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Research Paper

mTOR inhibitor and bone-targeted drugs break the vicious cycle between clear-cell renal carcinoma and osteoclasts in an in vitro co-culture model

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ABSTRACT

The skeleton is one of the most common sites of metastatic spread from advanced clear-cell renal carcinoma (ccRCC). Most of the bone lesions observed in RCC patients are classified as osteolytic, causing severe pain and morbidity due to pathological bone destruction. Nowadays, it is well known that cancer induced bone loss in lytic metastasis is caused by the triggering of a vicious cycle between cancer and bone resident cells that leads to an imbalance between bone formation and degradation. Targeting the mammalian target of rapamycin (mTOR) is an efficient treatment option for metastatic renal carcinoma patients. Moreover, bone targeted therapy could benefit bone metastatic cancer patients caused by advanced RCC. However, more data is needed to support the hypothesis of the beneficial effect of a combined therapy. The aim of this work is to investigate the effect of targeting mTOR and the sequential combination with bone targeted therapy as a strategy to break the vicious cycle between ccRCC cells and osteoclasts. A previously optimized fully human co-culture model is used to mimic the crosstalk between Caki-2 cells (ccRCC) and osteoclasts. Cells are treated at fixed timing with everolimus, zoledronic acid and denosumab as single or sequential combined treatment. We show that Caki-2 cells can induce osteoclast cells differentiation from isolated human monocytes, as demonstrated by specific tartrate-resistant acid phosphatase (TRAP) staining and f-actin ring formation, in a statistically significant manner. Moreover, differentiated osteoclasts proved to be functionally active by pit formation assay. Caki-2 cells co-cultured with osteoclasts acquire a more aggressive phenotype based on gene expression analysis. Interestingly, the sequential combined treatment of everolimus and zoledronic acid is the most effective in the inhibition of both Caki-2 cells survival and osteoclastogenic potential, making it an effective strategy to inhibit the vicious cycle of bone metastasis. At preclinical level, this observation confirms the value of our co-culture model as a useful tool to mimic the bone microenvironment and to assess drug sensitivity in vitro. A better understanding of the molecular mechanisms involved in tumor-bone cells crosstalk will be investigated next.

1. Introduction

Renal cell carcinoma (RCC) is one of the most common kidney cancer found in adults, accounting for 90%−95% of neoplasms originating from the kidney [1,2]. It derives from the renal tubular epithelium and the clear-cell RCC (ccRCC) is the most frequent histopathological variant [3]. Although the systemic therapy is progressing steadily, whenever metastasis occurs clinical management is complicated by the lack of an effective therapy [4,5]. Bone is one of the most common sites of ccRCC diffusion and the mechanisms fostering its pathogenesis are similar to breast cancer [6,7]. The nature of bone metastasis from ccRCC is mainly osteolytic, causing significant morbidity in patients affected with it due to severe pain, pathological fractures, spinal cord compression and hypercalcemia [8]. Nowadays, it is well known that tumor cells don't act alone in the establishment of metastasis. Indeed, the microenvironment plays a crucial role, and osteolytic bone relapse is the end result of a positive feedback interaction between tumor and bone resident cells [9,10]. This “vicious cycle” leads to a

Abbreviation: RCC, renal cell carcinoma; ccRCC, clear-cell renal cell carcinoma; M-CSF, macrophage colony-stimulating factor; RANK-L, receptor activator of nuclear factor-kb ligand; OPG, osteoprotegerin; VEGF, vascular endothelial growth factor; mTOR, mammalian target of rapamycin; Eve, everolimus; Zol, zoledronic acid; Deno, denosumab

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substantial break in bone physiological homeostasis microenvironment towards a pathological bone resorption [11]. Bone degradation and formation are two processes strictly regulated in physiological conditions in order to maintain the structural integrity of the tissue [12,13]. The cells responsible for bone degradation are osteoclasts, giant cells derived from hematopoietic cells of the monocyte-macrophage lineage. In physiological conditions their action is strictly controlled by osteoblasts cells, responsible for the bone formation. Specifically, osteoblasts can secrete macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-kB ligand (RANK-L), cytokines which activate different signaling pathways that promote the survival and differentiation of monocytes into osteoclast cells. In order to control this process, other factors can be secreted by stromal cells, such as osteoprotegerin (OPG), the decoy receptor of RANK-L, able to inhibit the pre-osteoclasts maturation. In lytic lesions, when tumor cells arrive in the bone, they induce an imbalance in bone homeostasis towards an uncontrolled bone resorption that leads to an excessive release of soluble factors, such as transforming growth factor-beta and bone morphogenetic protein, which sustain the tumor cell growth [14,15]. The classical therapeutic strategy adopted to inhibit the pathological bone resorption is bone-targeted therapy. With different mechanisms of action, bisphosphonates (*i.e.* zoledronic acid) and denosumab are the well established drugs in the treatment of bone metastasis. In particular, while denosumab is a monoclonal antibody against RANK-L and it inhibits the transition from pre-osteoclasts to osteoclasts, zoledronic acid, when internalized by osteoclasts cells, inhibits various metabolic processes, such as prenylation, leading to cell apoptosis [14].

Recently, the introduction of molecular-targeted therapy significantly improved the prognosis of advanced RCC patients [16]. A deeper understanding of the RCC pathogenesis has led to the development of targeted drugs able to inhibit specific biological pathways involved in RCC tumor progression. Among these, vascular endothelial growth factor (VEGF) and the mammalian target of rapamycin (mTOR) are key regulators of RCC cell proliferation, neoangiogenesis and tumor cell migration and the respective inhibitors have demonstrated significant clinical activity [17].

In addition to the involvement in RCC pathogenesis, recent evidence has highlighted the role of the mTOR/S6K in regulating several aspects of bone homeostasis [18,19]. Indeed, activation of this pathway by soluble factors can modulate the physiological bone remodeling directly acting on osteoblast and osteoclast differentiation and proliferation [20,21].

According to this evidence, inhibition of the mTOR pathway has been confirmed as an effective strategy to break the vicious cycle of bone metastasis from breast cancer in a preclinical in vitro model [22,23]. However, it has never been investigated in the interactions between bone cells and renal carcinoma.

In order to mimic the molecular interactions involved in the cross communication of lytic bone metastasis from renal carcinoma, we used our preclinical in vitro co-culture model to evaluate the osteoclastogenic potential of Caki-2 cells, a primary clear-cell renal carcinoma cell line. Afterwards, this model enables us to investigate the effects of Everolimus, an mTOR inhibitor, as a single agent and in combination with the standard bone-targeted therapies, zoledronic acid (Zol) and denosumab (Deno), in the break of bone lytic vicious cycle.

