtubule-stabilizing protein that may be involved in the regulation of microtubule dynamics and cytoskeletal organization. [UniProtKB: Q9Y6Q1]	Xq23	-2,09876	YES	YES	Integrin-mediated cell adhesion
FBXL7	Substrate recognition component of a SCF E3 ubiquitin-protein ligase complex. It mediates the ubiquitination and proteasomal degradation of AURKA, causing mitotic arrest. It also regulates mitochondrial function by mediating the ubiquitination and proteasomal degradation of the apoptosis inhibitor BIRC5. [UniProtKB: Q9UJT9]	5p15.1	-1,94951	NO	YES	Metabolism of proteins; Loss of proteins required for interphase microtubule organization from the centrosome; Cell Cycle, Mitotic; Innate Immune System
FTH1	Stores iron in a soluble, non-toxic, readily available form. Has ferroxidase activity. Role in delivery of iron to cells. [UniProtKB: P02794]	11q12.3	-1,93217	NO	YES	Ion metabolism disorders; Ferroptosis; Nuclear receptors meta pathway
CKAP2L	Microtubule-associated protein required for mitotic spindle formation and cell-cycle progression in neural progenitor cells. [UniProtKB: Q8IYA6]	2q14.1	-1,90484	NO	YES	2q13 copy number variation syndrome
SNX32	May be involved in several stages of intracellular trafficking. [UniProtKB: Q86XE0]	11q13.1	2,90680	YES	NO	Endocytosis
CNGB1	Subunit of cyclic nucleotide-gated (CNG) channels, nonselective cation channels, which play important roles in both visual and olfactory signal transduction. [UniProtKB: Q14028]	16q21	3,06125	NO	NO	Ciliopathies; Bardet Biedl syndrome
COL11A2	May play an important role in fibrillogenesis by controlling lateral growth of collagen II fibrils. [UniProtKB: P13942]	6p21.32	3,74446	NO	NO	Focal adhesion PI3K Akt mTOR signaling pathway
H2AC6	Core component of nucleosome. [UniProtKB: Q93077]	6p22.2	4,60400	NO	NO	RNA Polymerase I Promoter Opening; Packaging of telomere ends; Assembly of the pre-replicative complex; Meiosis; Gene expression (Transcription)

4.3 RNA quantification

SK-N-BE(2) cells were transfected for 72h using two distinct delivery systems, Lipofectamine 2000 (LF 2000) or Quatsomes (QS). This procedure enabled the collection of cell pellets which total RNA was subsequently extracted.

Four replicates have been quantified for each different transfection agent from independent experiments, which will subsequently be used for primer validation and quantification of candidate genes expression levels (Table 4). The RNA purity of the samples is measured using the ratio between absorbance at 260 nm and absorbance at 280 nm. All samples exhibit enough concentration and acceptable purity values within the range of 1.8 to 2.1, for proceeding with cDNA retro-transcription.

Table 4. *Total RNA concentration (ng/µL) and the purity for each RNA extraction measured by the absorbance ratio between 260 nm and 280 nm from SK-N-BE(2) cells transfected with miRNA (miR-Control or miR-323a-5p) using different delivery systems (Lipofectamine 2000 (LF2000) or Quatsomes (QS)). Absorbance measured by NanoDrop™ Lite Spectrophotometer.*

	n	Sample	Concentration (ng/µL)	A260/280
LF 2000 n=4	1	**miR-CT**	171.24	2.08
		miR-323a	144.76	2.09
	2	**miR-CT**	178.68	2.10
		miR-323a	55.12	2.09
	3	**miR-CT**	128.40	2.07
		miR-323a	87.76	2.01
	4	**miR-CT**	150.60	2.08
		miR-323a	90.20	2.08
QS n=4	1	**miR-CT**	177.68	2.07
		miR-323a	288.16	2.08
	2	**miR-CT**	397.74	2.08
		miR-323a	293.16	2.08
	3	**miR-CT**	283.52	2.00
		miR-CT	306.52	2.05
	4	**miR-CT**	366.28	2.07
		miR-323a	341.64	2.07

4.4 Validation of selected gene primers

To validate the specificity of the designed primers, a PCR reaction with two sets of primers per gene was set up with cDNA of SK-N-BE(2) cells and visualized in an agarose gel by electrophoresis (Figure 4).

