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The Exosomes-derived EGFR Ligand Amphiregulin (AREG) is a new key player in Multiple Myeloma Bone Destruction

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Abstract

Background: Multiple myeloma (MM) is a clonal plasma cell malignancy associated with osteolytic bone disease. Recently, the role of MM-derived exosomes in the osteoclastogenesis has been demonstrated although the underlying mechanism is still unknown. Since exosomes-derived epidermal growth factor receptor ligands (EGFR) are involved in tumor-associated osteolysis, we hypothesize that the EGFR ligand Amphiregulin (AREG) can be delivered by MM-derived exosomes and participate in MM-induced osteoclastogenesis.

Methods: Exosomes were isolated from the conditioned medium of MM1.S cell line and from bone marrow (BM) plasma samples of MM patients. The murine cell line RAW264.7 and primary human $CD14^+$ cells were used as osteoclast (OCs) sources.

Results: We found that AREG was specifically enriched in exosomes from MM samples and that exosomes-derived AREG led to the activation of EGFR in pre-OCs, as showed by the increase of mRNA expression of the downstream target *SNAIL* in both RAW264.7 and $CD14^+$ cells. The presence of neutralizing anti-AREG monoclonal antibody (mAb) reverted this effect. Consequently, we showed that the effect of MM-derived exosomes on osteoclast differentiation was inhibited by the pre-treatment of exosomes with anti-AREG mAb. In

addition, we demonstrated the ability of MM-derived AREG-enriched exosomes to be internalized human mesenchymal stromal cells (MSCs) thus blocking osteoblast differentiation, increasing MM cell adhesion and the release of the pro-osteoclastogenic cytokine interleukin-8. Accordingly, anti-AREG mAb inhibited the release of interleukin-8 by MSCs suggesting that both direct and indirect effects are responsible for AREG-enriched exosomes involvement on MM-induced osteoclastogenesis.

Conclusions: In conclusion, our data indicate that AREG is packed into MM-derived exosomes and implicated in OCs differentiation through an indirect mechanism mediated by osteoblasts.

Summary

Il Mieloma Multiplo (MM) è caratterizzato dalla presenza di aree osteolitiche dovute alla presenza di plasmacellule (PC) neoplastiche che causano l'interruzione della normale omeostasi ossea, promuovendo l'attività degli osteoclasti (OC) ed inibendo la funzione degli (OB). Al momento, uno dei campi di ricerca nella fisiopatologia del MM è proprio lo studio dei meccanismi che regolano l'attività degli OC e degli OB così come l'identificazione di target terapeutici per la prevenzione ed il trattamento delle lesioni osteolitiche. Negli ultimi anni è emerso che le vescicole extracellulari (EV), ed in particolare gli esosomi, giocano un ruolo fondamentale nel mediare il cross-talk tra le cellule tumorali ed il microambiente. Gli esosomi sono microvescicole aventi un diametro di 40-100 nm di origine endocitica che sono rilasciate nell'ambiente extracellulare da diversi tipi cellulari in condizioni sia fisiologiche che patologiche. Nel MM mediano la comunicazione cellula-cellula tra le plasma cellule e le cellule stromali midollari, influenzando la crescita e la sopravvivenza tumorale, l'apoptosi, l'invasione, l'angiogenesi e la resistenza ai farmaci. Inoltre, dati in letteratura dimostrano che gli esosomi isolati da cellule di MM sono coinvolti nel differenziamento degli OC.

The epidermal growth factor receptor (EGFR) è una glicoproteina transmembrana con un'intriseca attività tirosin chinasica, stimolata da diversi fattori di crescita quali amphiregulina (AREG) betacellulina (BTC), fattore di crescita epidermico (EGF), epigen (EPGN), epiregulina (EREG), il fattore di crescita EGF-simile legante l'eparina (HBEGF) e il fattore di crescita trasformante α (TGF-α). Il pathway di EGFR agisce a livello di diversi processi fisiologici cellulari avendo così degli effetti pleiotropici su proliferazione, differenziazione e motilità. Dati di letteratura dimostrano che l'asse EGFR- EGFR ligands stimola la proliferazione dei precursori osteoclastici e dei precursori osteoblastici, inibendo la differenziazione di quest'ultimi in vitro. Zhu et al. riportano che EGF-like ligands stimolano l'osteoclastogenesi attraverso l'attivazione degli OC.
Scopo del presente studio è stato quello di valutare il coinvolgimento dell'asse EGFR-EGFR ligands nella malattia ossea indotta da MM ed in particolare valutare la presenza dei ligandi di EGFR all'interno degli esosomi.

Per prima cosa abbiamo isolato gli esosomi rilasciati da una linea cellulare di MM (MM1.s) e dal plasma midollare di 4 pazienti affetti da MM attivo mediante protocollo di ultracentrifugazione. Abbiamo visto che gli esosomi isolati dalle MM1.s e da 3 pazienti su 4 risultano arricchiti della proteina AREG, che sembra invece poco presente nel mezzo condizionato delle cellule deprivato di esosomi. Il risultato supporta l'ipotesi che AREG possa essere rilasciato dalle cellule per mezzo degli esosomi.

Il modello sperimentale utilizzato è costituito da due linee cellulari: le RAW 264.7 (cellule murine macrofagiche, precursori degli osteoclasti) e monociti CD14+ isolati, tramite metodo immunomagnetico, da buffy-coat di sangue periferico di donatori sani.

Per dimostrare il coinvolgimento degli esosomi rilasciati dalle cellule di MM nell'induzione dell'osteoclastogenesi attraverso l'attivazione di EGFR pathway, abbiamo

valutato attraverso RT-PCR i livelli di espressione di SNAIL, un fattore di trascrizione dipendente dall'attivazione di EGFR, nelle RAW 264.7 e CD14+ trattati con esosomi isolati dalle cellule di MM ed esosomi pre-trattati con anticorpo neutralizzante anti-AREG. Abbiamo dimostrato che i livelli di espressione di SNAIL aumentano in seguito a trattamento con gli esosomi di mieloma mentre tale effetto è parzialmente revertito dopo pre-trattamento con anticorpo neutralizzante anti-AREG. Nello stesso setting sperimentale abbiamo valutato mediante RT-PCR i marker di differenziamento osteoclastico.

Il trattamento con esosomi induce, sia a livello di mRNA che a livello proteico, un aumento dei markers di osteoclastogenesi come MMP9, Cathepsin K e TRAP. Anche questi risultati dimostrano che gli esosomi isolati da pazienti affetti da MM inducono un effetto pro-osteoclastogenico ma tale effetto viene revertito in seguito al pretrattamento degli esosomi con anticorpo neutralizzante anti-AREG. Inoltre abbiamo validato il potenziale ruolo dell'asse EGFR/EGFR ligands nella formazione di OC, tramite esperimenti di osteoclastogenesi *in vitro* a partire sia da RAW 264.7 che da CD14+ in presenza di esosomi isolati dalle cellule di MM ed esosomi pre-trattati con anticorpo neutralizzante anti-AREG. La presenza di esosomi induce un maggior differenziamento osteoclastico rispetto alle cellule non trattate e rispetto alle cellule trattate con l'anticorpo neutralizzante anti-AREG. In seguito abbiamo valutato gli effetti degli esosomi su cellule mesenchimali (MSC). E' stato visto che gli esosomi isolati dalle cellule di MM vengono internalizzati dalle MSC indipendentemente dalla presenza dell'anticorpo neutralizzante AREG, inibiscono la differenziazione osteoblastica, aumentano l'adesione delle cellule di MM e inducono il rilascio di citochine pro-osteoclastogeniche come IL-8 da parte delle MSC. L'inibizione della produzione di IL-8 dopo il trattamento con l'anticorpo neutralizzante anti-AREG suggerisce che gli esosomi isolati dalle cellule di MM e

arricchiti della proteina AREG inducono l'osteoclastogenesi, sia attraverso un meccanismo diretto sugli OC che attraverso uno indiretto mediato dalle MSC.

In conclusione, i nostri dati indicano che gli esosomi isolati dalle cellule di MM e arricchiti della proteina AREG giocano un ruolo nell'osteoclastogenesi MM indotta attraverso l'attivazione del pathway di EGFR.

CHAPTER 1

Background

1.1 Multiple Myeloma

Multiple myeloma (MM) is a clonal plasma cell (PC) malignancy characterized by the abnormal accumulation of malignant MM cells within the bone marrow (BM) which leads to osteolytic bone disease[1, 2]. MM PCs secrete a monoclonal immunoglobulin called M-protein, usually IgG or IgA and detectable by serum protein electrophoresis, or only circulating k or λ-free light chains. MM is preceded by the asymptomatic precursor phases, including monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), which are characterized by the presence of M-protein but lack of end-organ damage or tissue dysfunction, including osteolytic lesion, hypercalcemia, renal insufficiency, anemia, and bone disease[3]. Furthermore MGUS and SMM share some of the genetic features of myelomas that require treatment, supporting a multistep development model where MGUS progresses to SMM and finally to symptomatic intramedullary MM[4].

1.2 Role and modifications of bone marrow microenvironment

Many studies have shown that the interaction between MM cells and the bone marrow (BM) microenvironment plays a crucial role in MM pathogenesis[5, 6]. BM microenvironment is very important in the growth, survival and migration of malignant PCs.

In the normal niche, the bone marrow microenvironment consists of:

- the extracellular matrix component (ECM), including fibrous proteins, proteoglycans, glycosaminoglycans;
- the cellular component, including hematopoietic stem cells (HSCs), erythroid cells, immune cells, as well as BMSCs such as mesenchymal stem cells (MSCs), marrow adipocytes, fibroblasts, osteoblasts (OBs), osteoclasts (OCs) and endothelial cells (ECs).

Interaction between MM cells and BM cells is mediated through several adhesion molecules and cell-surface receptors such as integrins, cadherins and selectins. Moreover, these interactions upregulate the release of cytokines and growth factors, such as Interleukin (IL)-6, IL-1β, , IGF-1, VEGF, B cell–activating factor, fibroblast growth factor (FGF) and tumor necrosis factor α (TNF-α), inducing microenvironment modifications that support the growth, migration, survival and drug resistance of myeloma cells[7] as well as BM angiogenesis[8] and osteoclastogenesis[5].

1.3 Osteolytic lesions: osteoclasts activation and osteoblasts suppression

In the normal niche, the bone marrow microenvironment consists of OCs, that mediate old bone resorption and OBs which mediate new bone formation, with the contribution of cytokines. Bone homeostasis is maintained by a balanced OBs and OCs functional activity[9]. During MM progression, in contrast to normal bone remodeling, the functional balance between OCs and OBs is definitively perturbed [10]. The increased osteoclastic activity is associated with alterations in the production of *Receptor Activator of Nuclear factor KB Ligand* (RANKL) and osteoprotegerin (OPG). RANK is a trans-membrane signaling receptor, member of the tumor necrosis receptor superfamily, expressed on OCs progenitor and mature OCs. Its ligand, RANKL, is a polypeptide of 217 amino acids produced by MSCs, pre-osteoblasts and activated T lymphocytes. RANKL directly induces osteoclastogenesis with macrophage colony-stimulating factor (M-CSF), through the activation of many intracellular signaling pathways (MAPK, NF-kB, JNK pathways, etc.), which promote differentiation, activation and survival of OCs[11].

OPG is a member of the TNF receptor family which is secreted by BM MSCs and OBs and functions as a decoy receptor by binding to RANKL. When OPG binds RANKL prevents RANK-RANKL binding and in this way inhibits OCs maturation and bone resorption[12]. In contrast, binding of MM cells through α4β1 to VCAM-1 on BMSCs decreases secretion of OPG and increases RANKL expression, thereby promoting bone resorption and osteolysis. Consequently, malignant PCs affect the OPG/RANKL ratio in favour of RANKL, and also promote an upregulation of pro-osteoclastogenic cytokines, such as IL-1, IL-3, IL-6, TNFα, M-CSF and MIP-1α, produced by MSCs, OBs and MM cells, favouring osteoclast formation and promoting lytic bone lesions[13]. Importantly, the deregulation of the RANKL/OPG system occurs in MM but not in MGUS[5].

Furthermore, OCs through an increased production of IL-6 and osteopontin (OPN) can promote PCs growth, thus contributing to the maintenance of a vicious circle[14, 15]**.** Other soluble factors are involved in imbalance of osteoclast/osteoblast activity in MM. IL-8, a member of the CXC chemokine family, has been studied for its role in promoting tumor angiogenesis, cell motility and invasion[16]. This cytokine has been described as activator of bone destruction in metastatic and MM bone disease. Indeed, IL-8 is responsible for the increased osteolysis observed in metastatic bone disease and that its release, following the interaction between MM cells and human MSCs, contributed to in vitro OC formation[17, 18].

Concurrently with the increase of osteoclast activity, osteoblast activity is markedly suppressed in MM; this is mainly due to the block of the osteogenic differentiation process of MSCs induced by MM cells[15]. OBs originate from MSCs and are responsible for bone matrix synthesis. The major transcription factor regulating osteoblast commitment and osteogenic differentiation of MSCs is Runt-related transcription factor 2 (Runx2), also named Cbfa1. The direct cell-cell contact between osteoprogenitor cells and MM cells inhibits the activity of Runx2/Cfba1 in osteoprogenitor cells. The event is mediated by the binding of VLA-4 on MM cells and VCAM-1 on osteoblast precursors [19]. Runx2 induces the production of OPG, therefore it is possible that the inhibition of RUNX2 activity also affects the OPG/RANKL ratio inducing osteoclastogenesis. Moreover, soluble factors expressed by MM cells, such as DKK1 and IL-7, may also contribute to the inhibitory effects of MM cells on osteoblast differentiation and Runx2 activity. In MM, several studies demonstrated the role of DKK-1 in the pathogenesis of osteolytic lesions[20-22]. In fact, DKK1 is elevated in BM and blood of MM patients with osteolytic lesions[21] and it has been demonstrated that patients without bone lesions have lower

DKK1 levels than do patients who have bone lesions[23]. DKK-1, a 28672 kDa protein secreted by OBs and osteocytes, plays an important role in regulating bone metabolism by acting as a negative modulator of the Wingless (WNT)/β-catenin signaling pathway.