2. Materials and methods

2.1. Cell cultures

The experiments were performed on Caki-2 cells, a human clear-cell renal carcinoma cell line (ccRCC) obtained from the America Type Culture Collection (MD, USA). Caki-2 cells were maintained as a monolayer in 75 cm^2 flasks at 37 °C in D-MEM High Glucose Medium (PAA, NJ, USA) supplemented with 1% glutamine (PAA), 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere.

2.2. Osteoclastogenesis assay

Human osteoclasts were generated from the differentiation of monocytes collected from PBMCs (peripheral blood mononuclear cells) of healthy donors who gave written informed consent to take part in the study [22]. Briefly, monocytes were isolated from buffy coats by Ficoll density centrifugation. EDTA whole blood (50 mL) was diluted 1:1 with phosphate-buffered saline, layered on lymphocyte separation medium (Lymphosep, Biowest, Nuaillé, France) in a Leucosep tube (Leucosep™ Centrifuge Tubes, Greiner Bio-One, Frickenhausen, Germany) and centrifuged without brakes at 1000 g for 20 min. The PBMCs layer was collected and washed twice with phosphate-buffered saline (PBS) and treated for 4 min on ice with ACK solution in order to induce the lysis of red blood cells. After having washed them twice with PBS, cells were resuspended in Alpha-MEM (LONZA, Basel, Switzerland) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamine (complete Alpha-MEM).

Immediately after isolation, PBMCs were counted and plated at a concentration of 1,500,000 PBMCs/well in a 24-well plate. After 24 h, the medium was replaced. In the positive control condition (Ctrl+), complete Alpha-MEM supplemented with 20 ng/mL of M-CSF (Peprotech, NJ, USA) was added in order to induce the maturation of monocytes into pre-osteoclasts. From day 6–7 of culture, differentiation medium was supplemented with RANK-L (20 ng/mL) soluble factor capable of inducing the differentiation of pre-osteoclasts in osteoclasts. The medium was changed every 2–3 days and TRAP+ polycarion cells were identified after 14 days of differentiation [22,24–26].

2.3. Tumor cells - osteoclast co-culture in vitro model

In order to assess the effect of cancer cells on osteoclastogenesis, direct co-culture with Caki-2 cells was performed. Briefly, 4,000 Caki-2 cells were seeded in 6.5 mm diameter, 0.4 μm transwell inserts (Corning Ltd, London, UK) on day 0 of the assay. Cells were then allowed to adhere to their supports and after 24 h Caki-2 cells-seeded inserts were placed over the monocyte cultures till the end of the assay [22,26]. Tumor cells were cultured (as a control group for the direct co-culture), under the same conditions, in a 96-well plate (Caki-2 cells cultured alone) since the growth area of a transwell insert is comparable.

2.4. Quantification of TRAP-positive multinucleated cells

Mature osteoclasts were fixed after 14 days of differentiation by incubation in 4% Paraformaldehyde (Electron Microscopy Sciences) for 20 min at room temperature and then stained for tartrate resistant acid phosphate (TRAP kit, Sigma-Aldrich, Steinheim, Germany) staining. Osteoclasts were counted with a magnification of 10x in an Axiovision Microscope in 5 fields/well as multinucleated cells (more than 4 nuclei) TRAP-positive cells. Images of mature osteoclasts were acquired at different magnifications with EVOS XL Cell Imaging System (Thermo Fisher Scientific, MA, USA). Each experiment was performed in quadruplicate and repeated at least 3 times.

In order to evaluate drug-treatment induced osteoclastogenesis inhibition, the number of treated osteoclasts was normalized to the respective untreated condition. Each experiment was performed in quadruplicate and repeated at least three times. Inhibition of osteoclastogenesis after drug exposure was reported as percentage (%) of osteoclastogenesis normalized to the respective untreated control.

2.5. Pit formation assay

This assay has been performed in order to assess the activation and functionality of mature osteoclasts. Briefly, Corning® Osteo Assay Surface, coated with a proprietary synthetic inorganic bone mimetic

substance, has been used as support plate for the osteoclastogenesis assay of human monocyte cultured alone or in co-culture with Caki-2 cells. At the end of the assay (day 14), the medium was aspirated. After incubation with 10% bleach solution for 5 min at room temperature, the wells were washed twice with distilled water and allowed to dry at room temperature for 3 to 5 h. Individual pits were observed using EVOS XL Cell Imaging System (Thermo Fisher Scientific) at 10x magnification [27].

2.6. Phalloidin staining

Phalloidin staining to detect F-actin rings in differentiated osteoclasts was performed as follows: cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, PA, USA) for 20 min at room temperature followed by permeabilization with 0.1% Triton X-100 for 5 min. The cells were subjected to immunofluorescence staining with phalloidin stock solution (1:40 in PBS) (Life Technologies, CA, USA) for 20 min in dark conditions at room temperature. Cells were then washed 3 times with PBS and nuclei were counterstained with DAPI. Cells were then mounted with Prolong Gold (Thermo Fisher Scientific) and examined by fluorescence microscopy with EVOS XL Cell Imaging System (Thermo Fisher Scientific) [22].

2.7. Drugs

Everolimus (Eve) and zoledronic acid (Zol) were kindly provided by Novartis (NJ, USA). A stock concentration of 50 mM in sterile water was obtained, filtered and stored at −20 °C. Denosumab 120 mg in 1.7 mL (Xgeva, CA, USA) was stored at 4 °C. Drugs were diluted up to the desired concentration in complete Alpha-MEM containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine immediately before use.

2.8. Drug treatment

Drug dose and exposure time were established based on plasma levels from pharmacokinetic clinical data [22,28,29]. Tumor cells and osteoclasts cells were exposed to drugs for 72 h. On day 3 of the osteoclastogenesis assay, cells were treated with Eve at a concentration of 0.1 μg/ml (peak plasma value). After 7 days' differentiation, cells were exposed to Deno or Zol. Deno was used at a concentration of 5 μg/mL (peak plasma value) while Zol was tested at a concentration of 10 μM. Zol was administrated at a higher dose compare to the peak plasma value since bisphosphonates can accumulate in the bone mineralized matrix where they can reach a 10-fold higher concentration than in plasma [30,31].

2.9. Evaluation of drug effect on cancer cells

Caki-2 cells were treated with drugs as previously described. Percentage of survival was then assessed by MTT assay (Sigma Aldrich) according to the manufacturer's instructions, as previously reported [32]. Sensitivity to drugs was calculated as normalization to the respective untreated condition.