For each pair of primers, two negative controls were loaded corresponding to a sample without retrotranscriptase when performing the retrotranscription and a sample without cDNA. L27 primers were used as a positive control.

Figure 4A shows that both primer pairs of *FTH1* present a clear band corresponding to the expected molecular size of the primers' product. Primer pair nº1 was selected. For *FBXL7*, primer pair nº1 was chosen since was the only one to show clear band with the expected size.

In Figure 4B, for *CAPN6*, a clear band is observed in both primer pairs. Thus, as previously done, primer pair nº2 is selected due to its shorter amplification product. For *CKAP2L*, amplification is also observed in both primer pairs. However, more than one band is observed in primer pair nº2, indicating that the primer might amplify other genomic regions besides the gene of interest. Therefore, primer pair nº1 is chosen for this gene.

In the case of the upregulated genes *H2AC6* and *SNX32* (Figure 4C), amplification bands are observed in both primer pairs for both genes. For H2AC6, the primers amplifying the smaller product were selected, which corresponds to primer pair nº2. For *SNX32*, a faint band apart from the one corresponding to the expected size was observed in the first primer pair, indicating that these primers amplify another fragment that does not correspond to this gene.

Finally, for *CNGB1* and *COL11A2* (Figure 4D), none of the designed primer pairs show a clear PCR product, thereby indicating that none of the primers can be used or that the levels of these genes are below detection levels.

In summary, primer pair nº1 was selected for the genes *FTH1*, *FBXL7* and *CKAP2L*, and primer pair nº2 was selected for *CAPN6*, *H2AC6* and *SNX32*. None of the designed primers could be validated for *CNGB1* and *COL11A2*.

