UNIVERSITY OF VERONA

DEPARTMENT OF MEDICINE

DOCTORAL SCHOOL OF LIFE AND HEALTH SCIENCES

PhD PROGRAM IN BIOMOLECULAR MEDICINE

Clinical Proteomics and Genomics Curriculum

XXXIII CYCLE

IDENTIFICATION OF NEW TARGETS AND THERAPEUTIC STRATEGIES FOR THE TREATMENT OF MELANOMA

S.S.D. MED/09

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ABSTRACT

Malignant melanoma is the deadliest type of skin cancer. Its incidence is still increasing and when not diagnosed at early stages its prognosis is poor. Such aggressiveness is due to its metastatic invasive features, high genetic mutation rate and resistance to chemotherapeutic treatments. The attempt to find possible genetic oncotargets and pharmacological treatments aiming to reduce the aggressivity of this cancer and ameliorate its prognosis is very challenging. In this work, by exploiting a human metastatic melanoma xenograft zebrafish model, both aspects were explored, studying on one side the effects of genetic engineering on transcription factor activity in melanoma and on the other side how pharmacological treatments based on a fungal molecule can be exploited to restrain the invasive potential of this tumor. Indeed, several transcription factors affect cellular processes by modulating gene expression in cancers. In particular, it has been reported a higher expression of RUNX2 in melanoma cells than in normal melanocytes. To investigate the role of this transcription factor in melanoma development, a deletion in the Runt domain of RUNX2 was obtained with Crispr/Cas9 in a human metastatic melanoma cell line. The present study pointed out that cells harbouring the deletion in the Runt domain when transplanted in zebrafish had reduced abilities to proliferate, to migrate and to form metastases. Furthermore, the deletion affected neoangiogenesis indicating the potential role of RUNX2 and particularly of its Runt domain as a new melanoma oncotarget. On the other side, a hint came from studies that underlined the anti-neoplastic potential of vegetal and fungal lectins due to their specific binding to the T antigen of human cancer cells. In fact, it has been shown that a fungal lectin purified from the fruiting bodies of *Boletus edulis* mushrooms named BEL β-trefoil was able to decrease viability and proliferative ability of human metastatic melanoma cells *in vitro*. The present work showed the ability of BEL β-trefoil to spread in the tissue of treated zebrafish xenografts and to reduce melanoma cell migration and formation of metastases, indicating this lectin as an effective biomolecule with potential of counteracting melanoma disease progression.

SOMMARIO

Il melanoma maligno è il più letale tra i tumori cutanei. La sua incidenza è in costante aumento e quando non diagnosticato nelle fasi iniziali la sua prognosi è infausta. Tale aggressività è dovuta alle sue caratteristiche di invasività metastatica, all'elevato tasso di mutazione genetica e alla resistenza ai trattamenti chemioterapici. Il tentativo di trovare possibili oncotarget genetici e trattamenti farmacologici volti a ridurre l'aggressività di questo tumore migliorandone la prognosi rimane una sfida aperta. Nel presente lavoro, tramite l'utilizzo di un modello di xenotrapianto di cellule di melanoma umano in zebrafish, sono stati esplorati entrambi gli aspetti, studiando da un lato gli effetti dell'ingegneria genetica sull'attività di un fattore di trascrizione potenziale oncotarget nel melanoma e dall'altro la possibilità di sfruttare molecole fungine come trattamento farmacologico volto a ridurre la capacità invasiva di questo tumore. Infatti, diversi fattori di trascrizione influenzano i processi cellulari regolando l'espressione genica nel cancro. In particolare, è stata riportata una maggiore espressione di RUNX2 nelle cellule di melanoma rispetto ai melanociti normali. Per indagare il ruolo di questo fattore di trascrizione nello sviluppo di questo cancro, è stata ottenuta una delezione nel dominio Runt di RUNX2 tramite Crispr/Cas9 in una linea cellulare di melanoma. Il presente studio ha poi evidenziato che le cellule aventi la delezione nel dominio Runt trapiantate in zebrafish presentano ridotte capacità di proliferare, migrare e formare metastasi. Inoltre, è emerso che tale delezione influenza anche il processo di neoangiogenesi, indicando il potenziale ruolo di RUNX2 e in particolare del suo dominio Runt come nuovo oncotarget nel melanoma. D'altro canto, un spunto è venuto da studi che hanno evidenziando il potenziale antineoplastico delle lectine vegetali e fungine grazie al loro specifico legame con l'antigene T delle cellule tumorali umane. In particolare, è emerso che una lectina fungina purificata dal comune fungo *Boletus edulis* e denominata BEL β-trefoil era in grado di diminuire la vitalità e la capacità proliferativa delle cellule di melanoma *in vitro*. Il presente lavoro, ha mostrato la capacità di BEL β-trefoil di diffondersi nel tessuto dei pesci trattati e di ridurre la migrazione delle cellule di melanoma trapiantate e la formazione di metastasi, indicando il potenziale di questa lectina nel contrastare la progressione del melanoma.

1- INTRODUCTION

1.1 Melanoma

Cutaneous malignant melanoma comprises less than 5% of malignant skin tumors; however, it is one of the most aggressive malignancies in humans and it is responsible for almost 60% of lethal skin neoplasia (1). In the last two decades, major advances have been made in the understanding of melanoma, reporting new predisposition genes and key somatic events directly translated into therapeutic management (2).
However, melanoma incidence has increased nearly fourfold in the past three decades (3) and is still rising faster than that of any other solid tumor (2). This can be explained by the complex epidemiological features underling the onset of melanoma, which involve genetic predisposition, somatic mutations, environmental factors and lifestyle habits (4).
An early detection of skin lesions and subsequent local tumor excision before cells dissemination represent successful therapeutic approaches. However, after metastases spreading, effective interventions against melanoma are limited, resulting in poor prognosis. In fact, metastatic melanoma is refractory to existing therapies and, even if an initial positive response may be observed, cells acquire the ability to evade apoptosis and also become resistant to chemotherapy (5). Furthermore, the latent period from initiation of melanoma carcinogenesis to clinical presentation can be decades long (2). Taken together, all these elements help to explain why the incidence rates are still increasing in most countries and why it is still of great interest to unravel the biology that underlies melanoma in order to find new oncotargets and to provide targeted drugs and new therapeutic approaches.
Many studies conducted in animal models and in primary tumors shed light on the complex background involved in the development of melanoma and on its metastatic progression.
Melanoma is an aggressive cancer of the melanocyte lineage (1). Melanocytes are pigment-producing cells, in fact they contain melanosomes which produce melanin. Melanocytes derive from precursor cells named melanoblasts which originates from neural crest and then migrate in their niches located in the integument, eye (uvea and retina), inner ear, central nervous system, heart and muscle (4). Melanocytes in the

integument are responsible for hair and skin colour variations and are located amongst the basal layer of the dermal-epidermal junctions, hair follicles, sebaceous glands and dermis. In the skin, melanocytes interact with keratinocytes through a cytokine, hormonal and growth factor signalling net (4). The most common type of cutaneous melanoma in western populations is associated with melanocytic nevi, which are aggregates of melanocytes that can undergo neoplastic transformation (1). This process is due to a very complex interaction between genetic risk factors, lifestyle habits and environmental factors, such as cumulative UV exposure (4).

On the biomechanical point of view, the tumor initiation is characterized by the melanoma precursor losing its adhesiveness both to the basal laminae, caused by deregulation mechanisms of adherent junctions, and to adjacent keratinocytes, consequent to a downregulation of E-cadherin (6). Thus the melanoma enters the radial growth phase with cells proliferating in the epidermis, invades the dermis, and start the vertical growth phase (5).

On the molecular point of view, in the last years it has been reported that mutation rate and gene modulation in malignant melanoma are higher than in other solid malignancies. The most known altered genes in melanoma are *NRAS*, the first melanoma oncogene to be discovered (4), and *BRAF*. Indeed the Ras/Raf/MEK/ERK pathway regulates cell fate decisions and is a key regulator of melanoma cell proliferation (5). In fact, the most frequently mutated gene of this pathway is *BRAF*, particularly at the position 600 with a glutamic acid for valine substitution, known as V600E (7). This mutation is involved in several aspects of melanoma onset and progression since it leads to constitutive ERK signalling, stimulates other genes such as *MITF* (microphthalmia-associated transcription factor) (8) and *p16* (5), and promotes autocrine VEGF (Vascular Endothelial Growth Factor) secretion (9). Another component of this signalling pathway commonly affected in melanoma is *NRAS*, which gain-of-function mutation leads to hyperactivation of *ERK*. Interestingly, *NRAS* and *BRAF* mutations are mutually exclusive (5).

Another key gene expressed in most human melanomas and used as a diagnostic marker for the disease is MITF (Microphthalmia Transcription Factor). It is a basic helix–loop–helix leucine zipper transcription factor that is considered to be the master regulator of melanocyte biology. It regulates melanoblasts survival and melanocyte lineage commitment, and it is a key player in melanoma, where continue MITF

expression is required for melanoma cell proliferation and survival (10).
Other studies identified also mutations in cyclin-dependent kinase inhibitor 2A (*CDKN2A*), cyclin-dependent kinase 4 (*CDK4*), BRCA1 associated protein 1 (*BAP1*) and in genes safeguarding telomere maintenance (3, 11, 12).
A relevant signalling pathway in melanoma is the phosphoinositide-3-OH kinase (PI(3)K) pathway, which regulates cell survival, proliferation, increase in cell mass and motility, and it is hyperactivated in a high proportion of melanomas (5), through gain-of-function mutations in PI(3)K, overexpression of its effector protein kinase B (Akt) (13) and loss of function of the downstream effector PTEN (Phosphate and Tensin Homologue) (14).
Studies focus on the discovery of genes, pathways and mechanisms involved and altered in tumor development and progression because every gene mutations in a cancer, including somatic mutations which in melanoma occur in particularly high number, may become potential targets to counteract cancer progression and in fact they are often referred to as oncotargets.