2.10. Quantitative real-time reverse transcription-PCR (RT-qPCR)

Caki-2 cells and osteoclasts, either cultured alone or co-cultured were lysed in 800 μl of TRIzol reagent (Invitrogen, CA, USA) and the total RNA was extracted according to the manufacturer's instructions. Five hundred nanograms of RNA were reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad, CA, USA). Real-Time PCR was performed on the 7500 Real-Time PCR System (Applied Biosystems, CA, USA) using either TaqMan or SYBR green chemistry (Applied Biosystems) according to the specific target gene assay. TaqMan gene expression assay was performed for cancer cells marker analysis, using as reference the stably expressed endogenous B-actin and HPRT genes. The following markers were analyzed: RANK (receptor activator of nuclear factor κ B), RANK-L (receptor activator of nuclear factor kappa-B ligand), OPG (osteoprotegerin), VIM (Vimentin) and CDH1 (E-Cadherin) (Life Technologies).

For osteoclast marker analysis, SYBR green chemistry (Applied Biosystems) was adopted. The stably expressed endogenous B-actin and HPRT genes were used as reference. The following markers were analyzed: CTSK (cathepsin K), OSCAR (osteoclast-associated immunoglobulin-like receptor), NFATC1 (nuclear factor of activated T cells 1), CAII (carbonic anhydrase II) and CALCR (calcitonin receptor). Gene expression was quantified by the Delta Delta CT method.

2.11. Western blot

Caki-2 proteins were isolated by direct lysis with M-PER lysis buffer (Life Technologies). The protein content was quantified using the BCA protein assay kit (Thermo Fisher Scientific). An equal amount of protein from each sample was separated on Bolt™ 10% Bis-Tris Plus Gels (Life Technologies) and transferred to polyvinylidene fluoride membranes through Trans-Blot® Turbo™ blotting system (Bio-Rad). The membranes were blocked for one and a half h in 5% non-fat dry milk (Bio-Rad) diluted in PBS with 0.1% Tween 20 (Sigma-Aldrich) at room temperature and incubated with primary antibody overnight at 4 °C. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. The following primary antibodies were used: anti-IKB-α (1:1000) (Cell Signalling Technology), anti-Vinculin (1:1000) (Thermo Fisher Scientific).

2.12. Statistical analysis

Each experiment was repeated at least three times with 4 technical replicates for each condition. Data are presented as mean ± SE (standard error) or as percentage (%) of osteoclastogenesis or percentage (%) of survival. Student's *t*-test was used as appropriate and accepted as significant at $p \leq 0.05$.

3. Results

3.1. Caki-2 cell-derived factors induce osteoclastogenesis from PBMCs

In order to evaluate the osteoclastogenic potential of Caki-2 cells, monocytes isolated from peripheral blood mononuclear cells were seeded on day 0 and they were led to differentiate for 14 days while co-cultured with Caki-2 cells. Our optimized model allows us to evaluate whether tumor cells can secrete soluble factors able to induce osteoclast differentiation. Osteoclastogenesis assay lasts for 14 days and it consists of two sequential phases: the differentiation of monocytes in pre-osteoclasts (day 0-day 7) and the differentiation of pre-osteoclasts into osteoclasts (day 7-day 14). Therefore, we analyzed whether and in which phase Caki-2 – derived paracrine factors act predominantly in the osteoclasts differentiation (Fig. 1A). Caki-2 cells are able to induce osteoclasts differentiation in a significant manner, even if at a lower extent compared to the positive control (Ctrl+), where in the differentiation is induced by directly adding M-CSF and RANK-L to the medium (Fig. 1B). The pro-osteoclast activity of Caki-2 cells did not appear to be crucial in a specific phase of differentiation, therefore, we decided to optimize our model with a co-culture that would last for the whole length of the assay. This strategy enabled us to better mimic the interactions between tumor and osteoclasts. In an independent assay, we confirmed that Caki-2 cells are able to induce osteoclast differentiation in a comparable manner to the Ctrl+ (Fig. 1C).

The mean number of osteoclast cells (with >4 nuclei and positive to TRAP staining, as described in the experimental section) for microscopic field was 10.6 ± 1.27 (Ctrl-); 74.7 ± 8.9 (Ctrl+); 41.7 ± 4.6 (Caki-2 Co-Culture). Student's *t*-test showed significant difference