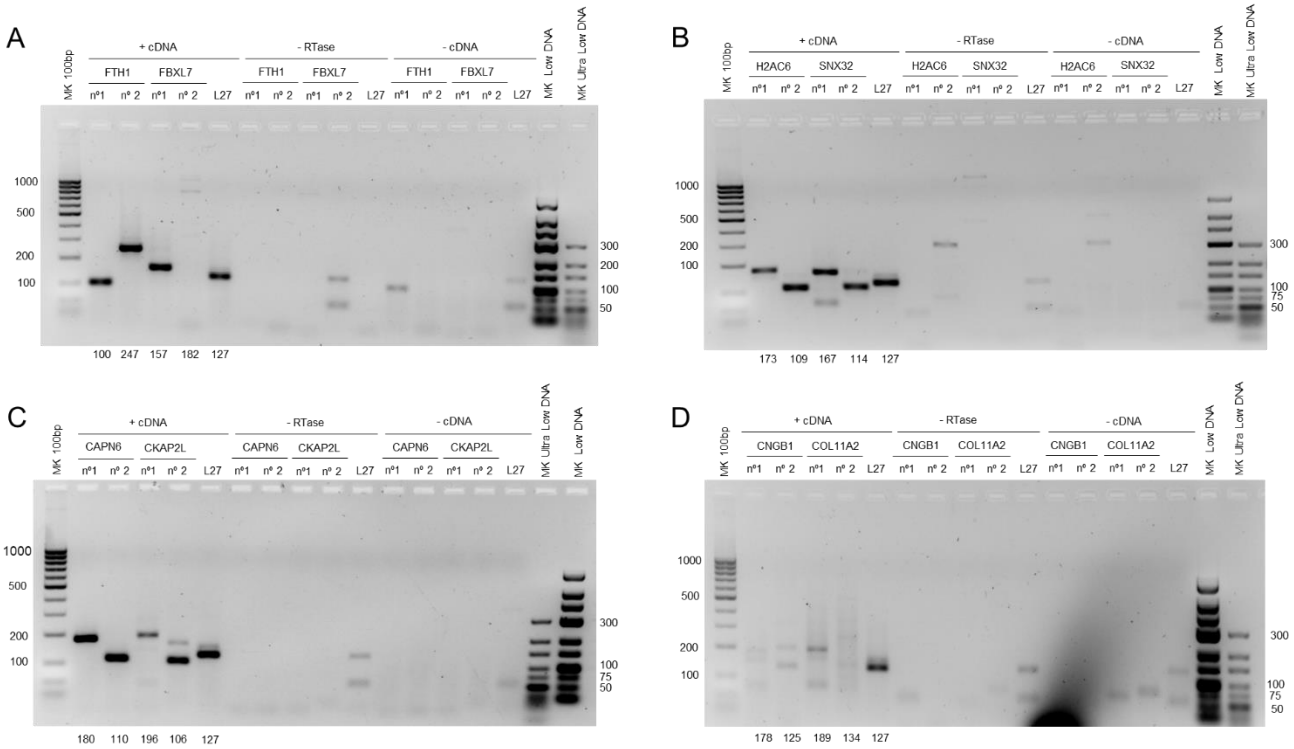

Figure 4. *Gene primer pairs designed were validated by PCR. Agarose gel electrophoresis (2%) of PCR products validating the primer genes selected was performed using a pool of cDNA from SK-N-BE(2) cells transfected with miR-Control. In lanes 2 and 3, primers 1 and 2 for FTH1 (A), CAPN6 (B), H2AC6 (C) and CNGB1 (D) are found. In lanes 3 and 4, primers 1 and 2 for FBXL7 (A), CKAP2L (B), SNX32 (C), and COL11A2 (D) are found, all with cDNA. As negative controls, from lane 7 to 11, the respective primers of each gel were used with samples without RTase during the retrotranscription and from lane 12 to 16, no cDNA was loaded. L27 primer was used as a positive control. Scale is shown in base pairs (bp).*

4.5 Validation of RNA-seq results by qPCR

Genes selected from the RNA-seq analysis were validated by qPCR using samples of SK-N-BE(2) cells transfected with miR-323a-5p (Figure 5). Figure 4 compares the RNA-seq with the qPCR results of the expression based on the Log_2FC of cells transfected with miR-323a-5p versus miR-Control. *CCND1* and *CHAF1A* are genes that were previously demonstrated to be downregulated after miR-323a-5p transfection.

QPCR results for downregulated genes follow the same trend than RNA-seq analysis results although the high variability between samples observed for *FTH1* and *FBXL7* prevent us from confidently asserting that these similarities are accurate. For *H2AC6* and *SNX32*, the qPCR values show a slight upregulation, although not reaching the levels observed in the RNA-seq data. For *CHAF1A*, the values obtained from RNA-seq and qPCR are very similar. In contrast, *CCND1* appears to be more downregulated in the qPCR samples.

Overall, the selected genes exhibit slightly more differential expression compared to the genes previously used as response biomarkers (e. g. *CCND1* and *CHAF1A*).