1.2 RUNX2 and the Runt domain

Interesting oncotargets in cancer are often transcription factors that can affect multiple cellular processes and trigger cellular transformation by acting on gene expression of downstream effectors. Among transcription factors, Runt-related transcription factor 2 (RUNX2) exhibits marked overexpression in melanoma and in many other cancers as emerged in several studies.
RUNX2 belongs to the RUNXs family, where the systematic name RUNX is an abbreviation for the term "Runt-related protein" and include, in humans, RUNX1 and RUNX3 (15). The members of this family share a defining characteristic: the Runt domain, a highly conserved motif of 128 aa-long located at the N-terminal end of the protein. The name itself "Runt" gives clues about the high level of conservation of this domain through evolution: indeed, it derives from the fact that the pair rule gene *Runt* in *Drosophila melanogaster* is the founding member of the Runt domain family of transcription factors (16). Runt is the DNA-binding and protein-protein domain and is also responsible for the heterodimerization of RUNX proteins subunits (17). In fact, RUNX proteins are able to bind DNA both as monomers of DNA-binding subunit

α or as heterodimer with the β subunit (18). The association with the non-DNA binding β subunit both enhances the DNA binding affinity of RUNX proteins and stabilises the interaction between the α subunit and the DNA (18).

The human *RUNX2* gene was identified and localised on chromosome 6p21, spanking a region of approximately 200kb, and it comprises 8 exons (19). *RUNX2* gene expression is transcriptionally regulated by alternative splicing. Two promoters are present, leading to two different *RUNX2* mRNA isoforms where the Runt domain remains unchanged and the difference is focused in the 5' region: type I RUNX2 is a 513-amino acid protein starting with the N-terminal amino acid sequence MRIPVD, and type II RUNX2 is a 521-amino acid protein starting with the N-terminal amino acid sequence MASNSL (17, 18).

RUNX2 is widely known as a master transcription factor of bone formation since it induces the commitment of mesenchymal stem cells to osteogenic fate and osteoblasts differentiation (20). However, RUNX2 activity extends beyond the processes of bone formation and its involvement in tumor development is largely documented. For example, RUNX2 is overexpressed and amplified in osteosarcoma, the most common bone malignancy in the pediatric population (21) and contribute to the tumor growth in bone and to osteolytic lesions formation in other tumors such as breast and prostate cancers (22).

Remarkably, several studies revealed that *RUNX2* is overexpressed also in various types of non-bone related cancers such as prostate and breast cancer (23), thyroid papillary carcinoma (24), pancreatic cancer (25), lung cancer (26) and melanoma (27). These studies also showed that RUNX2 involvement is not limited to the formation of metastasis in bone and to the osteolytic damage, but that it is directly involved in the oncogenesis of the primary tumor and metastasis formation through the regulation of components of fundamental pathways associated with tumor development and progression. Indeed, RUNX2 overexpression upregulates transcription factors *SOX9* and *SMAD3* and participates to a reciprocal interaction with PI3K/AKT signalling pathway thus conferring to the cancer an increased migration ability and invasiveness by promoting the Epithelial-to-Mesenchymal-Transition (EMT) process (28). RUNX2 is also involved in other fundamental signalling pathways of the tumorigenesis process, such as WNT and transforming growth factor beta (TGF β) (29). It also act as a

negative regulator of the p53-Dependent DNA damage Response process (30). Furthermore, RUNX2 exerts pro-angiogenic effects through stimulation of endothelial cell proliferation, migration and invasion (31), induction of VEGF expression (31) and physical and functional interaction with Hypoxia Inducible Factor 1-a (HIF1-a) (32), which are major actors in the angiogenesis process.
In melanoma, RUNX2 is overexpressed and promotes migration and invasion abilities (33) by regulating the the EMT process (27) and the PI3K pathway (28), which is one of the major pathways altered in melanoma (5) as previously described.

1.3 Zebrafish model

Most of the studies in the cancer research field have been historically carried out in tissue culture, cancer cell lines and rodent models. However, in the last decades another animal model has emerged thanks to its unique features and interesting advantages: *Danio rerio*, which is usually referred to as zebrafish.

Zebrafish emerged as a model organism for developmental genetics in the 1960s, described by George Streisinger and became more popular starting from the 1980s especially in the field of developmental biology. It is characterized by small size, making it easy to house and handle, high fecundity with about 200 embryos or more per week upon adult mating, external development, embryonic transparency and rapid maturation time. Furthermore its genome has been fully sequenced finding that about 70% of all human genes and 82% of disease-causing human genes have at least one ortholog in zebrafish (34), and as vertebrates, zebrafish share nearly all organs with mammals, including the brain, eyes, heart, intestines, pancreas, kidneys and liver, even though they are simpler than the human counterparts (35).
By virtue of these peculiarities, zebrafish has then gained attention also as a robust cancer model that complements what can traditionally be achieved in mice and cell culture systems (36). The evolutionary conservation of cancer-related programs between human and zebrafish is striking and allows translation of research outcomes obtained in fish back to humans (37). In fact, tumors in zebrafish strongly resemble human tumors at the histological, transcriptional and genomic levels (35). Furthermore, the optical transparency at early stages and the availability of transparent adult strains (38) with the use of fluorescent labels allows detailed and dynamic *in vivo* visualization

of cancer growth and progression (39) on large numbers of animals at reasonable costs. Zebrafish cancer models offer also the possibility to investigate the dynamics of tumor plasticity, cell invasion and metastasis (40), as well as the interaction with the tumor microenvironment (41) and the tumor-dependant angiogenesis process which are not easy to analyse otherwise.

Zebrafish modelling human cancer can be obtained in different ways such as site-specific mutagenesis of genes predisposing to cancer, chemical mutagenesis, oncogene expression by stable transgenesis or inducible/selective gene expression of mutant alleles in somatic tissues (36). Another approach is to transplant fluorescently labelled human cancer cells into zebrafish embryos which offer the advantage of having an incomplete immune system lacking a proper adaptive immune response (42) and therefore preventing the need for immunosuppression in the first 14 days after fertilization (40). Human cancer cells for xenotransplant can be either common cancer cell line or patient-derived (43), that can be marked with a fluorescent stain and then analysed in detail in a three-dimensional environment within an engrafted tumor. Despite the microenvironment of the tumor is not fully static since the recipient is not mature and is still undergoing organogenesis (35), this is still a powerful approach providing a 'whole-organism test tube' (41) offering speed and resolution not otherwise achievable for a primary screening tool (35). Furthermore, the xenograft of patient-derived tumor cells in zebrafish is emerging as a valid model for personalized medicine, allowing to generate a sort of humanised cancer model for each patient, by injecting patient-derived tumor cells and eventually testing pharmacological treatments This approach provides new opportunities for anticancer personalized therapy (35), especially for the possibility to evaluate patient-derived xenograft cell invasiveness (37) and to test drug sensitivity directly from patient biopsy (44). In fact, zebrafish is a model of election also for chemical compounds screening since its high fecundity merged with the possibility to deliver molecules of interest directly in the swimming water or through injection, provide an ideal platform for high-throughput screening of drug panels to find new potential pharmacological treatments (45).

Zebrafish models are nowadays available for several human cancers such as blood tumors (acute myeloid leukaemia, B-cell and T-cell B-cell acute lymphoid leukaemia), liver and testicular cancer, neuroblastoma, pediatric brain tumor, pancreatic cancer and melanoma (41).

The zebrafish model has recently contributed to major insights in the progression of melanoma (46). Human melanoma is among the most genomically abnormal of all cancers, with a mutation rate so high that makes it tricky to distinguish between few driver mutation and a lot of somatic and transient mutations (35). To this purpose the zebrafish is particularly useful for the ease of genetic manipulation, useful to find novel cancer markers or to recapitulate mutation found in human cancers (41). For example, there is high conservation of the most frequent oncogenes in melanoma, *BRAF*^{V600E} and *NRAS*^{Q61K} in zebrafish models of cancer (35). Microinjection of human genes under tissue-specific promoters leads to tumors that are similar to the human disease and a stable *BRAF*^{V600E} under *mitfa* promoter transgenic line is available which spontaneously produces melanocytic lesions that develop into an invasive melanoma resembling that of humans. (47). This and other transgenic lines allowed to study melanoma initiation showing the neural cell progenitors state re-emergence as a key event in this process (46). This led to the test of the inhibitors of dihydroorotate dehydrogenase (DHODH), that was found to selectively abrogate neural crest development in zebrafish as well as melanoma growth in mouse xenografts and human cell lines (48) and thereafter the DHODH inhibitor leflunomide (FDA approved drug commonly used to treat rheumatoid arthritis) was tested in phase I/II clinical trials, suggesting the potential of zebrafish as a useful model to translate the pharmacological test from the lab to clinical investigation (36). Also the xenotransplant of melanoma cells in embryos allowed very interesting studies, being zebrafish an invaluable model to image cell motility, metastases formation and tumor-microenvironment (49) as well as neoangiogenesis (49-51).

1.4 Lectins and BEL

In melanoma the challenge to develop new and more effective therapeutic tools is still in full progress since the prognosis for melanoma is still poor and its incidence is still rising, as previously mentioned.

