

## **Crosstalk at multiple myeloma microenvironment: role of exosomes-associated miRNAs.**

### **Introducion**

Multiple myeloma (MM) is a hematologic malignancy characterized by abnormal proliferation of plasma cells within the bone marrow which leads to paraprotein release in the serum, osteolytic bone disease, anemia and renal failure <sup>1</sup>. Even though, rapid advances in the therapeutic options in bone disease treatment have been accomplished in the recent years, the progression of MM bone disease is rarely halted <sup>2</sup>. Additional studies to understand the complex mechanisms adopted by MM cells to influence tumor microenvironment will be needed to overcome the limits of current pharmacology. MM bone disease is sustained by a supportive microenvironment based on complex cross-talk involving stromal, immune, endothelial and bone cells, as well as extracellular matrix components. In bone marrow, MM cells modify the normal microenvironmental conditions and in turn require host modifications for their survival <sup>3</sup>. In contrast to normal bone remodelling, during multiple myeloma progression the functional balance between osteoclasts (OCs) and osteoblasts (OBs) is definitively perturbed <sup>4</sup>. A variety of cytokines, growth factors, adhesion molecules, extracellular matrix proteins and RNA species are secreted by both tumor and non-tumor cells, mediating cell-to-cell communication within the tumor microenvironment and providing a suitable niche for cancer cell growth and survival. Recently, extracellular vesicles (EVs) have emerged as novel vehicles of tumor-derived molecules and thus players in the crosstalk between cancer cells and the tumor microenvironment <sup>5, 6, 7</sup>. EVs are plasma membrane fragments that include, among several others, microvesicles (MV) and exosomes. Exosomes are small vesicles of 40-100 nm diameters that are initially formed within the endosomal compartment and are secreted when a multivesicular body (MVB) fuses with the plasma membrane. These vesicles are released by many cell types including cancer cells and are considered messengers in intercellular communication <sup>5</sup>. In MM, cell derived microvesicles are considered mediators for myeloma angiogenesis, while BMSC-derived exosomes significantly act on viability, survival, migration and drug resistance of MM cells <sup>8, 9</sup>. Furthermore, preliminary data from Prof. Alessandro's group show that MM cell-derived exosomes play a relevant functional role in OCs differentiation <sup>10</sup>.

miRNAs are noncoding RNA molecules that functionally modulate mRNA expression in a wide range of biological process. Once thought to operate only inside the cell, it is now known that miRNA can be exported and function outside the cell <sup>11</sup>. In MM, specific miRNA signatures have been associated to different steps of MM <sup>12</sup> and a strong relationship between deregulated expression of miRNAs and the tumor phenotype has been demonstrated <sup>13</sup>. More recently, miRNAs beyond their key role in MM pathogenesis, are emerging as potential tools for the targeting the miRNA network as a novel therapeutic strategy providing a novel rationale and a new venue of investigation in this disease. Circulating miRNAs can be shielded from degradation because they exist in complex with RNA-binding proteins or other extracellular structures, such as EVs <sup>14</sup>. EVs that contain miRNAs may be an important means of cell-cell communication within the tumor microenvironment <sup>15</sup>.

Understanding the nature of these nanovesicles and the manner in which their miRNAs affect tumor progression will have significant implications for therapeutic intervention against cancer. Recent studies show that EVs mediate the transfer of functional miRNAs that are implicated in osteolytic bone metastasis<sup>14</sup>. Furthermore, EV-enclosed miR-192 confers anti-metastatic activity in lung cancer model, thus reducing osseous metastasis<sup>16</sup>. The presence of EVs-enclosed miRNAs in cancer tissues provides new opportunities to identify the role of cancer-derived EVs in the interactions with the tumor-microenvironment; furthermore, it would provide a rich source of new biomarkers that will allow prediction of disease progression and a means to follow patient responses to therapies.

### **Preliminary results / Preliminary study**

In bone disease, myeloma cells have significant effects on recruitment and proliferation of osteoclast (OC) progenitors. Data from Prof. Alessandro's group show that the treatment of Raw264.7, a cellular model of osteoclast formation, with MM-derived exosomes increased the expression of OCs specific markers, as TRAP, Cathepsin K and MMP9. Furthermore, they evaluated if exosomes released by MM cells might control OCs formation and activity. TRAP staining and the formation of multinucleated cells were used as markers of differentiated cells. In presence of MM cell-derived exosomes, the number of TRAP-positive multinucleated OCs was significantly higher compared to control cells.

### **Aims**

Several findings indicate that exosomes are released in circulation, they are markers of metastatic disease and contain miRNAs. Exosome miRNA cargo can mark lethal disease and participate in modulating the response of microenvironment to the tumor during formation of metastatic niche. I will identify a specific miRNA profile in exosomes from different multiple myeloma cell lines and patients; I will determine whether exosome-enclosed miRNAs affect metastatic properties *in vitro* and metastasis formation *in vivo*. This study will lead to a better understanding of the molecular and functional roles of these EVs.

The aims of the proposal are:

- During the first year of the PhD, I will attempt to identify molecular signature of miRNAs found in exosomes from different MM cell lines and in the circulation of patients with different grade of MM disease, in order to isolate miRNAs contained in exosomes from cultured cells with different malignant phenotype (drug resistance, aggressiveness) and from the serum of patients affected by MM and age-matched controls (n= 20 per group). Exosome miRNAs will be described using miRNA microarrays and next generation sequencing (NGS).