1.4 Exosomes: Biogenesis and secretion

In the last years, several studies highlighted a role of tumor-derived extracellular vesicles (EVs) in disease progression[24, 25]. EVs are a heterogeneous family of membranous vesicles released by various cell types into the extracellular space and can be subdivided in microvesicles, exosomes and apoptotic bodies[26]. Exosomes have been found secreted by different cell lines as well as in various body fluids including plasma, saliva, urine, malignant ascites and amniotic fluid[27]. These nanovesicles originate from multivesicular bodies (MVBs). MVBs originate from the inward budding of late endosome with the accumulation of intraluminal vesicles (ILVs) and are released by the fusion of MVBs with the plasma membrane[28]. Two mechanisms are important in MVB formation, secretion and exosomes release process: the Endosomal sorting complexes required for transport (ESCRTs)-dependent mechanisms and the ESCRTs-independent mechanisms[29]. The ESCRT is formed by four distinct complexes : ESCRT-0 regulates the cargo clustering in a ubiquitin dependent manner, ESCRT-I and ESCRT-II is necessary for bud formation, ESCRT-III regulates the vesicle scission from the MVBs membrane and the associated ESCRT protein, such as ALIX and Tsg101, has been shown to be involved in this process. Instead, the ESCRTs-independent mechanisms involve lipids such as ceramide that has been shown to drive formation, loading and secretion of exosomes, but also tetraspanins or heat shock proteins[30, 31]. Specific surface markers such as tetraspanins (CD9, CD63, CD81), heat shock 70kDa protein 4 (Hsp70), ALG-2-interacting protein X (Alix), tumor susceptibility gene 101 (Tsg101), and MHC classes I and II are used as

markers to characterize the purified vesicles as exosomes[32]. This feature is attributed to their ability to actively transport mRNA, miRNA, proteins and growth factors towards target cells, modifying their behavior as well as the microenvironment[33].

1.5 Exosomes in Multiple myeloma

The involvement of EVs in the crosstalk of MM cells with BM microenvironment cells[34] and in the progression of the disease[35] has been reported in MM patients. Several studies, have showed that tumor-derived exosomes may "educate" BM-derived cells to modify the BM microenvironment, thus leading to the promotion of tumor progression.

In MM, cell derived EVs are considered mediators for myeloma angiogenesis, while BM stromal cell-derived exosomes significantly act on viability, survival, migration and drug resistance of MM cells[36]**.** Caivano *et al* analyzed level of the serum EVs in patients with different types of hematological malignancies and found that it is significantly elevated in MM patients[37]. Exosomes derived from MM cells are enriched of antigen presenting molecules, adhesion molecules and MM-related antigens, such as the ectoenzyme CD38 and CD138 which are markers of plasma cells[38]. It has been reported that exosomes released by MM cells induced angiogenesis promoting the expression and secretion of VEGF[39]. Furthermore, exosomes-derived MM also play a role in OCs formation and activation. Raimondi *et al* showed that MM cell-derived exosomes play a relevant functional role in OCs differentiation. In particular, it has been found that exosomes treatment increased the expression of OCs specific markers, such as Tartrate-resistant acid phosphatase (TRAP), Cathepsin K (CTSK) and Matrix metalloproteinase 9 (MMP9) and directly control OCs formation and activity[40]. These result highlight the ability of exosomes to directly affect OCs differentiation and function.

The microRNAs (miRNAs) are non-coding RNA molecules that functionally modulate mRNA expression in a wide range of biological process. MiRNAs play their function through the RNA-induce silencing complex (RISC), which bind in the specific 3'-untraslated regions (3'-UTR) of the target genes, resulting in the inhibition or in the alteration of the gene expression. Once thought to operate only inside the cell, it is now known that miRNAs can be exported and function outside the cell[41]. It has been demonstrated the potential role of exosomal miRNAs as biomarker in MM. In a recent study, the authors identified 158 differentially expressed exosomes-derived miRNAs (including let-7 family members, miR-17/92 and miR99b/125a clusters) in MM compared to normal healthy controls[42]. In another study, specific miRNA signatures have been associated to different steps of MM[43] and a strong relationship between deregulated expression of miRNAs and the tumor phenotype has been demonstrated[44]. Circulating miRNAs can be shielded from degradation though the complex with RNA-binding proteins or other extracellular structures, such as EVs. miRNA contained in exosomes could be an important means of cell-cell communication within the tumor microenvironment[45]. Recent studies show that EVs mediate the transfer of functional miRNAs that are implicated in osteolytic bone metastasis[46]. Nevertheless, the specific content of MM-derived exosomes (proteins and/or miRNAs) is not yet identified and its role in MBD is still unknown.

1.6 The Epidermal Growth Factor System

The epidermal growth factor receptor (EGFR), is a transmembrane glycoprotein of 170 kDa, with intrinsic tyrosine kinase activity. It is formed by a an extracellular ligand-binding domain (ectodomain), a transmembrane domain, a short juxtamembrane section, a tyrosine kinase domain and a tyrosine-containing C-terminal tail. This protein can be bound and activated by a family of seven peptide growth factors consisting of amphiregulin (AREG), betacellulin (BTC), epidermal growth factor (EGF), epigen (EPGN), epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF) and transforming growth factor-alfa (TGFA). The binding of soluble ligand induces homo- and/or heterodimerization of the receptor that leading to *trans*-autophosphorylation and subsequent activation of several cellular signaling transduction pathway including Ras/MAPK, PI3K/AKT and STAT. This network is involved in a variety of cellular physiological processes such as proliferation, differentiation and motility[47]. Recent studies have reported that this signaling network plays an essential role in bone metabolism by affecting both OBs and OCs[48]. EGF-like ligands are able to stimulate OC formation by decreasing the expression of OPG and by increasing the expression of RANKL and monocyte chemoattractant protein 1 (MCP1) in OBs, in an EGFR dependent manner, consequently stimulating TRAP-positive OC formation[49]. Moreover, EGF and AREG stimulate pre-osteoblastic cell proliferation but inhibit their differentiation into osteoblastic cells, inducing decreased mature osteoblast number and, in an EGFR dependent manner, consequently stimulating TRAP-positive OC formation promoting osteolytic lesions[49]. In MM, it has been demonstrated that EGFR ligands are a growth factor for malignant PCs. In particular, AREG supports MM cells growth through the IL-6 production by BMSCs[50].

1.7 Rational and Objectives

MM cell-derived exosomes play a relevant functional role in the induction of OCs differentiation and activity. Recent data indicate that EGFR system is involved in bone remodeling and that EGF-like ligands stimulate osteoclastogenesis by acting at least in part on OBs. On the basis of these evidences the main goals of this project were to identify EGFR ligands, such as Amphiregulin (AREG), in MM cell-derived exosomes and to investigate their possible role in bone microenvironment modulation. We hypothesize that AREG can be delivered by MM-derived exosomes and participate in MM-induced osteoclastogenesis.

The specific aims of this study were:

1. To isolate exosomes, by ultracentrifugation, from a MM cell line (MM1.s) and from BM plasma sample of patients with active MM.
2. To demonstrate that AREG is packed into MM-derived exosomes.
3. To investigate the involvement of AREG enriched MM-exosomes in osteoclast differentiation through EGFR pathway activation.
4. To evaluate the effects of MM-derived exosomes in osteoblast differentiation.

CHAPTER 2

Materials and Methods

Data set analysis

The expression of AREG at mRNA level on PCs from 11 monoclonal gammopathy of undetermined significance, 133 MM patients at the diagnosis, 9 primary plasma cell leukemia (PCL) and 4 healthy donors (GSE16122)[51], generated on AffymetrixGeneChip HG-U133A arrays (Affymetrix, Santa Clara, CA, USA), were extracted from CEL files using RMA normalization procedure and custom CDF annotation package (GeneAnnot v2.2.1, Rehovot, Israel), as previously described[52].

Reagents

Recombinant AREG (R&D Systems, Abingdon, UK) was reconstituted at 0.1 mg/ml in sterile PBS, aliquoted and stored at −20 °C. Neutralizing anti-AREG monoclonal antibody (mAb) (R&D Systems, Abingdon, UK) was reconstituted at 0.2 mg/ml in sterile PBS, aliquoted and stored at −20 °C.SB225002 (Cayman Chemical, Michigan, USA) was solubilized at 10 mM stock solution in DMSO and stored at 20°C.

Cells and cell culture conditions.

Cell lines. The human myeloma cell line (HMCL) MM1.S was purchased from Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM) and antibiotics (100 U/ml penicillin, and 100 μg/ml streptomycin) all obtained from ThermoFisher Scientific (Waltham, MA, USA).

Murine macrophage RAW 264.7 cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Euroclone, Milan, Italy) and differentiated to OC as previously described[53]. The human telomerase reverse transcriptase transduced mesenchymal stromal cell line (hTERT-MSCs) was kindly gifted by Dr Giuseppe Gaipa (Monza, Italy). hTERT-MSCs were cultured in Mesenchymal Stem Cell Growth Medium (MSCGM™ Bullet Kit, Lonza, Walkersville, MD, USA) to maintain them into an undifferentiated condition and in Mesenchymal Stem Cell Osteogenic Differentiation Medium to induce osteogenic differentiation (MSC Osteogenic Differentiation BulletKit™, Lonza).

$CD14^+$ monocytes isolation. Human peripheral mononuclear cells (PBMCs) were isolatedby Ficoll-Paque (GE Helthcare-Bio Science, Uppsala, Sweden) from whole blood of the healthy donors in accordance with the Declaration of Helsinki guidelines and University of Palermo Ethics committee. Once isolated, cells were washed with MACS isolation buffer for monocyte isolation. PBMCs were then incubated with human CD14

microbeads (MiltenyiBiotec, BergischGladbach, Germany) for 15 min at 4 °C. The magnetic separation was performed using LS columns (MiltenyiBiotec), and the bound cells were then washed and suspended in α-MEM medium supplemented with 10% FBS, for further experiments.

Exosome isolation. Exosomes released by MM1.S after a 48-hour culture period in presence of FBS previously ultracentrifuged (exosome-free FBS), were isolated from conditioned culture medium by differential centrifugation, as previously described[54]. Briefly, culture medium was centrifuged subsequently for 5 min at 300xg, 15 min at 3,000xg, 30 minutes at 10,000xg and ultracentrifuged 90 min at 100,000xg in a Type 70 Ti, fixed angle rotor.

Exosomes were isolated from bone marrow (BM) plasma of 4 MM patients (3 newly diagnosed and 1 relapsed). All patients provided written informed consent in accordance with the Declaration of Helsinki. The Institutional Review Board of the University of Parma (Italy) approved this part of the study. Exosomes were isolated from human plasma and prepared as described above. Exosome pellets were washed, suspended in PBS and exosome protein content was determined by the Bradford assay.

Cells treatment. Exosomes (50 μg/ml) previously isolated from either MM1S or BM plasma MM samples, were treated or not with anti-AREG mAb (50 μg/ml) for 2 hours at 37°C. Both human primary $CD14^+$ monocytes and RAW264.7 cells were incubated for 3 and 6 days in osteoclastogenic medium (recombinant human (rh)RANKL 25 ng/ml and MCSF 25 ng/ml) with exosomes treated or not with anti-AREG mAb, and with rhAREG (50 μg/ml) as positive control. The media were changed every 3 days. At the end of the culture period, OC differentiation and EGFR activation were assessed as described below.

Human primary $CD14^+$ monocytes purified from PB were also treated with rh IL-8 and with the conditioned medium of hTERT-MSCs treated with MM1.S exosomes in the presence or not of CXCR1-CXCR2 inhibitor (SB225002). At the end of the culture period, OC differentiation was assessed.

OB differentiation. Lastly, in other experimental setting, hTERT-MSCs were used to evaluate the role of MM exosomes on OB differentiation. hTERT-MSCs were treated for 10 and 14 days with exosomes from MM1.S or from MM plasma patients in undifferentiating or osteogenic differentiation medium; the media were changed every 3 days. At the end of the culture period, osteogenic differentiation, exosome uptake and EGFR activation were assessed.

*OC differentiation***.** OC differentiation of RAW 264.7 and human PB CD14+ were evaluated after 6 days of culture conditions by the detection of tartrate-resistant acid phosphatase (TRAP) activity, according to the manufacturer's protocol (Acid Phosphatase, Leukocyte (TRAP) Kit; Sigma–Aldrich, USA) and evaluated by light microscopy. Three independent experiments were performed in triplicate; cells from 5 different fields were counted for each condition.

Atomic Force Microscopy (AFM)

Fresh cleaved mica was incubated with a vesicle solution diluted in PBS to a final concentration of 30 ng/µl for 15 minutes at room temperature. Sample was gently rinsed by PBS and Tapping mode AFM measurements were carried out in liquid by using a Nanowizard III scanning probe microscope (JPK Instruments AG, Germany) equipped with a 15-µm scanner, and AC40 (Bruker) silicon cantilevers (nominal spring constant 0.1

N/m, typical tip radius 10 nm, resonance frequency 55kHz, scan rate 1.5 Hz, free oscillation amplitude 7 nm).

Dinamic light scatter (DLS)

Exosome size distribution was determined by dynamic light scattering (DLS) experiments. Collected MM-exosome patient samples were diluted to avoid inter-particle interaction and placed at 20°C in a thermostatic cell compartment of a Brookhaven Instruments BI200-SM goniometer, equipped with a Brookhaven BI-9000 correlator and a solid-state laser tuned at 532 nm. Scattered intensity autocorrelation functions were analyzed by using a constrained regularization method or alternatively a gamma distribution[40, 55] in order to determine the size distribution (namely the z-averaged hydrodynamic diameter distribution).