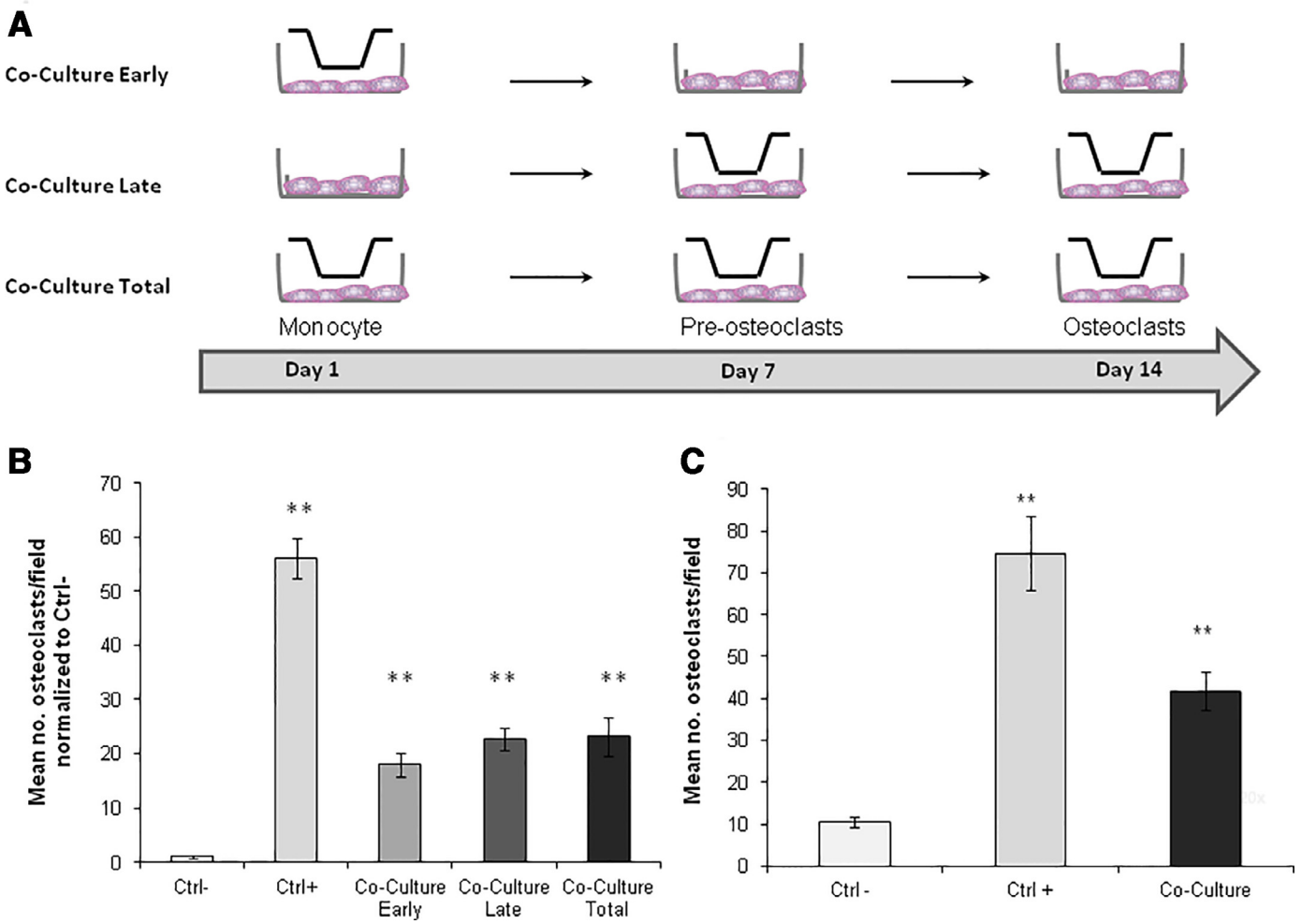

Fig. 1. Co-Culture *in vitro* model. **(A)** Experimental design of Co-Culture optimization model. We evaluated 3 different conditions based on the stage of the osteoclastogenesis assay: 1. Co-Culture Total: direct co-culture for 14 days with PBMCs; 2. Co-Culture Early: direct Co-Culture for the first 7 days; 3. Co-Culture Late: direct Co-Culture for the last 7 days. Co-Culture was obtained through transwell inserts (Corning) which enable medium sharing between Caki-2 and PBMCs. **(B)** Mean number of osteoclasts per microscopic field. Mean number was normalized with respect to Ctrl- in order to disregard spontaneous osteoclastogenesis. **(C)** Mean number of osteoclasts per microscopic field in another independent assay. Significance $p^* \leq 0.05$; $p^{**} \leq 0.01$. Error bars: SE.

between Ctrl+ and Ctrl- ($p < 0.01$) and between Ctrl- and Co-Culture ($p < 0.01$).

TRAP staining confirmed the presence of osteoclasts in all conditions, also in the Ctrl-, due to the spontaneous osteoclastogenesis that can occur as previously reported [33–35] (Fig. 2A). Since activated macrophage polycarions could be included in the PBMCs and could express TRAP [22,24], we assessed the presence of F-actin rings as hallmarks of osteoclast differentiation and we evaluated the functionality of mature osteoclasts through the Pit Formation Assay (Fig. 2B and C). Thus, we confirmed that Caki-2 cells can secrete soluble factors capable of inducing differentiation of functionally active osteoclasts.

3.2. Effect of co-culture and mTOR inhibitor on osteoclastogenesis

We investigated the effect of Eve on the osteoclastogenic potential of Caki-2 cells. In a previous work, we confirmed that mTOR blockage is more effective in the inhibition of osteoclasts maturation at an early administration, suggesting a key role of mTOR pathway in the transition from monocytes to pre-osteoclasts [22]. We thus decided to treat cells with Eve on day 3 of the assay at the plasmatic peak level (0.1 μg/ml). At the end of the assay, TRAP staining was performed and TRAP positive cells with more than 4 nuclei were counted (Fig. 3A). All the conditions were significantly sensitive to the mTOR inhibition. In particular, the mean number of osteoclasts for microscopic field decreased in the Ctrl+ condition from 74.7 ± 8.9 to 24 ± 5.5 and in the Co-Culture condition from 41.7 ± 4.6 to 26.8 ± 4.6 (Fig. 3B). Eve treatment was less effective in samples conditioned with cancer cells than in those in the Ctrl+ condition. In particular, compared to the respective condition, the decrease in osteoclastogenesis was 35.9% when conditioned with cancer cells and 67.9% in the Ctrl+ condition. This observation suggests that Caki-2 derived factors can support osteoclast survival to Eve treatment. Moreover, we evaluated the gene expression modulation of osteoclasts markers (Fig. 3C and D). In the Ctrl- condition all osteoclasts markers expression decreased significantly after Eve treatment, instead, in the Ctrl+ condition, only the expression of CTSK, NFATC1, CAII decreased significantly compared to Ctrl+ not treated. However, in the co-culture conditions, except for CTSK gene, the expression of all osteoclast markers did not change significantly compared to the respective untreated control group, confirming the ability of Caki-2 soluble factors to support osteoclast survival and differentiation after Eve treatment (Fig. 3D).

3.3. Effect of co-culture and mTOR inhibitor on cancer cells

First, we observed that co-culture with osteoclasts is able to induce a trend towards a proliferative advantage on Caki-2 cells suggesting that osteoclasts can secrete factors able to support the tumor cells proliferation (Fig. 4A). One of the features that give tumor cells a better

