Growth Inhibition of Retinoblastoma Cell Line by Exosome-Mediated Transfer of miR-142-3p

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Introduction: Retinoblastoma (Rb) is the most common ocular paediatric malignancy and is caused by a mutation of the two alleles of the tumor suppressor gene, RB1. The tumor microenvironment (TME) represents a complex system whose function is not yet well defined and where microvesicles, such as exosomes, play a key role in intercellular communication. Micro-RNAs (mRNAs) have emerged as important modifiers of biological mechanisms involved in cancer and been able to regulate tumor progression.

Methods: Co-culture of monocytes with retinoblastoma cell lines, showed a significant growth decrease. Given the interaction between Rb cells and monocytes, we investigated the role of the supernatant in the cross-talk between cell lines, by taking the product of the co-culture and then using it as a culture medium for Rb cells.

Results: miR-142-3p showed to be particularly over-expressed both in the Rb cell line and in the medium used for their culture, comparing to control cell line and the normal supernatant, respectively. Therefore, we provided evidence that miR-142-3p is released by monocytes in the co-culture medium’s exosomes and that it is subsequently up-taken by Rb cells, causing the inhibition of proliferation of Rb cell line by affecting cell cycle progression.

Conclusion: This study highlights the role of exosomic miR-142-3p in the TME of Rb and identifies new molecular targets, which are able to control tumor growth aiming the development of a forward-looking miR-based strategy.

Keywords: tumor microenvironment, microvesicles, microRNAs, co-culture, monocytes, cross-talk

Dr Dino Amadori passed away in 2020. His contributions to this study were essential and pivotal. His scientific contributions to the retinoblastoma disease in Africa will be remembered from generation to generation.

Introduction

Retinoblastoma (Rb) is the most common ocular paediatric malignancy that arises from the retina, it is caused by a mutation of the two alleles of the tumor suppressor gene RB1. and it is the first to be recognized as being connected to hereditary genetic defects. Therapeutic approaches include surgery (enucleation), chemotherapy (systemic, intrarterial and in some cases periocular) and radiotherapy. The incidence is of almost one in 15,000–20,000 births, resulting in a cancer type with a substantial social impact.

RB1 gene is a central regulator of cell cycle and its tumor suppression function is widely known due to the inhibition of the E2F1 transcription factor, which is responsible for the cell’s transition from G1 to S phase of the cell cycle. Rb can be unilateral or bilateral. Non-heritable cases are caused by somatic inactivation of both alleles of the RB1 gene, while heritable Rb presents a germline mutation of RB1, which is followed by a second somatic inactivation of the other allele.

Studies have already demonstrated that the tumor microenvironment (TME) has a pivotal role in the progression of many types of cancer including Rb. The inflammatory infiltrate associated with many tumors is able to modulate the biology of
cancer cells, both with anti- and pro-tumoral effects. In particular, macrophages, that surround the tumor and are able to release growth factors and cytokines, stimulate angiogenesis, tumor growth and its metastatic capacity.14–20 MicroRNAs (mRNAs) are small non-coding RNAs (ncRNAs) which have emerged as important modifiers of a plethora of biological mechanisms including those involved in cancer,21–28 and specifically in Rb.29

As previously introduced, the tumor microenvironment assumes, in this already complex system, a key function not yet well defined in intercellular communication that develops also through important micro-vesicles, including exosomes.30,31 Exosomes are extracellular vesicles with diameter less than 150nm, which are released after fusion of late endosome multivesicolar bodies (MVBs) with the plasma membrane. They contribute to inter-cellular paracrine signalling mechanisms, within TME, by carrying and transferring their cargo of RNA, DNA and proteins from one cell to another.32,33

It was also demonstrated, that exosomal miRNAs contribute to cancer proliferation and drug resistance.30,34,35

These inspirational discoveries identified a pivotal mechanism of action of miRNAs, which, through their transport from exosomes, are able to modify the cancer cell progress.