Lectins are carbohydrate-binding proteins that form reversible and non-covalent bind with specific sugars of polysaccharides, glycoproteins and glycolipids (52) without modifying them enzymatically (53). The first to be discovered in 1888 was the hemagglutinin from the seeds of *Ricinus communis* (54). Lectins are ubiquitous in nature, in fact they can be found in plant roots and seeds such as soybean and legumes,

fungi, bacteria, algae, invertebrate's body fluids and mammalian cell membranes (55). They exert different roles depending on the organism they belong to. For example in legumes they are involved in the symbiotic association with nitrogen fixing bacteria, while seed lectins are implicated in the reserve of substances, defence against pathogens, insects and fungi (56). Furthermore they are often responsible of processes related to microbes phagocytosis and bacterial toxicity (56). They have also a role in normal and tumor cell adhesion, developmental processes and cell differentiation (55).

In fungi, whose survival rely on the symbiosis with a variety of hosts, lectins are considered to be enrolled in such interaction through the recognition of a specific glycoconjugate (57). In edible mushrooms, the presence of lectins is known since very long and many have been studied and characterized (56). The reason for the increasing interest in these biomolecules is that their ability to target carbohydrates in glycoproteins, such as Galactose (Gal) and N-Acetilgalactosammine (GalNAc), is emerging as a potential powerful tool in medicine as anti-cancer treatment. Fungal lectins can act specifically on malignant cells thanks to the presence of the Thomsen-Friedenreich antigen (T-antigen) on the surface of neoplastic cells, of the disaccharide Galβ1-3GalNAc. This disaccharide, that is hidden in normal cells, allows selective binding of lectins through the serine or threonine of glycoproteins (58). Moreover, lectins can bind to ribosomes and inhibit protein synthesis. They also affect the cell cycle by inducing non-apoptotic G1-phase accumulation mechanisms and G2/M phase cell cycle arrest, they affect apoptosis through interaction with Bcl-2 and p53 signalling pathways and the caspase cascade; furthermore, lectins can also downregulate telomerase activity and inhibit angiogenesis (59).

The lectin present in the common and very widely diffused edible mushroom *Agaricus bisporus* (Agaricus Bisporus Lectin, ABL), for instance, has been shown to selectively inhibit the proliferation of human malignant epithelial cell lines without any evident toxicity for normal cells (60). More recently, also a lectin obtained from the common edible mushroom *Boletus edulis* showed antineoplastic properties *in vitro* (57), thus representing an interesting tool for therapeutic applications against cancer. This protein is a dimer and each monomer folds as β-trefoil domain, with three trefoil subunits made of four β subunits each and therefore it has been named Boletus edulis lectin (BEL) β-trefoil (61). Notably, the *in vitro* treatment of A375 and MeWo melanoma cell lines with different concentrations of BEL β-trefoil resulted in

reduction of the proliferation ability and inhibition of the migration capacity of treated cells (62) which make this molecule interesting to be investigated as a potential opponent of metastatic melanoma progression *in vivo* in a whole organism.

2- AIMS OF THE STUDY

Metastatic melanoma is one of the most aggressive cancer in humans and its incidence is still increasing. When not detected and removed at early stage, it acquires a metastatic phenotype for which the prognosis is poor since it become also resistant to chemotherapy. Therefore, the biomedical research is still challenged to find new oncotargets as well as new molecules to prevent or reduce the cellular migratory ability in order to counteract melanoma.

The aims of the study were the following:

- investigation of a possible melanoma oncotarget by deepening the knowledge on the role of the DNA-binding domain Runt of the transcription factor RUNX2 in human metastatic melanoma cells. In particular, the study focused on the effects on proliferation and migration abilities, metastases formation and neoangiogenesis process;
- investigation of the possible effects of a lectin obtained from edible mushroom *Boletus edulis* with *in vitro* demonstrated anti-proliferative properties in reducing the migration ability and metastases formation of melanoma cells *in vivo*.

In order to achieve these purposes, the study exploited a xenograft zebrafish model of human metastatic melanoma, which is a proven tool to model human cancer biology for it has peculiar characteristics and experimental advantages making it unlike all other *ex vivo* assays and a top choice for *in vivo* imaging and chemical screening.

3- MATERIALS AND METHODS

3.1 Cell culture

A375 melanoma cells (purchased from American Type Culture Collection) and A375 CRISPR/Cas9 clone (3G8) and A375RED melanoma cells were cultured under humidified atmosphere of 5% CO_2 and passaged in growth medium DMEM/F12 containing 10% foetal bovine serum (FBS) supplemented with antibiotics (1% penicillin and streptomycin) and 1% glutamine (Gibco, Thermo Fisher Scientific).

3.2 Cell staining

Cells were then detached and harvested by scraping, washed and counted using Invitrogen CountessII (Thermo Fisher Scientific) suspended at a density of 1×10^6/mL and stained with Vybrant Cell-Labeling Solution (Molecular Probes) a lipophilic fluorescent dye that in zebrafish is stable for at least two weeks according to literature (49). Staining either in red (DiL stain) or green (DiO stain) was performed for 20 minutes at 37°C according to the protocol provided by the manufacturer.

3.3 Plasmid DNA transfection

To obtain a A375 cell line with stable red staining, A375 cells were harvested by scraping, washed and counted with Invitrogen CountessII. 5000cells/ml/well were seeded in a 24-well plate. Once 60-80% confluence was reached (1 day), cells were transfected with 0.5 μg/μL DNA plasmid expressing RFP using Lipofectamine 2000 reagent prepared following manufacturer's instructions (Lipofectamine™ 2000 Transfection Reagent, Invitrogen Thermo Fisher Scientific). During the following day, the cells were expanded to 6-well plates, T-25 and T-75 flasks.

3.4 Cell and single-cell sorting

At 100% confluence in T-75 flasks, A375 RFP-transfected cells were harvested by scraping, washed and counted with Invitrogen CountessII and resuspended at a concentration of $1x10^6$cells/100 μL in PBS-EDTA 0.5mM. Single-cell sorting of RFP-positive A375 cells were performed at the CPT Centre of the University of Verona using FACSAria Fusion (Becton Dickinson) after excluding dead cells and doublets through morphologic evaluation. The 5% highest RFP expressing cells were selected

and plated in 24-well plates and then passed and expanded in T25 and T75 flasks. Then sorted again with the same protocol. The purity of each fraction was > 90-95%. The third time, cells were single-cell sorted in 96-well plates. Once colonies formed, they were evaluated for intensity of the signal using EVOS FL Auto Imaging System (Invitrogen Thermo Fisher Scientific). The 5 most intense colonies were selected and grown and then the most homogeneous was selected and expanded as A375RED line.

3.5 BEL β-trefoil:GFP

BELβ-trefoil:GFP in aliquots of 0.25 mM each, in PBS with 10% DMSO was provided by the Biocristallography Lab (Department of Biotechnology, University of Verona) and obtained as previously described (62).

Briefly, the protein coding sequence (57) was inserted into the pET22b plasmid used to transform the *E. coli* BL21 C41 expression strain with a standard heat shock protocol. The protein expression was induced in liquid LB media by adding 0.25 mM IPTG and shacking overnight at 20°C. Harvested cells were lysed by sonication and the soluble fraction was loaded onto a Nickel–Sepharose column previously equilibrated with buffer 20 mM Tris-HCl pH 7.5, 500 mM NaCl and 10 mM imidazole and the protein was eluted with a gradient of imidazole, from 10 to 350 mM. The histidine tag was cleaved, adding thrombin to the protein solution, and the tag-free protein was concentrated and loaded onto a Superdex G75 column equilibrated with PBS (0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The protein solution was then sterilized on a 0.2 µm syringe filter. The fluorescent BEL β-trefoil was obtained by cloning the protein-coding sequence into the pWaldo-GFP plasmid as previously reported in order to obtain the expression of the recombinant BEL β-trefoil fused with a C-terminal GFP. The engineered plasmid was used to transform *E. coli* BL21 DE3 strain and the protein expression was induced in Terrific Broth media by adding 0.25 mM IPTG and shacking overnight at 20°C. The chimeric protein was purified using the above protocol of the recombinant alone BEL β-trefoil:GFP.

3.6 BELβ-trefoil:GFP in vitro treatment

A375RED cells, were harvested by scraping, washed and counted with Invitrogen CountessII and plated again in growth medium, 600 cells/well in 96 well plates. Once 80% confluence of A375RED cells was reached, BELβ-trefoil:GFP was

added at 1:10 dilution in cell growth medium.

3.7 Cell viability

Cell viability was evaluated by XTT test (Cell proliferation kit II – XTT Roche), a colorimetric assay based on the reduction of the tetrazolium salt sodium 3I-(1-phenylamino-carbonyl-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (XTT) by mitochondrial dehydrogenase of viable cells to a formazan dye. XTT test was performed after 24 h of BELβ-trefoil:GFP exposure. Briefly, 100 µl XTT labelling mixture was added to each well and the cells were incubated at 37°C under a humidified atmosphere with 5% CO_2 for 4 h. The spectrophotometric absorbance of the samples was measured every hour (four measurements) using a microtitre plate (ELISA) reader at a wavelength of 450 nm.

3.8 Zebrafish handling

Zebrafish embryos were obtained from natural spawning of nacre (*mitfa* $^{w2/w2}$) adults (ZFIN database ID:ZDB-ALT-990423-22) and *kdrl:GFP* adults, raised according to the standard protocols of "The zebrafish book" by Westerfield's and staged according to Kimmel et al. (63).