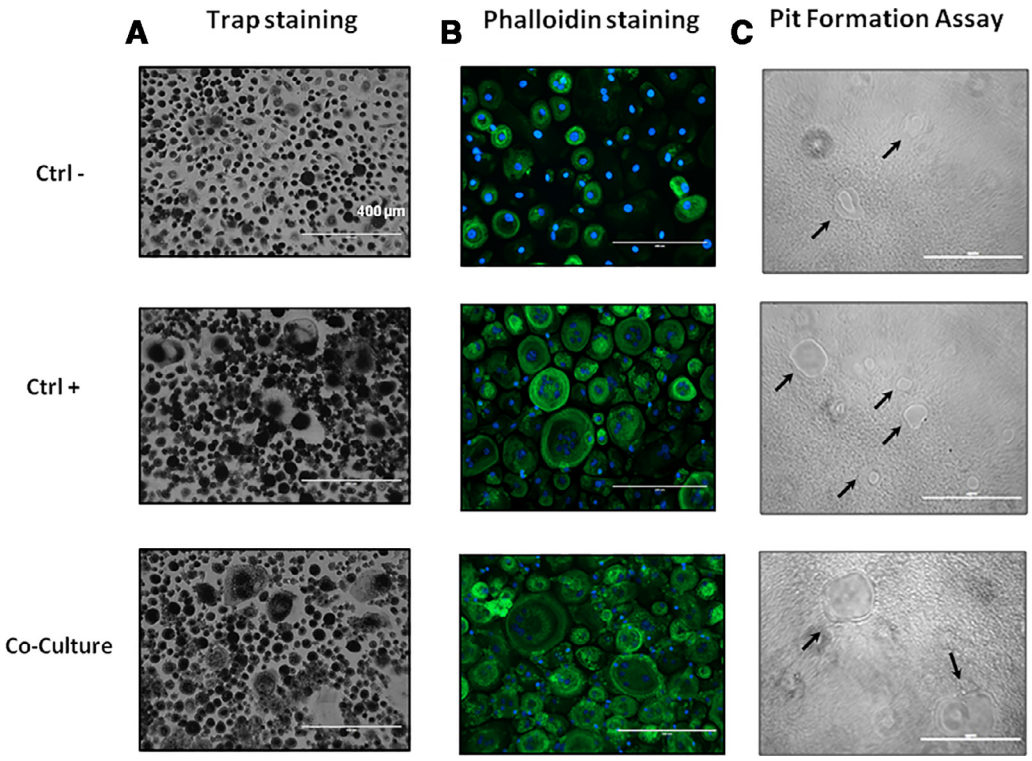

Fig. 2. Representative images of PBMCs-osteoclasts after 14 days of osteoclastogenesis assay. **(A)** TRAP Staining in all the different conditions. **(B)** Phalloidin staining (green) to detect F-actin rings on osteoclasts in all the different conditions. **(C)** Pit Formation Assay for all the tested conditions to evaluate the functionality of mature osteoclasts. Pits are highlighted by arrows. 10x magnification. Scale bar: 400 µm.

chance of survival and proliferation into the bone tissue is osteomimicry, *i.e.* the ability to acquire a bone cell phenotype [36]. For this reason, we next performed gene expression analysis on Caki-2 cells to evaluate the modulation of different markers of osteomimicry and EMT (epithelial-mesenchymal transition), a hallmark of malignancy. Osteoclasts can induce the increase of RANK-L, RANK expression (normally expressed by bone resident and by stromal cells) and the decrease of E-cad (CDH1), suggesting that cancer cells can acquire a more aggressive phenotype (Fig. 4B and C).

The effect of mTOR inhibition was evaluated on Caki-2 cells cultured alone or co-cultured with osteoclasts. The inhibition of Caki-2 survival by Eve treatment, normalized to the respective control, was 34.9% if cultured alone, while 20.3% in osteoclasts-culture condition (Fig. 4A). Caki-2 cells surviving Eve treatment showed no interesting modulation if cultured alone, while when co-cultured with osteoclasts Caki-2 showed a decrease in RANK expression and an increase in OPG expression compared to the untreated Co-Culture condition, even if not statistically significant (Fig. 4B and C).

Given the strong interconnection between mTOR and Nf-kB pathways, we investigated whether Everolimus could indirectly impact on the activation of this pathway. We showed that mTOR inhibition is able to block the Nf-KB pathway, as suggested by the increase of the unphosphorylated form of Ik-B, inhibitor of the transcription factor Nf-kB (Fig. 4D). Interestingly, this increase is lower in the co-culture condition.

3.4. Inhibition of osteoclastogenesis induced by Eve and bone-targeted therapy

Deno and Zol are two bone targeted drugs with a different mechanism of action. Deno is known to inhibit the RANK-L binding in the transition from pre-osteoclasts to mature osteoclasts, meanwhile Zol can induce apoptosis in mature osteoclasts, acting in the second phase of the osteoclastogenesis assay. Since Eve is more effective in the first phase, we tested a sequential combined treatment with Eve and bone-targeted therapy (Deno, Zol). Deno and Zol, tested as single agents, can induce a significant osteoclastogenesis inhibition (Fig. 5A and B). In the Ctrl+ condition, the percentage of osteoclastogenesis was 29% and 13% respectively compared to the untreated Ctrl+ condition. For PBMCs co-cultured with Caki-2 cells, the osteoclast differentiation was 53% and 33%, respectively. The combined treatment with Eve was effective in the inhibition of osteoclastogenesis. The combination of Eve and Zol was significantly different in the condition of osteoclasts co-cultured with Caki-2 cells compared to the single Eve treatment ($p = 0.005$). Interestingly, in all the treatment conditions, osteoclasts co-cultured with Caki-2 cells showed an increased survival compared to the respective Ctrl+ condition.

3.5. Effect of bone-targeted therapy on Caki-2 cell survival

Next, we evaluated the survival of Caki-2 cells after bone-targeted treatment as single agent or in combination with Eve through MTT analysis (Fig. 5C). Interestingly, we observed that Caki-2 cells, when co-cultured with osteoclasts, showed an increased sensitivity to Zol treatment and to the combined Eve + Zol treatment (Fig. 5C).

4. Discussion

This study aimed to investigate a novel pharmacological strategy to break the cross-communication between ccRCC cells and osteoclasts in the establishment of osteolytic bone metastasis through our optimized, fully human preclinical in vitro model [22,24–26]. To date, the molecular mechanisms involved in this crucial interaction are not well clarified and a better understanding could help the identification of a potential therapeutic strategy more effective in the inhibition of the "vicious cycle" of bone metastasis. It is difficult to recapitulate the full cellular complexity of this intercommunication in vitro and the fact that our study mimics only osteoclasts and tumor cells interactions can be considered a technical limitation. However, the accessibility and a reliable reproducibility of the 2D co-culture model enable us to dissect the molecular interactions involved between two cell types, simplifying the complex network of the bone metastatic microenvironment and simplifying the identification of an efficient therapeutic strategy.