In this study, we tried to elucidate the cross talk through co-culture experiments between an Rb cell line and monocytes. We noticed that the medium derived from their co-culture contains exosomes, which act as miR-vectors and transfer miR-142-3p, already identified as an exosome transported miR.36 Rb cell line uptakes these miR, which is able to modulate, significantly, cells’ proliferation activity.

Among other genes, we identified TGFβR1 gene as one of the direct target genes of miR-142-3p, as reported also in other studies.37,38 Previous reports have demonstrated that TGFβ pathway is associated with the development of various types of cancer.39–42

Considering the clinical relevance of these findings, we decided to develop an embryonic zebrafish model, for its anatomic characteristics,43–45 which could offer the opportunity to study the tumor progression and invasiveness using retinoblastoma cell lines.

To our knowledge, this study is the first to investigate using a zebrafish model, how miRNAs can regulate tumor development in retinoblastoma

**Materials and Methods**

**Cell Preparation**

Human CHLA-215 cells were gently provided by Dott. David Cobrinik (Children’s Hospital Los Angeles, Los Angeles, California, 90027, USA). The cell line was cultured in IMDM (American Type Culture Collection, ATCC 30–2005) supplemented with 20% final concentration of FBS exosome depleted (Gibco Ref. A27208-03), 100mg/mL penicillin/streptomycin and MycoZap Prophylactic (Lonza) to a final concentration of 0.002%. Cells were tested free of mycoplasma and other contaminants.

Monocytes were isolated from peripheral blood samples obtained by healthy volunteers. Briefly, human blood was diluted with PBS 1X solution at 1:2 dilutions. Next, the diluted sample was layered onto Leucoscept tube and separated by Ficoll density gradient centrifugation to isolate the mononuclear cell fraction. In order to remove the excess of erythrocytes, we used ACK 1X lysing buffer (8.29g NH4Cl, 1g KHCO3, 37.2mg EDTA) to lyse red blood cells and obtain a sample, which contains principally white blood cells. Monocytes were isolated from PBMCs by exploiting their ability to adhere to glass or plastic and the additional use of Monocyte Attachment Medium (PROMOCELL C-28051). For that purpose, PBMCs were cultured for 4 hours in RPMI 10% FBS exosome depleted, 100mg/mL penicillin/streptomycin and MycoZap Prophylactic (Lonza) to a final concentration of 0.002%. Finally, it was possible to isolate attached monocytes by removing the culture media including the rest of lymphocytes.

We determined the purity of isolated monocytes by staining the cells with Human CD14 PerCp antibody Miltenyi (cod. 130-094-969) and analysing them using fluorescence-activated cell sorting (FACS) (Appendix 1). All experiments were repeated 3 times recruiting different healthy donors in order to guarantee experiment’s reproducibility.

All the cell lines above, were grown at 37°C in a 5% CO₂ atmosphere, unless indicated otherwise.

**Cell Counting Method**

We count cells using TC20 AUTOMATED CELL COUNTER and we assessed cell viability based on cell permeability via trypan blue exclusion.
Macrophages and Rb Cell Co-Culture
Co-cultivation of macrophages and retinoblastoma cell line was performed in 6-well plates (Corning). Retinoblastoma cell lines were seeded on the 0.4μm inserts (Corning), which are permeable to supernatants but not to cellular components.

Monocytes were seeded in the lower chambers (CHLA-215/monocytes ratio 1:25) and both were grown for the indicated periods of time. Cells were harvested at the indicated timepoints and supernatants were conserved for further analysis.

Exosome Purification
Exosome isolation was based on Size Exclusion Chromatography Method (SEC) using the q-EV columns according to the manufacturer’s recommendations (Izon). Briefly, cell supernatant, from ten 6-well plates, was collected and both cells and cellular debris were removed by centrifugation at 3000g for 15min. The resulting supernatants were also filtered through a 0.22 μm filter and then were transferred to a filter concentrate disposal (Centricon-Plus 70) in order to reduce sample volume.

Briefly, q-EV column was equilibrated with PBS 1X. Previously concentrated cell supernatant was loaded onto the column and 500μL fraction collection started immediately adding PBS as elution buffer. We collected for each sample, a total of 20 fractions.