Uptake of MM-derived exosomes by hTERT-MSCs.

MM1.S exosomes were labeled with PKH26 according to supplier's information. Briefly, exosomes collected after the 100,000 × g ultracentrifugation, were incubated with PKH26 for 10 min at room temperature. Labeled exosomes were washed in PBS by ultracentrifugation, the pellets were suspended in low serum medium and incubated with hTERT-MSCs for 3 hours. hTERT-MSCs were grown on coverslips coated with COL1A1 (Calbiochem, Darmstadt, Germany) and were treated with 50μg/ml of exosomes pre-treated or not with anti AREG mAb. hTERT-MSCs were stained with Actin Green (Molecular Probes, Life Technologies, Carlsbad, California, U.S) that binds actin with high affinity. Nuclei were stained with Hoechst (Molecular Probes, Life Technologies,

Carlsbad, California, U.S) and analyzed by confocal microscopy. Fluorescence intensity was measured using IMAGE J software (http://imagej.nih.gov/ij/).

Adhesion assay

Adhesion assay was performed as previously described by our group[56]. Briefly, hTERT-MSCs monolayer was incubated for 48 h with 50 μg/ml of MM1.S exosomes pre-treated or not with anti AREG mAb . After treatments, cells were washed with PBS and MM1.S cells were added for 3.5 h at 37°C. Adherent cells were stained with haematoxylin/eosin, each test group was assayed in triplicate; five high power (400×) fields were counted for each condition.

OsteoImage Bone Mineralization Assay

The amount of *in vitro* mineralization of hTERT-MSCs, seeded in 96-well tissue culture plates and treated for 10 and 14 days with exosomes from MM1.S cells in undifferentiating medium or in osteogenic differentiation medium, was evaluated using the OsteoImage Mineralization Assay Kit (Lonza, Walkersville, MD), according to supplier's information. Briefly, after each culture time point, media was removed, cells were washed in PBS and fixed. After fixation, cells were washed in the appropriate buffer and the staining reagent added. Mineralization was quantitated on a fluorescent plate reader at a 492/520 nm ratio.

Flow cytometry

Phosphorylation levels of EGFR in hTERT-MSCs incubated for 48 h with 50 μg/ml of MM1.S exosomes pre-treated or not with anti AREG mAb were determined by flow cytometry. Cells were fixed and permeabilized with Leucoperm kit (AbDSerotec). EGFR- or phospho-EGFR unconjugated primary antibody (Cell Signalling Technology, Lane Danvers, MA, USA) were added; cells were washed and a FITC secondary antibody was added. Stained cells were analyzed on a FACS Calibur (Becton Dickinson) using Cellquest software.

Western blot assay

Total proteins from MM1.S cells lysates, MM1.S exosome, conditioned medium of cells deprived of exosomes, patient's exosomes and RAW 264.7 lysates were extracted and analyzed by SDS-PAGE followed by Western blotting. The amount of proteins loaded per lane was 50 μg. Antibodies used in the experiments were as follows: anti-EGFR, pEGFR (Cell Signalling Technology, Lane Danvers, MA, USA), anti-AREG (Novus Biologicals), anti-GAPDH (Santa Cruz Biotechnology, CA, USA).

RNA extraction and real-time PCR

RNA was extracted using the commercially available IllustraRNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to manufacturer's instructions. Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-QPCR was performed in 48-well plates using the Step-One Real-Time PCR system (Applied

Biosystems). For quantitative Sybergreen real-time PCR, reaction was carried out in a total volume of 20 µl containing 2× SYBR Green I Master Mix (Applied Biosystems), 2 µl cDNA and 300 nM forward and reverse primers. Primer sequences, obtained from Invitrogen (Foster City, CA, USA), were as follows:

Human GAPDH (5'ATGGGGAAGGTGAAGGTCG3',5'GGGTCATTGATGGCAACAATAT3')

Human SNAIL (5'GCGAGCTGCAGGACTCTAAT3',5'CCCGCAATGGTCCACAAAAC3')

Human MMP9 (5'CGCTACCACCTCGAACTTTG3', 5'GCCATTCACGTCGTCCTTAT3')

Human TRAP (5'GATCCTGGGTGCAGACTTCA3', 5'GCGCTTGGAGATCTTAGAGT3')

Human CATH K (5'ACCGGGGTATTGACTCTGAA3', 5'GAGGTCAGGCTTGCATCAAT3')

Human IL8 (5'GAATGGGTTTGCTAGAATGTGATA3',5'CAGACTAGGGTTGCCAGATTTAAC3')

Human ALP (5'ACCGGGGTATTGACTCTGAA3', 5'GAGGTCAGGCTTGCATCAAT3')

Human OCN (5'GAGGGCAATAAGGTAGTGAA3', 5'CATAGATGCGTTTGTAGGC3')

Human COL1A1 (5'AAGGTGTTGTGCGATGACGTG3', 5'CACGTCATCGCACAACACCTT3')

Human OPG (5'GGCAACACAGCTCACAAGAA3', 5'CTGGGTTTGCATGCCTTTAT3')

Mouse GAPDH (5'CCCAGAAGACTGTGGATGG3', 5'CAGATTGGGGGTAGGAACAC3')

Mouse TRAP(5'GCGACCATTGTTAGCCACATACG3',5'CGTTGATGTCGCACAGAGGGAT3')

Mouse CATH-K (5'GCGTTGTTCTTATTCCGAGC3', 5'CAGCAGAGGTGTGTACTATG3')

Mouse MMP9 (5'GCTGACTACGATAAGGACGGCA3', 5'GCGGCCCTCAAAGATGAACGG3')

Mouse SNAIL (5'GCGAGCTGCAGGACTCTAAT3', 5'CCCGCAATGGTCCACAAAAC3').

Human RANKL gene expression was assessed using the TaqMan Gene Expression Assay (Life Technologies, Milan, Italy)

Real-time PCR was performed in triplicates for each data point. Relative changes in gene expression between control and treated samples were determined using the ΔΔCt method. Levels of the target transcript were normalized to a GAPDH endogenous control, constantly expressed in all samples (ΔCt). For ΔΔCt values, additional subtractions were performed between treated samples and control ΔCt values. Final values were expressed as fold of induction.

ELISA assay

MMP-9 levels were quantified by Human MMP-9 ELISA assays (Invitrogen) for CD 14^+ monocytes and with mouse total MMP9–enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN) for RAW 264.7 cells according to the manufacture's protocol. Furthermore, the levels of ALP and IL8 secreted by hTERT-MSCs were quantified respectively by ALP ELISA assay (Cloud-Clone Corp ELISA KIT assay, Cloud-Clone Corp. Houston, TX) and Human IL8 ELISA assay (R&D Systems, Minneapolis, MN) according to the manufacture's protocol.

Statistical analysis

Data are expressed as means ± SD of three independent experiments. Statistical analysis was done with a paired sample t-test. Differences were considered significant when $p \leq 0.05$.

CHAPTER 3

Results

3.1 The EGFR ligand AREG is expressed by MM cells and enriched in exosomes

By analyzing the mRNA expression level of EGFR ligands in a published dataset (accession number GSE16122), we found that $CD138^{+}$ cells expressed AREG at high level. We did not find a significant difference across the different type of monoclonal gammopathies although MM patients showed higher expression level (Fig.1A). Consistently, we found that HMCLs expressed AREG mRNA (data not shown) by real-time PCR.

Exosomes were then isolated from one HMCL MM1.S and characterized by the atomic force microscope (AFM) in order to confirm that we are working with vesicles of about 80 nm (Fig. 1B). We found that AREG was specifically enriched in exosomes as confirmed by its low abundance in MM1.S cells and in the exosomes-deprived conditioned medium (Fig. 1C).

3.2 MM1.S-exosomes induce the activation of EGRF pathway in OC progenitor cells

We next investigated whether MM1.S-exosomes treatment induced the activation of EGFR pathway in OC progenitors. RAW 264.7 cells treated with MM1.S cell line exosomes under osteoclastogenic conditions, showed an increase in the phosphorylation of EGFR (Fig. 2A). The treatment with exosomes derived from MM1.S cells pretreated with anti-AREG mAb reduces the phosphorylation of EGFR. Subsequently, we found that the 6-day treatment with MM1.S-derived exosomes induced a significant increase in the mRNA expression level of *SNAIL*, a downstream target of EGFR, both in RAW 264.7 (Fig. 2B, upper panel) and in pre-osteoclast human $CD14^+$ (Fig. 2B, lower panel). The presence of anti-AREG mAb reverted this effect (Fig. 2B).

3.3 AREG-enriched MM cell-derived exosomes induced OC differentiation.

Raimondi *et al.* demonstrated that MM-derived exosomes directly induce the expression of OC specific markers[40]. We confirmed that the treatment with MM-derived exosomes from MM1.S increases OC specific markers, such as TRAP, CTSK and MMP9 at mRNA level both in RAW 264.7 (Fig. 3A) and PB human $CD14^+$ cells (Fig. 3B). This effect was confirmed at protein level for MMP-9 (Fig. 3C) and by TRAP staining (Fig.3D). The pro-osteoclastogenic effect of MM-derived exosomes was significantly abrogated by the pre-treatment with anti-AREG mAb (Figure 3 A-B-C-D) suggesting a direct effect of MM exosomes-derived AREG on OC differentiation.

On the basis of these data, we investigated whether ex *vivo*, exosomes from MM patients deliver the EGFR ligand. Exosomes were isolated from BM aspirates of MM patients. The vesicles were analyzed with DLS, showing a clear distribution with peak at about 100 nm(Fig. 4A) and with western blot for TSG101 (Fig. 4B). AREG was enriched in the exosomes obtained from 3 out of 4 patients, thus confirming the exosomal packaging of the ligand. As observed with exosomes from MM1.S cell line, we found that also patient-derived exosomes increase the expression of SNAIL in pre-OCs, while the presence of anti-AREG mAb abolished this effect (Fig. 4C). Similarly, the pro-osteoclastogenic effects of exosomes obtained from MM patients were abrogated by the pre-treatment with the anti-AREG mAb at mRNA (Fig. 5A) and protein level as shown for MMP9 (Fig. 5B) and TRAP staining (Fig. 5C). Overall, these data indicate that the EGFR ligand AREG is packed into MM-derived exosomes and directly involved in OC differentiation.

3.4 MM-derived exosomes are internalized into human BM mesenchymal cells blocking osteogenic differentiation and increasing the release of the pro-osteoclastogenic cytokines through the activation of EGFR pathway.

To further investigate the mechanism by which AREG- enriched exosomes from MM cells are involved in MM-induced alteration of bone remodeling, we evaluated the effect of exosomes on hTERT-MSCs. We demonstrated that MM1.S-derived exosomes are internalized into hTERT-MSCs independently by the neutralization of AREG (Fig. 6A). Exosomes internalization by hTERT-MSCs induced the activation of EGFR pathway as demonstrated by the increase of the tyrosine kinase receptor phosphorylation. EGFR activation was blocked by the treatment of anti-AREG mAb (Fig. 6B).

Interestingly, under osteogenic conditions, the treatment of hTERT-MSCs with MM1.S-derived exosomes for 14 days reduces the mRNA levels of OB differentiation markers (Fig 7A). In addition to gene expression, the ability of MM exosomes to inhibit ALP release was evaluated at the protein level (Fig. 7B). OB differentiation is characterized by the formation of mineralized nodules. Therefore, we performed an *in vitro* mineralization assay in order to functionally evaluate the effect of MM exosomes on bone-like nodules deposited by cells. As shown in Fig.7C, 14 days of treatment of hTERT-MSCs with MM1.S exosomes under osteogenic conditions, reduces the formation of mineralized nodules. No differences were observed in exosomes-treated MSCs maintained into an undifferentiated state. To further confirm the observed exosomes-mediated inhibition of OB differentiation, we performed a qRT-PCR of cells treated with exosomes from the BM aspirates of MM patients. Accordingly, 14-day treatment of hTERT-MSCs under osteogenic conditions decreases OB differentiation marker at the mRNA (Fig.7D) and protein level (Fig. 7E).

Finally, we found that the treatment of hTERT-MSCs with exosomes increases the adhesion of MM1.S cells to the mesenchymal monolayer and that this effect is abrogated by the presence of the anti-AREG mAb (Fig. 8A), Moreover, we found that exosomes are able to reduce OPG mRNA and to increase RANKL mRNA levels by hTERT-MSCs (Fig. 8B). .

Interestingly, a significant increase in the production of the pro-osteoclastogenic cytokine IL-8 by hTERT-MSCs was observed at both mRNA (Fig. 8C) and protein level at 24 and 48h (Fig. 8D). These effects were abrogated by the pre-treatment with anti-AREG mAb (Fig.8C-D).

To correlate the increased expression and secretion of IL8 from exosomes-treated MSCs with the exosomes-dependent increase of OC function, we co-treated human CD14+ with (i) recombinant IL8 (rIL8) and (ii) with the conditioned medium of hTERT-MSCs treated with MM1.S exosomes, in the presence or absence of the IL8 receptor (CXCR1–2) inhibitor, SB225002 (SB). The treatment with rIL8 induced the expression of MMP9 and CTSK mRNA (Fig 8E). Consistently, the treatment with conditioned medium of hTERT-MSCs pre-treated with MM exosomes, increased the expression of the osteoclastogenic markers. This effect was abrogated by the use of SB (Fig 8E) thus confirming the role of IL8 released by MSCs after exosomes stimulation, in the activation of OC differentiation.