To test whether miRNAs are involved in tumor-specific functional categories detected by gene ontology-enrichment analysis, such as "metastatic potential", we will create a miRNA-mRNA interaction network that will help us to identify candidate miRNAs for further studies. I will then generate stable knockdown (KD) multiple myeloma cell lines for selected exosome-enclosed miRNAs to evaluate their biological function, by using antisense miRNA cloned into GFP expression lentivector. Transfected cells will be FACS sorted and puromycin-selected colonies propagated.

- During the second year of the PhD, in order to determine whether exosome-enclosed miRNAs affect osteoblast and osteoclast functions *in vitro*, I will test the miRNA species identified in plasma exosomes for their ability to functionally modulate the tumor microenvironment by affecting the function of OCs and OBs. In the normal niche, the bone marrow microenvironment consists of osteoblasts (OB) and osteoclasts (OC), which play an important role in remodeling the bone. Bone homeostasis is maintained by a balanced production of OB and OC<sup>19</sup>. The usual balance between bone resorption and new bone formation is lost in many cases of MM, resulting in bone destruction and the development of osteolytic lesions<sup>20</sup>. Bone destruction is mediated by factors produced or induced by tumor cells that stimulate formation and activation of osteoclasts, the normal bone-resorbing cells. Preliminary data show that MM-exosomes are responsible for the promotion of osteolytic lesions but the mechanism by which this occurs is still understood. In order to test if MM-exosomes potentiate the metastatic ability of MM cells through the transfer of one or more miRNAs, *in vitro* models of monocytes/macrophages cell lines, preosteoclast committed, (murine RAW267.7, human PBMC treated with RANK-L and MCS-F before exosome treatment) will be used as target cells and treated with exosomes from MM cells with and without selected miRNA-KD. I will test markers of osteoclast differentiating phenotype, in EV-treated cells, the levels of Cathepsin K (CTSK), Matrix Metalloproteinases 9 (MMP9) and Tartrate-resistant Acid Phosphatase (TRAP), indicators of MM-mediated osteoclastogenesis, will be evaluated by RT-PCR and ELISA assays. The number of mature osteoclasts in response to exosome treatment will be assessed using TRAP staining. This staining and the formation of multinucleated cells will be used as markers of differentiated cells. We will next investigate the ability of the Raw 264.7 TRAP-positive cells to resorb bone by dentine pit formation assay by stimulating for 6 days osteoclasts formation on synthetic dentine discs with RANKL (positive ctrl) or MM-derived exo. To examine the functional role of EV-enclosed miRNAs in osteoclastogenesis, we will ectopically express the miRNAs in pre-osteoclast cells prior to induce osteoclast differentiation by RANKL. Data in literature showed that osteogenic potential and activity of osteoblasts in MM patients were significant decreased<sup>21</sup>. Therefore, I will test the ability of MM-derived exosomes to modulate osteoblast differentiation. Osteoblast-like cells MG-63 or human BM osteoprogenitor cells (PreOBs), cultured in the specific osteogenic medium, will be treated with exosomes and the expression of osteoblast markers collagen I, alkaline phosphatase (ALP), and osteocalcin (OC) will be evaluated.
- During third year of the PhD, I will attempt to determine the *in vivo* capability of exosome to induce formation of metastatic lesions. Nu/Nu mice will be injected intratibially with exosomes from MM cells with and without selected miRNA-KD. Exosome injection will start one day before intratibial injection of MM cells in both hind limbs per animal in all groups. Formation of osteolytic lesions will be monitored weekly by employing high-resolution positron emission tomography-computed tomography (PET-CT). I expect that mice treated with KD-miRNA-exosomes delay progression of the lesions induced by wild type exosomes. Furthermore, we will assess selected miRNA kinetics during bone metastasis progression in the serum of animals in order to identify miRNAs whose expression correlates with the presence of osteolytic lesions. Human MM lesions from the bone of nude mice treated with exosome will be harvested and processed for histology and

immunohistochemistry. Tissue sections will be analyzed for the expression of metalloproteinases and TRAP. I will then ask if the *in vivo* effects driven by exosome on osteoclast activity is due to a specific exosome distribution and cell tropism. For this reason, in order to test, for the first time, the biodistribution of MM exosomes, I will incubate exosomes with the lipophilic fluorescent tracer DiR (30 minutes), and inject them, after washing, in the tail vein of nu/nu mice. Recently, this approach has been used in Prof. Alessandro's laboratory to label exosomes and follow their route *in vivo*<sup>22</sup>. Mice will be imaged, using an IVIS Optical Imaging System, at different time points (15 minutes, 1h, 8h, 24h) and up to 48h post injection. Mice will then be sacrificed and organs harvested in order to measure ex-vivo fluorescence. Furthermore, selected organs (bone marrow, lung, liver, spleen, kidney, heart, thymus, brain) will be fixed, and exosome uptake observed by confocal microscopy. This will allow us to determine the specific distribution of exosomes and to evaluate in which organs their accumulation and uptake occur.

## Conclusion

This study represents a new concept in the multiple myeloma field, with no pre-existing literature. The possibility that miRNA stability is preserved in the bloodstream in exosomes is emerging as an area of important investigation and opens up the possibility of using this EV fraction in blood as a minimally invasive source of markers of multiple myeloma aggressiveness. Furthermore, this study will provide the first *in vivo* evidence for a functional role for MM exosome in bone metastasis. Our findings will open up novel avenues for translational applications in the clinical management of bone metastasis in patients with MM.

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