All fractions were further analysed with NANOSIGHT in order to determine the concentration of isolated vesicles in each one. Evaluation of EV distribution by NTA (Nanoparticle Tracking Analysis), showed an enrichment of fractions 7, 8, 9.

Nanoparticle Tracking Analysis (NTA)
Exosomes were analyzed using a NanoSight NS300 instrument equipped with a 405 nm laser (NanoSight) at 25°C. Particle quantification and movement was tracked by NTA software (version 3.2). Vesicle’s concentration (in millions), for each size, was obtained analyzing each track. In the end of analysis, three videos of 60 seconds were recorded for each sample. Data analysis was performed with NTA 3.2 software (Nanosight). When fractions contained high numbers of particles, we further diluted them in PBS before analysis and the final concentration was then calculated according to each dilution factor. For each sample were recorded three videos of either 30 or 60 sec and data are presented as the mean ± SD of the three video recordings.

MACSPlex Analysis
The MACSPlex Exosome Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) permits the detection of 37 exosomal surface epitopes (CD3, CD4, CD19, CD8, HLA-DR, CD56, CD62P, CD11c, CD81, MCSP1, CD146, CD41b, CD42a, CD24, CD86, CD44, CD326, CD133/1, CD29, CD69, CD142, CD105, CD2, CD1c, CD25, CD49e, ROR1, CD209, CD9, SSEA4, HLA-BC, CD63, CD40 CD45, CD31, CD20, and CD14) plus two isotype controls (REA and IgG1) (Appendix 2).

EV-containing samples were processed as follows: we used 120μL of EVs isolated from cell supernatant. After overnight incubation at room temperature protected from light, we proceed with flow cytometry sample acquisition using a BD FACSCanto (Becton Dickinson, San Diego, CA, USA), which is equipped with two lasers, 630nm and 488nm, capable of detecting the necessary fluorescence signals. To normalize data, we used one negative control sample (MACSPlex Buffer only) in each run experiment to determine and subtract non-specific signals. Finally, we obtained exosomal surface epitope concentrations calculating the ratio of beads + EVs + Ab to the corresponding controls (beads + Ab) Values below the corresponding control were regarded as negative. All samples were processed following the manufacturer’s instructions. Data analysis was performed using the corresponding software (BD FACSDiva).

RNA Isolation, qRT-PCR Analysis and microArray Profiling
Total RNA was isolated from cells and exosomes using mirVana kit (Life Technologies) and miRneasy kit as well (QIAGEN). Reverse transcriptions were performed using the Taqman Advanced miRNA cDNA Synthesis Kit (cod. A28007) and qRT-PCR was performed with Applied 7500 following the manufacturer's protocol.

For normalizations, GAPDH mRNA or miR-26a was used for the expression of TGFβR1 or mature miRNAs respectively. All reactions were carried out in triplicate. miRNA or gene expression levels were calculated using the ΔΔCt method. Specifically, for each sample is calculated the difference between the Ct values (ΔCt) of the gene of interest and the housekeeping
gene. Afterwards, we calculated the difference in the ΔCt values between the experimental and control samples ΔΔCt. The mean fold change in expression of the gene of interest between the two samples is then equal to \(2^{-\Delta\Delta C_{\text{t}}}}\).

miRNA expression profiling of retinoblastoma cell lines and exosomes was performed using the Affymetrix Genechip miRNA 4.0 array, according to the manufacturer’s instructions (Appendix 2).

**Protein Expression Analysis**

In order to evaluate the expression of TGFβR1 and CD9 we used Western blotting method. Proteins, after denaturation, were separated by electrophoresis using Criterion TGX Stain Free Gel Precast 4–20% (Bio-Rad Laboratories) gel and Laemmli Sample Buffer (Bio-Rad) added in ratio 1:1, 5% of β-mercaptoethanol (Carlo Erba Reagents). After electrophoretic run, proteins were transferred, using the Trans-Blot Turbo System (Bio-Rad Laboratories), on a PVDF membrane (Trans-Blot Transfer Turbo midiformat 0.2µm; Bio-Rad Laboratories). Following, in order to prevent non-specific binding of antibodies, we incubate the membrane with 5% non fat dried milk diluted in TPBS buffer for 3 hours.