CHAPTER 4

Discussion

In this study we focus on unveiling the molecular mechanism by which MM exosomes are able to affect OC differentiation. We demonstrate that the EGFR ligand AREG is packed into exosomes from MM cell line as well as from the BM aspirates of patients and that its presence is responsible for the exosome-induced osteoclastogenesis. The activation of the EGFR pathway has been correlated to metastatic bone diseases and in particular to the increased bone resorption observed in these tumors[57, 58]. In particular, in breast cancer Mercatali*et al.* showed that the crosstalk between MSCs and cancer cells promoted osteoclastogenesis by stimulating RANK and EGFR signaling pathways[59]. Furthermore, EGFR deficiency impaired OCs recruitment in EGFR-deficient mice[60]. In MM, Mahtouk and colleagues showed that, among the EGFR ligands, AREG is significantly over-expressed by MM cells as compared to normal PCs and that it is able to stimulate cell growth[50].

It has already been demonstrated that cancer exosomes contain the EGFR ligands, including AREG, suggesting that the EGFR system contributes to the exosome-mediated communication within the tumor microenvironment[61-63]. For example, Taverna et al.

demonstrated that non-small cell lung cancer- derived exosomes, containing AREG, induce EGFR pathway activation in pre-OCs leading to the increased expression of RANKL[62]. Here we found that AREG is abundantly present in MM exosomes partially explaining previously published data by Raimondi *et al.*[40].

Since the presence of AREG is directly responsible for the activation of OC function in exosome-treated pre-OCs, we further assessed whether the presence of the ligand in the exosomes was able to modulate MSCs phenotype and to activate OC formation indirectly through MSCs. We found that the treatment of MSCs with MM exosomes increased the release of IL8 in the conditioned medium, while AREG depletion abrogated the effect. Similarly, recent data show that the ligand–receptor interaction between AREG produced by leukemic cells, and EGFR by BM stromal cells, modulates leukemic and stromal cells bidirectional crosstalk[63]. In addition, in chronic myeloid leukemia model, we have previously shown that AREG is involved in the activation of EGFR downstream signaling in mesenchymal stromal cells leading to the expression and release of IL8[56].

IL-8 is responsible for the increased osteolysis observed in metastatic bone disease[17] and that its release, following the interaction between MM cells and human MSCs, contributed to in vitro OC formation[18]. Here we found that exosomes, through the activation of the EGFR pathway, may also indirectly contribute to the induction of osteoclastogenesis by promoting the release of IL8 by MSCs; in fact, IL8-enriched conditioned medium induces the expression of OC specific markers in human pre-OCs.

Based on our data showing that MM-derived exosomes block osteogenic differentiation of MSCs, it is conceivable to hypotheses that MM exosomes contribute to increase the number of undifferentiated MSCs and consequently the production of pro-osteoclastogenic

cytokines as IL-8[18] and RANKL[64]. Accordingly we show that MM-derived exosomes increased the RANKL mRNA expression and decreased that of OPG. Clearly this effect can be involved in the indirect pro-osteoclastogenic effect of MM-derived exosomes.

Moreover exosomes may contribute to the block of bone formation process through the activation of EGFR pathway. In a recent study, Kumar and colleagues in vivo demonstrated that exosomes from acute myeloid leukemia modulate the BM niche; in particular authors showed that exosomes suppress osteogenic differentiation of mesenchymal stromal progenitors[65]. Other authors observed that MM cells-derived exosome contain the lncRNA RUNX2-AS1 which is responsible for the decreased expression of RUNX2 in MSCs, leading to the osteogenesis suppression[66]. Although in this study authors identified one of the molecular interactor of the exosome-mediated osteogenic inhibition, further studies need to be conducted in order to characterize MM exosome content and fully understand how MM exosomes contribute to the uncoupled bone remodeling by the inhibiting bone formation.

The observation that MM cell-derived exosomes induced the activation of EGFR pathway in both OC progenitors and in MSCs suggests the possibility to use EGFR inhibitors such as erlotinib and gefitinib to impair the cross talk between MM cells and the bone microenvironment and potentially the development of bone lesions. Consistently, it was reported that erlotinib inhibits osteolytic bone invasion of non-small lung cancer[67] and that gefitinib inhibits the ability of MSC to induce OC differentiation[68].

In conclusion, our data indicate that MM-derived exosomes could be responsible for the uncoupled bone remodeling increasing OCs differentiation both directly and indirectly

through at least in part the release of IL-8 by MSCs. Thus AREG packed into MM-derived exosomes, may represent a potential new player in MM-induced osteoclastogenesis.

CHAPTER 5

Figure legends

Fig. 1. (A) Box plot represents the median level of AREG expression of 11 monoclonal gammopathy of undetermined significance (MGUS), 133 MM patients at diagnosis and 9 plasma cell leukemia (PCL) (GSE16122).**(B)** Representative AFM image of MM1.S exosomes. **(C)** Total proteins were extracted from MM1.S cells, MM1.S exosomes (exo) and from exosome-deprived conditioned medium (cm-exo) and 50 μg of protein per lane were subjected to western blot analysis with an antibody against AREG.

Fig. 2. (A) Western blotting analysis of pEGFR and EGFR in whole lysates of RAW 264.7 cells incubated, for 6 days, with MM1.S derived exosomes (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml), with rhAREG (50 μg/ml) and rhRANKL (25 μg/ml) as positive control. The histogram on the right represents the ratio pEGFR/EGFR, based on densitometric analysis normalized versus GAPDH, used as loading control. **(B)** Evaluation by quantitative Real Time PCR of mRNA expression of SNAIL in RAW 264.7 incubated, for 3 and 6 days, with MM1.S derived exosomes (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml), rhRANKL (25 μg/ml) and with rhAREG (50 μg/ml) as positive control. Human PB $CD14^+$ cells incubated, for 6 days in osteoclastogenic medium (rhRANKL 25 ng/ml and MCSF 25 ng/m)l, with MM1.S derived exosomes (50 μg/ml)

treated or not with anti-AREG mAb(50 μg/ml).* Exo vs untreated (*p ≤ 0.05; **p ≤ 0.01); # Exo+AREGnAb vs Exo (#p ≤ 0.05; # # #p ≤ 0.001).

Fig. 3. Evaluation by quantitative Real Time PCR of mRNA expression of TRAP, Cathepsin K and MMP9 in **(A)** RAW 264.7 incubated, for 3 and 6 days, with MM1.S derived exosomes (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml), rhRANKL (25 μg/ml) and with rhAREG (50 μg/ml) as positive control. **(B)** Human PB $CD14^+$ cells incubated, for 6 days in osteoclastogenic medium (rhRANKL 25 ng/ml and MCSF 25 ng/m), with MM1.S derived exosomes (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml).* Exo vs untreated (*p ≤ 0.05); # Exo+AREGnAb vs Exo (#p ≤ 0.05). **(C)** Human Metalloproteinase-9 (hMMP9) protein level was measured by ELISA in the conditioned medium of Human PB $CD14^+$ cells incubated, for 6 days in osteoclastogenic medium (rhRANKL 25 ng/ml and MCSF 25 ng/m), with MM1.S derived exosomes (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml). * Exo vs untreated (*p ≤ 0.05); # Exo+AREGnAb vs Exo (#p ≤ 0.05). **(D)** Trap staining of Human PB $CD14^+$ seeded in 96 well plate in presence or absence of MM1.S derived exosomes (50 μg/ml) treated or not with nAb AREG and rhAREG anti-AREG mAb(50 μg/ml) for 6 days.

Fig. 4. (A) MM patient exosomes size distribution was determined by DLS analysis. **(B)** Total proteins were extracted from exosomes isolated from the BM plasma of MM patients and were subjected to western blot analysis with antibody against AREG and Tsg101. The table below indicates the clinical information of the four MM patients analyzed. **(C)** Evaluation by quantitative Real Time PCR of mRNA expression of SNAIL in Raw 264.7 cells incubated with exosomes from MM patients (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml). * Exo vs untreated (**p ≤ 0.01); # Exo+AREGnAb vs Exo (##p ≤ 0.01).

Fig. 5. (A) Evaluation by quantitative Real Time PCR of mRNA expression of TRAP, Cathepsin K and MMP9 in Raw 264.7 cells incubated with exosomes from MM patients (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml). **(B)** Murine Metalloproteinase-9 (mMMP9) protein level was measured in the conditioned medium of Raw 264.7 cells incubated, for 6 days, with exosomes from MM patients (50 μg/ml) treated or not with anti-AREG mAb(50 μg/ml), rhRANKL (25 μg/ml) and with rhAREG (50 μg/ml) as positive control. * Exo vs untreated (*p ≤ 0.05; **p ≤ 0.01); # Exo+AREGnAb vs Exo (#p ≤ 0.05; # #p ≤ 0.01). **(C)** Trap staining of RAW 264.7 (1000 cells/well) seeded in a 96 well plate in DMEM with 10% FBS in presence or absence of exosomes from MM patients (50 μg/ml) or not with anti-AREG mAb(50 μg/ml) and with rhAREG (50 μg/ml) as positive control for 6 days**.** At the end of the treatment period, the OCs were identified as multinucleated cells when they contain more than three nuclei positive for tartrate resistant acid phosphatase.

Fig. 6. (A) Analysis at confocal microscopy of hTERT-MSCs treated for 4 hours with MM1.S exosomes pretreated or not with nAb AREG, compared with untreated hTERT-MSCs (Ctrl). hTERT-MSCs were stained with phalloidin Alexa Fluor (green), nuclear counterstaining was performed using Hoescht (blue), exosomes were labelled with PKH26 (red); histogram shows fluorescence intensity expressed as ratio between a.u. and number of hTERT-MSCs treated with MM1.S exosomes and MM1.S exosomes pretreated with nAb AREG. **(B)** Levels of EGFR and phospho-EGFR were determined by FACS analysis in hTERT-MSCs after 48h treatment with MM1.S exosomes pretreated or not with nAb AREG.

Fig.7. (A) Evaluation by quantitative Real Time PCR of mRNA expression of ALP, OCN and COL1A1 in hTERT-MSC treated for 10 and 14 days with MM1.S exosomes under

undifferentiating medium (ctrl) or in osteogenic differentiation medium (diff). **(B)** ALP protein release was evaluated by ELISA assay in the conditioned medium of hTERT-MSC treated for 14 days with MM1.S exosomes under undifferentiating medium (ctrl) or in osteogenic differentiation medium (diff). **(C)** Quantification of in vitro osteoblast mineralization in the hTERT-MSC treated for 10 and 14 days with MM1.S exosomes under undifferentiating medium (ctrl) or in osteogenic differentiation medium (diff) was evaluated using OsteoImage Mineralization Assay Kit. Values are expressed as fluorescence units (RFU; 492 nm excitation/520 nm emission wavelengths).* Diff vs ctrl (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001); # Diff+ Exo vs diff (#p ≤ 0.05; # # p ≤ 0.01). **(D)** Evaluation by quantitative Real Time PCR of mRNA expression of ALP, OCN and COL1A1 in hTERT-MSC treated for 14 days with exosomes isolated from BM plasma of 3 MM patients in osteogenic differentiation medium (diff). **(E)** ALP protein release was evaluated by ELISA assay in the conditioned medium of hTERT-MSC treated for 14 days with exosomes isolated from BM plasma of 3 MM patients in osteogenic differentiation medium (diff).# Diff+ Exo vs diff (#p ≤ 0.05; # # p ≤ 0.01; ### p ≤ 0.001).

Fig. 8. (A) Adhesion assay of MM1.S exo on hTERT-MSCs: pre-treatment of hTERT-MSCs cells with MM1.S exo for 48h increases MM1.S cell adhesion to mesenchymal cells**.** Treatment with exosomes pretreated with anti-AREG mAb reduces this effect. Right panel: a representative phase contrast micrograph showing the adhesion of MM1.S cells to exosome-treated hTERT-MSCs monolayer. (B) Evaluation by quantitative Real Time PCR of mRNA expression of OPG and RANKL in hTERT-MSC treated for 14 days with MM1.S exosomes in osteogenic differentiation medium (diff) (*p ≤ 0.05; **p ≤ 0.01). (**C**) IL8 mRNA expression was evaluated by Real Time-PCR in hTERT-MSCs treated for 24 or 48h with MM1.S exosomes pretreated or not with anti-AREG mAb for 24 and 48h. **(D)**

IL8 protein release was evaluated by ELISA assay in the conditioned medium of hTERT-MSCs monolayer after 48h treatment with MM1.S exosomes pretreated or not with anti-AREG mAb. * Exo vs untreated (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001); # Exo+AREGnAb vs Exo (#p ≤ 0.05; # # p ≤ 0.01). **(E)** Evaluation by quantitative Real Time PCR of mRNA expression of Cathepsin K and MMP9 in $CD14^+$ monocytes untreated or treated for 6 days with rIL8, with the conditioned medium of BMMSC cells treated with MM1.S exosomes with or without SB225002 (*p ≤ 0.05; **p ≤ 0.01).

CHAPTER 6

Figures

Fig.1 The EGFR ligand AREG is expressed by MM cells and enriched in MM1.s-derived exosomes.