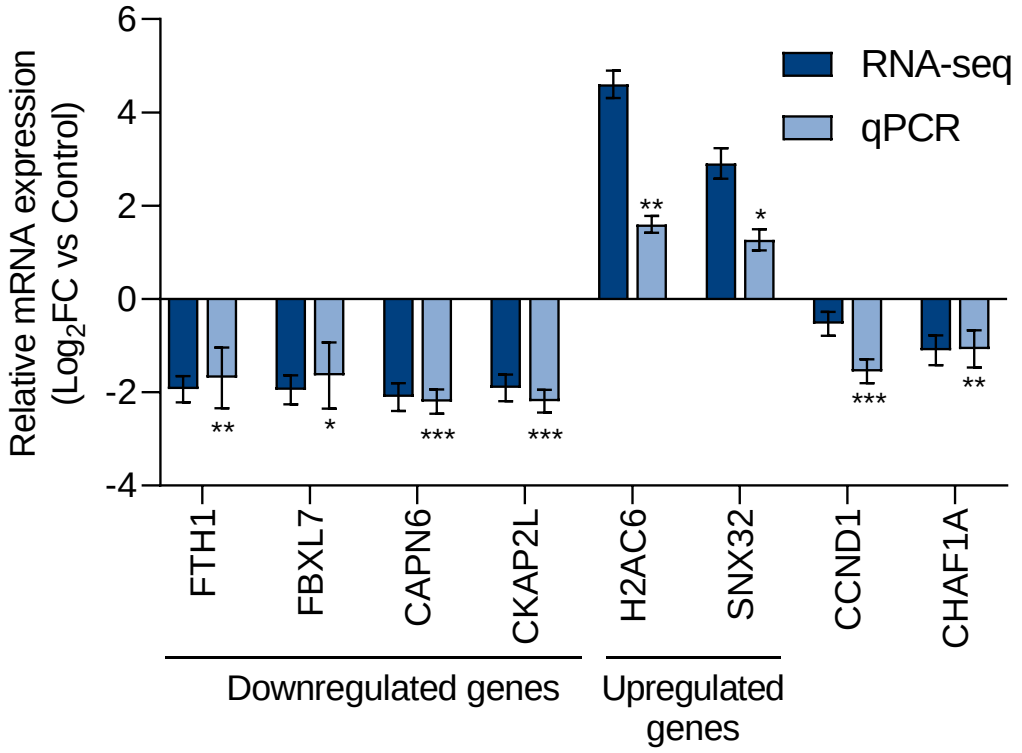

Figure 5**. Predicted miR-323a-5p target genes by RNAseq were validated by qPCR. Relative gene expression of miR-323a-5p target genes detected by qPCR compared to RNAseq data in SK-N-BE(2) cells transfected with the LF2000-miR-323a-5p versus LF2000-miR-Control. FC means fold change and samples were normalized using the housekeeping gene L27. Graph represents the mean ± SEM of four independent experiments. * denotes statistical significance difference compared to control. * means p < 0.05,** p < 0.01, p < 0.001.*

4.6 Comparison of differentially expressed miR-323a target genes using two different delivery systems

To determine if the potential of the selected genes as response biomarkers is the same regardless of the transfection system used to overexpress miR-323a-5p in NB cells, we compared the expression levels of the selected genes in NB cells transfected using Lipofectamine 2000 (LF 2000) or Quatsomes (QS).

Figure 6 represents in Log_2FC the modulation of candidate genes (*FTH1*, *FBXL7*, *CAPN6*, *CKAP2L*, *H2AC6*, and *SNX32*) and some of the genes used as markers so far (*CCND1* and *CHAF1A*) normalized to the housekeeping gene *L27*.

Focusing on the downregulated genes *FTH1*, *FBXL7*, *CAPN6* and *CKAP2L*, their expression does not decrease as much as expected and a decrease in differential expression is observed in QS compared to LF 2000. The variability observed in *FTH1*

and *FBXL7* makes it difficult to confirm if differential expression is affected using QS. However, these observed differences are significant for *CAPN6* and *CKAP2L*, which are genes where it cannot be significantly stated that transfection using QS modulates their expression compared to the control. That is, transfecting the cells with these nanoparticles influences the reduction in the differential expression of these genes compared to the control.

Regarding *H2AC6* and *SNX32*, genes that should be upregulated, they do not appear to be in the RNA extracts of cells transfected with QS. In contrast, both genes are significantly upregulated when using LF2000. Therefore, we can conclude that the transfection with QS was less efficient.

For *CCND1* and *CHAF1A*, no significant modulation was also observed in QS-transfected samples, showing that QS transfection is less effective compared to LF2000.