We used the following primary antibodies: Vinculin clone FB11 (IgG1) monoclonal Ab (Biohit) 1:1000, TGFβR1 (Origene cod. APO1457P0-N) 1:1000, CD9 (D8O1A, Cell Signaling) Antibody 1:1000, beta IV Tubulin (TUBB4A) Mouse Monoclonal Antibody (HRP conjugated) [Clone ID: OTI5C1] 1:1000.

Secondary antibodies and dilutions used were the following: Precision Plus Protein Western C StrepTactin-HRP Conjugate (Bio-Rad) 1:10000, Goat anti-rabbit IgG-HRP.

Images were acquired using Chemidoc (Bio-Rad) and analyzed using ImageJ Software.

**Cells Transfection**

CHLA-215 cell line were seeded at 1×10⁶ per flask and after they were transfected with miR142-3p mirVana mimic or inhibitor (Ambion by Life technologies) and the corresponding Negative Control #1 at the final concentration of 25nM according manufacturer's protocol. For the miRNA transfection, we used TransIT-X2 Dynamic non-liposomal polymeridic delivery system (Mirus) following manufacturer's specifications.

**Zebrafish Husbandry**

AB wild-type zebrafish strain was handled in compliance with local animal welfare regulations (authorization n. prot. 18311/2016; authorization for zebrafish breeding in IRST facility released by the “Comune di Meldola”, 09/11/2016) and in conformity with the Directive 2010/63/EU. Fertilized eggs were obtained by natural spawning and maintained in embryo water with 0.1% of Methylene Blue at 28°C, according to Kimmel et al.⁴⁶

Figure 1 Decreased proliferation activity of retinoblastoma cell line CHLA-215 after co-culture with monocytes Mo. ****P< 0.0001.
**Tumor Xenograft in Zebrafish Embryos**

AB zebrafish embryos were dechorionated at 48 hours post-fertilization (hpf). We used 23 embryos for each condition. Before manipulation, embryos were anesthetized in 0.02% tricaine solution (Sigma-Aldrich). CHLA-215 retinoblastoma cells were collected by centrifugation, stained with CellTracker™ CM-Dil (Invitrogen) and resuspended in PBS at a concentration of 2.5 × 10^9 particles/ml. 

**Figure 2**

**A** Fr. 7

Mean 112.2 nm
Mode: 80.3 nm
Concentration: 5.91 × 10^9 particles/ml

**Fr. 8**

Mean 128.1 nm
Mode: 94.6 nm
Concentration: 1.16 × 10^10 particles/ml

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**B**

- **CD9**
- **CD63**
- **CD81**

**Figure 2 Continued.**
10^7/µL. After anesthetization, 300/500 cells were implanted in the yolk sack of embryos at 48 hpf. Embryos that show cancer cells in circulation were excluded. The three groups injected with CHLA-215 control (CTR), transfected with miR-142-3p mimic cells and transfection negative control were incubated at 34°C. At 24h post-injection (hpi), cancer masses were analysed using a fluorescence stereomicroscope (Nikon SMZ 25 equipped with NIS Elements software).

Data Analysis
We performed statistical analysis using GraphPad Prism version 6 statistical software (GraphPad Software, San Diego, CA, USA), using Multiple t test. Statistical significance was indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001.

qRT-PCR data, were analyzed using 7500 Software v2.0.6 for 7500 Real Time PCR System and relative expression levels were calculated using the method of comparative Ct (ΔΔCt).

Results
Co-Culture of Retinoblastoma Cells with Monocytes Decrease Rb Cell Proliferation Rate
We performed co-culture experiments between Rb cell line (CHLA-215) and monocytes from healthy donors. We observed that cell proliferation activity decreased markedly for up to 72h (Figure 1).