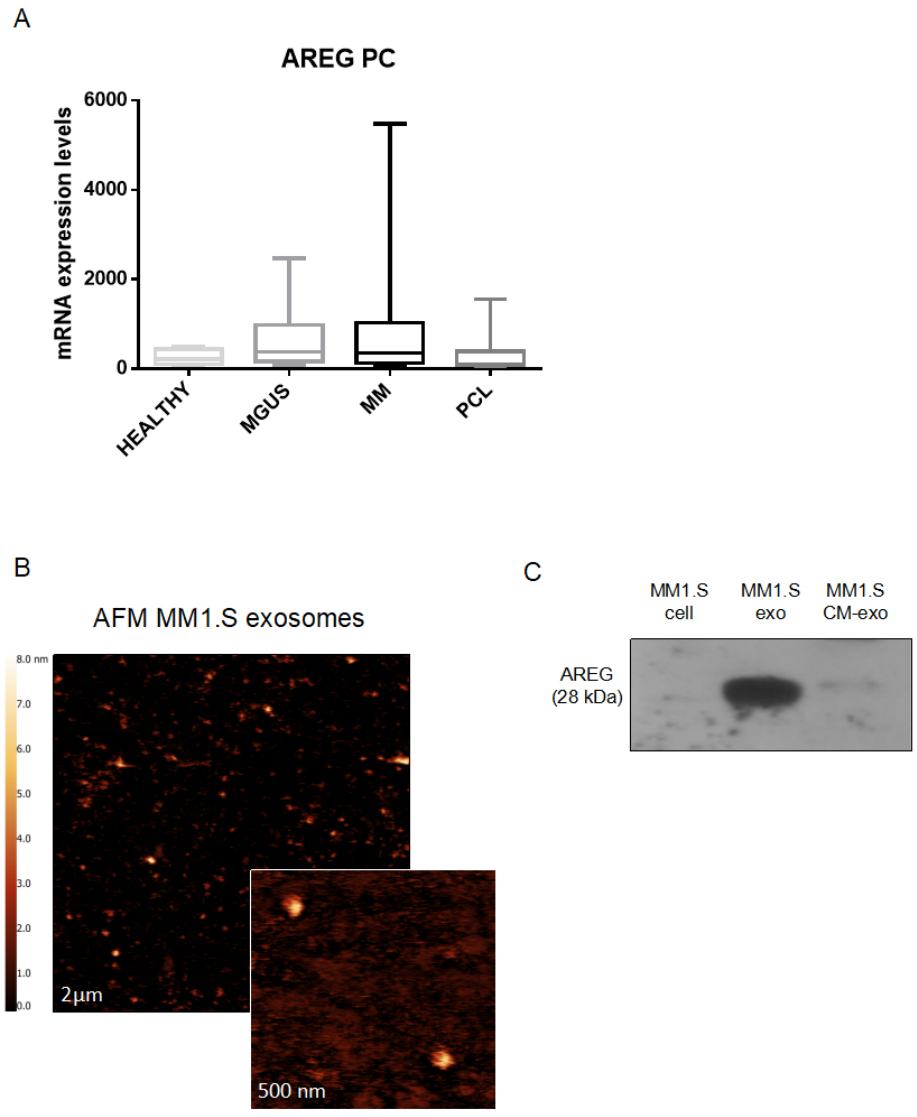


Fig.2 MM1.S-derived exosomes induce the activation of EGRF pathway in OC progenitor

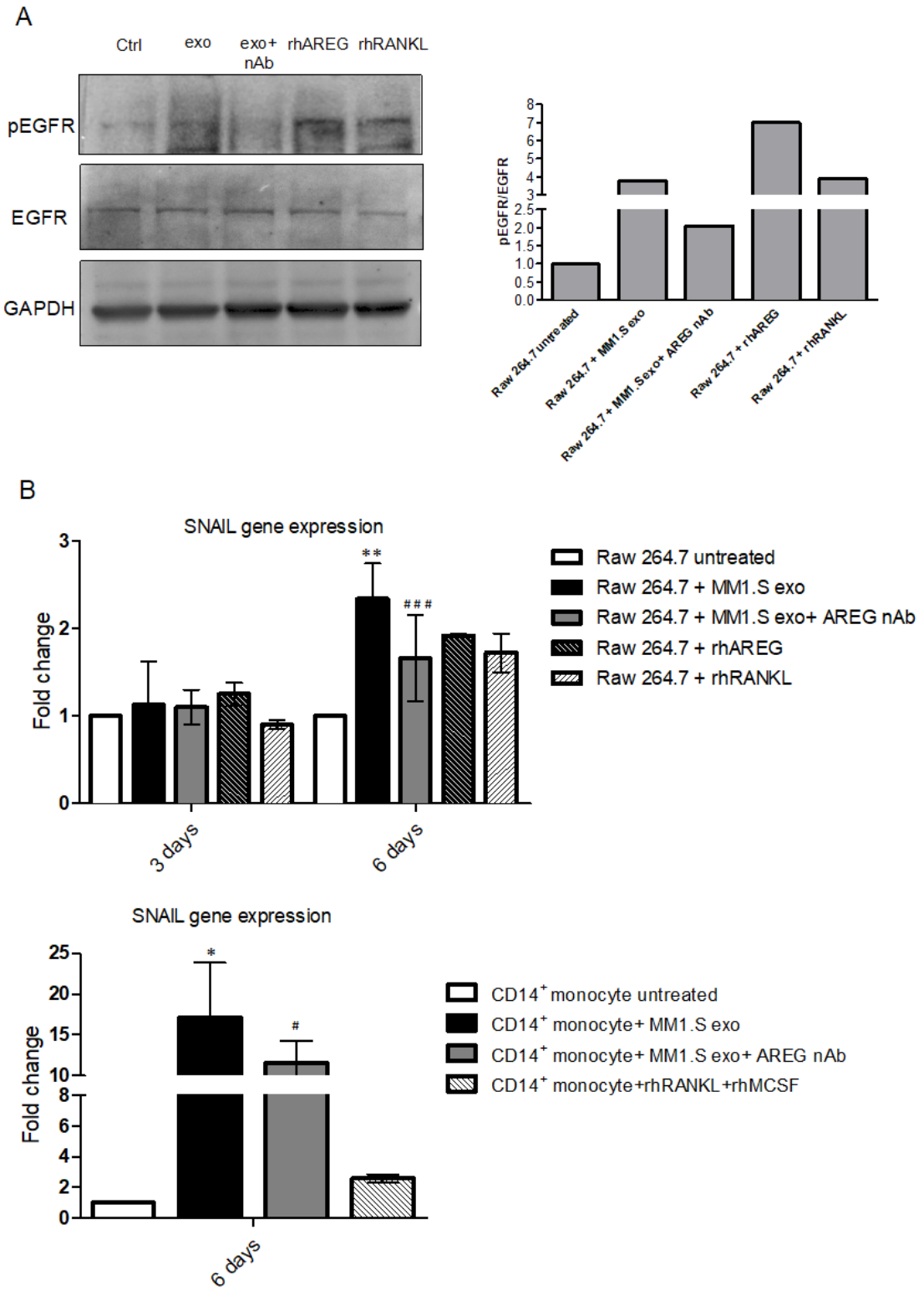


Fig.3 AREG enriched MM1.s-derived exosomes induced osteoclast differentiation.

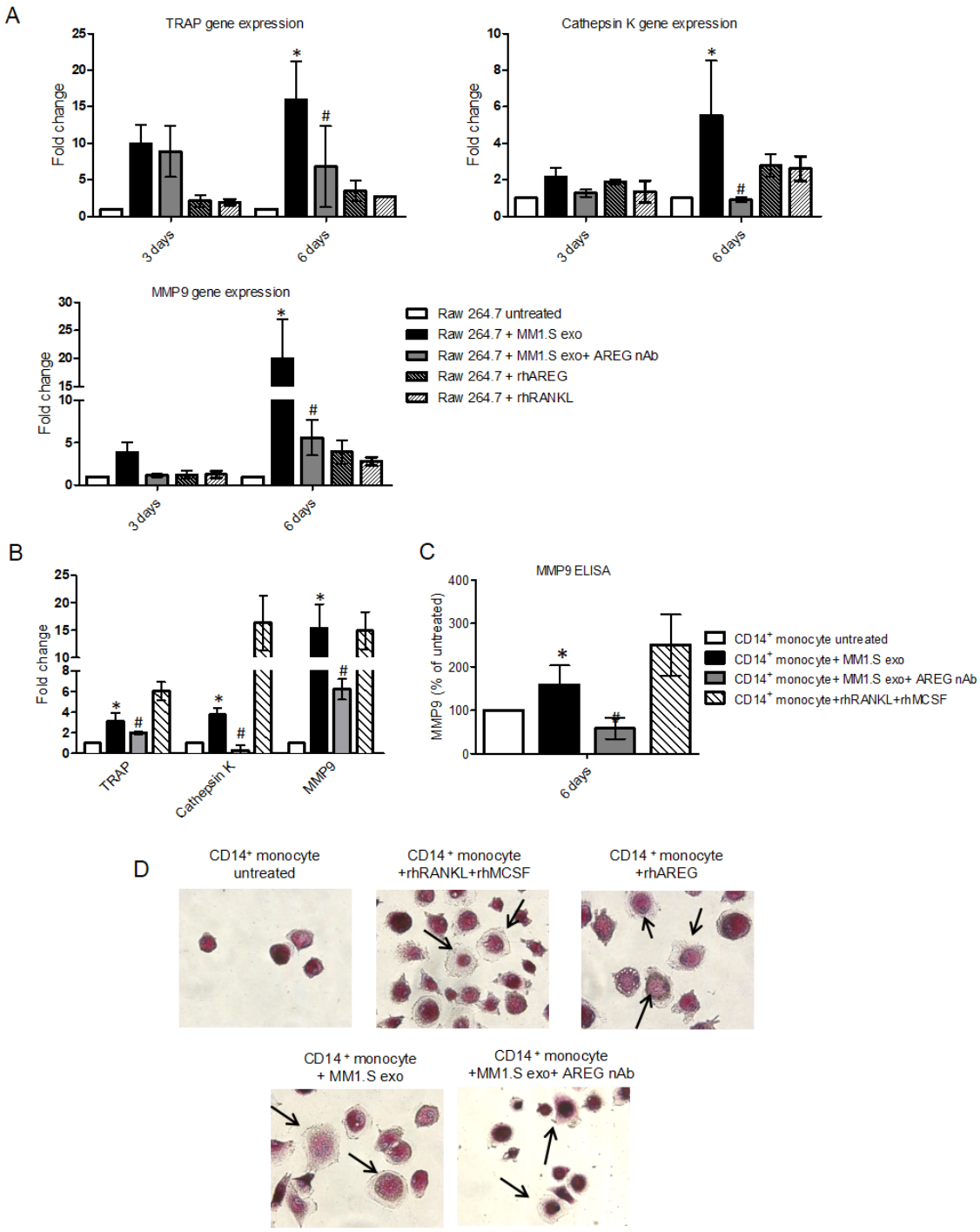
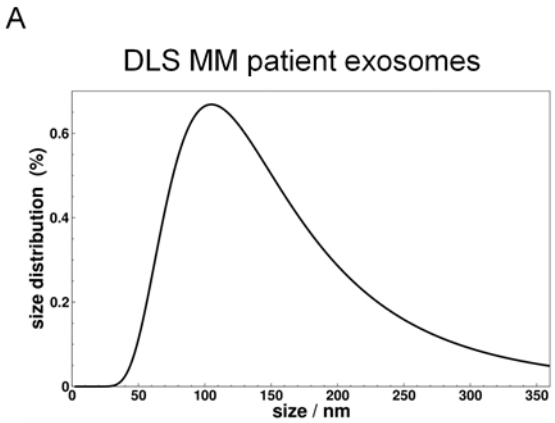
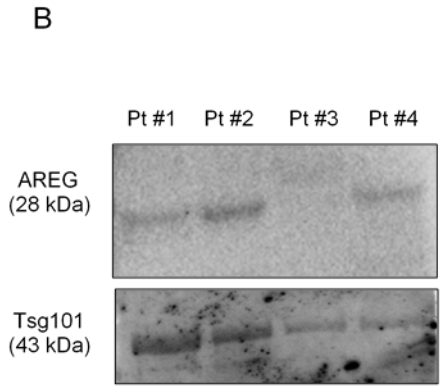


Fig.4 AREG enriched MM patients-derived exosomes induce the activation of EGFR pathway in OC progenitor





PATIENT	DIAGNOSIS	AGE	SEX	ISS
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# 2	MM ND	79	M	III
# 3	MM R	75	F	
# 4	MM ND	76	M	III

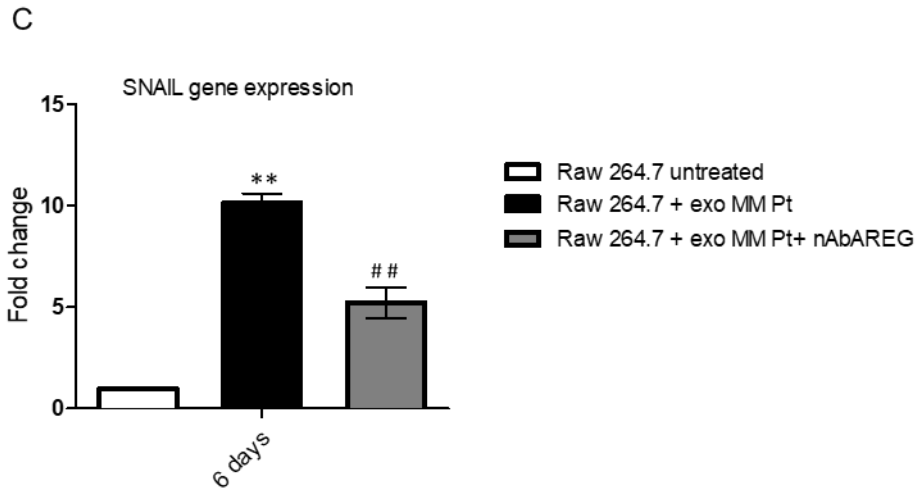

Fig.5 AREG enriched MM patients-derived exosomes induced osteoclast differentiation