3.9 Zebrafish xenotransplantation with human melanoma cells and BELβ-trefoil:GFP injection

For xenotransplantation, nacre zebrafish embryos were mechanically dechorionated at 2 dpf (days post-fertilization), anesthetized with Tricaine (Sigma) 0.16 mg/ml and placed along plastic lanes on a transplantation mould, immersed in 2% methylcellulose (Sigma) in fishwater. Stained cells (either A375, A375RED or 3G8 melanoma cells) were loaded in a borosilicate glass capillary needle and microinjected into the yolk (about 30 cells/embryo), using FemtoJet 4x microinjector and InjectMan micromanipulator (Eppendorf). Xenotransplant embryos were grown at 33°C, monitored daily and documented from 1 day post injection (dpi) up to 1 week (experimental endpoint, 9dpf). For lectin treatment experiment, BELβ-trefoil:GFP 0.25 mM was loaded in a borosilicate glass capillary and injected at 3dpf, one day after the injection of A375RED cells, with the same machineries mentioned above. Imaging was performed using Leica FluoCombi III M165FC fluorescence microscope.

3.10 RNA extraction and quantification

At the experimental endpoint (9dpf), larvae were euthanized and collected. Samples were disrupted with Precellys24 tissue homogenizer (Bertin Instruments). Then, total RNA was extracted using RNeasy Protect Mini kit (Qiagen) following the protocol provided by the manufacturer. RNA concentration was determined with Qubit3 Fluorometer using the "Qubit RNA HS Assay Kit" (Invitrogen) according to the manufacturer's protocol. RNA samples obtained were stored at -80°C.

3.11 RT-PCR

Reverse transcription with random primers was performed with 1ug of RNA in a total volume of 20 µL using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instruction and running the RT-PCR reaction on SimpliAmp™ Thermal Cycler (Invitrogen, Thermo Fisher Scientific). cDNA samples obtained were stored at -80°C.

3.12 qPCR

Real-time PCRs were performed in a total volume of 20 µL containing PCR Master with ROX premixed with SYBR Green and 1 µL of cDNA from each sample. The following custom-designed SYBR green primer sets (Invitrogen) were used:
runx2a(fwGACGGTGGTGACGGTAATGG, rvTGCGGTGGGTTCGTGAATA);
runx2b (fw CGGCTCCTACCAGTTCTCCA, rv CCATCTCCCTCCACTCCTCC);
rank (fw GCACGGTTATTGTTGTTA, rv TATTCAGAGGTGGTGTTAT);
alp (fw CAAGAACTCAACAAGAAC, rv TGAGCATTGGTGTTATAC);
actb1 (fw CCCAAAGCCAACAGAGAGAA, rv ACCAGAAGCGTACAGAGAGA)
as housekeeping gene. The amplification reaction program included 10' at 95°C for the enzyme activation step and 40 cycles of 15" at 95°C and 1' at 60°C.
Thermocycling and signal detection were performed with QuantStudio 3 Real-Time PCR System (Applied Biosystems). C_t (threshold cycle) values for each reaction were obtained using QuantStudio Design and Analysis software (Applied Biosystems) that came with the instrument. For each amount of RNA tested, triplicate C_t values were averaged and three independent experiments were performed. To calculate relative gene expression levels between different samples, the analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

4- RESULTS

In order to deepen our knowledge on the implications of the Runt domain on RUNX2 functions and activities and to explore a possible new oncotarget in melanoma, a deletion in the Runt domain was obtained. The CRISPR/Cas9 editing technique allowed the deletion of a region within the Runt domain in the A375 metastatic melanoma cell line, generating a clone harbouring a 114 bp in-frame deletion, corresponding to 38 amino acids. This deletion leads to the synthesis of a shorter but functional RUNX2 protein, in which 1/3 of the middle Runt region is missing. The clone with the Runt deletion (del- Runt) derived from the A375 cell line is called 3G8 cell line. The cell line functional characterization *in vitro* analysed the parameters of viability, proliferation, migration ability and apoptosis. The viability was evaluated with an in vitro cytotoxicity test performed with XTT, a colorimetric assay in which the tetrazolium salt XTT in the viable cells undergoes a reduction reaction forming a detectable formazan derivative orange-coloured. This showed reduced cell viability of the 3G8 cell line: in fact, while 100% of the wildtype (A375) cells are vital compared to 60% of del-Runt (3G8) cells. The Ki-67 test, based on the expression of the protein Ki-67 in the active phases of the cell cycle, revealed that the 3G8 clone has a lower proliferation ability since 80% of A375 cells nuclei expressed Ki-67 compared to 61% of 3G8. The clone with the Runt deletion had also a reduced migration ability, which was evaluated through scratch test and measured in terms of relative migration distance (RMD) after 48 hours of cell growth. It emerged a lower ability to migrate of 3G8 clone (21.8%; SD±7%) compared to A375 metastatic melanoma cells (33.4%; SD±10%). Furthermore, it has also been shown an increased apoptosis evaluated through the Tunel assay, in which the fluorophore binds to double-strand DNA breaks which are typical of Caspase cut in apoptosis. This experiment showed that in del-Runt 3G8 cells the percentage of positive apoptotic nuclei is higher (30%) compared to WT (12%).

To evaluate these promising results *in vivo* the zebrafish xenotransplantation model was identified as particularly suitable to mimic human melanoma as described in literature (35, 49), also because it doesn't have a fully developed immune system in the early stages, so immunosuppression is not required when injecting exogenous material (35).

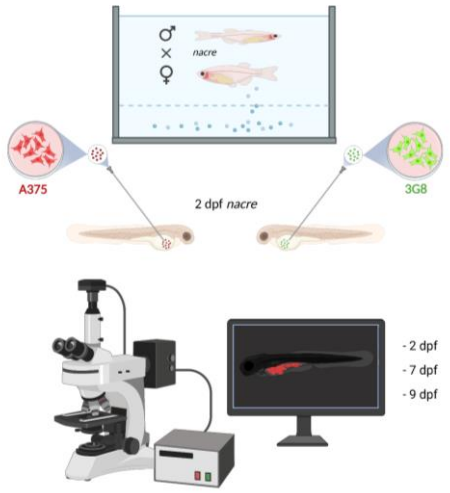

Figure 1 – Experimental setup overview for zebrafish xenotransplant with melanoma cells. Human metastatic melanoma cells were labelled with fluorescent vital stains, wildtype A375 in red and Runt-deleted 3G8 in green. Then, cells were injected in 2dpf nacre larvae and imaged with a fluorescence microscope until 9 dpf. Three timepoints were considered: 2 dpf, 7 dpf, 9 dpf.
(Created with BioRender.com)

Wildtype A375 metastatic melanoma cells were labelled in red and the relative clone with the Runt deletion 3G8 in green, with lipophilic fluorescent vital stains that are stable for several cell cycles even after transplantation in zebrafish for at least two weeks (49). The recipients were nacre zebrafish, a line encoding an inactivating mutation of the melanocyte inducing transcription factor a (*mitfa*) gene, therefore they are devoid of melanocytes (35) and thus extremely transparent and very useful for the fluorescent tracking of the transplanted cells. Stained A375 and del-Runt 3G8 cells were injected in the yolk of zebrafish embryos at 2 days post fertilization (dpf) (Figure 1). They were monitored every day until the 7th day after injection and three more relevant timepoints were then taken into account. The first timepoint was 0 days post injection (dpi) in the 2 dpf larvae, when transplanted cells were still located in the yolk. In the following days they started to move and at the second timepoint, 5 dpi, cells appeared more dispersed in the yolk of 7 dpf larvae. At the third timepoint, which was also the experimental endpoint, 7 dpi/9 dpf, initial metastases were detectable in A375 and, to a lesser extent, in 3G8 transplanted larvae (Figure 2). At the experimental endpoint, a lower migration ability and lower number of metastases were observed for the 3G8 clone with the deletion in the Runt domain. Indeed, A375 wild type melanoma cells migrated in 81% and remained *in situ* in 19% of transplanted larvae, forming an average number of 2.07 metastases. Conversely, del-Runt 3G8 cells migrated in 58% and were still *in situ* in 42% of transplanted larvae, generating on average 0.83 metastases (Figure 3).