Exosomes Derived from Rb-Monocytes Co-Culture Medium, Decrease Proliferation Rate of Rb Cells
In order to assess whether co-culture medium’s exosomes (Figure 2) were responsible for the cell growth decrease, we isolated exosomes after 48h from the conditioned medium, mentioned above, and we added them in the normal culture medium. We noticed a significant growth decrease for up to 72h compared to the control cell line, as well (Figure 2).

Effect of Exosomic miR-142-3p Transferred from Human Monocytes to Retinoblastoma Cell Lines Mediated Rb-Monocytes Co-Culture Conditioned Medium
To reveal exosome’s content and understand which is the key-molecule responsible for growth decrease, we performed a microRNA profiling of exosomes isolated from the supernatant and retinoblastoma cell lines as well.

We noticed that miR-142-3p was not expressed in retinoblastoma cell lines but after their culture using the conditioned medium, miR-142-3p expression was increased.
In order to identify exosomes and their cargo as the main reason of growth inhibition of Rb, we depleted by ultracentrifugation, exosomes from the supernatant isolated after 48h and we observed reduced expression levels of miR-142-3p in CHLA-215 cells after culture with conditioned medium for up to 72h (Figure 3).

These data indicate that in absence of exosomes, miR-142-3p is non-present in the medium and consequently not expressed in Rb cells (Figure 4).

To further confirm that miR-142-3p is responsible for the growth rate decreased of retinoblastoma cells, we decided to transflect Rb cell line with corresponding miR mimic in order to assess whether modulation of miR-142-3p is related with cancer cell growth. Given that TGFβR1 is a direct target of miR-142-3p and their expression is conversely correlated, we used this gene to assess the cell transfection efficiency (Figure 5A–C). Cell growth curves indicated that cells transfected with miR-mimic manifest a significant growth decrease (Figure 6).

Figure 3 Exosomic miR-142-3p mediated cross-talk between retinoblastoma cell lines and monocytes. Quantitative real time polymerase chain reaction (q-RTPCR) for miR-142-3p in CHLA-215 after 48h culture with conditioned medium (c/m) and exosome-depleted medium (depl/m) by ultracentrifugation. Relative levels of miRNAs expressions were normalized to miR-26a. Data are presented as mean SD of experiments conducted in triplicate. CTR vs CM **P= 0.002 and CTR vs DEPL.MEDIUM ***P=0.0045.

Figure 4 Exosomic miR-142-3p expression in supernatants of monocytes control (Mo CTR), CHLA-215 control and supernatant, product of their co-culture (c/m). miR-142-3p is present as exosome cargo in the conditioned medium (c/m). Quantitative real time polymerase chain reaction (q-RT PCR) for miR-142-3p in supernatants’ exosomes after 72h of culture. Relative levels of miRNAs expressions were normalized to miR-26a. Data are presented as mean SD of experiments conducted in triplicate. s/n Mo CTR vs s/n CHLA-215 C.C ***P= 0.0002 and s/n CHLA-215 CTR vs s/n CHLA-215 C.C ***P=0.0001.
These data confirmed that miR-142-3p as monocyte’s exosome cargo, is able to decrease the proliferative ability of Rb cell line.

**Tumor Progression of CHLA-215 Cell Line Transfected with miR142-3p Mimic in Embryonic Zebrafish Model**

In order to investigate in vivo tumor development regulation by miR-142-3p, we transfected CHLA-215 cell line with miR-142-3p mimic and then we injected the cells to zebrafish embryos. After 24h, measuring the fluorescence signal, we noticed a significant tumor growth inhibition in the embryos injected with mimic-transfected cells. This data confirmed that miR-142-3p acts as tumor suppressor in vivo model, as well (Figure 7).