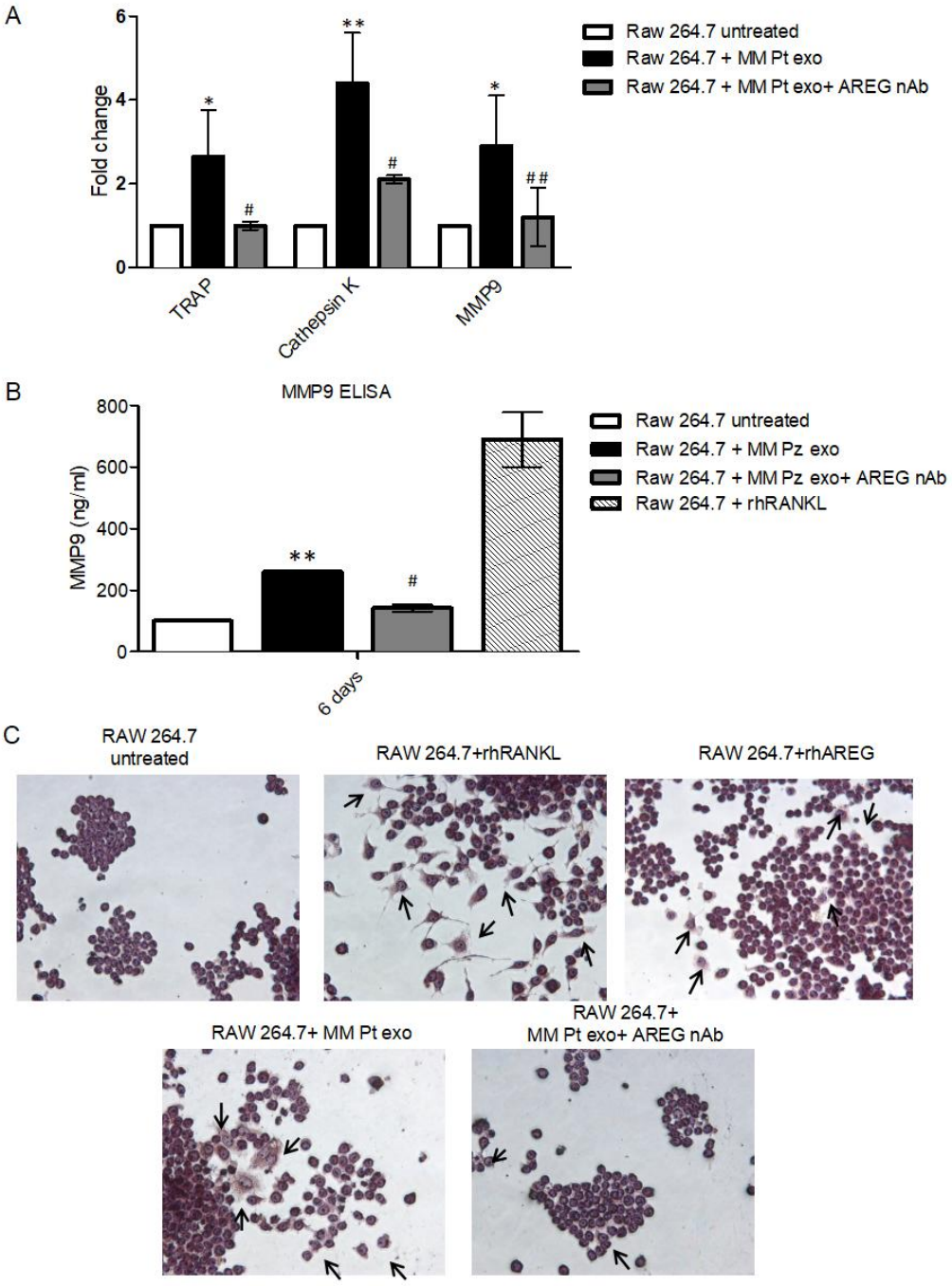

Fig.6 MM-derived exosomes are internalized into human MSCs and induced the activation of EGFR pathway

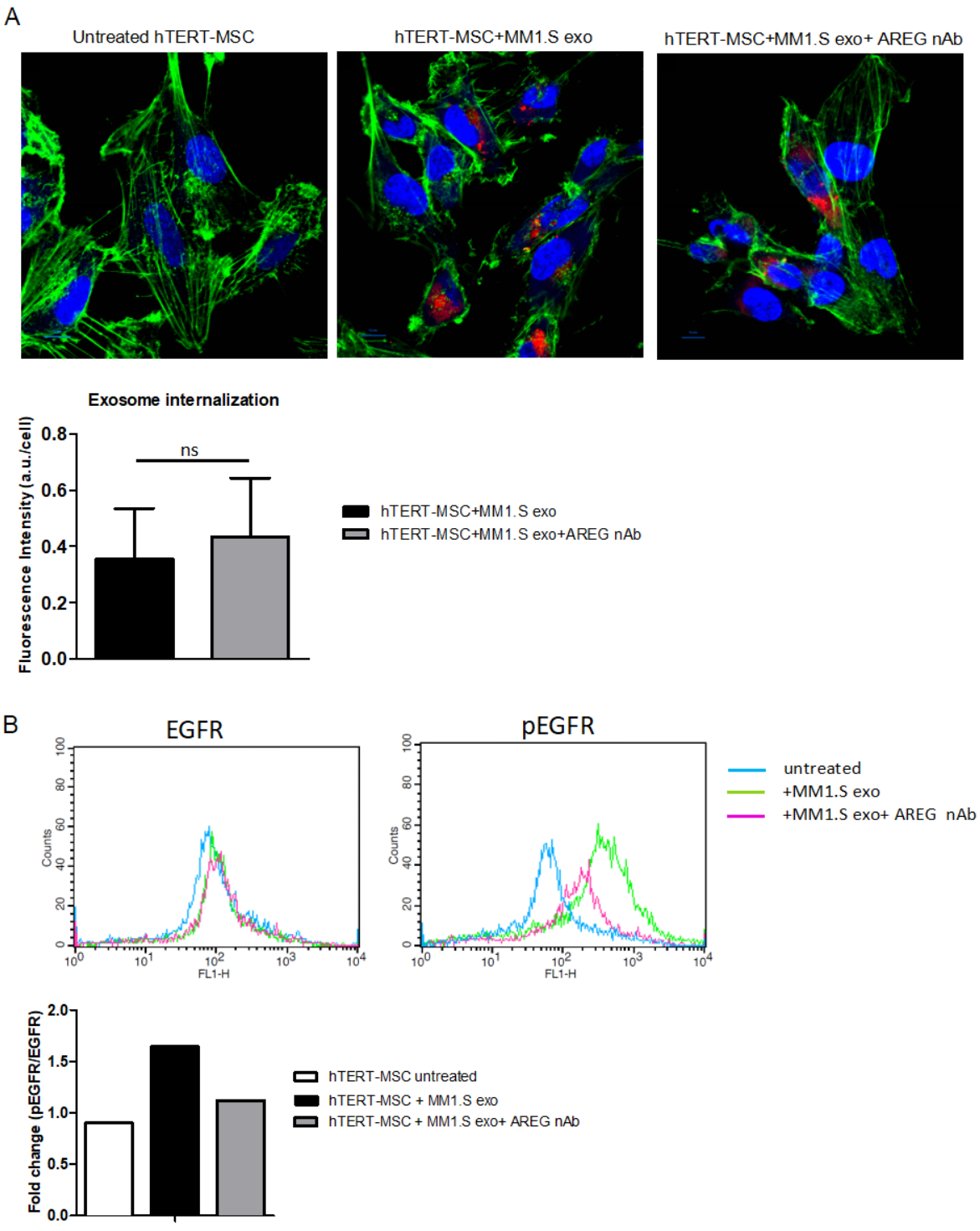

Fig.7 MM-derived exosomes reduced the expression of osteoblast markers in human MSCs

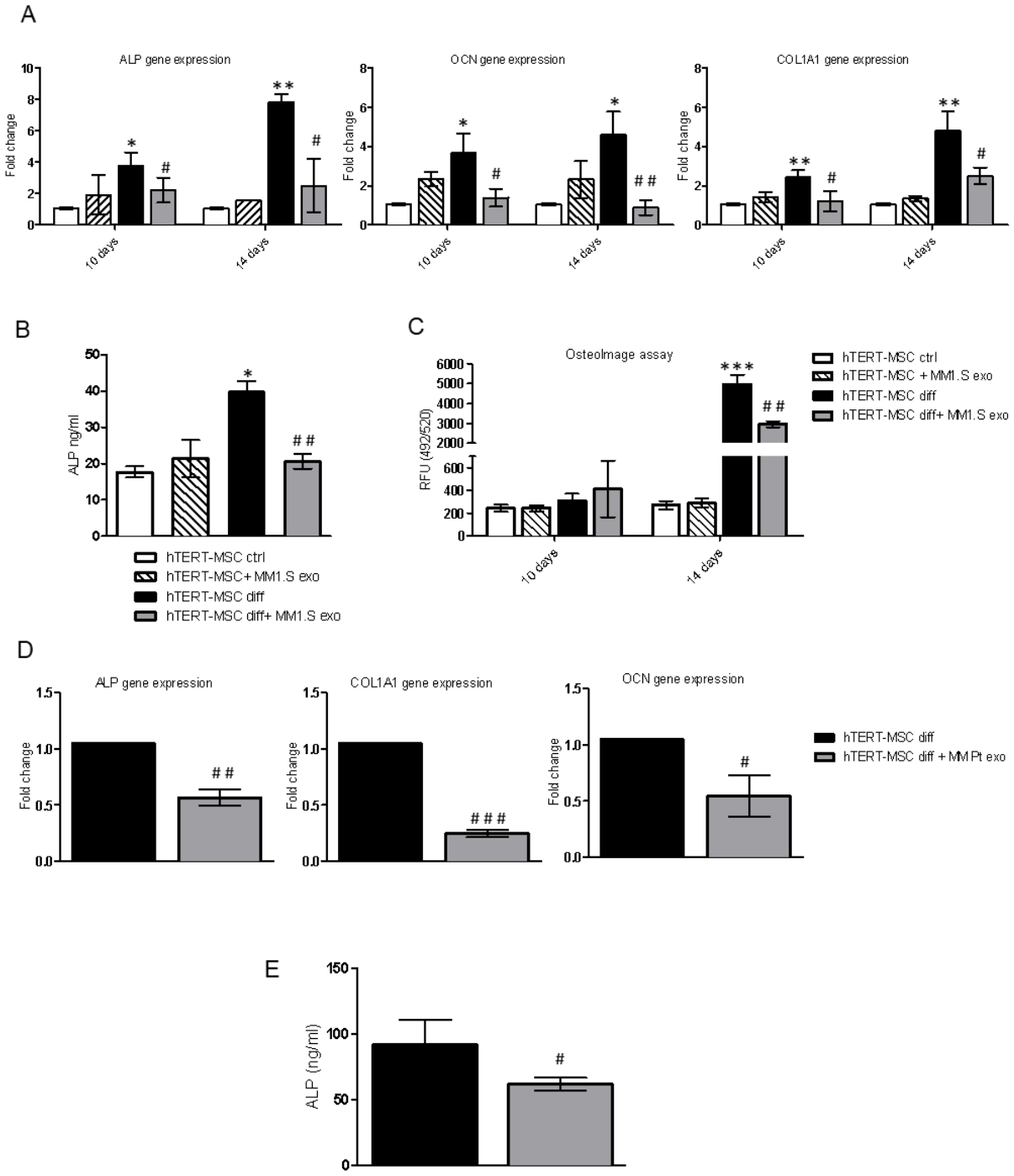

Fig.8 Effects of MM-derived exosomes on human MSCs.