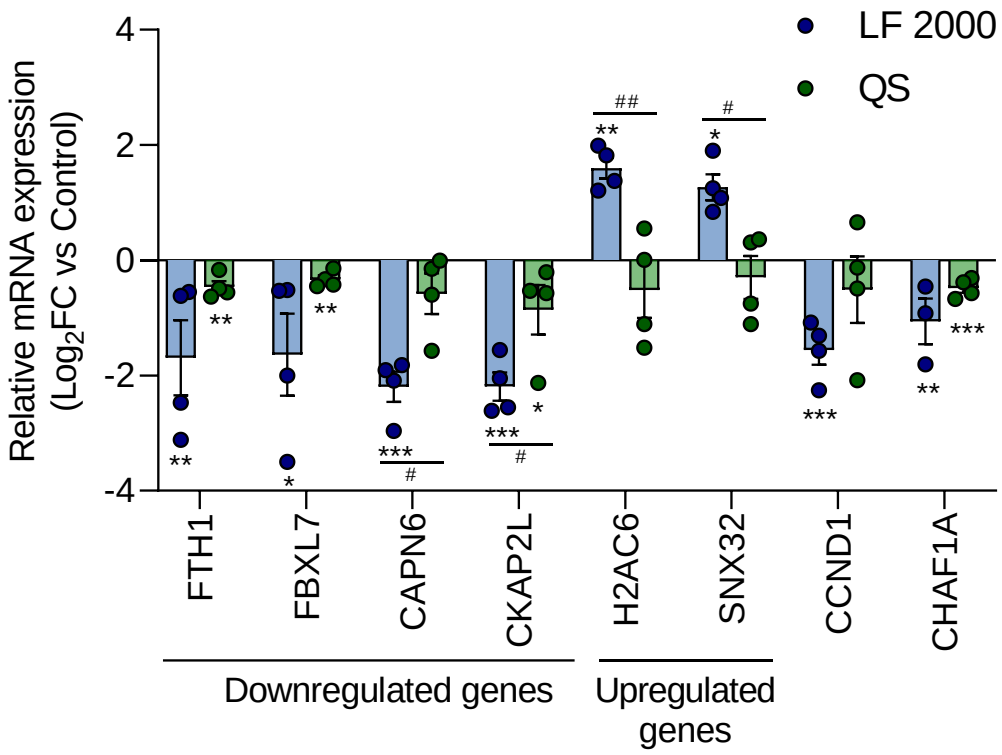

Figure 6. *Comparison of miR-323a-5p target genes relative expression versus control in NB cells transfected using different delivery systems. Gene expression is detected by qPCR in SK-N-BE(2) cells transfected with the miR-323a-5p using Lipofectamine 2000 (LF 2000) or Quatsomes (QS). FC means fold change and samples were normalized using the housekeeping gene L27. Data are presented as the mean ± SEM of four independent experiments. * denotes statistical significance difference compared to control. # denotes statistical significance difference between delivery systems. * and # means p < 0.05,** and ## means p < 0.01,*** and ### means p < 0.001.*

5. Discussion

MiR-323a-5p exerts tumor suppressor functions in neuroblastoma, making it a promising therapeutic candidate for neuroblastoma treatment. It has been shown that this microRNA intervenes in cell cycle pathways causing cell cycle arrest and promoting cellular differentiation (Soriano et al., 2019). Therefore, overexpression of miR-323a-5p in NB cells can have a great effect in reducing these cancerous cells proliferation. Certain genes such as CCND1, CHAF1A or p27 involved in the cell cycle are regulated by miR-323a-5p. Measuring the expression levels of these gens or quantifying their protein levels can phenotypically reflect the effects of treatment with this microRNA. However, these genes do not optimally reflect the treatment's efficacy. Consequently, this study aimed to identify genes that show greater modulation in response to miR-323a-5p, in order to find more effective response biomarkers than those identified so far. This exploration is made possible through RNA-seq analysis followed by validation using qPCR, considering different transfection systems to evaluate the consistency of the results.