![Figure 5](https://doi.org/10.2147/CMAR.S351979)

**Figure 5** (A) CHLA-215 cell line transfected with miR-142-3p mimic. Quantitative real time polymerase chain reaction (q-RT PCR) for miR-142-3p in CHLA-215 after 48h. **P=0.0032.** (B and C) TGFβR1 inversely correlated to miR-142-3p expression after CHLA-215 transfection with miR-142-3p mimic or inhibitor. **P=0.0035, C.** **P=0.0051.** Quantitative real time polymerase chain reaction (q-RT PCR) gene expression for TGFβR1 in CHLA-215 cell line after 48h. Relative levels of miRs and gene expression were normalized to miR26a and GAPDH respectively. (D) Western Blot analysis of TGFβR1 expression inversely correlated to miR-142-3p. Data normalized using vinculin. Data are presented as mean SD of experiments conducted in triplicate.
**Figure 6** Cell growth curves indicated that cells transfected with miR-142-3p-mimic, manifest a significant growth decrease. ****P< 0.0001.

**Figure 7** (A) Representative fluorescence microscopy images of zebrafish embryos xenotransplanted with CHLA-215 cell transfected with miR-142-3p mimic. Images of embryos at 2 and 24 hours post injection, scale bar 100 µm. (B) Mean fluorescence signal CHLA-215 cell transfected with miR-142-3p mimic xenotransplanted embryos arbitrary units. *P< 0.03. (C) Tumor growth inhibition rate between CTR neg transf. and mimic transf. conditions. *P< 0.01.
Discussion

Retinoblastoma is a rare paediatric tumor with a great impact on patients, families and society.\textsuperscript{5,7} However, despite its importance, its low incidence has narrowed the fields of research regarding some aspect of this cancer.

Nevertheless, this type of cancer can be either hereditary or non-hereditary and although early detection provides the opportunity of controlling the primary tumor with effective therapies, metastatic activity remains fatal.

In the last years, tumor microenvironment has attracted the attention of the wide scientific community, given that it is already proved that cancer is a multifactorial disease, which does not involve only cancer cells but interacts significantly also with surrounding cells.\textsuperscript{9–13}

The herein presented investigation aimed to shed some light on the tumor microenvironment by applying approaches that enabled us to overcome the experimental obstacles hindering the identification of the cross talk between cancer cells and the “system” surrounding them, opening a path towards a potentially new therapeutic perspective. The TME of Rb either can promote or inhibit cancer cell growth, affecting this cancer’s clinical behaviour.\textsuperscript{13} Macrophages, one of the principal elements of inflammatory infiltrate, originate from precursor monocytes and are highly plastic cells, capable of responding to slight changes in the microenvironment by initiating several immunological activation programs (referred to as polarization of macrophages), which can induce tumor reprogramming.\textsuperscript{18–20}

Micro-RNAs are small non-coding RNAs that are able to regulate gene expression and modulate many biological processes. They appear to be dysregulated in almost all human cancers, including retinoblastoma and consequently they might be exploited as predictive tools as well.\textsuperscript{29,49}

In particular, miR-142-3p showed a highly specific role for myeloid’s and hematopoietic’s cells both formation and differentiation.\textsuperscript{50,51} Nevertheless, it achieves different effects depending on the involved tumor. In fact, whilst on the one hand, it has a role as tumor suppressor in various cancers like colorectal,\textsuperscript{52} breast,\textsuperscript{53,54} hepatocellular\textsuperscript{55,56} and bladder,\textsuperscript{57} cancer, on the other hand Qi et al underlined its onco-miR function in nasopharyngeal carcinoma\textsuperscript{58} due to its effects on tumor cell proliferation.

TGF\(\beta\) is a family of extracellular signalling molecules with a central role in many cellular processes like cell growth, differentiation, death and migration. In normal cells, it acts as a tumor suppressor, as it inhibits their growth and transmits signal through cell-surface serine threonine receptors to the intracellular transcription factor, Smad. Being more specific, TGF\(\beta\) dimer binds with TGF\(\beta\)R II on the cell surface. Afterwards, the dimer binding leads to the formation of type I and type II receptor complex where type II receptor triggers the phosphorylation and activation of type I receptor. The activated ligand-receptor complex binds to the intracellular Smad proteins, which consequently dimerizes and moves to the nucleus where they can activate transcription of target genes.\textsuperscript{39,59–61}