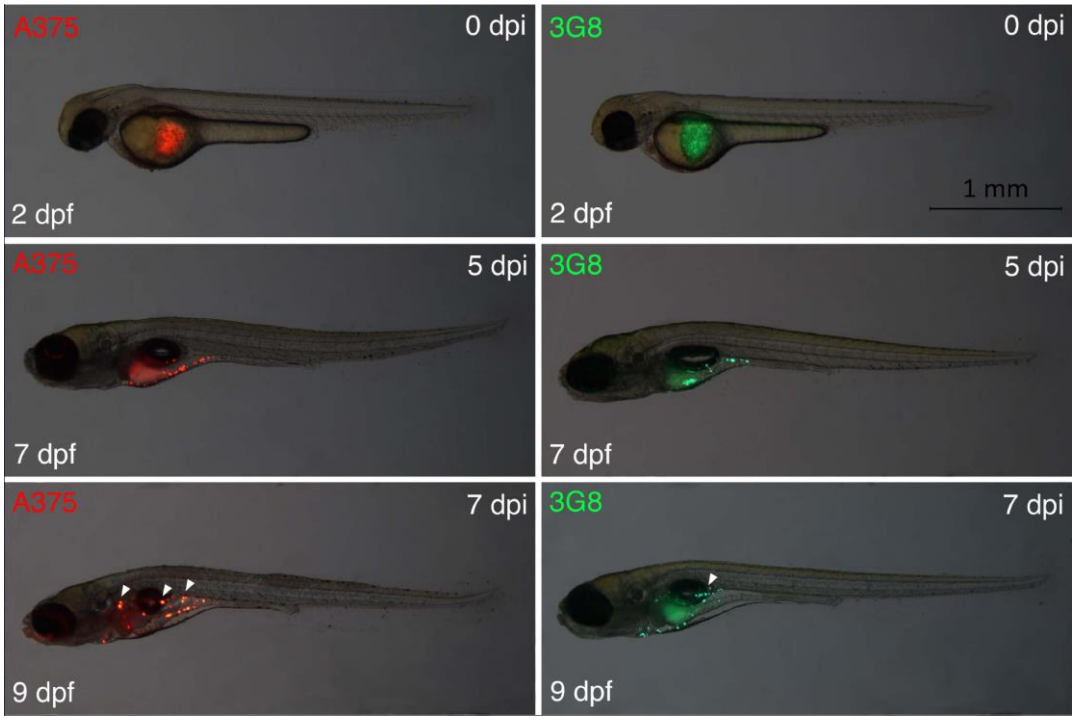

Figure 2 - Differential migratory ability of A375 vs. 3G8 melanoma cells in zebrafish xenotransplants. Representative images (20X magnification) for each timepoint show wild type (A375, stained in red, on the left) and del- RUNT (3G8, stained in green, on the right) melanoma cells migratory and proliferative behaviour in transplanted zebrafish embryos. At 2 days post fertilization (dpf) and day 0 post injection (dpi), transplanted cells are still located in the yolk. At 5 dpi, cells appear more dispersed in the yolk of 7 dpf larvae. At 7 dpi/9 dpf, initial metastases (indicated by white arrowheads) are detectable mainly for A375 than for 3G8 xenotransplant larvae. All views are lateral, anterior to posterior from the left to the right.

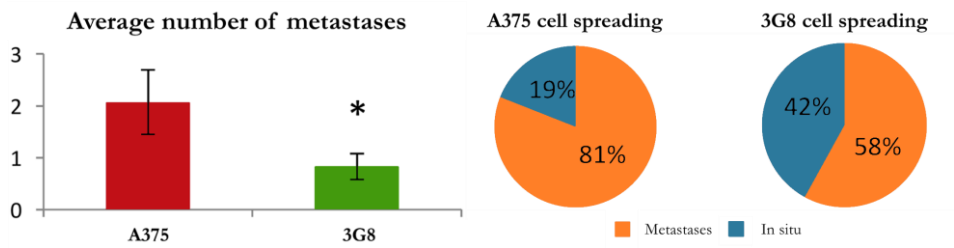

Figure 3 - Charts with average number of metastases (left) and cell spreading (right) in A375 vs. 3G8 xenotransplants. At the experimental endpoint (7dpi/9dpf), in A375 (n=22) and 3G8 (n=12), 2.07 metastases were observed in the A375 xenotransplants and 0.83 in the 3G8 xenotransplants (chart on the left, * $p < 0.05$). Cell spreading were observed in 81% of the A375 xenotransplants and in 58% of the 3G8 xenotransplants (chart on the right).

The results indicate that Runt domain deletion reduce the probability of cell spreading as well as the proliferation and metastases formation ability. The number of transplanted zebrafish that remained in the whole experimental time and thus were considered was 22 for A375 cells (red) and 12 for 3G8 cells (green).

After investigating the processes of cell proliferation, migration and metastases formation, another key process in metastatic melanoma progression, in which Runx2 is known to be involved, is angiogenesis. It was interesting to evaluate the impact of the RUNT domain also in this process, using the RUNT-deleted clone 3G8. A proteome analysis made by collaborators showed a specific protein signature of 3G8 cells related to apoptosis and migration and pointed out the involvement of the Runt domain in the neoangiogenesis process. In addition, VEGF gene expression was reduced in 3G8 as compared to A375 cells and HUVEC co-cultured with 3G8 cells expressed lower levels of CD105 and CD31 neoangiogenic markers. Furthermore, the tube formation assay revealed down-regulation of capillary-like structures in HUVEC co-cultured with 3G8 in comparison to those co-cultured with A375 cells.
These findings provide new insights into Runx2 molecular details which can be crucial to possibly propose it as an oncotarget of melanoma.
The xenotransplant model of zebrafish emerged as a useful tool also in this case, to evaluate whether a difference in the formation of vasculature was visible *in vivo* or not. The recipients came from the *kdrl:GFP* reporter line, in which the fluorescent protein GFP is expressed under the promoter of the vascular endothelial growth factor receptor kdr-like (*kdrl*) gene. *kdrl* encodes for the receptor for VEGF, it has a tyrosine-protein kinase activity and it is required for the development of different blood vessel types in the embryo (64). It is also involved in angiogenesis, specifically in VEGF-induced sprouting of new blood vessels and in artery morphogenesis (64). Therefore, the reporter line *kdrl:GFP* allows to follow the expression of the *kdrl* gene thanks to the green fluorescent signal from the GFP and to image the vessels in the embryo. As previously described, A375 wildtype metastatic melanoma cells and RUNT-deleted 3G8 clones were stained, both in red to be able to distinguish them from the green signal of the blood vessels in the *kdrl:GFP* larvae (Figure 4). One control group of 55 larvae received no cell injection and was used as reference of the normal vein development. The cells were injected at 2 dpf in the yolk, in 55 embryos per

experimental group (A375 n=55, 3G8 n=55), and monitored for 48 hours after the injection, until 2dpi/4 dpf. The area of interest for the evaluation of the formation of blood vessels was the sub-intestinal vein plexus (Figure 4 C-D), a highly angiogenic district widely employed for investigating the molecular mechanisms of tumor angiogenesis, which is also particularly useful in the case of tumor cell engraftment to follow new blood vessel development in zebrafish embryo in order to study the interplay between tumor and host microenvironment (65).

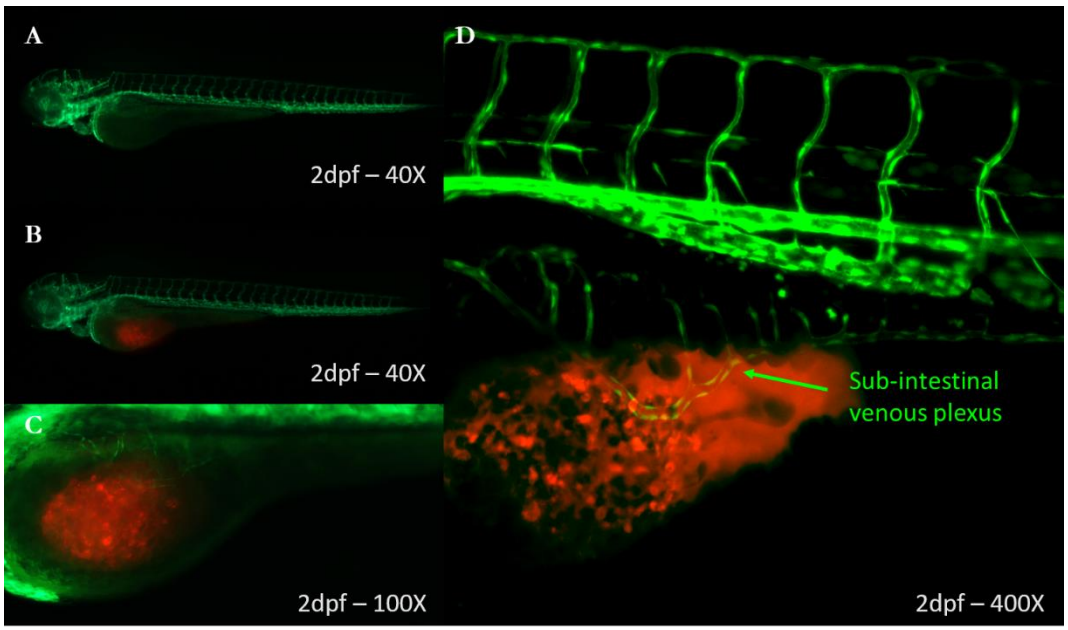

Figure 4 - Experimental background for the evaluation of neoangiogenesis. In a *kdrl:GFP* larva of 2 dpf (A), human metastatic melanoma cells (either A375 or 3G8 stained in red) are transplanted (B), which is shown also in greater magnifications (C and D) to highlight the area of interest, the sub-intestinal venous plexus (D), which is located in the abdominal area in correspondence with the central-caudal part of the yolk.

In the first time point, 1 day post injection (dpi) in the 3 dpf larvae, the A375 xenotransplants showed an increased ramification of the veins in the sub-intestinal plexus. The trend was maintained also the following day, at the experimental endpoint (2dpi/4dpf). In particular, the ramifications development toward and around the implanted cells was more evident in the A375 xenotransplants than in the 3G8 ones (Figure 5). The A375 xenotransplant zebrafish showed an increase of +80% ramifications compared to the controls and +50% compared to 3G8 xenotransplants, while the latter had +20% ramifications than the controls (Figure 6).