5.1 Identification and characterization of potential candidates as response biomarkers

Through RNA-seq analysis, the eight most differentially expressed genes were identified. This selection includes read counts thresholds and statistical significance cutoffs to ensure that this gene list is as robust and relevant as possible. The inclusion of both upregulated and downregulated genes was also considered to give a broader view of the role of miR-323a-5p in the various pathways where it may be associated. *FTH1*, *FBXL7*, *CAPN6* and *CKAP2L* were the candidates selected as downregulated genes and *H2AC6*, *SNX32*, *CNGB1* and *COL11A2* were the upregulated candidate genes. Each of the selected genes was characterized with detailed information on their main function, genomic location or main cell signaling pathways where they participate. A clear relationship between the functions carried out by the different genes as well as the pathways in which each of them is involved could not be established. This reflects the wide variety of metabolic pathways in which miR-323a-5p may be involved.

Information on the possible implications of these genes in cancer has also been included, highlighting *CAPN6*, whose activity has been linked to different types of cancer (Liu et al., 2011), *FBXL7*, which has been reported to function as either an oncoprotein or a tumor suppressor in different cancers (Sanchez-Diaz et al., 2017; Y. Wang et al., 2022), *FTH1*, that has been shown to be involved in various cancers acting as either an oncogene or a tumor suppressor (Ali et al., 2021; Di Sanzo et al., 2011, 2020) and *CKAP2L*, which has been reported to have an oncogenic role related to cell-cycle

progression (Q. Li et al., 2022; Y.-F. Li et al., 2020; P. Wang & He, 2020). However no significant information was obtained within the context of NB.

This compilation also included the regulatory role of miR-323a-5p on the genes, with only *CAPN6* and *SNX32* among the total of eight genes were predicted to be direct targets of the miRNA. The question could arise as to how the remaining genes are modulated if miR-323a-5p is not predicted to bind. This is answered by the wide variety of indirect regulatory mechanisms by microRNAs, such as modulating gene expression by regulating their transcription factors or even acting on some of their cofactors. They can also act by controlling cell signaling, affecting signaling cascades and consequently the transcriptional activity of various genes. MiRNAs are also capable of influencing chromatin and exert epigenetic modifications that clearly affect gene expression (Cai et al., 2009). Some of these mechanisms, among others, explain how miR-323a-5p could indirectly regulate the expression of the studied genes.

It was also found that most of the selected genes exhibit lower expression levels in SK-N-BE(2) (cell line used in the study) compared to other NB cell lines (Uhlen et al., 2017). This may not be clinically representative and suggests that future studies should also consider testing the detection of these genes in different neuroblastoma cell lines.

5.2 Assessing potential response biomarkers by qPCR

The RNA-seq results were validated using qPCR. The primers designed for *CNGB1* and *COL11A2* did not show amplification in the primer validation test prior to qPCR, a fact that could be related to the low levels of expression of these genes, especially in the samples transfected with miR-Control or even to a non-optimal primer design. Attempts were made to amplify these genes with samples transfected with miR-323a-5p (instead of the control samples used with all the other genes), which were expected to have higher expression levels, and even with other cell lines, but no amplification band was observed in either case. Consequently, we think that this amplification process could be improved in the future by optimizing the PCR conditions, such as adjusting the annealing temperatures or even testing different primer sequences to ensure that the lack of amplification is not due to the low expression levels of the genes. However, for the remaining genes it was possible to select a pair of primers that efficiently amplified the corresponding gene during the qPCR.

Validation from qPCR generally confirmed the RNA-seq results, especially in downregulated genes (*FTH1*, *FBXL7*, *CAPN6* and *CKAP2L*). *H2AC6* and *SNX32*, which were expected to be upregulated, showed a trend of increased differential expression compared to the control in the qPCR, but did not reach the levels observed in the RNA-

seq. Nevertheless, high variability was observed that could potentially affect results reliability. This is attributed to the variability among replicates, which were originated from four independent experiments. Even though the same conditions were followed in the procedures, processes such as cell culture and transfection were conducted at different times, were technical factors like normalization, the efficiency of retro-transcription, variability in RNA extraction or biological factors such as transfection efficiency or intrinsic cellular variability could be responsible for this variation. Regarding the normalization used, in this work, the housekeeping gene L27 was used to normalize the expression of the study genes. Housekeeping genes should not initially show any variation in expression between samples transfected with miR-323a-5p and the control samples, or if it did, this would be due to cell-to-cell variability as they are expected to maintain expression levels in all conditions (Eisenberg & Levanon, 2013). However, in this study, in some cases some variability in L27 expression levels was detected, which could have influenced in some way this variability between replicates and at some point, the detection of the most modulated genes. In Mercatelli et al. (2021), ACTB and B2M were used as housekeeping genes in SK-N-BE(2), where they report detecting high expression and very low variance, suggesting the potential use of these housekeeping genes in further studies as an improvement proposal.