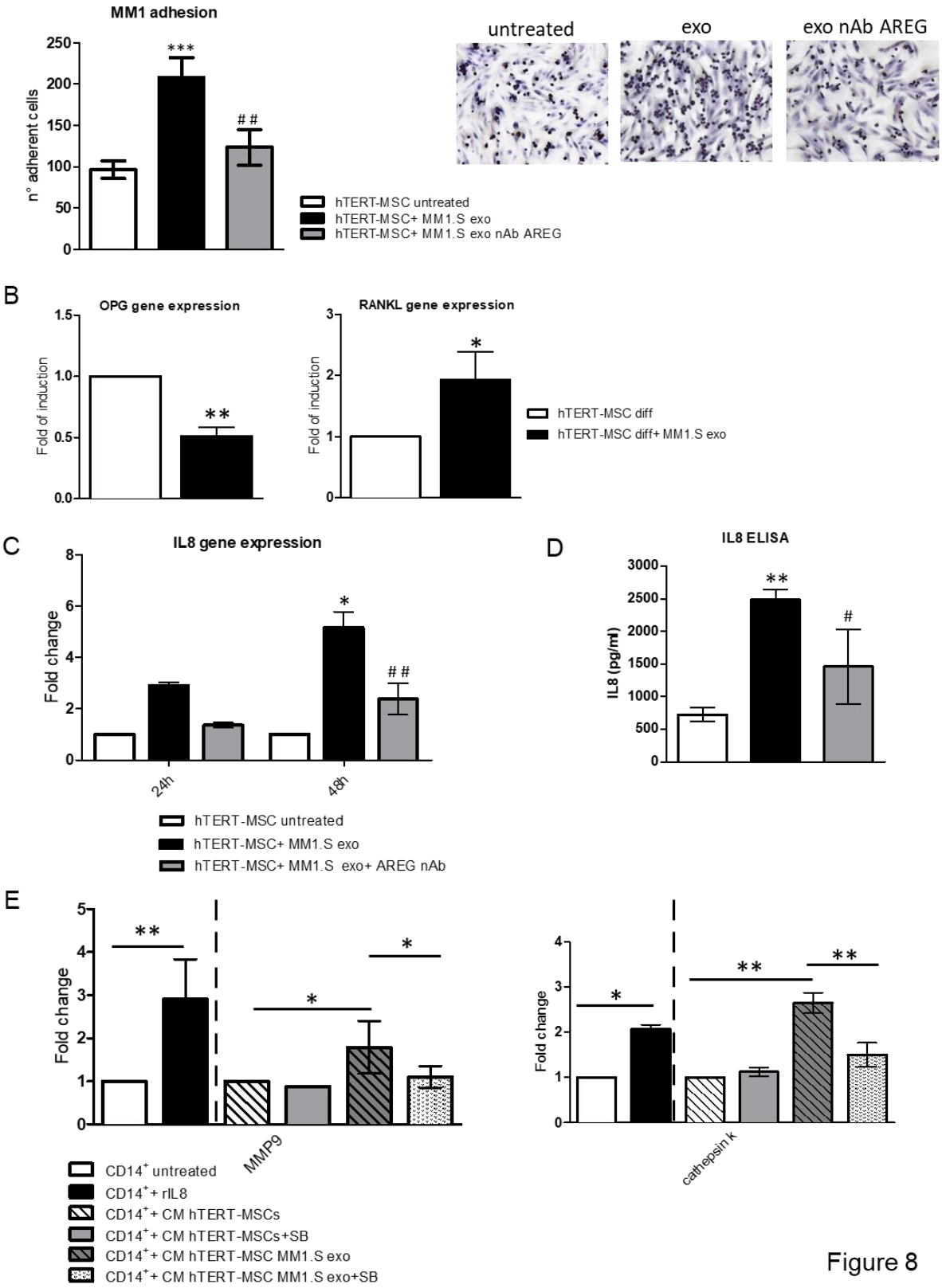

Figure 8

Bibliography

[1] N. Raje, G.D. Roodman, Advances in the biology and treatment of bone disease in multiple myeloma, Clin Cancer Res, 17 (2011) 1278-1286.
[2] M. Rossi, M.T. Di Martino, E. Morelli, M. Leotta, A. Rizzo, A. Grimaldi, G. Misso, P. Tassone, M. Caraglia, Molecular targets for the treatment of multiple myeloma, Curr Cancer Drug Targets, 12 (2012) 757-767.
[3] A. Palumbo, K. Anderson, Multiple myeloma, The New England journal of medicine, 364 (2011) 1046-1060.
[4] R.A. Kyle, E.D. Remstein, T.M. Therneau, A. Dispenzieri, P.J. Kurtin, J.M. Hodnefield, D.R. Larson, M.F. Plevak, D.F. Jelinek, R. Fonseca, L.J. Melton, 3rd, S.V. Rajkumar, Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma, The New England journal of medicine, 356 (2007) 2582-2590.
[5] K.C. Anderson, R.D. Carrasco, Pathogenesis of myeloma, Annu Rev Pathol, 6 (2011) 249-274.
[6] G.W. Basak, A.S. Srivastava, R. Malhotra, E. Carrier, Multiple myeloma bone marrow niche, Curr Pharm Biotechnol, 10 (2009) 345-346.
[7] T. Hideshima, C. Mitsiades, G. Tonon, P.G. Richardson, K.C. Anderson, Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets, Nat Rev Cancer, 7 (2007) 585-598.
[8] D. Ribatti, B. Nico, A. Vacca, Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma, Oncogene, 25 (2006) 4257-4266.
[9] G.A. Rodan, Bone homeostasis, Proc Natl Acad Sci U S A, 95 (1998) 13361-13362.
[10] A. Hameed, J.J. Brady, P. Dowling, M. Clynes, P. O'Gorman, Bone disease in multiple myeloma: pathophysiology and management, Cancer Growth Metastasis, 7 (2014) 33-42.
[11] S. Lentzsch, L.A. Ehrlich, G.D. Roodman, Pathophysiology of multiple myeloma bone disease, Hematol Oncol Clin North Am, 21 (2007) 1035-1049, viii.
[12] A.P. Trouvin, V. Goeb, Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: maintaining the balance to prevent bone loss, Clin Interv Aging, 5 (2010) 345-354.
[13] W.S. Simonet, D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Luthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. RenshawGegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee, W.J. Boyle, Osteoprotegerin: A novel secreted protein involved in the regulation of bone density, Cell, 89 (1997) 309-319.
[14] L.C. Hofbauer, M. Schoppet, Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases, JAMA, 292 (2004) 490-495.
[15] G.D. Roodman, Pathogenesis of myeloma bone disease, J Cell Biochem, 109 (2010) 283-291.
[16] Y. Ning, P.C. Manegold, Y.K. Hong, W. Zhang, A. Pohl, G. Lurje, T. Winder, D. Yang, M.J. LaBonte, P.M. Wilson, R.D. Ladner, H.J. Lenz, Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models, Int J Cancer, 128 (2011) 2038-2049.
[17] M.S. Bendre, D.C. Montague, T. Peery, N.S. Akel, D. Gaddy, L.J. Suva, Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease, Bone, 33 (2003) 28-37.

[18] A.B. Herrero, A. Garcia-Gomez, M. Garayoa, L.A. Corchete, J.M. Hernandez, J. San Miguel, N.C. Gutierrez, Effects of IL-8 Up-Regulation on Cell Survival and Osteoclastogenesis in Multiple Myeloma, Am J Pathol, 186 (2016) 2171-2182.
[19] N. Giuliani, S. Colla, F. Morandi, M. Lazzaretti, R. Sala, S. Bonomini, M. Grano, S. Colucci, M. Svaldi, V. Rizzoli, Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation, Blood, 106 (2005) 2472-2483.
[20] R.T. Franceschi, G. Xiao, Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways, J Cell Biochem, 88 (2003) 446-454.
[21] E. Tian, F. Zhan, R. Walker, E. Rasmussen, Y. Ma, B. Barlogie, J.D. Shaughnessy, Jr., The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma, The New England journal of medicine, 349 (2003) 2483-2494.
[22] N. Giuliani, V. Rizzoli, Myeloma cells and bone marrow osteoblast interactions: role in the development of osteolytic lesions in multiple myeloma, Leuk Lymphoma, 48 (2007) 2323-2329.
[23] U. Heider, M. Kaiser, M. Mieth, B. Lamottke, J. Rademacher, C. Jakob, E. Braendle, D. Stover, O. Sezer, Serum concentrations of DKK-1 decrease in patients with multiple myeloma responding to anti-myeloma treatment, Eur J Haematol, 82 (2009) 31-38.
[24] A.K. Ghosh, C.R. Secreto, T.R. Knox, W. Ding, D. Mukhopadhyay, N.E. Kay, Circulating microvesicles in B-cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression, Blood, 115 (2010) 1755-1764.
[25] L. De Luca, G. D'Arena, V. Simeon, S. Trino, I. Laurenzana, A. Caivano, F. La Rocca, O. Villani, G. Mansueto, S. Deaglio, I. Innocenti, L. Laurenti, S. Molica, G. Pietrantuono, A. De Stradis, L. Del Vecchio, P. Musto, Characterization and prognostic relevance of circulating microvesicles in chronic lymphocytic leukemia, Leuk Lymphoma, 58 (2017) 1424-1432.
[26] B. Gyorgy, T.G. Szabo, M. Pasztoi, Z. Pal, P. Misjak, B. Aradi, V. Laszlo, E. Pallinger, E. Pap, A. Kittel, G. Nagy, A. Falus, E.I. Buzas, Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles, Cell Mol Life Sci, 68 (2011) 2667-2688.
[27] S. Keller, J. Ridinger, A.K. Rupp, J.W. Janssen, P. Altevogt, Body fluid derived exosomes as a novel template for clinical diagnostics, J Transl Med, 9 (2011) 86.
[28] R.M. Johnstone, M. Adam, J.R. Hammond, L. Orr, C. Turbide, Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes), J Biol Chem, 262 (1987) 9412-9420.
[29] S. Stuffers, C. Sem Wegner, H. Stenmark, A. Brech, Multivesicular endosome biogenesis in the absence of ESCRTs, Traffic, 10 (2009) 925-937.
[30] N. Kosaka, H. Iguchi, Y. Yoshioka, F. Takeshita, Y. Matsuki, T. Ochiya, Secretory mechanisms and intercellular transfer of microRNAs in living cells, J Biol Chem, 285 (2010) 17442-17452.
[31] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brugger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes, Science, 319 (2008) 1244-1247.
[32] A. Bobrie, M. Colombo, G. Raposo, C. Thery, Exosome secretion: molecular mechanisms and roles in immune responses, Traffic, 12 (2011) 1659-1668.
[33] C. Corrado, S. Raimondo, A. Chiesi, F. Ciccia, G. De Leo, R. Alessandro, Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications, Int J Mol Sci, 14 (2013) 5338-5366.
[34] J. Wang, S. Faict, K. Maes, E. De Bruyne, E. Van Valckenborgh, R. Schots, K. Vanderkerken, E. Menu, Extracellular vesicle cross-talk in the bone marrow microenvironment: implications in multiple myeloma, Oncotarget, 7 (2016) 38927-38945.
[35] B.K. Arendt, D.K. Walters, X. Wu, R.C. Tschumper, D.F. Jelinek, Multiple myeloma dell-derived microvesicles are enriched in CD147 expression and enhance tumor cell proliferation, Oncotarget, 5 (2014) 5686-5699.

[36] J. Wang, A. Hendrix, S. Hernot, M. Lemaire, E. De Bruyne, E. Van Valckenborgh, T. Lahoutte, O. De Wever, K. Vanderkerken, E. Menu, Bone marrow stromal cell-derived exosomes as communicators in drug resistance in multiple myeloma cells, Blood, 124 (2014) 555-566.
[37] A. Caivano, I. Laurenzana, L. De Luca, F. La Rocca, V. Simeon, S. Trino, F. D'Auria, A. Traficante, M. Maietti, T. Izzo, G. D'Arena, G. Mansueto, G. Pietrantuono, L. Laurenti, P. Musto, L. Del Vecchio, High serum levels of extracellular vesicles expressing malignancy-related markers are released in patients with various types of hematological neoplastic disorders, Tumour Biol, 36 (2015) 9739-9752.
[38] V. Quarona, V. Ferri, A. Chillemi, M. Bolzoni, C. Mancini, G. Zaccarello, I. Roato, F. Morandi, D. Marimpietri, G. Faccani, E. Martella, V. Pistoia, N. Giuliani, A.L. Horenstein, F. Malavasi, Unraveling the contribution of ectoenzymes to myeloma life and survival in the bone marrow niche, Ann N Y Acad Sci, 1335 (2015) 10-22.
[39] Y. Liu, X.J. Zhu, C. Zeng, P.H. Wu, H.X. Wang, Z.C. Chen, Q.B. Li, Microvesicles secreted from human multiple myeloma cells promote angiogenesis, Acta Pharmacol Sin, 35 (2014) 230-238.
[40] L. Raimondi, A. De Luca, N. Amodio, M. Manno, S. Raccosta, S. Taverna, D. Bellavia, F. Naselli, S. Fontana, O. Schillaci, R. Giardino, M. Fini, P. Tassone, A. Santoro, G. De Leo, G. Giavaresi, R. Alessandro, Involvement of multiple myeloma cell-derived exosomes in osteoclast differentiation, Oncotarget, (2015).
[41] J.A. Weber, D.H. Baxter, S. Zhang, D.Y. Huang, K.H. Huang, M.J. Lee, D.J. Galas, K. Wang, The microRNA spectrum in 12 body fluids, Clin Chem, 56 (2010) 1733-1741.
[42] A. Seckinger, T. Meissner, J. Moreaux, V. Benes, J. Hillengass, M. Castoldi, J. Zimmermann, A.D. Ho, A. Jauch, H. Goldschmidt, B. Klein, D. Hose, miRNAs in multiple myeloma--a survival relevant complex regulator of gene expression, Oncotarget, 6 (2015) 39165-39183.
[43] L. Raimondi, N. Amodio, M.T. Di Martino, E. Altomare, M. Leotta, D. Caracciolo, A. Gulla, A. Neri, S. Taverna, P. D'Aquila, R. Alessandro, A. Giordano, P. Tagliaferri, P. Tassone, Targeting of multiple myeloma-related angiogenesis by miR-199a-5p mimics: in vitro and in vivo anti-tumor activity, Oncotarget, 5 (2014) 3039-3054.
[44] M. Lionetti, M. Biasiolo, L. Agnelli, K. Todoerti, L. Mosca, S. Fabris, G. Sales, G.L. Deliliers, S. Bicciato, L. Lombardi, S. Bortoluzzi, A. Neri, Identification of microRNA expression patterns and definition of a microRNA/mRNA regulatory network in distinct molecular groups of multiple myeloma, Blood, 114 (2009) e20-26.
[45] S. Taverna, V. Amodeo, L. Saieva, A. Russo, M. Giallombardo, G. De Leo, R. Alessandro, Exosomal shuttling of miR-126 in endothelial cells modulates adhesive and migratory abilities of chronic myelogenous leukemia cells, Mol Cancer, 13 (2014) 169.
[46] B. Ell, L. Mercatali, T. Ibrahim, N. Campbell, H. Schwarzenbach, K. Pantel, D. Amadori, Y. Kang, Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis, Cancer Cell, 24 (2013) 542-556.
[47] T. Holbro, G. Civenni, N.E. Hynes, The ErbB receptors and their role in cancer progression, Experimental Cell Research, 284 (2003) 99-110.
[48] T. Yi, H.L. Lee, J.H. Cha, S.I. Ko, H.J. Kim, H.I. Shin, K.M. Woo, H.M. Ryoo, G.S. Kim, J.H. Baek, Epidermal growth factor receptor regulates osteoclast differentiation and survival through cross-talking with RANK signaling, J Cell Physiol, 217 (2008) 409-422.
[49] J. Zhu, X. Jia, G. Xiao, Y. Kang, N.C. Partridge, L. Qin, EGF-like ligands stimulate osteoclastogenesis by regulating expression of osteoclast regulatory factors by osteoblasts: implications for osteolytic bone metastases, J Biol Chem, 282 (2007) 26656-26664.
[50] K. Mahtouk, D. Hose, T. Reme, J. De Vos, M. Jourdan, J. Moreaux, G. Fiol, M. Raab, E. Jourdan, V. Grau, M. Moos, H. Goldschmidt, M. Baudard, J.F. Rossi, F.W. Cremer, B. Klein, Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells, Oncogene, 24 (2005) 3512-3524.