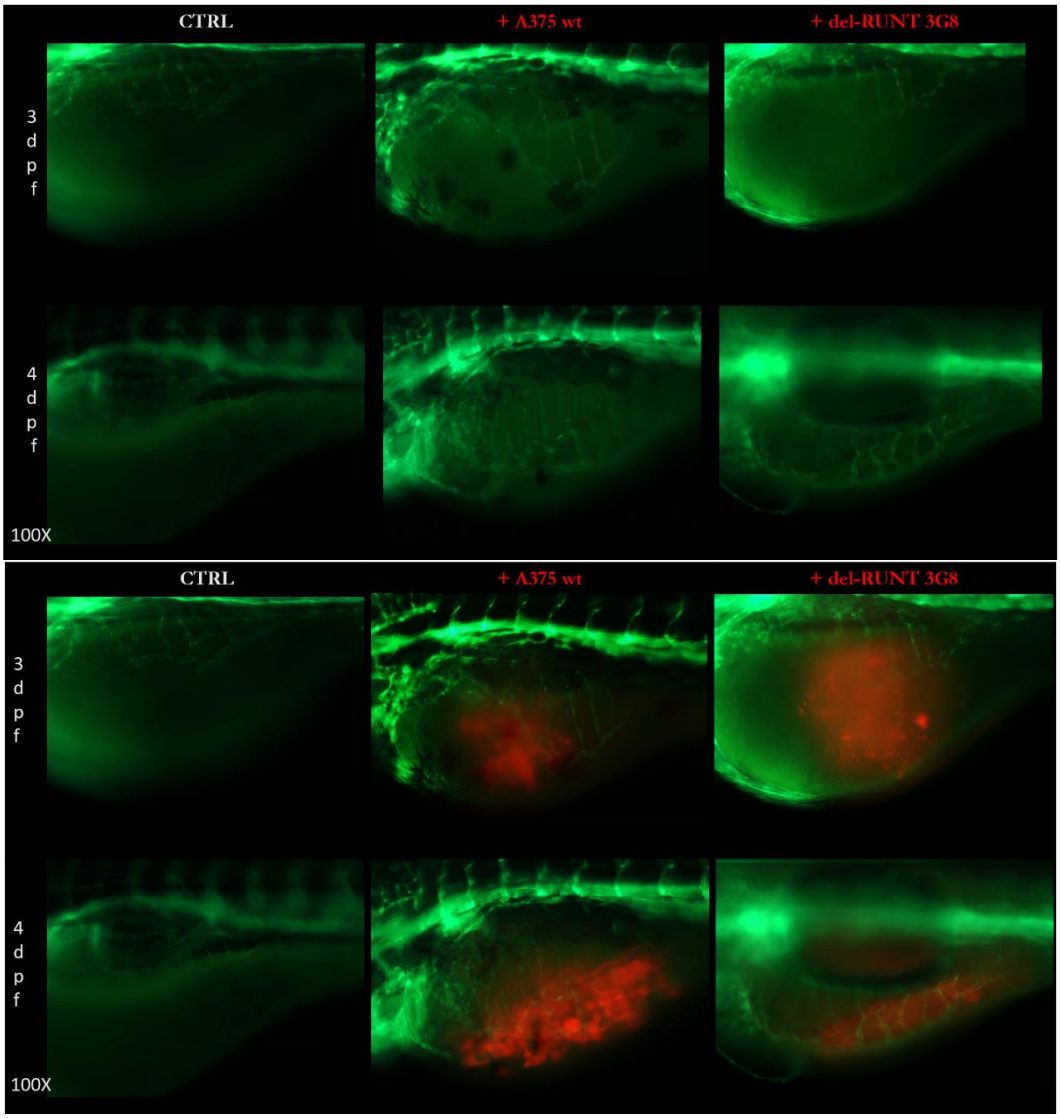

Figure 5 - *In vivo* evaluation of neoangiogenesis. In the panels are displayed the most representative acquisitions (100X magnification) for each group. In the first column are the control, in the second the A375 xenotransplants, in the third the 3G8 xenotransplants. The panel on the top represents only the green channel with GFP signal from vessels in the *kdrl:GFP* line. The panel on the bottom represent also the merge with the red channel with red stain signal either from A375 or 3G8 cells. On the first row at the timepoint of 3dpf, 1 day post injection, A375 xenotransplant already show a significative ramification of veins in the sub-intestinal plexus with respect to 3G8 xenotransplant, which is particularly evident in the first panel without red channel merging. This difference is even more evident at 4dpf-2dpi, on the second row.

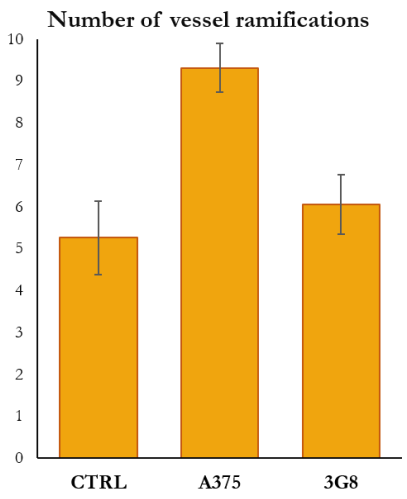

Figure 6 - Number of vessel ramifications. The chart represents the average number of veins and ramifications in the district of the sub-intestinal vein plexus in the three groups. In the A375 xenotransplants there is an increment of 50% more ramification compared to 3G8 xenotransplants (controls n=55, A375 xenotransplants n=55, 3G8 xenotransplants n=55).

In order to evaluate a potential molecule to affect and counteract melanoma proliferation and migration ability, the mushroom lectin BEL β-trefoil:GFP was tested *in vivo* as a potential opponent of the spreading ability of metastatic melanoma human cells transplanted into zebrafish. In order to be able to perform live imaging *in vivo* following the presence of the lectin, a chimeric BEL β-trefoil:GFP was used, which was fused with GFP.

Firstly, A375 metastatic melanoma human cells were transfected with a plasmid expressing Red Fluorescent Protein (RFP) in order to obtain a stable A375 line expressing permanent red fluorescent signal thus allowing to avoid the passage of staining with fluorescent dyes (Figure 7).

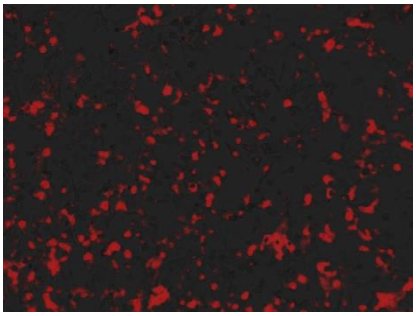

Figure 7 - A375RED line. A375 human metastatic melanoma cell line expressing RFP (10X magnification).

Secondly, to test possible effects of the chimeric protein BEL β-trefoil:GFP on cellular viability, A375 cells were treated for 24 hours with two different concentrations of this lectin, 0.012 μM and 0.025 μM and a XTT test was performed. This showed a 40% of reduction in cell viability for the treated cells: in fact, 100% of the wildtype (A375) cells were vital, compared to 63% of the cells treated with 0.012 μM BEL β-trefoil:GFP and 60% for those treated with 0.025 μM BEL β-trefoil:GFP (Figure 8). This result recapitulates what already emerged in previous works (62).

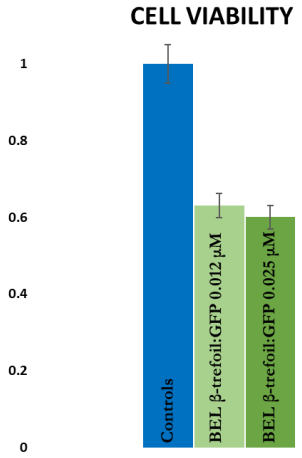

Figure 8 - Cell viability for BEL β-trefoil:GFP *in vitro* treatment. A375 melanoma cells were treated with two different concentrations of the chimeric protein BEL β-trefoil:GFP. After 24 hours, the untreated A375 cells (Controls, blue) were 100% vital, compared to 63% of 0.012 μM BEL β-trefoil:GFP treated cells (light green) and 60% of 0.025 μM BEL β-trefoil:GFP treated cells (dark green).

Furthermore, BEL β-trefoil:GFP was injected at 3 dpf cell xenotransplant in zebrafish without cell xenotransplant (n=15), to evaluate the persistence of the chimeric lectin alone in the larval tissues, which was confirmed at 5 days post injection by microscopy observation of the green signal from GFP (Figure 9).

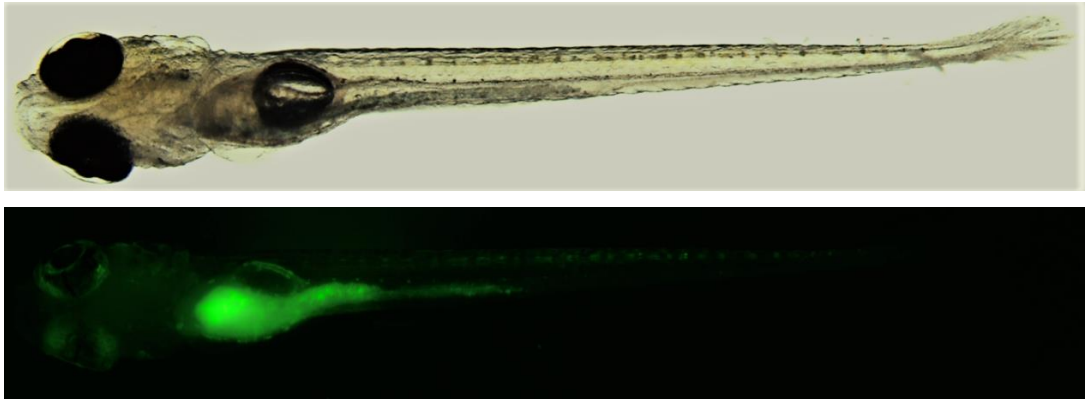

Figure 9 - On the top, brightfield photograph of a 7 dpf larvae at 5 dpi of BEL β-trefoil:GFP. On the bottom, fluorescence image showing the green fluorescent signal of BELβ-trefoil:GFP still present in the larva after 5 days from injection. 20X magnification.