We used Caki-2 cells, a primary clear cell-renal carcinoma cell line

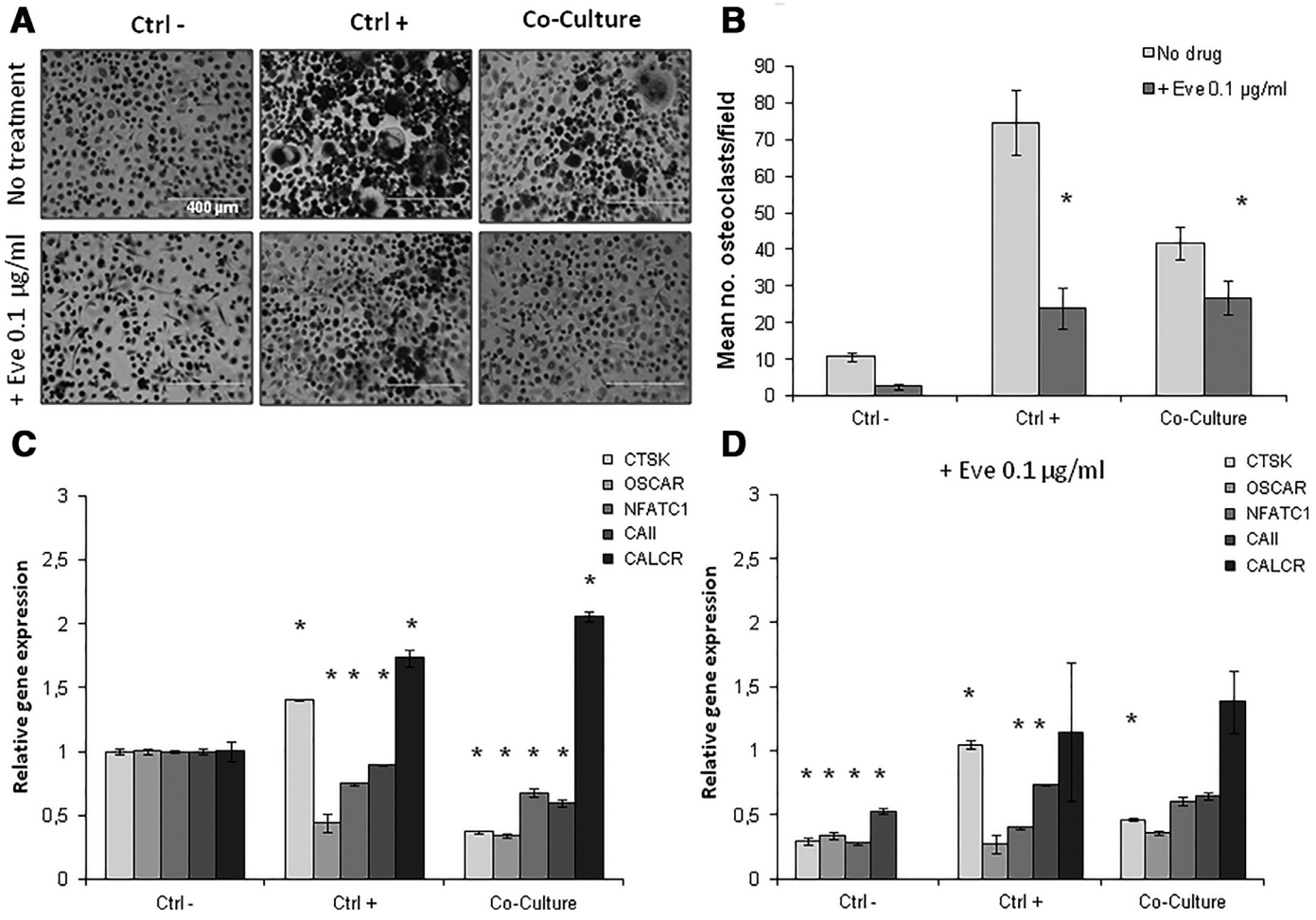

Fig. 3. Effect of Co-Culture and Eve treatment effect on osteoclastogenesis. **(A)** Pictures representing TRAP staining in osteoclasts after 14 days of differentiation. 10x magnification. **(B)** Osteoclastogenesis assay; mean number of osteoclasts per microscopic field. **(C and D)** Relative gene expression of osteoclast markers compared to Ctrl- untreated condition. Error bars: SE. Significance $p^* \leq 0.05$; $p^{**} \leq 0.01$. Significance is related to the Ctrl- or to the respective untreated condition for Eve-treated cells.

because more than 90% of patients with metastatic RCC have clear cell RCC [37]. To our knowledge, this study represents the first preclinical evidence that renal carcinoma cells can induce the differentiation of human monocytes in functionally active osteoclasts, indicating a direct action in the modulation of bone remodeling. Indeed, when renal carcinoma cells spread to the bone site, they can frequently cause an osteolytic bone metastasis, towards excessive bone degradation [38,39]. Nowadays, it is still not clear how renal carcinoma cells can induce the imbalance in bone homeostasis. However, one hypothesis is that tumor cells can affect the balance necessary for the regulation of bone homeostasis by acting on the RANKL-RANK-OPG system [40,41]. Different studies have investigated the role of RANKL-RANK-OPG system in RCC [42,43]. Mikami et al. found that RANKL and RANK expression positively correlate with the primary tumor stage [42]. Moreover, it has been demonstrated that high RANK-L and RANK expressions are significant predictors of bone metastatic recurrence from RCC [43]. In line with this, our results showed an increased expression of RANK gene when Caki-2 cells are co-cultured with osteoclast cells. These results suggest that Caki-2 cells can secrete soluble factors able to directly induce osteoclast differentiation. However, we still need to identify the key soluble effectors of this process.

Another interesting data is that in our model, osteoclasts seem to increase the proliferation of Caki-2 cells, which display more aggressive features and osteomimicry properties. This suggests that osteoclast cells can induce an environment supportive of the tumor growth. It is well known that osteoclasts can create an acidic extracellular microenvironment through the external secretion of hydrogen ions (H^+) [44,45]. If tumors have successfully adapted to this condition, then a more aggressive phenotype might be induced [45]. Moreover, osteoclasts could secrete soluble growth factors that directly impact on the Caki-2 cells phenotype [46,47]. We hypothesized that the survival advantage can depend on both mechanisms.