Despite these drawbacks and even the validation of RNA-seq results, no significant improvements in modulation have been observed compared to *CCND1* and *CHAF1A*, which prevents the selection of a clear candidate as a response biomarker that improves upon the biomarkers currently used in *in vivo* studies.

5.3 Comparison of delivery methods

The efficiency of two different delivery systems to transfect cells with miR-323a-5p was compared: LF 2000, a commercially-available formulation, commonly used in *in vitro* studies, and QS, an experimental non-liposomal lipid nanovesicles with an unilamellar structure, which provide a stable and efficient platform for clinical delivery of miRNAs, protecting them from RNases and allowing delivery to the tumor site (Boloix et al., 2022). Our results showed significant differences in gene modulation between the two delivery systems. QS do not achieve the same efficiency in gene modulation as LF2000, showing reduced differential expression for downregulated genes and high variability in upregulated genes. This could be related to differences in miRNA delivery efficiency, miRNA-QS complex stability or differences in the cellular internalization pathway used for QS-miRNA complexes. Consequently, a clear candidate for a response biomarker could not be selected using QS as the delivery method. It should be noted that we are currently working on the RNA-seq analysis of *in vitro* samples transfected with miR-323a-

5p using QS, which will serve for a future comparison of differentially expressed genes with what has been observed using LF 2000.

5.4 General insights and future improvements

Although observing that selected genes are modulated in response to miR-323a-5p, the results obtained *in vitro* do not reveal a clear candidate response biomarker that significantly improves those actually used (i. e. *CHAF1A* and *CCND1*). Furthermore, Quatsomes, employed in *in vivo* experiments, showed reduced gene modulation and thus, not useful to detect the effects of miRNA transfection efficiently. To fully complete this study, experimental validation of the selected genes in *in vivo* models is necessary to confirm or refute their biological and therapeutic relevance as response biomarkers.

For future investigations, several options can be considered for implementation. It would be interesting to extend RNA-seq analysis using different cell lines and samples from *in vivo* experiments to validate the generalizability of the identified genes. Moreover, while our focus has been on protein-coding genes, it is worth noting that there are also many non-coding RNAs that might serve as better biomarkers. Incorporating the analysis of these non-coding RNAs in future studies could improve the identification of new potential response biomarkers. Additionally, the implementation of multi-omics analysis would be enriching. Targeting the transcriptome might not be the optimal way to detect the miR-323a-5p effects, so considering proteomics, metabolomics or even combining them with transcriptomics could provide a more complete vision of the impact of miR-323a-5p on NB cells.

6. Conclusions

- RNA-seq analysis identified 8 genes that were modulated by miR-323a-5p that could act as potential response biomarkers.
- QPCR analyses confirmed most of the results obtained by RNA-seq, especially for the downregulated genes (*FTH1*, *FBXL7*, *CAPN6* and *CKAP2L*). For the genes expected to be upregulated (*H2AC6* and *SNX32*), the same levels of modulation were not detected in qPCR.
- Significant differences in gene modulation were detected between the two delivery systems with QS showing lower gene modulation efficiency compared to LF2000.

Although the study has identified and validated several genes exhibiting modulation in response to miR-323a-5p, a clear response biomarker that significantly improves those currently used *in vivo* was not found. The differences between the delivery systems and the experimental variability highlight the need to implement *in vivo* testing, to perform additional studies with other NB cell lines, to incorporate non-coding RNAs analysis and to consider proteomic, metabolomic or even multi-omic approaches for a better understanding of the impact of miR-323a-5p in NB.

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