Using the xenotransplant model of zebrafish, A375RED human metastatic melanoma cells were injected in 2dpf embryo yolk. The day after, at 3dpf, in the larvae of the treated group BEL β-trefoil:GFP 0.25 μM in PBS was injected in the yolk, while in the larvae of the untreated group PBS only was injected. The individuals were then monitored each day and cell spreading and metastases formation was reported between 5 to 7 dpi (7-9 dpf) of human melanoma cells both for untreated (A375) and treated (A375+ BEL β-trefoil:GFP) larvae. At the experimental endpoint 9 dpf, after 6 days post treatment (dpt) with BEL β-trefoil:GFP, both the GFP signal from the chimeric lectin and the cells were detected together in treated larvae (Figure 10). At 9 dpf, fluorescence microscope acquisitions showed that in untreated zebrafish the cells were dispersed farther from the site of injection than in the treated zebrafish, where most of the cells were still in the yolk area upon lectin treatment and cell spreading appeared reduced as well as the metastases formation (Figure 11). In fact, the number of cells spreading in BEL β-trefoil:GFP treated zebrafish was lower (average number of spread cells=38) compared to the untreated ones in which more cells (average number of spread cells=45) were able to migrate away from the site of injection and to form initial metastases (Figure 12A). On this regard, not only the average number of metastases was higher in untreated zebrafish (3.3) than in the untreated (2.3) (Figure 12B), but also 80% of untreated xenotransplant zebrafish showed metastases compared to 67% of treated ones, while 33% of treated individuals presented in situ cells, versus 20% of untreated (Figure 13).

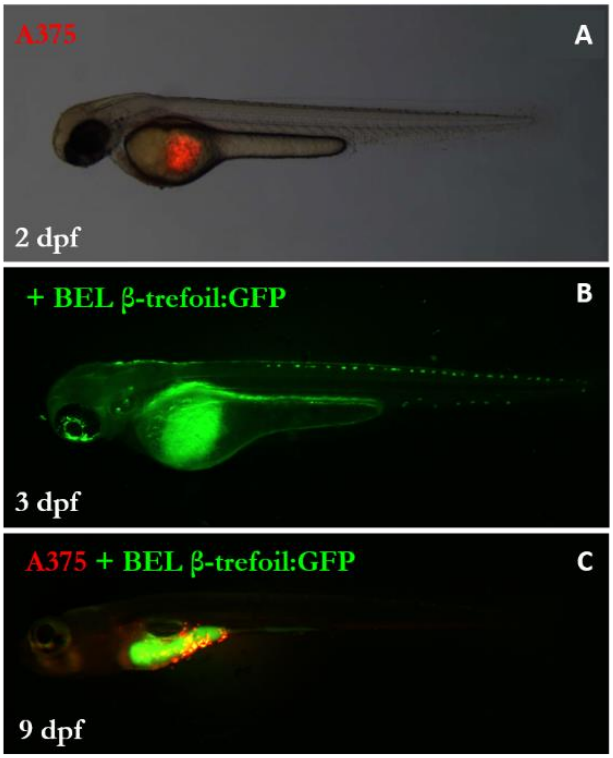

Figure 10 - Experimental timeline. At 2 dpf, A375RED human metastatic melanoma cells were injected in the yolk of the larvae (A). At 3 dpf, in the larvae of the treatment group also BEL β-trefoil:GFP 0.25 μ M was injected in the yolk (B). Merged images at 9 dpf, showing both the red signal from the cells and green signal from the lectin(C). 20X magnification.

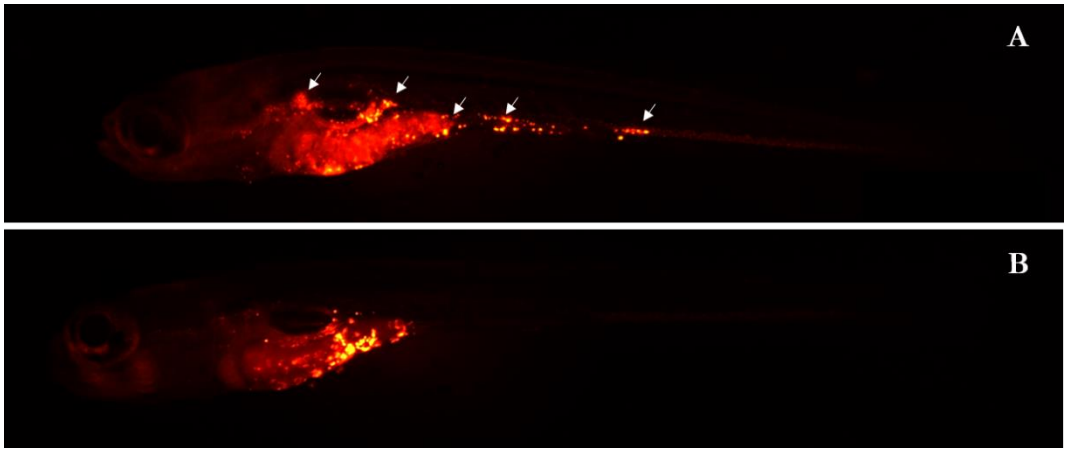

Figure 11 - Effects of BEL β-trefoil treatment on the migratory ability of A375 melanoma cells in zebrafish xenotransplants. Representative images showing untreated (A) and BEL β-trefoil:GFP 0.25 μM treated (B) zebrafish xenotransplant of A375RED human melanoma cells. At 9 dpf, significant cells spreading and initial metastases (indicated by white arrows) are detectable in untreated larvae, while most cells are still in the yolk area in treated zebrafish (40X magnification).

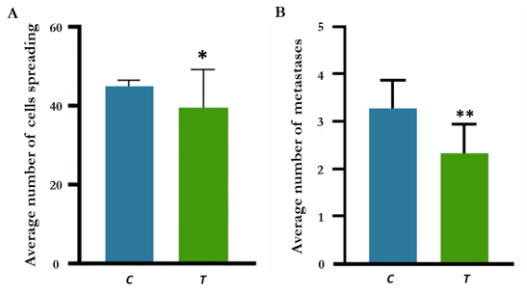

Figure 12 - Effects of BEL β-trefoil:GFP treatment on numbers of cell spreading and metastases. The average number of cell spreading (A) was 38 in BEL β-trefoil:GFP 0.25 μM treated zebrafish (T, green) and 45 in the untreated controls (C, blue). *P<0.05. The average number of metastases (B) was 3.3 for untreated zebrafish and 2.3 in treated zebrafish **P<0.01.

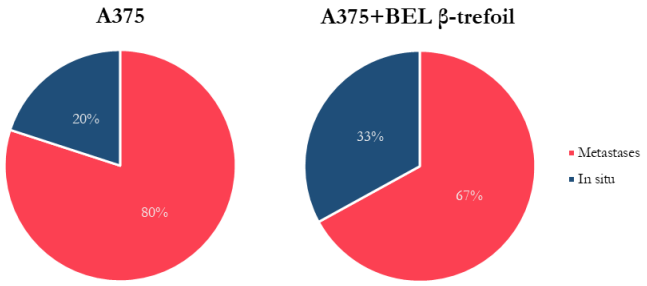

Figure 13 - Melanoma cells migration: untreated vs. BEL β-trefoil:GFP treated xenotransplants. In zebrafish transplanted with A375 cells and untreated (chart on the left), at 9dpf melanoma cells migrated in 80% and remained in situ in 20% of larvae. In zebrafish transplanted with A375 and treated with BEL β-trefoil:GFP 0.25 μM (chart on the right) melanoma cells migrated in 33% and were still in situ in 80% of transplanted zebrafish.

These data highlight the reduced migratory ability of metastatic melanoma cells in the presence of the lectin BELβ-trefoil.

In order to investigate a possible effect of BEL β-trefoil on the expression of endogenous *runx2*, at the experimental endpoint (9 dpf), total RNA was collected from BEL β-trefoil:GFP 0.25 μM treated as well as from untreated larvae. The expression of the zebrafish duplicated orthologs for the human Runx2, *runx2a* and *runx2b* were analysed. The qPCR results showed that lectin treatment affected the expression of

endogenous *runx2*, since both *runx2a* and *runx2b* genes were downregulated (Figure 14). Consequently, additional genes involved in bone remodelling were analysed. In particular, the expression of Receptor Activator of Nuclear Factor- kappaβ (*rank*) gene and of Alkaline Phosphatase (*alp*) gene. The results showed a statistically significant upregulation of *alp* in lectin-treated zebrafish. We also observed an increased but not significant expression of *rank*. (Figure 13). The relative gene expression levels between different samples was performed using the $2^{-\Delta\Delta CT}$ method and considering the untreated A375 xenotransplants as calibrator.

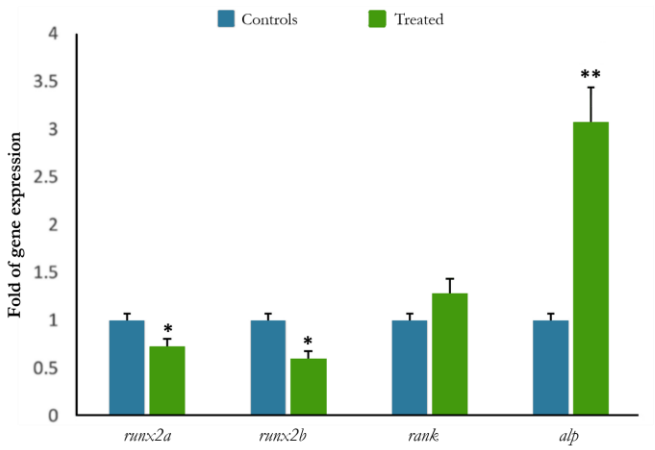

Figure 14 - Gene expression of *runx2*, *rank* and *alp* upon BEL β-trefoil:GFP treatment. Zebrafish xenotransplant treated with BEL β-trefoil showed reduced expression levels of both zebrafish endogenous *runx2a* and *runx2b* genes and a statistically significant over-expression of *alp*; *rank* expression was upregulated without reaching statistical significance. *P < 0.05; **P < 0.01.