Once confirmed that Caki-2 cells can induce the mature differentiation of osteoclasts, we investigated the efficacy of Eve in the inhibition of this crucial crosstalk. The introduction of targeted agents, such as vascular endothelial growth factor tyrosine kinase inhibitors (VEGF-TKIs) and mTOR inhibitors, has led to a significant improvement in the clinical management of advanced RCC patients showing better response and progression free-survival [48,49]. The mTOR pathway is a key regulator of ccRCC tumor progression [50]. Indeed, Eve is indicated for the treatment of advanced ccRCC patients after VEGFR-tyrosine kinase inhibitor failure [51,52]. The use of Eve decreased after the regulatory approval of Cabozantinib, an oral inhibitor of tyrosine kinases including MET, VEGFR and AXL (tyrosine kinase receptor), which increased overall survival, delayed disease progression and improved objective response rates with respect to Eve in advanced ccRCC patients in progression after antiangiogenic treatment in the randomized phase 3 METEOR trial [53]. The survival benefit with Cabozantinib was confirmed after long-term follow-up [54]. In another study, pre-specified subgroup analysis based on the presence of baseline bone metastasis in advanced ccRCC patients revealed that Cabozantinib was superior to Eve in terms of improving clinical- and bone-related outcomes

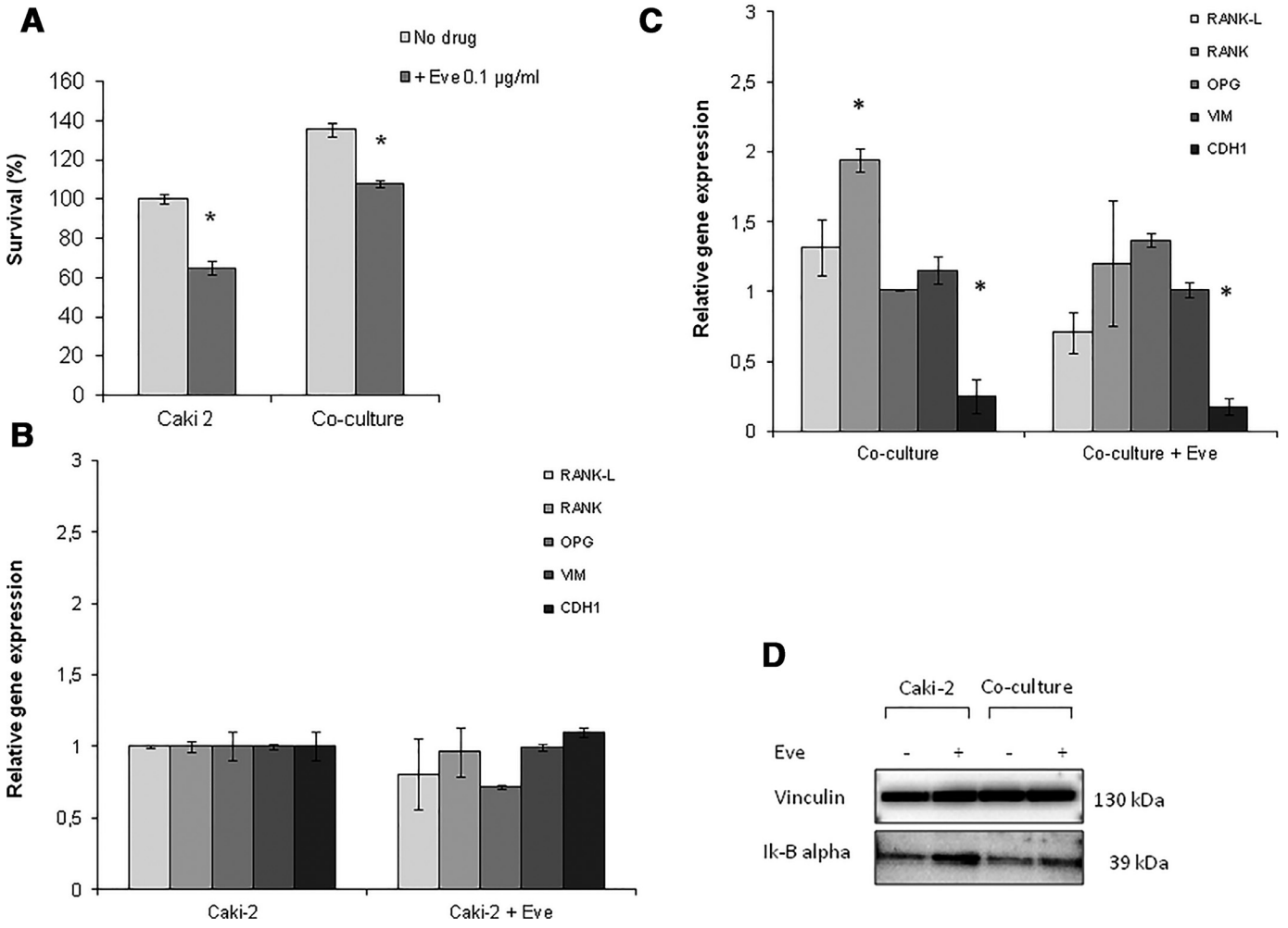

Fig. 4. Effect of Co-Culture and Eve treatment on Caki-2 cells. **(A)** MTT analysis of Caki-2 cells (absorbance at 550 nm). Data are expressed as a percentage (%) of survival normalized with respect to the proliferation rate of Caki-2 cultured alone. **(B and C)** Gene expression analysis of Caki-2 cells with respect to untreated Caki-2 cultured alone. Markers of EMT (VIM-CDH1) and osteomimicry (RANK-L, RANK, OPG) were analyzed.**(D)** Western blot analysis of Caki-2 cells to detect Vinculin expression as loading control and Ik-B alpha to evaluate Eve effect on Nf-kB pathway. Error bars: SE. Significance $p^* \leq 0.05$.

[55]. The approval of new drugs, including Cabozantinib, has substantially increased the therapeutic options for advanced ccRCC patients in progression after VEGFR-targeted therapy, and the use of Eve in clinical practice is now reserved for third- and subsequent-line treatment. Herein, we decided to investigate the effect of Eve because we have previously demonstrated for bone metastatic breast cancer that it could be a suitable candidate for the inhibition of the vicious cycle involved in the bone metastasis pathogenesis [22]. The mTOR pathway presents a dual role in cancer cells regulation and in osteoclast proliferation and survival [19,56,57]. Our results confirmed that Eve is able to inhibit the osteoclastogenesis induced by renal carcinoma cells. Interestingly, we showed that soluble factors expressed by Caki-2 cells can support the osteoclast survival. As regards cancer cells, our results showed and confirmed the interconnections of the mTOR and Nf-kB pathway [23]. Eve showed to inhibit the Nf-kB pathway, and this blockage is lost in the co-cultured condition with osteoclasts. This suggests that persistence of Nf-kB pathway in the co-culture could contribute to the lower sensitivity of Caki-2 cells to Eve treatment.