[51] L. Agnelli, L. Mosca, S. Fabris, M. Lionetti, A. Andronache, I. Kwee, K. Todoerti, D. Verdelli, C. Battaglia, F. Bertoni, G.L. Deliliers, A. Neri, A SNP microarray and FISH-based procedure to detect allelic imbalances in multiple myeloma: an integrated genomics approach reveals a wide gene dosage effect, Genes Chromosomes Cancer, 48 (2009) 603-614.
[52] L. Agnelli, M. Forcato, F. Ferrari, G. Tuana, K. Todoerti, B.A. Walker, G.J. Morgan, L. Lombardi, S. Bicciato, A. Neri, The reconstruction of transcriptional networks reveals critical genes with implications for clinical outcome of multiple myeloma, Clin Cancer Res, 17 (2011) 7402-7412.
[53] F. Costa, D. Toscani, A. Chillemi, V. Quarona, M. Bolzoni, V. Marchica, R. Vescovini, C. Mancini, E. Martella, N. Campanini, C. Schifano, S. Bonomini, F. Accardi, A.L. Horenstein, F. Aversa, F. Malavasi, N. Giuliani, Expression of CD38 in myeloma bone niche: A rational basis for the use of anti-CD38 immunotherapy to inhibit osteoclast formation, Oncotarget, 8 (2017) 56598-56611.
[54] S. Raimondo, L. Saieva, C. Corrado, S. Fontana, A. Flugy, A. Rizzo, G. De Leo, R. Alessandro, Chronic myeloid leukemia-derived exosomes promote tumor growth through an autocrine mechanism, Cell Commun Signal, 13 (2015) 8.
[55] R. Noto, M.G. Santangelo, S. Ricagno, M.R. Mangione, M. Levantino, M. Pezzullo, V. Martorana, A. Cupane, M. Bolognesi, M. Manno, The tempered polymerization of human neuroserpin, PLoS One, 7 (2012) e32444.
[56] C. Corrado, S. Raimondo, L. Saieva, A.M. Flugy, G. De Leo, R. Alessandro, Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an interleukin 8-dependent survival of leukemia cells, Cancer Lett, 348 (2014) 71-76.
[57] C.H. Hou, F.L. Lin, K.B. Tong, S.M. Hou, J.F. Liu, Transforming growth factor alpha promotes osteosarcoma metastasis by ICAM-1 and PI3K/Akt signaling pathway, Biochem Pharmacol, 89 (2014) 453-463.
[58] A. De Luca, A. Carotenuto, A. Rachiglio, M. Gallo, M.R. Maiello, D. Aldinucci, A. Pinto, N. Normanno, The role of the EGFR signaling in tumor microenvironment, J Cell Physiol, 214 (2008) 559-567.
[59] L. Mercatali, F. La Manna, G. Miserocchi, C. Liverani, A. De Vita, C. Spadazzi, A. Bongiovanni, F. Recine, D. Amadori, M. Ghetti, T. Ibrahim, Tumor-Stroma Crosstalk in Bone Tissue: The Osteoclastogenic Potential of a Breast Cancer Cell Line in a Co-Culture System and the Role of EGFR Inhibition, Int J Mol Sci, 18 (2017).
[60] K. Wang, H. Yamamoto, J.R. Chin, Z. Werb, T.H. Vu, Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment, J Biol Chem, 279 (2004) 53848-53856.
[61] J.N. Higginbotham, M. Demory Beckler, J.D. Gephart, J.L. Franklin, G. Bogatcheva, G.J. Kremers, D.W. Piston, G.D. Ayers, R.E. McConnell, M.J. Tyska, R.J. Coffey, Amphiregulin exosomes increase cancer cell invasion, Curr Biol, 21 (2011) 779-786.
[62] S. Taverna, M. Pucci, M. Giallombardo, M.A. Di Bella, M. Santarpia, P. Reclusa, I. Gil-Bazo, C. Rolfo, R. Alessandro, Amphiregulin contained in NSCLC-exosomes induces osteoclast differentiation through the activation of EGFR pathway, Sci Rep, 7 (2017) 3170.
[63] C. Corrado, L. Saieva, S. Raimondo, A. Santoro, G. De Leo, R. Alessandro, Chronic myelogenous leukaemia exosomes modulate bone marrow microenvironment through activation of epidermal growth factor receptor, J Cell Mol Med, 20 (2016) 1829-1839.
[64] N. Giuliani, R. Bataille, C. Mancini, M. Lazzaretti, S. Barille, Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment, Blood, 98 (2001) 3527-3533.
[65] B. Kumar, M. Garcia, L. Weng, X. Jung, J.L. Murakami, X. Hu, T. McDonald, A. Lin, A.R. Kumar, D.L. DiGiusto, A.S. Stein, V.A. Pullarkat, S.K. Hui, N. Carlesso, Y.H. Kuo, R. Bhatia, G. Marcucci, C.C.

Chen, Acute myeloid leukemia transforms the bone marrow niche into a leukemia-permissive microenvironment through exosome secretion, Leukemia, (2017).
[66] F. Morandi, D. Marimpietri, A.L. Horenstein, M. Bolzoni, D. Toscani, F. Costa, B. Castella, A.C. Faini, M. Massaia, V. Pistoia, N. Giuliani, F. Malavasi, Microvesicles released from multiple myeloma cells are equipped with ectoenzymes belonging to canonical and non-canonical adenosinergic pathways and produce adenosine from ATP and NAD(), Oncoimmunology, 7 (2018) e1458809.
[67] K. Furugaki, Y. Moriya, T. Iwai, K. Yorozu, M. Yanagisawa, K. Kondoh, K. Fujimoto-Ohuchi, K. Mori, Erlotinib inhibits osteolytic bone invasion of human non-small-cell lung cancer cell line NCI-H292, Clin Exp Metastasis, 28 (2011) 649-659.
[68] N. Normanno, A. De Luca, D. Aldinucci, M.R. Maiello, M. Mancino, A. D'Antonio, R. De Filippi, A. Pinto, Gefitinib inhibits the ability of human bone marrow stromal cells to induce osteoclast differentiation: implications for the pathogenesis and treatment of bone metastasis, Endocr Relat Cancer, 12 (2005) 471-482.

Scientific Products: publications, book chapters, acts in congress and awards.

Publications:

- Stefania Raimondo†, Laura Saieva†, **Emanuela Vicario**†, Marzia Pucci, Denise Toscani, Mauro Manno, Samuele Raccosta, Nicola Giuliani and Riccardo Alessandro. Multiple myeloma-derived exosomes are enriched of Amphiregulin (AREG) andactivate the Epidermal Growth Factor pathway in the bone microenvironment leading to osteoclastogenesis. *Journal of Hematology and Oncology 2019*.

- Bolzoni M, Toscani D, Costa F, **Vicario E**, Aversa F, Giuliani N. The link between bone microenvironment and immune cells in multiple myeloma: Emerging role of CD38. Immunology letters 2018.

- Taverna S, Fontana S, Monteleone F, Pucci M, Saieva L, De Caro V, Cardinale VG, Giallombardo M, **Vicario E**, Rolfo C, De Leo G, Alessandro R. Curcumin modulates chronic myelogenous leukemia exosomes composition and effect angiogenic phenotype, via exosomal miR-21. Oncotarget.

Book chapters :

- Identificazione dei microRNA in esosomi/microvescicole. S. Raimondo, **E. Vicario**, R. Alessandro. Ligandassay 2016

Acts in congress:

- Denise Toscani, Martina Chiu, Giuseppe Taurino, **Emanuela Vicario**, Valentina Marchica, Fabrizio Accardi, Anna Benedetta Dalla Palma, Paola Storti, Gaetano Donofrio, Franco Aversa, Ovidio Bussolati and Nicola Giuliani. Myeloma-Induced Alterations of Glutamine Metabolism Impair Bone Microenvironment Niche in Multiple Myeloma Patients. Accepted for 60th ASH Annual Meeting & Exposition, San Diego, CA.

- Federica Costa, Marina Bolzoni, Rosanna Vescovini, Fabrizio Accardi, Anna Benedetta Dalla Palma, Federica De Luca, Valentina Marchica, Denise Toscani, **Emanuela Vicario**, Paola Storti, Franco Aversa and Nicola Giuliani. Relationship between Bone Marrow PD-1 and PD-L1 Expression and the Presence of Osteolytic Bone Disease in Multiple Myeloma Patients. Accepted for 60th ASH Annual Meeting & Exposition, San Diego, CA.

- Paola Storti, Rosanna Vescovini, Valentina Marchica, Marina Bolzoni, Federica Costa, **Emanuela Vicario**, Denise Toscani, Anna Benedetta Dalla Palma, Fabrizio Accardi, Fabio Malavasi, Franco Aversa and Nicola Giuliani. $CD14^{+}CD16^{+}$ Monocyte Binding to Myeloma Cells Is Required for Daratumumab Dependent Killing in Multiple Myeloma Patients. Accepted for 60th ASH Annual Meeting & Exposition, San Diego, CA.

- **Vicario Emanuela**, Stefania Raimondo, Riccardo Alessandro and Nicola Giuliani. Multiple Myeloma-derived exosomes promote bone destruction through the delivery of amphiregulin. Accepted as Poster "Multiple Myeloma and related malignancies". Bari 2018

- **Vicario E**, Raimondo S, Saieva L, Costa F, Marchica V, Toscani D, Bolzoni M, Storti P, Aversa F, Alessandro R and Giuliani N. The Exosomes-Derived EGFR Ligand Amphiregulin (Areg) Is A New Key Player In Multiple Myeloma Bone Destruction. Accepted as Oral Comunication " XV Congresso Nazionale della Società Italiana di Ematologia Sperimentale". Rimini 2018

- Plasma Cells Transcriptional Profile In Smoldering Myeloma Patients In Relationship To Progression To Active Multiple Myeloma. P. Storti , B. Dalla Palma, K. Todoerti, L. Agnelli, V. Marchica, M. Bolzoni, F. Costa, D. Toscani, **E. Vicario**, G. Todaro, G. Sammarelli, F. Accardi, I. Manfra, L. Notarfranchi, F. De Luca, F. Aversa, A. Neri, N. Giuliani. Accepted for XV Congresso Nazionale della Società Italiana di Ematologia Sperimentale. Rimini 2018

- Use Of Bovine Virus As Alternative Tools In Multiple Myeloma Oncolytic Virotherapy. V. Marchica, R. Vescovini, V. Franceschi, G. Barbarito, M. Bolzoni,

D. Toscani, F.Costa, P. Storti, **E. Vicario**, B. Dalla Palma, F. Accardi, G. Tebaldi, I. Airoldi, G. Donofrio, F. Aversa and N. Giuliani. Accepted for XV Congresso Nazionale della Società Italiana di Ematologia Sperimentale. Rimini 2018

- Study Of Plasma Cell Trascriptome In Smoldering Myeloma Patients In Relationship To Progression To Active Multiple Myeloma. Paola Storti, Benedetta dalla Palma, Katia Todoerti, Luca Agnelli, Valentina Marchica, Marina Bolzoni, Federica Costa, Denise Toscani, **Emanuela Vicario**, Gabriella Sammarelli, Fabrizio Accardi, Ilenia Manfra, Laura Notarfranchi, Federica DeLuca, Luisa Craviotto, Franco Aversa, Antonino Neri and Nicola Giuliani. Accepted for 23RD CONGRESS EHA. Stockholkm 2018

- Raimondo S, Saieva L, **Vicario E**, Costa F, Giuliani N, Alessandro R. Multiple myeloma-derived exosomes carry EGFR ligand and are responsible for the uncoupled bone remodeling. Accepted for the ISEV2018 Annual Meeting.

- **Vicario E**, Raimondo S, Saieva L, Costa F, Tinnirello V, Accardi F, Aversa F, GiulianiN and Alessandro R. The Exosomes-Derived EGFR Ligand Amphiregulin (AREG) Is a New Pro-Osteoclastogenic Factor in Multiple Myeloma. Accepted for 59th ASH Annual Meeting & Exposition, Atlanta, GA

- Taverna S, Fontana S, Monteleone F, Pucci M, Saieva L, De Caro V, Cardinale VG, Giallombardo M, **Vicario E**, Rolfo C, De Leo G, Alessandro R. Curcumin modulates chronic myelogenous leukemia exosomes composition and affects angiogenic phenotype, via exosomal miR-21. Accepted for XI Annual Congress Of The Italian Proteomics Association, (Perugia, May, 16-19, 2016).

AWARDS

- Winner of the 1st prize for the abstract "Multiple Myeloma-derived exosomes promote bone destruction through the delivery of amphiregulin" presented at Congress "Multiple Myeloma and related malignancies". Bari 2018

- Travel Grant “Multiple Myeloma and related malignancies” novembre 2018 for the abstract “Multiple Myeloma-derived exosomes promote bone destruction through the delivery of amphiregulin”. Bari 2018

- Travel Grant XV Congresso Nazionale della Società Italiana di Ematologia Sperimentale Ottobre 2018 for the abstract “The Exosomes-Derived Egfr Ligand Amphiregulin (Areg) Is A New Key Player In Multiple Myeloma Bone Destruction”. Rimini 2018

- Abstract Achievement Award at 58th ASH Annual Meeting for the abstract “The Exosomes-Derived EGFR Ligand Amphiregulin (AREG) Is a New Pro-Osteoclastogenic Factor in Multiple Myeloma”. Atlanta, GA