5- DISCUSSION

Metastatic melanoma is one of the most aggressive types of cancer when not detected properly and its incidence is still increasing (3). Among factors underlying melanoma onset and progression there is the high mutation rate and the impairment of gene expression affecting the processes of cellular proliferation, migration and neoangiogenesis which promote tumor growth (2). Of particular interest in this case are the transcription factors, that can guide cell signalling processes triggering cellular malignant transformation. Therefore, it is crucial to find alternative ways to counteract this cancer and new possible pharmacological treatments. This study aimed to approach the problem from two points of view. The first was to act at the gene expression level on a transcription factor that can impact cellular growth and transformation and neoangiogenesis, the second was to test a molecule with negative effects on cellular proliferation and migration.

RUNX2, the master transcription factor for osteogenic differentiation, has been investigated in several studies that demonstrated its involvement in non-bone related tumors including pancreatic cancer, breast cancer, ovarian epithelial cancer, prostate cancer, lung cancer, thyroid cancer (23-27) and in melanoma, where it mediates cell invasion and migration (33). These results made RUNX2 an interesting possible oncotarget in melanoma. However, given its involvement in signalling pathways that are fundamental also for the growth and development of normal cells, such as NOTCH, TGF- β /activin, MAPK, WNT and PI3K/AKT pathways, it is not easy to perturb melanoma through a complete disruption of the whole RUNX2 transcription factor. Therefore, since up to now detailed data about the involvement in cancer progression of its Runt domain are lacking, it has been considered interesting the option to investigate the impact of a deletion in the Runt DNA-binding domain on human metastatic melanoma. A 114-bp deletion in Runt was obtained with CRISPR/Cas9 in human metastatic melanoma cell line A375 leading to the production of a shorter but functional RUNX2 protein in the Runt-deleted 3G8 clone. The effects were then analysed *in vitro*, showing reduced viability, increased apoptosis and reduced proliferation and migration ability in Runt-deleted 3G8 cells compared to wildtype A375 (66). Furthermore, RUNX2 is known to be involved in the regulation of angiogenesis in cancer, but evidence in melanoma is lacking. Indeed, RUNX2 turned

out to be involved in the neoangiogenesis process since HUVEC cocultured in 3D matrix with Runt-deleted 3G8 cells showed reduced ability to form capillary-like and net-like structures compared to co-cultures with WT A375 melanoma cells. Furthermore, 3G8 cells showed also reduced VEGF expression (67).

The effects of the impairment of the Runt domain on metastatic melanoma progression were investigated also *in vivo* taking advantage of the xenograft zebrafish model. Cancer cell transplantation models in zebrafish have been widely used in the last two decades to study tumor angiogenesis, invasion and metastasis by engrafting dyed or fluorescently-labelled tumor cells. Furthermore, zebrafish is standing out as a useful and reliable model in the melanoma research field (35, 37, 46) and the possibility to transplant human cancer cells without need for immunosuppression in the early stage of the development (40) is an emerging tool that have already allowed the investigation of dynamics of melanoma onset and progression in several studies (43, 49, 68). In fact, human metastatic melanoma cells injected in the zebrafish embryo survive, exhibit motility, divide and, even though they do not form complete tumors, they become scattered throughout the embryo in interstitial spaces, reflecting the dedifferentiated state of the cancer cells (68) even forming metastases (49). Therefore in the present study human metastatic melanoma A375 cells and their derived Runt-deleted cells 3G8 were injected in 2-days-post-fertilization (dpf) zebrafish embryos in order to develop xenograft models of human melanoma. Differences in terms of proliferation and migration abilities were then evaluated. 3G8 cells, carrying the partial deletion of Runt, showed impaired ability to proliferate and migrate. This was evaluated by counting the number of cells able to evade the site of injection (the yolk), and the number of initial metastases formed and the number of individuals in which these events occurred. All these characteristics occurred in higher number in wildtype A375 cells than in Runt-deleted 3G8 cells zebrafish xenotransplants, supporting the initial hypothesis of the involvement of Runt domain in the pro-proliferation and pro-migration roles of RUNX2 which emerged also from the *in vitro* analyses (66).

The zebrafish xenotransplant model was further used to investigate the tumor-related neoangiogenesis. Studies in literature using this approach evolved from the evaluation of angioblasts activation with immunohistochemistry using specific antibodies (49), to the study of the extravascular migratory metastasis formation and the interaction between melanoma cancer cells and the external surface of zebrafish

vessels through in vivo imaging coupled with 3D reconstruction (50).
The approach in this study was adapted from literature protocols for zebrafish tumor xenograft angiogenesis assay (69). Thus, red stained A375 wildtype melanoma cells and 3G8 Runt-deleted cells were transplanted in the zebrafish transgenic reporter line *kdrl:GFP* in order to be able to observe the vasculature marked with specific GFP signal. The use of a transgenic reporter line for the vasculature is more advantageous than immunohistochemical/alkaline phosphatase stain of endothelial cells because the specificity of the signal is much higher (69). The number of ramifications in the sub-intestinal vein plexus of larvae (65) with 3G8 cells engrafts were halved with respect to wildtype A375 cells xenotransplants. This result allows to infer the involvement of the Runt domain in the pro-angiogenic effects of RUNX2 in melanoma consistently with the outcome of the *in vitro* experiments (67). Furthermore, in the A375 xenotranplants the ramifications develop toward and around the implanted cells in a more evident way than in 3G8 xenografts, consistently with a reduced pro-migratory effect of 3G8 cells as observed *in vitro* and *in vivo* in the previous part of the present study. Therefore, the results strongly suggest that Runt domain of RUNX2 is involved in increasing the risk of tumor proliferation and migration and promotes neoangiogenesis in metastatic melanoma cells both *in vitro* and *in vivo*, hence it may be considered an interesting oncotarget to counteract this cancer progression.

Therefore, considering that the melanoma aggressiveness may be imputed to its high ability to acquire a metastatic phenotype, the study pursued the interest in finding potential therapeutic molecules to counteract the proliferative and migratory ability of melanoma cells. Thus, the xenotransplant model of zebrafish was used in virtue of its high suitability for drug screening (45), in order to evaluate the properties of a potential anti-proliferative molecule in the context of metastatic melanoma. The focus was on mushroom lectins, particularly BEL β-trefoil from the common edible mushroom *Boletus edulis*, which recently emerged to be able to reduce cell viability and RUNX2 expression in melanoma cell lines in a dose-dependent fashion (57, 62). A chimeric BEL β-trefoil:GFP lectin was used to treat A375 cells confirming the lectin dose-dependent ability to reduce cell vitality after 24h of treatment. Afterwards, A375 metastatic melanoma cells were injected in 2 dpf zebrafish embryos and one group was treated with injection of BEL β-trefoil:GFP at 3dpf. The results showed that BEL

β-trefoil can spread in the tissues and remains detectable for several days; the results showed as well that BELβ-trefoil treatment can impact melanoma cell spreading in treated zebrafish xenografts, since after 7 days from melanoma cell injection in untreated zebrafish the number of spreading cells and the number of metastases was higher compared to xenotransplant treated with BELβ-trefoil for 6 days. In the presence of this lectin, most of the melanoma cells remained in the injection area (yolk area) and cell were able to migrate and form metastases in lower number compared to untreated controls. These results recapitulate what expected from the previous studies *in vitro* with this lectin (57, 62) and also from other studies on zebrafish xenotransplant with vegetal lectins (70). Expression of endogenous *runx2* was also evaluated, since we know it to be highly expressed in the melanoma cells we used. Often the orthologs of human genes in zebrafish are duplicated because in fish a partial genome duplication occurred during evolution (34), in fact both *runx2a* and *runx2b* expressions were analysed resulting downregulated in BELβ-trefoil:GFP treated larvae. Considering the fundamental role of RUNX2 in bone formation and remodelling also two of its downstream genes involved in bone processes were analysed. The gene *alp*, which encodes for a glycoprotein involved in osteoblast differentiation was upregulated, while *rank*, which encodes for a membrane receptor expressed by osteoclasts, showed a non-significant increase in the expression. Downregulation of *runx2* and upregulation of *alp* are one of the signatures of osteogenic maturation rather than osteogenic differentiation in zebrafish. These findings suggest that the BEL β-trefoil lectin can affect bone remodelling and thus, although it may be considered a beneficial molecule for reducing the metastatic ability of melanoma cells, particular attention should be paid to the concurrence of bone disorders and further studies should be performed to better understand this mechanism.

In conclusion, the study exploited a xenotransplant zebrafish model of human metastatic melanoma and the results highlight the role of the Runt domain of RUNX2 in vivo in reducing the migratory and proliferative abilities as well as the metastases formation and affecting neoangiogenesis. Moreover, the study also provide evidence for the role of BELβ-trefoil in reducing the migration ability and metastases formation of melanoma cells and at the current state it is the first demonstration of such property in an *in vivo* model.

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APPENDIX

PUBLICATIONS ON PEER REVIEWED JOURNALS

1. Carbonare LD, Bertacco J, Marchetto G, Cheri S, Deiana M, Minoia A, Tiso N, Mottes M, Valenti MT. **Methylsulfonylmethane enhances MSC chondrogenic commitment and promotes pre-osteoblasts formation**. *Stem Cell Res Ther.* (2021) Jun 5;12(1):326. doi:10.1186/s13287-021-02396-5.

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