Moreover, we aimed at investigating the efficacy of Eve in combination with conventional bone-targeted therapy (Deno and Zol) to inhibit osteoclastogenesis. Bone targeted therapy can be considered a standard treatment for bone metastatic renal carcinoma patients [58]. Clinical trials showed that Deno is not inferior to Zol in the prevention of skeletal-related events [59]. In our system, Deno showed to be effective in the inhibition of osteoclastogenesis induced by Caki-2 cells, however it did not prove to be effective in decreasing the survival of Caki-2 cells. Conversely, Zol can inhibit osteoclastogenesis and decrease the Caki-2 cells survival even as single agent. Interestingly, this effect is substantiated when Caki-2 cells are co-cultured with osteoclasts. These results are confirmed by previous preclinical and clinical data which suggest that bisphosphonates have potential direct antitumor effects and prevent tumor progression of RCC [60,61]. Moreover, we showed that the combination of Eve and Zol is significantly more effective in the inhibition of osteoclastogenesis compared to treatment with Eve as a single agent. These results give a preclinical rationale for the use of Eve plus Zol in the treatment of patients with bone metastases from ccRCC, as a strategy to inhibit the vicious cycle of lytic lesions. Our data are confirmed by a clinical study conducted by Broom et al. that has demonstrated improved results of adding Zol to Eve in patients with RCC bone metastasis [62].

5. Conclusions

In conclusion, we confirm, supported by clinical observations, the value of our in vitro preclinical model as a useful tool to mimic the molecular interactions between tumor and bone cells. Taking into account the limitation of the system, it can be useful for studying drug effects on the complex bone microenvironment, in order to identify new

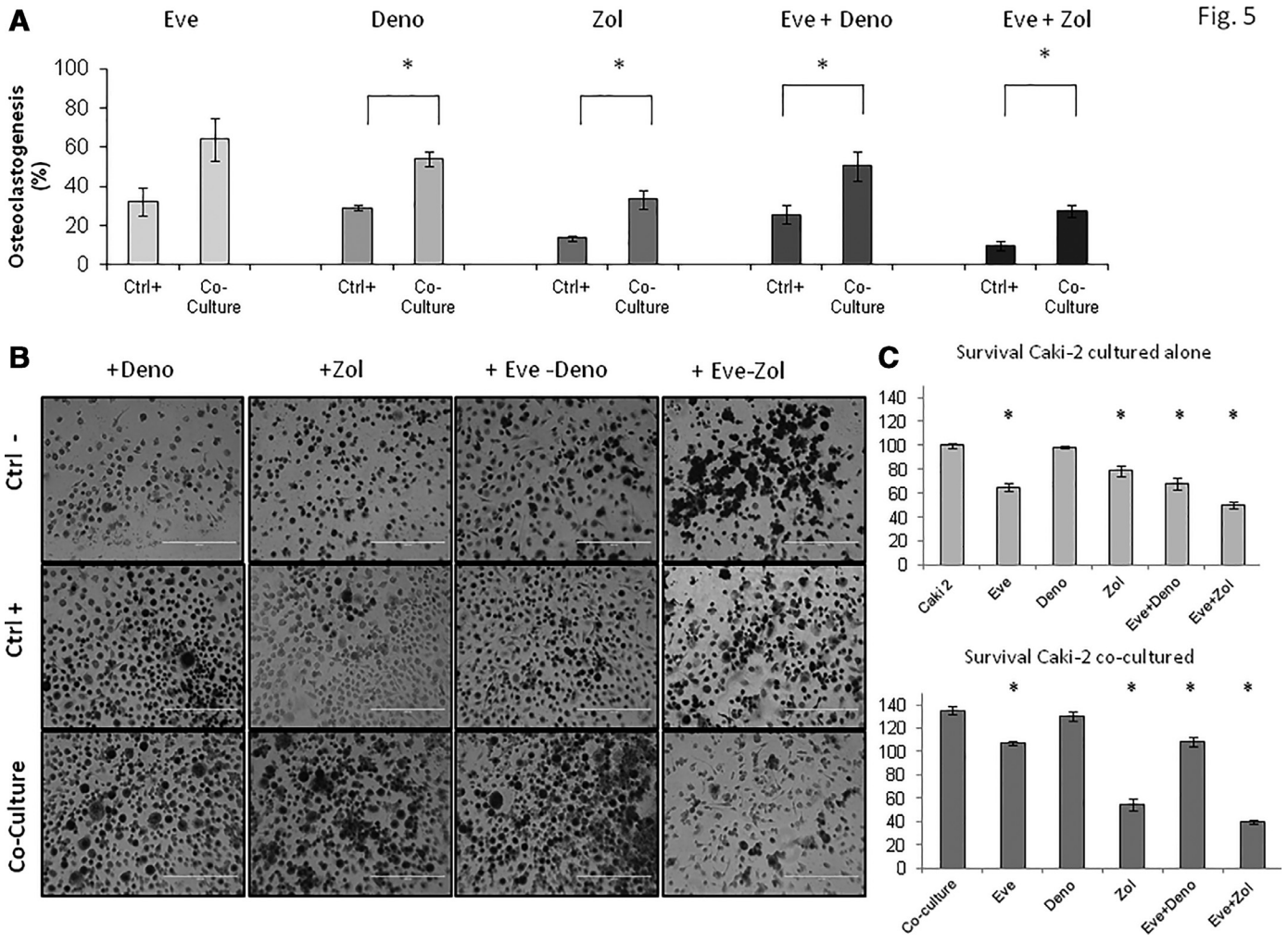

Fig. 5. Effect of drugs on osteoclasts and Caki-2 cells. **(A)** Drug effect on osteoclastogenesis; percentage of osteoclastogenesis compared to untreated conditions after Eve and other bone-targeted drugs; significance $p^* \leq 0.05$; Error bars: SE. **(B)** Representative pictures of TRAP staining in osteoclasts after 14 days of differentiation. 10x magnification. **(C)** MTT analysis on Caki-2 cells (absorbance at 550 nm). Data are expressed as a percentage (%) of survival normalized with respect to the proliferation rate of Caki-2 cultured alone. Significance is related to the corresponding untreated samples $p^* \leq 0.05$.

strategic therapeutic interventions able to inhibit the vicious cycle of bone metastasis. Moreover, it can help in improving the understanding of the biology of bone metastasis from renal carcinoma for the identification of soluble factors that could be therapeutically targeted.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Author Contributions

Chiara Spadazzi, Federica Recine and Toni Ibrahim conceived the idea for the study. Chiara Spadazzi and Laura Mercatali designed the experiments. Chiara Spadazzi, Giacomo Miserocchi, Chiara Liverani and Alessandro De Vita performed the experiments and acquired the data. Alberto Bongiovanni and Valentina Fausti contributed to data interpretation and revised the manuscript. Chiara Spadazzi drafted the paper. All authors read and approved the final version of the manuscript for submission.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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