Exosomes are lipid-bilayer-based extracellular vesicles (EVs) that are produced in the endosomal compartment of most eukaryotic cells microvesicles and can act as versatile mediators facilitating the paracrine cell to cell communication mediated their RNA, DNA and protein cargo. Therefore, they are linked to the development of various physiological processes, including cancer progression.\textsuperscript{32}

A plethora of studies exposed a correlation between immune system cells and a favourable antitumor response.\textsuperscript{30,62,63} This correlation prompts further investigations whether the paracrine exchange of miRs as exosomal cargo between Rb and surrounding monocytes, can or cannot influence tumor progression.

This study identified exo-miRs able to be transferred from monocytes to the recipient Rb cell and to modulate its proliferation capacity.

Specifically, we first observed that co-culture with monocytes decreased Rb cell-line proliferation activity. Isolating exosomes from Rb-monocytes co-culture medium, we assessed that they were responsible for this effect. Afterwards, we investigated exosome’s cargo trying to identify the key molecules that could be able to cause growth diminution.

Hence, using a microRNA profiling, we individuated miR-142-3p whose expression was upregulated in monocytes, it was subsequently transferred to the supernatant by exosomes and was finally uptaken by Rb cells, managing to diminish their proliferative activity.

The use of multiple bioinformatics tools (http://www.targetscan.org, http://mirdb.org) allowed us to predict miR-142-3p possible target genes in order to correlate its onco-suppressive function to a pathway and understand better the mechanism.
underlying the diminution of proliferative activity. The fact that we identified TGFβR1 as one of miR-142-3p target genes, raised our interest and could be an ulterior hint for further researches. TGFβ pathway, it is considered to have a pivotal role in cell proliferation and consequently it is related to cancer development. To be more precise, the principal cause was confirmed to be the dysfunction of receptor pathways, which leads to incapability to regulate cell growth.

Embryonic zebrafish (Danio rerio) model has identified as a valid alternative of murine model. Some of its characteristics, namely the transparent body and the embryo’s incomplete immune system that prevent the rejection of injected tumor cells, are very useful and offer the opportunity to detect cancer processes. The fluorescent circulatory system is another feature, which permits the detection of metastatic activity.

Moreover, zebrafishes share a fair number of characteristics with mammals, which represents an added value especially when applied experiments demand to closely imitate human organisms and physiological procedures.

Those qualities confirmed the herein exposed cell dynamics also in vivo. Indeed, the cells injected with transient miR-142-3p overexpression, confirmed the tumor suppressor function of this miR also when planted in zebrafish, showing a limited tumor progression and dissemination outside the injected area comparing to the control Rb injected cells.

Moreover, previous researches reinforced our early hypothesis by showing how exosomal miR-29a and miR-21 released by non-small cell lung cancer succeed to bind to human TLR8 receptor in macrophages of microenvironment and induce an increased secretion of IL-6-mediated NF-κB and TNF-α pathway, which then leads to cancer cell enhanced growth and metastatic activity.

To our knowledge, the present study represents the first one that shed some light on retinoblastoma progression and regulation using zebrafish in vivo model. Further analysis could ultimately try to extend obtained results to other tumor’s types.

**Conclusion**

The above presented data, suggest a role of exosomic miR-142-3p in retinoblastoma. We propose that the cross-talk between monocytes and Rb cell lines could be able to modulate cancer cell proliferation and further confirmation was gathered by using zebrafish model as well. In conclusion, our study shed lights on the role of miR-142-3p and opens the path towards the investigation of this miR as a potential therapeutic approach in retinoblastoma, aimed at inhibiting tumor progression.

**Ethics Statement**

The study involved healthy donors, which were enrolled for the study protocol approved by IRST-Area Vasta Romagna Ethics Committee, approval no. 2411/2020/5.1, 27 March 2020.

The study was conducted according to the Good Clinical Practice standard operating procedures and 1975 Helsinki declaration.

All healthy donors gave informed consent for participation in the research study.

**Disclosure**

The authors report no conflicts of interest in this work.

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